Study Protocol

Antibiotic-mediated Improvements in Vigilance: Mechanisms of Action of Clarithromycin in Hypersomnia Syndromes

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Abstract

Pathologic sleepiness in the central disorders of hypersomnolence is associated with the presence of an endogenous, cerebrospinal fluid (CSF) peptide that can positively, allosterically modulate GABA-A receptors. We frequently prescribe the macrolide antibiotic clarithromycin for patients with hypersomnolence that is refractory to standard of care agents, as clarithromycin is a negative allosteric modulator of GABA-A receptors. We have published a randomized, placebo-controlled trial that provides evidence that clarithromycin improves sleepiness in patients with these central nervous system hypersomnia syndromes.

While the most parsimonious explanation for clarithromycin's efficacy is antagonism of an endogenous PAM of GABA-A receptors, several alternate mechanism may explain its wake-promoting effects: 1) state-dependent changes in connectivity within the brain's default mode network; 2) interference with the production of soporific cytokines; or 3) alteration of gastrointestinal microbiota composition, which is itself increasingly recognized as a modulator of neural excitability. Differentiating the anatomic and molecular mechanisms underlying clarithromycin's promotion of vigilant wakefulness will advance our ability to treat excessive sleep and pathologic sleepiness common to many neurologic conditions. We therefore propose a randomized, controlled trial of clarithromycin for excessive daytime sleepiness in hypersomnia patients, to evaluate each of these potential mediators of its improvement of pathologic sleepiness.

Introduction and Background

Excessive daytime sleepiness and long sleep durations are common features of many neurologic disorders, including myotonic dystrophy, Parkinson's disease, and the central nervous system hypersomnia syndromes (1-3). These latter syndromes are a group of disorders with overlapping clinical phenotypes and, except in the case of narcolepsy due to hypocretin deficiency (narcolepsy type 1), potentially shared pathophysiology (4-9).

Pathologic daytime sleepiness in these disorders impairs occupational performance, limits quality of life, and more than doubles motor vehicle and other accident risk (10, 11). Because the underlying cause of the majority of these hypersomnia syndromes is not known, treatments are aimed at increasing monoaminergic signaling involved in wake promotion. Yet, at least one-fourth of patients with hypersomnia syndromes cannot achieve satisfactory control of symptoms with these treatments (4) and disability or medical leaves of absence are often necessary (12). ***There is a clear need for novel treatments for excessive daytime sleepiness to resolve this failure of the current standard of care***.

The development of more effective treatments for pathologic sleepiness will require understanding of the mechanisms involved. Our group has demonstrated an increase in GABAergic signaling in patients with these hypersomnia syndromes, which serves as compelling explanation for their impaired vigilance. A small (0.5-3.0 kDa) peptide or peptide fraction within cerebrospinal fluid (CSF) acts as a positive allosteric modulator (PAM) of GABA-A receptors in vitro (12, 13). The potentiation of GABAergic chloride currents by this PAM is markedly increased by the CSF of patients with hypersomnia syndromes compared to that of non-sleepy controls (12). Work is ongoing to confirm the causal role of this PAM in the sleepiness observed by patients with these disorders, but such **potentiation of GABAergic signaling** is a biologically plausible cause of sleepiness based on the known role of GABA in natural sleep and pharmacologic sedation (14-17).

Based on this proposed pathophysiology of excessive daytime sleepiness, we and others have begun testing negative allosteric modulators and antagonists of GABA-A receptors for the treatment of excessive daytime sleepiness in patients with hypersomnia syndromes (12, 18-20). We have demonstrated that intravenous flumazenil reduces both subjective sleepiness and objective deficits in vigilance in hypersomnolent patients compared to placebo (12). Unfortunately, flumazenil is currently FDA-approved only in intravenous form and has a short, < 1 hour half-life, making it impractical and infeasible for long-term use by patients with hypersomnia. Compounded versions are available at only three pharmacies within the United States and are cost-prohibitive in many cases. Therefore, a more accessible GABA modulator or antagonist is needed.

Clarithromycin is one such GABA modulator. Clarithromycin causes increases in excitability of hippocampal CA3 pyramidal neurons, driven primarily by clarithromycin's dose-dependent reduction of GABA signaling (21). In [C^{11}]flumazenil positron emission tomography (PET) on non-human primates, clarithromycin delays the dissociation of [C^{11}]flumazenil from GABA-A receptors, further indicating modulation of GABA-A receptors by clarithromycin (22). ***Clarithromycin's allosteric modulation of GABA-A receptors may contribute to its reductions in excessive daytime sleepiness.***

Following reports of profound insomnia caused by clarithromycin, we piloted use of clarithromycin for patients with hypersomnia syndromes whose sleepiness was refractory to currently available treatments (23). In these 53 patients, who had previously failed treatment with an average of 2.6 (+/- SD 1.4) medications, 64% reported improved alertness and reduction in sleepiness with clarithromycin.

We then performed a placebo-controlled, double-blind, crossover trial of clarithromycin in patients with hypersomnia syndromes, 85% of whom were treatment-refractory, with persistent, severe sleepiness despite available medications (18). In this 20 subject trial, clarithromycin and matched placebo were administered twice daily for 2 weeks, separated by a 1 week washout period. ***Clarithromycin resulted in significant, clinically meaningful improvements in sleepiness severity, sleepiness-related limitations in extended activities of daily living, and sleepiness-related quality of life***. Long sleep durations and sleep inertia, both ancillary symptoms of hypersomnia disorders that contribute to functional impairments, were also improved with clarithromycin.

Reductions in daytime sleepiness via clarithromycin's modulation of GABA-A receptors is a parsimonious explanation for its treatment benefit in hypersomnolent patients. However, our preliminary data provide several reasons to suspect that the benefit of clarithromycin is not solely or mainly mediated by its actions on GABA-A receptors. Although direct application of clarithromycin clearly modulates the effect of GABA, as detailed above, our repeated sampling of CSF in two patients prior to and during treatment with clarithromycin has not shown a consistent reduction in GABA-A receptor potentiation by CSF. Both hypersomnia patients improved dramatically with clarithromycin, remaining on treatment for 6.5 years and 2.5 years, respectively. CSF from the first subject demonstrated only minimal reduction in potentiation while on treatment with clarithromycin compared to pre-treatment potentiation (95.2% +/- 21.3% pre-treatment, 89.5% +/- 23.8% on clarithromycin). In the other patient, there was no decrease in potentiation between the two CSF samples (95.8% +/- 16.1 pre-treatment, 96.3% +/- 21.5% on clarithromycin). This lack of change in CSF potentiation of GABA-A receptors needs to be confirmed or refuted in a sufficiently powered sample, but does suggest that alternate or additional mechanisms contribute to clarithromycin's reduction of sleepiness. Further, while both flumazenil and clarithromycin modulate GABA-A receptors in vitro, clinical responses to these two agents are distinct. In a group of 137 hypersomnolent patients treated separately with both clarithromycin and flumazenil, 26% of those who responded to clarithromycin reported no benefit from flumazenil, and response to the two agents was significantly different (Fisher exact test $p = 0.0002$) . This implies that a portion of clarithromycin response is being driven by mechanisms distinct from those possessed by flumazenil, which are more limited to actions at GABA-A receptors. ***Understanding these additional mediators of clarithromycin's effects on sleepiness will inform the development of novel therapeutics for these poorly understood disorders.*** Attractive candidate mediators for clarithromycin's reduction in sleepiness are modulation of default mode connectivity, reduction of soporific cytokines, and improvement in gastrointestinal dysbiosis. These potential mediators are not mutually exclusive. We will systematically assess clarithromycin's effects on each of these systems.

Functional neuroimaging provides opportunities to visualize networks that may underlie impairments in vigilant attention in the hypersomnia disorders. The body of literature on functional neuroimaging in hypersomnia disorders has focused almost exclusively on patients with narcolepsy due to hypocretin deficiency, rather than the larger group of hypersomnia disorders for which hypocretin levels are normal and GABA-A receptor potentiation by CSF is increased. This existing work has generally demonstrated reductions in cerebral blood flow and/or hypometabolism of the frontal and cingulate cortices, and increased activity in the nucleus accumbens, insula, amygdala, and inferior frontal gyri (24), but at times has revealed discordant findings in otherwise similar studies (25, 26). To some extent, differences in findings across similar studies may reflect the lack of objective monitoring for vigilance during testing, e.g., with simultaneous EEG, as unquantified, inadvertent sleep can profoundly bias functional imaging results (27).

Studies in patients with other hypersomnia disorders have been minimal to date. Patients with idiopathic hypersomnia have decreased regional blood flow to the medial prefrontal cortex compared to controls, via 99mTc-ethyl cysteinate SPECT (28), and patients with idiopathic hypersomnia or narcolepsy type 1 may have regional hypermetabolism of the anterior and middle cingulate and the insula using FDG-PET (26). Relevant to several of these areas is our ongoing investigation into the role of the default mode network (DMN) in hypersomnolent patients.

The DMN consists of a group of highly correlated brain regions most active during quiet rest (i.e., task-negative network). Its connectivity changes with sleep states (29-32) and it is increasingly implicated in the symptomatology of sleepiness. Among healthy participants undergoing resting state fMRI (rs-fMRI), level of sleepiness is strongly correlated with connectivity across the DMN (33, 34). During resting state, sleep

deprived participants demonstrate reduced connectivity with the DMN, particularly across midline nodes (35, 36). Experimental sleep deprivation of healthy subjects also results in decreased task-related deactivation of the DMN during psychomotor vigilance testing (37). Sleep deprivation-related changes in DMN connectivity during tasks are more prominent during trials with performance impairment, implying that these changes are mediating the impairments in performance caused by sleep deprivation (38). Changes in DMN connectivity caused by the sleep disorder, obstructive sleep apnea, are reversible with treatment (39). ***We hypothesize that alterations of DMN connectivity contribute to the sleepiness experienced by patients with hypersomnia syndromes and that clarithromycin's effect on sleepiness is partially mediated by changes in DMN connectivity.***

Multiple pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, are actively transported into the CNS (40) and are strongly implicated in producing sleepiness and/or increasing sleep duration. Such cytokines may affect sleep-wake regulation in a number of ways. First, somnogenic cytokines decrease production of wake-promoting dopamine and serotonin, via increases in microglial expression of indoleamine 2,3-dioxygenase and decreases in tetrahydrobiopterin (41). Second, somnogenic cytokines increase production of several sleep-promoting substances. IL-1β and TNF-α activate nitric oxide synthetases, which increase nitric oxide production, inducing sleep (42). IL-1β increases production of adenosine, a key signal for the homeostatic regulation of sleep (42). IL-1β and TNF-α activate nuclear factor kappa-light-chain enhancer of activated B-cells (NF-κB), which in turn enhances production of sleep-producing cyclooxygenase-2 and adenosine A1 receptors (42). Third, somnogenic cytokines decrease the firing rates of wake-promoting brainstem cholinergic and serotonergic neurons (40) and increase the firing rate of sleep-promoting neurons in the ventrolateral preoptic area (42).

In human studies, the relationship between inflammation and excessive daytime sleepiness has been most extensively studied in the sleep disorder, obstructive sleep apnea (OSA). Levels of TNF-α are higher in OSA patients than in controls, exhibiting a dose-response relationship with OSA severity (43), and antagonism of TNF-α via etanercept results in a substantial reduction in objectively-measured sleepiness (44). IL-6 levels are also elevated in patients with OSA and correlate with severity of daytime sleepiness (45). Pro-inflammatory cytokines have been implicated in the pathophysiology of sleepiness or long sleep times in a wide range of human diseases.

A pro-inflammatory state has not been definitively established in the hypersomnia disorders, but several converging lines of evidence suggest one is likely. First, patients with idiopathic hypersomnia or narcolepsy type 2 have increased rates of inflammatory, allergic, and autoimmune diseases (46). Second, there is a strong phenotypic overlap between the hypersomnia disorders and atypical depression, including long sleep times (47), and systemic inflammation is particularly associated with atypical depression rather than melancholic depression (41). Third, among the hypersomnolent patients in our randomized, controlled trial of clarithromycin, measures of sleepiness showed trends toward moderate correlations with cytokine levels. Trends for TNF-alpha were seen with the Stanford Sleepiness Scale ($r = 0.24$), the Functional Outcomes of Sleep Questionnaire ($r = -0.28$), and lapses of at least 500 msec on the psychomotor vigilance task ($r = 0.48$, $p = 0.03$). Similar trends were seen for the relationship between IL-6 and sleepiness (Stanford $r = 0.28$, lapses $r = 0.69$, $p = 0.0005$ for lapses) and between IL-1β and measures of sleepiness (lapses $r = 0.32$).

Clarithromycin has a strong anti-inflammatory effect, reducing these soporific cytokines in a variety of tissue types. Our preliminary data supports the hypotheses that such reduction in cytokines contributes to clarithromycin's beneficial treatment effects. Within our clarithromycin trial, the change in multiple self-reported and objective measures of sleepiness showed trends toward correlations of moderate strength with the changes in plasma cytokine levels during clarithromycin use. Reductions in IL-1β correlated with improvements in Epworth Sleepiness Scale scores ($r = 0.36$), Functional Outcomes of Sleep Questionnaire scores ($r = 0.41$), and SF-36 vitality subscales ($r = 0.47$). Reductions in IL-6 levels correlated with reduction in lapses of psychomotor vigilance ($r = 0.39$). Reductions in TNF-α correlated with improvements in Stanford Sleepiness scale scores ($r = 0.37$) and psychomotor vigilance test reaction times ($r = 0.44$). This clinical trial was powered for efficacy, not cytokine changes, and we recognize the limitations inherent in these post-hoc, discovery analyses performed within our clarithromycin trial data. However, the consistent effect across different cytokines and multiple measures of sleepiness, in the context of existing literature, leads us to ***hypothesize that reduction in soporific cytokines is one of the mechanisms by which clarithromycin reduces pathologic daytime sleepiness in hypersomnia disorders.***

In addition to inflammation, gastrointestinal microbiome homeostasis can have profound effects on CNS function. Recent accumulating evidence in this regard has been driven by advances in sequencing technology that have made it possible to perform detailed investigations of brain-gastrointestinal microbiome interactions. In healthy individuals, microbiome composition is largely stable over time, determined predominantly by a combination of diet (48) and environment (49). Yet dysbiosis, i.e., an imbalance of the microbiota composition with an increased ratio of pathogenic: beneficial organisms and a reduction in diversity, is observed in multiple disease states. These include systemic disease such as obesity (50-52) and diabetes (51, 53), but also neuropsychiatric syndromes including anxiety (54-56) and neurodevelopmental conditions (54, 55, 57-59).

There are several established pathways by which gastrointestinal flora influence CNS function that are particularly relevant for the regulation of sleep-wake. In particular, both immunomodulation and changes in specific neurotransmitter levels, including GABA, are mechanisms by which gastrointestinal dysbiosis results in changes central nervous system function (55, 56, 60, 61), and in turn both of these are implicated in sleep-wake regulation, as discussed above. Investigations directly testing the relationship of the microbiome and sleep-wake regulation have been minimal to date, but based on these known gut-brain communication pathways, there is strong biological plausibility for such regulation.

Even short-term administration of clarithromycin changes microbiome composition. Triple therapy for H. pylori, including clarithromycin, amoxicillin, and a proton-pump inhibitor, results in relative increases in Bacteroidetes and reductions in Firmicutes, as well as increases in Proteobacteria (62-64). While available studies have evaluated the effects of clarithromycin in combination with another antibiotic, effects on microbiome are likely to be additive. As an exploratory aim, we hypothesize that gastrointestinal dysbiosis contributes to excessive daytime sleepiness in patients with hypersomnia disorders and that ***clarithromycin-induced changes in microbiome composition will mediate the beneficial effects of clarithromycin on reducing excessive daytime sleepiness.***

Objectives

Aim 1: To identify central nervous system mediators of clarithromycin's ability to promote wakefulness and reduce sleepiness, among patients with central hypersomnia syndromes.

Hypothesis 1a: Reversal of the ability of CSF to enhance GABA-A receptor function in vitro mediate clarithromycin's improvements in quality and quantity of wakefulness.

Hypothesis 1b: Changes in resting state connectivity between nodes of the default mode network during wake will be associated with improvements in self-reported and objectively measured sleepiness.

Aim 2: To probe extra-neuronal mechanisms by which clarithromycin may reduce sleepiness, including changes in systemic inflammation and changes in gastrointestinal microbiota composition, in patients with central hypersomnia syndromes.

Hypothesis 2a: Improvement in sleepiness with clarithromycin use will be positively associated with reductions in systemic inflammation, especially reductions in levels of tumor necrosis factor-α.

Hypothesis 2b (exploratory): Improvement in sleepiness with clarithromycin use will be positively correlated with modulation of gastrointestinal dysbiosis.

Study Design & Methods

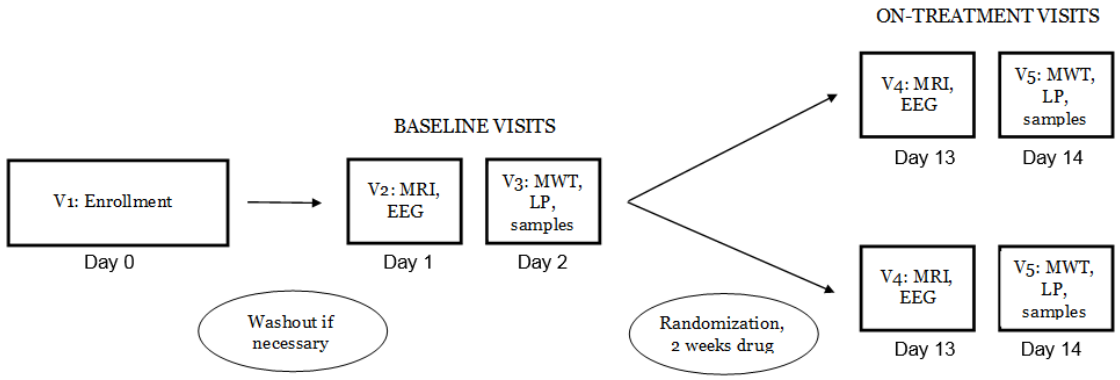
Data for both aims will be collected simultaneously via a randomized, double-blind, parallel group, placebo-controlled trial (see Figure 8). All measures will be collected at baseline and at the conclusion of fourteen days of treatment. Participants will have five study visits across a 2-4 week period. Visit 1 will involve discussion of the study, informed consent, enrollment, instruction regarding wake-promoting medication discontinuation, if necessary, and will be given a wrist actigraphy to wear until visit 3. Visits 2 and 3 will be used for collection of baseline data and Visits 4 and 5 for on-treatment data collection. Visit 2 will be performed at least 5 half-lives after discontinuation of any wake-promoting medication. Visit 2 will entail MRI

with simultaneous EEG and instruction in the collection of stool sample. MRI visits will be scheduled within a 2 hour period (1000-1200) for all subjects, to avoid potential differences based on time of day. The following day, participants will undergo Visit 3, which will consist of MWT, lumbar puncture, and blood draw. The blood draw will be performed on arrival. In addition to being used for testing of cytokines, the blood draw will include a pregnancy test (serum or urine) for women of childbearing potential. The lumbar puncture will be performed after the completion of MWT, to minimize effects on sleep latency and to ensure consistent time of day (1600-1800) for CSF collection.

Between visits 3 and 4, following collection of baseline data, participants will be randomized to the placebo or clarithromycin arm. The personnel performing the randomization will not have access to any baseline data, i.e., randomization will be independent of baseline characteristics. However, randomization will be performed after collection of baseline data to ensure all participants who are randomized are able to tolerate and perform all study procedures. In particular, claustrophobia preventing MRI scans, difficulty remaining motionless resulting in above-threshold movement artifact in rs-fMRI, and inability to collect CSF with bedside lumbar puncture are relatively infrequent but are expected to occur in a minority of enrolled participants. To preserve sample size, participants will not be randomized if they cannot complete these baseline procedures.

After randomization, participants will be provided with study drug from an Investigational Drug Service courier and will be given another wrist actigraphy to wear until visit 5. Two weeks following randomization, while still taking study medication, participants will return for the same two-day testing described for Visits 2 and 3. Participants will discontinue use of study medication upon completion of Visit 5. Two weeks after discontinuing the study medication, participants will be called and asked about any adverse events and when/if their sleepiness returned to baseline after stopping the intervention. If sleepiness is still not back to baseline, they will be called two weeks later with the same query, until sleepiness returns to baseline (or 2 months, whichever comes first).

Figure 8: Study protocol



Abbreviations: V = Visit; MRI = resting-state and task-based functional MRI; EEG = electroencephalography; MWT = maintenance of wakefulness testing and other measures of sleepiness; LP = lumbar puncture for cerebrospinal fluid collection; samples = collection of blood and stool samples

Clarithromycin will be dosed as 500 mg twice daily, once upon awakening and once with lunch. This is the dose most often used in our clinical practice and previously shown to be effective in our randomized, controlled trial (18, 23). Matched placebo will be dosed in the same manner. Medications and packaging will be prepared by the Emory Investigational Drug Service so as to appear identical.

Procedures will include those to measure sleepiness and those to measure potential mediators of clarithromycin's reduction of sleepiness. Measures of sleepiness will include: Epworth Sleepiness Scale (65), Maintenance of Wakefulness test (MWT) (66), EEG power, self-reported sleep duration via sleep log, fatigue severity via the Fatigue Severity Scale (67) and the Multidimensional Fatigue Inventory (68), and sleep inertia via the Sleep Inertia Questionnaire (69) and a 10-point Likert scale used in our prior clarithromycin clinical trial. Measures of mediators will include: cerebrospinal fluid for measurement of GABA-A receptor potentiation, functional MRI of the brain for DMN connectivity analysis, simultaneous EEG during MRI to exclude inadvertent sleep during scanning, blood draw for measurement of inflammatory markers, and stool

sample for microbiome analyses. Details on procedures and analyses for these individual mediators is as follows:

During the research slow-down created by the COVID19 pandemic, all study procedures were placed on hold. In anticipation for research ramp up, modifications will be made to study visits and procedures to allow continued recruitment and data collection while remaining in compliance with the University's guidelines relative to social distancing.

Moving forward, study staff will omit certain study procedures if necessary. However, study staff will make a determination on a case-by-case basis.

GABA-A receptor potentiation

CSF will be collected via lumbar puncture performed by a board-certified neurologist (Dr. Trotti or Rye) and is a procedure with very low complication rates in experienced hands. We have collected and banked 671 CSF fluid samples as part of our ongoing hypersomnia research projects, at a rate of approximately one per week for the last 10 years. Although most of our existing protocols have not required repeat lumbar puncture, we have found patients to be willing to undergo repeat lumbar puncture when asked, with 29 of these individuals having undergone at least two. This speaks to the motivation of our hypersomnia patient population to participate in advancing research into their disease and makes us confident that the lumbar puncture component of the current protocol will not be a substantial disincentive to participation.

Effects of participant CSF on GABA-A receptors will be assessed via patch-clamp assay by co-Investigator Dr. Jenkins, as previously described (12). Briefly, CSF will be applied to human embryonic kidney (HEK293) cells expressing GABA-A receptor ($\alpha1\beta2\gamma2s$) receptors. Cells will be whole-cell voltage clamped and superfused with extracellular saline. Solutions of GABA, GABA plus CSF at baseline, and GABA plus CSF collected during treatment will be separately applied for 2 second intervals and magnitude and duration of chloride current will be measured. Careful attention will be paid to keeping the GABA concentration sufficient to activate a response that is 10% of the maximum effective concentration for each cell and testing each sample 2-3 times on 3-5 cells each, as we have demonstrated that these factors are critical to ensure reproducibility of these assay findings (13, 70). The difference between measured current with GABA alone and the current measured with GABA + CSF will yield a measure of potentiation for each CSF sample in each condition.

CSF will also be tested for hypocretin levels (which are used to distinguish the two types of narcolepsy included in this study) at an Emory research lab using a standard commercial kit (Phoenix) with Stanford reference sample.

rs-fMRI for DMN connectivity analyses

MRI images will be acquired on a Siemens 3.0 Tesla Magnetom TIM Trio MRI scanner (Malvern, PA). Anatomic images will include standard T1-weighted 3D MPRAGE (176 sagittal slices; TR = 2300 msec; TE = 2.89 msec; inversion time (TI) = 800 msec; flip angle (FA) = 8°; field of view (FOV) = 256 × 256 mm; image resolution = 1 x 1 x 1 mm). Functional MRI images will be acquired with standard gradient echo EPI (44 axial slices; thickness = 3.5 mm; TR/TE/FA = 2000 msec/24 msec/80°; FOV = 220 mm x 220m; 3 x 3 x 3.5 mm resolution). Functional scans will include a 10 minute resting state scan, during which participants will be asked to look at a fixation marker, remain motionless but awake, and not to think of anything in particular, and a 10 minute task-based scan, during which subjects will complete the n-back test with 1, 2, and 3 back levels.

Simultaneous EEG will be recorded throughout the MRI scanning session using the BrainVision system (BrainProducts, Germany), which includes a 64-channel EEG cap, MRI compatible hardware, and software allowing real-time visualization of artifact-filtered EEG. We have extensive experience with simultaneous EEG-MRI recording using this system, performed as part of Dr. Trotti's K23 award. EEG will be continually viewed by a registered polysomnographic technician during MRI scanning. Participants who fall asleep (defined as two consecutive 30 second epochs of N1 sleep or a single epoch of any other stage) will be given a verbal cue to wake up, and images within 60 seconds surrounding the time of sleep onset and offset will be excluded from analyses to prevent bias induced by sleep-related changes in functional connectivity (27).

MRI image pre-processing will follow our standard pipeline for rs-fMRI studies and will include: registration of anatomic data to MNI space, skull stripping of anatomic data, segmentation of anatomic data into grey matter, white matter, and CSF, slice time correction of functional data, motion correction of functional data, registration of functional data to standard space, skull stripping of functional data, signal regression of nuisance regressors (motion, white matter signal, CSF signal, heart rate, and respiration) for functional data, temporal filtering of functional data, and z-scoring of functional data. Because global signal can differ across groups (71, 72), we will separately examine the temporal standard deviation and the power

spectra of the global signal by group before determining whether or not to include global signal as a nuisance regressor. This pre-processing pipeline uses FSL (Oxford University) and MATLAB (MathWorks, Massachusetts, USA).

Soporific cytokines

Serum will be collected and analyzed for soporific cytokines, including IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-15, IL-18, TNF-α, TNF-β, and INF-α, via multiplexed immunoassay. The cytokine of primary interest, based on our preliminary data and existing literature, is TNF-α. All blood samples will be collected at 0800, with the same scheduled time for each phlebotomy procedure chosen to avoid time of day variation in cytokine levels (73).

Microbiome 16S rRNA and *Drosophila* model

For microbiome analyses, participants will be instructed in standardized collection and storage of stool samples, to be collected once prior to beginning study medication and once at its completion. 16S rRNA sequencing of the microbiome will be performed and analyzed using Emory standard analysis pipeline through Emory's microbiome core laboratory. Comparison of changes in microbiome composition via 16S rRNA sequencing results will be our primary outcome for microbiome analyses.

Co-Investigator Dr. Jones and collaborator Dr. Freeman will extend these analyses by using fecal samples to humanize *Drosophila* for each of the three conditions (baseline, clarithromycin, and placebo), to more directly assess for causality. Germ-free flies will be created following standard protocols, which consist of washing embryos with bleach and ethanol and then raising flies on autoclaved medium (74). Microbiota transfer to "humanize" flies with the gut microbiome of subjects in different conditions will then be achieved at day 2 by adding fecal homogenate to the medium of germ-free flies. Sleep phenotyping will be performed at day 4, using validated measures. Sleep length, consolidation, and circadian timing will be assessed with the *Drosophila* Activity Monitor (TriKinetics). The assay will be conducted in triplicate with a minimum of 60 flies total in each condition.

Potential risks to participants will include risks from study medications and risks from study procedures. Half of participants will receive inert placebo and half will receive clarithromycin. Common but non-serious risks of clarithromycin include: diarrhea, nausea, vomiting, taste changes, abdominal pain, dyspepsia, headache, and rash. Rare but serious risks of clarithromycin include: antibiotic resistance, superinfection, C. difficile colitis, hepatotoxicity, interstitial nephritis, pancreatitis, QT prolongation, ventricular arrhythmias, thrombocytopenia, leukopenia, neutropenia, seizures, psychosis, hallucinations, anaphylaxis, Stevens-Johnson syndrome and other drug rashes, hypoglycemia, myasthenia gravis exacerbation, and rhabdomyolysis. Inclusion/exclusion criteria have been selected to minimize risks associated with clarithromycin use.

The Actiwatch provides information about general activity, sleep schedule, naps, wake episodes, as well as information about sleep quantity and quality.

The MWT is a routine neurodiagnostic procedure with minimal risk. It is non-invasive and well tolerated. There is a rare, non-serious risk of skin irritation from leads or other equipment. Waking EEG will be collected simultaneous to MWT for EEG power analyses.

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Participants with treated OSA who have not previously had a clinical MSLT will be scheduled for an additional study visit. During this visit, participants will come to the Sleep Center at approximately 7 am and remain until approximately 5 pm to complete a "multiple sleep latency test" (MSLT), taking naps five times during the day. For the week leading up to the MSLT, the participant will keep a sleep log and wear actigraphy; both should demonstrate at least 6 hours of sleep the night before MSLT. In participants using CPAP, the CPAP should be worn at least 6 hours the night before MSLT
The grant will cover the cost of the MSLT and actigraphy.

Participants will be instructed in the collection of a stool sample. Samples will be collected by participants in their own homes, to minimize collection-related discomfort. Participants will be provide appropriate supplies to ensure sterile collection procedures. Rare, non-serious risks include discomfort with collection procedure.

Blood will be collected via venous phlebotomy for the analysis of peripheral cytokine levels. Common, non-serious risks associated with phlebotomy include: discomfort, bruising, and skin reactions to adhesive tape. Rare, moderate risks include syncope or presyncope from vasovagal reaction. Rare, serious risks include infection.

Participants will undergo brain MRI for functional connectivity analyses. Common, non-serious risks include claustrophobia.

Lumbar puncture will be performed by a board-certified neurologist, Dr. Trotti or Dr. Rye, each of whom have at least 17 years' experience performing this procedure. Lumbar puncture will be performed to allow collection of cerebrospinal fluid for analysis of GABA-A receptor potentiation. Common, non-serious risks of lumbar puncture include: post-dural puncture headache and back pain. Uncommon, non-serious risks include headache that does not resolve without further intervention, necessitating either prescription of gabapentin or blood patch. Rare, serious risks include nerve damage, spinal cord damage, hemorrhage, infection, and herniation.

Private, identifiable information will be collected on all participants for the purpose of determining eligibility and performing the study. There is a rare, serious risk of loss of confidentiality of health information.

Community Participation

We plan to recruit participants through several patient groups, including Atlanta Hypersomnia Support, the Hypersomnia Foundation, and the Narcolepsy Network. In gratitude for recruitment support and in acknowledgement of the importance of including participants, patients, and their supporters in study result dissemination, we will offer to provide presentations of the study results to each of these groups.

Participant Selection

Adult participants with idiopathic hypersomnia or narcolepsy will be recruited to participate. These two hypersomnia disorder share a common phenotype and both are associated with increases in potentiation of GABA-A receptors by CSF (4-8, 12). Our recruitment goal is 92 participants who complete baseline and on-treatment testing, 46 in each treatment condition. To obtain this number, we anticipate enrolling up to 150 people, to account for participants who chose not to continue participation (i.e., drop-out) or who are screen failures, including due to difficulty tolerating baseline procedures (e.g., too much movement during MRI).

Inclusion criteria will include: 1) diagnosis of idiopathic hypersomnia, established via International Classification of Sleep Disorders, third edition (ICSD-3) criteria (75), including objective confirmation via multiple sleep latency test mean sleep latency $\leq$ 10 minutes, actigraphic daily sleep times > 660 minutes, averaged over at least 7 days, and/or polysomnographic sleep time > 660 minutes in $\leq$ 24 hour monitoring, participants with a clinical diagnosis of idiopathic hypersomnia not meeting the testing criteria, will be eligible (per ICSD-3 testing exception); or diagnosis of narcolepsy type 1, established via ICSD-3 criteria including multiple sleep latency test mean sleep latency $\leq$ 10 minutes in the presence of at least 2 sleep onset REM periods and cataplexy and/or CSF hypocretin-1 concentration of ≤110 pg/mL; narcolepsy type 2, established via ICSD-3 criteria including multiple sleep latency test mean sleep latency $\leq$ 10 minutes in the presence of at least 2 sleep onset REM periods; 2) age 18-60); 3) free of wake-promoting medication or willing to discontinue current wake-promoting medication for at least 5 half-lives prior to baseline measures; 4) free of pre- or probiotic supplements for at least six months prior to baseline measures.

Participants who judge they cannot discontinue wake-promoting medication will be allowed to continue them, as long as all of the following are true:

- *persistent report of sleepiness despite medication;*
- *Epworth Sleepiness Scale (ESS) score ≥ 10;*
- *Stable medicine dose for at least 2 months before enrollment and throughout participation in the study.*

Exclusion criteria will include: 1) other potential causes of hypersomnolence, including untreated moderate or severe sleep apnea,, severe periodic limb movement disorder with arousals, uncontrolled metabolic disorders, hypocretin deficiency, or cataplexy; 2) contraindication to clarithromycin; and 3) contraindication to any of the study procedures.

Participants who are taking a medication that has a potential interaction with clarithromycin because both medications can cause QTc prolongation will be eligible if either: 1) they can safely discontinue the potentially interacting medication prior to and throughout the study (in conversation with the prescribing physician), or 2) they undergo an an electrocardiogram (EKG) prior to baseline visits that shows a normal QTc interval while on the potentially interacting medication.

Participants with treated moderate to severe OSA, who have a diagnosis of narcolepsy type 1, narcolepsy type 2, or idiopathic hypersomnia, will be considered for enrollment if the following are true:

Treated OSA-

- *Documentation that OSA is being effectively treated (in the case of PAP, device-generated data should show an AHIf <10 or simultaneous HSAT/PAP titration at current pressure or mean auto pressure should show AHI/pAHI/REI4 < 10. In the case of other treatments, HSAT or in-lab PSG should show AHI/pAHI/REI4 < 10)*
- *Documentation that participant is using prescribed therapy (in the case of PAP, documentation will come from PAP generated data; in the case of other treatments, documentation will come from participant self-report)*
- *Documentation of IH, NT1, or NT2 diagnosis either established prior to development of moderate to severe sleep apnea (e.g., PSG/MSLT confirming diagnosis of hypersomnia disorder at a time when AHI <15) or MSLT conducted after treatment of OSA demonstrates diagnosis of IH, NT1, or NT2*

Participants will be recruited in several ways. Most of the hypersomnia patients seen clinically by Drs. Trotti, Rye, and Bliwise are enrolled in a longitudinal study of hypersomnia, in which they have provided consent to be contacted for participation in other hypersomnia research protocols. These patients will be individually contacted for possible recruitment. To ensure enrollment of patients seen by other providers, fliers will be posted in each clinic room, informing potential participants about the study and providing contact information for the study team. All providers within the Sleep Center will be given a presentation on the study aims and protocol and asked to directly refer any potentially-appropriate patients. Providers will also be given a pocket-sized, laminated study reference guide, with an emphasis on inclusion criteria, for easy access during patient care. Providers will be given reminders of the study and need for patient referrals at monthly Sleep Center faculty meetings. We will search Emory's billing database to identify patients with idiopathic hypersomnia or narcolepsy type 2 for contact. Although the latter strategy will only be employed if necessary, we have used this strategy successfully in prior hypersomnia disorder studies, given that both idiopathic hypersomnia and narcolepsy type 2 are rare diseases.

Separate from clinic-based recruitment, we will also recruit patients through three patient support groups. These include Atlanta Hypersomnia Support, the national Hypersomnia Foundation, and the national Narcolepsy Network. Drs. Trotti, Rye, and Bliwise have existing relationships with all three of these organizations. Dr. Trotti successfully recruited patients from each of these three organizations for her ongoing K23 award. We will also recruit participants via a brochure and a website (on Emory's scholarblogs platform).

Participants will be given reasonable compensation, without undue inducement, for their time and participation expenses.

Informed Consent

Informed consent will be obtained from participants in-person, through discussion, time for questions and time for consideration of whether or not to participate, and review of the written consent form. This will take place at the Emory Sleep Center. Person obtaining consent will ensure comprehension by answering all questions and presenting both verbal and written information.

During the research slow-down created by the COVID19 pandemic modifications will be made to the consent process to allow continued recruitment while protecting the health of participants, study staff, and the population:

1) The consent process will be completed over the phone or virtually, rather than in-person. The video platform that will be used to complete virtual consents is Zoom. This platform is currently approved by Emory.
2) The subject will be sent a legible and currently approved version of the consent form via encrypted email.

3) Email will be used to obtain a clear wet signature copy of the signature page of the current, IRB-approved version of the ICF from the subject.

We request a HIPAA waiver for the identification of potential eligible subjects, through review of Emory Sleep Center billing records and medical records, because the studied diseases are rare and such screening for eligible participants will be necessary to identify sufficient participants.

Incidental findings on MWT will be reviewed by Dr. Bliwise, who is board certified by the American Board of Sleep Medicine. Findings of potential clinical significance will be discussed with Dr. Trotti, who will alert the participant so that appropriate clinical diagnostic testing may be obtained. Incidental findings on MRI will be reviewed by the Medical Director of the Center for Systems Imaging, with a similar protocol for notifying Dr. Trotti and participants in the event of findings of potential clinical significance.

Compensation for Time & Effort

Reasonable compensation will be provided for participant time and travel expenses, at a rate of $400 per participant, pro-rated for completed procedures in the case of participants who do not complete all visits. Breakdown of reimbursement is:

-Enrollment visit: $10
-Visit 2 (MRI): $90
-Visit 3 (MWT): $100
-Visit 4 (MRI on treatment): $100
-Visit 5 (MWT on treatment): $100

Statistical analyses

Power calculations were performed in consultation with a clinical trials statistician via the local CTSA network (supported by NIH UL1-TR000424). The study was powered based on our primary mediator of interest, systemic inflammation. In our prior clarithromycin trial, changes in inflammatory markers were correlated with changes in sleepiness measures. These preliminary data were used to power the current study. Assuming a two-tailed alpha level of 0.05, 80% power, and correlations between change scores of 0.40, we will need 46 patients per group for our primary outcome of TNF-α. A correlation strength of 0.40 is consistent with that seen in our preliminary data for TNF-α, IL-1β, and IL-6, in which correlations ranged from 0.36 to 0.47. Correlations will be performed within and between groups.

Measurement of GABA-A receptor potentiation, levels of TNF-alpha and other cytokines, and microbiome 16S rRNA composition will yield continuous variables. These will be inspected with descriptive analyses to ensure normal distribution. When necessary, e.g., for levels of cytokines, variables will be mathematically transformed. Change scores (pre vs post treatment) will be calculated for each potential mediator and each measure of sleepiness.

The association between changes in sleepiness and changes in inflammatory markers and other potential mediator will be investigated individually using scatterplots within treatment group. Pearson correlation and linear regression will be used to test these associations. Fisher's transformations will be used to test whether the correlations are equal between treatment groups. Multiple linear regression will be used to test whether or not there is any effect modification.

For mediational analyses, bootstrapping will be utilized (76). Bootstrapping is a nonparametric statistical technique that does not utilize normal distribution assumptions during calculations. Each mediator and measure of sleepiness will first be compared individually. Significant mediators will be combined in a multivariate mediation model. P-values < 0.05 will be deemed statistically significant, after correction for multiple comparisons.

For functional connectivity analyses, each functional scan will be parceled into the 273 regions of interest (ROIs) contained in the Brainnetome Atlas (77) and mean timecourse will be calculated for each ROI. Pearson correlations between each pair of ROIs will be calculated, to determine the strength of functional connectivity between each pair of regions. This will yield a functional connectivity matrix for each functional scan. These correlation matrices will be Fischer z-transformed and averaged across each condition (baseline, placebo, clarithromycin) to create a mean functional connectivity matrix for each condition. For our primary analyses, only ROIs within the DMN will be considered, and will be compared by condition using ANOVA, correcting for multiple comparisons by controlling false discovery rate (78). In the case of significant differences in DMN connectivity by group, mediation models will be created, as described by Lindquist (79).

Following DMN analyses, the full correlation matrix will be evaluated for extra-DMN connectivity changes between groups, as an exploratory analysis.

Data and Safety Monitoring and Reporting

The study PI, Dr. Trotti, will be responsible for overseeing all aspects of this Data and Safety Monitoring Plan. The Study Coordinator will remain in close contact with participants during their study enrollment. He will assess for any adverse events on a scheduled basis, by phone calls one day and one week after Visit 3. This phone assessment will include a structured assessment for common and rare risks, as detailed in the Protection of Human Subjects section. In addition, coordinator will encourage participants to report any possible adverse events that are not included in this structured assessment or that occur in between scheduled assessments.

All adverse events will be collected in a standardized log provided by Emory's Institutional Review Board (IRB). Dr. Trotti and coordinator will meet weekly to review all reported adverse events. Non-serious adverse events will be reported annually to the IRB at the time of continuing review. In the case of serious adverse events, coordinator will immediately notify Dr. Trotti, to ensure management of the adverse event and appropriate reporting.

Once a month, Dr. Trotti and coordinator will meet to review data quality, including results from all baseline and on-treatment procedures. At these meetings, Dr. Trotti will perform spot checks of protocol compliance and data verification.

There will be no pre-specified stopping rules, as this is not a safety nor efficacy trial.

The study will be reviewed by Emory's Conflict of Interest office prior to study start, to ensure appropriate management of any possible conflicts.

Reporting of adverse and other reportable events

Dr. Trotti will be responsible for all event reporting. Reporting of events to the Emory IRB will be performed using a secure, electronic reporting system and will conform to Emory guidelines, including:

1) Unanticipated problems. Unexpected events that are judged to be related to study procedures that involve risk to participants, even in the absence of an adverse event, will be reported within 10 business days;

2) Deaths. Any deaths judged to be related to study participation will be reported within 10 business days; deaths judged to be unrelated will be reported annually at continuing review;

3) Substantive protocol deviations. Deviations that adversely affect the rights and welfare of participants, the safety of participants, the willingness of participants to continue participation, or the integrity of the research data will be reported within 10 business days;

4) Serious adverse events that are related to the study but are not unanticipated. These events will be reported annually at continuing review.

Similarly, we will follow all IC-specific rules about reporting events to the NIH. If this study is judged non-exempt by the FDA, reporting to the FDA will also occur based on FDA timelines.

Data Safety Monitoring Board

Per the Emory IRB, a formal Data Safety Monitoring Board (DSMB) is recommended in the case of:

1) large or multisite studies;
2) trials intended to provide definitive information about effectiveness or safety;
3) prior data indicating the possibility of unacceptable toxicity; or
4) study evaluating mortality or other major safety or effectiveness endpoints.

As the proposed study does not meet these criteria, we intend careful monitoring of safety by PI Dr. Trotti through this Data Safety Monitoring Plan, rather than a separate DSMB.

Independent Safety Monitor

At the recommendation of the NIH review panel, we have added an independent safety monitor, Dr. Anandi Sheth, to provide additional monitoring of safety for this study. An infectious disease physician, clinical trialist, and associate professor at Emory University (in the Department of Internal Medicine), Dr. Sheth has the necessary experience with antibiotic use and clinical trials to act in this role. She will review blinded data regarding AEs at least once every 6 months.

Confidentiality

All protected health information will be maintained on password-protected, HIPAA compliant servers and/or encrypted computers. Whenever feasible, participants and participant samples will be identified by code rather than by identifiable information. All data will be stripped of identifiers prior to publication.

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