Research Proposal
Development of Diagnostic and Therapeutic Approaches to Trimethylaminuria

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Background:
Clinical and laboratory diagnosis of trimethylaminuria:
The consequences of trimethylaminuria (TMAU) were recognized by Shakespeare (The Tempest, Act 2. Scene 2), and as elegantly stated in Trinculo's monologue, once the diagnosis has been made it is like a bolt from the blue for affected individuals and their families.

Excess dietary choline is metabolised by anaerobic micro-organisms in the large intestine to trimethylamine, which in turn is converted to odourless trimethylamine N-oxide by the last step in the choline degradative pathway, flavin mono-oxygenase 3 (FMO3)(1). Primary or secondary accumulation of trimethylamine has no deleterious physical effect, but can cause devastating social debilitation, because trimethylamine when eliminated in urine, sweat or breath, saliva and other body fluids has a very distinctive odour of decaying fish. The odour becomes more prominent during periods of stress, with fever and with strenuous exercise as a consequence of increased sweating(1). In addition, dietary intake of marine fish exacerbates symptoms since these animals contain large amounts of trimethylamine-N-oxide (which is believed to have antifreeze properties), which can be converted back to trimethylamine by gut bacteria(2).

Primary TMAU is most often caused by a functional defect of FMO3(3), and the genetic disorder is inherited in an autosomal recessive manner as a consequence of mutations in the FMO3 gene. At least 30 different mutations have been reported within the 9 coding exons of the FMO3 gene, which is located on the short arm of chromosome 1(4-6), and of those about a quarter are nonsense mutations(7), although the proportion of patients with nonsense mutations is unknown. The incidence of TMAU due to FMO3 deficiency is not precisely known, but it has been suggested that it may range between 1 in 100 and 1 in 1000(5). What is certain is many people remain undiagnosed for unacceptably long periods of time(8).

Secondary TMAU has been described in patients with severe liver disease (which is the major site of activity of the FMO3 enzyme)(9), chronic renal disease (as a consequence of bacterial overgrowth in the gut)(10), and in patients treated with large doses of betaine for disorders of cobalamin or homocysteine metabolism or possibly L-carnitine for organic acidopathies and fatty acid oxidation disorders(1). In addition, transient TMAU has been reported in a preterm infant who was fed with
choline-rich food supplements, such as egg yolk. Soy and liver(11), and has been reported in some women just at the onset of menstruation(9).

The key to establishing the diagnosis is suspecting it in the first place. TMAU sufferers have endured their disorder for years or even decades, often subject to ridicule by their peers and doubted by their health care professions, before the diagnosis has finally been established. Quantitation of trimethylamine and trimethylamine-N-oxide in a random urine sample will confirm clinical suspicions, however it should be remembered that excessive trimethylamine excretion may be intermittent, so a normal single result does not rule out the disorder(9). The diagnosis can be more firmly established by conducting a choline or marine fish load test(1), or by FMO3 mutational analysis.

**Current approaches to the management of trimethylaminuria:**
The optimum management of TMAU usually needs to include a combination of approaches(1, 9, 12) including:

- dietary restriction of choline-containing foods (including egg yolk, liver and other organ meats, legumes, and products containing lecithin [322] and choline [1001], which are put into processed foods as emulsifiers) and marine fish (including cephalopods like octopus and squid and crustaceans like lobster, crab, prawns and balmain bugs)
- low pH (5.5 – 6.5) soaps (eg goat’s milk soap), deodorants and body lotions (eg Lactcyd™)
- copper-chlorophyll or activated charcoal, which are not absorbed across the gut, and which can irreversibly bind to trimethylamine in the gut thereby limiting its systemic absorption
- probiotics to change the balance of gut flora
- intermittent oral antibiotics to reduce the gut bacterial load

These treatments, however, are not perfect, and can be difficult to maintain consistently. No new approaches to the treatment of trimethylaminuria have been developed in recent decades. An important component of the development of new therapies is to have appropriate cell biological and animal models of the disorder, so that efficacy and safety of proposed new treatments can be tested.

**New strategies for the treatment of trimethylaminuria:**
Read through of premature termination mutations:
Premature termination or nonsense mutations arise as a result of a single nucleotide change in a gene where the change leads to the conversion of an amino acid in the protein sequence to a premature stop codon. Such mutations often result in the protein losing most if not all of its functional capacity. It was recognised a number of years ago that aminoglycoside antibiotics can force the transcriptional machinery to read through the premature stop mutations, and allow the normal protein to be made, restoring activity of the protein(13). However, aminoglycoside antibiotics have significant side effects and are not a viable therapeutic option. More recently a new class of drugs has been developed that has the capacity to promote read through of premature termination mutations, and which appear to be totally non-toxic(14). One in particular, PTC124, has been shown to result in the production of normal dystrophin in the mdx mouse model of Duchenne muscular dystrophy(14), and has been used in clinical trials in human subjects with cystic fibrosis, with clear benefits being found(15). In addition, there are a number of other read-through agents currently being evaluated for potential in vitro and in vivo use. An inborn error of metabolism like TMAU would be an excellent candidate for this type of therapy, as an increase of enzyme activity to perhaps as little as 10% of normal should be enough to overcome the biochemical block.

*We propose to use an in vitro (cell culture model) approach to determine whether PTC124 and other read-through agents are of potential therapeutic value in this proportion of TMAU patients.* If we
demonstrate potential in vitro efficacy, we will then go on to study the efficacy of read-through agents in the mouse model. This mouse model will have a premature termination mutation of FMO3 deficiency for testing of new therapies for trimethylaminuria.

Other strategies for metabolising TMA in the small intestine:
Anaerobic gut bacteria can contribute to the trimethylamine load in patients with TMAU by enhancing the metabolism of choline in food to trimethylamine in the gut(1). As stated above, one form of therapy of TMAU, albeit in more extreme cases, is to treat patients with antibiotics aiming to reduce the intestinal load of these bacteria. However, the antibiotics that need to be used have potentially serious side effects, and so can only be used for short periods of time.

An alternative strategy for reducing the gut trimethylamine load would be to colonise the gut with harmless bacteria that are capable of metabolising trimethylamine. One such micro-organism is Methylophilus methylotrophus. This is an aerobic monoflagellate bacterium that uses methanol as the sole source of carbon and energy(16). It was initially thought to be of potential commercial value in the single-cell protein production industry, but it proved to be a nonfinancial venture. When cultured in trimethylamine, the enzyme trimethylamine dehydrogenase is induced, which converts trimethylamine to dimethylamine and formaldehyde(17). Extensive studies have shown that this micro-organism is non-pathogenic and non-toxic in animals(18, 19). Therefore colonisation of the gut with Methylophilus methylotrophus in individuals with TMAU could be of potential therapeutic utility.

We propose to study the potential therapeutic benefit of Methylophilus methylotrophus in our mouse model of TMAU.

Research Plan:
Aims:
1. To develop an in vitro system for testing whether PTC124 and other read-through agents can correct the functional consequences of premature termination mutations of the FMO3 gene.

Using standard cloning techniques that are well established in our laboratories, we will generate a human FMO3 expression vector, and then use site-directed mutagenesis to generate all of the reported FMO3 nonsense mutations. We will then express them in a mammalian cell system (such as COS or HEK293 cells). In addition, we will use CRISPR technology(20) to generate FMO3 nonsense mutations in a suitable FMO3-expressing hepatic cell line. We will develop a functional assay of the FMO3 enzyme, using previously reported spectrophotometric methods(21), and then confirm that the mutations cause non- or dysfunctional FMO3 enzyme. CI’s Christodoulou and Carpenter have extensive experience in the use of spectrophotometric enzyme assays, have the necessary equipment to be able to establish this specific assay, and do not anticipate any major hurdles in establishing this method.

We will also perform western analysis of the wildtype and mutant proteins using commercially available antibodies (both Abcam and Abnova have an antibody against the human FMO3 protein which has been successfully used for western analyses) to identify those mutations which result in a stable but truncated protein and those mutations which result in the production of an unstable protein. Western analysis is a standard technique, and is very well established in the Christodoulou and Tam laboratories.

Having done these initial functional studies, we will then expose cells to varying concentrations of PTC124 and other read-through agents, and assay for improvement in functional activity, and perform westerns to determine whether full length protein is now being made.
2. To develop a mouse model with a premature termination mutation of FMO3 deficiency, and to study the biochemical and phenotypic consequence of this mutation in the mouse.

The outcome of the cell culture study in Aim 3 will inform us of the most specific nonsense mutation that will cause a premature termination of transcription and can respond to the read-through activity mediated by read-through agents to restore the normal protein function. We will create a similar mutation in the mouse genome by inserting the specific single-nucleotide change into the Fmo3 gene. This will be achieved by gene targeting techniques on mouse embryonic stem cells. The engineered cells will be used to generate live mice that carry the specific mutation. The genetically modified mice will be assessed for the levels of trimethylamine and trimethylamine-N-oxide, using a mass spectrometric technique (21) that we will develop in-house (CI Carpenter is the head of the NSW Biochemical Genetics Service, and is an international expert in the development of mass spectrometric methods to analyse metabolites) to ascertain that they display the biochemical features of trimethylaminuria.

3. To test the in vivo efficacy of read-through agents in our mouse model of FMO3 deficiency.

Having developed a mouse model with a nonsense mutation of the Fmo3 gene and demonstrating that recapitulates the human TMAU disorder, we will be in an excellent position to explore the in vivo efficacy of various read-through agents.

We will quantitate trimethylamine and trimethylamine-N-oxide levels in urine samples from wildtype and mutant mice fed on normal chow, and if necessary a chow rich in choline. We will also carefully monitor the health and behaviour of the mice, although we do not expect to find any physical or behavioural abnormalities in the mutant mice. The mice will then be euthanised, livers harvested, and we will go on to then evaluate FMO3 enzyme activity in the livers of wildtype and mutant mice, the organ which primarily expresses FMO3 (22), and quantitate FMO3 the level of and the size of the wildtype and mutant FMO3 protein extracted from liver samples.

We will then administer the read-through agent of choice to wild-type and mutant mice at varying doses and time intervals. During this time we will monitor the health of the mice, and collect urine samples for quantitation of trimethylamine and trimethylamine-N-oxide. We will then euthanise the mice, collect their livers, and assay for FMO3 activity and examine the FMO3 protein by western to test whether mutant mice are now able to generate a full length functional FMO3 protein.

4. To test the therapeutic efficacy of Methylophilus methylotrophus as a therapeutic adjunct in our mouse model of FMO3 deficiency.

An aliquot of the Methylophilus methylotrophus micro-organism will be sourced and culture stocks will be established. We will apply the same methodology as in aim 5 to examine the potential therapeutic effects of intragastrically delivered Methylophilus methylotrophus, again given at varying doses and intervals. We have experience with intragastric administration of microorganisms in mouse models through other work where we are developing a new therapeutic of another inborn error of metabolism, phenylketonuria.

Conclusions:
As a result of the research program outlined in this proposal we will have developed the unique resource of a mouse model for TMAU, which will be of great value in assessing new therapeutic approaches to the disorder. We will also have demonstrated whether PTC124 and other read-through agents are able to correct functional defects of FMO3 for subset of mutations, and will have shown whether it is also of in vivo efficacy in our mouse model. Finally, we will have examined whether the micro-organism Methylophilus methylotrophus is of potential therapeutic value in our mouse model for
TMAU. We believe that these outcomes will represent a major advance on the current state of play with regards to the diagnosis and treatment of patients with TMAU worldwide.

**Time-lines for the project** (at half-yearly milestones):

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<th>Study component</th>
<th>Year 1</th>
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<tr>
<td>Generation of the mouse model for FMO3 deficiency, &amp; biochemical and phenotypic characterisation</td>
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<tr>
<td>Generation of a range of <em>FMO3</em> nonsense mutations, &amp; their functional analysis in a cell culture system</td>
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<td>Evaluation of the efficacy of read-through agents in our <em>in vitro</em> cell culture system</td>
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<td>Evaluation of the efficacy of read-through agents in our mouse model system</td>
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<td>Evaluation of the therapeutic utility of <em>Methylophilus methylotrophus</em> in our mouse model system</td>
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**References:**


Proposed Budget:

Staffing:

Given to wide range of techniques, their complexity and the volume of work that will need to be undertaken, funding for one postdoctoral research scientist is required. It is expected that this individual will have at least five years postdoctoral research experience, and will be adept in general molecular and cloning techniques, mouse experimental work, bacterial and mammalian cell culture work, and the various biochemical and protein based studies that will need to be undertaken.

This individual will need the assistance of a PhD student, who we would plan to recruit into the project

Research scientist will be employed at Research Officer HSM1 level:
Annual salary (including base salary, and all on costs) $95,468

A full time PhD student will be recruited to this project:
Annual stipend $28,000

[$123,468 per year]

Molecular Biological Reagents:
dNTPs, Taq polymerase, restriction enzymes, ligases, kinases, agarose, "clean-up" kits, antibodies for westerns, plasmid miniprep kits, MW markers, oligos for sequencing and PCR [$12,000 per yr]
General Cell Culture Reagents:
Plasticware and tissue culture reagents: for culturing mammalian cell lines and their manipulation, including DMEM/F12 media, PBS, FBS, etc. [$7,500 per year]

General Laboratory Reagents:
Buffers, solvents, salts, acrylamide, microfuge tubes, pipette tips, foil, cleaning supplies, gloves, syringes, parafilm, reagents for FMO3 enzyme assays, etc.  [$5,500 per yr]

Mouse Agistment Costs:
1 box (houses 10 mice) = $7.00/week
1 box (houses mating pair or pregnant mice) = $7.00/week
15 boxes (to house neonatal, juvenile and adult mice) for 20 weeks - 15 x $7.00/week x 20 = $2100
6 boxes (to house female mice in preparation for breeding) for 52 weeks - 6 x $7.00 x 52 = $2184
10 boxes (to house a breeding pair then the pregnant mouse) for 52 weeks - 10 x $7.00 x 52 = $3640
Total holding cost $7924
Animal health monitoring costs $1200/yr
[Total Animal Cost = holding + health monitoring = $ 9124/yr]

Annual budget requested:  $157,592AUD

Total budget requested for the life of this 3-year research proposal:  $472,776