Genetic abnormalities and signaling pathways altering urinary calcium excretion

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Abstract

Kidney stone disease is a common and serious problem with increasing incidence and frequency across the world. Hypercalciuria is an independent risk factor for the most common type of kidney stones i.e. calcium oxalate and calcium phosphate stones. Annually, billions of dollars are spent on diagnosis and treatment. A gene-wide association study linked hypercalciuria and claudin-14 with kidney stones. For this project, we have two inter-related goals, to identify novel genetic causes of idiopathic hypercalciuria and to delineate the signaling pathway downstream of calcium sensing receptor (CaSR) activation mediating increased claudin-14 expression. For the first part of this project we have extracted DNA from blood samples of patients suffering from idiopathic hypercalciuria and sequenced a few candidate genes. We identified a SNP that was found with significantly increased frequency in our patients compared to the 5008-allele control data (NCBI). However, when functional studies were performed with this SNP no significant difference in expression was seen. Interestingly, while performing these studies another SNP in the claudin-14 gene was reported to associate with kidney stones. When we cloned this SNP into a reporter construct we found a significant increase in expression, (N.B. this is a reporter for claudin-14 expression) suggesting this is a disease-causing variation. The second part of this project is to delineate the CaSR signalling pathway leading to increased claudin-14 expression. For this we used a cell culture model and luciferase reporter assays. We found that two signalling molecules PKC and cdc42 are present downstream the CaSR activation leading to attenuation in the transcription factor SP1 expression which ultimate results in increased claudin-14 expression.

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- **CaSR** Calcium sensing receptor
- **c-AMP** Cyclic adenosine phosphate
- **Cdc-42** Cell division control protein 42
- **CLDN-14** claudin-14 gene
- **IBMX** 3-isobutyl-1-methylxanthine
- **IH** Idiopathic hypercalciuria
- **mcldn 14 V1** mouse claudin-14 variant 1
- **MEK** Mitogen-activated protein kinase kinase
- **PLC** Phospholipase-C
- **PKC** Protein kinase-C
- **PCR** ploymerized chain reaction
- **SNP** single nucleotide polymorphism
- **SP1** specificity protein 1 transcription factor
- **TAL** thick ascending limb

Chapter 1. INTRODUCTION

1.1 Renal physiology

The kidney plays a variety of important roles in the body. It excretes waste products produced by different metabolic reactions including urea produced from protein metabolism, uric acid produced by nucleic acid metabolism, creatinine from muscle metabolism and bilirubin from hemoglobin metabolism. It also excretes ingested chemicals including pesticides, food additives, toxins and drugs from the body. Further, the kidney produces hormones such as erythropoetin, 1,25-dihydroxycholecalciferol (Vitamin D), renin and urokinase. Some peptide hormones including insulin and angiotensin II are metabolized and excreted by the kidney. They not only play an important role in the regulation of acid-base balance by excreting acids and reabsorbing bicarbonate but are also the only means of excreting non-volatile acids from the body. During hours of prolonged fasting the kidneys along with the liver have the ability to synthesize glucose from precursors such as amino acids. This process is called gluconeogenesis. By controlling the rate of water and electrolytes excretion, kidneys play an important role in the regulation of arterial blood pressure.

1.2 Kidney stones

Kidney stone disease is a common and serious disease whose incidence is increasing across the world. In Canada, 1 out of 10 individuals are at risk of kidney stone disease. (Kidney foundation of Canada). Kidney stones are associated with a variety of serious chronic medical issues which may include diabetes mellitus, hypertension, chronic kidney disease, cardiovascular diseases and increase risk of stroke (Alexander et al. 2014, Alexander et al. 2012). Furthermore, treatment of kidney stones carries a huge

economic burden on the health care system. Annually around $5 billion are spend on the diagnosis and treatment of this disease in the United States alone (Sakhaee et al. 2012).

1.2.1 Types of kidney stones

1.2.1.1 Calcium kidney stones

The most common type of kidney stones formed across the world are composed of calcium. There are two types of calcium kidney stones, calcium oxalate and calcium phosphate. Of the two types, the calcium oxalate type of kidney stones is more common consisting up to 85% (Oreopoulos et al. 2003) whereas, calcium phosphate consists only 15% of the total percentage (Sakhaee et al. 2012). Calcium based kidney stones therefore comprise approximately 80% of all types of kidney stones (Hess, B. 2003) **(**see **Table 1)**. There are two important concepts relevant to stone formation, supersaturation and metastability of solutes in liquids. Both these theories help us better understand how stones are formed in the urine. Supersaturation of solutes in liquids explains how a salt can form a nidus upon which further layers of salts can precipitate eventually forming a solid substance i.*e.* a stone. The second idea, that of metastability of solutes in liquids explains the metastable zone, which stresses that the precipitation of the solute occurs when the concentration of a salt is above its solubility level. Patients diagnosed with kidney stone disease tend to have a lower metastability limit, which helps in understanding their increased tendency to form kidney stones (Miller et al. 2007).

1.2.2.1 Non- calcium kidney stones

1. Uric acid stones:

Uric acid stones comprise approximately 10% of all kidney stones **(**see **Table 1)**. However, this percentage is not consistent across the globe, suggesting that some populations are more prone to develop this kind of kidney stones than others. Risk factors causing this difference in incidence and prevalence are not well identified (Cameron et al. 2007). This difference in incidence across the globe appears to be independent of the pathophysiology of uric acid stones formation (Moe et al. 2002). One group found that the most common risk factor associated with this type of kidney stones is the metabolic syndrome (Sakhaee et al. 2012). The metabolic syndrome is defined as a combination of different biochemical disorders and include other associated risk factors such as hypertension, dyslipidemia and obesity. These risk factors ultimately lead to different metabolic diseases such as type 2 diabetes mellitus and atherosclerosis contributing to an increased risk of developing cardiovascular abnormalities and stroke. The same group also performed a retrospective study in Dallas, that traced back the medical records of idiopathic uric acid nephrolithiasis patients and found many features consistent with the metabolic syndrome (Sakhaee 2009). There are two common forms of uric acid stones, pure uric acid stones or mixed uric acid stones. Most patients diagnosed with uric acid stones have a low pH of the urine (acidic acid) and low urine volume, which contributes to stone precipitation. However, they typically do not have abnormal uric acid levels.

2. Cystine stones

Cystine stones comprise less than 1 % of all pediatric kidney stones **(**see **Table 1)**. These types of stones are more common among children and adolescents than adults.

Cystine stones are seen exclusively in patients with cystinuria. Cystinuria is caused by an inherited defect in either SLC3A1 or SLC7A9 resulting in defective renal tubular cystine reabsorption. It is inherited as either an autosomal recessive or autosomal dominant disease. Low solubility of cystine in urine causes cystine stones in both types of inherited defects. Cystinuria is classified into three groups: type A, type B and type AB. Type A is caused by homozygous mutations in SLC3A1, type B is caused by homozygous mutations in SLC7A9 and type AB caused by heterozygous mutations in both SLC3A1 and SLC7A9 gene (Sakhaee et al. 2012).

3. Struvite stones

Up to 10% of all type of kidney stones consist of struvite **(**see **Table 1)**. Struvite stones are also known as infection related stones. Most of the struvite stones are composed of magnesium, ammonium and phosphate. Struvite stones are related to several urinary tract infections. Various bacteria such as Proteus, Klebsiella, Staphylococcus Epidermidis, Pseudomonas, Providencia, Ureaplasma urealyticum and Entercoccus contribute to the acquisition of urinary tract infection (Shortliffe et al. 1986). Struvite stones have a staghorn structure which make it hard for them to pass on their own. They usually need help of surgical procedures for their complete removal along with broad spectrum antibiotic coverage (Flannigan et al. 2014). Protease drug inhibitors are often associated with the development of rare forms of kidney stones such as dihydroxyadenine and ammonium urate (Sakhaee et al. 2012).

No.	Type of kidney stones	Percentage
1.	Calcium oxalate/Calcium phosphate	80% (Calcium oxalate 85% & Calcium phosphate 15 %)
2.	Uric acid	10 %
3.	Stuvite	10 %
4.	Cysteine	< 1 %

Table 1. Types of kidney stones and their relevant percentages.

1.2.2 Pathophysiology

Kidney stone formation is a complex process and various risk factors are associated with the pathophysiology. They can occur due to genetic, environmental or a combination of both genetic and environmental factors. Kidney stones are associated with various systemic disorders like hypertension, type 2 diabetes mellitus, metabolic syndrome, endocrine disorders, vascular diseases such as coronary heart diseases, ischemic stroke and chronic kidney diseases. Many theories have been proposed to explain the pathogenesis behind stone formation. One of the most well-known theories is the tissue injury theory. This theory argues that kidney stone formation occurs because crystals are retained due to tissue injury (Miller et al. 2007). Hyperoxaluria increases excretion of urinary enzymes such as N-acetyl-β-glucosaminidase, gamma-glutamyl transpeptidase and alkaline phosphatase which can cause renal epithelial cell injury (Miller et al. 2007). Further, overwhelming the antioxidant system through renal

epithelial cell injury and the production of reactive oxygen species leads to oxidative stress. Renal epithelial injury also results in the secretion of macromolecules such as CD44, phosphatidylserine, hyaluronan and osteopontin, which further enhances the adherence of crystals to the renal epithelial cell surface (Miller et al. 2007). Crystals favorably adhere to injured renal epithelial cells rather than healthy cells, specifically calcium oxalate crystals adhere to the injured surface of cells (Miller et al. 2007). A different theory revolves around Randall's plaque formation that mainly localizes to the basement membrane of the thin descending limb of the loop of Henle. In this theory calcium and phosphate ions anchor to the basement membrane that is composed of collagen and mucopolysaccharides (Sakhaee et al. 2009). These crystals become a nidus for calcium oxalate deposition along the urothelium, ultimately forming kidney stones (Sakhaee et al. 2009). A significant role of Randall's plaque in kidney stone disease was suggested through data collected from human cortical and papillary biopsies in patients with kidney stones (Miller et al. 2007). Uric acid kidney stones are associated with hyperuricosuria, low urine volume and aciduria. Of which the most important risk factor is low urine pH as uric acid crystalizes at a low pH. Higher uric acid concentration is another risk factor which can be either genetic or acquired. However, acquired causes are more common than genetic causes. Acquired causes of hypercalciuria are secondary to chemotherapy treatment for malignancy or due to the use of uricosuric medicines. Patients with diseases such as short bowel syndrome or those who have an ileostomy are at risk of developing kidney stones because of chronic diarrhea ultimately leading to low urine volume (<2L/day or lower).

In summary, kidney stone formation is a complex process and there are many different

types of kidney stones. Even though many theories attempt to explain the process of stone formation we still don't know the exact mechanism behind kidney stone formation.

1.2.3 Risk factors

There are various risk factors for developing kidney stones. They can be metabolic, genetic, environmental or a combination of all three of them. Hypercalciuria is the most common risk factor for the development of calcium kidney stones, which collectively constitute approximately 80% of all types of kidney stones. Several genes have been linked to hypercalciuria and kidney stones including **claudin-14**, calcium sensing receptor (CaSR), vitamin D receptor, ALP (alkaline phosphate), SLC34A1, TRPV-5, claudin-16 and -19, soluble adenylate cyclase. Uric acid stones are the next most common type of kidney stones. Hypocitraturia and hyperoxaluria are other risk factors for calcium oxalate and calcium citrate type of kidney stones. Hyperuricosuria, which can be secondary to ingestion of purine rich food and urate overproduction is a common risk factor for developing uric acid kidney stones. Disturbance in urinary pH either low or high is also a risk factor for developing calcium kidney stones (Sakhaee et al. 2012). Different stones precipitate at a different urine pH e.g calcium oxalate stones have a strong tendency to precipitate at an acidic pH whereas calcium phosphate stones tend to precipitate at an alkaline pH. Low urinary pH as seen with severe dehydration or chronic diarrhea is the leading cause for uric acid stone formation as it increases uric acid secretion in the presence of low urine volume (Hochreiter et al. 2003). In another study, a group proposed that idiopathic uric acid nephrolithiasis is either associated with or due to insulin resistance (Moe et al. 2002). Cystinuria, secondary to a defect in

tubular reabsorption can lead to the cystine type of kidney stones. Cystine has a strong tendency to form kidney stones in the presence of urinary pH below 8, as cystine has low solubility at this pH which promotes supersaturation (Hochreiter et al. 2003). Infection-related kidney stones (magnesium-ammonium-phosphate) or a staghorn calculus are mostly formed due to bacterial infections. Bacteria producing urease enzymes can split urea into ammonia hence raising the urinary pH. This promotes magnesium-ammonium-phosphate stone formation, which can grow into a staghorn calculus. These latter types of stones are notorious for obstructing the kidney tubules especially the collecting duct (Hochreiter et al. 2003).

1.2.4 Treatment for kidney stones

Low urine volume is one of the most important risk factors leading to kidney stone formation. So, the first and foremost step in the treatment for all types of kidney stones is increasing the daily intake of oral fluids up to 2.5 liters. Hypercalciuria is another common reason for kidney stone formation. Medications such as thiazide diuretics are frequently used for the treatment of hypercalciuria in kidney stone patients. In patients with acidic urine, potassium citrate can be used to alkalinize the urine. Kidney stones are frequently associated with decreased bone mineral density and an increased frequency of bone fractures. It has been seen that treatment with diuretics and alkalization of urine increases bone mineral density (Sakhaee et al. 2012). Allopurinol is used for decreasing urinary uric acid concentration by preventing the conversion of xanthine to uric acid (Sorensen et al. 2002). Hence it is used to treat patients who excrete excessive amount of uric acid in their urine. To relieve obstruction caused by

uric acid stones or in cases where surgical intervention is contraindicated, irrigation of the bladder can be performed using a catheter with an alkaline solution (Moe et al. 2002). For cystine stones drug treatment include penicillamine and α-mercaptopropionylglycine. These drugs can be used to split cystine molecules into more soluble compounds for excretion into the urine (Sakhaee et al. 2012). For the treatment of struvite kidney stones antibiotic coverage is required during all stages of therapy from removal of stone(s) to the treatment of urinary tract infection. Various surgical procedures are used including extracorporeal shockwave lithiotripsy, ureteroscopy and anatrophic nephrolithiotomy (Flannigan et al. 2014). However, percutaneous nephrolithiotomy (PCNL) is considered the gold standard method of treatment for these types of stones (Flannigan et al. 2014). Acetohydroxamic acid can be used as treatment for struvite stones where treatment with antibiotics and surgical removal are ineffective. Acetohydroxamic acid inhibits the urease enzyme produced by bacteria, preventing an increase in urinary pH and rise in NH_4^+ level (Sakhaee et al. 2012).

1.3 Calcium handling by the nephron

1.3.1 Calcium reabsorption in the proximal tubule

Almost two-third of the filtrate entering Bowman's capsule is reabsorbed in the proximal tubule. Consequently, the proximal tubule is often referred as the major reabsorptive segment of the nephron (Jianghui H et al. 2013). Most of the filtered water, ions (Na^{+}, Cl^{-}, Ca^{++}, Mg^{++} etc.) and organic solutes like glucose and amino acids are reabsorbed in this segment (Bergsland KJ et al 2013). In an average 70 kg healthy individual the glomerular filtration rate is approximately 170 liters per 24 hours. Of the calcium that enters the nephron almost 98 to 99 % of it is reabsorbed by the renal tubules. Which means if 10 g of calcium is filtered in 24 hours only 100-200 mg of calcium will be excreted in the urine and the rest is reabsorbed. Approximately 65% of calcium is reabsorbed in the proximal tubule by both a passive paracellular pathway and an active transcellular pathway. However, approximately 80% of the calcium is reabsorbed mainly by passive diffusion and solvent drag. As it is observed that the ratio of calcium in the proximal tubule fluid is very similar to that of calcium in the glomerular filtrate, suggesting that most of this reabsorption is via the paracellular pathway (Jianghui H et al. 2013). This active transport accounts for approximately 20% of calcium reabsorption. This transcellular active pathway is regulated by the two regulatory hormones, parathyroid hormone (PTH) and calcitonin.

1.3.2 The thick ascending limb (TAL) and electrolyte homeostasis

Approximately 90% of calcium is reabsorbed in a paracellular fashion, down the electrochemical gradient in the first two segments of the renal tubule i.e. proximal tubule

and thick ascending limb. Of the calcium filtered by the glomerulus approximately 25% is reabsorbed from the thick ascending limb. And almost 10% of the calcium is reabsorbed from the last two segments of the nephron (distal convoluted tubules and the collecting duct). Thick ascending limb (TAL) has cation selective tight junctions with relatively higher resistance than that of proximal tubule (Mount DB. 2014). The thick ascending limb (TAL) reabsorbs 50%-60% of filtered magnesium and 30%-35% of filtered calcium (Greger R et al. 1985). Of which approximately 20% of filtered calcium is reabsorbed via paracellular pathway. This paracellular reabsorption is facilitated by the trans-epithelial positive charge generated by ROMK channel present on the apical surface of the thick ascending limb. It plays an important role in the recycling of K^+ into the lumen of the TAL (Boim MA et al. 1995). Passive diffusion of water along the thin ascending limb, leaving behind higher concentration of ions entering the TAL plays a significant role in creating chemical concentration gradient facilitating calcium reabsorption through paracellular diffusion where calcium follows movement of Na^+ and water along the tight junction pores (Jianghui H et al. 2013).

Paracellular cation pores formed by the interaction of claudin-16 and 19 facilitate calcium reabsorption in TAL. Consistent with their role in paracellular ion transport, mutations in either the claudin-16 gene (Simon et al. 1999) or claudin-19 gene (Konrad et al. 2006) can cause autosomal recessive familial hypomagnesaemia with hypercalciuria and nephrocalcinosis. This renal disorder highlights the fact that expression of claudin-16 and claudin-19 (which are exclusively expressed in the thick ascending limb (TAL) of the nephron) is necessary for the reabsorption of a significant percentage of divalent cations via the paracellular pathway (Greger et al. 1985).

Increased circulatory load of calcium (sensed by calcium sensing receptor (CaSR) expressed along the basolateral surface of TAL) leads to the activation of an internal signaling cascade that ultimately leads to greater expression of claudin-14 on the tight junctions forming paracellular barrier (Mount DB. 2014). Thus, claudin-14 is a cation blocker tight junction protein and its expression along the apical surface inhibits calcium reabsorption hence leading to calciuria.

1.3.3 Vitamin D metabolism and CYP24A1

Calcium homeostasis is maintained in the body at both the cellular and organ level. The kidney, intestine and bone play an important role in maintaining this balance. Falling blood Ca^{2+} stimulates the parathyroid gland to secrete parathyroid hormone which in turn stimulates Ca^{2+} release from the bones and Ca^{2+} uptake by the kidney. The kidneys make 1,25-dihydroxyvitamin D_3, which acts to increase Ca^{2+} uptake from the intestine and hence bring blood calcium levels back to the physiological range.

Cholecalciferol is a steroid hormone that can be synthesized under the skin in the presence of ultraviolet light from its precursor 7-dehydrocholestrol. It can also be obtained from food products. Cholecalciferol or Vitamin D from either source (diet or skin) is hydroxylated at position 25 in the carbon chain in the presence of the hepatic enzyme 25-hydroxylase resulting in the formation of 25-hydroxyvitamin D or calcidiol (Bikle DD. 2014). 25-hydroxyvitamin D once released from the liver enters the circulation and travels to the kidney. In the kidney, 1 alpha-hydroxylase converts 25-hydroxyvitamin D into $1,25(OH)_2D$, the hormonally active form of vitamin D **(**see **Figure**

1) 24-hydroxylase converts the 1,25$(OH)_2$D into 24,25-dihydroxyvitamin D or calcitroic acid (Bikle DD. 2014). Calcitroic acid, the inactive form of vitamin D is excreted from the body through bile. Kidneys play an important endocrine role by strictly regulating homeostasis through the synthesis and release of stimulating and suppressing hormones (Bikle DD. 2014). The physiologically active form of vitamin D 1,25$(OH)_2$D, is responsible for calcium absorption from the intestines. If there are mutations in CYP24A1 (the gene regulating 24-hydroxylase expression) it can lead to the accumulation of active form of vitamin D, resulting in increased calcium absorption from the intestines leading to hypercalcemia and ultimately hypercalciuria because of an increase in the filtered load and decrease in calcium reabsorption by the kidney. More than 20 mutations in CYP24A1 gene have been described. They can result in either reducing or eliminating the activity of the 24-hydroxylase enzyme and are associated with idiopathic infantile hypercalcemia a disease characterized by hypercalcemia, hypercalciuria and nephrocalcinosis (https://ghr.nlm.nih.gov/gene/CYP24A1).

Conversely, a positive feedback exists when plasma Ca^{2+} levels are high. This stimulates the thyroid gland to secrete calcitonin, which is a counter regulatory hormone for parathyroid hormone. Calcitonin stimulates Ca^{2+} deposition in the bones and reduces calcium uptake from the kidneys, bringing blood Ca^{2+} levels back within the normal physiological range.

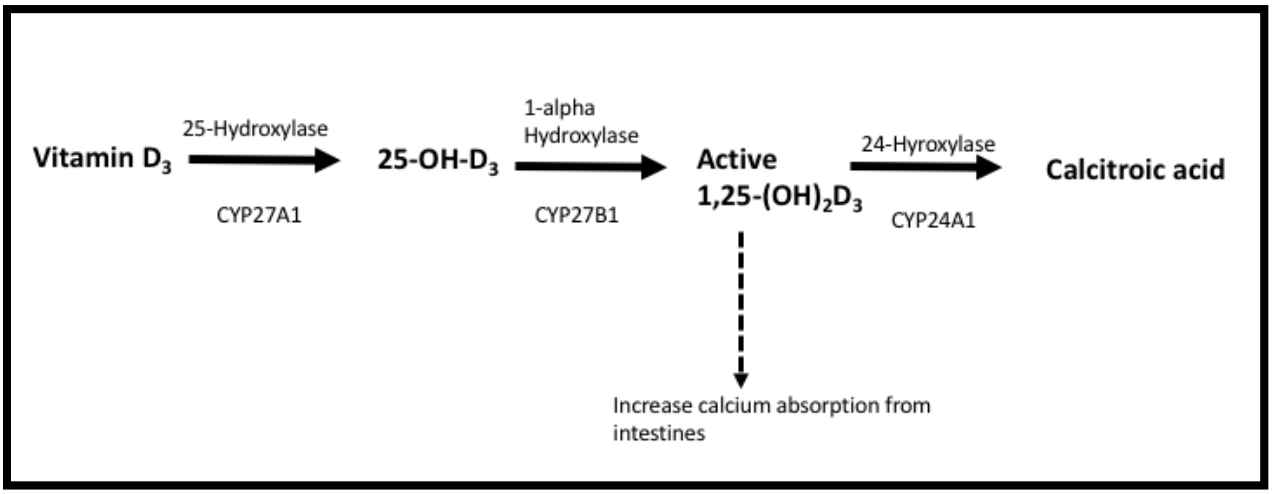


Figure 1.Vitamin D metabolism and CYP24A1.

A diagrammatic representation of the role of CYP24A1 in calcium homeostasis. 1,25 di-hydroxyvitamin D_3 ($1,25(OH)_2D_3$), the hormonally active form of vitamin D, is responsible for increasing calcium absorption from intestine. Mutations in CYP24A1 leads to accumulation of the active form of vitamin D, resulting in increased calcium absorption from the intestine, resultant hypercalcemia and ultimately hypercalciuria.

1.3.4 The CaSR

Calcium is important for a variety of cellular functions ranging from maintaining membrane stability to intra-cellular signaling. It also plays a critical role in neuronal transmission, blood coagulation and maintain bone structure. Calcium concentration is therefore maintained within a tight range in the plasma from 2.2 to 2.7 mmol/L, largely with the help of calcium sensing receptor (CaSR) is expressed in the parathyroid gland and the kidney along the renal tubule (Tennakoon, S. et al. 2016). In the kidney, the CaSR senses changes in the plasma calcium levels and when calcium concentration in the plasma is too high it mediates an increase in urinary calcium excretion, by increasing the expression of claudin-14 which blocks tubular calcium reabsorption (Gong et al. 2012). However, the signaling pathway between CaSR activation and claudin-14 expression is completely unknown.

The calcium sensing receptor (CaSR) is a member of G protein-coupled receptors family, also known as seven transmembrane receptors. They constitute a large family of receptor proteins that detect stimulus outside the cell and activate internal cell signaling pathways ultimately leading to cellular responses (Tennakoon, S. et al 2016).

Intestinal calcium absorption is regulated by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], which determines how much calcium will be absorbed into the body. In contrast the kidneys regulate how much calcium will leave the body. This triad between intestine, kidney and bones is regulated largely by parathyroid hormone (PTH), calcitonin (CT) and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (Riccard D et al. 2009). The calcium sensing receptor (CaSR) is one of the essential components in maintaining calcium homeostasis. It is expressed in different tissues which include the parathyroid hormone

(PTH)- secreting parathyroid glands, calcitonin (CT)-secreting thyroidal cells, intestines, kidneys and bone (Tfelt-Hansen J et al. 2005). The CaSR is a transmembrane protein containing a 612-amino acid long extracellular domain (ECD), seven transmembrane helices (TMD) consisting of 250-amino acids and an approximately 200-aminoacid long carboxyl terminal (C) tail (Tfelt-Hansen J et al. 2005). It is predicted that the CaSR's ECD exhibits a venus flytrap (VFT)-like motif (Hu J et al. 2003, Silve C et al. 2005) (Tennakoon, S. et al 2016) exposing a key binding site for extracellular calcium that responds to fluctuation in plasma calcium levels. It is presumed that when calcium binds to this VTF binding site it initiates a conformational change starting a cascade of internal cell signaling events. Regulation of the calcium sensing receptor (CaSR) gene is effected by different factors which upregulate its expression, including an increase in the extra-cellular calcium lead, this increased expression in turn leads to CaSR activation (Yarden N. et al. 2000). CaSR gene expression is also positively regulated by CaSR agonists, vitamin D via binding the vitamin D response elements (VDRE) in the two promoters of the CaSR gene (Canaff L. et al. 2002), and the cytokines interleukin-1β (Nielsen PK. et al. 1997) and interleukin-6 (Canaff L. et al. 2008).

1.3.5 Genetic variations of the CaSR are associated with kidney stones

The calcium sensing receptor (CaSR) regulates sodium and divalent cation transport across the thick ascending limb. The ROMK channel present on the apical surface of the thick ascending limb plays an important role in the recycling of K^+ into the lumen of the TAL (Boim MA et al. 1995), a process necessary for sodium transport across this segment through the Na+-K+-2Cl− cotransporter (NKCC2). Hypercalcemia results in the

dissipation of the lumen-positive trans epithelial voltage across the TAL by inhibiting ROMK channels (Wang WH et al. 1996), and limiting the rate of Na+-K+-2Cl− cotransport by reducing the availability of luminal K^+. The rate of dissipation of lumen-positive charge is dependent on the degree of hypercalcemia. This whole process is mediated by the CaSR activation. During hypercalcemia, calcium stimulates the basolateral CaSR, which has an inhibitory action on ROMK channels in the TAL (Wang WH et al. 1996), reducing the availability of luminal K^+ by limiting the rate of Na+-K+-2Cl− cotransport. There is also decrease in paracellular Na^+, Ca^{2+}, and Mg^{2+} transport, producing a "Bartter-like" phenotype (Vargas-Poussou R et al. 2002). Prostaglandins and P-450 metabolites regulate the downstream pathway of CaSR activation that leads to the inhibitory action on NKCC2 and ROMK (Hebert SC et al. 2007, Wang W et al. 1997, Wang WH et al. 1996).

a) Familial hypocalciuric hypercalcemia (FHH)

This is an autosomal dominant, benign disease characterized by excessive plasma calcium levels and associated with low urine calcium excretion. It is caused by disturbance in the regulation of parathyroid and renal function by plasma calcium (Law WM et al. 1985, Law WM et al. 1981). It is inherited as a heterozygous inactivating mutation of the CaSR gene, residing on the long arm of chromosome 3 (3q13.3–q21) and referred to as hypocalciuric hypercalcemia, familial, type 1 (HHC1, 145980) in the Online Mendelian Inheritance in Man (OMIM)]. Several different mutations lead to this disorder, missense mutations being the most common. So far more than 100 mutations

associated with Familial hypocalciuric hypercalcemia (FHH) have been described (http://www.casrdb.mcgill.ca/). (Riccard D et al. 2009)

b) Neonatal severe primary hyperparathyroidism (NSHPT)

NSHPT is commonly inherited as a homozygous condition (Hauache OM et al. 2001, Pollak MR et al. 1994, Pollak MR, et al. 1994) and only rarely as compound heterozygous mutations in the CaSR gene (Kobayashi M et al. 1997). It's a severe condition presenting with severe symptoms within the first 6 months of life (Harris SS et al. 1989, Hauache OM et al. 2001, Marx SJ et al. 1985, Rhone DP et al. 1975). It is associated with severe hyperparathyroidism leading to PTH-dependent hypercalcemia and bony changes. Clinical manifestation of the disease includes polyuria, dehydration, hypotonia, and failure to thrive (Eftekhari F et al. 1982, Heath D et al. 1989, Marx SJ. et al. 1985). The disease effects bones severely leading to multiple disabling fractures in many different bone types from long bones to rib fractures (Eftekhari F. 1982).

c) Autosomal dominant hypoparathyroidism

This is an autosomal dominant form of hypocalcemia/hypoparathyroidism caused by an activating mutation in the CaSR gene. Patients suffering from these mutations are often asymptomatic (Hauache OM et al. 2001, Hendy GN et al. 2000, Pearce SH et al. 1996). Some patients may present with mild-moderate hypocalcemia, with low-normal or significantly low PTH levels (Pearce SH et al. 2002). Others may present with complications seen in hypoparathyroidism that include neuromuscular irritability, basal ganglia calcification, and seizures (Pearce SH et al. 1996). If the condition is not well

treated patients may suffer from relative or significant hypercalciuria, nephrocalcinosis and renal insufficiency (Baron J et al. 1996, Pearce SH et al. 1996, Winer KK et al. 2003).

d) **Bartter syndrome**

Several causes of this syndrome have been identified so far based on the pattern of inheritance and associated gene mutations **(**see **Table 2).** One of the well-known types is Bartter syndrome type V caused by activating mutations in the CaSR gene and is inherited in an autosomal dominant manner (Vargas-Poussou R et al. 2002, Watanabe S et al. 2002). Patients suffering from activating CaSR mutations have a lower Ca^{2+} concentration in their plasma than typically observed along with hypokalemia due to renal K^{+} wasting and sufficient volume depletion to cause hyperreninemia, and hyperaldosteronemia. The other classic feature of Bartter syndrome, hypochloremic metabolic alkalosis is also present.

No.	Name/ Bartter Syndrome types	Pattern of inheritance	Associated gene mutations	Defect
1.	Neonatal Bartter's syndrome or Bartter's syndrome type 1	autosomal recessive	SLC12A1	Na-K-2Cl symporter
2.	Neonatal Bartter's syndrome or Bartter's syndrome type 2	autosomal recessive	KCNJ1	thick ascending limb K^+ channel
3.	Classic Bartter's syndrome or Bartter's syndrome type 3	autosomal recessive	CLCNKB	Cl^- channel
4.	Bartter's syndrome with sensorineural deafness or Bartter's syndrome type 4	autosomal recessive	BSND	Cl^- channel accessory subunit
5.	Bartter's syndrome associated with autosomal dominant hypocalcemia or Bartter's syndrome type 5	autosomal dominant manner	CASR	activating mutation of the calcium-sensing receptor

Table 2. Types of Bartter syndrome classified by the associated gene mutation.

1.4 Claudins

Claudins are 4-pass membrane proteins that localize to the tight junction of epithelia (Jianghui Hou et al. 2013). The family consists of 27 members in humans that can act as cation pores or barriers thereby determining the permeability properties of an epithelium. When acting as a cation pores they usually form paracellular channels permitting the selective reabsorption of ions (Tsukita et al, 2001). They play a key role in the regulation of the movement of substances between epithelial cells, a process referred to as paracellular transport. Paracellular transport is the predominant route for transepithelial cation transport in the kidney (Jianghui Hou et al. 2013). Claudins range in molecular mass from 21–28 kD and have charged extracellular loops. The cytoplasmic C-terminus of most claudins contains a PDZ (postsynaptic density 95/discs large/zonula occludens-1) binding domain that is critical for interaction with the sub-membrane scaffold protein ZO-1 and for the correct localization to the tight junction (Jianghui Hou et al. 2013).

1.4.1 Role of claudin 2 & claudin-12 in the proximal tubule

The proximal renal tubule is responsible for the reabsorption of most water and solute entering as glomerular filtrate which occurs through paracellular channels in the tight junction. Claudins form these paracellular channels acting either as cation/anions pores or barriers. Claudin-2 is expressed in both the early and late proximal tubule segments with high levels being expressed in the late proximal tubule (Enck AH et al. 2001, Kiuchi-Saishin Y et al.2002) where they form small cation pores responsible for selective reabsorption of solutes (Amasheh S et al. 2002) including calcium. Most of the

calcium reabsorption occurs in a paracellular fashion down the concentration gradient where calcium follows the movement of sodium along with water. This paracellular calcium reabsorption is facilitated on most part by the claudin-2 present along tight junction in the proximal tubule (Enck AH et al. 2001).

To examine the role of other claudins in the nephron, specifically in the proximal tubule the Alexander lab recently started working on claudin-12 to see if it is expressed in this segment and plays any functional role in maintaining calcium homeostasis. To that end, they found that claudin-12 is expressed in the proximal tubule where it forms a cation permeable pore (unpublished data). Furthermore, functional studies suggested that claudin-12 plays an important role as a cation permeable pore responsible for selective reabsorption of solutes in the proximal tubule including calcium reabsorption. However, genetic deletion of claudin-12 in mice didn't reveal any alteration in urinary calcium excretion (unpublished data). Hence claudin-12 data is in very preliminary stages and whether claudin-12 interacts with other claudins to mediate calcium reabsorption or if there is any physiological compensation for the loss of function of claudin-12 gene is still unknown. Regardless defective calcium reabsorption is seen in the proximal tubule in patients with idiopathic hypercalciuira (Bergsland KJ et al. 2013). Therefore, the loss of function of tight junction proteins is likely responsible for defects seen in calcium reabsorption in the proximal tubules. To date specifically claudin-2 and-12 are the only cation selective claudins known to be present in the proximal tubule and thus they might be responsible for defective reabsorption of calcium seen in this segment of nephron.

1.4.2 Role of claudin-14 in the thick ascending limb (TAL)

Claudin-14 is expressed in the thick ascending limb (TAL) when calcium levels are high in the blood. The thick ascending limb (TAL) reabsorbs a significant percentage of filtered divalent cations (30–35% Ca++ and 50–60% Mg++) (Greger R et al. 1985). In this segment both Ca++ and Mg++ are reabsorbed down their electrochemical gradient by the interaction of claudin-16 and claudin-19 that form a cation permeable pore in the tight junction. This paracellular transport is driven by a lumen positive transepithelial voltage (Vte) (Jianghui Hou et al. 2013). The Vte is generated by a positive charge effluxion across the apical surface (apical K^+ recycling). The other driving force is the chemical concentration gradient for calcium generated by water removal from the ultra-filtrate in the thin descending limb. (Jianghui Hou et al. 2013). Claudin-14 acts as a cation blocker that prevents calcium reabsorption down its electrochemical gradient in the thick ascending limb through the claudin-16 and -19 pore. Hence its expression in the TAL lowers plasma calcium by preventing calcium reabsorption, thereby increasing urinary calcium excretion.

1.4.3 Alterations in claudin-14 are associated with kidney stones

A gene wide association study (GWAS) linked idiopathpathic hypercalciuria and kidney stones with the claudin-14 gene (Thorleifsson et al. 2009). However, the identified SNPs in this study were in the coding region of claudin-14 and did not change the amino acid sequence. Moreover, claudin-14 knockout mice (Wilcox et al. 2001, Ben-Yosef et al. 2003) and patients with null mutations in claudin-14 are deaf (Bashir et al. 2013, Lee et al. 2012) and had no disturbance in calcium regulation. This inferred that it

is likely a gain of function of the claudin-14 protein rather than loss of function that leads to imbalance in calcium homeostasis leading to hypercalciuria and kidney stone formation.

Work done in the Alexander lab supports that claudin-14 expression is transcriptionally regulated and that intronic sequences in the claudin-14 gene play an important role in the regulation of gene expression. Further it is clear that claudin-14 expression is strongly influenced by CaSR signaling. This interaction has been named 'the CaSR-CLDN14 axis'. That claudin-14 expression is regulated by CaSR activation has been demonstrated on multiple occasions. A huge (40-fold) increase in claudin-14 expression was seen when the CaSR agonist (Cinacalcet) was given to the wild-type mice (Dimke et al. 2013a). Another group observed less urinary calcium excretion in claudin-14 knockout mice on a high calcium diet compared to the wild type mice on the same diet (Gong et al. 2012), suggesting that increase in claudin-14 expression in the TAL leads to calciuria. (Gong et al. 2012). Further that claudin-14 acts as a paracellular cation blocker and plays an important role in renal calcium handling was also demonstrated in other cell culture models (MDCK and OK cells) (Dimke et al. 2013a). Thus, when the plasma calcium concentration increases it stimulates CaSR activation, expressed on the basolateral surface in the TAL (Riccardi et al. 1996) and leads to increase in claudin-14 expression in the tight junctions. This newly synthesized cation barrier (claudin-14) expressed in the tight junction prevents Ca^{2+} reabsorption, leading to increased calcium excretion in urine, which is also known as calciuria **(**see **Figure 2).**

Thus, the likely pathogenesis resulting in hypercalciuria and kidney stones in persons with alterations in the claudin-14 gene is a gain of function rather than a loss of function

or expression in the TAL (Jianghui Hou. et al. 2013). Consistent with this no disturbance in calcium homeostasis was seen in either claudin-14 knock-out mice or patients with null mutations in claudin-14 (Wilcox et al. 2001; Ben-Yosef et al. 2003). In fact, autosomal recessive deafness can be caused by claudin-14 coding mutations (Wilcox et al. 2001). Thus, suggesting transcriptional regulation of claudin-14 likely plays an important role in maintaining calcium homeostasis rather than mutations in the coding region of this gene.

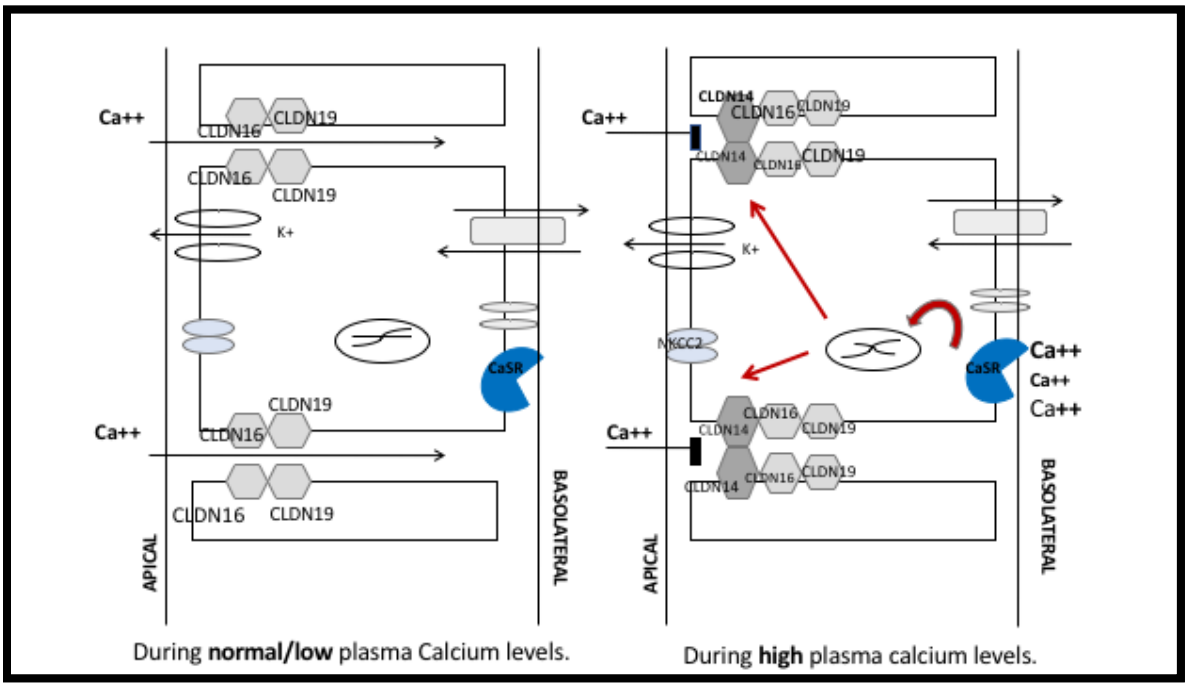


Figure 2. The CaSR-CLDN14 axis. A novel pathway for renal calcium handling.

A diagrammatic representation of the role of claudin-14 in calcium homeostasis. Left side normal serum calcium, right side high serum calcium. High plasma calcium levels stimulate the CaSR on the basolateral membrane of the TAL. This results in a cascade of internal signaling events ultimately leading to the expression of claudin-14 at the tight junctions leading to calciuria. On the other hand, during periods of low to normal plasma calcium levels, calcium reabsorption is facilitated through the interaction of claudin-16 and claudin-19 which form cation pores.

1.5 Rationale and Hypothesis

Activation of the calcium sensing receptor (CaSR) leads to increased claudin-14 expression. Abundant genetic evidence suggests that this pathway is inappropriately increased in patients with hypercalciuria and kidney stones. To identify ways to better treat these patients we must first understand how activation of the calcium sensing receptor increase claudin-14 expression. The calcium concentration in plasma is maintained within a narrow limit. Any fluctuation in plasma calcium levels can have drastic effects. Whenever plasma calcium levels are high it stimulates the CaSR in the TAL leading to a cascade of internal cell signaling events that ultimately leads to increase transcription and later increased expression of claudin-14 protein in the tight junction. Claudin-14 is a cation blocker and its appearance along the tight junction blocks calcium reabsorption leading to calciuria. However, the internal signaling pathway from CaSR activation to claudin-14 expression is completely unknown.

The claudin-14 gene is transcriptionally regulated and SP1 mediates increased expression of different genes post CaSR activation. We hypothesized that **activation of the CaSR attenuates SP1 activity to increase claudin-14 expression (**see **Figure 3)**. Genetic variations in claudin-14 associating with kidney stones are non-coding or in intronic regions. Given the biology of the CaSR–CLDN14 axis we therefore hypothesized that **the most common variations in CLDN14 gene associated with kidney stones lead to inappropriate increase in the expression of claudin-14.**

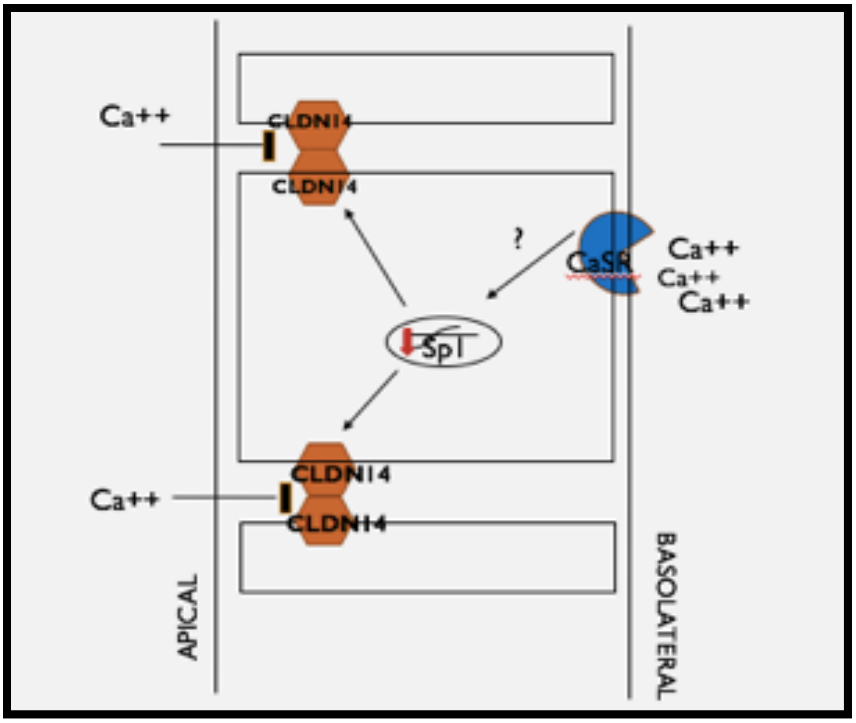


Figure 3. Activation of the calcium sensing receptor (CaSR) attenuates SP1 activity to increase claudin-14 expression.

SP1 regulates increased expression of different genes post CaSR activation we therefore hypothesize that activation of the CaSR attenuates SP1 activity which ultimately results in the increase in claudin-14 expression along the tight junction membrane in thick ascending limb (TAL). Claudin-14 (CLDN-14), CaSR (calcium signaling receptor), SP1 (transcription factor) & serum calcium (Ca^{++}).

1.6 Specific Aims

Literature review suggests that alterations in claudin-14 gene are linked with both kidney stones and hypercalciuria. Also, previous work done in the Alexander lab has shown that an intronic claudin-14 variant inappropriately increases gene expression, this variant occurs with a significantly higher frequency in idiopathic hypercalciuria kidney stone formers compared to the controls (Megan Ure et al. 2017).

Furthermore, Alexander lab has also revealed that high serum calcium levels activates the CaSR leading to a cascade of internal signaling events that ultimately increase claudin-14 expression. However, this internal signaling pathway from CaSR activation to increase in claudin-14 expression is completely unknown. Thus, this project has two inter-related goals;

1) To Identify novel genetic causes of Idiopathic Hypercalciuria

and

2) To Delineate the signaling pathway between calcium sensing receptor (CaSR) activation and claudin-14 transcription.

Although there is no known cause for idiopathic hypercalciuria, a review of the literature reveals that certain genes frequently associate with this disease. Of these genes **claudin-14** has been the most frequently associated with both hypercalciuria and kidney stones (Thorleifsson et al.2009). Activation of the calcium sensing receptor (CaSR) leads to an increase in claudin-14 transcription and greater expression of the

claudin-14 protein at the tight junction in the thick ascending limb (TAL) of the nephron. However, the signaling pathway leading to this increased transcription is completely unknown and delineation of this signaling pathway is thus the focus of this project. Luciferase activity assays performed in the Hek-293 cell culture model was used to delineate part of this signaling pathway. By investigating already established signaling pathways we identified PKC signaling and the transcription factor SP1 to be **present downstream the CaSR activation pathway in TAL (**see **Figure 4)**. We were also able to exclude a role for other signalling molecules. Specifically, phospholipase C (PLC), a membrane associated enzyme that responds to a variety of external stimuli such as hormones, neurotransmitters, growth factors etc (Putney JW et al. 2012) and is present downstream CaSR activation pathway (Godwin SL et al. 2002) was examined. Further we examined a role for protein kinase C (PKC) and mitogen-activated protein kinase kinase MEK (Tennakoon, S et al. 2016) both of which are present downstream of CaSR activation pathway and play an important role in stimulating internal cell signaling events. We also examined the role of the cell division control protein (cdc42), a small GTPase hydrolase enzyme that regulates a variety of signaling pathways and cellular functions depending on the type of stimuli. Finally, we worked with specificity protein 1 (SP1) transcription factor, SP1 regulates expression of different genes post CaSR activation (Gill et al. 1994, Li et al. 1991) where it can act both as an activator or a repressor **(**see **Figure 3)**. All these signaling molecules are discussed in detail in the relevant chapter **(**see **chapter 4).**

The second part of this thesis deals with the Identification of novel genetic causes of idiopathic hypercalciuria. To do so we worked with a cohort of patients with idiopathic

hypercalciuria and sequenced several candidate genes that were selected based on their location and function in different segments of the nephron. More specifically, we examined claudin-2, -12, -14 & CYP24A1.They are discussed in the relevant section **(**see **chapter 3).**

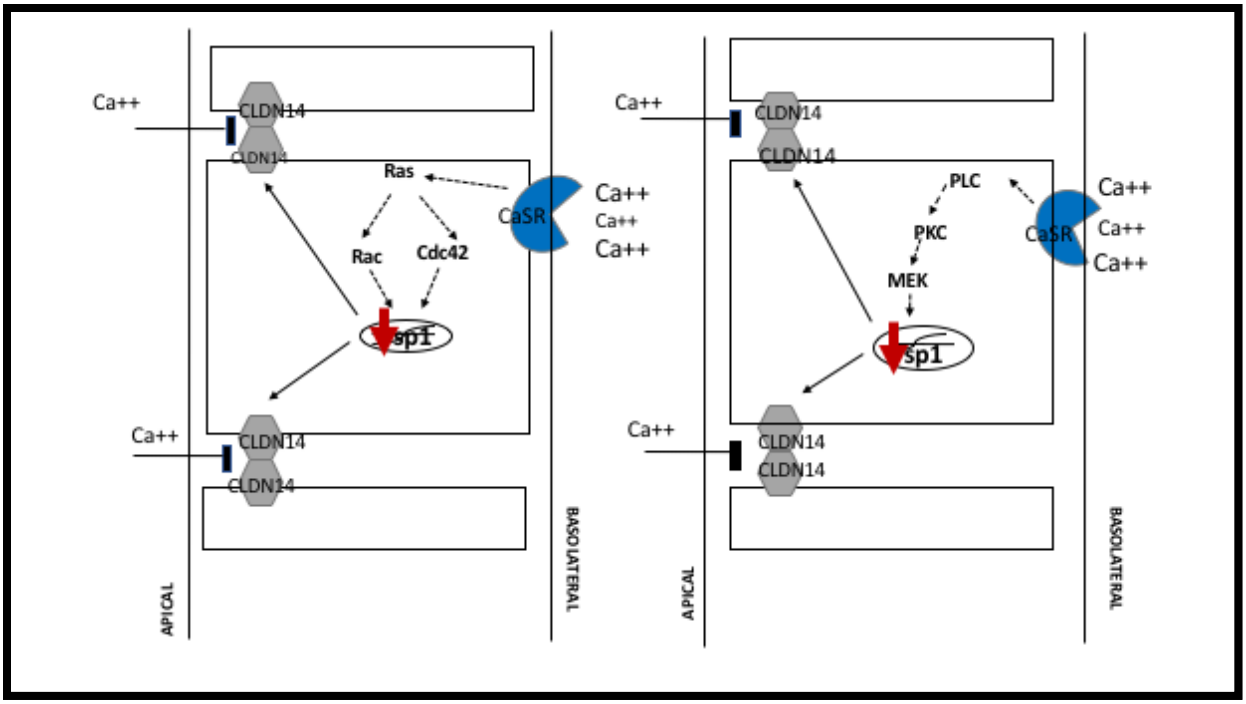


Figure 4. Proposed pathways for activation of the CaSR and increased expression of claudin-14.

Considering already established CaSR activation pathways, we predicted several interlinked signaling molecules that can signal downstream CaSR activation.
Claudin-14 (CLDN-14), CaSR (calcium signaling receptor), SP1 (transcription factor), serum calcium (Ca^{++}), Protein kinase-C (PKC), Phospholipase-C (PLC), Mitogen-activated protein kinase kinase (MEK), Cell division control protein 42 (cdc-42), Rac & Ras signaling GTPases

Chapter 2. METHODS

2.1 Methodology used to identify novel genetic causes of idiopathic Hypercalciuria.

We employed a cohort of patients with idiopathic hypercalciuria that have three characteristics in common, all patients displayed normocalcemia, normal PTH and hypercalciuria in the absence of any disease that causes excessive calcium excretion. Specifically, children with rare genetic disorders (Bartter Syndrome, Dent's disease, distal renal tubular acidosis, or Williams syndrome), which can cause hypercalciuria were excluded from the study. Hypercalciuria was defined as a urine Ca: Cr (mmol: mmol) ratio >0.56 (1–18years) on two separate samples.

Ethics approval for this project was obtained from the University of Alberta (Pro00018459) and the University of Saskatchewan (Bio 11–126). A cohort of children (0 to 18 years) with idiopathic hypercalciuria were recruited from the Pediatric Kidney Stone Clinic at the Stollery Children's Hospital Edmonton, Alberta, Canada or from the Pediatric Nephrology clinic at the Royal University Hospital, Regina, Saskatchewan.

DNA was extracted from the blood of patients and then polymerase chain reaction (PCR) using specific primers for the genes of interest was performed on the candidate genes (claudin-2, -12-14 & CYP24A1). These samples were then purified using a PCR purification kit and sent for sequencing. Finally, data analyses were done by comparing variants found in our idiopathic hypercalciuria cohort to the 5008-allele data from NCBI data base.

2.1.1 Genomic DNA isolation

DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used for extracting human DNA from blood samples as per the manufactures instructions. 2 mL of human blood (anti-coagulated) was centrifuged at maximum speed for 30 min at 4 degrees Celsius. After that, the supernatant was transferred to a different tube and 250 uL of pellet was added to another tube. In a 2-mL tube, 250 uL of blood pellet and 100 uL of proteinase K was added along with 500 uL of PBS and 1 mL of AL buffer (Qiagen kit, Hilden, Germany). This mixture after vortexing was left at 56 degrees Celsius overnight in a water bath to dissolve the mixture. Then the mixture was transferred into a DNeasy mini spin column and centrifuged at 8000 rpm for 1 min, next AW1 then AW2 buffers (Qiagen kit, Hilden, Germany) were added. Elution was done using 50 uL of autoclaved double distilled water.

2.1.2 PCR

Specific gene fragments (claudin-2, -12, -14 & CYP24A1) were amplified from human genomic DNA (gDNA) using specific primers (IDT, Oralville, IA, USA) (Supplemental table 1) by PCR. The PCR reaction consisted of: 94 °C for 1 min, 94 °C for 30 seconds, primer melting temperature (55-65 °C) for 30 seconds, 72 °C for 90 seconds, 44 cycles then 71°C for 10 mins and finally 4 °C forever, was carried out with a C1000 Thermal cycler (BioRad, Mississauga, ON, Canada). The PCR product was later run on a 1.5% agarose gel containing ethidium bromide.

Table 3. Sequencing primers for CYP24A1.

Exons	Forward primer	Reverse primer
1	5'- GCGCGAAAAGGGGTTTAC -3'	5'- GCCAGCAGCATCTCATCTAC -3'
2	5'- CCGTGGACCGACTCTAATCTG -3' 5'- CTGTACAAGAGCTCAGGGTTG -3'	5'- CACGACACCCTGGTAAACCC -3'
3	5'- CCATTGCTCCCTCATTGTG -3'	5'- CCCCCTTTTACCGCTAGG -3'
4	5'- GCAGCAATAATGCCTGTTTAC -3'	5'- GTGACTTCAGGATGAATAACA -3'
5	5'- CTATGCGAGGAATCTATGTTC -3'	5'- GTGATGAGTCATTGGACCTGG -3'
6	5'- TTTGTGTATGCTGGGGCAATC -3'	5'- TCCAGTGGAAAATCATCCCC -3'
7	5'- GCACATGAAGTCTCCTCCTA -3'	5'- GATATTGCAAGAAGGAGTTTGG -3'
8	5'- CGAGAACAGTGTTCTAACACAT -3'	5'- GCTATAAGGACGCGTGAAAGAG -3'
9	5'- GCTTTAGAATTGTGCACC -3'	5'- GAACTTAGACTGTGCTCAC -3'
10	5'- TTCTCACTACCTTGCAGA -3'	5'- TGGTGCACAATTCTAAAGC -3'
11	5'- CATAGCTCATCCCTCGTC -3'	5'- GGGTAAAACCTTTACCG -3'

Genomic DNA corresponding to 137,230,757 – 137,236,554, OMIM ID. 126065 from genomic draft variant GRCh38.p7. on chromosome 9, a region encompassing the 11 coding exons of CYP24A1 were amplified by PCR with overlapping primers.

2.1.3 DNA Gel Electrophoresis

1.5% (1.5 g in 100 mL water) agarose (Invitrogen, Carlsbad, CA, USA) was poured into and allowed to solidify in a gel electrophoresis apparatus (Electrophoresis system, Fisher Scientific Company, Ottawa, ON, Canada). The gel was prepared in 1 X TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH = 8) and heated for 2 mins in a microwave (Danby, Canada). The solution was then allowed to cool for 10 mins before 1.5 µL of ethidium bromide (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was added. Each well in the solidified gel was loaded with 2 µL of the samples (PCR product), 3.5 µL of 2X loading dye (Fermentas Canada Inc, Burlington, ON, Canada) and was mixed uniformly.10 bp or 10 Kbp Plus DNA (Generuler, Fermentas Canada Inc, Burlington, ON, Canada) ladder was used as a control to determine the fragment size. The gel was run at a current of 150 volts for 30 mins and visualized with a gel imager (Gel Doc EZ Imager, BioRad, Mississauga, ON, Canada) **(**see **Figure 5).**

2.1.4 Generation of luciferase constructs for SNP rs199565725 and rs128494 of CLDN-14

a) Constructs containing SNP rs199565725

DNA segment from intronic region of claudin-14 gene containing the wild-type (WT) or variant (V) sequence of rs199565725 was amplified using forward and reverse primer 5'-CTATATTTCCTAGTGTTTTTTTTTGTTTGTTTGCTTGTTTTGTTTTTTTA-3' and 3-

CCATGGATATAAAGGATCACAAAAAAAAACAAACAAACGAACAAAACAAAAAAATCT AGA-5', respectively. These primers contained restriction enzyme sites at their ends

corresponding to Kpn1 and BglII. After digestion with Kpn1 and BglII enzymes (New England Biolabs Inc., Ipswich, MA), the sequences were ligated into the pGL3 promoter Vector (Promega Corp., Madison, WI) and later construct containing the insert was verified through sequencing.

b) Construct containing SNP rs128494

DNA segment from intronic region of claudin-14 gene containing the wild-type (WT) or variant (V) sequence of rs128494 was amplified using forward and reverse primer 5'- GGGGTACCCC- GGT GGT TGG TGT GGC AAT -3' and 5'- GAAGATCTTC-CTAAACCTCTCCCTGCC -3, respectively. These primers contained restriction enzyme sites at their ends corresponding to Kpn1 and BglII (Supplemental table 3). After digestion with Kpn1 and BglII (New England Biolabs Inc., Ipswich, MA), the sequences were ligated into the pGL3 promoter Vector (Promega Corp., Madison, WI) and later construct containing the insert was verified through sequencing.

c) Sequencing for SNP rs199565725

SNP rs199565725 is present in the intronic region of claudin-14 gene approximately 1500 bp away from the coding exon. In a genome-wide association study it was predicted that this sequence has a protective role and can regulate the claudin-14 gene expression (Oddsson A. ,2015). For this we sequenced our idiopathic hypercalciuria cohort for this SNP by using specific primers **(**see **Table 4).**

SNP ID.	Forward primer	Reverse primer
rs199565725 (for patient samples)	5'- GGG ACC TCC TAA TGC TCG C -3'	5'- CCT GGC CAA CAT GGT GAA ACC -3'

Table 4. PCR primer set for sequencing patient samples encompassing region having SNP rs199565725.

d) Sequencing for SNP rs128494

SNP rs128494 is present in the intronic region of claudin-14 gene approximately 200 bp away from the coding exon. We sequenced our idiopathic hypercalciuria cohort for the identified linkage disequilibrium block by using specific primers **(**see **Table 5).**

In a comparison study between our idiopathic hypercalciuria cohort and 1000 genome data (NCBI data base) we found that this SNP is found in higher frequency in our idiopathic hypercalciuria cohort compared to the control p=<0.05. So, we cloned this SNP rs128494 into a reporter vector to observe any difference in claudin-14 expression.

SNP ID	Forward primer	Reverse primer
rs128494 (for cloning and for patient samples)	5'- GGGGTACCCC- GGT GGT TGG TGT GGC AAT -3'	5'- GAAGATCTTC- CTAAACCTCTCCCTGCC -3

Table 5. PCR primer set for sequencing patient samples (and for cloning) encompassing the region having SNP rs128494.

Highlighted base-pairs in purple are the restriction sites.

2.1.5 Sequencing of human DNA variants

The regions of interest for the candidate genes which includes claudin-2, claudin-12, claudin-14 and CYP24A1 were amplified by PCR with overlapping primers (Supplemental table 1). The PCR product, after clean-up with the Qiaquick PCR Purification Kit (Qiagen, Canada) was sequenced with both the forward and reverse PCR primers at The Genomics Core at the University of Alberta (https://tagc.med.ualberta.ca). When a variation was noted between the patient's DNA and that reported (NCBI data base), the PCR and sequencing reaction were repeated to confirm the altered sequence or the reported SNP.

2.2 Methodology used for to delineate the signaling pathway between calcium sensing receptor (CaSR) activation and claudin-14 transcription.

For this part of the project we worked with the cell culture model Hek-293 cells, which were transfected with calcium-phosphate to transiently express the luciferase constructs of interest. We then performed luciferase activity assays on these samples. We also performed immunoblotting with different primary antibodies to demonstrate successful transfection with the relevant plasmid or to quantify protein expression.

2.2.1 Cell culture

Hek-293 cells were originally obtained from ATCC (Rockville, MD, USA). They were regularly passaged every 48 to 56 hours, once cell reached 80 to 100% confluence. Cell were kept at 37 degrees Celsius in a 5% CO2 incubator. Hek-293 cells were maintained in DMEM-1X, containing 10% FBS and 5% penicillin, streptomycin and glutamine.

2.2.2 Transfection

The calcium phosphate method was used for transfection of Hek-293 cells (Jordan M. et.al 1998). Cells were plated in a 10-cm petri-dish 3 to 6 hours before transfection. At the time of transfection, we added 590 uL of 2x HEPES (NaCl 8.0 mM, Na2HPO4 0.105 mM, HEPES 6.5 mM and H2O to make it to 500 mL, adjust pH to 7.0 and filter sterilize) into 507 uL H_2O and 72 uL of $CaCl_2$ in a microcentifuge tube. This solution was incubated for 15 mins before adding it dropwise to the plated cells. We transfected 3000 ng of the DNA of interest and 100 ng of PRL-TK. Before assaying, transfected cells

were incubated for 48 hours to allow time for the expression of the transiently transfected DNA.

2.2.3 Immunoblotting

After transfection cells were plated on a 10-cm petri-dish and then were incubated for another 48 hours. Cells were later washed with 1 X phosphate buffer saline (8.0g NaCl, 0.2g KCL, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, add H_2O to make total volume upto 1 liter, adjust pH to 7.4). After washing, the cells were trysinized with 0.5 mL trypsin and 1 mL of culture medium was added. Cells were transferred along with medium into a 1.5 mL tube and were centrifuged at 4000 rpm for 5 minutes at 4 degrees Celsius. The pellet was again washed with PBS and centrifuged again at 4000 rpm for 5 minutes at 4 degrees Celsius. To lyse cells RIPA buffer was added to the pellet. Protease inhibitor and pheylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor was added to prevent protein degradation. All the protein concentrated in the supernatant was collected from the mixture and the pellet containing cell debris was discarded. Using a nanodrop 2000c (Thermo Scietific, USA), the absorbance was measured at a wavelength of 280-nm and for standardization of samples protein concentration was calculated using a bovine serum albumin (BSA, Sigma-Aldrich A-4503) standard curve. 50 ug of protein was mixed with 2x Laemmli sample buffer (130 mM Tris-HCL pH 6.8, 4.6% SDS, 0.02% Bromphonel Blue, 20% Glycerol, 2% 2-Mercaptoethanol [Sigma-Aldrich M-7522],1% protease inhibitor cocktail, in double distilled water) in a 1:1 ratio and incubated at room temperature (RT) for 10 mins. The mixture containing the protein sample and 2x Laemmli sample buffer was loaded onto an acrylamide gel (30%

Acrylamide/Bis Solution, 29:1 [Bio Rad, Cat #: 161-0156], 0.5 M Tris-HCL pH 6.8, 20% SDS, 0.1% TEMED Omnipur [Calbiochem 8920], and 10% APS) and a 25 amp/gel current applied for 75 mins. Protein in the gel was then transferred onto an Immobilon PVDF membrane (Cat No. IPVH00010) pre-wetted with methanol at RT and were run 100 V for 1 hour at room temperature. Membranes were then blocked with 5% milk in Tris-Buffered Saline (Tris 6.05 g and NaCl 8.76 g in 800mL of H2O, adjust pH to 7.6) containing Tween (TBST) overnight at 4 degrees Celsius to reduce non-specific binding. Later membranes were left for another overnight period at 4 degrees Celsius in the primary antibody diluted 1/1000 in TBST with 5% milk, then washed with fresh TBST 3 times for 10 mins at room temperature. Specific primary antibodies used were mouse anti-gapdh from thermofisher, rabbit anti-CaSR from thermofisher and rabbit anti-SP1 from thermofisher. Membrane was next incubated for 2 hours at room temperature with secondary antibody conjugated to horseradish peroxidase (HPR) diluted 1/5000 in TBST with 5% milk and later washed with fresh TBST 3 times for 10 mins at room temperature. Enhanced chemiluminescence (ECL) (Western Blotting reagents, GE Healthcare 45000875) was then added to the membranes for 2 mins. The Bio-Rad Chemi-Doc imaging system was used to visualize membranes and record images of the blots. After imaging the SP1 signal the membranes were washed with double distilled water for 4 mins and then stripped with 0.2 M NaOH for 10 mins before being washed with water for 5 mins. Image J software was used for semi quantification of the relative band intensity.

2.2.4 Dual luciferase assay

Three types of co-transfections were performed for these experiments. For the first set of experiments we co-transfected (Myc-DDK-tagged) Human SP1, transcript variant 1 (from Origene) with the intronic 1500 bp claudin-14 construct inserted in a PGL3 Enhancer vector (Promega, Madison, WI, USA) upstream of the luciferase gene (luc+) and incubated for 48 hours before collecting cell lysate for luciferase assay. For the second set of experiments we co-transfected intronic 1500 bp claudin-14 construct with a construct containing the human CaSR (hCaSR-PCMV6-DDK-MYC) (OriGene, Rockville, MD, USA) into Hek-293 cells and added different drugs after 24 hours of incubation **(**see **Table 6).** And finally, we co-transfected intronic 1500 bp claudin-14 construct (this construct was developed by our former lab member Jawad F. Alzamil's; it consists of an intronic mouse claudin-14 V1 1500bp region that has a promoter activity and is sensitive to CaSR activation) with a construct containing the human CaSR (hCaSR-PCMV6-DDK-MYC) (OriGene, Rockville, MD, USA) and cdc42 wild-type or mutant sequence (cdc42 mutant tagged with GFP is an autosomal dominant point mutation T4N, leading to functional inactivation) into Hek-293 cells. In all the experiments Hek-293 cells were co-transfected the PRL-TK vector (a kind gift from Dr. Michael Walter), which was used as an internal control for the vector expression. Purified DNA was prepared in double distilled autoclaved water and the plasmid DNA used for the transfections had a A260/A280 ratio of 1.7–1.9. Cell extracts were prepared after incubation with 1 mL (passive lysis buffer) for 20 minutes (Promega, Madison, WI, USA) for each 10-cm dish and analyzed for luciferase assay activity. For analysis, we mixed 10-100 µL of cell lysate and 100 µL of Luciferase Assay Reagent and measured

the light emitted with a luminometer (DLR Ready, TD- 20/20 Luminometer, Turner Design). While measuring, the light produced by this protocol the first signal is the firefly luciferase activity (i.e. our construct of interest) then we added 100 µL stop and glo buffer into the mixture and measured the second light signal again. This second reading is from the activity of renilla luciferase which is our internal expression control, PRL-TK. As per the manufacturer's instructions (Promega, Madison, WI, USA) for analyzing the results. Firefly activity was normalized to Renilla activity (PRL-TK). Cinacalcet HCl (Santacruez, USA), U-73122 (Sigma, USA), U0126 ethanolate (Sigma, USA) or Staurosporine (Sigma, USA) was added 24 hours after transfection in some cases. For these experiments luciferase activity was measured 48 hours after transfection. Each reporter assay was done a minimum of two times with consistent results.

Table 6. List of drugs used and their working concentration.

Drugs	Drug target	Working Concentration
Cinacalcet	CaSR agonist	12 μM
U-73122	PLC inhibiotor	10 μM
Staurasporine	PKC inhibitor	20 nM
U0126	MEK inhibitor	1.25 μM

Chapter 3. RESULTS OF HUMAN COHORT SEQUENCING

Idiopathic hypercalciuria (IH) is the excretion of urine with too much calcium when plasma calcium levels are normal. It is the most common metabolic abnormality in children causing an increased risk of kidney stones. IH is a polygenic trait with increased incidence in first-degree relatives. Children with IH fail to reabsorb calcium from the proximal part of the nephron and some have increased circulating 1,25-dihydroxyvitamin D_3 levels. Most the calcium filtered by the nephron is reabsorbed from the proximal tubule in a paracellular fashion down its concentration through tight junction proteins. Claudin-2 and -12 are tight junction proteins in the proximal tubule conferring calcium permeability. Moreover, increased claudin-14 expression prevents proximal calcium reabsorption. We hypothesized therefore that loss of function mutations in **claudin-2 or -12**, or gain of function mutations in **claudin-14** would cause hypercalciuria and kidney stone formation. Moreover, a loss of function mutation in **CYP24A1**, the enzyme that inactivates active vitamin D, would cause hypercalciuria by increasing 1,25 dihydroxyvitamin D levels, intestinal calcium absorption and consequently urinary calcium excretion. This chapter describes the sequencing results for these genes on a cohort of children with idiopathic hypercalciuria.

3.1 Sequencing results

DNA was extracted from blood samples of patients suffering from idiopathic hypercalciuria (IH) and sequenced for claudin-2, claudin-12, claudin-14 and CYP24A1. We sequenced the coding exon of claudin-2, claudin-12 and CYP24A1 and the 5' intronic region identified as the linkage disequilibrium block in a previous GWAS in the claudin-14 gene. Specific regions were amplified by PCR (polymerized chain reaction) using overlapping primers listed in the methods section **(**see **Table 3)**. The PCR product, after clean-up with the PCR purification kit, was sequenced using both the forward and reverse sequence primers. Whenever a variation was noted between the patient's DNA and the reported wild-type allele (5008-allele NCBI data base), the PCR and sequencing reaction were repeated to confirm the altered sequence. No SNPs were identified in claudin-2, one non-coding SNP was found in claudin-12 and five different SNPs were identified in each of claudin-14 and CYP24A1 **(**see **Table 7).**

All the SNPs that were identified in claudin-12 & CYP24A1 were synonymous SNPs except for the one SNP found in exon 9 in CYP24A1 **(**see **Table 8 & 9).** However, this SNP has already been published and is reported to be a disease-causing variant causing Idiopathic Infantile Hypercalcemia (Schlingmann, K P et al. 2011). All the SNPs identified in the linkage disequilibrium block in the claudin-14 gene were assessed by comparing minor allele frequency (MAF) between our idiopathic hypercalciuria cohort and the 5008 allele NCBI data base **(**see **Figure 6).**

3.1.1 Summary of sequencing results

Gene	No. of patients sequenced	No. of different SNPs found
Claudin-2	15	0
Claudin-12	24	1
Claudin-14	15	5
Cyp24a1	30	5

Table 6. Summary of sequencing results for the candidate genes and number of SNPs found in the idiopathic hypercalciuria Cohort.

3.1.2 Representative DNA gels of PCR amplified claudin-2, -12 & -14.

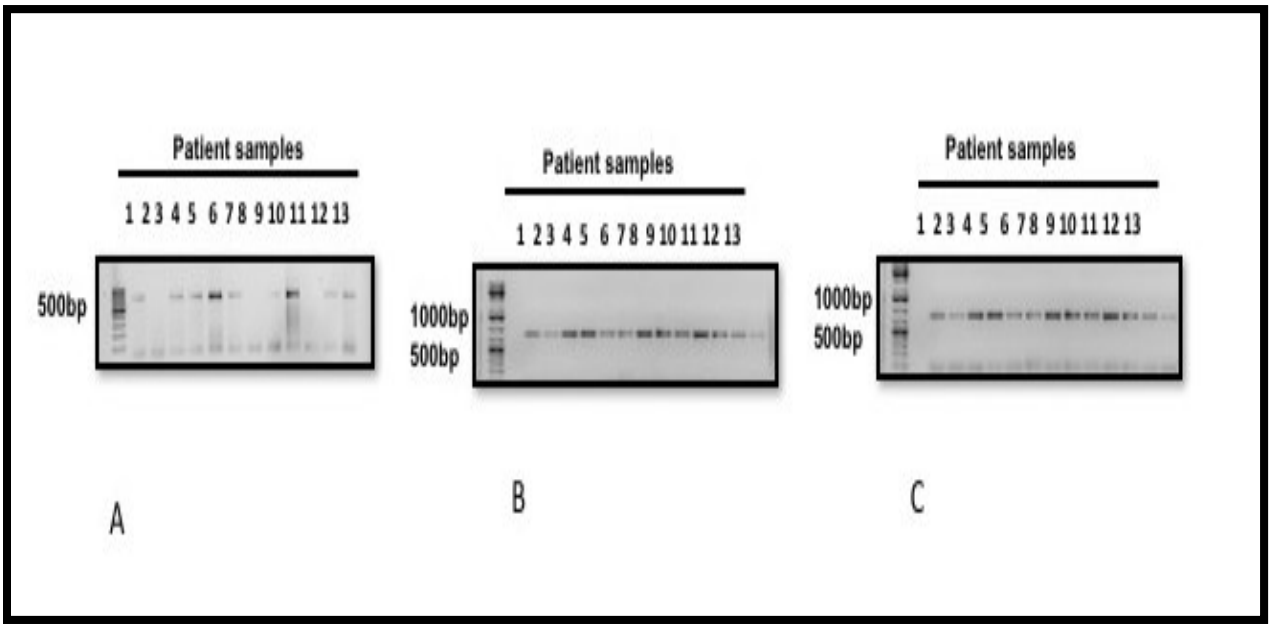


Figure 5. Representative DNA gel electrophoresis results for the claudin-2, -12 & -14 amplified by PCR before being sequenced. The ladder is loaded on the left.

A) Claudin-2 B) Claudin-12 C) Claudin-14. Besides the ladder each band corresponds to a different patient sample indicated by their ID number above the gel.

3.1.3 SNPs identified in claudin-12 & -14.

No	SNP ID	Allele	Cohort (N)	1000 genomes (N)	Locus	Position	p-value	Amino-acid change
1.	Rs17862175	C	47	4947	CLDN12	7:90412862	0.58773	Non-coding
		T	1	61				
2.	RS128494	C	25	3140	CLDN14	21:36461960	0.01972	Non-coding
		T	5	1868				
3.	RS219776	G	24	3737	CLDN14	21:36462537	0.49947	Non-coding
		C	6	1271				
4.	RS219777	C	25	4084	CLDN14	21:36462423	0.80167	Non-coding
		A	5	924				
5.	RS219778	T	24	3738	CLDN14	21:36462343	0.50095	Non-coding
		G	6	1270				
6.	RS117560775	C	29	4896	CLDN14	21:36461633	0.68582	Non-coding
		T	1	112				

Table 7. SNPs identified in Claudin-12 & -14.

SNP ID and characterization are based on the NCBI data base. The SNP identified in claudin-12 is in the coding region however it's a synonymous SNP (i.e. it does not cause a change in the amino acid). All the SNPs found in claudin-14 were in the intronic region. Note: No SNPs were found in claudin-2. Claudin-2 OMIM ID. 300520. Genomic draft variant GRCh38.p7. Claudin-12 OMIM ID. 611232. Genomic draft variant GRCh38.p7. Claudin-14 OMIM ID. 605608. Genomic draft variant GRCh38.p7.

No.	SNP ID	Allele	Cohort (N) Alleles=60	1000 genomes (N)	Locus	Position	p-value	Amino-acid change
1.	RS6068816	C	54	4179	CYP24A1	20:54164552	0.17373	Non-coding
		T	6	829				
2.	RS2296241	G	58	2714	CYP24A1	20:54169680	0.0001	Non-coding
		A	2	2294				
3.	Rs2296239	C	51	3027	CYP24A1	20:54158989	0.0001	Non-coding
		T	9	1981				
4.	Rs6068812	A	59	5007	CYP24A1	20:54158096	0.0000001	**coding**
	Pathological allele	G	1	1				
5.	Rs35873579	C	58	4965	CYP24A1	20:54171651	0.04223	Non-coding
		T	2	43				

Table 8. Characterization of SNPs found in CYP24A1 (Exons 1 to 11).

SNP ID and characterization are based on the NCBI data base. The SNPs found in CYP24A1 are in the coding region, however, all of them are synonymous SNPs except rs6068812 in exon 9 that causes a base pair change from leucine to serine. OMIM ID. 126065. Genomic draft variant GRCh38.p7.

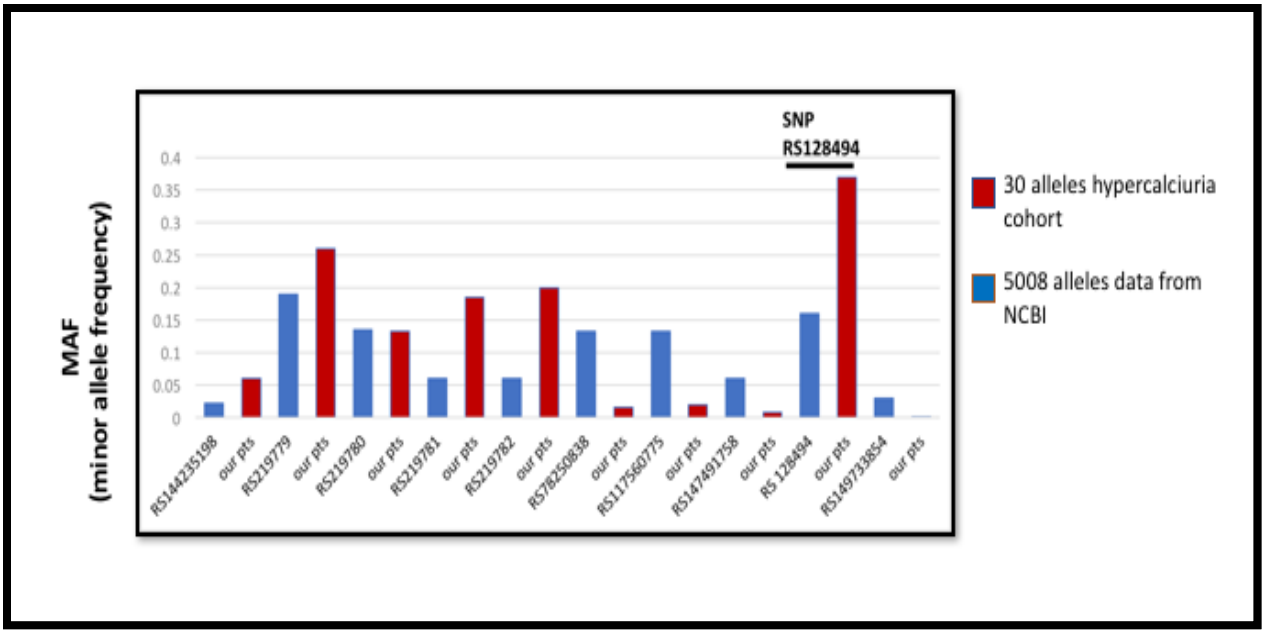


Figure 6. Sequence analysis of claudin-14 (n=30).

We identified 10 non-coding SNPs in an intronic region of the claudin-14 gene. Of the 10 variations identified, we observed that the single nucleotide polymorphisms (SNP) rs144235198, rs219779, rs219781, rs219782 & rs128494 occurred in a higher frequency in our idiopathic hypercalciuria cohort compared to the 5008-allele data (NCBI) where $p < 0.05$ by chi-squared.

Of all the variants that were identified in this genomic region **(SNP) rs128494** was the most enriched variant, found in 25/30 children (20=homozygous and 5=heterozygous). This frequency is higher than expected when compared to 1868 control subjects from 1000 genome project**, p=0.019725** by chi-squared.

Note: Red bars represent our idiopathic hypercalciuria cohort whereas blue bars represent 5008 allele data (NCBI). The X-axis displays different SNPs from the idiopathic hypercalciuria cohort and 5008 allele data and y-axis represent minor allele frequency (MAF).

3.2 SNP rs128494 does not increase claudin-14 expression.

Statistical analyses were done comparing SNPs found in the linkage disequilibrium block of the intronic region of claudin-14 and the 5008-allele data from the NCBI data base. Based on this analyses five SNPs; rs144235198, rs219779, rs219781, rs219782 & rs128494 were found in higher frequency in our Idiopathic hypercalciuria cohort compared to the 5008-allele data (NCBI). However, of all the variants that were identified and analyzed SNP **rs128494** was the most enriched variant (**with p=0.019725** by chi-squared). Therefore, we hypothesized that rs128494 might alter gene expression as it occurred with significantly increased frequency in our patients relative to the 1000 genomes data published in NCBI **(**see **Figure 6).** Therefore, we cloned this intronic claudin-14 SNP into the pGL3 promoter vector, expressed it in Hek-293 cells and used this tool to determine if it had any influence on claudin-14 expression.

We expected that this luciferase assay would show significantly increased activity when the variant rs128494 was expressed. However, no significant increase in claudin-14 expression was seen when the construct was expressed in Hek-293 cells relative to empty vector or wild-type sequence in the absence of the calcium sensing receptor **(**see **Figure 7).** We also repeated a similar experiment in the presence of the calcium sensing receptor in all three groups. However, no significant difference was seen between the groups even in the presence of the calcium sensing receptor **(**see **Figure 8).**

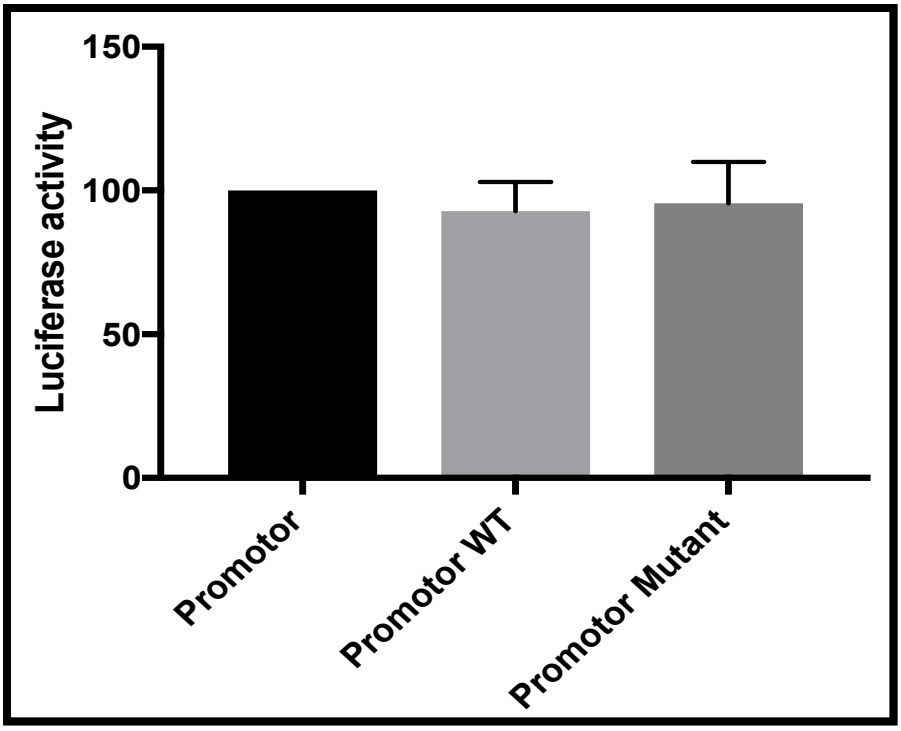


Figure 7. No significant difference in reporter activity was observed with SNP rs128494 promoter construct.

We measured the luciferase signal produced by the pGL3 promoter vector containing SNP rs128494 transiently transfected with the PRL-TK control vector into Hek-293 cells. The firefly luciferase activity produced by the pGL3 vector was normalized to Renilla luciferase activity from the PRL-TK control vector, then values were expressed as a percentage of the activity of the empty promoter vector. We were therefore able to compare the wild type sequence in a pGL3 promoter vector with the variant sequence for rs 128494. Results are displayed as mean +/- SEM; n=6. No significant difference was noted between the groups.

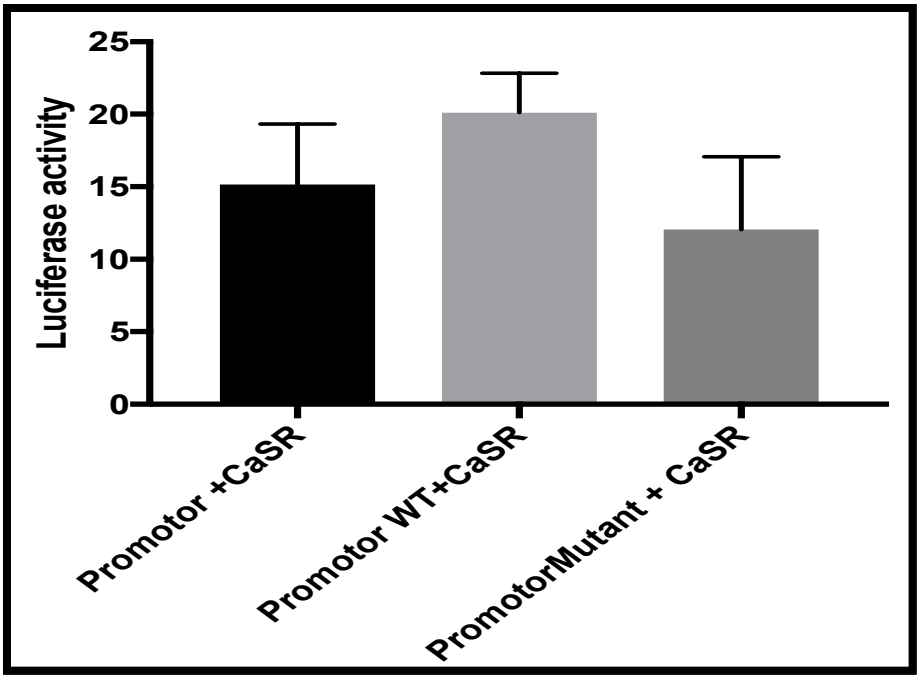


Figure 8. No significant difference in reporter activity was observed when SNP Rs 128494 promoter construct was co-transfected with the CaSR.

Activation of the CaSR is known to enhance clauidn-14 activity. We therefore repeated the above experiment however, this time also transfected the CaSR with both the pGL3 vector (containing either no insert, the wild-type or variant rs128494 sequence) into hek-293 cells. Firefly luciferase activity was normalized against Renilla luciferase activity, values are expressed as a percentage of the activity of the empty promoter vector. We did not observe a significant difference between the wild type sequence and the variant for SNP rs128494. The results are displayed as mean +/- SEM; n=4. No significant difference was noted among the groups.

3.3 SNP RS 199565725 increases claudin-14 expression.

The genome-wide association study (GWAS) conducted in Iceland among 5,419 cases of kidney stone formers and 279,870 controls, yielded four loci (CLDN14, SLC34A1, ALPL and CaSR) that influence kidney stones formation (Oddsson A et al. 2015). Statistical analysis was done to find an association between identified sequence variants and kidney stones (Oddsson, A et al. 2015). Of the variations identified for the claudin-14 (CLDN14) gene, the strongest signal association with kidney stones was seen with a two-base pair deletion in one of the intronic regions of claudin-14 (CLDN14) rs199565725 [del AC] (Oddsson, A et al. 2015). This paper also demonstrated an imbalance in biochemical profile resulting in increased serum magnesium, decreased serum potassium and PTH seen with rs199565725 [del AC]. Moreover, claudin14 knockout mice on a high-calcium diet have significantly higher serum magnesium levels than wild-type (Gong, Y et al. 2012). This suggest that rs199565725 [del AC] may mediate a decrease of CLDN14 gene function. Moreover, this intronic claudin-14 sequence variant rs199565725 [del AC] was also associated with reduced bone mineral density (Oddsson, A et al. 2015). Based on this large genome wide association study (GWAS) and the similarities between biochemical profile among cases in this study and our hypercalciuria cohort, we hypothesized that rs199565725 [del AC] might be the effected sequence altering the function of the claudin-14 gene leading to hypercalciuria. N.B. the AC deletion was found to be protective. Therefore, we cloned this intronic claudin-14 sequence variant rs199565725 [del AC] into the promoter vector to observe if there is any significant difference in reporter expression. Secondly, we determined the frequency of this SNP in our Idiopathic hypercalciuria cohort. Not surprisingly we found

that none of the children with idiopathic hypercalciuria carried the protective SNP **(**see **Table 10)**. However, we were surprised to find that the SNP increased, not decreased the reporter activity **(**see **Figure 9).**

3.3.1 Sequencing results for SNP rs199565725.

Gene	SNP ID	#. of patients sequenced	#. Of SNPs found/ total number of patients
Claudin-14	RS 199565725	11	0/11

Table 9. Sequencing results from the idiopathic hypercalciuria cohort.

None of the patients sequenced for this region carries the SNP rs199565725.

3.3.2. Luciferase assay activity.

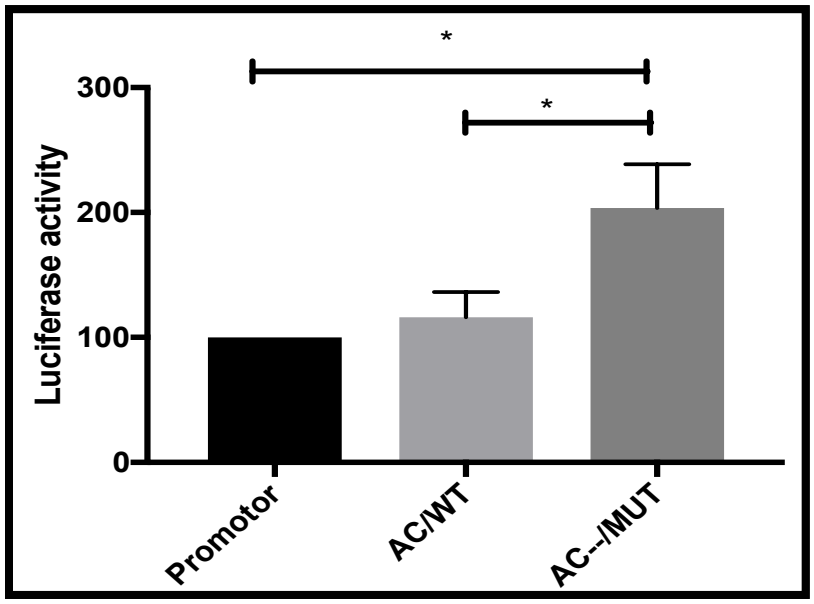


Figure 9. Rs199565725 increases reporter activity.

We examined the luciferase signal produced by the constructs containing the wild-type and variant SNP rs199565725 sequence cloned into the PGL3 promoter vector. All constructs were transiently transfected with the PRL-TK control vector into Hek-293 cells. Firefly luciferase activity was normalized to Renilla luciferase activity and the values are expressed as a percentage of the activity of the empty promoter vector. The wild type sequence was then compared to the empty vector and the variant rs199565725 sequence. Results are displayed as mean +/- SEM; n=13. *A significant difference was observed between empty promoter and the pGL3 vector with the mutant sequence. Also, a significant difference was observed between the wildtype and the mutant sequence cloned into pGL3 promoter vector.

Chapter 4. RESULTS: DELINEATON OF THE CaSR SIGNALLING PATHWAY THAT INCREASE CLAUDIN-14 EXPRESSION.

NOTE: Section 4.1 Identification of the CaSR sensitive claudin-14 promoter is republished with permission. (reference: Jawad F. Alzamil's Master's Thesis 2014).

A large genome wide association study (GWAS) linked SNPs in the claudin-14 gene to an increased risk of hypercalciuric nephrolithiasis. (Thorleifsson et al. 2009). Multiple studies demonstrate that claudin-14 expression is transcriptionally regulated and plays a central role in calcium homeostasis and the formation of kidney stones (Gong Y. et al 2012, Thorleifsson et al. 2009). Activation of the calcium sensing receptor (CaSR) regulates the transcription of claudin-14. However, the signaling pathway downstream of CaSR activation mediating this effect is completely unknown. Claudin-14 consists of 4 different splice variants all coding for the same protein. The variants are different in the 5' untranslated region. To identify the variant regulated by the CaSR we first performed real-time PCR using specific primers for the different variants. We identified variant 1 as the variant regulated by the CaSR **(**see **Figure 10, 11 & 12).** To identify the promoter region for this variant we cloned increasingly longer fragments 5' to the promoter into the PGL3 basic vector and then performed luciferase assays on these constructs. We identified a region encompassing 1500 bp 5' of the 5'UTR of variant 1 of claudin-14 to contain a promoter region sensitive to CaSR activation **(**see **Figure 13 & 14).**

With this tool, which was identified and created by a former MSc student in the laboratory, Mr. Jawad Alzamil, we set out to delineate the signaling pathway between CaSR activation and increased claudin-14 expression. SP1 has been shown to regulate the expression of other genes post CaSR activation (Gill et al. 1994, Li et al. 1991), we therefore hypothesized that the 'activation of the CaSR attenuates SP1 activity' **(**see **Figure 15).**

4.1 Identification of the CaSR sensitive claudin-14 promoter (republished with permission)

(N.B. All data shown in section 4.1 is a summary from Jawad F. Alzamil's Master's Thesis 2014)

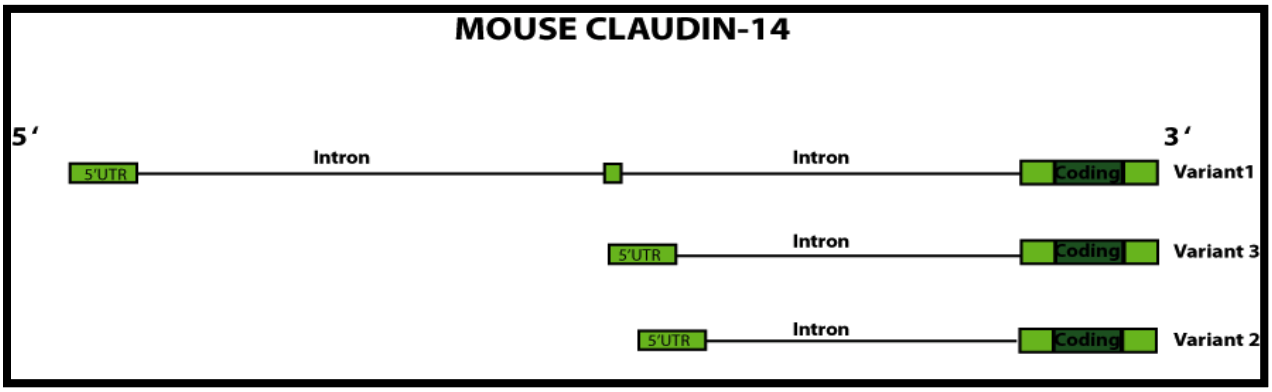


Figure 10. Pictorial representation of the mcldn14 variants based on the mouse claudin-14 gene in the NCBI data, RefSeq, Oct 2009.

All the variants have the same coding region, consequently they all code for the same protein, claudin-14. Variant 1 is the longest transcript and has 3 exons containing the 5'UTR, a coding region and a 3' UTR. The 3 variants differ from each only by having a different 5'UTR (reference: Jawad F. Alzamil's Master's Thesis 2014).

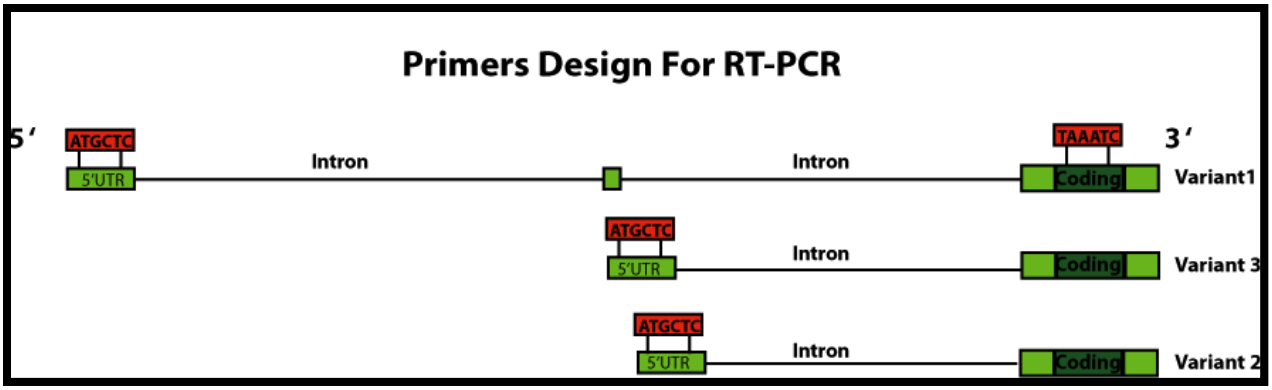


Figure 11. Forward and reverse primers were designed and used to target the 5'UTR of each variant.

For the 5' UTR of each variant, a forward and reverse primer and probe were designed to complement this unique sequence. A forward (F) and reverse (R) primer was also designed to complement the coding region as a positive control since all three variants share the same coding region **(**reference: Jawad F. Alzamil's Master's Thesis 2014).

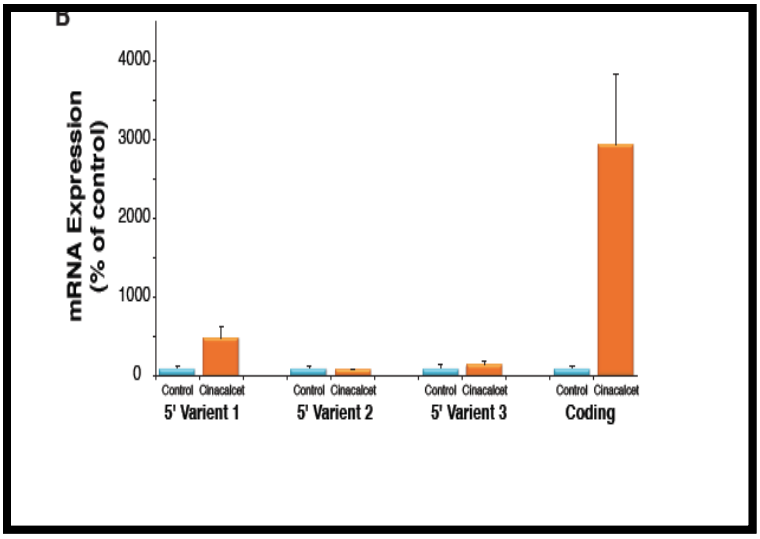


Figure 12. Quantitative real time PCR to determine which mouse claudin-14 transcript variant was regulated by CaSR activation.

Quantitative real-time PCR was performed on cDNA made from mRNA isolated from two groups of mice; one group was treated with vehicle (displayed in blue bars) and the other group was treated with cinacalcet HCL (a CaSR agonist) for 5 days (shown in orange bars). Primers for the coding region were also employed and used as a positive control. Statistical analyses found an approximately 30-fold increase in mRNA expression of claudin-14 in the group that was treated with cinacalcet HCL compared to the control. Out of the 3 variants only variant 1 showed a significant increase in the cinacalcet HCL treated group compared to the control, consistent with variant 1 being upregulated by CaSR activation. Expression was normalized to GAPDH &18S, * represents $P < 0.001$. (reference: Jawad F. Alzamil's Master's Thesis 2014).

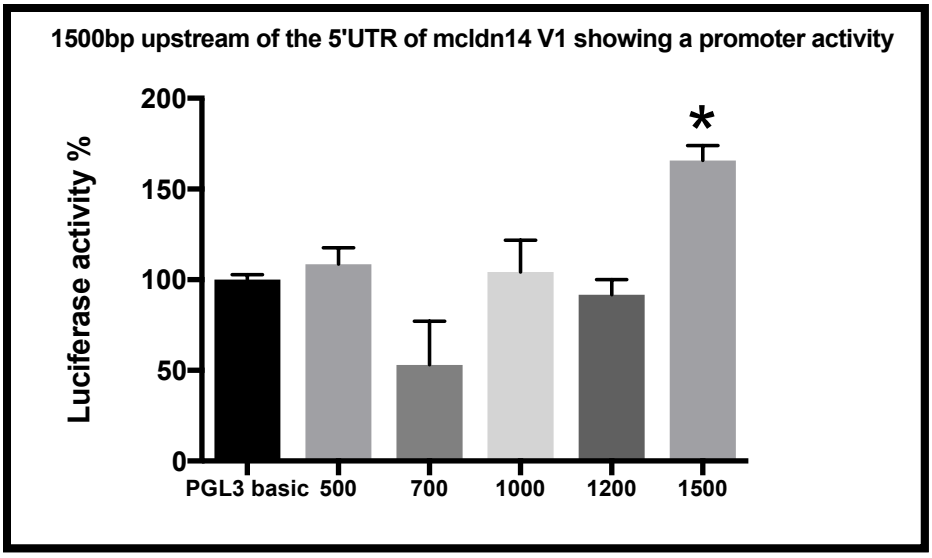


Figure 13. The fragment 1500bp 5' to the mcldn14 V1 first exon contains the promoter.

Analysis of the luciferase signal produced by different length constructs (listed on the x axis) 5' UTR of the mouse claudin-14 variant 1 (mcldn14V1) first exon cloned into the PGL3 Basic vector. Each of these constructs was transiently transfected with the PRL-TK control vector into a cell line stably expressing the CaSR. Only 1500 mcldn14V1 construct demonstrated a significantly increased luciferase activity relative to the empty vector. Firefly luciferase activity was measured and normalized to Renilla luciferase activity (produced by the PRL-TK control vector) and the data is expressed as a percentage of the activity of the negative control PGL3 Basic. The mean +/- SEM; n=6 is displayed. * Indicates a significant difference from the negative control, PGL3 Basic: $p<0.01$; a one-way ANOVA with Gesser-Green house correction was used for comparison (reference: Jawad F. Alzamil's Master's Thesis 2014).

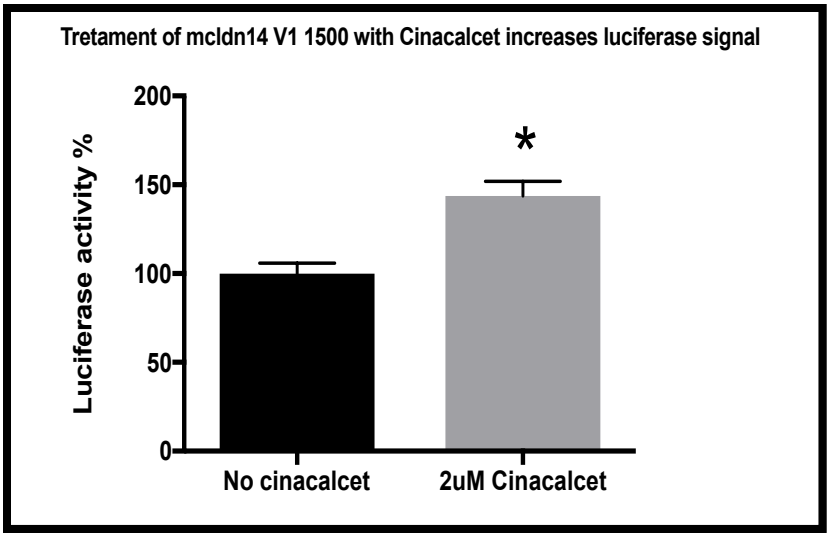


Figure 14. Cinacalcet HCl further increases mcldn14 V1 1500bp construct luciferase activity.

Analysis of the luciferase signal produced by the 1500bp fragment upstream of the first exon of mcldn14 V1 cloned into PGL3 Basic vector treated with either vehicle or with 2 uM cinacalcet HCL. This construct was transiently transfected with the PRL-TK control vector into a cell line stably expressing the CaSR. Firefly luciferase activity was measured and normalized to Renilla luciferase activity and the data is expressed as a percentage of the activity of the 1500bp mcldn14V1 expression in the absence of cinacalcet HCL treatment. The results are displayed as the mean +/- SEM; n=5. * Indicates a significant difference from the negative control (no treatment): $p<0.0025$; comparison was made with a two-sample equal variance ttest with two-tailed distribution (reference: Jawad F. Alzamil's Master's Thesis 2014).

4.2 Effect of SP1 expression on the CaSR mediated increase in claudin-14 promoter activity.

We first sought to examine the effect of SP1 expression on our 1500 bp mcldn14V1 construct expression. To do so we co-transfected Hek-293 cells with the 1500 bp mcldn14V1 construct and either cDNA encoding SP1 or the empty vector, pCMV6. As a positive internal control, we expressed the 1500 bp mcldn14V1 reporter construct with the CaSR and treated it with either vehicle or cinicalcet HCL. Treatment with cinacalcet HCL greatly increased reporter expression **(**see **Figure 15 B).** Expression of the empty vector had no effect on the expression of the reporter construct, however, co-expression of SP1 with the reporter, significantly attenuated baseline expression. This data is consistent with SP1 being a negative regulator of claudin-14 variant 1 expression. Later we also performed immunoblots on the above transfected groups using primary anti-CaSR antibody, the right size band on immunoblot at 130 kDa suggests successful transfection of Hek-293 cells with CaSR plasmid **(**see **Figure 15 A).** As an internal positive control immunoblot was also stripped and re-probed using primary anti-GAPDH antibody **(**see **Figure 15 A).**

We next sought to determine if SP1 is downstream of CaSR activation in the pathway increasing claudin-14 expression. To this end, we transfected the 1500bp fragment of mcldn14V1 with the CaSR into Hek-293 cells in the presence and absence of SP1 and treated the cells with either vehicle or cinacalcet. We observed a significant increase in reporter activity when the empty vector was transfected and treated with cinacalcet **(**see **Figure 16 B)**. Again, expression of SP1 attenuated the expression of the 1500 bp fragment construct. Treatment of the cells expressing SP1 with cinacalcet increased

expression from the attenuated levels but not anywhere near to the level that cells expressing the empty construct did **(**see **Figure 16 B).** This data is consistent with SP1 inhibiting claudin-14 expression and with cinacalcet, at least in part regulating claudin-14 expression through SP1. Later we also performed immunoblots on the above transfected groups using primary anti-SP1 antibody, the right size band on immunoblot at 110 kDa suggests successful transfection of Hek-293 cells with SP1 plasmid (see **Figure 16 A).**

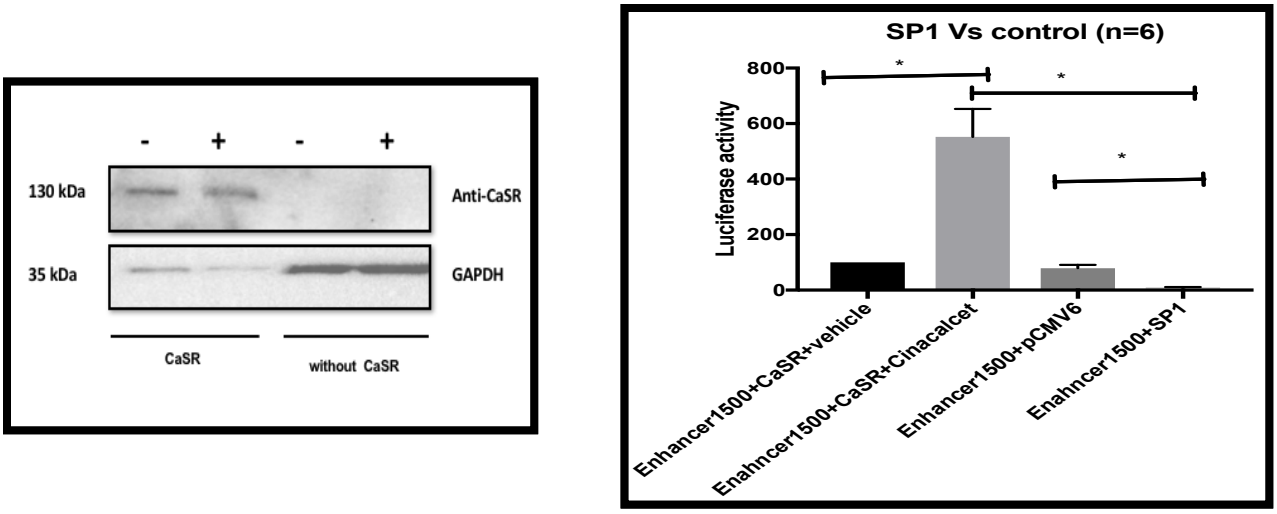
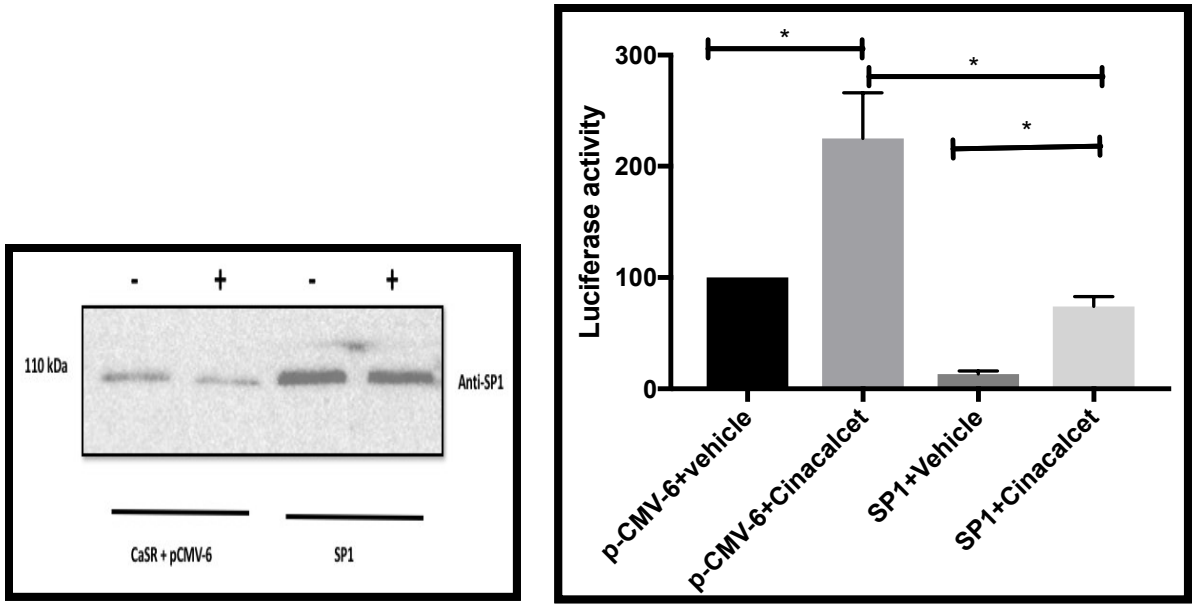


Figure 15. A) Immunoblot showing successful transfection with the CaSR. B) SP1 inhibits expression of the 1500 bp claudin-14 promoter construct.

A) The first two groups were co-transfected with 1500 bp mcldn-14V1 construct and the CaSR whereas, the last two groups were co-transfected with 1500 bp mcldn-14V1 construct and Pcmv-6 or SP1. Both groups were treated with Cinacalcet or vehicle. Immunoblotted with an anti-CaSR antibody and GAPDH. Note: -ve shows no cinacalcet & +ve shows added cincalcet at 24 hours after transfection. Cell lysate was collected 48 hours after transfection. B) Analysis of the luciferase signal produced by the 1500 bp fragment upstream of the 5'UTR of mouse claudin-14 variant 1 (1500 bp mcldn14V1) cloned into pGL3 enhancer vector. All constructs were transiently co-transfected with the PRL-TK control vector into Hek-293 cells. The first two constructs were also co-transfected with the CaSR and the last two with pcmv-6 and SP1 respectively. Firefly luciferase activity was measured and normalized to Renilla luciferase activity, these values were then expressed as a percentage of the 1500 bp mcldn14V1 expression in the absence of any drug (first group). Addition of cinacalcet increased reporter expression significantly. The third and fourth group were not treated with drug. The results are displayed as mean+/- SEM; n=6. * Indicates a significant difference from negative control (no treatment group) to Cinacalcet HCL treated group and between pcmv-6 (empty vector) and SP1. Comparisons were made using one-way ANOVA and doing multiple comparisons (comparing mean of each column with mean of every other column). * represents $p < 0.05$



A **B**

Figure 16. A) Immunoblot showing successful transfection with SP1. B) SP1 expression attenuates the increase in reporter activity of the 1500 bp CLDN14V1 promoter construct induced by CaSR activation.

A) The first two groups were co-transfectd with 1500 bp mcldn-14V1 construct, pCMV-6 and the CaSR whereas, the last two groups were co-transfected with 1500 bp mcldn-14V1 construct, SP1 and the CaSR. Both groups were treated with Cinacalcet or vehicle. Immunoblotted with an anti-SP1 antibody. Note: -ve shows no cinacalcet & +ve shows added cincalcet at 24 hours after transfection. Cell lysate was collected 48 hours after transfection. B) Analysis of the luciferase signal produced by the 1500 bp fragment upstream of the 5'UTR of mcldn-14 V1cloned into PGL3 enhancer. All constructs were transiently transfected with the PRL-TK control vector and the CaSR into Hek-293 cells. These constructs were either transfected with pcmv-6 or SP1 and then treated with either vehicle or cinacalcet. Firefly luciferase activity was measured and normalized to Renilla luciferase activity and the values are expressed as a percentage of the activity of the 1500 bp mcldn14V1 expression in the absence of any drug (first group). Results are displayed as mean+/- SEM; n=6. P=<0.05* Indicates a significant difference from negative control (no treatment group) or between pcmv-6 (empty vector) and SP1. Comparisons were made using one-way ANOVA and doing multiple comparisons (comparing mean of each column with mean of every other column).

4.3 Effect of the PKC inhibitor (Staurosporine) on CaSR mediated increase in claudin-14 promoter activity.

We have demonstrated that PGL3 Enhancer 1500 bp construct 5' to the UTR of mcldn14 V1 has a region that contains an element sensitive to CaSR activation. Moreover, CaSR activation leads to greater expression of claudin-14 through a mechanism that appears at least in part to depend on SP1, potentially via its attenuation. This lead us to hypothesize that activation of the CaSR attenuates SP1 activity leading to an increase in claudin-14 expression. To determine the signaling pathway between CaSR activation and attenuated SP1 activity we employed several different pharmaceutical inhibitors (PKC inhibitor, PLC & MEK inhibitor) in our Hek-293 cell culture model system.

First, we used the PKC inhibitor (Staurosporine). PKC has been identified as a downstream signaling molecule and negative regulator of CaSR signaling (Lazarus S et al. 2011). In other words, CaSR activation attenuates PKC activity. To assess if increased CLDN14 expression downstream of CaSR activation is mediated through PKC, we co-transfected the PGL3 Enhancer 1500bp construct and the CaSR into Hek-293 cells and added 20 nM staurosporine, the PKC inhibitor (Chae H.J et al. 2000) and cinacalcet 24 hours after transfection. We then performed a luciferase assay the following day (i.e. we assayed luciferase activity 48 hours after transfection). This experiment revealed that the luciferase signal from the PKC inhibitor treated group were significantly higher than the group treated with vehicle. However, this increase was not greater than that of cinacalcet HCL alone **(**see **Figure 17).** This suggests that PKC is constitutively attenuating the expression of CLDN14 and that activation of the CaSR

inhibits PKC activity, thereby preventing this attenuation and allowing CLDN14 expression **(**see **Figure 17).**

To implicate altered SP1 expression in this pathway we performed immunoblots for SP1 on the above treated samples. We observed that both cinacalcet and staurosporine decreased SP1 expression, however the decrease was not greater in the group treated with both drugs **(**see **Figure 18 A & B)**. This data is consistent with PKC activity supporting the expression of SP1. Activation of the CaSR appears to both attenuate PKC activity and consequently SP1 expression allowing for claudin-14 expression.

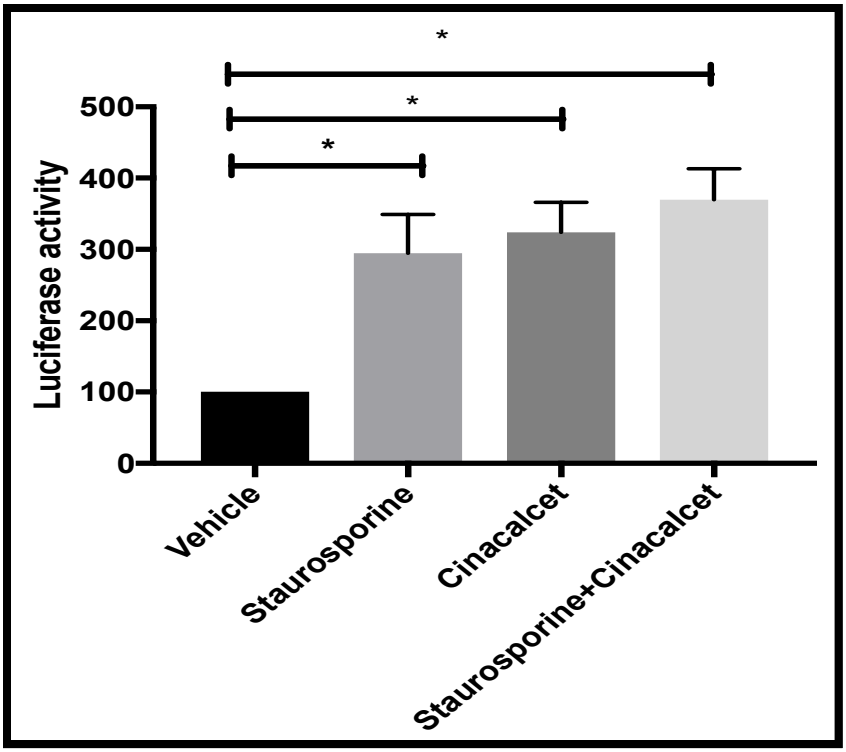
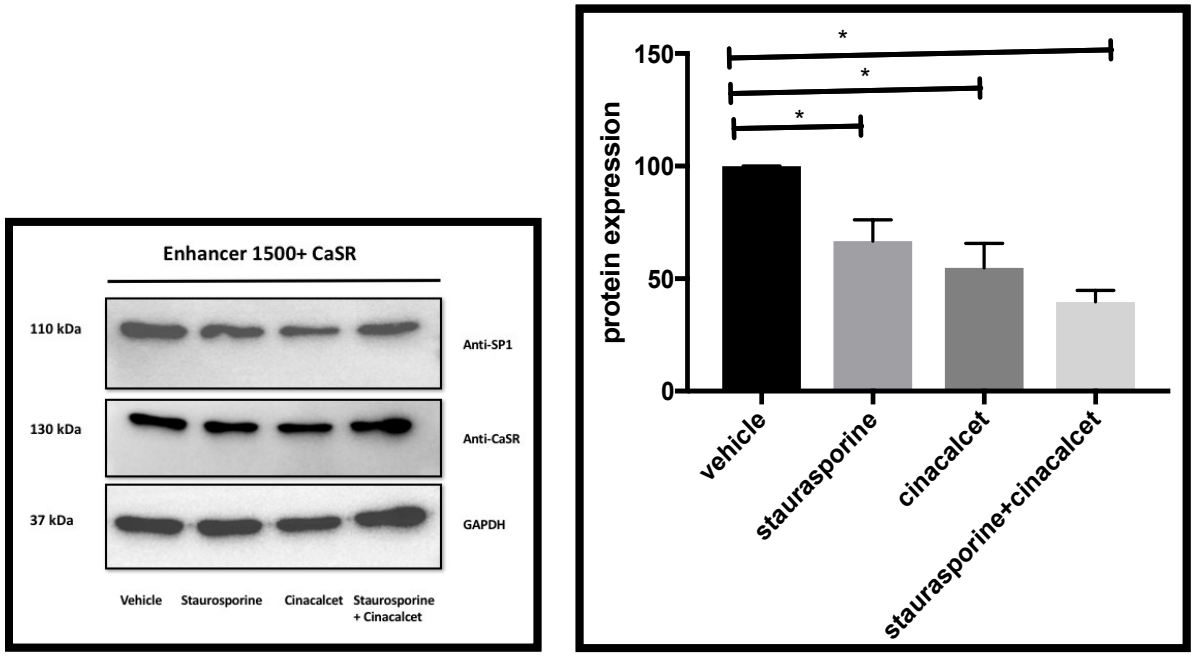


Figure 17. PKC inhibitor (Staurosporine) increases claudin-14 expression but not more than that of Cinacalcet HCL.

The 1500 bp fragment upstream of the 5'UTR of mcldn-14 V1 cloned into PGL3 enhancer was transiently transfected with the PRL-TK control vector and the CaSR into Hek-293 cells and treated with the drugs listed below the graph. Firefly luciferase activity was measured and normalized to Renilla luciferase activity, and the data expressed as a percentage of the activity of the 1500 bp mcldn14 expression in the absence of any drugs. The results are displayed as mean+/- SEM; n=7. * Indicates a significant difference from negative control (no treatment group): $p<0.05$; using one-way Anova and performing multiple comparisons.



A **B**

Figure 18. A) Immunoblot showing SP1 protein expression. B) PKC inhibitor (Staurosporine) decreases SP1 protein expression.

A) All groups were transfected with mcldn-14 V1 1500 promoter construct (enhancer 1500) and CaSR. After 24 hours, the drugs listed below the graph were added. Cell lysate was collected 48 hours after transfection and immuonbloted for SP1, visualized and then stripped and re-probed with GAPDH to normalize the results. The correct size band on immunoblot at 110 kDa indicates SP1 protein expression whereas, GAPDH protein is shown at 37 kDa and CaSR at 130 kDa expected. B) Quantification of the representative immunoblots. * Indicates significant difference where $p<0.05$. n = 5. Hek-293 cell lysate collected at 48 hours after transfection. Each group was transfected with mcldn-14 V1 1500 promoter construct (enhancer 1500) and CaSR. After 24 hours of transfection cells were treated with different drugs mentioned below the graph. Results were normalized with GAPDH protein expression. Data analysis was done using one-way ANOVA and doing multiple comparisons between groups. Significant decrease in SP1 expression is seen with Staurosporine treated group compared to the vehicle. Significant decrease in SP1 expression is also seen with Cinacalcet and Staurasprine + cinacalcet treated groups compared to the vehicle. However, this decrease in SP1 protein expression is not significant between staurosporine and cinacalcet treated group suggesting that at least a part of claudin-14 regulation through CaSR activation is via attenuated SP1 activity.

4.4 Effect of MEK and PLC inhibitors on CaSR mediated increase in claudin-14 promoter activity.

To determine the signaling pathway between CaSR activation and attenuated SP1 activity we tried two other pharmaceutical inhibitors in our Hek-293 cell culture model. We used the MEK inhibitor, U-0126, and the PLC inhibitor U-73122. Further downstream of CaSR activation MEK and PLC activation has been observed (http://jcs.biologists.org/content/118/5/855) (Saidak Z et al. 2009). To assess if these pathways are involved in mediating increased claudin-14 expression we transfected the PGL3 Enhancer 1500bp construct into Hek-293 cells and added 1.25 μM MEK inhibitor (Ong Q et al. 2015) 24 hours after transfection, then did the luciferase assay the following day (i.e. we assayed luciferase activity 48 hours after transfection). This experiment revealed that the luciferase signal from the MEK inhibitor treated group was not significantly different the group treated with vehicle, nor did it affect the increase in expression induced by CaSR activation **(**see **Figure 19).** Thus, MEK activation does not appear to alter CLDN14 expression nor the increase in expression induced by CaSR activation **(**see **Figure 19)**.

Similarly, we transfected the PGL3 Enhancer 1500bp construct into Hek-293 cells and added 10 μM PLC inhibitor (MacMillan D et al. 2010) the day after transfection, then did the luciferase assay the following day (i.e. we assayed luciferase activity 48 hours after transfection), to assess the role of PLC, a known affecter of CaSR activation of CLDN14 expression. This experiment revealed that the luciferase signal from the PLC inhibitor treated group was not significantly different than the group treated with vehicle nor did this inhibitor affect the increase in expression induced by CaSR activation. Thus, PLC

inhibition does not alter claudin-14 reporter expression, nor does it alter the increase in expression induced by CaSR activation **(**see **Figure 20).**

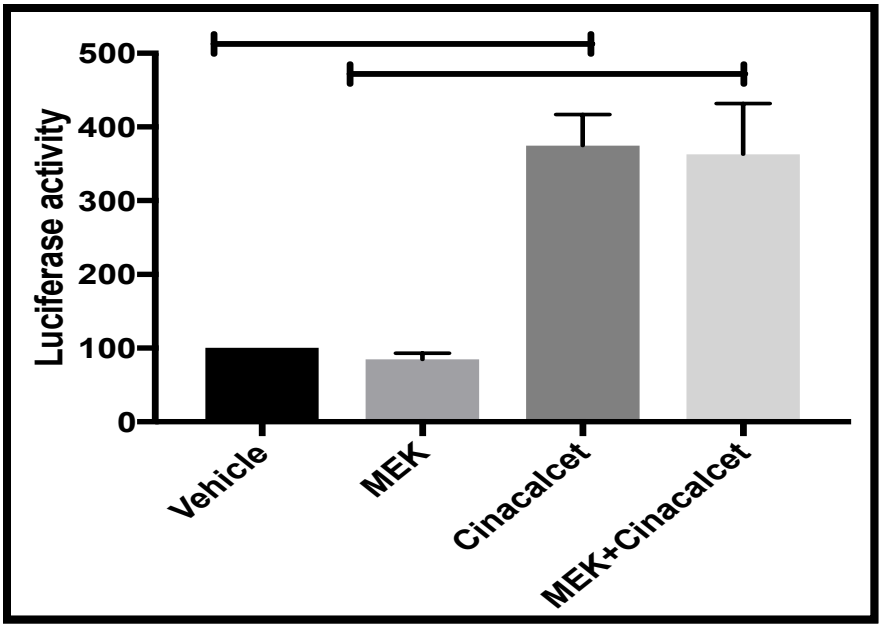


Figure 19. U-0126 (MEK inhibitor) did not alter claudin-14 reporter expression, nor did it alter the increase in expression induced by CaSR activation.

The 5'UTR of mcldn-14 V1cloned into PGL3 enhancer was transiently transfected with the PRL-TK control vector and the CaSR into Hek-293 cells. Firefly luciferase activity was measured and normalized to Renilla luciferase activity and the values expressed as a percentage of the activity of the 1500 bp mcldn14 expression in the absence of any drug. The drug treatment is listed below the x-axis and the results displayed as mean+/-SEM; n=6. $p<0.05$; using one-way Anova and doing multiple comparisons.

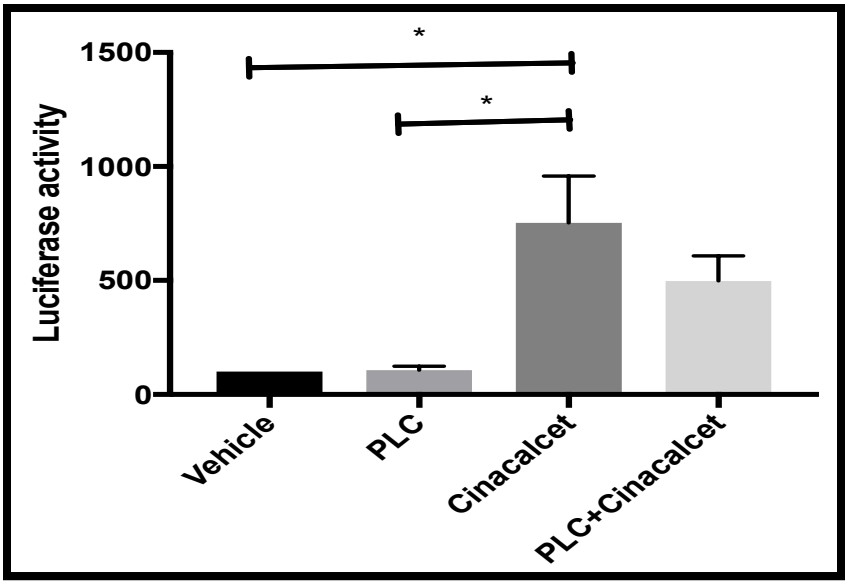


Figure 20. U-73122 (PLC inhibitor) did not alter claudin-14 reporter expression, nor did it alter the increase in expression induced by CaSR activation.

The 1500 bp fragment upstream of the 5'UTR of mcldn-14 V1 cloned into PGL3 enhancer was transiently transfected with the PRL-TK control vector and CaSR into Hek-293 cells. Firefly luciferase activity was measured and normalized to Renilla luciferase activity, and the data is expressed as a percentage of the activity of the 1500 bp mcld14 V1 expression in the absence of any drug. The drug treatment is listed on the x axis. The results are displayed as mean+/- SEM; n=6. * Indicates a significant difference from negative control (no treatment group). $p<0.05$; using one-way Anova and doing multiple comparisons.

4.5 Effect of cdc42 on the CaSR mediated increase in claudin-14 promoter activity.

To figure out what is downstream of PKC leading to attenuation of SP1 and increased claudin-14 expression we examined an established PKC signaling pathways. Cdc42 is a small cell signaling GTPase protein that belongs to the Rho family of Ras GTPase superfamily. It plays an important role in the regulation of several transcription factors that contribute important roles at various levels in different cell signaling pathways (Benitah et al., 2004). It can play an important role in protein kinases activation and regulation of the cell cycle. Cdc42 is suggested to be present downstream of PKC activation where it can act as an intercellular molecular switch that regulates various signaling pathways (Jamie M et al. 2011).

To find the downstream activation molecule after PKC activation, we transiently co-transfected the 1500 bp fragment upstream of the 5'UTR of mcldn-14 V1cloned into PGL3 enhancer, PRL-TK control vector and CaSR into Hek-293 cells. These constructs were also transfected with GFP or cdc42 wildtype or a dominant-negative cdc42 mutant tagged with GFP. Cdc42 is 1512 base pairs long and is translated into a 191-amino acid protein. The cdc42 mutant construct is an autosomal dominant negative point mutation T4N, where threonine is substituted with an asparagine at position 4 of the amino-acid chain leading to functional inactivation of cdc42 molecule. As a positive control, cells were also transfected with eGFP as these constructs are tagged with GFP. These groups were treated with Cinacalcet or the vehicle 24 hours after the transfection. Then the luciferase assay was done to determine expression. Expression of cdc42 wild-type

construct did not alter reporter expression. Furthermore, we observed a significant increase in expression when cdc42 wildtype expressing cells were treated with Cinacalcet. This increase was comparable to the cells expressing empty vector (eGFP) that were treated with cinacalcet. However, compared to the control expressing and wild-type cdc42 expressing cells treatment of the cells expressing the cdc42 mutant construct with Cinacalcet had an attenuated increase in expression **(**see **Figure 21).** This work suggests that cdc42 is signaling the increase in claudin-14 expression downstream of CaSR activation.

To confirm that the cells were transfected successfully with eGFP and cdc42 wildtype or mutant tagged with GFP we performed immunoblotting with a primary anti-GFP antibody to confirm the transfection. Cells transfected with eGFP showed the right size band on immunoblot at 27 kDa whereas, cdc42 wildtype or the mutant tagged with GFP showed a band at approximately 50 kDa as expected. These right size bands on the immunoblot suggested that cdc42 wildtype or mutant were transfected successfully **(**see **Figure 22).**

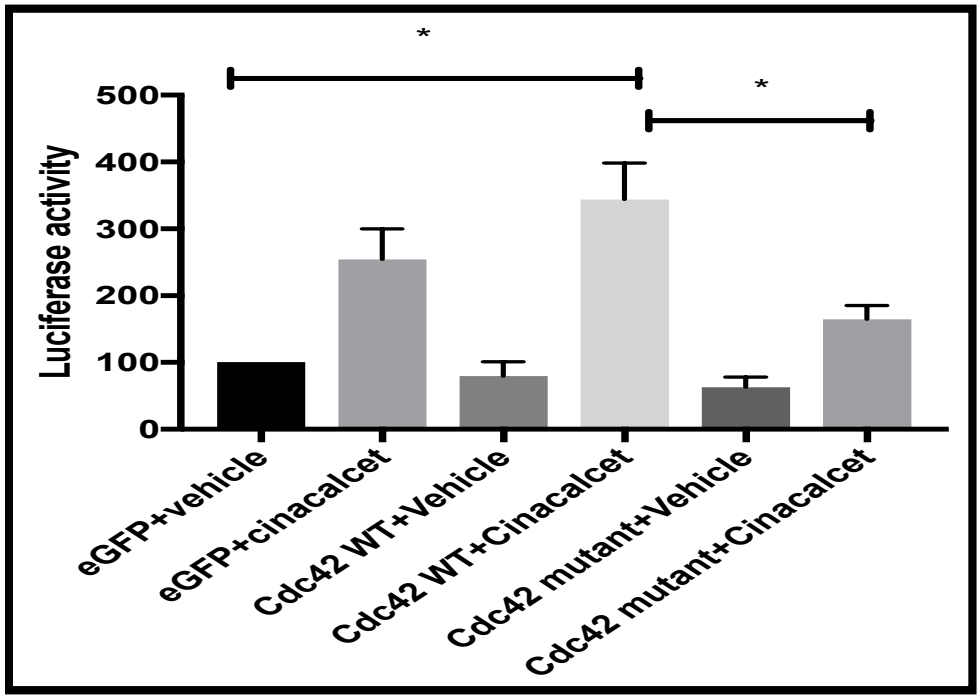


Figure 21. Cdc42 activity mediates the increase in reporter expression induced by Cinacalcet HCL.

The 1500 bp fragment upstream of the 5'UTR of mcldn-14 V1 cloned into PGL3 enhancer was transiently transfected with the PRL-TK control vector and the CaSR into Hek-293 cells. These constructs were transfected with GFP (left two columns), with cdc42 wild type labeled with GFP (middle two columns) or with the dominant negative cdc42 mutant tagged with GFP (right two columns). Firefly luciferase activity was measured and normalized to Renilla luciferase activity, and the results expressed as a percentage of the activity of the 1500 bp mcldn14 expression in the absence of any drug treatment. Note: each set of transfections (cdc42 WT or mutant) was treated with either vehicle or cinacalcet. Results are displayed as mean+/- SEM; n=5. * Indicates a significant difference from negative control (no treatment group). $p<0.05$; using one-way Anova and doing multiple comparisons.

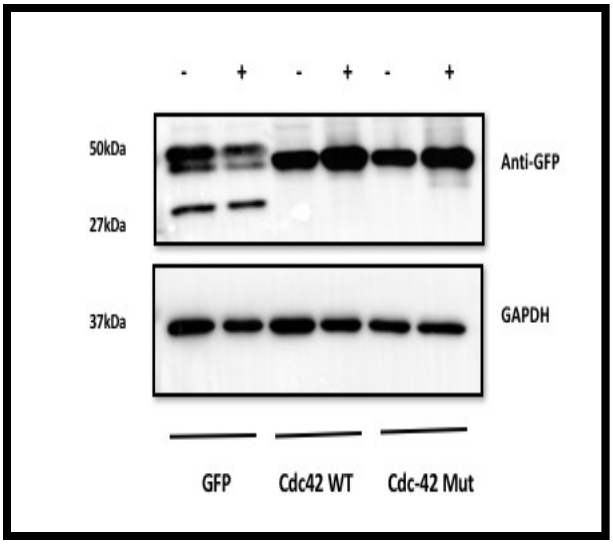


Figure 22. Immunoblot showing successful transfection with GFP marker and cdc42 wildtype or mutant tagged with GFP.

The 1500 bp mcldn-14 V1 construct co-transfected with the CaSR and eGFP, cdc-42 wildtype or mutant tagged with GFP and treated with Cinacalcet or vehicle. Immunoblotted with primary anti-GFP antibody. NOTE: -ve shows no cinacalcet & +ve shows added cincalcet at 24 hrs after transfection. Cell lysate was collected 48 hours after transfection.

Chapter 5. DISCUSSION

This project is focused on understanding the cause(s) of idiopathic hypercalciuria. Hypercalciuria is the leading cause of kidney stone formation in both genders across the world and among all age groups. Hypercalciuria can further be divided into 2 broad categories 1) secondary hypercalciuria and 2) idiopathic hypercalciuria. Secondary hypercalciuria is an increase in urinary calcium excretion due to a known pathological condition e.g. patients with Dent's disease, William syndrome and Barter's syndrome suffer from hypercalciura along with other clinical manifestations. Whereas in idiopathic hypercalciuria no cause has been identified after a complete medical examination and laboratory investigation. A large gene wide association study (GWAS) linked SNPs found in claudin-14 to kidney stones and idiopathic hypercalciuria (Thorleifsson et al.2009). However, both claudin-14 knockout mice and patients with mutations in the coding region of claudin-14 are deaf. Furthermore, the mice had no evidence of dysregulation in calcium homeostasis. Claudin-14 was identified as a tight junction protein present in the thick ascending limb of the loop of Henle (Gong et al.2012). Previous work done in the Alexander lab suggests that CaSR (calcium sensing receptor) activation leads to an increase in claudin-14 expression in the thick ascending limb (TAL). Claudin-14 is a paracellular cation blocker protein, when it is expressed in the tight junction it leads to an increase in the transepithelial resistance and decreases paracellular calcium flux (Henrik et al. 2013). Increased dietary calcium intake and prolonged treatment with active 1,25(OH)2D3 leads to an increase in claudin-14 expression. Furthermore, in vivo stimulation with Cinacalcet leads to activation of the CaSR and an increase in claudin-14 expression (Henrik et al.2013).

Although the activation of the CaSR resulting in an increase in claudin-14 expression has been observed by many research groups and this activation is associated with both idiopathic hypercalciuria and kidney stones, the signaling pathway mediating this remains completely unknown. Therefore, for this project, we set out two specific goals. 1) To delineate signaling pathway downstream of CaSR activation leading to increased claudin-14 expression, and 2) to Identify novel genetic causes of idiopathic hypercalciuria.

The kidneys play an important role in calcium homeostasis. Of the calcium filtered at the glomerulus the thick ascending limb (TAL) is responsible for 25% of the total reabsorption. Claudin-14 regulates this in response to CaSR activation. During periods of low to normal plasma calcium concentration, calcium reabsorption is facilitated through paracellular pores formed through interaction of claudin-16 and claudin-19. However, when plasma calcium concentrations are high calcium binds to the CaSR on the basolateral surface of the thick ascending limb and this leads to an increase in claudin-14 expression in the tight junction. Claudin-14 is a paracellular cation blocker, when expressed on the apical surface it blocks the reabsorption of calcium leading to an increased excretion of calcium in the urine. Inappropriately increased levels of calcium in the urine is called hypercalciuria, which is one of the greatest risk factors for developing kidney stones. Evidence from the literature supports the regulation of claudin-14 by a transcriptional mechanism. However, the exact pathway mediating this transcriptional regulation of claudin-14 is still unknown.

Interestingly one group has demonstrated a novel microRNA pathway regulating claudin-14 expression (Gong et. Al 2012). They showed that renal calcium handling is

regulated at the micro-RNA level and this regulation is largely effected by serum calcium levels (Gong et. Al 2012). This group proposed that the claudin-14 gene produces different splice variants which vary in number for different species. There are five for human, three for mouse and one for rat. These splice variants differ in their 5'-UTR sequences while their coding region and 3'-UTR sequences are conserved in all variants (Gong, 2012). They introduced a new concept of claudin-14 gene regulation at the micro-RNA level (Gong et. Al 2012). A novel micro-RNA site, miR-9 and miR-374 that targets the 3' UTR of the claudin-14 mRNA cause it to degrade and thus prevent its translation into the claudin-14 protein. Moreover, activation of CaSR decreases miR-9 and miR-374 levels preventing claudin-14 degradation and allowing translation into protein (Gong et. Al 2012). The same group also proposed that histone deacetylase (HDAC) permits increased transcription of renal microRNA-9 and microRNA-374, which cause degradation of claudin-14 mRNA expression in turn allowing the paracellular reabsorption of calcium. However, in contrast, another group showed that renal miR-9 and miR-374 do not play any role in claudin-14 regulation (Toka et al. 2012). They treated CaSR deficient mice and other control animals with supplemented calcium and didn't find a significant difference in the renal expression of miR-9 and miR-374 between CaSR deficient mice treated with calcium compared to the control. These leaves open the possibility of direct transcriptional regulation, which is supported by our data. To this end the Alexander lab performed a microarray on renal mRNA isolated from mice that were treated with cinacalcet HCL or control didn't show any significant difference in miR-9 expression (data unpublished). Therefore, this novel pathway suggesting that claudin-14 regulates renal calcium transport in response to CaSR signaling through a

novel microRNA pathway is still controversial. Therefore, the signaling pathway between CaSR activation and claudin-14 expression in the TAL is still unknown. And the first part of this project is therefore focused on delineating this pathway.

5.1 Proposed pathway for the claudin-14 activation

To delineate the CaSR signaling pathway mediating increased claudin-14 expression we created a cell culture model. To this end we used Hek-293 cells, transiently transfected with a mouse claudin-14 Variant 1 (mcldn-14 V1) 1500 promoter construct, which contained a CaSR responsive element, along with the calcium sensing receptor (CaSR) and added different pharmaceutical inhibitors 24 hours post transfection. Once cell lysate was collected (48 hours after transfection) a luciferase assay was performed. We first co-transfected the transcription factor SP1 with the mcldn-14 V1 1500 promoter construct. SP1 transcription factor has been seen to alter expression of multiple different genes post CaSR activation. (Gill et al. 1994, Li et al. 1991, Pugh, Tjian 1991, Su et al. 1991). SP1 is also involved in many cell regulating processes where it can act as an activator or a repressor. To determine if SP1 was involved in mediating increased claudin-14 expression post CaSR activation we co-transfected mcldn-14 V1 1500 promoter construct with SP1 and as a control we co-transfected mcldn-14 V1 1500 promoter construct with the empty vector. The luciferase assay was done after 48 hours of transfection. A significant decrease in expression was observed in the group transfected with SP1 (see **Figure 15 C**). Furthermore, a significant decrease in SP1 was also observed when both groups were co-transfected with the CaSR and Cincalcet was added later (24 hours post-transfection). These experiments suggest that not only

does SP1 inhibit claudin-14 expression independent of CaSR signaling, but it also attenuates the increased expression of clauidn-14 induced by the activation of the CaSR signaling pathway (see **Figure 16**). Based on these results we hypothesized that activation of the CaSR attenuates SP1 activity to increase claudin-14 expression.

Next we sought to use different pharmacological inhibitors of known signaling pathways activated by the CaSR to determine what is upstream of SP1. Therefore, Hek-293 cells were transfected with the promoter construct mcldn14 V1 1500 and the CaSR and different drug inhibitors were added (Staurosporine, a PKC inhibitor or a PLC inhibitor) 24 hours post transfection. We found that the PLC inhibitor and MEK inhibitor did not alter claudin-14 expression (see **Figure 19 & 20**). However, the PKC inhibitor (staurosporine) increased claudin-14 expression but not more than that of Cinacalcet (see **Figure 17**). These data suggest that PKC is present downstream CaSR activation pathway. Moreover, consistent with PKC altering claudin-14 expression through SP1, treatment with staurosporine significantly decreased SP1 expression as did treatment with the calcium sensing receptor Cinacalcet (see **Figure 18**). We also considered the possibility that either our transfection or treatment with PLC and MEK (1 & 2) inhibitors didn't work. To eliminate this possibility, we will also employ phospho-specific anti-body for PLC and MEK (1 & 2) inhibitor to make sure the drug is working.

We then sought to figure out what is downstream of PKC leading to attenuation of SP1 and increased claudin-14 expression. Cdc42 is a small cell signaling GTPase protein that belongs to the Rho family of Ras GTPases superfamily. It regulates a variety of cell signaling pathways and has been shown to be activated downstream of CaSR activation. It can play an important role in epithelial differentiation, protein kinases

activation, regulation of cell cycle and cell growth. It is found on chromosome 1 at location 22,235,157-22,292,024, it contains 6 exons and 1512 base pairs, which are translated into 191 amino acids. Our cdc42 mutant construct is an autosomal dominant negative point mutation, T4N, where threonine is substituted for an asparagine at position 4 of the amino-acid chain leading to functional inactivation. Cdc42 can play an important role in the regulation of several transcription factors that play an important role at various levels in different cell signaling pathways (Benitah et al., 2004). To determine if cdc42 was participating in the regulation of CLDN14 expression we transfected Hek-293 cells with the intronic 1500 mcldn14 V1 promoter construct, the CaSR and either cdc42 wild-type or cdc42 mutant construct. As a control, cells were also transfected with eGFP as the constructs were tagged with GFP. In all groups Cinacalcet or vehicle was added 24 hours post transfection. A significant decrease in claudin-14 expression was seen with the cdc42 mutant construct compared to cdc42 wild-type construct (see **Figure 21**). Later we performed immunoblotting using primary anti-GFP antibody to confirm that Hek-293 cells were successfully transfected with GFP marker and cdc-42 wildtype or mutant construct tagged with GFP (see **Figure 22**). We also tried to transfect Hek-293 cells with the intronic 1500 mcldn14 V1 promoter construct, CaSR and rac wild-type or rac mutant construct. Rac is also a small cell signaling GTPase protein that belongs to the Rho family of Ras GTPases superfamily that regulates a variety of cell signaling pathways and has been shown to be present downstream of CaSR activation pathway. However, we were not able to successfully transfect rac wild-type and mutant constructs into our cell culture model. Based on the work done so far for this project it suggests to us that PKC and cdc42 are present upstream of SP1 and

play an important role in the cell signaling pathway that mediates increased claudin-14 expression post CaSR activation (see **Figure 23**).

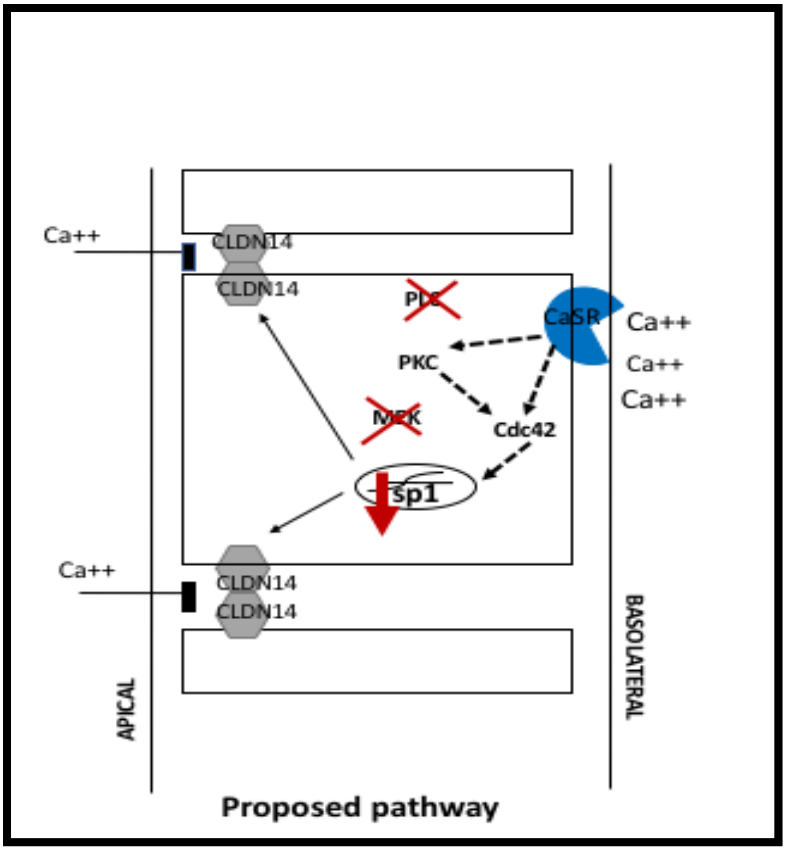


Figure 23. Proposed signaling pathway post calcium sensing receptor activation mediating increased claudin-14 expression.

Claudin-14 (CLDN14), CaSR (calcium sensing receptor), SP1 (specificity protein 1 transcription factor), serum calcium (Ca^{++}), Protein kinase-C (PKC), Phospholipase-C (PLC), Mitogen-activated protein kinase kinase (MEK), Cell division control protein 42 (cdc42) & Cyclic adenosine phosphate (c-AMP).

5.2 SNP rs199565725 [del AC] likely causes kidney stones by inappropriately increasing claudin-14 expression and causing calciuria.

This part of the project was focused on identifying novel genetic causes of idiopathic hypercalciuria. We worked with a cohort of patients with idiopathic hypercalciuria who had hypercalciuria (urine calcium: creatinine ratio >0.56mM/mM), normocalcemia (2.2 to 2.7mM) and normal serum PTH (1.1 to 6.8pM) levels without any known disease causing this condition. DNA was extracted from blood samples taken from these patients. DNA was amplified for claudin-2, -12, -14 and CYP24A1 using PCR (polymerized chain reaction). These genes were selected based on their location, expression and function in the nephron. Claudin-2 and -12 form paracellular cation pores in the proximal tubule, which is responsible for 65% of calcium reabsorption. Claudin-14 is present in the thick ascending limb (responsible for 25% of calcium reabsorption) and is a paracellular cation blocker which blocks reabsorption of calcium leading to calciuria. CYP24A1 encodes 24-hydroxylase, an enzyme that regulates the final step in vitamin D metabolism converting 1,25-(OH)2D3 (the hormonally active form of vitamin D) into calcitroic acid (the inactive form of vitamin D) which is excreted through bile from the body. Vitamin D plays an important role in maintaining calcium homeostasis. It does so by increasing calcium absorption from the intestine. This lead to our hypothesis that loss of function mutations in claudin-2, -12, CYP24A1 or gain of function mutations in claudin-14 cause kidney stones and hypercalciuria.

Sanger sequencing was done for different regions of these candidate genes. Of the SNPs that were found in higher frequency in our idiopathic hypercalciuria cohort compared with the control (5008-allele data from NCBI data base) we found that an

intronic claudin-14 SNP rs128494 was the most enriched variant (see **Figure 6**). Later we cloned this intronic claudin-14 SNP containing either rs128494 or the wild type sequence into a reporter vector to determine if it influences gene expression. These constructs were transfected into Hek-293 cells and luciferase assay activity was measured. No significant increase was seen in luciferase activity after 24 and 48 hours post transfection. This suggests to us that claudin-14 intronic SNP rs128494 does not alter claudin-14 expression (see **Figure 7 & 8**). However, transcription factors or signaling molecules present in the thick ascending limb might not be present in Hek-293 cells. So, these experiments do not completely exclude the possibility that this SNP might be pathogenic.

In 2015 a large genome-wide association study was conducted in Iceland with 5,419 cases and 279,870 controls (Oddsson, A. 2015). In this study four genes (CLDN14, SLC34A1, ALPL and CASR) were associated with kidney stones (Oddsson, A. 2015). Of the claudin-14 variations identified a two-base pair deletion in one of the intronic regions of claudin-14 (CLDN14) rs199565725 [del AC] had the strongest association with kidney stones (Oddsson, A. 2015). It was predicted that rs199565725 [del AC] is one of the responsible SNPs leading to a disease-causing mutation in these individuals. However, all the data presented in this paper was based on statistical analysis and no functional studies were done. So, we performed a functional study with this SNP. We used the same cell culture model that we used for the rs128494 variant. We cloned this intronic claudin-14 SNP containing either rs199565725 [del AC] or the wild type sequence into a reporter vector to determine if it influences gene expression. Luciferase activity was noted for Hek-293 cells transfected with constructs containing rs199565725

[del AC] and the wild type sequence. A significant increase in luciferase activity was seen after 48 hours of transfection (see **Figure 9**), suggesting that intronic SNP rs199565725 [del AC] does alter claudin-14 expression. Although the exact regulation is puzzling, as this intronic claudin-14 SNP rs199565725 [del AC] was associated with decreased kidney stone formation and as such we would have predicted that it mediates a decrease in claudin-14 expression. However, our results using the Hek-293 cell culture model and transiently transfecting these cells showed a significant increase in claudin-14 expression with the variant. These conflicting results might be a due to the cell culture model used for this experiment, as discussed above, the Hek-293 cells might be lacking the appropriate transcription factor or signaling molecules that suppress not increase expression in the TAL. Alternatively, the seemingly contradictory results might be due to the length of insert cloned into the reporter vector as we might be missing another regulatory molecule upstream or downstream of the SNP that leads to decreased expression relative to the wildtype sequence. Ultimately we also sequenced our hypercalciuria cohort for this intronic SNP rs199565725 [del AC] to see if any of our patients have this SNP. Interestingly we didn't find this SNP in any of our patients sequenced so far, consistent with it being protective (as all our patients have kidney stones).

5.3 Therapeutic considerations

The mechanism whereby altered claudin-14 expression underlying kidney stone pathogenesis can be explained as a gain-of-function effect of CLDN14 rather than a loss-of-function effect (Jianghui Hou. 2013). Understanding the calcium sensing receptor signaling pathway leading to increased clauidn-14 expression in some patients should therefore help design therapeutics to decrease urinary calcium excretion and help prevent kidney stone formation. We could specifically target signaling molecules in this pathway to decrease claudin-14 expression, thereby reducing calciuria and kidney stone formation risk. Further 1) this will help to provide a molecular explanation for the association between claudin-14 variations and kidney stones and 2) it also helps justify the therapeutic rationale to inhibit the calcium sensing receptor in patients with kidney stones.

5.4 Conclusion

Through sequencing and data analysis (using NCBI data base) of our idiopathic hypercalciuria cohort as well as from one study done in the Icelander population (Oddsson, A. 2015) we identified two intronic claudin-14 SNPs rs128494 and rs199565725 [del AC] that were associated with kidney stones and cloned these SNPs into a reporter vector. SNP rs128494 was found to be significantly increased in number in our hypercalciuria cohort compared to 5008 genome data (NCBI data base) (see **Figure 6**). However, rs128494 didn't show any significant increase in claudin-14 expression when introduced into a cell culture reporter model (see **Figure 7 & 8**). Whereas, rs199565725 [del AC] which was found in significantly increased number in Iceland's population showed a significant increase in claudin-14 expression when introduced in the same cell culture model (see **Figure 9**). Unfortunately, we expected this SNP to decrease expression not increase. More work is therefore necessary to sort this out.

Secondly, to delineate the signaling pathway between CaSR activation and claudin-14 expression we worked with the Hek-293 cell culture model using the luciferase assay for measuring claudin-14 expression. SP1 has been shown to regulate the expression of other genes post CaSR activation, we therefore hypothesized that the 'Activation of CaSR attenuates SP1 activity' (see **Figure 3**). When SP1 was transiently transfected in Hek-293 cells with mcldn-14 1500 promoter construct it attenuates expression, including post CaSR activation. To find the signaling molecules involved in this regulatory pathway we found a significant increase in claudin-14 expression when staurosporine (a PKC inhibitor) was added. However, this increase in expression is not more than that of

Cinacalcet, suggesting that PKC is present downstream of CaSR activation (see **Figure 17**). Also, transfecting Hek-293 cells with a cdc42 dominant negative mutant lead to a significant decrease in claudin-14 expression compared to the cdc42 wild type (see **Figure 19**) consistent with both PKC and cdc42 being present in the regulating pathway downstream of CaSR activation. Interestingly SP1 expression is altered by CaSR activation and staurosporine treatment (see **Figure 18**). We therefore propose that CaSR activation attenuates PKC activity which activates cdc42 to inhibit SP1 thereby increasing claudin-14 transcription (see **Figure 23**).

5.5 Future Directions

The next goal for this project after confirming the presence and localization of the transcription factor SP1 by western blot and immunoflourescence post cdc42 inacativation is to create stable cell lines for Hek-293 cells overexpressing the calcium sensing receptor (CaSR) and to repeat all these experiments with a Hek-293 stable cell line. Secondly we want to perform luciferase assay using other more physiological cell lines such as the rat TAL (thick ascending limb) cells. We will co-transfect other cell lines with transfection factor SP1 or pcmv-6 and the intronic 1500 mcldn-14 V1 promoter construct and comparing it with the controls. We also want to see the effect of Cinacalcet and staurosporine (PKC inhibitor) in the presence and absence of CaSR in other cell lines such as the rat TAL cells. We predict that transfecting SP1 with 1500 mcldn-14 V1 promoter construct in these cell lines will attenuate SP1 expression. Considering the possibility that SP1 can be endogenously expressed in these cell lines we plan to perform immunofluorescence in both cell lines to compare the difference between the two groups. Based on the results that we have so far our prediction is that activation of CaSR attenuates SP1 activity via PKC and ultimately leads to an increase in claudin-14 expression. We know that protein kinase enzymes specifically PKC can be regulated by c-AMP. To analyze this hypothesis that attenuation of SP1 is via PKC which is regulated by cAMP, we plan to add forskolin to increase cAMP and IBMX to inhibit dephosphorylation, to cells expressing SP1 and 1500 mcldn-14 V1 promoter constructs. We predict that addition of both forskolin and IBMX will stimulate the PKC activation by increasing the cAMP, leading to increase in SP1 activity resulting in decrease claudin-14 expression. To verify the predicted results of this experiment we

also plan to add Cinacalcet, forskolin and IBMX to SP1 and 1500 mcldn-14 V1 promoter constructs. We predict to see either no effect or decrease in claudin-14 expression if attenuation of SP1 leading to increase in claudin-14 expression is via PKC stimulation.

For the other part of this project that deals with the idiopathic hypercalciuria cohort we plan to make new constructs with the insert in the opposite orientation i.e 3' to 5' UTR segment, where the segment carrying the variant or wildtype sequence is inserted downstream of a reporter gene using luciferase reporter gene assay. These constructs will help us understand if the altered sequence is in fact inhibiting the binding of a transcription factor. We also want to co-transfect both intronic claudin-14 SNPs rs199565725 [del AC] and rs128494 with CaSR into Hek-293 cell model and later add cinacalcet to see if there is any significant difference in claudin-14 expression. Secondly non-radioactive electrophoretic mobility shift assay can be done to see any interaction between claudin-14 protein and the DNA containing the SNP. Also, we plan to do in-silico analysis for these two SNPs using the JASPAR vertebrate database to try to predict transcription binding sites that might be altered by the SNPs. We can also use other cell lines like rat TAL cells to transfect these SNPs or wildtype. In the future if we have enough evidence that these SNPs show a significant difference compared to the control, we can also design mouse knock-in models to further support our hypothesis. We also plan to collect more blood samples from idiopathic hypercalciuria children and continue to sequence more patients. We predict that there are more intronic SNPs in claudin-14 gene that are associated with idiopathic hypercalciuria that might alter its regulation and cause kidney stones.

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