COVID-FIS: A PHASE 2 PLACEBO-CONTROLLED PILOT STUDY IN COVID-19 OF FISETIN TO ALLEVIATE DYSFUNCTION AND EXCESSIVE INFLAMMATORY RESPONSE IN OLDER ADULTS IN NURSING HOMES

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I have read and agree to Protocol entitled "COVID-FIS: A PHASE 2 PLACEBO-CONTROLLED PILOT STUDY IN COVID-19 OF FISETIN TO ALLEVIATE DYSFUNCTION AND EXCESSIVE INFLAMMATORY RESPONSE IN OLDER ADULTS IN NURSING HOMES". I am aware of my responsibilities as an investigator under the guidelines of Good Clinical Practice (GCP), local regulations (as applicable) and the study protocol. I agree to conduct the study according to these guidelines and to appropriately direct and assist the staff under my control, who will be involved in the study.

The Principal Investigator at local site: _____ Print name	_____ Signature	__ __ / __ __/ __ __ __ __ Date
Primary Principal Investigator: Dr. James L. Kirkland, MD, PhD _____ Print name	_____ Signature	__ __ / __ __/ __ __ __ __ Date

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List of Abbreviations

AE	Adverse Event/Adverse Experience
AFFIRM	Alleviation by Fisetin of Frailty, Inflammation, and Related Measures Trial
ALT	Alanine Aminotransferase
ARDS	Acute Respiratory Distress Syndrome
AST	Aspartate Aminotransferase
BAP	Biospecimens Accessioning and Processing Laboratory
CFR	Code of Federal Regulations
CK	Creatine Kinase
CoV	Coronavirus-19
CRF	Case Report Form
CTCAE	Common Terminology Criteria for Adverse Events
CVA	Cerebrovascular Accident
D	Dasatinib
DAMP	Damage-Associated Molecular Profile
DSMB	Data and Safety Monitoring Board
ECMO	Extra-Corporeal Membrane Oxygenation
eGFR	Estimated Glomerular Filtration Rate
ESR	Erythrocyte Sedimentation Rate
FDA	Food and Drug Administration
γ-H2.AX	γ-Histone 2.A DNA Damage Foci
GCP	Good Clinical Practice
GCSF	Granulocyte Colony Stimulating Factor
GMP	Good Manufacturing Practice
HIPAA	Health Insurance Portability and Accountability Act
HUVEC	Human Umbilical Vein Endothelial Cell
IB	Investigator's Brochure
IL (*e.g.,* IL-6)	Interleukin (*e.g.,* Interleukin-6)
IND	Investigational New Drug Application
IP	Investigational Product
IPF	Idiopathic Pulmonary Fibrosis
IRB	Institutional Review Board
LAR	Legally Authorized Representative
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein-1
MDS	Minimal Data Set
MHV	Mouse Hepatitis Virus, a Mouse β-Coronavirus
MIP1-α	Macrophage Inflammatory Protein 1-α
miRNA	Micro-RNA
MMP	Matrix Metallopeptidase
MSC	Mesenchymal Stem Cell
NME	Normal Microbial Experience
PAMP	Pathogen-Associated Molecular Profile
PHI	Protected Health Information
PI	Principal Investigator
Q	Quercetin
SA-βGal	Senescence-Associated β-Galactosidase
SAE	Serious Adverse Event/Serious Adverse Experience

SASP	Senescence-Associated Secretory Phenotype
SCAP	Senescent Cell Anti-Apoptotic Pathway
sHLH	Secondary Hemophagocytic Lymphohistiocytosis
SOP	Standard Operating Procedure
TAF	Telomere-Associated DNA Damage Foci
TGN	Translational Geroscience Network
TNF-α	Tumor Necrosis Factor-α
U of M	University of Minnesota
UPIRSTO	Unanticipated Problems Involving Risk to Subjects or Others
WCBP	Women of Childbearing Potential

Study Summary

Title	COVID-FIS: A Phase 2 Placebo-Controlled Pilot Study in COVID-19 of Fisetin to Alleviate Dysfunction and Excessive Inflammatory Response in Older Adults in Nursing Homes
Running Title	**COVID-FIS**: **COVID-**19 Pilot Study of **Fis**etin to Alleviate Dysfunction and Inflammation
Protocol Number	20-008867
Phase	Phase II
Methodology	Randomized, placebo-controlled, double-blind secondary prevention trial
Overall Study Duration	32 months
Subject Participation Duration	Subject participation duration 6 months (screening, 4 days treatment, follow-up visits, unblinded at the end of the study)
Single or Multi-Site	Multi-Site
Objectives	• To prevent COVID-19 (CoV) disease complications by a 7 point score adapted from the WHO Ordinal Scale for Clinical Improvement of CoV (Appendix 1) • To evaluate safety and tolerability of Fisetin in this patient population • To reduce progression of severity of SARS-CoV-2 infections with no, mild, or moderate to severe or critical symptoms. To decrease senescent cells, inflammation, and physical dysfunction (frailty)
Number of Subjects	250 enrolled and screened, 150 accrued and randomized
Diagnosis and Main Inclusion Criteria	Women or men age ≥ 65 years in a nursing home with mRNA-PCR test-proven CoV infection. SpO2 ≥ 85% (on room air or ≤ 2 L of supplemental oxygen) at time of enrollment.
Study Product, Dose, Route, Regimen	This study will involve a 2-day oral or NG or D tube course of Fisetin twice (~20 mg/kg/day for 2 consecutive days; Days 0, 1, 8 and 9).
Duration of Administration	4 out of 10 days
Reference therapy	Placebo-controlled
Statistical Methodology	Assuming 75 subjects/ group with a significance level of 0.05, the study will have 80% power to detect an odds ratio of 2.24 comparing the placebo- to Fisetin-treated group, using the 7-point severity score at Day 14 as the endpoint in an ordinal logistic regression model.

1 Introduction

This document is a protocol for a human research study. This study will be carried out in accordance with applicable United States government regulations and Mayo Clinic's and other participating institutions' research policies and procedures.

1.1 Background

Coronavirus-19 (CoV): Coronaviruses constitute the subfamily *Orthocoronavirinae*, in the family *Coronaviridae*, order *Nidovirales*, and realm *Riboviria*. They are enveloped viruses with a positive-sense single-stranded RNA genome and a nucleocapsid of helical symmetry. They are the largest among known RNA viruses. As of December 22, 2020, there have been at least 1.71 million confirmed deaths and more than 77.6 million confirmed cases in the β-coronavirus-19 (CoV) pandemic. CoV can cause physical dysfunction (frailty) and death from hyper-inflammation, cytokine storm, and associated acute respiratory distress syndrome (ARDS), myocarditis with troponin leak, and multi-organ failure, particularly in older or chronically-ill individuals, with 25-50% of nursing home residents who test positive (PCR) dying from CoV complications[10].

Cellular Senescence: Senescent cells were discovered in 1961[11] when serially-subcultured human embryonic fibroblasts were shown to lose replicative capacity, yet remain viable. This prompted work to test if aging leads to accumulation *in vivo* of pre-senescent cells, which have limited remaining replicative potential, and senescent cells, which cannot replicate but are metabolically active. Senescent cell burden is very low in young individuals but increases with aging in several tissues, including adipose tissue, lung, skeletal muscle, heart, kidney, brain, bone, and skin[8,12-18]. For example, we found the number of primary adipocyte progenitors (preadipocytes) with limited replicative potential (pre-senescent and senescent cells) cloned from adipose tissue of rats that were isogenic and raised under controlled conditions rises across the age spectrum[6]. Cellular senescence contributes to age-related dysfunction and multiple diseases throughout the lifespan. Senescent cells appear at pathogenic sites of many major diseases, including in the lung in chronic lung disease, asthma, smoking, and idiopathic pulmonary fibrosis (IPF), adipose and other tissues in diabetes/ obesity, the heart and vessels cardiovascular diseases, brain in Alzheimer's disease, around cancers, bone in osteoporosis and osteoarthritis, kidney in renal disease, and liver in cirrhosis and steatosis in preclinical species and humans[8,15,17], all conditions that are associated with physical dysfunction (frailty), hyper-inflammation Cytokine storm), and CoV morbidity and mortality.

Senescence is essentially a cell fate, like differentiation, proliferation, apoptosis, or necrosis. External and internal signals can contribute to driving a cell into senescence. These are generally cell or tissue damage-related, including DNA alterations (dysfunctional telomeres, strand breaks, *etc*.), metabolic dysfunction (reactive oxygen species [ROS], high glucose, bioactive lipids, mitochondrial dysfunction), protein alterations (aggregates, misfolding, failed autophagy), inflammatory signals, mechanical/shear stress, damage-associated molecular pattern factors (DAMPs; tissue damage signals [extracellular nucleotides, *etc*.]), cellular damage signals (DNA damage, telomeric dysfunction, protein aggregates, mitochondrial dysfunction), oncogenes, mitogens (insulin-like growth factor-1, *etc*.), and pathogen-associated molecular pattern factors (PAMPs), including viruses, bacterial/fungal proteins, lipopolysaccharide (LPS), *etc*. Once initiated, senescence takes from 1 to 6 weeks to become established through transcription factor cascades that may, but do not always, include $p16^{INK4A}$/ retinoblastoma protein and/or $p53/p21^{CIP1}$. These cause extensive changes in gene expression, histone modifications, altered organelle function (*e.g*., mitochondria, endoplasmic reticulum, nucleolus, nuclear envelope), elevated protein production due to increased mTOR and decreased autophagy, and profound

morphological and metabolic shifts[15,19].

There are no fully sensitive or specific markers for identifying senescent cells. These cells are generally large, may have high $p16^{INK4A}$ and/or $p21^{CIP1}$ (but not always), and exhibit DNA damage foci, particularly in telomeres (telomere-associated DNA damage foci [TAFs]), senescence-associated distention of pericentromeric satellite DNA, and increased β-galactosidase activity (SA-βGal)[15]. Like cancer cells, senescent cells frequently are metabolically-shifted from fatty acid utilization toward glycolysis, resulting in ROS generation, lipid accumulation, lipotoxicity, and dysdifferentiation into adipocyte-like but insulin-resistant "MAD" (mesenchymal adipocyte-like default) cells[20]. Senescent cells are resistant to apoptosis[21].

Senescent Cell Characteristics: Cellular senescence leads to inflammation, fibrosis, DNA damage, mitochondrial dysfunction, ROS generation, NAD^+ depletion, protein aggregation, failed autophagy, lipotoxicity, and stem and progenitor cell dysfunction. In turn, cellular senescence can be caused by other fundamental aging processes, including DNA damage, low NAD^+, mitochondrial dysfunction, ROS, protein aggregates, and lipotoxicity. Thus, from our and others' data, fundamental aging processes appear to be interlinked, findings that led us to formulate a "Unitary Theory of Fundamental Aging Processes", which posits that interventions, such as senolytics, targeting any one of these processes, such as cellular senescence, will affect many of the rest of the aging processes that predispose to aging phenotypes, frailty, hyper-inflammation, chronic diseases, and morbidity and mortality from acute insults, including viral infection such as CoV. Therefore, effects of the study agent in our clinical trial will be ascertained on senescent cells as well as other fundamental aging processes.

Senescence-Associated Secretory Phenotype (SASP): Accumulation of senescent cells can cause local and systemic inflammation, tissue destruction, immune system inhibition, and stem and progenitor cell dysfunction due to their SASP[3,4,15,22,23]. Generally 30-70% of senescent cells develop a SASP that entails release of pro-inflammatory, pro-apoptotic cytokines (TNFα, IL-1α, IL-1ß, IL-6, IL-7, IL-8, IFNγ, and many more), chemokines (*e.g.*, MCP-1, MIP1-α, RANTES, RARRES2, IP-10) that attract, activate, anchor, and sequester immune cells (including macrophages, dendritic cells, T-lymphocyte subsets, and neutrophils), tissue-destroying proteases (MMP-3, -9, -12, *etc.*), pro-coagulant factors (PAI-1, *etc.*), factors that impede stem cell/ progenitor function and cause fibrosis (Activin A, TGF-β-related proteins), ferritin, growth factors (*e.g.*, GCSF), bioactive lipids (bradykines, saturated ceramides, prostanoids), micro-RNA's (miRNA's), tissue-damaging non-coding nucleotides (including release of mitochondrial DNA, which attracts, activates, anchors, and sequesters dendritic cells[24]), microvesicles including exosomes, and other factors that cause tissue necrosis, systemic inflammation, immunological dysfunction, stem cell/ progenitor dysfunction, fibrosis, and spread of senescence to non-senescent cells. <u>These SASP factors include those linked to frailty, hyper-inflammation/ cytokine storm/ ARDS/ myocarditis with troponin leak, fibrosis, and multi-organ failure in CoV patients[10]</u>.

Likely due to their SASP, only a small number of senescent cells can cause considerable dysfunction. In pre-clinical experiments, transplanting senescent cells around the knee joints of young mice was sufficient to induce an osteoarthritis-like phenotype, while transplanting non-senescent cells did not[25]. Transplanting 10^6 radiation- or chemotherapy-induced senescent autologous ear fibroblasts or syngeneic adipocyte progenitors intraperitoneally into lean, adult mice, so that fewer than 1/10,000 of all cells in the transplanted mice were senescent, induced impaired physical function (frailty) and premature death due to early onset of the same age-related diseases that cause death in naturally-aged mice[25]. This also occurred after human

senescent (*vs*. non-senescent) cells were transplanted into immune-deficient (SCID) mice. When labelled senescent cells were transplanted intraperitoneally, they remained within the peritoneum, yet the transplant recipients developed non-labeled senescent cells in their limbs, demonstrating that senescence can spread from cell to cell, even at a distance[25].

Clearance of Senescent Cells by the Immune System: Senescent cells are usually cleared by the immune system[26]. They can attract, activate, anchor, and sequester (deplete) immune cells, including macrophages, dendritic cells, T-lymphocytes, and neutrophils through such SASP chemokines as MCP-1, MIP1-α, RANTES, RARRES2, and IP-10, cytokines such as IL-6, and TNFα, extracellular mitochondrial DNA, possibly miRNAs, and other factors[24-27]. However, above a threshold burden, senescent cells interfere with the immune system and its ability to remove them. For example, IL-6, a SASP component, interferes with macrophage migration, MMPs can cleave FAS ligand and other immune cell surface proteins, senescent cells cause fibrosis (impeding immune cell infiltration and trapping immune cells within inflammatory foci), and senescent cells can express "don't eat me" signals[26]. Consistent with this, senescent cells can drive cancer initiation and progression, which senolytics are highly effective at preventing, with substantial delays in death from multiple forms of cancer in mice[7,25,56]. Fisetin, which selectively clears senescent cells, delays death of older mice, 50% of which die from cancer, by over 17%[56].

Threshold Theory of Senescent Cell Burden: Based on the above, we formulated a "Threshold Theory of Senescent Cell Burden", which holds that once senescent cell abundance is sufficient to cause spread of senescence that exceeds capacity of the immune system to keep up with clearing these cells, an accelerated aging-like state with frailty, increased inflammation, and multi-organ failure ensues[25,26]. Consistent with this: 1) the number of senescent cells that needs to be transplanted to cause frailty, limit healthspan, and cause premature death, including from cancer, is higher in young than old mice and in young lean than young obese mice. Old mice or young, insulin-resistant, obese mice have more pre-existing senescent cells than young, lean mice[25]. 2) Senescent cell abundance remains low in skin until early old age in humans, followed by an upward inflection in senescent cell burden in subjects who are in their late 60's through the mid-70's[28]. This precedes the age-related increase in multi-morbidity[29]. 3) The lag between induction of senescence by chemotherapy and development of age-related morbidities is longer in childhood cancer survivors than adults who received higher chemotherapy doses in preparation for bone marrow transplantation[30,31]. These findings support the hypothesis that there is a threshold above which senescent cell burden due to spread of cellular senescence becomes self-amplifying. This causes increased risk for senescence- and age-related phenotypes, physical dysfunction/ frailty, hyper-inflammation, fibrosis, and diseases, perhaps contributing to age-related multi-morbidity[29]. Potentially, attraction, activation, anchoring, and sequestering of immune cells is accelerated after senescent cells exceed this threshold, predisposing to excessive inflammation in frailty and during viral infection, plus perpetuating and amplifying production of the SASP factors that underlie frailty, hyper-inflammation/ cytokine storm/ immune dysfunction, ARDS, cardiomyopathy, and multi-organ failure as occurs in fatal CoV cases, as well as subsequent fibrosis, *e.g.*, of the lungs.

Pathogen-Associated Molecular Profile Factors (PAMPs): Of note, the SASP is modifiable: drugs, hormones, and especially the PAMPs associated with infections such as coronaviruses, can modify the SASP. We found that PAMPs can exacerbate the SASP in previously relatively quiescent human senescent cells to a much greater extent than the effect of cytokines on the SASP. This leads to greatly increased secretion of factors underlying cytokine storm (IL-6, IL-8, MCP-1) and increased attraction and activation of innate immune cells, potentially driving the frailty, hyper-inflammation, cytokine storm, and ARDS caused by coronavirus (Fig. 1).

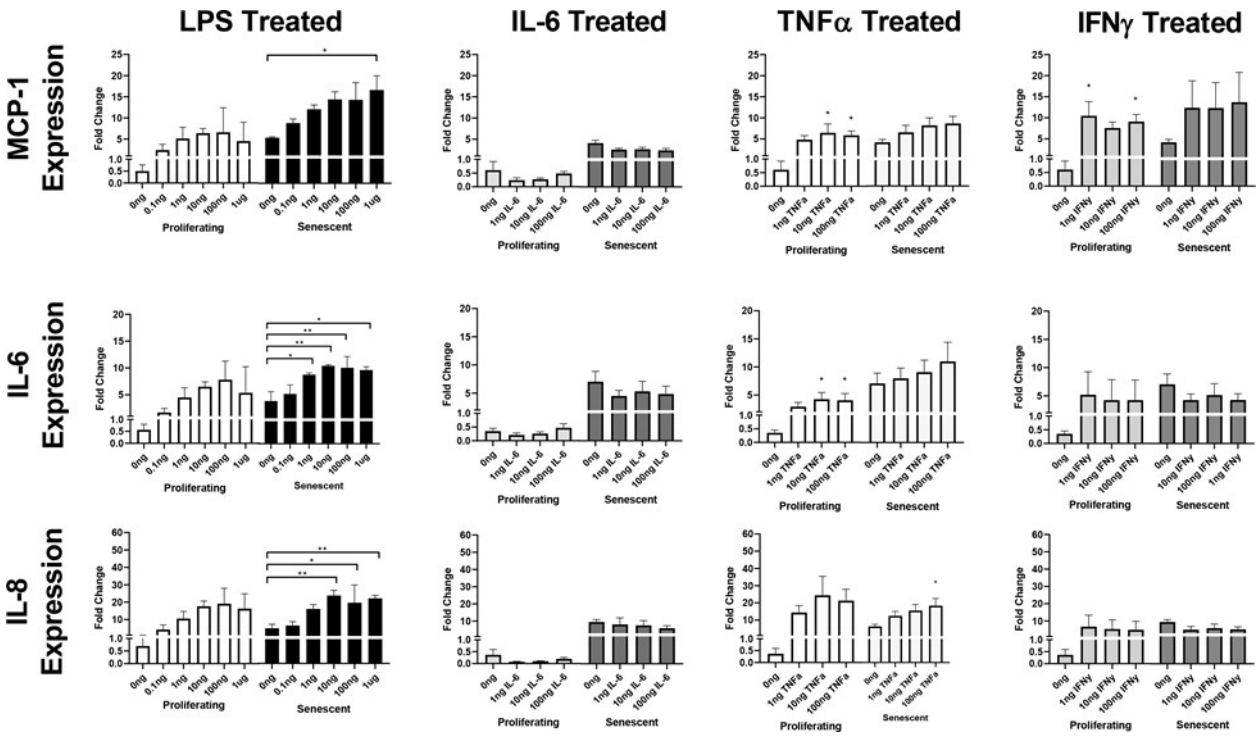


Fig. 1. Senescent cells challenged with PAMPs highly express SASP factor mRNAs. Human subcutaneous adipocyte progenitors from different subjects were irradiated or not to generate senescent or proliferating cells, respectively (n=3). Then cells were treated with different concentrations of the prototype PAMP, LPS, for 3 hours before mRNA isolation. One-way ANOVA and Dunnett multiple comparison post-tests were performed against the control (0 ng) for each condition (non-senescent or senescent). * = $p<0.05$, ** = $p<0.01$. Human non-senescent and radiation-induced senescent preadipocytes were treated with human recombinant IL-6, TNF-α, and IFN-γ for 3h. Note that the y-axis denotes *fold* change.

Similar results were obtained *in vivo* when progeroid mice, which have senescent cells in the same numbers and tissues as aged mice, were challenged with the PAMP, LPS (Fig. 2). In two tissues examined (kidney and liver), levels of senescence markers ($p16^{Ink4}$, $p21^{Cip1}$) and the SASP (*Il1β*, *Il6*, *TNFα,* and *Mcp1*) were greater in the progeroid mice compared to age-matched controls, illustrating amplification of the response to PAMPs in organisms with increased senescent cell burden. In another study focused on neuroprotection in the face of LPS challenge, rats were administered the senolytic agent, Fisetin, 20 i.p. mg/kg/day x 14 days, while being challenged with LPS[32]. In Fisetin-treated animals, inflammation and neurodegeneration were suppressed, while memory improved.

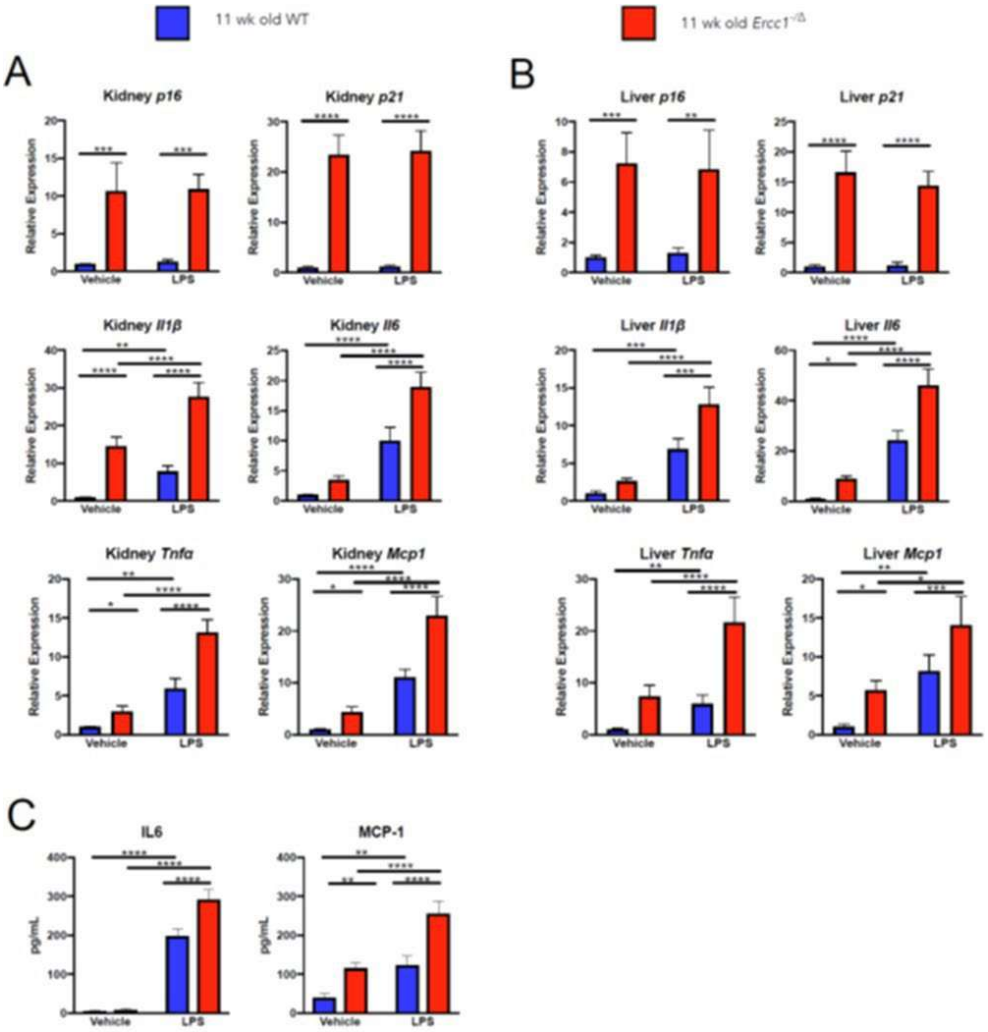


Fig. 2. Pre-existing senescence causes an exaggerated response to pathogens *in vivo*. Eleven-week-old wild-type (blue) and progeroid *Ercc1*$^{-/\Delta}$ (red) mice were injected with 500 ng/kg of LPS or vehicle (PBS) i.p. (N=4/ group). Twenty-four hours post-injection, mice were euthanized and tissues were collected. Total RNA was isolated from tissues and senescence and SASP marker expression was quantified in the (**A**) kidney and (**B**) liver by real-time qPCR using the ΔΔCt method. *Gapdh* was used as a control for gene expression analysis. (**C**) Circulating levels of IL-6 and MCP-1 were quantified in serum by ELISA. All values represent the mean ± SD. Statistical significance was determined by two-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001. (unpublished data, M. Yousefzadeh, B. Hughes, P. Robbins, L. Niedernhofer).

Amplifier/ Rheostat Hypothesis of Cellular Senescence: The SASP depends on the type of cell that became senescent, how senescence was induced, and its *milieu*. The SASP changes over time in response to internal (*e.g.*, transposons) and external cues (*e.g.*, PAMPs, DAMPs, glucocorticoids, SASP inhibitors such as rapamycin or metformin). PAMPs are particularly effective at exacerbating the SASP (Figs. 1&2), perhaps accounting for severe morbidity in previously highly-functioning elderly individuals with an increased (but quiescent) senescent cell burden that is activated by infections such as CoV or septicemia. Based on these considerations, we advanced an "Amplifier/ Rheostat Hypothesis" of cellular senescence: that the increased senescent cell burden in elderly and chronically ill patients contributes to their excessive, frailty/morbidity/mortality-inducing hyper-inflammatory (cytokine storm) response to infections that have much less cytokine-inducing effects in younger, previously healthy people (Fig. 3).

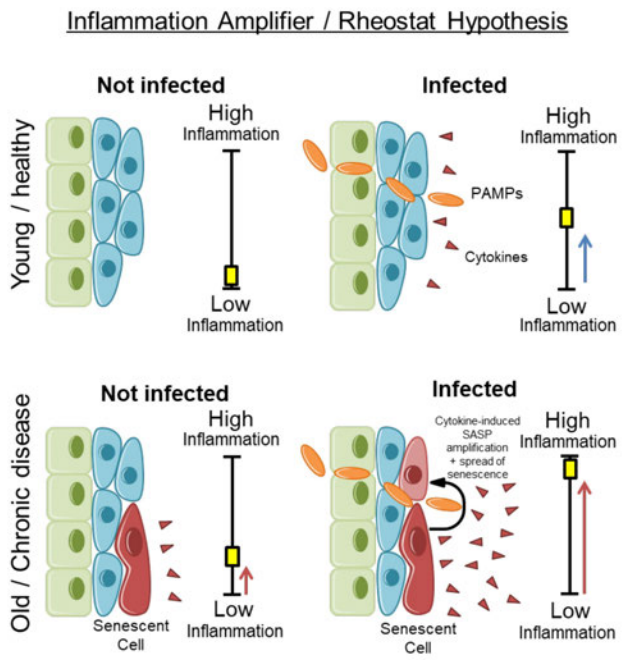


Fig. 3. SASP Amplifier/Rheostat Hypothesis. Among the 10 leading causes of mortality in the elderly are influenza, pneumonia, genitourinary infections, and septicemia. The elderly and patients with cellular senescence-associated diseases (*e.g.*, diabetes, dementias, smoking, asthma) are more susceptible to adverse outcomes in response to infections (*e.g.*, ARDS, cytokine storm, myocardial damage with troponin leak, delirium, hypotension, thrombosis, delayed recovery) and develop a more exaggerated inflammatory state than young patients without pre-existing chronic diseases. These adverse outcomes of infections in the elderly or patients with senescence-associated chronic diseases have generally been attributed to immune system dysfunction or to chronic inflammation. Our "Amplifier/ Rheostat" Hypothesis is that PAMPs (*e.g.*, LPS, viral antigens) cause a shift in the SASP of pre-existing senescent cells (SC) into a highly inflammatory, pro-apoptotic, pro-fibrotic SASP. This may not only exacerbate acute systemic inflammatory responses and cytokine release by innate immune system cells (macrophages, dendritic cells, T lymphocyte subsets, neutrophils), but could also amplify spread of senescence to other cells. The resulting additional senescent cells may act to exacerbate and prolong inflammation, attenuate or delay recovery, lead to persistent frailty, cause long term tissue fibrosis if the afflicted individual survives long enough, and contribute to multi-organ failure (L. Langhi, T. Tchkonia, J.L. Kirkland, October, 2018; unpublished).

Consistent with our Amplifier/Rheostat Hypothesis, we found CoV PAMPs exacerbate the SASP in quiescent human senescent cells, increasing secretion of factors underlying cytokine storm (IL-1α, IL-6, IL-8), potentially contributing to frailty, hyper-inflammation, and ARDS (Fig. 4). This is consistent with the findings that rapalogs, glucocorticoids, and metformin, all of which inhibit the SASP, lessen CoV cytokine storm[33-35]. However, unlike senolytics, these drugs have to be administered continuously, adding to off-target effects, especially in ill elderly patients. We also discovered SASP factors impair key viral defenses in human non-senescent cells (Fig. 5) and increase expression of the CoV viral entry proteins, ACE II and TMPRSS2 (Figs. 6&7).

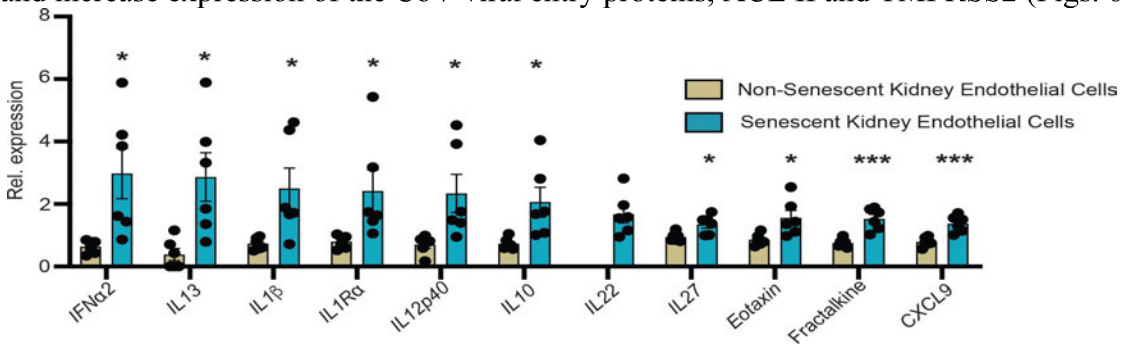


Fig. 4. CoV antigens exacerbate the SASP in cultured human senescent preadipocytes. Human kidney endothelial cells (n=6 replicates) were induced to undergo senescence with 10 Gys of ionizing radiation (Snc) or not (non-Snc) and then treated with 500ng SARS-CoV-2 antigen spike protein 1 (S1) for 24 hours. Forty-eight SASP-related proteins were measured in conditioned media (CM) by Luminex xMAP technology. Overall, effects of S1 were significantly more pronounced on Snc than non-Sncs. Each data point represents fold change in S1-treated cells relative to untreated ones. Paired t-tests were used to test differences within the Snc and non-Snc cell types. A mixed effects model was run to test for an interaction between Snc/non-Sncs and treatment, taking into account replicate measures within a subject (raw and p values for each cytokine are in Suppl. Tables 1& 2). Effects of S1 were more pronounced on Sncs than non-Sncs (cumulative $p<0.007$).

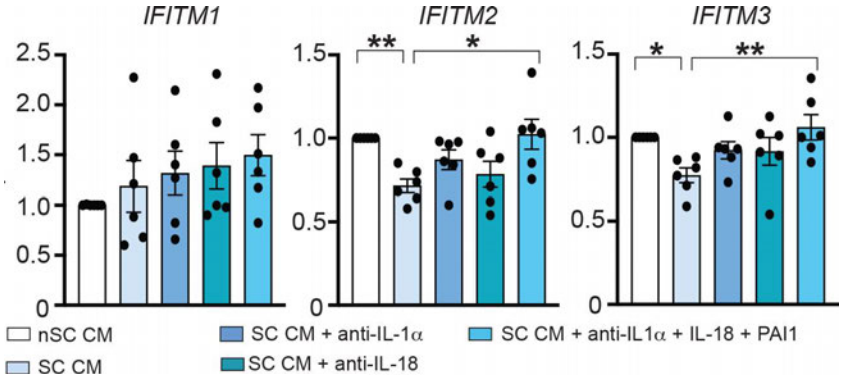


Fig. 5. The SASP inhibits innate anti-viral defense mechanisms. Primary human endothelial cells were irradiated to induce senescence or were untreated. Twenty days later, CM (n=4) was collected and used for treating primary human non-senescent kidney endothelial cells, either with or without antibodies to IL-1α, IL-18, and PAI-1. The impact on expression of interferon-induced transmembrane (IFITM) genes related to SARS-CoV-2 pathogenesis was measured by qPCR in RNA isolated from the treated cells. One-way ANOVA. *p<0.05, **p<0.01.These anti-viral defenses can also be enhanced in older patients by rapalog SASP inhibitors[7].

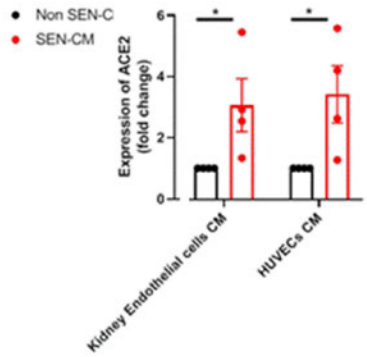


Fig. 6. The SASP causes increased expression of ACE II CoV viral binding/entry protein in human lung cells. Human non-senescent lung epithelial cells were treated with conditioned medium collected from human non-senescent and senescent kidney endothelial cells and HUVECs for 48 hrs. ACE II mRNA in lung epithelial cells was assayed by PCR (n=4).

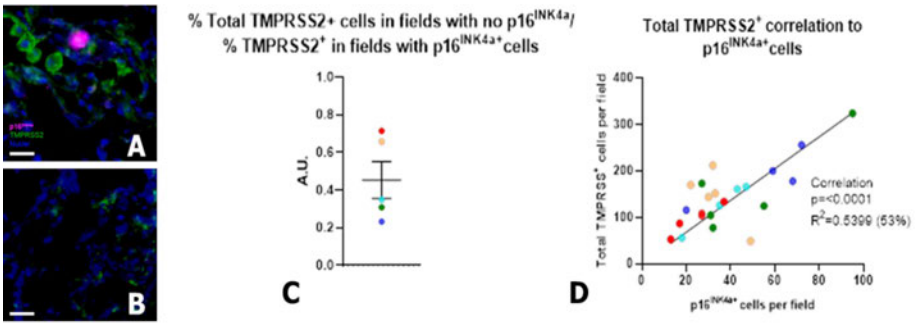


Fig. 7. The SASP causes increased expression of TMPRSS2, a CoV viral binding/entry protein in human lung. Human lung biopsies were stained for TMPRSS2, $p16^{INK4a}$, and total nuclei (N=5 subjects). TMPRSS2, was higher in representative fields containing any $p16^{INK4A+}$ cells (**A**) than in fields without $p16^{INK4A+}$ cells (**B**). Scale bar = 20 μm. (**C**) $TMPRSS2^{+}$, $p16^{INK4A+}$, and total nuclei were counted in each field. Fields with and without $p16^{INK4A+}$ cells were selected and the percent is shown of $TMPRSS2^{+}$ cells as a function of all cells. The fraction of $TMPRSS2^{+}$ cells in fields without $p16^{INK4A+}$ cells is significantly lower than the fraction of $TMPRSS2^{+}$ cells in fields with $p16^{INK4A+}$ cells, consistent with induction of TMPRSS2 protein in cells by $p16^{INK4A+}$ senescent cells. p<0.005; T-test. (**D**) $TMPRSS2^{+}$ and $p16^{INK4a+}$ cells/field are linked (p<0.0001; linear regression). N=5 (colors), 4-5 images/subject (total 23 images).

Old But Not Young Mice Die After MHV β-Coronavirus Exposure: In late 2019, our collaborators at the University of Minnesota (U of M) took advantage of a unique facility, a BSL-3 animal facility that brings experimental mice in contact with pet store mice to provide a "normal microbial experience" (NME). Female mice were co-housed with female pet store mice (Fig. 8). Because they fight, male mice were exposed to dirty bedding from pet store mice. Aged wild-type (WT) barrier-reared mice were obtained from the NIA and exposed to NME. Nine of 10 aged female mice exposed to pet store mice died rapidly. Exposing aged male mice to dirty bedding was also sufficient to kill the mice rapidly and remarkably synchronously. A key fomite transferred by pet store mice is a β-coronavirus similar to CoV, Mouse Hepatitis Virus (MHV), which causes an epidemic murine illness with high mortality, especially among colonies of laboratory mice[36]. Additionally, like many elderly humans dying from CoV, the old infected mice had cytokine storm (Fig. 9).

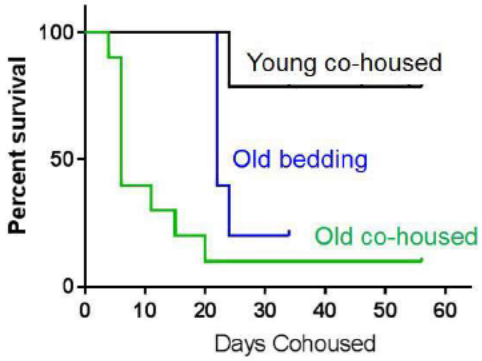


Fig. 8. Old mice are more susceptible to dying from acute exposure to mouse β-coronavirus than younger mice. 20 young (<6 months old; 10 female, 10 male) and 20 old mice (24 months, ~80 years in humans; 10 female, 10 male) were introduced into a β-coronavirus (Mouse Hepatitis Virus [MHV]) -contaminated facility. Old female mice were co-housed with contaminated female mice (green) and old male mice were exposed to contaminated bedding (blue). Mice at 24 months have senescent cell accumulation across tissues, including aorta, adipose tissue, liver, large intestine, kidney, pancreas, spleen, brain, lung, and skin[5,6], while mice <15 months of age have few if any. Consistent with our above Amplifier/ Rheostat Hypothesis, within a short period, almost all of the old and few of the young mice died (M. Yousefzadeh, S. Hamilton Hart, L. Niedernhofer 2019; unpublished).

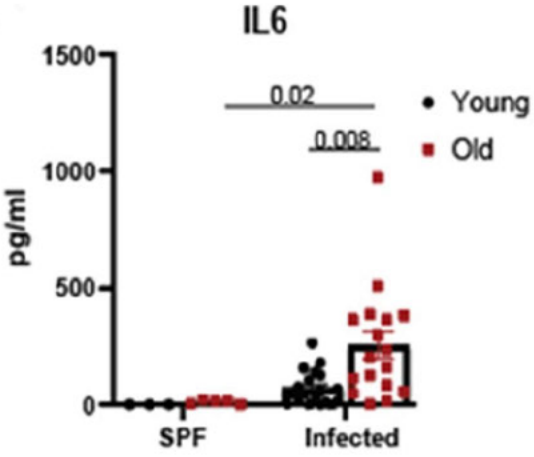


Fig. 9. Old β-coronavirus-infected mice develop higher IL-6 than old uninfected mice or young infected or uninfected mice. Blood IL-6 levels were assayed 5 days after infection with mouse β-coronavirus (mouse hepatitis virus). This is much like elderly humans who are at greater risk of a CoV-induced hyper-inflammatory response and cytokine storm than humans who were healthy and younger before being infected. SPF: specific pathogen-free.

Senolytics: In 2004 Sharpless *et al.*[37] showed that senescent cell accumulation is delayed in Ames dwarf mice (with pituitary hormone deficiencies) and calorically-restricted mice, models with increased healthspan and lifespan. This was critical in prompting us to test the hypothesis that targeting senescent cells may alleviate multiple age-related disorders. Our efforts to discover senolytics, drugs that selectively eliminate senescent cells, began in 2004/5, with initial attempts to create fusion proteins comprising a senescent cell surface-binding domain coupled to a toxin, high throughput compound library screens for candidates that eliminate senescent but not non-senescent cells, and other approaches. These traditional approaches were not initially successful, so we turned to a hypothesis-driven discovery paradigm to discover the first senolytic drugs[15,38,39]. We hypothesized that since senescent cells kill cells around them and damage tissues due to their inflammatory, apoptotic SASP, yet these senescent cells do not die, they must have pro-survival networks that defend them against their own SASP: senescent cell anti-apoptotic pathways (SCAPs)[8,38-40].

From proteomic/transcriptomic databases and using bio-informatics methods, we discovered the SCAP network[38]. Through RNA interference analyses, we demonstrated that transiently disabling this SCAP network leads to apoptosis of senescent cells, while normal cells without a SASP are not affected, the "Achilles' heel" of senescent cells. We next used bioinformatics approaches to identify drugs and natural products that act on critical SCAP nodes. Among these are Fisetin, Quercetin (Q), Dasatinib (D), and now over 20 others. We found these agents selectively eliminate senescent cells in mice and humans. They alleviate multiple conditions in mice, including diabetes and its complications, cardiovascular disorders (including heart failure and vascular dysfunction), dementias, pulmonary dysfunction (including bleomycin-induced lung disease), diabetes and its complications, frailty, kidney and liver damage or dysfunction,

osteoporosis, and osteoarthritis and delay cancer and extend healthspan and median lifespan[8,15-18].

We intentionally selected drugs for further development from among those identified as being senolytic. We focused on drugs that: 1) were already FDA-approved or were natural products with a history of safe human use, 2) could be administered orally, and 3) have a short elimination half-life (T½). Fisetin met these criteria. It is a natural product with a favorable safety profile, is effective orally, and has an elimination T½ ~3 hours, meaning it is cleared within a half day.

The target of senolytics is senescent cells, not a single receptor, enzyme, or biochemical pathway. By targeting pro-survival networks instead of single molecules, specificity for senescent cells can be increased, side-effect profiles flattened, and off-target effects on non-senescent cells reduced. Drugs that have single or limited targets, such as Navitoclax (ABT-263), which we discovered is senolytic at the same time as another group[41,42], or Nutlin3a, have off-target apoptotic effects on multiple non-senescent cell types, making them "panolytic". Navitoclax, a BCL-2/ BCL-w/ BCL-xL inhibitor, eliminates only a restricted range of senescent cells, *e.g.*, BCL-2 inhibitors are not senolytic against human adipocyte progenitors, one of the most abundant senescent cell types in aged humans or people with diabetes and obesity. Our strategies for developing senolytics have more in common with approaches for developing antibiotics than the conventional one-drug/one-target/one-disease drug development approach.

Description of the population to be studied. Women and men at or over age 65 with mRNA test-proven CoV infection who are nursing home residents will be screened for inclusion in this study. Patients themselves, if competent, or patients whose LAR have given informed consent and who meet screening criteria will be randomized to receive Fisetin 20 mg/kg/day orally (capsules or powder dissolved in water or added to apple sauce, pudding, or yogurt) or by nasogastric (NG-tube) or transabdominal duodenal tube (D-tube; powder dissolved in water or enteral hydration/ nutrition solutions) in pulsed doses (within 30 minutes) to achieve transient, high tissue Fisetin levels in a "hit-and-run" approach twice for 2 consecutive days (Days 0, 1, 8, and 9) or placebo. The study will be unblinded at the end of the study. The primary outcome is a 7 point ordinal severity scale. Secondary outcomes will include measures of senescent cell abundance/ inflammation (blood markers of senescent cells, including SASP factors), physical dysfunction/ frailty (including data from the Minimal Data Set [MDS] that is collected every quarter or 100 days from most nursing home residents as a Medicaid/Medicare requirement), safety/ tolerability, progression to severe or critical CoV, oxygenation and oxygen requirement, cell lysis syndrome, CBC, kidney and liver function, antibodies, and viral load, body temperature, chest imaging, need for acute hospital transfer, palliative care, intubation, or ICU care, and mortality. Blood and urine tests will be conducted before and after initiating treatment with Fisetin or placebo if possible.

1.2 Investigational Product (IP)

An investigational product is defined as a pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical study, including products already with a marketing authorization but used or assembled (formulated or packaged) differently than the authorized form, or used for an unauthorized indication, or when used to gain further information about the authorized form. The investigational products will be stored in a secure area according to local regulations. Investigators will have responsibility for ensuring that investigational product is only dispensed to study subjects. The investigational products (Fisetin and placebo) will be dispensed only by authorized personnel according to regulations that apply at pharmacies associated with the subjects' nursing homes, Mayo Clinic Rochester and the other Translational

Geroscience Network (TGN) sites. In this protocol, the investigational products are Fisetin and placebo.

Fisetin: Fisetin (3,3',4',7-tetrahydroxyflavone) is a member of the flavonoid family, a family of naturally-occurring polyphenolic compounds. As a natural product, it is widely sold, including by Amazon, from which it is available in containers of 30 capsules of 100 mg Fisetin/ capsule at <$20 per container and also containers of 500 mg capsules. The structure of Fisetin is similar to that of Q, which is one of the first two senolytic drugs discovered[38] (Fig. 10). Fisetin (Fig. 11), a high Trolox-equivalent antioxidant, is present in many fruits and vegetables such as apples, persimmon, grapes, onions, and cucumbers, with the highest concentration found in strawberries (160 μg/g)[43,44]. The average dietary intake of naturally-occurring Fisetin was approximately 0.8 mg/day in an earlier Japanese study[45,46]. However, the average total intake of polyphenols, comprised mainly of flavonoids, is around 1 gram per day, although it may be quite variable[47-49]. Fisetin has little or no odor or taste.

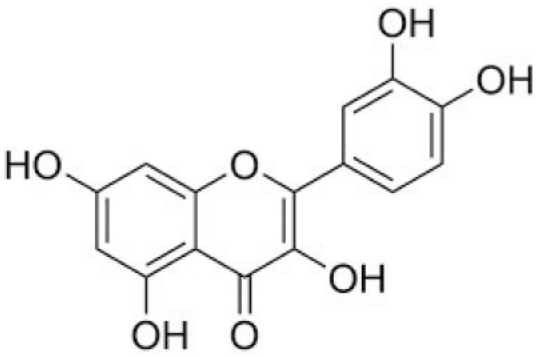


Fig. 10. Structure of the senolytic flavonol, Q. Quercetin, one of the first two senolytic drugs identified (along with Dasatinib [D]), and which is present in apple peels, has a bitter taste and is less bioavailable and has a longer elimination T½ than Fisetin.

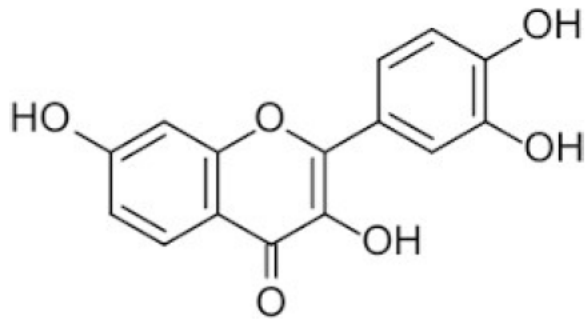


Fig. 11. Structure of Fisetin. Fisetin differs from Q by a hydroxyl group. It has little or no odor or taste. Its terminal T½ in mice is ~3.1 hrs[1]. This short half-life makes Fisetin ideal as a senolytic drug to ablate pro-inflammatory, pro-apoptotic, non-dividing senescent cells in a "hit-and-run" fashion[8]. ***Fisetin 100 and 500 mg capsules have been widely sold by Amazon and other vendors and have caused no proven side effects in over-the-counter sales in any reports that we can find.***

Due to its being a hydrophobic compound, Fisetin easily penetrates cell membranes, entering into cells to exert antioxidative and other effects[50,51]. Other promising biological activities include anti-hyperlipidemic[51-53], anti-inflammatory[52], neurotrophic, apoptotic[54], and antiproliferative effects[55].

There are reports of genotoxicity due to some flavonols, but Fisetin may exert little or no genotoxic effects *in vivo*[56]. Indeed, Fisetin has been noted to be anticarcinogenic[43,57,58]. We found that chronic Fisetin administration delays cancer in mice and increases remaining lifespan in old mice by over 17%[59]. Since up to 50% of most mouse strains die from cancers, this suggests that the beneficial effects of Fisetin on such carcinogenic factors as senescent cell burden and tissue chronic inflammation more than counteract any theoretical effects of flavonol-associated genotoxicity. In the same mouse study, Fisetin was administered to a murine model of a human progeroid syndrome[59]. These mice are profoundly sensitive to genotoxic agents[60], yet their health and lifespan were improved by long-term, high dose Fisetin[59]. *Thus, Fisetin is anti-carcinogenic.*

Fisetin is available as a dietary supplement. Other flavonols such as Q have been safely used in clinical trials in amounts up to 500 mg twice daily for up to 8 weeks. To date, no reports of significant toxicity for Fisetin *in vivo* have been made. Since Fisetin, along with other flavones and flavonoids, has been shown to inhibit cytochrome P450 2C9, CYP3A4, and others (Section 3.9.4), precaution will be taken to avoid significant drug-drug interactions, *e.g.*, with warfarin[61].

We demonstrated that Fisetin is a senolytic agent and alleviates multiple senescence-associated disorders in mice, decreases physical dysfunction if administered to old mice, attenuates age-related tissue damage and pathology, and extends remaining lifespan by 17%[39,59]. We have not found any adverse effects in mice (at up to 25 times the dose used in the mouse effectiveness studies [below] or the dose for humans proposed here) or old *Rhesus* monkeys (at up to 5 times the dose for humans proposed here). We obtained a full IND for Fisetin from the FDA and have a GMP drug supply handled by Mayo Research Pharmacy. We are currently conducting a clinical trial of Fisetin *vs*. placebo in elderly subjects with multi-morbidity and impaired mobility living in the community but without CoV to alleviate frailty, AFFIRM (Alleviation by Fisetin of Frailty, Inflammation, and Related Measures in Older Adults; ClinicalTrials.gov identifier NCT03430037; IND 134052; >50 subjects enrolled; no severe or serious adverse events so far).

Here, Fisetin 100 mg capsules or Fisetin (with cellulose) powder (in water, other chilled or room-temperature drinks, apple sauce, pudding, yogurt, or enteral hydration/ nutrition solutions) *vs*. placebo will be administered orally or by NG or D tube (~20 mg/ kg/ day) twice for 2 consecutive days on study Days 0, 1, 8, and 9 to elderly CoV positive nursing home residents (by mRNA-PCR within 10 day prior to randomization). To reduce senescent cell abundance by a "hit-and-run" approach, the full daily doses of Fisetin will be administered within 30 minutes. The investigational supplies will be obtained from Vital Nutrients, a contracted GMP manufacturer. Fisetin capsules supplied by Vital Nutrients are opaque blue in color. Fisetin powder is a green yellow color. We established stability parameters for Fisetin in collaboration with Mayo Clinic's Department of Laboratory Medicine and Pathology. We acquired an IND for Fisetin after establishing botanical sourcing, GMP isolation by Vital Nutrients, assurance of lack of contaminating pesticides, heavy metals, and solvents, and stability and degradation. We are currently conducting a clinical trial of Fisetin *vs*. placebo in hospitalized, non-ventilated elderly CoV patients to prevent progression of respiratory dysfunction, COVID-FISETIN (A Phase 2 Placebo-Controlled Pilot Study in COVID-19 of Fisetin to Alleviate Dysfunction and Excessive Inflammatory Response in Hospitalized Older Adults; IND 149813).

1.3 Preclinical Data

In our article about the first senolytics discovered, we demonstrated D+Q reduces senescent cell burden in multiple tissues and improves function in naturally-aged animals[38]. Since then, D+Q, Fisetin (which is closely related to Q), Navitoclax, and other senolytics have been shown to alleviate multiple diseases and disorders in animal models (Table 1). Among effects are: restoration of mesenchymal stem cell (MSC) function; improved cardiac ejection fraction, fractional shortening, and regeneration in old mice; enhanced vascular reactivity in old mice, decreased vascular calcification and restored vascular reactivity in hypercholesterolemic, high fat-fed *ApoE*$^{-/-}$ mice; decreased loss of intervertebral disc glycosaminoglycans and spondylosis in progeroid *Ercc1*$^{-/\Delta}$ mice; decreased gait disturbance in mice following radiation damage to a leg and hematological dysfunction caused by whole body radiation; decreased age-related changes in skin; improved pulmonary function and reduced pulmonary fibrosis in mice with bleomycin-induced lung damage, a model of IPF, plus decreases in: liver fibrosis in *Mdr*$^{-/-}$ cirrhotic mice; hepatic steatosis, insulin resistance, and renal dysfunction in high fat-fed mice; neuropsychiatric dysfunction and impaired neurogenesis in high fat-fed mice; neuro-inflammation, impaired

neurogenesis, microvascular impairment, and cognitive dysfunction in mouse models of dementia; age-related osteoporosis; uterine fibrosis; physical frailty; and muscle weakness in naturally-aged, progeroid, radiated, and senescent cell-transplanted young mice, as well as delayed cancer and increased healthspan and lifespan in old mice[8,14-17,25,27,38,42,62-69].

Condition	References
Diabetes/ Obesity	27,67,70-73
Cardiac Dysfunction	62,63,74
Vascular Hyporeactivity/ Calcification	63
AV Fistulae	75
Frailty	25,38,59,64
Age-Related Muscle Loss (Sarcopenia)	76
Chemotherapy Complications	25,31,42,64,77
Radiation Complications	78
Cancers	25
Bone Marrow Transplant Complications	31
Organ Transplantation Complications	24,79
Myeloma/ MGUS	80
Age-Related Cognitive Dysfunction	81
Alzheimer's Disease	82,83
Parkinson's Disease	84
Amyotrophic Lateral Sclerosis	85
Ataxia	38
Obesity-Related Neuropsychiatric Dysfunction	86
Renal Dysfunction	87,88
Urinary Incontinence	38
Osteoporosis	89-91
Osteoarthritis	92
Age-Related Intervertebral Disk Disease	38,93
Idiopathic Pulmonary Fibrosis	64,94
Hyperoxic Lung Damage	95
Chronic Obstructive Pulmonary Disease	96
Tobacco	97
Hepatic Steatosis	66
Cirrhosis	65
Primary Biliary Cirrhosis	98
Progerias	38,59
Pre-eclampsia	99
Macular Degeneration	100,101
Glaucoma	102-104
Cataracts	105
Prostatic Hypertrophy	106-108
Psoriasis	109
Healthspan	25,38,59
Lifespan	25,38,59

Table 1. Conditions with emerging evidence for a causal contribution of cellular senescence or that are alleviated by senolytic drugs. Cellular senescence contributes to age-related dysfunction and diseases throughout the lifespan. Senescence can contribute to local and systemic inflammation, dysfunction, and spread of senescence through release of cytokines, chemokines, proteases, ferritin, stem cell/progenitor toxins, reactive metabolites, miRNA's, mitochondrial DNA, other nucleotides, and extracellular vesicles, the SASP[2-4]. Pre-senescent cells, which have limited remaining replicative potential, and senescent cells, which cannot replicate, accumulate to a greater extent *in vivo* in skin biopsies from older than younger subjects[9]. Senescent and pre-senescent adipocyte progenitors cloned from adipose tissue increase with aging in rats raised under controlled conditions[6]. Senolytics have been shown to alleviate many of these conditions by us (**bold**) and others.

Most studies of senolytics for various disease states, including in humans, have been with D+Q since D and Q were the first senolytics discovered. However, while D+Q is relatively safe, it is likely not as safe as Fisetin for very ill patients. Unlike Fisetin, D cannot be administered safely to patients with a prolonged QTc (>450 msec). Many elderly and chronically-ill nursing home residents have a prolonged QTc, meaning they would be excluded from studies involving treatment with D. Additionally, there are multiple other drug interactions with D that would result in fewer patients being able to be treated than with Fisetin. Navitoclax has a poorer safety profile than Fisetin or D+Q: unlike D, which was approved by the FDA in 2006, Navitoclax has failed to gain FDA approval so far as a generally-available prescription drug, largely because of extensive and unpredictable induction of apoptosis in non-senescent cell types – "panolytic effects". For example, Navitoclax can cause life-threatening neutropenia or hemorrhage due to platelet depletion. Fisetin so far has had an exceptional safety profile in our clinical trials for elderly patients with multi-morbidity. Therefore, we decided to analyze Fisetin here rather than D+Q or Navitoclax.

"Hit and run": D+Q or Fisetin are effective if administered once every few days or weeks since they do not need to be continuously present to occupy a receptor or interfere with an enzyme, reducing off-target effects (Figs. 28&29). Brief disruption of pro-survival pathways is sufficient to kill senescent cells in mouse and human cell cultures, *in vivo* in mice and monkeys, and in

human adipose explants freshly-isolated from obese subjects[15,38,59,64]. In mice, monthly D+Q is as effective as daily administration to alleviate age-related osteoporosis[91], reducing potential side-effects. These points, together with satisfying a modified set of Koch's postulates (Table 2), show that senolytics alleviate dysfunction by removing senescent cells, not other off-target mechanisms. Unlike microbes or cancer cells, since senescent cells do not divide, they are unlikely to acquire replication-dependent drug resistance. Fisetin is well-suited for a "hit-and-run" administration protocol since it has an elimination T½ of ~3 hours, we demonstrated that it takes only 30 minutes of exposure of human tissues for senolytics to initiate the apoptosis process[25], and senescent cells take over 1 week to re-accumulate. Of note, no steady state levels for Fisetin need to be achieved and the goal is to eliminate senescent cells in a "hit-and-run" fashion, not to achieve a steady state blood level. The critical outcome here is target engagement, *i.e.*, do we kill senescent cells?, not do we achieve a particular drug or tissue level? Therefore, as in our other trials, traditional blood pharmacokinetic parameters will not be assayed. Rather, the critical pharmacodynamic measure, senescent cell abundance, will be assayed. This approach has been tacitly agreed to by the FDA regarding our other clinical trials of senolytics, including with Fisetin.

Table 2. A Modified Set of Koch's Postulates

To establish causation:

- Are senescent cells present in animals or humans with the disorder?
- Do individuals without senescent cells have the disorder?
- Is the disorder reproduced by inducing local accumulation of senescent cells (*e.g.*, by transplanting senescent cells, focal irradiation, or tissue-specific genetic approaches)?
- Does removing these transplanted or induced senescent cells prevent or alleviate the disorder?
- Does targeting naturally-occurring senescent cells alleviate the disorder?
- Does administering the potentially senolytic candidate have few or no effects related to the disorder being tested in individuals with few or no senescent cells (*e.g.*, young mice)?
- Does the potentially senolytic candidate alleviate the condition if given intermittently, at intervals longer than the drug's half-life, since senescent cells can take 10 days to 6 weeks to re-accumulate (at least in culture)? In the case of D+Q, senolytic drugs are as effective if administered monthly as continuously, at least in the case of age-related osteoporosis[91].
- Does the candidate alleviate multiple age-related conditions? (If a candidate is truly senolytic and the Geroscience Hypothesis is true, then it should alleviate multiple age- and chronic disease-related disorders).

The above criteria have been met for senolytic drugs for frailty, diabetes, and age-related osteoporosis and many have been met for osteoarthritis and neurodegenerative diseases in mice.

Fisetin selectively causes apoptosis of human cultured senescent cells: Fisetin reduces viability of 30-70% of senescent human umbilical endothelial cells (HUVECs) *in vitro*[39] (Fig. 12). Note that 30-70% elimination of senescent cells was achieved in our *INK-ATTAC* mice by activating the suicide gene expressed only in $p16^{Ink4a+}$ cells (many or most of which are senescent) in these mice[110]. This extent of elimination was sufficient to alleviate multiple age-related conditions in chronologically-aged and progeroid mice[14,63,64,110].

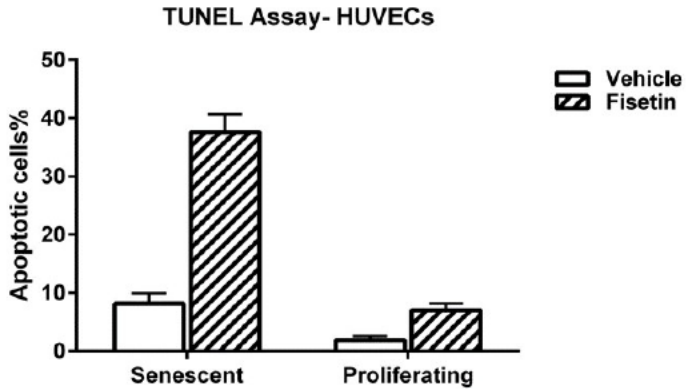


Fig. 12. Fisetin reduces human senescent cell viability through apoptosis. Senescent and non-senescent cultured HUVEC's were treated with Fisetin 20 μM or vehicle, respectively, for <12 hours and the percentage of apoptotic cells was measured by TUNEL assay.

Fisetin selectively eliminates senescent cells from mice: Fisetin reduces age-related senescent cell accumulation in mice (Figs. 13 & 14).

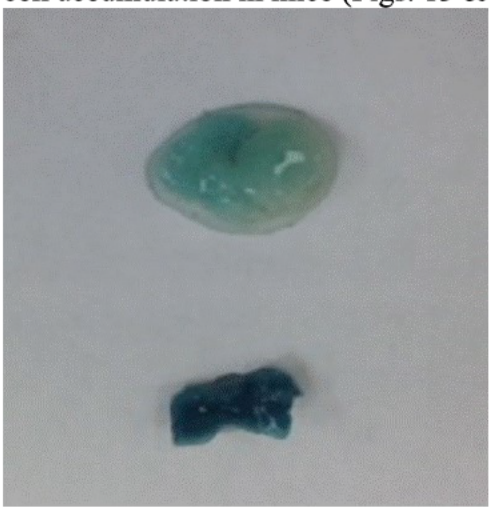


Fig. 13. Fisetin reduces senescent cell abundance in mouse adipose tissue. Inguinal adipose tissue from a 27 month old mouse that had been treated with Fisetin as daily pulse doses (tissue above) for 2 consecutive days once a month for 3 months (from age 24 months) was assayed for senescence-associated β-galactosidase (SA-βGal) activity 4 days after the last dose of Fisetin. Adipose tissue from a control 27 month-old littermate treated with vehicle is shown below. Fisetin also attenuated age-related lipodystrophy, as is evident by the larger adipose depot in the treated mouse. SA-βGal makes adipose tissue appear blue. Representative of 3 pairs of Fisetin and vehicle-treated littermates. A 27 month-old mouse is equivalent to an 80-90 year old human.

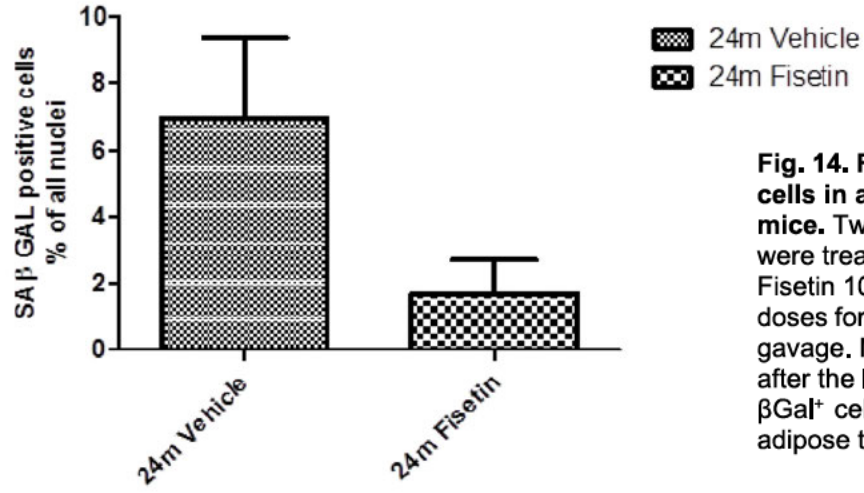


Fig. 14. Fisetin reduces SA-βGal⁺ cells in adipose tissue from old mice. Twenty-four month-old mice were treated with vehicle (N=3) or Fisetin 100 mg/kg (N=2) as daily pulse doses for five consecutive days by oral gavage. Mice were sacrificed 3 days after the last dose. Numbers of SA-βGal⁺ cells were assayed in inguinal adipose tissue.

Fisetin selectively eliminates senescent cells from monkeys: (Fig. 15).

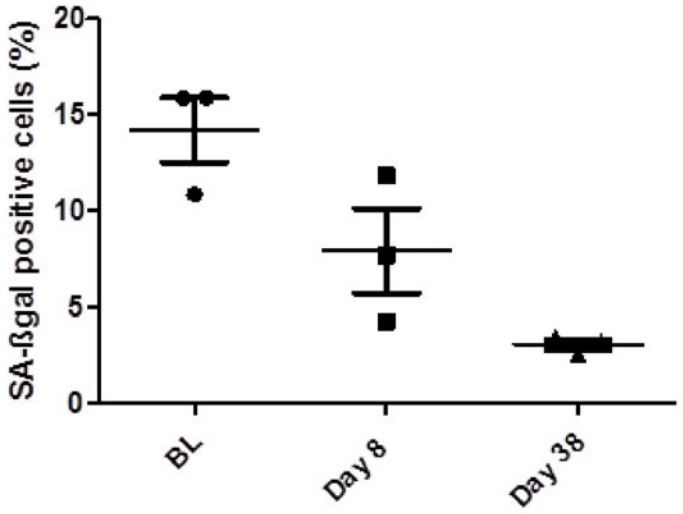
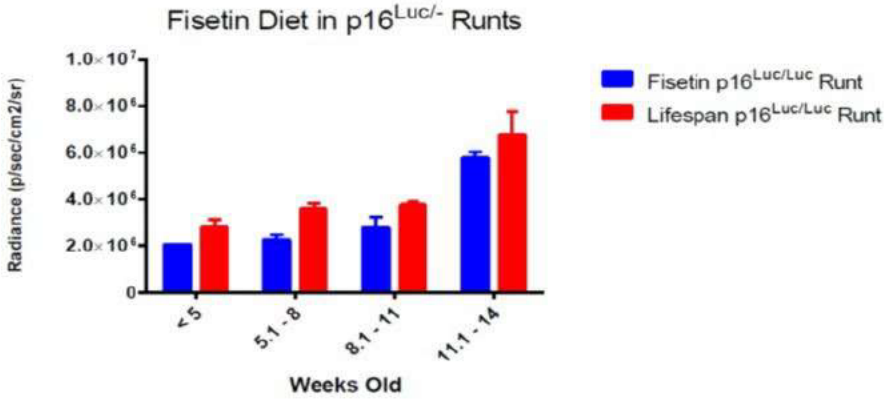


Fig. 15. SA-βGal⁺ cell number in subcutaneous adipose tissue biopsies of *Rhesus* macaques at baseline and after treatment with pulse doses of Fisetin. There is a greater decrease of senescent cells in the adipose tissue of *Rhesus* macaques at day 38 than at day 8 after the first dose of Fisetin 100 mg/kg. Animals were treated with Fisetin as daily pulse doses for 3 consecutive days. Biopsies were done at baseline, 8 days, and 38 days after the first dose of Fisetin.

Fisetin alleviates cellular senescence-related phenotypes and extends healthspan and lifespan in mice: *Ercc1*$^{-/\Delta}$ mice, which model human XFE progeria, have features resembling accelerated aging, with a lifespan of 6 months[111]. They develop progressive age-related chronic degenerative diseases including cancers (in at least 50%)[112] and accumulate senescent cells in the same tissues as aged wild-type mice, albeit more rapidly[5]. To track senescent cell abundance *in vivo*, *Ercc1*$^{-/\Delta}$ mice were crossed with *p-16-luc* transgenic mice. *p-16-luc* mice express luciferase under the control of the *p16*^{Ink4a} promotor (*p16*^{Ink4a} is increased in many or most senescent cells). After administering luciferin i.v., *p16*$^{Ink4+}$ cells expressing the transgene emit light and can be seen and quantified *in vivo* using a light-detection box. The light signal (radiance) indicates the intensity and location of *p16*$^{Ink4+}$ cells. Note that oral exposure to Fisetin at this dose, which is higher than that proposed for our human studies, does not cause toxicity even in these frail mice. There were no adverse effects on mobility, feeding, appearance, or weight.

Fisetin decreased senescent cell burden in *Ercc1*$^{-/\Delta}$*; p16-luc* mice as determined by decreased radiance (Fig. 16). *Ercc1*$^{-/\Delta}$*; p16-luc* mice on C57Bl/6 Albino:FVB background at 3-4 weeks of age were fed *ad libitum* with a standard Teklad 2020 chow diet ("Lifespan" diet; N=15) or the standard Teklad 2020 chow diet containing Fisetin 500 ppm (N=2). Fisetin 60 mg/kg/day was consumed by each mouse based on Fisetin 500 ppm (500 mg/kg) being in the diet and 3-5 g chow eaten/ day. Senescent cell burden was measured at baseline and biweekly in control (lifespan) and Fisetin-treated mice. Imaging of luciferase activity indicates a significant decrease in senescence signal.



	P16 L/-;Ercc1 -/? Fisetin			P16 L/-;Ercc1 -/? Lifespan			
Weeks Old	Mean	Std Dev	n	Mean	Std Dev	n	p-Value
< 5	2.03E+06		1	2.79E+06	6.46E+05	4	0.3718
5.1 - 8	2.26E+06	3.91E+05	3	3.56E+06	1.05E+06	15	0.0557
8.1 - 11	2.76E+06	6.60E+05	2	3.76E+06	4.83E+05	10	0.028
11.1 - 14	5.77E+06	352639	2	6.76E+06	4072220	17	0.7405

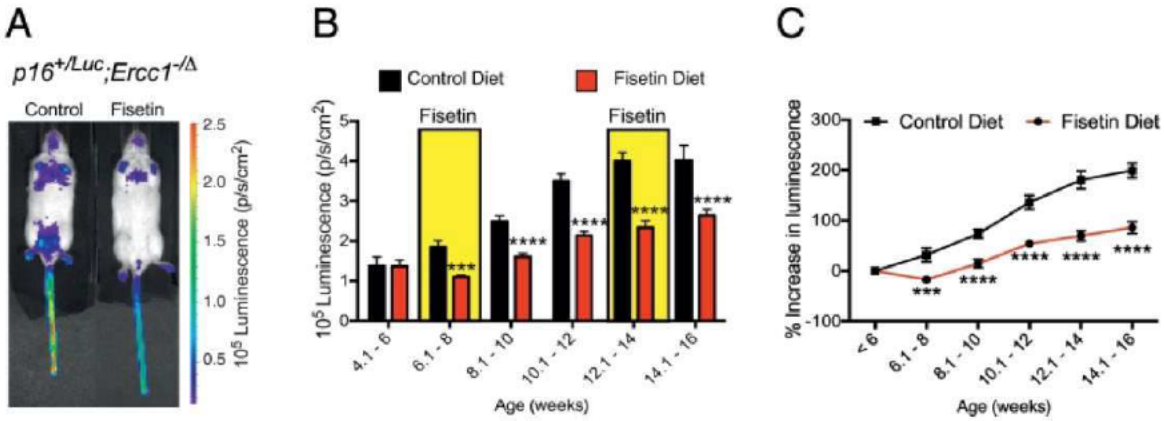


Fig. 16. Fisetin reduces age-related senescent cell accumulation in *p16$^{Luc/-}$;Ercc1$^{-/\Delta}$* mice. Intermittent treatment of progeroid mice with Fisetin reduces senescent cell burden. (**A**) Representative image of age-matched, 12 week-old male *p16^{+}/Luc;Ercc1$^{-/\Delta}$* mice fed a diet containing Fisetin 500 ppm (500 mg/kg), or drug-free control diet. (**B**) Luciferase signal was measured biweekly in *p16^{+}/Luc;Ercc1$^{-/\Delta}$* mice fed either control chow or chow containing Fisetin 500 ppm, N = 4-10 mice/ group and time point. The Fisetin was administrated intermittently for two weeks at a time (yellow bars). Otherwise the mice were fed a control diet. (**C**) The same data as seen in (B), but plotted as the percent change in luciferase signal as the animals aged. Values represented as the mean ± SEM. Two-tailed unpaired Student's t-test. ***p < .001, ****p < .0001.

Fisetin increased skin hydration and elasticity and increased healthspan in *Ercc1$^{-/\Delta}$* mice (Figs. 17-19).

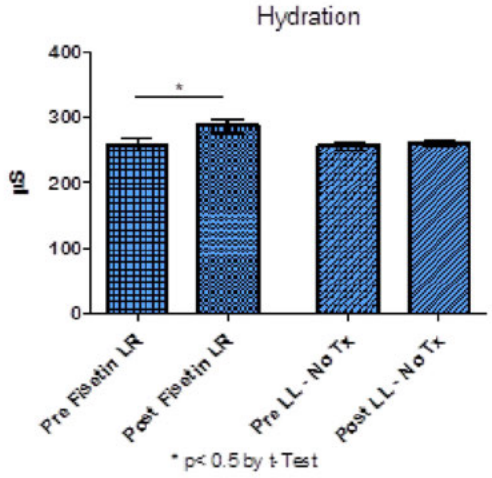


Fig. 17. Skin hydration decreases with aging. Fisetin alleviated age-related loss of skin hydration. Hairless *Shk$^{-/-}$;K14-Cre$^{+/-}$;Ercc1$^{-/fl}$* mice lack the DNA repair protein ERCC1 in the epidermis, so that they are photosensitive. Mice were UV-irradiated 3X/ week at a dose that provides the equivalent of 30 years of photo-aging in humans. Mice (N=3) were treated topically with Fisetin 5 mg/ml in a gel (100 µL), 3X/ week. No Tx = UV + Vehicle only. Fisetin caused a significant increase in skin hydration.

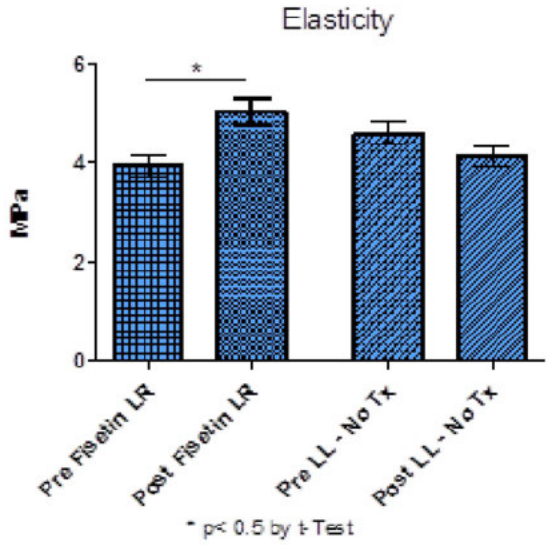


Fig. 18. Fisetin alleviated the age-related decline in skin elasticity. Mice (N=3) were treated topically with Fisetin 5 mg/ml in a gel (100 µL), 3X/ week. No Tx = UV + Vehicle only. Fisetin caused a significant increase in skin elasticity.

Fisetin Diet improves healthspan in *Ercc1*$^{-/\Delta}$ mice

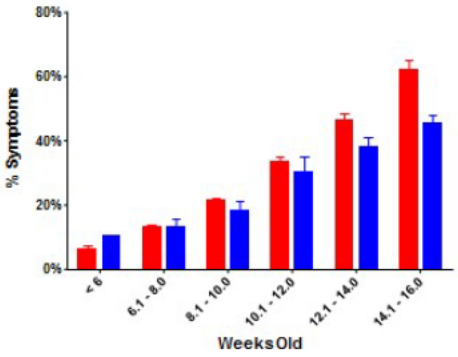


Fig. 19. Fisetin extends healthspan measured by a composite frailty score in the *Ercc1*$^{-/\Delta}$ mouse model of accelerated aging. At 3-4 weeks of age, *Ercc1*$^{-/\Delta}$ mice on a *C57Bl/6:FVB* background were fed *ad libitum* with a standard Teklad 2020 chow diet (N=5) or standard Teklad 2020 chow diet containing Fisetin 500 ppm (N=5). Fisetin 60 mg/kg/day is consumed by each mouse based on Fisetin 500 ppm (500 mg/kg) in the diet and 3-5 g of chow eaten/ day. The composite frailty score for mice includes assays of mobility, cognition, appearance, continence, tremor, gait disorder, and other variables. This score was measured in both groups at baseline and biweekly by an investigator blinded as to treatment group.

Fisetin increases remaining lifespan in old mice, partly through delaying cancers, which account for 50% of deaths in mice, and other causes of death (Fig. 20).

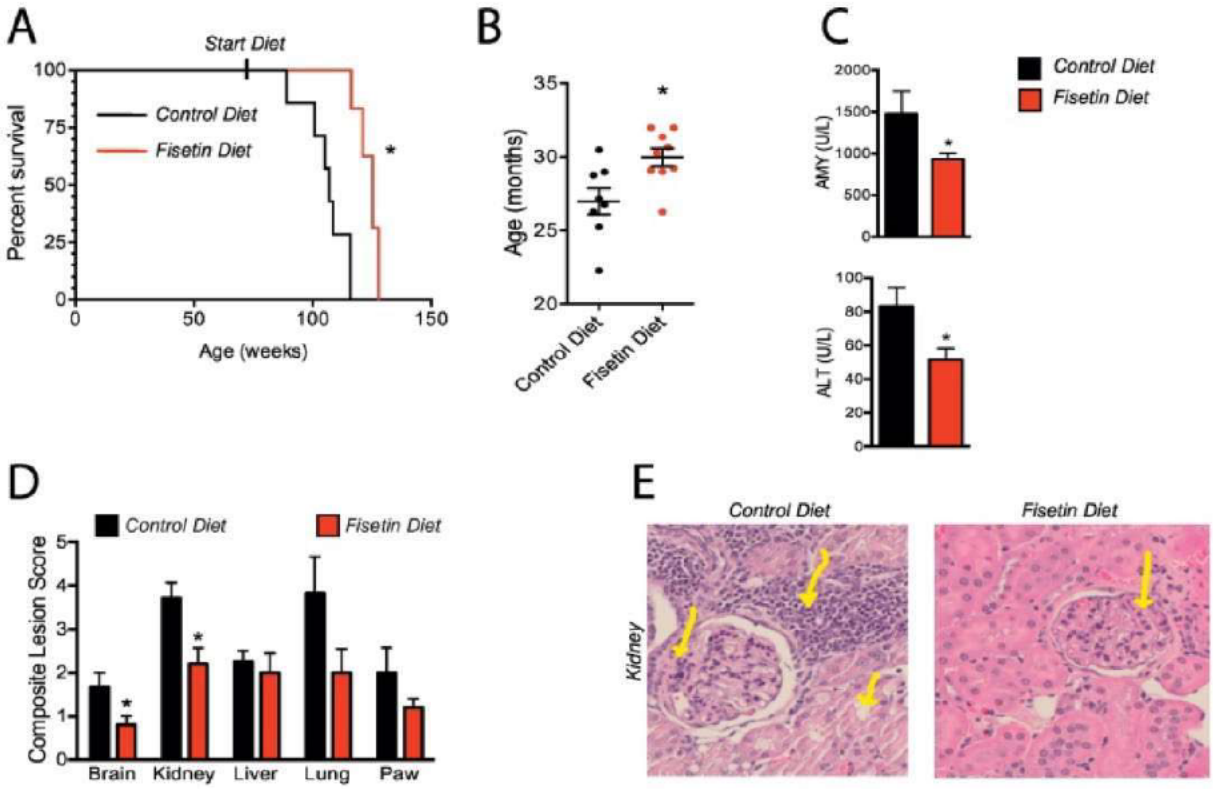


Fig. 20. Fisetin increases survival in chronologically-aged wild-type mice. Late-life intervention with Fisetin in aged wild-type mice extends health span and lifespan. (**A**) At 85-weeks of age (>20 months; equivalent to age >70 years in humans), male and female mice were administered a diet containing Fisetin 500 ppm (500 mg/kg) or a control diet with no drug. Lifespan was measured. N = 8–9 mice/ group. Log rank (Mantel-Cox) test. (**B**) Median lifespan of the same cohort of mice. Each dot represents an individual animal. Black bars indicate the mean ± S.E.M. Two-tailed unpaired Student's t-test. (**C**) Clinical chemistry of blood from the above mice to measure markers of liver (alanine aminotransferase [ALT]) and pancreatic (amylase [AMY]) dysfunction. N = 3–6 mice/ group. Two-tailed unpaired Student's t-test. (**D**) Composite lesion scores for aged-related pathologies in multiple tissues determined by histopathologic analysis according to the criteria of the Geropathology Grading Platform[113]. N = 3–8 mice/ group. Two-tailed unpaired Student's t-test. (**E**) Representative images of the kidney of a mouse fed control or Fisetin chow. In the control mouse, arrows (from left to right) indicate increased cellularity at a segment of the glomerular capsule border, moderate levels of lymphoid aggregates, and tubular cell vacuolization. In the Fisetin-treated mouse, the arrow indicates only mild segmental cellularity at the glomerular capsule border and a few scattered lymphoid cells near the glomerulus (200× magnification). *p < .05.

Idea generation: The generation of this study began with our Amplifier/ Rheostat Hypothesis (Fig. 3), which we validated in September, 2018 by testing if PAMPs exacerbate the SASP (Fig. 1). Together with the finding that coronavirus causes deaths from cytokine storm/ ARDS, we formulated the hypothesis that the senolytic drug, Fisetin (Fig. 11), could alleviate complications of coronavirus due to an excessive inflammatory reaction in February, 2020. This led us to search for a mouse model of coronavirus. Our collaborators at the U of M had an infected colony and had done an experiment in December, 2019 showing that young mice survive, but old mice die beginning 7 days after exposure to the mouse β-coronavirus, Mouse Hepatitis Virus (MHV; Fig. 8) and develop high circulating IL-6, unlike infected young mice (Fig. 9). We administered Fisetin *vs*. vehicle to old mice and sent them to the MHV-infected colony at U of M (Fig. 21). We and our collaborators discovered that Fisetin substantially increased survival of β-

coronavirus-infected old mice (Figs. 22, 24, 26, 28, &29).

Aged, infected prevention results: We conducted an experiment in which 10 male and 10 female mice >24 months old, equivalent to humans aged >80 years, were administered Fisetin 20 mg/kg as daily pulse doses by oral gavage for 2 days and 10 control >24 month old male and 10 female mice were administered vehicle at Mayo Clinic. After 2 more days, the mice were moved from Mayo Clinic to the mouse β-coronavirus-contaminated facility at U of M. Since they fight, the males were placed in bedding in which infected male mice had been housed. The females were placed directly in contact with contaminated female mice, and so had a much higher exposure than the males. Similarly to the male experiment, in a second experiment, 10 female mice pretreated with Fisetin and 10 female mice with vehicle at Mayo Clinic were placed in bedding in which infected female mice had been housed. Those mice pretreated with Fisetin or vehicle by gavage before being transferred from Mayo Clinic to the infected colony at U of M were administered Fisetin 500 ppm or vehicle, respectively, in food while they were in the contaminated facility (Fig. 21).

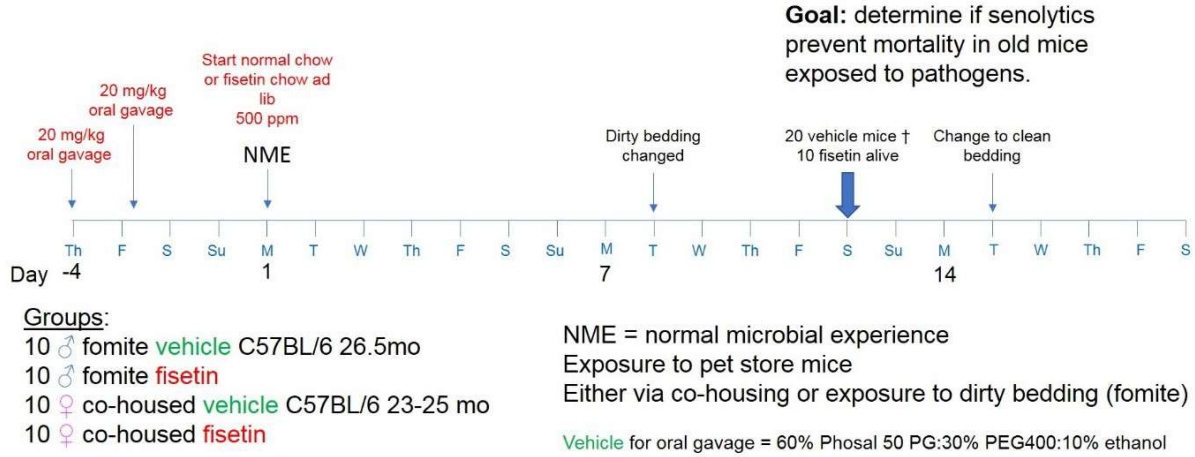


Fig. 21. Mouse coronavirus Fisetin experiment design.

All of the 20 vehicle-treated mice in the first experiment died within 12 days of pathogen exposure. However, 5 of the 20 Fisetin-treated bedding-exposed male ($p<0.0001$) and infected mouse-exposed female mice ($p=0.2$) were still alive after 15 days of continuous infection exposure plus a further 12 days after being switched to clean bedding at Day 16 (Fig. 18). In the second experiment (Fig. 20), all of the 10 vehicle-treated bedding-exposed female mice died within 10 days of infection exposure. However, 7 of the 10 Fisetin-treated female mice were still alive ($p<0.0002$) after 13 days of continuous infection exposure. Thus, Fisetin prevents mortality in old mice caused by viral exposure that includes the mouse β-coronavirus. All mice tested had blood test-proven mouse β-coronavirus infection. Tests for multiple other pathogens were negative.

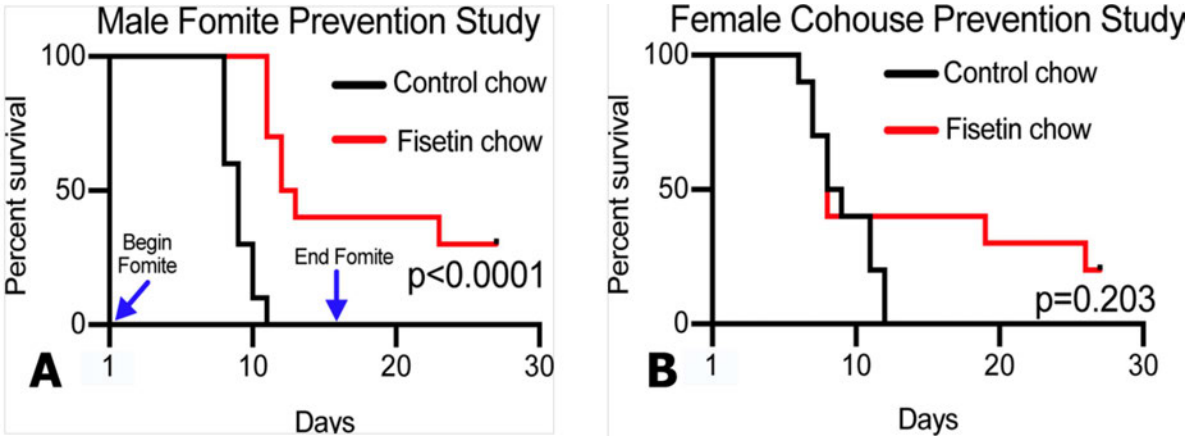


Fig. 22. Survival of male mice exposed to bedding contaminated with mouse β-coronavirus is increased substantially by Fisetin. (A) Old male mice (age>24 months; N=10) were administered Fisetin 20 mg/kg/day as daily pulse doses, and 10 control old male mice vehicle, by oral gavage beginning 4 days before being transported from Mayo Clinic to a contaminated facility at U of M, where they were exposed to bedding from β-coronavirus-infected mice. Males cannot be exposed directly to infected mice because they fight. At the U of M facility, the Fisetin-treated mice were maintained on a Fisetin 500 ppm diet and the vehicle-treated mice were fed regular chow. Male mice treated with Fisetin had significant improvement in survival *vs*. vehicle-treated old male mice in this prevention study ($p < 0.0001$; log-rank Mantel Cox test). **(B)** Old female mice (age >24 months; N=10) were also administered Fisetin at Mayo Clinic beginning 4 days before being transported to U of M and exposed directly to infected female mice (likely resulting in a much higher viral load than in the males described above that were exposed only to infected bedding). The females exposed directly to infected female mice tended to have better survival *vs*. vehicle-treated old female mice (N=10; p=0.2). Of note, by day 11, when the vehicle-treated mice had died, the only pathogen that the female co-housed mice had antibodies against was the mouse β-coronavirus, MHV (Fig. 23).

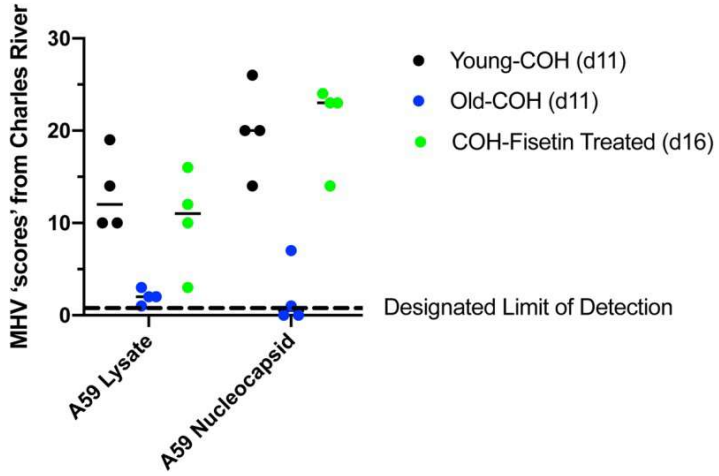


Fig. 23. Fisetin increases the antibody response of old female mice co-housed with mouse β-coronavirus-infected mice. Old female mice (24 months) had less antibody response against coronavirus than young mice by 11 days after both groups were co-housed with mice infected with mouse β-coronavirus. However, at Day 16, the 24-month old female mice treated with Fisetin (as in Fig. 21) had an antibody response similar in magnitude to the young mice. All old female mice treated with vehicle had died by Day 16. Thus, Fisetin treatment does not prevent antibody response of old mice to coronavirus infection and allows time for the older mice to develop an antibody response similar to that of young mice.

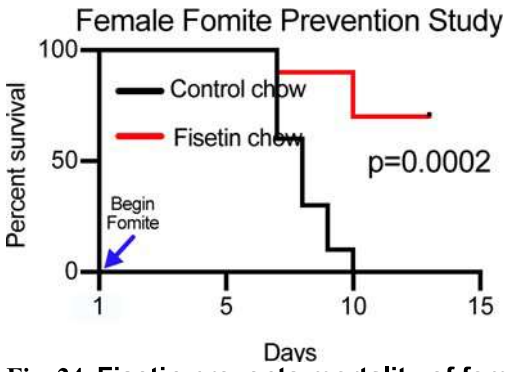


Fig. 24. Fisetin prevents mortality of female mice exposed to bedding contaminated with mouse β-coronavirus. As for males in the above prevention experiment (Fig. 22A), old female mice (age >24 months; N=10) were administered Fisetin 20 mg/kg/day as daily pulse doses or vehicle (N=10) by oral gavage beginning 4 days before being transported from Mayo to the contaminated facility at U of M, where they were continuously exposed to bedding from β-coronavirus-infected mice. At the U of M facility, Fisetin-treated mice were fed Fisetin 500 ppm chow and vehicle-treated mice were fed regular chow. Like males, female mice treated with Fisetin had significant improvement in survival *vs*. vehicle-treated mice in this prevention study ($p < 0.0002$; log-rank Mantel Cox test).

Aged, infected treatment results: In this experiment, we tested if Fisetin intervention in old mice after they have been infected and as they become symptomatic slows death. Old mice (24 months) were transferred from Mayo Clinic to the contaminated facility at U of M and, 3 days after introduction to that facility from Mayo Clinic (study Day 0), were treated for 3 consecutive days with Fisetin 20 mg/kg/day as daily pulse doses *vs*. vehicle by gavage on study Days 3 to 5, 10 to 12, and 17 (Figs. 25&26). After each series of high daily Fisetin doses by gavage, they were maintained on Fisetin 500 ppm in food or regular chow, respectively, on study Days 6 to 9 and 13 to 16.

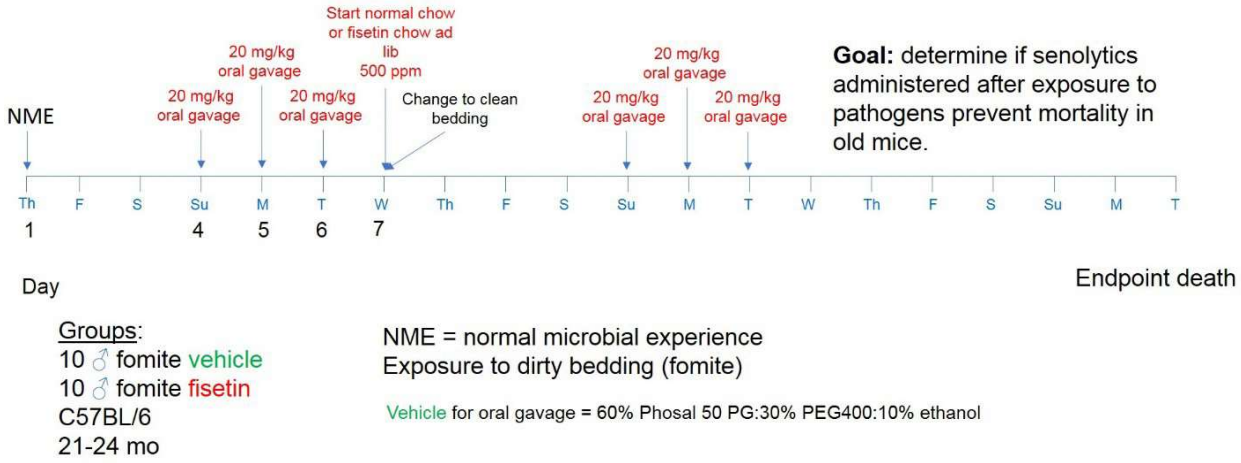


Fig. 25. Experiment to determine if senolytics administered after exposure to β-coronavirus slows mortality in old mice.

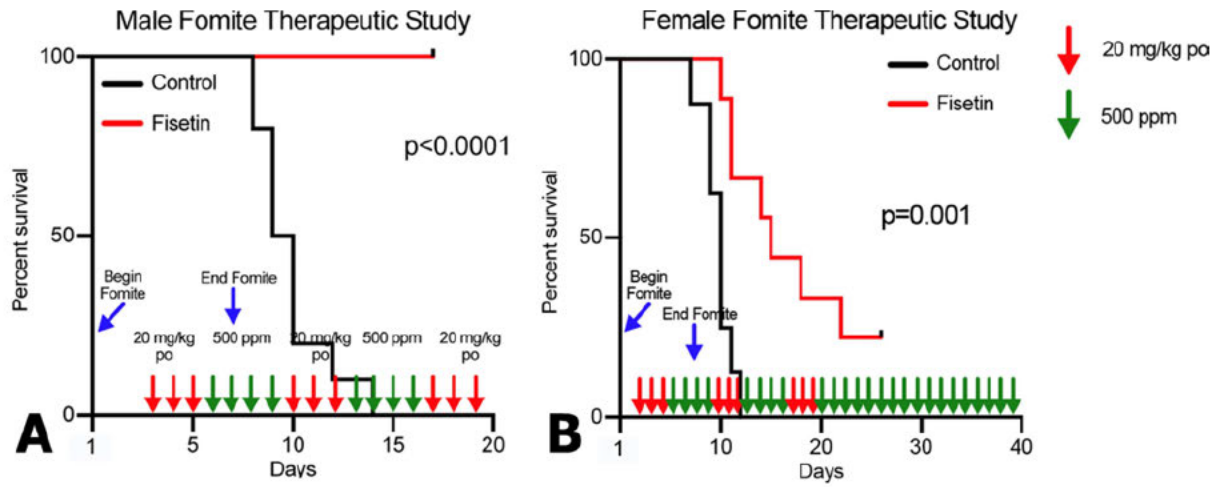


Fig. 26. Fisetin treatment slows death of old mice infected with β-coronavirus. (A) Male 24 month old mice were transported from Mayo Clinic to the mouse β-coronavirus contaminated colony at U of M, depicted here as Day 0. All mice were exposed to bedding used by infected male mice. Beginning 3 days after transfer from Mayo Clinic, they were treated for 3 days with Fisetin 20 mg/kg/day as daily pulse doses *vs.* vehicle by oral gavage (N=10 mice/ group) on Days 4 to 6. On Days 7 to 9, the Fisetin-treated mice were fed with chow containing Fisetin 500 ppm and the vehicle-treated mice were fed chow only. On Days 10 to 12 and 13 to 16, animals were treated with Fisetin 20 mg/kg as daily pulse doses *vs.* vehicle by oral gavage on Days 10 to 12 and 13 (all vehicle-treated mice were dead at Day 14). On Day 17 animals were treated with Fisetin 20 mg/kg as daily pulse doses. **(B)** Female 24 month old mice were treated in the same way. Fisetin significantly increased survival of β-coronavirus-infected mice (males: $p < 0.0001$; females: $p < 0.001$; log-rank Mantel Cox test).

Fisetin treatment of old, β-coronavirus-infected male as well as female mice slowed death (Fig. 26). Similarly, targeting *p16^{Ink4a}*-expressing cells, many of which are senescent, by treating old MHV-infected *INK-ATTAC* mice with AP20187[13], delayed death (Fig. 27). Of note, in separate, parallel studies in which animals were euthanized after cohousing or exposure to dirty bedding, transmission of MHV was 100% and antibodies against the virus were detected by day 11 (Fig. 23). No antibodies to other common mouse pathogens were detectible. Additionally, only 2 days Fisetin significantly decreased mortality in old female mice infected by β-coronavirus (Fig. 28). Mortality began to increase at Day 7, leading us to test 2 courses of 2 days Fisetin (Fig. 29).

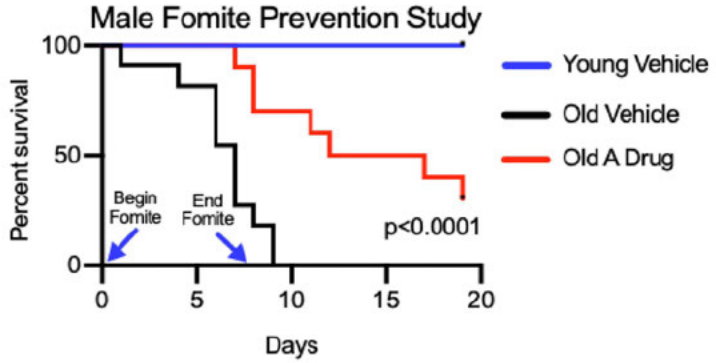


Fig. 27. Targeting highly p16^{Ink4a}-expressing cells slows death of old mice infected with β-coronavirus. Old (24 month) *INK-ATTAC* mice[13,107], which express ATTAC, a vFKBP-caspase fusion protein whose cell death-inducing activity can be activated by the drug, AP20187 (A Drug), which has no effect on wild-type mammalian cells, were treated with AP20187 *vs.* vehicle i.p. for 2 days/ week beginning 2 weeks before being exposed to mouse β-coronavirus in the contaminated colony at U of M. p16^{Ink4a} is increased in many senescent cells. Depleting p16^{Ink4a}-expressing cells decreased mortality, consistent with the pro-survival effects of Fisetin in old mice infected with mouse β-coronavirus being a consequence of decreasing senescent cell abundance as opposed to other potential effects of Fisetin.

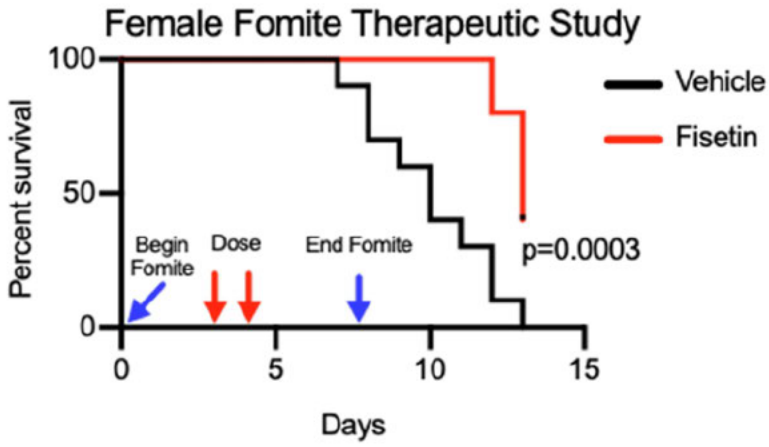


Fig. 28. Only 2 days Fisetin administration decreases β-coronavirus mortality in old mice. 24m old female mice were administered Fisetin (20 mg/kg/day) for 2 days by oral gavage (N=10) *vs*. vehicle (N=10) from the 3rd to the 5th days after being infected by mouse β-coronavirus, which decreased deaths significantly (p<0.0003; log-rank Mantel Cox test), This is further consistent with beneficial effects of Fisetin in old CoV-infected mice being due to senescent cell ablation rather than other effects of Fisetin that would require continuously high Fisetin blood levels and continuous administration of Fisetin. Mortality began to rise again at Day 7.

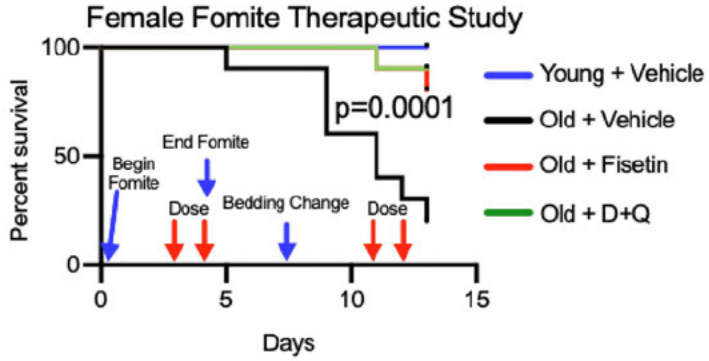


Fig. 29. Senolytics administered following the protocol proposed in this clinical trial decrease mortality from transient exposure to coronavirus in old mice. Old and young female mice were exposed to fomite bedding contaminated with mouse β-coronavirus for 4 days. Old mice were given Fisetin, D+Q, or vehicle (N=10/ group) by gavage on days 3 and 4. Day 4 was the last day of β-coronavirus exposure (in the experiments above, mice were continuously exposed to β-coronavirus).

Using the same protocol as the study proposed here, Fisetin substantially decreased mortality in mice infected with β-coronavirus (but, like the patients to be examined in this clinical trial, were exposed *transiently*, not continuously), supporting the design of our trial. Since D+Q also reduced mortality (as did AP20187 in *INK-ATTAC* mice; Fig. 27), our hypothesis that targeting senescent cells will delay, prevent, or treat complications of β-coronavirus infection is supported.

1.4 Clinical Data to Date

Senescent cell clearance from humans: In the first clinical study published about senolytics, a pilot, open-label study in 14 patients with IPF, we found that 9 pulse-doses of oral D+Q over 3 weeks led to improved 6-minute walk distance, walking speed, ability to rise from a chair, and short physical performance battery by 5 days after the final dose[114]. These results led to initiation of a Phase IIb randomized, placebo-controlled, double-blind trial that is currently underway.

Interim results of a Phase 1, open-label, clinical trial of D+Q for subjects with diabetic kidney disease (DKD) underway at Mayo (ClinicalTrials.gov Identifier: NCT02848131) showed a 3 day oral course of D+Q administered as pulse doses in 9 subjects with DKD reduced adipose tissue senescent cell burden assayed by $p16^{INK4A+}$ and SA-βGal^{+} cells by 11 days after the last dose, compared to biopsies before D+Q administration[72]. A composite score of 10 circulating SASP factors was significantly decreased 11 days after completing the 3 day D+Q intervention. Furthermore, activated $CD68^{+}$ macrophage tissue infiltration was significantly decreased. This trial is continuing (goal=30) to test effects of senolytics on adipose tissue and skin senescent cell abundance, blood and urine SASP factors, metabolic and renal function, inflammation, quality of life, safety (drug toxicity), and tolerability. No serious drug side effects have emerged so far. Evidence continues to show clearance of senescent cells. Each subject will be followed for 4

months after the single course of D+Q to provide data for a larger Phase IIb randomized, placebo-controlled, double-blind trial of senolytics for DKD.

The senolytic drug to be used in our study here is Fisetin. We found frailty in elderly women is associated with senescent cell burden and adipose tissue inflammation[94]. Additionally, senolytics alleviated frailty in the subjects in our IPF trial[115]. Therefore, a Phase IIb double-blind, placebo-controlled clinical trial of Fisetin to reduce senescent cell burden and alleviate frailty and inflammation, AFFIRM, has commenced (ClinicalTrials.gov Identifier: NCT03430037). Oral Fisetin 20 mg/kg/day for 2 days *vs*. placebo is being administered as daily pulsed doses for frailty (gait speed <0.8 m/sec) and inflammation in elderly women with multi-morbidity. This trial at Mayo Clinic is FDA-approved and has enrolled over 50 subjects who have received Fisetin or placebo. The trial is not yet unblinded. No participants have had severe or serious adverse events so far at the same daily doses of Fisetin as proposed here for older CoV patients.

1.5 Dose Rationale

Others have treated mice with Fisetin 50 mg/kg/day for weeks without evidence of toxicity[116]. We treated 4 male and 4 female 8 month old C57/Bl6 mice with Fisetin 500 mg/kg/day (25-fold higher than the dose to be used in this clinical trial) by oral gavage for 2 consecutive days compared to 4 male and 4 female mice treated with vehicle. We found no evidence of substantial toxicity, as assessed by monitoring activity, food intake, and respiratory quotient in metabolic cages (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments) for 48 hours after the last Fisetin dose. We administered 100 mg/kg/day for 2 consecutive days by mouth as daily pulse doses, 5 times the dose proposed in in this clinical trial, to 2 older *Rhesus* monkeys (ages 19 and 28 years, which is old for this species) at the National Primate Center. In over 1 month of close monitoring, neither monkey had adverse effects such as reduced appetite, altered activity, vomiting, or diarrhea. Acknowledging the higher rates of drug metabolism in mice compared to humans and differences in physiology between non-human primates and humans, in an effort to avoid side effects, a daily dose of ~20 mg/kg was selected here. We verified that this dose is sufficient to reduce senescent cell burden in mice. Our studies in very old coronavirus-infected mice demonstrated that this dose of Fisetin dramatically enhances survival (Figs. 22, 24, 26, 28, and 29). We will administer this dose, 20 mg/kg/day for 2 consecutive days, at Days 0 and 1 and 8 and 9, since senescent cells can re-accumulate by 10 days, at least in culture, and mortality rises several days after completing the first round of Fisetin in mice (Fig. 28). We found anti-CoV antibodies in old mice by 16 days after initiating Fisetin treatment. Hence, here two courses of 2 consecutive days of Fisetin 20 mg/kg/day will be administered, as in mice in Fig. 29).

1.6 Risks and Benefits

There appear to be few, if any, risks of Fisetin at the doses to be used that we have evidence for, provided the subjects meet the exclusion criteria in the protocol regarding medications that Fisetin could interfere with. We have not seen serious or severe adverse events so far in elderly frail women enrolled in our AFFIRM studies from Fisetin 20 mg/ kg/ day for 2 consecutive days in the over 50 subjects randomized to Fisetin or placebo. We did not see adverse effects such as altered food intake, weight, or behavior in old *Rhesus* macaques given 5 times the dose proposed here or in mice given 25 times the dose. *Fisetin has been widely sold as a nutritional supplement in 100 and 500 mg capsules for many years by Amazon and other vendors without prescription and without any reported side effects that we could find.*

Based on our studies in mice, there may be decreased morbidity and mortality due to β-coronavirus infection after Fisetin administration. All subjects in this study will be elderly and

have proven CoV infection that could progress to severe or critical disease. The potential benefit to individual subjects in this trial and other people might be large and risks from Fisetin are low or negligible.

In this study, we will examine the effect of oral/ enteral Fisetin as a senolytic agent in humans, which may have immense potential for and reducing frailty/ physical dysfunction and inflammation and alleviating morbidity and mortality from CoV or other infections in older subjects and those with other conditions and diseases associated with accumulation of senescent cells. As an additional, advantageous, off-target effect, removal of senescent cells may allow for functional improvement of neighboring or distant tissues and cells, including MSC's, accelerating recovery. We found that senolytics restore function of MSC's[14,69]. Our collaborator, G. Ellison, working with investigators in China, noted that transplanting healthy MSC's can alleviate symptoms and signs in severely ill CoV-infected patients[117]. Thus, an additional benefit of senolytics for CoV could be this alleviation of stem cell and progenitor dysfunction.

Based upon review of the published literature and our pre-clinical data, we do not anticipate the occurrence of serious adverse events with the prescribed regimen in this study. We have identified a variety of drug-drug interactions (*e.g.*, current use of warfarin) for which we established exclusion criteria or modification plans to minimize associated risks. We could find no reports of Fisetin toxicity in the literature. Some flavonols have been associated with DNA damage *in vitro*, but Fisetin exerts little or no genotoxic effects[56]. To the contrary, Fisetin has been noted to be anticarcinogenic[43,57,58]. Indeed, we found that chronic Fisetin administration delays cancer in mice and increases remaining lifespan in old mice as well as increasing lifespan of DNA damage/ genotoxicity highly-prone *Ercc1*$^{-/\Delta}$ mice by over 17%[59]. Since up to 50% of most mouse strains die from cancers, this shows that the beneficial effects of Fisetin on such pro-carcinogenic factors as senescent cell burden and tissue chronic inflammation appear to more than counteract any theoretical effects of flavonol-associated genotoxicity. **Fisetin actually reduces cancer risk**, as opposed to increasing it.

Overall, we do not anticipate substantial risk from the natural product, Fisetin, which is present in many foods, including cucumbers, strawberries, and other fruits and vegetables, albeit at levels lower than will be used here. CoV infection causes 25 to 50% mortality in nursing home patients. Thus, the risks of using this drug are likely minimal compared to the anticipated benefits for nursing home patients with a limited life expectancy, even without CoV infection.

2 Study Objectives

2.1 Primary Objectives

The primary objective is to test the efficacy of the senolytic drug (Fisetin) in preventing or delaying progression of CoV severity in nursing home patients with proven CoV according to a 7 point scale for nursing home CoV severity adapted from the WHO Ordinal Scale for Clinical Improvement of CoV*:

- Not hospitalized nor on additional oxygen (compared to baseline), at baseline level of function (as assessed by activities of daily living).
- Not hospitalized nor on additional oxygen, but below previous level of function.
- Hospitalized or moved to a more intensive unit within the nursing home because of additional dysfunction (not simply because of positivity), but not requiring additional oxygen nor placed on an antiviral drug for worsening CoV symptoms.
- Hospitalized or moved to a more intensive unit within the nursing home and requiring additional oxygen (*vs*. baseline) and/ or placed on an antiviral drug for worseningCoV symptoms.

- Hospitalized or moved to a more intensive unit within the nursing home and requiring additional oxygen by ECMO or ventilator.
- Transferred to palliative care to provide end-of-life care.
- Death.

*The WHO Ordinal Scale for Clinical Improvement of CoV (Appendix 1) is scored from 0 to 8 with 0 = no evidence of infection to 8 = death. Our adapted scale does not include scores 0 or 1, since no subjects in COVID-FIS will meet these criteria (*e.g.*, all subjects have proven CoV infection, so none have "no infection").

2.2 Secondary Objectives

A secondary objective is to assess the safety and tolerability of oral/enteral Fisetin in older nursing home residents. These elderly adult nursing home residents will have proven CoV infection.

Further objectives are to test the efficacy of the senolytic drug (Fisetin) in preventing, delaying, or alleviating physical dysfunction (frailty and other functional parameters in the MDS which is completed every quarter or 100 days for nursing home residents nationally), inflammation; 2), reducing senescent cell abundance in blood, increasing oxygen requirement (assessed by changes in daily mean skin pO_2 as a function of inhaled oxygen [SpO_2]/FiO_2), decreasing escalation in oxygenation device, intubation, non-invasive ventilation, cytokine storm/ ARDS/ myocarditis with troponin leak, death, delayed recovery, poor clinical condition, and progression to fibrotic lung disease and enhancing antibody response using data available from nursing home/ hospital records and data and samples from subjects collected where feasible.

3 Study Design

3.1 General Description

This study is a pilot, randomized, placebo-controlled, multi-site study of Fisetin in elderly nursing home participants. A total of 250 participants will be enrolled in this study. Study staff and participants will be blinded with regards to groups. Subjects will be randomized to placebo or treatment. Fisetin 20 mg/kg/day or placebo will be made available by the study staff for oral or enteral administration on study Days 0, 1, 8, and 9.

If Remdesivir or another anti-viral agent or immunization should become standard of care in nursing home CoV patients (*e.g.*, approved by the FDA) during the study, patients being treated with such agents may be enrolled, with randomization stratified based on whether subjects are on such an anti-viral agent. We conducted studies of mice (N=6 old mice; age 22.9±0.7 months [means±SEM]; females) on higher doses of Fisetin than to be administered here (100 mg/ kg/ day for 2 days) by gavage and Remdesivir (50 mg/ kg/ day) i.p. We did not find Fisetin plus Remdesivir resulted in increased toxicity (changed body weight, food intake, activity, grooming, appearance, or frailty score) during any of the 4 days following administration *vs*. Remdesivir alone (Table 3; 100 mg/ kg/ day for 2 days by gavage; N=6 old mice; age 23.4±0.7 months). Therefore, if patients already in COVID-FIS are started on Remdesivir, they will not be withdrawn from the study. During statistical analyses, patients will be analyzed regarding Remdesivir administration *post hoc*.

Table 3. Remdesivir + Fisetin is Not More Toxic than Remdesivir Alone

Treatment	Age (Mo.)	Frailty Score	Body Wt. (g)	Body Temp. (°C)	Body Wt. (g)	Frailty Score	Body Wt. (g)	Body Temp. (°C)	Body Wt. (g)
Date	**5/4**	**5/4**	**5/4**	**5/4**	**5/9**	**5/10**	**5/10**	**5/10**	5/11
RDV+Fisetin	25.03	0.357	27.2	31	26.00	0.37	25.7	31.73	26.1
RDV+Fisetin	22.20	0.125	34.2	32	34.9	0.185	33.8	32.07	34.1
RDV+Fisetin	22.35	0.143	30.1	32	28.5	0.259	27.6	32.83	28.2
RDV+Fisetin	19.47	0.21	29.5	32	27.3	0.278	27.6	31.9	27.8
RDV+Fisetin	24.18	0.20	42.2	32	41.5	0.204	39.8	30.9	39.7
RDV+Fisetin	24.18	0.161	28.3	31.7	27.9	0.204	27.9	32.17	28.3
Mean	**22.90**	**0.2**	**31.9**	**32**	**31.02**	**0.25**	**30.4**	**31.93**	**30.7**
SEM	**0.752**	**0.031**	**2.08**	**0.14**	**2.24**	**0.026**	**2.00**	**0.24**	**1.93**
RDV	24.01	0.214	27.6	31.7	27.1	0.241	26.6	31.67	26.8
RDV	23.59	0.232	27.9	32.1	27.9	0.296	27.8	32.6	28
RDV	22.20	0.268	30.9	31.6	29.9	0.241	29.1	32.3	29.3
RDV	22.20	0.125	34.5	32.4	34.1	0.185	33.8	32.77	33.5
RDV	24.18	0.148	27.8	31.2	27.8	0.212	26.9	31.33	27
RDV	24.18	0.178	30	32.3	29.3	0.259	28.9	32.03	28.5
Mean	**23.39**	**0.194**	**29.78**	**31.9**	**29.35**	**0.239**	**28.85**	**32.12**	**28.85**
SEM	**0.353**	**0.020**	**1.00**	**0.17**	**0.95**	**0.014**	**0.98**	**0.20**	**0.92**

Mice were administered Remdesivir (RDV)+Fisetin or RDV alone for 2 days beginning on 5/8/20.

Indices of senescent cell abundance, inflammation (circulating cytokines and SASP factors), physical function/ frailty, overall health status, mobility, nutritional state, adverse event screening, temperature and vital signs, medication use, oxygenation status (SpO_2/FiO_2, increase in oxygen requirement, or changes in oxygenation device), and need, based on disease severity, change of care plan to end-of-life palliative care or being transferred to an intensive nursing home unit, hospital, or ICU care will be recorded daily (if feasible at the time of similar evaluations in the course of providing usual care for residents of nursing homes and/ or such records will be abstracted from the patient's nursing home record, where feasible within 1 week). Before the initial Fisetin or placebo administration and at the intervals shown in Appendix 2, blood and urine will be collected if feasible for assaying the following: C-reactive protein, ESR, SASP factors and immunologic markers (including IL-6, IL-7, IL-8, IL-1α&β, IL-1αR, TNF-α, TNF-αR1&2, MCP-1&3, MIP1-α, IP-10, Activin A, Ferritin, D-dimer, MMPs 3, 9, and 12, among others, miRNA's, circulating and urinary cell-free mitochondrial/ nuclear DNA and exosomes, immune response and immune cell profile), oxygenation (venous or arterial SaO_2), and safety tests (blood glucose, CBC, bilirubin, liver and renal function). Lung imaging may be done if feasible. If any of these tests are conducted as part of the patients' nursing home care (*e.g.*, chest x-rays), such results will be recorded instead of being repeated for this study.

If positive, this study would provide strong rationale for larger-scale clinical trials to determine effects of Fisetin on the following outcomes: 1) delay or prevention of progression to severe complications of CoV, including cytokine storm, ARDS, myocarditis with troponin leak, and long-term lung fibrosis, or for other infections in elderly or chronically-ill individuals, 2) treatment of severe complications of CoV or other infections in elderly or chronically-ill individuals, 3) treatment of nursing home participants who are infected with CoV (positive PCR test) despite immunization, and 4) delay, prevention, or treatment of multiple cellular senescence-associated conditions in the elderly, including individuals with chronic diseases.

3.2 Number of Subjects

Enroll and screen 250 subjects to accrue and randomize 150 men and postmenopausal women aged ≥65 years old to be administered Fisetin (N=75) or placebo (N=75).

3.3 Duration of Participation

The study is estimated to complete enrollment within 18 months from study initiation; however, enrollment will remain open until the study goal is met. The duration of this study for each subject will be a maximum of 6 months. Study treatment will take approximately 4 out of 10 days. Subjects will be followed for up to 6 months or death, whichever comes first, including any available laboratory and chest imaging results if done as part of nursing home care. This will be done through study staff and health care providers in the nursing home or, if transferred to hospital, another facility, or home, by study staff contacting patients, their LAR, and/or their health care providers daily if feasible from Days 0 to 14 and at Days 30, 90 and 180 (±14; unblinding) after Fisetin *vs*. placebo was commenced if feasible, or from nursing home or hospital records. Adverse event reporting will be collected daily if feasible from the time of consent through Day 14. Adverse events will be assessed again at Days 30, 90 and 180, if possible. Subjects will not be required to return to the nursing home if discharged home or to another care setting.

3.4 Primary Study Endpoints

In this pilot study, the primary endpoint will be decreased progression of proven CoV infection in nursing home patients assessed using a 7 point scale adapted from the WHO Ordinal Scale for CoV Improvement (Appendix 1; Section 2; Primary Objectives) with unblinding at the end of the study.

3.5 Secondary Study Endpoints

In this pilot study of elderly adults in nursing homes who have proven CoV infection and no, mild, or moderate symptoms (Table 4), secondary endpoints will be: 1) safety and tolerability; 2) decreased blood senescent cells (blood $CD3^+$ T cell assay[34] for $p16^{INK4A}$ and SASP factors[69]; if feasible); 3) cognitive function (from MDS section C, Cognitive Patterns, items C0100 – C1310; 4) function status/activities of daily living (from MDS Section G, Functional Status, items G0110-G0900; 5) functional abilities/frailty (from MDS section GG, Functional Abilities and Goals, items GG0130-0170 improved physical and cognitive function; 6) decreased circulating inflammatory markers and mediators (if feasible); 7) prevention or delay of deterioration in oxygenation status (SpO_2/FiO_2) if feasible; 8) reduced progression to intubation/ transition to end-of-life palliative care because of worsening condition, transfer to special nursing home unit admission because of worsening condition, hospital, ICU admission/ progression to ARDS/ myocarditis with troponin leak, death, delayed recovery, and delayed antibody response, and 9) decreased lung fibrosis during or at the end of the 6 month time subjects are in the study.

3.6 Primary Safety Endpoints

The primary safety endpoints are incidence of serious adverse events and hypersensitivity reactions. Safety assessments are undertaken with the measurement of safety laboratory tests and procedures, vital signs, and recording of adverse events.

3.7 Identification of Source Data

Source data will be obtained from the nursing home record and documented in study-specific case report forms(paper and/or electronic data capture). No information in source documents about the identity of the subjects will be disclosed.

4 Subject Selection, Enrollment, and Withdrawal

4.1 Inclusion Criteria

Subjects must meet *all* of the following inclusion criteria to be eligible for enrollment:

1. Men or post-menopausal women age ≥65 years.
2. Current nursing home resident.
3. CoV severity of moderate or less OR SpO2 ≥ 85% (on room air or ≤ 2 L of supplemental oxygen) at time of enrollment.
4. SARS-CoV-2 infection confirmed by mRNA-PCR test at Mayo Clinic or other CLIA certified laboratory, within 10 days before randomization.
5. Willing and able to provide written informed consent or have a legally authorized representative (LAR) who will provide informed consent.

4.2 Exclusion Criteria

Subjects with *any* of the following exclusion criteria will not be eligible for enrollment:

4.2.1. General Exclusion Criteria

1. Presence of any condition that the Investigator or the subject's attending physician, physician's assistant, or nurse-practitioner believes would put the subject at risk or would preclude the patient from successfully completing the trial.
2. Pregnancy (note that only post-menopausal women will be enrolled).

4.2.2. Laboratory Exclusion Criteria

3. Total bilirubin >3X upper limit of normal or as *per* clinical judgment.
4. Serum aspartate transaminase (AST) or alanine aminotransferase (ALT) >4x the upper limits of normal or as *per* clinical judgment.
5. Hemoglobin <7 g/dL; white blood cell count ≤2,000/mm^3 (≤2.0 x 10^9/L) or ≥20,000/mm^3 (≥20 x 10^9/L); platelet count ≤ 25,000/μL (≤25 x 10^9/L); absolute neutrophil count ≤1 x 10^9/L; lymphocyte count <0.3 x 10^9/L at screening or as *per* clinical judgment.
6. Unstable (as *per* clinical judgment) major cardiovascular, renal, endocrine, immunological, or hepatic disorder.
7. eGFR <25 ml/ min/ 1.73 m^2 or as *per* clinical judgment.
8. Plasma and/or serum glucose >300 or as *per* clinical judgment.

4.2.3. Clinical History Exclusion Criteria

9. Human immunodeficiency virus infection.
10. Known active hepatitis B or C infection.
11. Invasive fungal infection.
12. Uncontrolled (as *per* clinical judgment) pleural/pericardial effusions or ascites.
13. History of diverticulitis or diverticulosis with GI bleeding, as *per* clinical judgment.
14. New/active invasive cancer except non-melanoma skin cancers as *per* clinical judgment.
15. Known condition associated with major immunodeficiency as *per* clinical judgment.

4.2.4. Medication Exclusion Criteria (see Investigator Brochure for additional information)

16. Known hypersensitivity or allergy to Fisetin.
17. Subjects taking any of the medications listed in the Investigator Brochure may participate if they are otherwise eligible AND the medication can be safely held

during the following times:

- Immediately before the 1st IP administration (Day 0) until at least 10 hours after the 2nd IP administration (Day 1).
- Immediately before the 3rd IP administration (Day 8) until at least 10 hours after the 4th IP administration (Day 9).

18. Participation in other clinical trials involving treatment for COVID-19 (unless reviewed and approved by the Principal Investigator). *Note that institutional standard of care treatment of COVID-19 including glucocorticoids, hydroxychloroquine, azithromycin, remdesivir, anti-spike antibodies, and/or convalescent plasma are not excluded from the study.*

4.3 Subject Recruitment, Enrollment, and Screening

Recruitment will be of patients who are residents of nursing homes who have proven CoV infection. Subject recruitment will be by medical, nursing, and other nursing home staff and/or research staff. Permission will be obtained from each location before recruitment begins.

Trained clinical coordinators will contact patients, health care proxies (as appropriate), and responsible nursing home staff (including the attending physicians, nurse-practitioners, or physician's assistants) and assist in recruitment and carry-through of the protocol. For each nursing home, during discussions leading to participation with their administrators and medical and nursing leadership, a key contact will be identified who will ensure access, consent, data and sample collection, and data integrity and identify and assist in resolving issues as the study proceeds. In most cases, this will be the Medical or Nursing Director or their designees. Written and informed consent will be obtained by appropriately trained study personnel with support from the nursing home staff as needed. In the pandemic situation where access to nursing homes and hospitals is limited, if allowed, digital/electronic consent can be obtained from the patient's LAR and must be documented by the investigator or the authorized designee (see Section 6.1). Documentation of recruitment and enrollment efforts will be maintained in a secure institutionally-supported database. Clinical judgment regarding inclusion and exclusion will be *per* the study physicians and/ or the patient's physicians, nurse-practitioners, or physician's assistants.

4.4 Early Withdrawal of Subjects

When and How to Withdraw Subjects

All subjects will be assessed during the initial day of medication administration, subsequent days of medication administration, and all study visits. If a severe adverse event (SAE) occurs at any time during administration of the drug regimen, a formal review will occur. If 4 or more events accrue, the pilot study will be held and either the dosing regimen revised or discontinuation of the study protocol will occur. Other interventions will be as *per* the direction of the Food and Drug Administration (FDA), Mayo Clinic Institutional Review Board (IRB), and policies of the patient's nursing home.

4.4.1. Study Completion:

For each subject in the study, the end of study will be reached when treatment and post-treatment safety follow-up periods have been completed, including follow-up of patients discharged from the nursing home to home, other care settings, or hospital, *i.e.* subjects will be followed if

feasible, irrespective of their location, by telephone or in person until the 180 (±14) day follow-up visit or death.

4.4.2. Treatment Discontinuation:

A subject's treatment may be discontinued prior to that subject completing the 4 days of drug treatment. Subjects whose treatment has been discontinued will remain in the study and, if feasible, will continue to be assessed for all planned efficacy and safety endpoints and will be included in statistical analyses. Some reasons may include:

- Subject safety issues
- Failure of subject or staff to adhere to protocol requirements
- Subject voluntarily withdraws from treatment
- Subject is unable to tolerate continued IP administration
- Subject experiences a Grade 3 or higher adverse event attributed to the IP by the investigator
- Subject experiences a Grade 3 or higher clinically significant laboratory abnormality attributed to the IP by the investigator
- Treating physician determines that continuing study treatment would not be in the subject's best interest
- Subject becomes lost to follow-up
- The investigator or the subject's nursing home attending physician, physician's assistant, or nurse-practitioner may discontinue a subject's treatment (without regard to the subject's or their LAR's consent) if they believe that continued treatment would be contrary to the best interests of the patient.

If premature treatment discontinuation occurs for any reason, the reason for premature treatment discontinuation, along with who made the decision (subject, LAR, responsible nursing home physician, physician's assistant, nurse-practitioner, investigator) will be recorded in the CRF.

4.4.3. Subject Withdrawal:

A subject may be withdrawn from the study prior to that subject's completing all of the study related procedures if the subject or LAR decides to withdraw from the study (withdrawal of consent). Withdrawn subjects may not reenter the study.

4.4.4. Premature Withdrawal from Study:

Subjects or their LAR may voluntarily withdraw from the study for any reason at any time. Subjects are considered withdrawn if they or their LAR state an intention to withdraw further participation in all components of the study or if subjects die. If premature withdrawal occurs for any reason, the reason for premature withdrawal from the study, along with who made the decision (subject, LAR, responsible nursing home physician, physician's assistant, or nurse-practitioner, investigator) will be recorded in the CRF.

4.4.5. Reporting of Serious Adverse Events and Unanticipated Problems:

When an adverse event has been identified, the study team will take appropriate action necessary to protect the study subject and then complete the Study Adverse Event Worksheet and log. The investigator will evaluate the event and determine necessary follow-up and reporting required.

4.4.6. Subject Replacement:

Subjects withdrawn (due to withdrawal of consent) from the study will not be replaced and analytic methods will be by intent-to-treat.

4.5. Data Collection and Follow-Up for Withdrawn Subjects

- For withdrawn subjects (due to withdrawal of consent) not undergoing any study intervention, no additional follow-up will be done.
- For withdrawn subjects (due to withdrawal of consent) receiving Fisetin (Intervention), any additional evaluation will be for subject safety only. Research data will not be collected on subjects after withdrawal of consent.

5. Investigational Product (IP)

5.1. Description

While Fisetin is currently widely available and marketed as a dietary supplement at daily doses of 100 mg or more, this study will use a product manufactured under GMP conditions. The Fisetin will be supplied in 100 mg capsules or powder dissolved in water, apple sauce, pudding, yogurt, or enteral hydration/ nutrition solutions to be administered orally or enterally (NG or D tube). The investigational supplies will be obtained from Vital Nutrients, a contracted GMP manufacturer. Fisetin capsules supplied by Vital Nutrients are size #3 or 00 opaque blue in color.

Current inventory lot (20E44) of Fisetin capsules from manufacturer Vital Nutrients were tested by the ICL using mass spectroscopy. The sample results lead us to believe that all of the active capsules contain Fisetin active ingredient. The placebo comparator used for this study was manufactured using the same size opaque blue capsules by Vital Nutrients or cellulose powder with food dye to approximate the appearance of Fisetin/ cellulose powder as closely as possible. Each placebo capsule is mainly composed of cellulose along with some coloring agents to approximate the appearance of the active capsule contents. None of the current inventory lot (20E44) of the placebo capsules contained Fisetin that exceeded the acceptance criterion (≤2 mg Fisetin *per* capsule).

5.2. Treatment Regimen

5.2.1. Fisetin/Placebo

The treatment dosing regimen is based on the preclinical information available about Fisetin along with information about similar clinical drug trials using the alternative senolytic compounds D and Q. Participants will be administered Fisetin over two 2-day courses of treatment during the study duration. On study each of Days 0 and 1 and Days 8 and 9, subjects will receive a target dose of 20 mg/kg of body weight. Therefore for an adult with a body weight of 75 kg, on each day the participant will take 15 of the 100 mg capsules or an equivalent amount of Fisetin powder mixed with water, other room temperature or chilled drinks, foods such as apple sauce or pudding, or in enteral solutions, at room temperature or chilled. Dosing should be completed in as short of a time as possible, with a goal of 30 minutes to complete ingestion of the capsules.

Dosing will be done during 2 sets of 2-day oral or NG or D tube treatments with Fisetin (~20 mg/kg/day for 2 consecutive days) on Days 0 and 1 and Days 8 and 9.

There are no recommended dosing adjustments for kidney or liver dysfunction. For identified drug-drug interactions, subjects will either be excluded from the study or drug modifications will be requested.

5.2.2. Concomitant Therapies

"Concomitant therapies" refers to treatment that subjects receive during the conduct of the study. All concurrent therapies, including medications, intubation, and oxygen will be recorded.

5.2.3. Standard of Care

Subjects will continue to receive institutional standard of care for the treatment of COVID-19 pneumonia and other conditions. The use glucocorticoids, Hydroxychloroquine, Azithromycin, Remdesivir, or Metformin is permitted. Subjects administered Rapamycin or other mTOR inhibitors, should they become standard of care, will be withdrawn unless the Rapamycin or other mTOR inhibitors are held for ≥5 days before the first dose of Fisetin on Day 0 until 1 hour after the final Fisetin dose on Day 9. Subjects started on other treatments for COVID-19 will continue to be followed if possible until the end of the study.

5.3 Method for Assigning Subjects to Treatment Groups

Subjects will be randomized to either Fisetin or placebo using an internet-based, web browser randomization procedure maintained by the data management team at Wake Forest School of Medicine. Study coordinators access the randomization application through the trial website. Access to this application is password-protected and its communications are encrypted. Once security requirements are satisfied, consent and eligibility of the participant are verified, and randomization will occur. Randomization is controlled centrally based on each potential participant's consent and eligibility status. ONLY eligible participants will have the randomization button displayed. It will be impossible to randomize an ineligible person, or a person for whom screening or consent is incomplete. We will use a permuted block randomization scheme with varying block sizes and without stratification so that group assignment is balanced as the study proceeds, but study personnel will not be able to predict treatment assignment.

5.4 Preparation and Administration of IP

The request for the IP will be sent to the research or general pharmacy associated with the nursing home and individually prepared for each subject.

If a subject becomes hospitalized, the research staff will contact the hospital staff to determine the feasibility of the IP being administered in the hospital.

5.5 Subject Compliance Monitoring

Patient adherence to study treatment will be monitored by study and/ or nursing home staff (or hospital staff) when they administer the IP.

5.6 Prior and Concomitant Therapy

Drugs listed as part of the exclusion criteria are not permitted during each of the two 2 days courses of treatment with Fisetin (Days 0 & 1 and Days 8 & 0). If patients are required to initiate these medications within these periods, then they will be removed from the study primarily due to risk of drug-drug interaction.

5.7 Packaging

The study medication will be prepared and dispensed by the research or general pharmacy associated with the nursing home with appropriate labeling to include a statement that these products are for investigational use only.

5.8 Masking/ Blinding of Study

In order to minimize the study bias, this study will use a process for randomized assignment to either active Fisetin or placebo (see Section 5.3). The investigational drug blind will be maintained using the processes of the research or general pharmacy associated with the nursing home for preparation of the investigational product. All subjects and study personnel except for those directly involved with IP preparation will be blinded to IP assignment until the end of the study. All unblinded dosing information must be maintained in a secured area, accessible only by unblinded personnel.

5.9. Unmasking/ Unblinding Procedure

The investigational drug blind shall not be broken by the investigator unless information concerning the investigational drug is necessary for the medical treatment of the subject. In the event of a medical emergency, if possible, the sponsor-investigator or PI should be contacted before the investigational drug blind is broken to discuss the need for unblinding.

If the PI or patient's attending physician, nurse-practitioner, or physician's assistant decides emergency unblinding is medically necessary, the PI or patient's attending physician, nurse-practitioner, or physician's assistant needs to contact the research or general pharmacy associated with the nursing home. The sponsor-investigator must be notified as soon as possible if the investigational drug blind is broken. The date, time, and reason the blind is broken must be recorded in the source documents, CRFs, or Event forms/logs.

5.10 Receiving, Storage, Dispensing, and Return

5.10.1. Receipt of IP

The IP for this study will be delivered to and managed by the research or general pharmacy associated with the nursing home according to their established standard procedures.

5.10.2. Storage of IP

IP should be stored between the temperatures of 2-30 degrees C.

5.10.3. Dispensing of IP

The IP is to be used exclusively in the clinical study according to the instructions of this protocol and directions for use. The Principal Investigator's designee is responsible for providing nursing home staff caring for the subjects with the IP and instructions for dosing and proper storage of the IP. The Principal Investigator's designee will record the amount of IP dispensed, date of dispensing, as well as the amount of IP returned and IP remaining.

5.10.4. Return or Destruction of IP

At the completion of the study, there will be a final reconciliation of IP shipped, IP dispensed, IP returned, and IP remaining. This reconciliation will be logged on the IP reconciliation form, signed, and dated. Any discrepancies noted will be documented and investigated, prior to return or destruction of unused IP. IP destroyed on site will be documented in the study files.

6 Study Procedures

The study procedures to be conducted for each subject enrolled in the study are presented in tabular form in Appendix 2 and described in the text that follows.

STUDY STAFF WILL HAVE DIRECT CONTACT WITH STUDY SUBJECTS; STUDY STAFF WILL OBTAIN A MAJORITY OF THE DATA FOR THIS STUDY BY COMMUNICATING WITH NURSING HOME STAFF AND ABSTRACTING INFORMATION FROM THE SUBJECT'S NURSING HOME CHARTS.

For each nursing home, during discussions leading to participation with their administrators and medical and nursing leadership, a key contact will be identified who will ensure access, data and sample collection, and data integrity and identify and assist in resolving issues as the study proceeds. In most cases, this will be the Medical or Nursing Director or their designees.

Biosafety Level 2 (BSL2) containment practices will be followed for sample transport.

6.1 Screening Visit (Days -2 to 0)

Patients will be pre-screened prior to being recruited for the study. Subjects will be screened within 3 days before randomization to determine eligibility for participation in the study.

Written informed consent will be obtained from the nursing home resident or from a Legally Authorized Representative (LAR) who can provide informed consent. Sections B to E (B: Hearing, Speech, and Vision, C: Cognitive Patterns, including the Brief Interview for Mental Status, D: Mood, and E: Behavior) of the most recent (within the last 100 days) Minimum Data Set (MDS) will be utilized to determine participants' ability to provide a truly informed consent and such an ability should be verified by staff with access to the nursing homes (*e.g.*, Medical or Nursing Director, nurse practitioner, physician's assistant) and if the participant is not able, their LAR will be asked. All MDS datasets for each subject will be collected throughout their inclusion in the study (up to 194 days) and the date(s) and type of vaccinations for CoV will be recorded.

In the pandemic situation where access to nursing homes and hospitals is limited, if allowed, digital/electronic consent will be obtained from the patient's LAR and will be documented by the investigator or the authorized designee. For any subject, it is the responsibility of the investigator or study team member to obtain consent prior to performing any protocol-mandated assessment.

After the informed consent and agreement by the patient's nursing home attending physician, nurse-practitioner, or physician's assistant, the following assessments are performed to determine eligibility requirements as specified in the inclusion and exclusion criteria. Assessments performed as part of the routine care of the subject by nursing home staff may be used to assess eligibility.

- Demographics.
- Focused medical history including the following information if feasible: date of first symptoms, overall symptoms, exposure source, demographics, baseline characteristics, allergies, medical history, reasons for nursing home admission (diagnoses, key symptoms and signs at and since admission as abstracted from the most recent MDS or available nursing home records).
- Determination of Ordinal Scale score.
- Review of concomitant medications including any COVID-19 vaccinations.
- Review of adverse events.
- Vital signs – heart rate, temperature, blood pressure, body weight, oxygenation (use of supplemental oxygen, amount of supplemental oxygen and Sp02 (if possible)) and

respiratory rate. Preferably, height will be obtained during the Screening visit (but may be obtained at a later visit, if it cannot be obtained during the Screening visit).
- SARS-CoV-2 mRNA-PCR/Influenza & RSV Rapid Test (during the influenza season) in a Mayo Clinic or other CLIA certified laboratory prior to randomization.
- Safety Labs must be collected and assessed within 48 hours prior to randomization. These labs include: bilirubin, creatinine, AST, ALT, hemoglobin, white blood cells, platelet count, absolute neutrophil count, lymphocyte count, plasma or serum glucose, and eGFR.
- If clinical chest imaging (X-ray, CT, MRI) is performed during the subject's participation in the study or within 14 days prior to enrollment, results may be obtained.
- Record any SAEs and all AEs related to protocol-mandated procedures occurring after completion of the consent form.

Subjects who qualify should be immediately randomized. Randomization and dosing should occur on the same day if possible.

6.2 Baseline (Day 0 & Day 1) – IP Administration

The following evaluations are to be completed at the screening or Day 0 visit. The investigator must have confirmed eligibility before proceeding with randomization on the Day 0 visit. Participants should complete the following assessments before being administered IP if feasible.

Patients who meet eligibility criteria, including a positive SARS-CoV-2 PCR and, during the influenza season, a rapid influenza and RSV test at a Mayo Clinic or other CLIA certified laboratory before randomization, and do not have evidence of unstable (as *per* clinical judgment) anemia, diabetes, liver disease, or renal dysfunction:

- Eligibility confirmation.
- Determination of Ordinal Scale score.
- Review of concomitant medications.
- Review of adverse events.
- Vital signs – heart rate, temperature, blood pressure, body weight, oxygenation (use of supplemental oxygen, amount of supplement oxygen used and Sp02 (if possible)) and respiratory rate. Collection of most recent (at least within 100 or fewer days) Minimal Data Set (MDS) on record for that participant).
- If clinical chest imaging (X-ray, CT, MRI) is performed during the subject's participation in the study or within 14 days prior to enrollment, results may be obtained.
- The 1st IP administration will occur on Day 0. The 2nd IP administration will occur on Day 1 (12 to 36 hours after the 1st IP administration).

6.3 Day 8 & Day 9 – IP Administration

- Verify eligibility to re-dose.
- Determination of Ordinal Scale score.
- Review of concomitant medications.
- Review of adverse events.
- Vital signs – heart rate, temperature, blood pressure, body weight, oxygenation (use of supplemental oxygen, amount of supplement oxygen used and Sp02 (if possible)) and respiratory rate. If clinical chest imaging (X-ray, CT, MRI) is performed during the subject's participation in the study, results may be obtained.
- The 3rd IP administration will occur on Day 8. The 4th IP administration will occur on Day 9 (12 to 36 hours after the 3rd IP administration).

6.4 Day 14 (±2 days)

- Determination of Ordinal Scale score.
- Review of concomitant medications.
- Review of adverse events.
- Vital signs – heart rate, temperature, blood pressure, body weight, oxygenation (use of supplemental oxygen, amount of supplement oxygen used and Sp02 (if possible)) and respiratory rate. Safety Labs – if possible, collect blood for bilirubin, creatinine, AST, ALT, hemoglobin, white blood cells, platelet count, absolute neutrophil count, lymphocyte count, plasma or serum glucose, and eGFR.
- If clinical chest imaging (X-ray, CT, MRI) is performed during the subject's participation in the study, results may be obtained.
- Research Labs 15 mL of blood and Collection of Biospecimens for Future Research – if possible, additional blood up to 85 mL (minimum of 35 mL) and 40 mL of urine will be collected.

6.5 Day 30 (-2 to +7 days)

- Determination of Ordinal Scale score.
- Review of concomitant medications.
- Review of adverse events.
- Vital signs – heart rate, temperature, blood pressure, body weight, oxygenation (use of supplemental oxygen, amount of supplement oxygen used and Sp02 (if possible)) and respiratory rate. Collection of most recent (at least within 100 or fewer days) Minimal Data Set (MDS) on record for that participant).
- If clinical chest imaging (X-ray, CT, MRI) is performed during the subject's participation in the study, results may be obtained.

6.6 Day 90 (±14 days)

- Determination of Ordinal Scale score.
- Review of concomitant medications.
- Review of adverse events.
- Vital signs – heart rate, temperature, blood pressure, body weight, oxygenation (use of supplemental oxygen, amount of supplement oxygen used and Sp02 (if possible)) and respiratory rate. Collection of the most recent Minimal Data Set (MDS) on record for that participant.
- Safety Labs – if possible, collect blood for bilirubin, creatinine, AST, ALT, hemoglobin, white blood cells, platelet count, absolute neutrophil count, lymphocyte count, plasma or serum glucose, and eGFR.
- If clinical chest imaging (X-ray, CT, MRI) is performed during the subject's participation in the study, results may be obtained.

6.7 Day 180 (±14 days) - Unblinding

- Determination of Ordinal Scale score.
- Review of concomitant medications.
- Review of adverse events.
- Vital signs – heart rate, temperature, blood pressure, body weight, oxygenation (use of supplemental oxygen, amount of supplement oxygen used and Sp02 (if possible)) and

respiratory rate. Collection of the most recent Minimal Data Set (MDS) on record for that participant.

- Safety Labs – if possible, collect blood for bilirubin, creatinine, AST, ALT, hemoglobin, white blood cells, platelet count, absolute neutrophil count, lymphocyte count, plasma or serum glucose, and eGFR.
- If clinical chest imaging (X-ray, CT, MRI) is performed during the subject's participation in the study, results may be obtained.

7 Study Procedure Details

7.1 Assessments

Study staff will obtain relevant information pertaining to physical examinations/assessments by communicating with nursing home staff and abstracting information from the subject's nursing home charts and Minimal Data Sets (MDS).

7.2 SARS-CoV-2 Testing

Subject must have a SARS-CoV-2 infection confirmed by PCR test at Mayo Clinic or other CLIA certified laboratory prior to randomization. A SARS-CoV-2 test may be ordered for research purposes after research consent is obtained. During the influenza season, a rapid influenza and RSV test will also be obtained at a Mayo Clinic or other CLIA certified laboratory.

7.3 Determination of Ordinal Scale

The subject's score on the Ordinal Scale for Clinical Improvement (Appendix 1) will be recorded daily (if feasible) through day 14 using the subject's worst assessments of the day. In addition, the score will be recorded on days 30, 90 and 180, if possible.

7.4 Safety Labs

Safety labs should be collected and assessed within 48 hours prior to randomization: bilirubin, creatinine, AST, ALT, hemoglobin, white blood cells, platelet count, absolute neutrophil count, lymphocyte count, plasma or serum glucose, and eGFR. If possible, safety labs will also be collected at the other time points listed in Appendix 2. Results from laboratory testing performed as part of nursing home care may also be obtained.

Treatment-emergent AEs and laboratory abnormalities will be summarized using descriptive statistics and listed by subject.

7.5 Research Labs

If possible, at each of the time points listed Appendix 2, 15 ml of blood will be collected to measure $p16^{INK4A}$ positive $CD3^{+}$T lymphocytes - a biomarker of senescence and chronological aging. Blood will be tested for the CoV N and S1 antibodies by ELISA. The N antibody, if positive, indicates a past infection with CoV and is not affected by the RNA vaccines. The S antibody can become positive due to past CoV infection or CoV RNA vaccination. This will allow us to test both response to the vaccine in those subjects who are vaccinated as well as verifying previous infection/ adequate immune response to CoV infection. As is the case for MCL (Mayo Clinic Labs), samples will be handled in BSL-2 conditions.

7.6 Biospecimens for Future Research

If possible, at each of the time points listed Appendix 2, up to 40 ml of urine and up to 85 ml of blood will be collected and stored for future research. The goal will be to evaluate and answer pertinent questions related to the biology of CoV infection in the elderly, aging, and senescence. Studies utilizing these samples will allow us to gain a greater understanding of the pathophysiology pathophysiology of CoV infection in the elderly, accelerated aging, and the potential for us to develop new therapies to improve patient outcomes.

8 Statistical Plan

8.1 Sample Size Determination

Power calculations for the comparison of the Fisetin and control groups were made assuming an equal distribution among the 7-point scale categories at Day 14 among the controls using a two-sided significance level of 0.05 (alpha). With 75 subjects per group, the study would have 80% power to detect a log odd's ratio of 0.81 which translates to an odd's ratio of 2.25 comparing the control to Fisetin group. Power calculations were run using the PASS software package.

8.2 Statistical Methods

All participants who are randomized will be included in the primary efficacy analysis. The analysis will follow the ITT principle (*i.e*. according to the treatment arm they are randomized to). For the primary analysis using the values of the 7-point scale, we will test the coefficient of the treatment effect (as a fixed effect) in an ordinal logistic regression model using a 2-sided 0.05 level Wald test after adjusting for key baseline variables such as baseline COVID severity status, age, and sex that show imbalance between the groups. Subjects who withdraw early but received at least some treatment will have their last known values carried forward until they complete the study at Day 180±14. All participants who receive any dose of drug (Fisetin or placebo) will be included in the safety and tolerability evaluation. The definitions of adverse events are described in Sections 9.1-9.3. Participants will be assessed over the entire period of drug administration. Frequency tables will be generated to summarize the occurrence of AE's by treatment arm. Comparisons of the rates of individual AEs will be done using tests of proportions such as the Fisher's exact test or Chi-squared test.

8 Analysis plans for secondary endpoints:

- Rate- or proportion-based endpoints: Several measures will be evaluated in patients that will be summarized as incidence rates or the proportion of patients who have an event or incident of interest; *e.g*., mortality within 14 days. For these endpoints, we will calculate the proportions of patients who have these incident events of interest for each of the treatment arms and calculate 95% confidence intervals for these outcomes. These will be graphically and quantitatively compared using chi-square tests. Logistic regression models will be used to assess the influence of treatment arm on the incidence of these events while adjusting for other factors of interest.
- Time-to-event endpoints: Similarly, several measures will be analyzed in a secondary manner that will evaluate the distributions of the time to events of interest. These include time to hospital discharge, survival, or symptom improvement. For these outcomes, Kaplan-Meier methods will be used to graphically evaluate potential differences between treatment arms or other groups of interest. Further, these methods will be used to estimate median times as well as time-specific rates (*e.g*., rate of transfer to hospital at 14 days). Cox proportional hazards models will be used to evaluate the influence of treatment arms, adjusting for other factors of interest.

- Oxygen supplementation and intubation: We will assess the incidence of oxygen supplementation or intubation and time to initiation of oxygen supplementation or intubation. In those who are given oxygen supplementation, we will summarize the duration of oxygen supplementation. Similarly, we will characterize and summarize the need for and duration of intubation. These will be analyzed in a manner similar to that described for the proportion/rate-based variables as well as the time-to-event variables but will specifically be based on a subset of patients given the nature of the outcome of interest. As such, these evaluations will be exploratory in nature.
- Influence of demographic factors on outcomes: These endpoints of interest will tie in with earlier stated analyses, where we will overall assess how these demographic factors such as age, biological sex, and race and ethnicity may influence the outcomes. Since this study will be done at multiple different nursing homes that see different races and ethnicities of patients, these analyses can help to assess if there are any differential outcomes or interactions with factors on these outcomes.
- Senescent cell markers: The distribution of changes in the senescent cell markers will be summarized graphically stratified by treatment arms. Differences between the groups will be assessed using linear regression analysis at the time of interest, adjusting for baseline values. Mixed effects models, where feasible, will also be used to assess the treatment affect using all available measurements.

9 Safety and Adverse Events

Safety evaluations will include adverse event (AE) and serious AE (SAE) reporting. Adverse event reporting will be conducted throughout the study for all subjects. The reporting period begins at the time of informed consent and continues through study completion. Adverse events will be assessed at every visit by study or nursing home staff.

Definitions, documentation, and reporting of AEs are described in Section 9.1-9.4.

9.1 Definitions

9.1.1 Unanticipated Problems Involving Risk to Subjects or Others (UPIRTSO)

Any unanticipated problem or adverse event that meets the following three criteria:

- Serious: Serious problems or events that result in significant harm (which may be physical, psychological, financial, social, economic, or legal), or increased risk for the subject or others (including individuals who are not research subjects). These include: (1) death; (2) life threatening adverse experience; (3) hospitalization - inpatient, new, or prolonged; (4) disability/incapacity - persistent or significant; (5) breach of confidentiality, and (6) other problems, events, or new information (*i.e.* publications, DSMB reports, interim findings, product labeling change) that in the opinion of the local investigator may adversely affect the rights, safety, or welfare of the subjects or others, or substantially compromise the research data, **AND**
- Unanticipated: (*i.e.* unexpected) problems or events are those that are not already described as potential risks in the protocol, consent document, not listed in the Investigator's Brochure, or not part of an underlying disease. A problem or event is "unanticipated" when it was unforeseeable at the time of its occurrence. A problem or event is "unanticipated" when it occurs at an increased frequency or at an increased severity than expected, **AND**
- Related: A problem or event is "related" if it is possibly related to the research procedures.

9.1.2 Adverse Event

An Adverse Event (AE) is any untoward or medical occurrence in a patient or clinical investigation subject administered a study product and which does not necessarily have a causal relationship with this treatment. Therefore, an AE is any unfavorable and unintended sign (including abnormal laboratory finding), symptom, or disease, which is temporarily associated with the use of an investigational product in a patient or research subject, whether or not considered related to the investigational product.

An AE also includes any worsening (ie, any clinically significant change in frequency and/or intensity) of a pre-existing condition that is temporarily associated with the use of the study drug.

9.1.3 Serious Adverse Event

Adverse events are classified as serious or non-serious. Serious problems/events can be well defined and include:

- Death - includes all deaths, even those that appear to be completely unrelated to study drug (eg, a car accident in which a patient is a passenger).
- Life threatening adverse experience - in the view of the investigator, the patient is at immediate risk of death at the time of the event. This does not include an AE that had it occurred in a more severe form, might have caused death
- Hospitalization - Inpatient hospitalization is defined as admission to a hospital or an emergency room for longer than 24 hours. Prolongation of existing hospitalization is defined as a hospital stay that is longer than originally anticipated for the event or is prolonged due to the development of a new AE, as determined by the investigator or treating physician.
- Inpatient, new, or prolonged; disability/incapacity.
- Persistent or significant disability or incapacity.
- Birth defect/congenital anomaly.

and/or *pe*r protocol may be problems/events that in the opinion of the sponsor-investigator may have adversely affected the rights, safety, or welfare of the subjects or others, or substantially compromised the research data.

All adverse events that do not meet any of the criteria for serious will be regarded as **non-serious adverse events**.

Criteria for reporting SAEs must be followed for these events. See section regarding reporting of SAEs for more information on recording and reporting SAEs.

All study subjects will be provided with study team contact information 24/7. They will be educated on contacting the study team if any of these events occur. This will allow the study staff to be contacted if a serious adverse event occurs between study visits.

9.1.4 Adverse Event Reporting Period

For this study, the study treatment period is defined as 10 days, but subjects will be monitored in the nursing home, hospital, another care facility, or at home directly by study staff or the patient's care providers or by telephone contact by study staff for Day 180±14. The adverse event reporting period ends when the subject completes the study at Day 180±14.

9.1.5 Preexisting Condition

A preexisting condition is one that is present at the start of the study. A preexisting condition will be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

9.1.6 Medical History Findings

At screening, any clinically significant abnormality will be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event will also be recorded and documented as an adverse event. All admission diagnoses and reasons for hospital admission will be recorded.

9.1.7 Post-Study Adverse Event

All unresolved adverse events will be followed by the Principal Investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit or follow-up telephone call (Day 180±14), the PI will instruct each subject to report, to the PI, any subsequent event(s) that the subject, or the subject's nursing home or personal physician, physician's assistant, or nurse practitioner believes might reasonably be related to participation in this study.

9.1.8 Abnormal Laboratory Values

A clinical laboratory abnormality will be documented as an adverse event if it changes from a value within the normal range (or as *per* clinical judgment) before treatment to one outside and worse than the normal range after treatments. Such changes will prompt a repeat test, telephone call to check the subject's status, a repeat visit, and/or referral to the subject's primary care physician.

9.1.9 Hospitalization, Prolonged Hospitalization, or Surgery

Any adverse event that results in hospitalization or prolonged hospitalization will be documented and reported as a serious adverse event unless specifically instructed otherwise in this protocol. Any condition responsible for surgery will be documented as an adverse event if the condition meets the criteria for an adverse event.

Neither the condition, hospitalization, prolonged hospitalization, or surgery will be reported as an adverse event in the following circumstances:

The following hospitalizations will not be considered SAE for this study:

- a visit to the emergency department or other hospital department <24 hours, that does not result in admission (unless considered an important medical or life-threatening event).
- elective surgery planned prior to signing consent.
- admissions as *per* protocol for a planned medical/surgical procedure.
- routine health assessment requiring admission for baseline/trending of health status (*e.g.*, routine mammogram).
- medical or surgical admission other than to remedy ill health and planned prior to entry into the study. Appropriate documentation will be obtained in these cases.

9.2 Recording of Adverse Events

At each contact with the subject, the study team will seek information on adverse events by specific questioning and, as appropriate, by examination. Information on all adverse events will be recorded immediately in the source document, and also in the appropriate adverse event section of the case report form (CRF) or in a separate adverse event worksheet. All clearly related signs, symptoms, and abnormal diagnostic, laboratory, or procedure results will be

recorded in the source document.

All adverse events occurring during the study period will be recorded. The clinical course of each event will be followed until resolution, stabilization, or until it has been ultimately determined that the study treatment or participation is not the probable cause. Serious adverse events that are still ongoing at the end of the study period will be followed up to determine the final outcome. Any serious adverse event that occurs during the Adverse Event Reporting Period and will be considered to be at least possibly related to the study treatment or study participation will be recorded and reported immediately.

9.3 Reporting of Serious Adverse Events and Unanticipated Problems

When a serious adverse event has been identified, the study team will take appropriate action necessary to protect the study participant and then complete the Study Adverse Event Worksheet and log. The Principal Investigator will evaluate the event and determine the necessary follow-up and reporting required. The PI will notify the Sponsor-Investigator within 24 hours of becoming aware of the serious adverse event and report the event to the Mayo Clinic IRB within 5 working days.

9.3.1 Principal Investigator Reporting: Notifying the Mayo Clinic IRB

The Principal Investigator will report to the Mayo Clinic IRB any UPIRTSOs and Non-UPIRTSOs according to the Mayo Clinic IRB Policy and Procedures.

Information collected on the adverse event worksheet (*and entered in the research database)*:

- Subject's name:
- Disease/histology (if applicable):
- The date the adverse event occurred:
- Description of the adverse event:
- Relationship of the adverse event to the research: Not Related, Possible, Probable, Definitely Related.
- If the adverse event was expected:
- The severity of the adverse event: Mild, Moderate, Severe.
- If any intervention was necessary/action taken:
- Resolution: (was the incident resolved spontaneously or after discontinuing treatment).
- Date of Resolution:

The Principal Investigator will sign and date the adverse event report when it is reviewed. For this protocol, only directly related SAEs/UPIRTSOs will be reported to the IRB. The Sponsor-Investigator will review all adverse event reports to determine if specific reports need to be made to the FDA.

9.3.2 Relationship

The relationship of an AE to the Investigational Drug is a clinical decision by the Principal-Investigator based on all available information at the time of the completion of the CRF and is graded as follows:

0. Not related: a reaction for which sufficient information exists to indicate that the etiology is unrelated to the study drug; the subject did not receive the study medication or the temporal sequence of the AE onset relative to administration of the study medication is not reasonable or the event is c1early related to other factors such as the subject's clinical state, therapeutic intervention or concomitant therapy.

1. Possible: a clinical event, including laboratory test abnormality, with a reasonable time sequence to administration of the drug but which could also be explained by concurrent disease or other drugs or chemicals; information on drug withdrawals may be lacking are unclear.

2. Probable: a clinical event including laboratory test abnormality, with a reasonable time sequence to administration of the drug, unlikely to be attributed to concurrent disease or other drugs or chemicals and which follows a clinically reasonable response on withdrawal (de- challenge): re-challenge information is not required to fulfi1 this definition.

3. Definitely Related: a reaction that follows a reasonable temporal sequence from administration of the drug, or in which the drug level has been established in body fluids or tissues, that follows a known or expected response pattern to the suspected drug, and that is confirmed by improvement on stopping or reducing the dosage of the drug, and reappearance of the reaction on repeated exposure (re-challenge).

9.3.3 Severity

The maximum intensity of an AE during a day should be graded according to the definitions below and recorded in details as indicated on the CRF. If the intensity of an AE changes over a number of days, then separate entries should be made having distinct onset dates.

1. Mild: AEs are usually transient, requiring no special treatment, and do not interfere with patient's daily activities.

2. Moderate: AEs typically introduce a low level of inconvenience or concern to the patient and may interfere with daily activities, but are usually ameliorated by simple therapeutic measures.

3. Severe: AEs interrupt a patient's usual daily activity and traditionally require systemic drug therapy or other treatment.

9.4 Sponsor-Investigator Reporting: Notifying the FDA

The Sponsor-Investigator will report to the FDA all unexpected, serious suspected adverse reactions according to the required IND Safety Reporting timelines, formats, and requirements.

Unexpected fatal or life threatening suspected adverse reactions where there is evidence to suggest a causal relationship between the IP and the adverse event will be reported as a serious suspected adverse reaction. This will be reported to the FDA on FDA Form 3500A no later than 7 calendar days after the sponsor-investigator's initial receipt of the information about the event.

Other unexpected serious suspected adverse reactions where there is evidence to suggest a causal relationship between the IP and the adverse event, will be reported as a serious suspected adverse reaction. This will be reported to the FDA on FDA Form 3500A no later than 15 calendar days after the sponsor-investigator's initial receipt of the information about the event.

Any clinically-important increase in the rate of serious suspected adverse reactions over those listed in the protocol or product insert will be reported as a serious suspected adverse reaction. This will be reported to the FDA on FDA Form 3500A no later than 15 calendar days after the sponsor-investigator's initial receipt of the information about the event.

Findings from other studies in human or animals that suggest a significant risk in humans exposed to the drug will be reported. This will be reported to the FDA on FDA Form 3500A, no later than 15 calendar days after the sponsor-investigator's initial receipt of the information about the event.

9.5 Unmasking/ Unblinding Procedures

The IP blind shall not be broken by the investigator unless information concerning the IP is necessary for the medical treatment of the subject. In the event of a medical emergency, if possible, the sponsor-investigator should be contacted before the IP blind is broken to discuss the need for unblinding.

If the Sponsor-Investigator and PI decides emergency unblinding is medically necessary, the PI needs to contact the Research Pharmacy. The sponsor-investigator must be notified as soon as possible if the IP blind is broken. The date, time, and reason the blind is broken must be recorded in the source documents, CRFs, or Event forms/logs.

9.6 Stopping Rules

All patients will be assessed during the 10 days of drug (Fisetin or placebo) administration and during each visit following administration of the drugs. If a severe adverse event occurs at any time during administration of the 10-day drug regimen, a formal review will occur and subsequent patients will be enrolled one at a time using the same regimen. If four or more events accrue, the pilot study will be held and either a potentially alternative dosing regimen considered or discontinuation of the study protocol will occur. Other interventions will be as *per* the direction of the FDA and Mayo (and other participating institutions) IRB.

Toxicity Stopping Rule: The study will be terminated if the number of treatment-related deaths in the patients already registered and treated in the trial exceed 10%.

9.7 Medical Monitoring

It is the responsibility of the Principal Investigator to oversee the safety of the study. This safety monitoring will include careful assessment and appropriate reporting of adverse events as noted above, as well as the construction and implementation of a data and safety-monitoring plan (see section 11 "Study Monitoring, Auditing, and Inspecting"). Medical monitoring will include a regular assessment of the number and type of serious adverse events.

9.8 Internal Data and Safety Monitoring Board (DSMB)

A DSMB appointed by the National Institutes of Health not affiliated with the study will be responsible for evaluating the progress of the study and will be provided unblinded data on a regular basis to monitor patient safety. This committee will communicate by meeting monthly or as decided by the National Institutes of Health. Data will be reviewed by the DSMB in an unblinded fashion. Randomization codes for each enrolled patient will be provided to the DSMB as requested. The Committee will make its recommendations by monitoring progress, data, outcomes, toxicity, safety, and other confidential data, and may recommend stopping the clinical trial if an excessive number of serious adverse events are observed.

10 Data Handling and Record Keeping

10.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study.
- Who will have access to that information and why.
- Who will use or disclose that information.
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (long term survival status that the subject is alive) at the end of their scheduled study period.

10.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in the clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: nursing home records, hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

10.3 Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. Case reports in the form of completed checklists will be kept to assure inclusion/exclusion criteria and review of adverse events/toxicity. All data requested on the CRF will be recorded. All missing data must be explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, write "N/D". If the item is not applicable to the individual case, write "N/A". All entries will be printed legibly in black ink. If any entry error has been made, to correct such an error, a single straight line will be drawn through the incorrect entry and the correct data will be entered above it. All such changes will be initialed and dated. Errors will not be erased and "white-out" will not be used to correct errors. For clarification of illegible or uncertain entries, the clarification will be printed above the item, initialed, and dated. If the reason for the correction is not clear or needs additional explanation, details will be added related to the justification for the correction.

10.4 Data Management

The data will be housed in both hard copy case report forms (CRFs) and eCRFs.

10.5 Data Security and Confidentiality

Source documents and CRFs and original consents will be stored in secured locations. All data will be entered into a password protected, limited access database. Individually identifiable patient history and medical record information will be stored in a database under coded accession numbers. Clinical laboratory values will be stored in the electronic medical record system, requiring protected password access. These data are monitored regularly for access and a formal policy regarding protection of personal privacy is in place. The key to identification of subjects will be maintained in a secure office environment under the direction of the principal investigators.

10.6 Data Quality Assurance

Manual and computerized quality checks will occur during data collection and analyses and any

discrepancies will require Case Report Form (CRF) review and validation of correct data.

10.7 Records Retention

The investigator will maintain records and essential documents related to the conduct of the study. These will include subject case histories and regulatory documents. The sponsor-investigator will retain the specified records and reports for:

1. Until 2 years after the last shipment and delivery of the drug for investigational use is discontinued and the FDA has been so notified. OR
2. As outlined in the Mayo Clinic (and other participating institutions) Research Policy Manual –"Retention of and Access to Research Data Policy"

[redacted]

Whichever is longer.

11 Study Monitoring, Auditing, and Inspecting

11.1 Study Monitoring Plan

The investigator will allocate adequate time for such monitoring activities. The Investigator will also ensure that the monitor or other compliance or quality assurance reviewer is given access to all the study-related documents and study related facilities (*e.g.*, nursing home, pharmacy, diagnostic laboratory, *etc.*), and has adequate space to conduct the monitoring visit.

As a service to the sponsor-investigator, this study may be monitored during the conduct of the trial by staff from the Mayo Clinic Office of Research Regulatory Support (and other participating institutions). Clinical trial monitoring may include review of the study documents and data generated throughout the duration of the study to help ensure the validity and integrity of the data along with the protection of human research subjects. This will aid in complying with FDA regulations.

11.2 Auditing and Inspecting

The investigator will permit study-related monitoring, audits, and inspections by the IRB, the sponsor, and government regulatory agencies, of all study related documents (*e.g.*, source documents, regulatory documents, data collection instruments, study data, *etc.*). The investigator will ensure the capability for inspections of applicable study-related facilities (*e.g.*, nursing home, pharmacy, diagnostic laboratory, *etc.*).

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable compliance offices.

12 Ethical Considerations

This study will be conducted according to United States government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted local Institutional Review Board (IRB), in agreement with local legal prescriptions, for formal approval of the study. The decision of the IRB concerning the conduct of the study will be made in writing to the sponsor-investigator before commencement of this study.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the IRB

for the study. The formal consent of a subject, using the Approved IRB consent form, will be obtained before that subject undergoes any study procedure. The consent form will be signed manually or electronically by the subject or the subject's LAR and the individual obtaining the informed consent.

13 Study Finances

13.1 Funding Source

National Institutes of Health funding will be used to support this study.

13.2 Conflict of Interest

Any study team member who has a conflict of interest with this study (patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, *etc*.) must have the conflict reviewed by a properly constituted Conflict of Interest Committee with a Committee-sanctioned conflict management plan that has been reviewed and approved by the study sponsor-investigator prior to participation in this study.

13.3 Subject Stipends or Payments

Subjects will not receive remuneration.

14 Publication Plan

The principal investigators, Drs. Kirkland and Khosla, hold the primary responsibility for publication of the results of the study.

We will register with ClinicalTrials.gov within 21 days of the first subject being enrolled. We will post results to ClinicalTrials.gov within 12 months of final data collection for the primary outcome.

Appendix 1. Ordinal Scale

Ordinal Scale for Clinical Improvement

Patient State	Descriptor	Score
Uninfected	No clinical or virological evidence of infection	0
Ambulatory	No limitation of activities	1
	Limitation of activities	2
Hospitalized Mild disease	Hospitalized, no oxygen therapy	3
	Oxygen by mask or nasal prongs	4
Hospitalized Severe Disease	Non-invasive ventilation or high-flow oxygen	5
	Intubation and mechanical ventilation	6
	Ventilation + additional organ support – pressors, RRT, ECMO	7
Dead	Death	8

https://www.who.int/blueprint/priority-diseases/key-action/COVID-19_Treatment_Trial_Design_Master_Protocol_synopsis_Final_18022020.pdf

Appendix 2. Schedule of Events

Procedures	Screening[1]	Baseline / Treatment	Treatment	Follow-up	Long-Term Follow-up		
Day(s)	-2 to 0	0 & 1	8 & 9	14 (±2 days)	30 (-2 to +7 days)	90 (±14 days)	180 (±14 days)
Verify Inclusion/Exclusion Criteria	X	X	X				
Informed Consent	X						
Demographics & Medical History	X						
Randomization		X					
IP Administration[2]		X	X				
Ordinal Scale	X	X	X	X	X	X	X
Concomitant Medications[3]	X	X	X	X	X	X	X
Adverse Events	X	X	X	X	X	X	X
Vital Signs[4]	X	X	X	X	X	X	X
MDS (as available)		X			X	X	X
Chest Imaging[5]	X	X	X	X	X	X	X
SARS-CoV-2	X						
Influenza & RSV Rapid Test	X						
Safety Labs[6]	X			X		X	X
Research Labs[7] (blood and urine) & Biospecimens for Future Research Labs				X			

Footnotes for Schedule of Events

Procedures/Assessments in Green are MANDATORY
Procedures/Assessments in White will be performed (or results will be obtained) if possible

***Refer to Study Procedures section for detailed descriptions of Procedures/Assessments.**

1. Screening procedures may overlap with baseline procedures.
2. IP administration will involve a 2-day oral or NG or D tube treatment twice with Fisetin or placebo. The 1st IP administration will occur on Day 0. The 2nd IP administration will occur on Day 1 (12 to 36 hours after 1st IP administration). The 3rd IP administration will occur on Day 8. The 4th IP administration will occur on Day 9 (12 to 36 hours after 3rd IP administration).
3. Concomitant medication should include recording of any COVID-19 vaccinations.
4. Vital signs – heart rate, temperature, blood pressure, body weight, oxygenation (use of supplemental oxygen, amount of supplement oxygen used and Sp02 (if possible)), respiratory rate and height (preferably collected at screening but may be obtained at a late visit is not able to be obtained at screening).
5. If clinical chest imaging (X-ray, CT, MRI) is performed during the subject's participation in the study (or within 14 days prior to enrollment), results may be obtained.
6. Safety labs include: bilirubin, creatinine, AST, ALT, hemoglobin, white blood cells, platelet count, absolute neutrophil count, lymphocyte count, plasma or serum glucose, and eGFR. Safety labs collected at screening should be collected and assessed within 48 hours prior to randomization if feasible.
7. Research Labs 15 mL of blood and Collection of Biospecimens for Future Research – if possible, additional blood up to 85 mL (minimum of 35 mL) and 40 mL of urine will be collected.

References

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