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Can we predict treatment response in children with ADHD to a vitamin-mineral supplement? An investigation into pre-treatment nutrient serum levels, *MTHFR* status, clinical correlates and demographic variables

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ABSTRACT

Background: Intent-to-treat analyses from a randomized controlled trial showed significant between-group differences favouring micronutrient treatment on the Clinical Global Impression-Improvement, but no group differences on clinician, parent and teacher ratings of overall ADHD symptoms. There was an advantage of micronutrients over placebo in improving overall function, emotional regulation, aggression, and reducing impairment as well as improving inattention based on clinician but not parent observation. No group differences were observed on hyperactive-impulsive symptoms. We investigated predictors of response defined by pre-treatment variables.

Method: We conducted analyses of data from a clinical trial of children (7–12 years) with ADHD, whereby participants were randomized to receive micronutrients or placebo for 10 weeks followed by a 10 week open-label (OL) phase. We included only children who had been exposed to micronutrients for a full 10 week period and demonstrated satisfactory adherence, either in RCT phase (n = 40) or OL phase (those who received placebo during RCT phase; n = 31). Seven outcomes were examined: change in ADHD symptoms (clinician/parent), ADHD responder, overall responder, change in mood, change in functioning, and change in aggression. Demographic, developmental variables, current clinical and physical characteristics, *MTHFR* genotype at two common variants, and pre-treatment serum/plasma levels (vitamin D, B_{12}, folate, zinc, copper, iron, ferritin, potassium, calcium, magnesium, and homocysteine) were all considered as putative predictors.

Results: Substantial nutrient deficiencies pre-treatment were observed only for vitamin D (13%) and copper (15%), otherwise most children entered the trial with nutrient levels falling within expected ranges. Regression analyses showed varying predictors across outcomes with no one predictor being consistently identified across different variables. Lower pre-treatment folate and B_{12} levels, being female, greater severity of symptoms and co-occurring disorders pre-treatment, more pregnancy complications and fewer birth problems were identified as possible predictors of greater improvement for some but not all outcome measures although predictive values were weak. Lower IQ and higher BMI predicted greater improvement in aggression.

Conclusions: This study replicates Rucklidge et al. (2014b) showing the limited value of using serum nutrient levels to predict treatment response although we cannot rule out that other non-assayed nutrient levels may be more valuable. Additionally, no specific demographic or clinical characteristics, including *MTHFR* genetic status, were identified that would preclude children with ADHD from trying this treatment approach.

1. Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder affecting approximately 5% of children (American Psychiatric Association, 2013) that is associated with ongoing psychiatric problems in adulthood, unemployment, school failure and incarceration (Klein et al., 2012; Molina et al., 2009; Hechtman et al., 2016). The most evidence-based treatments for ADHD are

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pharmacological; however, because of potential side effects and failure to prevent or alter long-term course, they can be perceived as an unattractive choice for some families (Swanson et al., 2017). As such, attention has widened to investigate other treatment options.

Consideration of the role that nutrition plays in the expression of ADHD has re-emerged over the last few years with food dyes, processed foods, and low consumption of fruit and vegetables all shown to have an association with ADHD symptom severity (Howard et al., 2011; Pelsser et al., 2011; Rios-Hernandez et al., 2017). However, other variables are also potentially relevant to brain health. For example, poor gut health, microbiome composition (Dinan & Cryan, 2017; Dinan et al., 2018; McNally et al., 2008), inflammation (Oddy et al., 2018), genetic variants that influence metabolism (Ames et al., 2002), and mitochondrial dysfunction (McNally et al., 2008; Toker & Agam, 2015; Kaplan et al., 2015), have all been identified as possible factors that may influence psychiatric disorders and contribute to the need for more nutrients than might be available in consumed food. The presence of any or all of these factors could effectively reduce the availability of nutrients for optimal brain health. Considering all these factors, supplementation may need to be considered over and above the manipulation of diet.

To date, the treatment of ADHD with a single nutrient approach has resulted in small and inconsistent findings (Hariri & Azadbakht, 2015). Based on research conducted in our lab, we have speculated whether multi-ingredient treatment approaches may result in more consistent treatment effects (Gordon et al., 2015; Rucklidge et al., 2011; Rucklidge et al., 2018; Rucklidge et al., 2014a). Given ADHD is a complex heterogeneous disorder, it has been suggested that intervening with one nutrient is highly unlikely to yield large effects (Mertz, 1994). A number of factors also lend support to the hypothesis that multinutrient approaches are worthy of investigation. From a physiological perspective, multiple nutrients are required in biological processes such as the methylation cycle and the Krebs cycle and it may be advantageous to combine nutrients to maximize metabolic function. Neurotransmitters, including dopamine and nor-adrenalin, which are implicated in ADHD (Thapar & Cooper, 2016), undergo several metabolic steps in relation to synthesis, uptake, and breakdown. Each of these steps is dependent upon multiple co-enzymes (cofactors), most of which include a variety of vitamins and minerals. Therefore, it appears reasonable to investigate a combination of a comprehensive range of micronutrients at doses expected to be sufficient to elicit a possible response without being likely to cause adverse effects in the majority of participants.

When investigating any new treatment, not only is it important to establish safety and efficacy, but also to establish who may benefit the most from the treatment. Understanding what treatments work for whom and what pre-treatment factors predict treatment outcome are common investigations in clinical trials, although predictors often tend to be weak and not replicated across studies. For example, ADHD subtype predicted change in behavioural regulation to a cognitive training intervention for those with combined subtype showing greater change relative to inattentive subtype (van der Donk et al., 2016). Based on the Multimodal Treatment Study of Children with ADHD (MTA) trial, comorbid anxiety appears to increase response to behavioural treatments but gender and comorbid disruptive disorders did not moderate treatment outcome (The MTA Cooperative Group, 1999). Antshel and Remer (2003) found that conduct and oppositional defiant disorder symptoms predicted poorer response to social skills training but other studies don't replicate this finding (Ollendick et al., 2008). Buitelaar et al. (1995) determined that younger age, high IQ, lower symptom severity, greater inattention, and low rates of anxiety predicted better response to methylphenidate. However, other studies have not found symptom severity to be a useful predictor. For example, Johnston et al. (2015) found that reduced impulse control and comorbid conduct disorder predicted response to methylphenidate but symptom severity proved less useful in prediction. Overall, no one variable stands out as a consistent predictor to both pharmacological and nonpharmacological treatments in ADHD research.

Biochemical markers (biomarkers) have been increasingly studied in attempts to identify those who might be at risk for ADHD as well as who might benefit from a treatment (Scassellati et al., 2012). Some biomarkers are modifiable and may lead to targeted treatments. With nutritional interventions, it is therefore important to determine whether pre-treatment nutrient levels might assist with determining response to a broad-spectrum combination of nutrients. Although many nutritional deficiencies have been associated with ADHD symptoms such as magnesium, zinc, iron, vitamin D, vitamin B_2, B_6 and B_9 (Kamal et al., 2014; Bener & Kamal, 2013; Bener et al., 2014; Greenblatt & Delane, 2017; Landaas et al., 2016), to date only one study has looked at nutrient biomarkers as predictors of treatment outcome (Rucklidge et al., 2014b). That study found that lower levels of vitamin D and copper were possible predictors of response but overall effects were small and inconsistent across different outcome measures.

This current study presents a replication of Rucklidge et al. (2014b), analyzing whether nutrient biomarkers taken prior to micronutrient treatment are useful for predicting treatment response in children with ADHD. These predictors were explored alongside more commonly investigated predictors including demographic variables, developmental history, *MTHFR* genotype at two common variants, and clinical correlates. This current investigation into predictors is based on a fully blinded RCT that showed benefit for one of three primary outcomes as well as a number of secondary outcomes. Specifically, there was an advantage of micronutrients over placebo in improving overall function, emotional regulation, aggression, and reducing impairment as well as improving inattention based on clinician but not parent observation. No benefit of nutrients over placebo was observed for hyperactive/impulsive symptoms (Rucklidge et al., 2018).

2. Methods

2.1. Study design

The study was approved by the university and national institutional review boards. After describing the experimental nature of the trial and explaining the other treatment options available in the community, written informed consent/assent was obtained. The trial was prospectively registered (ACTRN12613000896774).

Comprehensive study details have been described previously (Rucklidge et al., 2018). In brief, this was a 10 week double-blind (participants and investigators), parallel–group RCT designed to assess the efficacy and safety of a broad spectrum micronutrient formula (Daily Essential Nutrients (DEN)) compared with placebo, followed by a 10 week open-label (OL) trial with DEN in 93 medication-free children with ADHD, 7–12 years. Participants had to meet criteria for ADHD based on the Kiddie Schedule for Affective Disorders and Schizophrenia Lifetime Version (K-SADS-PL) (Kaufman et al., 1997), as well as parent and teacher Conners Rating Scales-Revised (CRS-R; T score $>$ 65 on parent form and $>$ 60 on teacher form) (Conners, 1997). The K-SADS was also used to identify co-occurring conditions.

The K-SADS has excellent instructions for ratings and previous research has shown robust reliability and validity data (Kaufman et al., 1997). The K-SADS interviews were conducted by doctorate-level clinical psychologists or clinical psychology graduate students and trained on appropriate administration of the interview via training videos as well as through observation by a clinical psychologist. All interviewers had established excellent interrater reliability through training. Interviews are regularly reviewed by a second rater to maintain and review reliability of the diagnoses. For cases not observed for reliability, every case is reviewed with the PI (a clinical psychologist) prior to a diagnosis being made. Participants were also seen by our study psychiatrist.

Participants took three capsules per day initially, divided into three doses to be taken with meals and water, increasing to six capsules per

day after three days, divided into three doses. On the 7th day, the dose was further increased to 12 capsules per day, in three doses of four capsules. For some, the titration went more slowely. The placebo and DEN (see Appendix Table S1 for DEN ingredients) were similar in appearance, used the same coating, and the placebo included riboflavin in order to mimic the smell and change in urine colour associated with taking vitamins. Following the 10-week double-blind RCT phase, participants could choose to enter a 10-week open-label (OL) phase using DEN. The titration regimen used for the RCT was repeated for all participants at the beginning of the OL phase.

At baseline and at the end of the RCT phase, fasting laboratory blood screening tested thyroid function, serum lipids, prolactin, fasting glucose, blood clotting, plasma nutrient levels (25 hydroxycholecalciferol (vitamin D), zinc, copper) and serum nutrient levels (vitamin B_{12}, folate, iron, ferritin, potassium, calcium, magnesium, and homocysteine). Affordability issues affected the ability to evaluate more expensive and less commonly assayed micronutrients including other B vitamins.

2.2. Genotyping MTHFR

Patient saliva samples were collected using the Oragene-DNA collection kit (DNA Genotek, Canada, OTT) and stored at room temperature. Human genomic DNA was then extracted according the manufacturer's directions. DNA amplification was carried out by polymerase chain reaction (PCR), performed in a total reaction volume of 50 µL containing 1 x PCR reaction buffer with 1.5 mM MgCl2 (Roche Diagnostics), 0.5 µM of each primer (IDT, Singapore, Table 1), 0.2 µM each deoxynucleoside triphosphate (dNTP), 1 M betaine, 0.5 U Fisher Taq-ti polymerase (Fisher Biotec, Wembley WA, Australia) and ~20 ng of genomic DNA. For amplification of DNA encompassing rs1801133, the thermal cycling conditions consisted of an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 63 °C annealing for 15 s and 72 °C for 45 s with a final extension of 72 °C for 5 min. For amplification of DNA encompassing rs1801131 the touchdown thermal cycling conditions consisted of an initial denaturation step of 95 °C for 2 min, 15 cycles of 95 °C for 30 s, 65 °C annealing for 15 s and 72 °C for 45 s with a temperature decrease of 1 °C per cycle, followed by 20 cycles of 95 °C for 30 s, 65 °C annealing for 15 s and 72 °C with a final extension of 72 °C for 5 min.

Sanger DNA sequencing was carried out on PCR products that were purified using AcroPrep (PALL Corporation, New York, USA) 96 well filter plates (omega 30 K), and then re-suspended in water. Purified PCR amplicons (~10 ng) were sequenced with the appropriate primer (Table 1) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturers protocol. Sequencing reaction products were run on an AB3130xl fragment analysis system equipped with a 50 cm capillary using POP7 polymer.

2.3. Sample selection

In order to increase power to detect associations with changes in outcome measures over ten weeks, we combined the data from the RCT and OL samples together. Data from the RCT phase for those randomized to DEN and data from the OL phase for those randomized to placebo during the RCT were used. Two participants who dropped out of the RCT phase while taking DEN were not included as well as eight participants from the placebo group who did not complete the entire OL phase of the trial. Seven children who had been randomized to the placebo no longer met study entry at the start of the OL phase and were not included (scores on the parent CRS-R had dropped below a T-score of 65). Six were identified as inadequately adherent with the micronutrients (< 75% consumption) and were also excluded. These exclusions reduced our final sample to 71 participants (40 from RCT phase, 31 from OL extension phase).

Table 1
Oligonucleotide sequences used for PCR and Sanger sequencing.

Name	Sequence 5′ - 3′	Target SNP
A1298C F MTHFR	CCCTCTGTCAGGAGTGTGCC	rs1801131 (A1298C)
A1298C R MTHFR	ACTCCAGCATCACTCACTTTGTG	rs1801131 (A1298C)
677 F MTHFR	CCTCTCCTGACTGTCATCCC	rs1801133 (C677T)
677 R MTHFR	GAACTCAGCGAACTCAGCAC	rs1801133 (C677T)

2.3.1. Outcome measures

All participants were monitored in both phases by face-to-face meetings with a clinical psychologist, or senior clinical psychology graduate student under a clinical psychologist's supervision. To capture the breadth of psychiatric symptoms monitored (including the primary outcome measures (POM)), but also considering the outcomes where clinically meaningful changes in excess of the placebo effect were identified (Rucklidge et al., 2018), seven different outcome measures were considered: 1) change in clinician-rated ADHD symptoms (POM), 2) ADHD responder, 3) overall responder status (POM), 4) change in overall functioning, 5) change in parent-rated ADHD symptoms (POM), 6) change in emotion dysregulation, and 7) change in aggression. These outcome measures were assessed at baseline, end of RCT and end of OL using the following measures:

2.3.2. Clinician ratings

1) The ADHD Rating Scale IV – (ADHD-RS-IV) – clinician version (Zhang et al., 2005; Faries et al., 2001) was used to assess change in clinician-rated ADHD symptoms. The ADHD-RS-IV contains 18 items directly linked to DSM-IV diagnostic criteria for ADHD covering symptoms of both inattention and hyperactivity/impulsivity. The ADHD-RS-IV has been found to have high internal consistency (0.92 for total; 0.86 for the inattention subtype; 0.88 for the hyperactivity-impulsivity subtype) and test-retest reliability (0.85 for total; 0.78 for inattention; 0.86 for hyperactivity-impulsivity) (Dupaul et al., 1998; Collett et al., 2003).
2) The score on the clinician ADHD rating scale was used to determine a dichotomous outcome variable, named ADHD responder, based on a ≥ 30% decrease in *either* the attention or hyperactivity/impulsivity subscales of the ADHD-RS-IV, a standard percentage change in ADHD ratings used in the ADHD literature (Sprich et al., 2016).
3) Overall responder was determined by the Clinical Global Impressions – Improvement (CGI–I) ratings (Guy, 1976), a scale that ranges from 1 (very much improved) to 7 (very much worse) as compared with pre-treatment functioning. These ratings were then used to classify an individual as either a responder (1 or 2, much or very much improved) or non-responder (3 or more). CGI-I ratings were significantly correlated with change in other measures ($p < .001$), suggesting the ratings were valid.
4) The Children's Global Assessment Scale (CGAS) (Shaffer et al., 1983) was used by the clinician to assess the overall level of functioning of the child as compared with pre-treatment functioning. It is a single numerical scale from 1 to 100 with a higher score indicative of better functioning. The CGAS has been found to have a test-retest reliability around 0.85 and high joint reliability of 0.83–0.91 in research settings (Rush et al., 2008).

2.3.3. Parent ratings

5) The CPRS-R (Conners, 1997) (long version: 80 items) was used to assess change in parent-rated ADHD symptoms. The DSM-IV Combined subscale (18 items) was used for assessing change in parent-rated ADHD symptoms. High internal consistency coefficients for the CPRS-R:L subscales have been found (Cronbach α 0.87–0.91).

The CPRS-R:L's validity has been calculated to have 92% sensitivity, 95% specificity, 94% positive predictive power and 93% negative predictive power (Rush et al., 2008). The CPRS-R pre-treatment ratings on the DSM subscales were significantly correlated with the K-SADS diagnoses of ADHD ($p < .001$).

6) The Child Mania Rating Scale, Parent Version (CMRS-P) was used to assess change in emotion dysregulation. It is a 21-item rating scale based on DSM-IV criteria for mania (Pavuluri et al., 2006). Items cover symptoms such as feeling irritable, racing thoughts, rage attacks and rapid mood swings. This measure was chosen to capture change in emotion dysregulation, given that these behaviour challenges are increasingly recognized to be significant features of ADHD (van Stralen, 2016; Faraone et al., 2018). Internal consistency and retest reliability were 0.96. Correlation of the CMRS-P with the Washington University Schedule for Affective Disorders and Schizophrenia Mania Rating Scale, and the Young Mania Rating Scale has been identified as high (0.78–0.83) (Pavuluri et al., 2006).
7) The Strengths and Difficulties Questionnaire (SDQ) (Goodman, 2001) was used to assess change in aggression. The SDQ provides a subscale assessing conduct problems (SDQ-CD) using a 3-point scale (not true, somewhat true and certainly true). Research has shown that the SDQ-CD (parent version) has acceptable internal consistency (range 0.46–0.76) and test-retest reliability (range 0.52–0.89). In terms of concurrent validity, the SDQ-CD has been shown to be highly correlated (mean 0.71) with the Child Behavior Checklist (CBCL) externalizing scale (Stone et al., 2010). Based on our dataset, the SDQ-CD pre-treatment ratings were significantly correlated with the diagnoses of CD and CD symptom count based on the K-SADS interview ($p < .001$).

Change was calculated as change in outcome measures (pre-post) over the relevant 10 week period being assessed (i.e. RCT phase for those randomized to micronutrients or OL extension for those initially randomized to placebo). As such, the pre-treatment for those in RCT was baseline, and for those in the OL phase was the end of RCT data.

2.4. Predictors

The following predictors were considered as potentially associated with treatment response: 1) Exposure to DEN (RCT/OL), 2) demographic variables including age, gender, family income, estimated IQ, education level, family status (single/dual parent home), 3) dietary patterns assessed using a brief dietary intake questionnaire modified from Baker et al. (2003): a "healthier" dietary pattern was one consisting of eating more fruit, vegetables, breakfast, and infrequently eating sweets, desserts and fast foods (a higher total score is indicative of healthier eating), 4) total positive responses to questionnaires assessing developmental risk factor groups including gestational (e.g., toxaemia, bleeding, high blood pressure, illness in mother, substance use), delivery (e.g., low birth weight, premature, breech, foetal distress), developmental (e.g., slow to reach developmental milestones such as walk/talk/dress, motor coordination problems and learning problems), sensory risk factors (e.g. sensitivity to noise, light, food, texture), and medical risk factors (e.g., allergies, diabetes, asthma, meningitis), 5) temperamental risk factors as assessed by the Early Adolescent Temperament Questionnaire – Revised (Parent) (Muris & Meesters, 2009) using three subscales: frustration, surgency, and inhibitory control, 6) total number of current co-occurring disorders (as assessed by the KSADS-PL at baseline), 7) past psychotropic medication use (e.g. stimulants), 8) Body Mass Index (BMI) percentile (age and gender adjusted), 9) *MTHFR* genotype at two common variants, and 10) pre-treatment fasting nutrient levels (vitamin D, B_{12}, folate, zinc, copper, iron, ferritin, potassium, calcium, magnesium, and homocysteine).

2.5. Statistical analyses

To provide an indication of the strength of the changes in the continuous outcome measures over ten weeks, pre and post outcome measure scores were compared using paired *t*-tests. Correlations were also calculated between pre-treatment values of outcome measures and nutrient levels.

The following statistical strategy was adopted to identify independent pre-treatment predictors of response to DEN. Firstly *t*-tests, Pearson's correlation coefficients, and chi-square analyses as appropriate were used to determine the univariate associations between putative pre-treatment predictors and treatment responses.

Hierarchical forward stepwise linear and logistic regression analyses were conducted for each outcome measure using any predictor that showed some association ($p < .10$) from the univariate analyses with a treatment response outcome. Whether DEN was taken during the RCT or OL extension phase was entered into the model alongside sex regardless of whether their contribution was statistically significant. This was to ensure that if there were group differences between those taking the nutrients during the RCT phase versus the OL phase, entering it into the model allowed us to determine if group was influencing the results. The pre-treatment assessment of the outcome measure was also included in the model (e.g. when assessing predictors of change for CGAS, pre-treatment CGAS was included in the model). Predictors that entered into the model with a significant contribution of $p < 0.05$ were considered independently associated with treatment response.

3. Results

3.1. Patient characteristics

Pre-treatment demographics, clinical features and serum nutrient levels are shown in Tables 2 and 3. Most participants entered the trial with serum nutrient levels within the normal reference ranges, excepting vitamin D deficiency (13%), copper deficiency (15%) and copper excess (6%). Correlations between nutrient levels and pre-treatment psychiatric variables (e.g., mood, ADHD scores, CGAS) revealed only one significant association: higher pre-treatment copper was significantly correlated with higher pre-treatment aggression levels as measured on the SDQ-CD ($r = 0.255$, $p = 0.032$).

We genotyped two common variants (rs1801131/A1298C, rs1801133/C677T) of *MTHFR* gene for 66 children (93%) in the sample. For A1289C, 37 (56%) individuals were homozygous for the A allele (AA), 27 (41%) were heterozygous (AC) and two (3%) were homozygous for the C allele (CC). For C677T, 32 (49%) individuals were homozygous for the C allele (CC), 30 (46%) were heterozygous (CT) and four (6%) were homozygous for the T allele (TT). The study did not contain individuals who were double homozygous for the minor variants, and allele frequencies were consistent with the European population in the Ensembl database (Yates et al., 2016).

3.2. Safety assessments

Full efficacy and safety of this combination of micronutrients for children have been reported in the primary outcome paper (Rucklidge et al., 2018) which showed benefit of micronutrients over placebo in improving overall function, reducing impairment and improving inattention (clinician-rated), emotional regulation and aggression, but not hyperactive/impulsive symptoms, with minimal side effects and with no group differences in side effects. We did not observe any serious adverse events during the open-label phase and reports of side effects were consistent with those reported during the RCT phase.

3.3. Effectiveness

Pre- and post-treatment paired *t*-tests are illustrated in Table 4,

Table 2
Pre-treatment demographic and clinical characteristics of study participants.

Characteristic	Total sample (n = 71)	
Demographics	mean ± SD or n(%)	Range within the sample
Male	55(77)	
Ethnicity		
European decent	56(79)	
Māori	15(21)	
Parents: Married/common-law	56(79)	
Age	9.7 ± 1.5	7.0–12.9
Estimated IQ[a]	97.0 ± 15.1	65–126
Socio-economic status[b]	46.9 ± 14.8	10–74
Dietary patterns[c]	29.9 ± 4.3	21–37
BMI	17.9 ± 2.5	14.3–24.8
BMI percentile	60.1 ± 26.6	4–98
Developmental risk factors		
Gestation risk (scores ranges 0–9)	1.4 ± 1.3	0–5
Delivery Risk (scores range 0–9)	1.2 ± 1.5	0–6
Developmental Risk (scores range 0–8)	2.0 ± 2.3	0–8
Medical History Risk (scores range 0–11)	1.8 ± 1.5	0–6
Sensory Risk (scores range from 6 to 42)	21.9 ± 8.7	6–42
Temperament (parent report)[d]		
Frustration	3.9 ± 0.7	2.2–5.0
Surgency	3.3 ± 0.8	1.4–5.0
Inhibitory Control	2.4 ± 0.6	1.0–3.6
Clinical characteristics		
ADHD type		
Inattentive	13(18)	
Hyperactive/Impulsive	4(6)	
Combined	54(76)	
Any anxiety disorder	27(38)	
Any disruptive behavioural disorder	39(55)	
Any mood disorder	8(11)	
Tics	5(7)	
LD	14(20)	
Enuresis	8(11)	
encopresis	6(8)	
Any co-occurring psychiatric disorder - current	61(86)	
Total co-occurring Psychiatric Disorders -Current	1.8 ± 1.3	0–6
History of past psychiatric medications	22(31)	
Pre-treatment outcome measures		
Clinician ADHD-IV-RS DSM Combined	43.7 ± 7.0	28–54
Parent CPRS-R:L DSM Combined	41.2 ± 7.1	22–53
CMRS	22.8 ± 12.0	4–46
CGAS	48.7 ± 7.4	25–70
SDQ- conduct problems	5.1 ± 2.3	0–10

BMI = Body Mass Index, IQ = Intelligence Quotient, LD = Learning Disability, DSM = Diagnostic and Statistical Manual, CMRS = Child Mania Rating Scale, CGAS = Children Global Assessment Scale, SDQ = Strengths and Difficulties Questionnaire.

[a] Assessed using Block Design and Vocabulary subtests of the WISC-IV (Wechsler, 2004).

[b] Based on the NZSEI which ranks occupations from 10 to 90 (Milne et al., 2013) with a higher score indicative of a higher economic status.

[c] Assessed using a brief dietary intake questionnaire (Baker et al., 2003) with higher numbers indicative of healthier eating.

[d] Measured using the Early Adolescent Temperament Questionnaire.

showing significant changes for the outcome variables. Based on the CGI—I, 35 (49%) of the participants were identified as "much" to "very much" improved. Response defined a priori as a ≥ 30% decrease in either attention or hyperactivity/impulsivity on the ADHD-RS-IV, identified 33 (46%) of participants as ADHD responders. We also confirmed that there were no significant group differences ($p > 0.05$) on changes in the outcome measures between those exposed to the nutrients during the RCT phase and those exposed to the nutrients during the OL phase, supporting the decision to combine data from both phases. Of note, there was no change in reported eating patterns ($p = 0.443$) during the course of the trial.

3.4. Nutrients

For those participants with pre-treatment and post-treatment serum nutrient levels (n = 39; only those consuming nutrients during the RCT phase could be included as blood was not taken at end of OL), nutrient assays showed significant increases for vitamin D ($p = 0.008$), B_{12} ($p < 0.001$), and folate ($p < 0.001$) levels, but not for ferritin, iron, zinc, copper, potassium, calcium, and magnesium. Homocysteine levels dropped significantly ($p < 0.001$). The copper to zinc ratio did not change significantly (see Table 4).

3.5. Predictors of treatment outcome

Table 5 shows the p-values from the univariate analyses comparing the putative predictor pre-treatment variables and the changes in outcome measures. As this was an exploratory study, any association that had a $p \leq 0.10$ (shown in bold) led to the predictor variable being included in the hierarchical stepwise regression analysis or logistic regression analysis for that outcome variable. Table 6 shows the significant predictors across the outcome variables.

3.6. Clinician ratings of ADHD

The predictor variables included for change in clinician-rated ADHD symptoms identified from the univariate analyses were pre-treatment clinician-rated ADHD symptoms, group, sex, gestational risk, delivery risk, number of current psychiatric comorbidities, and pre-treatment folate levels. In the final multiple regression model, three variables remained significant ($R^2_{adj} = 0.170$, $p = 0.004$). Greater number of co-occurring disorders ($\beta = 0.318$, $p = 0.008$), lower delivery risk ($\beta = -0.242$, $p = 0.031$) and being female ($\beta = 0.285$, $p = 0.013$) contributed significantly to the model and resulted in greater change in clinician-rated ADHD symptoms. Comparing means between males and females showed that the greatest difference in the change was associated with ratings of inattention by the clinicians ($F(1, 69) = 7.126$, $p = 0.009$). Females showed a greater improvement in the inattention symptoms over the 10 week period compared with males.

A logistic regression was performed to ascertain the effects of group, sex, number of co-occurring conditions, pre-treatment folate, pre-treatment B_{12}, and pre-treatment homocysteine levels on the likelihood that participants were identified as ADHD responders. The logistic regression model was statistically significant, $\chi^2(4) = 14.885$, $p = 0.005$. The model explained 28.9% (Nagelkerke R^2) of the variance in response status. Only pre-treatment folate and number of co-occurring disorders contributed significantly to the model. Lower folate levels pre-treatment and greater number of co-occurring disorders were associated with being identified as an ADHD responder. However, the level of folate pre-treatment for the ADHD responders (mean = 24.0, SD = 9.0) was still within the normal reference range for folate (> 8 nmol/L).

Fig. 1 presents the pre-treatment folate levels divided into tertiles in relation to ADHD responder status. Although there were more ADHD responders in the lowest tertile for pre-treatment folate (56%) as compared with those identified in the highest tertile of pre-treatment folate levels (40%), this difference was not significant ($\chi^2(2) = 1.296$, $p = 0.523$). Due to the long history of research interest in copper, zinc, copper:zinc ratios, and iron levels in relation to ADHD behaviours, the other nutrients were also divided into tertiles to illustrate the relationship between serum levels of these nutrients and response to the treatment (see Fig. 1). Although the graphs generally show a pattern

Table 3
Pre-treatment nutrient levels of study participants.

Pre-treatment nutrient levels	Total sample (n = 71)			
	Mean ± SD	Range within the sample	Deficient (identified as below reference range): n(%)	Elevated (defined as above reference range): n(%)
Plasma 25 Hydroxy Vitamin D (nmol/L): Reference range: 50–150	73.1 ± 22.6	40–110	9(13)	0(0)
Vitamin B_{12} (pmol/L): Reference range: 130–650	453.7 ± 165.2	171–820	0(0)	8(11)
Folate (nmol/L): Reference range: > 8.0	26.0 ± 8.1	9–34	0(0)	0(0)
Ferritin (μg/L): Reference range: 15–200	39.0 ± 21.4	14–115	1(1)	0(0)
Iron (μmol/L): Reference range: 6–25	15.3 ± 4.8	5–24	1(1)	1(1)
Plasma Zinc (μmol/L): Reference range: 10–17	12.5 ± 1.5	9.5–17.1	1(1)	1(1)
Plasma Copper (μmol/L): Reference range: 13.2–21.4	16.0 ± 3.0	7.1–17.3	11(15)	4(6)
Copper:Zinc ratio	1.3 ± 0.3	0.55–2.24	–	–
Potassium(μmol/L): Reference range: 3.5–5.2	4.1 ± 0.3	3.6–5.0	0(0)	0(0)
Calcium (μmol/L): Reference range: 2.2–2.6	2.5 ± 0.1	2.3–2.7	0(0)	1(1)
Magnesium (μmol/L): Reference range:0.6–1.2	0.8 ± 0.1	0.7–0.9	0(0)	0(0)
Homocysteine (n = 61) Reference range: 5–15	5.2 ± 1.4	3–11	29(48)	0(0)

that those in the lowest tertile have higher response rates compared with those in the highest tertile, comparisons of the three tertiles across all serum nutrient levels did not reveal any significant group differences.

3.7. Children's global assessment scale (CGAS)

Univariate predictors of change in CGAS included pre-treatment CGAS, group, sex, pregnancy risk factors, and number of co-occurring disorders. The final model was significant (R^2_{adj} = 0.140, p = 0.018), with pre-treatment CGAS (β = 0.258, p = 0.029) and being female (β = 0.240, p = 0.041) identified as significant predictors. Children who entered the trial with a lower rating on the CGAS (identifying worse functioning) showed the greatest improvement during the trial. Females showed significantly greater improvements on the CGAS (F (1, 69) = 5.773, p = 0.019) as compared with the males; the mean improvement on the CGAS was almost twice that of the males (10.13 versus 5.25).

3.8. Overall responder (based on CGI-I ratings)

A logistic regression was performed to ascertain the effects of group, sex, BMI percentile, gestational, delivery, and developmental risk factors, B_{12}, and zinc on the likelihood that participants were identified as responders ("much" to "very much" improved) to the active ingredients. The logistic regression model was statistically significant, χ^2 (7) = 17.871, p = 0.003. The model explained 30.0% (Nagelkerke R^2) of the variance in response status. More pregnancy complications, lower B_{12} levels and lower delivery risk were associated with an increased likelihood of responding to the nutrients. Given the focus on the effects of pre-treatment nutrient levels, we considered response across B_{12} tertiles. Among those in the lowest tertile (< 373 pmol/L) for B_{12},

Table 4
Pre-treatment and post 10-week exposure to nutrients on continuous outcome measures and nutrient levels.

Variable	Total sample (n = 71)								
	Baseline		Post		Change from pre-treatment (pre minus post)	confidence interval	t-value	p	ES[a]
	Mean	SD	Mean	SD					
Outcome variables									
Clinician ADHD-IV-RS DSM Combined	43.7	7.0	35.3	11.2	8.4	6.0 to 10.7	7.182	< **0.001**	1.20
Parent DSM Combined	41.1	7.1	33.2	11.5	7.9	5.3 to 10.6	6.000	< **0.001**	0.75
CMRS	22.8	12.0	15.1	10.4	7.8	5.4 to 10.1	6.556	< **0.001**	0.78
CGAS[b]	48.7	7.4	55.1	8.8	−6.4	−8.1 to −4.6	−7.257	< **0.001**	0.88
SDQ- conduct problems	3.9	2.8	3.4	2.6	0.5	0.0 to 1.0	2.050	< **0.05**	0.30
Changes in nutrient levels (n = 39; pre and post only available for those randomized to nutrients)									
Vitamin D (nmol/L)	72.5	22.3	81.5	16.9	−9.0	−15.5 to −2.5	−2.807	**0.008**	0.46
Vitamin B_{12} (pmol/L)	461.2	186.2	906.9	300.8	−439.2	−516.2 to −375.2	−12.814	< **0.001**	2.39
Folate (nmol/L)	26.3	8.5	53.6	22.3	−27.3	−34.6 to −19.9	−7.511	< **0.001**	1.38
Ferritin (μg/L)	41.1	22.0	40.3	24.0	0.8	−6.0 to 7.5	0.230	0.820	0.04
Iron (μmol/L)	15.8	4.7	16.3	5.0	−0.5	−2.5 to 1.4	−0.550	0.586	0.09
Zinc (μmol/L)	12.5	1.8	12.6	1.6	−0.2	−0.7 to 0.4	−0.532	0.598	0.06
Copper (μmol/L)	16.0	3.4	15.5	3.2	0.5	−0.4 to 1.4	1.149	0.258	0.18
Copper/zinc ratio	1.3	0.3	1.2	0.2	0.1	0.0 to 0.2	1.567	0.125	0.26
Potassium (μmol/L)	4.1	0.3	4.1	0.3	0.0	−0.1 to 0.07	−0.716	0.478	0.11
Calcium (μmol/L)	2.5	0.1	2.5	0.1	0.0	−0.04 to 0.01	−1.360	0.181	0.21
Magnesium (μmol/L)	0.8	0.1	0.8	0.1	0.0	−0.03 to 0.01	−1.071	0.290	0.16
Homocysteine (μmol/L)	5.5	1.6	3.8	0.8	1.7	1.1 to 2.2	6.055	< **0.001**	1.21

DSM = Diagnostic and Statistical Manual, CGAS = Children's Global Assessment Scale, CMRS = Child Mania Rating Scale, SDQ = Strengths and Difficulties Questionnaire.
Bolded indicate significant group differences.
[a] Cohen's d measured as the mean change/SD change.
[b] An increase is indicative of better functioning.

Table 5
Univariate associations (*p*-values) between the outcome measures and putative pre-treatment predictor variables.

	Δ Clinician total ADHD	≥30% Δ ADHD Clinician (yes/no)	Responder: CGI-I	Δ CGAS[a]	Δ Parent total ADHD	Δ CMRS	Δ aggression
Demographic variables							
Sex (F)	**0.051**	0.144	0.230	**0.019(−)**	0.137	0.991	0.928
Ethnicity (European decent/Māori)	0.736	0.571	0.819	0.235	0.036	0.894	0.897
Group (RCT/OL)	0.892	0.845	0.893	0.947	0.131	**0.010**	0.398
Parental marital status (two parents/single parent)	0.547	0.549	0.819	0.854	0.646	0.533	0.262
Age	0.438	0.281	0.779	0.407	0.279	0.897	0.970
Estimated IQ	0.230	0.393	0.217	0.407	**0.060(−)**	0.393	**0.008(−)**
NZSEI	0.269	0.387	0.120	0.496	0.576	0.241	0.994
Dietary patterns	0.824	0.354	0.545	0.734	0.996	0.579	0.214
BMI percentile	0.242	0.316	**0.042**	0.441	0.152	0.206	**0.001**
Developmental risk factors							
Gestation risk	**0.027**	0.176	**0.032**	**0.054(−)**	**0.056**	**0.012**	**0.097**
Delivery Risk	**0.051(−)**	0.136	**0.027(−)**	0.029	0.814	0.729	0.631
Developmental Risk	0.307	0.306	**0.062**	0.616	0.975	0.579	0.352
Sensory Risk	0.208	0.447	0.224	0.452	0.966	0.986	0.760
Medical History Risk	0.876	0.271	0.803	0.592	0.652	**0.016**	0.311
Temperament (parent report)[b]							
Frustration	0.662	0.613	0.853	0.940	0.756	0.738	0.322
Surgency	0.447	0.535	0.111	0.262	0.236	**0.058**	0.698
Inhibitory Control	0.947	0.523	0.785	0.713	0.817	0.566	0.404
Clinical characteristics							
Number Psychiatric comorbidities - Current	**0.009**	**0.072**	0.533	**0.048(−)**	0.132	0.238	0.128
Past Medication (yes/no)	0.797	0.908	0.664	0.925	0.438	0.960	0.774
MTHFR status							
A1289C (AA versus AC/CC)	0.130	0.684	0.336	0.559	0.227	0.200	0.951
C677T (CC versus CT/TT)	0.822	0.822	0.221	0.514	**0.080(−)**	0.518	0.921
Nutrient levels							
Vitamin D	0.776	0.363	0.398	0.975	0.202	0.379	0.595
Vitamin B_{12}	0.186	**0.093(−)**	**0.050(−)**	0.929	0.169	0.329	0.459
Folate	**0.094(−)**	**0.051(−)**	0.294	0.898	0.221	0.430	0.514
Ferritin	0.496	0.720	0.543	0.608	0.948	0.471	0.866
Iron	0.354	0.335	0.909	0.461	0.555	0.618	0.499
Zinc	0.935	0.874	**0.090**	0.220	0.306	**0.081**	0.408
Copper	0.951	0.343	0.341	0.958	0.432	0.461	0.477
Copper/zinc ratio	0.954	0.412	0.109	0.565	0.307	0.959	0.249
Potassium	0.415	0.354	0.650	0.386	0.175	0.832	0.155
Calcium	0.345	0.953	0.770	0.251	0.920	0.319	0.808
Magnesium	0.520	0.377	0.217	0.599	0.790	0.393	0.120
Homocysteine	0.112	**0.052**	0.244	0.559	0.163	0.625	0.916

CMRS = Child Mania Rating Scale, CGAS = Children's Global Assessment Scale, SDQ = Strengths and Difficulties Questionnaire, CGI-I = Clinical Global Impression – Improvement, NZSEI = New Zealand Socio-Economic Index, RCT = Randomized Controlled Trial, OL = Open Label, BMI = Body Mass Index percentile.
Bolded p values are less than or equal to 0.1.
(−) indicates a negative association between the pre-treatment factor or level and outcome for those variables considered for input into the regression models.
[a] An increase in CGAS scores identifies improved functioning.
[b] Based on early Adolescent Temperament Questionnaire-Revised.

67% were responders versus 35% in the highest tertile (> 510 pmol/L), although the group difference across the tertiles was only marginal (χ^2 (2) = 4.841, $p = 0.089$).

3.9. Parent ratings of ADHD

Univariate predictors of change in parent-rated ADHD scores were pre-treatment parent ADHD scores, sex, group, estimated IQ, gestational risk factors, and genetic status for C677T (those who were heterozygous (CT) showed less change compared with those who were homozygous (CC)). The model was not significant ($R^2_{adj} = 0.048$, $p = 0.113$).

3.10. Ratings of emotion dysregulation (CMRS)

Univariate predictors of change in CMRS ratings from pre-treatment to end of treatment included pre-treatment CMRS scores, group, sex, gestational risk, medical history, surgency temperament, and pre-treatment zinc. The final model was significant ($R^2_{adj} = 0.342$, $p < 0.001$). Pre-treatment CMRS ($\beta = 0.587$, $p < 0.001$) contributed significantly to the model with higher pre-treatment CMRS being associated with greater change on the CMRS.

3.11. Conduct problems

Univariate predictors of change in conduct problems included pre-treatment conduct problems, group, sex, pregnancy risk factors, estimated IQ, and pre-treatment BMI. The final model was significant ($R^2_{adj} = 0.328$, $p < 0.001$), with pre-treatment conduct problems ($\beta = 0.381$, $p < 0.001$), pre-treatment BMI ($\beta = 0.360$, $p = 0.001$), and pre-treatment estimated IQ ($\beta = -0.238$, $p = 0.027$), identified as significant predictors. Greater aggression pre-treatment, lower IQ and higher BMI percentile were associated with greater reduction in symptoms of aggression. Inspection of the tertiles for IQ identified that children in the low (< 91 IQ) and middle tertile (between 91 and 103) showed greater change in aggression compared with the children in the highest tertile for IQ (> 103 IQ), F (2, 67) = 3.955, $p = 0.024$. In contrast, those in the lowest tertile (< 49th) for BMI percentile showed

Table 6
Significant predictors associated with outcome measures based on regression analyses.

Outcomes	Significant predictors	β	*P* value	R^2_{adj}	P value
Clinician rated ADHD symptoms				0.170	0.004
	Delivery risk	−0.242	0.031		
	Being female	0.285	0.013		
	Number of co-occurring disorders	0.318	0.008		
ADHD responder				0.289	0.005
	Number of co-occurring disorders	0.653	0.016		
	Pre-treatment Folate	−0.079	0.030		
Overall responder				0.431	< 0.001
	Gestational risk	0.502	0.033		
	Delivery risk	−0.579	0.028		
	Pre-treatment B_{12}	−0.004	0.026		
CGAS[a]				0.140	0.018
	Pre-treatment CGAS	0.258	0.029		
	Being female	−0.240	0.041		
Mood				0.342	< 0.001
	Pre-treatment CMRS	0.587	< 0.001		
Aggression				0.328	< 0.001
	Pre-treatment conduct problems	0.381	< 0.001		
	Pre-treatment BMI percentile	0.360	0.001		
	Pre-treatment estimated IQ	−0.238	0.027		

CMRS = Child mania Rating Scale, CGAS = Children's Global Assessment Scale, IQ = Intelligent Quotient, BMI = Body Mass Index.
[a] Increase in CGAS identifies improvement.

almost no change in aggression whereas those in the middle and highest tertiles for BMI showed much greater change, F (2, 68) = 4.231, $p = 0.019$ (see Fig. 2).

4. Discussion

This study investigated predictors of response to a micronutrient treatment for children with ADHD which has previously been shown to improve psychological functioning across a number of different areas as compared with placebo. Given that the treatment involved a broad range of micronutrients, pre-treatment nutrient levels were of particular interest. However, only two pre-treatment micronutrient levels, B_{12} and folate, showed a possible association with treatment response. Lower pre-treatment folate levels predicted who would be classified as an ADHD responder while lower pre-treatment B_{12} contributed to the prediction as to who responded more globally to the treatment. No other relationships between individual pre-treatment nutrient levels and treatment response were identified, although there appeared to be a fairly consistent pattern where participants in the lower tertile for nutrients tended to have higher levels of response than those in the top tertile.

The results indicated that there were no specific demographic variables (e.g., age, socio-economic status, marital status, ethnicity, education) that would act as contraindications for micronutrient treatment of ADHD in children. The only two developmental variables that were identified as possible predictors of response were higher number of gestational risk factors and lower number of birth complications. Temperament did not affect outcome. We also observed that being female, poorer general functioning, higher levels of aggression, greater emotional dysregulation, co-occurring disorders, higher BMI and lower estimated IQ were shown to lead to greater improvement for some but not all outcome measures.

There has been substantial speculation involving the contribution of *MTHFR* gene mutations in abnormal methylation and neuropsychiatric disease, such as autism, schizophrenia and ADHD. This hypothesis is not strongly supported by the scientific literature (Ergul et al., 2012), and in our sample of ADHD children we did not observe unusual genotype frequencies. Furthermore, *MTHFR* status did not appear to significantly predict response, suggesting that this genetic variant does not usefully predict who will respond to a broad-spectrum intervention; however, had the intervention been contained to only those nutrients relevant to the methylation cycle, it may have proved more useful. We also established that those who were double heterozygote for both SNPs were no more likely to respond relative to those who were either single heterozygote or double homozygote.

4.1. Pre-treatment nutrient levels and outcomes

Lower pre-treatment folate predicted who would be identified as an ADHD responder. This may represent a signal but requires replication. It was also a relatively weak predictor as those who entered the trial with higher folate levels still showed a reasonable response rate of 40% versus those who entered with lower folate levels at 56%. Of note, no associations between folate levels and outcome were identified in the adult ADHD predictor study (Rucklidge et al., 2014b). In terms of possible mechanisms, lower folate levels are inversely correlated with homocysteine levels, a marker of oxidative stress (Karababa et al., 2017). Decreasing homocysteine has been shown to be associated with improvement in mental health status (Mech & Farah, 2016). In this study, pre-treatment homocysteine levels were not found to be an important predictor of change and, consistent with a recent publication (Karababa et al., 2017), we identified that many of the children in the sample already had a *low* homocysteine level at baseline.

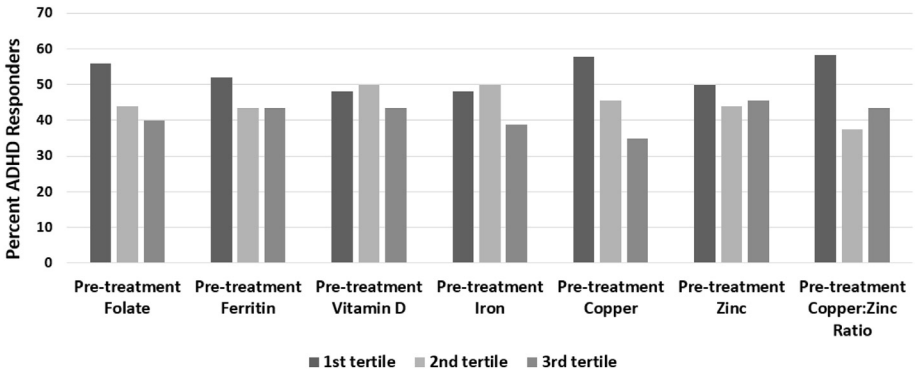

Fig. 1. Pre-treatment nutrient levels converted to tertiles (1st tertile is the lowest) and compared with per cent ADHD responders. There were no significant group differences across the tertiles.

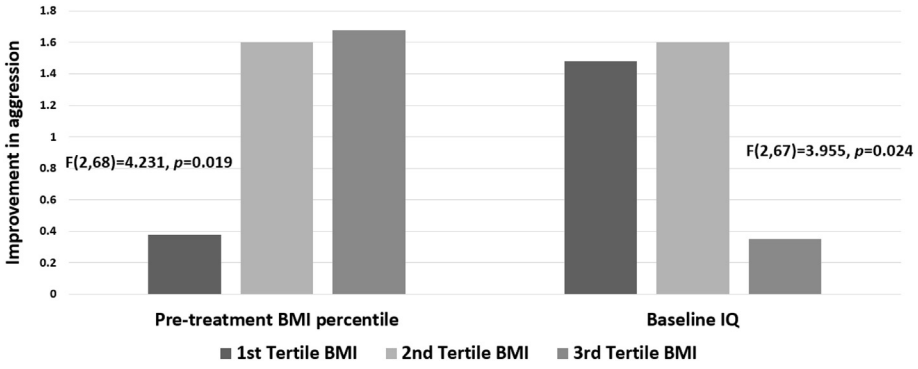

Fig. 2. Improvement in aggression based on tertiles for BMI percentile and estimated IQ.

Relatedly, *lower* levels of pre-treatment B_{12} predicted a better global response to the intervention suggesting that those who enter the trial with relatively lower B_{12} levels, may benefit more from the intervention, with almost twice as many children responding if they were in the lowest tertile (67% responded) for B_{12} versus the highest tertile (35% responded). Both B_{12} and folate are important nutrients for the folate-methylation cycles to function as they both act as methyl donors. Indeed, evidence from a subsample of this group identified that the nutrients lead to greater increase in methylation over 10 weeks relative to placebo (Stevens et al., 2018). It may be that genetic variants, other than *MTHFR*, such as betaine-homocysteine methyltransferase (*BHMT*), could aid with the prediction of who may benefit the most from the intervention. Future research could usefully investigate multiple genetic variants alongside nutrient levels and treatment response (Saha et al., 2018). It is important to note, however, that even the children in the lowest tertile did not display any deficiencies in folate or B_{12} based on reference ranges, raising questions around the usefulness of reference ranges for identifying who could benefit from additional nutrients based on deficiencies.

Contrary to some studies on ADHD (Konofal et al., 2004; Arnold et al., 2005), but not all (Rucklidge et al., 2014b; Donfrancesco et al., 2013), we did not observe significant correlations between any of the nutrient levels and ADHD behaviours pre-treatment. Because the risk factors for ADHD are so heterogeneous, the likelihood of finding significant correlations with any single nutrient is small. Additionally, some recent research has challenged the use of serum nutrient levels as serum nutrient levels are a marker only of peripheral rather than brain status. For example, an important study found that treatment with folinic acid of cerebral folate deficiency ascertained by examination of the cerbrospinal fluid (despite normal serum folate levels) led to significant improvement of treatment refractory depression in 13 patients (Pan et al., 2017). Another study noted *elevated* serum levels of B_6 in children with autism despite finding that the children responded positively to B_6 treatment. This suggests that the serum markers were not discriminating between the different metabolic forms of B_6 and the observed excess of B_6, resulted from the presence of an impaired enzyme (pyridoxal kinase) that converts pyridoxine to pyridoxal 5 phosphate (Adams et al., 2006).

The term “deficiency”, as is often used in the ADHD literature when discussing nutrient levels, may also be problematic. Although research often shows that the ADHD group mean nutrient levels are often below control group means (Landaas et al., 2016), means are typically within the normal reference range, potentially challenging the use of the term “nutrient deficiency” when attempting to investigate causes of ADHD and in relation to predicting response to nutrients. Given that reference ranges are generally defined as the set of values that 95% of the normal population falls within (Marshall et al., 2014), this does not necessarily mean that these ranges are best equipped to identify what is required for optimal health for any particular individual. Had functional ranges (the range used to assess risk for disease before the disease develops) been used in this study, many more would have been identified with “deficiencies”. An important hypothesis which requires further investigation is that some individuals may have *suboptimal* nutrition despite having nutrient levels within the reference range, meaning they might have a nutrient deficiency *relative to their metabolic needs* (Kaplan et al., 2007) rather than relative to general population levels.

4.2. Comorbidity and severity of symptoms

This study found greater benefit on ADHD measures for individuals with a greater number of co-occurring disorders. Given that co-morbidity with ADHD is the rule rather than the exception, it is reassuring that this intervention should generalize to typical ADHD referrals in the community, a finding that hasn't always been observed for other treatments, such as behavioural parent training (van den Hoofdakker et al., 2010), although other studies show no effect of co-morbidity on treatment response (Ollendick et al., 2008). This finding may also be consistent with observations of benefit of nutrients across a range of symptoms, including mood, anxiety and aggression (Gordon et al., 2015; Sole et al., 2017; Hambly et al., 2017). Relatedly, greater symptom severity (specifically poorer general functioning, higher levels of aggression, and greater emotional dysregulation) as assessed by both clinicians and parents at pre-treatment, was predictive of a better outcome. Although greater symptom severity is especially susceptible to regression towards the mean and therefore may have played a role in this finding, the fact that the RCT component of our study showed greater change on these measures in the active group versus the placebo group acts against regression to the mean being the primary driver for change (Rucklidge et al., 2018).

4.3. Gender

Our finding that females were more likely to respond to the treatment than males across two measures needs to be cautiously interpreted given that there were only 16 girls in the sample. Nevertheless, the findings are consistent with previous findings where non-pharmacological treatments for ADHD appear to be more efficacious for females (Hodgson et al., 2014). Further, given that females with ADHD are at high risk for continued impairment into adulthood (Hinshaw, 2018), determining whether these observations hold in the long term needs to be further investigated.

4.4. Body Mass Index

It was intriguing to observe that those children who fell in the lower percentile for BMI were less likely to show change in aggression relative to those in a higher BMI percentile. Obesity has been described as an inflammatory state (Saltiel & Olefsky, 2017) and micronutrients might help combat inflammation, presumably through enhanced mitochondrial production of ATP (Kaplan et al., 2015). A meta-analysis of

children's diets suggested that a healthier diet is associated with a lower BMI (Dallacker et al., 2018). It is possible that this finding may apply to our participants. Similarly, large longitudinal data have found that children who consume a "processed" dietary pattern ingest food that is energy dense but nutrient poor relative to those who consume "health conscious" or "traditional" diets (Cribb et al., 2013). It is also possible that those with a higher BMI have a lower level of antioxidants which may result in a higher requirement for micronutrients (Hosseini et al., 2017; Gust & Logomarsino, 2017). As such, those falling in the higher percentile for BMI may benefit more from a nutritional intervention due to a suboptimal diet.

Interestingly, dietary reports from parents of the children in the highest tertile did not suggest that their children ate any more poorly than those in the lowest tertile. It may be that our measure of dietary patterns was too crude to detect relatively subtle differences in food choices and/or that the parents did not report accurately what their children were eating. There is evidence that the latter is an issue with parents' report of their children's diet (Leech et al., 2018). Further research using more accurate and comprehensive measures of dietary patterns may elucidate this preliminary observation. We also need to be cautious about these findings as BMI percentiles, while better than BMI for this sample as they adjust for age and gender, do not take into account Tanner stages or diversity associated with ethnicity.

4.5. IQ

Children in the highest tertile for IQ (> 103) showed smaller changes in aggression as compared with children with lower IQ. This finding was not driven by higher aggression scores in the children at baseline in the lower IQ range. This finding should be interpreted with caution given that the association was observed for only one outcome variable (aggression).

4.5.1. Limitations

While this is the first exploratory study examining serum nutrients as predictors of response to micronutrient treatment in children with ADHD, it has a number of limitations including the relatively small sample size, the small number of females, the lack of assessment of Tanner stage, rudimentary assessment of dietary patterns, and the reliance on parent recall to assess childhood risk factors. Regrettably, we were not able to use teacher ratings as an insufficient number were returned following the open-label phase. The measures used to define response may also have limited the opportunity to detect predictors given that they were all based on behavioural outcomes. The fact that half the participants were in an open-label extension (i.e., participants and raters were not blind) raises the possibility that some reported change could have been due to observer/rater bias; however, the lack of differences between these two phases and the fact that we have previously reported that both the parents and clinicians were no better than chance at guessing group allocation during the RCT phase (Rucklidge et al., 2018) suggests rater bias is unlikely to explain all changes in outcome measures. Nevertheless, not including the placebo condition makes it more challenging to attribute any effects directly to the micronutrient intervention. Importantly, given that this was an initial exploratory study, a large number of analyses have been undertaken and therefore, there is a very real possibility of type I errors. As such, our results are exploratory and further research is required, preferably with a placebo arm, to establish whether our findings are able to be replicated and whether these predictors are veritable moderators of outcome.

Unfortunately, financial considerations prevented us from doing more extensive nutrient testing on less commonly assayed and more expensive biomarkers including thiamine, riboflavin, vitamin A, C and E and other minerals such as selenium, iodine, and chromium; we are unable to determine whether these biomarkers may be more useful predictors. However, the nutrient assays we conducted included most of the micronutrients most commonly investigated in relation to ADHD (Ahn et al., 2016) and are representative of those typically conducted by nutritionists and integrative physicians (Bradstreet et al., 2010; Villagomez & Ramtekkar, 2014). Similarly, examination of nutrient levels in other tissues could be considered in future studies, such as hair or red blood cells. Serum and/or plasma levels, while relatively easily assayed, may not represent total body nutrient levels accurately (Villagomez & Ramtekkar, 2014). For example, plasma zinc is not generally seen as a good measure of zinc stores (Arnold et al., 2011). Red blood cell zinc (Villagomez & Ramtekkar, 2014), or elemental zinc from hair may be alternative options (Elbaz et al., 2017; Tippairote et al., 2017).

5. Conclusions

This is the first study to investigate the associations between serum nutrient levels and other pre-treatment variables and treatment response to a micronutrient intervention for ADHD in children. Overall, only a couple of weak associations with baseline single nutrient levels were identified. Whether pre-treatment folate and B_{12} are important serum nutrient predictors of treatment response needs to be further investigated in future research. Indeed, our results suggest that if micronutrient treatment is being considered, the individual serum nutrient levels that we assessed are unlikely to play a critical role in the decision to proceed. Other nutrient levels, however, may prove to be more fruitful. Future research is required to further evaluate the utility of nutrient levels in relation to the aetiology and treatment of ADHD more broadly, including the evaluation of nutrient levels in other tissues.

Our findings suggest that if children with ADHD and multiple comorbidities do not respond well to established ADHD medications, micronutrients may well have an important therapeutic role. Additionally, it was reassuring that no variables were identified that appeared to act as a contraindication to using this treatment approach. This, and the documented safety of micronutrients, suggest that they may also be worthy of consideration along with other more established ADHD treatment options.

Contributor statement

All authors were involved in the conception and design, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the submitted version.

Ethical statement

This trial was registered prospectively with the Australian New Zealand Clinical Trials Registry (ANZCTR). The ANZCTR is recognized as an ICMJE acceptable registry (http://www.icmje.org/faq.pdf) and a Primary Registry in the WHO registry network (http://www.who.int/ictrp/network/primary/en/index.html).

The product Daily Essential Nutrients was provided free by the manufacturer; however, the company did not provide financial assistance for the study; they were not involved in any aspect of the study, including design, writing and data analysis. They have not read a copy of this manuscript. All financial assistance came from the University of Canterbury, UC Foundation, the GAMA Foundation, Canterbury Medical Research Foundation, Foundation for Excellence in Mental Health Care, the Carney Centre for Pharmacogenetics and Vic Davis Memorial Trust.

All authors have shared in all stages of formulating and writing of this article. None of the authors have any financial disclosures or any commercial affiliation. Copyright Transfer forms will be completed if/when the paper is accepted for publication.

We warrant that the material contained in the manuscript

represents original work, has not been published elsewhere, and is not under consideration for publication elsewhere. This is our second submission on this RCT. We have complied with the ethical procedures of APA in the treatment of our sample for research purposes.

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