

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 sweating¹. 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 bacteria².

Primary TMAU is most often caused by a functional defect of FMO3³, 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 1⁴⁻⁶, and of those about a quarter are nonsense mutations⁷, 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 1000⁵. What is certain is many people remain undiagnosed for unacceptably long periods of time⁸.

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 disorders¹. 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 liver¹¹, and has been reported in some women just at the onset of menstruation⁹.

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 disorder⁹. The diagnosis can be more firmly established by conducting a

choline or marine fish load test¹, or by *FMO3* mutational analysis. Currently, there is only one laboratory in Australia offering biochemical testing for trimethylaminuria, but we believe this is suboptimal because this laboratory does not routinely quantitate trimethylamine-N-oxidase, potentially missing cases of the disorder. Best practice guidelines suggest that both trimethylamine and trimethylamine-N-oxidase should be measured¹. Similarly, mutation screening of the *FMO3* gene is not routinely available in Australia.

We propose to develop a comprehensive national service for the accurate biochemical and molecular screening for trimethylaminuria.

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 protein¹³. 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 (Welch ref). One in particular, PTC124, has been shown to result in the production of normal dystrophin in the mdx mouse model of Duchenne muscular dystrophy¹⁴, and has been used in clinical trials in human subjects with cystic fibrosis, with clear benefits being found¹⁵. 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 is 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 PTC124 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 gut¹. 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 energy¹⁶. 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 formaldehyde¹⁷. 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 comprehensive biochemical screening for trimethylaminuria
Previous methods have used an HPLC approach to quantitated trimethylamine in urine samples. This is a labour intensive method that has the added disadvantage that it does

not easily lend itself to the quantitation of trimethylamine-N-oxide as well. We plan to use a mass spectrophotometric approach similar to that described by Johnson²⁰. Co-chief investigator, Kevin Carpenter, head of the NSW Biochemical Genetics Service based at the Children's Hospital at Westmead, is an international authority on the use of mass spectrometric techniques in the diagnosis of inborn errors of metabolism. He will oversee the development of the new more complete biochemical testing for TMAU. We have urine samples from patients with TMAU already in storage, and these will be used as positive controls for the development phase. This new testing procedure, coupled with a marine fish or choline load as needed¹, will provide a very powerful means for diagnosis of the majority of TMAU patients.

2. To develop complete molecular genetic screening of the FMO3 gene for individuals suspected of having trimethylaminuria.

John Christodoulou has nearly two decades of experience in the analysis of gene mutations, and his research laboratory has all of the facilities to be able to develop comprehensive mutation testing of the *FMO3* gene. Once this testing has been developed to a robust stage, the methodology will be transferred to the molecular genetics diagnostic laboratory at the Children's Hospital at Westmead, for which he is the administrative head, and so will be available as a routine diagnostic test on referral by clinicians in Australasia.

3. To develop an in vitro system for testing whether PTC124 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). We will develop a functional assay of the *FMO3* enzyme, using previously reported spectrophotometric methods²¹, and then confirm that the mutations cause non- or dysfunctional *FMO3* enzyme. CI 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 assay for improvement in functional activity, and perform westerns to determine whether full length protein is now being made. Initial discussions with senior staff from Genzyme Therapeutics, the current patent holder of PTC124, suggest that we will be able to obtain as supply of the drug for our studies.

4. 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 PTC124 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 the mass spectrometric techniques developed for Plan 1, to ascertain that they display the clinical features of trimethylaminuria.

5. To test the *in vivo* efficacy of PTC124 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 PTC124.

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 FMO3²², and quantitate FMO3 the level of and the size of the wildtype and mutant FMO3 protein extracted from liver samples.

We will then inject PTC124 intraperitoneally into wildtype 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.

6. 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.

Conclusions:

As a result of the research program outlined in this proposal we will have established a comprehensive national diagnostic service for TMAU. In addition, 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 is 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 in Australia.

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

Study component	Year 1		Year 2		Year 3	
Development of urinary quantitation of TMA & TMAO	X	X				
Development of molecular screening of <i>FMO3</i> gene	X	X				
Generation of the mouse model for FMO3 deficiency, & biochemical and phenotypic characterisation	X	X	X	X		
Generation of a range of <i>FMO3</i> nonsense mutations, & their functional analysis in a cell culture system	X	X				
Evaluation of the efficacy of PTC124 in our <i>in vitro</i> cell culture system			X	X		
Evaluation of the efficacy of PTC124 in our mouse model system					X	X
Evaluation of the therapeutic utility of <i>Methylophilus methylotrophus</i> in our mouse model system					X	X

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Proposed Budget:

Staffing:

Given to wide range of techniques, their complexity and the volume of work that will need to be undertaken, funding for two postdoctoral research scientists is required. It is expected that these individuals 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.

Each scientist will be employed at Research Officer HSM1 level:

Annual salary (including base salary, and all on costs) \$88,573

[\$177,146 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 large box (houses 10 mice) = \$8.00/week

1 small box (houses mating pair or pregnant mice) = \$5.50/week

15 small boxes (to house neonatal, juvenile and adult mice) for 20 weeks - 15 x

\$5.50/week x 15 = \$1650

6 large boxes (to house female mice in preparation for breeding) for 52 weeks - 4 x \$8.00 x 52 = \$2496

10 small boxes (to house a breeding pair then the pregnant mouse) for 52 weeks - 10 x

\$5.50 x 52 = \$2860

Total holding cost \$7006

Animal health monitoring costs \$2214/yr

[Total Animal Cost = holding + health monitoring = \$ 9220/yr]

Mouse experimental work will take place over 3.0 yr (total cost = \$27660).

Annual budget requested: \$211,266

**Total budget requested for the life of this 3-year research proposal:
\$633,798**