BMJ Open

# Serial Paediatrics Omics Tracking in Myalgic Encephalomyelitis (SPOT-ME): protocol paper for a multidisciplinary, observational study of clinical and biological markers of paediatric myalgic encephalomyelitis/chronic fatigue syndrome in Australian adolescents aged 12–19 years

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**To cite:** Thomas N, Chau T, Tantanis D, *et al*. Serial Paediatrics Omics Tracking in Myalgic Encephalomyelitis (SPOT-ME): protocol paper for a multidisciplinary, observational study of clinical and biological markers of paediatric myalgic encephalomyelitis/chronic fatigue syndrome in Australian adolescents aged 12–19 years. *BMJ Open* 2024;**14**:e089038. doi:10.1136/bmjopen-2024-089038

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (https://doi.org/10.1136/bmjopen-2024-089038).

Received 21 May 2024
Accepted 01 November 2024



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## ABSTRACT

**Introduction** Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a disabling condition that can affect adolescents during a vulnerable period of development. The underlying biological mechanisms for ME/CFS remain unclear and have rarely been investigated in the adolescent population, despite this period representing an age peak in the overall incidence. The primary objective of this is to provide a foundational set of biological data on adolescent ME/CFS patients. Data generated will be compared with controls and over several time points within each patient to potentially develop a biomarker signature of the disease, identify subsets or clusters of patients, and to unveil the pathomechanisms of the disease.

**Methods and analysis** This protocol paper outlines a comprehensive, multilevel, longitudinal, observational study in paediatric ME/CFS. ME/CFS patients aged 12–19 years and controls will donate biosamples of urine, blood, and peripheral blood mononuclear cells for an in-depth omics profiling analysis (whole-genome sequencing, metabolomics and quantitative proteomics) while being assessed by gold-standard clinical and neuropsychological measures. ME/CFS patients will then be provided with a take-home kit that enables them to collect urine and blood microsamples during an average day and during days when they are experiencing postexertional malaise. The longitudinal repeated-measures study design is optimal for studying heterogeneous chronic diseases like ME/CFS as it can detect subtle changes, control for individual differences, enhance precision and boost statistical power. The outcomes of this research have the potential to identify biomarker signatures, aid in understanding the underlying mechanisms, and ultimately, improve the lives of children with ME/CFS.

## STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ We outline an Australian study of paediatric myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) that will comprehensively analyse metabolic, physiological, proteomic, genomic, clinical and neuropsychological measures from children and adolescents diagnosed with ME/CFS and controls.
⇒ The Serial Paediatrics Omics Tracking in Myalgic Encephalomyelitis (SPOT-ME) study was designed in consultation with an ME/CFS advisory committee consisting of adolescents with ME/CFS and parents whose children have ME/CFS.
⇒ ME/CFS diagnosis will be confirmed based on the stringent criteria outlined in both the Canadian Consensus Criteria paediatric case definition and the Paediatric ME/CFS Primer.
⇒ The longitudinal component of SPOT-ME intends to track changes in identified, potential biological markers of ME/CFS over a 4-month period in patients, with additional biological collections scheduled for 'crash' episodes (ie, a severe but generally time-limited worsening of ME/CFS symptoms).
⇒ Consistent with the unpredictable nature of ME/CFS, there are risks of missing data and attrition at various stages of study participation There may also be some variability in the integrity of the samples collected by longitudinal families at home.

**Ethics and dissemination** This project was approved by the Royal Children's Hospital's Human Research Ethics Committee (HREC 74175). Findings from this study will be disseminated through peer-reviewed journal publications and presentations at relevant conferences. All participants

will be provided with a summary of the study's findings once the project is completed.

## INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a clinical condition characterised by unexplained severe fatigue that is accompanied by a variety of symptoms related to cognitive, immunological, endocrinological and autonomic dysfunction.[1] In addition to persistent fatigue, individuals also experience postexertional malaise (PEM), which is defined as the worsening of ME/CFS symptoms following physical or mental exertion that can last for days or even weeks. The incidence of ME/CFS has been observed to peak between the ages of 10–19 years and 30–39 years and there is a notable female preponderance in both groups.[2 3] As this condition impacts individuals of all age groups, genders, races and socioeconomic backgrounds,[4] it constitutes a debilitating and costly public health issue with an enormous associated burden for both patients and caregivers.[4]

### Prevalence and illness onset

The prevalence of paediatric ME/CFS is estimated at 0.1%–0.5%[5 6] with differences in the illness process observed between males and females.[2 7–9] Nevertheless, the onset of paediatric ME/CFS during middle-to-late adolescence occurs during a crucial period of educational, social, emotional, hormonal and physical development.[7 10] Evolving data in adults suggest the onset arises from the complex interaction between genetic predisposition, host biology and environmental factors. Known triggers include viral infections, physical or emotional stressors, and environmental toxins.[11] A gradual onset of the condition is more common in adolescents, with symptoms developing over months or years.[12] Clinical management may help to improve symptom severity over time; however, a large majority of adolescents continue to show a persisting or fluctuating symptom pattern, and meet diagnostic criteria for ME/CFS at least 2 years following initial diagnosis).[13 14]

As a result of the timing and multidimensional complexities of this illness process, paediatric ME/CFS is associated with significant, negative impacts on school attendance, physical and psychological functioning, and overall quality of life.[12 15] Yet, despite the impact that this condition can have during this major developmental phase, there has been limited research focusing on the causative factors behind this condition within the adolescent population.[2]

### Pathophysiology of ME/CFS

Drawing predominantly from the adult literature, available data suggest central nervous system, genomic, proteomic and metabolomic contributions to ME/CFS. Central nervous system dysregulation, along with resulting chronic neuroinflammation, is thought to underlie fatigue, cognition, pain and sleep difficulties that are common in ME/CFS.[16] Several studies have also suggested that affected individuals may be genetically predisposed to ME/CFS.[17–19] Expression and regulation of proteins in ME/CFS have been shown to be altered in adults, including various proteins involved in the inflammatory cascade, oxidative stress processes, mitochondrial processes and neuroendocrine pathways.[11] Studies have also revealed the dysfunction of the hypothalamus–pituitary–adrenal (HPA) axis in ME/CFS, with recent meta-analysis showing that levels of HPA axis cortisol are reduced.[4] Furthermore, several in-depth proteomics and metabolomics profiling experiments have revealed that energy, mitochondrial, lipid and amino acid metabolism pathways are all dysregulated in adult ME/CFS.[20–22]

Although these findings provide us with valuable clues about the potential underlying pathophysiology of paediatric ME/CFS, further research within a paediatric population is required to establish age-appropriate diagnostic biomarkers and effective therapeutic approaches.

### Aims and objectives

We aim to address this clinical need through a detailed examination of the biological, neuropsychological and clinical impacts of ME/CFS on children and adolescents using genomics, metabolomics, proteomics and clinical assessments as part of the 'Serial Paediatrics Omics Tracking in Myalgic Encephalomyelitis' (SPOT-ME) study.

The objectives of this study are to:

1. Characterise the biological differences between paediatric ME/CFS patients and controls using an in-depth omics profiling analysis (whole-genome sequencing, metabolomics and quantitative proteomics) of urine, blood and peripheral blood mononuclear cells (PBMCs).
2. Characterise differences in ME/CFS clinical symptomatology and neuropsychological functioning between the ME/CFS and control groups.
3. Examine relationships between clinical and neuropsychological functioning and metabolomic, proteomic and genomic markers in ME/CFS patients and controls.
4. Characterise the temporal relationship between metabolites, physiological parameters and clinical symptomatology in paediatric ME/CFS patients across a singular, consecutive 4-month period in the longitudinal, ME/CFS-only arm of the study.

## METHODS AND ANALYSIS

### Study design

#### Patient and public involvement

An ME/CFS advisory committee, comprising three young people with paediatric ME/CFS and two parents, was consulted in the planning stages of the study design to help determine feasibility and acceptability of the baseline study visit and the longitudinal components of SPOT-ME for ME/CFS adolescent patients. The committee was established prior to the commencement

of data collection and will continue to be consulted on occasion during data collection.

All committee members and study participants will be provided with a summary of the study's findings once the project is completed.

## Participants

### Recruitment

This study aims to recruit 50 paediatric ME/CFS patients and 25 control participants aged 12–19 years between November 2021—November 2024. This sample size was determined based on previous exploratory studies in adults that identified significant changes in ME/CFS compared with controls in various omics-based studies.[23] We view n=25 as a suitable control cohort. Due to anticipated heterogeneity in the ME/CFS cohort, n=25 is also considered a suitable minimum sample size for the ME/CFS group. To maximise the study's statistical power, we intend to continue recruiting both patients and control participants even after the goal sample size for each group has been achieved (subject to logistical constraints).

Potentially eligible ME/CFS patients and their caregiver(s) will be informed about SPOT-ME by a member of their clinical team, such as their paediatrician or general practitioner. If interested, a researcher-designed and clinician-designed questionnaire incorporating the study eligibility criteria and other relevant clinical information (ie, illness duration, symptom information, trigger for onset) will then be administered by their treating clinician, or a paediatric physician from the research team, to confirm their ME/CFS diagnosis and study eligibility. In the questionnaire, criteria for diagnosis were adapted from and based on the Canadian Consensus Criteria paediatric case definition[24] and the Paediatric Primer clinical diagnostic worksheet[12] (see online supplemental appendix A: Clinical Questionnaire).

Controls will be recruited through invitations to participate in SPOT-ME that are displayed on printed flyers around the main hospital site and/or online bulletin board notices for site staff, in addition to word-of-mouth recruitment from existing study participants.

### Inclusion criteria

All participants must be adolescents aged between 12 and 19 years at enrolment who either (1) have an existing diagnosis of ME/CFS as defined by the Canadian Consensus Criteria paediatric case definition[24] and the Paediatric ME/CFS Primer[12] or (2) are currently healthy and have never been diagnosed with a chronic illness (control group). Participants must have sufficient English to complete a neuropsychological assessment and complete self-reported clinical symptom questionnaires. Participants must also be able to nominate one caregiver with sufficient English to complete caregiver questionnaires regarding the young person's emotional and behavioural well-being.

### Exclusion criteria

Exclusion criteria for both patients and controls include (1) insufficient English to complete clinical questionnaires and assessments of cognition, (2) the presence of primary major depressive and/or anxiety disorder that adequately explains the fatigue experienced and (3) the diagnosis of a pre-existing neurodevelopmental disorder or brain injury.

## Procedure

The study commenced in November 2021 and is planned to conclude by the end of February 2025. Eligible participants and their nominated caregivers will be asked to provide written consent to participate in the SPOT-ME Study. A summary of the subsequent study procedure is shown in figure 1. Overall, the SPOT-ME study consists of two parts: an in-person clinical study visit and an at-home longitudinal component.

### Part 1: clinical study visit (all participants)

All participants will undertake a 3.5-hour clinical study visit at the Murdoch Children's Research Institute, located within the Royal Children's Hospital (Melbourne, Australia), to complete baseline blood and urine collections as well as a cognitive assessment (figure 1). During this visit, the participant's nominated caregiver will also complete two questionnaires that ask about the young person's emotional and behavioural well-being. The participant will be asked to complete their own online questionnaire about their overall well-being prior to attending. Further details about these questionnaires and assessments are described in the Materials section.

Fasted whole blood samples will be collected in sodium heparin anticoagulant vacutainers, serum vacutainers and PAXgene tubes (figure 1). On the day of collection, the blood samples will be processed by research team members into whole blood, plasma, 'buffy coat' (which contains PBMCs), and red blood cells to obtain biochemical assays of protein and metabolite markers. Blood collected in the PAXgene tube will be used for subsequent genomic analysis. Urine samples will be analysed for biochemical markers (metabolomics, proteomics).

### Part 2: longitudinal study (ME/CFS participants only)

At the conclusion of part 1, interested ME/CFS participants will also have the option of consenting to complete the at-home, 4-month, longitudinal component of the SPOT-ME study (figure 1).

### Collection days

Participants will have eight fortnightly 'collection days' scheduled for the days when they feel that symptoms are at baseline (ie, a 'normal' or 'non-crash' day without obvious symptom exacerbation). They will also be asked to conduct six collections on 'crash' days (ie, days when they are experiencing significant PEM or a generalised 'crash' in their ME/CFS symptoms). The determination of what constitutes a 'crash' or 'non-crash' day will be led

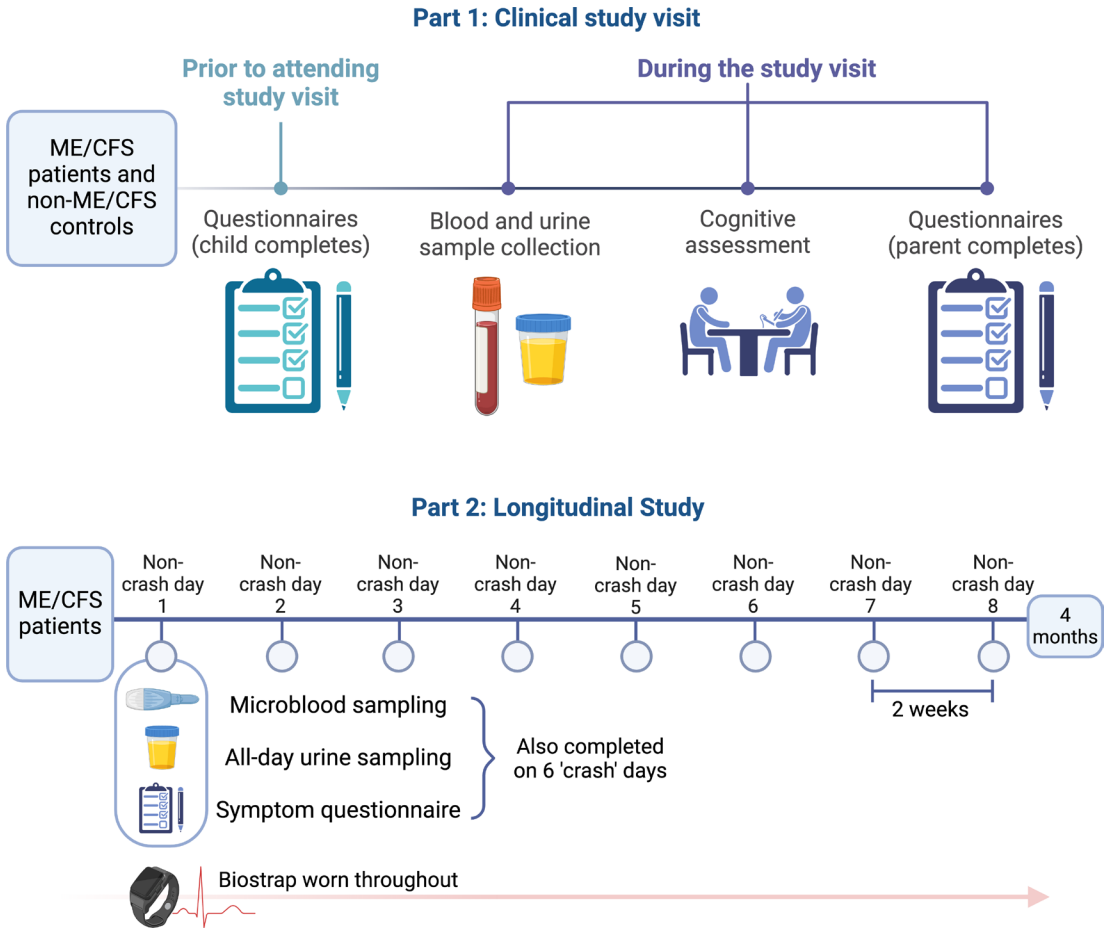


**Figure 1** Outline of the study workflow. Part 1 of the protocol is the clinical study visit. ME/CFS patients (n=50) and healthy controls (n=25) will complete questionnaires, donate blood and urine samples and undergo a cognitive assessment. Part 2 is the optional longitudinal study. Patients (n=47) will conduct at-home collection of biological samples and complete symptom questionnaires on 14 collection days (eight 'non-crash' days and six 'crash days', see definitions below) and wear a Biostrap activity watch throughout the course of the study. Figure created with BioRender.com. ME/CFS, myalgic encephalomyelitis/chronic fatigue syndrome.

by the participant and their family according to their own personal experiences of ME/CFS symptom fluctuations.

On the morning of a collection day, participants will collect a fasted, capillary (ie, finger prick) microblood sample. Participants will also retain a 1.5 mL urine sample each time they urinate on the collection day. Both blood and urine samples will then be stored in the participant's freezer until the conclusion of their longitudinal study participation.

### Clinical Symptom Questionnaires (modified De Paul Symptom Questionnaire–Paediatric Version)

At the end of each collection day, participants will complete an online symptom questionnaire. The questionnaire contains 10 questions adapted from the De Paul Symptom Questionnaire–Paediatric Version (DSQ-Ped)[25] that relate to the symptom domains of fatigue, PEM, pain, cognitive dysfunction, sleep dysfunction, influenza-like symptoms and autonomic dysfunction (see table 1 for a brief summary; refer to online supplemental appendix B for a full copy of the modified questionnaire).

### Physiological measures

Participants will be provided with a Biostrap activity watch to wear continuously throughout their longitudinal study participation to record their heart rate, heart rate variability, respiratory rate, oxygen saturation levels and objective sleep quality.

## Materials

### Clinical and neuropsychological measures

A summary of the questionnaires and cognitive assessments that will be completed by participants and/or caregivers can be found in table 1. The questionnaires, as well as any data collected from the cognitive assessments, will be securely hosted and managed on the REDCap electronic data capture platform.[26 27]

## Analysis

### Biological measures

#### *Exploratory biological experimental assays*

Biological samples from both part 1 and part 2 will be analysed using omics techniques and cell assays (figure 2).

Table 1 Clinical Questionnaires and Neuropsychological Assessment Measures

| Measure | Description |
|---|---|
| DePaul Symptom Questionnaire–Paediatric Version (DSQ-Ped)[25] | The DSQ-Ped is a validated 79-item self-report questionnaire that assesses all domains of ME/CFS symptoms as specified by the Canadian Consensus Criteria paediatric case definition.[24] Children and adolescents are asked to provide a 5-point Likert scale rating (0 indicating 'none of the time' or 'no problem' and four indicating 'always' or a 'very big problem') regarding their symptom frequency and severity over the past 3 months, in addition to providing responses to broad questions about their symptom onset, aetiology and impact on everyday functioning.<br>The modified version of the DSQ-Ped (see online supplemental appendix B) consists of 10 items that have been adapted from the original DSQ-Ped to capture a high-level summary of the severity of fatigue, postexertional malaise, aches and pains, cognitive difficulties, autonomic issues, mood and sleep-related difficulties. This modification was necessary to minimise the burden of longitudinal participation on participants and the risk of missing data. |
| Paediatric Quality of Life Inventory (PedsQL) 4.0 Generic Core Scales, Teen Self-Report Version[38] | The PedsQL 4.0 Generic Core Scales are a 23-item questionnaire that assesses four domains of health-related quality of life: physical, emotional, social and school functioning (teen-self report). Participants will provide responses on a 5-point Likert scale (0 indicating 'never' and 4 indicating 'always') regarding the frequency of health-related problems over the past month. |
| Paediatric Quality of Life Inventory Multidimensional Fatigue Scale, Child and Teen Self-Report (PedsQL MFS)[38] | The PedsQL MFS questionnaire consists of 18 items that ask the respondent to rate, on a 5-point Likert scale (0 indicating 'never' and four indicating 'almost always'), the frequency of fatigue symptoms that may have impacted their overall health-related quality of life over the past month. The three fatigue dimensions assessed are general fatigue, sleep/rest fatigue and cognitive fatigue. |
| Pittsburgh Sleep Quality Index (PSQI)[39] | The PSQI is a self-report questionnaire of subjective sleep quality and quantity over the past month. Participants are asked to describe their sleep/wake habits, the frequency of sleep disruptions, and any impacts of daytime sleepiness. |
| The Hospital Anxiety and Depression Scale (HADS)[40] | The HADS is a 14-item self-report measure of state-based anxiety and depression, assessed along a 4-point Likert scale. The questionnaire demonstrates good validity and reliability in adolescent populations[41] and has been recommended as a mood screening tool in ME/CFS.[42] |
| The Behaviour Assessment System for Children-Third Edition, Parent Rating Scale (BASC-3 PRS)[43] | The BASC-3 PRS is a 173-item parent-rated questionnaire on emotional and behavioural well-being in children and adolescents. Responses are provided along a 4-point Likert scale (ie, never, often, sometimes, almost Always) to capture different dimensions of emotion and behaviour, such as internalising problems, externalising problems and adaptive behaviours. |
| The Behaviour Rating Inventory Of Executive Function-Second Edition (BRIEF-2)[44] | The BRIEF-2 asks parents to rate their child's executive functioning skills across 63 items that encompass domains such as inhibition, working memory and planning and organisation. Responses are provided along a 3-point Likert scale (ie, never, sometimes, often). The BRIEF-2 is only administered to participants aged 12–18 years. |
| Wechsler Intelligence Scale for Children-Fifth Edition: Australian and New Zealand Standardised Edition (WISC-V)[45] | The WISC-V is a clinician-administered battery of tasks for children aged 6–16 years that assess overall intellectual functioning, including its constituent components: verbal intellectual skills, visual-spatial skills, fluid reasoning, working memory and information processing speed. The WISC-V will be administered to participants aged 12–16 years. |
| Wechsler Adult Intelligence Scales-Fourth Edition: Australian and New Zealand Language Adapted Edition (WAIS-IV)[46] | The WAIS-IV is a clinician-administered battery of tasks to assess overall intellectual functioning, which is composed of four domains: verbal intellectual skills, perceptual reasoning, working memory and information processing speed. The WAIS-IV will be administered to participants aged 17–19 years. |

ME/CFS, myalgic encephalomyelitis/chronic fatigue syndrome.

Blood samples will be analysed using whole genome sequencing, MS-based metabolomics and proteomics, nitrogen assays and NMR-based metabolomics. PBMCs will be transfected to become lymphoblasts, which will be grown in $^{13}$C-Glucose and $^{13}$C,$^{15}$N-Glutamine media for 24 hours prior to MS-based metabolomics and nitrogen assays. Urine will be analysed using $^{1}$H NMR metabolomics and nitrogen assays.

### *Biological sample processing*

Four 6 mL sodium heparin vacutainers and one 4 mL serum vacutainers will be collected at Royal Children's

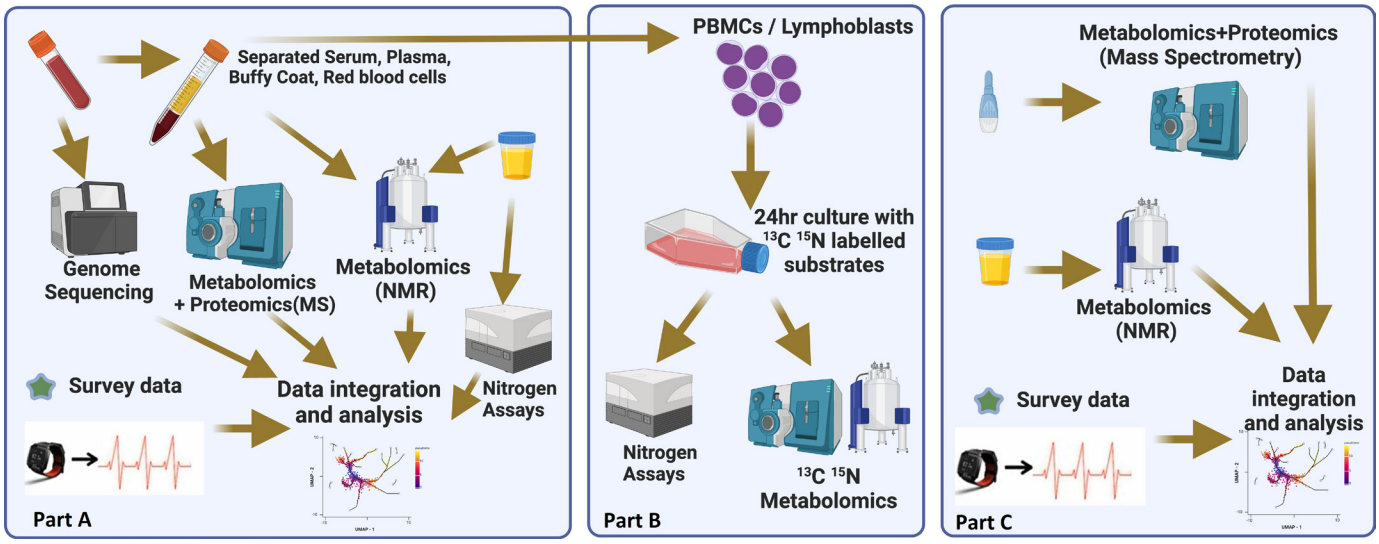


**Figure 2** Overview of the biological experimental workflow. (A) Blood samples collected during the clinical study visit will be processed into serum, plasma, buffy coat, red blood cells and whole blood. These blood fractions will be analysed using whole genome sequencing, Mass Spectrometry (MS) proteomics and both MS and nuclear magnetic resonance (NMR) metabolomics. In addition, urine will be analysed using NMR metabolomics and nitrogen assays. (B) PBMCs will be isolated from the blood collected from the clinical study visit, cultured in labelled media and will be analysed using nitrogen assays, MS and NMR metabolomics. (C) Micro-blood samples and urine collected from the longitudinal study will be analysed using MS proteomics and metabolomics, and NMR metabolomics, respectively. In both (A, C), survey and activity data will be integrated with biological data to perform a multimodal data analysis. Created with BioRender.com. PBMC, peripheral blood mononuclear cell.

Hospital pathology service. These vacutainers will be transferred to the processing laboratory at room temperature (RT) and centrifuged on arrival, avoiding any freezing step prior to processing. The processing protocol is as follows: four 6 mL aliquots of fasted whole blood collected in sodium heparin vacutainers will be centrifuged at 1000×g for 10 min at RT to separate plasma, buffy coat and red blood cells. These will be stored in cryotubes, in approximately three 1 mL aliquots of plasma, two 0.5 mL aliquots of buffy coat and two 0.6 mL aliquots of red blood cells expected per participant. The 4 mL GEL vacutainer will be processed to serum after clotting for 30 min at RT, followed by centrifugation at 1000×g for 10 min at RT, and stored in two 0.6 mL aliquots per participant. Urine samples will be divided into three 1 mL aliquots in 1.5 mL cryotubes. After processing, all samples will be stored at −80°C until analysis. The PaxGene RNA tube will be left at RT for at least 2 hours, then stored at −30°C for 24 hours, and finally at −80°C until analysis, following the manufacturer's instructions.

### *Genomics*

Whole genome sequencing will be performed to identify single nucleotide polymorphisms in the paediatric ME/CFS cohort. Sequencing will be conducted by The Advanced Genomics Collaboration at the University of Melbourne. Briefly, genomic DNA will be extracted from whole blood and sequences will be generated using the NovaSeq 6000 System.[28] Sequence processing, quality control, variant calling and annotation will follow the DRAGEN-GATK pipeline.[29]

### *Quantitative proteomics*

We will use a whole cell label-free method.[30 31] It uses sodium deoxycholate solubilisation, denaturation, alkylation, extraction, offline high-pH fractionation of peptides and sequential analysis of concatenated fractions by online nano-high-performance liquid chromatography (HPLC)-electrospray ionisation-MS/MS on one of several Thermo Scientific Q Exactive Plus instruments.

### *MS-based metabolomics*

Biofluids will be resolved on a ZIC-pHILIC column (5 µm particle size, 150×4.6 mm, Merck SeQuant) connected to an Agilent 1260 (Santa Clara, California, USA) HPLC system. Metabolites will be detected by electrospray ionisation using an Agilent 6545 Q-ToF MS system (Santa Clara, California, USA) in negative ionisation mode. Samples will be analysed in batches according to clinic visits and per individual for the longitudinal study. Metabolite identification will be based on mass, retention time and tandem mass spectrometry (MS/MS) fragmentation patterns, and the relative abundance of metabolites will be obtained using MassHunter Quantitative Analysis B 0.7.00 (Agilent).[32 33]

### *NMR-based metabolomic and proteomics*

A $^{1}$H nuclear magnetic resonance (NMR) protocol was established by members of the research team to monitor the metabolites in blood and urine samples of individuals with ME/CFS.[22 34] A significant advantage of NMR is that it involves minimal and non-destructive sample handling. One and two-dimensional $^{1}$H data of blood and urine samples will be obtained using high sensitivity

NMR spectrometer (700 MHz Bruker Avance NEO spectrometer) equipped with a cryogenically cooled probe that increases the sensitivity of the instrument 3–4 fold, and a cooled auto-sampler (SampleJet) for best sample maintenance. Sample acquisition will follow standard protocols.[22 35]

### Nitrogen Assays

Ammonia, nitrate and nitrite (breakdown products of nitric oxide) cannot be measured by metabolomics techniques. We will, therefore, conduct assays to identify these metabolites in the case–control blood plasma, PBMCs and longitudinal urine samples. The nitrite/nitrate assay is based on nitrate reductase converting nitrate to nitrite, followed by the addition of 2,3-diaminonapthalene, which converts nitrite to a fluorescent compound. The ammonia assay is based on the o-phthalaldehyde method in which the reagent reacts with ammonia/ammonium ion-producing a fluorometric result.[36]

### Clinical and neuropsychological measures

Summary scales and composite indices from clinical and neuropsychological measures that were collected prior to the study visit or on the day of the clinical study visit (refer to table 1 for outline) will be used for subsequent between-groups statistical analyses. Responses to the modified DSQ-Ped, completed as part of the longitudinal component only, will be analysed at an item level.

### Statistical methods

All planned analyses will be conducted using the Statistica, R and Stata software packages. Data will first be cleaned and patterns of missing data will be analysed using Little's Missing Completely at Random (MCAR) Test.[37] For data that is likely missing at random (MAR) or MCAR, the statistical methods will proceed as planned. For data that is neither MAR nor MCAR (ie, missing not at random), joint multiple imputation will be used to impute the missing data. A maximum likelihood estimator will be used for the linear regression and mixed-effects analyses to ensure robust results regardless of the nature of our missing data.

To address the first and second objectives of this study (ie, comparing baseline biological differences between ME/CFS patients and controls), point estimates of the means and SDs of each identified metabolic, proteomic and genomic marker, and the summary variables from the clinical and neuropsychological measures will be reported for both the ME/CFS and control groups. The mean difference between these values and the 95% CI around this mean difference will also be reported. These point estimates will also be generated for males and females in each group to determine whether the group-level differences vary by sex. Separate correlational analyses between subjective clinical, emotional and behavioural well-being symptom ratings and objective cognitive outcomes will also be undertaken.

To investigate the impact of metabolomic, proteomic and genomic markers on neuropsychological functioning and clinical symptomatology, the findings from the previous analyses will be used to inform the independent and dependent variables of separate univariate linear regression models for each marker shown to differ significantly between ME/CFS and control groups. These analyses will be performed for each group and further stratified by sex. The results may also be used to inform the development of provisional classification models based on the strongest biological predictors of clinical difference between adolescents with and without ME/CFS. The classification performance of these models would be evaluated through techniques such as the area under the receiver operating characteristics curve.

A compilation of the rich omics data will be used to develop an enriched pathway analysis. Linear mixed-effects models will be used to observe changes over time in the longitudinal study data, with comparisons of baseline and PEM days being used as a case–control comparison. Principal component analysis, partial least squares regression and hierarchical cluster analysis will be performed to identify biomarkers of significance. Finally, another set of linear mixed-effects models will be generated to explore the temporal relationship between sex, metabolites, physiological parameters and clinical symptomatology (ie, modified DSQ-Ped) in the ME/CFS-only longitudinal arm of this study with appropriate corrections for multiple comparisons.

## DISCUSSION

The onset of paediatric ME/CFS during middle adolescence disrupts a significant period of physical, emotional, social and academic development for many children worldwide. The current absence of reliable diagnostic biomarkers compounds the effect of this illness by prolonging each young person's time for diagnosis and treatment. This protocol paper outlines a novel Australian observational study designed to identify potential diagnostic biomarkers for paediatric ME/CFS through comprehensive biological, clinical and neuropsychological phenotyping. The study ensures a homogeneous patient population by applying both the Canadian Consensus Criteria paediatric case definition and the Paediatric ME/CFS Primer. This approach enhances diagnostic accuracy, reduces misclassification and strengthens the validity of paediatric ME/CFS research, contributing to a clearer understanding of this often-misunderstood condition.

The power of multidisciplinary, multilevel analysis in complex, heterogeneous conditions like ME/CFS lies in its ability to simultaneously explore data across various levels, including peripheral biomarkers, brain imaging, neurocognitive markers and clinical symptoms. Unlike traditional methods, this approach integrates diverse data to uncover interactions that might otherwise be missed. By capturing these nuances, multilevel analysis offers

more accurate insights and better-targeted interventions, ultimately improving patient care.

Furthermore, the longitudinal tracking of biological and clinical symptom changes is, as far as we are aware, the first of its kind within the paediatric ME/CFS population. This approach captures the fluctuating symptoms (and associated biomarkers) characteristic of ME/CFS. By using repeated measures, each participant serves as their own control, allowing for more accurate assessments of the condition's heterogeneous variability. Additionally, biological samples are collected during 'crash' episodes—periods of severe but temporary symptom worsening—enabling comparisons between 'good' and 'bad' days and effectively capturing individual symptom variability.

Consistent with research conducted within other chronic illness populations, there is a risk of missing data and attrition from the in-person clinical study visit and throughout the longitudinal component of the study because of fluctuating symptom levels within the ME/CFS group. The consultation with our advisory committee was an attempt to mitigate as much of this risk as possible, however, it is recognised that ME/CFS remains an unpredictable condition. Additionally, the collection of blood and urine samples by participating children and their families may lead to some variability in the integrity of the samples. Participants and their caregivers will be advised to record any deviations from the protocol, to manage such variability as effectively as possible.

The outcomes of the SPOT-ME study are anticipated to aid in the prognosis of ME/CFS in the development of effective treatments, and ultimately to improve the lives and futures of children with ME/CFS.

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**Contributors** CWA is the guarantor. NT and DT drafted the original manuscript. All authors were involved in the subsequent drafting of this manuscript and reviewing it critically for important intellectual content. Conception of the study and/or substantial contribution to the study design: CWA, NT, PRG, SJK, EKJ and AS. Acquisition of data: TC, DT, NT, KH and CWA. All authors approved the final version of this manuscript to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Funding** This study was supported by an Australian Government National Health and Medical Research Council grant (number 1200292).

**Competing interests** None declared.

**Patient and public involvement** Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

**Patient consent for publication** Not applicable.

**Provenance and peer review** Not commissioned; externally peer reviewed.



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## REFERENCES

1 Carruthers BM, Jain AK, De Meirleir KL, *et al*. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. *J Chronic Fatigue Syndr* 2003;11:7–115.
2 Bakken IJ, Tveito K, Gunnes N, *et al*. Two age peaks in the incidence of chronic fatigue syndrome/myalgic encephalomyelitis: a population-based registry study from Norway 2008-2012. *BMC Med* 2014;12:167.
3 Knight S, Harvey A, Lubitz L, *et al*. Paediatric chronic fatigue syndrome: complex presentations and protracted time to diagnosis. *J Paediatr Child Health* 2013;49:919–24.
4 Cortes Rivera M, Mastronardi C, Silva-Aldana CT, *et al*. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: A Comprehensive Review. *Diagnostics (Basel)* 2019;9:91.
5 Nijhof SL, Maijer K, Bleijenberg G, *et al*. Adolescent chronic fatigue syndrome: prevalence, incidence, and morbidity. *Pediatrics* 2011;127:e1169–75.
6 Tate WP, Walker MOM, Peppercorn K, *et al*. Towards a Better Understanding of the Complexities of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Long COVID. *Int J Mol Sci* 2023;24:5124.
7 Blakemore S-J. The developing social brain: implications for education. *Neuron* 2010;65:744–7.
8 Lacerda EM, Geraghty K, Kingdon CC, *et al*. A logistic regression analysis of risk factors in ME/CFS pathogenesis. *BMC Neurol* 2019;19:275.
9 Thomas N, Gurvich C, Huang K, *et al*. The underlying sex differences in neuroendocrine adaptations relevant to Myalgic Encephalomyelitis Chronic Fatigue Syndrome. *Front Neuroendocrinol* 2022;66:100995.
10 Ausubel G. Theory and problems of adolescent development: iuniverse. 1954.
11 Deumer U-S, Varesi A, Floris V, *et al*. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS): An Overview. *J Clin Med* 2021;10:4786.
12 Rowe PC, Underhill RA, Friedman KJ, *et al*. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome Diagnosis and Management in Young People: A Primer. *Front Pediatr* 2017;5:121.
13 Josev EK, Cole RC, Scheinberg A, *et al*. Health, Wellbeing, and Prognosis of Australian Adolescents with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS): A Case-Controlled Follow-Up Study. *J Clin Med* 2021;10:3603.
14 Rowe KS. Long Term Follow up of Young People With Chronic Fatigue Syndrome Attending a Pediatric Outpatient Service. *Front Pediatr* 2019;7:21.
15 Knight SJ, Politis J, Garnham C, *et al*. School Functioning in Adolescents With Chronic Fatigue Syndrome. *Front Pediatr* 2018;6:302.
16 Mackay A, Tate WP. A compromised paraventricular nucleus within a dysfunctional hypothalamus: A novel neuroinflammatory paradigm for ME/CFS. *Int J Immunopathol Pharmacol* 2018;32:2058738418812342.
17 Dibble JJ, McGrath SJ, Ponting CP. Genetic risk factors of ME/CFS: a critical review. *Hum Mol Genet* 2020;29:R117–24.
18 Albright F, Light K, Light A, *et al*. Evidence for a heritable predisposition to Chronic Fatigue Syndrome. *BMC Neurol* 2011;11:62:1–6:.

19 Underhill RA, O'gorman R. Prevalence of Chronic Fatigue Syndrome and Chronic Fatigue Within Families of CFS Patients. *J Chronic Fatigue Syndr* 2006;13:3–13.
20 Germain A, Ruppert D, Levine SM, *et al*. Prospective Biomarkers from Plasma Metabolomics of Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome Implicate Redox Imbalance in Disease Symptomatology. *Metabolites* 2018;8:90.
21 Germain A, Ruppert D, Levine SM, *et al*. Metabolic profiling of a myalgic encephalomyelitis/chronic fatigue syndrome discovery cohort reveals disturbances in fatty acid and lipid metabolism. *Mol Biosyst* 2017;13:371–9.
22 Armstrong CW, McGregor NR, Lewis DP, *et al*. Metabolic profiling reveals anomalous energy metabolism and oxidative stress pathways in chronic fatigue syndrome patients. *Metabolomics (Los Angel)* 2015;11:1626–39.
23 Annesley SJ, Missailidis D, Heng B, *et al*. Unravelling shared mechanisms: insights from recent ME/CFS research to illuminate long COVID pathologies. *Trends Mol Med* 2024;30:443–58.
24 Jason LA, Jordan K, Miike T, *et al*. A Pediatric Case Definition for Myalgic Encephalomyelitis and Chronic Fatigue Syndrome. *J Chronic Fatigue Syndr* 2006;13:1–44.
25 Jason LA, Sunnquist M. The Development of the DePaul Symptom Questionnaire: Original, Expanded, Brief, and Pediatric Versions. *Front Pediatr* 2018;6:330.
26 Harris PA, Taylor R, Thielke R, *et al*. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009;42:377–81.
27 Harris PA, Taylor R, Minor BL, *et al*. The REDCap consortium: Building an international community of software platform partners. *J Biomed Inform* 2019;95:103208.
28 Modi A, Vai S, Caramelli D, *et al*. The Illumina Sequencing Protocol and the NovaSeq 6000 System. *Methods Mol Biol* 2021;2242:15–42.
29 DePristo MA, Banks E, Poplin R, *et al*. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet* 2011;43:491–8.
30 Stroud DA, Surgenor EE, Formosa LE, *et al*. Accessory subunits are integral for assembly and function of human mitochondrial complex I. *Nature New Biol* 2016;538:123–6.
31 Lake NJ, Webb BD, Stroud DA, *et al*. Biallelic Mutations in MRPS34 Lead to Instability of the Small Mitoribosomal Subunit and Leigh Syndrome. *Am J Hum Genet* 2017;101:239–54.
32 Bartho LA, Keenan E, Walker SP, *et al*. Plasma lipids are dysregulated preceding diagnosis of preeclampsia or delivery of a growth restricted infant. *EBioMedicine* 2023;94:104704:104704:.
33 Bartho LA, McKeating DR, Walker SP, *et al*. Plasma metabolites are altered before and after diagnosis of preeclampsia or fetal growth restriction. *Sci Rep* 2024;14:15829.
34 Armstrong CW, McGregor NR, Lewis DP, *et al*. The association of fecal microbiota and fecal, blood serum and urine metabolites in myalgic encephalomyelitis/chronic fatigue syndrome. *Metabolomics (Los Angel)* 2017;13:8.
35 Dona AC, Jiménez B, Schäfer H, *et al*. Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. *Anal Chem* 2014;86:9887–94.
36 Rehman MZ, Melamed M, Harris A, *et al*. Urinary Ammonium in Clinical Medicine: Direct Measurement and the Urine Anion Gap as a Surrogate Marker During Metabolic Acidosis. *Adv Kidney Dis Health* 2023;30:197–206.
37 Little RJA. A Test of Missing Completely at Random for Multivariate Data with Missing Values. *J Am Stat Assoc* 1988;83:1198–202.
38 Varni JW, Burwinkle TM, Katz ER, *et al*. The PedsQL in pediatric cancer. *Cancer* 2002;94:2090–106.
39 Buysse DJ, Reynolds CF III, Monk TH, *et al*. The Pittsburgh sleep quality index: A new instrument for psychiatric practice and research. *Psychiatry Res* 1989;28:193–213.
40 Zigmond AS, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983;67:361–70.
41 White D, Leach C, Sims R, *et al*. Validation of the Hospital Anxiety and Depression Scale for use with adolescents. *Br J Psychiatry* 1999;175:452–4.
42 Deale A, Husain K, Chalder T, *et al*. Long-term outcome of cognitive behavior therapy versus relaxation therapy for chronic fatigue syndrome: a 5-year follow-up study. *Am J Psychiatry* 2001;158:2038–42.
43 Reynolds CR, Kamphaus RW. Behaviour Assessment System for Children3rd ed. Bloomington, MN: Pearson, 2015.
44 Gioia GA, Isquith PK, Guy SC, *et al*. Behaviour Rating Inventory of Executive Function2nd ed. Lutz, FL: PAR Inc, 2015.
45 Wechsler D. Wechsler Intelligence Scale for Children, Fifth Edition: Australian and New ZealandStandardised Edition. Australia: Pearson, 2016.
46 Wechsler D. Wechsler Adult Intelligence Scale - Fourth Edition: Australian and New Zealand Language Adapted Edition. Australia: Pearson, 2008.