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The effect of mTOR inhibitor rapamycin on a dietary Drosophila melanogaster model of calcium oxalate nephrolithiasis

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Abstract

Impaired cellular tolerance of reactive oxygen species (ROS) has been suggested as a common mechanistic link associated with aging in both metabolic syndrome and nephrolithiasis. The mechanistic (mammalian) target of rapamycin (mTOR) activity is characteristic of metabolic syndrome. When nutrients are abundant, mTOR is active. Conversely, fasting inhibits mTOR. Metabolic syndrome is correlated with an increased risk of self-reported or imaging findings of nephrolithiasis. At the individual level, patients with a higher BMI have an increased prevalence of recurrent symptomatic nephrolithiasis, 24-hour urinary excretion of oxalate, sodium, uric acid, calcium, and phosphorous as well as lower pH. Calcium oxalate crystals produce ROS in renal epithelial cells and upregulate ROS and also mTOR activity. Rapamycin pharmacologically inhibits mTOR leading to autophagy – a natural defense mechanism against ROS – and drives sub-cellular recycling machinery to a net catabolic state. Ideally, inhibiting mTOR limits the duration and degree of damage done by ROS and subsequent inflammatory process. Additionally, improved clearance of damaged organelles prevents runaway generation of ROS by mitochondrial dysfunction associated with calcium oxalate crystal adherence and internalization. Taken together, this may prevent the progression of calcium oxalate urolithiasis. In this study, we examined the effect of short-term intermittent rapamycin treatment on the area of calcium oxalate concretion in the Malpighian tubules of a *Drosophila melanogaster* fed a lithogenic diet containing 0.1% sodium oxalate.

Keywords: nephrolithiasis, rapamycin, mTOR, Drosophila melanogaster, calcium oxalate, sodium oxalate, Malpighian tubule, aging, metabolic syndrome, reactive oxygen species, autophagy.

Lay Summary

We do not know why obesity and aging are risk factors for kidney stones. With obesity and aging, there is opportunity for damage and stress on the body to keep organs like the kidneys from working like they should. As a result, we think that this may lead to more risk of forming kidney stones. A pathway called the mTOR pathway might help explain why older and more obese people form more stones compared to younger fit people. Our project found that using a drug (rapamycin) to slow down this pathway could lower the ability kidney stones to form in a fruit fly animal model. The fruit flies given this drug tended to live longer in stressful environments compared to equivalent flies that did not get this drug. This finding suggests we should design more experiments to block this pathway either through specific diets, exercises, and/or drug treatments that might one day be tried in humans.

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Abbreviation List

Abbreviation	Description
ROS, RNI	Reactive oxygen species, Reactive nitrogen species
mTOR	Mechanistic (or mammalian) target of rapamycin
BMI	Body mass index
DM, *Drosophila, D. melanogaster*	*Drosophila melanogaster*; fruit fly
CKD	Chronic kidney disease
ESRD	End stage renal dysfunction
GFR	Glomerular filtration rate
QoL	Quality of life
NHANES	National Health and Nutrition Examination Survey
PTH	Parathyroid hormone
THP	Tamm Horsfall protein; uromodulin
TOS	TOR signaling motif
TSC	Tuberous sclerosis complex
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
ATP	Adenosine triphosphate
Raptor	Rapamycin associated protein of TOR
CASTOR	Cellular Arginine Sensor of TOR
S6K	S6 Kinase; mRNA transcription initiation
SREBP	Sterol responsive element binding protein
HIF1a	Hypoxia inducible factor 1 alpha
Rictor	Rapamycin insensitive companion of TOR
SGK1	Serine/theronine-protein kinase 1
MT	Malpighian tubule; fly nephrons (minus glomeruli)
CAFE	Capillary Feeder
Lithogenic diet, 0.1% NaOx, 0.1% Oxalate	Contain 0.1% Sodium Oxalate w/v
Nonlithogenic diet, NF	Do not contain 0.1% Sodium Oxalate

Chapter 1

Aim

Excessive caloric intake and low physical activity have been associated with a wide variety of age-related diseases associated with reactive oxygen species including metabolic syndrome and nephrolithiasis.[1] Indeed, elevated body mass index (BMI) is an independent risk factor for recurrent symptomatic kidney stone episodes.[3] Previous studies have correlated higher BMI to abnormal urinary nephrolithiasis parameters and recurrent symptomatic stone episodes.[2] A major regulator of overall growth in relation to nutrient availability is the mechanistic/mammalian target of rapamycin (mTOR) pathway.[3] When active, the mTOR complex coordinates efficiency of cellular processes depending on nutrient availability, cell stress and growth factors. Essentially, active mTOR is seen as a nutrient "checkpoint", indicating to the cells that the net environmental conditions are favourable to allow for growth and reproduction. Conversely, processes associated with lower mTOR activity are involved in breaking down macromolecules. Lower mTOR activity leads to increased cellular senescence, apoptosis and protein/organelle turnover through increased autophagy. There is mounting evidence that in complex age-associated diseases like metabolic syndrome, mTOR is overactive.[3–8]

Recently in a dietary rodent model fed *ad libitum*, calcium oxalate crystal formation was associated with activation of mTOR activity and promoted stone formation.[9] Importantly mTOR activity can be inhibited, either pharmacologically with rapamycin and via dietary restriction. Damage of upstream and downstream genes in the mTOR pathway may also modulate mTOR responsivity and act in concert with ROS to age related conditions like metabolic syndrome and nephrolithiasis. *Drosophila melanogaster* (DM) is a convenient model organism for studying nephrolithiasis due to homology between human nephrons and DM Malpighian tubules. If altering mTOR activity can be shown to modulate nephrolithiasis, DM is a uniquely positioned model organism to interrogate how the mTOR pathway may help prevent nephrolithiasis. In the following thesis, we will determine whether the mTOR inhibitor rapamycin has an effect on calcium oxalate concretion formation in a dietary model of calcium oxalate nephrolithiasis in DM.

Introduction

The importance of managing nephrolithiasis

Nephrolithiasis, commonly known as “kidney stone disease”, can be devastating. In addition to pain, nephrolithiasis can harbour bacteria and cause bacterial sepsis, particularly if there is blockage of urine flow by the stone. With each episode of stone, the possibility for acute kidney injury, ureteral stricture, and complications from treatments can occur. Chronic kidney disease, defined as a 3 month period of glomerular filtration rate (GFR) less than 60 ml/min/1.73m^2, elevated markers of kidney damage[10], can occur from prolonged obstruction by stone, by stricture, or by recurrent kidney infections. Observational studies suggest patients with a history of kidney stones are at least twice as likely as other patients to develop chronic kidney disease (CKD).[11] Prospective cohort multivariate analyses have demonstrated that the risk of end-stage renal dysfunction (ESRD) requiring renal replacement therapies like dialysis are more than twice as likely in female patients reporting a history of stones (OR 2.37, 95%CI 1.13-4.96).[11,12]

A significant contributor to the potential morbidity of stone disease it its recurrent nature; as any patient with a first episode of stone has around a 50% chance of having a recurrent episode within 5 years.[13] The indirect morbidity of nephrolithiasis is difficult to quantify yet is significant. A vast majority of trials examining quality of life in kidney stone patients demonstrate lower quality of life (QoL), bodily pain and general health is worse in kidney stone patients compared to control patients.[14] Longitudinal follow up with a follow up of 18 months, suggests that there is no difference patient reported QoL as time from the initial renal colic episode increases.[15] There is also a further loss of QoL related

to repeat procedures as a result of recurrent stone episodes, as well as potentially unsuccessful procedures, and complications related to both stone disease and prior surgical intervention. An estimated 2.1 billion dollars are expended annually in the United States for the direct management of urolithiasis, a figure that has increased 50% since 1994.[27] As nephrolithiasis has its peak in 40s-60s, there is certainly a decrease in work productivity associated with kidney stones.[16] For an average episode of renal colic managed in the outpatient setting, patients may miss anywhere from 5 to 48 hours of productive working time.[17] If the condition of the patient is such to warrant inpatient management, the average patient will miss 2-5 days of work.[17] Altogether, a review of indirect costs from privately insured billings show that the total indirect cost of stone disease may be upwards of $775 million US dollars and 3.1 million days off work per year.[18,19]

Epidemiology of nephrolithiasis

Nephrolithiasis is increasing in prevalence (Figure 1.1).[20] In North America, the prevalence of urolithiasis from 2007 to 2010 was estimated to be 8.8% using the Nutrition Heath Examination Survey (NHANES) data set.[21] Previously, male sex and advanced age were described as risk factors for stones, but from a global perspective there appears to be an increase in stone prevalence regardless of sex, ethnicity or age.[20,22] Kidney stone prevalence increased by 16% annually between 1997 and 2012, a pace that surpassed the increase in obesity over the same time period.[23] The increase in stone prevalence can be partially attributable to the increasing use of cross-sectional and US imaging. Indeed, the Icelandic group led by Edvardsson found an increase in the incidence of urolithiasis from 108 per 100 000 people in 1985 – 1990 to 138 per 100 000 people despite no increase in symptomatic stone rates.[24]

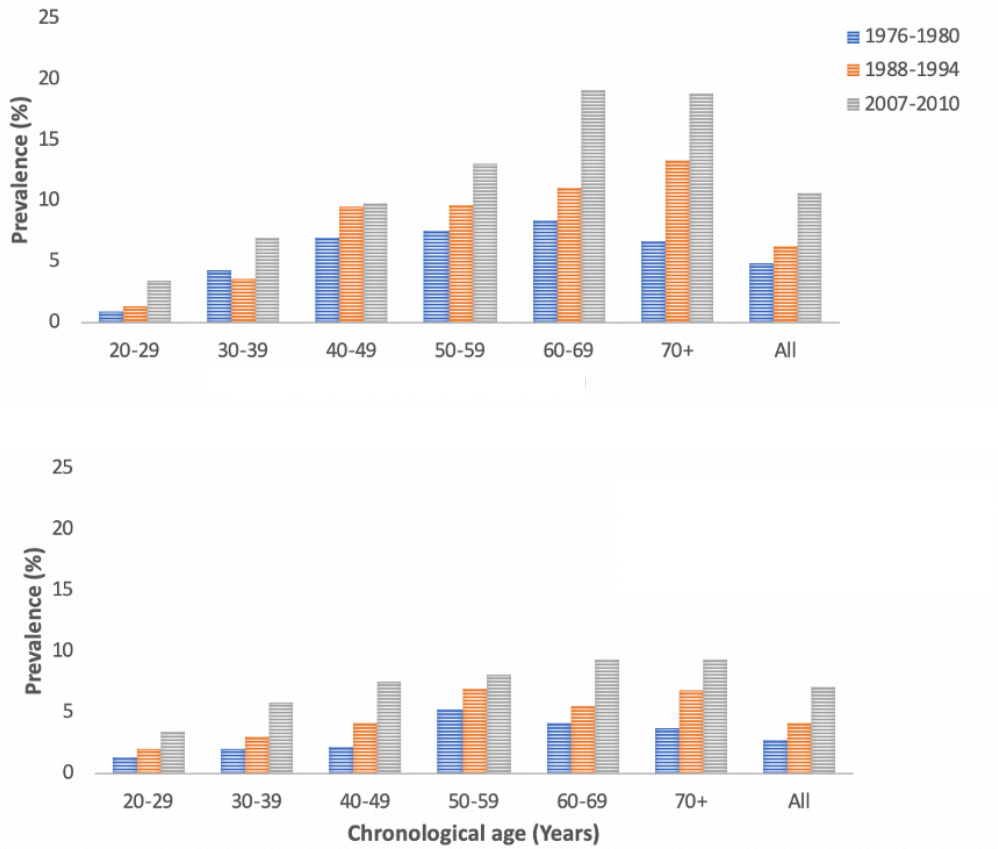


Figure 1. 1 Prevalence of nephrolithiasis in the United States of America by decade from three time periods of NHANES data collection.

Percent prevalence of stones in USA has increased in all age groups by decade in both (A) men and (B) women. The coloured bars indicate different periods of time that prevalence data was collected. Figure generated on Microsoft Excel v 16.44 using data from NHANES I, NHANES II, and NHANES III collection periods. NHANES = National Health and Nutrition Examination Survey.[25,26]

Pathophysiology of nephrolithiasis

Interestingly, Maloney et al have suggested that urolithiasis tends to cluster around regions more so than ethnicity; therefore, metabolic abnormalities and dietary habits may outweigh the genetics shared by a particular ethnic group.[27] It is also well known that nephrolithiasis is more prevalent in hot, arid, dry climates like mountains, deserts or the tropics where people are prone to dehydration. The physical chemistry and pathogenesis of stones are not fully understood; however, reduced fluid intake is inarguably an important mediator of stone formation. At a minimum the supersaturation and nucleation of solutes in urine is required.[28] Nephrolithiasis formation begins at the level of the nephron with the filtration of blood and supersaturation of stone-forming ions in urine. Supersaturation occurs when the concentration product exceeds the solubility product of solutes in urine. Importantly, stone inhibitors in urine greatly increase the solubility product of solutes in urine compared to aqueous solutions lacking these inhibitors. When the concentration product exceeds the metastable range of urine with stone inhibitors in solution, crystal precipitation may occur. This is known as the formation product. In other words, below the solubility product stone crystal formation will not occur, and concentrations of solute above the formation product will lead to stones. Increasing the range of the metastable zone is therefore the focus of prevention of kidney stones.[29]

Once the formation product level of salt-forming substances is reached, crystals require an anchoring site to provide time for crystal growth in order for crystals to reach a clinically relevant size. Under normal conditions, the time required for filtered blood to become urine and pass though the renal collecting system into the bladder via the ureter does not provide sufficient time to allow for significant crystal growth.[30] However, blockage or "plugging" the collecting ducts of the nephron, local areas of urinary stasis, established stone/crystals, damaged cells or even foreign/native materials not normally in the urine can contribute to nidus for stone growth by providing an anchoring site.[28,30]

Non-calcium nephrolithiasis

Stones are classified according to their composition, and are broadly divided into calcium containing stones (oxalate, hydroxyapatite, brushite) and non-calcium containing stones (uric acid, struvite, cystine, triamterene, ammonia, silica, drug and matrix stones).[31] For each of these types of stones, the chemistry of stone formation varies greatly and inhibitors for one type may not impact others. Although the metabolic syndrome may be associated with both uric acid and calcium oxalate stones, for the purpose of this thesis only calcium-based stones will be considered.

Calcium based nephrolithiasis

Calcium based stones are responsible for approximately 80% of urolithiasis cases in the industrial world.[32] Calcium oxalate stones may be either monohydrate or dihydrate and may have a minor or major component of phosphate. Primary calcium phosphate monohydrate (brushite) stones are more rare than calcium oxalate stones but make up approximately 15% of patients with nephrolithiasis.[33] Calcium homeostasis is a complex process involving the parathyroid glands, kidneys, small intestine, and bone. [34] Calcium is absorbed in its ionic state; therefore, impaired calcium absorption can occur when complexed with phosphate, citrate, oxalate, sulfate and fatty acids from diet. As a result, about 600 – 1200mg of calcium intake will lead to about 100 – 300 mg of absorption, primarily by the small bowel. When calcium is low, parathyroid hormone (PTH) through a vitamin D-dependent pathway is required to enhance the transcellular absorption of calcium at the brush border of intestinal epithelia and maximize calcium resorption from kidney. Only about 1-3% of filtered calcium is excreted in the urine due to calcium resorption by the kidney; and areas of the kidney most active in calcium homeostasis include the proximal tubule (60-65%) and thick ascending limb of loop of Henle (25-30%) with the rest being reabsorbed transcellularly in the distal convoluted tubule. Hypercalciuria, or elevated calcium in the urine, is the most common metabolic abnormality associated with calcium-based stones and may result from any abnormality of the usual calcium homeostasis mechanism.[35]

Randall's plaques and progression of nephrolithiasis

There are two main theories regarding the growth of kidney stone crystals. Free particle stone theory requires the crystallization to occur within the nephron. The former theory is supported by the observation that nephron dimensions and physiologic parameters of crystal growth are biologically possible.[30] Furthermore, there is evidence of intermedullary collecting duct "plugs" in certain stone-formers.[36–38] Fixed particle growth theory assumes that crystal adherence occurs around a stationary "anchor site", providing a favorable environment for growth.[39] The stationary site may be exogenous (foreign body) or endogenous in the form of different types of crystals (calcium oxalate stone developing on a calcium phosphate or matrix nucleus), a previous collecting duct "plug", or on a Randall's plaque. Randall's plaques are thought to provide a potential nidus for stone formation. Important work by Evans *et al.* on renal papillae specimens following percutaneous surgery to treat idiopathic calcium oxalate stones revealed that a Randall's plaque may be sub-epithelial and localized to the thin ascending loop of Henle at the level of the renal papilla.[36] They are composed of matrix and calcium apatite material which erode through the urothelium of the renal papilla in order to contact urine and produce stones. Indeed, of 5000 calcium oxalate stones analyzed, calcium apatite stone nidus was present almost universally in calcium oxalate stone formers.[40]

Randall's plaques are thought to be vascular in origin and vascular injury and subsequent repair to the vasa recta or conversion of smooth muscle in nearby blood vessels from a contractile to a secretory phenotype may contribute to the process of erosion through the urothelium.[41,42]

Khan *et al.* provided a unified theory combining both elements of free and fixed crystal theory.[42] In this unified theory, an excess of stone forming substrate, or a lack of stone inhibitors may lead to free crystals. Upon contact with these free crystals, renal epithelial cells may become overwhelmed with maintaining homeostasis causing an increase in oxidative stress which subsequently results in paracrine signalling, inflammation and de-differentiation of nearby cells. Some cells may then acquire a secretory phenotype and deposit calcium phosphate crystals, and this combined with increased sub-epithelial metabolism of connective tissue may promote the growth of a plaque. With time and further metabolism, the plaque erodes through the epithelium where it can be exposed to urine and stone may form.[42]

Reactive oxygen species, and nephrolithiasis

Reactive oxygen species (ROS) are unstable oxygen containing molecules that easily react with other macromolecules.[43] At high levels, they damage organelles and lead to mitochondrial dysfunction contributing significantly to inflammation and cell death.[44] Autophagy – the process a cell uses to break down its own macromolecule polymers – is a mechanism for resistance to cellular stress created by ROS.[44] Adhesion and internalization of calcium oxalate crystals in the renal tubular epithelium results in upregulation of several genes involved in energy metabolism including a major source of ROS, NADPH oxidase, at the level of the mitochondria.[45] Excessive apoptosis, inefficient autophagy and locoregional inflammatory signalling exacerbates calcium nephrolithiasis.[46]

Inflammation may trigger mineralization of renal interstitial collagen, assisting the growth of Randall's plaques and eventual renal epithelial sloughing.[47] Previous authors have shown that treatment strategies directed at reducing generation of ROS like administration of antioxidant N-acetylcysteine or NADPH oxidase inhibitor apocynin can reduce ROS, prevent mitochondrial dysfunction and subsequently reduce nephrolithiasis progression in a lab model.[48]

Inhibitors of nephrolithiasis

The presence of naturally occurring stone inhibitors in solution explains why supersaturated urine does not necessarily lead to crystal nucleation, growth, aggregation and adherence to renal epithelium.[28] Indeed non-stone formers appear to have more stone inhibitors in their urine compared to those who form stones.[49,50] These inhibitors range from ions to large multi-residue amino acids produced at the level of the nephron. Small molecules that have shown the ability to raise the solubility of calcium-based stones include inorganic citrate, pyrophosphate, and, magnesium.[51] In an *ex*-vivo model of supersaturated calcium phosphate solution, the combination of inorganic pyrophosphate, citrate and magnesium contributed to approximately 20% of the inhibitory effect of whole urine on calcium phosphate crystal formation. The role of citrate in stone prevention relies on its presence in urine as a complex with calcium, thereby preventing association of calcium with oxalate or phosphate. Citrate also leads to alkalinization of urine and exacerbate calcium phosphate stones if urine pH is too alkaline.[35] Magnesium may chelate with oxalate with a much higher affinity than calcium, reducing the opportunity for calcium and oxalate molecules to complex.[52]

Macromolecules involved in stone inhibition include glycosaminoglycans, acid mucopolysaccharide, RNA, and glycoproteins.[28,53] In this class of inhibitors, extensive intracellular processing in the rough endoplasmic reticulum and post-translational modifications prior to secretion are required for proper physiologic function. Heparin sulfate is the most commonly referenced stone-inhibiting glycosaminoglycan, and is consistently found in lower concentrations in recurrent calcium oxalate monohydrate stone formers compared to non-stone formers.[54,5549,50] Conversely, glycosaminoglycans appear to be a significant component of apatite matrix in plaques found in Randall's plaques.[56] Others have suggested that this observation may be simply non-selective presence in matrix due to their relative abundance in urine as albumin and uromodulin are also present in high amounts.[49] Rather, highly anionic proteins and cationic proteins normally found in the intracellular or nuclear compartments represent a disproportionate percentage of stone matrix compared to their abundance in urine.[49]

There are also several glycoproteins that may act as stone inhibitors; however, the two most important are nephrocalcin and Tamm Horsfall protein (THP) or uromodulin.[57] Nephrocalcin is a highly acidic protein with four isoforms produced by renal epithelial cells in the proximal tubule and thick ascending loop of Henle.[28] Different isoforms of nephrocalcin have been shown to have different gamma-carboxygltamic acid residues and thus varying affinities for calcium. This may be clinically relevant as human stone formers have been shown to fewer of the strong-inhibitor isoforms and instead produce more weak-inhibitor isoforms.[58,59] In addition, all isoforms sampled from stone-formers had fewer gamma-carboxyglutamic acid residues compared to equivalent isoforms from non-stone formers.[58,60]

THP is the most common protein found in human urine and is produced by the renal epithelia of the thick ascending loop of Henle and distal convoluted tubule. It is a membrane-anchored protein that requires cleavage by phospholipase or protease for release into urine where it likely has multiple roles including prevention of urinary tract infections (UTIs), inhibition of calcium oxalate monohydrate crystal aggregation in alkaline urine, and as a regulator of endocytosis of TRPV5 and TRPV6 channels required for transcellular Ca^{2+} resorption in the kidney.[28,59,61] Indeed genome-wide association studies as well as animal knockout models suggest that THP is a significant inhibitor of calcium oxalate nephrolithiasis.[41,62,63]However, in acidic urine, THP has the potential to change conformation and polymerize such that it encourages crystal aggregation, facilitating potential nidus for stone.[53]

General prevention of nephrolithiasis

Preventative strategies have in the past focused on addressing dietary , anatomical and metabolic risk factors; however, the complete pathophysiology underlying these recommendations is not known.[28,64] General advice includes increased fluid intake to produce >2L urine per day after first stone episode, avoiding dehydration (occupational or adverse arid climate exposure), reducing dietary sodium/animal products, and identifying underlying medical or anatomic reason for stone formation.[35] Unfortunately, there is currently no evidence in favor of the primary prevention of nephrolithiasis.[65] Secondary prevention can be achieved through the targeting of identifiable metabolic stone parameters, for example increasing citrate intake, moderating oxalate intake, and ensuring adequate calcium intake.[35] Much of the difficulty in providing new effective recommendations, particularly when there is no identifiable risk factor, relates to the relative lack of animal models of nephrolithiasis in the context of reactive oxygen species generated by metabolic syndrome.[64]

mTOR and nephrolithiasis

The association of metabolic syndrome with calcium oxalate urolithiasis is an active area of current research.[64] In particular, ROS associated with metabolic syndrome may increase biomineralization on Randall's plaques and exacerbate oxidative damage from hyperoxaluria.[41,66] Calcium oxalate crystal adherence to renal urothelium triggers an inflammatory cascade and has been shown to exacerbates stone formation in both *in vivo* and *in vitro* models.[9,67] Furthermore, ROS may upregulate epithelial to mesenchymal transition (EMT) of renal epithelial cells to osteoblast-like cells and the ensuing formation of Randall's plaques.[47] In addition, further mesenchymal activity results in the excessive production of matrix degrading proteins and enzymes thereby allowing the plaques to erode through the urothelium and produce a nidus for stone formation.

Interestingly, mTOR has been described to affect both EMT as well as inflammatory responses in various other tissues including cervical[68], pulmonary[69] and renal tissues affected by diabetic nephropathy[70]. Although there is a paucity of data in the literature, there have been suggestions that mTOR is implicated in the pathogenesis of obesity-associated renal tubulointerstitial inflammation and urolithiasis.[41,71] In one study comparing 27 non-obese to 35 obese patients, urinary markers of tubulointerstitial inflammation including monocyte chemoattractant protein 1 (MCP-1) and neutrophil gelatinase associated lipocalin (NGAL) were elevated in the obese cohort.[41] Furthermore, examination of kidneys from a high-fat diet rat model of obesity showed evidence of increased inflammation that was reversed by rapamycin treatment.[71]

Metabolic syndrome, aging, and, nephrolithiasis

Interestingly, stone disease is increasing in parallel with the increasing prevalence of metabolic syndrome, which affects 1 in 4 Americans and is rising in pediatric populations. [1,72] Metabolic syndrome has multiple definitions. However, it is universally defined by the presence of insulin resistance, obesity, hypertension, and dyslipidemia and leads to an increased risk of cardiovascular disease and accelerated atherosclerosis.[1,72,73] A meta-analysis of large retrospective datasets suggests that metabolic syndrome is correlated with increased risk of self-reported or imaging findings of urinary stone disease.[73] At the individual level, patients with a higher BMI have increased prevalence of recurrent symptomatic urolithiasis, 24-hour urinary excretion of oxalate, sodium, uric acid, calcium, and phosphorous as well as lower urine pH.[74,75] Hyperinsulinemia, an eventual end result of the metabolic syndrome, has been associated with hypercalciuria in obese patients who form stones.[76,77] The risk of calcium and uric acid based stones is higher for obese individuals.[2,78] Furthermore, even with accounting for confounding risk factors, nephrolithiasis has been associated with the complications of metabolic syndrome, specifically myocardial infarction and accelerated atherosclerosis.

There exists differences in the rates of urinary stone disease amongst different racial groups. Despite all ethnicities suffering the burden of urolithiasis, the highest prevalence of nephrolithiasis is in Caucasians and is positively correlated with income and a sedentary lifestyle.[25] For example, Soucie et al has shown that the highest prevalence of stone disease in Caucasians followed by Hispanic, Asian, and, African Americans.[79] Examination of emergency department demonstrate differences in the gender predominance of stone disease in different ethnicities. For example, Dall'era et al showed a male to female ratio of 2.05 for Caucasians compared to 1.17 for Hispanics. Furthermore, Sarmina et al in 1987 described an incidence in symptomatic nephrolithiasis with a male: female ratio of 2.03 in Caucasians vs a 0.63 ratio in African Americans.[80] This finding of increased male predominance appears to hold true today, however the authors of a study in South Carolina in 2019 have suggested that the greatest increase in stone disease were observed in adolescents, females and African American patients.[23] Strikingly, the prevalence of stone disease doubled in children and there was a 45% increase in lifetime risk in women over the course of this study period.[23]

Age has an interesting relationship with both nephrolithiasis and metabolic syndrome. According to Stats Canada, the percentage of adults who are obese increases with increasing age.[81] Between the ages of 40 and 60, over 70% of men and 60% of women are obese or overweight in Canada.[81] In contrast, 8.5% of children aged 5 to 9 years old are obese; however the prevalence appears to be rising.[82] Historically, the incidence of nephrolithiasis peaks beginning at age 40 and is more common until age 60; however, in parallel to the increasing rates of obesity, it is becoming more common for kidney stones to affect people under the age of 20.[28].[83,84] Interrogation into a national pediatric database including all admissions, emergency and outpatient visits from 1999 – 2008 related to nephrolithiasis revealed an increase in paediatric patients treated for stones from 18.4 per 100 000 in 1999 to 57 per 100 000 in 2008.[85] Although consistent evidence exists for a trend in the annual increase in pediatric nephrolithiasis, the pathophysiology behind this observation has been elusive. Ultimately, obesity and metabolic syndrome have been suggested as comorbid conditions that may have a role in promoting nephrolithiasis, or share common risk factors, with nephrolithiasis formation.[2,23]

mTOR pathway and metabolic syndrome

Recently, the mTOR pathway has been suggested as a key pathway at the crossroads of urolithiasis and metabolic syndrome. mTOR is a highly conserved serine/threonine protein kinase recognized as a central coordinator of growth and metabolism which is found from yeasts to higher order organisms including insects and mammals (Figure 1.2).[86]

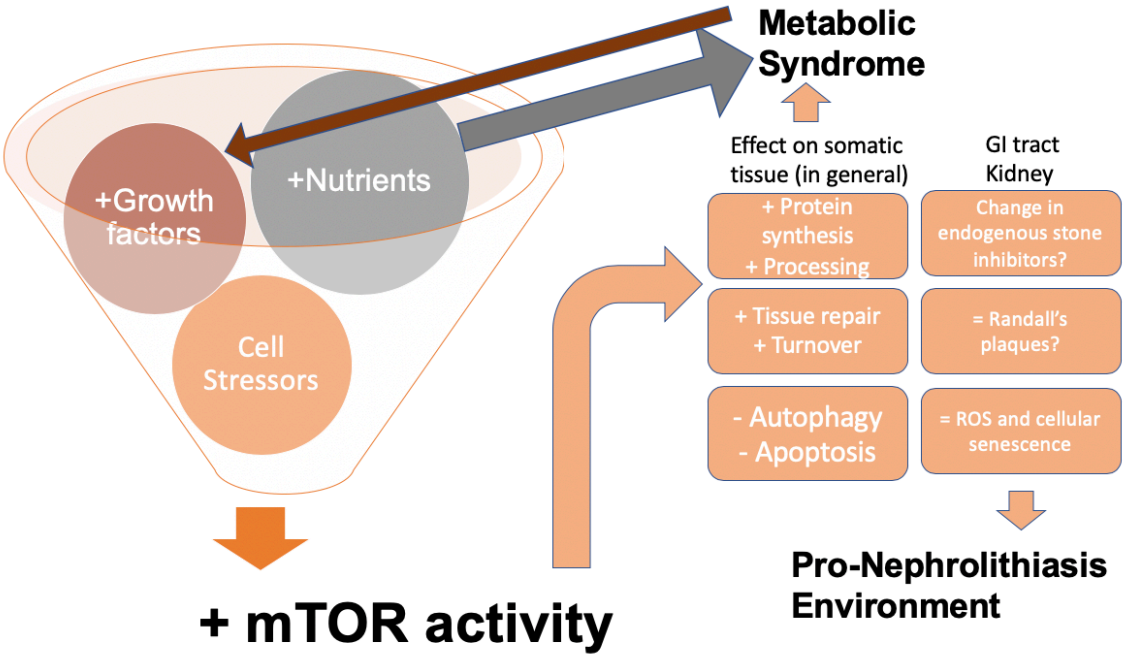


Figure 1. 2 An overview of the mTOR pathway as a mediator of the correlation between metabolic syndrome and nephrolithiasis.

The positive influence of various pro-growth signals including growth factors, abundant micronutrients and environmental stimuli lead to an increase in mTOR activity. The aggregate effects of mTOR activity are pro-anabolic at the cellular level. In general, tissue growth leads to the metabolic syndrome phenotype. In parallel, the effects of mTOR specifically at the local level of the kidney or gastrointestinal (GI) tract may promote nephrolithiasis by several mechanisms correlated with increased mTOR activity. For example, an increase in protein synthesis and post-translational processing may lead to an increase in defects in the processing of macromolecules.Defective macromolecules may still function reasonably well in most cases, but stone inhibitor proteins that are associated with nephrolithiasis might not provide the same protection against kidney stones. The impact of reduced mTOR activity on tissue repair and turnover may accelerate the erosion of Randall's plaques. Altered reactive oxygen species and

epithelial barrier function of GI tract and kidney urothelium may also be affected by mTOR inhibition or rapamycin directly.

When mTOR is activated, its core protein (Figure 1.3A) joins other proteins to form one of two complexes: mTORC1 or mTORC2 (Figure 1.3B and 1.3C). The appropriate subcellular location and specification of mTORC1 is defined by the binding of mTOR to the regulatory protein associated with mTOR (Raptor).[3] Raptor is a scaffolding protein that facilitates and stabilizes protein-protein interactions for mTOR substrate proteins to the TOR signaling motif (TOS). In addition, mTOR associated protein, LST8 homolog (mLST8) and Deptor proteins are also required for mTORC1 activity, as it helps stabilize the active catalytic site of mTOR protein kinase domain (Figure 1.3B).[3,87,88]

Tuberous sclerosis complex (TSC) protein is an upstream regulator of mTORC1 (Figure 1.4A). [3] When TSC is active, it inhibits mTORC1. Canonical signaling cascades impacting mTORC1 activity include IGF-1/AKT/PTEN, MAPK-Erk, Wnt and TNF-alpha pathways.[87] In particular, ATP deprivation during starvation activates AMPK. The active AMPK protein then inhibits mTORC1 via Raptor phosphorylation. Ultimately, ATP deprivation leads to activation of TSC2 through the signalling cascade above.[88,89]

CASTOR is another component of mTORC1 that allows for detection of lysosomal amino acid concentrations.[90] When amino acids are present in adequate amounts, RagGTPases bind to CASTOR. When CASTOR is bound by RagGTPase, there is a conformational change that occurs increases Raptor's affinity its mTOR binding site, stabilizing the mTORC1 complex. Therefore, effective mTORC1 signaling is only possible with appropriate growth signals and nutrient availability.[86]

mTORC1 activity couples growth signals with nutrients available in the extracellular milleu in order to promote anabolic cellular activities (Figure 1.4B). S6 Kinase (S6K) is downstream of mTORC1 activity and is the protein responsible for the initiation of mRNA translation/processing.[61] Transcription factors required for de novo lipid synthesis are regulated by mTORC1 through the sterol responsive element binding protein (SREBP), which is activated through the activation of S6K and the inhibition of Lipin 1.[86] In addition, *de novo* purine and pyrimidine synthesis is upregulated through mTORC1 activity.[3,4] Finally, through hypoxia inducing factor alpha (HIF1a) and c-Myc mTORC1 activity leads to increased glycolysis. Through this process, NADPH and other intermediates of the citric acid cycle are produced which use abundant environmental energy and macromolecules and promote organism growth.[91]

Importantly, mTORC1 activity is associated with reduced autophagy. When nutrients are abundant, mTORC1 acts via AMPK to phosphorylate and inhibit ULK1, which subsequently serves to inhibit autophagy. In addition to impairment of apoptosis under inappropriate conditions, lysosomal biogenesis and creation of proteins required for autophagy machinery is inhibited by mTOR through protein TFEB. Therefore, active mTORC1 appropriately limits macromolecule recycling through autophagy in settings where building blocks for such macromolecules are available in the environment (Figure 1.5A).

In contrast, mTORC2 is responsible for cellular proliferation, cell-cell interactions and cytoskeletal rearrangement (Figure 1.4B and 1.5B). mTORC2 is defined by mTOR binding to rapamycin insensitive companion of mTOR (RIctor) in addition to mLST8. The rapamycin-FKBP12 complex does not bind to mTOR when it is bound to Rictor as mTORC2, however chronic use of rapamycin may limit the number of mTOR molecules and lead to insulin resistance as it can inhibit the downstream AKT/PKB pathway.[92] Downstream actions of mTORC2 includes cytoskeletal remodeling and insulin signalling. In addition, mTORC2 is also involved in cell survival, endocytosis/exocytosis, and ion transportation through the regulation of serine/theonine-protein kinase-1 (SGK1) (Figure 1.5B).[3]

In summary, the physiologic role of mTOR is conserved in a wide range of species. These are accomplished through two protein complexes: mTORC1 and mTORC2 that have different downstream pathways. Common intracellular processes involving mTOR include glucose homeostasis, adipogenesis, lean mass homeostasis, immune function, autophagy associated with aging and cellular proliferation.[3,86] Since the mTOR pathway controls the cell recognizing cues to grow in its environment, manipulation of this pathway may influence the development of metabolic syndrome and likely other physiologic processes dependent on the trafficking of solutes such as nephrolithiasis (Figure 1.2).

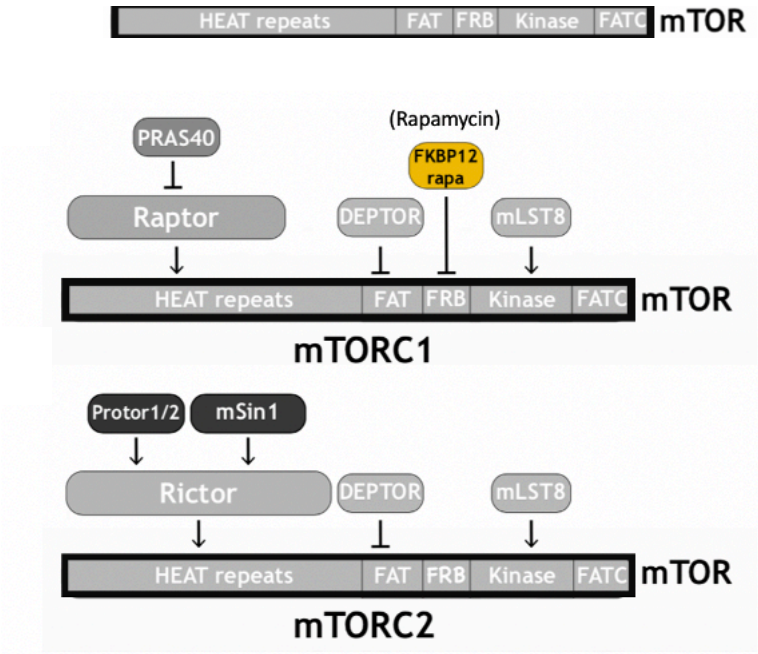
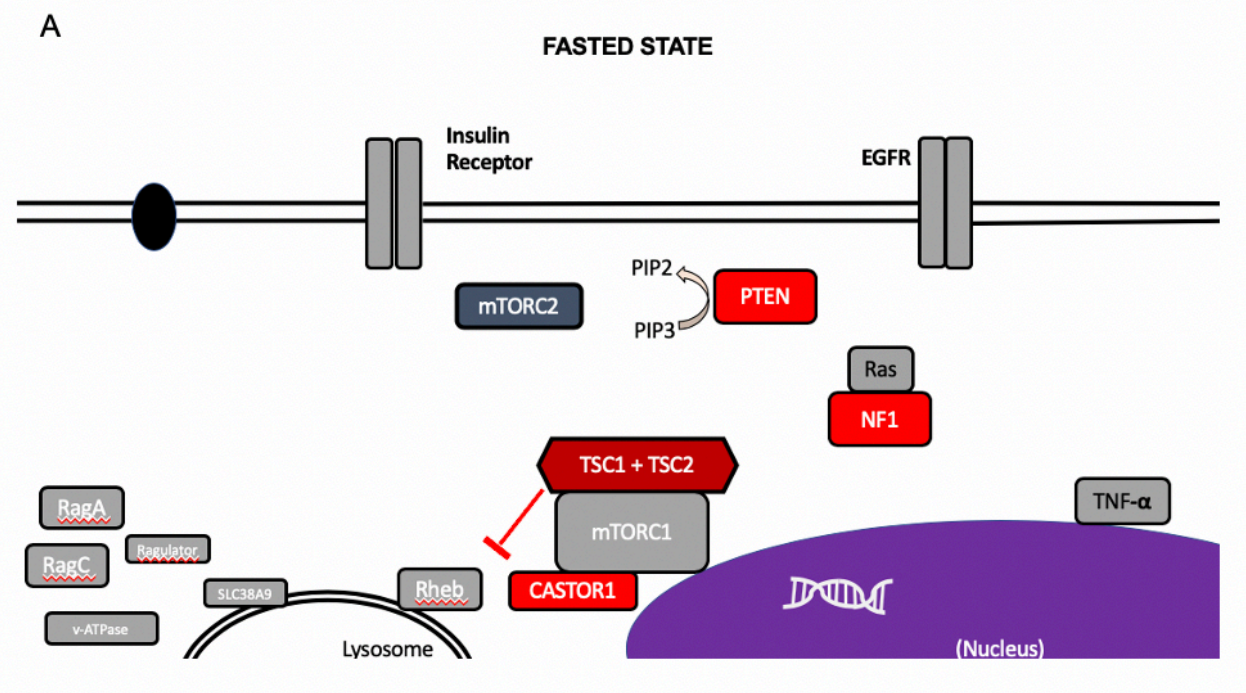


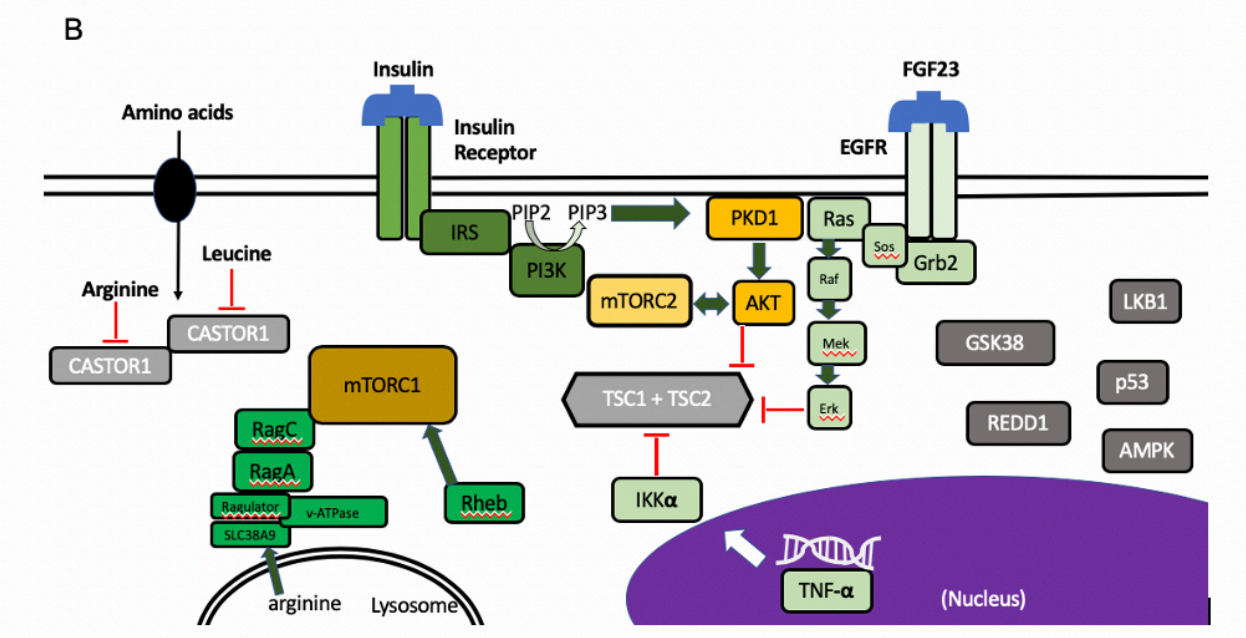
Figure 1. 3 (A) The core mTOR protein has several domains required for proper function and regulation.

The mTOR core protein executes its function via its kinase domain near the c-terminus of the protein. HEAT = Huntingtin, Elongation Factor 3, Protein Phosphatase 2A, mTOR (acronym of four proteins in which this repeat structure is found), FAT = Focal Adhesion kinase Targeting, FRB = FKBP12-Rapamycin Binding, FATC= Focal Adhesion kinase Targeting C-terminal. (B) The mTORC1 complex is defined by the interaction of the mTOR core protein with a scaffold protein called Raptor. Rapamycin inhibits mTORC1 complex activity by complexing with FKBP12 and binding the mTOR core protein FRB domain. The regulation of mTORC1 is complex and can occur through interactions at other domains. For example, the PRAS40 protein inhibits Raptor's ability to complex with the mTOR core protein. Other proteins known to inhibit mTORC1 complex activity independent of Rapamycin-FKBP12 include Deptor and mLST8. Raptor = Regulatory associated protein of mTOR. PRAS40 = proline rich Akt (acutely transforming retrovirus AKT8 in rodent T cell lymphoma) substrate of 40 kDa. FKBP12 = FK506/Tacrolimus

Binding Protein 12. (C) The mTORC2 complex is defined by interactions with the Rictor protein.

In order for Rictor to complex with the mTOR core protein, Protor1/2 and mSin1 must be present. Note: Rapamycin-FKBP12 does not directly inhibit mTORC2. Other proteins known to inhibit both the mTORC1 and mTORC2 complexes, independent of Rapamycin-FKBP12. These include Deptor and mLST8. Raptor = Regulatory associated protein of mTOR. Rictor = Rapamycin insensitive companion of mTOR, Protor1/2 = Protein observed with Rictor 1 and 2, mSin1 = mammalian stress-activated protein kinase interacting protein 1, Deptor = DEP (Dishevelled, Egl-10, Plekstrin) domain containing mTOR-interacting protein. mLST8 = mammalian Lethal with SEC13 protein 8.[3]


A
FASTED STATE
Insulin Receptor
EGFR
PIP2
PIP3
PTEN
mTORC2
Ras
NF1
TSC1 + TSC2
mTORC1
RagA
RagC
Regulator
SLC38A9
v-ATPase
Rheb
CASTOR1
TNF-α
Lysosome
(Nucleus)


B
Insulin
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FGF23
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Sos
Grb2
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Raf
Leucine
Arginine
mTORC2
AKT
LKB1
CASTOR1
CASTOR1
Mek
GSK38
mTORC1
TSC1 + TSC2
Erk
p53
RagC
REDD1
RagA
AMPK
Regulator
v-ATPase
Rheb
IKKα
SLC38A9
arginine
Lysosome
TNF-α
(Nucleus)

Figure 1. 4 Negative regulators of mTORC1 and mTORC2.

In the absence of amino acids and growth factor activity, mTORC1 is sequestered by CASTOR1 protein in the cytoplasm of the cell. Furthermore, scaffold protein Rictor is not activated by Rheb and lysosomal scaffold composed of Rag proteins are not assembled. Inactivity of mTORC2 is maintained by conversion of PIP3 to PIP2 by PTEN and Ras sequestering by NF1. (B) Major positive regulators of mTORC1 and mTORC2. Amino acids, particularly arginine and leucine are necessary but not sufficient for mTOR activity by preventing CASTOR protein sequestering of mTORC1. Insulin, FGF and EGF are key growth factors that promote mTOR activity via their G-coupled protein receptors and downstream intracellular signalling pathways involving phosphoinositol metabolism and Ras cascade. The active mTORC2 affects Akt and vice versa, potentiating the downstream effects of both insulin-response and mTORC2 signalling pathways. Finally, the insulin and fibroblast/epidermal growth factor pathways, as well as the products of inflammatory signalling via NF-κB, lead to inhibition of the TSC1/2 complex and permit mTORC1 localization at level of the lysosome whereby it is required to function. CASTOR = Cytosolic arginine sensor for mechanistic target of rapamycin complex 1, Rag = Recombination activating gene, Ragulator = Regulator of Rag, v-ATPase = v-Type adenosinetriphosphatase, SLC38A9 = Solute carrier family 38 member 9, Rheb = Ras homolog enriched in brain, IRS = insulin response substrate, PI3K = Phosphoinositol-3-kinase, PIP = Phosphatidylinositol, PIP-2 = Phosphatidylinositol 4,5-bisphosphate, PIP-3 = Phosphatidylinositol 2,4,5-trisphosphate, AKT = Ak strain transforming, PKD1 = Polycistin 1, Ras = Rat sarcoma protein, Raf = Rapidly accelerated fibrosarcoma protein, MEK = Mitogen-activated protein kinase/extracellular signal-regulated kinase, ERK = Extracellular regulated kinase, TSC1/2 = Tuberous sclerosis complex 1 and 2, TNF-α = Tumour necrosis alpha, IKK α = IκB kinase alpha, GSK3B = Glycogen synthase kinase 3B, LKB1 = Liver kinase B1, AMPK = AMP-activated protein kinase, PTEN = Phosphatase and tensin homolog protein, NF-1 = Neurofibromatosis type 1 protein.[3]

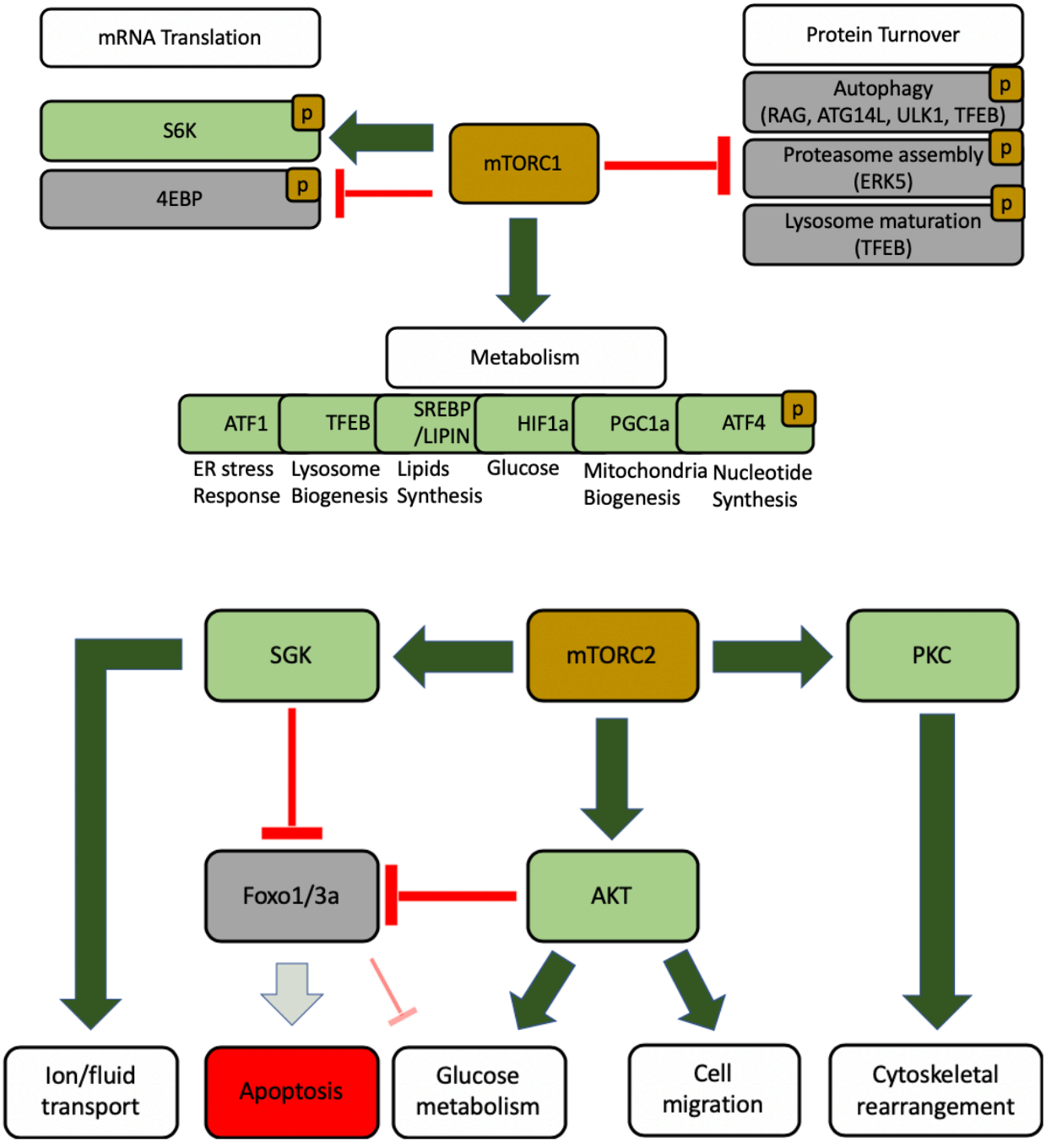

mRNA Translation
S6K
p
4EBP
p
mTORC1
Protein Turnover
Autophagy
(RAG, ATG14L, ULK1, TFEB)
p
Proteasome assembly
(ERK5)
p
Lysosome maturation
(TFEB)
p
Metabolism
ATF1
TFEB
SREBP
/LIPIN
HIF1a
PGC1a
ATF4
p
ER stress
Response
Lysosome
Biogenesis
Lipids
Synthesis
Glucose
Mitochondria
Biogenesis
Nucleotide
Synthesis
SGK
mTORC2
PKC
Foxo1/3a
AKT
Ion/fluid
transport
Apoptosis
Glucose
metabolism
Cell
migration
Cytoskeletal
rearrangement

Figure 1. 5 The downstream effects of mTORC1 and mTORC2 signalling normally complement one another.

(A) The mTORC1 complex is responsible for the increase in basic anabolic properties of mTOR activity like increased glucose utilization, mRNA translation, positive net protein balance and cellular supply of nucleotides, sterols. S6K = Serine-6-Kinase Protein, 4EBP = 4E-Binding Protein, ATF1 = Activating Transcription Factor 1, TFEB = Transcription factor EB, SREBP = Sterol Regulatory Element Binding Proteins/, HIF1α = Hypoxia Inducible Factor 1-alpha, PGC1 α = Peroxisome proliferator activated receptor Gamma Coactivator 1-alpha, ATF4= Activating Transcription Factor 4. (B) The mTORC2 complex is chiefly involved in survival processes of the cell undergoing stress including cell motility and ion/fluid regulation. SGK = Serine/Threonine-protein Kinase, Foxo1/3a = forkhead box class-O protein 1 and 3a, PKC = Protein Kinase C, AKT = Ak strain Transforming protein.[3]

Autophagy and mTOR

Autophagy is a catabolic process that lies at the interface of reactive oxygen species and cell stress response.[93] The goal of autophagy is to degrade damaged organelles or proteins in order to release stored energy or re-purpose nutrients that the cell requires.[94] The first step in autophagy involves ROS or reactive nitrogen species (RNS) triggering signalling cascades.[95] Subsequently, rearrangement of cytoplasm compartmentalizes the substance of interest to be degraded into autophagosomes.[94] Autophagosomes then fuse with lysosomes where hydrolase enzymes catalyze degradation.[96]

mTOR dependent phosphorylation of ATG13 suppresses ULK1 and prevents activation by AMPK a key activator of autophagy.[94,95] mTOR regulates lysosomal function

and biogenesis through phosphorylation of TFEB that keeps it in a cytosolic location when active.[90]

Autophagy can also be regulated by ROS and other pathways in a non-mTOR dependent manners, adding to the complexity of this process.[97] Age-related diseases like metabolic syndrome and cardiovascular disease exhibit reduced efficiency of autophagy.[98] Inefficient autophagy results in impaired tolerance of ROS and subsequent cell death or further damage[99]

Rapamycin and inhibition of mTOR

Rapamycin is a molecule isolated from *Streptomyces hygroscopius* found in the soil of Easter Island (Rapa Nui).[100] Rapamycin requires a protein called FKBP12 in order to inhibit mTOR.[101] The rapamycin-FKBP complex is an allosteric partial inhibitor of mTORC1 kinase and binds directly to mTOR at its FRB domain[3]. Therefore, when mTOR is inhibited by rapamycin, catabolic processes such as autophagy are upregulated and drive sub-cellular recycling machinery. In addition, PI3 kinase signaling required for insulin signaling is inhibited by rapamycin.[102,103] The benefits of mTORC1 inhibition may be limited to acute dosing of rapamycin; however, as chronic exposure to rapamycin may exacerbate diabetogenic phenotypes.[104] With less mTOR protein available to form mTORC2, cells cannot orchestrate subsequent processes required for appropriate glucose homeostasis.[105]

Clinical Uses of Rapamycin and Side-Effects

Rapamycin is indicated in the treatment of lymphangioleiomyomatosis,[106] as part of immunosuppression regime for renal transplant[107] and a component of drug eluting stents for percutaneous coronary artery revascularization after myocardial infarction.[108] Rapamycin has also been used off-label to treat autosomal dominant polycystic kidney disease (ADPKD),[109] renal angiomyolipomas,[110] and other solid-organ transplants (heart, lung, and liver).[111] Renal transplant immunosuppression regimes may include rapamycin mycophenolate mofetil but usually include prednisone induction, a calcineurin inhibitor (cyclosporin or tacrolimus) or mycophenolate mofetil.[112] To our knowledge, rapamycin (or immunosuppression in general) has never been suggested as part of a preventative strategy for nephrolithiasis.

Importantly, rapamycin has well described serious complications from chronic use: type 2 diabetes mellitus, hypertriglyceridemia, bone marrow toxicity (anemia 12-76%, leukopenia 11%, thrombocytopenia 30%), gastrointestinal irritation (15-20%), oral ulcers (10-19%), thromboembolic diseases (17%), interstitial lung disease (4-17%), angioedema/lymphedema (2.2-15%), proteinuria (10%), glomerulonephritis (2%).[111] The therapeutic range of rapamycin to avoid toxicity while preventing renal transplant rejection is between 4-20 µg/L.[113] Oral bioavailability of sirolimus is approximately 15% with a large volume of distribution in humans including in red blood cells and fatty tissues (5.6-16.7 L/kg).[114] The half-life of rapamycin is dependent on metabolism by cytochrome P-450 3A4 (CYP3A4).[114] Rapamycin is rapidly absorbed, metabolized with time to peak dose of 1 hour, and has a half-life between 39.3 – 86.5 hours.[115] CYP3A4 has many substrates including estradiol and may explain some of the variation in drug clearance between men and women.[114,115]

Specifically, rapamycin has been associated with obesity and metabolic syndrome in the kidney transplant population.[116] Several studies have shown an increased risk of developing new-onset type 2 diabetes mellitus (NOTDM) after conversion from a calcineurin inhibitor to rapamycin (sirolimus) as part of immunosuppression rejection prophylaxis.[117–119] Additionally, NOTDM has been associated with an increased cardiovascular mortality and hypertriglyceridemia after kidney transplant.[120] Despite a high prevalence of obesity and metabolic syndrome in the kidney transplant population, the incidence of kidney stones is reported between 1-1.7% it is generally a rare complication and some argue lower than in the general population.[121,122]

Clinical correlate of overactive mTOR: Autosomal dominant polycystic kidney disease and risk of nephrolithiasis

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease with a prevalence of 1 in 1000 to 1 in 2500 individuals.[123] It is characterized by multiple cysts affecting both kidneys. Individuals with ADPKD typically develop urolithiasis earlier than the general population, at a median age of 37.[83,123] The formation of urolithiasis in ADPKD patients has been associated with higher rates of hyperoxaluria and decreased ammonia excretion.[85] Mao and colleagues have shown that the formation of urolithiasis in ADPKD patients is associated with higher rates of hypocitraturia, hypomagnesuria, low urine pH, impaired uric acid metabolism, and hypophosphatemia compared to stone formers without ADPKD.[124] Additionally, ADPKD patients have been shown to have an increased incidence of post-transplant diabetes mellitus, lipid abnormalities and cardiovascular disease risk compared to all renal transplant recipients.[124]

A multitude of genes are associated with this condition, however the most classical genes are PKD1 and PKD2 and are typically inherited in an autosomal dominant manner.[125] However, ADPKD has also been described to occur without a positive family history; implying a possible de-novo mutation, germline/somatic mosaicism or epigenetic changes to PKD1/PKD2 gene products.[126]

Loss of these proteins lead to reduced intracellular calcium and reduced PDE1 activity, resulting in excessive cAMP production and cystogenesis through activation of proliferation and secretion pathways.[127] In the absence of PKD1, there is aberrantly active mTOR, and in fact high levels of mTOR activity have been observed in models of ADPKD.[88]This may result through the interactions of PC1 and TSC2 which typically inhibit mTORC1 formation and is known to be inactivated in certain models of ADPKD.[88]

Historically, the development of nephrolithiasis in ADPKD has been thought to be related to CKD and mechanical obstruction from the size of cysts.[128] Recommendations for these individuals includes maintaining a healthy lifestyle and diet, avoiding smoking and non-steroidal anti-inflammatory medications (NSAIDs).[129] Vasopressin receptor inhibition, either through drinking water or tolvaptan, leads to decreased cAMP and may prevent downstream activation of mTOR and other pro-growth pathways; thereby decreasing cystogenesis.[129] Initial murine models investigating mTOR inhibition were promising in preventing the progression of PCKD; however, phase 3 clinical trials assessing everolimus and rapamycin failed to demonstrate decreased disease progression indicated by total kidney volume.[109,128]

Calcium oxalate crystals activate mTOR and exacerbate nephrolithiasis

Unno *et al* recently suggested that autophagy is crucial in ensuring an appropriate homeostatic response to calcium oxalate monohydrate exposure and that this can be perturbed by an abundance of calcium oxalate monohydrate in the environment. In addition, less calcium oxalate monohydrate was required in the presence of an autophagy inhibitor 3-methyladenine. Finally, a more select mTORC1 inhibitor called Torin1 was shown to increase autophagy and may protect cells from the effect of downregulated autophagy caused by calcium oxalate monohydrate exposure. With more efficient autophagy, less inflammation and crystal aggregation was observed.

Importantly, when mice were treated with rapamycin after 4 days of oxalate administration, they had less calcium oxalate crystal deposition in their kidneys. The authors concluded that increased mTOR activity is a major cause of compromised autophagy by oxalate administration and that this can be remedied by mTOR inhibition *in vivo*. [9] The presence of phosphorylated SQSTM1, which serves as a marker of impaired autophagy, has been shown to be present in the renal mucosa of stone-forming kidney specimens, and conversely lacking in non-stone forming samples.[9]

Drosophila melanogaster as a model of nephrolithiasis

DM has been used as a model organism to study the effects of rapamycin and mTOR as well as obesity, heart disease, insulin signaling and ROS generation in metabolic syndrome-like states.[130–132] When DM is subjected to excess exogenous fat, cardiac cells and liver cells accumulate fat in addition to elevated hemolymph triglycerides which is analogous to humans.[130] In addition, insulin leads to activation of GLUT4 insulin-dependent glucose transporter and a feed-forward insulin release in both DM and mammals; therefore, DM also a physiologically relevant model for diseases of insulin-resistance despite initial concerns regarding different sugar metabolism than in humans.[107] Indeed, the mTOR pathway has shown to be overactive in a high-fat diet induced obesity model using DM.[130] In this model, inhibition of mTOR via rapamycin has been shown to protect the heart and internal organs in DM from elevated hemolymph triglycerides.[130]

The DM model of renal stone disease is a well validated model to study urolithiasis due to its remarkable homology to humans (Figures 1.6, 1.7, and, 1.8).[133,134] The basic role of the urinary system is to filter, reabsorb and secrete solutes from the blood to and from the urine. Once created, the urine is to be eliminated. In humans, the formation of urine is accomplished by the nephron: which is comprised of the glomerulus, proximal tubule, loop of Henle, distal tubule and collecting duct which does all of the filtering, resorption and secretion (Figure 1.6A).[34] The human glomerular vasculature is lined by podocytes. Fluid then passes through the podocyte filter based on net interstitial pressure, luminal pressure, interstitial osmolarity and luminal osmolarity (Figure 1.6B).

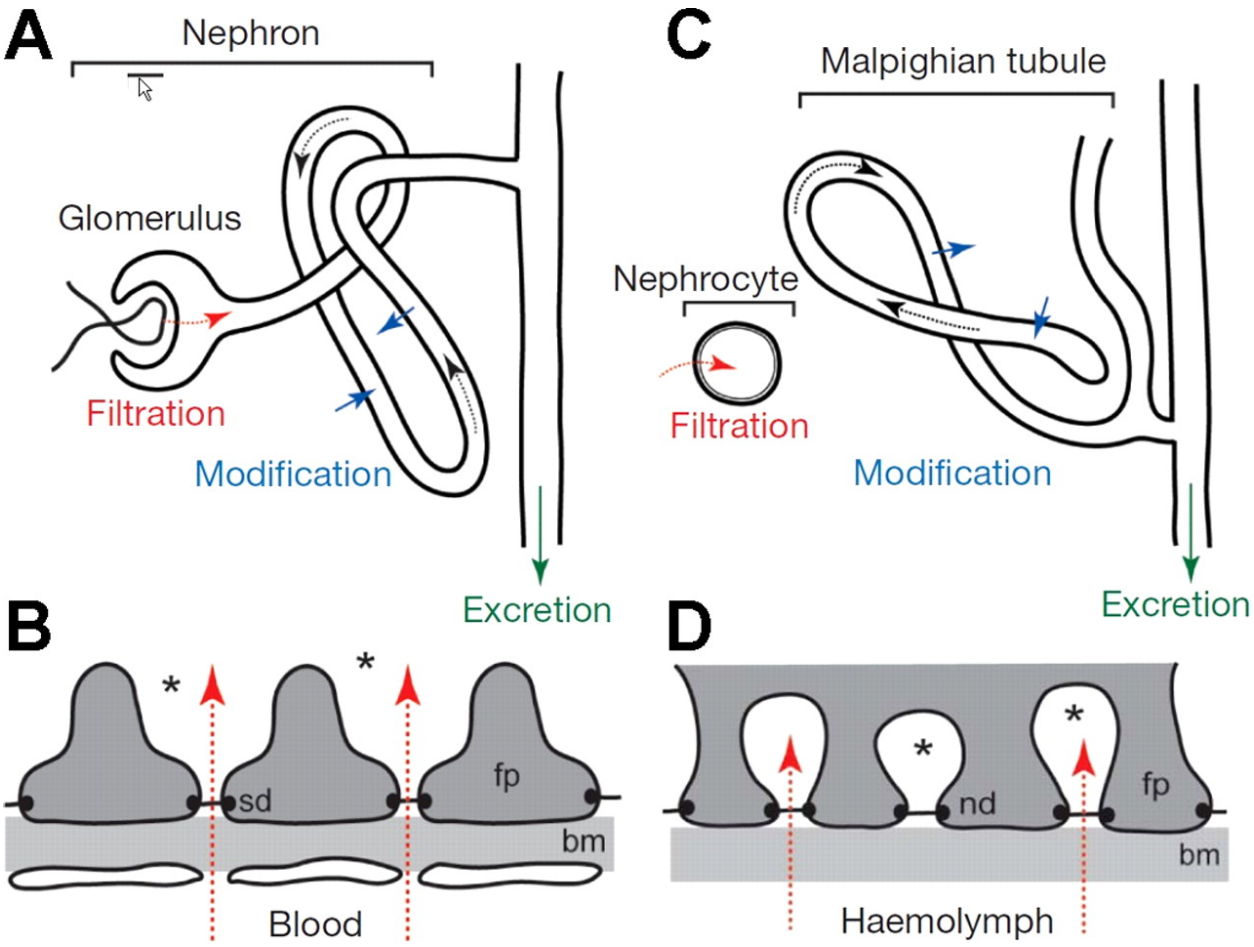


Figure 1.6 Functional homology and structural differences between mammalian nephrons and arthropod Malpighian tubules

(A) Mammalian nephron and (C) arthropod Malpighian tubule. (B) Podocytes and (D) Nephrocytes importantly share fenestration properties that permit them to act as a semi-permeable selective filtration barrier. (Weavers, Helen et al. “The Insect Nephrocyte Is a Podocyte-like Cell with a Filtration Slit Diaphragm.” Nature 457.7227 (2009): 3220326. PMC.) fp = foot process, sd = slit diaphragm, nd = nephrocyte diaphragm, bm = basement membrane.

Renal epithelial cells that make up the nephron then act to modify the concentrations of substrates in the ultrafiltrate produced by the glomerulus.[34] Urine is then sent via the renal papilla into the minor calyces, major calyces, renal pelvis, ureter and finally to the bladder to be eliminated when convenient per urethra. In contrast to humans, DM circulatory system is avascular. Instead, DM have a complex fluid called “hemolymph” that moves nutrients, wastes and hormones between structures in the fly.[133] DM have an analogous structures comparable to the glomerulus that filter hemolymph, called nephrocytes (Figure1.6 C).[133] The nephrocytes are located in the pericardium and have a similar diaphragm, with small foot processes spaced 30nm apart with an underlying basement membrane. Akin to the human podocytes, the nephrocyte foot processes form a barrier to particle size and charge, resulting in filtration of the hemolymph (Figure 1.6 D).[135] The filtered hemolymph then enters the Malpighian tubules, which is homologous to the human tubular system of the nephron.[135] There are four Malpighian tubules in a DM, each comprised of three segments called the initial, transitional and main segments.[133,136] These segments are composed of either principal or stellate cells which manage the ion concentration of the luminal aspect of Malpighian tubules. Finally, each pair of Malpighian tubules join together to form a short “ureter” that connects the tubule to the fly hindgut or cloaca (Figure 1.7).[133]

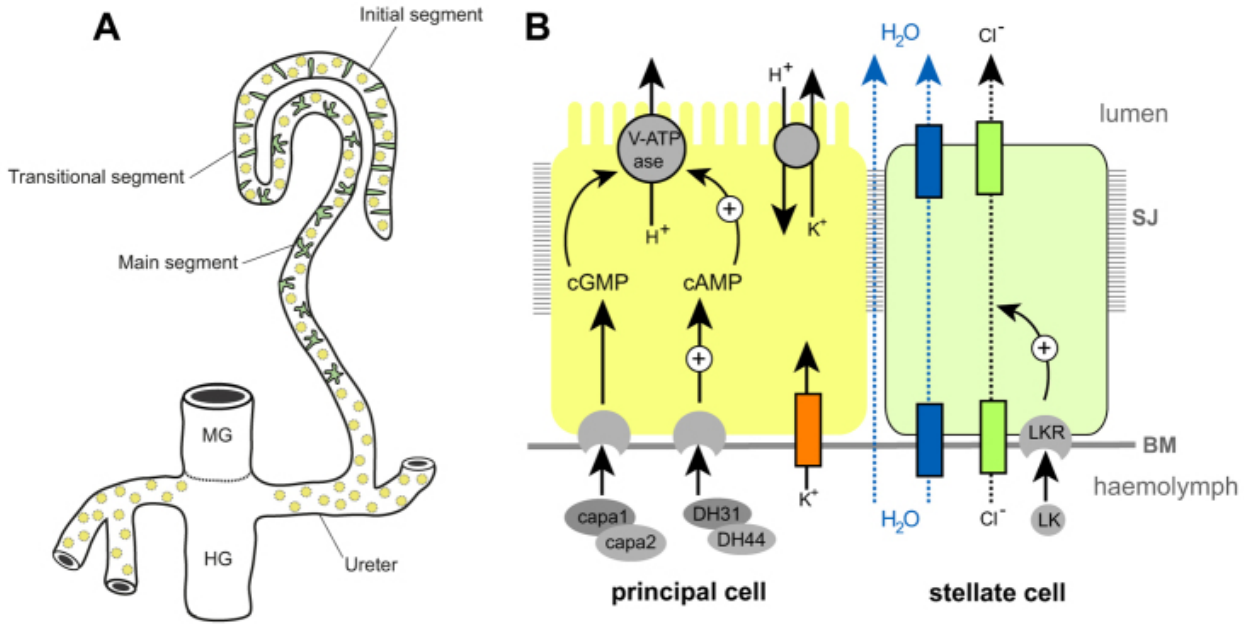


Figure 1.7 Malpighian tubule (MT) segments.

(A) Principal cells dominate the initial and earl transitional segment of the MT whereas the stellate cells are primarily found in the distal half of the transition and throughout the main MT segments. (B) Principal and stellate cells with their respective ion and solute transport mechanisms. Osmosis occurs transcellularly. (Denholm, Barry et al. “The tiptop/teashirt genes regulate cell differentiation and renal physiology in Drosophila.” Development (Cambridge, England) 140.5 (2013):1100-1110. PMC). MG = Midgut, HG = Hindgut. V-ATPase = V-type adenosine-triphosphatase, cGMP = cyclic guanosine monophosphate, cAMP = cyclic adenosine monophosphate, capa 1/2 = Capability peptides 1 and 2, DH31/DH44 = Diuretic hormone 31 and 44, LK = Leucokinin, LKR = Leucokinin receptor, BM = Basement membrane, SJ = Septate junction.

DM have been used as a model for calcium oxalate nephrolithiasis since 2011.[137] Although urolithiasis in this model is not spontaneous, it is relatively easy to form stones via supplementation of lithogenic agents like ethylene glycol or sodium oxalate.[133,138] Tubular concretions of calcium oxalate crystals are analogous to urinary stones and have been confirmed by scanning electron microscopy and energy-dispersive x-ray spectroscopy.[138] Furthermore, due to the evolutionary conservation of ion channels and cellular pathways between the Malpighian tubules and human nephrons, administration of drugs known to impact certain types of stone formation like hydroxycitric acid have worked in this model.[139] Further advantages of this model include its high throughput ability; consequently, DM can be an extremely economical *in vivo* model with low-maintenance husbandry requiring relatively rudimentary equipment.[114] In terms of the study of stone burden, DM tubules are transparent, thus simple light microscopy allows for the rapid and easy observation of calcium oxalate concretions without specialized staining.

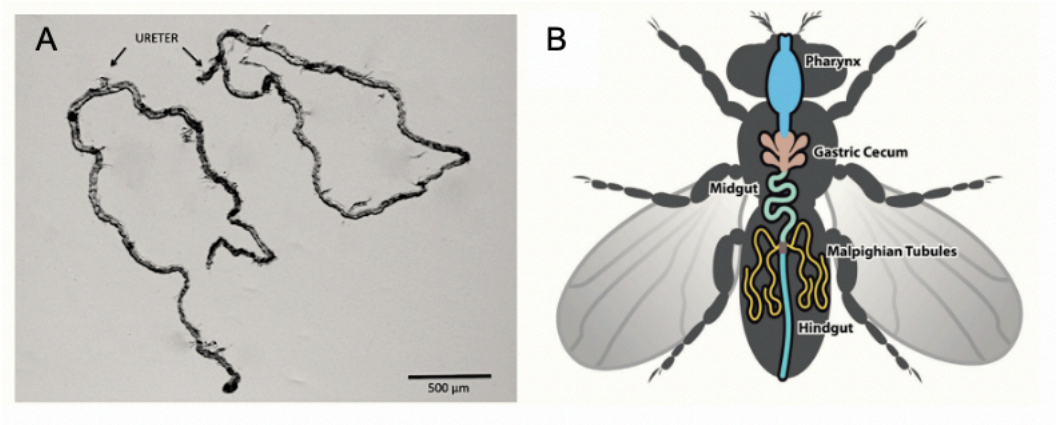


Figure 1. 7 DM Malpighian Tubule anatomy.

A) Light micrograph of dissected (B) Artistic rendition of relevant DM anatomy for study of nephrolithiasis. Each pair of Malpighian tubules are connected to the hindgut via a very short (100 micrometer) “ureter”.[133]

Hypothesis

Calcium oxalate crystals can activate mTOR and exacerbate the effect of ROS on the formation and progression of calcium oxalate kidney stone disease.[9] Therefore, we hypothesize that calcium oxalate concretion formation in the Malpighian tubules of DM treated with intermittent doses of rapamycin (an mTOR inhibitor) will be significantly less than individuals not treated with rapamycin. We also hypothesize that treatment groups fed a lithogenic diet while intermittently fasting every 24 hours on agar will have less calcium oxalate concretions in their Malpighian tubules compared to a lithogenic alternating with NF (*ad libitum*) diet early in the life of the fly. Additionally, mTOR inhibition has also been associated with an increase in life expectancy in animal models.[3] We hypothesize that life expectancy of DM treated with rapamycin will be significantly longer compared to control groups with and without lithogenic diets. Finally, we expect that treatment groups fed a lithogenic diet while intermittently fasting every 24 hours on agar early in the life of the fly will have longer life expectancy compared to *ad libitum* diets.

Chapter 2

Materials and Methods

Ethics

DM are an invertebrate organism and as such experiments are exempt from requiring animal ethics board approval. All insects were handled according to the Canadian Council on Animal Care (CCAC) Guidelines[140]

DM Husbandry

CantonS (Stock #64349) wild type strain *Drosophila* flies were obtained from Bloomington Stock Center, Indiana, USA (http://fly.bio.indiana.edu). All stocks were maintained in plastic 50mL Erlenmeyer wide fly vials (Genesee Scientific Corporation, CA). Stocks were stored separate from sample flies to avoid mite infestation. Twenty-five to fifty DM individuals were mated for 18-24 hours on standard media with yeast paste and grape agar. Flies that were no longer required were disposed of, in an Erlenmeyer flask containing 70% ethanol. Eclosed (recently having emerged from the pupal state) DM were allowed to mature 24 hours prior to first dose of anaesthesia, or treatment. Wide fly vials (GEN32-121, Genesee Scientific, California, USA) were used for housing samples during experimentation. At all times except for changing flies from vial to vial, stocks were maintained in a DM incubator (DigiThermâ *Drosophila* Incubator, Tritech Research Inc.) at a humidity of 40%, a temperature of 25°C, and were exposed to a 12-hour light and 12-hour dark cycle.

DM were transferred under gentle carbon dioxide (CO_2) anaesthesia (Flystuff Flowbuddy Flow Regulator with Ultimate Flypad, 590122BCU) for 5 seconds, for a total of 10 seconds of hypoxia, from food medium to treatment agar in fly vials every 24 hours. DM were observed for 10 minutes after anaesthesia transfer and any dead or escaped individuals were censored from life-expectancy data. Food media and agar tubes were changed every 5 days.

Production of DM food media

Individual batches of food media were produced in one-litre volumes. Ingredients were weighed using a digital scale (Sartorius AG, Goettingen, Germany) and heated using a hotplate in a non-sticking commercially available cooking pot. Freshly prepared media was pipetted into the wide polypropylene vials using a disposable pipette. A grade 50 cheesecloth was placed to reduce the risk of contamination while cooling prior to placement for storage. Vials were covered by cellulose acetate plugs (Flugs GEN49-101, Diamed Inc., Ontario, Canada) and left to cool overnight to reduce condensation prior to storage at 4°C. Unused vials of media were discarded if not used within 28 days.

Standard food DM media and agar

Standard food as defined in this study was prepared according to a previously utilized formula (Bloomington #1) which provided DM with adequate nutrients for development in lab environment.[141,142] (Table 2.1). One-thousand mL of distilled water was brought to a rolling boil. Agar was added and stirred until clear. For agar tubes, this mixture was allowed to cool and then poured into wide vials in 10mL aliquots. For all other media, cornmeal was then stirred in until a thick formula formed. Yeast was then added and stirred until dissolved. Corn syrup was then added prior to cooling. The mixture was allowed to cool prior to adding propionic acid to media as a preservative. Finally, the mixture was decanted in 10mL aliquots into wide vials as described previously.

INGREDIENT	QUANTITY
Distilled Water	1000 ml (total)
Agar (66-103, Diamed Inc.)	5.76 g
Yeast Powder (51475-2.5KG, Sigma Inc.)	17.3 g
Cornmeal, yellow (62-100, Genesee Scientific)	73 g
Corn syrup, solid (62-109, Genesee Scientific)	76.9 g
Propionic acid (1368, Sigma Inc.)	5 ml
Tegosept (20-258 Diamed Inc.) 30% solution (optional)	5 ml

Table 2.1 Ingredients used in the preparation of standard Drosophila lab media ("Normal Food").

For 0.1% sodium oxalate, 10mg oxalate was added to the mixture prior to pouring.

Lithogenic DM media

Lithogenic food as defined in this study is 0.1% (weight/volume) sodium oxalate (NaOx) dissolved in the standard DM food prior to cooling to room temperature. For each replicate, normal media was separated by 50% volume. Five-hundred mL was poured into a 1L beaker and 0.5mg of sodium oxalate was added to the mixture and stirred thoroughly until fully dissolved. The ensuing lithogenic mixture was then decanted in 10mL aliquots into wide vials.

Microcapillary rapamycin dosing

The CAFE method of drug delivery in DM has been previously described (Figure 2.1).[143] Briefly, 5% agar is poured into the base of fly tubes. The cotton lids are pierced by a 100 µL pipette and the same pipet is used to hold a fine capillary inside the vial through the pipette tip. Using capillary action, approximately 5-10 µL aliquots of rapamycin solution were loaded into the capillary. The fly tubes are then closed and the capillary containing rapamycin and vehicle, or vehicle alone are placed in the pipette. Flies in the experimental group were fed normal food media with dissolved 0.1% (weight/volume) sodium oxalate (lithogenic media). The experimental group alternated daily between a vial containing lithogenic media and a vial of non-nutritious 5% agar with 100 uM rapamycin dissolved in 10 µL of 100% ethanol and 5% sucrose (weight/volume) accessible by a microcapillary. Our control groups were fed normal media alternating every 24-hours on an agar tube. Each agar tube contained a capillary filled with 10 µL ethanol and 5% sucrose (weight/volume) solution +/- 100 µM rapamycin (Figure 2.2). Our positive control group alternated between standard and lithogenic media every 24h without use of CAFE (Figure 2.2). All DM were placed on 5% agar (weight/volume) to avoid desiccation.

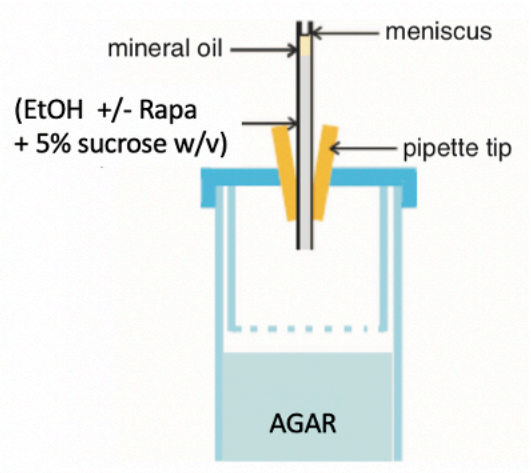


Figure 2.1 Diagram of the Capillary Feeder (CAFE) assay.

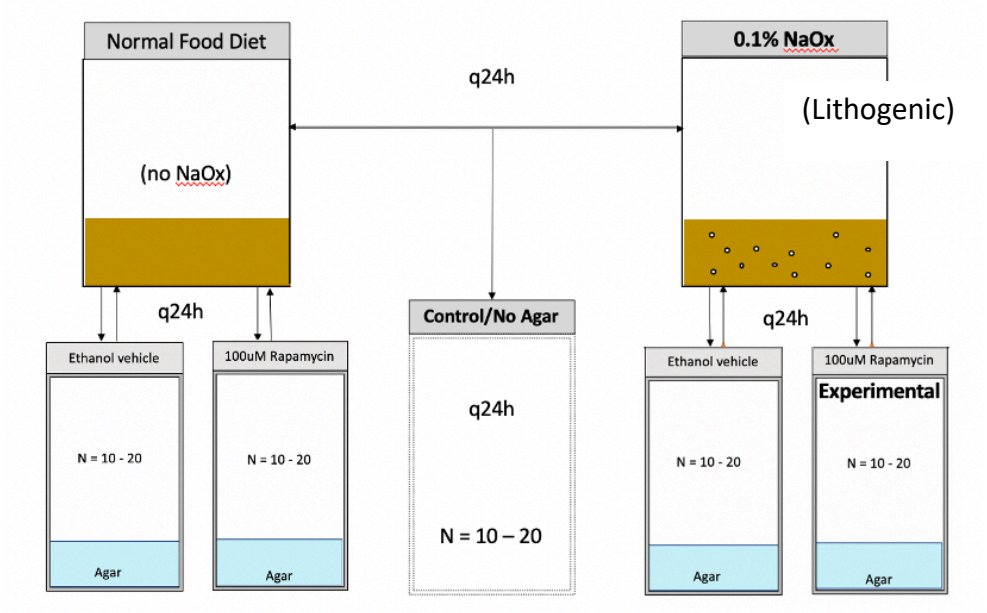
In this method of feeding, DM climb up the sides of the vial filled with agar and take up substances within the capillary housed within the pipette tip. EtOH = 100% ethanol, w/v = weight by volume.[143]

Study design

Experimental and control diet groups were studied with both males and females separated. Twenty flies per replicate were included in the positive control groups (Figure 2.2A). All experiments were performed over a duration of 10 days. *Drosophila* in in this group were fed lithogenic food on day 1, anesthetized at the beginning of day 2 and transferred to normal food for 24 hours. This was repeated for a total of 10 days.

Flies were fed lithogenic food on day 1, and then transferred to tubes containing agar and a capillary with vehicle (ethanol + 5% sucrose) on a 5% agar base on day 2. These media tubes were then alternated every 24 hours for 11 days post eclosure (Figure 2.2B). The negative control group was fed normal food on day 1, and then maintained on a capillary containing ethanol with 5% sucrose on a 5% agar base for the remainder of the experiment. The first experimental group alternated between normal food every 24 hours with 100μM rapamycin on 5% agar. The second experimental group alternated between lithogenic food and 100μM rapamycin on 5% agar (Figure 2.2A). Five replicates of each control and treatment group were performed to achieve an adequate number of Malpighian tubules for analysis. No formal power calculation was calculated; however, the number of flies required was determined based on previous study sample sizes in the literature.[132,137,138,144–147]

A



B

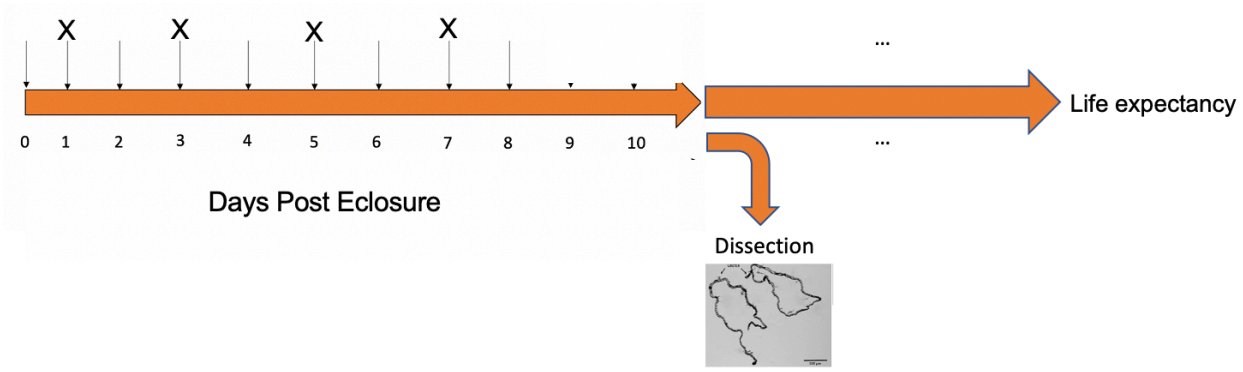


Figure 2. 2Diagram of control and treatment groups.

(A) Daily alternating lithogenic (0.1% NaOx), or normal food with agar or normal food identified by arrows. (B) Flowchart for experiments. The fly tubes were changed every 24 hours as marked by the X. On the 11th day post eclosure, each group was dissected for Malpighian tubule analysis, whereas life expectancy vials were to continue until endpoint or censoring was reached. X = Treatment day with rapamycin or vehicle on agar. NaOx

= sodium oxalate, q24h = every 24 hours. Optimal dosing of rapamycin was not determined in this study.

Malpighian tubule isolation

CO_2 was used to euthanize DM at the completion of the experiment at 10 days. DM were then transferred to a 1 mL Eppendorf tube containing 99% ethanol for 3 minutes, and then to a Petri dish (Pyrex 3160100, Sigma-Aldrich Inc) lined with Sylgard (Dow Corning Inc., MI, USA) that contained 10mL of phosphate-buffered saline (PBS) via fine tipped forceps. DM dissections (Figure 2.4) were completed within 45 minutes of immersion in PBS and placed in a solution of 37% formaldehyde diluted 9:1 with PBS for one hour prior to being fixed on a slide for examination under the microscope.

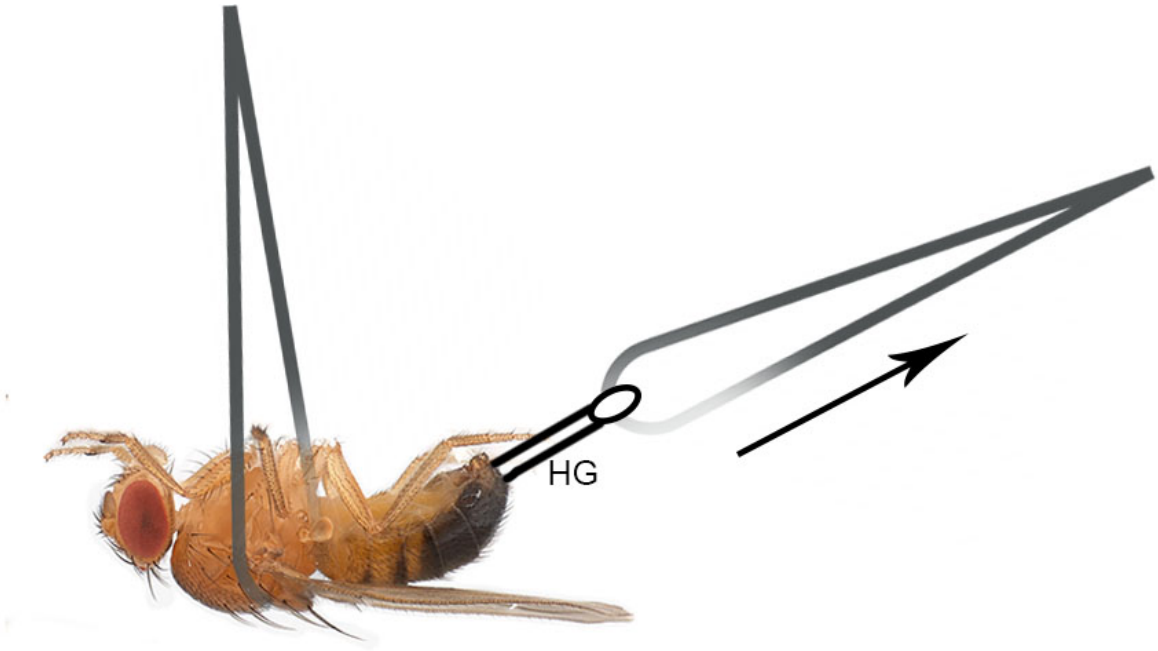


Figure 2.3 Malpighian tubule dissection via hindgut traction (HG). Malpighian tubules are attached laterally (not shown) to the hindgut. (Drosophila image courtesy of Nicolas Gompel, adapted for use under Creative Commons Attribution NC 3.0 Unported.)

Quantification of birefringent concretions in DM Malpighian tubules

Each group of DM were incubated for 10 days total. Flies received 5 days of the lithogenic or nutrient containing food, and five days were spent with a daily dose of rapamycin with 5% sucrose in ethanol or 5% sucrose in ethanol alone. All groups began on non-lithogenic food for consistency. All Malpighian tubules were dissected on day 10, where the last 24-hour period was on lithogenic to ensure maximal stone burden. The degree of DM calcium oxalate concretions was defined as the total birefringent pixel area per Malpighian tubule.

Statistics

Life expectancy was analyzed via Kaplan-Meyer Curve, Cox Proportional Hazard model and Log-Rank Test on R (Version 1.3.1093 © 2009-2020 RStudio, PBC). Malphigian tubule concretion birefringence intensity was measured via ImageJ [ImageJ Version 1.51(100)) pixel analysis (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018]. When appropriate, pixels were adjusted by dividing the total number of pixels by the number of specimens represented in the image field. In order to approximate a normal distribution, the number of pixels per MT in view for all groups were normalized by taking the square-root of each measurement. Statistical analysis using SPSS (IBM SPSS Statistics v25) for mean normalized pixel/MT for each group was analyzed with an analysis of variance (ANOVA) with significance set at a p value < 0.05.

Chapter 3

Results

DM Survival Analysis

In total 288 of 300 (96%) male and 299 of 300 (99.6%) female DM had end events through 86 days of analysis (Table 3.1). Male DM on oxalate + rapamycin CAFE treatment had lower survival compared to female DM on same treatment (23 days vs 49 days, Table 3.1; HR 0.57, 95%CI: 0.40 – 0.82, p <0.001 Figure 3.2 and 3.3). Male DM on oxalate + rapamycin CAFE treatment had significantly shorter median survival compared to male DM on normal food + rapamycin CAFE treatment (23 vs 35 days, Table 3.1; HR 0.61, 95% CI: 0.41 – 0.89, p-value = 0.01, Figure 3.1 and 3.3). Male DM on oxalate + rapamycin CAFE treatment had significantly shorter median survival compared to male DM on oxalate + normal food *ad libitum* treatment (23 vs 48 days, Table 3.1; HR 0.52, 95% CI: 0.36 – 0.75, p-value = <0.001, Figure 3.1 and 3.3). Male DM on oxalate + rapamycin CAFE treatment had longer median survival compared to the oxalate + vehicle CAFE treatment but did not reach threshold for significance (23 days vs 23 days, Table 1; HR 1.43, 95%CI: 1.00 – 2.05, p-value = 0.052, Figure 3.1 and 3.3).

Male DM on normal food + rapamycin had significantly longer life expectancy than male DM on oxalate + vehicle treatment (35 days vs 23 days, Table 1; HR: 2.34, 95%CI: 01.60 – 3.43, Figure 3.4). Male DM on normal food + rapamycin CAFE treatment had significantly shorter median survival compared to female DM equivalent treatment (35 days vs 37 days, Table 1; HR 0.38, 95%CI: 0.26 – 0.95, p = 0.026, Figure 3.4). Male DM treated with oxalate + vehicle had significantly shorter survival than female DM treated with equivalent treatment (31 days vs 23 days, Table 1; HR 0.49, 95%CI 0.34 – 0.71, p < 0.001, Figure 3.3). No significant difference in median survival was seen between male DM on oxalate + rapamycin CAFE treatment compared to male DM on normal food + vehicle. Male DM on *ad libitum* lithogenic diet had no significant difference in survival compared to male DM on normal food + rapamycin CAFE treatment (48 days vs 35 days, HR 1.17, 95%CI: 0.80 – 1.7, p = 0.42, Figure 3.5). Male DM on *ad libitum* lithogenic diet had longer median survival than male DM on normal food + vehicle CAFE treatment (HR 2.04, 95%CI 1.42 – 2.90, p < 0.001, Figure 3.5).

There was no significant difference in median survival between female DM on oxalate + rapamycin CAFE treatment compared to other females (Figure 3.1 and 3.6). Female DM on oxalate + vehicle treatment had significantly lower survival compared to female DM on normal food + vehicle (31 days vs 33 days, Table 1; HR 0.66, 95%CI: 0.46 – 0.95, p = 0.024, Figure 3.6). Female DM on normal food + rapamycin CAFE treatment had longer median survival compared to male DM on the same treatment (37 days vs 35 days, Table 3.1; HR 1.5, 95%CI: 1.04 – 2.20, p = 0.029, Figure 3.6). There was no significant difference in median survival seen between male and female DM on *ad libitum* lithogenic diet (Figure 3.2 and 3.7).

Diet	N	Event (%)	Median Survival Days (95%CI)
M Ox Rap	60	59 (98%)	23 (23 - 34)
M Ox V	60	60 (100%)	23 (23 - 25)
M NF Rap	60	51 (85%)	35 (33 - 40)
M NF V	60	58 (97%)	23 (23 - 28)
M Ox NF	60	60 (100%)	48 (23 - 61)
F Ox Rap	60	59 (98%)	49 (23 - 53)
F Ox V	60	60 (100%)	31 (23 - 37)
F NF Rap	60	60 (100%)	37 (24 - 48)
F NF V	60	60 (100%)	33 (23 - 50)
F Ox NF	60	60 (100%)	30 (30 - 51)

Table 3.1 Kaplan-Meier survival data for Male and Female DM survival analysis.

N = sample size, Event = death, Ox Rap = 0.1% sodium oxalate + rapamycin, Ox V = 0.1% sodium oxalate + vehicle (no rapamycin). NF Rap = normal food + rapamycin. NF V = normal food + vehicle. Ox NF = 0.1% sodium oxalate + normal food (*Ad libitum* lithogenic diet).

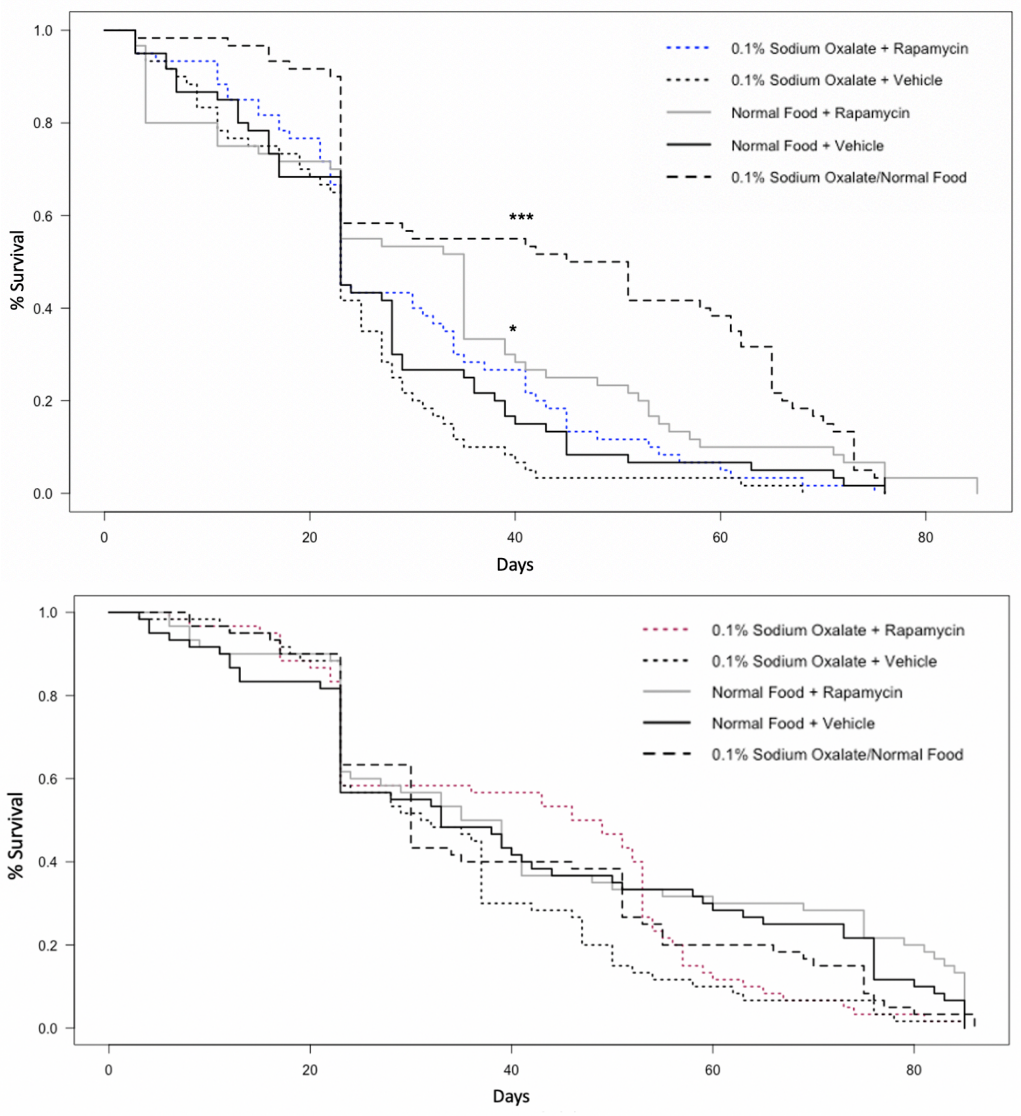


Figure 3.1 Kaplan-Meier survival curves for male (top) and female (bottom) *D.* melanogaster given lithogenic or non-lithogenic diets, with or without rapamycin.

Vehicle denotes *D. melanogaster* cohorts that were fed non-nutritious solution without rapamycin by CAFE. Reference cohort for comparison is 0.1% sodium oxalate + rapamycin group for both males and females. Significance by Log-Rank test, levels: * = $p < 0.05$, *** = $p < 0.001$.

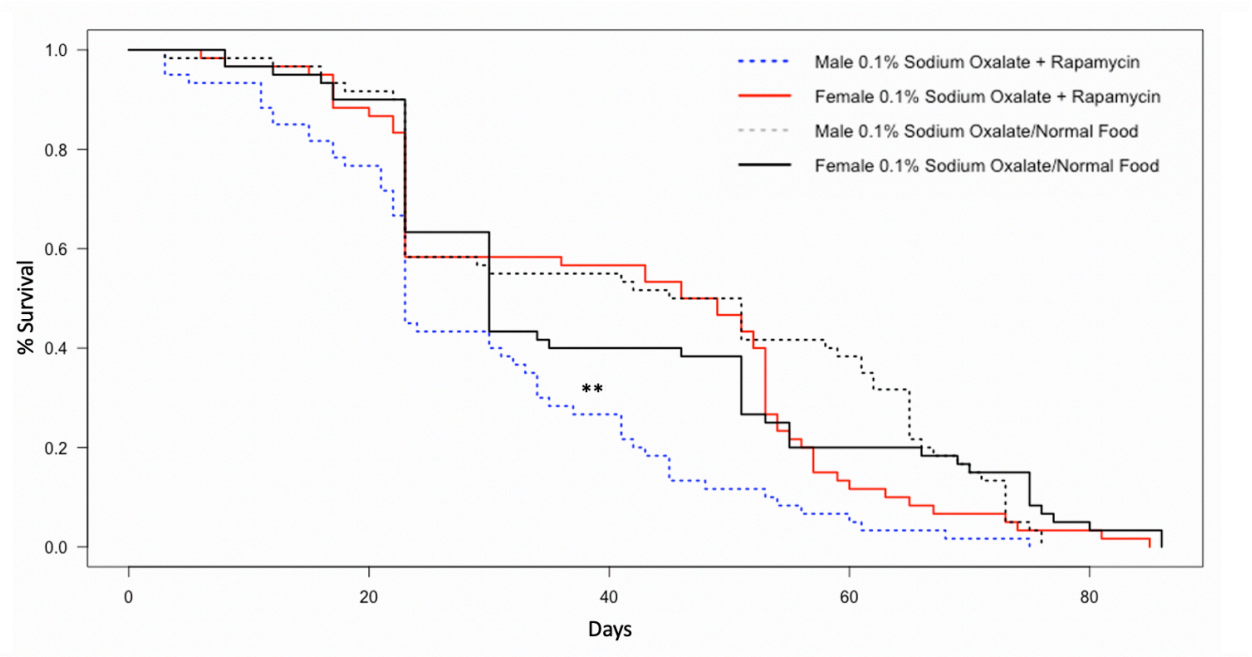


Figure 3.2 Kaplan-Meier survival curves comparing *ad libitum* lithogenic diet treatment to CAFE lithogenic treatment + rapamycin in female and male *D. melanogaster*.

Female 0.1% sodium oxalate + rapamycin group is reference curve for significance testing by Log-Rank test. HR 1.76 (95%CI: 1.22 – 2.2.5). Significance level ** = $p < 0.002$.

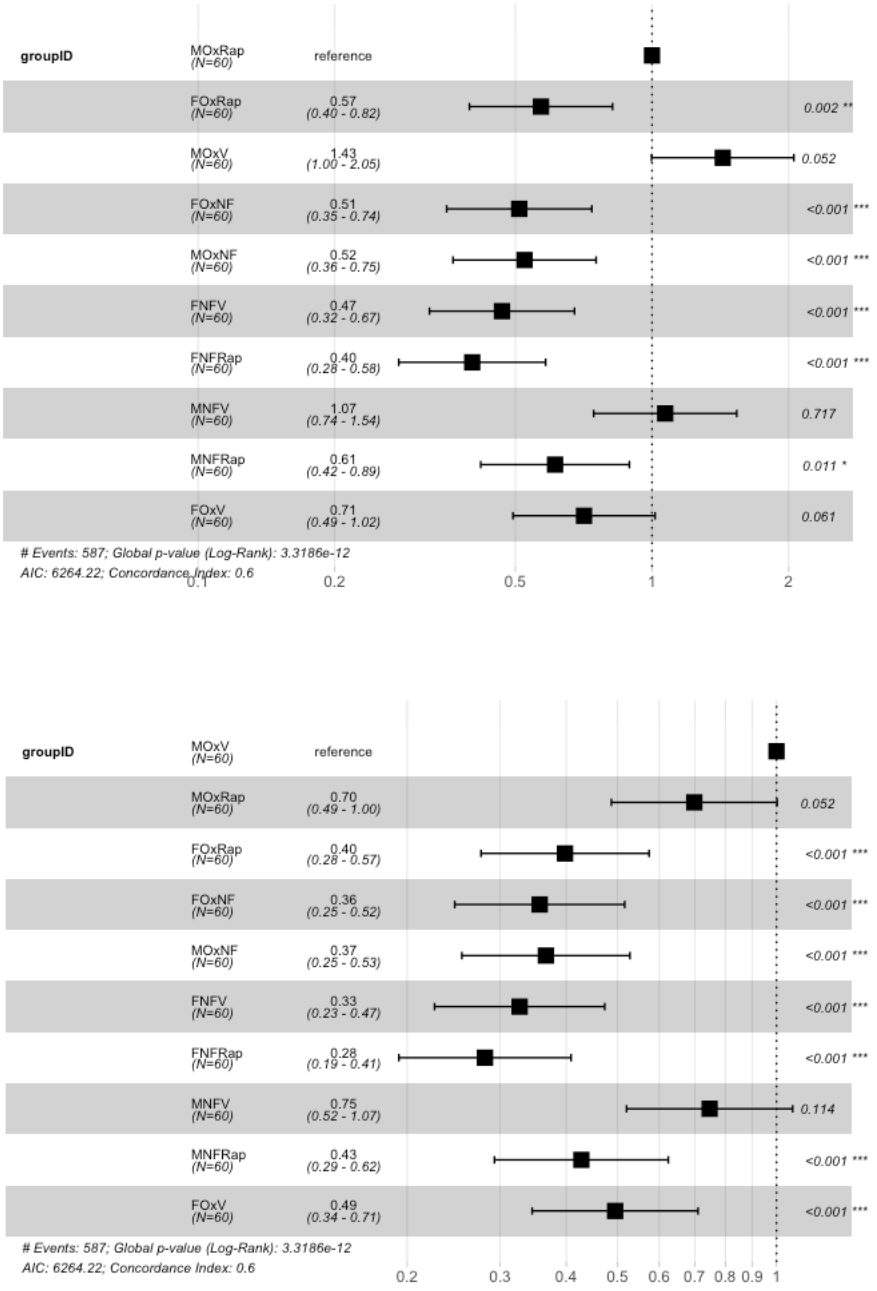


Figure 3.3 Forest plots of Hazard Ratios for male DM on lithogenic + rapamycin (top) or vehicle (bottom) CAFE treatments.

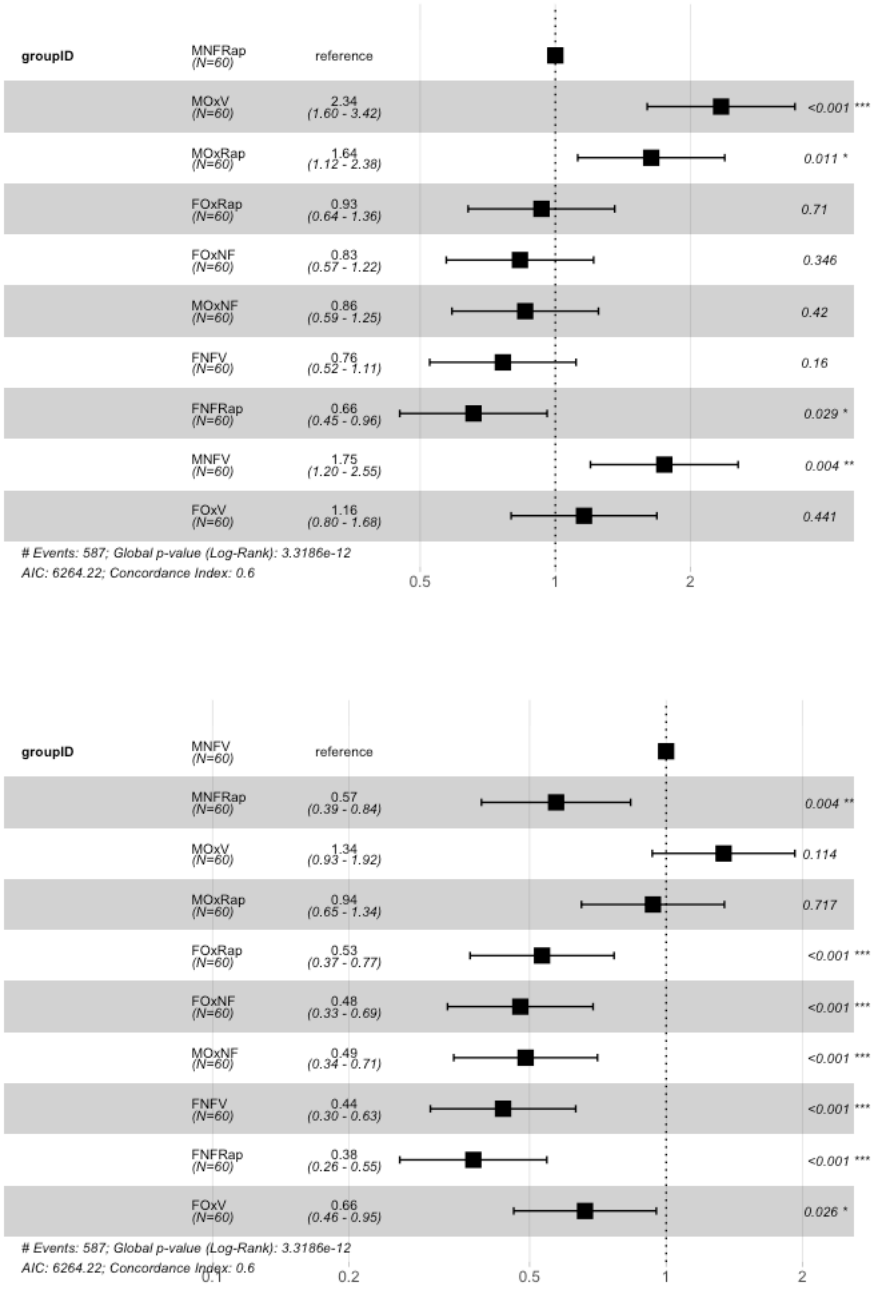


Figure 3.4 Forest plots of Hazard Ratios for male DM on normal food + rapamycin (top) or vehicle (bottom) CAFE treatments.

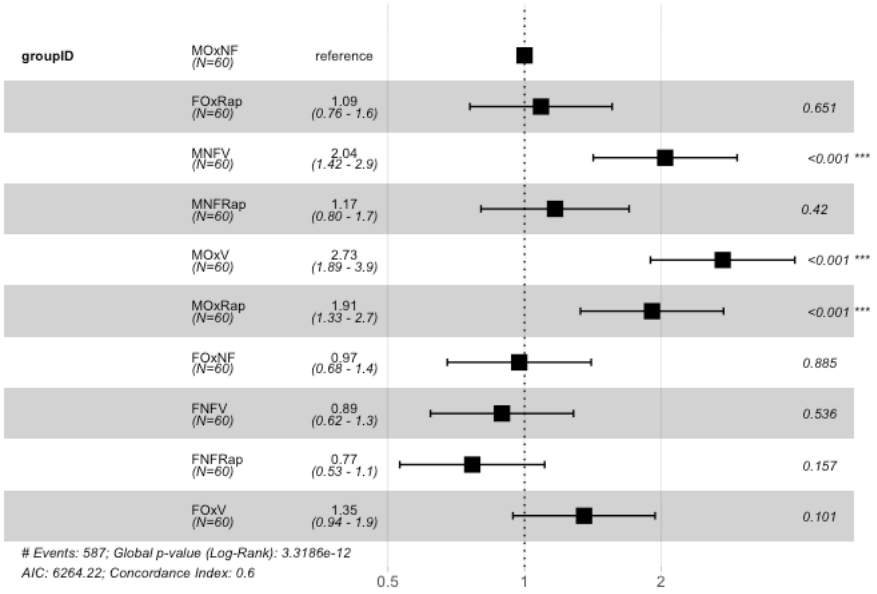
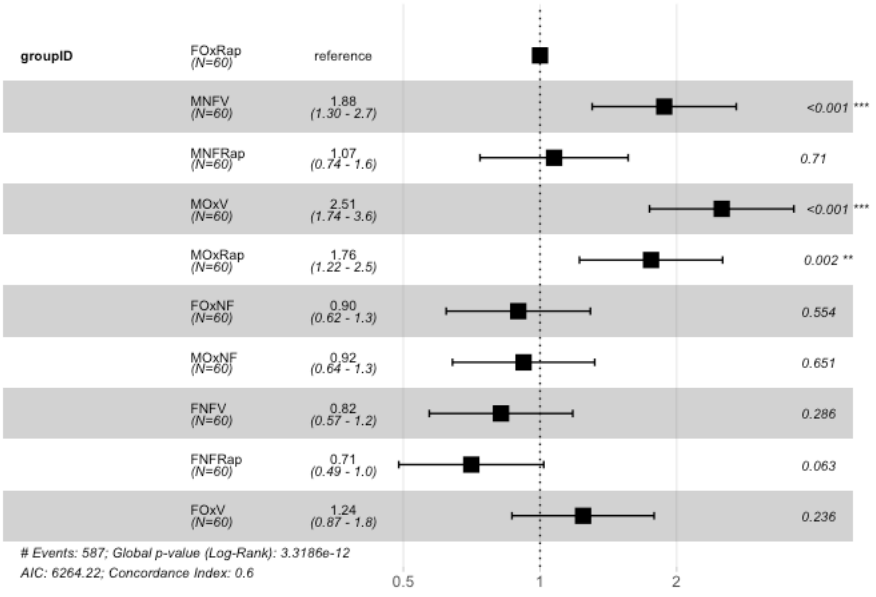


Figure 3.5 Forest plot of Cox Proportional Hazard Ratios for male DM on *ad libitum* lithogenic treatment.



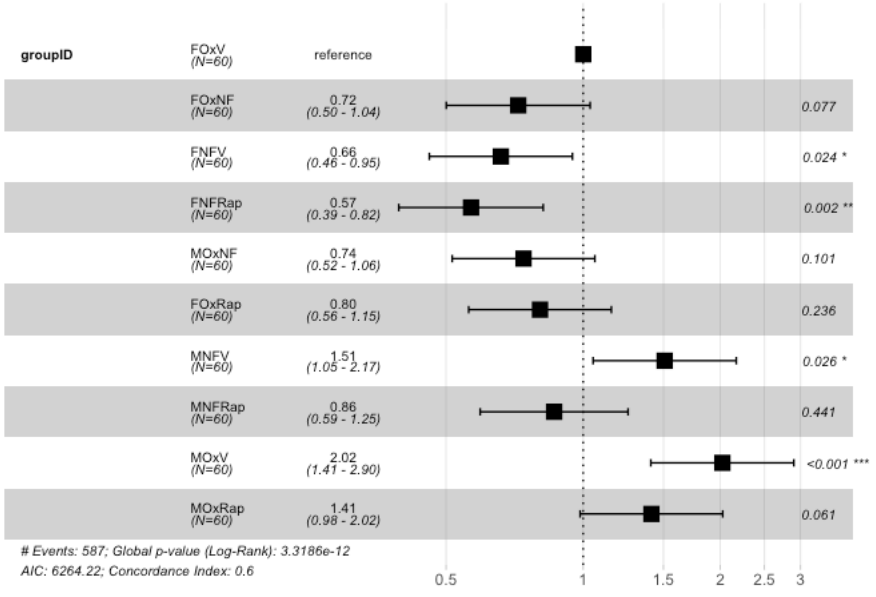


Figure 3. 6 Forest plots of Hazard Ratios for female DM on lithogenic + rapamycin (top) or vehicle (bottom) CAFE treatments.

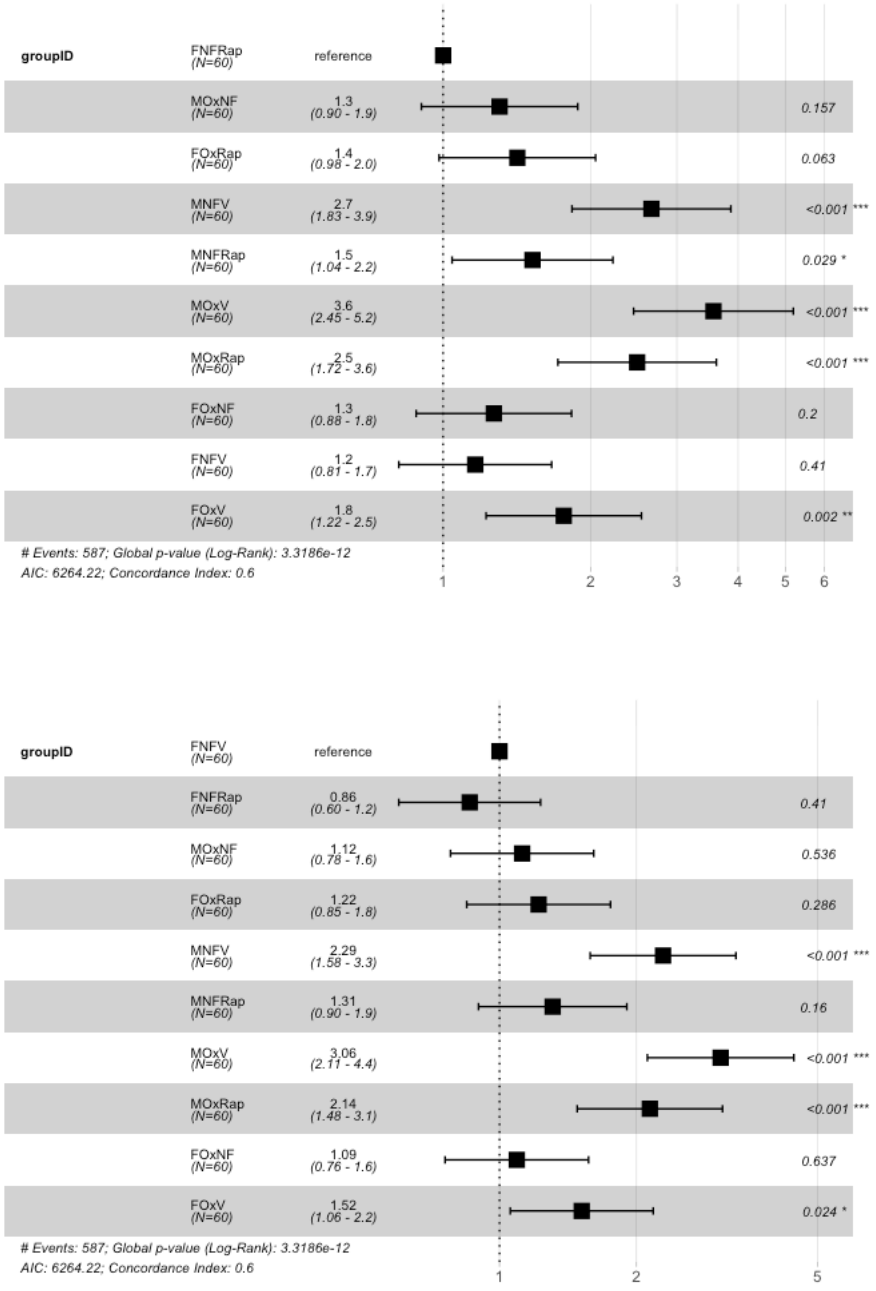


Figure 3.7 Forest plots of Hazard Ratios for female DM on normal food with rapamycin (top) or vehicle (bottom) CAFE treatments.

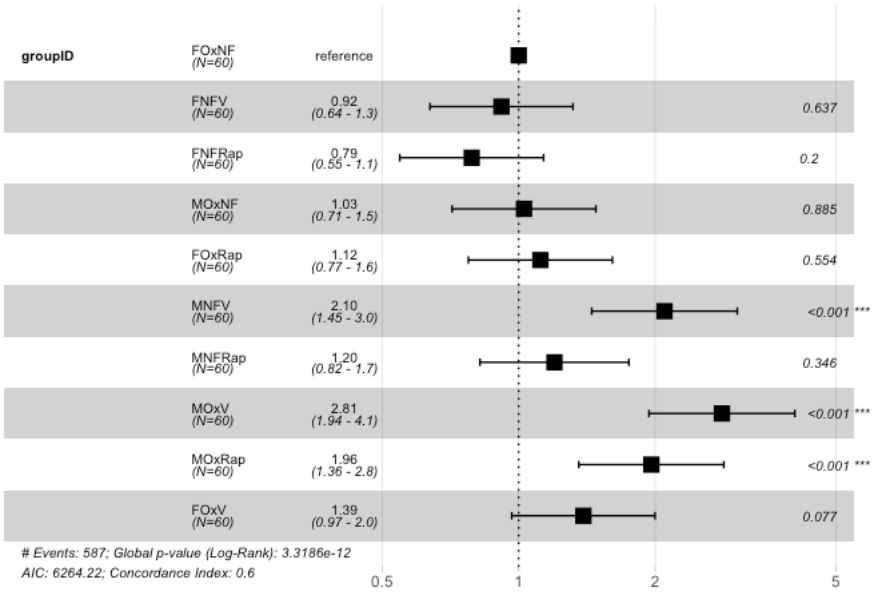


Figure 3.8 Forest plots of Cox Proportional Hazard Ratios for female DM on *ad libitum* lithogenic diet.

Ex vivo imaging of Malpighian tubules and effect of rapamycin on concretion birefringence.

Female DM who were available for full MT analysis ranged between 21 and 43 individual DM per treatment group (Table 3.2). The total of dissected MT for analysis ranged from 67 to 104 per group in female DM and the median number Malpighian tubules isolated intact is displayed in Table 3.5.

	0.1%/NF	NF/+Rapa	NF/-Rapa	0.1%/+Rapa	0.1%/-Rapa
Sample size	32	21	24	43	38
Malpighian tubules	74 (4)	71 (1)	67 (4)	104 (4)	85 (2)
Birefringence Median pixels (IQR)	2212 (2045)	49 (87)	20 (91)	647(580)	1254 (2322)
Birefringence per tubule Median pixels (IQR)	1161 (1059)	4 (3)	2 (5)	16 (9)	29 (15)
Normalized birefringence per tubule Mean (95%CI)	33 (29-37)	4 (3-5)	3 (2-5)	16 (14-19)	30 (25 - 35)

Table 3.2 Descriptive statistics of female DM Malpighian tubules dissections for each diet. Birefringence measured by square root transformed pixels per Malpighian tubule.

0.1%/NF = 0.1% sodium oxalate – normal food protocol. NF/+Rapa = normal food – rapamycin protocol. NF/-Rapa = normal food without rapamycin. 0.1%/+Rapa = 0.1% sodium oxalate – rapamycin protocol. 0.1%/- Rapa = 0.1% sodium oxalate without rapamycin.

Male DM in each group who were viable for full MT analysis ranged between 18 and 39 individual DM per group (Table 3.3), and had a total number of dissected MT of 45 to 122 per group for analysis. The median number of intact MT isolated per fly are demonstrated in table 3.6.

	0.1%/NF	NF/+Rapa	NF/-Rapa	0.1%/+Rapa	0.1%/-Rapa
Sample size	28	19	18	35	39
Malpighian tubules Frequency (median)	95 (4)	45 (1)	81 (4)	122 (4)	83 (2)
Birefringence Median pixels (IQR)	1754 (2591)	0 (55)	17 (55)	993 (1386)	1511 (1399)
Birefringence per tubule Median pixels (IQR)	1161 (1059)	0 (5)	5 (20)	407 (394)	897 (693)
Normalized Birefringence per Tubule (mean, 95%CI)	28 (25-31)	2 (1-3)	4 (2-5)	19 (17-21)	28 (25-32)

Table 3.3 Descriptive statistics of male DM Malpighian tubules dissections for each diet.

0.1%/NF = 0.1% sodium oxalate – normal food protocol. NF/+Rapa = normal food – rapamycin protocol. NF/-Rapa = normal food without rapamycin. 0.1%/+Rapa = 0.1% sodium oxalate – rapamycin protocol. 0.1%/- Rapa = 0.1% sodium oxalate without rapamycin.

DM that did not get exposure to oxalate displayed very little birefringence compared to groups treated with oxalate-based diets (Figure 3.9 D-E, I-J). Sex did not affect Malpighian tubule birefringence when DM were fed equivalent diets (Figure 3.9). There was no difference in MT birefringence in DM fed normal food without oxalate, regardless of rapamycin treatment status. Furthermore, there was no difference in birefringence of DM on oxalate alternated with 24h of normal food (0.1%/NF) or vehicle without rapamycin (0.1%/-Rapa).

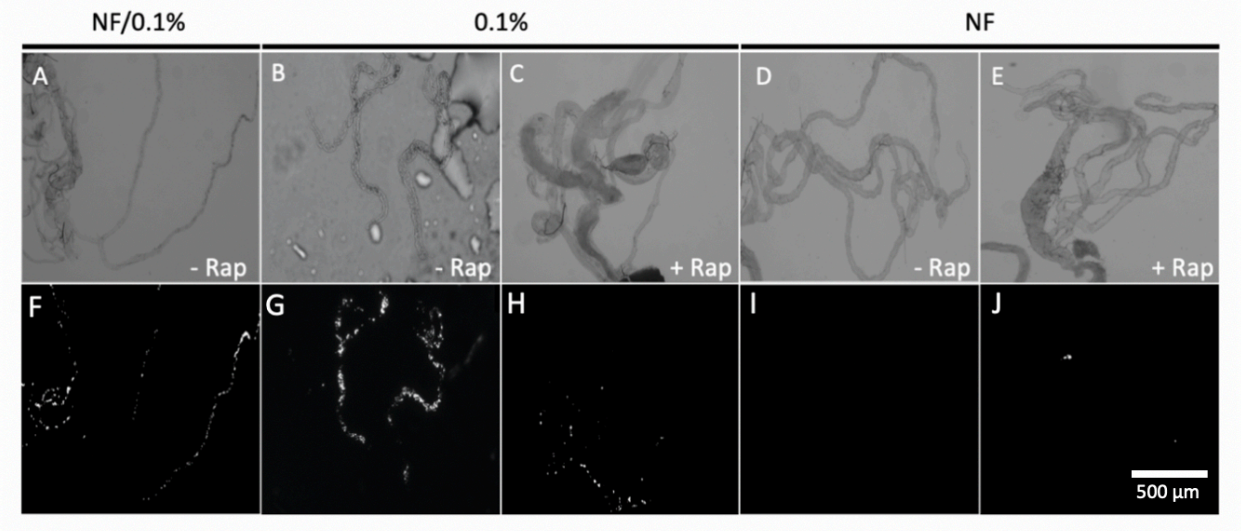


Figure 3.9 Representative images of Malpighian tubules.

(A-E) Bright-field and (F-J) plane-polarized light microscopy at x 40 magnification for dissected male and female DM Malphigian tubules at eleven days post-eclosure. NF/0.1% = 0.1% sodium oxalate – normal food protocol. 0.1% = 0.1% sodium oxalate protocol. NF = normal food protocol. Rapamycin treatment indicated by "+ Rap" (C, E, H, J). White bar represents standardized distance of 500 µm.

The oxalate/rapamycin dietary protocol resulted in significantly less mean MT birefringence per Malpighian tubule compared to DM on both oxalate/normal food (16 vs 33, $p < 0.01$, Figure 3.10) and oxalate/vehicle protocols (16 vs 30, $p < 0.01$, Figure 3.10). DM fed oxalate/rapamycin had significantly higher MT birefringence compared to DM fed normal food with rapamycin (16 vs 3, $p < 0.01$, Figure 3.10) and normal food without rapamycin (16 vs 4, $p<0.01$, Figure 3.10*).* Drosophila fed 0.1% oxalate/normal food had significantly more MT birefringence compared to both normal food/vehicle (30 vs 3, $p <0.01$, Figure 3.10) and normal food/rapamycin (30 vs 4, $p<0.01$, Figure 3.10) groups.

Male DM fed 0.1% oxalate/rapamycin had significantly lower MT birefringence compared to 0.1% oxalate/normal food (19 vs 28, $p < 0.01$, Figure 3.10) and compared to 0.1% oxalate/vehicle (16 vs 28, $p <0.01$, Figure 3.10). The oxalate/rapamycin protocol had significantly greater mean normalized MT birefringence compared to DM fed normal food protocols with rapamycin (16 vs 2, $p<0.01$, Figure 3.10) and without rapamycin (19 vs 4, $p < 0.01$, Figure 3.10).

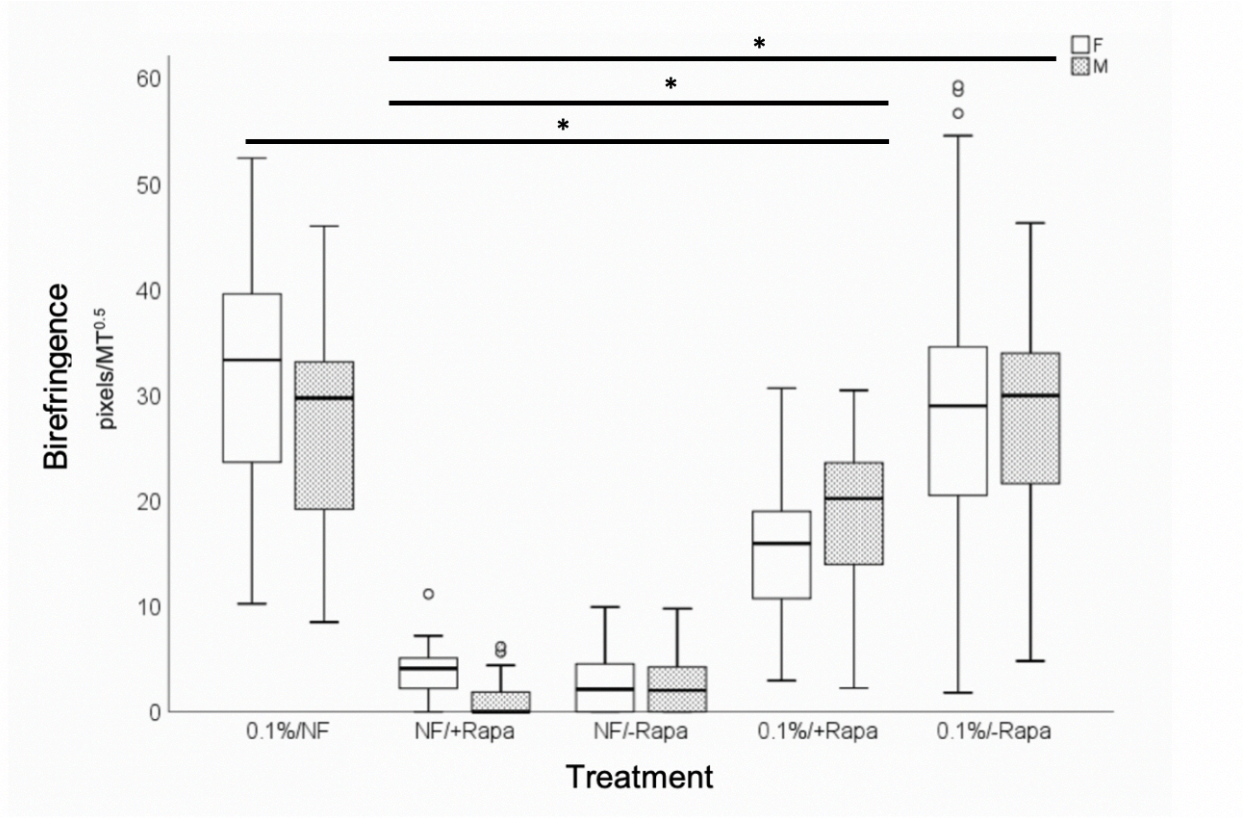


Figure 3.10 Box plot of DM normalized Malpighian tubule birefringence under plane-polarized light microscopy.

Female = white and male = grey. 0.1%/NF = 0.1% sodium oxalate/normal food protocol. NF/+Rapa = normal food/rapamycin protocol. NF/-Rapa = normal food without rapamycin. 0.1%/+Rapa = 0.1% sodium oxalate – rapamycin protocol. 0.1%/- Rapa = 0.1% sodium oxalate without rapamycin. Significance indicated by *, $p < 0.01$.

Chapter 4

Discussion

Survival analysis

Measuring life expectancy is simpler than measuring overall decline in function of DM with advancing age and can be used as a crude surrogate for stress.[148] The degree of DM calcium deposition in the Malpighian tubule (MT) is associated with both oxalate load and reduced life expectancy. Other studies have shown that MT concretions are inversely correlated with life expectancy.[138] As expected, calorie restricted lithogenic diets are associated with significantly lower median survival compared to calorie restricted non-lithogenic treatments in male DM. Intermittent dosing of rapamycin via CAFE extends relative median survival of male DM fed a lithogenic diet compared to vehicle. Since mTOR inhibition by rapamycin is known to induce autophagy[86], we hypothesized that this treatment would help reverse calcium oxalate crystal damage by improving the ability of DM to clear ROS and damaged proteins.

We hypothesized that increased clearance of dysfunctional proteins and lower ROS following rapamycin treatment would lead to an increase in life expectancy and tolerance of calcium oxalate crystals. However, compared to available literature our treatment did not improve life expectancy.[138] However, our finding that rapamycin extends the relative median life expectancy of wild type DM compared to vehicle is consistent with other studies. For example, Wang et al. described a median survival advantage of a mitochondrial dysfunction mutant of *Drosophila* treated with 100 μM of rapamycin daily compared to vehicle (71 vs 62 days, a 14.5% increase).[149]

Oxalate does also appear to be toxic to DM, resulting in lower relative life expectancy. In these cases, rapamycin may improve survival independent of sodium oxalate treatment. For context, the average life expectancy of DM ranges between 60 – 90 days and our DM given rapamycin on a lithogenic diet have a median survival of 33 days. Ali et al. reported very few DM fatalities before 20 days with median survival of about 35 days for DM on an *ad libitum* lithogenic diet with 0.1% sodium oxalate.[138,150] The male DM given a lithogenic diet in our study have median survival of 33 and 27 days in the rapamycin and vehicle groups respectively. In fact, the median ages of our DM in all groups are shorter than in the literature.[149–151] Our DM also appear to perish earlier in their life cycle, with nearly 60% of deaths before 20 days of age.

We noted shorter overall life span and increased premature death in our DM across all treatment groups compared to the available literature.[138,150] Reduced life expectancy may be related to the daily exposure to anaesthesia in order to change to agar for the purpose of administering rapamycin via CAFE. Specifically, recurrent CO_2 narcosis may have made our flies more susceptible to other causes of death such as infection. Hypercarbia has been shown to decrease host resistance to bacterial infection in *Drosophila* via upregulation of metabolic genes and reduced innate immune response to infection.[152] Unregulated host metabolism and poor resistance to infection may produce ROS and impair the epithelial lining integrity of the *Drosophila* gut, in addition to altering the homeostasis of Malpighian tubules.[47,153]

Fan et al. have shown that rapamycin can rescue intestinal epithelial integrity and improve life expectancy in a DM model of aging.[153] Rapamycin and mTOR inhibition can limit the harmful effect of reactive oxygen species by influencing the proliferation rate of intestinal stem cells and immune cells,.[153] Therefore, improvement in intestinal integrity is a mechanism by which rapamycin may have improved relative median survival between in DM fed a lithogenic diet. A competing hypothesis is that rapamycin acted on its microenvironment to improve median survival. Rapamycin has known antifungal properties that help ward off fungal organisms with pathologic potential in DM like *C. neoformans*.[100,154] Importantly, no macroscopic evidence of fungal infection was observed in our study.

Unexpectedly, male DM fed *ad libitum* calorie diet with alternating lithogenic days had the longest median life expectancy. In other studies, lithogenic diet treatment has a dose-dependent effect on reducing median life expectancy.[138] Additionally, environments of low nutrient availability should reduce mTOR activity, removing a barrier for autophagy to occur.[94] In particular, the rapamycin groups undergoing brief periods of dietary restriction were expected to have longer life expectancy as seen by others who have studied life span extension by by dietary restriction with and without rapamycin.[82,155] In the literature, rapamycin and dietary restriction are not equivalent, as they appear to act in some cases synergistically to improve life expectancy.[145,156] However, the CAFE method of drug delivery in DM is known to have lower survival compared to equivalent strains fed *ad* libitum.[157] Additionally, our dietary restriction treatment may have allowed for differential bacterial survival and create a hostile microenvironment. For example alphaproteobacteria has been suggested as a bacterial taxon associated with longevity in aging flies.[158] It is known to be impacted by rapamycin treatment, which has subsequently been linked to a mechanism for reduced mortality and health decline in aged flies.[158]

DM fed *ad libitum* alternating every other day with a lithogenic diet may have had a the longest median survival in part due to the constant presence of propionic acid in its environment. Propionic acid is a short-chain fatty acid (SCFA) that acts as a potent anti-fungal agent by inducing mercaptase-dependent apoptosis in eukaryotes.[159,160] Furthermore, *Propionibacterium* and *Lactobacilli* are known to produce propionic acid as a preservative by anaerobic fermentation of carbohydrates.[161] Propionic acid derived from food is also required for proper phospholipid membrane homeostasis in DM.[162] Without propionic acid supplementation, it is possible *Lactobacilli and Propionibacterium* are outcompeted by other endogenous non-propionic acid producing microorganisms leading to inefficient lipid metabolism and disordered epithelial defences. Other researchers have suggested that changes in endogenous microflora leading to dysbiosis may have a deleterious effect on DM lifespan via impaired immune or stem cell function, epithelial barrier function or through direct infection by microorganisms.[163–165]

Intermittent treatment with rapamycin did not increase median survival for female DM fed any diet. Furthermore, treatment-for-treatment, median life expectancy was greater for female DM compared to male DM. It is a known phenomenon that male DM are more sensitive to starvation and stress due to a greater tendency to move and compete for limited resources compared to females, leading to quicker wasting of stored lipids and proteins.[166]

Additionally, it is know that male and female DM harbour different microbiota in their environment.[164] Therefore, they may have responded differently to our treatments due to differences in microorganisms in their environment that was not controlled between genders. This could be mitigated in the future by using gnotobiotic (a "known microbiome") or axenic ("germ free") DM. Interestingly, the DM endogenous parasite *Wolbachia spp.* is associated with increased larval male-specific death and an increase in female median survival likely due to impaired oogenesis, reproductive function steroid hormone metabolism and energy conservation.[167,168] Furthermore, mTORC1 signalling has been shown to suppress *Wolbachia* titres in oocytes, leading to reduced ecdysone (steroid hormone) signalling that has been shown to significantly increase life expectancy in DM.[169] Since meiosis and reproduction is a non-life extending, energy-consuming processes, inhibiting this process may allow female DM to tolerate stressful situations better than male DM in our study.[168]

Another competing explanation is differential compensatory "binge eating" when placed back in an *ad libitum* diet condition. This is in agreement with work by Farhadian et al. that showed both male and female DM transiently increase their rate of food intake following fasting.[170] In fact, the female compensatory increase in rate of food intake lasted for approximately 24-hours in their study; whereas, males returned to baseline feeding in 12-hours, a finding that has been reproduced by others.[170,171] In our study, since females lived longer it may be because of a higher overall intake of nutritious or antimicrobial compounds following a period of fasting, helping them prevent confounding causes of death from infection.

Finally, it is possible that a fasting environment during treatment with either rapamycin or vehicle of 24-hours duration is not adequate to achieve benefits of mTOR inhibition. Furthermore, short intermittent fasting may be beneficial or harmful at different points in the DM life cycle.[155] Additionally, other authors have studied fasting durations ranging from 24-hours to 7 days in duration.[155,171,172] It is reasonable to assume therefore that our fasted DM may not have received the full physiologic benefits of reduced mTOR activity. Therefore, fasted DM would be at a survival disadvantage given less nutrients to support on-going metabolic demands.

Ex vivo crystal analysis

Drosophila melanogaster exposed to 0.1% sodium oxalate in their diet form highly birefringent calcium oxalate monohydrate crystals in their Malpighian tubules.[138] In agreement with our hypothesis, rapamycin treatment resulted in fewer visible MT calcium oxalate concretions compared to DM on other lithogenic diets. This result suggests that intermittent rapamycin may reduce nephrolithiasis in our dietary *Drosophila* model of calcium oxalate nephrolithiasis.

Our findings are primarily hypothesis generating for future study of the role of mTOR in nephrolithiasis. First, calcium and oxalate are small substrates that can travel across epithelial cell boundaries into the body through a paracellular mechanism.[173,34] Calcium oxalate crystallization is dependent on the concentration of oxalate and calcium in solution; therefore, gut homeostasis and effective oxalate uptake is an interesting target for the prevention of nephrolithiasis. Worsening epithelial barrier function often leads to increased paracellular transport of solutes.[174] Indeed, impaired epithelial barrier function in humans is seen following antibiotic treatment, fiber-poor diets high in processed foods, patients with metabolic syndrome and obesity, and aging.[175] Therefore, direct toxic insult on epithelia and/or alterations of the gut microbiome may lead to a higher systemic oxalate load and filtration into the Malpighian tubule lumen through increased oxalate absorption from the gut.[176] Fan *et al.* have demonstrated that rapamycin can rescue epithelial stem cell function in older Drosophila with impaired gut barrier function and homeostasis.[153] Further study is required to confirm whether modulation of mTOR in DM may impact oxalate uptake from a dietary source or whether it acts at the level of the Malpighian tubule itself.

An advantage of the DM model organism includes the possibility for future organotypic studies via the UAS-GAL4 system.[133] By employing a renal-specific (*uro*) and UAS-GAL4 system targeting one of the interacting proteins in the mTOR pathway or mTOR itself, one could elucidate whether mTOR activity is important at the level of oxalate transport in the gut or via its action at the Malpighian tubule (Figure 4).

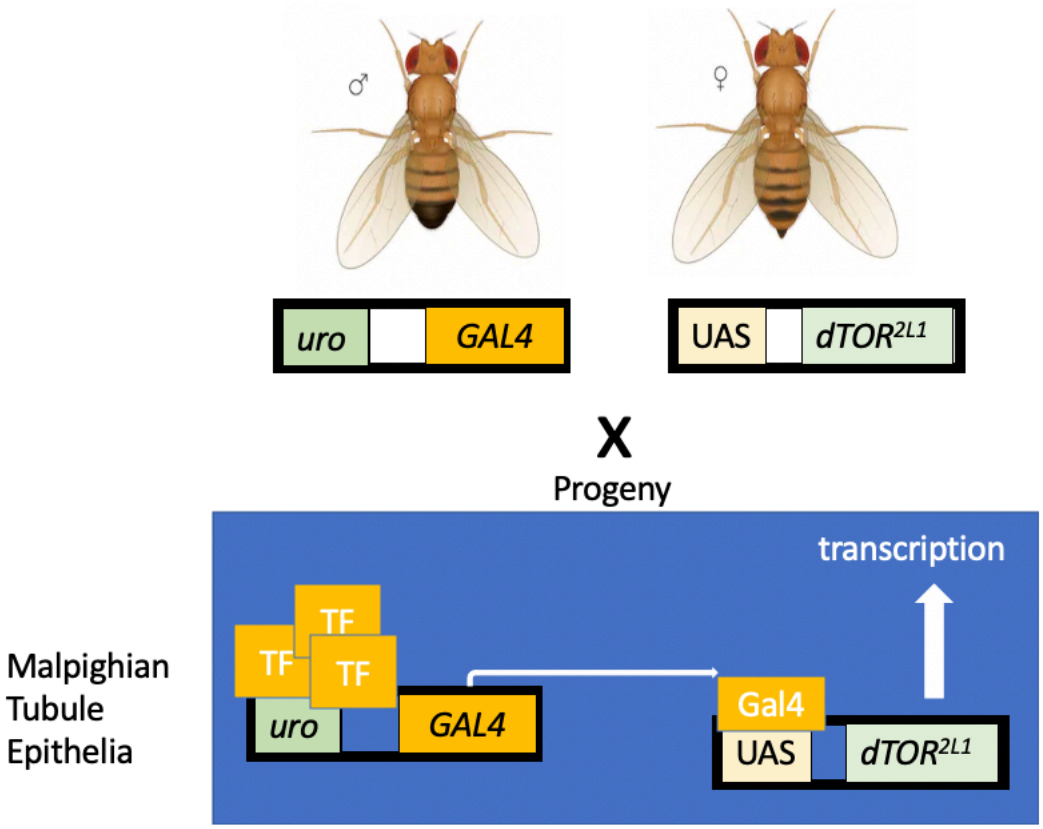


Figure 4 UAS-GAL 4 system with proposed Malpighian tubule tissue promoter *uro* genes producing null mTOR mutant. This technique requires isolation of pure male DM with the uro-GAL4 construct gene and pure female containing UAS-dTOR^{2L1}. Upon activation of uro by endogenous inducing transcription factors (TF), GAL4 is produced. The protein product of Gal4 then interacts with the UAS region of the gene construct carrying the mTOR mutant. This interaction leads to increased transcription of the desired gene specifically at the site of the Malpighian tubule in all progeny.

Second, rapamycin may upregulate autophagy leading to reduced calcium oxalate MT concretion area. Though we did not quantify autophagy in our study, our results support previous experiments by Unno *et al.* who showed improved efficiency of autophagic flux in renal tubular cells exposed to calcium oxalate when treated with rapamycin.[9] Overall this led to decreased luminal calcium oxalate stone formation and improved clearance of calcium oxalate crystals. There was a significant difference in Malpighian tubule concretions in DM fed non – lithogenic *ad libitum* and all DM fed 0.1% oxalate without rapamycin treatment. Therefore, rapamycin reduced the amount of calcium oxalate concretions in Malpighian tubules, but it may not inhibit the crystallization process completely. In other words, calcium oxalate nephrolithiasis still occurs; however, the further aggregation and growth of calcium oxalate crystals may be hindered by rapamycin treatment in our DM model.

In some settings, autophagy may actually exacerbate stone formation. In contrast to apoptosis which is a process of controlled cell death, autophagy is the process by which cells recycle macromolecules.[93] Duan *et al.* showed that chloroquine inhibition of autophagy reduced tubular inflammation and calcium oxalate deposition.[179] Indeed, their study found that calcium oxalate crystals led to more immature apoptotic vesicle formation at very high concentrations of oxalate (0.75 mM for 48h), a finding that was not seen in the study by Unno *et al.* who used a nephrocalcinosis model of calcium oxalate formation exacerbated by glycolate administration.[9,179] The latter study may be more relevant for the physiology of idiopathic calcium oxalate kidney stones in man. The study by Duan et al used chloroquine in order to inhibit autophagy. Although chloroquine administration does lead to inhibition of autophagy it also directly impairs cytoskeletal rearrangement required for the management endoplasmic reticulum stress.[180] The endoplasmic reticulum is important for processing secreted proteins like Tamm-Horsfall protein, thus potentially explaining why this mechanism of apoptosis inhibition may confound the study of nephrolithiasis inhibtiors.[63,180,181] However more research needs to be done on timing of autophagic activation/inhibition in relation to serum oxalate load and mTOR inhibition. Further support for rapamycin being positively associated with nephrolithiasis protection includes work describing a reduction in tubulointerstitial inflammation and fibrosis in obesity related renal conditions.[71,182]

Importantly, there was some variability in the degree of stone burden within individual DM of the rapamycin group fed a lithogenic diet. Furthermore, in both the rapamycin and vehicle lithogenic diet groups, there is a 24-hour period where flies are essentially calorically restricted to just a small amount of sucrose and either capillary containing vehicle or rapamycin solution. There does not appear to be significantly lower calcium oxalate MT concretions in calorically restricted compared to *ad libitum* caloric intake on a lithogenic diet. This finding casts doubt on our hypothesis that fasting between 0.1% sodium oxalate loads reduces Malpighian tubule concretions through inhibition of the mTOR pathway. However, it is possible that the duration of fasting in our study was not adequate to inhibit mTOR similar to rapamycin treatment. As a result, we did not note significant differences between our *ad libitum* diet conditions and those experiencing a period of fasting on agar. It is known that the metabolic effects of dietary restriction, of which fasting is a common strategy, is a complex endocrinologic process. Indeed, others have found that the physiologic benefits of fasting may be enhanced by rapamycin and are not purely mediated by mTOR activity.[158]

Additionally, timing of dietary restriction in the life of DM may be important. According to the pleiotropic hypothesis regarding the benefits surrounding autophagy and mTOR inhibition, actions that are detrimental earlier in life may be beneficial later (and vise versa).[94] Interestingly, Catterson et al. found that if DM were fed only twice a week until age 30 days, they had significantly longer life expectancy compared to DM who were either always fasting on a 2:5 schedule until death or always ate *ad libitum*.[155] DM that fasted throughout life actually had the shortest life expectancy in the aforementioned study.[155] The fact that fasting is not a universally protective activity suggests a possible epigenetic mechanism for its benefits.[183] Given the correlation between non-metabolic syndrome phenotype, aging, and relative protection from nephrolithiasis, further study on this topic is needed to determine whether the benefits of different periods of fasting has any true impact on nephrolithiasis pathogenesis.

Another hypothesis may be that rapamycin promotes the production of effective natural stone-inhibitors. Theoretically, mTOR inhibition should improve autophagy and rapidly clear dysfunctional or misfolded proteins in cells affected by inflammation.[95] The net result would be properly folded stone-inhibiting proteins secreted into urine. However, this is a less likely hypothesis as primary cilia are absent in *Drosophila* renal tubules.[177] Since primary cilia increases surface area for exchange of solutes, this suggests that there may be lower bulk secretory and resorptive processes at the level of the Malpighian tubule in DM compared to mammals.[177] Indeed, in our literature search we could not identify any articles suggesting a beneficial effect of rapamycin or mTOR inhibition on the production of molecules like osteopontin, Tamm Horsfall protein or other endogenous stone inhibitors involved in the dietary DM model of urolithiasis.

Taken together, our findings suggest that calcium oxalate deposition in DM Malpighian tubules does not require daily exposure to sodium oxalate and that the amount concretion is reduced by rapamycin. Although fasting did not appear to influence overall survival or Malpighian tubule calcium oxalate concretions, our data is inconclusive to claim an effect on nephrolithiasis and further study is needed to clarify this. Treatment with rapamycin appeared to help mitigate the adverse effect of sodium oxalate on both median survival and Malpighian tubule concretions, and the mechanisms underlying this are likely multifactorial. In addition, the gut microbiome and the effect of different genders may have also impacted our results and should be accounted for in future studies. Furthermore, rapamycin treatment may reduce mTOR activity exacerbated by calcium oxalate crystals adherence to renal tubule-like epithelial cells. Reduced mTOR may permit net clearance of damaged proteins that lead to inefficient clearance of lithogenic substrates.

Limitations and assumptions of study

Although we could have censored individuals that clearly died on transfer or by trauma, it is not possible to know the cause of death of all DM subjects for certain and we analyzed our life expectancy data in a non-informed manner to avoid bias. Furthermore, we cannot definitively state whether or not confounding factors which affect life expectancy influenced the weak correlation between stone formation and life expectancy in our model. We do assume by equal treatment of all groups that confounding variables should be minimized. We also cannot guarantee equal drug or nutrient uptake amongst our *Drosophila* subjects. Since it is a closed environment with limited resources, some individual DM may outcompete others for resources like sucrose in our CAFE system. Therefore, some may be overdosed while others may be underdosed. This may explain a rather large variation in stone burden seen in treatment groups containing 0.1% NaOx.

Observation of large cohorts of age-matched Canton S *Drosophila melanogaster* allows for population level information about the progression of aging and how treatments impact this process.[184] Arking *et al* found that longer *Drosophila* life span was related to increased resistance to oxidative stress and by reducing the impact of oxidative damage due to increased gene expression of antioxidant genes she could extend life expectancy.[185] In contrast to diets leading to metabolic syndrome, rapamycin and dietary restriction have been shown to improve life expectancy in yeast, flies as well as mice in part by limiting ROS.[86,149] Though life span extension is associated with slower senescence, shorter life spans are not necessarily associated with a "premature aging" phenotype.[186,187] In addition, body size and therefore mTOR activity may also vary in CantonS DM themselves. Others have shown that variation in body size alone can explain 40% of variation in life expectancy.[188] This variance may have produce a significant amount of noise in the interpretation of life expectancy data in relation to unequal baseline mTOR activity.[188] We did not see extended life expectancy, therefore, the relative improvement in survival seen in our study may be confounded by DM succumbing to stressors unrelated to ROS, calcium oxalate concretions and/or mTOR inhibition.

We chose pharmacologic inhibition of mTOR over a genetic model in Drosophila for several reasons. Rapamycin is more readily available in reagent stock than available mutants of mTOR in *Drosophila* that are not fatal before adulthood when the Malpighian tubules are available for dissection. *De novo* screening for mTOR mutants would be required in order to achieve this and this was not reasonably possible within the time frame of this project. However, there are significant disadvantages when using pharmacologic inhibition of mTOR with rapamycin. Due to the low water solubility, short half-life in acidic environment such as DM food, uncertain compatibility in solution with sodium oxalate and temperature instability of rapamycin in solution; rapamycin in our model had to be delivered via CAFE capillary aliquots from a stock solution.[189] An inevitable complication of the CAFE method is unequal dosing between flies of the same vial and survival stress. However, we would expect this to be randomly distributed in each vial and not dependent on treatment group. Notably, the group with the greatest survival was treated *ad libitum* without CAFE. Therefore, caloric restriction in the setting of a lithogenic diet in future studies should be attempted without use of CAFE assay if possible.

In contrast to rodent models, *Drosophila* have an open circulatory system, and as a result endothelial/vascular cell dysfunction is not easily modelled.[133] Since Randall's plaques are thought to be of vascular origin, we cannot interrogate this area of nephrolithiasis in DM.[36,56,138,147] Given that mTOR activity is required for wound healing and epithelial-to-mesenchymal cell transformation, this is an important short-coming of the dietary DM model of nephrolithiasis and mTOR action. Furthermore the immune system of *Drosophila* also differs greatly from humans.[190] While others have shown the activation of autophagy by mTOR inhibition may reduce Randall's plaques, DM do not form Randall's plaques. Therefore, rapamycin may also act in other ways to prevent nephrolithiasis.[9]

Though *Drosophila* is an excellent genetic model organism, "strong" direct mutants of mTOR lead to arrest in embryological development and may not survive long into adulthood.[191] In addition, rapamycin also indirectly affects mTORC2 activity, which can lead to a diabetic phenotype in face of increased sugar loads via relative β cell hypoplasia of the pancreas depending on chronicity of use and expression of FKB-506 binding proteins.[192,193] Therefore, for control of when and for how long we could initiate mTOR inhibition we decided to use a pharmacologic intervention in addition to our sodium oxalate dietary strategy. Considering another alternative would be to repeat this study protocol with a complete competitive antagonist of mTORC1 such as Torin1 or a water soluble version of rapamycin – everolimus.[194]

However, rapamycin is only a partial allosteric antagonist of mTORC1 at treatment doses.[101] The pharmacokinetic profile of rapamycin in DM is unknown, but the likely distribution and therefore half-life is difficult to ascertain limiting efforts to optimize a dosing schedule. For one, they have an erythrocyte analogue (hemocyte), it represents less than 10% of its total hemolymph cells.[195] In total, 95% of rapamycin is bound to immunophilins in human erythrocytes. DM do have an analogue to cytochrome P450 3A4, and therefore may have similar enzymatic processing of rapamycin into up to 16 different metabolites.[196] DM do not have an important role for estrogens, and females are generally larger than males; therefore, from gender is expected to play a lesser role in the tissue distribution and metabolism of rapamycin compared to humans.[107,114,197]

The DM model itself for the study of nephrolithiasis is not without its limitations. Due to the lack of a vascular system and lymphoid structures, the biochemical and inflammatory environment in DM are not analogous to humans. Furthermore, DM normally feeds on high-sugar nutrients and has evolved adaptations necessary to prevent hyperglycemia, such as storage of glucose as hemolymph trehalose.[131] In addition, utilizing a dietary model of DM stone disease requires a large sample size of flies in order to reduce the variation in feeding habits of individual flies. Though DM are easy to house, they are sensitive to dehydration and temperature changes[198].

Drosophila are also too small to ensure that each individual receives the same amount of any dietary or drug intervention. The CApillary FEeding (CAFE) assay is a common, well established method to circumvent the difficult problem of dosing medications that may have low half-lives at specific temperatures or pH levels (Figure 2.1).[143] Finally, DM hatch from a larval phase whereby they are constantly are feeding and ambulatory, followed by a period of 3-4 days of fasting while awaiting to pupate into their final fly form.[198] Consequently, when studying mTOR which may be sensitive to states of fasting, the results may not be applicable to humans as we do not typically have these types of dietary patterns.

We are unable to claim that our rapamycin treatment reduced calcium oxalate concretions due to direct mTOR inhibition. We also must assume that rapamycin did not affect the feeding behaviour of DM to feed on the capillary solution. It has been shown that DM do not consume all drugs administered by CAFE at the same rate.[143] We also assumed that the reason for improvements in life expectancy, as well as calcium oxalate concretions, was due to direct DM mTOR inhibition rather than an effect on microbial environment by rapamycin, a compound with known antibiotic and immunosuppressive properties.[100]

Ultimately, other studies are required to elucidate whether it is a drug phenomenon of rapamycin on the environment, or, mTOR inhibition in DM that exacerbates nephrolithiasis. For example, should a null-mutant mTOR *Drosophila* strain such as $dTOR^{2L1}/dTOR^{l(2)k17004}$ that is phenotypically smaller than CantonS show similar results to our study it would strengthen the hypothesis that mTOR inhibition directly acts on DM cells to influence nephrolithiasis rather than the environment. Additionally, if we repeated our study in germ free DM it may refute the idea that rapamycin worked by modulating the microbiological environment of our subjects leading to relative protection from nephrolithiasis. In our fasting conditions, we may be missing the optimal duration or time for fasting to prevent nephrolithiasis, limiting our ability to assess its impact. Furthermore, without a measurement of apoptosis or protein synthesis, we cannot conclude which mechanisms are important in stone prevention via rapamycin administration.

Chapter 5

Conclusions and future directions

In conclusion, we are the first to demonstrate that intermittent rapamycin administration by CAFE is effective at reducing calcium oxalate Malpighian tubule burden in a dietary model of DM fed a 0.1% sodium oxalate diet every other day. In terms of mTOR inhibition and its role in nephrolithiasis, our study supports the recent findings of Unno et al. who suggested that rapamycin and mTOR inhibition reduce calcium oxalate crystal growth in mice.[9] Our study is novel as it suggests that intermittent dosing of rapamycin may be adequate to inhibit calcium oxalate nephrolithiasis. Furthermore, this work can be used as a pilot for further studies in Drosophila examining the mTOR pathway in calcium nephrolithiasis. Though longer life expectancy did not occur with rapamycin treatment, this may be confounded by limitations innate to using CAFE for drug delivery including the lack of propionic acid supplementation, feeding rate controls, and the possibility of microorganism involvement with a different gender predominance which may have impacted our findings. In order to study whether fasting can resilience mitigate calcium oxalate concretion formation, we can design relatively affordable optimization studies to look at the optimal microenvironment, duration of fasting and/or oxalate loading to reduce confounders.

In order to confirm our statement that rapamycin affects calcium oxalate stone formation, either at the level of the gut or Malpighian tubule epithelia, we would need to measure the output of DM mTOR activity via p-S6K or p-eIFB4 to ensure DM had physiologically relevant mTOR inhibition. However, systemic administration of rapamycin would not allow us to elucidate if nephrolithiasis is protected by mTOR inhibition at the level of the Malpighian tubular epithelia or gut in DM. To solve this, we would also need to create tissue-specific mutants in order to achieve knowledge of the tissue most important for prevention of nephrolithiasis in our model.

To accomplish the goal of identifying where mTOR is most needed in the prevention of nephrolithiasis, DM can be used generate tissue-specific mutants via the UAS-GAL4 system.[177] In this genetic method, a tissue-specific promotor region of DNA is combined with the GAL4 gene, a transcription factor.[199] Once GAL4 is produced in the tissue of interest, it can bind the UAS promoter and drive the expression of any gene of interest. For example, we could generate UAS-GAL4 flies with a *uro* promoter region, and drive the expression of a weak functionally null-mutant of mTOR like $dTOR^{2L1}$ only in the Malpighian tubule (Figure 4). This is possible because of the specificity of *uro* for Malpighian tubule structures. If this model were to show reduced calcium oxalate burden, it would strengthen the hypothesis that mTOR activity is important at the level of the Malpighian tubule rather than elsewhere. We could also examine the effect of pharmacologic inhibition of mTOR on a knockout model of autophagy to determine the necessity of autophagy for protection by mTOR inhibition.

Finally, to date mTOR inhibition has only been interrogated for calcium-based urolithiasis. Since metabolic syndrome is associated with uric acid stones as well as calcium oxalate stones, it would be interesting to determine the impact of rapamycin treatment or genetic mTOR inhibition in non-calcium oxalate stones that are also associated with aging and metabolic excess. This could be accomplished with a DM uric acid model of urolithiasis, which our group has previously developed.[134]

Overall, we conclude that inhibition of mTOR activity shows promise in reduction of calcium oxalate stone prevention in a dietary *Drosophila melanogaster* model of nephrolithiasis. In particular, we are the first to show that calcium oxalate concretions can be created with intermittent sodium oxalate treatment. Furthermore, we show that these concretions can be significantly reduced in *Drosophila melanogaster* by intermittent rapamycin treatment delivered via a capillary feeding system. This is important evidence as it supports the creation of genetic DM models to further investigate which metabolically active pathways downstream from the mTOR pathway are most strongly associated with prevention of calcium oxalate concretions. Once the downstream mTOR pathways with the most potential impact on calcium oxalate crystal formation are identified, these may then be targeted for further study in mammalian models. Ultimately, this pilot project will inform future work on incorporating mTOR pathway in our dietary DM model of nephrolithiasis.

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Curriculum Vitae: Michael Pignanelli

EDUCATION

Master of Science in Surgery *2019 – 2020*
Candidate – Western University

Resident in Urology *2018 – Present*
PGY3, Schulich school of Medicine & Dentistry – London Health Sciences Centre

Doctor of Medicine *2014 – 2018*
Schulich School of Medicine & Dentistry – Windsor Campus, Western University

Bachelor of Medical Sciences, Honours *2010 – 2014*
Western University – London, Ontario
Specialization in Medical Sciences, with honours. Four-year Dean's List scholar.

HONOURS, AWARDS, RECOGNITION

Resident Research Day Best Project Finalist 2019
Awarded honorarium as runner-up in annual research competition amongst Western urology residents.

Fundamental Sciences & Surgical Innovation Node - "Dragon's Den" Winner 2019
Awarded $5000 seed funding to assist in development of fail-safe catheter adaptor to prevent injury from accidental Foley catheter removal with an intact balloon left inflated.

Fred N. Hagerman Award for Merit in Surgical Clerkship 2018
Awarded annually to one student who shows most promise for a career in surgery based on highest composite of clinical performance and academic merit

Erie St. Clair LHIN Genitourinary Conference Honorarium *2017*
For commitment and presentation of robot assisted laparoscopic radical prostatectomy outcomes at annual health network conference on genitourinary oncology.

R. John and Agnes M. Adams Scholarship *2016*
Awarded annually by The Heart & Stroke Foundation to one medical student at each medical school in Canada based on potential impact of their research project to the field of cardiology.

Academic works

Selected Publications

Pignanelli M, Spierling A, Al K, Burton J, Bjazevic J, Razvi H. Intermittent dosing of rapamycin decreases calcium oxalate stone burden in a dietary Drosophila melanogaster model of urolithiasis, moderated poster session, CUAJ June 2020 available online at: file:///Users/michaelpignanelli/Downloads/6732-Article%20Text-32138-1-10-20200602.pdf

Stern N, Pignanelli M, Welk B. *The management of an extraperitoneal bladder injury associated with a pelvic fracture*. *Can Urol Assoc J.* 2019 n;13(6 Suppl4):S56-S60. doi: 10.5489/cuaj.593

Pignanelli M, Just C, Bogiatzi C, Dinculescu V, Gloor G, Allen-Vercoe E, Reid G, Ruetz K, Velenosi T, Ruetz K, Urquhart B, Spence JD. *Mediterranean Diet Score: Associations with Metabolic Products of the Intestinal Microbiome, Carotid Plaque Burden, and Renal Function.* Nutrients. 2018 Jun 16;10(6). pii: E779.

Bogiatzi C, Gloor G, Allen-Vercoe E, Reid G, Wong R, Ruetz K, Velenosi T, Urquhart B, Dinculescu V, Pignanelli M, Spence JD. *Metabolic products of the intestinal microbiome and extremes of atherosclerosis.* Atherosclerosis. 2018 Jun;273:91-97.

Patent
United States Provisional Application No. 62/772,686. November 29, 2018.

METHODS TO PREVENT TRAUMA UPON SUDDEN TENSION ON OR REMOVAL OF A URINARY CATHETER CONTAINING AN INFLATED BALLOON
CGO Ref. 68015-001 PRV.

OTHER

Certificate in Developing Essential Skills in Clinical Research *Winter 2017*
Harvard Medical School – Online Global Academy

PROFESSIONAL MEMBERSHIPS

Canadian Urologic Association (Associate Member) *2017 – Present*
American Urologic Association (Resident/Fellow Member) 2018 – Present