Action for M.E. PhD Studentship Application Form

Section 1 - PhD Student (*leave blank if not yet appointed*)	
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Appointment held (if any)	
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Section 2 – Principal Investigator (PI)	
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Details of Co-Applicants (name/job role/university/contact details)	Dr Jethro Johnson Deputy Director, Oxford Centre for Microbiome Studies Kennedy Institute of Rheumatology, University of Oxford, UK jethro.johnson@kennedy.ox.ac.uk Dr Beata Godlewska Clinical Researcher, Honorary Consultant Psychiatrist, Department of Psychiatry, University of Oxford, UK Beata.godlewska@psych.ox.ac.uk Prof Wei Huang Department of Engineering Science University of Oxford, UK wei.huang@eng.ox.ac.uk

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Section 3 – The Research

Title of project	Is there a role for the microbiome and leaky gut in driving ME/CFS and other conditions associated with chronic fatigue?
Number of years funding required (1 – 3 max)	3
Total support requested (£)	£45,000
Proposed start date	Oct 1st 2021

Scientific Abstract (200 words max)

Microbial imbalance of the intestinal biome is observed as a key associated factor in many chronic conditions including ME/CFS. In this proposal we will explore this association in detail in multiple cohorts associated with fatigue examining not only the gut but also the currently unexplored blood biome. We propose that the blood biome and its association with a leaky gut could be a pivotal factor in a number of chronic conditions including ME/CFS, CLD, Long Covid and endometriosis. The tools to study rare L-form bacteria in our blood and tissues are now available and we will utilise new technologies in two research centres. This PhD will recruit ME/CFS patients using current Institute of Medicine (IOM) criteria with follow up clinical assessments in collaboration with experienced ME/CFS clinical researchers. Assessments of pathogen load and microbial genomic composition will be carried out by specialist laboratories with the PhD student involved in patient diagnosis and bio-informatic analysis. The final part of the PhD will involve the

characterisation of L-form bacteria in pure cultures derived from ME/CFS patients using Raman Spectro-microscopy. This technique will look for unique L-form fingerprints which we can be used to identify L-forms present in blood cells from patients.

Why the research is important for people with M.E. (300 words max) and how you will ensure meaningful involvement

Our key focus is to not only understand more about the biology of ME/CFS but to open up treatment options. If a leaky gut is a key component of ME/CFS restoring a normal gut microbiome balance with current approaches such as Faecal Microbiota transplantation (FMT) could be life changing treatment options. Identifying opportunistic pathogen organisms in the blood with levels reflecting symptom severity in individual patients with the ability to grow these organisms in the laboratory will open up new opportunities for patient specific approaches to treatment. Our collaborator SoftCell biologicals have been developing this approach using L-form cultures from patients with chronic urinary tract infections (CURTIs), testing standard antibiotics in the laboratory and feeding back to clinicians treating the patients. Clinical trials using this approach would allow existing antibiotics tested in the lab to then be then given to patients in a blinded trial setting.

Even if L-form pathogens do not strongly correlate with disease severity or specific symptoms increasing our understanding of the gut dysbiosis in ME/CFS will open up new research avenues to explore. Oxford is very strong in microbiome and microbiology research and as we move forwards, this project will provide a great stimulus to bring other top researchers into the ME/CFS field. Our study of post exertional malaise (PEM) and finding simple less intensive ways through which it can be induced and measured during standard clinical practise will be extremely important both in research and also in patient diagnosis. Diagnosis of ME/CFS with a quantitative PEM assessment could have a fundamental impact on how the condition is managed by clinicians; potentially preventing the development of more severe ME/CFS if steps are put in place early on to appropriately manage the condition. In this and other clinical research projects we will work closely with the local OMEGA ME group and the ME Association to help with recruitment.

Details of research project (three A4 sides max):

A) Aims

This project will test the hypothesis that enhanced gut permeability linked to gut dysbiosis, associated levels of low grade infection in the body and high levels of L-form organisms in blood are contributing factors in ME/CFS. We will also determine if changes in the above correlate with symptom severity over a 6 month period in a sub set of patients. In addition to a healthy control group we will also investigate endometriosis, Chronic Lyme Disease (CLD) and Long Covid all of which have many symptoms in common with ME/CFS.

Working Hypothesis: ME/CFS, Long Covid and CLD are triggered by an infection which triggers Gut dysbiosis and a failure in gut wall integrity. In individuals who develop ME/CFS, Long Covid and CLD, when the primary infection clears, a dysbiotic gut remains with associated deficiencies in key gut bacteria. Over time the lack of key bacteria components in the bowel results in a breakdown of gut wall integrity allowing leakage of opportunistic pathogens from the gut into the body. The diversity and composition of the gut microbiota plays a key role in the maintenance of intestinal homeostasis and the induction of immunity (see [1] for review). Once in the body the immune system recognises and attempts to neutralise opportunistic pathogens. Recent evidence shows that once taken up by our immune cells certain bacteria can undergo a transition, entering an L-form, wall-less state [2]. These L-forms can persist inside our immune cells, invisible to the immune system and not triggering antibody or cytokine responses. In vivo data suggests that L-forms can readily transform from the wall less to the walled state [3]. This could result in high levels of localised pathogens in tissues, which would trigger an immune /inflammatory response.
Questions and objectives: In this PhD project we will examine a series of questions on 4 disease cohorts: ME/CFS (Mild, Mod and Severe), Long Covid, CLD and Endometriosis.

1) Is there evidence for gut dysbiosis with associated gut leakiness, low-grade blood infection and aberrant immune cell function in these chronic conditions?
2) In a longitudinal study in a subset of patients over 6 months. Do levels of dysfunction in 1) correlate with symptom severity?
3) Are L-forms found in the blood of patients and can their presence be validated using different approaches in two independent laboratories?
4) In the longitudinal study in 2. Do either the abundance or types of detectable L-forms correlate with symptom severity in patients?

B) Work which has led to the project (including pilot data)

Although immune dysfunction may associate with ME/CFS there has been inconsistency in data linked to specific immune cell activation and cytokine profiles associated with pathogenic agent(s) [2, 4-6]. Active virus or "classic" bacterial/fungal infections have not been detected suggesting that if pathogenic agents are present their effects are more subtle or organ localised. In this proposal we suggest that L-form organisms lacking a cell wall play a key role in the development of a range of chronic diseases including ME/CFS. Recent data from SoftCell, examining the blood of over 2000 individuals, many suffering from chronic diseases, has identified the presence of a large number of potential pathogens in blood. The number of L-form bacteria that SoftCell can culture tends to be higher in patients suffering from chronic conditions like ME/CFS and Fibromyalgia (Figures 1, 2, see appendix 3). This is a new medical field linked to the growing awareness that our blood is not sterile containing a vast array of micro-organisms and latent infectious agents which could all have an impact on health [7].

Gut dysbiosis [8, 9] with evidence of gut leakage [8] and possible low-grade tissue/blood infections [10] are emerging as important areas in ME/CFS. Considering many ME/CFS, Endometriosis and Chronic Lyme disease patients suffer from irritable bowel (IBS) and other long-term gut issues, restoring gut function could have an impact on at least gut symptoms. Preliminary research by the Dove Clinic (Hampshire) exploring the potential of Faecal microbiota transplantation (FMT) is showing real clinical benefit in ME/CFS patients [11]. If a leaky gut is the predominant driver of symptoms, a simple solution would be to "plug" the holes either pharmaceutically or by restoring the gut microbiome balance. This could give patients who have a weakness in their gut or immune system an opportunity to re-set the balance of L-forms in the body. Much of the research into ME/CFS has focused on trying to understand the complex biology underlying the condition and its symptoms. Disease heterogeneity, challenges with diagnosis and lack of research funding have made this extremely difficult. Could a therapeutic approach as simple as restoring the health of the gut make a difference? This project through fully exploring the microbiome and gut health in ME/CFS and other conditions will look to connect abnormalities in the gut and the immune system with patient symptoms.

C) Experimental design and methods

Diagnosis and recruitment (please see appendix for more detail)

i)ME/CFS: We aim to recruit 70 female ME/CFS patients into the study with a range of severities. At least 10 subjects will be have severe and chronic symptoms. We will also recruit a healthy control group of 30 healthy women (18-55 years). In addition we will recruit women with several different disorders (see below) - all patients and healthy controls will have the microbiome of samples of their stool examined. ME/CFS, Long Covid, recovered Covid and healthy controls will undergo an exposure to a PEM stressor with follow up assessment as outlined in appendix 1.

ii)Long Covid and individuals who have fully recovered from Covid : We will recruit 40 female patients with active Long COVID and 20 who have recovered and assess them against IOM ME/CFS criteria. The study selection criteria requires a positive PCR test for Covid with mild symptoms (no hospital admission). We will define Long Covid patients as those who have been suffering with post viral fatigue/ME/CFS for at least 6 months.

iii)Chronic Lyme Disease (CLD) patients: Forty patients with baseline and post long-term antibiotic treatment samples taking part in an ongoing University College Dublin study (Professor

Jack Lambert) will be recruited into the study. CLD is a condition with many similar symptoms to ME/CFS. See appendix 1 for more details.

iv)Endometriosis: For this cohort we recruited 40 patients with endometriosis and 40 patients referred for laparoscopy without evidence of endometriosis. We will make use of an existing bank of plasma samples collected by Professor Christian Becker and Professor Krina Zondervan, WRH. New stool samples will be collected from 30 endometriosis and 30 non endometriosis patients.

Experimental sections

Part 1 Exploring the presence of a leaky gut, low grade infection phenotype and gut dysbiosis

This section will determine if a leaky gut and low-grade blood/tissue infection are common themes across all the cohorts. Lipopolysaccharide (LPS) levels will be determined as a measure of leaky gut as described previously in ME/CFS Giloteaux et al [8] . Low-grade infection will be assessed by measuring levels of the cathelicidin antimicrobial peptide (CAMP) recently shown to be elevated in ME/CFS patients [10]. LPS and CAMP assays will be used as a cost effective first line screen on all plasma samples (n=320). Gut dysbiosis previously shown to be a feature in ME/CFS patients [8, 9] will be assessed by 16sRNA sequencing of stool samples in all groups (n=300) (Dr Jethro Johnson Kennedy Institute, Oxford). Of particular interest is Faecalibacterium Prausnitzii a bacteria shown to be reduced in ME/CFS patients [9] and severe Covid patients [12]. F.Prausnitzii, a potent butyrate producer, has been shown to produce the microbiobial anti-inflammatory molecule (MAM) [13] with a recent paper showing a lack of F.Prausnizii and loss of the derived MAM protein being linked to a leaky gut in mouse models of obesity [14]. Re introducing the MAM protein to the gut improved gut integrity with a reduction in LPS levels in the blood stream [14].

Part 2 Are pathogens present in the blood of ME/CFS, CLD and Long Covid patients?

This work will be carried out by Softcell Biologicals (SB) (Utah, USA) using an established metagenomic whole genome shotgun (mWGS)' sequencing on blood samples. Based on the findings in part 1, a pilot study of five ME/CFS, Long Covid and endometriosis patients with evidence of a leaky gut and/or low grade infection and 5 controls will be selected to investigate the presence of associated L-form organisms. Studies will include a baseline assessment and two further samples taken when symptoms worsen or improve (captured with the Solve ME smartphone app and a modified version of the De Paul PEM Questionnaire). Levels of LPS and CAMP will again be assessed tests (n=60 Oxford samples). For the CLD cohort we anticipate 70% of our CLD patients will show a good response to long term antibiotics based on previous data by Professor Lambert and published data [15] . For this cohort we will select 10 subjects, 7 who respond well to treatment and 3 who do not, sending frozen blood samples at baseline, mid and post antibiotic treatment to SB for mWGS sequencing (n=30). This will allow us to look at changes in blood pathogens with treatment and look for a correlation with symptoms. L-form cultures will be established by SB on all fresh blood shipped using a patented protocol. Initially this will involve the fresh Oxford samples but we plan to send CLD bloods for culturing when collected in follow up work. In collaboration with Dr Jethro Johnson (Kennedy institute) we will look to validate the data generated by SB on 10 randomly selected samples from the 90 shipped to SB. We will use Metagenomic Shotgun sequencing at a high depth to identify pathogens and also 16S seq at a lower depth to determine if pathogens reach higher enough levels for this approach to be used (see attached quotation)[16]. The 16S sequencing if successful is a much more cost effective approach.

Part 3 Evidence of abnormal immune cell function and the presence of L-form organisms in ME/CFS patient blood cells

On the Oxford blood samples taken in part 2 (n=60, 5 ME/CFS x3, 5 Long Covid x3, 5 normal control x 3, 5 x endometriosis x3) we will also explore immune cell activation using a standard protocol using the Sysmex XN1000 analyser and a research protocol using CytOF analysis [17] (see appendix 1). Our WRH collaboration with Sysmex currently provides technical support in new disease areas to study immune cell function. In this project we will use the white blood cell differentiation (WCD) and white precursor and pathological cell (WPC) channel panel[18]. Using the WCD and WPC channels we are able to tag individual immune cells and assess their functional state. Running as a pilot study (due to high costs) we will also use the CytOF system to provide an

in depth immune cells analysis on a small sample set (10 patients). To identify L-form organisms directly in patient cells we will use pure L-form cultures established in part 2 and determine specific L-form Raman micro-spectroscopy profiles. This technique has been utilised to study differences in PBMC between ME/CFS patients and controls [19] and more recently to discriminate peripheral blood mononuclear cells (PBMC) from ME/CFS patients and multiple sclerosis (MS) patients (Figure 3). Material can be cryopreserved in fetal calf serum /dimethyl sulfoxide at -80^{o}C with fixing in 5% paraformaldehyde before analysis. In this study fixed L-form organisms will be shipped to the UK for analysis. Our recent data has shown unique Raman spectra in isolated mitochondria (Figure 4). We will look for similar unique signatures in L-form organisms and then look for the same signatures in the blood cells from the same samples the L-forms were derived. We are confident our machine learning approaches [19] will allow us to differentiate similar spectra even if only slightly different. The amplification steps required to detect low levels of L-form pathogens in blood samples has led to them to be considered by many as artefacts. By using the variety of approaches outlined above we hope to reach a higher level of certainty of their existence and their association with chronic disease.

e)Timetable and milestones (see appendix 2)

f)Justification for financial support

Of the £152,962 (+ $50,000) required to run the PhD project we are applying for £45,000 from Action for ME. This money will be used to pay for assay kits £9,547, blood brain barrier breakdown research £10,000, Microbiome assessment and blood biome sequencing costs at the Kennedy Institute £29,383 and £5000 to cover research nurse time to take blood samples and contribute to the immune cell function cost 90 samples Sysmex (£180) and 10 samples CyTOF (8,000). We are also submitting grants to the Caudwell Trust (£30K) with money also available from Oxford ME/CFS patient donations. *NB: SB have provide a $50,000 in kind contribution for work carried out at SB, see attached letter of support.*

Communication and dissemination plan (200 words max)

Our initial route of communication will be via published papers in peer-reviewed journals. It is essential for ME/CFS research to gain respect from other disciplines and the wider research community if ME/CFS is to get the attention it deserves. We will also attend CMRC meetings, these are great opportunities to discuss our research with fellow researchers and patients. As we expand our knowledge, connecting our research in Oxford with other ME/CFS groups working in the UK has the potential to identify projects suitable for larger budgets. We need to be mindful about over hyping our data before it is published. We are potentially breaking new ground with this proposal and need to be sure of our results before making exaggerated claims. As we move our ME/CFS research to a sustainable clinical research programme we are very keen to work/engage with patients in webinars and other social media activities. With Jamie Strong, a ME/CFS patient and key researcher in my group we plan to set up a patient advisory group. As we move forwards on our journey in understanding the biology of ME/CFS it is really important that we take the patients with us.

Lay summary in simple language for the non-expert (500 words max)

It is becoming clear we are not in as much control of our bodies as we previously thought. We have known for some time that our diet and lifestyle can have a major impact on our health, but we really had no idea that most of the cells in our bodies are not human. The bacteria of our gut known as the microbiome contains more living cells that all of our organ systems put together. Aging, antibiotics, diet and infections can all influence our gut microbiome moving the profile into a less healthy state. Recent studies have shown that the gut microbiome of ME/CFS patients lacks the diversity of a normal gut and in one study lacked levels of a specific bacteria which appears fundamental to gut health. This bacteria is also lacking in a mouse genetic model of diabetes and interestingly these animals also show evidence of a leaky gut. The bacteria makes a protein which helps stabilise the gut wall reducing gut leakiness. A leaky gut profile has also been shown in ME/CFS with patients showing high levels of bacterial components in the blood stream.

If bacteria are entering the body could this be a problem? Our immune system can deal with pathogens as they enter the body with the activation of specific immune cells to deal with the

invaders. As evidence in ME/CFS does not support an ongoing infection what could be going on? Our co-applicant SoftCell Biologicals have identified high levels of L-form, wall less pathogens in a range of chronic conditions including ME/CFS. In a wall less state, L-form pathogens have "stealth like" properties essentially invisible to the immune system, hiding in cells and not triggering an inflammatory response. Once inside cells bacteria can persist in an L-form state reverting to a normal wall form if conditions allow. As immune cells containing L-forms can be long lived and travel all over the body with L-form's suddenly switching to wall form state, localised inflammation could be triggered.

This proposal aims to address some key questions. Firstly is there evidence of a leaky gut in ME/CFS and similar conditions and can we find elevated levels of an infection marker protein recently identified in ME/CFS? Can we find further evidence of an abnormal gut microbiome in ME/CFS and do we see evidence of low levels of the bacterial know to restore gut wall integrity? Secondly, in patients showing high levels of a leaky gut and or evidence of low-grade infection do we find high levels of L-form organisms and can these be cultured in the lab? In a long-term study over 6 months in patient's showing high levels of L-form pathogens is there a correlation between L-Form levels and symptoms and is there any correlation with leaky gut infection markers? The answers to these questions will be highly informative as to the whether the gut is a fundamental player in ME/CFS and other chronic diseases.

Section 4 - References (full citation)

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3. Markova, N., *Dysbiotic microbiota in autistic children and their mothers: persistence of fungal and bacterial wall-deficient L-form variants in blood.* Sci Rep, 2019. **9**(1): p. 13401.
4. Brenu, E.W., et al., *Immunological abnormalities as potential biomarkers in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis.* J Transl Med, 2011. **9**: p. 81.
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13. Quevrain, E., et al., *Identification of an anti-inflammatory protein from Faecalibacterium prausnitzii, a commensal bacterium deficient in Crohn's disease.* Gut, 2016. **65**(3): p. 415-425.
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18. Schuff-Werner, P., et al., *Performance of the XN-2000 WPC channel-flagging to differentiate reactive and neoplastic leukocytosis.* Clin Chem Lab Med, 2016. **54**(9): p. 1503-10.
19. Xu, J., et al., *A new approach to find biomarkers in chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) by single-cell Raman micro-spectroscopy.* Analyst, 2019. **144**(3): p. 913-920.
20. Nepotchatykh, E., et al., *Profile of circulating microRNAs in myalgic encephalomyelitis and their relation to symptom severity, and disease pathophysiology.* Sci Rep, 2020. **10**(1): p. 19620.
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Section 5 – Financial Breakdown (total costs)

	Year 1	Year 2	Year 3
Student stipend	**£15,000**	**£15,000**	**£15,000**
University / college fees	**£8,290**	**£8,787**	**£9,314**
Conference & training allowance	**£500**	**£500**	**£2000**
Materials & consumables (please list) **Test kits LPS & CAMP (390 samples)** **Blood brain barrier breakdown assays (D Sutherland Tasmania (ME/CFS, Long Covid and CLD,**	**LPS £3827 & CAMP £5,720** **£10,000**		

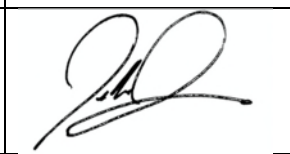
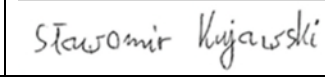
Plus controls) 160 samples **Kennedy costs: Gut microbiome (n=300)** **Blood biome 10 subjects validation samples** **Blood biome 16 S (90 subjects)** **Patient diagnosis private consulting room hire. Witney Balance Studios £120 per month 4 hrs one morning per week. 12 months** **Massage Cuff for PEM induction** **Cognitive PEM inducing questionaires** **MOXY Sensor and software** **Research Midwives taking blood JR OT time taking blood with severes (10 patients)** **Softcell Biologicals Pathogen whole genome sequencing from blood plus setting up L-form culture. Approximate.** **Sysmex/CytoF assessment (90 samples)** **CytOF (panel set up & 10 samples)**	**£15,043** **£1440** **£3000** **£200** **£871** **£2500**	**£8,113** **£2500** **$50,000** **£180** **£8,950**	**£6,227**

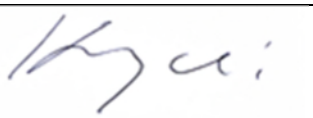
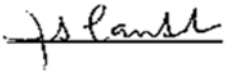
Raman costs access charges 3 months **Raman slides** **Bioinformatics training** **General consumables Blood collecting tubes etc**	**£2500**	**£3000** **£1000** **£1000** **£2500**	
Total	**£68,891**	**£51,530 + $50,000 SB**	**£32,541**
Total (£) match funding required/secured (please indicate which and identify where from or plan to secure including timescales)		Caudwell trust £30,000 to be confirmed, SoftCell Biologicals in kind contribution $50,000 (Agreed), Dr Inga Williams will self-fund her D Phil, donations for ME/CFS research Oxford £15,000 (available)	

Section 6 – Peer Review

If you are able to, please suggest up to 5 peer reviewers and email addresses (if known) who have no conflicts of interest with you/PI and the research (*please note this is not mandatory or assessed*)	Dr Jo Elson joanna.elson@newcastle.ac.uk> Dr Julia Newton Prof Elisa Oltra elisa.oltra@ucv.es> Prof Warren Tait warren.tate@otago.ac.nz Dr Avindra Nath avindra.nath@nih.gov

Section 7 – Signatures (please sign and date)

PI		**Date**	16-2-2021
Co-Applicant (s)		**Date**	
Jethro Johnson			17-2-2021
Beata Godlewska			16-2-2021
Pawel Zalewski			16-2-2021
Slawomir Kujawski			16-2-2021

Wei Huang			16-2-2021
Kim Midwood			17-2-2021
Jack Lambert			17-2-2021
Confirm SHORT CVs of all applicants attached		YES	

Please return to: research@actionforme.org.uk by 12pm Monday 22 February 2021