CLINICAL TRIAL PROTOCOL

THE EFFECT OF REGULAR EXERCISE & INTERMITTENT RAPAMYCIN DOSING ON MUSCLE PERFORMANCE IN OLDER ADULTS

Study Product	Sirolimus (Rapamycin)
Development Phase	2a
Sponsor	Add details once confirmed (update section 5.3)
Protocol version	Original
Protocol date	23 February 2022
Compliance	This study will be conducted in accordance with standards of Good Clinical Practice (as defined by the International Council for Harmonisation), ethical principles that have their origin in the Declaration of Helsinki, and all applicable national and local regulations

Commercial in Confidence

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1. PROTOCOL SYNOPSIS

Protocol Title	The Effect Of Regular Exercise & Intermittent Rapamycin Dosing On Muscle Performance In Older Adults
Protocol Number	
Study Phase and Type	Phase 2a Proof-of-concept study
Target Population	Male or female adults aged 65-85 years who are medically stable and do not already perform weekly, strenuous exercise
Primary Objective	The safety, tolerability, feasibility, and trial design of weekly Sirolimus (Rapamycin) 6mg or placebo dosing over a 13-week period, in combination with thrice-weekly at-home exercise programs.
Primary Endpoint	The Primary Endpoint is improvement in 30-Second Chair-Stand Test after a 13-week exercise training program
Secondary Objectives	To evaluate the efficacy of Sirolimus (Rapamycin) on: 1. the safety and tolerability of Sirolimus (Rapamycin) administered weekly over a 13-week period in subjects aged 65-85 years who are medically stable 2. muscle strength 3. muscle endurance 4. community balance and mobility 5. lean muscle mass 6. improvement in insulin resistance 7. general physical activity
Secondary Endpoints	• Treatment-emergent adverse events (TEAEs), including adverse events of special interest (AESI) from the time of first dose of IP through End of Study (EOS) • Observed change from baseline in handgrip strength through EOS • Observed change in 6-minute walk test from baseline through EOS • Observed change Community Balance & Mobility Scale from baseline through EOS • Observed change from baseline in lean muscle mass by DEXA scan through EOS • Full Blood Count, U&Es, LFTs, HbA1c, lipids, serum IGF-1 • DNA methylation clocks • Observed change from baseline in insulin resistance as determined by Homeostatic Model Assessment through EOS

Study Design:	A 13-week, randomised, double-blind, placebo-controlled, Phase 2a Proof of concept trial of 40 participants, with 20 in the placebo arm and 20 in the Sirolimus (Rapamycin) arm. Participants will complete a thrice-weekly at-home exercise program. Medication will be taken once a week. Before the trial begins, exercycles will be delivered to the participants' house, and once the trial begins the participants will complete a standardised exercycle program.
Subjects	20 adults in the placebo group, 20 in the Sirolimus (Rapamycin) group
Clinical visits	4
Investigational product	Sirolimus (Rapamycin) 6mg weekly dosing, or a placebo
Investigational products administration	Oral administration
Inclusion/Exclusion Criteria	Inclusion: • Males and females aged ≥65 years and ≤85 years at the time of signing informed consent • Medically stable and do not already perform regular strenuous exercise • Capable of providing written informed consent and willing and able to adhere to all protocol requirements • Participants must be reliable, adherent, and agree to cooperate with all planned trial evaluations as explained in detail during the informed consent process and to be able to perform them. Exclusion: • Already participating in strenuous activity enough to cause a noticeable increase in breathing more than twice a week • Anaemia - Hg < 9.0 g/dl, Leukopenia - white blood cells (WBC) < 3,500/mm^3 , Neutropenia - absolute neutrophil count < 2,000/mm^3 , or Platelet count - platelet count < 125,000/mm^3 • Older adults scheduled to undergo major surgery in the next 12 months • Any uncontrolled or serious disease, or anything medical (e.g. known active infection or major haematological, renal, metabolic, gastrointestinal, or endocrine dysfunction) or surgical that, in the opinion of the investigator, may

	interfere with participation in the clinical study and/or put the patient at significant risk. • Malignancy (except non-melanoma skin cancers, cervical carcinoma in-situ) within the last 5 years. • Known hypersensitivity, allergy, or any contraindication to Rapamycin or its excipients • Fibromyalgia or Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, Breast Implant Illness • Congestive heart failure: self-assessed functional status of heart failure New York Heart Association (NYHA) classification III or IV • COPD Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification III or IV • Impaired renal function, as defined as glomerular filtration rate eGFR < 30 • Type 1 or Type 2 Diabetes (HbA1c must be <50 mmol/mol) • Substance, alcohol or drug abuse within the 3 months prior to informed consent that would interfere with trial participation leading to decreased compliance with trial procedures or study medication intake in the opinion of the investigator • Psychological, familial, sociological, or geographic factors potentially hampering compliance with the protocol, visits, or trial procedures or any other clinical condition that would jeopardise patient safety while participating in the clinical trial in the opinion of the investigator • Those who have taken metformin, Rapamycin, or rapalogs in the past 6 months
Study Procedures	See Time & Events Table below
Sample Size Determination	40 in total
Statistical Consideration	**Analysis sets** Intention-to-treat set: All randomised participants Per protocol set: All participants reporting having undertaken at least 2/3 of the exercise sessions and, if in the Sirolimus arm, having taken at least half of the stipulated doses. Safety Set: All randomised participants.

	Inferential analyses: Generalised linear mixed models (patient as random intercept) as appropriately selected during blind review, adjusted for baseline outcome values. Standard errors obtained from the wild bootstrap. Fifteen multiply imputed copies of the data to offset missingness. No interim analysis for efficacy is planned. **Demographics and baseline characteristics** Reported by arm and overall (mean and standard deviation, or proportion), without p-values. **Safety analysis** Negative binomial of counts of adverse events regressed on allocation arm in interaction with adjudicated severity. Adjudicated relatedness will be regressed on the allocation arm to test for equivalence.
Data Monitoring Committee	MMCT to add
Plan Study duration	Total Study Duration: 13 weeks

1.1. TIME & EVENTS TABLE

Visit	**1**	**2**	**3**	**4**
Study Day	**Pre**	**0**	**6 weeks**	**13 weeks**
Informed consent	x			
Review of Subject Eligibility	x	x		
Demographics	x			
Medical/travel /contact history	x			
Physical Exam	x		x	x
Vital signs	x	x	x	x
ECG	x		x	x
Haematology & Biochemistry	x		x	x
Urine albumin/creatine ratio	x		x	x

DEXA Scan	x			x
Demonstration of 30s Chair-Stand Test	x			
Randomization		x		
Allocation of weekly medication		x	x	
Distribute subject diary card		x	x	
Collect subject diary cards			x	x
30s Chair-Stand Test		x	x	x
Hand Grip Strength		x	x	x
Community balance & mobility scale		x	x	x
6-minute walk test		x	x	x

2. Brief Hypothesis

Periods of time where the mechanistic target of Rapamycin (mTOR) pathway is activated via exercise, combined with alternate periods of time where mTOR is inhibited using Sirolimus (Rapamycin), will result in greater muscle performance in older adults compared with just exercise alone.

3. Background

3.1. Aging & Muscle Strength

Human skeletal muscle inevitably undergoes remarkable changes with aging, characterized by a decline in muscle mass and strength of about 1% per year from the age of around 40 years [1]. A growing body of evidence suggests that muscular strength is inversely and independently associated with all-cause and cardiovascular mortality even after adjusting for cardiorespiratory fitness [1]. Ultimately, muscle wasting contributes significantly to weakness, disability, increased hospitalization, immobility, and loss of independence. Interventions for sarcopenia (the loss of skeletal muscle mass and strength as a result of aging) include exercise and nutrition because both have a positive impact on protein anabolism but also enhance other aspects that contribute to well-being in sarcopenic older adults, such as physical function, quality of life, and anti-inflammatory state [1].

3.2. Rapamycin

Rapamycin (also known as Sirolimus or the drug name Rapamune) is a natural macrocyclic lactone produced by the bacterium *Streptomyces hygroscopicus*. It is FDA-approved for the treatment of various conditions including as an immunosuppressant to prevent organ transplant rejection, and in drug-eluting coronary stents to inhibit vascular smooth muscle cell proliferation, thereby preventing restenosis in coronary artery lesions. Additionally, based on its antiproliferative effects, Rapamycin is used in the treatment of various cancer types and the rare lung disease: lymphangioleiomyomatosis [2].

3.3. THE MTOR PATHWAY

The mechanistic target of Rapamycin, or mTOR, is a protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and interacts with several proteins to form two distinct types of multiprotein complexes, named mTOR complexes 1 and 2 (mTORC1 and mTORC2) [3, 4]. Both these complexes play vital roles in cellular regulation, and, therefore, dysregulation of this signalling pathway disrupts cell homeostasis and may lead to pathologies associated with aging. mTORC1 and 2 are not equally sensitive to Rapamycin, with mTORC1 being highly sensitive and mTORC2 only inhibited after prolonged exposure [5]. mTORC1 is a nutrient-sensing protein kinase that can activate protein synthesis, drive lipid and nucleotide synthesis, and represses catabolism and autophagy.

Given its functionality, mTOR activity is now accepted as a major driver of the aging process. Indeed, in multiple organisms inhibition of the mTORC1 pathway has been shown to extend life. Studies in which mTOR or Raptor (a subunit of mTORC1) were depleted showed life-expanding effects in yeast [6] , nematodes [7, 8], flies [9], and mammals [10].

3.4. PRECLINICAL STUDIES

3.4.1. MOUSE

Treatment of mice with Rapamycin has been shown to increase the median and maximum lifespan of mice of both sexes when treatment was started at middle age (270 of 600 days of age) [11]. More specifically, treatment with Rapamycin for only 3 months at middle age was sufficient to increase life expectancy by up to 60% and improve measures of healthspan in middle-aged mice [12]. Another study found that the extension of the median survival was higher in female mice (18%) than in males (10%) [13]. Furthermore, Rapamycin treatment in mice was shown to increase alveolar bone levels [14], improve cardiovascular function [15-17], decrease body weight [13] , and remodel the microbiome [12] .

3.4.2. RAT

In rats it was shown that Rapamycin treatment reduces food intake and body weight [17], increases grip strength, and attenuates the decline in maximum running distance [18]. Daily dosing in food did cause some diabetes-like symptoms [18], but repeated low doses do not seem to influence glucose homeostasis with long term use [17].

3.4.3. DOG

Companion dogs are used in anti-aging research as a model that may be more representative of human aging, as even though dogs age faster than humans; they do get similar aging-associated diseases. A study by Urfer *et al*., showed that low dose (0.05 mg/kg and 0.10mg/kg) Rapamycin treatment 3 times a week over a period of 10 weeks in middle aged dogs was well tolerated, with no clear adverse effects except for a decrease in the volume of red blood cells. The dogs had improvements in their left ventricular cardiac function and had increased activity [19].

3.5. CLINICAL STUDIES

The current FDA-approved use of Sirolimus (Rapamycin) is immunosuppression for organ transplant patients, and the treatment of cancer. The FDA-approved doses used for organ

transplantation are much higher than the doses proposed for this study (6mg total, once a week) and are associated with high levels of adverse events:

- FDA-approved dose for kidney transplant rejection prophylaxis:
    - Low dose: 3 mg/m^2 per day, ↑ up to 40 mg/day max
    - Medium dose: 6 mg/m^2 per day, ↑ up to 40 mg/day max
    - High dose: 15 mg/m^2 per day, ↑ up to 40 mg/day max

Common side effects at the FDA-approved doses include stomatitis, diarrhoea, nausea, anaemia, cytopenia, hyperglycaemia, and hyperlipidaemia, as well as the (intended) immunosuppressive effect. These side effects would not be acceptable for use as an anti-aging treatment. However, the doses used for these conditions are relatively high (loading doses up to 15mg, followed by 2-5 mg/daily), when compared to the doses observed to have an anti-aging effect.

For this reason, we have chosen to focus on a low dose protocol as it has already been shown to be safe as described below. The other consideration is Sirolimus (Rapamycin)'s relatively long half-life in humans (64 hours). Therefore, to have periods of mTORC1 inhibition and alternative periods of activation, Sirolimus (Rapamycin) should not be administered more frequently than once a week.

Human studies into the anti-aging effects of Sirolimus (Rapamycin) have been performed to test its safety in a healthy, older population. For this purpose, the consensus is that treatment should be at lower dosing levels than the FDA-approved treatment regimen, to induce inhibition of mTORC1, but not affect mTORC2 signalling. Indeed, low dose, or even a one-time dose can inhibit mTOR signalling. A single dose of 6 mg in young, healthy men of a normal weight was shown to partially inhibit mTOR-mediated signalling and stimulate insulin-mediated glucose uptake [20]. However, many human studies performed to date are restricted in evaluation of efficacy due to a low number of study subjects. For instance, in a study in which 13 patients over 60 years with coronary artery disease were treated with low daily doses (0.5, 1, 2 mg/day) of Rapamycin, it was shown that it was well tolerated. While the number of patients was low, there was some correlation between senescence markers and physical performance, but no improvement in frailty was detected [21].

In a randomized controlled trial of 14 patients aged between 70 and 93 years of age who received an oral dosing of 1 mg/day of sirolimus, the treatment was considered safe. Side effects were considered mild at this dose with few cases of facial rash, stomatitis, gastrointestinal issues, decreases in erythrocyte parameters, and increases in myeloid and regulatory T cells. No increase in blood glucose concentration or insulin secretion and sensitivity were observed [22].

Regarding the potential immunosuppressive effects of Sirolimus (Rapamycin), a phase 2a randomized, placebo-controlled clinical trial was performed to determine whether low-dose mTOR inhibitor therapy would ***enhance*** immune function and decrease infection rates in 264 elderly subjects given the study drugs for 6 weeks. Low dose treatment with a Rapamycin analogue (RAP001) was safe and was associated with a significant ($P = 0.001$) decrease in the rate of infections reported by elderly subjects for a year after study drug initiation. In addition, that trial demonstrated an improved response to influenza vaccination of 20%, as well as a reduction in the number of $CD4^+$ and $CD8^+$ T cells expressing PD-1, which inhibits T cell

signalling and is found at higher levels with increasing age [23, 24]. These studies tested a variety of doses of RAP001 (0.5 mg/daily, 5mg/weekly, and 20 mg/weekly) and found very limited adverse events. The most common adverse event were mouth ulcers, which were mild in most cases, except for one patient treated with a dose of 20 mg/week who had severe mouth ulcers.

In a cohort of patients above 40 years of age, topical administration of Rapamycin led to a reduction in cellular senescence and improvement of histological markers of aging in the skin [25].

Importantly when considering the combination of exercise and Sirolimus (Rapamycin), a study of sixteen healthy young males found that 16mg Sirolimus (Rapamycin) taken before resistance exercise impaired mTORC1 signalling and muscle protein synthesis after exercise [26]. This observation is important for our proposed trial design, whereby Sirolimus (Rapamycin) should be taken on a non-exercise day to maximise the time-periods where mTORC1 is switched on by exercise, and off by Sirolimus (Rapamycin).

Given the existing safety data available for the use of Rapamycin in humans, alongside the animal data suggesting that Rapamycin can be used to reduce symptoms of age-related decline, a crowdfunded trial called Participatory Evaluation (of) Aging (With) Rapamycin (for) Longevity Study (PEARL) is currently underway (ClinicalTrials.gov Identifier: NCT04488601). This is a randomized, double-blind, placebo-controlled trial assessing the effects of low, medium, and high doses of intermittent Sirolimus (Rapamycin) dosing on a weekly schedule over a period of 6-12 months. The researchers aim to establish a long-term safety profile, determine the long-term efficacy of rapalogs in reducing clinical aging measures, and biochemical and physiological endpoints associated with declining health and aging in healthy older adults aged 50-85 years. The primary endpoint is changes in visceral fat as measured by dual-energy x-ray absorptiometry (DEXA) scan, and will use Sirolimus (Rapamycin) dosages up to 5mg/day twice per week. Due to the study design, the PEARL trial will not be able to answer what effect the combination of exercise and intermittent Rapamycin dosing will have on the muscle performance of older adults. Alternating periods of mTORC1 activation via exercise, and inhibition via Sirolimus (Rapamycin) appears to be a plausible way to improve muscle performance in older adults compared with just exercise alone.

3.6. SELECTION OF ORALLY BIOAVAILABLE RAPAMYCIN ANALOGUE

Pure Rapamycin has poor bioavailability, which lead to the development of the rapalogs: Sirolimus and Everolimus. Everolimus is a second generation Sirolimus derivative specifically developed to have improved pharmacokinetic properties including, but not limited to, facilitated oral formulation, higher oral bioavailability and better metabolic stability in comparison to Sirolimus. As these regions of the Sirolimus and Everolimus molecules are structurally similar, it was hypothesized that both molecules would have the same effects on the mTOR pathway; however this is not the case. Like Sirolimus, Everolimus inhibits mTORC1, but at the clinically relevant concentrations tested, Everolimus was much more effective at inhibiting mTORC2 [27]. It is important to note that the lifespan-enhancing effects of mTOR inhibitors have been linked to mTORC1 inhibition, whereas inhibiting mTORC2 might even be detrimental, because mTORC2

controls insulin-mediated suppression of hepatic gluconeogenesis [28]. Therefore for this study, Sirolimus has been selected as the preferred Rapamycin analogue as it does not inhibit mTORC2 to the same extent as Everolimus.

3.7. CURRENT STANDARD OF CARE AND ALTERNATIVE TREATMENTS FOR AGING

Aging is not currently defined as a disease by the FDA, EMA, or WHO. Therefore, no treatments are approved to treat or prevent aging. However, aging-related diseases have now been included in the International Classification of Diseases, describing those as "caused by pathological processes which persistently lead to the loss of an organism's adaptation and progress in older ages".

In addition to Rapamycin, the drug Metformin has also been suggested to reduce aging-related morbidities. Metformin has been used for decades as an antihyperglycemic drug in type 2 Diabetes Mellitus. Its mechanisms are multifaceted as it has multiple sites of action and molecular mechanisms, including inhibition of mTOR, reducing insulin levels, and decreasing IGF-1 signalling. Research has suggested that Metformin may be beneficial against multiple aging-related morbidities, such as obesity, metabolic syndrome, cancer, and cardiovascular disease[29]. However, Metformin indirectly inhibits mTORC1 via the activation of another nutrient sensing enzyme called AMPK, unlike Rapamycin which is a direct inhibitor.

3.8. RATIONALE AND HYPOTHESIS

Given its capability of blocking mTORC1 signalling, Rapamycin could be used to restore the mTORC1 balance and thereby improve exercise performance in older adults. mTORC1 is activated by branch-chain amino acids such as leucine or in response to an anabolic stimulus via exercise. Overactivation of mTORC1 has been observed in aged human muscles, but this overactivation does not induce protein synthesis. Instead, chronic mTORC1 activation in old muscle leads to muscle atrophy mainly due to the inability to induce autophagy, suggesting the importance of mTOR-induced regulation of autophagy in aged muscle [30]. Therefore, intermittent dosing with Rapamycin may restore the mTORC1 balance, whereby there are periods of mTORC1 activation and therefore protein synthesis, but also periods of mTORC1 inhibition with Sirolimus (Rapamycin) leading to autophagy. Plausibly, this approach may lead to an improvement in muscle performance of older adults. Furthermore, with a low and intermittent dosing regime, we aim to inhibit primarily mTORC1, thereby reducing the potential of adverse events.

The current FDA-approved use of Sirolimus (Rapamycin) for, among others, the use as an immunosuppressant in organ transplants and the treatment of cancer is at doses that cause high levels of adverse events. However, as described above, two previous trials of older, but otherwise healthy patients showed that side effects were rare at doses of 0.1 mg/day, 0.5 mg/day, and 5 mg/week [23, 24].

For this reason, we have chosen to focus on a low dose protocol as it has already been shown to be safe in humans. The other key consideration is the relatively long half-life of Sirolimus

(Rapamycin) in humans (64 hours). Therefore, to have periods of mTORC1 inhibition and alternative periods of activation, Sirolimus (Rapamycin) should not be administered more frequently than once a week.

3.9. Outcome Justification

3.9.1. Primary Outcome

To measure the effect exercise and weekly Sirolimus (Rapamycin) dosing will have on the muscle performance of older adults, we have selected the 30-Second Chair-Stand Test (30CST) as our primary outcome.

When measuring muscle performance, it is important to first define muscle strength, muscle power and muscle endurance. Muscle strength refers to "the amount of force a muscle can produce with a single maximal effort". Muscle strength should be differentiated to muscle power which is defined by "the ability to exert a maximal force in as short a time as possible, as in accelerating, jumping and throwing implements" and to muscle endurance which is defined as "the ability of muscles to exert force against resistance over a sustained period of time" [31]. Compared to muscle strength, power concerns work rate (work done per unit time). In healthy older people, muscle power declines earlier and faster compared to muscle mass and strength. Leg power has been shown to be highly correlated with physical performance tests such as gait speed, chair stand test and stair-climb time, and several comparative studies have found that muscle power is a better predictor of mortality compared to muscle strength. Muscle power can be assessed across a range of muscle groups, but most often the leg press and knee extension exercises are used to measure muscle power. The 30CST developed by Rikli and Jones is one of the most important physical performance clinical tests because it measures lower body power, balance and endurance and relates it to the most demanding daily life activities. The 30CST has been widely used in many studies not only to evaluate functional fitness levels but also to monitor training and rehabilitation [31].

The original 30CST paper published in 1999 by Rikli and Jones provided the mean scores by age-group[32]:

Age-group	Mean	Standard Deviation
60-69	14.0	2.4
70-79	12.9	3.0
80-89	11.9	3.6

We also have data on the change in the 30CST after exercise. A study of twenty healthy women aged between 65-79 demonstrated that after 12-weeks of a combined exercise intervention program with extra emphasis on balance and muscle strength found that the 30CST increased by 13.5% (14.8 ± 4 to 16.8 ± 3.4)[33]. A subsequent trial of 29 older adults included both males and females with an average age of 76 demonstrated a 20% improvement in the 30CST after a 12-week training program.[34]

3.9.2. SECONDARY OUTCOMES

It is also important to measure balance, but unfortunately the most commonly used balance tests suffer from ceiling effects in young seniors. A 2019 systematic review of performance-based clinical tests of balance and muscle strength used in young seniors concluded: "Based on the findings in this review, there seems to be only one promising scale for adequately assessing balance in healthy young seniors, i.e. showing no ceiling effects and having measures of high validity and reliability, namely the Community Balance & Mobility (CBM) scale" [35]. For this reason, the CBM will be assessed as a Secondary Objective for balance.

Epigenetic clocks are based on the methylation status of a set of genomic positions and provide an accurate age estimate in humans. Samples can be obtained from various cell types, including white blood cells, cheek cells obtained via a cheek swab, brain, the colon and other organs (hence it is considered a biomarker for the age of almost every part of the body). This sets the method apart from tests that rely on biomarkers of age that work in only one or two tissues, including the gold-standard dating procedure, aspartic acid racemization, which analyses proteins that are locked away for a lifetime in tooth or bone. In human DNA, methyl groups most often attach at CpG sites, where a cytosine precedes a guanine in the DNA. This process is catalyzed by at least three DNA methyltransferase (DNMTs). A typical human genome contains more than 28 million such sites. For this study, a genome-wide assessment of DNA methylation will be performed by TruDiagnostics via blood and saliva samples.

Given that we are using Sirolimus (Rapamycin) to influence mTORC1, it is important to measure the levels of mTORC1 activity. The measurement of the phosphorylation status of p70 S6 kinase (S6K) offers a robust way to measure mTORC1 activity from a blood sample[36]. TruDiagnostics also has the ability to measure S6K phosphorylation levels, and they will conduct this measurement in addition to the DNA methylation measurements as outlined above

3.10. PHASE 2A FEASIBILITY TRIAL & SUBSEQUENT PHASE 3 TRIAL

As described in section 3.7, the hypothesis of improved muscle performance in older adults relies on periods of mTORC1 activation via exercise, alternating with periods of inactivation via Sirolimus (Rapamycin). Before exploring this idea with a much larger, phase 3 superiority trial, there are several feasibility issues that should be addressed with an initial Phase 2a trial:

- Safety and tolerability of weekly 6mg Sirolimus (Rapamycin) dosing, particularly that muscle performance is not worsened by the intervention
- Recruitment rate
- Adherence to thrice-weekly exercise program
- Preliminary efficacy data, particularly the improvement and standard deviation of the 30-Second Chair Stand Test over a 13-week exercise program. This preliminary data would allow for an accurate power calculation for the Phase 3 Superiority Trial.
- Assessment procedures

Once the above feasibility issues have been thoroughly tested in a Phase 2a trial, we can move to a much larger, Phase 3 Superiority Trial. The Phase 3 trial would be a 1:1 randomised, placebo-

controlled trial, also of weekly 6mg Sirolimus (Rapamycin) dosing and a thrice-weekly exercise program. The numbers needed in the Phase 3 trial would likely necessitate a multi-centre trial.

4. Study Objectives

4.1. Primary Objective:

Assess the safety, tolerability, feasibility, and trial design of weekly Sirolimus (Rapamycin) 6mg or placebo dosing over a 13-week period, in combination with thrice-weekly group exercise programs.

4.2. Secondary objective:

Assess the improvement in the 30-Second Chair-Stand Test after a 13-week exercise training program. This will enable an appropriate power calculation to be conducted to inform the number of patients required for a superiority clinical trial.

5. Experimental Plan

5.1. Study Design

This study is a test of an FDA-approved drug, Sirolimus (Rapamycin), being repurposed for the treatment of muscle aging in combination with exercise. The first phase is a proof-of-concept trial, designed to assess the safety, tolerability, feasibility, and trial design of weekly Sirolimus (Rapamycin) 6mg or placebo dosing over a 13-week period, in combination with thrice-weekly group exercise programs. The trial will also assess improvement in the 30-Second Chair-Stand test after a 13-week exercise training program. This will enable an appropriate power calculation to be conducted to inform the number of patients required for a subsequent, superiority clinical trial.

Participants can be within 65-85 years of age at the time of enrolment, with either no co-morbidities or a well-controlled chronic condition, who do not already perform weekly, strenuous exercise. Participants will provide informed consent and undergo initial screening. If eligible, patients will be randomised in a 1:1 ratio to either the once-weekly placebo or Rapamycin group. In parallel to the oral medication, patients will engage three times a week in an at-home exercycle fitness program. The exercycles will be hired and delivered to the participants' homes. Fitness-dependent exercise standardisation is important to minimise variance, particularly given that the primary outcome is a measure of exercise performance and can therefore be greatly influenced by the level of exercise training. To that end, here is the proposed exercycle training program:

Week	Warm-up	Training	Cooldown
1	2 min @ 50RPM	3 min @70-80RPM, very low resistance	2 min @ 45RPM
2	2 min @ 50RPM	4 min @70-80RPM, very low resistance	2 min @ 45RPM
3	2 min @ 50RPM	5 min @70-80RPM, very low resistance	2 min @ 45RPM
4	2 min @ 50RPM	6 min @70-80RPM, very low resistance	2 min @ 45RPM
5	2 min @ 50RPM	7 min @70-80RPM, low resistance	2 min @ 45RPM
6	2 min @ 50RPM	8 min @70-80RPM, low resistance	2 min @ 45RPM
7	2 min @ 50RPM	9 min @70-80RPM, low resistance	2 min @ 45RPM
8	2 min @ 50RPM	10 min @70-80RPM, low resistance	2 min @ 45RPM
9	2 min @ 50RPM	11 min @70-80RPM, medium resistance	2 min @ 45RPM
10	2 min @ 50RPM	12 min @70-80RPM, medium resistance	2 min @ 45RPM
11	2 min @ 50RPM	13 min @70-80RPM, medium resistance	2 min @ 45RPM
12	2 min @ 50RPM	14 min @70-80RPM, medium resistance	2 min @ 45RPM
13	2 min @ 50RPM	15 min @70-80RPM, medium resistance	2 min @ 45RPM

All warm-ups and cooldown phases should be completed with a very low resistance setting.
If participants are unable to complete the exercycle training program due to difficulty, the program can be adjusted whereby the resistance setting is lowered. If a participant still cannot complete the training program, then they should aim to ride for as long as they can before moving to the cooldown phase.
Patients will record their exercise program in an exercise diary, and it will include the length of training, the RPM, and the resistance setting.

The weekly schedule is as follows:

Day of the week	**Action**
Monday	Exercycle training program
Tuesday	Rest
Wednesday	Exercycle training program
Thursday	Rest
Friday	Exercycle training program
Saturday	Placebo/Sirolimus in the morning
Sunday	Rest

To measure adherence, the Sirolimus (Rapamycin) will be blister-packed and the packs collected at the 6-week and 13-week interval.

5.2. SUBJECT SELECTION

5.2.1. NUMBER OF SUBJECTS

40 subjects, divided in a 1:1 ratio between the placebo and Rapamycin groups

5.2.2. INCLUSION CRITERIA

- Age 65-85 years
- Any sex
- Any ethnicity
- Interest in taking Sirolimus (Rapamycin) off-label
- Willing to undergo blood and urine tests, fitness tests, and complete thrice weekly exercycle training program.
- Relatively good health with only well-managed chronic diseases (hypertension, coronary artery disease, etc.), clinically stable
- Adequate cognitive function to be able to give informed consent

5.2.3. EXCLUSION CRITERIA

- Already participating in strenuous activity enough to cause a noticeable increase in breathing more than twice a week
- Anaemia - Hg < 9.0 g/dl, Leukopenia - white blood cells (WBC) < 3,500/mm3 , Neutropenia - absolute neutrophil count < 2,000/mm3 , or Platelet count - platelet count < 100,000/mm3
- Older adults scheduled to undergo major surgery in the next 12 months
- Active malignancy
- Patients with impaired wound healing or history of a chronic open wound
- Impaired hepatic function, measured by alkaline Phosphatase (ALP), alanine aminotransferase (ALT), Albumin, or T. Bili, whereby the levels are 1.5x greater than the normal upper limit.

- HIV/AIDS, chronic Lyme, Babesia, Ehrlichiosis, Anaplasmosis, or other chronic infections that require ongoing treatment or monitoring
- Allergy to Rapamycin
- Any form of clinically relevant primary or secondary immune dysfunction or deficiency (e.g. X-linked agammaglobulinemia (XLA), common variable immunodeficiency (CVID))
- Chronic oral corticosteroid or immunosuppressive medication use (e.g. Enbrel, Humira, methotrexate).
- Fibromyalgia or Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, Breast Implant Illness
- Congestive heart failure: self-assessed functional status of heart failure New York Heart Association (NYHA) classification III or IV
- COPD Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification III or IV
- Impaired renal function, as defined as glomerular filtration rate (GFR) < 30
- Diabetes Type 1 or 2 (pre-diabetes is ok)
- Substance abuse disorder either untreated or if treated within the last 5 years
- PTSD, Bipolar disorder, Schizophrenia, or any other untreated or poorly controlled mental health or mood disorder, or history of hospitalization due to mental health condition
- Those who have taken metformin, Rapamycin, or rapalogs in the past 6 months

5.2.4. Withdrawal Of Participants From The Study

Participants are free to withdraw from the study at any time. They can do so by notifying the Principal Investigator by email or phone call. Participants will provide consent as to whether data obtained up to that moment can continue to be used.

5.3. Schedule of Assessments and Procedures

5.3.1. Study Recruitment

Recruitment shall be the responsibility of the lead clinical centre. Participants will be identified from the research centre database, referrals from Primary Care, and advertising through local newspapers and social media. The study co-Principle Investigator, Dr Brad Stanfield, can also promote the study on his YouTube channel and other social media platforms.

5.3.2. Screening and Consent

A video explaining the trial protocol will be publicly available on YouTube as part of the advertising campaign. Potential participants that are interested in the trial can then contact Middlemore Clinical Trials directly, where they can organise a face-to-face appointment for further explanation of the trial and complete their consent form. Once the participant has given their consent, they can continue their enrolment application which will include a series of questionnaires, including past medical history, allergies, previous injuries, medications, current exercise training levels, and then specific questions related to the inclusion/exclusion criteria. If at this point it appears that the participant would be a good candidate, then the baseline assessments as described in section 5.3.3 will be performed, and a second face-to-face discussion will be arranged with the Principal Investigator. At the second visit the Principal Investigator,

after reviewing the participants' data and answering any remaining questions, will determine the participants' eligibility and prescribe the drug. Muscle performance measurements will then be taken at that second clinic visit.

5.3.3. First Clinic Visit: Baseline Testing

Once consent is provided, the participant will undergo the following tests:

1. **Medical history:** Past medical history, allergies, medications, will be reviewed and confirmed.
2. **Physical examination:** Resting heart rate, blood pressure, and oxygen saturations. In order to screen for undetected cardiac abnormalities that may hinder the patient's ability to complete the exercise program, the heart will be auscultated to screen for any heart murmurs, and an ECG shall be performed.
3. **Body composition testing:** A DEXA scan will assess lean muscle mass
4. **Blood tests:** Full Blood Count, Creatinine, Sodium, Potassium, Creatinine Kinase, Liver Function Tests, Lipids, HbA1c, IGF-1, hs-CRP, Fasting Blood Glucose, and Insulin Levels, S6k phosphorylation, TNFa, Interleukins 1, 6, 8 (IL-1, IL-6, IL-8), Nicotinamide adenosine dinucleotide (NAD); β-galactosidase (β-gal); Reactive oxygen metabolites (ROM); Total antioxidant capacity (TAC)
5. **DNA Methylation Age:** Blood and saliva samples will be sent to TruDiagnostics (based in USA) to perform the analysis.

The exercycle bike will be delivered to the participants' house, and participants will be instructed not to use the bike until the trial has begun.

Before the participant leaves, they will be taught how to do the 30-Second Chair-Stand Test to reduce the learning effect. They will also be provided with a video of the 30s Chair-Stand Test, and encouraged to practice 3 sets of 3 repetitions at home.

5.3.4. Second Clinic Visit: Muscle Performance Measurements

One week after completing the baseline testing, the participant will return to the clinic, their eligibility for the trial confirmed, vital signs taken, and then their baseline muscle performance measured.

To warm up for the physical tests, the participant should complete this 5-minute program as outlined in this video: https://youtu.be/m2Bni2lcrWw .

The examiner will demonstrate the 30s Chair-Stand Test (https://www.physio-pedia.com/30_Seconds_Sit_To_Stand_Test), ensure correct patient technique, and then the test will be performed.

After a 5 minute break, hand grip strength will be measured in the dominant hand via a dynamometer.

After a 3 minute break, the 6-minute walk test will be performed (https://www.physio-pedia.com/Six_Minute_Walk_Test_/_6_Minute_Walk_Test), followed by a 10 minute break.

Finally, the Community Balance & Mobility Scale test will be performed (https://www.physio-pedia.com/Community_Balance_and_Mobility_Scale).

At this point, participants will be randomised to either the placebo or Sirolimus (Rapamycin) group (see 6.3) and the drug (blinded to the patient and the trial co-ordinator) will be given to the participant in a 6 week blister pack. A 1-page instruction handout outlining the weekly medication dosing and thrice weekly exercycle program will also be given to the participants. Finally, participants will be given an exercise diary that they need to complete after each workout.

5.3.5. Third Clinic Visit: 6 weeks

For the next six weeks, the patients will follow the outlined program. Once the 6 weeks are completed, the participants will return to the clinic for another round of physical tests using the same protocol as outlined for the baseline visit, including measurement of vital signs and ECG.

To measure adherence to the trial protocol, we will collect the used medication blister pack and exercise diary. A 7-week medication blister pack as well as another exercise diary will then be issued.

5.3.6. Fourth & Final Clinic Visit: 13 Weeks

Participants will continue their thrice weekly training program and weekly medication dosing, then report to the clinic for a final round of examinations. This will involve collecting the used medication blister-pack and exercise diary, checking vital signs, ECG, blood tests, DEXA scan, then the full battery of physical tests as outlined above.

5.4. Feasibility Endpoints

- Participant recruitment rate from a single centre.
- Completion of a 13-week exercise program for adults who do not already engage in frequent exercise.
- Outcome variance estimation by arm.
- Adherence to once-weekly medication.
- Safe side effect profile to Sirolimus (Rapamycin) in combination with exercise.
- Reliable measurement of muscle performance

5.5. Primary & Secondary Efficacy Endpoints

5.5.1. Overview

Primary endpoint: 30-second (30-s) Chair Stand Test

Secondary endpoints:

- Muscle strength
    - Handgrip strength
- Muscle Endurance
    - 6-minute walk test
- Community Balance & Mobility (CBM)
- Lean muscle mass as measured by DEXA scan
- Homeostatic Model Assessment for Insulin Resistance
- Full Blood Count, U&Es, LFTs, HbA1c, lipids, serum IGF-1 to ensure safety
- DNA methylation clocks
- S6k phosphorylation

6. INVESTIGATIONAL PRODUCT

6.1. INVESTIGATIONAL DRUG

Rapamycin (also known as Sirolimus and Rapamune) is an antibiotic produced by the bacterium *Streptomyces hygroscopicus*. It is a macrolide consisting of a 29-membered ring containing 4 trans double bonds, three of which are conjugated, with molecular formula $C_{51}H_{79}NO_{13}$ and a molecular weight of 914.2 g/mol.

Rapamycin is FDA-approved for use as an immunosuppressant to prevent organ transplant rejection, and in drug-eluting coronary stents to inhibit vascular smooth muscle cell proliferation, thereby preventing restenosis in coronary artery lesions. Additionally, based on its antiproliferative effects, Rapamycin is used in the treatment of various cancer types and the rare lung disease lymphangioleiomyomatosis [2].

Sirolimus is manufactured by Pfizer, who will provide both the Sirolimus. CompoundLabs (New Zealand) will provide the matching placebo.

6.2. CONTROL DRUG

The placebo drug capsules will appear identical to the Rapamycin capsules. The patient, the administering physician and the patient evaluator will be blinded as to whether the patient will be getting the placebo or the Rapamycin drug. The placebo will be given with the exact same dose frequency as the Rapamycin drug.

6.3. RANDOMIZATION TO TREATMENT

Randomisation will be carried out using block randomisation. The algorithm producing the schedule will be coded using an R script by the trial statistician, who will keep the blocking scheme secret. The code will be seeded with a randomly generated number and run by a third party. The resulting schedule will consist of a password-protected Excel spreadsheet containing participant study identifiers and corresponding allocation arms. The third party will transmit this schedule to the pharmacy service, which will prepare the placebo and Sirolimus blister packs

accordingly and identify them with study identifiers. These identifiers will be assigned to participants sequentially in order of recruitment. Concealment under block randomisation will be upheld by the double-blind nature of the study and by the block scheme secrecy.

6.4. Dosing and Administration

Participants will receive 6 mg one day per week, taken as a prepared capsule. Discontinuation of the drug can be made based on adverse events in a participant, which will be discussed between the participant and primary investigator.

6.5. Blinding

The study participants and research coordinators will be blinded to the treatment groups. A third party not otherwise involved in the study will perform the randomization and code the blister packs of the drugs. They will allocate the compounded capsules or placebo capsules into the bottles with unique codes.

6.6. Storage

Capsules should be stored at 20°C to 25°C. Capsules should be protected from light, and are dispensed in a blister-pack.

6.7. Concomitant Medications

If a participant fits the eligibility criteria but are on other medications they should continue to take those at the prescribed dosages. The research team will inform the participant's GP of their participation in the trial. Any changes to concomitant medications or any planned surgery should be discussed with the PI immediately.

7. Adverse Event Reporting

7.1. Adverse Events Definitions

Adverse drug reaction (ADR): a noxious and unintended reaction to a drug at doses normally used in humans for prophylaxis, diagnosis, therapy of diseases, or for the modification of physiological function. A causal relationship is at least reasonably possible.

Adverse event (AE): an adverse occurrence experienced by a study subject during the clinical trial that is not necessarily associated with the drug. An AE can, therefore, be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of the investigational product, whether related to the investigational product. When an AE has been determined to be related to the investigational product, it is considered an adverse drug reaction.

7.2. SERIOUS ADVERSE EVENTS

Serious adverse events (SAE): any AE that results in death, is life-threatening, requires inpatient hospitalization, is persistent, or causes significant disability/incapacity.

Adverse events will be graded according to the system below:

- Grade 1: Asymptomatic or mild symptoms; clinical or diagnostic observations only; no intervention indicated (e.g. headache, nausea, abdominal discomfort)
- Grade 2: Moderate; minimal, local or non-invasive intervention (e.g. vomiting, diarrhoea, shortness of breath)
- Grade 3: Severe; or medically significant but not immediately life threatening; hospitalization or prolongation of hospitalization indicated; disabling (e.g. dehydration, hypotension)
- Grade 4: Life-threatening ; urgent intervention indicated (e.g. respiratory failure, myocardial infarction, liver failure)
- Grade 5: Death related to an AE

7.3. REPORTING OF ADVERSE EVENTS

7.4. ROUTINE

The principal investigator will be notified about any adverse events. The Principal Investigator will document this information in the participants' medical record. Participants will be asked to report any serious adverse event immediately to the Principal Investigator; this can be done by email or phone (after hours phone number available).

AEs will be documented using standard AE reporting (FDA regulations 21CFR314.80 and 21CFR213.32(s)). Both expected (already known) and unexpected AEs will be reported.

7.5. EXPEDITED

Serious adverse events that occur while the participant is actively participating in the research study will be reported to the Ethics Committee.

8. STATISTICAL AND OTHER ANALYSES

8.1. EFFICACY ANALYSES

As some of the feasibility issues are related to the efficacy analyses, they are described first. The efficacy analysis plan is intended to match the analysis plan of the full trial closely.

The intention-to-treat analysis set will be the analysis set for the primary analysis. It is defined as the set of all randomised patients with their original randomised allocation.

The per-protocol analysis set will be the analysis set for a sensitivity analysis. It is defined as the set of all patients who will have reported to have undertaken at least 2/3 of the exercise sessions and, if in the Sirolimus arm, to have taken at least half of the protocol-stipulated doses.

The general inferential analysis plan consists in regressing outcomes at 6 and 13 weeks on the indicator of the intervention group in interaction with the assessment time point, adjusting for the baseline value of the outcome. (Other covariates may be identified during the blind review and used for adjustment, after inclusion in the Statistical Analysis Plan.) In this manner, the baseline-adjusted effect of the intervention on the change from baseline is being estimated. The regression approach will take place using appropriate generalised linear mixed models (GLMM), selected during a blind review of the data, absent all knowledge about allocation. The patient ID will be entered as a random intercept to account for the correlation between measurements at 6 and 13 weeks.

The final analyses will be carried out using the selected GLMMs. Parametric assumptions will be allayed using the wild bootstrap [37, 38]to estimate standard errors. In a larger trial, standard sandwich estimators of the variance would be used in lieu of the bootstrap.

Missing data will be dealt with by multiple imputations to 15 completed copies of the data. The nominal significance level for this Phase 2a study will be 0.1; however, in line with current thinking regarding preliminary studies, the results will deemphasise testing and emphasise point and interval estimation, producing confidence intervals for active arm effect having between 75% and 95% confidence levels in increments of 5 percentage points[39]. Sensitivity analyses will be carried out in the per-protocol analysis set and in the complete-data-only set (excluding cases variable-wise).

8.2. SAFETY ANALYSES

The safety analysis set consists of all randomised patients. Adverse events will be allocated on an as-treated basis: events occurring before a participant ceases treatment or completes the trial will be assigned to the randomised arm; events occurring after cessation to the placebo arm.

Counts of adverse events will be regressed on the allocation arm as described above in interaction with the graded severity using negative binomial regression. Dichotomised relatedness to the intervention (Possible, Probable, Definite relatedness to the intervention) will be regressed on the allocation arm using a quasi-Binomial framework with logarithmic link (relative risk regression) or a relative risk working model (e.g. Poisson with bootstrap estimation of the variance) to test for non-inferiority of the Sirolimus arm. Standard errors will be estimated using the boostrap, resampling participants. The modalities of non-inferiority will be detailed in the final analysis plan.

8.3. FEASIBILITY ANALYSES

The overall and weekly screening and randomisation rates will be reported (as number of events per week) with Poisson-based 95% confidence intervals.

Rates of drug treatment cessation, exercise programme cessation and study withdrawal will be reported and compared across randomisation arms using a log-rank test.

The residual variance of each outcome will be estimated and used to inform the sample size and analytical design of the Phase 3 trial.

Adherence to the medication will be reported by arm at 6 and 13 weeks using mean percentage of supply used, and compared between arms using Beta regression with bootstrap-based standard errors and p-values[40]. The causal effect of the medication on the primary outcome will be estimated using 2-stage residual inclusion estimation, with the randomised allocation as the instrumental variable[41].

The rate of adverse events will be compared between arms using quasi-Poisson regression with the log of the follow-up time as offset.

Reliability of the muscle performance measurements will be assessed by considering the standardised muscle-performance-related outcomes (30CSTest, handgrip strength and 6MWT) at each visit as a single battery of test, and assessing their factor loadings in a repeated congeneric model using confirmatory factor analysis[42]. The communalities will be reported at each visit, as well as the result of a test restricting all loadings to be equal.

8.4. SAMPLE SIZE JUSTIFICATION

The sample size was selected primarily to satisfy logistic constraints of this feasibility study. As this study is not confirmatory, we do not predicate the sample size primarily on power considerations: arm sizes of 20, assuming no withdrawals, will provide 80% and 90% power, respectively, to detect fairly large Cohen's effect sizes of 0.8 and 0.9, respectively, at a nominal significance level of 0.1.
In regard to safety, the arm sizes and post-randomisation follow-up time will enable to detect rate ratios of adverse events between the Sirolimus and placebo arms varying between 2.4 and 3.7, dependent upon the rate in the placebo arm and the dispersion of the data.
Sample sizes of 12 per arm have been indicated as sufficient for pilot or feasibility studies[39]. However, our feasibility objective of variance estimation makes it preferable to aim for a total sample size of 40, as a 95% confidence interval for the variance will then have expected half-width of less than half the true variance, providing a reasonably precise estimate.

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