Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS): Clinical trials, medical treatment and pathomechanisms

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Scientific environment

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Ingrid Gurvin Rekeland, December 2023

Abstract in English

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is an under-researched disease affecting 0.2-0.8 % of the population, of unknown aetiology, with high symptom burden, no validated specific and sensitive biomarker, and no standard approved effective treatment. The interest in ME/CFS in our cancer ward started in 2007, with observations of several patients with long-standing ME/CFS who got cancer and who independently reported that the cancer drug treatment had beneficial effects on their ME/CFS symptoms. The treatments included the cytotoxic drug cyclophosphamide and/or the monoclonal B-cell–depleting anti-CD20 antibody rituximab. The observations led to the working hypothesis that ME/CFS in a subgroup could be a variant of an autoimmune disease, with an immunological trigger, often with a post-infectious onset and with a role for B-cells/plasma cells and antibodies. The ME/CFS research group at Haukeland University Hospital have worked for 15 years trying to elucidate pathomechanisms, discover biomarkers and perform clinical trials to assess possible treatments.

In this project, we aimed to assess the therapeutic potential and possible side effects of rituximab and cyclophosphamide in two clinical trials, RituxME and CycloME (papers II and III). The placebo-controlled phase III RituxME study with 151 patients did not show a significant clinical benefit from rituximab compared to placebo. The open-label study with cyclophosphamide, CycloME, with 40 included patients, showed a beneficial effect on ME/CFS symptoms after treatment with a response rate of 55%. The 6-year follow-up study (Paper V) of both RituxME and CycloME showed that a significant number of patients treated with cyclophosphamide reported sustained, clinically meaningful improvement after 6 years. As part of the rituximab investigations, we retrospectively measured rituximab concentrations and anti-drug antibodies (ADAs) in serum samples from patients enrolled in a previous open-label phase II rituximab maintenance study (KTS-2-2010) to investigate possible associations with clinical and biochemical data (Paper I). We did not find that rituximab concentration and kinetics were significantly associated with patient symptoms or response in the trial. Finally, we investigated the combined use of

activity monitoring and patient-reported outcome measures (PROMs) in a cohort of ME/CFS patients with no intervention, to assess symptom variation over time, attempting to develop new non-invasive tools to monitor patients and to improve future outcome measures in a trial context (Paper IV).

The effect of an immunomodulatory drug on symptoms supports the underlying hypothesis that in a subgroup of patients the immune system is involved in the pathomechanisms of ME/CFS.

Abstract in Norwegian

Myalgisk encefalomyelitt/kronisk utmattelsessyndrom (ME/CFS) er en underprioritert sykdom som rammer 0,2-0,8 % av befolkningen, med ukjent etiologi, høy symptombelastning, ingen biomarkør, og ingen godkjent effektiv behandling. Interessen for ME/CFS ved vår kreftavdeling startet i 2007, da vi observerte flere pasienter med langvarig ME/CFS som fikk kreft, og som uavhengig av hverandre rapporterte at kreftbehandlingen hadde gunstig effekt på ME/CFS-symptomene. Behandlingene inkluderte cellegiften cyklofosfamid og/eller det monoklonale anti-CD20-antistoffet rituksimab. Observasjonene førte til arbeidshypotesen om at ME/CFS i en undergruppe kan være en variant av en autoimmun sykdom, utløst av en infeksjon eller annet immunologisk stimuli, og med B-celler/plasmaceller og antistoffer som viktige i sykdomsmekanismen. ME/CFS-forskningsgruppen ved Haukeland universitetssykehus har i 15 år arbeidet med å belyse sykdomsmekanismer og finne biomarkører, og har gjennomført kliniske studier for å finne mulig medikamentell behandling.

I dette prosjektet testet vi det terapeutiske potensialet og mulige bivirkninger, til rituksimab og cyklofosfamid i to kliniske studier, RituxME og CycloME (artikkel II og III). Den placebokontrollerte fase III-studien RituxME med 151 pasienter viste ingen signifikant klinisk nytte av rituksimab sammenlignet med placebo. Den åpne studien med cyklofosfamid, CycloME, med 40 inkluderte pasienter, viste en gunstig effekt på ME/CFS-symptomer etter behandling, med en responsrate på 55 %. Den seksårige oppfølgingsstudien (artikkel V) av både RituxME og CycloME, viste at et betydelig antall pasienter behandlet med cyklofosfamid rapporterte vedvarende, klinisk meningsfull forbedring etter seks år. Som en del av rituksimab-undersøkelsene målte vi retrospektivt rituksimab-konsentrasjoner og antistoffer mot legemidler (ADA) i serumprøver fra pasienter som deltok i en tidligere vedlikeholdsstudie med rituksimab (KTS-2-2010) for å undersøke mulige sammenhenger med kliniske og biokjemiske data (artikkel I). Vi fant ingen sammenheng mellom rituksimab-konsentrasjon og pasientenes symptomer eller respons i studien. I artikkel IV undersøkte vi bruk av aktivitetsarmbånd (Fitbit) i

kombinasjon med pasientrapporterte utfallsmål (PROMs) i en kohort av ME/CFS-pasienter uten intervensjon. Målet var å kartlegge symptomvariasjoner over tid, og forsøke å utvikle objektive verktøy for å følge pasienter og forbedre fremtidige utfallsmål i kliniske studier.

Gunstig effekt på ME/CFS-symptomene av cyclofosfamid, et immunmodulerende medikament, støtter den underliggende hypotesen om at immunsystemet er involvert i sykdomsmekanismen hos en undergruppe av ME/CFS pasientene.

List of Publications

Paper I
***Rekeland IG**, Fluge Ø, Alme K, Risa K, Sørland K, Mella O, deVries A, Schjøtt J*
Rituximab serum concentrations and anti-rituximab antibodies during B-cell depletion therapy for Myalgic Encephalopathy/Chronic Fatigue Syndrome.
Clin Ther 2019 May;41(5):806-814. Epub 2018 nov 28
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Paper II
*Fluge Ø, **Rekeland IG,** Lien K, Thürmer H, Borchgrevink Pc, Schäfer C, Sørland K, Assmus J, Ktoridou-Valen I, Herder I, Gotaas ME, Kvammen Ø, Baranowska KA, Bohnen LMLJ, Martinsen SS, Lonar AE, Solvang AH, Gya AES, Bruland O, Risa K, Alme K, Dahl O, Mella O*
B-Lymphocyte depletion in patients with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: A randomized, double-blind, placebo-controlled trial.
Ann Intern Med 2019 05 07;170(9):585-593. Epub 2019 apr 2
PMID: 30934066

Paper III
***Rekeland IG**, Fosså A, Lande A, Ktoridou-Valen I, Sørland K, Holsen M, Tronstad KJ, Risa K, Alme K, Viken MK, Lie BA, Dahl O, Mella O, Fluge Ø*
Intravenous cyclophosphamide in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. An open-label phase II study.
Front Med (Lausanne) 2020;7():162. Epub 2020 apr 29
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Paper IV
***Rekeland IG**, Sørland K, Bruland O, Risa K, Alme K, Dahl O, Tronstad KJ, Mella O, Fluge Ø,*
Activity monitoring and patient-reported outcome measures in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome patients.
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Paper V
***Rekeland IG**, Sørland K, Neteland LL, Fosså A, Alme K, Risa K, Dahl O, Tronstad KJ, Mella O, Fluge Ø,*
Six-year follow-up of participants in two clinical trials of rituximab or cyclophosphamide in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome
Submitted

Contents

Abbreviations

ADAs	Anti-drug Antibodies
AE	Adverse Events
BAFF	B-cell activating factor
CBT	Cognitive behavioural therapy
CFS	Chronic Fatigue Syndrome (CFS)
CRPS	Complex regional pain syndrome
COMPASS-31	Composite Autonomic Symptom Score-31
COVID-19	The corona virus disease 2019
CPET	Cardiopulmonary exercise testing
CTCAE	Common Terminology Criteria for Adverse Events
DSQ-SF	DePaul Symptom Questionnaire Short Form
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
FM	Fibromyalgia
FMD	Flow-mediated dilation
FMT	Faecal microbiota transplantation
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
FSS	Fatigue Severity Scale
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation
GET	Graded exercise therapy
GLM	General linear model
GWAS	Genome-wide association study
HADS	Hospital Anxiety and Depression Scale
HHV	Human Herpes virus
HLA	Human Leukocyte Antigen
HUH	Haukeland University Hospital
iCPET	Invasive cardiopulmonary exercise testing

IGHV genes	Immunoglobulin heavy chain variable region genes
IOM	The US Institute of Medicine
LDN	Low dose Naltrexone
mAChRs	Muscarinic acetylcholine receptors
ME	Myalgic Encephalomyelitis
ME/CFS	Myalgic Encephalomyelitis/Chronic Fatigue Syndrome
MS	Multiple Sclerosis
NICE	The British National Institute for Health and Care Excellence
NK-cell	Natural killer cell
NO	Nitric Oxide
OUH	Oslo University Hospital
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PEM	Post exertional malaise
PORH	Post-occlusive reactive hyperemia
POTS	Postural orthostatic tachycardia syndrome
PROMs	Patient reported outcome measures
SARS-CoV-2	The severe acute respiratory syndrome coronavirus 2
SEID	Systemic exertion intolerance disease
SF-36	Short Form-36
SF-36 PCS	SF-36 physical component summary
SF-36 PF	SF-36 physical function subscale
sGC	Soluble guanylate cyclase
SLE	Systemic Lupus erythematosus
TRPM3	Transient Receptor Potential Melastatin 3
UNN	University hospital of northern Norway
WASF3	Wiskott-Aldrich Syndrome Protein Family Member 3
α1AR	α1 adrenergic receptors
ß1AR	ß1 adrenergic receptors
ß2AR	ß2 adrenergic receptors

1 Introduction to ME/CFS

1.1 History

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disease of unknown aetiology characterized by severe fatigue and post-exertional malaise (PEM), cognitive disturbances, pain, sleep problems, sensory hypersensitivity and several symptoms related to immune and autonomic function.

"Chronic fatigue" or "chronic fatigue syndrome" is not a new medical condition. During the last centuries there have been different names describing clinically similar conditions. "Febricula" is known from the 17th century and "Neurasthenia" from the 19th century. "DaCosta's syndrome" was first described in 1871, and during the first decades of the 20th century DaCosta's syndrome was called the effort syndrome, neurocirculatory asthenia, and the autonomic imbalance syndrome. The syndrome described exhaustion in soldiers; with fatigue, breathlessness, palpitations, dizziness, chest pain, headaches, digestive disturbances and sleep difficulties (1). Several outbreaks of "epidemic neuromyasthenia" were described from different parts of the world during the 19th century (2, 3). Two outbreaks were described in the military in Switzerland in the late 1930s, three different outbreaks in Iceland from 1948 to 1949 related to an epidemic of poliomyelitis (of which the Akureyri outbreak is best known), and one outbreak in Adelaide, Australia 1949-1951, also after poliomyelitis. In the United States, several outbreaks were reported around 1950, with students, nurses and hospital staff describing symptoms quite similar to the criteria used for ME/CFS (2). The term "benign myalgic encephalomyelitis" was first used to describe an outbreak amongst doctors and nurses at the Royal Free Hospital in 1955 (4). This name has been disputed by clinicians, with the argument that the course of the disease is not benign but disabling for the patients, and that encephalomyelitis is often a specific and potentially lethal neuro-pathological process.

In recent literature, the terms chronic fatigue syndrome (CFS) and myalgic encephalomyelitis (ME) are often used together in the umbrella term ME/CFS, and this is the name used in this thesis.

In 1970, two psychiatrists in the United Kingdom reviewed the reports of 15 outbreaks of "benign myalgic encephalomyelitis" and concluded that these were the results of a psychosocial phenomenon or "mass hysteria" and suggested the name "myalgia nervosa" for this "functional" disorder (5). This proposed psychological etiology created great controversy. However, in the last 10 years, there has been some movement in the field towards greater agreement on the disease with a biomedical focus.

The US Institute of medicine (IOM) published a large report about ME/CFS in 2015: "Beyond Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: Redefining an Illness" (3). The primary message of the committee's report was "that ME/CFS is a serious, chronic, complex, systemic disease that often can profoundly affect the lives of patients". The report suggests a new name: "Systemic exertion intolerance disease" (SEID), and new diagnostic criteria. The IOM report concludes that PEM is a key symptom that can differentiate ME/CFS from other diseases, that there is sufficient evidence to support the existence of an immune dysfunction, and the assumption that ME/CFS can be triggered by an infection.

In 2021, The British National Institute for Health and Care Excellence (NICE) published its updated guidelines for ME/CFS. NICE concluded that the psychological approach to treatment with cognitive behavioural therapy (CBT) is only an adjunctive and not a curative treatment, and that graded exercise therapy (GET) is possibly harmful and should not be used regularly as treatment for ME/CFS (6).

In Norway there is still an ongoing debate, both on aetiology and about treatment recommendations. The climate of debate among clinicians in the media has not been constructive and is unlikely to benefit patients. The Norwegian research institutes SINTEF and Fafo have in recent years performed a large study among 660 people with a diagnosis of ME/CFS, exploring their experience with the Norwegian health

and social security system (7). They concluded that patients experience a public system that has too little knowledge about the disease and offers treatments that are of little benefit and sometimes have negative effects. For example, most patients reported being sicker after rehabilitation and work assessment programmes. The authors concluded that this study should serve as a reminder to decision-makers of the "do no harm" principle for this vulnerable group of patients, until new research reveals alternative treatment strategies (7).

Studies show a significant gender gap in ME/CFS, with 3 out of 4 patients being women. Notably, this ratio mirrors other immuno-inflammatory diseases such as systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis and Sjögren's syndrome. While there is limited research on the gender and sex-specific aspects of ME/CFS, discernible differences between men and women have been reported in areas such as clinical characteristics, age, quality of life and triggering factors (8, 9). Historically, ME/CFS has been stigmatised, particularly in the context of changing societal views on gender. In the medical literature, long-term fatigue has been transformed from a somatic condition caused by modern civilisation to a self-inflicted psychiatric condition. At the same time, it changed from a high-status male condition to a low-status female condition (10). This highlights the need to dissociate ME/CFS from status and gender stereotypes and to broaden our medical understanding of the disease.

1.2 Diagnosis

Over the past few decades, different criteria and guidelines for ME/CFS have been used in both clinical and research settings (3, 11-13). The range of diagnostic criteria has presented a challenge to both clinicians and researchers and has impeded the comparison of results across different studies. The main symptoms are profound fatigue, PEM (14, 15), sleep disturbances with inadequate restitution (16), pain and sensory hypersensitivity, orthostatic intolerance, cognitive difficulties and several other symptoms.

The Fukuda case definition for CFS (1994) (11) requires prolonged or chronic fatigue that persists or relapses for ≥ 6 months and four or more of the following symptoms concurrently present for ≥ 6 months: impaired memory or concentration, sore throat, tender cervical or axillary lymph nodes, muscle pain, multi-joint pain, new headaches, unrefreshing sleep or PEM. PEM is not mandatory in this definition.

The Canadian criteria for ME/CFS (2003) made by Carruthers et al. (13), with minor changes in the revised version of 2011 (12), have four required symptoms: fatigue, PEM and/or fatigue, sleep dysfunction and pain. In addition, two or more neurological and/or cognitive manifestations are required, as well as at least one symptom from two of the following categories: autonomic, neuroendocrine or immune. The illness must have lasted ≥ 6 months, and the revised version specifies that symptoms may not be "lifelong". Both the International Consensus Criteria for ME (2011) penned by Carruthers and colleagues (12), and NICE clinical guidelines for ME/CFS (2021) (6) are quite similar to the original Canadian criteria (2003) (13). All criteria include a list of exclusionary conditions. The report from IOM in 2015 also included a set of criteria (3). Three symptoms are mandatory: reduction in function level more than 6 months (not lifelong), PEM and unrefreshing sleep. One of the two following symptoms are also required: cognitive impairment or orthostatic intolerance. Both the revised Canadian criteria, NICE criteria and IOM criteria have PEM as a mandatory criterion.

Case definition is an important issue, and stringent criteria are necessary to differentiate ME/CFS from general fatigue, which is a prevalent symptom in the general population, affecting at least ten times more people than ME/CFS (17).

We have used the Canadian consensus criteria (2003) in Paper II-IV (18-20). The first paper in this thesis, concerning the analysis of rituximab concentrations (21) used samples from a previous phase II trial assessing rituximab maintenance treatment (22). Although the Fukuda criteria were used as inclusion criteria in this study, retrospective analysis confirmed that the patients included also met the Canadian criteria.

Table 1. Summary of the Canadian Consensus Criteria (CCC) for myalgic encephalomyelitis (ME/CFS) (2003)

Required symptoms:
The criteria define persistent or recurring chronic fatigue as lasting for over 6 months, but not a lifetime. • Fatigue or exhaustion • Post-exertional malaise • Unrefreshing sleep • Pain (or discomfort), myalgia, arthralgia and/or headaches (often of new type, pattern, or severity)
Two or more neurological/cognitive manifestations are required:
• Impaired short-term memory (confusion) • Impaired vision • Hypersensitivity • Photophobia • Ataxia • Muscle weakness/muscle twitches.
At least one symptom from two of the following three categories:
• Autonomic: orthostatic intolerance, POTS • Neuroendocrine: thermolability, bladder dysfunction • Immune: tender lymph nodes, recurrent flu-like symptoms

1.3 Post Exertional Malaise (PEM)

PEM is a hallmark symptom of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). PEM is an exacerbation of some or all symptoms after physical or mental exertion. Typically, the patients experience increasing fatigue, malaise, dizziness and nausea, with flu-like symptoms, pain and cognitive dysfunction. Patients describes PEM as a post-exertional "crash," "exhaustion," "flare-up," "collapse," "debility," or "setback." (3). Deterioration can be delayed by hours or days, and often lasts for days to weeks, and sometimes months. (3). Studies using repeated cardiopulmonary exercise testing (CPET) on consecutive days show that previous exercise negatively affects oxygen uptake and exertion effort in ME/CFS the second day (23-25), and that ME/CFS subjects needed an average of about two weeks to recover from a two-day CPET, whereas sedentary controls needed only two days (26).

PEM is one of the core symptoms both in the NICE guideline (6), the diagnostic SEID criteria from the IOM report (3), and the "Canadian" or International Consensus Criteria (12, 13).

1.4 Severity of ME/CFS

Severity definitions vary somewhat between studies in the literature. In our studies, we have used a set of criteria classifying mild to very severe disease based on examples of different function levels (full version provided in supplementary to Paper II). According to National Institutes of Health (NIH) guidelines, activity levels are reduced by at least 50% compared to pre-symptom levels, even in the mild group. People with mild ME/CFS care for themselves and may do some light housework. People with moderate ME/CFS have usually given up work or education, need periods of rest and are usually housebound. People with severe ME/CFS are unable to do any activity for themselves or can only do minimal daily tasks. They may spend most of their time in bed and are often very sensitive to light and sound. People with very severe ME/CFS are confined to bed all day and depend on care for all daily tasks. Some even need tube feeding. (6). Patients with very severe disease are often

not enrolled in clinical trials because it is difficult to transport them to a trial centre, and the physical strain of participating in a trial and/or transportation can cause a severe deterioration of their symptoms.

1.5 Epidemiology and prognosis

1.5.1 Epidemiology

ME/CFS has profound impact on quality of life for patients and their caretakers (27-29) and the socio-economic costs are very high (3).

Different studies have estimated that ME/CFS affects 0.1-0.8% of the population (12, 17, 30, 31). Carruthers et al (12) use an estimated prevalence of 0.2% based on the Fukuda criteria (11). In 2011 Nacul and coworkers estimated that the minimum prevalence rate of ME/CFS was 0.2% meeting any case definition, and 0.1% for the Canadian consensus definition. They based their calculations on 143.000 individuals aged 18-64 years registered by primary care services in three regions of England (17). In 2019, Valdez and coworkers presented data from a large database of medical and facility claims from commercially insured patients in the U.S. and estimated the prevalence of ME/CFS to be 0.85% or roughly 2.8 million patients in the U.S. (30). In 2022, Orji and coworkers published a large study from Australia with more than 2.2 million patients from general practice and estimated the prevalence to be 0.09 to 0.14%. The authors expected these rates to underestimate the true prevalence of ME/CFS in the Australian general practice setting. They also pointed out that the timing of the study was important (2015-2019) as it was before Long Covid and other sequelae of Covid-19 infection (31).

1.5.2 Prognosis and natural course of disease

There is limited literature describing prognosis and disease course over time. An epidemiologic study from Norway found two age peaks in the incidence of ME/CFS, with the first peak at age 10 to19 and the second at age 30 to 39 (32). A study describing onset and disease course of ME/CFS in a United States-based cohort with 150 patients, found that 59% described a fluctuating course over time, while 4%

reported steadily improving symptoms (33). A study from an outpatient clinic in France describing 168 patients with ME/CFS, reported full recovery in 8.3% and improvement in 4.8%, in total 13.1%. The study was retrospective, and the patients were followed from one to nine years. The authors concluded that the prognosis is generally poor (34). A systematic review from 2005 included 14 studies of participants with CFS and found, with a broad range between studies, that a median 5% had a full recovery, while a median 39.5% experienced some improvement over a time span of one to five years (35). In 2020, the Norwegian ME association performed an internet-based questionnaire survey of ME/CFS patients. The 5822 participants classified the course of their disease up to the time of participation: 12% reported improvement, 23% a stable course, 29% large variations, and 35% worsening. Only 2% reported a full recovery. Irrespective of the overall pattern of disease, the majority described a disease course characterized by variation over time (36). Such online surveys may underestimate the degree of recovery over time, as patients who recovered may be less interested in following the ME/CFS-related internet pages and social media groups used to promote the survey.

1.6 Etiology

1.6.1 Genetics

The presence of an inherited component in ME/CFS is supported by an increased risk of ME/CFS among relatives (13, 37, 38). A recent review concluded that a large genome-wide association study (GWAS) is the best way to determine a putative genetic association in ME/CFS and understand the aetiology (39). The challenge is the large number of participants needed to conduct an adequately powered GWAS. They conclude that at least 10.000 participants and an equal or greater number of healthy controls are needed. DecodeME is an ongoing genomic study recruiting up to 25.000 people in the UK with a clinical diagnosis of ME/CFS (40). Questionnaires are completed online or on paper and participants' saliva DNA samples are acquired by post. Genetic data and all relevant documents will be available online, via open access. The results of this large genomic study are awaited. There is some evidence

for a genetic predisposition in ME/CFS (41, 42). The immunologically important human leukocyte antigen (HLA) genes have previously been studied in small ME/CFS cohorts, and certain class II alleles have been found more prevalent (43, 44). A recent study of a larger Norwegian cohort of patients and controls identified two potential HLA risk alleles, namely HLA-C*07:04 and HLA-DQB1*03:03 (45). This study was part of Asgeir Lande's PhD thesis.

1.6.2 Post infectious onset – post viral

Several findings support an immunological mechanism in a subset of ME/CFS patients. Different studies report infectious onset in 60-75% of the cases (33), in line with our studies (18, 19, 22) . Epstein-Barr virus (EBV), Human Herpes virus (HHV-6), Human Parvovirus B19, Enterovirus, Chlamydia and Mycoplasma pneumonia are the most frequently reported triggers of the disease (12, 46). In a study of CFS risk after infectious mononucleosis among adolescents, 13%, 7%, and 4% met the Fukuda diagnostic criteria for CFS after 6, 12, and 24 months, respectively (47). Parasites such as Giardia Lamblia were reported to be a trigger for chronic fatigue, as described ten years after an outbreak in Norway in 2004 (48). A recent review links prior outbreaks of ME/CFS to enteroviruses (49). The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the corona virus disease 2019 (COVID-19) pandemic, and even patients with asymptomatic or mild disease have experienced fatigue, dyspnoea, cognitive impairment, and other symptoms lasting for months after infection. These symptoms of long COVID syndrome are similar and sometimes overlapping with ME/CFS (50, 51). A study from Charite university in Germany, published in 2022, showed that 50% of long Covid patients fulfilled the Canadian Consensus Criteria for ME/CFS (52). A study from the same university hospital showed associations with autoimmune-related gene variants among ME/CFS patients with an infectious onset (53).

A recent review describes different post-acute infection syndromes, both viral and non-viral. Examples of viruses are SARS-CoV-2, Ebola, Dengue, Polio, SARS, EBV, influenzae and others (54). This review offers four possible explanations for the chronic post-infectious state; (I) Chronic stimulation of the immune system as a result

of persistent infection or persistent unviable pathogen structures; (II) Immune activation targeting self-antigens either through infection-induced impairment of regulatory T (Treg) cell function, molecular mimicry, or other mechanisms; (III) Chronic dysregulation of the microbiota–gut–brain axis, and (IV) In some syndromes the symptoms can be explained by permanent organ damage (54) which is unusual in ME/CFS.

1.6.3 Infections and autoimmunity

The link between infections and autoimmune diseases is well established; EBV and SARS-CoV-2 are examples of viruses associated with autoimmunity (55, 56). The hyperstimulated state of the immune system triggered by such viruses, in a genetically predisposed individual, may trigger an autoimmune disease. Several systemic autoimmune diseases have been associated with EBV, best documented for multiple sclerosis (MS) and systemic erythematosus (SLE) (56, 57). Also, lymphoma might develop as a consequence (55, 58). Elderly patients with ME/CFS have an increased risk of B-cell lymphomas indicating a chronically activated B-cell system, and especially marginal zone lymphomas, which are known to be associated with autoimmunity and chronic infections (59). There are several studies with findings supporting B-cell involvement; skewed B-cell receptor gene usage and an upregulation of specific immunoglobulin heavy chain variable region (IGHV) genes that correlated to infection at onset (60), and increased serum B-cell activating factor (BAFF) (61).

1.6.4 Autoantibodies in ME/CFS and other autoimmune diseases

Based on the available literature and the empirical observations that immune-modulatory treatments may be beneficial for patients, our research group has established a pathomechanistic model suggesting that ME/CFS in a subset of patients is associated with a pattern of autoantibodies that appear after triggers such as systemic infections (62). Such anti-self antibodies may have a beneficial and protective role combating infections, but may also be associated with the development of autoimmunity, as described for other autoimmune diseases (63). A

study from Wang and coworkers from 2021 showed that SARS-CoV-2 induced a rapid increase and high prevalence of autoantibodies against many different immunomodulatory proteins in healthy individuals, implicating a variety of immune pathways (64). Thus, we suspect that the autoantibodies in ME/CFS are not complement-activating, nor associated with the histologic inflammation and tissue damage that are seen in many classic autoimmune diseases. Instead, the antibodies may be functional, either agonistic or antagonistic, naturally occurring autoantibodies persisting beyond the expected time course after the initial infection. Endogenous self-reactive autoantibodies targeting G-protein coupled receptors (GPCR) may be involved (65). A report from an international symposium on autoantibodies concluded that GPCR autoantibodies are also present in healthy individuals and may be involved in regulatory networks associated with different physiological states and diseases, including infectious (Covid-19), autoimmune and other diseases (66). Many GPCR autoantibodies are allosteric modulators and exhibit a broad range of pharmacological properties, altering both receptor signalling and trafficking. The relative new knowledge about GPCR autoantibodies and their function is important in different diseases, but also interesting as a potential target for therapeutic interventions to modulate GPCR signalling (65).

Agonistic autoantibodies to ß2 adrenergic receptors (ß2AR) and muscarinic (mAChR) 3 receptors have been demonstrated in orthostatic hypotension (67), a characteristic symptom frequently seen in ME/CFS. In postural orthostatic tachycardia syndrome (POTS), which also affects a subgroup of ME/CFS patients, an autoimmune basis has been suggested by the presence of several functional autoantibodies towards GPCRs affecting blood pressure and heart rate regulation, such as antibodies to α1AR, ß1AR and ß2AR (68). The role of autoantibodies in ME/CFS is not completely understood, but studies have shown elevated levels of antibodies to adrenergic and muscarinic receptors (69, 70), as was also the case in samples from patients in one of our early rituximab trials (22) published in the study by Loebel et al (69). Interestingly, a study showed improvement of ME/CFS symptoms after immunoadsorption to remove antibodies (71), and this has also been demonstrated in long Covid (72).

The observations described above, and several other studies, support that immune dysregulation and autoimmunity are possible pathomechanistic factors in a subgroup of ME/CFS patients (73-75). A review (46) article summarizes data indicating autoimmunity as an aetiological factor.

1.6.5 Endothelial dysfunction and cardiovascular dysfunction

ME/CFS involves a broad spectrum of symptoms affecting many organ systems. What might be the target of the autoimmune response? The immune system itself is one possible explanation, with autoantibodies against many immunomodulatory proteins, as described shortly after SARS CoV-2 infection (64). The vascular system connects all organs of the body and is also a possible target. It is possible that an autoimmune mechanism can affect the autonomic control of blood vessel tone and flow autoregulation. Many of the characteristic symptoms may result from an antibody-mediated functional disturbance in blood flow autoregulation, causing tissue hypoxia on exertion. There is increasing evidence of endothelial dysfunction and cardiovascular symptoms in ME/CFS (76-82). In patients from the RituxME and CycloME studies, we found reduced Flow-mediated dilation (FMD) and Post-occlusive reactive hyperaemia (PORH) compared to healthy controls (76, 82). Endothelial dysfunction has been demonstrated by other groups (80, 83), and was recently also demonstrated in long Covid (84).

The overlap between ME/CFS and POTS has been reported in several studies (85, 86). Van Campen and coworkers found that 86% of ME/CFS patients had orthostatic intolerance symptoms during daily life, and 90% had abnormal cerebral blood flow during a 30 min head-up tilt table test (87). The same group showed that severely ill patients had a significant reduction in cerebral blood flow provoked only by sitting upright, and patients previously diagnosed with POTS had the largest reductions (87). They also concluded that orthostatic intolerance was not caused by deconditioning (88). A recent study focused on the overlap between long COVID, ME/CFS and POTS in adolescents (85). Symptoms of orthostatic intolerance (OI) were very common also among long COVID patients that did not formally meet the criteria for

POTS. Moreover, many POTS patients had overlapping symptoms with long COVID, including fatigue, cognitive difficulties, headaches and more (85). The short time frame since SARS-CoV2 infection argue against deconditioning as a major determinant of symptoms. A direct effect of the virus on central autonomic networks has been postulated, but for those patients developing symptoms more than two weeks after infection, an autoimmune pathogenesis is also possible. This is in line with the pre-pandemic observations of elevated G-protein coupled adrenergic, and in some cases both adrenergic and muscarinic, autoantibodies in POTS patients (68, 89).

A study by Joseph et al described invasive CPET in 160 patients who fulfilled ME/CFS criteria and described two types of neurovascular dysregulation that probably contribute to ME/CFS exertional intolerance (81). One subgroup of ME/CFS patients, who had reduced right atrial pressure and venous return, with reduced cardiac output on exertion ("preload failure") were labelled "low flow" patients. The other subgroup had normal or high cardiac output on exertion, with evidence of impaired systemic peripheral oxygen extraction. Compared to healthy controls, these patients had higher mixed venous SaO2 in the pulmonary artery during exercise, suggesting systemic microcirculatory dysfunction with impaired oxygen delivery to muscle tissue as a mechanism. Small vessel arterio-venous shunting is a possible explanation, as one third of patients in this cohort had small fibre neuropathy, and arterio-venous shunting with blood flow dysregulation is seen in patients with small fibre neuropathy (81).

Reduced central venous pressure with reduced venous return in ME/CFS has been recognized for decades. In comparison, Ehlers-Danlos syndrome is a heterogeneous group of inherited connective tissue abnormalities characterized by skin hyperextensibility, joint hypermobility, and connective tissue fragility. These patients may to some extent overlap with ME/CFS, often with fatigue and orthostatic intolerance or POTS, probably due to the loose connective tissue in venous vessel walls, with more distended veins followed by venous pooling and reduced central venous pressure, and with decreased venous return and thus reduced cardiac output on exertion (90).

1.6.6 Metabolism

Several studies have reported metabolic changes in ME/CFS patients. Mitochondrial dysfunction and metabolic abnormalities, with altered metabolism of substrates for energy metabolism, have been repeatedly hypothesised as disease mechanisms in ME/CFS. Findings include alterations in serum amino acids and fatty acids for tricarboxylic acid cycle (TCA) fuelling, increases in pyruvate and lactate, and impaired pyruvate dehydrogenase (PDH) function. (9, 23, 80, 91-96).

A major part of these metabolic adaptations may be secondary and caused by an underlying tissue hypoxia in ME/CFS (62). However, such metabolic changes may also represent important effector mechanisms for the symptomology of ME/CFS. For instance, it is likely that tissue hypoperfusion due to endothelial dysfunction and other vascular effects, compromises energy metabolism in muscle cells and other tissues, and thereby causes fatigue, PEM and other neuro-muscular effects. This was supported by a serum metabolomics study by our group using samples from the RituxME and CycloME studies, which showed different metabolic phenotypes in subsets of ME/CFS patient, with associations to symptom severity (97). This study also found metabolic changes that appeared to be uniform within the patient group, and which theoretically may point to common pathomechanistic elements. Overall, the findings were interpreted as possible effects of disturbed cellular energy metabolism, in a manner that agrees with mechanisms of tissue hypoperfusion and hypoxia. A recent study by Wang and colleagues demonstrated increased levels of Wiskott-Aldrich Syndrome Protein Family Member 3 (WASF3) protein in skeletal muscle biopsy samples obtained from a cohort of ME/CFS patients (98). Increased WASF3 is associated with endoplasmic reticulum (ER) stress and reduced mitochondrial function. WASF3 induction by ER stress using endotoxins, is known to be associated with fatigue in humans (98) and hypoxia increases WASF3 expression (99). In total, these findings may support the hypoxia and hypoperfusion hypothesis in a subgroup of ME/CFS patients.

An inadequate autoregulation of blood flow on exertion, would be expected to result in tissue hypoxia with earlier switch from aerobic to anaerobic metabolism,

accompanied by accumulation of lactic acid. Several studies have shown increased lactate in brain or cerebrospinal fluid (100, 101). A study with regular arterial blood sampling during exercise, using two-day CPET protocol, demonstrated earlier increase in lactate the second day, as compared to healthy (23).

In another study from our group, we found a reduction in serum levels of energetic substrates entering metabolic oxidation downstream of the pyruvate dehydrogenase enzyme (PDH) (91). This suggests that pyruvate catabolism may be impaired in ME/CFS patients and that cells use the ketogenic amino acids, which can be converted to acetyl-CoA as energy fuel independently of PDH. We also found significant mRNA upregulation of pyruvate dehydrogenase kinase (PDK)1, PDK2, and PDK4 in peripheral blood mononuclear cells from ME/CFS patients. The function of the PDKs is to inhibit PDH by phosphorylation and reduce PDH activity. Reduced PDH activity will increase lactate, especially on exertion, in line with previous mentioned studies showing increased serum lactate.

1.6.7 Working hypothesis

Our research group has been working on the hypothesis that ME/CFS could be a variant of an autoimmune disease, with a role for B-cells, plasma cells and antibodies. This is the underlying assumption for our research efforts, and particularly our clinical trials. In 2021, our research group published a viewpoint article in the Journal of Clinical Investigation focusing on pathomechanisms and possible interventions in ME/CFS (62). The proposed model of pathomechanisms for the initiation and maintenance of ME/CFS is divided into three steps. The first step involves an immune response to an infection or other immunological trigger. Secondly, the functional autoantibodies target the vasculature, possibly involving G-protein coupled receptors (GPCRs). Antibody-mediated functional disturbances may include endothelial dysfunction in large and small arteries with impaired autoregulation of blood flow, arteriovenous shunting with impaired peripheral oxygen delivery, and impaired venous return. Such inadequate autoregulation of blood flow is expected to result in tissue hypoxia, particularly during exertion. The third level involves secondary compensatory efforts to restore homeostasis and energy balance. These

include autonomic adaptations, often with sympathetic activation, and metabolic adaptations, which may resemble alterations seen in tissue hypoxia and also during endurance exercise in healthy subjects.

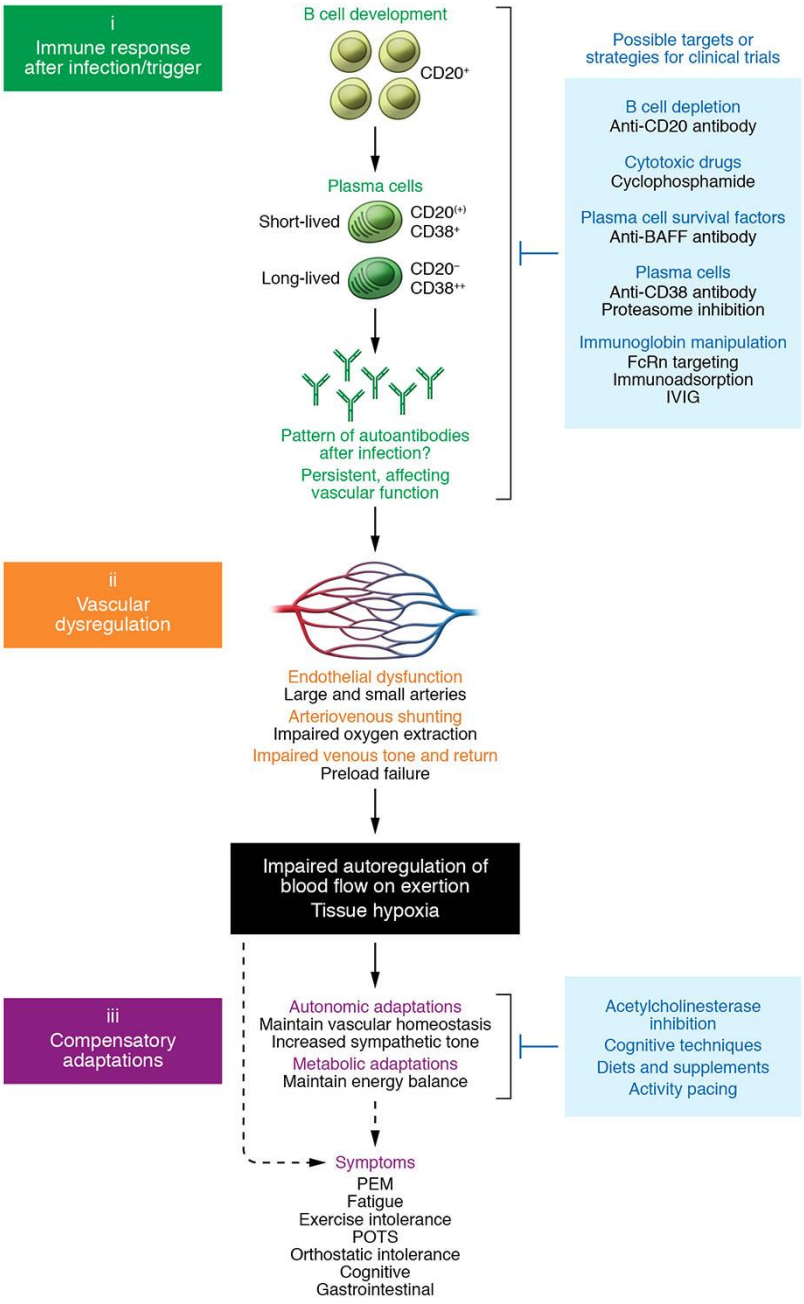


Figure 1. Proposed model for ME/CFS pathomechanisms (62).
(Fluge et al. Pathomechanisms and possible interventions in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) JCI 2021)

1.6.8 Comorbidities and autoimmune diseases

Comorbidities of ME/CFS with various established autoimmune or putative immune-mediated diseases are known, including fibromyalgia (FM), Hashimoto's thyroiditis and Postural orthostatic tachycardia syndrome (POTS). The overlap between ME/CFS and POTS is described above (1.6.5, "Endothelial and cardiovascular dysfunction"). In the RituxME and CycloME studies the occurrence of comorbidities were quite similar, with 7% of the participants in both trials reporting comorbid fibromyalgia, 5% and 10% Hashimoto´s thyroiditis, 9% and 10% depression, and 11% and 10% anxiety, respectively (see supplemental tables in published trials, RituxME and CycloME (18, 19)). Other studies have reported a higher comorbid occurrence of fibromyalgia among ME/CFS patients, with up to 77% of overlap with fibromyalgia, which is also s a comorbidity with 50% prevalence in rheumatoid arthritis and SLE. A family history of autoimmune diseases in ME/CFS is common (46). In the RituxME and CycloME trials there were high occurrences of autoimmunity among first-degree relatives of the participants, 40% and 55% respectively (19).

An Australian epidermiologic survey found that approximately 40% of the ME/CFS patients had abdominal pain, nausea, bloating and symptoms of 'irritable bowel', with alternating constipation and diarrhoea (102). A substudy from the RituxME trial concluded that the ME/CFS patients commonly reported fullness/bloating, abdominal pain and nausea with signs of impaired gastric accommodation by ultrasound investigation, and visceral hypersensitivity, but in conclusion their symptoms had more similarities to functional dyspepsia than to irritable bowel syndrome (103).

Overlap in mechanisms between small fibre neuropathies, complex regional pain syndrome (CRPS) and ME/CFS has also been discussed (104-106), with functional autoantibodies to G-protein coupled receptors or vasoactive mediators and vascular dysfunction as possible common factors.

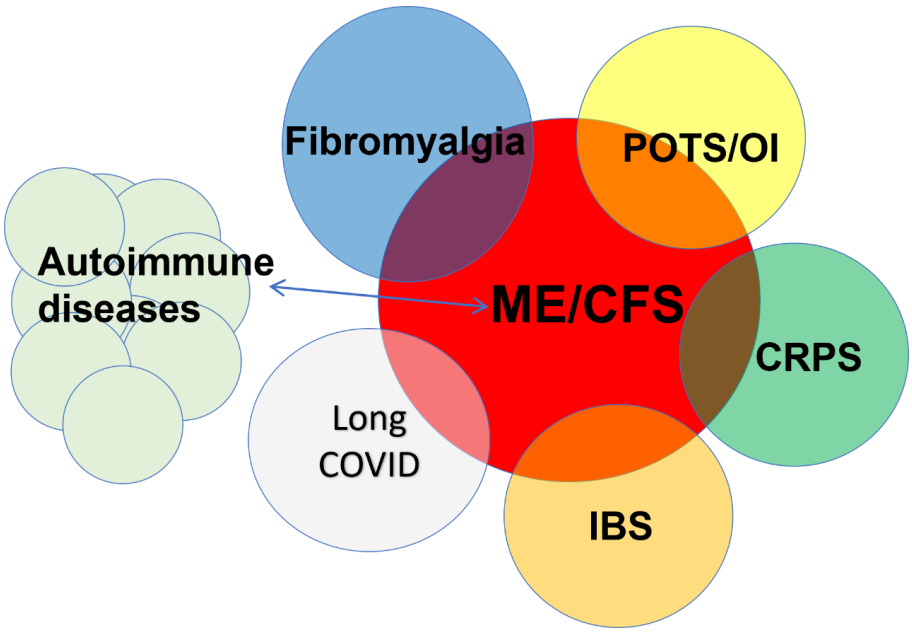


Figure 2. Comorbidities in ME/CFS: Fibromyalgia, Postural orthostatic tachycardia syndrome (POTS)/Orthostatic intolerance (OI), Complex regional pain syndrome (CRPS), Irritable bowel syndrome (IBS), Long Covid and established autoimmune diseases

1.6.9 Psychological aspects of the disease

As fatigue is common in depression and chronic fatigue can lead to depression, the overlapping characteristics of depression and ME/CFS continue to be discussed. A recent study concluded that psychiatric status was not an important causal contributor to symptoms or symptom burden in CFS (107). However, as with other comorbidities, it is important to identify and treat any psychological conditions because of their possible impact on a patient's mental health and quality of life (107). In Norway there is still an ongoing debate, both on aetiology and, as a natural consequence, on treatment recommendations. To date, there is no sensitive and specific biological marker or PROM that can identify whether a patient might or might not, benefit from cognitive therapies or other interventions. The British National Institute for Health and Care Excellence (NICE) published updated guidelines for ME/CFS in 2021, concluding that the psychological approach of treatment with cognitive behavioural therapy (CBT) should be considered an adjunctive and not a curative treatment, and that graded exercise therapy (GET) is possibly harmful and should not be used as treatment for ME/CFS (6).

A recent study by the Norwegian research institutes SINTEF and Fafo showed that based on which diagnostic criteria the patients fulfilled, there were differences in whether the patients perceived CBT as helpful or not (7). Only 16% of patients complying with the Canadian consensus criteria found CBT helpful for their ME/CFS symptoms, compared with more than half of patients who complied with the Fukuda criteria, and this difference was statistically significant. Also, a higher PEM score correlated with lack of effect of CBT (7). These observations underline the importance of strict patient characterisation when recruiting and reporting trials.

1.7 Medical treatment

At present, there is no established standard treatment for ME/CFS. There is limited evidence of the benefit of any pharmacological intervention, but some drugs can be useful to manage symptoms, such as pain and sleep (6). It is important to take into account that ME/CFS patients can be more intolerant for drugs, it is therefore

generally recommended to start at a lower dose than in usual clinical practice, and slowly but gradually increase.

A systematic review of randomised controlled trials (CFS/ME) evaluated studies up to April 2019 and found 25 randomised controlled trials (RCTs) with 22 different pharmacological interventions, 29 RCTs of 19 non-pharmacological interventions and two RCTs of combined interventions (108). They concluded that there is no definitive effective intervention that can be considered consistent and reproducible.

A literature-based commentary on pharmaceutical interventions in CFS, also from 2019, described various interventions such as antivirals (valacyclovir/acyclovir), rintatolimod, analgesics, antidepressants and other treatments such as hydrocortisone, antihistamines, and coenzyme Q and NADH supplementation (109). They concluded that there is no single drug with convincing clinical benefit in the majority of patients.

1.7.1 Immune system as therapeutic target

1.7.1.1 B-cell depletion – anti-CD20 antibody

Rituximab is a therapeutic monoclonal antibody that targets the CD20 molecule on the surface of B-lymphocytes. By binding to the CD20 protein, rituximab induces cell-mediated and complement-mediated cytotoxicity resulting in rapid and reversible B-cell depletion (110) During maturation of B-cells to plasma cells, CD20 is lost. The absence of CD20 expression in stem cells preserves the stem cell pool, and this is important for reconstitution after B-cell depletion. The production of antibodies by differentiated long-lived plasma cells is also unaffected by rituximab treatment (110), as rituximab will only target the mature B-cells and to some extent the short-lived plasmablastss (See Figure 1). Rituximab is used in treatment of B-cell lymphoma, leukemia, rheumatoid arthritis and other autoimmune diseases such as SLE (110) and multiple sclerosis (111).

1.7.1.2 Cytotoxic drugs – cyclophosphamide

The main mechanism of action of cyclophosphamide is its ability to covalently bind an alkyl group, primarily affecting the DNA (112). This irreversible interaction results in the inhibition of DNA replication and apoptosis, leading to cell death in both resting and dividing white blood cells, and subsequently impairs humoral and cellular immune responses. (113). Rapidly proliferating cells such as haematopoietic cells, intestinal epithelial cells and skin fibroblasts are most sensitive to cyclophosphamide (114). This property is used in cancer therapy, but also in various immune-mediated diseases by influencing activated immune cells (115). Dosing is very important when using cyclophosphamide, as the effects and side effects of cyclophosphamide are highly dose-dependent. Lower doses have a greater affinity for T-cells, specifically T-regulatory cells (T-regs). In contrast, higher doses can completely eliminate hematopoietic cells. T-regs are more impacted due to their higher proliferation rate compared to other T-cell subsets, such as T-helper (Th) cells. However, cyclophosphamide also affects B-cells and other components of the immune system (114), and may inhibit activated B-cells from becoming plasmablasts, and subsequently the production of antibodies. T-regs are thought to be important in autoimmune diseases as they help to down-regulate the activity of Th cells and maintain self-tolerance (116). Some studies have reported a greater frequency of T-regs in ME/CFS patients in comparison to healthy controls (117-119). The T-reg markers are also markers of general T-cell activation (116). Thus, the potential effect of cyclophosphamide in ME/CFS may be to interfere with the balance between immune cell subsets and possibly counteract a pathogenic immune cell environment. Cyclophosphamide is still frequently used in cancer (120), and also used to treat immune-mediated diseases like systemic lupus (SLE), rheumatoid arthritis, vasculitis, and multiple sclerosis (115, 121-123).

1.7.1.3 Plasma cells – anti-CD38 antibody

The CD38 protein is highly expressed on plasmablasts, short- and long-lived plasma cells, but also with weaker protein expression on subsets of macrophages, B-cells including regulatory B-cells, and T-lymphocytes (124). If autoantibodies are

produced mainly in CD38-positive, CD20-negative, long-lived plasma cells, rituximab will not reduce autoantibody production and will have no effect on symptoms, unless treatment period is prolonged. Daratumumab is a humanized IgG monoclonal antibody directed against the CD38 protein, and treatment reduces antibody production. Other mechanisms may may also be important related to CD38 and autoimmune inflammation, as the complexity and role for CD38 in immune cells are not completely understood (124). The drug is approved for use with chemotherapy or as monotherapy for patients with multiple myeloma (125), and has been used in treatment-refractory autoimmune diseases (126).

1.7.1.4 Intravenous gammaglobulin

Intravenous gammaglobulin has been tested in ME/CFS, but the results of the studies were inconsistent, and after a negative trial in 1997 (127), the general interest declined. There is still some off-label use and based on information from patients. In a review article from 2021 (128), which reanalysed data from the original trials with immunoglobulins and the authors concluded that a subgroup of patients had some improvement, and that the search for subgroups and research on immunoglobulins should continue.

1.7.1.5 Immunoadsorption

Interestingly, a study demonstrated improvement of ME/CFS symptoms after immunoadsorption to remove antibodies in ME/CFS patients (71). Based on the observations of elevated autoantibodies against ß2 adrenergic receptors, and muscarinic 3 and 4 acetylcholine receptors in subsets of patients, they treated 10 patients with post-infectious CFS/ME with immunoadsorption. Seven patients had improvement of symptoms, and three patients had long-lasting symptom improvement. This has also been demonstrated in long Covid, with similar response rate (72).

1.7.1.6 Low dose naltrexone (LDN)

One drug often used as off-label treatment for ME/CFS patients is low dose naltrexone (LDN), mainly reported to have beneficial effects on sleep and pain. There

is no literature confirming the efficacy of LDN for ME/CFS patients, only case reports (129). One study based on the theory that ME/CFS has immune dysfunction and abnormalities in Natural killer (NK) cell functions, tested the effect of LDN in vitro (130). They found that LDN restored function of Transient Receptor Potential Melastatin 3 (TRPM3) ion channel in Natural killer cells, with a possible positive effect on NK-cell functioning.

1.7.1.7 Rintatolimod (Ampligen®)

Rintatolimod (Ampligen®) is an immunomodulatory double-stranded RNA drug, which has been tested in a randomised trial. In a study from 2020, the authors concluded that more than half of the patients improved in physical performance and quality of life, but only in the subset of patients with disease duration between two to eight years (131). Another review concluded that rintatolimod appeared to be well tolerated, but with a minor symptom improvement (132).

1.7.2 Neurovascular and metabolic targets for intervention

1.7.2.1 Pyridostigmine (Mestinon®)

Joseph and colleagues tested pyridostigmine (Mestinon®) based on the hypothesis that neurovascular dysregulation is important in the pathophysiology of ME/CFS (133). In a small, randomised trial, they showed that pyridostigmine improved peak VO2 in ME/CFS by increasing cardiac output and right ventricular filling pressures. This shows that it is possible to influence exercise haemodynamics in ME/CFS patients by cholinergic stimulation, but there were no reports of effects over time.

1.7.2.2 Nutrients and nutritional supplements

Nutrients and nutritional supplements have been explored for their potential benefit in ME/CFS. No evidence supports the use of these supplements with the possible exception of the mitochondrial modulating combination of NADH and coenzyme-Q10, which showed a small but statistically significant improvement in the Fatigue Impact Scale total score in the treatment group (134).

Possible targets or treatment strategies are also described in Figure 1.

1.7.3 Ongoing and planned trials

1.7.3.1 Pyridostigmine (Mestinon) and LDN

In a collaboration between Harvard and Uppsala university hospitals, a randomized, double-blind, and placebo-controlled trial in planned, aiming to investigate two particular drugs: Pyridostigmine (Mestinon) and LDN, separately and in combination. (https://www.omf.ngo/lift-trial/). There is also a planned study at Charite university hospital, to investigate improvement in the physical function domain in SF-36 with the soluble guanylate cyclase (sGC) stimulator vericiguat compared with placebo, in participants with post-COVID-19 syndrome. sGC is a receptor for nitric oxide (NO), and Vericiguat aims to improve endothelial dysfunction and microvascular perfusion, followed by increased blood flow. If this study turns out positive, Vericiguat will also be of interest to ME/CFS patients (ClinicalTrials no. NCT05697640).

1.7.3.2 The BC-007 aptamer

GPCR autoantibodies target one of the three extracellular loops of the 7-transmembrane receptors by allosteric binding, and may be either agonistic or antagonistic (65). Recently, a new therapeutic principle has been launched, describing the use of a 15-aptamer, a single-stranded oligonucleotide with defined sequence (BC-007), which binds to a common sequence in GPCR autoantibodies, and inhibits the binding of these functional autoantibodies to the receptor. The BC-007 (Berlin Cures) is intended for use in GPCR autoantibody-mediated diseases. A case report with a rapid, positive effect has been published in long Covid (135), and a randomised and placebo-controlled trial in long Covid patients is ongoing (ClinicalTrials no. NCT05911009).

1.7.3.3 Faecal microbiota transplantation (FMT)

In recent years there has been increasing interest in the gut microbiome and the link to the immune system, both regarding autoimmune diseases and ME/CFS. A small, randomized study with 11 participants published in 2023 evaluated faecal microbiota transplantation (FMT) in CFS patients (136). They concluded FMT to be safe but did not relieve symptoms or improve quality of life in this small cohort. This year, a

Norwegian randomised study on FMT with 80 participants completed enrolment (ClinicalTrials no. NCT03691987), and the results from this study will be important.

1.8 Background from the Department of Oncology, Haukeland University Hospital

In our oncology unit, we have over more than 15 years observed several patients with long-standing ME/CFS, who have reported significant improvement of their ME/CFS symptoms following chemotherapy for breast cancer, malignant lymphoma or testicular cancer. In total, at least 12 patients have independently reported such unexpected clinical effects. All of these patients had received chemotherapy, most with cyclophosphamide and some with the addition of the therapeutic anti-CD20 monoclonal antibody rituximab. The first pilot experiences (137) provided the basis for the study group's decision to pursue these observations in separate clinical trials (18, 22, 138).

2 Aims of the project

2.1 General aim

The overall aim of this thesis is to increase the general understanding of disease mechanisms in ME/CFS, and to test the hypothesis that development of ME/CFS is associated with a variant of an autoimmune pathomechanism with a role for B-cells/plasma cells and autoantibodies. If treatment with immunomodulatory drugs as rituximab and/or cyclophosphamide leads to convincing improvement of ME/CFS related symptoms, this hypothesis is strengthened.

Few clinical trials have been conducted on ME/CFS patients, and very few drug trials. Through clinical trials, we wanted to assess feasibility, toxicity, and possible therapeutic benefit from intervention. We also aimed to gain better knowledge of the natural course and variation of symptoms over time in ME/CFS patients, in order to improve outcome measures and endpoints in future trials in this patient group. In addition, we wanted to investigate subgroups for possible clinical or biochemical characteristics associated with a higher response rate to treatment.

2.2 Specific aims of the papers included in the thesis were:

Paper I: Rituximab concentrations: The aim was to examine the associations between rituximab serum concentrations and clinical improvement, clinical and biochemical data, and the relevance of anti-drug antibodies (ADAs) against rituximab. Serum samples from patients with ME/CFS sampled in a previous phase II trial assessing rituximab maintenance treatment were used (22).

Paper II: the RituxME trial: The objective was to verify or disprove the association between B-cell depletion with the monoclonal anti-CD20 antibody rituximab and clinical responses in patients with ME/CFS, as indicated in previous studies (22, 137, 138).

Paper III: the CycloME trial: The aim of the study was to evaluate immunomodulatory treatment with intravenous cyclophosphamide in patients with ME/CFS, focusing on feasibility, toxicity, response rate and duration of responses.

Paper IV: the Fitbit study: The aims were to explore natural symptom variation, the feasibility of continuous activity monitoring using the Fitbit activity watch in studies of ME/CFS patients, and to compare activity data with patient-reported outcome measures (PROMs).

Paper V: Follow-up study RituxME and CycloME: In the two trials, the patients were included in overlapping time periods and with similar inclusion criteria. The aim was to investigate long-term improvement and possible unexpected late side effects after treatment. We used patient-reported outcome measures (PROMs) and compared results from the RituxME and CycloME trials as well as the three groups rituximab-, placebo- and cyclophosphamide-treated patients in post-hoc analyses.

3 Material and methods

In order to facilitate the understanding of the "Methodological considerations" chapter, the patients and methods used in the different studies are briefly summarized here.

3.1 Patients

Following the first rituximab randomised trial in 2011 (138) and up to now, our ME/CFS research group has received referrals from physicians or direct requests from patients to be evaluated for inclusion in clinical trials. After screening of these referrals and selection of patients who appeared to meet the Canadian consensus criteria, the patients included in RituxME and CycloME trials were randomly selected and contacted for information, informed consent process, and thorough clinical evaluation. For the Fitbit study (paper IV), the recruitment was done by advertising through the local ME association's Facebook page, and the research group's e-mail newsletter.

3.1.1 Paper I – Rituximab concentrations

In this study we analysed samples from the KTS-2-2010 study published in 2015 (22). The measured rituximab concentrations and ADAs in serum samples included 23 patients for whom samples were still available in the biobank. All patients were included at Haukeland university hospital HUH, the age was 18-66 years (mean 39.7 years), and all patients fulfilled the Canadian criteria. There were 16 female and 7 male patients.

3.1.2 Paper II – The RituxME trial

151 patients were enrolled by five national trial centres (four university hospitals and one general hospital). All patients fulfilled the Canadian criteria. The age span was 18 to 65 years (mean 36.7 years), disease duration was at least 2 years (or at least 5 years if disease severity was mild) but less than 15 years. Patients with very severe disease were not included. There were 124 female and 27 male patients included.

3.1.3 Paper III - The CycloME trial

All 40 patients were included at the Department of Oncology and Medical Physics, HUH. Seven patients had parts of their treatment and follow-up at the Department of Oncology, Oslo University Hospital (OUH). All patients were diagnosed according to Canadian criteria, with age span 18–66 years (mean 41.4 years), disease duration at least two years. Patients with either mild or very severe disease (completely bedbound and in need of help for all basic activities of daily living) were not included. There were 31 females and 9 males included.

3.1.4 Paper IV – The Fitbit study

All 27 patients were included at the Department of Oncology and Medical Physics, HUH. All patients fulfilled the Canadian criteria. The age span was 18 to 65 years (mean age 42.3 years), disease duration more than two years, with mild to severe disease. There were only two male, and 25 female participants.

3.1.5 Paper V – Follow-up study RituxME and CycloME

Among the RituxME study patients, 112 out of 148 eligible patients (75.7%) participated; 77.3% of the rituximab and 74.0% of the placebo group. 16 out of 26 men (61.5%), and 96 out of 122 women (78.7 %) participated in the 6-year follow up study. For the RituxME cohort, patients with severe ME/CFS at baseline had a 91% rate of participation at the 6-year follow-up, compared to 72% in those with less severe disease. For the CycloME trial, 34 out of 36 (94.4%) available patients at 6 years participated, while one patient with severe and one with moderate disease did not participate.

3.2 Methods

3.2.1 Rituximab analyses

In Paper I, we retrospectively measured rituximab concentrations and antidrug antibodies (ADAs) in serum samples from patients included in an open-label phase II trial with maintenance rituximab treatment (KTS-2-2010), to investigate possible

associations with clinical and biochemical data. All serum samples used for rituximab measurements were collected immediately before the next scheduled rituximab infusion the interval between the maintenance doses (at 3, 6, 10, and 15 months). According to protocol the interval between infusions could vary 1-2 weeks. For comparability between patients, measurements were adjusted, using an estimated median t1/2 of 22 days according to the Summary of Product Characteristics (SPC) for MabThera®. Serum rituximab concentrations and ADAs were measured by the Biologicals Laboratory, Diagnostic Services Sanquin (Amsterdam, The Netherlands) using sandwich enzyme-linked immunosorbent assay (ELISA).

3.2.2 Drug intervention

In paper II, the RituxME trial, Smerud Medical Research International (Oslo, Norway) randomly assigned patients 1:1 to receive either rituximab or placebo. Patients received induction treatment with 2 infusions 2 weeks apart, of either rituximab (MabThera ®, Roche), 500 mg/m2 of body surface area (maximum of 1000 mg), or an equal volume of saline with added human albumin. In the maintenance phase, patients received a 500 mg fixed dose of rituximab or an equal volume of saline with human albumin at 3, 6, 9, and 12 months.

In paper III, the CycloME trial, cyclophosphamide was administered with 4-week intervals, in total six 30-minute intravenous infusions with 600 mg/m^2 at the first and 700 mg/m^2 at further cycles.

3.2.3 PROMS

In Paper II, the RituxME trial, patient reported outcome measures (PROMs) were recorded at baseline, using the Short Form 36 Health Survey (SF-36) ver. 1.2 in Norwegian translation (139), the Hospital Anxiety and Depression Scale (HADS) (140), the Fatigue Severity Scale (FSS) (141), and a modified DePaul Symptom Questionnaire (142). SF-36 forms were completed every 3 months, and FSS every 6 months. Function level was recorded at baseline and every second week and expressed as a percentage according to a table with examples, where 100% represents a completely healthy state and was recorded at baseline and every second week.

Patients recorded baseline scores of ME/CFS symptoms (PEM, fatigue, pain, cognitive symptoms, and other symptoms) using a scale of 1 to 10. The Fatigue score assessed self-reported symptom change from baseline, and was adapted from a Clinical Global Impression scale previously used in CFS (143). The relative scale for each symptom was 0 to 6, in which 3 denoted no change from baseline; 4, 5, and 6 slight, moderate, and major improvement, respectively; and 2, 1, and 0 slight, moderate, and major worsening, respectively. The primary variable Fatigue score (scale 0-6) was calculated as the mean of the four items: “Fatigue”, “Post-exertional exhaustion”, “Need for rest” and “Daily functioning”. Every second week for 24 months follow-up, patients recorded their changes from baseline in ME/CFS symptoms, i.e. the Fatigue score.

In Paper III, the CycloME trial, Fatigue score and Function level were recorded every second week as described for the RituxME-trial. SF-36 was reported at baseline and every 3 months during follow-up and at extended follow-up assessments at 24–30 and 38–48 months. Fatigue Severity Scale was recorded at 3-months intervals until 18 months.

In paper IV SF-36 and DePaul Symptom Questionnaire Short Form (DSQ-SF) for ME/CFS symptoms (144), were reported at baseline and every four weeks. The Norwegian translation of DSQ-SF is based on the translation of the complete DePaul Symptom Questionnaire (142). DSQ-SF examines the frequency and severity of 14 typical ME/CFS symptoms during the previous six months. Higher scores indicate higher symptom burden (score 0-112). During follow-up, patients completed the Composite Autonomic Symptom Score-31 (COMPASS-31) questionnaire, used to assess symptoms related to dysautonomia. One week after completing the study, the participants were asked to answer an evaluation of the study and the activity armband. We used an online survey from enalyzer.com. The answers were anonymous.

In the 6-year follow-up study, we used PROMs (SF-36 and DSQ-SF) and compared values at baseline, at 18 months, and at 6-year follow up for participants in the CycloME and the RituxME (rituximab and placebo groups) trials.

3.2.4 Outcome measures

The RituxME trial had two primary end points based on Fatigue score: (i) difference between treatment groups for repeated measurements of Fatigue score through 24 months, and (ii) overall rate of response, defined as a Fatigue score of at least 4.5 (scale 0-6) for at least 8 consecutive weeks.

The CycloME trial also had two primary end points based on Fatigue score: (i) overall response rate, defined Fatigue score of at least 4.5 for at least 6 consecutive weeks, and (ii) changes in Fatigue score compared to baseline through 18 months follow up. These endpoints were also analysed separately for the treatment-naïve patients (with no previous rituximab exposure).

Secondary outcome measures in both trials were based on SF-36 physical function subscale (SF-36 PF), SF-36 physical component summary (SF-36 PCS), FSS, self-reported Function level (percentage), and mean number of steps per 24 h.

Adverse events were registered continuously in both trials and summarized according to Common Terminology Criteria for Adverse Events (CTCAE) version 4.03.

3.2.5 Physical activity/Steps

In paper II and III the patients used an electronic SenseWear armband at home continuously for 5 to 7 days to record baseline level of physical activity (number of steps). In the RituxME trial this was repeated between 17 and 21 months, and in the CycloME trial repeated at 7–9, 11–12, 17–18, 24–30, and 38–48 months follow-up. In Paper IV we measured physical activity by continuous monitoring using Fitbit Charge 3 activity trackers for 6 months, including assessment of feasibility, and compared with the previously validated activity armband Sensewear used at baseline, 3 and 6 months.

3.2.6 HLA

The association analysis between HLA risk alleles and clinical response in the CycloME trial was not specified in the protocol and was performed post-hoc in the data analysis phase. High-resolution HLA genotyping was conducted as part of a larger study (45). In short, HLA-A, -B, -C, -DRB1, -DQB1, - DQA1, and -DPB1 alleles were genotyped at the Norwegian Sequencing Centre, Oslo. Only the potential HLA risk alleles identified by Lande et al. i.e., HLA-C∗07:04 and HLA-DQB1∗03:03 were investigated (45).

4 Summary of results

4.1 Paper I, rituximab concentrations

In paper I, we investigated if there were associations between rituximab concentrations and clinical and biochemical data in the KTS-2-2010 trial. None of the 23 patients had ADAs at any time point. Female patients had higher mean serum rituximab concentrations than males at 3 months (p=0.05). There was a significant negative correlation between B-cell numbers in peripheral blood at baseline and rituximab serum concentration at 3 months (r=-0.47, p=0.03). Clinical improvement of ME/CFS patients was not related to rituximab serum concentrations or ADAs (Figure 1, paper I), this was also in accordance with the RituxME trial, which refuted the beneficial effects of rituximab in ME/CFS (Paper II) (18).

4.2 Paper II, the RituxME trial

The RituxME trial was a national, multicentre, randomized, double-blind, placebo-controlled, phase III trial including 151 patients. The purpose was to compare active treatment with the B-cell depleting anti-CD20 antibody rituximab to placebo in a double-blind setting.

The study showed no significant differences in outcome measures between the rituximab and placebo groups during follow-up over 24 months. The primary outcome, repeated measurements of Fatigue score during follow-up, did not differ significantly between the treatment groups (P = 0.80). The difference in average Fatigue score between placebo and rituximab groups was 0.02 (Figure 2, paper II). Overall response rates did not differ significantly between the treatment groups by study centre. Regardless of treatment group, 30% (46 patients, 26 in the placebo group and 20 in the rituximab group) met the predefined criterion for overall response. Sustained worsening for at least 3 months was reported by 10% of patients. Neither clinical response nor clinical worsening was associated with rituximab intervention. In general, during 24 months of follow-up, both treatment groups had

slight but significant improvements over time in mean values for all self-reported outcome variables and for physical activity level.

Intervention tolerance was generally good, and few serious adverse events had a suspected or probable relation to the study drug. Serious adverse events (SAE) were detected in 26.0% of the rituximab group and in 18.9% of the placebo group, mainly due to prolonged hospital stay after infusions.

In conclusion, the RituxME trial showed no significant clinical benefit for rituximab compared to placebo.

4.3 Paper III, the CycloME trial

In the CycloME trial, 40 patients were included and treated with six infusions of cyclophosphamide. Follow-up was 18 months, plus additional follow-up visits at four and six and years. At 18 months, the overall response rate was 55%. Mean SF-36 PF increased from 33.0 at baseline to a maximum 51.5 at 18 months' follow-up ($p < 0.001$). Among 22 responders, mean SF-36 PF increased from 35.0 at baseline to 69.5 at 18 months ($p < 0.001$). For 18 non-responders, there was only a slight increase of SF-36 PF from 30.6 at baseline to a maximum of 34.4 at 3 months, and with no significant changes through the remaining study follow-up. After 4 years 37.5% of the patients were still in remission assessed by self-reported symptom and quality of life questionnaires and by physical activity measures of steps per day.

Clinical responses were associated with specific Human Leukocyte Antigen (HLA) alleles. Patients positive for HLA-DQB1*03:03 and/or HLA-C*07:04 ("risk alleles") ($n = 12$) had significantly higher response rate compared to patients negative for these alleles ($n = 28$), 83% vs. 43%, respectively.

We concluded that the open label study with cyclophosphamide showed a beneficial effect on ME/CFS symptoms in half of the patients after treatment. Response rate was higher in patients carrying the HLA risk alleles. Caution should be taken when interpreting the results of an open label study.

4.4 Paper IV, the Fitbit study

After evaluation of the above mentioned previous clinical trials with immunomodulatory drugs, we have considered aspects that may influence trial outcomes and conclusions. These include patient heterogeneity, patient inclusion criteria, case definitions, severity assessment, placebo mechanisms, natural symptom variation over time, and lack of objective outcome measures. There is limited knowledge about the variation and natural course of the ME/CFS disease over time.

In Paper IV, we conducted an observational study, in which twenty-seven participants used a Fitbit activity tracker continuously for six months, with regular recordings of symptoms and QoL questionnaires, with no intervention. The correlations between steps per day and self-reported SF-36 Physical function, social function, and DSQ-SF were significant. Use of the Fitbit activity watch recorded significantly higher number of steps than the SenseWear bracelet. Resting heart rates were stable during six months.

The study had a small number of participants (n= 27), too few to draw strong conclusions, but using the combination of SF-36 PF and DSQ-SF we identified a group of patients with milder disease that showed considerable variation in outcome measures (i.e., SF-36 PF) during follow-up compared to the remaining participants. We compared eight patients with milder disease (defined with baseline SF-36 PF > 50 or DSQ-SF < 55), to 19 patients with higher symptom burden (SF-36 PF < 50 and DSQ-SF > 55). The variation for SF-36 PF scores were 16.9 vs 3.4 points in these two groups, respectively.

We concluded that continuous activity registration with Fitbit Charge 3 trackers was feasible and useful in studies with ME/CFS patients to monitor steps and resting heart rate, in addition to self-reported outcome measures. The knowledge from this study could be useful for the design of study protocols and assessments of outcome measures in future interventional studies.

4.5 Paper V, follow-up study, RituxME and CycloME trials

Paper V is a follow-up study from the previous trials RituxME and CycloME. There were no unexpected side effects reported after six years. We found that mean SF-36 PF (scale 0-100) in the CycloME study increased from 35 at baseline to 54 at 18 months, and to 57 at six years. The RituxME rituximab group increased from mean SF-36 PF scores 33 at baseline, to 42 at 18 months and 46 at six years, and the placebo group increased from mean SF-36 PF scores 32 at baseline, to 46 at 18 months and 43 at six years.

In the CycloME trial, there was a mean improvement in SF-36 PF of 19 points (from 35.4 to 54.4) during the initial 18 months of the study, including an increase from 35.0 to 69.5 among the 22 patients (55%) registered as responders to cyclophosphamide. For the 34 participants at 6-year follow-up, there was a further slight increase in mean SF-36 PF of 2.3 (from 54.4 to 56.7) at six years. Mean SF-36 PF among 20 responders was 70.3 at 18 months, and 67.4 at 6 years.

At six years' follow-up, 44.1% of the CycloME patients, 27.6% of the rituximab group and 20.4% of the placebo group recorded a SF-36 PF of at least 70. With regards to worsening over time, 5.9% of cyclophosphamide-, 10.3% of rituximab-, and 14.8% of placebo-treated patients had a drop in SF-36 PF of 20 points or more from baseline to six years.

With significant individual variation in all study groups, a considerable number of patients treated with cyclophosphamide reported sustained, clinically meaningful improvement after six years. The improvements in SF-36 Physical Function, and in self-reported Function level, during six years follow-up, were significantly higher in the CycloME patients compared to participants in the RituxME trial. No unexpected long-term toxicity was reported.

5 Ethical considerations

The ME/CFS patient group represent 0.1-0.8% of the population, often with a high symptom burden and very low quality of life. ME/CFS has for many years had a low priority in medicine and in society. These patients deserve the same efforts in research and clinical trials as those suffering from other diseases.

We believe that the growing evidence for immune alterations in ME/CFS and the long-lasting high symptom burden with major consequences for the patients' quality of life, can justify the performance of clinical trials to assess immune modulating drugs with possible side effects.

Both rituximab and cyclophosphamide are well-known drugs used both in cancer treatment and in established autoimmune diseases. Toxicity and side effect profile in other diseases are therefore well known. In the oncology department, we have extensive experience with these drugs over a number of years.

Patients with ME/CFS is a group not previously treated with these drugs. ME/CFS patients generally express lower drug tolerance than other patient groups. Therefore, toxicity and adverse event registration has been an important focus. We have assessed adverse events systematically, particularly regarding fertility issues when using cyclophosphamide, as the age of many of the patients is the age when the ovaries are most vulnerable to such effects, and there is a risk of premature menopause and involuntary infertility. Although patients may assert at baseline that this risk does not represent a problem, as having children is not an issue due to the severity of the disease, we have found that in patients who experience an improved function level, these views may change. For this reason, this issue is carefully discussed during the pre-inclusion interviews so that patients are well informed both verbally and in writing before signing the consent form.

All studies were approved by the Regional Ethical Committee in Norway and by the Norwegian Medicines Agency. All patients gave their written informed consent. The trials were conducted in accordance with Good Clinical Practice (GCP).

Paper I: no 2010/1318-4. Paper II: no 2014/365. Paper III: no 2014/1672. Paper IV: no 28780. Paper V: amendments to the two clinical trials; RituxME: 2014/365, CycloME: 2014/1672.

6 Statistical analyses and considerations

6.1 Sample size

In the RituxME trial we estimated a sample size of 152 patients, using a presumed overall response rate of 50% in the rituximab group and 25% in the placebo group, with an expected distribution of a variable with seven categories corresponding to the Fatigue score (0-6), with a power of 0.80, a two-sided α-level of 0.05, and allowing for 5% withdrawal.

The CycloME study was a phase II trial with no placebo group. In addition to observation for possible clinical beneficial effects, the main aim was to assess the feasibility and toxicity from intravenous cyclophosphamide in ME/CFS patients. 40 patients were considered a sufficient number, but still not more than we could follow up properly.

In the Fitbit trial, we had planned to enroll 30 patients, but due to the coronavirus pandemic and our department's guidelines for trials during the pandemic, we had to stop at 27 enrolled patients.

6.2 Descriptive methods

We used descriptive methods to characterize the patients at baseline in the clinical trials. Means with standard deviation (SD) or 95% confidence interval (CI) for normally distributed data, or median with range (min-max) or interquartile range (IQR) for data with non-parametric distribution, as appropriate. Differences in distribution of parametric data between groups were tested by t-tests or analysis of variance (ANOVA), or by Mann-Whitney U test or Kruskal-Wallis test for non-parametric data. Differences in distribution of categorical data between groups were analysed by Chi-square statistics.

6.3 Missing data

In the RituxME trial (Paper II) (18), 151 patients were included, two patients withdrew during follow-up. Except for these two patients, the frequencies of missing data for the outcome measures were very low (in the range 0.4 – 1.3%), these were replaced by multiple imputation (details in Supplementary information at Annals.org). Primary and secondary outcome measures were tested by the intention-to-treat principle, for the RituxME trial including all patients who had received at least one infusion of rituximab or placebo. Difference in overall response rate between the rituximab and placebo groups was tested by Mantel-Haenszel test, adjusting for study centre.

In the CycloME trial (Paper III) (19), 40 patients were included and two of these withdrew within the 18 months initial trial follow-up (both were non-responders at the time of withdrawal), and one patient with severe ME/CFS (also non-responder) did not complete self-reported data from 4 months onwards. Except for these three patients, there were only 0.5% missing data for Fatigue score. Missing data in the CycloME trial were replaced using the last value carried forward (LVCF) method (i.e. the three patients with missing data were registered as non-responders from withdrawal throughout the study follow-up), and the data were analysed by the intention-to-treat principle.

In the follow-up study of RituxME and CycloME (Paper V), out of the patients available after six years, 75.7% of RituxME and 94.4% of CycloME patients participated, as described.

For paper IV an R-script was generated to download activity data from the participants. For Fitbit data we focused on steps per 24 hours and resting heart rate (measured at sleep during the night). The observed Fitbit activity and heart rate data were used as input for statistical analyses with no replacement for missing values (0.1% and 0.5% missing data, respectively). For SF-36 and DSQ-SF, missing data were replaced for one patient (missing 2 recordings out of 378) using the last value carried forward (LVCF) method.

6.4 General linear model (GLM) repeated measures

General linear model (GLM) repeated measures was used in analyses of data from the trials, to assess differences in course of outcome during follow up, between groups. In paper I GLM repeated measures was used to assess differences in course of adjusted serum rituximab concentrations, between patients with clinical improvement versus no improvement. In paper II, the RituxME trial, GLM repeated measures was used for the primary outcome Fatigue score over time, and for secondary outcomes. Time, intervention (treatment group), and their interaction were included as predictors, with study centre included as covariate (for details see Appendix Methods, available at Annals.org). In paper III, the open-label one-armed CycloME trial, GLM repeated measures were used to evaluate changes in outcome measures from baseline through 18 months follow-up. Simple contrasts in the time domain assessed changes from baseline to each specific time intervals or time points during follow-up, with the effect sizes from the parameter estimates (means and 95% CI). Between-group effects were analysed for sex, ME/CFS severity, ME/CFS duration, previous rituximab treatment, infection prior to debut of ME/CFS symptoms, and presence of specific HLA alleles.

Greenhouse-Geisser corrections were used for all GLM analyses with multiple levels of the dependent variable due to violations of the sphericity assumption. GLM repeated measures were also used in Paper V, for comparison of outcomes (SF-36 PF and Function level) over time (baseline, 18 months, six years), by trial (RituxME versus CycloME), or by treatment groups (cyclophosphamide, rituximab, placebo), adjusted for age, sex, study centre and baseline ME/CFS severity. Logistic regression was performed (method backwards stepwise) with SF-36 PF at six years (< 70 versus ≥ 70) as the dependent variable, and with age, sex, study centre, baseline SF-36 PF and clinical trial as predictor variables (for details, see Supplemental data, Plos One). In the Fitbit study we also used GLM repeated measures of variables, by groups (ME/CFS severity, categories of baseline SF-36 PF). Correlations between variables (Function level (%), SF-36 domains, DSQ-SF score, mean steps per 24 hours and resting heart rate, were performed by Spearman´s rho. Spearman analyses were also

used in Paper I to assess correlations between serum rituximab concentrations and B-cell numbers in peripheral blood.

6.5 Statistical considerations

The statistical tests used to present data and results from the clinical trials are considered standard, such as descriptive methods to characterize patient samples. However, the use of a statistical test to assess repeated measurements of outcome variables over time, and with comparison of between-group effects, should be discussed. This aspect has been thoroughly discussed with the editorial offices including statisticians in the journals (both Annals of Internal Medicine and Plos One). SF-36 PF (and other SF-36 domains, scale 0-100), DSQ-SF (scale 0-112) and Function level (%) used for repeated assessments during the trials, are in principle not continuous, but ordinal variables. To our knowledge, there are no universally accepted methods to compare groups (i.e. treatment group, or trial) for repeated measures of ordinal variables. We decided to use GLM repeated measures. The same considerations apply for alternative analyses using either repeated measures analysis of variance (RM-ANOVA) or Linear Mixed Model.

An alternative analysis of differences between-group (i.e. rituximab versus placebo, or severe versus moderate versus mild) at specific time points during follow-up, could be Mann-Whitney test or Kruskal-Wallis test for non-parametric data. However, the use of these non-parametric tests would not take into account the repeated measures design when assessing the outcome variables.

In the six-year follow-up study of RituxME and CycloME, we compared the two studies, and also the three treatment groups (rituximab, placebo, cyclophosphamide) by repeated measures (baseline, 18 months, 6 years) to assess the differences in course of the outcome variables SF-36 PF and Function level (%). This was a post-hoc analysis, and care should be taken in interpretation. However, the two trials had similar inclusion criteria, with inclusion in the same time period, but with RituxME as a multicentre trial. To adjust for these discrepancies, in the model we adjusted for

age, sex, study centre and baseline ME/CFS severity included as covariates. Similar adjustments were made when performing the logistic regression analysis with SF-36 PF as the dependent variable.

7 Methodological considerations

7.1 Paper I - Not designed as a pharmacokinetic study

The strengths of this study include a well-defined patient population with comprehensive follow-up according to the protocol for the clinical trial, standardized biobank sampling and validated methods for determination of serum rituximab concentrations and of ADAs. The study was not originally designed for the purpose of drug measurements and pharmacokinetics of rituximab in ME/CFS patients. No blood samples were taken shortly after rituximab infusions to capture peak concentrations, but immediately before next scheduled dose for assessment of trough concentrations. The intervals between the doses were gradually increasing during follow-up, in the maintenance phase with rituximab infusions at 3, 6, 10, and 15 months, i.e. the latest sample was taken 5 months after the preceding infusion, which means that rituximab concentrations at this time point were low. The differences in rituximab serum concentrations caused by minor differences in time intervals between rituximab doses, were adjusted presuming a rituximab half-life of 22 days in all patients and presuming a linear phase of elimination (all measurements at least three half-lives after the preceding dose). Assuming identical rituximab half-life and linear phase of elimination in all patients is a clear source of error, but this was still the best opportunity we had to compare patients in this retrospective setting.

7.2 Severity grading

Severity grading was based on the definition described in the Canadian consensus criteria (12, 13). In the clinical RituxME and CycloME trials and the Fitbit study, we used a table of examples describing levels of severity (Supplementary information at Annals.org, RituxME protocol). The grading is subjectively assessed by clinicians in collaboration with the patients and constitutes a possible bias in the studies. In the Fitbit trial (20) we thoroughly discussed grading and endpoints, steps per 24 hours, SF-36 PF, and self-reported Function level.

In the Fitbit study, mean steps per 24 hours showed a clear distinction between the severity groups with some overlap, in accordance with other studies. In the cyclophosphamide trial there was a clear difference between SF-36 PF during follow-up, between patients with moderate/severe versus milder disease severity, but more overlap between the mild/moderate and moderate groups.

7.3 PROMS

7.3.1 Fatigue score

The Fatigue score is explained under Methods, PROMs, in the RituxME study. The Fatigue score is based on the change from baseline in four fatigue-related symptoms: "fatigue", "fatigue after exertion", "need for rest" and "daily functioning". The relative scale for each symptom is 0 to 6, where 3 means no change from baseline, 4, 5 and 6 mild, moderate and major improvement, and 2, 1 and 0 mild, moderate and major worsening, respectively. Responses were defined as Fatigue score of at least 4.5 for at least 8 consecutive weeks in the RituxME trial, and for 6 weeks in both the KTS-2 trial (basis for Paper I) and CycloME trial.

When evaluating these trials, we have concluded that the Fatigue score is not an optimal outcome measure. All four fatigue-related symptoms are subjectively described at baseline on a scale of 0-10 at baseline, and during follow-up subjectively described for changes (scale 0-6) as compared to baseline. Different patients will, for example, define mild, moderate or major improvement of the symptom “need for rest” differently. A patient’s function level and disease severity at baseline can also influence how change is interpreted. When changes from baseline are described during follow-up, requiring retrospective recall for several years, this provides a basis for significant recall bias.

7.3.2 SF-36 PF

This questionnaire is commonly used in studies with ME/CFS patients as well as studies on other chronic diseases and is therefore useful for comparing different studies. The SF-36 contains 36 items on health-related quality of life. In the studies

we have used the SF-36 domains Physical Function, Bodily Pain, General Health, Vitality, Social Function and Mental Health (raw scores, scale 0-100) and focused on Physical Function (PF). The normal range for SF-36 PF in the population varies with age and sex, with higher scores in younger age groups and lower scores (indicating lower physical function) in women (145). Like many other questionnaires, the SF-36 has its challenges. For example, the patients are asked to assess whether various activities "limit me a lot", "limit me a little" or "do not limit me at all". A characteristic of ME/CFS patients is that they are often able to perform an activity, but if they do so, they must prioritise and possibly skip other activities, or they will have an increased symptom burden afterwards, which can last for hours, days or weeks. This post-exertional deterioration or PEM is not measured in this questionnaire.

7.3.3 Function level

Instructions for completion of all the patient-reported forms were included in the patient information folders. The self-reported Function level is expressed as a percentage of function compared to a completely healthy state (100%). To help patients choose their percent function level, they were given a list of different function levels with examples of daily tasks at each level. Despite the lists of examples, different patients' perception of their level of function is inevitably subjective, as is their interpretation of change over time. Nevertheless, it is a simple scale that can be used to assess each individual patient, although comparisons between patients are hampered with uncertainty.

7.4 Activity monitoring; Sensewear and Fitbit

Sensewear activity monitoring was used in the CycloME and RituxME trials, and in combination with continuous use of a Fitbit tracker in the Fitbit study. Activity measures and steps by Sensewear bracelets were registered for one week at baseline and again during follow-up. Due to unforeseen circumstances such as intercurrent illness or social obligations, the patients reported that the allocated weeks (when they were requested to wear the Sensewear) were not necessarily representative of their

activity level, as expressed by number of steps per 24 hours. Sensewear bracelets are worn on the upper arm, and some patients found them uncomfortable to wear. A few patients reported eczema. Another disadvantage was that the bracelets are not water resistant, and occasionally participants would forget to put them back on after a shower.

Since the use of these devices is growing in popularity, and many ME/CFS patients already wear some kind of activity tracker for their personal benefit, we wanted to compare with a common tracker used in the general population. When choosing a device for this project, our priorities were simplicity of use, performance on the basic functionalities (steps and heart rate), and privacy. Privacy and data protection issues were addressed in collaboration with the hospital's IT security manager and data protection officer. The Fitbit privacy terms and conditions were more specific on their compliance with the General Data Protection Regulation (GDPR) directive than several comparable trackers in the same price range. We used pseudonymisation toward third parties to protect participant´s privacy. Fitbit Charge 3 has been validated, and data in the activity range typical for ME/CFS patients were acceptable. However, several patients reported that their Fitbit devices recorded steps when they were not walking but engaged in other activities which involved arm movement or vibration, such as knitting, cooking and driving slowly in a car or electric wheelchair on bumpy roads. The technology is developing rapidly, and when we plan a new study, it is possible that other trackers will be better evaluated, both in terms of convenience and measurement accuracy.

Values from the two activity trackers correlated significantly at all three timepoints, but Fitbit recorded significantly higher numbers of steps as compared to Sensewear. Differences between activity trackers are important to keep in mind when comparing studies using different brands.

There is a general public perception that tracking leads to an increase in steps taken. A meta-analysis showed an increase in daily steps when wearing activity trackers (146). The experience from our studies is that for some patients with ME/CFS,

wearing a tracker may have the opposite effect to that seen in the general population, as some patients use activity trackers to monitor and pace their physical activity, partly as a tool to prevent PEM and 'crashes'. Our experience indicates that many patients find it useful to use a tracker to avoid such crashes, thereby increasing their overall level of function and improving their quality of life over time.

7.5 Outcome measures

As discussed under Fatigue Score and Function Level, none of these outcome measures are perfect because they are subjective and depend on recall of status at baseline for comparison.

As described above, the SF-36 is not a perfect questionnaire either, but it is validated and widely used in trials and is therefore useful for comparisons. A Norwegian group (Sommerfelt et al.) have developed a new questionnaire (FunCap), which takes into account the consequences of different activities. For example, do different activities lead to increased symptom burden, and how long does the exacerbation last? Using activity measures alone as an endpoint also has its limitations, as it does not address the potential for increased symptom burden with increased activity. ME/CFS is a disease with many symptoms, and patients will use an improvement in different ways. Some will use the extra energy to socialise, some will go for walks, others will read or spend energy on cognitive tasks. A combination of different questionnaires and activity measures to measure endpoints in trials is still the best option, until biological markers become available as objective outcome measures.

It is also useful for different research groups to agree on outcome measures, including which data collection tools to use, what should be considered a clinically relevant improvement, and how to characterize response in a trial. Such harmonisation of outcome measures would aid comparison of different trials, especially when considering the effects of intervention. Also, the distinction between improvement as a result from intervention or from natural variation of symptoms over time, can be

challenging. This is discussed in detail in the discussion section regarding the Fitbit study.

7.6 Dosing of rituximab and cyclophosphamide

In the RituxME trial, the rituximab maintenance doses were 50% to 60% lower than those in the previous maintenance study (KTS-2-2010). However, the two initial induction rituximab doses were higher and if the drug was effective in ME/CFS we would expect early responses to be more frequent in the rituximab than in the placebo group. Nevertheless, this change between the KTS-2-2010 rituximab maintenance study and the RituxME study led to some uncertainty as to whether higher doses might have resulted in more responders.

In the CycloME study, the doses used were in the range used to treat various autoimmune diseases (147) and in the same range as those used in adjuvant chemotherapy for breast cancer, usually combined with other chemotherapy drugs. The cumulative doses after six infusions of intravenous cyclophosphamide are approximately 6-9 g. The estimated risk of serious long-term toxicity other than the possible induction of menopause is low at these doses, but not negligible (148, 149).

8 Discussion

8.1 Discussion of results

8.1.1 Rituximab analyses

We performed a study characterizing rituximab concentration measurements using serum samples from participants in the previous KTS-2-2020 study (22) to investigate if the concentrations of rituximab had an effect on response status. We worked on this paper before the RituxME trial was unblinded in October 2017. It could be argued that measuring the concentrations of rituximab and looking at ADA became less important when a negative RituxME trial was obtained. When we concluded that rituximab concentration had no effect on response, this was in accordance with the negative outcome of the RituxME trial. Concentration measurements are still useful in clinical drug trials and can add important information. If the RituxME study had demonstrated significant differences between the rituximab and placebo groups, the scheduling of rituximab doses and intervals would be important to tailor further treatment trials with rituximab, and measurements of drug concentration would then be important. For future trials, such analyses should be specified in the protocol in advance, to ensure systematic sampling at predefined time points, in accordance with a pharmacokinetic study design.

8.1.2 The RituxME trial

There were high hopes for the randomised phase III trial of rituximab. This is one of the largest randomised drug trials performed in ME/CFS. Previous phase II rituximab trials by our research group, including the first randomized and placebo-controlled study (138) and the open-label rituximab maintenance study (22) had shown promising results, and it was important to do a double-blind and placebo-controlled trial to verify or refute the initial findings. However, the placebo and rituximab groups had a similar course during follow-up for both Fatigue score and SF-36 physical function (SF-36 PF), and the trial was negative with no significant

differences between the rituximab and placebo groups, in any of the outcome measures. We were not able to identify subgroups with a significant clinical effect from rituximab. There were responders in both the placebo and rituximab groups. In total, 30% met the criteria for a response, and both groups increased in SF-36 PF scores during follow-up, on average by 11.5 points (scale 0-100). We speculated that this limited average improvement represents some kind of "trial effect", indicating that the patients may have benefited simply from receiving regular medical follow-up and care. Overall, regardless of group allocation, there were higher clinical response rates among women than men, among patients with mild/moderate versus more severe disease, and among patients with shorter disease duration.

In retrospect, we believe that the RituxME response criteria were not stringent enough, and that this may have contributed to the effect on the response rates from natural variation in symptoms over time in both the rituximab and placebo groups. These considerations have been important in further work to improve outcome measures for future trials, including the design of the Fitbit study, which assessed ME/CFS symptom variation over time by different severity levels and also incorporated activity trackers.

The placebo mechanism is always of interest in a trial setting. After 6 weeks' follow-up, patients were asked to guess their allocation, and only 12.6% of the enrolled patients correctly guessed their intervention. Thus, the predictive value of patient expectations was uncertain.

Another important element when evaluating a trial is the group of patients included. Case definition, inclusion criteria and severity of disease are important aspects. We have used the Canadian consensus criteria (13). As we still do not have objective biological markers that can be assessed by clinical evaluation, a thorough medical disease history and strict inclusion criteria are the best measures to select a representative group of patients. Nevertheless, some heterogeneity in the patient group must be expected, and this can affect the results. In this trial, five centres with different investigators enrolled patients. There were some differences between the

study centres. Response rates were 43% at the University of Northern Norway (UNN) and between 25 and 31% at the other four centres (OUH, HUH, St. Olav and Notodden Hospital). By GLM repeated measures, the time-by-centre interaction was significantly different for Fatigue score (i.e. patients from UNN had better improvement in Fatigue score during follow-up), while the GLM repeated measures for SF-36 PF showed no significant difference between centres.

Rituximab targets CD20 positive B-cells. If autoantibodies are produced mainly in CD20 negative long-lived plasma cells, rituximab will not reduce autoantibody production and will have no effect on symptoms, unless treatment is prolonged. This means that ME/CFS may still be a disease driven by autoantibodies or an autoimmune mechanism, even if rituximab does not work in most patients. Patients with autoantibody production from early plasmablasts with some CD20 expression may respond, while a majority, if their autoantibodies stem from long-lived CD20 negative plasma cells, would be likely non-responders.

When we evaluated the RituxME trial, we found that the enrolment period was demanding for the patients. We had planned too many tests and substudies at baseline. As a result, patient-recorded outcome measures were visibly affected, and some patients took weeks to recover. In particular, the two-day CPET caused a great deal of PEM and some patients with high symptom burdens experienced a distinct worsening of symptoms for weeks, also described by Moore and coworkers (26) .

Following the coronavirus pandemic, some caution should be taken for the use of rituximab (150). Rituximab intervention results in profound B-cell depletion within a few days. Recovery of B-cell counts usually does not begin until 6-9 months after completion of therapy, and normal levels are often achieved after 9-12 months. While long-lived plasma cells are unaffected, and pre-existing antibody levels only slightly reduced after a limited rituximab infusion schedule (such as in the RituxME trial), B-cell depletion following rituximab reduces humoral immune responses to new antigens including infections and vaccines (150, 151). In addition, as a result of the prolonged depletion in memory B-cells, antibody production to recall antigens is also

reduced even 6-10 months after treatment (150). This reservation is relevant but was probably most important in the early years of the new pandemic. Because of the increasing immunity of the population due to repeated SARS-CoV2 infections and vaccines, one would expect the adverse effect of B-cell depletion on Covid-19 outcomes to be less pronounced. In the RituxME study, there were two cases of hospitalisation due to uncomplicated febrile neutropenia (late-onset neutropenia) with a probable association with rituximab, and two hospitalizations due to infections with an unlikely association with the drug (one appendicitis and one lung infection). In total, adverse events were seen in 63 patients in the rituximab group (81.8%) and 48 in the placebo group (64.9%). Serious adverse events (SAE) were detected in 26.0% of the rituximab group and in 18.9% of the placebo group, mainly due to prolonged stay in the hospital (after the outpatient clinic had closed) after infusions. The relatively large number of unrelated adverse events probably reflects the low tolerance for physical and cognitive strain in patients with ME/CFS. Any SAE with possible or probable relation to intervention was recorded in 8 patients (10.4%) in the rituximab group.

8.1.3 The CycloME trial

In an open-label study with a potentially toxic drug and a demanding treatment period, the duration of responses is important. The follow-up was originally planned for 12 months, but was extended twice, first to 18 months, then with an additional follow-up visit at 3-4 years after inclusion. After 18 months there were 22 responders (55%), with mean SF-36 PF increasing from 35.0 at baseline to 69.5 at 18 months. The response durations were sustained for most of the responders. Out of 22 responders, 20 completed the follow up at 3-4 years; 15 were still in remission. Seven even reported further improvement compared to their status at 18 months' follow-up. However, due to the lack of a placebo group, response data must be interpreted with caution.

The period of treatment with repeated cyclophosphamide infusions led to increased symptom burden and side effects in some patients, most commonly nausea and general malaise lasting 1-2 weeks after each infusion. At the scheduled doses of

cyclophosphamide, we did not record any events of haematological toxicity. When we surveyed patients at four years, the general opinion among the patients was that the treatment period was manageable, even among non-responders. We believe that the reduced quality of life of many patients prior to treatment justifies this intervention, if there is a possibility of long-term improvement.

8.1.4 HLA association

Samples from the CycloME study were analysed as part of a larger genetic study which found that the presence of two HLA alleles associated with ME/CFS (HLA-DQB1∗ 03:03 and HLA-C∗07:04) (45). Interestingly, these risk alleles were predictive of response to cyclophosphamide, which may support an autoimmune hypothesis if verified in new studies. Ten of the 12 patients (83.3%) positive for HLA alleles DQB1∗03:03 and/or C∗07:04 had a clinical response, compared to 12 out of 28 patients (42.9%) negative for these HLA alleles. HLA associations have been documented for virtually all established autoimmune diseases, some with very strong and others with moderate or weak associations, but an HLA association is not sufficient to determine the aetiology of a disease (152). Thus, this result from our study should not be interpreted as evidence for an autoimmune disease mechanism in ME/CFS.

8.1.5 The Fitbit study, natural variation and outcome measures

An important lesson from the Fitbit study is the impact of natural variation on changes in PROMs and activity tracker results over time. This is particularly important in a trial when defining outcome measures and deciding who is considered a 'responder'. As discussed in paper IV (20), some trials use a 10-point increase in the SF-36 PF as a criterion for "response". We found a group of patients with milder disease (baseline SF-36 PF > 50 or DSQ-SF < 55) with considerable symptom variation during 6 months' observation without any intervention, with a mean variation in SF-36 PF of 16.9 points (difference between lowest and highest value during four-week periods over six months), compared to the more severely ill group (SF-36 PF < 50 and DSQ-SF > 55) who had a mean variation in SF-36 PF of only 3.4

points. Although the Fitbit study was a relatively small study, it seemed quite evident that patients with higher symptom burden assessed by SF-36 PF and DSQ-SF had a more stable disease over time. Thus, inclusion of patients with milder disease may affect response rates due to more natural symptom variation, which again highlights the need for more stringent criteria defining clinical response. Conversely, in an intervention trial, inclusion of patients with higher symptom burden would make it easier to separate true effects from intervention due to less expected natural symptom variation. Importantly, these observations are not meant to trivialize the devastating effects mild ME/CFS can have on the quality of life of patients, but rather to reflect on how to improve the design and interpretation of interventional trials. Based on these observations, in upcoming studies we plan to include a "run-in period" with use of trackers and PROMs, in order to capture each patient's symptom variation over time before start of intervention, and we will probably include patients with moderate (mainly house-bound), moderate/severe and/or severe (mainly bedridden) ME/CFS in an attempt to avoid a major impact on response characterisation from natural symptom variation.

It is clearly important to consider the patient sample included, the baseline severity assessed by various variables, and the outcome levels when interpreting and drawing conclusions from a study. The mean increase of 10.8 points in SF-36 PF from baseline to 6 years seen in the placebo group from the RituxME trial, indicates that a 10-point increase of SF-36 PF is an inadequate criterion for "response" to an intervention in a study. Many clinical studies assessing the effects from cognitive behavioural therapy (CBT) have included patients with physical functioning assessed by SF-36 PF with mean values at baseline in the range of 50 to 60. We believe that baseline ME/CFS status with fatigue severity and physical impairment is important when interpreting the results from a trial, as discussed thoroughly in Paper IV (Fitbit study) (20). A recent review by Kuut et al. (153) also reported mean SF-36 PF of 55 in the CFS sample included in their eight CBT trials. In the active CBT group, the mean SF-36 PF after intervention was 73, as compared to 63 in the control group. The authors concluded that patients with less functional impairment and more fluctuating activity pattern were more likely to respond to CBT. This could be

compatible with a “trial effect” or natural variation among patients with milder ME/CFS, as described in the Fitbit study. Kuut et al. also concluded that severe functional impairment may reflect more severe disease, and that this subgroup of patients might need additional interventions or more intensive treatment.

There is also an argument to be made for assessing relative rather than absolute changes; a 20-point increase in SF-36 PF from e.g. 10 to 30 is not directly comparable to a 20-point increase from e.g. 50 to 70, and would surely have a greater impact on the patient’s change in quality of life.

8.1.6 The follow-up study, RituxME and CycloME trials

The six-year follow-up of the RituxME and CycloME trials showed that patients in the CycloME study improved during the study period and continued to improve slightly from the end of the study to the six-year follow-up. This was measured by mean scores for SF-36 PF, DSQ-SF and Function level. Correspondingly, the mean scores for SF-36 PF and Function level in patients participating in RituxME remained relatively stable from the end of the study to six years. At six-year follow-up, the percentage of patients with mean SF-36 PF ≥ 70 was significantly higher in CycloME, compared to RituxME participants. In the cyclophosphamide group, 44.1% had an SF-36 PF ≥ 70 at six years, compared to 27.6% in the rituximab group and 20.4 % in the placebo group. We chose a cutoff for SF 36-PF at 70 points, because this value for SF-36 PF that would indicate a substantial improvement, taking into accord that the mean SF-36 PF at baseline was in the range 30-35. At six-year follow-up, SF-36 PF ≥ 90, i.e., scores close to the normal population range, was achieved by 17.6% of the cyclophosphamide patients, 8.6% of the rituximab patients, and 7.4% of the placebo patients. In general, there is sparse data on natural course and recovery among ME/CFS patients. However, the recovery rate reported for the cyclophosphamide patients is probably higher than expected, based on results from previous retrospective studies (34).

Data from the placebo group add knowledge on ME/CFS patients who were included in a trial, with regular visits and follow-up over time, but without receiving active

intervention. This group showed an increase in mean SF-36 PF and Function level during the first two years, possibly due to a "trial effect" indicating benefit from regular visits. After the end of the study, the mean values stabilised. After six years, 7% of the placebo group reported SF-36 PF ≥ 90, close to the normal range of physical function in the general population. This indicates that ME/CFS may be reversible, and that spontaneous recovery is possible. Although many patients experience a chronic course of illness over many years, such reversibility is also an important argument in favour of research to unravel disease mechanisms and to elucidate rational targets for intervention, with the aim to push the pathomechanisms in the direction of health.

Interestingly, when we used the DSQ-SF algorithm for defining Canadian consensus criteria in the follow-up study, we found that 16 patients (47.1%) in the CycloME trial no longer fulfilled the diagnostic criteria at six years, compared to one patient at baseline. In the RituxME trial at six years, 23 (40.4%) patients in the rituximab group failed to meet the criteria, and 17 (31.5%) in the placebo group. The DSQ-SF algorithm for defining Canadian consensus criteria was not used at baseline in the RituxME study, but all patients were clinically evaluated, and this should be more accurate than the use of an algorithm based on a self-reported questionnaire. When we evaluated the DSQ-SF and divided the patients in groups based on the algorithm for defining Canadian criteria, we found that the symptom burden was still considerable, and that almost half of the patients still reported core ME/CFS symptoms such as PEM, cognitive problems, fatigue or sleep disturbances at least to a moderate degree and at least half the time, even though the DSQ-SF algorithm indicated that they no longer fulfilled the diagnostic criteria.

Regarding employment, in the CycloME study 15.0% worked part-time and no-one worked full-time at baseline. At six years (among 34 participants), six were working part-time and four were working full-time (29.4% in total). In the RituxME trial, at baseline, four worked part-time, one worked full-time and three were students (5.3%). After six years (among 112 participants), one worked part-time, three worked full-time and two were students (4.5%). Doubling the number of people working part-

time or full-time in the CycloME trial after 6 years is important both for the individual patient and from a societal perspective.

8.1.7 Adverse events

The monitoring of toxicity and adverse events was an important focus in both the RituxME and CycloME trials. Importantly, no serious long-term toxicity was reported between the end of the trial and the six-year follow-up in either study.

Regarding fertility issues in the CycloME study, five women aged 42 to 51 years at enrolment entered menopause during or after the 18-month study period. Four other patients (one of whom was using hormonal contraceptives) had irregular menstrual bleeding between the end of the study and the six-year follow-up. On a positive note, one of the young women with possible premature menopause reported in the CycloME study, went on to give birth to two children, both without the use of assisted reproductive technology.

8.1.8 Biological markers

We have collected samples over the years, mostly as part of the clinical trials, and have built up a relatively large research biobank (Regional ethical commitee no. for biobank: 2018-1532 and 2019-00767). The biobank contains both baseline and follow-up samples, and analyses are supported by clinical data from a well characterised patient sample.

A major focus for all our clinical trials has been to identify predictors of response. Despite extensive efforts, we have not yet been able to identify such definitive clinical or biochemical predictors. The HLA risk alleles presented in the CycloME study, where the response rate was 83% in the “risk allele”-positive group versus 43% in the “risk allele”-negative group, may turn out to be a predictive marker if verified in future studies.

In the unpublished data analysing various immunological laboratory parameters from the clinical trials, there are some interesting findings. In the following I will briefly summarise some findings from the CycloME trial.

In the CycloME trial, the mean baseline IgG and IgG1 levels were predictive for clinical response, as the levels were significantly lower in the responder group (n=21) compared to non-responders (n=16) (Figure 3). Significantly lower baseline serum IgG levels were also found in responders compared to non-responders in a previous study from our group (61), analysing serum samples from previous rituximab trials (22, 138).

In the CycloME trial there were significant but modest decreases of IgG, IgM and IgA during follow up, especially from baseline to 12 months (Figure 3). CD3 (T-cells), CD19 (B-cells) and CD16/56 (NK-cells) lymphocytes in peripheral blood showed significant trends towards ME/CFS severity, with the highest lymphocyte counts in the severe group compared to moderate and mild/moderate (Figure 4). After cyclophosphamide intervention, all subsets of lymphocytes decrease from baseline to 6 months and GLM repeated measures analyses showed significant time effects during follow-up for all subsets (Figure 4), also described by Ahlmann et al (114). CD3, CD4, CD8 T-cells and CD19 B-cells were still lower at 18 months compared to baseline, while NK cells had returned to baseline levels (Figure 4).

The broad effects of cyclophosphamide on different immune cells make it difficult to pinpoint a precise mechanism for the clinical effect observed in ME/CFS. The cytotoxic effects on proliferating cells make inhibition of activated B-cells to plasmablasts and reduction of IgG levels - including autoantibodies - a plausible possibility. The described downregulation of T cells, effects on different subsets of lymphocytes and interactions between immune cells are other possible mechanisms of cyclophosphamide in this disease.

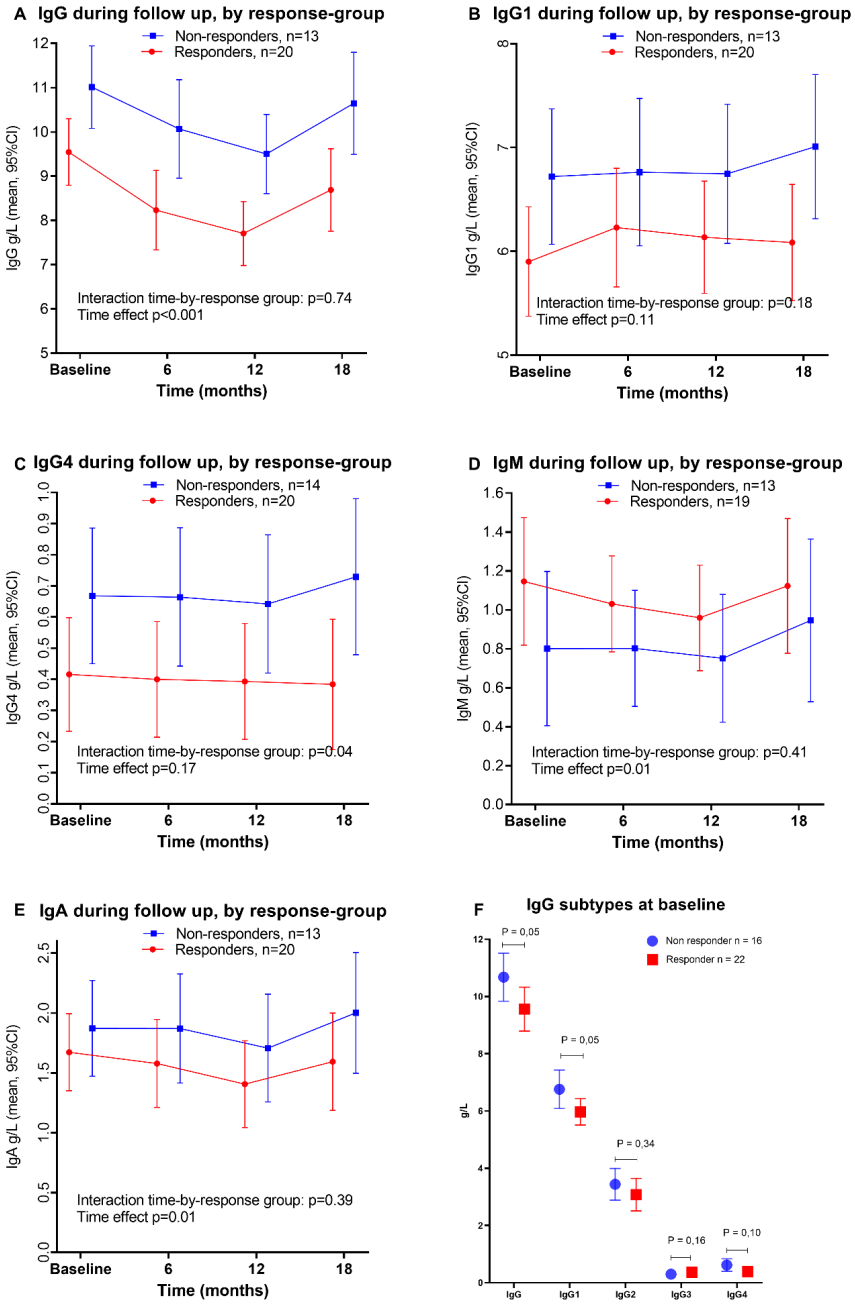
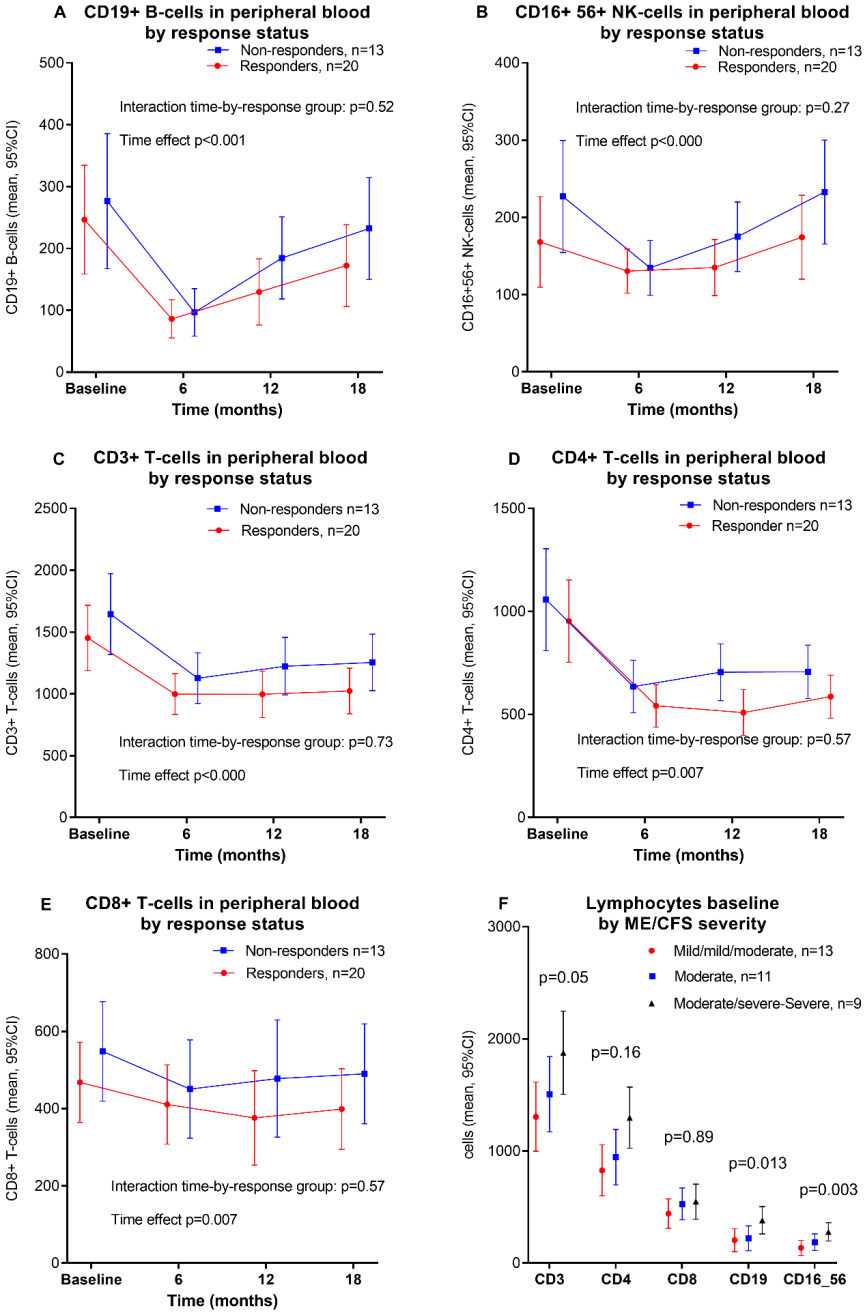


Figure 3. Panel A-E Immunoglobulins during follow-up in the CycloME trial, by response status. P-values from GLM repeated measures, for time effects and for interaction time-by-group. Panel F IgG subtypes at baseline, by response-status. P value from Mann-Whitney test.



Figur 4. Panels A-E: Lymphocytes subsets; CD19 (B-cells), CD16/56 (NK-cells), CD3 (T-cells), CD4 (helper T-cells), CD8 (cytotoxic T-cells) lymphocytes in peripheral blood during follow up in the CycloME trial. P-values from GLM repeated measures, for time effects and for interaction time-by-group. Panel F: Lymphocytes subsets at baseline by ME/CFS severity, P-value from Jonckheere trend test.

If the hypothesis that ME/CFS is an autoimmune disease is correct, a specific autoantibody could be a useful biomarker. Studies have assessed different known autoantibodies (69, 70) in ME/CFS patients, but without high specificity and sensitivity. During the last 15 years, our research group has performed experiments trying to define specific autoantibodies, but without success. As collaboration projects, we performed a study on neuronal autoantibodies (154), and also a study on immunoreactivity in serum to a random-sequence 125.000 peptide array, to search for specific immunosignatures in ME/CFS (155). Although there are interesting observations from these studies, we have not been able to identify a specific and sensitive autoantibody or immunosignature which could aid as a biomarker in ME/CFS.

These negative data also contribute to our speculations that ME/CFS may be a variant of an autoimmune disease, as stated above in the section describing hypothesis and pathomechanisms. Thus, ME/CFS may not be characterized by a few specific pathogenic autoantibodies as demonstrated in classic autoimmune diseases, often accompanied by complement activation, histologic inflammation and tissue damage. Rather, in ME/CFS there may be a functional disturbance from anti-self antibodies, naturally emerging after an immunological trigger, which disturb biological systems such as autoregulation of blood flow with tissue hypoxia on exertion. Such functional autoantibody responses, probably including GPCR autoantibodies, usually resolve after infection, but may persist in ME/CFS patients.

8.1.9 Mental health

A study from Nacul and coworkers from 2011 “The functional status and wellbeing of people with myalgic encephalomyelitis/chronic fatigue syndrome and their carers” is frequently referred in the literature (28). They reported that the scores for the SF-36 Physical and Mental Health Component Scores domains were considerably and consistently lower in people with ME/CFS, when contrasted with individuals with a range of other chronic diseases such as cancer, depression, and RA. This demonstrates that ME/CFS is not only physically disabling, but also has a significant

impact on mental health. Also, the mental health of carers was lower than expected, demonstrating the burden of this disease (28).

However, in our clinical trials (18-20) the Mental Health subdomain was only slightly reduced in study participants compared to the general population, whereas there were large reductions in SF-36 Physical Function, with mean baseline scores of 30-35 (scale 0-100).

8.2 Strengts and limitations

Strengths of these studies include well-defined ME/CFS patient samples with comprehensive follow-up according to the clinical trial protocols, and with standardised biobank sampling. All studies had very low levels of missing data.

Important strengths of the RituxME study include the considerable sample size of 151 patients, the multicentre design with different physicians involved in the study, and the randomisation and blinding performed by external parties.

CycloME was an open-label study, which is an important limitation. When the RituxME study turned out negative, we extended the CycloME study with additional follow-up, because we believed that data on long-term effect would strengthen the study, especially an 'open-label' study.

At the Department of Oncology, Haukeland University Hospital, both the oncology ward and the outpatient clinic have long experience using rituximab and cyclophosphamide, which is a strength and a reassurance when using these drugs in trials for a new group of patients.

As discussed under methodological considerations, one of the main challenges in our trials is the outcome measures and endpoints which are subjective. In particular, the Fatigue score has its limitations, as previously discussed, and we have therefore decided not to use this outcome measure in the Fitbit study, in the CycloME and RituxME six-year follow-up study, or for future trials. Throughout this period, and while working on new trials, we have sought to improve enrolment and outcome

measures, both through feedback from patients and by looking critically at our own research. The Fitbit study was designed to focus on the natural history and symptom variation of the disease in order to improve outcome measures for future studies.

The issue of what should be considered a real improvement in disease-related symptoms is carefully discussed in the Fitbit paper (20). We have concluded that in the absence of a perfect questionnaire and biological markers of response, a combination of clinical assessments, PROMs and activity trackers, with a “run-in period” before intervention, and repeated outcome measurements during follow-up, are important elements to improve response evaluations in a trial setting.

There are not many ME/CFS trials with long-term follow-up, and the six-year follow-up of the RituxME and CycloME trials is relatively unique in this context. Through the placebo group, we had the opportunity to describe the natural course of the disease in 54 participants who were enrolled in a trial but received no intervention.

A limitation for the follow-up study is the comparison of data from two different studies (RituxME and CycloME) with different patient groups. However, although the inclusion criteria were similar, and the patients were included during the same period.

When comparing the response data from the CycloME and RituxME trials, it is important to consider the very different mechanisms of action of the two drugs. Both drugs are used to treat cancer, but Rituximab is a "targeted therapy", and cyclophosphamide is a cytotoxic drug. Rituximab is a monoclonal antibody that selectively depletes B-cells that express the CD20 protein on their surface, while cyclophosphamide has a broader immunosuppressive effect on several subsets of lymphocytes. The main mechanism of action of cyclophosphamide is the ability to covalently bind an alkyl group, primarily affecting DNA. The toxicity profiles of the two drugs are also very different.

The main limitation of Paper I is the lack of design as a pharmacokinetic drug trial, and the small number of patients (n=23). The Fitbit study also had a small number of

participants (n=27), but part of the intention was to have close contact with all participants, as the focus was on the implementation and use of an activity tracker in a study setting, and with regular follow-up. With the added complication of the Covid-19 pandemic, we decided that a sample of 27 was a manageable number. In the post-trial analyses, we found that not all of the measures from the Fitbit wristband were equally reliable, so we decided to focus on the measures: steps per 24 hours and resting heart rate.

9 Conclusions

This thesis consists of five different papers with studies of ME/CFS patients: three clinical trials, one follow-up study and one study of rituximab pharmacokinetics using serum samples from one of the previous clinical trials. A total of 218 patients were involved in the clinical trials, plus blood samples from 23 additional patients in the drug trial. I would like to thank the patients for their participation and compliance in the studies. Thanks to the enrolled patients, we have very little missing data in the studies and therefore credible results in the studies. This is a very special group of patients to work with, with a great sense of gratitude and a desire to contribute. This made the work feel valuable and important.

The overall aim of the work was to increase the general understanding of the disease mechanisms in ME/CFS and to verify or dispute the hypothesis that ME/CFS is associated with an autoimmune pathomechanism, with a role for antibodies, B-cells and plasma cells.

Through the studies we have learnt a lot about ME/CFS, about the symptoms and progression of the disease over time, but also about how to conduct clinical trials in this patient group and the challenges involved. We have tried to share these experiences in the published articles.

The follow-up study shows that immunomodulatory treatment with cyclophosphamide led to long-term improvement in a significant group of patients, strengthening the hypothesis that the development of ME/CFS may be associated with a variant of an autoimmune pathomechanism. Although the rituximab trial was negative, ME/CFS may still be a disease driven by autoantibodies or an autoimmune mechanism. If the autoantibodies are produced in CD20-negative, long-lived plasma cells rituximab will not reduce autoantibody production, and one could speculate that targeted therapy against more mature plasma cells would be a treatment option.

10 Future perspectives

We want to continue our efforts to improve the understanding of the disease through clinical trials and laboratory studies. We believe it is possible to find the underlying mechanism and thus a rational treatment for this disease. Our research group is currently conducting a pilot study using the anti-CD38 antibody daratumumab to target CD38-positive, CD20-negative, long-lived plasma cells to more effectively reduce antibody levels (Regional Ethical Committee No. 445176). Based on the observations from the previous trials, in this pilot study we included a run-in period of three months before intervention and included patients with moderate (mainly housebound), moderate/severe or severe (mainly bedridden) ME/CFS.

If this pilot study shows adequate feasibility and toxicity, and indications of beneficial clinical effects in ME/CFS patients, we aim to conduct a new randomised clinical trial of plasma cell targeting versus placebo, taking into consideration the experience from the trials described in this thesis. We continue to expand our biobank, and with samples collected at baseline and at follow-up, we will be able to further investigate disease mechanisms, try to identify a biomarker, and continue the search for an effective rational treatment.

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Paper I

Brief Report

Rituximab Serum Concentrations and Anti-Rituximab Antibodies During B-Cell Depletion Therapy for Myalgic Encephalopathy/Chronic Fatigue Syndrome

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ABSTRACT

Purpose: Previous Phase II trials indicated clinical benefit from B-cell depletion using the monoclonal anti-CD20 antibody rituximab in patients with myalgic encephalopathy/chronic fatigue syndrome (ME/CFS). The association between rituximab serum concentrations and the effect and clinical relevance of antidrug antibodies (ADAs) against rituximab in ME/CFS is unknown. We retrospectively measured rituximab concentrations and ADAs in serum samples from patients included in an open-label Phase II trial with maintenance rituximab treatment (KTS-2-2010) to investigate possible associations with clinical improvement and clinical and biochemical data.

Methods: Patients with ME/CFS fulfilling the Canadian criteria received rituximab (500 mg/m^2) infusions: 2 infusions 2 weeks apart (induction), followed by maintenance treatment at 3, 6, 10, and 15 months. The measured rituximab concentrations and ADAs in serum samples included 23 of 28 patients from the trial.

Findings: There were no significant differences in mean serum rituximab concentrations between 14 patients experiencing clinical improvement versus 9 patients with no improvement. Female patients had higher mean serum rituximab concentrations than male patients at 3 months ($P = 0.05$). There was a significant negative correlation between B-cell numbers in peripheral blood at baseline and rituximab serum concentration at 3 months ($r = -0.47$; $P = 0.03$). None of the patients had ADAs at any time point.

Implications: Clinical improvement of patients with ME/CFS in the KTS-2-2010 trial was not related to rituximab serum concentrations or ADAs. This finding is also in line with a recent randomized trial questioning the efficacy of rituximab in ME/CFS. Rituximab concentrations and ADAs still offer supplemental information when interpreting the results of these trials. (*Clin Ther*. 2019;41:806–814)

Key Words: antidrug antibodies, B-cell depletion, chronic fatigue syndrome, myalgic encephalopathy, rituximab, rituximab concentrations.

INTRODUCTION

Myalgic encephalopathy/chronic fatigue syndrome (ME/CFS) is a disease of unknown etiology affecting ~0.2% of the population.[1] Patients with ME/CFS report a very low quality of life.[2] The main symptoms are profound fatigue, postexertional malaise, sleep disturbances with inadequate restitution, pain, impaired cognitive function, and several symptoms related to autonomic dysfunction and to the immune system.[3] Presently, there is no established standard interventional drug treatment for ME/CFS. Several observations support a

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role of immune disturbance in a subset of patients with ME/CFS: the female preponderance (3–4 times more common in women), an often abrupt start after infection (~70%), a genetic predisposition,[4] and studies indicating that partly overlapping syndromes such as postural orthostatic tachycardia syndrome[5] or complex regional pain syndrome[6] may have an autoimmune basis. A possible role of autoimmunity in ME/CFS has been suggested.[7–10]

Rituximab is a chimeric immunoglobulin G (IgG) monoclonal therapeutic antibody that targets CD20 and promotes a rapid and prolonged but reversible peripheral B-cell depletion,[11] with proven efficacy in lymphomas and in several rheumatic and autoimmune disorders.[12] B-cell depletion is associated with target-mediated elimination of rituximab.[13] Antidrug antibodies (ADAs) can also promote more rapid clearance of rituximab and change of clinical effect.[14] We have previously suggested a clinical benefit from B-cell depletion in patients with ME/CFS using the monoclonal anti-CD20 antibody rituximab in a small, randomized, placebo-controlled study (KTS-1-2008).[7] Prolonged responses were then shown in an open-label Phase II trial with maintenance rituximab treatment (KTS-2-2010).[15] However, we recently completed a multicenter, randomized, double-blind Phase III trial investigating rituximab maintenance treatment versus placebo (RituxME [B-Lymphocyte Depletion Using the Anti-CD20 Antibody Rituximab (Mabthera®) in Myalgic Encephalopathy/Chronic Fatigue Syndrome]; ClinicalTrials.gov identifier NCT02229942) and concluded that there were no significant differences in outcome measures between the rituximab and placebo groups (submitted).[16] The relationships between serum rituximab concentrations and efficacy have been studied in lymphomas[17–20] and in systemic autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus.[21–23] The associations of rituximab serum concentrations to effect, and the clinical relevance of antidrug antibodies (ADAs) against rituximab in ME/CFS, have not been described. A possible association would have been important when deciding doses and making new protocols for B-cell depletion in the future, should the treatment principle demonstrate clinical efficacy. However, rituximab is not an established treatment for ME/CFS, nor is B-cell depletion a proven cause of symptom improvement in these patients. Based on the available knowledge at the time of the study, and as part of a broader approach to better understanding the disease mechanisms and possible reasons why a subgroup of patients reported benefit after rituximab treatment, we analyzed serum samples from patients included in the KTS-2-2010 clinical trial.[15] We retrospectively measured rituximab concentrations and ADAs in serum samples harvested during follow-up to investigate possible associations with clinical improvement of ME/CFS symptoms, sex, and B-cell numbers in peripheral blood.

PATIENTS AND METHODS

Ethics, Trial Design, and Patient Cohorts

The clinical trial, including one amendment, was approved by the Regional Ethical Committee in Norway (no. 2010/1318-4) and by the National Medicines Agency. All patients gave written informed consent. The trial was conducted in accordance with Good Clinical Practice. The design and results of the rituximab maintenance trial have been previously reported.[15] KTS-2-2010 was a single-center, open-label, one-armed Phase II study (NCT01156909) that included 29 patients. The treatment schedule was rituximab (500 mg/m^2; maximum, 1000 mg) 2 infusions 2 weeks apart (induction), followed by maintenance rituximab infusions (same dose) after 3, 6, 10, and 15 months and with follow-up for 36 months. The inclusion criteria were a diagnosis of ME/CFS according to the Fukuda 1994 criteria[24] and age 18–66 years. All patients also fulfilled the Canadian criteria.[3] Further characterization of the inclusion and exclusion criteria is included in the trial results previously published.[15]

The present study analyzed serum rituximab concentrations in 23 patients for whom samples were still available in the biobank from the 28 patients who received rituximab maintenance infusions in the KTS-2-2010 trial. Six patients were not included for serum rituximab measurements: 2 pilot patients (no biobank sampling), 2 patients who withdrew from the study during follow-up (1 due to an allergic reaction and 1 due to intercurrent disease), 1 who changed treatment to the anti-CD20 antibody ofatumumab due to an allergic reaction during the third rituximab infusion, and 1 due to missing biobank samples. Of the 23 patients, 15 received six rituximab infusions, 6 received five infusions, and 2 patients received four infusions (Table 1). This scheme was according to protocol because patients with no signs of clinical

Table 1. Rituximab (RTX) serum concentrations and clinical data for 23 patients with myalgic encephalopathy/chronic fatigue syndrome (ME/CFS) in the KTS-2-2010 trial.

Sex, Age (y)	BSA (m^2)*	ME/CFS Duration (y) and Severity†	RTX Dose (mg)	No. of RTX Infusions	RTX Level (μg/mL)‡		RTX Level (μg/mL)‡		B Cells§ at Baseline	Clinical Improvement‖	Response at End of Study (3 Years)
					At 3 Months	At 6 Months	At 10 Months	At 15 Months			
F, 44	1.83	5 y, sev	925	6	16.0—16.0	10.0—10.0	1.1—5.2	4.8—1.1	201	Yes	No
F, 37	1.55	20 y, mod	800	5	9.3—8.2	11.0—9.7	3.5—3.1	1.2—1.1	282	No	No
M, 58	2.12	17 y, mod	1000	6	11.0—11.0	4.7—4.7	1.3—1.4	0.9—0.8	137	Yes	Yes
F, 26	1.79	3 y, mild/mod	900	5	10.0—10.0	8.6—8.6	2.3—2.0	0.4—0.4	851	No	No
F, 22	1.48	5 y, mod	750	6	14.0—14.0	11—9.7	2.5—2.8	0.7—0.8	146	Yes	Yes
M, 49	1.66	17 y, mod/sev	850	6	14.0—14.0	5.7—5.0	1.6—1.6	0.4—0.4	117	Yes	Yes
M, 20	1.80	8 y, mild	900	6	1.3—1.3	23—23.7	11.0—10.7	4.9—5.2	436	Yes	Yes
F, 28	1.71	12 y, mod	850	6	41.0—41.0	5.1—5.3	1.4—1.2	0.5—0.5	217	Yes	Yes
F, 37	1.75	10 y, mild	850	6	15.0—15.0	7.1—7.1	2.5—2.5	0.1—0.4	156	Yes	No
F, 32	1.55	9 y, mod/sev	800	6	11.0—11.0	4.7—4.7	1.6—1.6	13.0—0.6	m	No	No
F, 42	1.92	5 y, mild	950	4	12.0—10.9	7.2—6.4	1.5—1.3	m	365	No	No
F, 20	1.62	7 y, mod	800	6	22.0—14.2	5.3—7.4	1.3—1.2	0.7—0.7	110	No	No
M, 48	2.12	12 y, mod	1000	6	1.4—1.4	3.4—3.4	1.1—1.1	0.5—0.5	48	Yes	Yes
F, 46	1.61	13 y, mod	800	6	14.0—14.0	7.4—7.4	1.5—1.5	0.7—0.8	619	Yes	Yes
F, 25	1.74	11 y, mod	850	5	7.5—7.5	0.4—2.9	0.6—1.2	m	1151	Yes	Yes
F, 55	1.75	8 y, mod	850	5	20.0—20.0	12.0—7.7	3.2—3.9	m	m	No	No
M, 59	2.04	3 y, mod/sev	1000	6	5.3—5.3	3.5—4.5	1.1—0.9	0.3—0.4	286	Yes	No
F, 37	1.87	20 y, mild	900	6	11.0—11.0	2.0—3.1	9.5—2.5	1.3—1.4	79	Yes	Yes
F, 49	1.70	13 y, mod	850	4	14.0—14.0	11.0—17.1	m	m	222	No	No
F, 56	1.79	5 y, sev	875	5	26.0—22.9	11.0—11.0	5.8—5.8	m	293	No	No
M, 26	1.80	8 y, mild/mod	900	6	19.0—19.0	13.0—10.4	2.7—3.4	m	159	Yes	Yes
F, 47	1.63	4 y, mod	825	6	24.0—21.2	20.0—10.3	6.6—7.3	13.0—2.6	165	Yes	No
M, 50	2.08	1 y, mild	1000	5	10.0—10.0	3.3—1.7	3.5—2.8	m	241	No	No

BSA = body surface area; F = female; M = male.

* According to the Du Bois method.

† Categorized as mild, mild/moderate (mild/mod), moderate (mod), moderate/severe (mod/sev), or severe (sev).

‡ RTX serum concentrations, measured concentration - concentration adjusted to median time interval since last dose.

§ B-cell numbers in peripheral blood ($\times 10^6$/L) at baseline (m indicates missing sample).

‖ Clinical improvement according to predefined criteria in the KTS-2-2010 rituximab maintenance trial (ie, fatigue score >4.5 for 6 consecutive weeks; fatigue score with scale 0—6, in which 3 is no change from baseline and higher scores indicate less fatigue).

improvement at 10 months of follow-up could forgo the planned rituximab infusions at 10 and/or 15 months. The characteristics of the patients and response data are shown in the Table 1.

Measurement of Rituximab Concentrations and ADAs

All serum samples used for rituximab measurements were gathered immediately before the next scheduled rituximab infusion and frozen at −80 °C according to the standardized biobank procedure in the trial protocol.[15] For all 23 included patients, serum samples were available at 3 and 6 months' follow-up. At 10 months, there were 22 samples available, and at 15 months, 16 serum samples were available. In the protocol for the KTS-2-2010 trial, the interval between the maintenance doses (at 3, 6, 10, and 15 months) could vary 1−2 weeks, and in some patients, doses were postponed due to concomitant disease or other circumstances. Due to this naturalistic setting, the dosing interval and thus the sampling time could vary between patients at each new rituximab maintenance dose. Measured drug concentrations were adjusted according to an estimated median $t_{1/2}$ of 22 days according to the Summary of Product Characteristics for rituximab https://www.ema.europa.eu/documents/product-information/mabthera-epar-product-information_en.pdf.*

Assays for serum rituximab concentrations and ADAs were performed by the Biologicals Laboratory, Diagnostic Services Sanquin (Amsterdam, the Netherlands). Measurements were performed according to the International Organisation for Standardization 15189 guideline. Rituximab concentrations were determined by using sandwich ELISA. In short, anti−rituximab-idiotype antibodies were generated in rabbits by immunization with rituximab F(ab)2. After purification of IgG by using Protein A Sepharose (GE Healthcare, US), reactivity against human IgG was removed by passage over a Sepharose-IVIG column. IVIG is a therapeutic intravenous IgG preparation prepared from >1000 blood donors. Antibodies that did not bind to the column were unreactive with serum IgG but showed strong binding to rituximab but not to adalimumab, infliximab, or natalizumab. They were used for coating the ELISA plate and, after biotinylation, also as a detecting agent.

The detection limit of the assay is ~0.8 μg/L. Because sera are tested at 1:10 dilution or higher, the detection limit in serum is 8 μg/L. The accuracy of the test is 110% (precision, 11.3%). ADAs were detected in an antigen-binding test using Protein A Sepharose for catching patient serum IgG and ^{125} I-radiolabeled rituximab F(ab)2. Samples containing IgG antibodies against rituximab did not yield positive results in assaying for anti-adalimumab, anti-infliximab, or anti-natalizumab antibodies.

Statistical Analyses

Serum rituximab concentrations from patients with different dosing intervals were made comparable by an estimated median $t_{1/2}$ of 22 days according to the product monograph of rituximab. All blood samples were withdrawn ≥3 half-lives after each dose. Assuming similar rituximab terminal elimination kinetics between patients, and using the actual measured rituximab dose at the specified interval since last dose, we calculated adjusted rituximab concentrations corresponding to the median time intervals for each patient. We used the formula $N(t) = N_0\left(\frac{1}{2}\right)^{\frac{t}{t_{1/2}}}$, where N_0 is the initial concentration (calculated from $t_{1/2}$ and time interval), and $N(t)$ is the estimated concentration after time (t). This assessment was performed to generate comparable rituximab concentrations corresponding to the same time interval since the last rituximab dose, and these data were used for analyses.

Serum rituximab concentrations (adjusted) were correlated to B-cell numbers in peripheral blood at baseline and through follow-up. General linear model for repeated measures (GLM) was used, with the interaction term (time*group) assessing differences in course of adjusted serum rituximab concentrations, between patients with clinical improvement versus no improvement, and female patients versus male patients. Greenhouse-Geisser corrections were used. For GLM, samples from 22 patients at 3, 6, and 10 months were included; 15-month data were excluded because of missing samples. The Mann-Whitney U test for independent samples was used to assess differences in adjusted serum rituximab concentrations between

* Trademark in Norway: MabThera® (Roche, Basel, Switzerland).

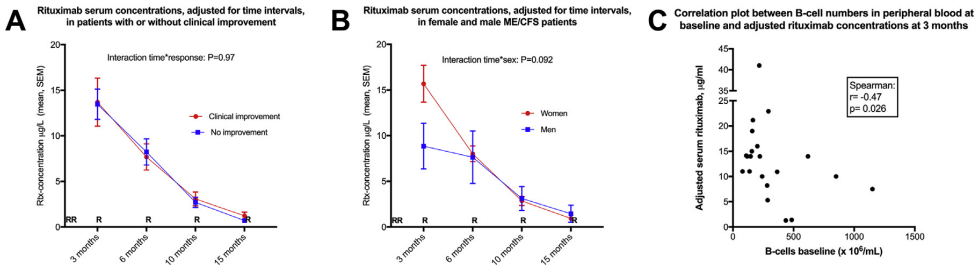

Figure 1. Rituximab serum concentrations during follow-up, adjusted for time intervals, in (A) 14 patients with and 9 without clinical improvement and in (B) 16 female and 7 male patients with myalgic encephalopathy/chronic fatigue syndrome (ME/CFS). The rituximab serum concentrations were assessed in samples taken at 3, 6, 10, and 15 months' follow-up, immediately before the scheduled infusion. The "R" in panels A and B indicate time points for rituximab infusions according to the trial protocol. P values from the general linear model for repeated measures are also shown. Error bars indicate mean with SEM. C, Correlation plot between B-cell numbers in peripheral blood at baseline and adjusted rituximab serum concentrations at 3 months' follow-up. Spearman correlations analysis between B-cell numbers in peripheral blood at baseline versus adjusted serum rituximab concentrations at 3 months' follow-up are shown in 21 patients with ME/CFS with available data.

groups at specific time points during follow-up, not taking into account repeated measures. Spearman analyses were used to assess correlations between serum rituximab concentrations and B-cell numbers in peripheral blood. A 2-sided P value < 0.05 was considered statistically significant.

RESULTS

Rituximab serum concentrations and clinical data for the 23 patients with ME/CFS are shown in the Table 1. Both measured value for rituximab serum concentrations and adjusted values to median time interval since last dose are presented. There were large interindividual differences in adjusted serum rituximab concentrations at all time points. Using GLM repeated measures (including 3, 6, and 10 months), there was no difference in repeated measures of adjusted serum rituximab concentrations between patients with clinical improvement versus no improvement during follow-up ($P = 0.97$), not for the course through follow-up nor at any of the specific time points of 3, 6, 10, or 15 months (Figure 1). None of the 23 patients had ADAs at any time point.

The adjusted serum concentrations of rituximab at 3, 6, 10, and 15 months according to sex are shown in the Figure 1. There was a trend for difference in repeated measures of adjusted serum rituximab concentrations between women and men assessed according to the interaction time*sex (P=0.092), with higher mean serum rituximab concentrations in female patients at 3 months ($P = 0.05$).

Higher B-cell numbers in peripheral blood at baseline correlated significantly with lower rituximab serum concentrations at 3 months ($r = -0.48$; $P = 0.03$). Correlation analyses (Spearman) revealed negative but not significant correlations between B-cell numbers in peripheral blood at 15 months and rituximab serum concentrations at 3 months ($r = -0.29$; $P = 0.22$), 6 months ($r = -0.03$; $P = 0.94$), and 10 months ($r = -0.04$; $P = 0.88$) of follow-up.

DISCUSSION

The current study is the first to examine the associations between rituximab serum concentrations and clinical improvement after B-cell depletion among patients with ME/CFS. The main finding was that any clinical effect of rituximab in patients with ME/CFS was not associated with serum concentrations or ADAs. We found a large interindividual variability for serum rituximab

concentrations at the different time points, in accordance with findings from other studies.[18,25,26] All patients had detectable serum concentrations of rituximab at 15 months (ie, 5 months after the last infusion). The lack of ADAs suggests a low risk of immunogenicity of rituximab in ME/CFS. Furthermore, ADAs could not explain the variability of rituximab concentrations or clinical effect.

Lack of associations between rituximab concentrations and clinical effect suggest that a concentration–effect relationship does not explain previously observed beneficial effects of the drug.[15] A recently completed (submitted) multicenter, randomized, double-blind Phase III trial investigating rituximab maintenance treatment versus placebo concluded that there were no significant differences in outcome measures between the rituximab and placebo groups.[16] This outcome casts doubt on the effects of rituximab intervention in ME/CFS in previous trials as well,[7,15] in which the improvements of ME/CFS symptoms could also have been caused by either placebo mechanisms or by natural variation over time. However, presently, we cannot exclude the possibility that selection mechanisms in previous trials could also be a relevant factor and that there may be a small subgroup of patients with ME/CFS with disease responsive to B-cell depletion. Thus, the assessment of associations between serum rituximab concentrations and clinical status of patients characterized as either responders or nonresponders, and presence of ADAs, is still interesting and offers supplemental information when interpreting the results.[7,15] In our opinion, it is highly relevant to include drug measurements when treating a new patient group off-label in clinical research.

Studies in patients with indolent lymphoma have suggested an association between higher serum rituximab concentrations and progression-free survival interval.[17–19] Serum trough concentrations of rituximab and AUC-time curves were higher for responders than for nonresponders in a study of aggressive B-cell lymphoma.[20] Results of studies in patients with rheumatoid arthritis have been inconclusive for the associations between rituximab serum concentrations and clinical responses. One study concluded that the variability in rituximab serum concentrations and ADA formation was not related to the clinical responses to rituximab,[26] whereas another study concluded that clinical responses depended on the degree of B-cell depletion but not on the rituximab doses given.[27]

Although the number of patients in the current study was low, we can now assume that the concentration of rituximab and the degree of B-cell depletion is not the main mechanism for symptom improvement in the patients with ME/CFS. This observation does not exclude the involvement of B cells or the immune system in the disease mechanisms. Body surface area (BSA) is mainly used for calculating induction and maintenance doses when treating lymphoma patients with rituximab intravenous infusions, whereas for the subcutaneous rituximab formulation, a fixed rituximab dose is common.[13] In systemic rheumatic diseases, different rituximab dosing regimens exist, but fixed doses with 6-month intervals are often used. One study concluded that sex and BSA explained ~32% of the interindividual variance for clearance, and 42% of the variance for the distribution volume.[25] In the KTS-2-2010 trial, we used BSA[28] when dosing rituximab; however, wide interindividual ranges of drug concentrations at each time point remained during follow-up.

Interestingly, female patients with ME/CFS had higher serum rituximab concentrations at 3 months of follow-up compared with male patients. Higher rituximab concentrations are known to be associated with female sex both in lymphoma treatment[13] and in rheumatoid arthritis.[25] Higher rituximab serum concentrations have previously been observed in women with rheumatic diseases, believed to be due to a higher distribution volume of the drug in men.[25,29] Data suggest that female patients with lymphoma benefit from rituximab-containing regimens more than men, possibly due to higher serum concentrations throughout induction and maintenance.[13]

None of the study patients had antibodies (ADAs) to rituximab at any time point. Antibody production represents an adaptive response and usually takes days to weeks following treatment exposure. The presence and extent of immunogenicity after monoclonal antibody administration vary and depend on several factors, most of which are related to the patients themselves, the antibodies, or the treatment regimen.[30] Monoclonal antibodies (mAbs) that deplete B cells, thereby attenuating the immune

response, seem to be at the lower end of the immunogenicity scale from other mAbs.[31] We only analyzed for ADA of IgG type, which are responsible for the majority of the ADA responses. The pharmacokinetic variability of mAbs is usually large and can partly be explained by ADAs, which accelerate mAb elimination,[27] but this theory could not explain the large interindividual variability in serum concentrations between patients in our cohort. A review article described no immunization with ADAs in patients with B-cell malignancies treated with rituximab, but a few patients with rheumatoid arthritis developed ADAs.[27] A study that compared intravenous and subcutaneous administration of rituximab in patients with follicular lymphoma detected ADAs in only 1 of 278 patients.[32]

B-cell numbers in peripheral blood at baseline were inversely correlated to rituximab serum concentrations at 3 months of follow-up. The association between a higher B-cell count before intervention and subsequent lower serum rituximab concentrations is expected and has been described by others,[25] possibly due to increased presence of the CD20 target and thus more rapid clearance of rituximab. Also, the effective B-cell depletion and reduction of CD20-positive cells after the first infusion result in a decrease in rituximab clearance following subsequent infusions due to the very low number of B cells present.[33] There were negative, but not significant, correlations between rituximab serum concentrations at 3 or 6 months and B-cell numbers in peripheral blood at 15 or 20 months of follow-up. However, the very low numbers of B cells at 15 months in most patients ($0-2 \times 10^6$/mL) makes these analyses uncertain. In the present study, both ME/CFS patients with or without clinical improvement during follow-up had adequate B-cell depletion, defined as $<5.0 \times 10^6$/mL CD19 + cells in peripheral blood.[22]

The strengths of the current study include a well-defined patient population with comprehensive follow-up according to the protocol for the clinical trial, standardized biobank sampling, and validated methods for determination of serum rituximab concentrations and of ADAs. The study was based on published clinical data with some limitations. It was not designed for the purpose of drug measurements and assessing the pharmacokinetic variables of rituximab in patients with ME/CFS. No blood samples were taken shortly after rituximab infusions to capture peak concentrations but immediately before the next scheduled dose for assessment of trough concentrations. The intervals between the doses were gradually increased during follow-up, with the latest sample taken at 15 months (5 months after the last infusion), which means that rituximab concentrations at this point were low. The differences in rituximab serum concentrations caused by minor differences in time intervals between rituximab doses were adjusted presuming a rituximab $t_{1/2}$ of 22 days in all patients and presuming a linear phase of elimination (all measurements at least 3 half-lives after the preceding dose).

CONCLUSIONS

The present study is the first to examine the associations between rituximab serum concentrations, ADAs, and clinical responses among patients with ME/CFS. The results are complementary to a recent trial[16] that questions the benefit of rituximab among patients with ME/CFS and adds to the search for disease mechanisms, effective drug therapy, and mechanisms related to improvement of ME/CFS symptoms.

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IGR and JS were responsible for the design of the study; ØF and OM were responsible for the design and conduct of the clinical KTS-2-2010 trial; KA and KR acquired the biobank serum samples; AdV analyzed the rituximab concentrations and ADAs; IGR, ØF, JS, and KS analyzed and interpreted the data; and IGR, ØF, JS, KS, KA, KR, and AdV wrote the manuscript and/or revised it critically for important intellectual content. All authors approved the final manuscript as submitted.

CONFLICTS OF INTEREST

The funders played no role in study design, data collection or analysis, decision to publish, or preparation of the manuscript. Haukeland University Hospital has patents and pending patent applications on the issue of B-cell depletion therapy for chronic fatigue syndrome (ME/CFS). The authors OM and ØF are mentioned as inventors in these applications. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

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Paper III

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CLINICAL TRIAL
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Intravenous Cyclophosphamide in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. An Open-Label Phase II Study

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Introduction: Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disease with high symptom burden, of unknown etiology, with no established treatment. We observed patients with long-standing ME/CFS who got cancer, and who reported improvement of ME/CFS symptoms after chemotherapy including cyclophosphamide, forming the basis for this prospective trial.

Materials and methods: This open-label phase II trial included 40 patients with ME/CFS diagnosed by Canadian criteria. Treatment consisted of six intravenous infusions of cyclophosphamide, 600–700 mg/m^2, given at four-week intervals with follow-up for 18 months, extended to 4 years. Response was defined by self-reported improvements in symptoms by Fatigue score, supported by Short Form 36 (SF-36) scores, physical activity measures and other instruments. Repeated measures of outcome variables were assessed by General linear models. Responses were correlated with specific Human Leukocyte Antigen (HLA) alleles.

Results: The overall response rate by Fatigue score was 55.0% (22 of 40 patients). Fatigue score and other outcome variables showed significant improvements compared to baseline. The SF-36 Physical Function score increased from mean 33.0 at baseline to 51.5 at 18 months (all patients), and from mean 35.0 to 69.5 among responders. Mean steps per 24 h increased from mean 3,199 at baseline to 4,347 at 18 months (all patients), and from 3,622 to 5,589 among responders. At extended follow-up to 4 years 68% (15 of 22 responders) were still in remission. Patients positive for HLA-DQB1*03:03 and/or HLA-C*07:04 ($n = 12$) had significantly higher response rate compared to patients negative for these alleles ($n = 28$), 83 vs. 43%, respectively. Nausea and constipation were common grade 1–2 adverse events. There were one suspected unexpected serious adverse reaction (aggravated POTS) and 11 serious adverse events in eight patients.

Conclusion: Intravenous cyclophosphamide treatment was feasible for ME/CFS patients and associated with an acceptable toxicity profile. More than half of the patients

responded and with prolonged follow-up, a considerable proportion of patients reported ongoing remission. Without a placebo group, clinical response data must be interpreted with caution. We nevertheless believe a future randomized trial is warranted.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier: NCT02444091.

Keywords: myalgic encephalomyelitis, chronic fatigue syndrome, ME, CFS, cyclophosphamide, clinical trial, medical treatment, HLA

INTRODUCTION

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disease of unknown etiology characterized by post-exertional malaise (PEM) (1, 2), sleep disturbances with inadequate restitution (3), fatigue, pain and sensory hypersensitivity, cognitive and several other symptoms. The diagnosis relies on exclusion of other disorders associated with fatigue, and there are no confirmatory diagnostic tests. Using the Canadian consensus criteria (4), an estimated 0.1% of the population suffer from ME/CFS (5), affecting women 3–4 times more often than men. ME/CFS has profound impact on quality of life for patients and their caretakers (6, 7). The socio-economic costs are high, and there is an urgent need for elucidation of the disease mechanisms, for improved diagnostic approaches, and for rational treatment (8).

We hypothesized that ME/CFS could be a variant of an autoimmune disease, with a role for B-cells and possibly autoantibodies. Several observations suggest that immune dysregulation and low-grade inflammation may be involved in the pathogenesis of ME/CFS (9–11). A review (12) summarizes data indicating autoimmunity as a possible etiological factor. Mechanisms may include dysregulations of cytokines (13), alterations in lymphocyte subsets (14) and presence of autoantibodies (15–17). A study with peptide arrays demonstrated an immunosignature based on serum antibodies that separated ME/CFS cases from healthy controls (18). Also, elderly patients with ME/CFS have an increased risk of B-cell lymphomas, especially marginal zone lymphomas known to be associated with autoimmunity or chronic infections (19). Recent research suggests disturbed turnover of complex lipids, fatty acids and amino acids and impaired energy metabolism as possible features of ME/CFS (20–23), possibly linked to low-grade inflammation (24).

There is evidence for a genetic predisposition in ME/CFS (25, 26). The immunologically important Human Leukocyte Antigen (HLA) genes were previously investigated in small ME/CFS cohorts, and certain class II alleles have been found more prevalent among patients (27–29). A recent study of a larger Norwegian cohort of patients and controls, identified two potential HLA risk alleles, namely HLA-C*07:04 and HLA-DQB1*03:03 (30).

At present, there is no established treatment for ME/CFS. In our oncology unit, we have observed seven patients with long-standing ME/CFS, who reported significant improvement of their ME/CFS symptoms after chemotherapy for either malignant lymphoma or breast cancer. These seven patients all received chemotherapy including the cytotoxic drugs cyclophosphamide or ifosfamide, and one patient also received rituximab. We decided to pursue these observations in separate clinical trials.

Rituximab is a monoclonal antibody that targets CD20 on the surface of B-cells, resulting in reversible B-cell depletion (31). Initial small studies testing rituximab in ME/CFS (32–34) indicated that a subgroup could benefit from B-cell depletion. However, in a recent Norwegian multicenter, randomized, double-blind and placebo-controlled trial, we reported no significant outcome differences between the rituximab and placebo groups (35).

Cyclophosphamide, an alkylating agent widely used in cancer treatment (36), induces immunosuppression and is also used to treat immune-mediated diseases like systemic lupus (SLE), rheumatoid arthritis, vasculitis, and multiple sclerosis (37–40). Based on the assumed immune disturbance in ME/CFS, the observed improvement in ME/CFS symptoms could be due to the immunosuppressive effect of cyclophosphamide (41).

In 2014, we treated four ME/CFS patients with six infusions of cyclophosphamide every 4 weeks. Two of the patients reported substantial improvement of their ME/CFS symptoms, lasting more than 4 years for one of them. In these pilot experiences, there were no infections, neutropenia, thrombocytopenia or unexpected adverse events. We decided to conduct a prospective trial to further investigate feasibility, efficacy and safety of cyclophosphamide treatment in ME/CFS patients.

METHODS

Trial Design

The CycloME study (EudraCT no. 2014-004029-41, ClinicalTrials.gov NCT02444091) was designed as an open-label phase II trial comprising 40 patients with ME/CFS. The study was approved by the Regional Committees for Medical and Health Research Ethics (2014/1672) and by the National Medicines Agency in Norway. Originally planned for 18 months follow-up, the protocol was amended for prolonged observation of patients up to 4 years after start of treatment. The protocol is available as supporting information (**Data Sheet 1**).

Setting and Patient Inclusion

Since 2011 patients with a likely diagnosis of ME/CFS have been referred to the Department of Oncology and Medical Physics, Haukeland University Hospital (HUH), for possible inclusion in clinical trials. Based on available information and proximity to

the treating hospital, patients previously included in trials with rituximab and newly referred patients were invited to receive information about the trial. Following signed informed consent, the patients were screened for eligibility.

Inclusion criteria were: a diagnosis of ME/CFS according to the Canadian criteria (4); age 18–66 years; disease duration more than 2 years; and disease severity mild-to-moderate, moderate, moderate-to-severe, or severe. Patients with either mild or very severe disease (completely bedbound and in need of help for all basic activities of daily living) were not included. The exclusion criteria and pre-treatment evaluation are detailed in the trial protocol (**Data Sheet 1**).

Recruitment lasted from March 2015 until December 2015. All 40 patients were included at the Department of Oncology and Medical Physics, HUH. Seven patients had parts of their treatment and follow-up at the Department of Oncology, Oslo University Hospital (OUH).

Follow-up was originally completed in August 2017, with assessments for prolonged follow-up performed in January 2018 and April 2019.

Patient Registrations

At baseline, patients recorded severity of a range of common ME/CFS symptoms including PEM, fatigue, cognitive symptoms and pain, using a numerical rating scale of 1–10. During 18 months follow-up, patients were asked to complete a symptom questionnaire every 2 weeks, recording change or no change to the same range of symptoms. The relative scale for symptom change ranged from 0 to 6, in which three denoted no change from baseline; 4, 5, and 6 slight, moderate, and major improvement; and 2, 1, and 0 slight, moderate, and major worsening, respectively. This scale was adapted from the validated Clinical Global Impression Scale, which has been used previously in ME/CFS (42). The primary outcome variable Fatigue score, which has not been validated, was calculated every second week during follow-up as the mean change score for the four fatigue-related items: "Fatigue," "PEM," "Need for rest," and "Daily functioning." At baseline and every 2 weeks, patients also recorded their percent function level on a scale from 1 to 100%, where 100% denoted a completely healthy state. A set of examples was provided to facilitate this assessment. Samples of all questionnaires are enclosed under Supporting Information (**Data Sheet 1**). Outcome measures also included the Short Form 36 Health Survey (SF-36) ver. 1.2 in Norwegian translation (43, 44), at baseline, every 3 months during follow-up and at extended follow-up assessments at 24–30 and 38–48 months. Fatigue Severity Scale was recorded at 3-months intervals until 18 months (45, 46). Physical activity level was recorded using an electronic SenseWear armband continuously for 5 to 7 days in a home setting (47, 48), at baseline and repeated in the time intervals 7–9, 11–12, 17–18, 24–30, and 38–48 months after start of treatment.

Intervention and Follow-Up

Six 30-minute intravenous infusions of cyclophosphamide were administered at 4-week intervals with 600 mg/m^2 at the first and 700 mg/m^2 at further cycles. Patients received premedication with ondansetron 8 mg and dexamethasone 4 mg, when necessary enforced by aprepitant 125 mg day 1, and 80 mg days 2 and 3. Patients with hematuria or dysuria in previous cycles were given oral uromitexan (**Data Sheet 1**). Patients used cold-caps (Elasto-Gel®, Southwest technologies, North Kansas City, USA) during infusions to reduce hair thinning. Each infusion was preceded by routine blood tests, including hematology, and a visit with a physician or study nurse. After the first and second infusions, a nadir blood sample was collected between days 10 and 14 after infusion. If there were no signs of neutropenia or thrombocytopenia after the first two treatments, no further blood tests between treatments were required. Throughout the 18 months follow-up, patients attended consultations with an investigator every 3 months. Adverse events were registered continuously at each treatment visit and at follow-up every 3 months and summarized according to Common Toxicity Criteria for Adverse Events (CTCAE) ver. 4.03. The Viedoc® electronic CRF system (PCG Solutions) was used for data collection and management in the study. There were no interim analyses. The trial was externally monitored by the Department for Research and Development at HUH.

Outcomes

Response to treatment was defined as Fatigue score $\geq$4.5 for a minimum of 6 consecutive weeks, occurring at any time point during treatment or within 18 months follow-up. The trial had two primary endpoints based on this definition: (i) overall response rate and (ii) changes in Fatigue score compared to baseline through 18 months follow up. These endpoints were also analyzed separately for the treatment-naïve patients (with no previous rituximab exposure).

Secondary endpoints included: (i) response duration calculated as the sum of response periods each of at least six consecutive weeks with mean Fatigue score $\geq$4.5; and changes from baseline to specific timepoints of (ii) SF-36 scores for Physical Function subscale (SF-36-PF) and Physical component summary score (SF-36-PCS); (iii) self-reported percent function level; (iv) mean number of steps per 24 h. Adverse events during the 18 months of follow-up from start of treatment were an additional secondary endpoint.

HLA Typing

High-resolution HLA genotyping was conducted as part of a larger study (30). In short, HLA-A, -B, -C, -DRB1, -DQB1, -DQA1, and -DPB1 alleles were genotyped using NGSgo kits and NGSengine software from GenDX (Utrecht, the Netherlands), and 2 $\times$ 150 bp paired-end sequencing on a Miseq instrument (Illumina, San Diego, USA) at the Norwegian Sequencing Centre, Oslo. The association analysis between HLA risk alleles and clinical response was not specified in the protocol, and was performed retrospectively in the data analysis phase. Only the potential HLA risk alleles identified by Lande et al. (30), i.e., HLA-C*07:04 and HLA-DQB1*03:03, were investigated.

Statistical Analysis

Descriptive methods were used to characterize the sample, with mean and standard deviation (SD) for normally distributed data, and median with range [min-max, or interquartile range (IQR)] for skewed data. Primary and secondary outcome measures were analyzed by the intention-to-treat principle. Changes from baseline through 18 months follow-up were assessed by General Linear Model for repeated measures (GLM), including time as a predictor. Greenhouse-Geisser corrections were used for all GLM analyses because Mauchly's tests were significant ($p < 0.001$), indicating violations of the sphericity assumption. The changes through follow-up, compared to baseline, were assessed by the within-subjects effects for time. Simple contrasts in the time domain were used to assess the changes from baseline to each specific time interval or time point during follow-up, with the effect sizes from the parameter estimates [means and 95% confidence intervals (CI)]. To assess differences between groups GLM repeated measures were performed with p-value (Greenhouse-Geisser corrected) from the interaction time-by-group. Groups analyzed were sex, ME/CFS severity, ME/CFS duration, previous rituximab treatment, infection prior to debut of ME/CFS symptoms, and specific HLA alleles. The distribution of sex, ME/CFS severity and the proportion of responders among carriers and non-carriers of the two aforementioned HLA-alleles, were compared using Odds Ratio (OR) and Fisher's exact tests.

All tests were two-sided with a significance level of 0.05. Missing data were replaced using the last value carried forward (LVCF) method. All analyses were performed using IBM SPSS Statistics ver.25 (IBM Corp., Armonk, USA), and Graphpad Prism ver.8 (GraphPad Software, La Jolla, USA).

Role of the Funding Sources

The research group for ME/CFS at Department of Oncology and Medical Physics (HUH) has received funding from the Kavli Trust and the Norwegian Ministry of Health and Care Services. The HLA sequencing has received funding from the Kavli Trust and Norwegian Research Council. The funders had no role in trial design, data collection, analysis, decision to publish, or preparation of the manuscript.

RESULTS

Study Population

The flow chart for patient screening, inclusion, treatment and follow-up is shown in **Figure 1**. Among available referrals with adequate medical information, we randomly selected 50 patients for eligibility screening. Ten patients were excluded due to violation of eligibility criteria, or declined to participate. We included 25 rituximab-naïve patients and 15 patients with previous rituximab intervention.

Table 1 shows baseline characteristics for all included patients ($n = 40$), the rituximab-naïve patients ($n = 25$), and patients with ($n = 22$) or without ($n = 18$) a response to cyclophosphamide according to the definition of the primary endpoint of the study.

Medical history and concomitant diseases at baseline, and concomitant medication during study follow-up, are summarized in **Supplementary Tables 1, 3**. **Supplementary Table 2** shows previous treatment by trial participants. Some kind of cognitive therapy had been tried by 52.5%, graded exercise or other physical therapy by 45.0%, adaptive pacing by 37.5%, vitamin B12 injections by 40.0%, and low dose naltrexone by 37.5%. None of the patients received any alternative intervention aimed at ME/CFS during the trial.

Thirty-one patients received all preplanned six infusions, three patients received five infusions, four received four infusions, and two received three infusions (**Figure 1**). The reasons for omitting infusions were either withdrawal of consent (two cases after cycle 4), or high symptom burden (seven cases). All the decisions to omit infusions were in agreement with the trial investigators. Thus, nine patients (22.5%) deviated from the planned treatment protocol.

Missing Data

For the 18 months study period, there were missing data for the two patients who withdrew from study after ∼5 months (both non-responders at the time of withdrawal), and for one non-responding patient with severe ME/CFS who failed to complete self-reported forms from 4 months onwards. Except for these three patients, there were eight missing data items out of 1,560 raw data for the variable Fatigue score. SenseWear activity armband data were complete at baseline, and had missing data from the two withdrawals during follow-up.

Primary Outcome

The overall response rate, i.e., proportion of patients with Fatigue score $\geq$4.5 for at least six consecutive weeks, was 22 out of 40 patients (55.0%, 95% CI 39.8–69.3%). Among the rituximab-naïve patients, 14 out of 25 patients achieved a clinical response (56.0%, 95% CI 36.9–73.4%).

Changes in Fatigue score during 18 months follow-up, with comparisons of mean Fatigue score at each 3-month interval to baseline are shown in **Figure 2**, for all patients (**Figure 2A**), rituximab-naïve patients (**Figure 2B**), patients with a response (**Figure 2C**), and no response during follow-up (**Figure 2D**). Repeated measures of Fatigue score showed significant increases from baseline, with similar improvements among the rituximab-naïve patients as observed in all patients. The Fatigue score increased significantly from baseline to 9 months after start of treatment and further through 18 months follow-up. Among the 18 patients with no response, the Fatigue score decreased significantly from baseline to 3 and 6 months, and thereafter returned to near baseline level. **Figure 2** also shows the courses of mean Fatigue score through 18 months' follow-up, subgrouped by ME/CFS disease severity (**Figure 2E**), and by presence/absence of HLA risk alleles (**Figure 2F**) in which patients with HLA-DQB1*03:03 and/or HLA-C*07:04 reported higher improvements of Fatigue score through follow-up than those negative for these alleles ($p = 0.05$).

Secondary Outcomes

Changes of SF-36-PF and percent function level through each 3-month interval, and mean steps per 24 h (at baseline, 7–9, 11–12, and 17–18 months), are shown in **Figures 3A–L**. Outcomes

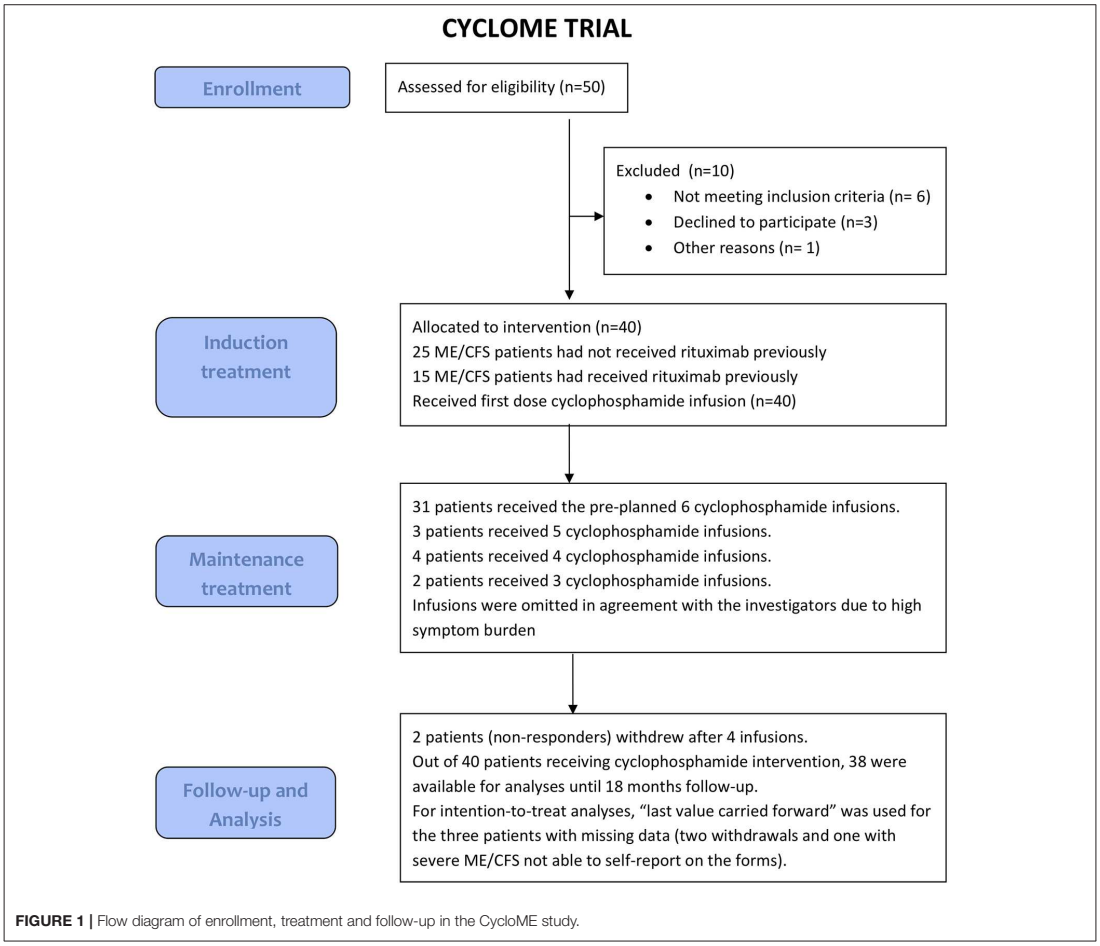

FIGURE 1 | Flow diagram of enrollment, treatment and follow-up in the CycloME study.

are shown for all patients and for the rituximab-naïve group, as well as for patients with and without response according to the study criteria. There were significant improvements of all outcome variables from baseline through 18 months follow-up among all 40 patients, with mean SF-36-PF increasing from 33.0 at baseline to a maximum 51.5 at 18 months follow-up ($p < 0.001$). Among 25 rituximab-naïve patients, mean SF-36-PF increased from 34.0 at baseline to 49.8 at 18 months ($p = 0.001$). Among 22 responders, mean SF-36-PF increased from 35.0 at baseline to 69.5 at 18 months ($p < 0.001$). For 18 non-responders there was only a slight increase of SF-36-PF from 30.6 at baseline to a maximum of 34.4 at 3 months, and with no significant changes through the remaining study follow-up. Similar patterns of significant changes were seen through follow-up, as compared to baseline, for percent function level and for mean steps per 24 h, and also for SF-36-PCS (not shown).

Figure 4 shows the courses of SF-36-PF by subgroups. There were no significant interactions time-by-group for sex, severity, disease duration, infection prior to ME/CFS, or previous treatment with rituximab, i.e., the changes in SF-36-PF over time were similar in all subgroups, except for HLA risk allele defined subgroups (see below). The reason for showing SF-36-PF in these plots was to enable comparison of data to other reported studies, in which SF-36-PF has often been used. There was no significant overall interaction between time and ME/CFS severity ($p = 0.51$), although the small group ($n = 6$) with severe disease had no clinically relevant increase in SF-36-PF, from 8.3 at baseline to a maximum of 11.7 at 12 months follow-up. The severe ME/CFS group included two patients with missing data (one withdrawal and one who failed to complete registration). However, seven patients with moderate-to-severe disease had similar improvements of the outcome measures

TABLE 1 | Baseline characteristics of the study population are shown for the intention-to-treat population, for rituximab-naïve patients and for patients with or without clinical response.

Characteristic	All patients ($n = 40$)	Rituximab-naïve[a] ($n = 25$)	Responders[b] ($n = 22$)	Non-responders[c] ($n = 18$)
Female, *n* (%)	31 (77.5)	18 (72.0)	18 (81.8)	13 (72.2)
Male, *n* (%)	9 (22.5)	7 (28.0)	4 (18.2)	5 (27.8)
Age, female pts, mean (min–max)	43.0 (25.0–61.1)	41.5 (26.6–54.6)	41.8 (25.0–60.3)	44.6 (26.6–61.1)
Age, male pts, mean (min–max)	37.6 (21.5–53.3)	35.1 (21.5–50.8)	39.5 (21.5–53.3)	36.0 (23.4–50.8)
BMI female pts[d], mean (min–max)	24.5 (17.1–33.1)	24.6 (17.1–33.1)	24.1 (17.1–32.7)	24.9 (19.0–33.1)
BMI male pts[d], mean (min–max)	24.5 (17.4–30.6)	23.4 (17.4–29.2)	25.9 (17.4–30.6)	23.4 (21.1–26.9)
Rituximab-naïve[a], *n* (%)	25 (62.5)	25 (100.0)	14 (63.6)	12 (66.7)
Previous rituximab treatment[e], *n* (%)	15 (37.5)	0	9 (40.9)	6 (33.3)
ME/CFS disease duration				
2–5 years, *n* (%)	7 (17.5)	7 (28.0)	5 (22.7)	2 (11.1)
5–10 years, *n* (%)	13 (32.5)	7 (28.0)	5 (22.7)	8 (44.4)
10–15 years, *n* (%)	9 (22.5)	4 (16.0)	6 (27.3)	3 (16.7)
>15 years	11 (27.5)	7 (28.0)	6 (27.3)	5 (27.8)
ME/CFS disease severity				
Mild/Moderate, *n* (%)	14 (35.0)	10 (40.0)	9 (40.9)	5 (27.8)
Moderate, *n* (%)	13 (32.5)	7 (28.0)	9 (40.9)	4 (22.2)
Moderate/severe, *n* (%)	7 (17.5)	5 (20.0)	4 (18.2)	3 (16.7)
Severe[f], *n* (%)	6 (15.0)	3 (12.0)	0	6 (33.3)
Infection prior to ME/CFS[g], *n* (%)	26 (65.0)	17 (68.0)	15 (68.2)	11 (61.1)
SF36 Physical Function[h], mean (min–max)	33.0 (0–65)	34.0 (0–65)	35.0 (10–65)	30.6 (0–65)
SF36 Physical component summary score[i], mean (min–max)	23.3 (13.5–41.6)	24.5 (14.6–41.6)	23.1 (13.5–41.6)	23.5 (14.6–31.0)
Steps, mean per 24 h, mean (min–max)	3,199 (568–9,637)	3,282 (568–9,637)	3,622 (1,083–8,178)	2,681 (568–9,637)
Total function level[j], mean (min–max)	16.9 (5–40)	17.0 (5–30)	19.3 (10–40)	14.1 (5–25)
HLA-DQB1*03:03 pos, *n* (%)[k]	10 (25.0)	6 (24.0)	9 (40.9)	1 (5.6)
HLA-C *07:04 pos, *n* (%)	4 (10.0)	2 (8.0)	3 (13.6)	1 (5.6)
HLA-DQB1*03:03 and/or HLA-C*07:04 pos, *n* (%)	12 (30.0)	6 (24.0)	10 (45.5)	2 (11.1)

[a] *Patients with no previous rituximab intervention.*
[b] *Clinically significant responders, including 18 patients with long response duration (≥30 weeks), three with moderate response duration (14–28 weeks) and one with marginal response duration (6–12 weeks).*
[c] *Patients with no clinically significant response.*
[d] *Body Mass Index (kg/m^2).*
[e] *Patients treated with rituximab in previous trial (KTS-2-2010) n = 14, or outside a clinical trial (n = 1).*
[f] *Two of six patients with severe ME/CFS withdrew from the study after four infusions.*
[g] *Self-reported infection prior to onset of ME/CFS disease.*
[h] *Short Form 36 (SF-36) physical function subscale (scale 0–100).*
[i] *SF-36 Physical Health Summary Score, norm-based with population mean 50.*
[j] *Baseline self-reported function level (scale 0–100%).*
[k] *HLA-types determined as part of a larger study (30).*

as patients with either moderate or mild-to-moderate disease. **Supplementary Figure 1** shows the courses during follow-up, for the SF-36 subscales Vitality, Social Function, and Bodily Pain (**Supplementary Figures 1A–F**), and also the Fatigue Severity Scale (**Supplementary Figures 1G,H**), all showing that the responders report improvement during follow-up which we interpret to be of clinical significance.

Out of nine patients included in the trial who had received previous rituximab treatment without reporting improvement of ME/CFS symptoms, four achieved a clinical response after cyclophosphamide intervention. Patients with HLA alleles HLA-DQB1*03:03 and/or HLA-C*07:04 reported higher improvements of SF-36-PF through follow-up than those negative for these alleles ($p = 0.05$) (**Figure 4F**).

Clinical Response Durations

Among the 22 patients with response, the total duration or response was median 44 weeks (range 6–70 weeks) within 18 months follow-up. The median ratio of clinical response duration to follow-up was 0.56 (range 0.08–0.90). Response duration was $\geq$ 30 weeks in 18 patients, 14–28 weeks in three patients, and 6–12 weeks in one patient.

The median time to first response was 22 weeks (range 2–42 weeks). There were no significant differences in time to first response by sex, disease severity, disease duration, infection prior to ME/CFS, or by previous rituximab treatment (data not shown).

Out of 22 responders, 17 patients (77.3%) reported a sustained response with Fatigue score of least 4.5 at the end of 18 months

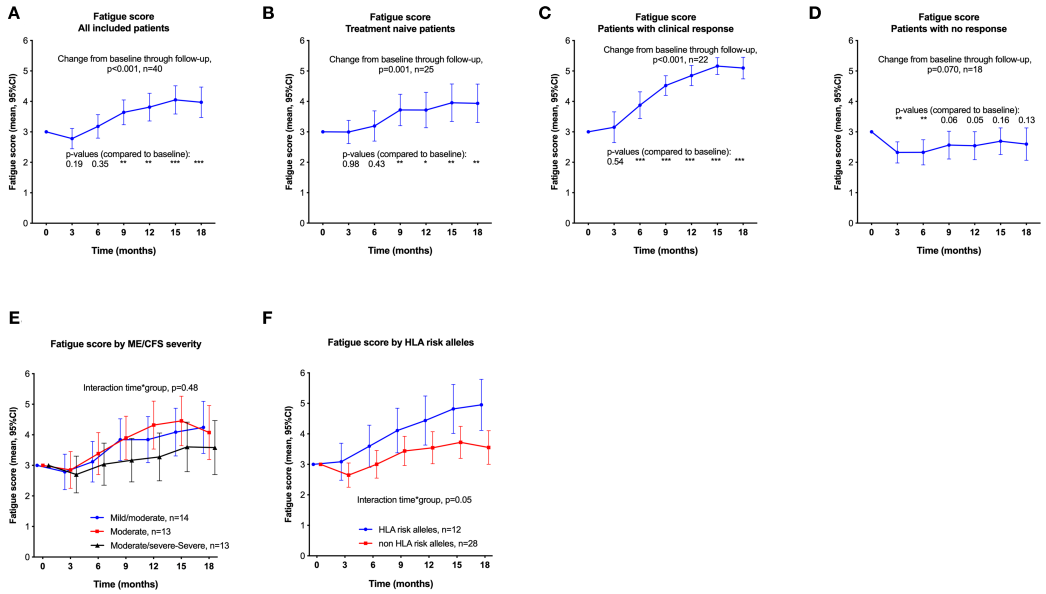

FIGURE 2 | Fatigue score (primary end point), means with 95% CI at time points through 18 months follow-up, from self-reported symptom scores every second week. The scale is 0–6, where 3 indicates no change from baseline and higher scores indicate less fatigue. **(A)**: All included patients ($n = 40$). **(B)**: Treatment-naïve patients (not previously exposed to rituximab, $n = 25$). **(C)**: Responders during follow-up ($n = 22$). **(D)** Non-responders during follow-up ($n = 18$). *P*-values from General Linear Model repeated measures assessing changes in Fatigue score from baseline. **(E,F)** show mean Fatigue score (with 95% CI) through 18 months' follow-up, subgrouped by ME/CFS disease severity **(E)**, and presence/absence of HLA risk alleles **(F)**. *P*-values from General Linear Model for interaction time-by-group, assessing difference between subgroups in repeated measures of Fatigue score over time compared to baseline. *P*-values: * <0.05; ** <0.01; *** <0.001. CI, confidence intervals.

follow-up. Among all 40 included patients, 21 (52.5%) reported a Fatigue score of at least 4.0 (slight improvement) at end of follow-up.

Prolonged Follow-Up

Following two approved protocol amendments, patients had additional visits or telephone interviews with recordings of SF-36 and percent function level and SenseWear physical activity measurements at 24–30 and 38–48 months follow-up. Due to the risk of recall bias, Fatigue score compared to baseline was not recorded at these late visits. Instead, patients were asked to self-assess whether their symptoms had relapsed, remained unchanged or had improved further since the end of trial (18 months). The changes of SF-36-PF, percent function level and mean steps, from baseline until extended follow-up at 38–48 months, by response status, are shown in **Figures 5A–C**.

At the 38–48 months visit, 36 out of 38 patients still in the study completed the interview including assessment of their percent function level, 35 recorded SF-36 forms and 32 completed SenseWear activity measurements. Out of 22 responders, 20 completed the interview; 15 were still in remission, while five reported a complete or partial relapse. For 20 responders with available SF-36 recordings at 38–48 months, the mean SF-36-PF was 70.8 (range 25–100) compared to mean 69.5 at 18 months. SenseWear activity registration was available for 19 out of 22 responders at 38–48 months with mean 6,415 steps per 24 h (SD 2,764), compared to mean 5,589 (SD 2,017) at 18 months (**Figure 5C**). Six patients with missing SenseWear data at 38–48 months included two responders in ongoing remission, one in relapse and three non-responders.

At baseline, only two of the responders had part-time work participation. During follow-up, at least nine out of 22 responders returned to either part-time of full-time work or studies.

HLA Data

Twelve of the 40 patients (30.0%) carried either of the two specific HLA risk alleles. Ten of the 12 patients (83.3%) positive for HLA alleles DQB1*03:03 and/or C*07:04 had a response, compared to 12 out of 28 patients (42.9%) negative for these HLA alleles (OR = 6.67; $p = 0.028$; **Figure 6**). The allele HLA-C*07:04 was present in four out of 40 patients (10.0%), and three (75.0%) of these were responders. HLA-DQB1*03:03 was detected in 10 out of 40 patients (25.0%), and 9 out of 10 (90.0%) were responders, compared to 13

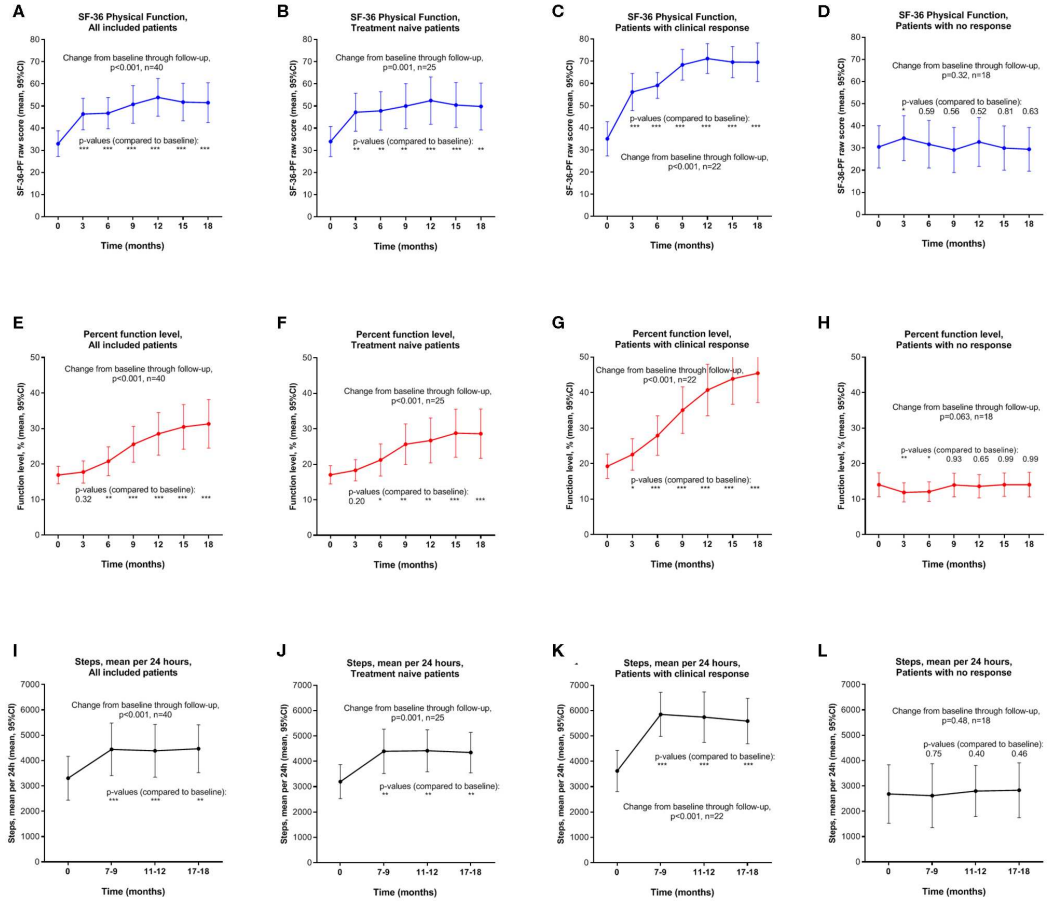

FIGURE 3 | SF-36 Physical Function (SF-36-PF) **(A–D)**, percent function level **(E–H)**, and mean steps per 24 h **(I–L)**, means with 95% CI, at time points through 18 months follow-up. Outcome data for all included patients ($n = 40$) **(A,E,I)**; Treatment-naïve (not previously exposed to rituximab, $n = 25$) **(B,F,J)**; Responders during follow-up ($n = 22$) **(C,G,K)**; Non-responders during follow-up ($n = 18$) **(D,H,L)**. *P*-values from General Linear Model repeated measures assessing changes in outcome variable from baseline. *P*-values: * <0.05; ** <0.01; *** <0.001. SF-36 Physical Function with scale 0–100, higher number indicates better function. CI, confidence intervals; SF-36, Short Form 36.

out of 30 HLA-DQB1*03:03 negative patients (43.3%); OR = 11.8, $p = 0.013$.

Among 12 patients with the HLA alleles DQB1*03:03 and/or C*07:04, 7 (58.3%) had mild-to-moderate, 3 (25%) had moderate, and 2 (16.7%) had moderate-to-severe ME/CFS. Contrary, among 28 patients without these HLA alleles, 7 (25.0%) had mild-to-moderate, 10 (35.7%) had moderate, and 11 (39.3%) had moderate-to-severe ME/CFS ($p = 0.05$). Eleven out of 12 patients (91.7%) with HLA alleles DQB1*03:03 and/or C*07:04 were female, compared to 20 out of 28 patients (71.4%) without these alleles ($p = 0.23$).

Adverse Events

Adverse events (AE) for the complete period of 18 months follow-up are shown in **Table 2**. Thirty-three patients (82.5%) reported AEs of CTCAE grade ≥ 2, of which gastrointestinal events such as nausea and constipation were the most common. Out of 16 grade 3–4 events in 11 patients, 11 resulted in hospitalization and were reported as serious adverse events (SAE, **Supplementary Table 4**). There was one suspected unexpected serious adverse reaction (SUSAR), in a female patient with moderate-to-severe ME/CFS who was a non-responder in the study. She experienced gradual worsening of postural orthostatic tachycardia syndrome (POTS) after

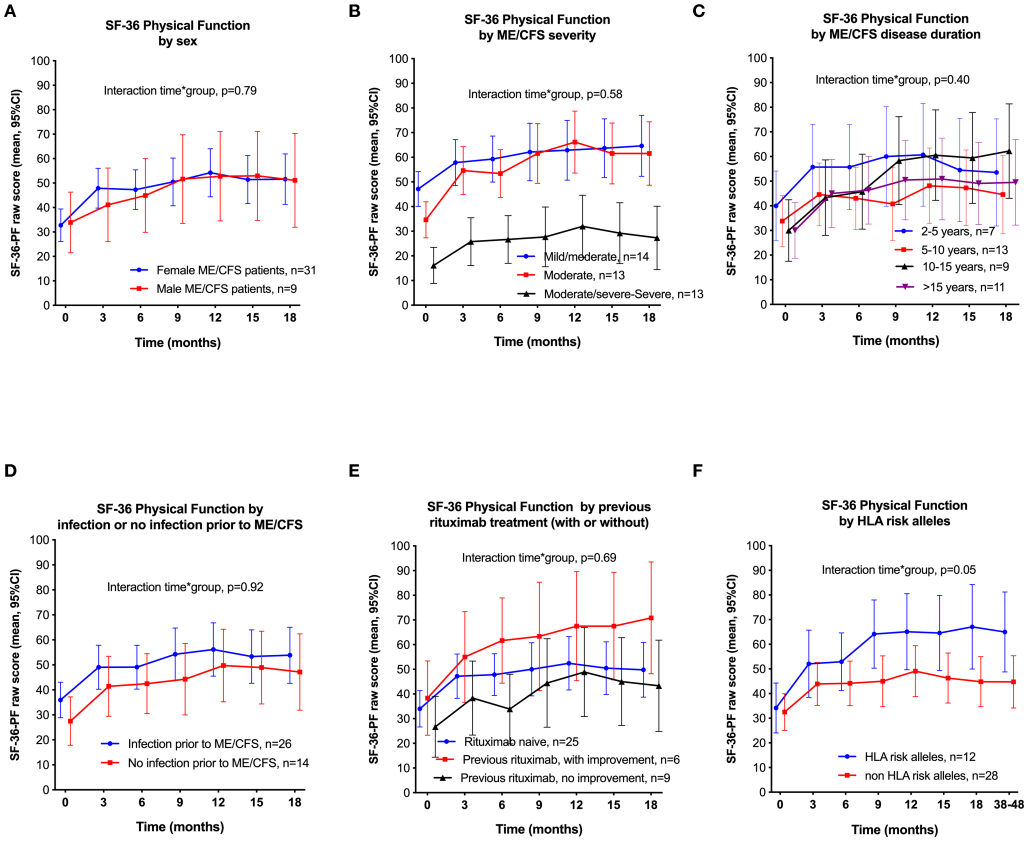

FIGURE 4 | SF-36 Physical Function (SF-36-PF), means with 95% CI through 18 months follow-up, by subgroups. *P*-values from General Linear Model for interaction time-by-group, assessing differences between the subgroups in repeated measures of SF-36-PF over time, compared to baseline. **(A)**: By men vs. women; **(B)** By ME/CFS severity; **(C)**: By ME/CFS disease duration; **(D)** With or without self-reported infection prior to ME/CFS; **(E)** With or without previous rituximab treatment; **(F)**: With or without HLA risk alleles (HLA DQB1*03:03 and/or HLA C*07:04). SF-36, Short Form 36; CI, confidence intervals; HLA, Human Leukocyte Antigen.

cycle 4, resulting in hospital admission for 2 weeks. She had experienced periods of similar POTS aggravations regularly since she became ill with ME/CFS 18 years before study inclusion. Her POTS symptoms gradually returned to baseline level, but study treatment was discontinued. With routine blood sampling before each cycle and after cycle 1 and 2, there was no sign of hematological toxicity. Two women both aged ≥41years at inclusion, experienced menopause after start of treatment, two others reported irregular menstrual bleeding that persisted to end of follow-up. One patient without a clinical response suffered a sudden death of unknown cause 4 years after inclusion in the study, i.e., 42 months after the last infusion, with no probable relation to the intervention.

DISCUSSION

The present open-label phase II study with cyclophosphamide infusions was well conducted with little missing data. More than half of the patients had clinical response according to the predefined criteria, many with long-lasting improvement of symptoms. At extended follow-up 3–4 years after inclusion 68% of responders were still in remission.

In general, the toxicity to cyclophosphamide infusions in ME/CFS patients was moderate, and there were few serious adverse events and no registered hematological toxicity. The most common side effects were nausea and general malaise lasting for 1–2 weeks after each infusion. ME/CFS patients reported more nausea and discomfort after cyclophosphamide

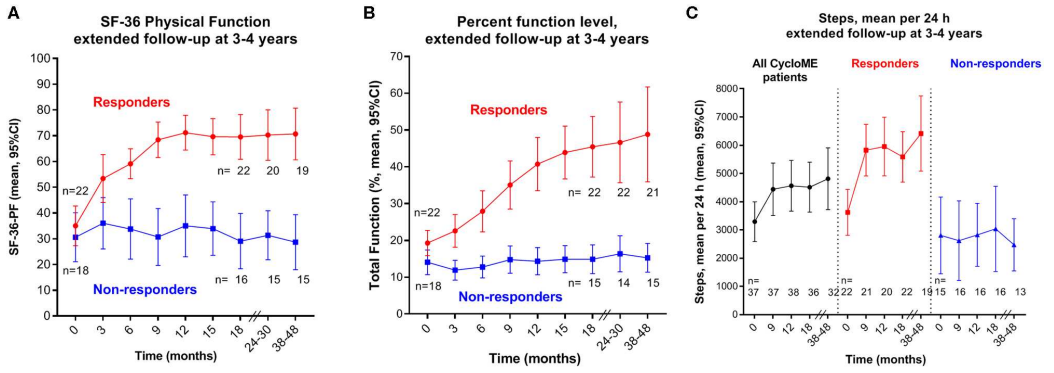

FIGURE 5 | Outcome variables at extended follow-up, by response status. Means with 95% CI at different time points including extended follow-up at 24–30 and at 38–48 months. **(A)**: SF-36 Physical Function (SF-36-PF); **(B)** percent function level; **(C)**: mean steps per 24 h. Numbers of patients at the different time points through follow-up are shown below the graphs. SF-36, Short Form 36; CI, confidence intervals.

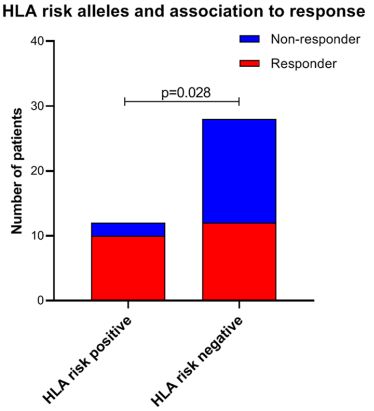

FIGURE 6 | Frequency of HLA risk alleles (HLA DQB1*03:03 and/or HLA C*07:04) in responders and non-responders during follow-up. *P*-value from Fischer's exact test. HLA, Human Leukocyte Antigen.

than cancer patients typically do at similar doses, in line with the generally low stress tolerance and sensitivity to drugs reported by many patients. We reinforced the anti-emetic regimen with aprepitant during the study in efforts to reduce the nausea experienced by the patients during the first days after infusion. Fertility concerns are an important toxicity issue with chemotherapy. Cyclophosphamide is an alkylating agent associated with ovarian failure and the risk increases with higher cumulative doses and with increasing age (49, 50). One study with intravenous infusions, applying similar cumulative doses (mean 9.1 gram) as in the present study, and mean age 31 years, reported ovarian failure in 13%, and transient amenorrhea in 20% of the patients (51). In our present trial, two women aged 41 and 46 years at inclusion experienced premature menopause, and two others reported irregular menstruation probably induced by the treatment at end of follow-up. In contrast to spontaneous premature menopause, chemotherapy associated ovarian dysfunction can resume over time (years) in some patients, even after a prolonged period of amenorrhea and elevated gonadotropin levels (52).

Since the 6-month initial treatment period with repeated cyclophosphamide infusions in some patients led to increased symptom burden and side effects, the extent and duration of improvement in ME/CFS symptoms are important aspects to justify the intervention. We therefore extended the follow-up period, and collected additional clinical data from participants, at 2–3 and 3–4 years after inclusion. The response durations were sustained for most of the responders. Out of 22 responders 82% were still in remission at 2–3 years and 68% at 3–4 years extended follow-up. Seven even reported further improvement compared to their status at 18 months follow-up. Also of note, three of the patients who registered relapse at 3–4 years still reported a 2-fold increase of their percent function levels as compared to baseline. Thus, responders' self-reported percent function levels, SF-36 Physical Function with increase from mean 35 at baseline to mean >70 at 12 months, and measured levels of physical activity (steps per 24 h), reflect clinically meaningful improvements of their abilities and activities of daily life. For comparison, the mean SF-36 Physical Function in the general population is 84.2 (95% CI 71.9–96.5) (53).

Compared to the randomized RituxME trial assessing rituximab vs. placebo in ME/CFS patients (35), the patterns of improvement among patients in the present CycloME trial seemed to be more homogeneous. In CycloME the clinical responses occurred earlier than in the RituxME trial; at median 22 weeks compared to 41 weeks. In the CycloME study the response rates were comparable between men and women, as

TABLE 2 | Patients with adverse events of CTCAE grade 1–4 during 18 months follow-up.

	≥1	≥2	3–4*	Related to study treatment†
Patients with ≥ 1 adverse event	39 (97.5%)	33 (82.5%)	11 (27.5%)	29 (72.5%)
Nausea	36 (90%)	15 (37.5%)	0	36 (90%)
Constipation	22 (55%)	9 (22.5%)	1 (2.5%)	19 (47.5%)
Diarrhea	7 (17.5%)	1 (2.5%)	0	6 (15%)
Stomach pain	9 (22.5%)	2 (5%)	1 (2.5%)	7 (17.5%)
Infections	24 (60%)	15 (37.5%)	3 (7.5%)	13 (32.5%)
Irregular menstrual bleeding	7 (17.5%)	3 (7.5%)	0	7 (17.5%)
Premature menopause	2 (5%)	1 (2.5%)	0	2 (5%)
Haematuria	6 (15%)	1 (2.5%)	0	6 (15%)
Urinary bladder symptoms**	5 (12.5%)	3 (7.5%)	0	5 (12.5%)
Hair loss	4 (10%)	0	0	4 (10%)
Rash or urticaria	6 (15%)	4 (10%)	1 (2.5%)	5 (12.5%)
Headache	12 (30%)	3 (7.5%)	1 (2.5%)	9 (22.5%)
Dizziness	6 (15%)	3 (7.5%)	0	5 (12.5%)
Edema of face or limbs	6 (15%)	0	0	5 (12.5%)
Palpitations or tachycardia	4 (10%)	2 (5%)	1 (2.5%)	2 (5%)

*11 grade 3–4 events for 8 patients were reported as SAE. See **Supplementary Table 4** for details.*

†*Possible, probable or very likely relation to study treatment.*

**Bladder/urinary tract pain or increased urinary frequency.*

opposed to higher response in women in the RituxME trial. The response rates were higher among patients with moderate or moderate-to-severe disease, compared to the 4 patients with severe ME/CFS who completed the intervention. In an ongoing addition to the trial (part B), feasibility and response rate are investigated in a small number of additional patients with severe ME/CFS, to gain experience and to decide whether severe patients may be included in a possible future randomized trial assessing cyclophosphamide intervention.

The response rates were similar among patients who were rituximab-naïve and patients who had participated in previous trials with rituximab intervention (32, 33). Also, four out of nine patients with no improvement after previous rituximab intervention experienced clinical benefit after cyclophosphamide in the present study.

Interestingly, the presence of either of the two HLA risk alleles, previously shown to be associated with ME/CFS (HLA-DQB1*03:03 and HLA-C*07:04) (30), was predictive for response to cyclophosphamide. In contrast there was no association between presence of these HLA alleles and clinical improvement among patients included in the RituxME trial (35) (data not shown).

The carrier frequency of any of these HLA risk alleles was 30% among ME/CFS patients in this trial, which is higher than the 19.1% reported in the recent study of 426 Norwegian ME/CFS patients (30). Western Norway is well represented in this large cohort, and the frequency of DQB1*03:03 and C*07:04 from Western Norway sources did not differ from the national frequency (data not shown). Therefore, geographical bias is not a probable explanation.

The association between cyclophosphamide response and the HLA risk alleles could be due to a true treatment effect in individuals carrying these alleles. There are several reports of associations between specific HLA alleles/haplotypes and responses to immune modulatory treatments (54–57), but to our knowledge this has not been demonstrated specifically for cyclophosphamide. Another possibility is that carriers of these HLA risk alleles constitute a subgroup among ME/CFS patients with an immune-driven pathomechanism generally responding better to immune modulating treatment. Finally, the observed association between the HLA risk alleles and response to cyclophosphamide could be coincidental, but warrants further investigation in a possible future randomized trial.

There are no biomarkers for ME/CFS or disease activity, and assessments of symptom changes consequently have to rely largely on self-recorded subjective variables. To increase the validity of the measurements, we used several different variables to measure symptom changes. These variables generally showed the same patterns of improvement and worsening of ME/CFS symptoms during the follow-up period. Self-reported improvements in Fatigue score, percent function level and SF-36 Physical Function scores correlated well, and with increased levels of physical activity. "Steps per 24 hours" is an objective measure, but not a perfect way to validate symptom improvement because individual patients will use their improved energy for different purposes. Some will walk, while some will prefer to read or increase the time for social activity.

The initial patient observations in our cancer clinic, of patients with long-standing ME/CFS who developed cancer, and who reported relief of ME/CFS symptoms after cancer treatment, included seven cases treated with cyclophosphamide (or ifosfamide), and in one case the combination of cyclophosphamide and rituximab. Our hypothesis was that ME/CFS in a subgroup of patients could be caused by an immunological dysfunction, possibly with a variant of an autoimmune pathomechanism. In the present study, the frequency of self-reported infection prior to ME/CFS debut (65%) was in line with other reports (58). Also, there was a high occurrence of autoimmunity among first-degree relatives (55.0%). Both observations may support an immunological basis for the disease. Initial phase II studies with rituximab (32, 33) suggested that a subgroup of patients could benefit from B-cell depletion therapy. Conversely, in the double-blind, placebo-controlled, multicenter, phase III RituxME trial there were no significant differences between the rituximab and placebo groups for any of the primary or secondary outcome measures (35). Taking the RituxME results into account, we have to interpret the data from the present open-label CycloME trial with caution. Patient selection, placebo mechanisms, patient's expectations in clinical trials, and natural variation of symptoms over time may be operative (59, 60). Until a randomized trial has been

performed, there is not sufficient evidence for a beneficial effect of cyclophosphamide in ME/CFS patients.

Other study limitations are self-referral, use of self-reported primary outcome measures with possible recall bias, and the inclusion of patients who had participated in previous studies with rituximab intervention. Although inclusion relied on strict diagnostic criteria, the unknown etiology of ME/CFS and lack of specific biomarkers could introduce unintended heterogeneity of the patient sample.

When comparing the response data from the CycloME and RituxME studies, it is important to consider the completely different modes of action of the two drugs. Rituximab is a monoclonal antibody which selectively depletes B-cells expressing the CD20 protein on their surface, while cyclophosphamide has broader immunosuppressive effects on several subsets of lymphocytes. The main mechanism of cyclophosphamide is the ability to covalently bind an alkyl group, affecting mainly the DNA (61). This interaction is irreversible and leads to inhibition of DNA replication and apoptosis, producing cell death amongst resting and dividing white blood cells and leading to impaired humoral and cellular immune responses (62). Rapidly proliferating cells are most sensitive to cyclophosphamide (41). This feature is utilized in cancer therapy, but also to influence activated immune cells that are present in different immune-mediated diseases (37). The effects and side-effects of cyclophosphamide are highly dose dependent. High doses can be used for the complete eradication of hematopoietic cells, but lower doses are relatively selective for T-cells, especially T-regulatory cells (T-regs). Cyclophosphamide affects T-regs, which have a generally higher proliferation rate than other T-cell subsets such as the T-helper (Th) cells, but also affects B-cells and other cells of the immune system (41). T-regs have an important role in down-regulating the effects of Th cells, and help prevent autoimmune diseases by maintaining self-tolerance (63). A higher frequency of T-regs in ME/CFS patients compared to healthy controls has been reported in some studies (64–66). The T-reg markers are also general T-cell activation markers (63). Thus, cyclophosphamide may interfere with the balance between immune cell subsets and possibly counteract a disease-facilitating environment.

Although the double blinded RituxME trial showed no significant differences between the rituximab and placebo groups for the outcome measures (35), there may still be a subgroup of ME/CFS patients that have an autoantibody-mediated disease where only few patients have autoantibody-production from early CD20-positive plasmablasts that can be targeted by rituximab. Other patients may still have autoantibody production, but from long-lived CD20-negative plasma cells. This mechanism is active in several rituximab refractory autoimmune diseases and could be compatible both with the total experience from our rituximab trials, and with the data from the present cyclophosphamide trial. Cytotoxic chemotherapy, such as cyclophosphamide, may inhibit B-cell activation and proliferation to new antibody-secreting cells, thus reducing the short-lived plasma cell compartment and recruitment of mature plasma cells (67).

If an autoantibody-mediated mechanism is operative in a subgroup of ME/CFS patients, the nature of possible endogenous targets for pathogenic immunoglobulins is still elusive. Increased serum levels of autoantibodies against several G-protein coupled receptors have been shown in ME/CFS (16). Clinical symptoms suggest inadequate regulation of autonomic functions and blood flow, also demonstrated in a recent study of reduced cerebral blood flow during head-up tilt test with orthostatic stress using Doppler flow imaging of carotid and vertebral arteries (68). Recent observations of patients with unexplained exertional intolerance and dyspnea demonstrated a subgroup with low ventricular filling pressure (preload failure) in upright position during cardiopulmonary exercise tests, related to reduced venous pressure (69, 70). Also, in patients with unexplained exertional intolerance, a subgroup had impaired systemic oxygen extraction, which may be associated with microcirculatory dysregulation or mitochondrial dysfunction (71). One might speculate on the possibility of an autoimmune process indirectly of directly affecting blood vessels, or against small nerve fibers including autonomic nerves regulating blood vessel function. Small fiber neuropathy (SFN) is associated with fatigue, postural orthostatic tachycardia syndrome (POTS), gastrointestinal disturbances and abnormal sweating (72). SFN has been demonstrated in 49% of fibromyalgia patients (73), and in up to 43% of patients with preload failure, many of whom had symptoms suggestive of ME/CFS (70). This could be associated with inadequate autoregulation of blood flow according to the demands of tissues, with local hypoxia and lactate accumulation on limited exertion, and with metabolic adjustments which could be secondary and compensatory in efforts to restore cellular energy balance (20, 21, 23, 74, 75). Microvasculopathy may also be reflected in arterial endothelial dysfunction which has been demonstrated in ME/CFS (76), and also investigated in substudies to the CycloME and RituxME trials (manuscripts in preparation).

The growing evidence for immune disturbances in ME/CFS, experience with cyclophosphamide in other autoimmune diseases, with broad immunosuppressive effects on several lymphocyte subsets including B-cells and T-regs, and the herein reported association between HLA risk alleles and clinical response to cyclophosphamide intervention, support that the observed relief of ME/CFS symptoms could be a drug effect targeting the underlying disease mechanisms. We strongly advise patients and physicians not to use cyclophosphamide for ME/CFS patients outside of clinical trials before a randomized trial has been conducted, to evaluate the possible benefits of the drug.

CONCLUSION

This study shows that cyclophosphamide intervention is feasible for ME/CFS patients. The growing evidence for immune alterations in ME/CFS and the high symptom burden with very low quality of life, we believe can justify use of an immune modulating drug with possible side effects. The treatment period was demanding for

most patients, but in total the toxicity was interpreted as acceptable. The treatment was associated with long-lasting improvements of ME/CFS symptoms for approximately half of patients. However, due to the lack of a placebo group, response data must be interpreted with great caution. In the further work to find effective treatment, we will consider a new multicenter, randomized, double-blind and placebo-controlled trial with cyclophosphamide. Should this trial prove cyclophosphamide to be beneficial for ME/CFS patients, this could also be important in the search for relevant disease mechanisms.

DATA AVAILABILITY STATEMENT

The datasets generated from this study are available on reasonable request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Regional Committees for Medical and Health Research Ethics (2014/1672), and by the National Medicines Agency in Norway. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IR, ØF, KS, and OM: conception and design. IR, ØF, AF, KT, AL, MV, BL, and OM: analyses and interpretation. IR, ØF, AF, IK-V, MH, KS, and OM: inclusion and follow-up of patients. IR, ØF, AF, IK-V, KS, KR, KA, MH, OM, AL, MV, and BL: collection and assembly of data. IR, ØF, KS, KR, KA, OD, and OM: administrative, technical, biobank and logistic support. IR, ØF, AF, KS, and OM: drafting the article. All authors: critical revision of the article and final approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00162/full#supplementary-material

Supplementary Figure 1 | SF-36 subscales (raw scores) and Fatigue Severity Scale during follow-up until 18 months, shown for the Intention-to-treat population ($n = 40$) in **(A,C,E,G)**, and for responders ($n = 22$) vs. non-responders ($n = 18$) in **(B,D,F,H)**. The SF-36 subscales for Vitality **(A,B)**, Social Function **(C,D)**, Bodily Pain **(E,F)**, and Fatigue Severity Scale **(G,H)** are shown. SF-36 subscales with scale 0–100, higher number indicates better function. Fatigue Severity Scale with scores 7–63, higher score indicates more fatigue. SF-36, Short Form 36; CI, confidence intervals; SD, standard deviation.

Supplementary Table 1 | Medical history and concomitant diseases reported at baseline, shown by System Organ Class (SOC) and CTCAE term.

Supplementary Table 2 | Previous treatments for ME/CFS, reported at baseline.

Supplementary Table 3 | Concomitant medication during 18 months follow-up (shown by ATC-code).

Supplementary Table 4 | Serious Adverse Events during 18 months follow-up (System Organ Class, CTCAE term, SAE category and relation to treatment).
Trial protocol.

Data Sheet 1 | Trial protocol.

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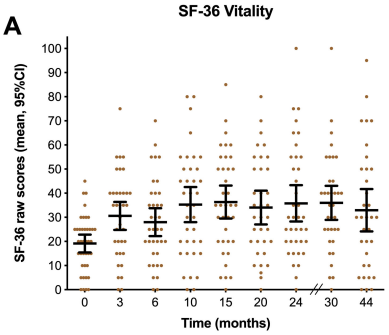
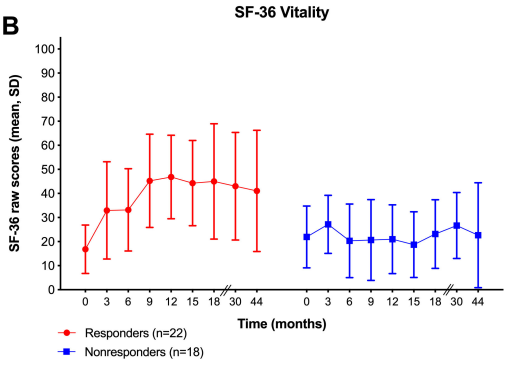
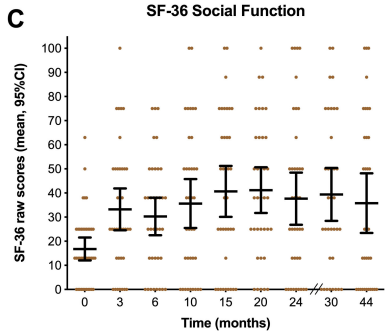
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.


A
SF-36 Vitality
SF-36 raw scores (mean, 95%CI)
Time (months)


B
SF-36 Vitality
SF-36 raw scores (mean, SD)
Time (months)
Responders (n=22)
Nonresponders (n=18)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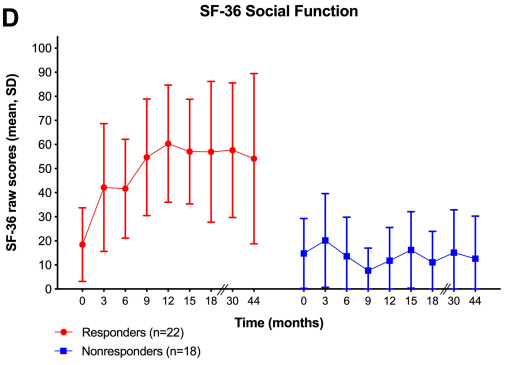
SF-36 Social Function
SF-36 raw scores (mean, 95%CI)
Time (months)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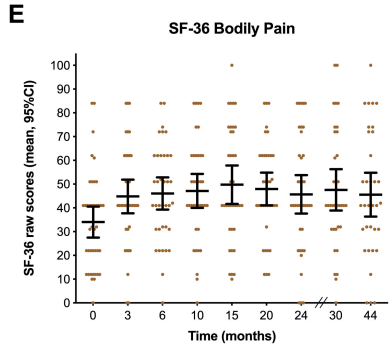
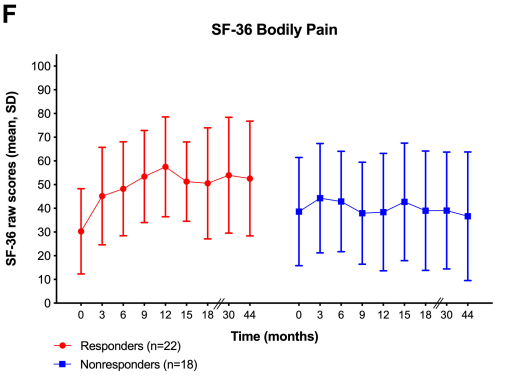
SF-36 Social Function
SF-36 raw scores (mean, SD)
Time (months)
Responders (n=22)
Nonresponders (n=18)


E
SF-36 Bodily Pain
SF-36 raw scores (mean, 95%CI)
Time (months)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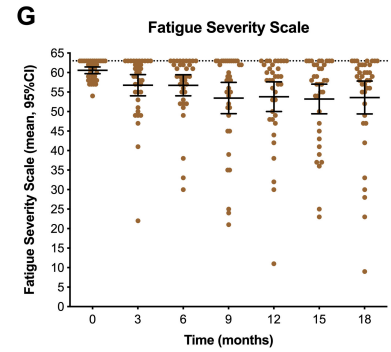
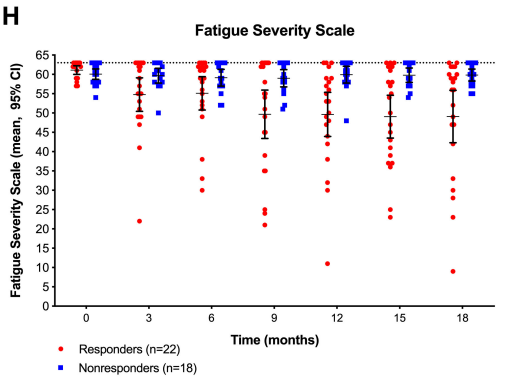
SF-36 Bodily Pain
SF-36 raw scores (mean, SD)
Time (months)
Responders (n=22)
Nonresponders (n=18)


G
Fatigue Severity Scale
Fatigue Severity Scale (mean, 95%CI)
Time (months)


H
Fatigue Severity Scale
Fatigue Severity Scale (mean, 95% CI)
Time (months)
Responders (n=22)
Nonresponders (n=18)

Supplementary Table 2. Previous treatments for ME/CFS, reported at baseline.

Type of treatment, n (%)	***n***	***%***
Cognitive therapy (CT)		
"Lightning Process" (LP)	13	32.5
Mindfulness	11	27.5
Other CT	4	10.0
Any CT (LP, Mindfulness, Other)	21	52.5
Physical therapy		
Graded exercise therapy (GET)	6	15.0
Other physical therapy	15	37.5
GET or other physical therapy	18	45.0
Activity management (adaptive pacing)	15	37.5
Not received any of these treatments	8	20.0
Not answered	1	2.5
Medical treatments		
Nexavir	2	5.0
Vitamin B12-injections	16	40.0
Long term antibiotics	10	25.0
Low dose naltrexone	15	37.5
Rituximab	15	37.5
Not received any of these treatments	7	17.5
Not answered	1	2.5

Supplementary Table 3. Concomitant medication during 18 months follow-up (shown by ATC-code)

ATC code	*Description*	*N=40*
A02	Antacids, n (%)	9 (22.5)
A03, A04	Antiemetics, n (%)	10 (25.0)
A06	Laxantia, n (%)	2 (5.0)
B01	Antithrombotic agents, n (%)	1 (2.5)
B03A, B03BB	Vitamin B12 supplements, n (%)	5 (12.5)
C07	Betablockers, n (%)	3 (7.5)
C08, C09	Antihypertensive agents, n (%)	4 (10.0)
C10	Statins, n (%)	2 (5.0)
G01, J01-05	Antibiotics, n (%)	13 (32.5)
G03A	Contraceptives (systemic), n (%)	4 (10.0)
H03	Thyroid hormone replacement, n (%)	4 (10.0)
M01A	NSAID, n (%)	17 (42.5)
N02A	Opioids[a], n (%)	11 (27.5)
N02B	Paracetamol, n (%)	15 (37.5)
N02C	Antimigraine agents, n (%)	5 (12.5)
N03A	Antiepileptic agents, n (%)	1 (2.5)
N05B	Anxiolytica, n (%)	4 (10.0)
N05C	Hypnotics and sedatives[b], n (%)	20 (50.0)
N06	Antidepressants, n (%)	6 (15.0)
R01, R03, R06A, S01G	Allergy and asthma medications, n (%)	20 (50.0)
	Other medications, n (%)	30 (75.0)
	Dietary supplements (non-ATC) , n (%)	10 (25.0)

[a]: 11 out of 40 patients received opioids at any time during follow-up. Among these, only 2 used tramadol daily on a regular basis, and 9 used codeine phosphate or tramadol on demand. None of the patients used any stronger opioids. [b]: 20 out of 40 patients had used hypnotics regularly or sporadically at any time during follow-up, 4 of whom had tried more than one type of hypnotic. Among the 20, 10 had used melatonin, 10 had used zopiclone and 4 patients had used nitrazepam or zolpidem.

Supplementary Table 4. Serious Adverse Events during 18 months follow-up (System Organ Class, CTCAE term, SAE category and relation to treatment)

System Organ Class	*CTCAE term*	*Grade*	*Adverse event category*	*Relation to treatment*
Cardiac disorders	Sinus tachycardia	3	SUSAR/ Hospitalization	Probable
	Sinus tachycardia	3	SAE/ Hospitalization	Unlikely
Gastrointestinal disorders	Stomach pain	2	SAE/ Hospitalization	Possible
General disorders	Other (ME/CFS symptom exacerbation)	3	SAE/ Hospitalization	Probable
Infections and infestations	Urinary tract infection	3	SAE/ Hospitalization	Possible
	Sepsis†	4	SAE/ Hospitalization	No
	Sepsis†	4	SAE/ Hospitalization	No
	Upper respiratory infection	3	SAE/ Hospitalization	No
Metabolism and nutrition disorders	Dehydration	3	SAE/ Hospitalization	Probable
Neoplasms	Other (olfactory meningioma) *	3	SAE/ Hospitalization	No
Renal and urinary disorders	Renal calculi†	3	SAE/ Hospitalization	No
Skin and subcutaneous tissue disorders	Urticaria	3	SAE/ Hospitalization	Possible

† 3 hospitalizations for one study patient; complications after elective daytime surgery.

* Elective hospitalizations/procedures

Paper IV

PLOS ONE

RESEARCH ARTICLE

Activity monitoring and patient-reported outcome measures in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome patients

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Abstract

Introduction

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disease with no validated specific and sensitive biomarker, and no standard approved treatment. In this observational study with no intervention, participants used a Fitbit activity tracker. The aims were to explore natural symptom variation, feasibility of continuous activity monitoring, and to compare activity data with patient reported outcome measures (PROMs).

Materials and methods

In this pilot study, 27 patients with mild to severe ME/CFS, of mean age 42.3 years, used the Fitbit Charge 3 continuously for six months. Patients wore a SenseWear activity bracelet for 7 days at baseline, at 3 and 6 months. At baseline and follow-up they completed the Short Form 36 Health Survey (SF-36) and the DePaul Symptom Questionnaire–Short Form (DSQ-SF).

Results

The mean number of steps per day decreased with increasing ME/CFS severity; mild 5566, moderate 4991 and severe 1998. The day-by-day variation was mean 47% (range 25%–79%). Mean steps per day increased from the first to the second three-month period, 4341 vs 4781 steps, $p = 0.022$. The maximum differences in outcome measures between 4-week periods (highest vs lowest), were more evident in a group of eight patients with milder disease (baseline SF-36 PF > 50 or DSQ-SF < 55) as compared to 19 patients with higher symptom burden (SF-36 PF < 50 and DSQ-SF > 55), for SF-36 PF raw scores: 16.9 vs 3.4 points, and for steps per day: 958 versus 479 steps. The correlations between steps per day and self-reported SF-36 Physical function, SF-36 Social function, and DSQ-SF were

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significant. Fitbit recorded significantly higher number of steps than SenseWear. Resting heart rates were stable during six months.

Conclusion

Continuous activity registration with Fitbit Charge 3 trackers is feasible and useful in studies with ME/CFS patients to monitor steps and resting heart rate, in addition to self-reported outcome measures.

Clinical trial registration

Clinicaltrials.gov: NCT04195815.

1. Introduction

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disease of unknown etiology with high symptom burden, no validated specific and sensitive biomarker, and no standard approved effective treatment. Defined by the Canadian consensus criteria, it affects 0.1–0.8% of the population [1, 2]. ME/CFS has a profound impact on the quality of life of both patients and caregivers, and entails high costs for society [3, 4].

Our working hypothesis is that ME/CFS in a subgroup could be a variant of an autoimmune disease. We have previously conducted clinical trials in patients with ME/CFS using the immunomodulatory drugs cyclophosphamide and rituximab [5–8]. In these studies, primary and secondary endpoints were based on various questionnaires for patient-reported outcome measures (PROMs) such as symptom change during follow-up and health-related quality of life. Activity monitoring using Sensewear armband were also used for secondary endpoints (mean steps per 24 hours); however, these armbands, although validated for clinical studies, had technical limitations and were used to record data for no more than 7 consecutive days at a time.

In the randomized and placebo-controlled trial assessing rituximab induction and maintenance infusions versus placebo in ME/CFS patients, the primary and secondary outcome measures were negative with no significant differences between the rituximab and placebo groups [7]. There were, however, fluctuations and changes in symptoms and activity level in both groups through follow-up. From our experience of these trials, we believe that there is a need for better knowledge and characterization of symptom variation over time in ME/CFS, independently of any treatment or intervention. An improved understanding of the natural course of ME/CFS over time would aid the development of better outcome measures and endpoints for future clinical trials, and contribute to distinguish natural symptom variation from changes in disease course which could be ascribed to trial intervention. Also, there is a need for objective parameters describing aspects of the ME/CFS disease, in addition to questionnaires designed to capture subjective and self-reported data.

In addition, comparison of data from different clinical trials are hampered by the use of different outcome measures and, importantly, inclusion of patient populations with different levels of symptom severity. The establishment of new, improved and broadly accepted outcome measures would aid such comparisons, which we believe are important when assessing trial outcomes. Until now, consensus on outcome measures is lacking, but there are some reports evaluating different PROMs in ME/CFS [9–15]. Several studies report data from the self-reported questionnaire for health-related quality of life Short Form 36 Health Survey (SF-36), with focus on the subscale Physical Function (SF-36 PF), and the DePaul symptom questionnaire (DSQ).

Aiming to improve the outcome measures for future clinical trials on ME/CFS, this study evaluates the use of data obtainable from activity trackers, which are growing in popularity. Several studies for evaluation of accuracy have been performed [16]. Both our group and others have used steps per 24 hours as an objective outcome measure in studies with ME/CFS, using different activity tracker technologies [5–8, 17–19]. The overall impression is that the measurement of steps in particular seems to have acceptable accuracy in controlled studies, and may be a useful readout in a clinical setting [16, 20, 21].

In this prospective observational study with no intervention, 27 participants used Fitbit activity trackers continuously throughout the six-month study period. We explored the feasibility of continuous activity monitoring in a clinical trial and whether the armbands could be used to assess levels of physical activity in ME/CFS patients. Further, we compared the continuous activity monitoring with PROMs for health-related quality of life and for ME/CFS symptoms, and asked the participants to assess which data best reflected their own perception of activity level and symptom severity. Data from the Fitbit monitor were compared with the SenseWear activity armbands, which have been validated and used in our previous clinical trials.

2. Materials and methods

2.1 Trial design

The study (ClinicalTrials.gov NCT04195815) was designed as a prospective observational study with continuous monitoring of physical activity using Fitbit Charge 3 activity trackers for 6 months, including assessment of feasibility, comparison with PROMs for quality of life (SF-36 ver.1.2) and for ME/CFS symptoms (DSQ-SF), and comparison with the validated activity bracelet Sensewear.

2.2 Setting and patient inclusion

Recruitment was performed by advertising via the local ME association's Facebook page, and the research group's e-mail newsletter. Of 122 candidates who applied for participation, 14 were excluded due to long travel distance to the study center, and 16 because they had participated in earlier studies. From the remaining 92 candidates, 30 were randomly selected and informed about the study by phone. Three patients decided not to participate, and the remaining 27 proceeded to clinical assessment and inclusion. Due to the Covid-19 pandemic, the inclusion process was closed after the first 27 included patients.

Inclusion criteria were: a diagnosis of ME/CFS according to the Canadian consensus criteria [22]; age 18 to 65 years; disease duration more than two years; and disease severity mild, mild-to-moderate, moderate, moderate-to-severe, or severe. For statistical analyses, these were lumped into three categories: mild (including mild and mild-to-moderate), moderate, and severe (including moderate-to-severe and severe). The exclusion criteria and baseline clinical evaluation with laboratory tests are detailed in the trial protocol (S1 File).

Recruitment and follow-up lasted from March 2020 until November 2020. All 27 patients were included and monitored at the Department of Oncology and Medical Physics, Haukeland University Hospital (HUH).

2.3 Activity armbands and data collection

The patients used Fitbit Charge 3 trackers on their non-dominant wrist (Fitbit, San Francisco, US) for continuous monitoring of physical activity, day and night. They were instructed to only remove the trackers for recharging, roughly for one hour per week.

A Data Protection Impact Assessment was performed prior to study start. In an effort to protect the participants' privacy, we used pseudonymisation toward third parties. Each participant Fitbit account was set up using a study-specific e-mail address, initials instead of name and a fictitious date of birth. Fitbit's terms of use comply with the General Data Protection Regulation (GDPR) directive. Fitbit activity data from each participant were downloaded at the study center weekly, using the Fitbit web API (https://dev.fitbit.com/build/reference/web-api/developer-guide/application-design/). For each participant we registered an Oauth 2.0 application with type set as "personal". The scopes were set to heartrate+profile+sleep+settings +activity+weight, with "time" set to 365 days.

An R-script was generated to facilitate downloading of data from all participants (S2 File). For more detailed information on data protection issues see trial protocol (S1 File).

In order to compare Fitbit activity data to the SenseWear device used in our previous studies, patients were instructed to wear a Sensewear armband on the non-dominant upper arm, continuously for 7 days, at baseline and two time points during follow-up (at 3 and 6 months). Sensewear armbands have been validated for use in clinical studies [23, 24].

During recent years, several clinical studies for validation of older generations of Fitbit trackers, in different diseases, have also been performed. The overall impression is that from the different measures available, number of steps had the best accuracy [16, 20, 21]. Sleep measures, energy expenditure and heart rate in the higher heart rate zones were found to be less accurate [25, 26]. In this study we focused on the measured "steps per 24 hours" and "resting heart rate" recorded during sleep or at inactivity during the day.

2.4 Self-reported questionnaires

At baseline, the patients recorded self-reported symptom score for Fatigue, PEM, and need for rest using a scale of 1 to 10 (higher number denotes more severe symptoms), and Function level (scale 1 to 100%) according to a table with examples in which 100% was completely healthy (S1 File).

At baseline and every four weeks, they completed Norwegian-language versions of the SF-36 questionnaire for health-related quality of life (Short Form 36 Health Survey ver. 1.2) [27], and the DePaul Symptom Questionnaire—Short Form (DSQ-SF) for ME/CFS symptoms [15], Norwegian translation of DSQ-SF is based on the translation of the complete DePaul Symptom Questionnaire [28]. (English versions in S1 File).

During follow-up, patients completed the Composite Autonomic Symptom Score-31 (COMPASS-31) questionnaire, used to assess symptoms related to dysautonomia.

One week after completing the study, the participants were asked to answer an evaluation of the study and the activity armband. We used an online survey from enalyzer.com. The answers were anonymous.

2.5 Statistics

For Fitbit data, steps per 24 hours and resting heart rate, with continuous data for 168 days (24 weeks), with no replacement for missing data, were used in the analyses. Means and standard deviations (SD) were calculated per 4-week period, and also dichotomized into days 1–84 vs days 85–168, to assess changes over time. Groups were compared by t-test (equal variance not assumed), or by Mann-Whitney test, as appropriate.

Repeated measures of variables, by ME/CFS severity and by categories of baseline SF-36 PF, were assessed by General Linear Model (GLM) for repeated measures.

Correlations (Spearman's rho) were performed between different variables describing aspects of ME/CFS such as Function (%), SF-36 domains, Compass sum score and domains, DSQ-SF total score, mean steps and resting HR.

All tests were two-sided with a significance level of $p < 0.05$. Missing data were replaced only for one patient at the 24-weeks recording for SF-36 and DFQ-SF (i.e. 2 recordings out of 378) using the last value carried forward (LVCF) method. All analyses were performed using IBM SPSS Statistics ver.26 (IBM Corp., Armonk, USA), and Graphpad Prism ver.9 (GraphPad Software, La Jolla, USA).

2.6 Ethics

The study was approved by the Regional Committees for Medical and Health Research Ethics in Norway (No. 28780). Concerns about patients' privacy are discussed under "Activity armbands" in Methods. A Data Protection Impact Assessment was performed in consultation with the IT Security Manager and Data Protection Officer for the Bergen Hospital Trust and user representatives. The user representatives have also assessed and contributed to the study protocol and all written information given to the participants. All participants gave written consent.

3. Results

3.1 Study population

Flow diagram of participants through enrollment, inclusion and follow-up and analysis are shown in Fig 1. After prescreening (described in Methods), 27 patients were included. All participants had an established ME/CFS diagnosis and met Canadian inclusion criteria by clinical assessment. Patients with a relatively mild degree of ME/CFS symptoms were somewhat overrepresented; this was accepted because the main purpose of the study was to assess the feasibility and usefulness of continuous activity monitoring and PROMs, and a representative sample was not required. Based on clinical assessment, participants were divided into three severity categories; mild (n = 11), moderate (n = 10) and severe (n = 6). Table 1 shows baseline characteristics for all included patients (n = 27), and also by severity group.

Except for one participant who started a low carbohydrate diet aimed at the ME/CFS symptoms, the participants did not undergo any intervention for their ME/CFS disease during the course of the study.

A dataset with clinical data, PROMS, steps and resting heartrate for all patients during follow up (S3 File) and TREND checklist for clinical studies (S4 File) are found in supplementary files.

3.3 Missing data

There were little missing data from the Fitbit recordings of activity level. For the 24-week study period, the mean number of days with valid recording of steps per 24 hours was 166 out of 168 days. There were recordings of steps per 24 hours for all 168 days in 20 patients, while 2 had recordings from 167 days, and 5 patients had valid recordings spanning from 152 to 166 days.

For resting heart rate, the mean number of days with valid recordings was 162. Sexteen patients had recordings from at least 166 days, and 11 patients had valid recordings spanning from 132 to 165 days. For steps per 24 hours and resting heart rate, the observed data were used as input for statistical analyses, with no replacement for missing values (approximately 0,1% and 0,5% missing data for steps per 24 hours and for resting heart rate, respectively).

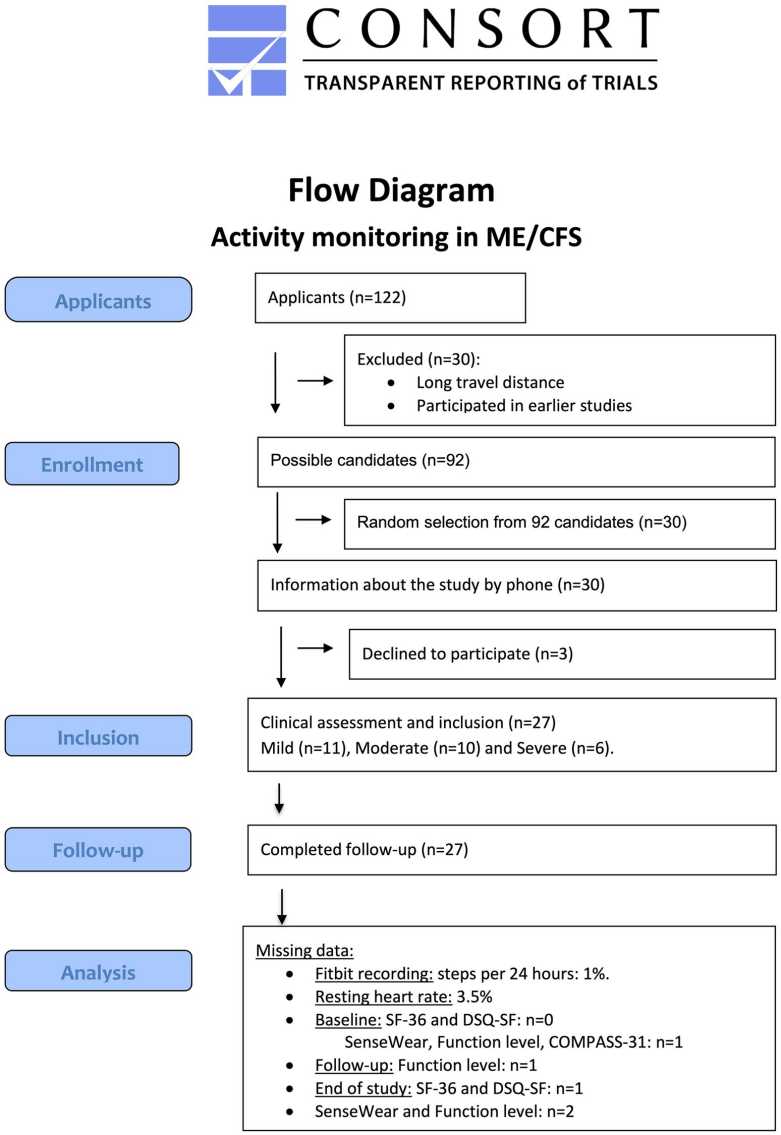

Fig 1. CONSORT flow diagram. Flow diagram of enrollment, follow-up and analysis in the study "Activity monitoring in ME/CFS".

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Table 1. Baseline characteristics of the study population, all patients and by severity.

Characteristic	All patients (n = 27)	Mild (n = 11)	Moderate (n = 10)	Severe (n = 6)	P-value (ANOVA)	P-value (Trend)[9]
Female, n	25	10	9	6	-	-
Male, n	2	1	1	0	-	-
Age, all patients, mean (min-max)	42.3 (20–62)	40.6 (20–58)	44.3 (20–62)	42 (31–60)	0.75	0.72
BMI[1] all patients, mean (min-max)	28.0 (20.0–44.0)	26.5 (21.4–37.6)	31.1 (20.6–44.0)	27.6 (21.5–31.7)	0.43	0.57
Systolic blood pressure, mean (min-max)	132 (104–170)	128 (104–154)	143 (122–170)	122 (107–141)	0.04	0.86
Diastolic blood pressure, mean, (min-max)	85 (63–105)	81 (63–94)	92 (81–105)	82 (66–101)	0.07	0.49
Mean resting HR[2]	68.5 (55–95)	67 (55–80)	71 (61–95)	65 (56–71)	0.40	0.55
ME/CFS disease duration						
2–5 years, n	6	4	2	0	-	
5–10 years, n	8	3	3	2	-	
10–15 years, n	7	1	4	2	-	
>15 years	6	3	1	2	-	
Self-reported Fatigue[3], mean (min-max)	7.1 (5–9)	6.4 (5–8)	7 (6–8)	8.5 (7–9)	<0.001	<0.001
Self-reported post-exertional[3] malaise, mean (min-max)	7.7 (6–10)	7.1 (6–9)	7.6 (7–8)	8.8 (8–10)	0.001	<0.001
Need for rest[3], mean (min-max)	7.2 (5–9)	6.5 (5–8)	7.4 (7–8)	8.2 (8–9)	<0.001	<0.001
Function level[4], mean (min-max)	18.0 (5–35)	26.4 (20–35)	16.2 (10–20)	8.0 (5–12)	<0.001	<0.001
SF-36[5] Physical Function, mean (min-max)	37.2 (5–70)	46.4 (25–70)	35.5 (25–65)	23.3 (5–45)	0.024	<0.01
SF-36[5] Bodily pain, mean (min-max)	38.3 (0–84)	44.7 (22–84)	39.4 (22–74)	24.7 (0–41)	0.093	0.04
SF-36[5] General Health, mean (min-max)	32.3 (10–65)	36.1 (15–65)	33.0 (10–50)	24.2 (10–45)	0.297	0.14
SF-36[5] Vitality, mean (min-max)	23.9 (0–70)	18.2 (0–40)	27.5 (10–35)	28.3 (10–70)	0.257	0.14
SF-36[5] Social Function, mean (min-max)	28.2 (0–75)	37.5 (13–75)	30.0 (0–75)	8.3 (0–25)	0.018	<0.01
SF-36[5] Mental health, mean (min-max)	78.4 (44–92)	76.0 (56–92)	81.6 (44–92)	77.3 (64–88)	0.600	0.7
DSQ-SF[6] total score, mean (min-max)	67.2 (38–106)	62.9 (42–87)	62.8 (38–81)	82.3 (63–106)	0.022	0.02
Steps per 24 hours[7], Fitbit mean (min-max)	4305 (756–8541)	5007 (3756–8199)	4927 (2895–8541)	1979 (756–4056)	0.001	0.02
Compass-31[8] sum score, mean (min-max)	41.9 13.3–63.7	36.8 13.3–54.9	42.5 33.5–57.6	49.4 38.2–63.7	0.114	0.04
Compass orthostatic[8], mean (min-max)	19.2 0–28.0	15.0 0–24.0	20.0 12.0–28.0	24.0 16.0-28-0	0.073	0.02

[1]Body Mass Index;

[2]Mean resting heartrate weeks 1–4,

[3]Self reported scale from 1–10 (higher number denotes more symptoms)

[4]Self-reported scale 1–100 (higher number denotes better function), according to a table with examples

[5]Short Form-36 Health Survey (SF-36) with raw scores (scale 0–100). Higher number denotes less symptoms.

[6]DePaul Symptom Questionnaire–Short Form (DSQ-SF), higher number denotes more symptoms.

[7]Steps mean per 24 hours, week 0–4.

[8]Composite Autonomic Symptom Score-31 (COMPASS-31); sum score and the domain "Compass orthostatic", higher number denotes more symptoms.

[9]ANOVA trend test

Missing data for one patient for Blood Pressure, self-reported Fatigue, PEM, Need for rest, Steps and Compass score.

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At baseline there were no missing data for SF-36 and DSQ-SF, and only missing data from one patient for SenseWear, Function level and COMPASS-31. During follow-up one patient failed to report Function level, and at 24 weeks (end of study) there were missing data for SenseWear and Function level for two patients, the observed data were used with no replacement for missing values.

For SF-36 and DSQ-SF one patient had missing data at the 24-week recording (i.e. 2 missing out of 378 recordings, 0.5%). These two data for SF-36 and DSQ-SF were replaced by the LVCF method.

3.4 Fitbit; steps and resting heart rate

Mean steps per 24 hours for all 27 patients during 168 days' follow-up were 4560. A representative profile for steps per 24 hours and resting HR throughout the study period is shown in Fig 2. The mean number of steps per 24 hours decreased with increasing severity with a significant ANOVA trend test; mild 5566 steps, moderate 4991 steps and severe 1998 steps ($p = 0.02$). The variation in mean steps per 24 hours (i.e. the difference between 4-week periods with highest and lowest values), were 1217 steps among patients with mild severity, 753 in moderate, and 240 in severe ME/CFS (Fig 3A). Baseline steps (week 0–4) for all patients and for the three severity groups are shown in Table 1.

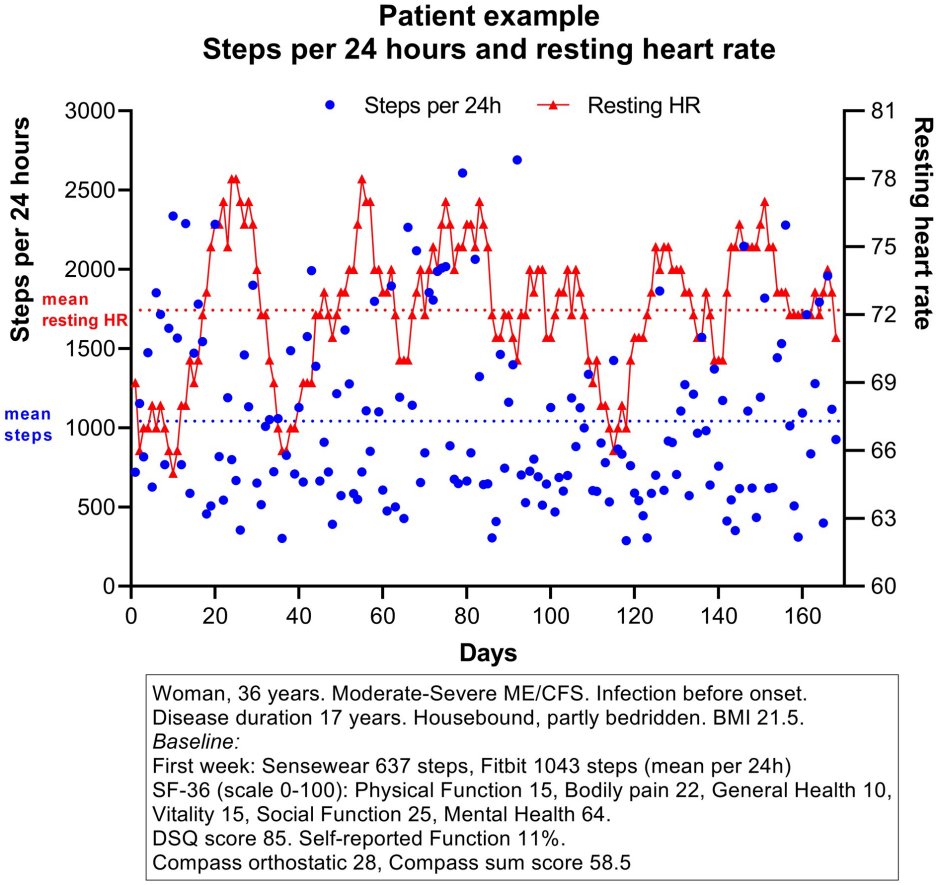

Fig 2. Patient example. Patient example with raw data for steps per 24 hours and resting heart rate, days 1–168.

https://doi.org/10.1371/journal.pone.0274472.g002

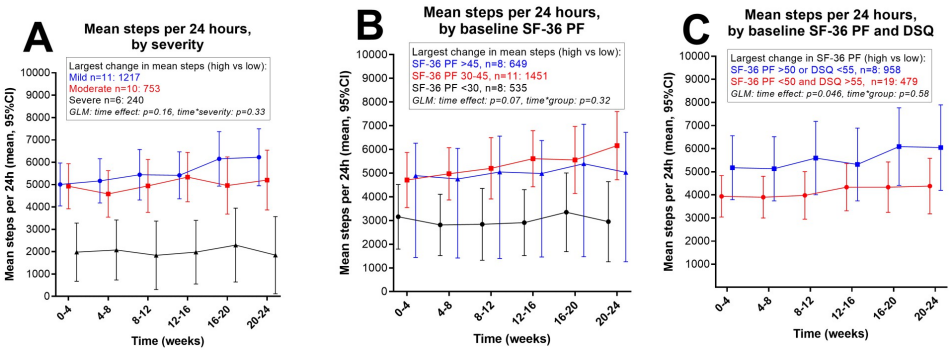

Fig 3. Steps per 24 hours, by severity, by SF-36 physical function, and by combination of SF-36 physical function and DSQ-SF. (A) Steps per 24 hours (mean, 95% CI) during follow-up, by severity categories; Mild, Moderate and Severe. (B) Steps per 24 hours (mean, 95% CI) during follow-up, by three categories based on baseline SF-36 PF. (C) Steps per 24 hours (mean, 95% CI) during follow-up, in two groups based on; SF-36 PF > 50 or DSQ-SF < 55, versus SF-36 PF < 50 and DSQ-SF > 55. The largest changes in mean steps between 4-week time periods, with difference highest versus lowest are indicated. General Linear Model (GLM) for repeated measures with p values for time effect and for interaction time-by-group are shown.

https://doi.org/10.1371/journal.pone.0274472.g003

For day-by day variation of steps per 24 hours, the mean coefficient of variation (CV, defined as SD/mean) was 47%, however with a broad range 25%–79% among patients, and with no significant difference between clinical severities, or between categories of SF-36 PF.

Mean steps per 24 hours for all 27 patients through days 1–84 compared to days 85–168, assessed by paired sample t-test, was significantly higher in the last period (4341 vs 4781 steps, p = 0.022). The absolute changes in mean steps per 24 hours (with minimum and maximum) from the first to the second half of the study were mean 723 steps (-960 to 4031) in patients with mild disease, mean 350 steps (-415 to 1236) in patients with moderate severity, and mean 68 (-263 to 291) in patients with severe ME/CFS. Although the increase in mean steps per day through follow-up was largest among patients with mild severity, or with higher baseline SF-36 PF, the changes were not significantly different by ME/CFS severity, (Fig 3A), or by categories of baseline SF-36 PF (Fig 3B) during follow up, assessed by GLM repeated measures. At baseline, mean steps per 24 hours (recorded weeks 1–4) correlated significantly with SF-36 PF (p = 0.01 by Spearman's rho) (Fig 4).

Mean resting heart rates were stable during follow-up in the three severity groups, and also by categories of baseline SF-36 PF. Correlation between steps per 24 hours and resting heart rate (recordings days 1–168) was not significant (p = 0.58). Note the considerable individual variation between minimum and maximum mean resting heartrate (Fig 5A).

A dataset with mean steps and resting heartrate per day for each patient is found in S5 File.

3.5 Comparison of Fitbit Charge 3 and SenseWear; steps

Recordings of steps per 24 hours using the Fitbit Charge 3 tracker (on the non-dominant wrist) were compared with recordings from the SenseWear activity device (on the non-dominant upper arm) for seven days at baseline, at 3 months and at 6 months. Values from the two activity trackers correlated significantly at all three timepoints, but Fitbit recorded significantly higher number of steps (Fig 5B). A Bland-Altman plot (Fig 5C) showed a systematic difference between the two devices, with a bias of 974 steps per 24 hours, (95% CI -542 to 2489), which corresponds to a bias of 27.5% (95% CI -5% to 60%).

A dataset with the comparison between Sensewear and Fitbit is found in S6 File.

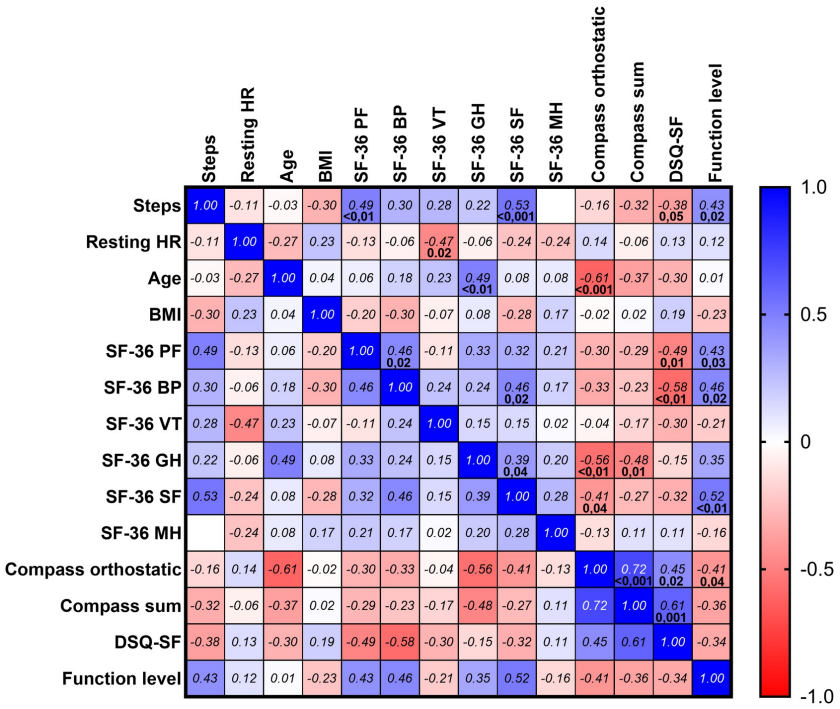

Fig 4. Correlations between baseline clinical data, PROMs, steps per 24 hours and resting heart rate. Spearman's correlation plot between baseline steps per 24 hour (mean, weeks 1–4), resting heart rate (mean, weeks 1–4), age, Body Mass Index. Short Form-36 Health Survey (SF-36); The raw scores (scale 0–100) for the six SF-36 domains (Mental health (SF36-MH), Physical function (SF-36 PF), Bodily pain (SF-36 BP), General health (SF-36 GH), Social function (SF-36 SF) and Vitality (SF-36 VF). Composite Autonomic Symptom Score-31 (COMPASS-31); sum and Compass orthostatic, DePaul Symptom Questionnaire–Short Form (DSQ-SF), and Function Level. Significant p-values are shown below Spearman's rho, with no adjustments for multiple correlations.

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3.6 Self-reported questionnaires for health-related quality of life

The raw scores (scale 0–100) for the six SF-36 domains (Mental health (SF-36 MH), Physical function (SF-36 PF), Bodily pain (SF-36 BP), General health (SF-36 GH), Social function (SF-36 SF) and Vitality (SF-36 VF), recorded at four-week intervals during follow-up, are shown in Fig 6A. The Mental health score was stable during follow-up, mean 78.4 (min 73.4—max 83.4) and close to reported values for women in the general population, i.e. mean 79.9 (SD 14.8) [29]. The five other SF-36 domains showed low scores, with the lowest reported for Vitality. Baseline values for all six SF-36 domains, for all patients and divided by the three severity groups are shown in Table 1. Fig 6B shows SF-36 PF in three severity categories during follow-up, and Fig 6C SF-36 PF by categories of baseline SF-36 PF (<30, 30–45, >45).

Mean steps per 24 hours correlated significantly with baseline SF-36 PF (p = 0.01), SF-36 SF (p = 0.03), and baseline DSQ-SF score (p = 0.007). DSQ-SF score correlated significantly with SF-36 PF, SF-36 BP, Function level and Compass sum score. All baseline correlations are shown in Fig 4.

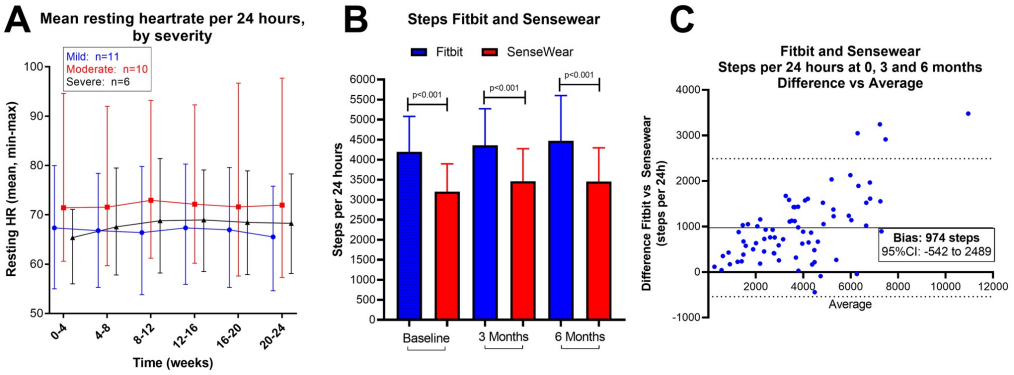

Fig 5. Activity data: Resting heart rate by severity, steps per 24 hours measured with Fitbit and SenseWear. (A) Resting heart rate, mean (min and max) levels, by three severity groups. (B) Steps per 24 hours measured for seven consecutive days by Fitbit and SenseWear, at baseline, 3 months and 6 months. (C) Bland-Altman plot showing difference (bias) between Fitbit and SenseWear devices for measured steps per 24 hours.

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Baseline DSQ-SF scores for ME/CFS symptoms are reported in Table 1. The mean scores for the mild and moderate groups were similar, but significantly higher for the severe group. Fig 7A shows DSQ-SF scores during follow-up categorized by baseline SF-36 PF.

In an explorative attempt to define groups of patients with the largest variations in outcome measures during six months' follow-up, we combined baseline SF-36 PF and DSQ-SF scores. One group of 8 patients with milder ME/CFS symptoms had either SF-36 PF $>$ 50 or DSQ-SF $<$ 55, while the remaining group of 19 patients with more pronounced ME/CFS symptoms had both SF-36 PF $<$ 50 and DSQ-SF $>$ 55. In this observational study with no intervention, the maximum differences between each 4-week period were more evident in the group of patients with milder ME/CFS, for mean number of steps per 24 hours (mean increase of 958 steps versus 479 steps, Fig 3C), for SF-36 PF scores (16.9 vs 3.4 points, Fig 7B), and for DSQ-SF (16.0 vs 3.0 points, Fig 7C).

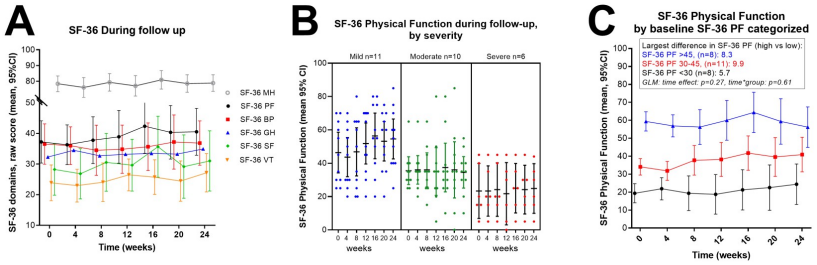

Fig 6. SF-36 subdomains (mean, 95% CI) during follow up; SF36 Physical Function (SF-36 PF) by severity categories, and by categories of baseline SF-36 PF. (A) SF-36 domains during follow-up; MH: Mental Health, PF: Physical Function, BP: Bodily pain, GH: General health, SF: Social function and VT: Vitality. Raw scores, scale 0–100, lower scores denote lower function. (B) SF-36 Physical Function (mean, 95% CI) during follow up shown in separate panels, for the severity categories. (C) SF-36 Physical Function (mean, 95% CI) during follow-up by three categories based on the baseline level of SF-36 PF; $<$ 30, 30–45, and $>$ 45. General Linear Model (GLM) for repeated measures with p values for time effect and for interaction time-by-group are shown.

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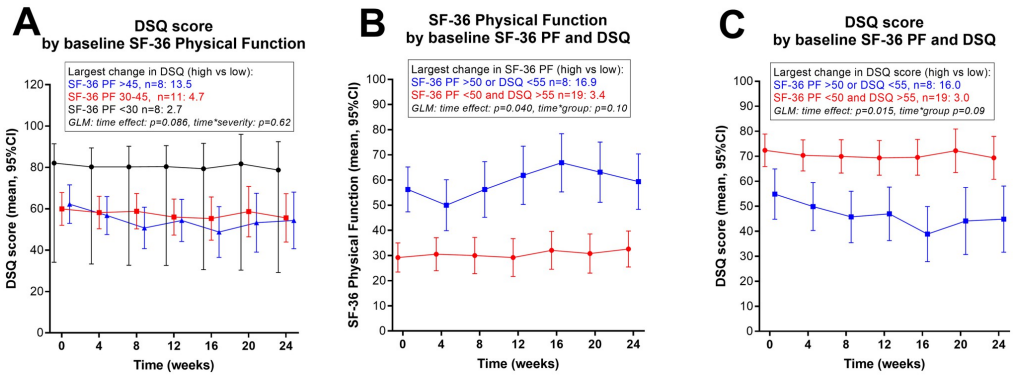

Fig 7. DSQ-SF and SF-36 Physical Function during follow-up, by ME/CFS categorized based on a combination of baseline SF-36 PF and DSQ-SF scores. (A) DePaul Symptom Questionnaire–Short Form (DSQ-SF) score (mean, 95% CI) during follow-up, by categories based on baseline SF-36 Physical Function; < 30, 30–45, and > 45. (B) SF-36 Physical function score (mean, 95% CI) during follow-up, by two groups based on: SF-36 PF > 50 OR DSQ-SF < 55 versus SF-36 PF < 50 AND DSQ-SF > 55. (C) DSQ-SF score (mean, 95% CI), by two groups based on; SF-36 Physical Function >50 OR DSQ-SF < 55 versus SF-36 Physical Function < 50 AND DSQ-SF > 55. The largest changes in mean scores between 4-week time periods, with difference highest versus lowest, are indicated. General Linear Model (GLM) for repeated measures with p values for time effect and for interaction time-by-group are shown.

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3.7 Compass-31

During follow-up the patients answered the questionnaire COMPASS-31 to map symptoms of autonomic dysfunction. COMPASS sum score and the domain "orthostatic intolerance" are shown in Table 1.

There were significant positive associations between the sum score of COMPASS-31 and of the domain "orthostatic intolerance," and the three levels of ME/CFS severity (p = 0.037 and p = 0.035, respectively). There were significant negative correlations between "Orthostatic intolerance" and the patient's age, the SF-36 domain "General health" and Function level (Fig 4).

3.8 Participant evaluation of the study

One week after completing the study the participants were asked to answer an evaluation of the study and the activity armband. We used an online survey from enalyzer.com. The answers were anonymous and 22 of the 27 participants answered (Table 2).

Several patients reported that their Fitbit devices recorded steps when they were not walking, but engaged in other activites which involved arm movement or vibration, such as knitting, cooking and driving slowly in a car or electric wheelchair on bumpy roads.

4. Discussion

The present study shows that continuous activity registration with Fitbit Charge 3 trackers is feasible in studies with ME/CFS patients. The mean number of steps per day decreased with increasing severity. Mean steps per day increased in the second as compared to the first half of the study. The correlations between steps per day and self-reported SF-36 Physical function, Social function, and DSQ-SF were significant. The study had a low number of participants, too few to draw firm conclusions, but using the combination of SF-36 PF and DSQ-SF we

Table 2. Participant evaluation of the study.

Question	Disagree[1] N, (%)	Agree[2] N, (%)	Undecided[3] N, (%)
Fitbit armband n = 23			
Fitbit app was easy to use	3 (13)	20 (87)	0
The Fitbit armband was comfortable to use	1 (4)	22 (96)	0
I experienced discomfort by using Fitbit armband	17 (74)	5 (22)	1 (4)
I had problems with the armband or the app, that I could not solve by simple troubleshooting	17 (74)	5 (22)	1 (4)
Using Fitbit affected my activity level	11 (48)	9 (39)	3 (13)
I used Fitbit as a tool to regulate my activity level	5 (22)	10 (44)	8 (35)
In my experience, the following measures reflected my activity level: n = 23			
Steps	4 (17)	17 (74)	2 (9)
Heartrate	0	22 (96)	1 (4)
Sleep	8 (35)	10 (44)	5 (22)
Active minutes	4 (17)	10 (44)	9 (39)
SF-36 n = 22			
The questionnaire was difficult to complete	16 (73)	5 (23)	1 (4)
Completing the questionnaire took a lot of effort	12 (54)	7 (32)	3 (14)
The questions were easy to understand	5 (23)	15 (68)	2 (9)
The questions were relevant for my situation	2 (9)	18 (82)	2 (9)
The questionnaire captured the changes in my condition	4 (18)	13 (59)	5 (23)
DePaul Symptom Questionnaire—Short Form n = 22			
The survey was difficult to answer	14 (64)	2 (9)	6 (27)
I used a lot of effort to answer * n = 23?	13 (57)	6 (26)	4 (17)
The questions were easy to understand	2 (9)	17 (77)	3 (14)
The questions were relevant for my situation	1 (4)	18 (82)	3 (14)
The survey captured the changes in my condition	2 (9)	14 (64)	6 (27)
Self-reported symptom change every two weeks n = 22			
The survey was difficult to answer	20 (91)	1 (5)	1 (5)
I used a lot of effort to answer	19 (86)	1 (5)	2 (9)
The questions were easy to understand	0	20 (91)	2 (9)
The questions were relevant for my situation	0	20 (91)	2 (9)
The survey captured the changes in my condition	1 (5)	17 (77)	4 (18)

[1]The column "Disagree" contains three levels of disagree; totally-, quite- and slightly disagree.

[2]The column "Agree" contains of three levels of agree; totally-, quite- and slightly agree.

[3]The column "Undecided" contains of "do not know" and "neither nor".

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identified a group of eight patients with milder disease that showed considerable variation in outcome measures during follow-up compared to the remaining participants.

In attempts to evaluate data from our previous clinical intervention trials of treatment involving the anti-CD20 B-cell depleting antibody rituximab [5–7, 30] or the cytotoxic drug cyclophosphamide [8], we have considered some aspects that may influence trial outcomes and conclusions. These include patient heterogeneity, patient inclusion criteria, case definitions, severity assessment, placebo mechanisms, natural symptom variation over time, and lack of objective outcome measures. There is limited knowledge about the variation and natural course of the ME/CFS disease over time. Moreover, it is not unlikely that patients could be subjected to a trial effect, i.e. the experience of benefit merely by the act of trial participation. In a patient group where the health system generally has little to offer, it is plausible that simply

the experience of receiving scheduled follow-up with regular doctor's appointments during study participation could have some impact on the disease course.

We wanted to evaluate and optimize the possible outcome measures for use in clinical trials by exploring the feasibility of continuous activity monitoring using the Fitbit Charge 3 armband to assess levels of physical activity in ME/CFS patients. Objective measures are important, but in view of the complexity of symptoms involved in this disease, it is necessary to combine the activity measures with self-reported questionnaires. An important question that should be further investigated in larger trials is which technologies and parameters are the most useful to reflect the ME/CFS symptoms and variations over time. Uniformly accepted outcome measures are lacking and criteria for improvement and recovery have been inconsistently defined across studies, making it difficult to assess treatment outcomes and compare different interventional studies.

In recent years, it has been quite common to combine patient-reported questionnaires with activity recordings such as number of steps per day as outcome measures, both in ME/CFS [7, 8, 17–19] and other chronic diseases [20, 21]. Wearable sensors can monitor and detect medical conditions [31], and one study described an alerting system for emerging COVID-19 infection and other stressful events [32]. Advanced studies describe wearable sensors that allow frequent and continuous measurements of different body functions, including HR, skin temperature, blood oxygen levels, physical activity, total gamma and X-ray radiation exposure, and glucose [33]. The use of these devices in the general population is growing in popularity, and many ME/CFS patients already wear some kind of activity device for their personal benefit. The possibilities in the future for wearable sensors in general health and research are vast.

When chosing a device for this project, our priorities were simplicity of use, performance on the basic functionalities (steps and heart rate), and privacy. The Fitbit privacy terms and conditions were more specific on their compliance with the General Data Protection Regulation (GDPR) directive than several comparable trackers in the same price range. Fitbit Charge 3 has been validated and showed acceptable accuracy during rest and treadmill activities, but performed poorly during sprint running and cycling. However, data in the range of activities typical for ME/CFS patients were acceptable [25].

The present study shows that it is feasible to use activity trackers for continuous registration of steps and resting heart rate in a study with ME/CFS patients. Our clinical impression from previous trials, and pilot experiences with ME/CFS patients, was that resting heartrate decreased when patients reported clinical improvement. We did not see the same tendency in this study with no intervention.

Continous Fitbit data for mean steps per 24 hours and for resting heart rate seemed useful, and may be used as outcome measures. However, due to the complexity of symptoms in individual patients, it is still important to also use the patient-reported outcome measures. Patients with ME/CFS cannot be evaluated based exclusively on measures of physical activity.

Generally, both PROMs and number of steps per 24 hours showed slight improvements during six months follow-up. By comparing mean steps per 24 hours in the first 12 weeks versus the last 12 weeks, we found a significant increase in the second part of the study. 23 of 27 patients were included between December and March, which means the first three-month period was winther to spring and the second three-month period was spring to summer. Some of the clinical improvement seen, could be explained by the fact that ME/CFS patients living in the Northern hemisphere often have less severe symptoms when the weather is warm.

The increase in steps and improvements in patient-reported measures were more evident among patients with mild disease. In patients with moderate (mainly housebound) or severe (partly bedridden) ME/CFS, there was little variation in symptom scores or number of steps over time. However, this observation may not be valid for ME/CFS patients in general, due to

the low number of patients in the present study. The cause of the increased activity during the study period is not certain. In a recent metaanalysis, feedback through activity monitors was found to increase the daily steps by 1235 in mixed groups of adults [34]. Such an effect could also be relevant for ME/CFS patients, yet with presumably smaller effect sizes due to the debilitating nature of their illness. Improvements in steps per 24 hours and in SF-36 PF scores (increase of 12 points) were also seen during two years follow-up in the placebo-group of the RituxME-trial [7]. We speculate that participation in a study with regular follow-up is in itself beneficial for the patients, and can explain some of the improvements during six months' follow-up. If the improvements seen in this and other studies can indeed be ascribed to a care effect, this is an important take home message to health services; even if there are limited treatment options available, these patients require qualified and regular support from health care professionals.

When including patients in clinical studies, self-reported medical history and clinical assessments are used for severity grading [35]. A recent study validated the ME/CFS severity by activity bracelets, cardiopulmonary exercise testing and SF-36 [18]. They found that the SF-36 Physical Function subscale (SF-36 PF) and the number of steps per day on an activity meter, showed a clear distinction between mild, moderate and severe ME/CFS patients, with some overlap between the groups. The mean steps by severity groups in this study are in accordance with other studies [17, 18]. For SF-36 PF and steps per day, this distinction between severity groups is similar to what we have seen in our previous studies [7, 8], and also to the present study. The correlation between SF-36 PF and steps was significant, in agreement with a previous study [17].

A systematic review of 56 randomized controlled trials (RCT) for chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) showed a total of 31 primary measurement tools used to assess the main outcome. The Checklist Individual Strength (CIS) was the most frequently used (35.7%), and others included the SF-36 (32.1%) [36].

We have used SF-36 in our studies, combined with other self-reported questionnaires. The general experience from our previous studies is that there are overall acceptable agreements between the clinical severity categories, questionnaires such as SF-36 PF and DSQ-SF and steps per day.

When validating and comparing different trials, the characteristics of the included study population are important, and may influence the outcome of the trial. Table 3 shows a selection of studies that have used the SF-36 PF subscale as an outcome measure. The mean baseline SF-36 PF ranged from 15 to 66 in these studies. In the present study, mean SF-36 PF for all patients was 37.2. There is no consensus as to what constitutes a clinically meaningful increase in SF-36 PF. Different studies define various changes in SF-36 PF as response criteria. In the studies described in Table 3, one study used an increase of 25 points in the SF-36 PF as a response criteria [19], while in other studies an increase of 10 [37] or even 7 points [38] was deemed sufficient to signify clinical response. To put this into perspective, the placebo group (n = 74) in our rituximab trial reported a mean increase in SF-36 PF score of 12 points during 24 months' follow-up (increase from 33 to 45) [7].

Although the SF-36 PF has major limitations for interpretation and is an imperfect measure, this item is often presented in clinical ME/CFS studies, both as a baseline characteristic and a possible measure of clinical outcome, as shown in Table 3. An additional concern is the use of an absolute change in SF-36 PF score as a response criterion, independent of the baseline score. One might argue that a 20-point increase in SF-36 PF from 10 to 30 would have a larger impact on quality of life as compared with an increase from 50 to 70.

As previously noted, in this study we made explorative efforts to identify groups of patients with larger symptom variations that would have had a significant influence on outcome

Table 3. Short Form-36 Health Survey, the domain "Physical Function" (SF-36 PF, raw scores, scale 0–100), in different ME/CFS studies.

Author	Year	Title		SF-36 PF Baseline	SF-36 PF Post intervention
Tummers et al [39]	2010	Effectiveness of Stepped Care (SC) for Chronic Fatigue Syndrome: A Randomized Noninferiority Trial. Care as usual (C)	N = 169 SC, N = 84 C, N = 85	 52 (SC) 54 (C)	 71 (SC) 72 (C)
White et al [40]	2011	Comparison of adaptive pacing therapy (APT), cognitive behaviour therapy (CBT), graded exercise therapy (GET), and specialist medical care (SMC) for chronic fatigue syndrome (PACE): a randomised trial	N = 641 APT N = 160 CBT N = 161 GET N = 160 SMC N = 160	 37 (APT) 39 (CBT) 38 (GET) 39 (SMC)	 46 (APT) 58 (CBT) 58 (GET) 51 (SMC)
Tummers et al [41]	2012	Implementing a minimal intervention for chronic fatigue syndrome in a mental health centre: a randomized controlled trial. Guided self-instruction (GSI), Waiting list (WL)	N = 123 GSI, N = 62 WL, N = 61	 50 (GSI) 51 (WL)	 65 (GSI) 59 (WL)
Fluge et al [6]	2015	B-Lymphocyte Depletion in Myalgic Encephalopathy/ Chronic Fatigue Syndrome. An Open-Label Phase II Study with Rituximab Maintenance Treatment	N = 27	40	67 (at 24 months) 68 (at 36 months)
Pinxsterhuis et al [42]	2017	Effectiveness of a group-based self-management program for people with chronic fatigue syndrome: a randomized controlled trial (I:Intervention, C: Control)	N = 137	46 (I) 46 (C)	48 (I) 51 (C)
Clark et al [43]	2017	Guided graded exercise self-help (GES) plus specialist medical care versus specialist medical care (SMC) alone for chronic fatigue syndrome (GETSET): a pragmatic randomised controlled trial	N = 211 GES, N = 107 SMC, N = 104	 47 (GES) 50 (SMC)	 56 (GES) 51 (SMC)
Crawley et al [37]	2018	Clinical and cost-effectiveness of the Lightning Process (LP) in addition to specialist medical care (SMC) for paediatric chronic fatigue syndrome: randomised controlled trial	N = 100	 56 (SMC) 53 (SMC+LP)	 72 (SMC) 86 (SMC+LP)
Stubhaug et al [38]	2018	A 4-Day Mindfulness-Based Cognitive Behavioral Intervention Program for CFS/ME. An Open Study, With 1-Year Follow-Up	N = 305	61 (all pts) 57 (CFS-CDC) 66 (CFS Oxford)	77 (all pts) 75 (CFS-CDC) 76 (CFS Oxford)
Fluge et al [7]	2019	B-Lymphocyte Depletion in Patients With Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: A Randomized, Double-Blind, Placebo-Controlled Trial	N = 151	 35 (Rituximab) 33 (Placebo)	 46 (Rituximab) 45 (Placebo)
Rekeland et al [8]	2020	Intravenous Cyclophosphamide in Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome. An Open-Label Phase II Study	N = 40	 33	 52 (at 18 months)
Castro-Marrero [44]	2021	Effect of Dietary Coenzyme Q10 Plus NADH Supplementation on Fatigue Perception and Health-Related Quality of Life in Individuals with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: A Prospective, Randomized, Double-Blind, Placebo-Controlled Trial	N = 174 Treatment N = 72 Placebo N = 72	 25 (Treatment) 28 (Placebo)	 29 (Treatment) 30 (Placebo)
Gotaas et al [45]	2021	Cognitive Behavioral Therapy Improves Physical Function and Fatigue in Mild and Moderate Chronic Fatigue Syndrome: A Consecutive Randomized Controlled Trial of Standard (S) and Short Interventions (SI). (C = waiting list)	N = 236	 53 (SI) 54 (S) 55 (C)	 63 (SI) 71 (S) 58 (C)
Scheibenbogen et al [19]	2021	Tolerability and Efficacy of s.c. IgG Self-Treatment in ME/CFS Patients with IgG/IgG Subclass Deficiency: A Proof-of-Concept Study	N = 12	 27	 42

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measures in a clinical study. Dividing the patients into clinical severity groups, we observed that the mild group tended to increase more in SF-36 PF and steps per 24 hours during six months' follow-up as compared to patients in the moderate and severe groups. When we combined SF-36 PF > 50 or DSQ-SF score < 55, we identified a group with the largest changes in SF-36 PF between four-week intervals during follow-up, i.e. 16.9 points among these 8 patients, compared to an increase of 3.4 points in the remaining 19 patients. Although we

cannot generalize from this small observational study with no intervention, our data underline the difficulties in distinguishing fluctuations in the natural course of the disease from the true effect of an intervention. If similar natural variations in patient-reported and physical activity measures occurred during a clinical trial, they could be wrongfully interpreted as response to an intervention, and could affect conclusions on response and effect sizes. Natural variation of symptoms over time, and associations with baseline disease severity, are therefore important to have in mind when planning clinical trials, and should also be included in the interpretation and discussion of clinical trial results.

In order to reduce the impact of natural symptom variation in future studies, one option could be to include a run-in period before start of intervention, to identify individual variation of symptoms e.g. over a time period of three to six months, and make it easier to interpret any changes occurring after active intervention.

The feedback from the patients assessing the use of Fitbit trackers was generally positive. Most found participation in the study useful, and they considered the Fitbit app and activity armband easy to use. Most patients reported that the Fitbit measures gave an accurate reflection of their activity level. Half of the patients reported that using the activity armband influenced their activity level. From the consultations with patiens in previous studies we have learned that some patients use activity trackers to monitor and pace their physical activity, partly as a tool to prevent post-exertional malaise (PEM) and "crashes". For some patients with ME/CFS, it is possible that wearing a tracker will decrease daily steps, at least in some periods, and give the opposite effect than the previously mentioned metaanalysis that showed increase in daily steps by wearing activity monitors [34].

The most important limitation of the study is the low number of participants in an observational study, too few to draw firm conclusions. The study population included more patients with mild disease than we usually include in our studies. There were little missing data both at baseline and follow-up. As discussed, the patients pointed at possible sources of error regarding Fitbit step registration, indicating that step count accuracy must be expected to vary between individuals as well as between devices. Nevertheless, the trackers can be a useful tool to monitor day to day changes for one individual, when the same technology is used over time.

5. Conclusion

In this study we have observed the course of 27 ME/CFS patients during 6 months' follow-up without any intervention. It is feasible to use activity trackers for the continuous registration of steps and resting heart rate in a study with ME/CFS patients. According to feedback from patients, the Fitbit trackers were easy to use, and gave a fair reflection of their physical activity levels. The correlations between steps per day and SF-36 PF, SF-36 SF, and DSQ-SF scores were significant. SF-36 PF has been reported in many studies of ME/CFS, with large differences in baseline values reflecting inclusion of patient populations which may not be easily comparable. After exploring different combinations of PROMs, activity measures and clinical assessment, we found that the combination of lower SF36-PF and higher DSQ-SF defined patients with more stable symptoms during follow-up in this study with no intervention. The knowledge from this study could be useful for the design of study protocols and assessments of outcome measures in future interventional studies. We propose including a run-in period with activity tracking and PROMs pre-intervention to evaluate normal fluctuations of the disease in individual patients. Due to the complexity of symptoms, it is necessary to combine the activity measures with patient-reported outcome measures to assess different aspects of disease.

Supporting information

S1 File. Study protocol.
(PDF)

S2 File. R script for the Fitbit web api.
(R)

S3 File. Clinical data, PROMS and Steps during follow-up.
(XLSX)

S4 File. TREND statement checklist for clinical trials.
(PDF)

S5 File. Mean steps and resting heartrate during follow up, for all participants.
(XLSX)

S6 File. Steps measured with SenseWear compared to Fitbit, data for all patients.
(XLSX)

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