The Role of Vitamin D and Omega-3 Long Chain Polyunsaturated Fatty Acids in Children with Autism Spectrum Disorder

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A Dedication to All the Kids with Autism and Their Families Who Participated in This Study

A story of hope…

"And the best thing is that she now says "Mommy" when she wants me. She never says this unless being prompted by me but now she does it on her own. I have been waiting for this for 5 years and I was so ecstatic and soooo happy when she did!"

Abstract

Background: The efficacy of vitamin D and omega-3 long chain polyunsaturated fatty acid (omega-3 LCPUFA), each individually, in Autism Spectrum Disorder (ASD) has been tested in a few trials and the results are inconclusive. Furthermore, several observational studies have observed low vitamin D and omega-3 LCPUFA status in populations with ASD. Children with ASD are susceptible to nutritional issues and poor diet quality due to sensory, behavioural and gastrointestinal issues associated with the condition, though no information regarding these children's nutritional status is available in New Zealand. Also, no validated nutritional quality assessment tools are available for this population.

Aim and Objectives: The overall aim of this study was to investigate the role of vitamin D (VID), omega-3 LCPUFA (OM), or both (VIDOM) in ASD in children through systematically reviewing literature and conducting an intervention trial with these nutrients. The primary objective was to investigate the efficacy of vitamin D, omega-3 LCPUFA, both on core symptoms and sensory issues after correcting major nutritional deficiencies and secondary objectives were to investigate the efficacy of intervention on irritability and hyperactivity, to study dietary adequacy/nutritional status of children with ASD, and to validate a Dietary Index of Children's Eating (DICE) questionnaire against 4-day estimated food record (4DFR).

Methods/Design: New Zealand children with ASD (age 2.5-8.0 years) participated in a 12-month randomised, double-blind, placebo-controlled, 2x2 factorial trial. Prior to trial entry, children's dietary adequacy and nutritional status were assessed by 4DFR, DICE questionnaire (designed based on New Zealand Ministry of Health Food and Nutrition guidelines), and nutritional biomarkers (25(OH)D, red blood cell fatty acids, iron, calcium, albumin, vitamin B_{12}, and folate). Data regarding dietary supplement use and special/exclusion diet, demographics and anthropometrics (height and weight) were also collected. Children then were randomly assigned to one of four treatment groups; daily 2000IU vitamin D_3, 722 mg docosahexaenoic acid (DHA), both supplements, or placebo, and behaviours were assessed. Core symptoms were assessed using Social Responsiveness Scale (SRS), sensory issues using Sensory Processing Measure (SPM), problem behaviours including irritability and hyperactivity using Aberrant Behaviour Checklist (ABC). Outcome measures were analysed pre- and post-intervention. Pair-wise mixed effects longitudinal models were used for data analysis.

Results: 309 families registered their interest in the study, of whom 190 families were either excluded or not enrolled. The children of remaining families (n=119) were screened for nutritional deficiencies and high serum 25(OH)D concentrations, of whom two children were excluded. Overall, 62% (73/117) of children completed the trial (placebo 16, VID 19, OM 23, VIDOM 15). The mean serum 25(OH)D concentrations (nmol/L) increased in the VID (27±14, P<0.001) and VIDOM (36±17, P<0.001) groups and changed slightly in the OM (1.1±14, P>0.05) and placebo (8.9±23, P>0.05) groups. The median omega-3 index (%) increased in the OM [4.4 (3.3, 5.9), P<0.001] and VIDOM [4.0 (2.0, 6.0), P<0.001] groups and decreased in the VID [-0.2 (-1.0, 0.1)] and placebo [-0.5 (-0.9, -0.1), P>0.05] groups. Compared to placebo, a greater improvement in multiple outcomes in the intervention groups was observed: SRS-social awareness for OM (0.4±2.9 *vs.* -1.4±2.3, P=0.03) and VIDOM (0.4±2.9 *vs.* -1.7±3.5, P=0.03); SRS-social communicative functioning for VIDOM (-5.6±10 *vs.* -16±24, P=0.07); SRS-total for OM (-5.8±12 *vs.* -17±18, P=0.08); SPM-taste and smell for VIDOM (-0.3±1.7 *vs.* -2.5±4.3, P=0.06), SPM-balance and motion for OM (-0.1±4.7 *vs.* -2.6±4.3, P=0.09), ABC-irritability for VID (0.8±6.1 *vs.* -4.0±4.9 P=0.01) and OM (0.8±6.1 *vs.* -5.0±5.0, P=0.001); and ABC-hyperactivity for VID (-0.8±5.6 *vs.* -5.2±6.3, P=0.047).

Out of 86 children whose food records were available, approximately 50% (39/86) reported taking dietary supplements and 15% (13/86) were on a special/exclusion diet. A large proportion of children had dietary intake for vitamin D below the Adequate Intake (AI, 96%), protein below the Average Macronutrient Distribution Range (AMDR, 65%), and iodine below the Estimated Average Requirement (EAR, 54%). Dietary intake of fibre (43%) and vitamin E (37%) was also below the AI by at least one third of children. All or most children exceeded the recommendations for sodium (100%), total saturated fat (80%) and sugar (52%). There was a significant and positive correlation (r=0.7; P<0.001) and good agreement (κ=0.6) between total scores from DICE (64±16) and 4DFR (58±11). Participants in the highest tertile of DICE had higher intakes of magnesium (P=0.02), vitamin A (P=0.03) and fibre (P=0.06).

Conclusions: Vitamin D and omega-3 LCPUFA, each individually or together, improved some behavioural symptoms of ASD. However, large attrition rates and resultant loss of statistical power preclude definitive conclusion and warrant further trials.

Also, the baseline assessment of nutrition confirms nutritional issues and poor diet quality in children with ASD. Given the importance of nutrition in growth and development and in the management of ASD, screening of the nutritional status of children with ASD for nutrient adequacy to reduce under- or over-consumption of nutrients is recommended. DICE is a valid tool for the assessment of diet quality in children with ASD living in New Zealand.

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Abbreviations

ABC	Aberrant Behaviour Checklist
AD	Autism Disorder
ADHD	Attention Deficit/Hyperactivity Disorder
ADI-R	Autism Diagnostic Interview-Revised
ADOS	Autism Diagnostic Observation Schedule
AI	Adequate Intake
ALA	Alpha-linolenic acid
AMDR	Acceptable Macronutrient Distribution Range
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
ARA	Arachidonic Acid
Anti-MAG	Anti-Myelin-Associated Glycoprotein
ASC	Autism Screening Questionnaire
ASD	Autism Spectrum Disorder
ATEC	Autism Treatment Evaluation Checklist
BASC	Behaviour Assessment System for Children
BDNF	Brain-Derived Neurotrophic Factors
CARS	Childhood Autism Rating Scale
CGI-I	Clinical Global Impression-Improvement
CGI-S	Clinical Global Impression-Severity
CI	Confidence Interval
D_2	Ergocalciferol
D_3	Cholecalciferol
DCS	Dendritic Cells
DHA	Docosahexaenoic Acid
DICE	Dietary Index of Children's Eating
DISCO	Diagnostic Interview for Social and Communication Disorders
DPA	Docosapentaenoic Acid
DSM	Diagnostic and Statistical Manual of Mental Disorder
EAR	Estimated Average Requirement
EPA	Eicosapentaenoic Acid
FABP	Fatty Acid Binding Protein
FADS	Fatty Acid Desaturase
FFQ	Food Frequency Questionnaire
HNRU	Human Nutrition Research Unit
ICD	International Classification of Diseases and Related Health Problems
IFN-γ	Interferon Gamma
IL	Interleukin
IQ	Intelligence Quotient
IQR	Interquartile Range
IU	International Unit
LA	Linoleic Acid
MIP2	Macrophage Inflammatory Protein-2
MOH	Ministry of Health
MUFA	Monounsaturated Fatty Acids

NGF	Nerve Growth Factor
NRV	Nutrient Reference Values
NZ	New Zealand
OM	Omega-3 Long Chain Polyunsaturated Fatty Acids
Omega-3 LCPUFA	Omega-3 Long Chain Polyunsaturated Fatty Acids
Omega-6 LCPUFA	Omega-6 Long Chain Polyunsaturated Fatty Acids
PDD	Pervasive Developmental Disorder
PDD-BI	Pervasive Developmental Disorder-Behaviour Inventory
PDD-NOS	Pervasive Developmental Disorder-Not Otherwise Specified
PGE2	Prostaglandin E2
PUFA	Polyunsaturated Fatty Acids
RBC	Red Blood Cell
RCT	Randomised Controlled Trial
RDI	Recommended Dietary Intake
RRB	Repetitive and Restricted Interests and Behaviour
SCQ	Social Communication Questionnaire
SD	Standard Deviation
SFA	Saturated Fatty Acids
SNP	Single Nucleotide Polymorphism
SPM	Sensory Processing Measure
SRS	Social Responsiveness Scale
TNF–α	Tumour Necrosis Factor Alpha
UL	Tolerable Upper Intake Level
US	United States
UVB	Ultra-Violate B
VABS	Vineland Adaptive Behaviour Scales
VDR	Vitamin D Receptor
VID	Vitamin D
VIDOM	Vitamin D + Omega-3 Long Chain Polyunsaturated Fatty Acids
WDHB	Waitemata District Health Board
1-OHase	25-hydroxyvitamin D-1α-hydroxylase
25(OH)D	25-hydroxyvitamin D
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
3di	Developmental, Dimensional, and Diagnostic Interview
4DFR	4-Day Estimated Food Record

List of Papers and Conference Presentations

Papers (Published, Submitted or to be submitted)

The following publications have been included in this thesis and incorporated as different chapters and sections in manuscript format. Therefore, in some cases, there may be replication.

Paper I Mazahery, H., C.A. Camargo, Jr., C. Conlon, K.L. Beck, M.C. Kruger, and P.R. von Hurst, Vitamin D and Autism Spectrum Disorder: A literature review. Nutrients, 2016. **8**(4): p. 236.

Incorporated as Chapter 2: Section 2.

Paper II Mazahery, H., W. Stonehouse, M. Delshad, M.C. Kruger, C.A. Conlon, K.L. Beck, and P.R. von Hurst, relationship between long chain n-3 polyunsaturated fatty acids and Autism Spectrum Disorder: systematic review and meta-analysis of case-control and randomised controlled trials. Nutrients, 2017. **9**(2).

Incorporated as Chapter 2: Section 3.

Paper III Mazahery, H., C. Conlon, K.L. Beck, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., B.J. Meyer, B. Tsang, O. Mugridge, and P.R. von Hurst, Vitamin D and omega-3 fatty acid supplements in children with Autism Spectrum Disorder: A study protocol for a factorial randomised, double-blind, placebo-controlled trial. Trials, 2016. **17**(1): p. 295.

Incorporated as Chapter 3

Paper IV Mazahery, H., M. Delshad, C. Conlon, K.L. Beck, M.C. Kruger, and P.R. von Hurst, Dietary adequacy and nutritional status of New Zealand children with Autism Spectrum Disorder and validation of a Dietary Index of Children's Eating (DICE) (Not submitted yet)

Incorporated as Chapter 4.

Paper V Mazahery, H., C. Conlon, K.L. Beck, O. Mugridge, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., B.J. Meyer, B. Tsang, B. Jones, and P.R. von Hurst, A randomised controlled trial of vitamin D and omega-3 long chain polyunsaturated fatty acids in the treatment of core symptoms of Autism Spectrum Disorder in children. J. Autism Dev. Disord., 2019 (online)

Incorporated as Chapter 5.

Paper VI Mazahery, H., C. Conlon, K.L. Beck, O. Mugridge, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., B.J. Meyer, B. Jones, and P.R. von Hurst, A randomised controlled trial of vitamin D and omega-3 long chain polyunsaturated fatty acids in the treatment of irritability and

hyperactivity among children with Autism Spectrum Disorder. The Journal of Steroid Biochemistry and Molecular Biology, 2018 (online).

Incorporated as Chapter 6.

Conference Presentations

I Mazahery, H. and O. Mugridge, Adaptations to standard nutrition research protocol for a nutritional supplement study in ASD children. South Pacific Congress, 2015.
Oral presentation

II Mazahery, H., C. Conlon, K.L. Beck, M.C. Kruger, O. Mugridge, C.A. Camargo, Jr., and P.R. von Hurst, Vitamin D status and its predictors in children with Autism Spectrum Disorder. The 20th workshop on Vitamin D, Orlando, 2017. Poster presentation

III Mazahery, H., C. Conlon, K.L. Beck, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., O. Mugridge, and P.R. von Hurst, A randomised, double-blind, placebo-controlled trial of vitamin D and n-3 long chain polyunsaturated fatty acids in the treatment of irritability and hyperactivity in children with Autism Spectrum Disorder. The 21st Workshop on Vitamin D, Barcelona, 2018.
Oral presentation. Recipient of "Claude McCarthy Fellowship Award" and "Trainee Travel Award".

IV Mazahery, H., C. Conlon, K.L. Beck, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., O. Mugridge, and P.R. von Hurst, Omega-3 long chain polyunsaturated fatty acids may modulate the effect of vitamin D supplementation on vitamin D status in children with Autism Spectrum Disorder. The 21st Workshop on Vitamin D, Barcelona, 2018.
Poster presentation.

V Mazahery, H., C. Conlon, K.L. Beck, O. Mugridge, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., Meyer, B.J., Jones, B., Tsang, B., and P.R. von Hurst, Vitamin D and omega-3 long chain polyunsaturated fatty acids improve behavioural symptoms in children with Autism Spectrum Disorder. Nutrition Society of New Zealand: Annual Scientific Meeting, Auckland, 2018

Oral presentation.

Chapter 1: Preface

This chapter starts with an introduction and provides justifications for this thesis, then followed by objectives, outcomes and hypotheses of this thesis. An outline of thesis structure is provided, followed by researchers' contribution report.

Introduction and Justification for Study

Autism Spectrum Disorder (ASD) is a complex neurodevelopment disorder with heterogeneous aetiology, clinical presentation, and treatment response. It is generally agreed that ASD is driven by an interaction between genetic and non-genetic factors [1]. This condition has been shown to have moderate genetic heritability, but a substantial shared twin environmental component [2]. Accumulating evidence from observational data point to a possible relationship between environmental factors, maternal diseases, and nutritional factors and ASD [3-6].

Similarly, the clinical presentation of ASD varies widely between individuals, and several behavioural, medical and biological conditions may co-exist with ASD core symptoms. Impaired social interaction and communication, repetitive and restricted interests and behaviour are the core symptoms of ASD. Among comorbidities, problem behaviours (e.g. irritability, hyperactivity and selective eating/mealtime issues) [7-9], intellectual disability [10], gastrointestinal symptoms [11-13], and biological abnormalities (e.g. oxidative stress and inflammation) [14-18] are common in children with ASD. Because the causes of ASD are not well understood and children exhibit a broad range of symptoms, there are no well-documented pharmacological agents and parents/caregivers often turn toward different ways to manage symptoms of ASD.

Besides behavioural intervention therapies and pharmacological treatment and despite knowing little about the nutritional status of children with ASD worldwide, dietary modification and supplementation are popular approaches used in these families. Recent public interest has focused on the role of vitamin D and omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFA) in the pathophysiology of ASD, however limited studies has examined the efficacy and safety of these nutrients in populations with ASD.

The identification of the vitamin D receptor and enzymes involved in vitamin D metabolism in different regions of brain [19] along with experimental data generally support the hypothesis that vitamin D has an important role in ASD [20,21]. These data are supported by reasonable evidence from observational studies suggesting that ASD prevalence is associated with the amount of Ultraviolet Beta (UVB) radiation and that vitamin D deficiency/inadequacy may be associated with pathophysiology of ASD. For example, Grant *et al.* (2013) reported a higher prevalence of autism in populations living at higher latitudes compared with lower latitudes and found a significant negative correlation between ASD prevalence in the United States population aged 6-17 years and UVB doses [22]. There is also evidence that problem

behaviours significantly increase during spring and decrease during autumn in adults with severe autism living in a farm community centre in Italy [23]. Due to limited vitamin D synthesis in the skin during winter months, vitamin D comes from the body stores and from dietary sources. Depletion of vitamin D in body stores by the end of winter and early spring may exacerbate the symptoms of ASD and resulting in increased problem behaviours.

Similarly, omega-3 LCPUFA seems to have the potential to positively affect ASD. Omega-3 LCPUFA are polyunsaturated fatty acids that the body can produce in limited amounts and should be mainly obtained from the diet. These fatty acids, mainly docosahexaenoic acid (DHA), are necessary for normal brain development and functioning [24], and long-term DHA depletion results in significant losses in brain DHA with consequent loss in brain function [25]. Observational data suggest that low omega-3 LCPUFA status may be associated with increased risk of ASD and the severity of symptoms [26]. Despite the promising evidence from experimental and observational studies, the findings from intervention trials are mixed and no definitive conclusion can be made.

Autism New Zealand states that one in every 66 (1.5%) New Zealanders has a condition somewhere on the autism spectrum. Parents/caregivers of children with developmental disabilities including ASD have been shown to experience lower quality of life and a substantial economic burden compared with the general population. Sufficient evidence now exists to justify investigation into the nutritional status (including vitamin D and omega-3 LCPUFA status) and the impact of improving vitamin D and omega-3 LCPUFA status on ASD symptoms. If an improvement in the status of vitamin D, omega-3 LCPUFA or both can be shown to reduce the symptoms of ASD (including both core symptoms and problem behaviours), even to a small degree, it is important that this be investigated. It is important to note that early intervention during the sensitive period of life when neural development is still occurring can lessen or potentially prevent the development of brain abnormalities and may have long-lasting benefits. Furthermore, even with a small improvement the benefit will be considerable because the lives of 1.5% of the population, and their families (an additional 3-8% of New Zealanders), not to mention their wider societal networks, will be positively affected.

Study Objectives

The VIDOMA commenced in Auckland, New Zealand, January 2015 and concluded in 2017. The name **VIDOMA** stands for **VI**tamin **D** and **OM**ega-3 in **A**utism.

The overall aim of this thesis was to investigate the role of vitamin D and omega-3 LCPUFA in ASD through systematically reviewing the literature linking these nutrients with ASD and an intervention trial examining the role of these nutrients in managing symptoms of ASD.

The primary objective of this study was to conduct a randomised double-blind, placebo-controlled, 2x2 factorial trial with vitamin D and omega-3 LCPUFA supplementation in children with ASD. We aimed to assess the effect of improved vitamin D and omega-3 LCPUFA status on core symptoms of ASD. To our knowledge, the VIDOMA trial is the first RCT with both vitamin D and omega-3 LCPUFA supplementation in children with ASD. However, since the conception of this trial, two RCTs with only supplemental vitamin D have been published, though with shorter study durations [27,28].

Because problem behaviours, irritability and hyperactivity, are so common in children with ASD, a secondary objective was to investigate the effect of improved vitamin D and omega-3 LCPUFA status on irritability and hyperactivity.

As the dietary adequacy and nutritional deficiencies were investigated at the screening stage to determine inclusion criteria for the RCT, the screening stage provided valuable information on dietary adequacy/nutritional status as well as other dietary, health and life style factors of children with ASD living in New Zealand. Furthermore, the collection of data regarding different aspects of diet using different tools provided an opportunity to validate a diet quality assessment tool (Dietary Index of Children's Eating, DICE) against food records (4 day estimated food record, 4DFR). This is the first descriptive study of this nature to be conducted in this population in New Zealand.

Outcomes

Primary outcome

Changes in vitamin D and omega-3 LCPUFA status and core symptoms of ASD including social and communicative functioning (Social Responsiveness Scale, SRS), repetitive and restricted interests and behaviours (SRS), and sensory profile (Sensory Processing Measure, SPM), in response to supplementation with vitamin D, omega-3 LCPUFA or both.

Secondary outcomes

1. Changes in co-existing problem behaviours including irritability and hyperactivity (Aberrant Behaviour Checklist, ABC), in response to supplementation with vitamin D, omega-3 LCPUFA or both.
2. Investigation of dietary adequacy and nutritional status of children with ASD.
3. Validation of DICE against 4DFR.

Hypotheses

1. Improving vitamin D and omega-3 status with supplementation has the potential to improve the core symptoms of ASD in children.
2. Improving vitamin D and omega-3 LCPUFA status with supplementation has the potential to improve co-existing problem behaviours in children with ASD.
3. Both vitamin D status and omega-3 LCPUFA will be below the optimum levels in children with ASD.
4. Dietary adequacy and nutritional status of children with ASD will be of concern.
5. Dietary Index of Children's Eating is a valid tool for assessing diet quality.

Thesis Structure

Following this preface chapter, chapter 2 consists of a review of the relevant literature. This chapter comprises of four sections, each dedicated to different topics. The first section provides an overview of ASD (including history, description, diagnostic criteria, and comorbidities), the prevalence of ASD, and psychosocial and economic burdens of ASD. This section is followed by a comprehensive review of scientific evidence (including observational, intervention and mechanistic evidence) linking vitamin D with ASD. This chapter was published in "Nutrients", and is presented here in manuscript format. An updated search was performed, and new information has been included in footnotes. To meet the requirement of this thesis, a figure (figure 1) – that is not included in the published paper – has been added. There are also some changes in formatting and referencing style to align with those of this thesis. Following this section, the relationship between omega-3 LCPUFA and ASD is systematically examined. This section was published in "Nutrients" as a systematic review and meta-analysis of case-control and randomised controlled trials and presented here in manuscript format. Similarly, an updated search was performed and new information has been included in footnotes. To meet the requirement of this thesis, a section discussing omega-3 LCPUFA metabolism, biomarkers and optimum levels has been added. Accordingly, there are some changes in reference numbers. To align with the style of this thesis, the formatting and

referencing style of published manuscript has also been changed. Finally, the role of vitamin D and omega-3 LCPUFA both together in ASD is discussed. This section is also presented in manuscript format but has not been published.

Chapter 3 includes all the details regarding the methodological procedures of the current trial, from the recruitment to the statistical analysis. This chapter was published in "Trials" and presented here in manuscript format. To note, the current thesis is part of a larger study and the published paper includes all the information relevant to that trial. The published paper has been amended to meet the requirement of this thesis and included here. These amendments include objectives, some of the outcome measures, and formatting and referencing style.

Chapters 4, 5, and 6 are the results sections, each including different outcome findings. These chapters are presented in the order of events in the trial. Chapter 4 includes the baseline dietary adequacy and nutritional status of the study population as well as the validation of DICE. This chapter is followed by chapter 5 reporting the primary outcome findings, the effect of intervention on core symptoms of ASD, and then chapter 6 that reports the secondary outcome findings, the effect of intervention on co-existing problem behaviours. Each chapter is presented in manuscript format.

Chapter 7 consists of an overview of findings and discussion (including an assessment of the strengths and limitations), followed by a brief conclusion, and recommendations for future research.

Appendices include each chapter's appendices as well as information sheet and consent forms.

Researchers' Contribution

Researchers	Contribution
Hajar Mazahery PhD researcher	Responsible for most aspects of the study including: recruitment and participant management, data collection, statistical analysis, and writing the research report Responsible for all aspects of the manuscripts including: conceptualisation and design of manuscripts, searching the literature, data extraction, data analysis (meta-analysis and trial outcome findings), drafting manuscript, and manuscript submission
Assoc Prof Pamela R von-Hurst Primary supervisor	Conceptualisation and design of trial, acquisition of funding and ethics approval, reviewing thesis, and reviewing all manuscripts
Dr Cath Conlon Secondary supervisor	Reviewing thesis and reviewing all manuscripts
Dr Kathryn L Beck Secondary supervisor	Reviewing thesis and reviewing all manuscripts
Prof Marlena C Kruger Secondary supervisor	Reviewing thesis, and reviewing all manuscripts
Owen Mugridge Trial manager	Recruitment and participant management, and data collection
Dr Welma Stonehouse Collaborator	Advice on omega-3 section, systematic review and meta-analysis and statistical analysis, and reviewing manuscripts (II, III, V, and VI)
Dr Carlos R Camargo Jr Collaborator	Advice on vitamin D section and statistical analysis, and reviewing manuscripts (I, III, V, and VI)
Dr Barbara J Meyer Collaborator	Advice on red blood cell fatty acids laboratory protocol, conducting the analysis, and reviewing manuscripts (III, V, and VI)

Researchers	Contribution
Dr Beatrix Jones Collaborator	Advice on statistical analysis and reviewing manuscripts (V and VI)
Dr Bobby Tsang Collaborator	Advice on autism, helping with recruitment, arranging hospital access for blood tests, and reviewing manuscripts (III, V, and IV)
Lindy Thomas Psychologist	Advice on assessment tools, reviewing the scorings of assessment tools and reports prepared for parents/caregivers
Dr Marilize Richter and Dr Martin Dickens	Randomisation (third party not involved in any aspect of trial)
Maryam Delshad	Duplicate data extraction for the systematic review – manuscript II, entry of some food diaries in foodworks, and validating Dietary Index of Children's Eating (DICE) – manuscript IV
Micaela G Makker	Entry of some food diaries in foodworks
Aimee J Waring	Entry of some food diaries
Nicole Taylor	Blind checking of blood biomarkers (at 6 and 12 months)

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Chapter 2: Review of the Literature

The first step in answering my research question "The Role of Vitamin D and Omega-3 in ASD" was to conduct a critical review of the available scientific evidence linking vitamin D and omega-3 to ASD. Before doing so, it was important to have a better understanding of ASD; how is diagnosed, what are clinical presentations, what is the prevalence, and what difficulties affected children and their parents have?

I start my literature review chapter with an overview of ASD, including the mentioned topics (section 1), followed by a comprehensive review of literature linking vitamin D to ASD (section 2), then by a systematic review and meta-analysis of case-control and randomised controlled trials of omega-3 long chain polyunsaturated fatty acids in ASD (section 3), and finally by a narrative review of literature investigating the potential synergistic effect of these nutrients on ASD (section 4).

Sections 2 and 3 have been published in scientific journals, and section 4 is written as a scientific journal paper but has not been published. Therefore, the reference list for each section is presented at the end of each section.

Section 1: Autism Spectrum Disorder: History, Diagnosis, Clinical presentation, Prevalence, and Burden

In this section, I will present an overview of the history of ASD, the diagnostic criteria for ASD, the clinical presentation of and comorbidities with ASD, the prevalence of ASD, and social, psychological and economic burdens of ASD.

History, Diagnostic Criteria, Clinical Presentation and Comorbidities

Autism Spectrum Disorder (ASD) is associated with impairment in social interaction and communication and with repetitive and restricted interests and behaviour (RRB). Autism was initially characterised by Leo Kanners (1943) [1] and Hans Asperger (1944) [2]. Leo Kanners (1943) described autism as having abnormal speech and monotonous, repetitive behaviour [1]. One year later, Han Asperger described the features of four cases with autism as all being gifted, having poor social and emotional relationships, lacking feelings for others, having stereotypic behaviours and special interests, being clumsy, and having idiosyncratic use of language but not language acquisition delay [2]. Since then, the identification of autism has undergone many changes, and many classification systems have been developed to help families in understanding their child's behaviour, clinicians in providing appropriate treatment, and researchers in conducting research in the field of autism.

There are three major classification systems, two of which are widely used for diagnosis of ASD; the International Classification of Disease series (ICD) and Diagnostic and Statistical Manual of Mental Disorders series (DSM). The Research Diagnostic Criteria (RDoC) was introduced in early 2009 in the US but has not been widely used. The RDoC has been developed by the National Institute for Mental Health as a research classification system, rather than as a routine clinical diagnostic system [3]. However, it has been argued that in its current form, the RDoC is of only limited use to autism researchers [4]. The reason for such argument is that RDoC does not cover all elementals of ASD; for example, its relevance to sensory issues are less clear [4].

The ICD and DSM have been developed as diagnostic systems and are frequently used in routine clinical settings. The ICD is a broad diagnostic system that includes all diseases and health related problems. The first version of ICD was introduced in 1893 and was developed and endorsed by the World Health Organisation. Autism was first mentioned in the 9th version of ICD in 1977 [5]. The ICD published its 10th revision in 1992 (ICD-10), and included an

overall category named as "Pervasive Developmental Disorders" and several subcategories including, childhood autism, atypical autism, Rett's syndrome, other childhood disintegrative disorder, overactive disorder associated with mental retardation and stereotyped movements, Asperger's syndrome, other pervasive developmental disorders and pervasive developmental disorders, unspecified [6]. The ICD-10 defines autism as the triad of impairments in social interaction, communication and atypical behaviour, a definition consistent with that of the DSM-IV [7].

The first version of DSM was a modified version of the ICD and was developed and endorsed by the American Psychiatric Association in 1952 [5]. The DSM has been used as a formal diagnostic system from the time when the third version, DSM-III, was published in 1980. Since then, the criteria for autism disorders have been modified and expanded four times (APA, 1987, 1994, 2000, 2013), with the major changes from DSM-IV to DSM-5 [7,8]. Highlights of the changes made to the DSM-5 are summarized below:

- Several separate categories in DSM-IV including autism disorder, childhood disintegrative disorder, Asperger's disorder and Pervasive Developmental Disorder Not Otherwise Specified (including atypical autism) (PDD-NOS) has been included into the one overarching category named "Autism Spectrum Disorder".
- The three domains of impairments in social interaction, impairments in communication and stereotyped behaviour in DSM-IV have become two, with the merging of the first two domains (deficits in social communicative functioning).
- Meeting all of the three criteria under the social communicative functioning domain is a diagnosis requirement – deficits in social emotional reciprocity, deficits in non–verbal communicative behaviours for social interaction and deficits in developing and maintaining relationships (see **Appendix 1**).
- The language delay criterion as a distinct criterion in DSM-IV has been removed, and it is considered as a specifier in DSM-5.
- The diagnosis age of three has been dropped and included manifestation of disorders either currently or historically – the symptoms must be present in early childhood but may manifest later in life when social demands exceed the individuals' limited capacity.
- Sensory processing issues has been added as a distinct criterion under the restricted, repetitive behaviours, interests or activities (RRB) domain – other criteria under this domain are stereotyped and repetitive speech, motor movement and use of objects; excessive adherence to routines or excessive resistance to change; abnormal highly

restricted and/or fixated interests. Meeting two of the above criteria is a requirement for diagnosis (see **Appendix 1**).

- Those diagnosed with ASD should receive a rating of severity, which is based on social communication impairment and repetitive and restricted patterns of behaviour (see **Appendix 2**).

Additionally, the symptoms must clinically cause significant impairment in social, occupational and other functioning and cannot be explained by intellectual disability and global developmental delay.

As the new criteria in DSM-5, clinicians are advised to describe other conditions which might be associated with ASD. These conditions include medical, behavioural and nutritional issues. The reason for describing these comorbidities is that they are so common in ASD [9], and the co-occurrence of these comorbidities and ASD is associated with lower quality of life, poorer adaptive functioning, higher psychiatric medication use, and lower responsiveness to standard treatments in individuals with both disorders than those with a single disorder [10-13].

Co-existing Problem Behaviours

In addition to core symptoms, children with ASD, may exhibit problem behaviours. Among problem behaviours, irritability and hyperactivity have been reported to be highly prevalent in ASD. Irritability is referred to vocal and motoric outbursts expressive of anger, frustration, and distress; these outbursts are often referred to by caregivers as "temper tantrums," "meltdowns," or "rages" [14]. In children with ASD, the term 'irritability' is often used to describe severe behavioural difficulties including, but not limited to, verbal and physical aggression, self-injury or property destruction. These behaviours have been included in the irritability subscale of the Aberrant Behaviour Checklist (ABC). Approximately 20% of people with ASD exhibit irritability and aggression at moderate to severe levels [15]. It has also been shown that boys with high functioning ASD had significantly higher irritability scores than healthy controls, and the pattern of irritability symptoms closely resembled that of severe mood disorder [16].

As neurodevelopmental disorders, both ASD and Attention Deficit Hyperactivity Disorder (ADHD) share similar phenotypic characteristics but are characterised by two distinct diagnostic criteria. While ASD is associated with impairment in social communication and repetitive and restricted patterns of behaviours, ADHD is associated with impairment in attention, hyperactivity, and impulsivity. Many studies show a high percentage of children

with ASD have ADHD comorbidity. Romero *et al.* (2016) showed that approximately 67% of children with ASD have clinical comorbidity with ADHD [17]. In another study of preschool children with autism disorder, Hoglund Carlsson *et al.* demonstrated that 33% of children exhibited severe hyperactivity/ADHD symptoms, and hyperactivity was one of the four most common disorders [9]. In another sample of 101 children with high functioning PDD, 75% had symptoms of ADHD at the moderate to severe range [18], with 95% having attention problems, 86% problems with regulation of activity level, 75% motor difficulty, and 50% had impulsiveness.

Nutritional Issues

There is a lack of New Zealand specific data on dietary adequacy/nutritional status of children with ASD. However, reports from other regions indicate that nutritional issues are highly prevalent in these children. Zimmer *et al.* (2012) demonstrated that children with autism with poor food variety had a lower total energy intake than both typically developing children (controls) and children with autism with a good food variety score [19]. Poor food variety was also associated with inadequate intake of calcium, zinc, vitamin D, vitamin B_{12}, folate, dairy and grains [19,20]. Although other investigations in children with autism have identified magnesium [21] and vitamin A [22] deficiency, Zimmer (2012) found a high dietary intake of both magnesium and vitamin A due to high supplement use [19]. Mari-Bauset *et al.* (2015) also found higher intake of vitamin E in children with ASD compared to typically developing controls [23]. In another study, vitamins A, D and K were identified to be consumed in inadequate amounts [24]. Furthermore, a large number of reports indicate that individuals with ASD have lower omega-3 LCPUFA status than typically developing individuals, and low dietary intake and disturbances in fatty acid metabolism and incorporation of these fatty acids into cellular membranes have thought to be the cause [25]. Finally, iron deficiency has been suggested to be highly prevalent in children with ASD [26], and this high prevalence has been attributed to inadequate dietary iron intake and malabsorption in these children [27]. Overall, these results suggest that nutritional issues are highly prevalent in children with ASD.

Nutritional issues in children with ASD could potentially be explained by several factors, including sensory problems [28], gastrointestinal issues [29] and odd eating habits/mealtime problems [30]. Sensory problems are a distinct criterion under the RRB domain of ASD and an important predictor of a child's nutritional status. Most children with autism have sensory abnormalities in multiple domains [28,31,32]. Over- and/or under-sensitivity to sensory factors such as smell, texture, colour and temperature may contribute to food selectivity and eating problems in children with ASD [33] and may put them at increased risk of nutritional

deficiencies. In a recent study of 90 children with ASD, almost two thirds had definite sensory issues and 21% probable sensory issues which were significantly associated with increased eating problems [33].

Gastrointestinal issues are another complication highly prevalent in children with ASD [22,29,34]. Mazurek *et al.* (2013) reported that of 2,973 children with ASD, 25% had at least one chronic gastrointestinal issue [29]. In another study by Wang *et al.* (2011), children with ASD had more GI problems (42%) than their unaffected siblings (12%) [34]. Gastrointestinal issues may relate to abnormal gut flora [35], decreased activity of digestive enzymes [36] or increased intestinal permeability [37]. Gastrointestinal issues have also been related to sensory abnormalities [29] and unusual eating habits [34].

Mealtime behaviours including food selectivity and picky eating are reported to be significant problems in children with ASD. These mealtime behaviours may result in the child taking a very limited range of foods, resulting in under- or over-consumption of nutrients. Schreck *et al.* (2004) in a large-scale study demonstrated that children with ASD refused significantly more food, had a less varied diet, ate fewer foods within each food group category and were more likely to select low-textured foods compared to typically developing controls [38]. Nadon *et al.* (2011) compared mealtime behavioural issues among children with autism and their healthy siblings and reported that children with autism had more than double the mealtime problems than their healthy siblings (13 *vs.* 5.0, respectively) [30].

Further complicating the issue is that some parents/caregivers choose to put their child on diets that exclude or restrict certain proteins, for example, gluten or casein. Based on a survey in 2006, 27% of families implemented an alternative diet for their children with ASD [39]. These diets are usually implemented among these children to reduce gastrointestinal issues, food sensitivity and/or allergies. These diets, if implemented without appropriate guidance, may result in compromised nutritional and health status. Dietary restriction has been shown to explain most of the differences in dietary adequacy between children with ASD and typically developing children [20]. Furthermore, a gluten-free casein-free diet that excludes dairy products may put the child at increased risk of calcium and vitamin D deficiency [19,24], and poorer bone density than those not on gluten-free casein-free diet [40].

Supplement use (including single nutrients and multi-nutrients) are among the most widely used adjunct treatment for ASD. Compared to the controls (25%), approximately two thirds of children with ASD (60%) regularly took a vitamin/mineral supplement [41]. It has also been reported that over-consumption of some nutrients in children with ASD could be

explained by dietary supplement use [19]. On the other hand, intake of nutrients above the tolerable upper limit may increase the risk of toxicity and be associated with many side effects.

Prevalence

There is a lack of New Zealand specific prevalence data, however according to Autism New Zealand, one person in 66 (1.5%) has ASD (including Asperger syndrome)[1]. The estimated prevalence rate of ASD in a nationally representative Australian sample has been suggested to be 2.5% using birth cohort and 1.5% using kindergarten cohort data [42]. In the US, the estimated prevalence rate for autism among 8-year-old children has been reported to range between one case in 34 (2.9%) and 59 (1.7%) children, which is 15-20% higher than the previous estimates (one case in 68 children, 1.5%) [43]. Differences in estimate prevalence have been attributed mainly to having increased access to school records. It has also been suggested that the prevalence estimate is slightly higher (4%) using the older definition of autism compared to DSM-5 [43]. These findings suggest that the new prevalence may underestimate the true prevalence among US children. On the other hand, it is important to note that the recent estimates have been suggested not to reflect the true prevalence due to the increased awareness among parents and clinicians, changes in screening and identification methods and reporting practices, changes in the sensitivity of diagnostic tools, changes in the diagnostic criteria, changes in the availability of services, and older populations receiving the diagnosis or avoiding seeking diagnosis [44,45].

Social, Psychological and Economic Burdens

Due to characteristics of ASD, both children on the spectrum and their parents/caregivers may experience various social, psychological and economic burdens. For example, children/adolescents with ASD have been reported to have fewer friendships, shorter duration of friendship, less frequent get-togethers [46], a less central position in social networks [47] and to experience a higher rate of bullying and victimisation [48,49]. Given these difficulties in social interaction, people with ASD have also been reported to have greater loneliness and more negative emotional and behavioural outcomes [50-52]. Furthermore, children with ASD have been reported to be two to three times more likely to experience an injury that needs medical attention than healthy controls, probably due the characteristics of condition, poor parental supervision and comorbidities [53,54]. In addition to these problems, many children with ASD experience poorer quality of life than their healthy counterparts [55], and have

[1] Retrieved from https://www.autismnz.org.nz/about_autism on 26th of July 2018.

below/low average achievement in at least one academic domain despite having average/above average intelligence quotient (IQ) [56].

Similarly, evidence suggests that parents/caregivers of these children report lower functioning of the family as a whole and reduced functioning as a family member [57], have problems with isolation from peers and their communities (mainly due to resource strain) [58] and have poorer quality of life [57,59]. Furthermore, ASD parents/caregivers have been reported to have higher level of parenting stress [57], more symptoms of depression and frequent use of Active Avoidance coping [60]. Finally, parents/caregivers of children with ASD may face a higher employment difficulties and financial burden than parents/caregivers of typically developing children [61].

ASD also imposes a large economic burden on families and society. It has been estimated that raising a child with ASD costs at least twice that of raising a typically developing child, because parents of these children earn less money, have higher expenses, smaller support networks, and considerable difficulties accessing child care, after-school care, and community services, as well as obtaining required services at school [62]. The cost of supporting an individual with ASD without intellectual disability has been estimated to average between US $1.4 to 2.4 million in the US and £0.9 to 1.5 million per child in the UK. Taking the prevalence of ASD into account, the societal cost has been estimated to be US $236 billion in the US and US $47.5 billion in the UK [63]. The cost estimate increases by approximately 75% if ASD co-exists with intellectual disability. The largest cost components are special education services, residential care or supportive living accommodation, individual productivity loss and medical costs for adults [63]. Leigh and Du (2015) suggested that the 2015 cost resembles the recent estimates for diabetes and ADHD and estimated that the cost will be US $461 billion in 2025 (including direct medical and non-medical costs as well as productivity cost) [64].

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Section 2: Vitamin D and ASD (Paper I)

Following a better understanding of ASD, in this section, I comprehensively review the evidence linking vitamin D to ASD. This evidence comes from epidemiological, observational, interventional and mechanistic studies.

This section was published in 2016 in "Nutrients.

Mazahery, H., C.A. Camargo, Jr., C. Conlon, K.L. Beck, M.C. Kruger, and P.R. von Hurst, Vitamin D and Autism Spectrum Disorder: A literature review. Nutrients, 2016. ***8****(4): p. 236.*

An update search was performed at the time of writing this thesis to see if there was any new evidence. In the case of finding such evidence, the information is included in footnotes.

To meet the requirement of this thesis "Figure 1" has been added here. Also, to align with the formatting and referencing style of this thesis, there are some changes in formatting and referencing style of published paper.

Vitamin D and Spectrum Disorder: A Literature Review

Abstract

Low vitamin D status in early development has been hypothesised as an environmental risk factor for Autism Spectrum Disorder (ASD), given the concurrent increase in the prevalence of these two conditions, and the association of vitamin D with many ASD-associated medical conditions. Identification of vitamin D-ASD factors may provide indications for primary and secondary prevention interventions. We systematically reviewed the literature for studies on vitamin D-ASD relationship, including potential mechanistic pathways. We identified seven specific areas, including: latitude, season of conception/birth, maternal migration/ethnicity, vitamin D status of mothers and ASD patients, and vitamin D intervention to prevent and treat ASD. Due to differences in the methodological procedures and inconsistent results, drawing conclusions from the first three areas is difficult. Using a more direct measure of vitamin D status – that is, serum 25(OH)D level during pregnancy or childhood – we found growing evidence for a relationship between vitamin D and ASD. These findings are supported by

convincing evidence from experimental studies investigating the mechanistic pathways. However, with few primary and secondary prevention intervention trials, this relationship cannot be determined, unless randomised placebo-controlled trials of vitamin D as a preventive or disease-modifying measure in ASD patients are available.

Introduction

Description of Autism Spectrum Disorder (ASD)

ASD is a neurodevelopment disorder characterized by impairment in socio-communicative functioning and by limited interests and repetitive and stereotypic behaviours [1]. Depending on the child's predominant symptomatology, ASD is comprised of different subgroups, including autism disorder, Asperger Syndrome and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) [2]. ASD usually appears during the first three years of life, but some cases appear later in life when social demands increase (regressive subtype) [1]. Individuals with ASD have: 1) difficulty in expressing and understanding certain emotions; 2) difficulty in understanding others' mood; 3) impairment in expressive language; 4) abnormal eye contact; 5) prefer minimal changes to routine, and 6) restricted ways of using toys and preference for isolated play, all of which make it difficult for individuals to establish relationships with others, to act in an appropriate way and to live independently [2]. Children with ASD also frequently experience behavioural and medical symptoms. Evidence of abnormalities in inflammatory markers, autoantibodies to the brain and glutathione in subsets of ASD patients has been reported; together, these findings suggest that ASD is a systemic condition with the likelihood of being a disease of inflammation, autoimmunity, and/or oxidative stress [3-16].

Over the past few years, the prevalence of ASD has increased dramatically. While previous prevalence studies identified fewer than 10 in 10,000 individuals [17], recent estimates suggest rates of 90 to 250 in 10,000 individuals [1,18-20]. Although this increase is, in part, attributed to the increased awareness and reporting of the disorder, as well as improved diagnostic criteria [21], a complex of genetic and environmental risk factors has also been implicated. It is now believed that ASD is genetically driven and can be triggered by environmental risk factors [22]. In 2008, Cannell published the hypothesis that low vitamin D status, either during foetal life or early childhood, is an important environmental risk factor for ASD [23]. Since then, the role of vitamin D in the aetiology of ASD has drawn epidemiologists' attention, but intervention research on the role of vitamin D as a preventive and disease-modifying measure is still in its infancy.

Vitamin D: Metabolism, Biomarker, and Optimum Level

Vitamin D, a fat-soluble vitamin, is a general name for a collection of steroid-like substances including ergocalciferol (D_2) and cholecalciferol (D_3) [24]. Vitamin D is present in the diet in limited amounts and is obtained mainly from exposure of skin to UVB radiation (**Figure 1**). During sun exposure, 7-dihydroxycholesterol which is present mainly in the layers of epidermis and to a lesser extent in dermis [25] absorbs UVB radiation and is converted to pre-vitamin D_3 [24]. Then pre-vitamin D_3 undergoes heat-induced isomerisation and forms vitamin D_3. Vitamin D_3 is derived from a cholesterol metabolite, and vitamin D_2 is the product of irradiation of plants [24]. Vitamin D binding protein (DBP) in the circulation is responsible for the transport of both vitamins D_2 and D_3. Carried by DBP, these vitamin D metabolites enter the liver where they are hydroxylated and converted to their respective 25-hydroxyvitamin D metabolites (e.g., 25(OH)D_3) [24,25]. These metabolites are inactive until they are converted to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] by 25-hydroxyvitamin D-1α-hydroxylase (1-OHase), and become biologically active [25,26]. At this point, vitamin D can bind to the vitamin D receptor (VDR) – found in most body tissues and organs [24,26] — and exert its musculoskeletal and non-musculoskeletal role in the body. However, because 1,25$(OH)_2$D synthesis is tightly regulated, circulating 25(OH)D has been suggested to be the best single indicator of vitamin D status; it reflects both dietary vitamin D intake and vitamin D synthesised by UVB radiation [27,28].

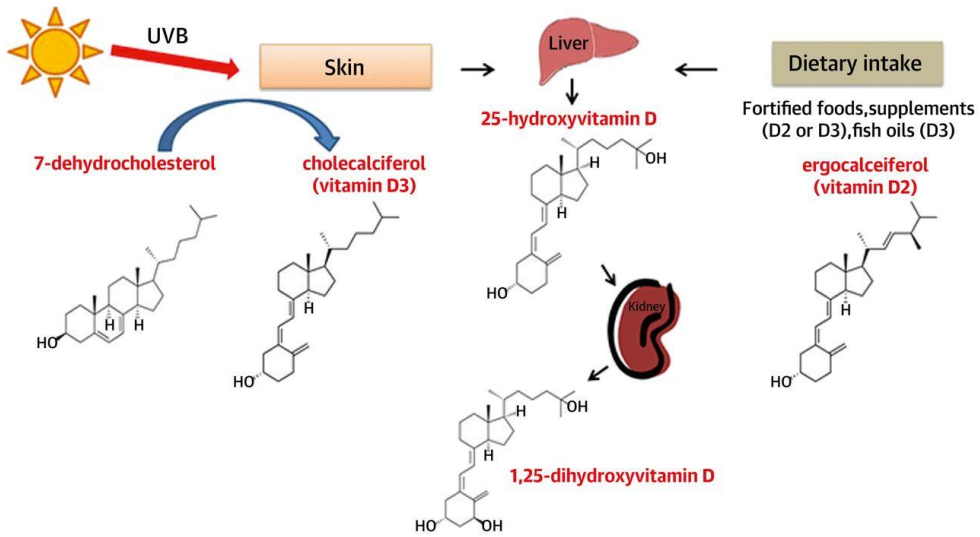

Figure 1: Vitamin D synthesis and Metabolism. Vitamin D is photosynthesized in the skin and is also obtained from diet. The active form of vitamin D, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], is formed by two hydroxylation steps in the liver and the kidney. Adapted from [25] and "reproduced with permission".

Vitamin D deficiency and insufficiency are controversial terms but generally defined as serum 25(OH)D concentration <25 nmol/L and 25-49.9 nmol/L, respectively [29]. Other cut-offs for

vitamin D deficiency and insufficiency are used by other groups, e.g., <50 and 50-74.9 nmol/L, respectively [30]. Until there is more clarity on these terms, we believe it is best to report the actual values rather to rely on ambiguous terms.

Objective

We aimed to investigate the relationship between vitamin D and ASD, and to systematically review the literature for all studies on this relationship, including studies on the mechanistic pathways that might underlie this relationship.

Materials and Methods

Firstly, we reviewed previous reviews [23,31,32] to identify vitamin D-related risk factors related to ASD. We grouped the identified factors into seven specific areas including 1) latitude; 2) season of birth and conception; 3) maternal migration and ethnicity; 4) vitamin D status of mothers; 5) vitamin D status of ASD patients; 6) maternal vitamin D intervention to prevent ASD; and 7) vitamin D intervention to treat ASD. According to their relationship with prevention stages, each of these areas were assigned to one of the three major areas of; 1) vitamin D-ASD-related areas providing indications for primary prevention; 2) vitamin D-ASD-related areas providing indications for both primary and secondary prevention; and 3) vitamin D-ASD-related areas providing indications for secondary prevention.

We performed a literature search covering studies published up to January 31 2016 in PubMed, the Web of Knowledge, EBSCO OVID, MEDLINE, PsycARTICLES, PsycINFO, SocINDEX and Google Scholars databases. Because it was impossible to cover all specific areas with one fixed term search we employed a distinct search strategy for each specific area. The search strategy was as follows: autism or Asperger or "Autism Spectrum Disorder" or ASD or "Pervasive Developmental Disorder" or PDD AND the following distinct search terms for each specific area;

- "incidence" or "prevalence" to search the literature in relation to latitude. For studies published between 1992-2012, we identified literature from a previous systematic review of autism disorder and PDD prevalence worldwide [33] to have a manageable dataset for searching. We excluded studies published before 1992 because case ascertainment was based on DSM-III and ICD-9.
- "season" or "month" AND "birth" or "conception" to search the literature in relation to Season of conception or birth.

- "migrant" or "immigrant" or "migration" or "immigration" AND "maternal" or "mother" to search the literature in relation to maternal migration and ethnicity
- "vitamin D" or ergocalciferol or "vitamin D2" or "cholecalciferol" or "vitamin D3"or "25-hydroxyvitamin D" or "25(OH)D" or "25ohd" to search the literature in relation to maternal vitamin D status, vitamin D status in ASD patients, maternal vitamin D intervention to prevent ASD, and vitamin D intervention to treat ASD.

We followed this summary with a presentation of the mechanistic pathways by which vitamin D may exert its role in either aetiology or pathobiology of ASD. The selection criteria for each of these specific areas varied and, for maximal clarity, the criteria are presented at the beginning of each specific area.

Results: Three Major Areas of Research

Vitamin D-ASD-Related Areas Providing an Indication for Primary Prevention

In this section, we will discuss vitamin D-related risk factors related to ASD which can be targeted for preventive purposes before ASD occurs. We found five specific areas including latitude, season of conception and birth, maternal migration and ethnicity, vitamin D status of mothers, and maternal vitamin D intervention to prevent ASD.

Risk of ASD – Latitude

We included studies that met the following criteria: 1) reported a prevalence estimate for either of the following: Autism Disorders (AD), ASD (including AD, Asperger's Syndrome, PDD-NOS and PDD (ASD plus Rett's disorder and Childhood Disintegrative Disorder); 2) case ascertainment was based on either Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV) or International Classification of Diseases and related health problems-Tenth Edition (ICD-10), and 3) included children <8 years of age. Studies that used medical registry or records only were excluded. There are several methodological factors that affect the prevalence estimates of ASD and these include classification systems, case ascertainment and participants' age. In studies using DSM-III, the prevalence is much lower than in those using DSM-IV or the Chinese classification system of Medical Disorders (CCMD) [34]. Also, some individuals previously diagnosed with ASD according to DSM-IV might miss out on a diagnosis with the new criteria outlined in DSM-5 because the definition of autism has gone through three major changes [35]. Several separate categories in DSM-IV including AD, Childhood Disintegrative Disorder, Asperger's Syndrome and PDD-NOS has

been included into the one overarching category named "ASD", the three domains of impairments in social interaction, impairments in communication and stereotyped behaviour in DSM-IV have become two, with the merging of the first two domains (deficits in social-communicative functioning), and finally the diagnosis age of three has been dropped and included manifestation of disorders either currently or by history [2].

Our literature search identified prevalence data for eight countries [17,36–45]. Four studies were from Asia (latitudes 24.46-39.91° N) [36,42,43,45], one from South America (34.28° S) [44] and six from Europe (51.24–63.91° N) [17,37-41]. We did not identify any study from the US, Africa and Australia/New Zealand that met our inclusion criteria. Among the included studies, different tools were employed, including eight screening instruments and seven diagnostic tools. There were seven studies that reported prevalence estimate for AD [17,36-41], four for PDD [42-45] and three for both AD and PDD [17,37,38]. Data regarding the prevalence estimate for AD and PDD are presented in Tables 1A and 1B, respectively. The AD prevalence estimate per 10,000 ranged from 11 in China [36] to 60 in Sweden [40]. The PDD prevalence estimate ranged from 6.3 in Iran [43] to 181 in Japan [45], and, on average, was >30 in Europe.

Two systematic reviews of epidemiological studies illustrated a regional variation in the prevalence estimate for autism [46], and a four-fold increase in the prevalence estimate for infantile autism in moving from Israel to Sweden latitudinally [47]. Consistent with these findings, we also observed a latitudinal variation in the prevalence estimate for AD and PDD, with those in the lower latitudes reporting lower prevalence estimates and vice versa. There were, however, a few exceptions; compared to Sweden [39], Finland had lower AD prevalence estimate despite being located at the quite similar latitude [41]. Mandatory vitamin D food fortification of certain foods and dietary supplementation policies may have improved vitamin D status in Finish populations [48-50], and may explain the lower prevalence estimate for AD in this country. The higher prevalence estimate for PDD in Japan compared with European countries may also, in part, be explained by the higher prevalence of low vitamin D status among Japanese women than European women. Approximately 95% of Japanese pregnant women have been reported to have 25(OH)D levels <50 nmol/L; the corresponding figures in the UK and Latin America have been reported to be 55% and 37%, respectively [51,52], a finding confirmed by a systematic review of global maternal and new born vitamin D status [53]. This may be due to lower dietary vitamin D intake and more sun—avoidance behaviour in Japanese population [54].

The latitude differences in prevalence estimates for autism should be interpreted with caution since they assume that latitude is a reasonable proxy measure for vitamin D status. If low vitamin D status is a risk factor for autism, it is expected to see high prevalence of autism in countries with high prevalence of low vitamin D status and vice versa. This relationship is well illustrated by both low prevalence estimates for PDD and vitamin D deficiency/insufficiency in Argentina (34.28° S). The prevalence estimate for PDD was 13 per 10,000, and for 25(OH)D levels <25 and <50 nmol/L was reported to be only 3% and 33% in young children and adults, respectively [55].

However, the low prevalence estimate for AD in China and PDD in Iran and UAE despite having high prevalence of low vitamin D status [56–58] may be due to methodological issues (screening and diagnostic characteristics) and underreporting. Translation of a screening or diagnostic instrument may adversely affect its psychometric properties and therefore its sensitivity in picking up the cues for autism [43]. This is an issue for non-English speaking developing countries such as Iran and UAE. Employing different screening cut-off ranges can also be a driving factor; using a wider cut-off range includes more children for the final diagnosis and thus fewer children are likely to miss the diagnosis. Furthermore, a shortage of service delivery, stigmatising effect of autism on parents, parental tolerance and expectations of children's behaviour and cultural influences and norms for atypical behaviours may be associated with underreporting of autism in developing countries [59].

Within each study population, it is also important to consider UVB doses reaching the population, differences in skin pigmentation, sun-avoidance or seeking behaviours, clothing style, and racial and ethnic differences. The prevalence of infantile autism in cohorts born before 1985 was reported to be in the earlier months to the North, perhaps partly, it is associated with the lower UVB doses and therefore lower vitamin D production [47]. Grant *et al.* (2013) reported an inverse relationship between the solar UVB radiation doses and the prevalence of ASD [60]. Accordingly, living at higher latitudes might be associated with lower doses of UVB radiation, if not compensated by more sun exposure or ingestion of vitamin D rich foods or supplements, and consequently increased risk of ASD.

Risk of ASD – Migration and Ethnicity

We included studies that 1) reported odds ratio (OR), prevalence ratio or relative risk for AD, ASD and/or PDD; 2) reported mother's country of birth or race/ethnicity; and 3) case ascertainment was based on formal diagnoses according to disease classification systems.

Our literature search identified 10 studies [61-70], with seven studies from Europe [61-65,67,70], two from the US [68,69] and one from Australia [66] (**Table 2**). The number of cases ranged from 250 in a study from Sweden [67] to 7540 in a study from the US [69]. Depending on the study design, statistical analysis were adjusted for a range of confounding factors, ranging from only one factor [63] to several factors in other studies [61,62,64-70], though the same factors were not considered by all studies.

Most studies showed increased risk of ASD among children of migrant parents [61–67,69,70], while some reported no association [61,65,68,69] or even decreased risk [61,68-70]. Williams *et al.* (2008) reported that the odds of having a child with ASD among mothers born outside Australia significantly increased compared with those born in Australia, 1.4 [95% CI, 1.0, 1.9], *P*=0.009 [66]. The mothers' original countries were not reported. Keen *et al.* (2010) reported an adjusted OR ranging from 1.2 [95% CI, 0.8, 1.9] in children of European mothers born outside the UK to 10 [95% CI, 5.5, 18] in children of Caribbean mothers compared with mothers born in the UK [63]. Magnusson *et al.* (2012) also reported an increased risk of having a child with ASD among mothers born outside Sweden as compared to those born in Sweden [61].

Magnusson *et al.* (2012) reported an increased risk of low functioning autism and decreased risk of high functioning autism in children of migrant parents in Sweden, 1.2 [1.0, 1.4] and 0.5 [0.4, 0.6], respectively [61], a finding confirmed by Williams *et al.* 2008 [66] and Becerra *et al.* (2014) [69]. The risk of having a child diagnosed with both autism and mental retardation increased two-fold in foreign-born black, Vietnamese and Filipino mothers [69]. In a study from the US, the proportion of children with ASD with intellectual disability was significantly different between black, Hispanic and white children: 48%, 38% and 25%, respectively [71]. A possible explanation is that mothers with darker skin are at increased risk of low vitamin D status and moving to northern latitudes with lower solar UVB radiation may exacerbate the condition and thereby lead to more severe disability among their children. It should be pointed out that having 25(OH)D levels in this study would address that explanation.

van der Ven *et al.* (2013), however, reported a decreased risk for ASD in children whose mothers were born in developing countries compared with those born in developed countries [70]. Within the ASD subtypes, a significant increased risk for AD and a decreased risk for Asperger Syndrome and PDD-NOS combined was reported in mothers of developing countries. These findings are consistent with several other studies reporting an increased risk for AD [62,64,65,67-69] and decreased risk for Asperger Syndrome [67] in children of mothers born outside the reference country. These findings are mirrored in studies

investigating the effect of child's ethnicity on prevalence estimate of ASD. While children of black ethnicity had increased risk for AD, 2.6 [1.3, 5.0], the risk for Asperger Syndrome and PDD-NOS decreased in these children 0.5 [0.2, 1.0] [72]. An explanation is that the aetiology of AD might be different from those of Asperger Syndrome and PDD-NOS in different race/ethnic group and that low vitamin D status which is highly prevalent in some race/ethnic groups, could exacerbate the condition, and cause AD.

The question remains why migration status is associated with no effect or decreased risk in some studies. Those showing no or decreased effect of maternal migration status mainly compared the prevalence estimates for autism among mothers born in the same region or of white ethnicity [61,64,65,69]. These populations share quite homogeneous characteristics with those of the reference country – the majority, if not all, have white skin colour and quite similar culturally accepted dressing code and attitudes toward sun exposure – that may affect vitamin D status of these populations.

The risk of AD was reported to be higher in children of mothers born in Mexico and in East Asian countries living in the US than those of mothers born in the US [68,69]. The decreased association reported in these studies may, in part, be attributed to the statistical analysis and the characteristics of minority groups. Although the relative risk of having a child with AD in mothers born in Mexico was 0.6 [95% CI, 0.5-0.7] in the Croen *et al.* study [68], it was 1.1 [95% CI, 1.0, 1.2] in another study by Becerra and colleagues [69]. In the latter study, statistical analysis was adjusted for more variables (13 *vs.* 7 variables, respectively), including diagnostic variability [68,69]. It is well documented that diagnostic variability contributes significantly to the prevalence estimates [34]. The effect of covariates on final findings is also illustrated by the lower crude rates for ASD in black and US-born Hispanics that was similar or slightly higher than the US-born whites when the statistical analysis was controlled for several covariates [69]. Furthermore, minorities might be under-identified for ASD due to lower socioeconomic status, cultural differences in views of typical and atypical behaviours, viewing communication and social skill delays as temporary that disappear with age, parent-health professional communication gap and lack of culturally sensitive screening and assessment tools [73]. Finally, not all studies controlled for sun exposure variables; therefore it is difficult to relate these results directly to vitamin D-ASD relationship.

Risk of ASD – Season of Conception and Birth

We included all studies that relied on formal diagnoses of autism (according to disease classification systems) and reported season/month of conception or birth in patients with autism (infantile autism, AD, and ASD).

Our literature search identified 20 studies (**Table 3**); with one study reporting the season/month of conception only [74], three reporting both the season/month of conception and birth [75–77], and 16 reporting the season/month of birth only [64,78–92]. The number of cases ranged from 54 in a Turkish study [79] to 19,328 in a US study [75]. Most participants in the control groups were live births (13 studies), two included siblings [78,85], one, both siblings and live births [89], three healthy age and sex matched children [78,79,81], and one compared cases with other neuro-developmental disorders [86]. Most of these studies did not control for any covariates in the statistical analysis [76,78,79,81,84,86-89,91,92]. Eight studies made adjustments for covariates ranging from two [82,83] to 12 covariates [64], though different factors were considered in these studies.

Children conceived during the winter months, when sun exposure is limited and vitamin D status is lower, were reported to have a higher risk of developing autism later in life than those conceived during the summer months [74]. Masumdar *et al.* (2012) [74] reported an increased rate of children later diagnosed with AD conceived in winter (final three weeks of November and first weeks of December) in California from 1994-1996 ($P<0.05$), though the seasonality finding disappeared between 1996 to 2000. The authors did not control for maternal residence at birth. While controlling for several variables including year of conception and maternal residence at birth, Zerbo *et al.* (2011) reported a 6% (OR 1.1, 95% CI 1.0, 1.1) increase in the risk of AD in children conceived during winter compared with summer months in California between 1990-2002 [75]. The incidence rate of AD increased steadily from the conception month of August to March, decreased from March to April and then remained unchanged from April to July. The rate of AD in November births was significantly higher compared to April.

By contrast, Hebert *et al.* (2010) reported an increased rate of children later diagnosed with ASD in those conceived during summer in the UK; with a conception rate of 9.5/1000 *vs.* 5.7/1000 in summer (June – August) and winter (December-February) months, respectively, with a corresponding peak in spring births, an OR of 1.9 [1.0, 3.4] [76]. The study had a small sample size of 86 cases, and the age, duration and follow up measures were not clearly stated. Moreover, there was no adjustment for potential confounding.

Table1A: Risk of Autism Disorder (AD) in relation to latitude.

Reference	Country, Area	Latitude	Age	Diagnosis	Prevalence/10,000	CI
[36]	China, Tianjin	39.13° N	8	DSM-IV/CABS-CV, CARS-CV	11	3.4, 25
[37]	UK, South East Thames	51.24° N	7	ICD-10/CHAT, CR, PDD-Q	31	23, 41
[38]	UK, Staffordshire and Cannock	52.80° N	4–6	ICD-10, DSM-IV/ multidisciplinary screening ADI-R	19	14, 25
[17]	UK, Staffordshire	52.80° N	2.5-6.5	DSM-IV/Clinical evaluation, ADI-R	17	11, 25
[39]	Sweden, Goteborg	57.70° N	3–6	ICD-10/Clinical evaluation, ADI-R	46	16, 77
[40]	Sweden, Karlstad	59.37° N	7	ICD-10/ASSQ (cut off teachers only: 17), Clinical evaluation, ADI-R	60	19, 141
[41]	Finland, Northern Ostrobothnia	63.91° N	8	DSM-IV-TR, DSM-5/ASSQ (cut off: 30 parents and teacher combined), ADI-R, ADOS-3	41	26, 64

Table 1B: Risk of Pervasive Developmental Disorder (PDD) in relation to latitude.

Reference	Country, Area	Latitude	Age	Diagnosis	Prevalence/10,000	CI
[42]	UAE, 3 regions	24.46° N	3	DSM-IV/ASC (cut off: 15), Clinical evaluation	29	0, 79
[43]	Iran, Country wide	32.00° N	5	DSM-IV/National screening, SCQ, ADI-R	6.3	5.8, 6.7
[44]	Argentina, San Isidro	34.28° S	0-5	DSM-IV/PRUNAPE, BDI, VABS, multi-disciplinary evaluation	13	-
[45]	Japan, Toyota	35.08° N	5-8	DSM-IV/ Integral screening system, Direct clinical evaluation	181	-
[37]	UK, South East Thames	51.24° N	7	ICD-10/CHAT, CR, PDD-Q	58	47, 71
[38]	UK, Staffordshire and Cannock	52.80° N	4-6	ICD-10, DSM-IV/ multidisciplinary screening, ADI-R	59	45, 75
[17]	UK, Staffordshire	52.80° N	2.5-6.5	DSM-IV/Clinical evaluation, ADI-R	62	50, 76

DSM-IV, Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition; CABS-CV, Clancy Autism Behaviour Checklist-Chinese Version; CARS, Childhood Autism Rating Scale; ICD-10, International Classification of Diseases and Related Health Problems (10th edition); CHAT, Checklist for Autism in Toddlers; CR, Checklist for referral; PDD-Q, Pervasive Developmental Disorder-Questionnaire; ADI-R, Autism Diagnostic Interview-Revised; ASSQ, Autism Spectrum Screening Questionnaire; ADOS-3, Autism Diagnostic Observation Schedule-Module 3; ASC, Autism Screening Questionnaire; SCQ, Social Communication Questionnaire; PRUNAPE, Prueba Nacional de Pesquisa; BDI, Battelle Developmental Inventory; VABS, Vineland Adaptive Behaviour Scales.

Lastly, Atladottir *et al.* (2007) found no association between season of conception or birth on incidence rate of ASD and ASD subcategories in a cohort born in Denmark between 1990 to 1999 [77]. The sample size was smaller (*n*=1860) than the sample size in the Masumdar *et al.* and Zerbo *et al.* studies (*n*=8,074 and 19,328, respectively), and the authors did not control for several variables included in the latter studies.

Season/month of birth and risk of autism has also been a research focus among epidemiologists. Spring birth has been associated with increased risk of autism in studies conducted in Canada [92], Japan [91], the US [82], Sweden [78,90], and the UK [76]. March birth as a risk factor for autism has also been reported by several studies from Canada [92], Denmark [80,88], the US [85] and Israel [87]. Lee *et al.* (2008) reported three peaks during spring, summer and autumn months in singleton and multiple births concordant with ASD (*n*=1051), and an 87% decreased risk for multiple births concordant for ASD in males in winter (December) compared to other seasons (relative risk 0.2, [95% CI 0.04, 0.8]; $P<0.05$) [82]. Consistent with these findings, Konstantareas *et al.* (1986) and Gillberg *et al.* (1990) also reported a significant deviation of season/month of autistic births from that of the general population among males, a pattern that was not evident in females (*n*=36 and 25, respectively) [90,92]. Konstantreas *et al.* commented that this finding could be due to the sampling artefact. With a male: female ratio of 4: 1 and an ASD multiple birth sample size of 161 [82], the results related to sex differences in the Lee *et al.* study (2008) may also be a sampling artefact.

In contrast, Maimburg *et al.* (2010) reported increased risk for infantile autism in those born in winter (October to March) [80]. Aggregation of different months into seasons or half years can result in different results or even loss of information. Bolton *et al.* (1992) reported different results by moving one month from a season and including it in the next month, for instance inclusion of December in the winter or autumn months [89]. A seasonal effect of birth on autism was reported when December was included in the winter; however, this pattern disappeared when December was included as an autumn month. As such, inclusion of both December and March in winter in the Maimburg *et al.* study (2010) [80], may explain the increased rate of autism births during this season.

Not all studies showed an association between season of birth and risk of autism [64,77,79,81,83-86]. The absent association in these studies may be attributed, at least in part, to the definition of season (discussed previously). Hultman *et al.* (2002) [64] categorised months into two seasons; four months in one season *versus* eight months in other season, a method that may have obscured the association. Furthermore, the small sample size in the

Meguid *et al.* (2010), Ugar *et al.* (2014) and Fernell *et al.* (2015) studies (all n <70) may have contributed to their non-significant findings.

The characteristics of the populations included in case and control groups, and case ascertainment may also mask the seasonal variability among groups. For instance, Fennell *et al.* (2015) did not find any seasonal deviations for ASD populations from the typically developing controls of Middle Eastern/African origin, though the season of birth difference was significant in cases and controls of Swedish origin (38% in spring *vs.* 18% in summer; P<0.05) [78]. This could be due to the narrow range and low year-round mean 25(OH)D concentrations in these populations (29-33 nmol/L in winter and summer, respectively) [93]. Kolevzon *et al.* (2006), identified cases through a screening programme for military services in Israel and Orthodox women were exempted from the military service. Therefore, some cases with autism might have been missed [83]. Landau *et al.* (1999) included cases with autism from different regions with different environmental influences and controls were individuals with mental retardation [86]. Disorders of neurodevelopment may share the same environmental influences and inclusion of these disorders in the control group may mask the seasonal variability among groups.

Interestingly, some studies did not find a season/month of birth effect on aggregated samples but reported seasonal deviations from the general population in autism with comorbidities such as language and developmental delay [84,85]. For example, Yeates Frederiks *et al.* (2000) did not find an effect of season of birth on autism rate in the aggregated sample, but they found a significant seasonal trend for low functioning autism (autism with IQ < 35) (increased rates for autistic births in the second quarter of the year) [84]. This reflects a trend similar to the Konstantreas *et al.* (1986) [92] and Stevens *et al.* (2000) [85] findings of a more pronounced seasonal effect on low functioning and or nonverbal autism. The differences in the seasonal effect of birth and risk of low and high functioning autism might be attributed to different etiological factors or to the same etiological factors but of different intensity. Whitehouse *et al.* (2012) reported a significant positive relationship between the severity of maternal vitamin D deficiency and the severity of the language impairment (mild and moderate to severe) in children at five and 10 years of age [94]. As such, the lack of season of birth variation does not exclude the possibility of an association if autism with comorbidities was considered.

Finally, seasonal of birth is a crude proxy measure for sun exposure and, more specifically, vitamin D status; many other potentially relevant factors vary by season. These environmental factors include application of pesticides [95], infection [96], nutritional factors [97] and

maternal biochemistry [98], all of which have been hypothesised to be implicated in the aetiology of ASD, and need to be taken into consideration when interpreting the literature on season of conception/birth and risk of ASD.

Risk of ASD – Vitamin D Status in Mothers

We included all studies reporting maternal vitamin D status and the risk for AD, Asperger Syndrome and or ASD in offspring. Our literature search identified two articles investigating the relationship between maternal vitamin D status and autism among offspring. Fernell *et al.* (2010) compared maternal 25(OH)D concentration of Swedish mothers with and without a child with autism (*n*=12 with vs 14 without) and Somali mothers with and without a child with autism (*n*=14 with vs 17 without) in autumn and spring [99]. More than 60% and 85% of mothers of Somali origin had 25(OH)D <25 nmol/L in spring and autumn, respectively, compared to only one mother of Swedish origin in both seasons. The difference in 25(OH)D concentrations was not significantly different between mothers with and without a child with autism for either ethnicity. This study is limited, however, by its small sample size and the long time period since pregnancy (average age of more than 4 years). Furthermore, a lack of difference could be also attributed to the very low and narrow maternal 25(OH)D concentration among mothers of Somali origin which does not allow the relationship between vitamin D status of mothers and risk of ASD to be addressed.

To overcome these limitations, Whitehouse *et al.* (2013) investigated the relationship between maternal 25(OH)D concentrations at 18 weeks of pregnancy and autism phenotype in the offspring at ages 5, 8, 10, and 14 years follow up [100]. The authors did not find any difference in 25(OH)D concentrations among mothers of children with and without clinical ASD (*n*=929) while controlling for several variables (maternal education, socioeconomic status, maternal race, age at conception, maternal smoking, alcohol intake during pregnancy, parity, infant health and sex). It should be noted that only three males were clinically diagnosed with ASD at follow up and extrapolating these results to the broader ASD population should be done with caution. Maternal 25(OH)D concentrations were not associated with the total score and four out of five subscales of Autism Like Quotient (autistic-like traits) among 406 offspring. However, compared to children of mothers in the higher tertiles, children of mothers in the lower tertile (25(OH)D <45 nmol/L) had higher scores on attention switching subscale (OR 5.5 [95% CI, 1.3, 23], $P<0.05$) while controlling for several potential confounders, including season of blood collection. A limitation of this study is that only 14% of offspring from the original cohort completed the study. The high attrition rate was biased toward the loss of mothers with lower socioeconomic status. It was shown that total autistic like trait score was

higher among offspring of mothers with low socioeconomic status and of mothers with low education levels. On the other hand, evidence shows that both lower socioeconomic status and lower maternal education are associated with lower vitamin D status [101,102].

Consistent with these findings, Whitehouse *et al.* (2012) and Morale *et al.* (2012) also showed that lower maternal 25(OH)D concentration in pregnancy (weeks 18 and 14, respectively) was associated with more language difficulties and mental and psychomotor outcomes in offspring, respectively [94,103]. While controlling for several variables, maternal 25(OH)D concentration <46 nmol/L was associated with significant language difficulties in the offspring at ages 5 and 10 years compared with those offspring of mothers with 25(OH)D concentrations >70 nmol/L [94]. Maternal 25(OH)D concentration >75 nmol/L in pregnancy was associated with increased mental and psychomotor scores in infants at 14 months compared to those with 25(OH)D concentrations <50 nmol/L [103].

By contrast, Gale *et al.* (2008) could not find any relationship between maternal 25(OH)D concentration during pregnancy and child's intelligence and psychological health at ages 9 months and 9 years [104]. The inconsistent findings could be, in part, attributed to important methodological issues and the timing of blood collection during pregnancy. At the follow-up, the sample size was smaller (n=178) and the attrition rate was higher (70%) in the Gale *et al.* study [104] than the Whitehouse *et al.* and Morales *et al.* studies (n=412 and 50% and n=1826 and 25%, respectively) [94,103]. Maternal 25(OH)D concentrations during pregnancy and infants' neuropsychological health might have differed between the many lost to follow-up and this could have biased the results.

Furthermore, while the blood sampling was done in late pregnancy (week 33) in the Gale *et al.* study [104], blood samples were collected during weeks 18 and 14±2.1 of pregnancy in the Whitehouse *et al.* and Morales *et al.* studies [94,103], respectively. These findings suggest that depending on the timing of the exposure to low vitamin D status, different brain areas might be affected and the consequence might be different neurodevelopment and cognitive outcomes in infants [105].

Vitamin D Intervention to Prevent ASD

We included all studies reporting using vitamin D supplements during or before pregnancy in relation to ASD in the offspring. We excluded those studies using vitamin D containing multivitamins or minerals only because those doses are relatively low and the effect of vitamin D *per se* cannot be determined from these interventions.

Table 2: Risk of autism in mothers born outside the reference country and according to mothers' ethnicity

Reference	Reference Country/Year	Cohort	Age (Year)	Cases Number	Cases Ascertainment	Mother's Country of Birth/Ethnicity	Odds Ratio [95% CI]	Covariates
[64]	Sweden 2002	1987-1994	<10	408	AD/ICD 9	Europe and North America Outside Europe and North America	1.1 [0.5, 2.5] 3.0 [1.7, 5.2]	Maternal age, parity, smoking during pregnancy, hypertensive disease, diabetes, pregnancy bleeding, mode of delivery, season of birth, gestational age, birth weight, Apgar score, congenital malformation
[68]	US/California 2002	1989–1994	-	4381	AD/DSM-III-R or DSM-IV	Other US states Mexico Other	0.9 [0.8, 1.0] 0.6 [0.5, 0.7] 1.1 [1.0, 1.2]	Sex, birth weight, plurality, birth order, maternal age, maternal race, maternal education RR
[65]	Denmark 2005	1984-1998	<10	818	AD, atypical autism/medical record registry/ICD-10	Scandinavia and Europe Outside Europe	1.0 [.8, 1.4] 1.4 [.1, 1.8]	Age, sex, interaction between age and sex, calendar year, history of autism or broader autism in siblings psychiatric disorders RR

Table 2: *Cont.*

Reference	Reference Country/Year	Cohort	Age (Year)	Cases Number	Cases Ascertainment	Mother's Country of Birth/Ethnicity	Odds Ratio [95% CI]	Covariates
[62]	Denmark 2006	1990-1999	<10	473	AD/ICD-8 and 10	Foreign Citizenship (not mentioned)	1.7 [1.3, 2.4]	Maternal and parental age, maternal citizenship, birth weight, gestational age, Apgar score, irregular foetal position, congenital malformation, psychoactive medicine use in pregnancy
[66]	Australia 2008	1990-1996	<5	368	ASD/Surveillance/DSM-IV	Outside Australia	1.4 [1.0, 1.9]	Sex, maternal age ≥35, gestational age <37
[67]	Sweden 2010	1980-2005	<25	250	AD and AS/DSM-IV or DSM-III or ICD-10/ADOS-G and ADI-R	Outside Nordic countries:		Year of birth, maternal age ≥40, gestational age <37, gestational age-adjusted birth weight
						AD	2.2 [1.6, 3.1]	
						AS	0.6 [0.3, 1.0]	
						Sub-Saharan Africa	5.6 [2.9, 11]	
						South or Central America	3.1 [1.3, 7.1]	
						East Asia	2.9 [1.4, 6.1]	
						Western Europe/USA	2.8 [1.2, 6.5]	
						Previous Eastern Europe	2.1 [1.3, 3.3]	
						Middle East/North Africa	2.0 [1.2, 3.2]	

Table 2: *Cont.*

Reference	Reference Country/Year	Cohort	Age (Year)	Cases Number	Cases Ascertainment	Mother's Country of Birth/Ethnicity	Odds Ratio [95% CI]	Covariates
[63]	UK 2010	1999-2005	<18	428 (267, Wandsworth and 161 in Lambeth)	ASD/2 boroughs/Multidisciplinary team assessment/ICD-10 using ADI-R, DISCO, ADOS	Other European		Family size RR
						L	1.3 [0.6, 2.8]	
						W	1.2 [0.8, 1.9]	
						African		
						L	7.9 [5.4, 12]	
						W	3.3 [2.4, 4.5]	
						Caribbean		
						L	10 [5.5, 18]	
						W	8.9 [5.9, 16]	
						Asian		
						L	4.0 [2.0, 7.8]	
						W	2.1 [1.3, 3.3]	
[61]	Sweden 2012	2001-2007	0–17	3918	ASD/Medical registry/Multidisciplinary teams/DSM-IV	African, American, Asian and European Low functioning	1.2 [1.0, 1.4]	Maternal and parental age, family disposable income, for subsample, birth weight, gestational age, Apgar score at 5 min after birth
						High functioning	0.5 [0.4, 0.6]	

Table 2: *Cont.*

Reference	Reference Country/Year	Cohort	Age (Year)	Cases Number	Cases Ascertainment	Mother's Country of Birth/Ethnicity	Odds Ratio [95% CI]	Covariates
[70]	Netherlands 2013	1998-2007	-	518	ASD/Psychiatric case registry/DSM-IV	Developing countries		Sex and paternal age RR
						ASD	0.6 [0.5, 0.9]	
						AD	1.4 [0.9, 2.4]	
						AS and PDD-NOS	0.4 [0.3, 0.6]	
						Developed countries		
						ASD	0.9 [0.6, 1.3]	
						AD	1.6 [0.8, 3.5]	
						AS and PDD-NOS	0.6 [0.4, 1.1]	
[69]	US/Los Angeles 2014	1995-2006	-	7540	AD/DSM-III-R/ICD-9-CM/ADOS	White foreign	1.0 [0.9, 1.2]	Maternal age, sex, birth year, birth type, parity, gestational age, birth weight, pregnancy complications, trimester pregnancy care began, maternal education, insurance and diagnostic variability (regional centres) RR
						Black foreign	1.8 [1.4, 2.2]	
						Mexico	1.1 [1.0, 1.2]	
						Central/South America	1.3 [0.9, 1.1]	
						China	0.7 [0.6, 0.8]	
						Japan	0.7 [0.5, 1.0]	
						Korea	1.0 [0.8, 1.2]	
						Philippines	1.3 [1.1, 1.4]	
						Vietnam	1.4 [1.2, 1.7]	

AD, Autism Disorder; ICD, International Classification of Diseases; US, The United States; DSM, Diagnostic and Statistical Manual of Mental Disorders; ASD, Autism Spectrum Disorders; ADOS, Autism Diagnostic Observation Schedule; ADI-R, Autism Diagnostic Interview-Revised; AS, Asperger; UK, The United Kingdom; DISCO, Diagnostic Interview for Social and Communication Disorders; W, Wandsworth; L, Lambeth.

Our literature search identified only one study reporting on the effect of vitamin D supplementation during pregnancy on the recurrence rate of autism in new born siblings [106] (**Table 4**). In an open-label prospective study, Stubbs, Henley and Green (2016) prescribed vitamin D at a daily dose of 5000 IU to mothers of children with ASD during pregnancy (majority during the second trimester, *n*=19), 7000 IU during breastfeeding, and 1000 IU to their new born infants during the first three years of life if they were not breastfed. The authors investigated the recurrence rate of autism in these children and compared it to other literature. The recurrence rate of autism was approximately four-fold less than that of other studies (5% *vs.* 20%). To note, this study had a small sample size and lacked a control arm.

Vitamin D-ASD-Related Areas Providing an Indication for both Primary and Secondary Prevention

In this section, we will synthesise the literature regarding a vitamin D-related measure – that is low vitamin D status in ASD patients – which can be a risk factor for ASD, and at the same time, a stage of ASD disease course.

Vitamin D Status in ASD Patients

We included all case-control studies of vitamin D status of individuals with ASD compared to healthy individuals, and participants of any age diagnosed with an ASD by either established diagnostic criteria, for example, DSM-IV or standardised established instruments.

Our literature search identified 15 studies reporting 25(OH)D concentrations in populations with ASD [78,79,81,107-119] (**Table 5**). Two of the 16 studies were excluded because either the full article was in Chinese [107], or cases were not compared with typically developing controls [119]. All 14 studies included in this review have been published over the past five years. Approximately half of the literature was from the Middle East [79,81,109,111,115,118], two from China [112,117], two from Europe [78,108], three from the US [113,114,116] and one from South America [110].

The number of cases ranged from 13 [115] to 254 [118]. Fernell *et al.* (2015) included siblings as controls [78], Saad *et al.* (2015) [111] and Kocovska *et al.* (2014) [108] included siblings, parents and typically developing children as controls, and the remaining studies included only non-family member typically developing children as controls. Children included as controls were thoroughly examined for medical and/or mental and developmental conditions in three

studies [81,111,112]. Controls were matched to cases on one or more attributes, including age, sex, ethnicity, socioeconomic status, season of birth, and/or season of blood collection. Season of blood collection was controlled in eight studies by either making adjustments in statistical analysis [108,113,114], performing all blood collections in one or two seasons [109,111,117], matching controls to cases on season of enrolment [79] or performing sub analysis for different seasons [112].

Of 14 studies, nine reported significant differences in 25(OH)D concentrations between cases and controls [78,81,108-112,117,118], one borderline difference [114], and four found no significant differences among study groups [79,113,115,116]. Tostes *et al.* (2012) reported that mean 25(OH)D concentration of children with ASD of different ethnic groups was lower by approximately 35 nmol/L than healthy age and sex matched controls ($P<0.001$) [110]. Neither ethnicity nor season of blood sampling was considered in the statistical analysis.

Bener *et al.* (2014) investigated several lifestyle and biochemical risk factors in relation to autism [118]. Children with ASD had significantly lower 25(OH)D concentrations than healthy age, sex and ethnicity matched controls (46±21 *vs.* 54±21 nmol/L, respectively, $P=0.004$). Circulating levels of 25(OH)D were inversely correlated with autism severity measured by the Autism Diagnostic Observation Schedule (ADOS). Factors associated with autism were serum calcium, 25(OH)D, blood relative, BMI, physical activity, child order, and ferritin. Despite the large sample size of 254 in each group, this study had several limitations; 1) cases were selected from a cohort study and controls were not examined for autism manifestations; 2) potential covariates or confounders were not controlled for in statistical analysis despite significant differences in socio-demographic characteristics of cases and controls; and 3) season/month of blood sampling was not controlled for in statistical analysis.

Table 3: Risk of autism according to seasonality of conception and birth

Reference	Country/ Year	Diagnosis	Case-Control Numbers	Case-Control Characteristics	Confounders/Covariates	Excess Conception	Excess Birth
[92]	Canada 1986	DSM-III	179-NR	Low functioning (IQ < 50) and high functioning (IQ > 50) autism, non-verbal and verbal autism from medical records from two different centres-live births			March *vs.* Sep-Feb Spring-early summer *vs.* winter and autumn (Aggregated sample) Spring *vs.* winter and autumn (Low functioning, nonverbal, male)
[91]	Japan 1988	DSM-III	80-71,013	Native infantile autism <8 years from clinic outpatients-children <8 years from annual reports			Second quarter of the year (corresponding to spring) *vs.* first and third quarter*
[90]	Sweden 1990	DSM-III-R	100-NR	Cryptogenic autism-populations born in Sweden (Central Bureau of statistics)	Cases with medical conditions and of mothers immigrated to Sweden from non-European countries were excluded		March
[89]	UK 1992	ICD-9, DSM-III	1435-196-121-24,957,169	National sample-clinic with autism sample-sibling controls-live births			Significantly deviated from the general populations' expected moth of birth (national sample) December, January, June, July and October
[88]	Denmark 1994	ICD-9	328-NR	Infantile autism-autism like disorder-borderline psychosis from clinic outpatients-live births			March and April *vs.* November December
[87]	Israel 1995	DSM-III-R	188-1,992,410	Infantile autism-live births			March and August

Table 3: *Cont.*

Reference	Country/ Year	Diagnosis	Case-Control Numbers	Case-Control Characteristics	Confounders/Covariates	Excess Conception	Excess Birth
[86]	International 1999	DSM-IV, ICD-10	620-284	Cases with autism from international multisite field trial for DSM-IV-Individuals with mental retardation from patients of a clinic			No association
[84]	Netherlands 2000	ICD-9	1031-NR	National registry of mentally retarded patients with AD and PDD-NOS (IQ < 35)-general population birth data			No association (month and season) (Aggregated sample) Second quarter of the year (Low functioning)
[85]	US 2000	DSM-III-R	175-123	High and low functioning autism (verbal IQ cut off of 65) recruited for a research project-full siblings and half siblings	Arbitrary assignations of month to season		No association (aggregated sample) March (more low functioning and socially passive autism) (Boston subset)
[64]	Sweden 2002	ICD-9	408-2040	Infantile autism <10 years from medical birth register-birth register	Maternal age, parity, smoking, mother's country of birth, hypertensive disease, diabetes, pregnancy bleeding, mode of delivery, gestational age, birth weight, Apgar score, congenital malformation		No association
[83]	Israel 2006	ICD-10	211-311,169	ASD adolescents (age of 17) from military medical registry-live births	Year of birth, socioeconomic status		No association

Table 3: *Cont.*

Reference	Country/ Year	Diagnosis	Case-Control Numbers	Case-Control Characteristics	Confounders/Covariates	Excess Conception	Excess Birth
[77]	Denmark 2007	ICD-10	1860-407,117	ASD and ASD subcategories from psychiatric registry-live births	General trend for increase in incidence over time, follow up time, length of gestation	No association	No association
[82]	US 2008	DSM-IV	1051-1,458,011	ASD singletons and multiple births from medical records-statistics data for singleton and multiple live births	Number of births and sex		Spring (April), summer (late July) and autumn (October) *vs.* winter (December and January) (Singletons and multiple births)
[80]	Denmark 2010	ICD-10	317-733,826	Infantile autism from medical birth register-live births	Sex, maternal smoking status, irregular fetal presentation, birth weight, gestational age, Apgar score, parental age, maternal citizenship, congenital malformation		Winter (October to March) *vs.* Summer (April to September), 2.2 [1.2, 3.9] *vs.* 1.0 [0.4, 2.5]
[81]	Egypt 2010	DSM-IV	70-40	ASD (recruited for the purpose of the study)-non ASD healthy controls			No significant difference June (27%) followed by March and April (11%,)
[76]	UK 2010	ICD-10	86–13,892	ASD from medical and educational records-live births		Summer *vs.* winter 2.1 [1.2, 3.7]	Spring *vs.* autumn (reference), 1.9 [1.0, 3.4)
[75]	US 2011	ICD-9	19,328-6,585,737	Full syndrome autism <6 years and live births <6 years from dataset	Sex, race/ ethnicity, Preterm birth, maternal age, maternal education, maternal place of residence at childbirth and maternal year of conception	Winter (January, February and March) *vs.* Summer, 1.1 [1.0, 1.1]	November *vs.* April (reference), 1.1 (1.1, 1.2]

Table 3: *Cont.*

Reference	Country/ Year	Diagnosis	Case-Control Numbers	Case-Control Characteristics	Confounders/Covariates	Excess Conception	Excess Birth
[74]	US 2012	DSM-IV	8,074-3,888,495	AD not comorbid with mental retardation-live births	Sex, parental age and education, race and ethnicity, insurance status, preterm birth and low birth weight	Winter (the last 3 weeks of November and first week of December), 2.1, 1.7 and 1.5 in 1994, 1995 and 1996, respectively	
[79]	Turkey 2014	DSM-IV	54-54	ASD (recruited for the purpose of the study)-non ASD healthy controls			No association
[78]	Sweden 2015	DSM-IV-TR	58-58	ASD (recruited for the purpose of the study)-non ASD siblings			No association in children of Middle Eastern/African ethnicity Spring *vs.* Summer, 38% *vs.* 10% in ASD and 18% *vs.* 35% in non ASD in children of Sweden and European ethnicity

Using siblings or family members as controls to minimize confounding by socio-demographic factors, Fernell *et al.* (2015) reported significantly lower mean 25(OH)D concentration at birth in children with autism of Scandinavian and other European origins than their non-ASD sibling controls [78]. On the other hand, the authors did not find any differences between cases and controls of Middle Eastern/African origin. The lack of association in Middle Eastern and African group may in part, be attributed to the small sample size and very low mean 25(OH)D concentrations in these two ethnic groups, 12±7.1 and 7.0±5.0 nmol/L, respectively. It should be also noted that season of birth was not significantly different between cases and controls in the latter ethnic groups, but it was significantly different across groups of Scandinavian and other European origins (10% *vs.* 35% of cases and controls in summer, respectively). Season of birth which corresponded to the season of blood sampling in this study was not controlled for in statistical analysis.

Kocovska *et al.* (2014) showed that ASD individuals had significantly lower 25(OH)D concentrations than their siblings, parents and healthy controls while adjusting for season of blood sampling (no one in ASD group had his/her blood sample drawn in winter) [108]. Furthermore, a larger proportion had 25(OH)D <50 nmol/L in cases than controls (88% in ASD group *vs.* 58% in sibling and parents each and 65% in healthy controls). A limitation to those studies including family members is that cases and controls cannot be matched on age and sex. Furthermore, inclusion of older siblings may confound the findings by younger parental age, and inclusion of apparently healthy younger siblings, on the other hand, may run the risk of including siblings without ASD who may develop ASD later in life in the control group [78,108]. For that reason, it is important that investigators examine family member controls more carefully for any autistic-like behaviour.

Examining both cases and controls for autistic-like features, Gong *et al.* (2013) reported a larger proportion of children with ASD having 25(OH)D concentrations <50 nmol/L as compared to healthy controls (58% *vs.* 33%, respectively P=0.01) [112]. Autism severity – measured by Childhood Autism Rating Scale (CARS) – negatively correlated with 25(OH)D concentrations (r= −0.4, P<0.001) while controlling for several covariates including age, sex, season, magnesium, BMI, calcium and phosphate. When vitamin D status of study groups was compared according to the four seasons, children with ASD had lower summer 25(OH)D concentrations than controls (P=0.02). It should be noted that doing sub-analysis in studies with small sample size results in even smaller sample sizes for each group, which could compromise the validity of the latter findings. As a general rule, it is preferable to control for season of blood sampling either in the statistical analysis or by limiting season/month of blood

collection rather than doing this by subgroup analysis, especially in studies with a small starting sample size.

Meguid *et al.* (2010) [81] and Saad *et al.* (2015) [111] also thoroughly examined all children, including both cases and controls, for medical conditions and mental disorders, and reported that two groups of Egyptian children with ASD had significantly lower levels of 25(OH)D compared to age, sex and socio-economic status matched healthy controls. The season of birth was taken into account in both studies but no significant differences were found in either group. Mean $1,25(OH)_2D$ and calcium concentrations was also lower in children with ASD than healthy controls [81], and serum 25(OH)D concentrations negatively correlated with CARS scores (r= -0.50, P<0.0001) [111]. Children on the severe end of the spectrum had lower mean 25(OH)D concentration than those with mild/moderate ASD (30±15 *vs.* 53±21 nmol/L, respectively). To avoid seasonal variations in 25(OH)D concentrations, all blood samples in this study were drawn during same two months (May and June).

Similarly, Mostafa and Al-Ayadi (2012) collected all blood samples from Saudi Arabian children with ASD and healthy controls during summer months [109]. Children with ASD displayed significantly lower 25(OH)D concentration than healthy controls. While 40% of children with ASD had 25(OH)D concentrations <25 nmol/L, no one from control group had 25(OH)D concentrations in that range. Cases also had significantly higher anti-myelin-associated glycoprotein (anti-MAG) anti-autoantibodies than controls. Furthermore, 25(OH)D concentrations correlated negatively with CARS scores, (r= −0.84, P<0.001) and anti-MAG anti-autoantibodies (r= −0.86, P<0.001). Children on the severe end of the spectrum had significantly higher anti-MAG anti-autoantibodies levels than those with mild/moderate ASD. These findings point to a link between low vitamin D status, autoimmunity and autism.

In a recent large study from China, Feng *et al.* (2016) reported a higher proportion of ASD children having 25(OH)D concentrations <25 nmol/L (13% *vs.* 0%, P=0.03) and <75 nmol/L (71% *vs.* 62%, P=0.03) than healthy typically developing controls [117]. To control for seasonal variation, the study lasted for six months; April – September corresponding to spring and summer months. To note, 25(OH)D concentrations of those entering the study in April could be different of those entering September due to a potentially limited sun exposure during winter months.

Neumeyer *et al.* (2013) [114] reported a higher proportion of boys with ASD having 25(OH)D concentrations <80 nmol/L than healthy boys (77% *vs.* 37%, P=0.02). Furthermore, cases had marginally lower serum 25(OH)D concentration than controls (67±5.0 *vs.* 79±4.0 nmol/L,

P=0.05). Despite comparable total energy intake and caloric intake from specific macronutrients among study groups, calcium and vitamin D intake was lower in cases than controls. Small sample size was a limitation of this study (*n*=18) with the sample size calculated based on differences in bone mineral density rather than 25(OH)D concentrations.

Hashemzadeh *et al.* (2015) failed to show any difference in 25(OH)D concentrations between children with ASD aged 3-12 years (*n*=13), and age and sex matched typically developing children (*n*=14) [115]. This study had the smallest sample size, a limited statistical power to detect differences among groups. Three other studies that also failed to show any relationship had somewhat larger sample sizes (ranging from 54 to 71 per group) and controlled for several covariates [113] and matched controls to cases on several attributes [79,116]. Molloy *et al.* (2010) [113] included boys with catheters placed for tonsillectomies as controls. Children were suffering from some form of acute inflammation, which potentially had an effect on 25(OH)D levels [120,121].

Although Adams *et al.* (2011) reported statistically significant differences in several biomarkers indicative of increased oxidative stress, reduced capacity for detoxification and energy transport, and vitamin deficiency in children with ASD than controls, plasma 25(OH)D did not differ across study groups [116]. The lack of difference could in part be attributed to some methodological issues; neither cases nor controls were assessed for autism manifestations by researchers and confounders such as ethnicity, season of blood sampling, medical conditions and medication use were not controlled for in statistical analysis. Approximately half of the cases were on different medications including psycho pharmaceuticals, central nerve system stimulants, anticonvulsants, gastrointestinal medication, asthma/allergies and insulin, and 11% of controls on either anti-inflammatory or anti-incontinence medications.

The study by Ugur *et al.* (2014) [79] was conducted in Turkey, where a national free vitamin D supplementation campaign has been implemented by government to eliminate vitamin D deficiency rickets [122]. The comparable levels of 25(OH)D among ASD cases and controls in this study could therefore be attributed to the nationwide vitamin D supplementation after initiation of ASD.

The cause and effect relationship from these case-control studies cannot be determined. Patients with ASD are likely to have medical conditions and use medications—affecting vitamin D metabolism or absorption – that have not been considered in these studies. Inflammation [120,121], gastrointestinal issues [123,124], antiepileptic drugs [125] and

genetics are among these factors. Furthermore, children with ASD may receive less sunlight due to lower outdoor activity and have inadequate intake of vitamin D [114,118,126-136], *i.e.*, reverse causality.

Along those lines, according to parental report, ASD children had significantly more sedentary behaviours than typically developing controls, a finding confirmed by Neumeyer *et al.* (2013) and Bener *et al.* (2014) [118]. Approximately 11% and 73% of ASD children and typically developing children had high physical activity levels, respectively [114], and fewer ASD children were exposed to sun than healthy controls (P=0.002) [118].

Moreover, due to repetitive and restricted dietary behaviours, ASD children are at increased risk of low dietary vitamin D intake [128,129]. Evidence suggests that a large proportion of children with ASD do not meet vitamin D requirements [126,132-136]. Using three-day food records, Stewart *et al.* (2015) reported a probable risk of inadequate intake of six micronutrients in >40% of children with ASD, one of which was vitamin D [126]. Even though vitamin D intake was improved in response to vitamin D supplementation, it did not reach the adequate intake in 30-40% of children. The scenario would be worse if more children of African American, mixed-race/ethnicity and less educated parents were enrolled because the socio-demographic characteristics of the study population was biased toward highly educated parents and those of white ethnicity who might have been more concerned about nutrition. Average vitamin D intake was 62% and 49% of dietary recommended intake (DRI) in boys with ASD aged 7-8 and 9-12 years, respectively [136]. Although these studies illustrated a large proportion of children with ASD not meeting the recommendations for vitamin D intake, they did not find any evidence that dietary intake of vitamin D in children with ASD differed from that of typically developing controls.

It has been argued that low dietary intake of vitamin D is a public health issue, and is not specific to children with ASD [131,137,138]. In a recent case-control study, Mari-Bausent *et al.* (2015) reported children with ASD having lower dietary vitamin D intake than healthy controls, though the difference was not statistically significant (79±60 *vs.* 104±97 IU/day, respectively, P=0.06) [131]. A large proportion of both groups did not meet the dietary recommended intake for vitamin D (88% of children with ASD and 75% of typically developing children, P=0.16), a finding confirmed by others [137,138].

However, several case-control studies have shown that children with ASD have lower vitamin D intakes than typically developing controls [114,128-130]. Emond *et al.* (2010) and Zimmer *et al.* (2012) reported that children with ASD had late introduction of solids after six months,

were difficult to feed and very choosy from 15-54 months, had less varied diet from 24 months, and ate fewer food on average at older ages, all of which affected their nutritional status [128,129]. Both studies showed that children with ASD had significantly lower dietary intakes of vitamin D than controls. Daily vitamin D intake was 199±176 IU and 320±119 IU in ASD and typically developing children, respectively ($P<0.05$) [129]. More children with ASD did not also meet the estimated average requirement (EAR) for vitamin D intake than healthy controls (79% *vs.* 55%, $P=0.01$) [130], and more selective eaters with ASD were at increased risk for inadequate intake (58% *vs.* 5%, $P=0.01$) [129]. Thus, low vitamin D status could be a risk factor for ASD if it occurs during critical period of development, or alternatively, could be a condition that appears as the disease progresses.

Vitamin D-ASD-Related Areas Providing an Indication for Secondary Prevention

Here, we will review the literature regarding a vitamin D-related measure – that is the treatment of low vitamin D status when ASD has already occurred – which can be considered as a disease modifier, and may reduce the symptoms of ASD in patients.

Vitamin D Intervention to Treat ASD

We included all studies reporting using vitamin D supplements among populations with ASD. We excluded those studies using vitamin D containing multivitamins or minerals only because those doses are relatively low and the effect of vitamin D *per se* cannot be determined from these interventions.

Our literature search identified six studies reporting on the effect of vitamin D supplementation on autism symptoms (**Table 4**); one clinical quality assurance practice [119], one case report [139], three open label intervention trials [111,117,140], and one randomised controlled trial [141]. The full text for one article was not available at the time of writing this review, as such the critical appraisal could not be employed, and the data presented here are based on the abstract[2] [141]. We found no randomised placebo-controlled trials, but three

[2] The full article was available at the time of writing this thesis. For more information refer to the footnotes in Table 4 where this article has been cited and in the same page where this study has been explained in more details.

ongoing studies[3] from Egypt (NCT02550912[4]), Ireland (NCT02508922[5]) and New Zealand (ACTRN12615000144516). Four studies were among children with ASD [111,117,139,141], one among developmentally delayed children with and without ASD [140], and one in adult patients with ASD and other psychiatric disorders [119]. Humble *et al.* (2010), in a clinical quality assurance project, reported a considerable improvement in psychosis or depression with the effective treatment of vitamin D deficiency in several patients. In their clinical practice they used 1600-4000 IU vitamin D_3 daily or 35,000–70,000 IU vitamin D_2 once weekly [119]. Patients with autism and schizophrenia had the lowest median 25(OH)D concentrations compared to other psychiatric disorders, 32 (25^{th}, 75^{th} percentile, 23, 39) nmol/L and 35 (24, 53) nmol/L, respectively, *vs.* >40 in other patients. No conclusion can be drawn from this study because it was not designed to assess the efficacy of vitamin D on autism and it was part of clinical practice, no placebo or control arm was included, different doses and types of vitamin D was used, and levels of 25(OH)D after treatment was not reported.

In a recent case report, Jia *et al.* (2015) reported that shifting serum 25(OH)D concentration in a 32 month old child with ASD from 31 nmol/L to 203 nmol/L after two months of high dose vitamin D_3 supplementation (150,000 IU per month administered intramuscularly plus 400 IU per day orally) improved core symptoms of autism [139]. Scores on Autism Behaviour Checklist, Childhood Autism Rating Scale (CARS) and Severity of Illness of Clinical Global Impression increased from 80, 35 and 6 at the baseline to 39, 28 and 4 at the follow up,

[3] A further RCT was available at the time of writing this thesis. In this trial, 42 children with ASD (<18 years) were randomly assigned to either vitamin D (2000 IU/day) or placebo for 20 weeks and 38 children completed the trial. Behaviours were assessed using Aberrant Behaviour Checklist (ABC), Social Responsiveness Scale (SRS), and Developmental Disabilities−Children' s Global Assessment Scale (DD-CGAS). Vitamin D supplementation had no effect on autism symptoms. Saad, K., *et al.*, Randomized controlled trial of vitamin D supplementation in children with Autism Spectrum Disorder. J. Child Psychol. Psychiatry, 2018. **59**(1): p. 20-29.

[4] This study was completed at the time of writing this thesis, but the findings were not available.

[5] This study was completed, and the findings were published at the time of writing this thesis. In this trial, 109 children with ASD (3-10 years) and serum 25(OH)D≥50 nmol/L were randomly assigned to one of treatment groups; 300 IU vitamin D_3/kg/day, not to exceed 5,000 IU/day or placebo for four months. The behaviours were measured using ABC, Autism Treatment Checklist (ATEC), CARS, and SRS. In response to vitamin D supplementation, the symptoms of ASD significantly improved, but placebo had no effect. Kerley, C.P., *et al.*, Lack of effect of vitamin D3 supplementation in autism: A 20-week, placebo-controlled RCT. Arch. Dis. Child, 2017. **102**(11): p. 1030-1036.

respectively. The findings from this single patient cannot be generalised to all patients with autism but it does encourage further research in this area. Following on from this case report, Feng and colleagues (2016) assigned 37 ASD children from a pool of 215 children with ASD (25(OH)D <75 nmol/L) to receive the same dose of vitamin D supplementation for three months [117]. The authors reported a significant improvement in scores on CARS (total) and Autism Behaviour Checklist (total and all subscales apart from sensory scores), which was more pronounced in 3 year old or younger children than older children.

Saad *et al.* (2015), in an open label trial, assigned 106 children with ASD and 25(OH)D concentrations <75 nmol/L to receive daily 300 IU/Kg not exceeding daily 5,000 IU for three months. This study was part of a case-control study investigating differences in 25(OH)D concentrations among children with autism and healthy age and sex matched controls (previously discussed). Symptoms of ASD were measured using CARS and Aberrant Behaviour Checklist (ABC). CARS scores decreased by 3.5-6.5 points in all 16 patients with final 25(OH)D >100 nmol/L, by 1.5-4.5 points in 31 out of 49 patients with 25(OH)D levels 75-98 nmol/L and no improvements in all 18 patients with 25(OH)D levels <75 nmol/L had improvements. With the exception of inappropriate speech subscale of ABC, all other measures (irritability, stereotypic behaviour, social withdrawal and hyperactivity) improved significantly after treatment. This study had a relatively large sample size and also limited seasonal effects by drawing all blood samples during the summer months, though the baseline and follow-up levels of 25(OH)D were not reported. Other limitations of this study were its drop-out rate of approximately 22% and the lack of a placebo or comparison arm.

In another study from Turkey, however, despite improvement in scores on ABC and Denver II in both treatment and control groups, Ucuz *et al.* (2015) failed to show any significant differences across groups [140]. To note, the improvement was more pronounced in those receiving vitamin D supplement. The authors recruited children with developmental delay with and without ASD, divided them into two groups; those with 25(OH)D concentration <50 nmol/L who received daily 5000 IU vitamin D supplement for one month and daily 400 IU vitamin D for further two months if 25(OH)D was not corrected at one month follow up, and ≥50 nmol/L who did not receive vitamin D supplements, and performed subcategory analysis for children with developmental delay with ASD and without ASD. This study had a small sample size of <11 participants per group. Furthermore, children were not randomly assigned to study groups and neither baseline nor follow up 25(OH)D concentrations were reported. It should be noted that the findings from this study cannot be extrapolated to children with ASD only. The brain chemistry of children with ASD may differ from that of children with

developmental delay [142], and as such the response to vitamin D supplementation – that may affect brain chemistry [143] – could vary.

In a randomised controlled trial including only children with ASD, Azzam *et al.* (2015), similarly, reported comparable improvements in scores on CARS, social IQ, and Autism Treatment Evaluation Checklist in both vitamin D supplemented (six months) and control groups[6] [141]. Although no quality appraisal can be performed for this study, what is clear is that this study had a small sample size and lacked a placebo arm. In summary, although these early interventional studies provided encouraging results, no firm conclusions can be drawn until randomised placebo-controlled trials with sufficient sample size are undertaken.

Potential Mechanistic Pathways

Vitamin D receptors (VDR) and enzymes involved in vitamin D metabolism have been identified in several regions of the brain including neurons and glial cells [144]. VDR has been shown to be present early in development, to increase during development, and to be present in the adult brain—all indicative of a role for vitamin D in the developing and adult brain [145]. Low vitamin D status has been implicated in pathophysiology of ASD in several ways. It has been hypothesised that ASD is a combination of both organ specific physiologic and systematic abnormalities such as *de novo* gene mutations, oxidative stress, impaired detoxification system, inflammation, immune dysregulation, abnormal neurotrophic factor and neurotransmitter levels, and seizures, at least in a subset of individuals with ASD [8]. Mounting evidence suggests that low vitamin D status is involved in the aetiology of the mentioned abnormalities [105].

[6] Updated information: 31 children with ASD (aged 2 to 12 years) participated in the trial but 21 children completed the intervention (intervention group: n=10 and control group with autism: *n*=11). A group of 23 apparently healthy and typically developing children were also included as another control group. Children with ASD were randomly assigned to either daily 2000 IU vitamin D for six months or no vitamin D. Children with ASD had lower serum 25(OH)D concentration than typically developing children, though the difference did not reach statistical significance (59±33 vs. 78±39 nmol/L, respectively). Although the change in serum 25(OH)D was significantly higher in children receiving vitamin D than controls, there was no difference in all outcome measures across two study groups after six months. To note, all children with ASD were kept on behavioural and speech therapy (30 minutes, three times/week) over the study period.

Table 4. Vitamin D interventions to prevent or treat Autism Spectrum Disorder (ASD).

Reference	Country /Year	Study Design/Country	Population Characteristics	Intervention	Baseline 25(OH)D (nmol/L)	Follow up 25(OH)D (nmol/L)	Outcome Measure
Vitamin D intervention to prevent ASD							
[106]	2016	Prospective open label intervention trial	Pregnant mothers of children with autism	Daily 5000 IU during pregnancy and 7000 IU during breastfeeding Daily 1000 IU during the first three years of life if child was not breastfed	All but two >50	70-198	Recurrence rate of autism in new born siblings was 5% which was lower than that reported in the literature (20%)
Vitamin D intervention to treat ASD							
[119]	Sweden 2010	Clinical quality assurance project	Autism and other psychiatric disorders (mean age of 44 years)	Daily 1600-4000 IU vitamin D_3 or 35,000 70,000 IU vitamin D_2 once weekly	32 (23, 39) in patients with autism and 45 (31, 60) in all patients *	-	Improvement in psychosis or depression
[139]	China 2015	Case study	A 32-month old male toddler	Monthly 150 000 IU IM and daily 400 IU orally for two months	31 **	203	ABC [1] (from 80 to 39) CARS (from 35 to 28) Severity of Illness of Clinical Global Impression (from 6 to 4).

Table 4. *Cont.*

Reference	Country /Year	Study Design/Country	Population Characteristics	Intervention	Baseline 25(OH)D (nmol/L)	Follow up 25(OH)D (nmol/L)	Outcome Measure
[111]	Egypt 2015	Open label intervention trial	106 children with autism with 25(OH)D <75 nmol/L	Daily 300 IU vitamin D_3/Kg not exceeding 5,000 IU/day for three months	<75	-	ABC[2]: Improvements in irritability (0.02), hyperactivity (0.03), social withdrawal (0.01) and stereotypic behaviour (0.04) No improvements in inappropriate speech CARS: Improvements in total (P<0.001), relating to people (P<0.001), imitation (p < 0.001), body use (P=0.01), object use (P=0.01), adaptation to change (P=0.004), listening response (P=0.01), visual response (P=0.003) and general impression (P<0.001) No improvements in fear, verbal communication, activity level, nonverbal communication and intellectual response The improvement was more pronounced in those with final 25(OH)D >100 nmol/L

Table 4. *Cont.*

Reference	Country /Year	Study Design/Country	Population Characteristics	Intervention	Baseline 25(OH)D (nmol/L)	Follow up 25(OH)D (nmol/L)	Outcome Measure
[141] ***	Egypt 2015	Randomised controlled trial	21 children with autism assigned to vitamin D or no treatment groups[7]	-[8]	-[9]	-[10]	CARS, social IQ and ATEC: Improved in both groups Improvement was not significantly different across groups
[140] ****	Turkey 2015	Open label intervention trial	Toddlers with developmental delay Toddlers with developmental delay with and without ASD (2-5 years old), and 25(OH)D <50 nmol/L (n=11, cases) and ≥50 nmol/L (n=10, controls)	Baseline 25(OH)D <37.5 nmol/L: daily 5000IU for one month and then daily 400IU for two months if 25(OH)D is between 37.5-50 nmol/L after one month Baseline 25(OH)D between 37.5–50 nmol/L: daily 400IU for one to three months depending on the level at one month	-	-	ABC and Denver II: Significant improvement in both groups (ABC, from 90±19 to 59±15 in cases and from 77±22 to 64±29 in controls; Denver II, from 64±13 to 72±17 in cases and from 73±11 to 80±12 in controls). Neither baseline nor endpoint scores were significantly different across groups, but improvement was more pronounced in cases

[7] n=10 cases with autism, n=11 controls with autism, and n=23 typically developing controls

[8] Daily 2000 IU vitamin D for six months

[9] Cases with autism: 47±20 nmol/L; controls with autism: 69±41 nmol/L; typically developing controls: 78±40 nmol/L

[10] Cases with autism: 71±35 nmol/L; controls with autism: 79±36 nmol/L; typically developing controls: 78±40 nmol/L

Table 4. *Cont.*

Reference	Country /Year	Study Design/Country	Population Characteristics	Intervention	Baseline 25(OH)D (nmol/L)	Follow up 25(OH)D (nmol/L)	Outcome Measure
[117]	China 2016	Open label intervention trial	37 children with autism with 25(OH)D <75 nmol/L	Monthly 150 000 IU IM and daily 400 IU orally for three months	-	-	CARS: Improvement in total scores ABC [1]: Improvement in total and social skills, body and object use, language, and social and self-help Improvement was more pronounced in younger children (≤3 *vs.* >3 years old).

ASD, Autism Spectrum Disorder; 25(OH)D, 25 hydroxyvitamin D; IU, international unit; D_3, cholecalciferol; D_2, ergocalciferol; ABC[1], Autism Behaviour Checklist; CARS, Childhood Autism Rating Scale; ABC[2], Aberrant Behaviour Checklist; ATEC, Autism Treatment Evaluation Checklist; Denver II, Denver Developmental Screening Test II. * Median (25^{th}, 75^{th} percentile); ** The values were ng/mL, they have been converted to nmol/L for easy comparison (1 ng/mL = 2.5 nmol/L); *** The full text was not available at the time of writing this manuscript and are information provided here are based on the abstract; **** The data for children with developmental delay and ASD are reported here

Table 5. Vitamin D status in ASD patients-Case-control studies.

Reference	Country/ Year	Case-Control Characteristics	Assessment Tools	Covariates/ Confounders	Boys: Girls	P-Value	25(OH)D Concentration (nmol/L)*	
							Cases	Controls
[81]	Egypt 2010	70 children with autism (mean age of 5.3±2.8) 40 age matched healthy controls of the same socioeconomic status controls (thoroughly examined)	DSM-IV	Season of birth	-	<0.001	71±41**	100±30
[113]	US 2011	71 Caucasian males with ASD (4–8 years old) 69 age matched typically developing controls	DSM-IV/ADOS	Covariates: age, BMI, use of supplement, antiepileptic medication, season of enrolment	Only male	n.s.	50 (range, 27, 78) ** (CFD)	43 (range, 20, 71)
						n.s	49 (range, 22, 77) (NCFD)	
[116]	US 2011	55 children with ASD (aged 5–16 years old) 44 age, sex and geographically similar distribution	Diagnostic confirmation from pediatrician or other professionals/PDD-BI (autism composite), ATEC, SAS		49: 6	n.s.	75±21**	72±21
[110]	Brazil 2012	24 children with ASD (mean age of 7.4±2.7 years) 24 age and sex matched healthy controls	DSM-IV		18:6	<0.001	66±8.7 **	101±7.8
[109]	Saudi Arabia 2012	50 children with ASD (5-12 years) 30 age and sex matched healthy controls	DSM-IV/CARS (severity)	All blood samples drawn in summer	39:11	<0.001	46 (IQR, 35) **	83 (IQR, 28)
[114]	US 2012	18 boys with ASD (mean age of 11±0.4 years) 19 boys without ASD	DSM-IV/ADOS	Season of blood collection, bone age	NA	0.06	67±4.8 **	79±4.0

Table 5. *Cont.*

Reference	Country/ Year	Case-Control Characteristics	Assessment Tools	Covariates/ Confounders	Boys: Girls	P-Value	25(OH)D Concentration (nmol/L)*	
[112]	China 2013	48 children with ASD (mean age of 3.7±1.2 years) 48 age and sex matched healthy controls (all examined thoroughly by paediatircian for any possible autistic features)	DSM-IV, CARS (all cases)	Season of blood sampling, all study population belonged to Chinese Han population	40:8	0.002	50±9.5 **	57±11
[118]	Qatar 2014	254 children with ASD (mean age of 5.5±1.6 years) 254 age, sex and ethnicity matched controls	ADOS		165:89	0.004	46±21**	54±2.0
[79]	Turkey 2014	54 children with ASD (mean age of 60±15 months) 54 age, sex, season of enrolment and societal status matched healthy controls	DSM-IV/ABC-T, ABC[21] CARS, ADSI, Stanford-Binet or WISC-R	Season of enrolment	47:7	0.069	63±28**	53±24
[108]	Faroe Island 2014	40 individuals with ASD (white European origin of different age) 62 typically developing sibling 77 parents 40 healthy age, sex and season of birth matched controls	ICD-10, DSM-IV/ADOS, DISCO, WISC-III, WAIS-R	Adjustment for month of blood collection	31:9	0.002 <0.001 <0.001	25 (IQR, 28)	38 (IQR, 32) (controls) 46 (IQR, 28) (siblings) 47 (IQR, 36) (parents)
[111]	Egypt 2015	122 children with ASD (mean age of 5.1±1.4 years) 100 age, sex and societal status matched healthy controls (all screened for any mental and autistic manifestations)	DSM-IV/CARS, ABC [2]	Blood samples collected during two months	75% male in control group	<0.0001	45±22**	106±24

Table 5. *Cont.*

Reference	Country/ Year	Case-Control Characteristics	Assessment Tools	Covariates/ Confounders	Boys: Girls	P-Value	25(OH)D Concentration (nmol/L)*	
[115]	Iran 2015	13 children with ASD (3-12 years) 14 age and sex matched controls	DSM-IV/CARS		11:2	0.35	13 (IQR, 9.6, 20)	12 (IQR, 4.9, 13)
[78]	Sweden 2015	58 multi-ethnic children with ASD diagnosed at the age of 4 or older 59 healthy siblings	Multi-professional expert team		51:6	0.01	24±20	32±28
[117]	China 2016	215 children with ASD (mean age of 4.8±1.0 years) 285 age and sex matched healthy controls (mean age of 5.1±1.1 years)	DSM-IV/ADOS	Blood samples collected during six months	173:42	0.02	-	-

ASD, Autism Spectrum Disorder; 25(OH)D, 25-hydroxyvitamin D; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition; US, The United States; ADOS, Autism Diagnostic Observation Schedule; BMI, body mass index; CFD, casein free diet; NCFD, non casein free diet; PDD-BI, Pervasive Developmental Disorder-Behaviour Inventory (autism composite); ATEC, Autism Evaluation Treatment Checklist;; SAS, Severity of Autism Scale; IQR, interquartile range; CARS, Childhood Autism Rating Scale; ADOS, Autism Diagnostic Observation Schedule; ABC-T, Aberrant behaviour checklist-Turkish version; ABC^1, Autism Behaviour Checklist; ADSI, Ankara Developmental Screening Inventory; WISC-R, Wechsler Intelligence for Children-Revised;.ICD-10, International Classification of Disease-Tenth Edition; DISCO, Diagnostic Interview for Social and Communication Disorders; WISC-III, Wechsler Intelligence Scale for Children-Third Edition; WAIS-R, Wechsler Adult Intelligence Scale-Revised; ABC^2, Aberrant Behaviour Checklist.

* All values reported as means ± standard deviation, otherwise stated; ** The values were ng/mL, they have been converted to nmol/L for easy comparison (1 ng/mL = 2.5 nmol/L)

Autism is considered to be driven genetically [146,147], though only a small percentage of cases are clearly linked to genetic causes [22]. The only study linking vitamin D metabolic gene variants to ASD risk is by Schmidt *et al.* [148]. The authors illustrated that the risk for ASD was increased in children inheriting the AA genotype of the GC gene (D binding protein), the GG genotype of the CYP2R1 gene (a catalyst enzyme involved in the transformation of vitamin D to 25(OH)D), and paternal *Taql* and *Bsml* genotypes of the VDR gene, highlighting the possible aetiological role of low vitamin D in ASD. All of these results support an association between vitamin D status and ASD.

Vitamin D regulates cell proliferation and differentiation and can protect the genome from daily life insults such as oxidative stress and toxins (for a comprehensive review refer to [149]). Treatment with vitamin D has been shown to decrease 8-hydroxy-2-deoxyguanosin, a marker of endogenous oxidative damage to DNA [150], increase Bax, a protein coding gene involved in apoptosis [150], regulates poly-ADP-ribose polymerase activity in the DNA damage response pathway [151], stabilize chromosomal structure and prevent double strand breaks [152].

There are presently several lines of evidence indicating oxidative stress and mitochondrial dysfunction are prevalent in individuals with ASD [3,10–16]. Recent evidence shows that children with ASD have lower levels of total glutathione and glutathione peroxidase (an enzyme involved in antioxidant defence and detoxification), higher levels of oxidised glutathione and F2-isoprostane (a marker of oxidative stress and lipid peroxidation) and reduced capacity for methylation than typically developing children [16,153]. Oxidative stress and reduced redox/antioxidant capacity in cerebellum and temporal cortex have been shown to have functional consequences for chronic inflammatory response, mitochondrial dysfunction and protein and DNA damage [3], and in peripheral tissue have been associated with anti-neuronal positivity [16] and clinical features of autism [15]. Evidence shows that 25(OH)D concentrations correlate significantly positively with glutathione levels in healthy adult populations [154], and low vitamin D status is associated with increased inflammatory, oxidative and endothelial activation biomarkers in obese individuals [155]. It has been documented that treatment with vitamin D has a protective effect on persistent biochemical features comparable to those reported clinically in patients with autism and ameliorates neurotoxicity, inflammation and DNA damage in propionic acid-intoxicated rats [156].

Exposure to heavy metals and impaired detoxification system, another abnormality in a subset of individuals with ASD, have also been involved in the aetiology of ASD [157]. Alabdali *et al.* (2014) reported children with autism having higher levels of mercury and lead than healthy

controls, and lower levels of glutathione-transferase and vitamin E in plasma [157]. Using cell culture models, Shinpo *et al.* (2000) and Garcion *et al.* (1999) indicated that pre-treatment of cells exposed to toxicants with $1,25(OH)_2D$ attenuated reactive oxygen species and nitric oxide, and enhanced intracellular gamma-glytamyl transpeptidase (an enzyme involved in glutathione synthesis) and glutathione in the intracellular pool [158,159].

Furthermore, evidence suggests that children with ASD have elevated levels of pro-inflammatory cytokines including, interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) in different tissues [3–7]. The activation of microglia, the unique resident cells of the central nervous system, is the prominent feature of autism [160] and is associated with increased several pro-inflammatory cytokines [161]. It is well known that vitamin D has an effect on immune system and can directly affect immune cells. For example, vitamin D metabolites have been shown to decrease the secretion of IL-6 and TNF-α [162], enhance the expression of anti-inflammatory cytokines such as interleukin 10 (IL-10) from activated *B* cells [163], and to direct dendritic cells (DCs) toward a more tolerogenic state [164].

Autism is also considered as an autoimmune disease in a subset of individuals. Elevated autoantibodies in the brain, specifically anti-myelin basic protein antibodies have been reported in autism [9]. Mostafa and Al-Ayadi reported a significantly higher proportion of children with ASD having anti-MAG anti-autoantibodies, anti-ganglioside M1, anti-neuronal and anti-nuclear antibodies than typically developing controls, that positively correlated with the severity of autism symptoms (CARS scores) [109,165–167]. Family history of autoimmune diseases was reported to be higher in children with ASD than controls [166,168]. Using a genome-wide data set of ASD and autoimmune disorders, Multiple Sclerosis (MS) was identified to be significantly associated with ASD [169]. Both conditions have been characterised by immunological abnormalities and dysfunctional myelination, at least in a subset of individuals [170,171]. Furthermore, anti-MAG anti-autoantibodies correlated negatively with serum 25(OH)D concentrations (r= –0.86, P<0.001) [109], and anti-nuclear antibody positive healthy controls were significantly more likely to be deficient in vitamin D than antibody negative healthy controls (71% *vs.* 22%, respectively, P=0.003) [172]. Vitamin D has also been shown to modify the expression of several genes involved in axogenesis and myelination [173]. These findings suggest an important role of vitamin D in autoantibody production and ASD pathogenesis, perhaps similar to other autoimmune diseases, such as MS and systemic lupus erythematosus [172,174].

Evidence suggests that vitamin D has a neuroprotectory effect through its neurotrophic and immunomodulatory properties. Neurotrophins are involved in the development, maintenance, survival and synapsis of neurons [105]. Abnormal levels of several neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factors (BDNF) have been reported in subset of individuals with ASD [175,176]. Vitamin D analogues have been shown to upregulate NGF in cultured glial cell and embryonic hippocampal cells [177,178], to decrease the percentage of cultured hippocampal cells undergoing mitosis [178], and to decrease BDNF and concomitantly to improve memory in postmenopausal women [179].

Multiple lines of evidence suggest an involvement of dysregulated neurotransmitter systems (serotonergic, oxytocinergic and dopaminergic systems) in autism. These systems play key roles in neurotransmission, brain maturation, cortical organisation and behaviours (including social behaviour and repetitive behaviour) [180-184]. Lower levels of plasma oxytocin [185] and abnormal serotonin concentrations in the brain and tissues outside the blood-brain barrier have been reported in populations with ASD [186,187]. While the binding of brain serotonin transporter was significantly lower in adults with high functioning autism than healthy controls, the binding of brain dopamine transporter was significantly higher in patients with autism [184]. Recently, two ASD associated independent gene variants, including a gene variants regulating STX1 phosphorylation (regulates neurotransmitter transporter) and dopamine transporter, that alter dopamine transporter function have been identified [188]. Vitamin D receptors have been identified in dopamine neurones in human's substantia nigra [145], and vitamin D response elements on genes involved in serotonin and oxytocin synthesis [189]. Pre-treatment with calcitriol has been shown to protect rats' striatum and accumbens from methamphetamine-induced reduction in dopamine and serotonin [143].

Finally, the above mentioned disorders may contribute to the aetiology of epilepsy, which is highly prevalent in populations with ASD [190]. According to retrospective follow up studies, 37 and 25% of the individuals with autism exhibited epilepsy and epileptic seizures, respectively [191,192]. Infants of mothers taking antiepileptic drugs during pregnancy have been shown to be at increased risk of being diagnosed with ASD and having low 25(OH)D concentrations [193]. Vitamin D and its analogues have been shown to increase seizure threshold, potentiate anticonvulsant activity of antiepileptic drugs, and to decrease the severity of seizures in animal studies [194–196]. Correction of vitamin D deficiency in patients with seizures, decreased the frequency of seizures by 30%-40% [197,198]. Vitamin D exerts its roles, in part, through its antioxidant and anti-inflammatory effects and also through its inhibitory effect on Ca^{2+} influx in the brain. Calcitriol upregulates the expression of calcium binding proteins (calbindin and parvalbumin) and inhibits the expression of L-type Ca^{2+}

channels [199]. It should be noted that at the same time, antiepileptic drugs impair vitamin D and calcium metabolism, leading to reduced levels of vitamin D and calcium [125], and these deficiencies can, on the other hand, exacerbate the condition.

Conclusions

Low vitamin D status in utero, postnatal and in early childhood has been hypothesised as a risk factor for neurodevelopmental disorders, specifically ASD. Animal and human cellular, biological, and physiologic studies have provided compelling evidence for numerous roles of vitamin D in various body processes, some of which are involved in the pathobiology of ASD. Our literature review identified a large number of observational studies but very few intervention trials investigating the relationship between vitamin D and ASD. Conclusions are not yet possible due to the inconsistent results, different methodological approaches employed, and very few trials in the current literature. However, there are some indications that early exposure to inadequate vitamin D may interact with other factors and contribute to the aetiology of autism, low vitamin D status might be highly prevalent in populations with ASD, and intervention with vitamin D might be beneficial in reducing autism symptoms among those who have ASD. Therefore, there is an urgent need for randomised controlled trials of vitamin D in populations genetically predisposed to ASD and in populations with ASD to confirm these findings and to generate evidence-based clinical recommendations for the prevention of ASD and management of ASD symptoms. Until better data are available, health care providers and researchers are advised to consider vitamin D-related factors as potential preventive and disease-modifying measures for ASD.

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DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hajar Mazaherey

Name/Title of Principal Supervisor: Assoc. Prof. Pamela von Hurst

Name of Published Research Output and full reference:

Mazahery, H., C.A. Camargo, Jr., C. Conlon, K.L. Beck, M.C. Kruger, and P.R. von Hurst, Vitamin D and Autism Spectrum Disorder: A literature review. Nutrients, 2016. 8(4): p. 236

In which Chapter is the Published Work: Chapter 2-Section 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Responsible for all aspects of the manuscript including: conceptualisation and design of manuscripts, searching the literature, data extraction, drafting manuscript, and manuscript submission.

Hajar Mazahery Digitally signed by Hajar Mazahery Date: 2018.11.15 00:30:19 +13'00'

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15/11/2018

Date

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Principal Supervisor's signature

20/11/2018

Date

GRS Version 3– 16 September 2011

Section 3: Omega-3 Long Chain Polyunsaturated Fatty Acids and ASD (Paper II)

After having a good understanding of the role of vitamin D in ASD, this section is devoted to covering the role of second element of this thesis, omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFA) in ASD. Herein, I systematically analyse case-control and randomised controlled trials of omega-3 LCPUFA in ASD.

This section was published in 2017 in "Nutrients".

Mazahery, H., W. Stonehouse, M. Delshad, M.C. Kruger, C.A. Conlon, K.L. Beck, and P.R. von Hurst, relationship between long chain n-3 polyunsaturated fatty acids and Autism Spectrum Disorder: systematic review and meta-analysis of case-control and randomised controlled trials. Nutrients, 2017. ***9****(2)*

An update search was performed at the time of writing this thesis to see if there was any new evidence. In the case of finding such evidence, the information are included in footnotes.

To meet the requirement of this thesis the following subsection " Omega-3 LCPUFA: Metabolism, Biomarker, Optimum level" has been added here. This subsection also includes a figure. Accordingly, reference numbers and figure numbers are different from those in the published paper. Also, to align with the formatting and referencing style of this thesis, there are some changes in formatting and referencing style.

Relationship between Long Chain n-3 Polyunsaturated Fatty Acids and Autism Spectrum Disorder: Systematic Review and Meta-Analysis of Case-Control and Randomised Controlled Trials

Abstract

Omega-3 long chain polyunsaturated fatty acid supplementation (omega-3 LCPUFA) for treatment of Autism Spectrum Disorder (ASD) is popular. The results of previous systematic reviews and meta-analyses of omega-3 LCPUFA supplementation on ASD outcomes were inconclusive. Two meta-analyses were conducted; meta-analysis 1 compared blood levels of LCPUFA and their ratios [arachidonic acid (ARA) to docosahexaenoic acid (DHA), ARA to eicosapentaenoic acid (EPA), or total omega-6 to total omega-3 LCPUFA] in ASD to those of typically developing individuals (with no neurodevelopmental disorders), and meta-analysis 2 compared the effects of omega-3 LCPUFA supplementation to placebo on symptoms of ASD. Case-control studies and randomised controlled trials (RCTs) were identified searching electronic databases up to May, 2016. Mean differences were pooled and analysed using

inverse variance models. Heterogeneity was assessed using I^2 statistic. Fifteen case-control studies (n=1193) were reviewed. Compared with typically developed, ASD populations had lower DHA (-2.1 [95% CI -3.2 to -1.1]; P<0.0001; I^2=97%), EPA (-0.7 [95% CI -1.3 to -0.2]; P=0.008; I^2=88%), and ARA (-0.8 [95% CI, -1.5 to -0.2]; P=0.01; I^2=96%) and higher total omega-6 LCPUFA to omega-3 LCPUFA ratio (0.4 [95% CI 0.1 to 0.8; P=0.02; I^2=74%). Four RCTs were included in meta-analysis 2 (n=107). Compared with placebo, omega-3 LCPUFA improved social interaction (-2.0 [95% CI -3.5 to -0.3]; P=0.02; I^2=0%) and repetitive and restricted interests and behaviours (-1.1 [95% CI -2.2 to -0.01]; P=0.05; I^2=0%). Populations with ASD have lower omega-3 LCPUFA status and omega-3 LCPUFA supplementation can potentially improve some ASD symptoms. Further research with large sample sizes and adequate study duration is warranted to confirm the efficacy of omega-3 LCPUFA.

Introduction

The prevalence of Autism Spectrum Disorder (ASD) has dramatically increased over the past few years. While previous prevalence studies of ASD identified less than 10 in 10,000 individuals [1], recent estimates suggest rates of 90 to 250 in 10,000 individuals [2-5]. ASD is a life-long neurodevelopment disorder that appears during the first years of life [6]. Depending on the child's predominant symptomatology, children with ASD exhibit difficulties with expressing and understanding certain emotions, understanding others' mood, expressive language, and maintaining normal eye contact, as well as preference for minimal changes to routine, restricted ways of using toys and isolated play, all of which make it difficult for individuals to establish relationships with others, to act in an appropriate way and to live independently [6]. In addition, children with ASD frequently experience behaviour problems and medical conditions, including inflammation, oxidative stress, and autoimmune disorders [7-12], and altered brain structure and function (in a subset of individuals) [13,14]. The rising ASD rates are ascribed, in part, to a complex interaction between multiple genes and environmental risk factors [15], among which omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFAs) is a strong candidate. LCPUFAs and their metabolic products have been implicated in ASD via their roles in brain structure and function, neurotransmission, cell membrane structure and microdomain organisation, inflammation, immunity and oxidative stress [16-20].

Blood polyunsaturated fatty acids (plasma, serum, red blood cell (RBC), and whole blood) levels are considered reliable biomarkers of their status [21]. Abnormality in blood levels of omega-3 LCPUFA has been reported in psychiatric disorders including, but not limited to, attention deficit hyperactivity disorder (ADHD) and ASD [22-24]. Explanations for such

abnormalities have been suggested to be lower dietary intake of omega-3 LCPUFAs, and disturbances in fatty acid metabolism and incorporation of these fatty acids into cellular membranes in populations with autism compared to healthy controls [24-26]. A smattering of reports indicate differences in omega-3 LCPUFAs, omega-6 LCPUFAs and/or omega-6 to omega-3 LCPUFA ratios between populations with autism and healthy controls [14,26], but a few also failed to show any differences [27,28]. The reason for such discrepancies is not well examined, and there have been no attempts to systematically compare these studies. Hence, systematic analysis and synthesis of the evidence are warranted to determine if there are any differences in these blood fatty acids levels among healthy versus individuals with ASD, and if so, whether omega-3 LCPUFA supplementation may be beneficial in reducing symptoms in ASD.

To our knowledge, the efficacy of omega-3 LCPUFA supplementation in ASD has been investigated by six open-label trials [29-34] and one case study [35], the majority of which (six out of seven studies) showed significant improvement in symptoms of ASD (Appendix 1). Despite this promising evidence, randomised controlled trials (RCTs) examining the beneficial effect of omega-3 LCPUFAs in reducing symptoms of ASD have yielded inconclusive results. For example, Amminger *et al.* (2007) showed that supplementation with omega-3 LCPUFA (EPA+DHA) was superior over placebo in reducing stereotypy, inappropriate speech and hyperactivity [36], while Mankad *et al.* (2015) failed to show any effect of omega-3 LCPUFA supplementation on autism severity symptoms, adaptive functioning, externalizing behavior or verbal ability [37].

To date, two systematic reviews of interventions with omega-3 LCPUFA in ASD have been published [38,39]. In the review by Bent *et al.*, published in 2009, authors set broad inclusion criteria and included all intervention trials of omega-3 LCPUFAs of any type, dose, and duration addressing core and associated symptoms of ASD [38]. They identified six studies; one randomised controlled trial, four open-label trials and one case-study and concluded that the evidence was insufficient to support clinical recommendations [38].

Two years later, James, Montgomery and Williams (2011) published a Cochrane review including only two RCTs and performing meta-analyses on three primary outcomes (social interaction, communication and stereotypy) and one secondary outcome (hyperactivity) [39]. The authors reached the same conclusion as the Bent *et al.* review [38], and identified four ongoing studies. At the time of writing this review, the findings of one trial were published

[37], one was terminated in 2014 (NCT01248130)[11], and no information was available regarding the recruitment status or the availability of data for two trials (NCT00467818 and NCT01260961).

An updated systematic review is timely; more studies are now available, the prevalence of ASD is increasing together with a greater interest in the medical community (health professionals) on the beneficial effect of omega-3 LCPUFA in the treatment of neurodevelopment disorders, as well as an increasing interest in using complementary and alternative medication in this population [40].

Omega-3 LCPUFA: Metabolism, Biomarker, Optimum level

Polyunsaturated fatty acids (PUFA) are fatty acids with more than one carbon-carbon double bond. Based on the position of the final double bound from the methyl end, they are classified into different series of PUFA, of which omega-6 and omega-3 are the most investigated. These fatty acids have a numeric name, starting with the number of carbon atoms, followed by the position of double bonds [41]. The shortest chained omega-6 and omega-3 PUFAs are linoleic acid (LA; 18:2, n-6) and α-linolenic acid (ALA; 18:3, n-3), and act as an important substrate for arachidonic acid (AA), and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively [42]. From a nutritional point of view, LA and ALA are considered essential fatty acids because our body cannot synthesise them, and as such must be obtained from diet [43]. The main dietary sources of LA are vegetable oil products such as soybean, canola, palm, margarine and shortening, and for ALA are green plants, walnuts, and flaxseed oil [44].

The longer chain of these PUFA can be obtained from dietary sources or synthesised in the body. The main dietary sources of AA are organ meats, eggs, poultry, and certain fish, and of EPA and DHA are seafood such as salmon, tuna and mackerel [41]. Daily dietary intake of LCPUFA can range from 50 mg to up to 840 mg of AA, from 42 mg to 691 mg of EPA, and from 98 mg to 991 mg of DHA in populations with a diet high in fish [45]. In New Zealand, approximately 31% of pregnant women have been reported to meet the recommendation for DHA (recommended 200 mg/d) [46]. Similarly, based on the recent Australian national nutrition survey, only 20% of the population met the recommended n-3 LCPUFA intakes and 10% of women of child bearing age met the DHA recommendation [47]. To prevent chronic

[11]Due to a change in the research priorities of the principal investigator in combination with low participant interest.

diseases, the suggested daily dietary intakes for omega-3 LCPUFA is 430 mg and 610 mg for women and men, respectively [48].

Besides dietary intakes, these LCPUFA can be synthesised from their precursors, LA and ALA (**Figure 1**) [49]. The biosynthesis process utilizes three enzymatic steps for the conversion of LA to ARA and seven steps for the conversion of ALA to DHA [49]. Linoleic acid undergoes a desaturation process utilising a desaturase enzyme encoded for by gene fatty acid desaturase 2 (FADS2) (converted to gamma-Linoleic Acid, GLA, 18:3), followed by an elongation process utilising an enzyme encoded for by elongase 5 (ELOVL5) (converted to Dihomo-gamma-Linoleic Acid, DGLA, 20:3), and then a further desaturation process utilising another desaturase enzyme encoded for by FADS1 to generate AA (20:4) [49]. Similarly, ALA undergoes three enzymatic processes (two desaturations and one elongation) to be converted to Eicosapentaenoic Acid (20:5), and four further steps (two elongation, one desaturation and one oxidation) to produce DHA (22:6) [49] The conversion process of LA and ALA to their corresponding products shares the same enzymatic processes and the same encoding genes.

Accordingly, these fatty acids compete with each other in the enzymatic process, with a more pronounced effect in the first two desaturation processes [49]. Animal and human studies have provided evidence for such a mechanism by showing that when levels of LA or its metabolic intermediates are increased dramatically, the biosynthesis of omega-3 LCPUA reduces and subsequently the ratio of omega-6:omega-3 increases [49,50-52]. It should be noted that the ratio of omega-6:omega-3 in the diet and in body tissues is of clinical significance; the increased ratios are associated with increased risk and with the severity of many diseases and disorders, while the lower ratios have been shown to be protective [42,53]. Depending on the diseases under investigation, the optimal ratio is suggested to vary from 2:1 – 5:1 [42].

On the other hand, it has also been suggested that the biosynthesis of ARA from LA is a saturable process. In a systematic review of human trials, Rett and Whelan (2011) demonstrated no correlation between dietary intake of LA and ARA levels in erythrocyte and plasma/serum phospholipids [54]. The biosynthesis of LCPUFA from their substrates is also believed to be influenced by other factors (e.g. sex, genetics and ethnicity) [52,55-59]. A comprehensive review of the literature on these factors is beyond the scope of this paper, but they will be briefly discussed in the “Discussion” section to explain the findings.

Once these LCPUFA have been synthesised or obtained from the diet, they are transported to different cells or tissues for further metabolism. These fatty acids are transported in the circulation bound to albumin or esterified to complex proteins such as triglyceride, cholesterol

esters, or phospholipids in lipoproteins. Through the lipid bilayer, it has been suggested that LCPUFA are transported via three pathways; passive diffusion, membrane associated proteins, or a combination of both, though the exact pathway remains to be elucidated. Fatty acid binding proteins (FABP), fatty acid transporter proteins (FATP), and a long chain fatty acid receptor (CD36) [60,61] are involved in the membrane associated pathway. Once inside the cell, LCPUFA are bound to cytoplasmic fatty acid binding protein, and when free fatty acids are activated to fatty acid acyl-CoA (utilising acyl-CoA synthetase) bound to acyl-CoA-binding protein. Fatty acid acyl-CoA, are then mainly directed to beta-oxidation, while some are stored, or converted into membrane phospholipids.

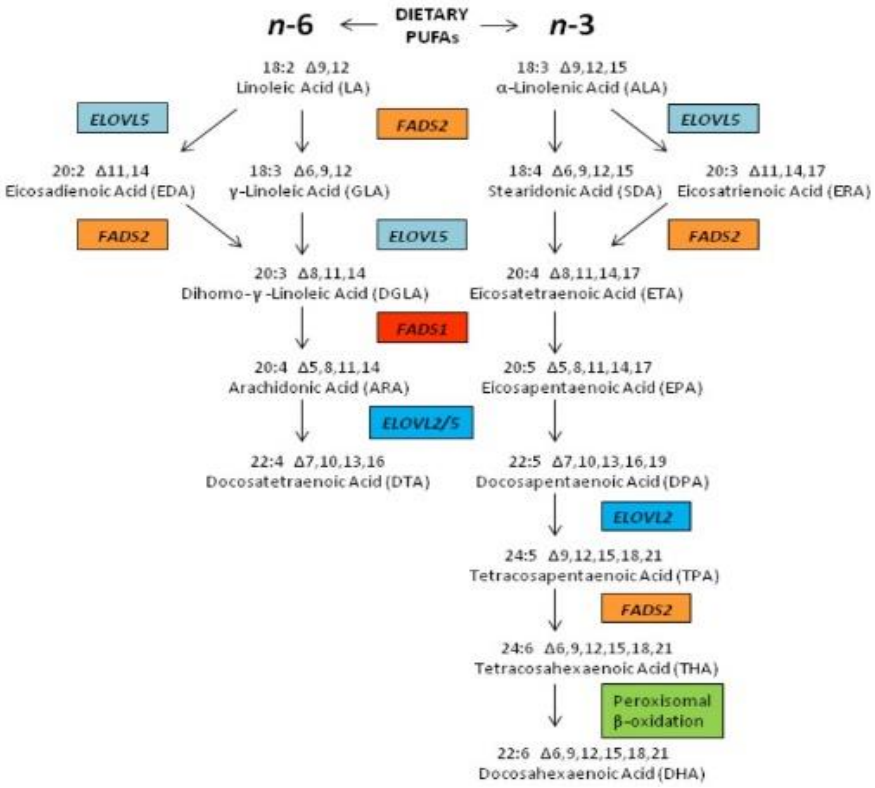


Figure 1: The biosynthetic process of LCPUFA. LCPUFA are derived from LA and ALA obtained from the mammalian diet by alternate desaturation (red/orange enzymes) and elongation (blue enzymes) steps. These enzymes utilize both omega-6 and omega-3 substrates. *Omega-3* LCPUFAs undergo further metabolism through a β-oxidation step (green box) to the generate DHA [49].

Blood levels of PUFA (plasma, serum, red blood cell, and whole blood) are considered biomarkers of PUFA status. While plasma and serum fatty acid profile reflects short-term intakes of the corresponding fatty acids, RBC fatty acid reflects long-term intakes and tissue levels. Whole blood fatty acid comprise both plasma and circulating cells (predominantly RBC). Red blood cell fatty acid and the omega-3 index (the EPA and DHA content of RBC membranes) expressed as a percent of total membrane fatty acids [62]) are believed to have several advantages over other mediums in the following regards [62]. RBC fatty acids have a

longer half-life (4-6 times longer) and are less variable than serum/plasma LCPUFA, are not influenced by the fasting or fed state, are responsive to increased intakes, and are resilient to pre-analytical variations.

According to the omega-3 index for mortality from cardiovascular disease, levels under 4% are associated with greater risk, and levels of 8-12% are shown to be protective [62]. Although the omega-3 index for neurodevelopment disorders has yet to be determined, it has been utilised in such studies [53,63,64]. The omega-3 index has been reported to be 1.4%, 3.2%, and 6.5% in children with ASD, ADHD, and typical development, respectively [53], indicating that levels under 4% may be considered as a risk factor for neuropsychological disorders.

Aim and Objectives

We aimed to conduct a current examination of evidence. We designed two systematic reviews and meta-analyses;

- Meta-analysis 1: a meta-analysis of evidence regarding blood omega-3 LCPUFA levels in populations with ASD compared to typically developing counterparts (with no neurodevelopmental disorders) of any age and sex. A secondary aim for meta-analysis 1 was to perform a priori subgroup analysis to investigate the influence of ASD on fatty acid composition across different age groups (studies including only young children < 12 yours old *vs.* studies also including children, teenagers, and adults).
- Meta-analysis 2: a meta-analysis of randomised controlled trials of omega-3 LCPUFA supplementation (of any type, dose and duration) in ASD populations (of any age and sex) to assess the clinical efficacy of omega-3 LCPUFAs treatment in reducing core symptoms of ASD and co-existing conditions.

Materials and Methods

All study procedures for both meta-analyses were pre-defined, but have not been registered or published elsewhere.

Eligibility Criteria

For meta-analysis 1, we included case-control observational studies that examined the differences in blood fatty acid levels between populations with ASD and healthy typically developing controls (with no neurodevelopmental disorders) of any age and sex. Studies were

excluded it they included non-typically developing controls, were non-English or unpublished. Because DHA, EPA and ARA are amongst the most reported fatty acids of omega-3 LCPUFAs and omega-6 LCPUFAs categories, respectively, and have been shown to be more biologically active in the brain and been linked to neurodevelopment disorders, we focused on these fatty acids as well as the ratio of ARA to EPA and DHA and the ratio of omega-6 LCPUFA to omega-3 LCPUFA [64,65]. We included studies that reported LCPUFA in various blood fractions expressed as either % of total fatty acids or in concentration units, including RBC, serum, plasma, plasma phospholipids and whole blood [21]. These fractions have been shown to be reliable markers for the general fatty acid pool [21].

For meta-analysis 2, we included RCTs of any dose, type, and duration of omega-3 LCPUFAs in participants with ASD of any age and sex who were randomised to receive either intervention or placebo, and reporting one of the following outcome measures: core symptoms of ASD including social interaction, communication, and repetitive restrictive behaviours or interests (RRB), and symptoms or behaviours associated with ASD including hyperactivity, irritability, sensory issues, and gastrointestinal symptoms. Unpublished and non-English studies were excluded.

Meta-analyses were performed if at least two studies employed the same assessment tool to measure the outcome of interest. There is a large variability in outcome assessment methods in ASD studies [66]. This use of different tools not only compromises the validity of a study by increasing the likelihood of type 1 error [67], but also complicates an effective comparison across studies.

Search Methods for Identification of Studies

We searched PubMed, MEDLINE, Web of Science, CINAHL, PsycINFO, PsycARTICLES and PsycNET up to May, 2016 to identify relevant studies in English. We employed broad search terms to include all potential studies that may fall within each of the mentioned reviews. The search strategy used the following terms: (“omega 3” OR “omega3” OR “omega-3” OR “polyunsaturated fatty acids” OR “polyunsaturated fatty acid” OR “essential fatty acids” OR “essential fatty acid”) AND (“autism” OR “autistic” OR “autism spectrum disorder” OR “Asperger”). We also reviewed the reference lists of all identified studies to identify additional studies. Results from each database were downloaded into EndNote (version X6, 2012, Thomson Reuters). Duplicates were removed and abstracts were screened. When an abstract met the eligibility requirements, it was assigned to one of two meta-analyses and the full article

was read to ensure the inclusion and exclusion criteria were met. The study identification was done by one investigator (H.M.).

Data Extraction, Management, and Quality Assessment

Two reviewers (H.M. and M.D.) independently performed data extraction from each study into pre-piloted extraction tables. Discrepancies in the data extraction were resolved by discussion and reaching consensus.

The following data were extracted for both meta-analyses: author, date of publication and setting, sources of funding, conflict of interest, aims, objectives and hypothesis, and population characteristics while extractions specific to each meta-analyses are described below.

For meta-analysis 1, the following data were also extracted: the mean and SD for blood omega-3 LCPUFAs (DHA, EPA or total), omega-6 LCPUFAs (ARA or total), and for omega-6 to omega-3 LCPUFA ratios (ARA to DHA, ARA to EPA, or total omega-6 to total omega-3 LCPUFA), fatty acid analysis method, the body tissue in which the fatty acid was measured, the unit of measure, and the significance value. If a study reported LCPUFA in two different blood tissues, the priority was given to RBC, followed by plasma phospholipids, serum/plasma, and whole blood. While RBC and plasma phospholipids LCPUFA reflects long-term fatty acid intake, serum/plasma or whole blood LCPUFA are influenced by recent intake of these fatty acids [21,68]. If a study reported both relative and absolute measures, the former measures were included in the meta-analysis to limit the methodological heterogeneity. The method by which blood fatty acid composition is expressed (relative *vs.* absolute) has been shown to modify the LCPUFA – disease relationship [69]. Inter-study variation in extraction and separation efficiencies in fatty acid analyses can be overcome by relative expression of fatty acids (expressed as a percentage of a fatty acid normalised to the total amount of all measured fatty acids in a sample) [69]. If more than two groups were included, only relevant groups were selected. A quality appraisal was performed in duplicate by two investigators (H.M. and M.D.) using the "Health Canada Quality Appraisal Tools for Observational Studies" [70]. A quality score of ≤ 6 was considered lower quality [70]. No studies were excluded based on quality scores, but sensitivity analysis was performed to assess the impact of these studies on the overall results.

For meta-analysis 2, study design, intervention (the dose of intervention was converted, where required, to gram from milligram for easy comparison), delivery method, compliance,

intervention period, outcome measures, assessment tools, results, conclusion, potential confounders and assessment of bias risk following Cochrane bias risk assessment including selection, performance, detection, attrition, reporting and commercial bias were also extracted [71]. A further quality appraisal was performed in duplicate by two investigators (H.M. and M.D.) using the "Health Canada Quality Appraisal Tools for Randomised Controlled Trials" to assess the quality of the individual studies [70]. A quality score of ≤ 7 was considered lower quality [70].

Statistical Analysis

Both meta-analyses were performed using Review Manager (RevMan, version 5.3, Copenhagen: The Nordic Cochrane Centre. The Cochrane Collaboration, 2014).

Meta-analysis 1

For each outcome, the mean and SD for each study group (cases and controls) was entered into Review Manager. If omega-3 to omega-6 fatty acid (total omega-3 to omega-6 LCPUFA, EPA to ARA or DHA to ARA) was reported, the ratio was converted to omega-6/omega-3 (1/ratio). The Review Manager calculator (between group differences) was employed to calculate the SD for these reverse ratios. The reverse ratio and SD were calculated for five studies [53,72-74,75].

The primary meta-analysis compared mean differences (95% confidence intervals (CI)) in outcomes across study groups. Due to significant heterogeneity a random effects model was used to calculate the forest plots with standardised mean differences and 95% CI. Standardised mean differences were calculated because blood levels of LCPUFA were measured and reported in different ways. A combination of Chi^2-statistic (P<0.1), I^2 statistics (I^2 0%-40%, low; 30%-60%, moderate; 50%-90%, substantial; 75%-100%, considerable heterogeneity), and considering the variation of point estimates and the overlap of CIs across different studies was performed to measure heterogeneity [71].

To avoid false positive or negative results, we limited the number of subgroup analyses to one (stratified by age < 12 years vs. other age groups) and sensitivity analyses to three (blood tissue type, study quality, and author's calculations). Then a priori subgroup analysis was performed using Chi^2-statistic with a P value of <0.05 taken to indicate statistical significance [71]. We could not conduct meta-regression to investigate the impact of the potential mediators (location, sex, and the way by which fatty acid composition is expressed) due to the

limited study numbers. To include one mediator in the analysis, at least 10 studies are required [71]. These variables were however carefully examined when the results were interpreted. Publication bias was examined using funnel plots in which the SE of the studies were plotted against their corresponding effect sizes.

Meta-analysis 2

For each outcome, the mean change and SD of change from baseline to endpoint for each intervention group (omega-3 LCPUFA and placebo) was entered into Review Manager. If only baseline and end data were available the mean change was calculated by deducting the baseline from the end value, and the SD was then imputed from a mean correlation coefficient for an outcome from other studies in the meta-analysis. Standard deviations were calculated for one study [76]. Study authors were contacted for missing data, and if no response was received the data was not included in the meta-analysis. Data was unable to be retrieved for three studies [77-79].

The primary meta-analyses compared mean (95% CI) differences (net change in scores) in each domain between omega-3 LCPUFA and control groups. Heterogeneity between studies was small hence a fixed-effects model was used to calculate forest plots with mean differences and 95% CI. Heterogeneity between studies was indicated using the same analyses employed in meta-analysis 1. No subgroup analyses or meta-regression were performed due to the limited number of studies included in the meta-analysis. However, one sensitivity analysis was performed to evaluate the impact of calculations (SDs) and major methodological differences on heterogeneity and the overall results. Publication bias was not assessed due to the small number of studies included in this meta-analysis.

Results

From the initial searches, 510 articles and from the cross-reference check, five articles were retrieved. Titles and abstracts of 254 articles were screened after non-English and duplicates were removed. At this level, 216 were excluded as not relevant to the current topic. The remaining 38 articles were read and categorised into two groups; 24 articles into the case-control studies group and 15 into the intervention group (**Figure 2**, PRISMA Flow Diagram).

Systematic Review and Meta-analysis 1

Of the 24 articles identified for meta-analysis 1, 15 were included in the meta-analysis. Reasons for exclusion were: not a case-control design [80,81], inappropriate control [25], the

data not reported in a form suitable for analysis [26,82-84], and double-reporting [85,86]. Characteristics of included studies can be found in **Table 1**.

The majority of studies were conducted in the Middle East (*n*=5; 2 Saudi Arabia (from one study group), 1 Oman, 2 Egypt) and Europe (*n*=4; 2 UK, 1 Belgium, 1 Italy), with others conducted in the US (*n*=2), Latin America (*n*=1), Canada (*n*=1), Asia (Japan, *n*=1), and Australia (*n*=1).

The 15 studies included 623 children and young people with ASD and 570 controls. Most studies included children under the age of 12, while a few included teenagers and adults also (*n*=3) [24,72,75]. One study included children and adults up to age 22 years [72]. Cases and controls were matched on both age and sex (*n*=8), two of which included other attributes such as IQ, home environment and dietary intake (*n*=1) or geographical region (*n*=1). Others matched two groups on either age (*n*=3) or sex (*n*=1), and one study included only males. Matching of cases and controls was not reported in two studies. In those studies including both sexes and reporting the sex distribution, the male:female ratio ranged from 2:1 to 12:1.

Most studies did not report the fasting state of blood samples while one study analysed non-fasting blood samples, and five studies fasting blood sample (ranging from 2 hours to overnight fasting) [28,72-74,87]. Fasting state is considered to affect fatty acid composition measured in plasma/serum but not in RBC [21,68]. While most studies reported serum/plasma fatty acid composition, four studies reported RBC levels [24,28,53,88] and two reported both [27,74]. Most studies reported relative levels while five studies reported absolute levels (all from the Middle East) [26,30,73,87,89], and one both levels [75]. Sensitivity analysis showed no impact of blood tissue type and the way by which fatty acid composition is expressed on the heterogeneity. However, the way by which fatty acid composition is expressed affected the overall effect size for some measures (Refer to the next section).

The majority of studies reported DHA, EPA and ARA levels while two studies did not report levels of EPA [26,89], and one ARA [89]. Five studies reported both total omega-3 LCPUFA and total omega-6 LCPUFA levels. One study did not report either of the mentioned measures but the ratio of ARA to DHA and ARA to EPA [73]. Total omega-6/omega-3 LCPUFA ratio was reported in six studies, of which two reported ARA/EPA ratio [27,53], and one reported both ARA/EPA and ARA/DHA also [74]. Of the remaining studies, two reported the ratio of ARA/EPA [28,88] and ARA/DHA each [26, 30], one both [75], and three no ratios [87,89,90]. Reverse ratios and SDs were calculated in five studies ([73] (only the ARA to EPA) and

[53,72,74,75]). With the exception of one study [73] (refer to the next section), sensitivity analysis showed no impact of calculation on the overall results.

All studies included in the review scored between four and nine points out of a possible 11 in our quality assessment tool, with three studies scoring ≤6 [24,88,89] (Appendix 2). It should be noted that the maximum score for the "Health Canada Quality Appraisal Tools for Observational Studies" is 12 [70] but because "measuring the exposure in duplicate or more" is of no relevance for case-control studies and all studies received a score of "0" for this criterion, the maximum score adds up to 11 for this review. Studies with scores of ≥7 are considered having higher quality. Sensitivity analysis showed no impact of removing studies with a quality score ≤6 on the heterogeneity or overall results.

Individual omega-3 LCPUFA (DHA, EPA, and ARA) and Their Ratios (ARA to DHA and ARA to EPA)

Significant differences were seen between those studies that recruited children only *vs.* those that also included teenagers and adults for blood levels of DHA and EPA (Chi^2=12, P=0.0006 and Chi^2=7.0, P=0.008, respectively) but not ARA (Chi^2=1.5, P=0.22) (**Figure 3**). Hence results for DHA and EPA for these subgroups were described separately. For ARA, the results described are from all studies combined.

Overall, in the younger age group (<12 years) studies, ASD children had significantly lower DHA and EPA levels than typically developing controls (standardised mean difference [95% CI] -2.1 [-3.2, -1.1], Z=3.9, P<0.0001 and -0.7 [-1.3, -0.2], Z=2.6, P=0.008, respectively). Considerable heterogeneity was seen for DHA (I^2=97%, P<0.00001) and EPA (I^2=88%, P<0.00001). Heterogeneity for DHA was not altered by removing any studies. Heterogeneity for EPA reduced slightly by removing the Parletta, 2016 study [53] (I^2=74%, P=0.0008). Removal of this study together with the Tostes, 2013 study [90] significantly reduced heterogeneity for EPA (I^2=0%, P=0.78) that was accompanied by a reduction in the difference between cases and controls (-0.3 [-0.5, -0.1], Z=2.7, P=0.006, n=356). These two studies were different with respect to some characteristics that may affect outcomes compared to other studies; children with ASD were significantly younger than typically developing children in the Parletta, 2016 study [53], and 88% of children with ASD in the Tostes, 2013 study were on psychotropic drugs [90].

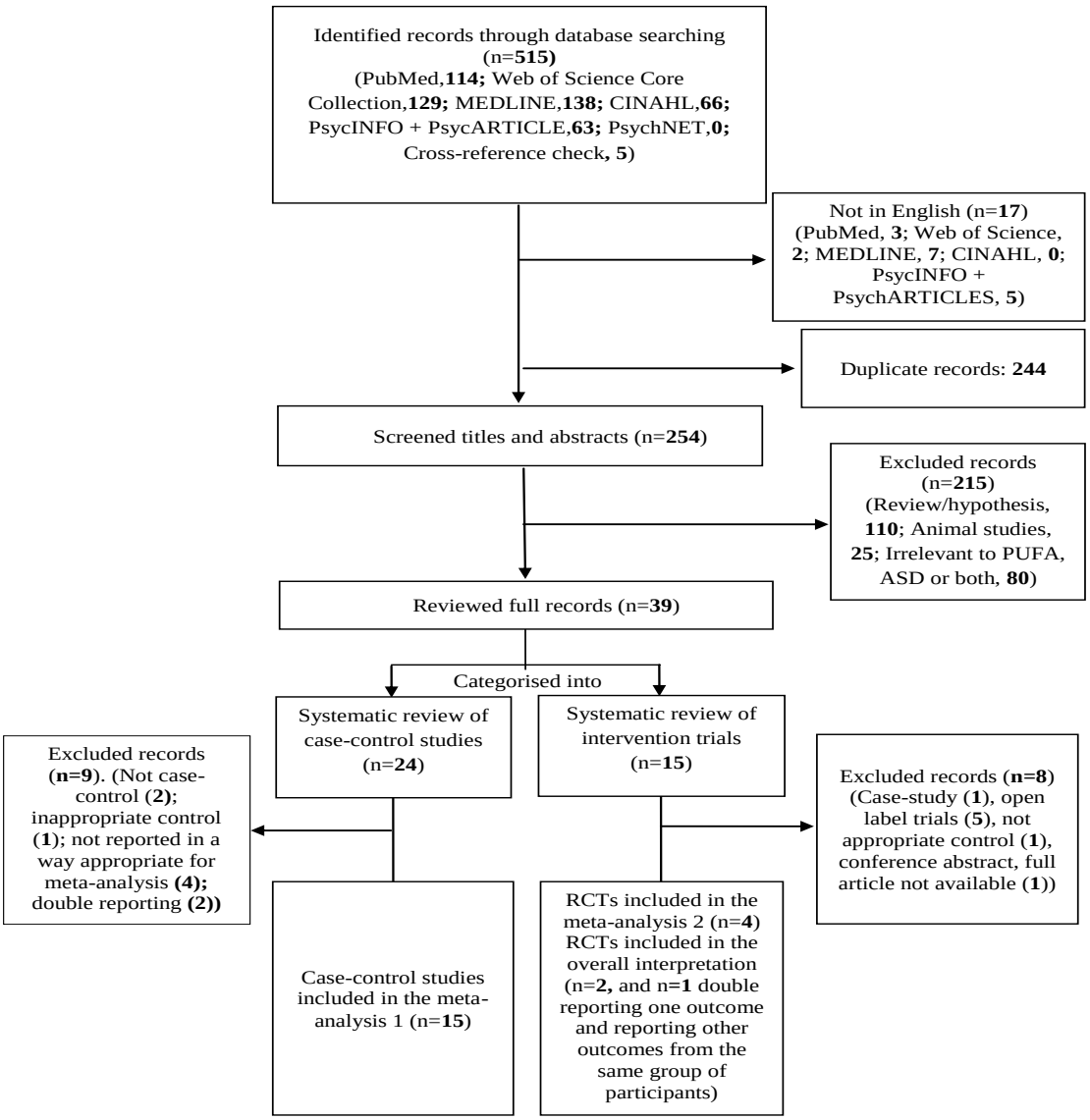


Figure 2. Flow diagram for selection of studies (PRISMA flow diagram)

In studies including all age groups (children, teenagers, and adults), no significant differences were seen in DHA and EPA levels between cases and controls (0.3 [-0.6, 1.2] and 0.3 [-0.2, 0.8], respectively). Heterogeneity for DHA and EPA was substantial (I^2=90%, P=0.0001 and I^2=68%, P=0.04, respectively). Removal of the Brigandi, 2015 study [24] reduced the heterogeneity significantly for DHA (I^2=0%, P=0.65) and slightly for EPA (I^2=64%, P=0.10). Removal of this study resulted in children with ASD having significantly higher DHA (0.7 [0.3, 1.1], Z=3.1, P=0.002, n=89) levels than typically developing controls but no impact on EPA. The Brigandi, 2015 study [24] was different from the other two studies in this subgroup in that both classic and regressive type ASD were included, cases and controls were not matched by any attributes, intellectual functioning of patients was not considered, and this study had a low quality appraisal score. The Sliwinski, 2006 [72] and Yui, 2016 [75] studies

included ASD patients with a borderline or normal intellectual functioning (IQ>70). The results should be interpreted with caution because the number of studies included is small (*n*=3).

With regard to ARA, children with ASD had significantly lower ARA levels than typically developing controls (-0.8 [-1.5, -0.2], *Z*=2.5, *P*=0.01), but heterogeneity between studies was substantial (I^2=96%, *P*=0.00001). Heterogeneity was not reduced by excluding any single study. However, removing studies that reported absolute levels [26,30,87] resulted in a smaller overall effect estimate (-0.2 [-0.9, 0.4], *Z*=0.7, *P*=0.48, *n*=840).

Only one study that included all age groups (children, teenagers, and adults) reported either ARA/DHA or ARA/EPA [75] thus the combined results of older and younger children are described (**Figure 4**). The ratio of ARA/DHA and ARA/EPA did not differ significantly between ASD populations and typically developing controls (*P*=0.94 and *P*=0.09, respectively). The heterogeneity was considerably high for both ratios. Heterogeneity was not reduced by excluding any studies (both ARA/DHA and ARA/EPA). With regard to ARA/EPA, however, the overall effect estimate changed considerably by the removal of El-Ansary's (2011a) study [73] (1.0 [0.3, 1.7], *Z*=2.9, *P*=0.004, I^2=91%). Cases and controls were not matched on sex in this study, the reverse ratio and SD was calculated, and the absolute level was reported.

Total omega-3 and omega-6 LCPUFA and Their Ratios

No significant differences were observed in total omega-3 and omega-6 LCPUFA between studies including young children only and those including all age groups (children, teenagers, and adults) (Chi^2=0.7, *P*=0.40 and Chi^2=3.8, *P*=0.05, respectively) thus the results for the combined groups are described (**Figure 5**).

Table 1: Characteristics of case-control studies included in meta-analysis 1

Reference and setting	Cases characteristics				Controls characteristics				Matching	Outcome				Quality score†
	N	Condition, classification system, tools$^{\delta}$	Age (years)*	Sex (M, F)	N	Health condition	Age (years)*	Sex (M, F)		Blood tissue type	Fasting state (length)	Values reported as	FA & ratios compared and the direction of difference	
Al-Farsi (2013) [89] Oman	40	Autism, based on DSM-IV, NR	4.1 (0.9)	NR	40	Healthy &TD	4.1 (0.8)	NR	Age Sex	Serum	NR	µg/ml	DHA↓	6
Bell (2004) [88] UK	29	11 classical autism & 18 regressive autism, NR, NR	NR	NR	55	Healthy & TD	NR	NR	None	RBC	NR	% of total FA	Total n3 ↓ DHA ↓ EPA ↓ Total ↔ ARA ↔ ARA/EPA ↑	4
Bell (2010) [27] UK	45	Autism, based on DSM-IV and ICD-10, ADI-R	7.5 (3.5)	39M, 5F	52	Healthy & TD	7.5 (3.6)	49M, 3F	Age Sex (45 pairs matched)	RBC Plasma	NR	% of total FA	Total n3[1]↔ DHA ↔ EPA ↔ Total ↔ ARA ↔ Total n6/n3[2]↑ ARA/EPA ↑	8

Table 1: *Cont.*

Reference and setting	Cases characteristics				Controls characteristics				Matching	Outcome				Quality score†
	N	Condition, classification system, tools$^{\delta}$	Age (years)*	Sex (M, F)	N	Health condition	Age (years)*	Sex (M, F)		Blood tissue type	Fasting state (length)	Values reported as	FA & ratios compared and the direction of difference	
Brigandi (2015) [24] US	121	Autism (but not Asperger or PDD-NOS), based on DSM-IV, CARS	3-17**	NR	110	Non autistic and developmentally delayed	3-17**	NR	NR	RBC	No	% of total FA	Total n3 ↓ DHA ↓ EPA ↔ Total n6 ↓ ARA ↓ Total n6/n3^{2}↑	6
Bu (2006) [28] US	40	Autism and regressive autism, based on DSM-IV and ICD-10, ADI-R & ADOS	3.6***	37M, 3F	20	TD	3.5***	16M, 4F	Age Sex Geographical residential area	RBC	Yes (2h)	% of total FA	Total n3 ↔ DHA ↔ EPA ↔ Total ↔ ARA ↔ ARA/EPA ↔	8
El-Ansari (2011a) [73] Saudi	25	Autism, NR, ADI-R, ADOS, 3di	4-12**	NR	16	Healthy &TD	4-11**	NR	Age	Plasma	Yes (10h)	mmol/l	ARA/DHA ↓	7

Table 1: *Cont.*

Reference and setting	Cases characteristics				Controls characteristics				Matching	Outcome				Quality score†
	N	Condition, classification system, tools$^{\delta}$	Age (years)*	Sex (M, F)	N	Health condition	Age (years)*	Sex (M, F)		Blood tissue type	Fasting state (length)	Values reported as	FA & ratios compared and the direction of difference	
El-Ansari (2011b) [87] Saudi Arabia	22	Autism, NR, ADI-R, ADOS, 3di	4-12**	NR	26	Healthy & TD	4-11**	NR	Age	Plasma	Yes (10h)	mmol/l	DHA ↓ EPA ↔ ARA ↓	7
Ghezzo (2013) [14] Italy	21	Autism, DSM-IV, ADOS & CARS	6.8 (2.2)	17M, 4F	20	Healthy & TD	7.6 (1.9)	14M, 6F	Age Sex	Serum	NR	% of total FA	DHA ↓ EPA ↓ ARA ↔ Total n6/n3 ↑	11
Jory (2016) [74] Canada	11	Autism, DSM (version NR), NR	3.9 (1.7)	8M, 3F	15	Healthy & TD	3.9 (1.1)	6M, 9F	Age	RBC Serum	Yes (NR)	% of total FA	DHA[1]↓ EPA ↓ ARA ↓ Total n6/n3 ↑ ARA/DHA ↔ ARA/EPA ↔	7
Meguid (2008) [30] Egypt	30	Autism, DSM-IV, clinical evaluations & CARS	3-11**	18M, 12F	30	Healthy & TD	NR	NR	Age Sex	Whole blood	NR	µg/ml	DHA ↓ ARA ↓ ARA/DHA ↓	7

Table 1: *Cont.*

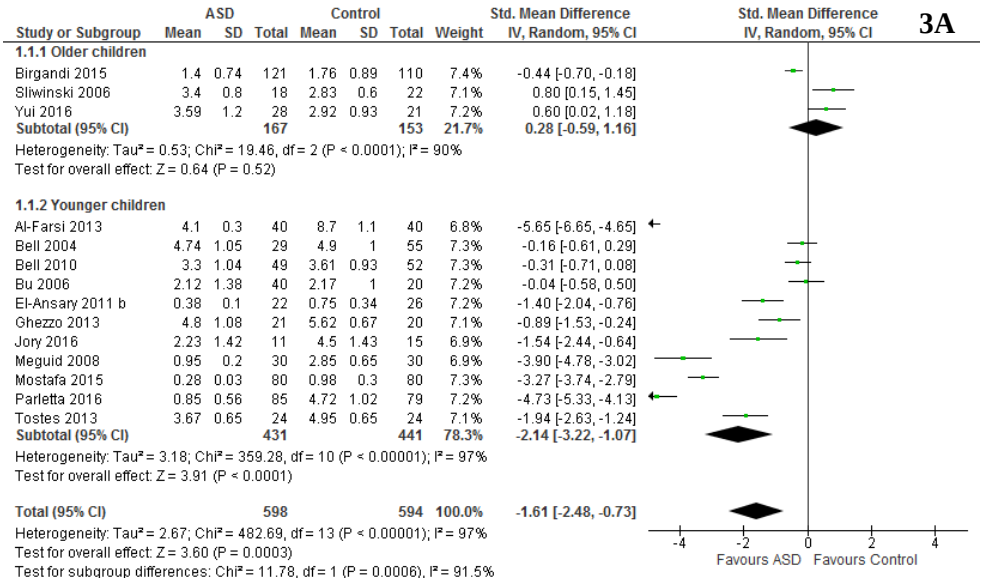
Reference and setting	Cases characteristics				Controls characteristics				Matching	Outcome				Quality score†
	N	Condition, classification system, tools$^{\delta}$	Age (years)*	Sex (M, F)	N	Health condition	Age (years)*	Sex (M, F)		Blood tissue type	Fasting state (length)	Values reported as	FA & ratios compared and the direction of difference	
Mostafa (2015) [26] Egypt	80	Autism, DSM-IV, clinical evaluation & CARS	7.4 (3.3)	66M, 14F	80	Healthy & TD	7.3 (3.1)	66M, 14F	Age Sex	Plasma	NR	mmol/l	DHA ↓ ARA ↓ ARA/DHA ↑	8
Parletta (2016) [53] Australia	85	Autism, clinical evaluation & CARS	5.3 (2.1)^	68M, 17F	79	Healthy & TD	8.3 (2.5)^	61M, 18F	Sex	RBC	NR	% of total FA	DHA ↓ EPA ↓ ARA ↓ Total n6/n3 ↑ ARA/EPA ↑	9
Sliwinski (2006) [72] Belgium	18	Autism with IQ>55 and post pubertal, DSM-IV, ADI-R	12-20**	Only male	22	TD post pubertal	12-22	Only male	None	Plasma	Yes (overnight)	% of total FA	Total n3 ↑ DHA ↑ EPA ↔ Total n6 ↔ ARA ↔ Total n6/n3^{2}↓	8
Tostes (2013) [90] Brazil	24	Autism, DSM-IV, clinical evaluation	7.4 (2.9)	18M, 6F	24	Healthy & TD	7.2 (1.8)	18M, 6F	Age sex	Plasma	NR	% of total FA	DHA ↓ EPA ↓ ARA ↑ ARA/DHA^{3}↑ ARA/EPA^{3}↑	9

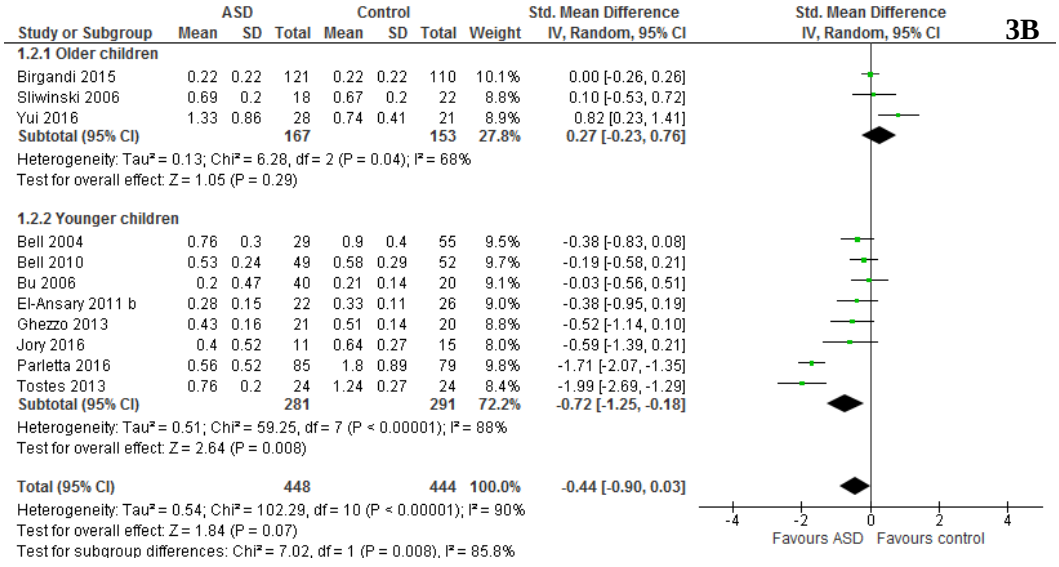
Table 1: *Cont.*

Reference and setting	Cases characteristics				Controls characteristics				Matching	Outcome				Quality score†
	N	Condition, classification system, tools[δ]	Age (years)*	Sex (M, F)	N	Health condition	Age (years)*	Sex (M, F)		Blood tissue type	Fasting state (length)	Values reported as	FA & ratios compared and the direction of difference	
Yui (2016) [75] Japan	28	Autism with IQ/70, DSM-IV, ADI-R	13.5 (4.7)	20M, 8F	21	Healthy &TD	13.9 (5.7)	15M, 6F	Age Sex IQ Eating habit Home environment	Plasma	NR	% of total FA & µg/ml	DHA[4]↑ EPA ↑ ARA ↓ ARA/DHA ↓ ARA/EPA ↓	8

ADI-R, Autism Diagnostic Interview-Revised; ADOS, Autism Diagnostic Observation Schedule; ARA, arachidonic acid; CARS, Childhood Autism Rating Scale; DHA, docosahexaenoic acid; DSM-IV, Diagnostic and Statistical Manual of Mental Disorder-Fourth Edition; EPA, eicosapentaenoic acid; F, female; FA, fatty acids; M, male; N, number of participants; NR, not reported; RBC, red blood cell; TD, typically developing; 3di, the Developmental, Dimensional, and Diagnostic Interview
[δ] Psychological assessment tools used to confirm ASD diagnosis. *Reported as mean (SD) unless otherwise stated. † Health Canada Quality Appraisal Tool for Observational Studies; A quality score of ≥ 7 was considered higher quality [70]. ** Inclusion criteria. *** Median. ^ Significantly different. ↓ Cases had lower levels than controls (P<0.05). ↑ Cases had higher levels than controls (P<0.05). ↔ No difference across groups (P>0.05). [1] RBC values are reported. [2] A borderline significance. [3]The significance was not reported.[4]% of total fatty acids is reported.

The pooled standard mean differences for the total omega-3 LCPUFA and total omega-6 LCPUFA between ASD and typically developing children were -0.16 [-0.54, 0.21] (I^2=73%, substantial heterogeneity) and 0.6 [-0.2, 1.3] (I^2=93%, considerable heterogeneity), respectively. Both were not statistically significant. Excluding comparisons that included teenagers and adults also [24,72] reduced heterogeneity in total omega-3 LCPUA (I^2=42%, P=0.18) (-0.3 [-0.7, 0.01], Z=1.9, P=0.06, n=245). Heterogeneity was reduced to an acceptable level when the Sliwinski, 2006 study [72] only was removed (I^2=14%, P=0.32) (-0.4 [-0.6, -0.2], Z=3.4, P=0.0007, n=472). The Sliwinski, 2006 study [72] was different from other studies with respect to several characteristics; it included post pubertal youngsters up to age 22 years, it was a male-only study, and included those with an IQ >55. Heterogeneity in total omega-6 LCPUFA was not altered by the removal of any studies.





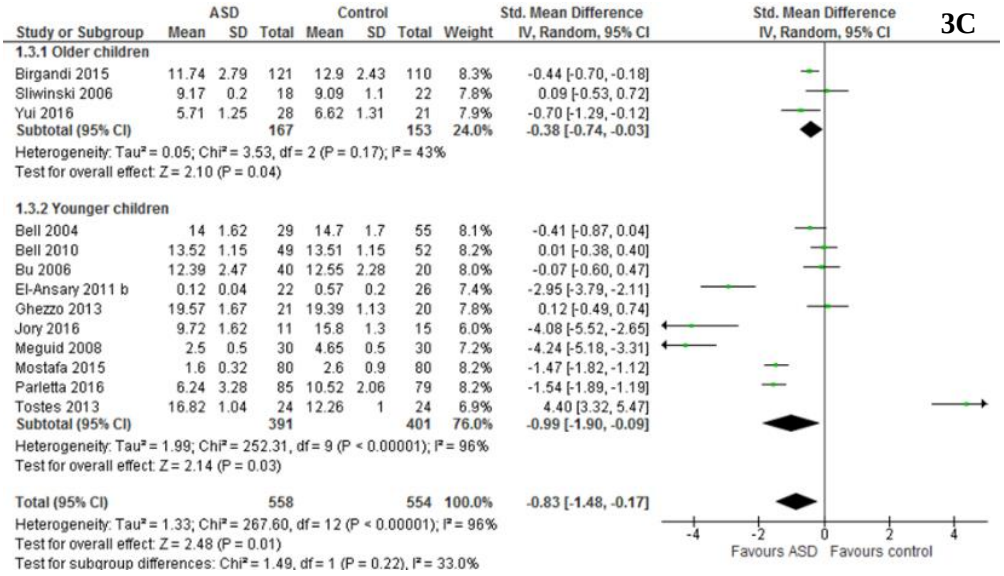
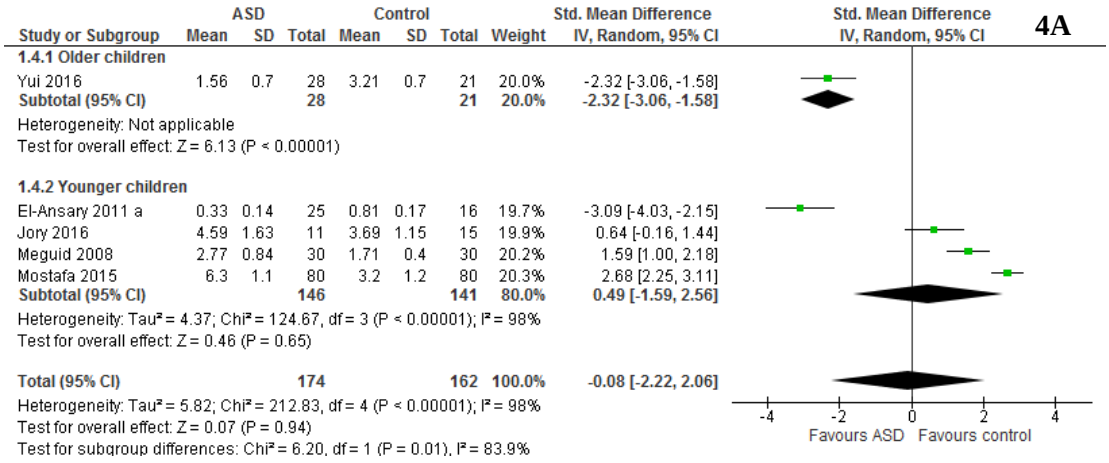


Figure 3: Forest plots of mean (95% confidence interval (CI)) weighted difference in blood levels of docosahexaenoic acid (DHA) (**3A**), eicosapentaenoic acid (EPA) (**3B**), and arachidonic acid (ARA) **(3C**) between populations with Autism Spectrum Disorder (ASD) and typically developing controls stratified for subgroups with studies including all age groups (children, teenagers, and adults) *vs.* those including children only. Direction of effect (negative, lower mean in ASD group; positive, lower mean in control group; zero, no difference between groups).

The ratio of total omega-6 LCPUFA to omega-3 LCPUFA did not differ significantly between studies including young children only and those including all age groups (children, teenagers, and adults) (Chi^2=3.04, P=0.08) thus the overall results are described here (**Figure 6**). Children with ASD had a significantly higher omega-6 LCPUFA to omega-3 LCPUFA ratio (0.4 [0.1, 0.8], Z=2.3, P=0.02). The heterogeneity was substantial and was decreased by the exclusion of comparisons that also included teenagers and adults [24,72] (I^2=29%, P=0.24). The difference between cases and controls as well as the effect size increased considerably, 0.7 [0.4, 0.9], Z=4.4, P<0.00001, n=328.

The funnel plots for DHA and ARA, indicated publication bias with a lack of smaller studies (studies with larger SEs) reporting negative results. Examination of the funnel plot for EPA indicated no evidence of publication bias.



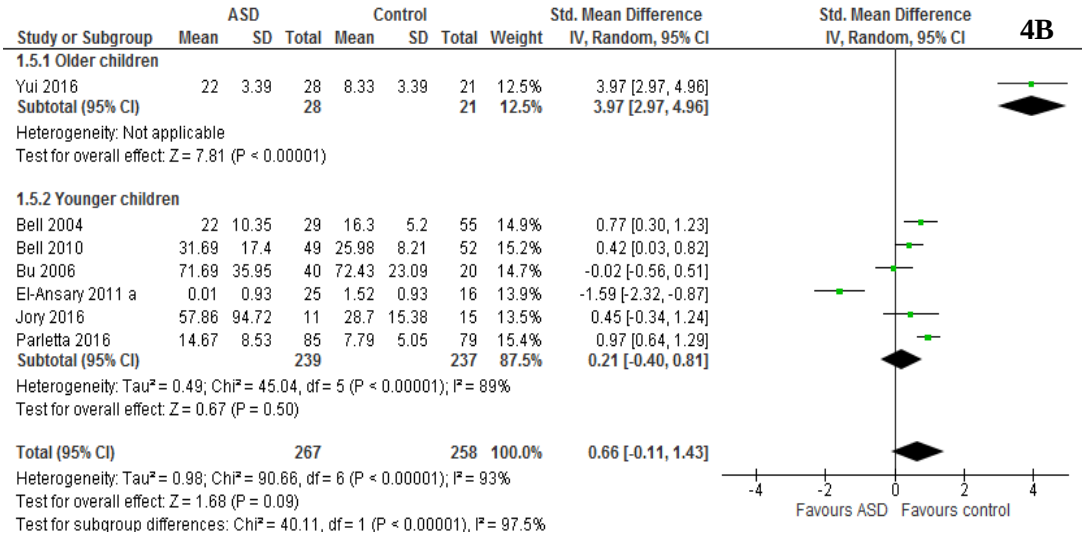


Figure 4: Forest plots of mean (95% confidence interval (CI)) weighted difference in the ratio of arachidonic acid (ARA) to docosahexaenoic acid (DHA) (**4A**) and the ratio of ARA to eicosapentaenoic acid (EPA) (**4B**) between populations with Autism Spectrum Disorder (ASD) and typically developing controls stratified for subgroups with studies including all age groups (children, teenagers, and adults) *vs.* young children only. Direction of effect (negative, lower mean in ASD group; positive, lower mean in control group; zero, no difference between groups).

Systematic Review and Meta-analysis 2

Of the 15 RCTs identified for systematic review and meta-analysis 2, four were included in the meta-analysis [36,76,91,92], two were included in the overall interpretation but not included in the meta-analysis [37,77], one was a double-reporting of one outcome [76] and reporting other outcomes from the same group of participants [79], and eight were excluded

(**Figure 2**). Reasons for not being included in the meta-analysis 2 but being included in the overall interpretation were: data not reported in a format suitable for analysis [77], and use of an assessment tool not used by others [37]. Reasons for complete exclusion were: a conference paper with unpublished results at the time of writing this review[12] [78], one open label randomised parallel intervention trial including a low sugar healthy diet as the control [29], and not being a RCT (*n*=6, one case-study and 5 open label trials [30-35]). Characteristics of included studies can be found in **Table 2** and of excluded studies in Appendix 1.

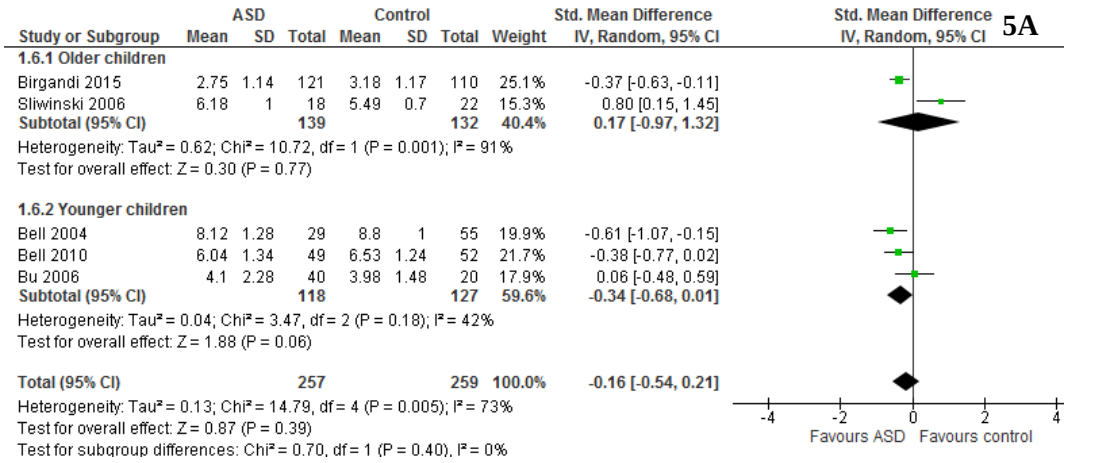
Of those included in the meta-analysis 2, two were conducted in the US [91,92], one in Austria [36], and one in Japan [76]. In the four studies, 55 participants with ASD received omega-3 LCPUFA supplements and 52 received placebo. The Bent, 2011 and 2014 studies included children under the age of 8 years [91,92], the Amminger, 2007 study included children under 17 years [36], and the Yui, 2011 study included children older than 6 years and adults up to 28 years [76].

Of the two studies that could not be included in the meta-analysis, one study was from the US [77] and one from Canada [37] together including 37 and 34 individuals in the intervention and placebo groups, respectively. Children under the age of 5 and 10 years were included in the Mankad, 2015 and Voigt, 2014 studies, respectively [77].

All but one (the Amminger, 2007 study included only males [36]) included both males and females. Study groups were not matched on sex in these trials. The male to female ratio ranged from 3:1 to 12:1.

[12] The article was published at the time of writing this thesis but the full article was not available for detailed review. However, based on the available abstract, Parellada *et al.* (2017) used a randomised, crossover, placebo-controlled study to investigate the effect of omega-3 (962mg/d for children and 1155mg/d for adolescents, for eight weeks, *n*=68) on RBC fatty acid composition (primary outcome) and behaviours (secondary outcome). It was demonstrated that treatment with omega-3 improved omega-6 to omega-3 ratio (primary outcome). Also, there was a within participant significant improvement in behaviours, but no treatment effect was detected. The lack of behavioural effect could be partly explained by carry over effect and short study period (Parellada, M., *et al.*, Randomized trial of omega-3 for autism spectrum disorders: Effect on cell membrane composition and behavior. Eur. Neuropsychopharmacol., 2017. **27**(12): p. 1319-1330.)

Of the RCTs included in the meta-analysis 2, the severity of autism at baseline was not considered in two trials [36,92], and the other two included patients with pre-defined severity (moderate severity and ABC social withdrawal subscale of >10 in the Bent, 2011 and Yui, 2011 studies, respectively) and IQ level (>50 and >80 in the Bent, 2011 and Yui, 2011 studies, respectively [76,91]). Of those included in the overall interpretation, the Voigt, 2014 study included patients with pre-defined severity (CARS score of >30 [77] and the Mankad, 2015 study equally distributed the severity across groups [37]. Co-existing problem behaviour was an inclusion criteria in two trials entered the meta-analysis 2 (hyperactivity in the Amminger, 2007 [36] and Bent, 2014 studies [92]).



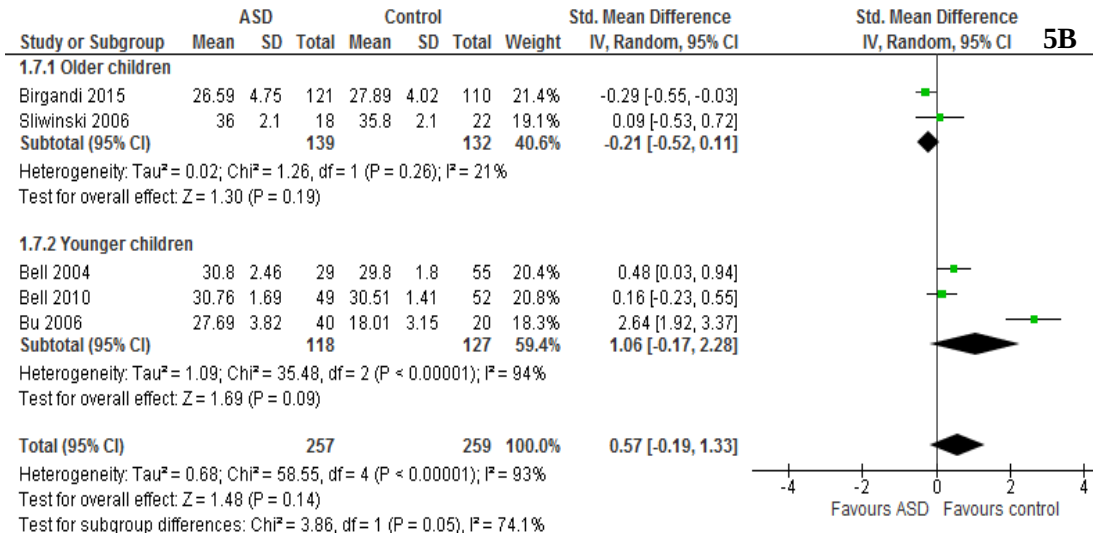


Figure 5: Forest plots of mean (95% confidence interval (CI)) weighted difference in the total n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) (**5A**) and total omega-6 long chain polyunsaturated fatty acids (omega-6 LCPUFA) (**5B**) between populations with Autism Spectrum Disorder (ASD) and typically developing controls stratified for subgroups with studies including all age groups (children, teenagers, and adults) *vs.* young children only. Direction of effect (negative, lower mean in ASD group; positive, lower mean in control group; zero, no difference between groups).

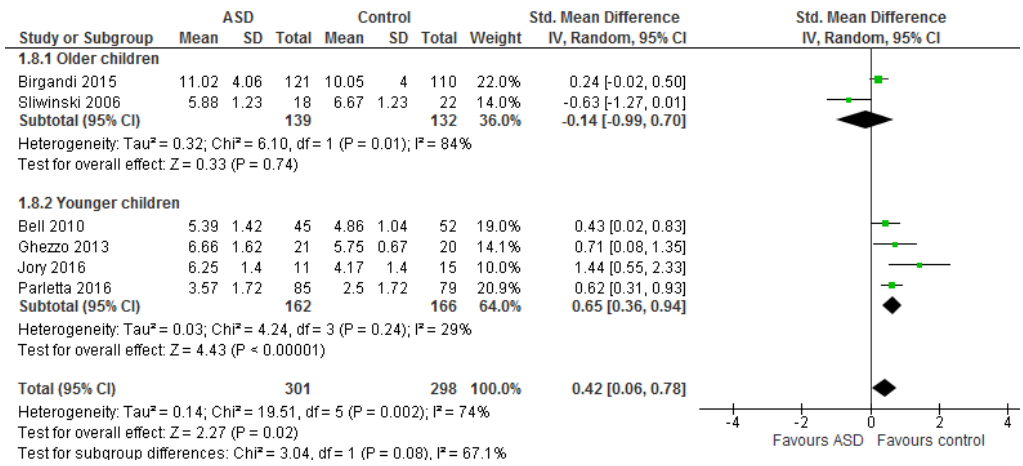


Figure 6: Forest plot of mean (95% confidence interval (CI)) weighted difference in the ratio of total omega-6 long chain polyunsaturated fatty acids (omega-6 LCPUFA) to total n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) between populations with Autism Spectrum Disorder (ASD) and typically developing controls stratified for subgroups with studies including all age groups (children, teenagers, and adults) *vs.* young children only. Direction of effect (negative, lower mean in ASD group; positive, lower mean in control group; zero, no difference between groups).

Study length ranged from 6-16 weeks in RCTs included in the meta-analysis 2 and was 26 weeks in RCTs included in the overall interpretation. The majority of participants were supplemented with both EPA and DHA. The Yui, study (2011 and 2012) [76,79] used a combination of DHA and AA and the Voigt, 2014 study used only DHA [77]. Intake of EPA and DHA ranged from 0.7 to 0.8 g/day and 0.2 to 0.7 g/day, respectively, with the Yui, 2011 (and 2012) and Voigt, 2014 studies having the lowest DHA dose [76,77,79]. The Mankad, 2015 study reported an initial total dose of EPA and DHA of 0.8 g/day for two weeks which was doubled when tolerability to that dose was determined [37]. Placebos used were olive oil [37,76,79], safflower oil [91,92], corn oil + soybean oil [77], and coconut oil [36].

Behaviours were assessed using a variety of assessment tools (ranging from one to six tools used in each study) including, Aberrant Behaviour Checklist (ABC) ([36,76,91,92] and [77] but the outcome was not reported), Social Responsiveness Scale (SRS) [79,91,92], Behaviour Assessment System for Children (BASC) [37,77,91], Clinical Global Impression (CGI) [37,77,91,92], and further several tools that were used in isolation . Of these, full data was available for only four studies using the same assessment tool (ABC) [36,76,91,92].

All studies included in the review were of good quality, scoring 11-14 points out of a possible 15 (Appendix 3). Studies with scores of >7 are considered as having higher quality.

Table 2: Study characteristics of randomised controlled trials (RCTs) included in systematic literature review

RCTs included in meta-analysis 2 (*n*=4)									
Reference and setting	**Age (years)**	**Sex distribution (M, F)**	**Sample size**	**Intervention**		**Duration**	**Outcome measure**	**Outcome**	**Quality score †**
				Active	**Placebo**				
Amminger (2007) [36] Austria Pilot	5-17	All male	Intervention (*n*=7) Placebo (*n*=6, 1 lost)	0.8 g/day EPA 0.7 g/day DHA	7 g/day coconut oil	6 weeks	ABC	No significant differences between groups at 6 weeks, but a greater change in hyperactivity and stereotypy subscale with a large effect size in the omega-3 group than placebo (7.0 and 2.4 units, effect size of 0.7 and 0.7, respectively). Well tolerated and safe.	10
Bent (2011) [91] US Pilot	3-8	24M, 3F	Intervention (*n*=14, 1 lost and 4discontinued) Placebo (*n*=13, 1 lost and 2 discontinued)	0.7 g/day EPA 0.5 g/day DHA	Orange flavoured pudding containing safflower oil	12 weeks	ABC PPVT EVT BASC SRS CGI-S	Significant increase in the percentage of serum omega-3 fatty acids. No significant differences in all measure across groups. Non-significant greater improvement in hyperactivity subscale in omega-3 group than placebo (2.7 *vs.* 0.3 units, effect size of 0.4). Decreases in some fatty acids correlated with decreased in hyperactivity. Well tolerated and safe.	13

Table 2: *Cont.*

Reference and setting	Age (years)	Sex distribution (M, F)	Sample size	Intervention		Duration	Outcome measure	Outcome	Quality score †
				Active	Placebo				
Bent (2014) [92] US Internet-based	5-8	50M, 7F	Intervention (*n*=29, 8 discontinued and 2 improper enrolment) Placebo (*n*=28, 4 discontinued and 1 improper enrolment)	0.7 g/day EPA 0.5 g/day DHA	Orange flavoured pudding containing safflower oil	6 weeks	ABC- (parent and teacher) SRS CGI-I	No significant differences in changes in SRS and CGI-I between groups. Non-significant greater improvement in hyperactivity subscale in omega-3 group than placebo (-5.3 *vs.* -3.4, effect size of 0.3). Significantly greater improvements in stereotypy and lethargy subscales (*P*=0.05 and 0.01, respectively). Well tolerated and safe.	13
Yui (2011& 2012)* [76,79] Japan Pilot	6-28	12M, 1F	Intervention (*n*=7) Placebo (*n*=6)	0.2 g/day DHA 0.2 g/day AA	Olive oil	16 weeks	ABC ADI-R SRS	Significant increase in plasma AA. No differences in plasma DHA and EPA. Significant improvement in social withdrawal subscale of ABC (*P*=0.04) and stereotyped and repetitive behaviours of ADI-R (*P*=0.04). Significant improvement in communication subscale of SRS reported in both treatment and placebo groups, though the effect size was more favourable for the treatment group than placebo group (0.9 *vs.* 0.4, respectively). Safe.	10

Table 2: *Cont.*

Reference and setting	Age (years)	Sex distribution (M, F)	Sample size	Intervention		Duration	Outcome measure	Outcome	Quality score †
				Active	Placebo				
Mankad (2015) [37] Canada	2-5	27M, 10F	Intervention (*n*=19, 4 drop outs) Placebo (*n*=19, 2 drop outs) Stratified by severity	1.5 g/day EPA+DHA	Refined olive oil in medium chain triglyceride	6 months	PDDBI BASC-2 CGI-I VABS-II PLS-4	No significant differences between groups in all measures at 6 months, but mild improvement in BASC-2 externalising subscale in placebo but worsening in omega-3 group (-3.0 *vs.* 3.0, respectively, *P*=0.02) Relatively well tolerated and safe	13
Voigt (2014) [77] US	3-10	40M, 8F	Intervention (*n*=24, 5 discontinued) Placebo (*n*=24, 9 discontinued) Stratified by age	0.2 g/day DHA	0.3 g/day corn oil + 0.3 g/day soybean oil	6 months	CGI-I CDI ABC BASC	431% increase in plasma phospholipid DHA No significant differences in the percentage with a positive response (CGI-I) across groups and in all other measures across groups. Well tolerated and safe	13

AA, arachidonic acid; ABC, Aberrant Behaviour Checklist; ADI-R, Autism Diagnstic Interview-Revised; BASC, Behaviour Assessment System for Children; CDI, Child Development Inventory; CGI-I, Clinical Global Impression-Improvement; CGI-S, Clinical Global Impression-Severity; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; EVT, Expressive Vocabulary Test; F, Female; M, Male; n, Number; PDDBI, Pervasive Developmental Disorders Behavioural Inventory; PLS-4, Preschool Language Scale; PPVT, Peabody Picture Vocabulary Test; SRS, Social Responsiveness Scale; US, United States; VABS-II, Vineland Adaptive Behaviour Scale

† Health Canada Quality Appraisal Tool for Experimental Studies; A quality score of ≥8 was considered higher quality [70]. * Different outcomes from the same group of participants were reported in two different papers.

The quality criteria failed by most studies were whether intention-to-treat or per-protocol analysis was conducted (though according to final sample size analysed – larger than the sample size with drop outs deducted – it was apparent that the majority employed intention-to-treat analysis), and controlling for potential confounders. The most prevalent potential confounding factors that were not reported or reported but not considered in statistical analysis were dietary intake of LCPUFA or baseline LCPUFA status (as a measure of LCPUFA status, either dietary intake or blood level of LCPUFA needs to be reported).With the exception of the Bent, 2011 study [91] which investigated the impact of baseline LCPUFA status on behavioural changes in response to supplementation, the majority of studies failed to examine such a relationship while assessing baseline LCPUFA status [37,76,77,79]. Further factors were compliance (not reported in most studies) and medical regimen (most studies recruited patients on a stable medical regimen but did not report the type of regimen and its distribution across groups). It is also worth noting that a small number of females were included in these studies (with a male:female ratio ranging from 1:3 to 1:12) which could be a limitation in terms of generalisability, though reflecting the sex distribution of ASD.

The risk of bias for each study is summarized in Appendix 4. Participants in the Amminger, 2007 and Yui 2011 (and 2012) studies were reported to be randomised but no details were available for random sequence generation [36,76,79]. Other studies used computer generated number [91,92] and block randomisation stratified by attributes including severity [37] and sex [77]. With the exception of the Amminger, 2007 study [36], randomisation was prepared by a third party in all trials [37,76,77,79,91,92]. The Amminger, 2007 provided no details regarding who performed the randomisation [36]. All studies were reported as double-blinded (both researchers/assessors and participants) [37,76,77,79,91,92]. However, in the Amminger, 2007 study it is unclear if the researchers/assessors were blinded [36]. It is also unclear when researchers/assessors and participants were unblinded in the Amminger, 2007, Yui, 2011 (and 2012), and Mankad, 2015 studies [36,37,76,79]. The blinding was kept for the entire study (including data analyses) in the Bent, 2011, Bent 2014, and Voigt, 2014 studies [77,91,92]. With the exception of the Yui, 2011 (and 2012, no drop outs) [76,79] and Bent, 2014 (all included in the final analyses) [92] studies, participants were lost to follow up in the Amminger, 2007 (one individual from control) [36], Mankad, 2015 (one individual from omega-3 LCPUFA group) [37], Bent 2011 (two individuals; one from each arm) [91], and Voigt, 2014 (five from omega-3 LCPUFA and nine from control) [77] studies. The reason for drop outs in the Voigt, 2014 study were difficulty with participation (four from omega-3 LCPUFA and three from control), trouble taking the supplements (one from omega-3 LCPUFA and three from control), and concerns about supplement side effects (three from

control) [77]. Regarding the latter concern, it is not clear if participants withdrew due to worsening behaviour, not observing any improvement, or because of an actual side effect during the intervention. With the exception of the Voigt, 2014 study [77], all outcomes in all trials were reported. The Voigt, 2014 study examined 52 behavioural subscales but only three outcomes were reported [77]. It is worth noting that the primary outcome was the measure of CGI which was completely reported. Other sources of bias including commercial bias were not apparent in any study.

Effect of omega-3 LCPUFA on Core Symptoms of ASD

Social interaction: The fixed mean difference for social interaction (assessed using ABC) significantly favoured omega-3 LCPUFA with small effect (-2.0 [-3.5, -0.3], Z=2.4, P=0.02) and no heterogeneity (I^2=0%, P=0.92) (**Figure 7A**). Removing the Yui, 2011 study [76] did not change the results. The Yui, 2011 study [76] differs from others in that their sample included older participants (2-28 years) and those with IQ >50, the daily dose of DHA was lower (0.2 g/day) and ARA (0.2 g/day) was added to the supplement. In addition, they used different dosing regimens for different age groups (half a dose for children aged 6-10 years) and SD was imputed resulting in a substantially greater SD in the omega-3 LCPUFA group compared to the placebo group and other studies. Using SRS social interaction sub-domains (social motivation, social cognition, and social awareness), the Bent, 2014 study [92] found no effect of omega-3 LCPUFA on social interaction (all domains >0.05). Similarly, the Yui, 2012 study [79] did not find any effect of omega-3 LCPUFA on any sub-domains of social interaction (measured by SRS, all P>0.05). The social interaction in response to intervention did not differ across groups in the Mankad, 2015 study [37] where the authors used other assessment tools. The mean change scores decreased (showing an improvement) in both treatment groups in these studies. Voigt *et al.* (2014) found a significant difference in BASC social skills, favouring omega-3 LCPUFA (-0.2 *vs.* 3.0, P=0.04), which disappeared after correction for multiple comparisons [77].

Communication: Communication scores (assessed using ABC) did not differ between omega-3 LCPUFA and placebo groups (-0.4 [-1.3, 0.6], P=0.42) (**Figure 7B**). Moderate heterogeneity was seen in the meta-analysis for communication (I^2=51%, P=0.11). Removing the Yui, 2011 study [76] reduced the heterogeneity to 0% but had no impact on the overall result. Bent *et al.* (2011) and Bent *et al.* (2014) also used other tools including Peabody Picture Vocabulary Test (PPVT), Expressive Vocabulary Test (EVT) and the communication sub-domain of SRS, respectively [91,92]. Neither study found any effect of omega-3 LCPUFA on communication. Similarly, the Mankad, 2015 study [37] did not find any differences across groups and the Voigt, 2014 study [77] reported worsened outcome

(reported by teachers) in response to omega-3 LCPUFA supplementation compared to placebo that showed improvement (1.4 *vs.* -4.5, *P*=0.02). However, the Yui, 2012 study [79] found greater improvements in SRS communication sub-scale scores in omega-3 LCPUFA group than the placebo group (-23 *vs.* -21, *P*=0.03).

Repetitive and restricted interests and behaviours: The fixed mean difference for RRB (assessed using ABC) favoured omega-3 LCPUFA with small effect (-1.1 [-2.2, -0.01], *Z*=1.9, *P*=0.05) (**Figure 7C**) and nil heterogeneity (I^2=0%, *P*=0.68). Removing the Yui, 2011 study [54] removed the significance (*P*=0.08) perhaps due to low statistical power. Using the Autism Diagnostic Interview-Revised (ADI-R) scale, Yui *et al.* (2011) also reported a significant improvement in one of four RRB sub-domains, stereotyped and repetitive motor movement (-1.7 *vs.* -0.7, *P*=0.04). However, Bent *et al.* (2014), using the RRB sub-domain of SRS, reported a trend favouring placebo, -2.9±12 (placebo) *vs.* -8.6±11 (omega-3 LCPUFA), *P*=0.08 [92]. Neither Yui *et al.* (2012) (using SRS RRB subscale) nor Mankad *et al.* (using Pervasive Developmental Disorders Behavioural Inventory (PDDBI) resistance to change subscale) showed an effect of omega-3 LCPUFA intervention on RRB (*P*>0.05) [37,79]. The mean scores improved in both treatment groups in these studies.

Effect of LCPUFA on Co-existing Conditions

Hyperactivity: Hyperactivity scores (assessed using ABC) did not differ between treatment groups (-2.1 [-4.9, 0.6], *P*=0.13) (**Figure 8A**) and heterogeneity was nil (I^2=0%, *P*=0.98). Sensitivity analysis by removing studies including older participants [36,76] had no effect on the overall result. Similarly, using BASC hyperactivity sub-domain, the Bent, 2011 study [91] did not find any difference between groups (*P*=0.83). Using the BASC externalizing behaviour scale, Mankad *et al.* (2015) reported a significantly worsened outcome in response to omega-3 LCPUFA supplementation compared to placebo (3.2 *vs.* -3.0, *P*=0.02) [37]. It should be noted that BASC externalizing behaviour is a composite measure of hyperactivity, aggression and conduct problems. The authors suggested that greater pre-existing gastrointestinal distress at baseline (8/19 *vs.* 1/19, in the omega-3 LCPUFA group *vs.* placebo group) may have predisposed the omega-3 LCPUFA group to higher externalizing behaviour.

Irritability: Irritability scores (assessed using ABC) did not differ between groups (0.1 [-2.1, 2.3], *P*=0.91) (**Figure 8B**) and heterogeneity was nil (I^2=0%, *P*=1.00). Sensitivity analysis by removing studies including older participants [36,76] had no effect on the overall result.

Sensory issues: The Mankad, 2015 study [37] was the only study that assessed the effect of omega-3 LCPUFA supplementation on sensory issues (using sensory/perceptual approach behaviour domain of PDDBI). Sensory symptoms comparably improved in both study groups. It should be noted that this domain has five clusters, all of which tap into a variety of repetitive behaviors.

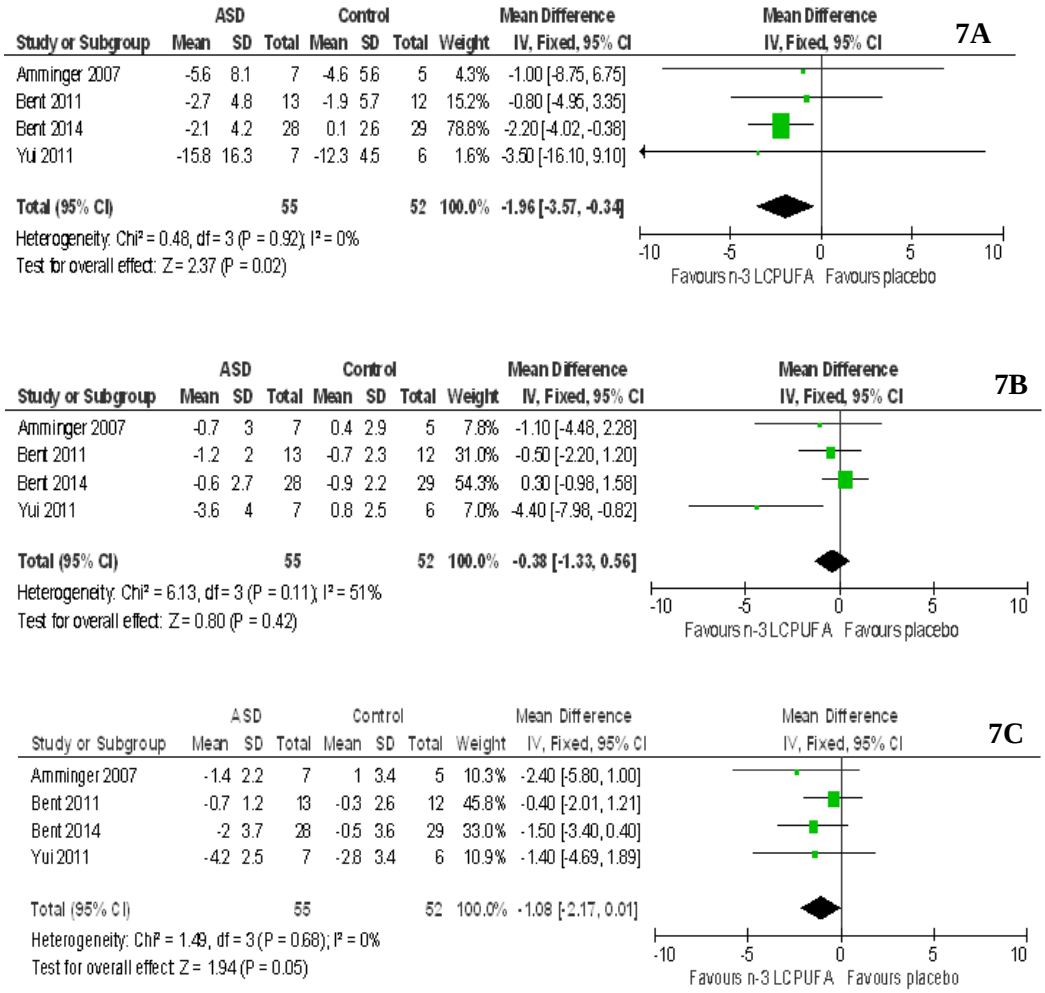


Figure 7: Forest plot of mean (95% confidence interval (CI)) fixed difference in change in social interaction (ABC) (**7A**), communication (ABC) (**7B**), and repetitive and restricted interests and behaviours (ABC) (**C**) in populations with Autism Spectrum Disorder (ASD) receiving omega-3 long chain polyunsaturated fatty acid supplementation (omega3 LCPUFA) and placebo. Direction of effect (negative, more improvement in omega-3 LCPUFA groups; positive, more improvement in placebo group; zero, no difference between groups).

Gastrointestinal symptoms: The Mankad, 2015 study [37] was the only study that assessed the effect of the intervention on gastrointestinal distress and found no differences across treatment groups (P>0.9) (assessed using CGI-I).

Publication bias could not be determined for any outcome measures due to small number of studies included in the meta-analysis (*n*=4).

Tolerability and Safety of LCPUFA Supplementation

All RCTs included in this review concluded that LCPUFA supplementation was well tolerated and safe. Adverse effects reported were not serious and were comparable across treatment groups.

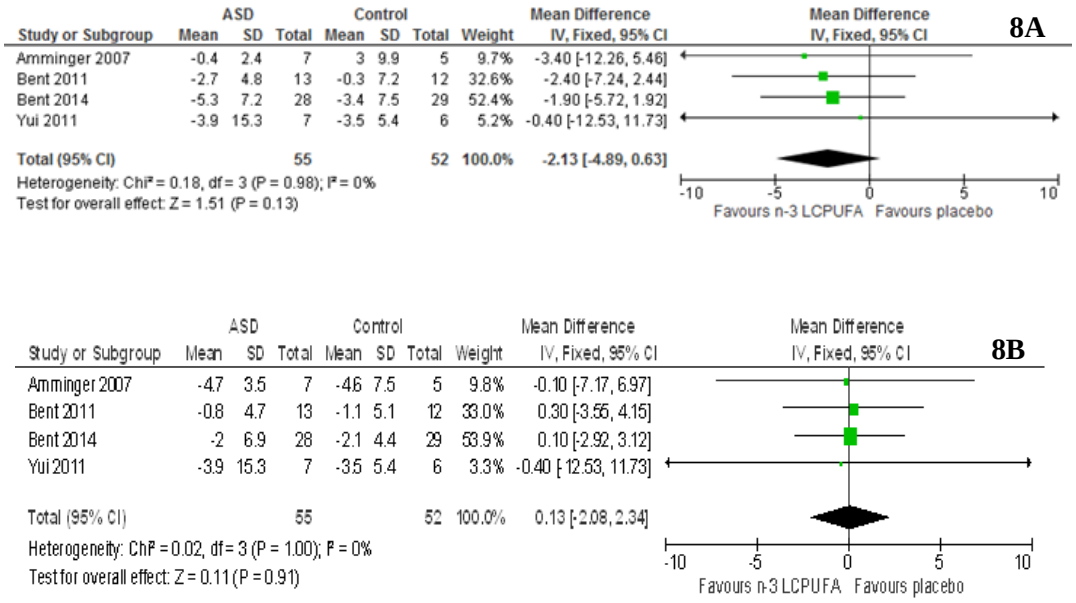


Figure 8: Forest plot of mean (95% confidence interval (CI)) fixed difference in change in hyperactivity (ABC) (**8A**) and irritability (ABC) (**8B**) in populations with Autism Spectrum Disorder (ASD) receiving n-3 long chain polyunsaturated fatty acid supplementation (n-3 LCPUFA) and placebo. Direction of effect (negative, more improvement in n-3 LCPUFA group; positive, more improvement in placebo group; zero, no difference between groups).

Discussion

The findings of each meta-analysis are individually discussed (starting with a discussion of findings of meta-analysis 1 and then meta-analysis 2) followed by a discussion on potential mechanistic pathways that might underlie the relationship between LCPUFA and ASD.

Systematic Review and Meta-analysis 1

The current study (meta-analysis 1), to our knowledge, is the first meta-analysis of case control studies of blood fatty acid levels in populations with ASD. The findings of this study were that children with ASD had lower levels of DHA, EPA, and higher total omega-6

LCPUFA to omega-3 LCPUFA ratio, but not ARA to DHA and ARA to EPA ratios, compared to typically developing children. However, the differences were only evident in studies that included children only and not in studies with wide age ranges that also included adolescents and adults. One should be cautious to make conclusions regarding the modulating effect of age on the relationship since the number of studies including homogenous samples of adolescents and homogenous samples of adults are limited.

Herein we compare the findings of the current study with those of meta-analyses in ADHD because there is an overlap in symptoms between ASD and ADHD. Our results are in agreement with recently published meta-analyses showing lower omega-3 LCPUFA levels, with larger effect size when DHA and EPA within each study were pooled than when these fatty acids were separately considered [93], and higher ratios of omega-6 LCPUFA to omega-3 LCPUFA [64] in patients with ADHD compared with healthy controls.

Another finding of the current study is that while the majority of included studies were of high quality, there was large methodological and clinical heterogeneity between studies, highlighting the importance of discussing the results in light of the study and population characteristics. The type of blood tissue in which the fatty acid composition is analysed has been suggested to affect the findings of case-control studies [27,74]. Bell *et al.* (2010) and Jory (2016) compared plasma/serum fatty acid composition with those of RBC [27,74]. The authors found significantly lower LA [27], ARA, DHA, and EPA [74] and higher omega-6 LCPUFA to omega-3 LCPUFA ratio [74] in RBC of children with autism compared with healthy controls. No polyunsaturated fatty acids in Bell *et al.*'s study [27] and only DHA in Jory's study [74] were found to be significantly different across groups when plasma/serum were compared. However, sensitivity analysis in the current study revealed no effect of removing studies that reported RBC fatty acids on heterogeneity.

The method by which blood fatty acid composition is expressed (relative *vs.* absolute) has also been shown to alter the findings of case-control studies investigating the fatty acid composition across groups and to modify the LCPUFA – disease relationship [69,75]. Yui *et al.* (2016) compared relative levels of plasma fatty acids with the same fatty acids expressed as absolute [75]. The authors found a significant difference in DHA, EPA, DPA, and arachidic acid, and the ratios of ARA to DHA and ARA to EPA (the reverse ratios were reported) across groups when relative levels were expressed. However, the significance disappeared for DHA and arachidic acid when absolute levels were compared. Although the removal of those studies reporting absolute levels had no impact on the heterogeneity, it resulted in the loss of significance across groups for ARA and in a significantly higher ARA

to EPA in populations with ASD than typically developing controls. These findings highlight the importance of taking the method by which blood fatty acid composition is expressed into account when blood fatty acid profile is investigated. A potential explanation for such findings could be the inter-study variation in extraction and separation efficiencies in fatty acid analysis [69].

The high heterogeneity reported in this meta-analysis could also be explained by populations' characteristics. The role of age and sex on fatty acid status has been well documented [82,94,95]. Using a linear model, Wiest *et al.* (2009) demonstrated that ARA level was modified by sex; while ARA level did not differ between male children with autism and healthy controls, female children with autism had significantly lower ARA than healthy controls [82]. Thus, an uneven distribution of age (particularly if a wide age range is included) and sex across groups could alter the results in different ways; mask the difference, change the effect direction, or influence the effect size, all of which may contribute to heterogeneity seen in this study. Lack of effect in the Bell, 2004 study (EPA, DHA, and ARA) [88], change of direction in the El-Ansary, 2011a (ARA/EPA and ARA/DHA ratios) and 2011b (ARA), and Sliwinski, 2006 studies (total omega-3 LCPUFA, DHA, and total omega-6/omega-3 LCPUFA ratio) [72,73,87], and the large effect size in the Parletta, 2016 study (EPA, DHA, ARA and their ratios) [53] may be explained by such characteristics.

The use of psychotropic medication can be another modifier. Psychotropic medications such as rispiradone may affect RBC fatty acid composition through their effect on oxidative stress and lipid peroxidation [23,96]. The Parletta, 2016 study [53] together with the Tostes, 2013 study [90] resulted in high levels of heterogeneity in EPA. ASD children in the former study were significantly younger than typically developing children [53], and approximately 88% of children with ASD in the Tostes, 2013 study were on psychotropic medication [90].

Location is another factor that may modify the fatty acid composition – autism relationship. With reference to those studies included in this meta-analysis, the Yui, 2016 [75] and Sliwinski, 2006 [72] studies were conducted in Japan and Belgium (among European countries) where the habitual dietary intake of fish and fish products are potentially high [97]. Both studies reported populations with autism having significantly higher DHA and EPA levels and lower omega-6 to omega-3 LCPUFA ratio than healthy controls, and both affected heterogeneity largely [72,75]. High consumption of omega-3 LCPUFA rich foods can mask the difference, and in populations who are potentially prone to disturbances in fatty acid metabolism can alter the effect direction [72,75].

Altered fatty acid composition in ASD has been suggested to be, in part, due to low dietary intake of LCPUFA. Children with ASD have very limited food preferences that may result in these children having limited intake of LCPUFA rich foods [89]. Only three studies included in this meta-analysis assessed the dietary intake of fatty acids [14,75,89]. Al-Farsi, 2013 reported a lower intake of ALA, assessed using a semi-quantitative food frequency questionnaire, in children with ASD than healthy controls [0.8 (0.2) *vs.* 1.2 (0.4) g/day, *P*=0.001] [89]. However, Ghezzo *et al.* (2013) [14] and Yui *et al.* (2016) [75] found no difference in LCPUFA intake across groups, pointing to the facts that these abnormalities could be due to disturbances in fatty acid metabolism rather than intake. For a brief discussion on the potential mechanistic pathways of LCPUFA in ASD refer to "Potential Mechanistic Pathways" section.

Systematic Review and Meta-analysis 2

Few RCTs have been completed and reported to date on omega-3 LCPUFA supplementation for ASD; only six trials were included in this review (four included in the meta-analysis and two in the overall interpretation), with a total of 178 participants. In this meta-analysis, a small but significant benefit of omega-3 LCPUFA supplementation was found for social interaction and RRB but not communication and co-existing behaviours and conditions. No evidence of significant heterogeneity between trials were found.

Four trials included in the present meta-analysis with a total of 107 participants are however insufficient to provide robust evidence. Furthermore, the findings cannot be generalised to all children on the autism spectrum because the included children predominantly comprised males, were of different age groups (less than eight years and up to 28 years), displayed moderate to severe symptoms [76,91] or high hyperactivity level [36,92].

The findings of this review to some extent contradict the results of previous systematic reviews and meta-analysis [38, 39]. These reviews did not find any statistically significant improvement in behaviour, but reported a larger positive effect on hyperactivity [38, 39] while the present review found a significant improvement in social interaction and no improvement in hyperactivity. Our results, however, are in line with those of previous reviews [38, 39] in that no improvement was identified in communication and irritability. With respect to RRB, sensory issues and gastrointestinal symptoms, no comparison can be made because they were not considered by these reviews. It should be noted that the small number of RCTs included in the review by Bent *et al.* (*n*=1) [38] and James *et al.* (*n*=2) [39] could have compromised the statistical power to detect any difference across groups.

Although case-control and open label studies provided evidence for a role of omega-3 LCPUFA in ASD, RCTs of supplementation with omega-3 LCPUFA yielded mixed results. One reason for such inconsistencies between studies may result from inadequately controlling for age, trial duration, habitual dietary intake of omega-3 LCPUFA and levels of these fatty acids in the circulation over the course of trial, participants' general health conditions at baseline and over the course of study, and outcome tools assessing behaviour. Response to omega-3 LCPUFA supplementation has been shown to be predicted by body weight adjusted dose, baseline omega-3 index (RBC DHA + EPA), sex, age, and physical activity level; with populations receiving larger doses, having a lower starting omega-3 index, older populations, females and those with higher physical activity levels experiencing a greater increase in the omega-3 index in response to supplementation [98].

Dietary intake of omega-3 LCPUFA rich foods in children with autism is low [67]. However, omega-3 fatty acid supplements are among the most commonly used complementary and alternative medication in ASD [40]. It is plausible to suggest that even though the mentioned trials excluded participants that used omega-3 LCPUFA supplements on their own initiatives at baseline, participants may have had high habitual dietary intake of these fatty acids due to their popularity, therefore responding differently to supplements which leads to diminishing the differences across treatment groups. An example of such implication could be the Voigt, 2014 study [77]; all children in this study had baseline LCPUFA levels above paediatric reference ranges for nutritional deficiencies and metabolic disorders (established at the Mayo Clinic [99]), and despite an increase of 431% in plasma DHA level, no improvement in behaviour was reported. Bent *et al.* (2011), on the other hand, showed that higher baseline level of some omega-6 and omega-3 polyunsaturated fatty acids including eicosadienoic acid (r=-0.8, P=0.02), docosadienoate acid (r=-0.7, P=0.03), and ALA (r=-0.6, P=0.03) were associated with reduction in hyperactivity [91]. In an open label trial of omega-3 LCPUFA in 41 children with autism aged 7-18 years, Ooi *et al.* (2015) showed an inverse correlation between autism mannerism severity and change in RBC fatty acids after 12 weeks of intervention and the severity was associated with baseline EPA level [32]. Unfortunately, the number of trials included in this review was inadequate to provide data on the effect of baseline omega-3 LCPUFA intake or status on behavioural changes in response to supplementation.

The sex differences in ASD might be partly explained by sex differences in fatty acid metabolism; males may be more vulnerable than females to deficiencies in LCPUFA because of hormonal reasons [100], and thus may respond poorly to supplements [98]. With the exception of one study [36], no trials included in this review stratified the randomisation by

sex. Unfortunately, no analysis could be performed for subgroups stratified by sex in this review because far more males than females were included and no studies reported behavioural changes in response to supplementation for males and females separately. With regard to the potential effect of age, three out of six studies included a wide age range (children and young people up to 28 years) which may have resulted in greater response variability. Due to the small number of studies included in this review, we could not perform subgroup analysis for different age bands to examine the effect of age on behavioural change in response to omega-3 supplementation.

The trial duration varied widely in studies included in this review (6-26 weeks). Evidence suggests that PUFA erythrocyte membrane reaches a steady state after 6 months [101] and at least 4 months is needed to demonstrate an effect on cognitive performance [41]. It has also been suggested that longer study periods of one year might be needed to demonstrate behavioural changes in response to omega-3 LCPUFA supplementation [102]. Furthermore, the majority of outcome assessment tools are retrospectively completed (and duration over which behaviour is assessed varies depending on the assessment tool used); for example, parents are required to consider the behaviour over the past four weeks when completing the ABC questionnaire while the timeframe for SRS is the past six months. It is of importance to avoid assessing and considering behaviour when the LCPUFA erythrocyte membrane has not yet reached its steady state. Therefore, wide variation and insufficient trial duration could explain the inconsistencies. Due to the small number of studies included in this review, we could not perform subgroup analysis for different intervention lengths to examine the effect of duration on behavioural change in response to omega-3 supplementation.

Gastrointestinal symptoms are highly prevalent in populations with ASD [103-105]. Mazurek *et al.* (2013) reported that of 2,973 children with ASD, 25% had at least one chronic gastrointestinal symptom [105]. Compared to typically developing children, developmentally delayed and ASD children were more likely to have at least one frequent gastrointestinal symptom [105]. ASD children with frequent abdominal pain, gaseousness, diarrhea, constipation or pain on stooling had worse scores in four (irritability, social withdrawal, stereotypy, and hyperactivity) out of five ABC subscales than ASD children with no frequent gastrointestinal symptoms [106]. The pain and discomfort caused by gastrointestinal distress can worsen the behaviour in people with ASD, more particularly in non-verbal individuals who cannot express their feelings. Mankad *et al.* (2015) reported significantly worsened externalizing behaviours in response to omega-3 LCPUFA supplementation which could be attributed to higher gastrointestinal distress reported in the active treatment group compared with the placebo (8/19 *vs.* 1/19, respectively) [37]. Thus, it is important to consider the

potential modulating effect of gastrointestinal symptoms over the course of study on behavioural changes in response to supplementation.

Additionally, a variety of assessment tools (ranging from one to six tools) were used in each study which complicated effective comparisons across studies resulting in the exclusion of three studies from the meta-analysis, and potentially compromised the validity of a study by increasing the likelihood of type 1 error [67]. With the exception of ABC, a widely-used tool to assess problem behaviours in pharmacological trials in ASD, the majority of assessment tools have been designed for diagnostic purposes and there is a lack of evidence regarding the sensitivity of these tools to slight changes in behaviour in response to intervention in ASD populations. It is also worth noting that the inappropriate speech subscale of ABC is not a comprehensive measure of communication compared to other tools like SRS (inappropriate speech subscale of ABC comprises of four questions while the SRS communication subscale consists of 22 questions). Thus, findings regarding communication (measured by ABC) should be interpreted with caution.

It is also worth noting that the small or lack of effect reported here could be due to large placebo response in many trials included in this review. The large placebo response may have limited our capacity to identify any differences in some behaviour across groups. It is documented that different factors [e.g. raters of outcome assessment tools (clinicians *vs.* parents), increased response to active intervention, location, pharmacological and adjunctive intervention, participants' age (younger *vs.* older), study duration (shorter *vs.* longer), and severity of condition (lower baseline severity *vs.* higher severity)] are associated with the increased placebo response in ASD and other neurodevelopment and psychiatric disorders [107-109]. However, the reason for observed improvement in placebo groups included in this review is unclear and could not be determined due to small number of studies included.

Potential Mechanistic Pathways

There are, though not very well understood, several potential biological pathways for a role of LCPUFA in ASD [110,111]. Approximately 60% of the brain's dry weight is fat, with DHA comprising 60% and 40% of the PUFA in the retina and brain, respectively [112], suggesting that it is structurally important. Evidence suggests that some individuals with ASD have abnormalities in the gray and white matter of brain regions that are involved in social interaction, RRB, and sensory processing [13,113-115]. Ingestion of DHA (through diet or supplementation) has been shown to be positively associated with gray matter volume and its functional integrity and with white matter microstructural integrity in healthy individuals [16,17,116].

At the cellular level, PUFA interact with and influence the functioning of integral membrane proteins, including enzymes, receptors, and ion channels [117,118]. Evidence suggests that the activity of Na+/K+-ATPase (an enzyme that controls ion transport produced by neurotransmission) and adenylate cyclase (an enzyme that catalyses the conversion of ATP to cyclic AMP and has been shown to modulate social behaviours [119]) is disturbed in individuals with ASD [14,120]. Ghezzo *et al.* (2013) reported children with ASD having a significant reduction in Na+/K+-ATPase activity, alterations in erythrocyte fatty acid membrane (a decrease in omega-3 LCPUFA and consequently an increase in omega-6 LCPUFA to omega-3 LCPUFA ratio, and an increase in monounsaturated fatty acids), and a reduction in erythrocyte membrane fluidity [14]. These alterations correlated with clinical features of ASD, particularly hyperactivity scores [14].

Further evidence for a relationship between omega-3 LCPUFA and ASD comes from studies investigating its role in neurogenesis and several neurotransmitter systems. LCPFA, particularly omega-3 series DHA, has been shown to favourably affect neurite survival, outgrowth and myelination in animal cultured cortical [121-123], sensory [124], and hippocampal neurons [18,125,126]. The development of axons and dendrites as well as myelination in multiple brain areas (involved in social behaviours, emotions, and RRB) has been reported to be impaired in individuals with ASD [127,128]. Similarly, an abnormal level of brain derived neurotrophic factor (BDNF, a protein that promotes the survival of neurons) in the circulation has been reported in children with ASD, which was associated with the severity of condition [110,129,130]. Docosahexaenoic acid administration normalised BDNF in the hippocampus, increased the growth of uninjured corticospinal and serotonergic fibers, and enhanced synaptic plasticity in an animal model of spinal cord injury [123,131].

Children with autism have been shown to exhibit significantly higher levels of several dopamine derivatives (in urine), dopamine transporter binding proteins, and serotonin in brain, and lower levels of serotonin transporter binding protein (in the brain), glutamine signal (in basal ganglia), and oxytocin than healthy controls [132-138]. Furthermore, within the ASD populations, basal ganglia glutamine signal and plasma oxytocin are negatively correlated with impaired behaviour [136,138]. In response to an omega-3 PUFA limited diet in rats, dopamine levels reduced and basal synaptic release of serotonin increased while the turn over metabolites of dopamine increased and those of serotonin decreased [19,139-141]. A DHA depleted diet also altered the glutamergic system in offspring female rats [142]. This alteration was associated with anxiety-like behaviours, memory deficit and exploratory behaviours during adulthood [142]. On the other hand, DHA treatment significantly

increased synaptic plasticity in hippocampal neurons and enhanced glutamatergic activity [143].

Another plausible mechanism supporting the association between LCPUFA and ASD is the anti- and pro-inflammatory properties of LCPUFA metabolic products. Eicosanoids (a collective name for prostaglandins, thromboxanes, leukotrienes and a variety of hydroxyl and hydroproxy fatty acid) are the enzymatic metabolic products of PUFA, and have important roles in inflammation [20]. While EPA or DHA derived eicosanoids have anti-inflammatory properties, those derived from ARA have pro-inflammatory properties [20]. Elevated levels of several peripheral pro-inflammatory cytokines and nuclear factor Kappa B (NF-κB, a transcription factor involved in inflammatory signaling pathways) have been reported in children with ASD [144-146]. Brigandi *et al.* [24] reported children with ASD having significantly higher plasma levels of prostaglandin E2 (PGE2) than healthy controls, a finding confirmed by El-Ansary & Al-Ayadhi (2012) [147] who also reported higher levels of leukotriene and 8-isoprostane together with PGE2 in children with ASD. In addition, lower levels of antioxidant proteins and increased levels of oxidative stress markers was associated with more severe ASD symptoms, including sensory issues [14,146,148]. Supplementation with omega-3 PUFA, on the other hand, decreased the gene expression of NF-κB, IL-12 and IL-13 [149], macrophage inflammatory protein-2 (MIP2), IL-6 [150] and tumor necrosis factor- α (TNF-α) [150-152].

Decreased antioxidant capacity and increased lipid peroxidation may result in RBC LCPUFA instability and decrease these fatty acids in autism [23,96]. Instability in RBC LCPUFA composition has been shown by a great loss in PUFA levels when the blood samples of children with autism were stored at -20°C, a finding not observed in the blood sample of healthy controls [84] [62]. The reason for such instability could be related to cellular phospholipase activity. Tostes *et al.* (2013) [90] and Bell *et al.* (2004) [88] reported children with ASD having significantly higher phospholipase A2 (PLA2) activity than typically developing controls that was reduced by EPA supplementation [66]. PLA2 is responsible for releasing fatty acids, more particularly ARA, from phospholipids [88].

Finally, the role of LCPUFA in ASD could be explained by defects in enzymes involved in the conversion of LCPUFA from their precursors or deficits in the process of incorporation of LCPUFA into the cell membrane [24-26]. Gene variants in fatty acid desaturase (FADS) – one of the strongest genome wide associated signals – have been shown to enhance the conversion of ARA from its precursor and to be sex- and ethnicity- specific [58,153]. The effect of FADS genotype has been shown to be more pronounced in African Americans than

Europeans (approximately two fold higher) [58]. The higher frequency of this genetic variant, but not the allelic effect of G allele, explained such a difference [58]. Also, while omega-6 aggregate desaturase indices was associated with multiple FADS2 SNPs (two SNP in males and nine SNP in females) in Caucasians, it wase associated with five FADS2 SNPs in East Asian Females [153]. In addition, carriers of APOE4 allele seems to have altered long chain omega-3 metabolism [154]. Compared to the non APOE4-carriers, the carriers have higher β-oxidisation rates of omega-3 LCPUFA [154]. Furthermore, Shimamoto *et al.* (2014) showed altered mRNA gene expression levels of fatty acid binding protein 7 (FABP7) in post-mortem ASD brains, and increased hyperactivity and anxiety-related phenotype (two common features in ASD) in FABP7 knockout mice [155]. Although the modifying role of genetic variants in enzymes involved in fatty acid metabolism in the LCPUFA-disease relationship has been well documented, in the context of ASD, it warrants further investigation.

Conclusions

The current meta-analysis of case-controls studies, to our knowledge, is the first to investigate fatty acid composition in populations with ASD. Future observational studies of omega-3 LCPUFA in children with ASD are encouraged while including a uniform biomarker (e.g. omega-3 index or percentage of omega-3 LCPUFA in RBC) and reporting method (e.g. relative or absolute), collecting dietary intake of both omega-3 and omega-6 LCPUFA, and matching cases and controls on potential modulating attributes (e.g. age, sex, severity of autism, genotype, and medication use). It is also critical to know whether inadequate LCPUFA status in ASD is attributed to inefficient or disrupted metabolism or other factors like LCPUFA consumption.

Based on the current evidence, omega-3 LCPUFA supplementation cannot be recommended as an alternative to support behavioural therapies for ASD children. However, it seems prudent that omega-3 LCPUFA could be used to complement other therapies in ASD populations given its long-term tolerability and acceptability (up to six months), potentially inefficient or disrupted LCPUFA metabolic pathways in this population, and its critical role in brain function and development, and various body processes some of which are involved in the pathobiology of ASD. It should be noted that this recommendation is made cautiously because the results of this study are based on a very small sample of studies (with methodological differences and limitations) and short duration of the interventions. Therefore, the generated statistics should not be over-interpreted but seen as indicative of the need to study the issue further (while controlling for potential modifying and confounding variables) to pursue the trends observed in this study. The effect of omega-3 LCPUFA on

behaviour may be modulated by background diet and baseline LCPUFA status, sex, age, trial duration, and gastrointestinal stress at baseline and over the study period, all of which are recommended to be considered in future research. Furthermore, an investigation of the potential modulating effect of genotype (e.g. APOE) on behavioural changes in response to LCPUFA supplementation is warranted. It is also recommended that future studies include a uniform assessment tool that is sensitive to minor behavioural changes in response to complementary/nutritional therapies. Finally, the potential placebo effect and the reasons for such effects are encouraged to be investigated and accounted for in the design stage of RCTs.

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DRC 16

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hajar Mazahery

Name/Title of Principal Supervisor: Assoc. Prof. Pamela von Hurst

Name of Published Research Output and full reference:

Mazahery, H., W. Stonehouse, M. Delshad, M.C. Kruger, C.A. Conlon, K.L. Beck, and P.R. von Hurst, relationship between long chain n-3 polyunsaturated fatty acids and Autism Spectrum Disorder: systematic review and meta-analysis of case-control and randomised controlled trials. Nutrients, 2017. 9(2).

In which Chapter is the Published Work: Chapter 2 - Section 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

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  Responsible for all aspects of the manuscripts including: conceptualisation and design of manuscripts, searching the literature, data extraction, data analysis, drafting manuscript, and manuscript submission

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Candidate's Signature

15/11/2018

Date

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20/11/2018

Date

GRS Version 3– 16 September 2011

Section 4: Vitamin D and Omega-3 Long Chain Polyunsaturated Fatty Acids together and ASD

Following the extensive review of literature investigating the role of vitamin D and omega-3, each individually, on ASD, this section discusses the literature that provide evidence for a synergistic effect of vitamin D and omega-3 on ASD.

This narrative review is written in a mnauscript format but has not been published.

Potential Synergistic Effect of Vitamin D and Omega-3 Long Chain Polyunsaturated Fatty Acids on Autism Spectrum Disorder

Abstract

Both vitamin D and omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFA) counteracts many of the same neurobiological abnormalities associated with Autism Spectrum Disorder (ASD), including, but not limited to, oxidative stress, inflammation, immune dysregulation, and neurotransmitter levels. Due to a clear gap in vitamin D and omega-3 LCPUFA-ASD clinical research, we reviewed the literature investigating vitamin D and omega-3 LCPUFA together via supplements or cod liver oil in diseases of inflammation and oxidative stress because they are of relevance to ASD, which is known to be a disease of inflammation and oxidative stress. Limited evidence from these studies are suggestive of a synergy between vitamin D and omega-3 LCPUFA on diseases of these natures, and potentially on ASD. Randomised, controlled clinical trials with these two nutrients in patients with ASD is warranted.

Introduction

Autism Spectrum Disorder (ASD) is a complex neurodevelopment disorder with heterogeneous clinical presentation and aetiology. ASD is associated with impairment of social interaction and communication and repetitive and restricted interests and behaviours as core symptoms; and several behavioural, medical and biological conditions as co-occurring symptoms. A large proportion of children with ASD have been shown to have intellectual disability [1], challenging behaviours (e.g. anxiety, irritability, depression, hyperactivity and mealtime issues) [2-4], gastrointestinal symptoms [5-7], abnormal levels of several biological and neurological markers including serotonin, gama-aminobutyric acid (GABA), oxytocin, dopamine and melatonin [8-11], oxidative stress [12], and inflammation

[13]. It has been proposed that ASD is a systemic illness in which inflammation and oxidative stress is involved, and dietary supplements or pharmacological agents that target abnormal biological markers of inflammation and/or oxidative stress have the potential to improve behaviours in this disorder [14,15].

It is generally agreed that ASD is driven by an interaction between genetic and non-genetic factors [16]. ASD is the most heritable of the neurodevelopment disorders because of the higher concordance rate of ASD among monozygotic twins compared with dizygotic twins [17]. This condition has been shown to have moderate genetic heritability, but a substantial shared twin environmental component [17]. Accumulating evidence points to a possible relationship between environmental factors (including heavy exposure to chemicals, pesticides, toxins, and air pollution), maternal diseases (including, but not limited to, viral infection and inflammation during pregnancy), and nutritional factors (including, but not limited to, vitamin D, omega-3 long chain polyunsaturated fatty acids (LCPUFA) and ASD [18-21]. It is important to note that these associations have been partially proven, and clearly additional clinical and basic scientific research into the causes of ASD, followed by intervention trials testing therapeutic options that target those identified causes is warranted.

In recent years, there have been some advances in our understanding of the potential role of vitamin D and omega-3 LCPUFA in the prevention and treatment of symptoms associated with ASD. The potential role of vitamin D and omega-3 LCPUFA, each individually, in ASD has been comprehensively reviewed elsewhere [20,21] and is not the focus of this review, although the key roles of each nutrient has been summarised in **Table 1** to set the scene for the discussion that follows.

With reference to the potential mechanistic role of vitamin D and omega-3 LCPUFA, it has been speculated these nutrients may improve ASD symptoms through their shared functions and/or each nutrient-specific role that complements the other nutrient's functions [22, 23]. However, due to a clear gap in vitamin D and omega-3 LCPUFA-ASD clinical research, we refer to indirect scientific evidence that are of relevance to ASD and have the potential to support the positive speculations about the synergy of vitamin D and omega-3 LCPUFA on ASD symptoms. Evidence is obtained from three different sources; (1) the recent paper by Patrick and Ames (2015) [23], (2) the scientific literature investigating vitamin D and omega-3 LCPUFA together in diseases of inflammation and oxidative stress, and (3) the scientific literature investigating the role of vitamin D and omega-3 LCPUFA supplementation via cod liver oil in diseases of inflammation and oxidative stress. Each of the sources of evidence will be briefly discussed in the following sections.

Table 1: The link between vitamin D and omega-3 LCPUFA and Autism Spectrum Disorder (ASD)*

ASD	The role of vitamin D	The role of omega-3 LCPUFA
Having a strong genetic component	Receptors are present in different brain regions (from early development to adulthood) Regulates cell proliferation and differentiation Metabolic gene variants (e.g. vitamin D binding protein and vitamin D receptor genotypes) are associated with ASD risk	
High prevalence of oxidative stress, reduced redox/antioxidant capacity, impaired detoxification system, and mitochondrial dysfunction	Has an immunomodulatory effect, enhances detoxification system, and is associated with inflammatory and oxidative stress biomarkers	Affects nuclear transcription factor, especially those involved in immunologic dysfunction Anti-inflammatory properties
Considered as an autoimmune disease	Involved in autoantibody production and may have important role in pathogenesis of autoimmune diseases Associated with the expression of several genes involved in axogenesis and myelination	Associated with autoimmune diseases (e.g. rheumatoid arthritis)
Structural abnormalities in some brain regions (involved in social interaction RRB, and sensory processing)		Structurally important (approximately 60% of the brain's dry weight is fat, with DHA comprising 60% and 40% of the PUFA in the retina and brain, respectively)
Abnormal levels of neurotrophic factors and neurotransmitter systems	Has a neuroprotective effect Receptors are present in dopamine neurones, and vitamin D response elements are found on genes involved in serotonin and oxytocin synthesis	Involved in neurogenesis and several neurotransmitter systems
Alterations in erythrocyte fatty acid membrane and erythrocyte membrane fluidity		Increase membrane fluidity and permeability

ASD, Autism Spectrum Disorder; omega-3 LCPUFA, omega-3 long chain polyunsaturated fatty acids; RRB, repetitive and restricted interests and behaviours
*The information summarised here are derived from two recent reviews [20,21].

Hypothesis by Patrick and Ames (2015)

Patrick and Ames (2015) [23] discussed the relevance of these nutrients in the aetiology and development of neuropsychiatric disorders (including ASD, depression, and hyperactivity) and the potential mechanistic links that explain how deficiencies in these nutrients can

interact with genetic pathways, such as the serotonin pathway, and thereby influences behavioural and neuropsychological outcomes [23] (**Figure 1**). The authors suggested that while vitamin D regulates serotonin synthesis (by regulating the conversion of the essential amino acid tryptophan to serotonin), eicosapentaenoic acid (EPA) increases the release of serotonin from pre-synaptic neurons (by reducing inflammatory signalling molecules in the brains; e.g. prostaglandin E_2), and docosahexaenoic acid (DHA) increases serotonin accessibility in post-synaptic neurons (by increasing cell membrane fluidity) [23]. Furthermore, serotonin is involved in a wide-range of cognitive functions and behaviours including mood, decision-making, social behaviour, impulsive behaviour, and social decision-making.

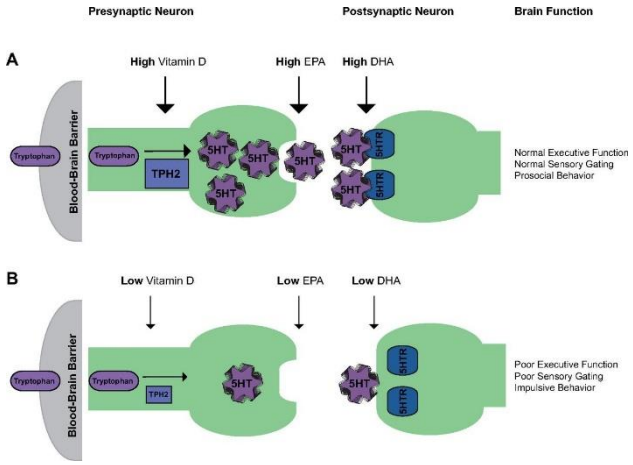

Figure 1: The role of vitamin D and omega-3 LCPUFA on serotonergic system and subsequently behaviour. Adapted from [23] and "reproduced with permission".

Both Supplements Together and Diseases of Inflammation and/or Oxidative Stress

Herein, we discuss the few clinical studies that have investigated the effect of vitamin D and omega-3 LCPUFA together on inflammatory and oxidative biomarkers and diseases of inflammation and oxidative stress such as diabetes. Evidence consistently suggests that decreasing inflammatory and oxidative biomarkers is associated with an improvement in disease symptoms and outcome. Accordingly, one can infer that if vitamin D and omega-3 LCPUFA together is effective in reducing inflammatory and oxidative biomarkers in a disease of inflammation or oxidative stress, it would have the same, more or less, effect on another disease that has an inflammatory or oxidative component. This is particularly true in the case of ASD because it is considered as a systematic illness in which inflammation and oxidative stress are involved, and the correction of inflammation and/or oxidative stress has been shown to improve behaviours in this disorder [14,15].

Our literature search identified four intervention trials that examined the effect of treatment with vitamin D and omega-3 LCPUFA together via supplementation on cognition (n=1) and

diseases with inflammatory and oxidative stress components (*n*=3, one type 1 diabetes and 2 gestational diabetes mellitus).

Hansen *et al.* (2017) designed a 6-week randomised, placebo-controlled, double-blinded pilot study to investigate the impact of vitamin D (400 IU/day), DHA (500 mg/day) and uridine (1000 mg/day) together with motor and cognitive training on motor and cognitive tests in 16 healthy children (aged 8-11 years) [24]. Although dietary supplementation increased blood concentrations of vitamin D and DHA, it had no effect on the trained motor and cognitive tasks (improvement was seen in all children from both treatment groups) [24]. It is important to note that the finding of this study was confounded by using training and uridine, and therefore the combined effect of vitamin D and DHA cannot be established. Uridine is known to synergistically act with omega-3 LCPUFA on cognitive function [25]. Furthermore, the lack of effect could be attributed to inadequate statistical power due to the small sample size, which was well below the authors' calculations (sample size of 26).

A recent case study described the effect of vitamin D and omega-3 LCPUFA together on type 1 diabetes [26]. Baidal *et al.* (2016) treated a 14-year old boy with new onset type 1 diabetes with high dose vitamin D (25,000 IU/week) and EPA (2.4 g/day) together and investigated the effect of treatment on beta-cell function, but not inflammatory markers, over a 12-month period [26]. Supplementation resulted in the preservation of beta-cell functions, and the authors concluded that this positive effect could be attributed to a significant decrease in the pancreatic beta-cell inflammatory response that leads to preservation of beta-cell mass and inhibition of adaptive and cell-mediated immune response, an explanation confirmed by others [27,28]. Type 1 diabetes – an organ-specific autoimmune and auto-inflammatory disease – is characterized by a progressive destruction of pancreatic beta-cells by the secretion of powerful pro-inflammatory cytokines which results in absolute insulin deficiency and the need for daily insulin treatment [29]. Gestational diabetes mellitus has been also suggested to be associated with chronic inflammation that leads to systematic insulin resistance [30].

Finally, a research group from Iran examined the effect of supplementation with vitamin D (50,000 IU/twice a month) and omega-3 LCPUFA (0.4 g/day EPA + 0.2 g/day DHA) for six weeks on inflammatory and oxidative stress biomarkers and glycaemic control and lipid profile in women with gestational diabetes [27, 28]. The authors demonstrated that as compared with each nutrient individually or placebo, supplementation with vitamin D and omega-3 LCPUFA together was associated with a greater decrease in high-sensitivity C-reactive protein and malondialdehyde and greater increase in glutathione and total

antioxidant capacity [27]. In addition, beneficial effects on glycaemic control (e.g. fasting plasma glucose, serum insulin levels, homeostatic model of assessment for insulin resistance, quantitative insulin sensitivity check index) and lipid profile (e.g. serum triglycerides and very low-density lipoprotein cholesterol levels) were observed [28].

In conclusion, evidence from these studies, though limited, are suggestive for a potential role of vitamin D and omega-3 LCPUFA if given together in ameliorating inflammation and oxidative responses in diseases of inflammation.

Cod Liver Oil and Diseases of Inflammation and Oxidative Stress

Further support for the role of vitamin D and omega-3 LCPUFA together can be obtained from cod liver oil studies as cod liver oil is a rich source of both vitamin D and omega-3 LCPUFA (EPA and DHA). However, one should be cautious in drawing inferences from such studies because other nutrients including vitamins A and E are found in large amounts in cod liver oil. Vitamin A has been shown to have biological properties of relevance for the prevention and treatment of inflammation and in the aetiology of oxidative stress if it is given in excess [31,32], and to have an antagonist effect on vitamin D actions in both humans and rats [33-35]. Vitamin A in cod liver oil and its potential interaction with vitamin D has been blamed for the null or negative findings of some studies [36,37]. It is also important to note that cod liver oil is not currently regulated or standardized, and the concentration of vitamins D and A can vary with time and among different manufacturers and preparations [38]. Vitamin E is also a potent antioxidant with anti-inflammatory properties [39] and may interact with omega-3 LCPUFA [40].

Our literature search identified seven observational studies that examined the relationship between vitamin D and omega-3 LCPUFA intake via cod liver oil and depression and anxiety (n=1), cardiovascular disease-related conditions (n=4; one coronary heart disease mortality, one several CVD-related conditions, and two type 1 diabetes mellitus), and asthma (n=2). In addition, 11 intervention trials were identified that examined the effect of vitamin D and omega-3 LCPUFA supplementation via cod liver oil on inflammatory/oxidative markers and endothelial functions (n=3) and inflammation-related joint pain (n=3) in human participants and on inflammatory and oxidative makers in animal experimental models of oxidative stress (n=3) and diabetes (n=2).

Observational studies: Evidence from one observational study suggests that cod liver oil consumption might be associated with depression with and without anxiety. Raeder *et al.* (2007) investigated the association between cod liver oil consumption and depression and

anxiety using data from a population-based cross-sectional health study in Norway [41]. The authors reported lower prevalence of high levels of symptoms of depression with and without anxiety among daily cod liver oil users. The prevalence was also lower among those who used cod liver oil for a longer period of time (in a subset of individuals aged 40-46 years). The cod liver oil consumed contained 0.3 g to 0.6 g DHA and 0.3 g to 0.6 g EPA, 250 μg vitamin A, 400 IU vitamin D, and 10 μg vitamin E. It is important to note that this study was a cross-sectional study where cod liver oil use was retrospectively assessed, and therefore a cause and effect relationship cannot be determined; one can postulate that individuals with depression might remember or report the cod liver use to a lesser degree than healthy counterparts.

With regard to associations between cod liver oil consumption and cardiovascular disease, the evidence is inconsistent. Egeland *et al.* (2001) failed to show an association between cod liver oil consumption and coronary heart disease mortality risk [42], while Lentjes *et al.* (2014) reported a negative correlation between cod liver oil consumption and self-reported cardiovascular disease-related conditions; including heart attack and stroke in men; and heart attack, stroke, high blood pressure, and diabetes in women [43]. The use of cod liver oil during pregnancy has been shown to be associated with lower risk of type 1 diabetes in offspring [44]. Stene and Joner (2003) reported a significant relationship between cod liver oil use during the first year of life and decreased risk of type 1 diabetes in young children [45].

Finally, in an Australian multicentre study, Hughes *et al.* (2011) reported an increased odds of having both hay fever and asthma together, but not each on their own, among adults (18-61 years) who consumed cod liver oil during their childhood [46]. Similarly, Mai *et al.* (2013) demonstrated that cod liver oil intake was associated with increased incidence of adult-onset asthma [37]. The positive association remained significant after adjusting for multiple potential covariates and across all age groups, sex, family history of asthma and body mass index subgroups. It is important to note that the analysis was based on more than 17,000 Norwegian adults aged 19-55 years and free of asthma at the time of baseline data collection (1995-1997) when the cod liver oil formula contained a high concentration of vitamin A (1000 μg per 5 ml). On the other hand, using a large population-based study including Northern European countries, Laerum *et al.* (2007) found a U shape relationship between adult cod liver oil consumption and asthma, with those never and daily taking cod liver oil having the highest risk of asthma [47].

Intervention trials: Cod liver oil consumption has been shown to have beneficial effect on inflammatory and oxidative stress and disease of inflammation endpoints. Using experimental animal models, it has been shown that supplementation with cod liver oil reduced sodium nitrite-induced hepatic injury by blocking the elevation of inflammatory cytokines, fibrosis mediators, and apoptosis markers [48]. Also, cod liver oil has been reported to reduce oxidative stress, block monocyte chemoattractant protein-1 (MCP-1) [49], reactivate mitochondrial function and reduce DNA fragmentation [50] in animal models of hepatic injury. In another animal model of oxidative stress, cod liver oil has been shown to attenuate carbon tetrachloride-induced toxicity and oxidative stress in the liver [51]. Cod liver oil has also been shown to be beneficial in diabetic rats; Haunkar *et al.* (2002) demonstrated that oxidative stress was prevented, and endogenous antioxidant enzyme activities was decreased in various tissues of diabetic rats treated with cod liver oil [53]. These rats also had a better control of glucose and lipid metabolism [52]. Finally, Cylan-Isik *et al.* (2007) demonstrated that cod liver oil supplementation improved cardiovascular and metabolic abnormalities in diabetic rats [53].

Some, but not all intervention trials in humans, have provided further support for cod liver use in diseases with an inflammation component. Supplementation with 30 ml cod liver oil daily (corresponding to 5.3 g omega-3 LCPUFA) for six weeks has been shown to decrease monocyte and neutrophil chemotactic responsiveness in healthy men (n=12) [54]. The concentration of vitamin D and A in the cod liver oil was not reported by the authors of study. Also, Papageoriou *et al.* (2011) demonstrated that acute intake of cod liver oil, olive oil, or soy oil was associated with lower TNF-α, as compared with acute intake of corn oil [55]. However, others reported that all types of oils had no effect on vascular inflammatory process and systemic oxidative stress but cod liver oil and soy oil improved endothelial function [56]. It is important to note that the acute effect of cod liver oil consumption on inflammatory and oxidative stress was assessed in the two latter studies and large doses of cod liver oil (corresponding to 0.008 g omega-3 LCPUFA, 5600 mcg vitamin A, and 2000IU vitamin D) was administered. Furthermore, Brunborg *et al.* (2008) compared short-time effect (14 days) of cod liver oil with that of seal oil on leukotriene B4 (LTB4, and inflammatory marker) and symptoms of illness in patients with inflammatory bowel diseases (n=38) [57]. Both supplements decreased leucotriene B4 and inflammatory bowel disease-related joint pain [57]. Galarraga *et al.* (2008) also reported that the use of anti-inflammatory medications was reduced in 39% of participants taking cod liver oil supplementation (corresponding to 2.2 g n-LCPUFA, 800 mcg vitamin A, and 200 IU vitamin D) for nine months, as compared with 10% of participants who received placebo [58]. However, others failed to show an effect of cod liver oil as an adjunct treatment to non-steroidal anti-inflammatory drugs in the

management of osteoarthritis in general practice [59]. No information about concentration of omega-3 LCPUFA (with the exception of EPA, 0.8 g) and vitamins A and D was available.

In conclusion, observational studies provide mixed evidence for a role of vitamin D and omega-3 LCPUFA via cod liver oil in diseases of inflammation and oxidative stress. The discrepancies found here could be attributed to the concentration of vitamin A and D in cod liver oil preparation and consequently the ratio of vitamin A to vitamin D, a well-established determinant of bone growth and calcium metabolism in poultry, though not well-studied in humans [38]. Also, due to the observational nature of these studies, recall bias should be taken into consideration.

With regard to intervention trials, studies of animal models of oxidative stress and diabetes provide more consistent findings of an effect of cod liver oil on inflammatory and oxidative biomarkers than human studies. The mixed findings from these studies could be attributed to the differences in concentrations of different nutrients in cod liver oil, study length, sample sizes and outcome measures of interest.

Conclusions

Both vitamin D and omega-3 LCPUFA counteracts many of the same neurobiological abnormalities associated with ASD, including, but not limited to, oxidative stress, inflammation, immune dysregulation, and neurotransmitter levels. Although there are a number of reviews evaluating the potential benefits of vitamin D and omega-3 LCPUFA in populations with ASD, this review along with Patrick and Ames study (2015) provide a scientific rationale for considering vitamin D and omega-3 LCPUFA together as an additional nutritional modifying agent for the management of symptoms in these populations. However, randomised, controlled clinical trials are warranted before making any definitive recommendations in populations with ASD.

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Chapter 3: Study Protocol – Paper III

Vitamin D and omega-3 fatty acid supplements in children with autism spectrum disorder: a study protocol for a factorial randomised, double-blind, placebo-controlled trial

Following an extensive review of literature, this chapter is devoted to covering the methods and study procedure used to answer the research question "The Role of Vitamin D and Omega-3 in ASD".

This chapter was published in 2016 in "Trials".

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The published study protocol includes all information about a larger trial, some parts of which are not relevent to this thesis. Accordingly, to meet the requirement of this thesis, the published paper has been amended and included here. The changes are attributed to the primary/secondary objectives (all predetermined) and outcome measures (those not relevant to this thesis are excluded). However, some objectives were developed aftere the commencement of the trial and writing the manucript. These objectives and relevant information are included in footnotes. There are also slight changes in formatting and referencing style to align with those of this thesis. To note, the manuscript was written and published perior to commencement of the trial, so is written in the future tense.

Abstract

Background: There is strong mechanistic evidence to suggest that vitamin D and omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFAs), specifically docosahexanoic acid (DHA), have the potential to significantly improve the symptoms of Autism Spectrum Disorder (ASD). However, there are no trials that have measured the effect of both vitamin D and omega-3 LCPUFA supplementation on autism severity symptoms. The objective of this 2x2 factorial trial is to investigate the effect of vitamin D, omega-3 LCPUFA or a combination of both on core symptoms of ASD.

Methods/design: Children with ASD living in New Zealand (n=168 children) will be randomised to one of four treatments daily: vitamin D (2000 IU), omega-3 LCPUFA (722 mg DHA), vitamin D (2000 IU) + omega-3 LCPUFA (722 mg DHA) or placebo for 12 months. All researchers, participants and their caregivers will be blinded until the data analysis is completed, and randomisation of the active/placebo capsules and allocation will be fully concealed from all mentioned parties. The primary outcome measures are the change in social-communicative functioning and sensory processing issues between baseline and 12 months. A secondary outcome measure is the effect on problem behaviours. Baseline data will be used to assess and correct basic nutritional deficiencies prior to treatment allocation. For safety measures, serum 25(OH)D and calcium will be monitored at baseline, 6 months and 12 months, and weekly compliance and gastrointestinal symptom diaries will be completed by caregivers throughout the study period.

Discussion: To our knowledge there are no randomised controlled trials assessing the effects of both vitamin D and DHA supplementation on core symptoms of ASD. If it is shown that either vitamin D, DHA or both are effective, the trial would reveal a non-invasive approach to managing ASD symptoms.

Trial registration: Australian New Zealand Clinical Trial Registry, ACTRN12615000144516. Registered on 16 February 2015.

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopment disorder usually diagnosed when developmental, educational and social demands increase [1]. ASD is believed to affect 1% of the New Zealand population [1]. Diagnostic criteria for ASD include delays or difficulties in socio-communicative functioning, restricted and repetitive behaviours/interests, sensory issues and aberrant behaviours [1,2]. ASD is also associated with medical conditions such as

gastrointestinal problems [1-5]. The clinical symptoms of individuals with ASD vary widely [5-7], suggesting that ASD is multi-factorial in nature.

It is generally agreed that both genetic and environmental factors contribute to the development of ASD. The high heredity of ASD has been shown by twin and familial studies [8,9]. However, it has been reported that only 30% of ASD cases are clearly associated with a syndrome or genetic markers leaving the aetiology of most cases without explanation [10].

Mechanistic evidence, as well as a scattering of ecological and cross-sectional studies, suggest that vitamin D may play an important role in the aetiology of ASD. Vitamin D receptors and 1α-hydroxylase have been identified in different regions of the brain and sensing neurons [11-13]. The active form of vitamin D has been shown to have an important role in the neuronal differentiation, structure, function and connectivity of the developing brain [14]. Vitamin D response elements have been identified on genes involved in serotonin and oxytocin synthesis [15]. Lower levels of plasma oxytocin [16] and abnormal serotonin concentrations in the brain and tissues outside the blood-brain barrier have been shown in populations with ASD [17,18]. Oxytocin and serotonin have been implicated in modulating social behaviour [19,20].

Serum level of 25-hydroxyvitamin D (25(OH)D), the best available marker of vitamin D status [21,22], has been shown to be significantly lower in individuals with autism than in their healthy counterparts [23,24]. Similarly, higher prevalence of ASD has been reported at higher latitudes and in individuals exposed to lower UVB radiation [24,25]. In adults with severe autism living in a community centre in Italy, problem behaviours significantly increased during spring and decreased during autumn [26]. Depletion of vitamin D in body stores by the end of winter and early spring seasons (due to lack of sun exposure) may have exacerbated the symptoms of autism and increased problem behaviours observed in this study.

The potential role of vitamin D deficiency in autism has received surprisingly little attention. Whilst a few case studies have reported beneficial effects of vitamin D supplementation on autistic core symptoms [27], no randomised, placebo-controlled trial with vitamin D supplementation has been conducted to date [28]. Jia *et al.* (2015) reported that shifting serum 25(OH)D concentration in a child with ASD from 31 nmol/L to 203 nmol/L after two months of high dose vitamin D supplementation (150,000 IU per month administered intramuscularly plus 400 IU per day orally) improved core symptoms of autism [27]. Although other trials investigating the effect of multivitamins/mineral supplements containing low doses of

vitamin D on autism symptoms have provided promising results [29,30], the individual effect of each nutrient cannot be determined from these studies.

Omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFAs) also have the potential to positively affect children with ASD. These omega-3 LCPUFAs, mainly DHA, are necessary for normal development and functioning of the brain and auditory and visual processing system [31-33]. Long-term DHA depletion results in significant losses in brain DHA with consequent loss in brain function [34]. Evidence shows that children with ASD have an increased omega-6 to omega-3 ratio in blood and low blood concentrations of omega-3 LCPUFAs which could be due to either low dietary intake or differences in fatty acid metabolism and incorporation into cellular membranes of children with ASD [35-37].

Reports on the benefits of omega-3 LCPUFAs in treating ASD are inconclusive. There are, to our knowledge, only four randomised controlled-placebo trials [38-41], three of which are small pilot studies. Bent *et al.* (2011) [40] and Amminger *et al.* (2007) [39] found that omega-3 supplementation was superior over a placebo (12 and 6 weeks, respectively) for reducing symptoms of hyperactivity and stereotypic behaviour in children with ASD. However, more recent studies have found that supplementation with omega-3 LCPUFA for 6 months had no beneficial effect on core symptom domains of ASD in children aged 2 to 5 years (n=38) [41] and 3 to 10 years (n=48) [38]. These studies are limited by their low participant numbers and short treatment periods.

In addition to these studies on the nutrients' effects when given individually, there are speculations that vitamin D and omega-3 LCPUFA may improve the ASD symptoms because of the shared functions and each nutrient-specific role that complement the other nutrient's functions [42,43]. Both nutrients are powerful anti-inflammatory agents, immune modulators and neuroprotectors [42]. Furthermore, evidence suggests while vitamin D regulates serotonin synthesis, omega-3 fatty acids increase serotonin release and membrane fluidity and thus increase serotonin accessibility [43]. ASD is associated with increased inflammation, oxidative stress, immune dysregulation and/or mitochondrial dysfunction in brain regions that are involved in social behaviour, sensory and motor coordination, memory, speech and auditory processing, and also with neurotransmitter dysregulation [17,18,44].

Unusual eating habits, a risk factor for nutrient deficiencies, are common in ASD [4]. Inadequate intakes of magnesium, zinc, folate, vitamin A, E, B_{12}, K and D as well as low intake of foods rich in omega-3 LCPUFAs have been reported in children with autism [3,45-

50]. However, a broad picture of the nutritional status of affected children in New Zealand is lacking.

Hypotheses[13]

1. Both vitamin D and omega-3 status, defined as omega-3 index (red blood cell (RBC) DHA + eicosapaentanoic acid (EPA)), will be low in children with ASD at the baseline (25(OH)D < 75 nmol/L [51] and omega-3 index of approximately 4 – 6% [52,53]).
2. Improving either vitamin D or omega-3 status with supplementation will reduce the severity of ASD symptoms in children with ASD.
3. Combined vitamin D and omega-3 LCPUFA supplementation will be more effective than either supplement alone or placebo in reducing the severity of ASD symptoms in children with ASD.

Aims

1. To establish vitamin D and RBC fatty acids status of children with ASD living in Auckland, New Zealand.
2. To investigate the effect of improving either vitamin D or omega-3 status in reducing the symptoms of ASD including socio-communicative functioning and sensory issues (primary outcomes) and aberrant behaviours (secondary outcome).
3. To establish the effectiveness of supplementation with combined vitamin D and omega-3 LCPUFA in reducing the symptoms of ASD including socio-communicative functioning and sensory issues (primary outcomes) and aberrant behaviours (secondary outcome).

Methods/Design

This study consists of two stages: Stage 1 will include recruitment and screening, while stage 2 is a vitamin D and omega-3 LCPUFA randomised double-blind placebo-controlled trial (**Figure 1**). The duration of the intervention is 12 months.

Stage 1 will provide the opportunity for a comprehensive description of the study population with respect to the nutritional status (biochemical indices and dietary intake), demographics,

[13] An objective/aim was developed after the commencement of trial and writing this manuscript; validation of a diet quality assessment tool (Dietary Index of Children's Eating, DICE) against food records (4 day estimated food record, 4DFR). It was hypothesised that DICE is a relatively valid tool for assessing diet quality.

and medical history. Stage 2 will demonstrate the efficacy of supplementation with vitamin D, omega-3 LCPUFA or both on reducing ASD symptoms.

Participants

This study is a collaboration between Massey University and the Waitemata District Health Board (WDHB), New Zealand. Caregivers of children who meet the criteria for the study will be approached in the first instance by the WDHB Developmental Coordinators.

We calculated that 42 participants (a minimum of 34 participants, and allowing for a 20% potential dropout rate) would be required for each arm of the trial to demonstrate a clinically significant difference at 80% power and 5% statistical significance. Power calculations were based on a 17 unit difference between supplemented groups and placebo in change from baseline to endpoint on the Social Responsiveness Scale (SRS) total score [54], on a mean SRS and standard deviation (SD) of 105 and 25 units in untreated children with ASD, respectively (from our 2015 pilot study, unpublished). The sample size was calculated using the formula below [55]:

$N=2\alpha^2K/(\mu_2-\mu_1)^2$

Where N is the Sample size required per group, α is the SD, K is the constant (7.9 denotes 80% power and 5% significance), and $(\mu_2-\mu_1)$ is the difference in SRS total score between groups.

To ensure that the study is adequately powered, a blinded interim analysis at >50% of initially planned enrolment will be performed by an independent third party to estimate the variance for potential sample size increase.

Inclusion/Exclusion criteria

Children will be eligible for this study if they are between 2.5 and 8.0 years, have a medical diagnosis of ASD confirmed by both a developmental paediatrician in accordance with the criteria listed at DSM-5 [2], and onset of symptoms after 18 months of age. The lower limit of 2.5 years has been chosen based on the age criteria of psychological assessment tools, and the upper limit of 8.0 years has been chosen to avoid the confounding effects of behavioural changes associated with pubertal stage. Caregiver's proficiency in English is a requirement (due to the nature of outcome assessment tools). Volunteers are excluded if they were diagnosed as having developmental delay since birth.

Additional inclusion criteria for the trial are: liver function within the normal range (albumin 34 – 48 g/L) and serum 25(OH)D < 75 + 10 nmol/L if they enter the trial in winter and < 105 nmol/L + 10 nmol/L if they enter the trial in summer. A 10 nmol/L variation was chosen because of the potential assay variability [56]. We have applied two different cut-off points for exclusion because there is a large seasonal variation in serum 25(OH)D concentrations in New Zealand ranging from 30 nmol/L [57] to 44 nmol/L [58].

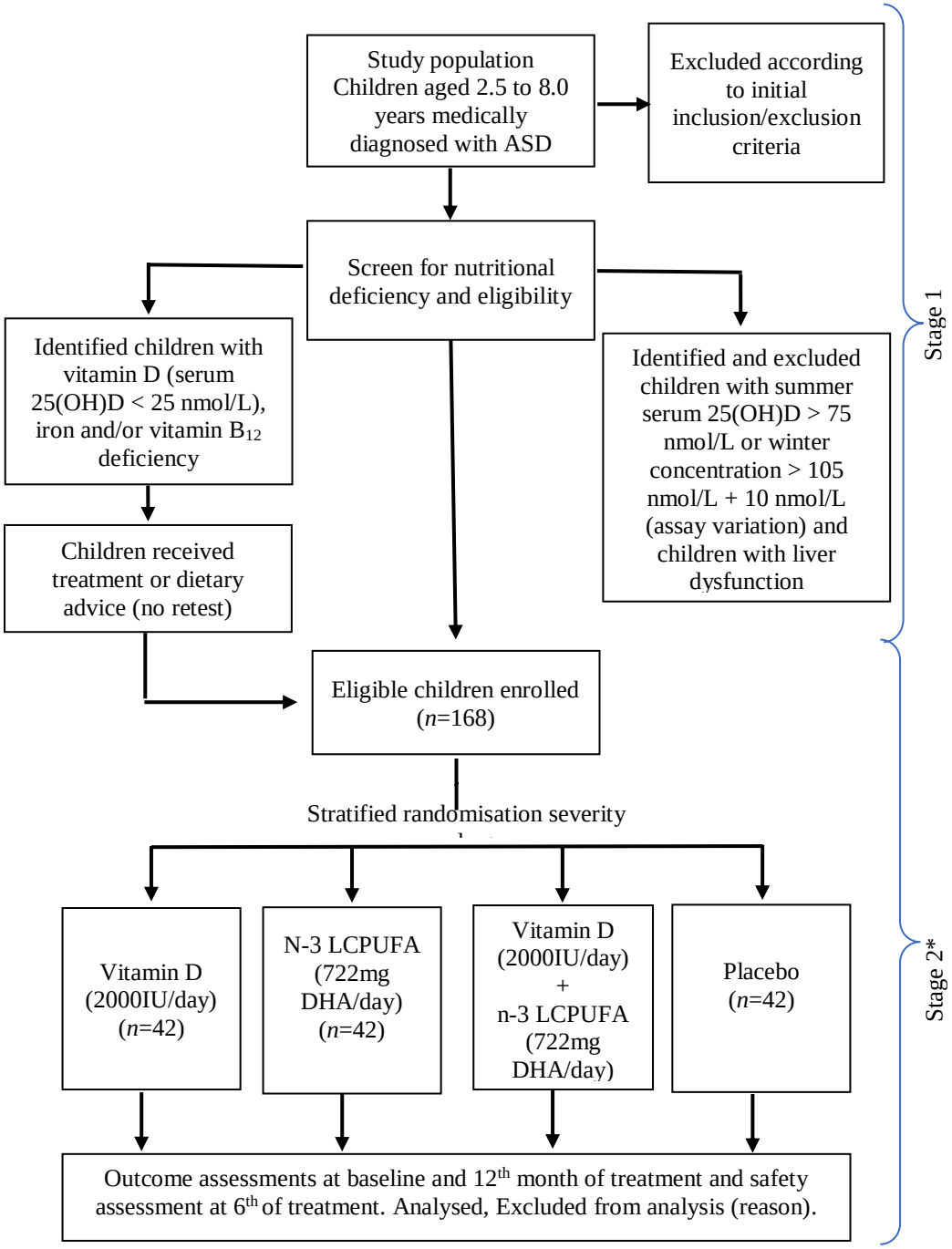

Figure 1: Schematic diagram of study design.

Funding and ethics

Partial funding for the study was provided by Massey University Strategic Innovation Fund, Massey University, NZ to cover the pilot study in 2015. Additional support has been provided by Douglas Nutrition, Pty Ltd, NZ who are supplying the active supplement and identical-appearing placebo, but who have no input into study design, implementation, data management, statistical analysis or reporting of results.

Ethical approval was granted by Health and Disability Ethics Committees, NZ, Reference NO. 14/NTA/113. Caregivers will sign informed consent forms (main and ancillary studies) for participation in this study that is collected by research coordinator (OM) or the investigator (HM) (See Appendices 1, 2 and 3).

The trial has been registered with the Australian New Zealand Clinical Trial Registry, ACTRN12615000144516.

The trial will be reported according to the Standard Protocol Items: Recommendations for Intervention Trials (SPIRIT).

Setting

The study will take place in Auckland, New Zealand. Non-fasted blood samples will be collected at the North Shore or Waitakere Hospitals in Auckland, NZ. Questionnaires and anthropometry will be undertaken at Massey University, Auckland, NZ. Auckland is New Zealand's largest city with a population of just over 1 million. It has been estimated that approximately 13,000 (33%) individuals with ASD reside in the greater Auckland region [1].

Stage 1 – Screening

Screening and recruitment (stage 1 of the study) will take place over a 24-month period, commencing January 2015. Children who meet the initial inclusion criteria will have a blood draw and will be screened for nutritional deficiencies. See **Table 1** and **Figure 2** for outcome measures, testing methods, and schedule of enrolment, intervention, and assessment, respectively.

Pre-intervention preparation

Prior to randomisation and inclusion in the trial, vitamin D, iron and vitamin B_{12} deficiencies will be addressed. Refer to **Table 2** for a list of nutritional deficiencies and the management strategies applied in this trial.

Stage 2 – Vitamin D and omega-3 LCPUFA intervention

The intervention consists of 2000 IU of vitamin D_3 per day, 722 mg of DHA per day, 2000 IU of vitamin D_3 plus 722 mg of DHA per day or placebo, in the form of 4 oral capsules. The treatment materials will be delivered in 750 mg gel capsules with a tear-off nozzle manufactured and supplied by Douglas Nutrition Ltd, NZ. Study capsules, vitamin D, omega-3 LCPUFAs and placebo, are identical in appearance, and all are tasteless and colourless. The child is required to consume the contents of 4 capsules per day mixed into their food of preference or by oral administration by syringe. Refer to **Table 3** for the total daily intake and contents of each capsule. Throughout the study period, children are allowed to have any therapy or medication for autism as well as any supplements provided that it does not contain vitamin D or omega 3.

Supplementation with 2000 IU vitamin D_3 has been shown to be a safe dose in infants [59,60], and the French Society of Paediatrics recommend 1000 to 1200 IU per day in breast fed infants [61]. The body mass of 2.5 – 8.0 year-olds in our trial will be considerably greater than infants which should further reduce the risk of adverse effect. Furthermore, 2000 IU per day is less than the safe upper limit of 2500 and 3000 IU/day suggested by the IOM for 1 – 3 and 4 – 8 year age groups, respectively [62].

The DHA dose of 722 mg/day is physiologically relevant and achievable through diet (equivalent to ~3 servings of fatty fish per week) and is comparable to doses used in other trials in children investigating the effects of omega-3 LCPUFA on behaviour and learning [63]. No side-effects have been reported in children with a dose of 600 mg DHA/day [64].

Randomisation, blinding and concealed allocation

Children will be randomly allocated to one of four groups, having been stratified for age and severity of ASD. Randomisation of the active/placebo capsules, the randomised sequence list, and group assignment will be fully concealed from the researchers, children, and caregivers for the entire study, including the data analysis. A third party not involved in any aspects of the study will generate a random block design in blocks of 4 and 8 using Randomization.com (http://www.randomization.com/). The third party will allocate a treatment code to a child once their eligibility for the intervention is confirmed and caregiver's consent is received. In an emergency situation where breaking of the study blind will be required, plans will be in place for the principal investigator to contact the third party responsible for randomisation to reveal the treatment assignment for a given participant.

Data collection

Participants (caregivers and children) will attend the Human Nutrition Research Unit (HNRU) at Massey University on two occasions, baseline and 12 months. Once recruited into stage 2 and prior to being given a 4-month supply of supplements, caregivers will complete some questionnaires on core symptoms of ASD (social and communicative functioning and sensory issues) and aberrant behaviours as the study outcome measures. Once the intervention is completed (12 months) baseline assessments of core symptoms of ASD and aberrant behaviours completed by caregivers will be repeated. Caregivers will also complete weekly gastrointestinal symptoms diaries over the study period.

Further information will be collected to describe the study population characteristics at the baseline. This information includes eating/mealtime behaviours, food diaries, sun exposure, skin colour and anthropometry (weight and height). At the final visit, children's weight/height will be measured. Refer to **Table 1** for outcome measures and testing methods.

Blood sampling and analysis

Children will have their blood samples drawn on three occasions, baseline (Stage 1 – Screening), 6 months (safety measures) and 12 months (end point). Refer to **Table 1** for nutritional biomarkers and testing methods. The non-fasted blood samples will be collected under the supervision of paediatric staff and processed at North Shore or Waitakere Hospitals of WDHB Labservice. Nutritional biomarkers will be assayed from a venous blood sample. These include the following: 25(OH)D, RBC fatty acids, calcium, albumin, iron studies, vitamin B_{12} and folate, and full blood count. With the exception of RBC fatty acids, all biomarkers will be analysed at North Shore Hospital. RBC fatty acids will be analysed at the University of Wollongong, Australia, and vitamin A in a laboratory at Massey University, New Zealand.

Questionnaires

The primary outcome measures are psychological assessments of core symptoms of ASD which are detailed in **Table 1**. The secondary outcome measure is the assessment of co-occurring problem behaviours. Standardised instructions will be given to all caregivers on how to complete the questionnaires during their visit to the HNRU. Before the participant departs the HNRU, researchers check all answers for completeness.

Social Responsiveness Scale™, Second Edition (SRS-2) [65]: The SRS-2 versions specific for age groups 2.5-4.5 and 4.5 through 18 years will be used. SRS-2 identifies social

impairment associated with ASDs and quantifies its severity in the domains of social awareness, social information processing, reciprocal social communication, social anxiety/avoidance, and stereotypic behaviour/restricted interests [65]. The clinical validity and sensitivity of SRS-2 has been determined in populations with ASD [66].

Sensory Processing Measures™ (SPM) [67]: The SPM versions specific for age groups 2-5 and 5-12 years will be used. SPM assesses sensory processing, planning and ideas (praxis) and social participation in children. The scales measure social participation, vision, hearing, touch, body awareness (proprioception), balance and motion (vestibular function) and planning and ideas (praxis) [67]. This tool has been standardised, validated and used in children with ASD [68,69].

Aberrant Behaviour Checklist (ABC) [70]: The ABC measures the variety of behaviour problems, namely irritability, social withdrawal, stereotypic behaviour, hyperactivity and inappropriate speech [71]. It has been validated in children with ASD [72] and has been widely used in treatment outcome studies of ASD [40].

Dietary Index of Children's Eating (DICE): The DICE is a simple diet quality assessment tool which has been developed by the research team, based on the New Zealand Ministry of Health Food and Nutrition Guidelines for Healthy Children and Young People [73]. This tool assesses the dietary intake of foods/drinks and comprises of 17 questions. It has been validated in a cohort of New Zealand healthy children aged 2 to 8 years (unpublished)[14], and will be validated in ASD children in this study against the 4-day estimated food record.

4-day estimated food record: Dietary intake data will be collected by a 4-day estimated food record, including one weekend day. Instructions on how to accurately complete the food record will be provided with the food record. Participants will be given a free-post, pre-addressed envelope for the return of the booklet. Average macro and micronutrient intake will be assessed over four reported days using FoodWorks Professional Edition 7 (Xryis Software, Brisbane, QLD, Australia, 2012).

In the present study, a 4-day food record was chosen because of the high respondent burden and time-consuming characteristics of longer food diaries such as a 7-day food record. Because of the high within person variation in nutrient intakes, it is recommended to record dietary intakes over a longer period of time to have a highly accurate estimate of intake.

[14] At the time of completing this thesis, this manuscript is under review.

However, if a 4-day food record covers different days randomly, it can provide accurate estimates of dietary intake [74].

Table 1: Summary of the study outcome measures and methods

Variables	Methods
Blood analysis*	
25(OH)D	Serum, Siemens ADVIA Centaur Vitamin D Total assay
RBC fatty acids	Erythrocytes, Shimadzu GC-17A flame-ionization gas chromatography (Rydalmere, NSW, Australia)
Calcium	Serum, Siemens Dimension Vista® System. CA Flex® reagent cartridge, Cat. No. K1023
Albumin	Serum, Siemens Dimension Vista® System. ALB Flex® reagent cartridge, Cat. No. K1013,
Iron studies	Ferritin: Serum, Siemens Dimension Vista® System. FERR Flex® reagent cartridge, Cat. No. K6440
	Iron: Serum, Siemens Dimension Vista® System. IRON Flex® reagent cartridge, Cat. No. K3085
	Total iron binding capacity: Serum, Siemens Dimension Vista® System. TIBC Flex® reagent cartridge, Cat. No. K3084
Vitamin B_{12}	Serum, Siemens Dimension Vista® System. B12 Flex® reagent cartridge, Cat. No. K6442
Folate	Serum, Siemens Dimension Vista® System. FOL Flex® reagent cartridge, Cat. No. K6444
Full blood count	Whole blood, Sysmex XE-5000^{TM} Automated Haematology System.
Questionnaires	
Primary outcome measures	
Socio-communicative functioning	Social Responsiveness Scale-second edition (SRS-2) [65]. Completed by caregiver at Massey University.
Sensory problems	Sensory Processing Measure (SPM) [67]. Completed by caregiver at Massey University.
Secondary outcome measure	
Problem behaviours	Aberrant Behaviour Checklist-Community (ABC-C) [70]. Completed by caregiver at Massey University.
Other measures	
Socio-demographics	Completed by caregiver.
Medical history	Completed by caregiver.
Dietary assessments	Dietary Index of Children's Eating (DICE). Completed by caregiver.
	Four-day estimated food record. Completed by caregiver. Analysed using FoodWorks 2007 (Xyris Software)
	Nutritional supplements and special dietary regimens. Completed by caregiver.
Sun exposure and skin colour	Completed by caregiver.
Medication/supplement use/incidence of adverse events (including gastrointestinal symptoms) and supplement compliance	Diary completed by caregiver.
Anthropometry	Weight: Tanita electronic scale; Height: Stadiometre; Measured by researcher at Massey University.

25(OH)D, 25-hydroxyvitamin D; RBC fatty acids, Red Blood Cell fatty acids; HPLC, high performance liquid chromatography

* All blood samples are collected and analysed at the North Shore and Waitakere Hospitals.

	STUDY PERIOD						
	Enrolment		Allocation	Post-allocation			
Timepoint	t_{-2}	t_{-1}	t_0	$t_{baseline}$	$t_{6\text{-}month}$	$t_{12\text{-}month}$	$w_{1\text{-}52}$
Enrolment:							
Initial eligibility screen	♦						
Informed consent	♦						
Nutritional deficiencies screen		♦					
Allocation			♦				
Interventions:							
Vitamin D				♦	♦	♦	
Omega-3 LCPUFA				♦	♦	♦	
Vitamin D + omega-3 LCPUFA				♦	♦	♦	
Placebo				♦	♦	♦	
Assessments:							
Nutritional biomarkers							
Serum 25(OH)D				♦	♦	♦	
RBC fatty acids				♦		♦	
Calcium				♦	♦	♦	
Albumin				♦	♦	♦	
Iron studies				♦			
Vitamin B_{12}				♦			
Folate				♦			
Full blood count				♦			
Primary outcome							
Social Responsiveness Scale-2				♦		♦	
Sensory Processing Measure				♦		♦	
Secondary outcome							
Aberrant Behaviour Checklist				♦		♦	
Dietary assessment							
Dietary Index of Children's Eating				♦			
4-day food record				♦			
Nutritional supplements and dietary regimen				♦			
Other assessments							
Socio-demographics				♦			
Medical history				♦			
Sun exposure and skin colour				♦			
Compliance and adverse events diary							♦

t, timeline; w, week; LCPUFA, long chain polyunsaturated fatty acid.
*According to SPIRIT statement: Defining Standard Protocol Items for Clinical Trials.

Figure 2: Schedule of enrolment, intervention, and assessment

Information regarding sun exposure and skin colour, nutritional supplements and any special dietary regimens followed will be collected by questionnaires specifically designed for the purpose of the current study. The sun exposure questionnaire includes a question on caregiver's beliefs and attitudes toward sun exposure as well as questions on country and city of residency in pregnancy, season of birth, and child's sensitivity to temperature and light extremes.

Compliance to medication/adherence to study protocol

Caregivers will receive weekly emails containing a link to the online compliance and gastrointestinal issues diary and a tip/fact about autism and nutrition matters. Caregivers will be contacted by telephone at one, three, six and nine months for morale purposes and to encourage compliance. Caregivers also will receive quarterly trial newsletters. The newsletters will include an update on the study, generic topics about ASD, a caregiver's experience in relation to that topic, and entertainments/competitions for the study children.

New intervention materials will be sent out to participants every 4 months and caregivers will be asked to place the bottles from the previous months aside, with any unused capsules in them, to be returned at their next visit (6 months or 12 months) at which stage unused capsules will be counted and recorded. Compliance to treatment will be analysed by counting each participant's remaining supplements once they have completed the intervention.

All participants will be recalled at 6 months for a blood test to check for hypervitaminosis D (serum 25(OH)D >225 nmol/l) and hypercalcaemia (serum Ca >2.7 mmol/l). Results will be checked by a third party who is un-blinded (but has no involvement in analysis of results). The third party will de-identify blood test results and send them to the trial paediatrician and senior investigator for review and recommendation if the child's serum 25(OH)D or calcium level is above the safe upper limit. If 25(OH)D concentrations are at or approaching 225 nmol/l, or if hypercalcaemia is present, dose administration will be adjusted.

Table 2: Nutritional deficiencies and their management strategies prior to entering the intervention trial**.**

Nutritional deficiency	Management
Vitamin D	Participants with serum 25(OH)D concentrations <25 nmol/L will be offered supplementation of 400IU per day*.
Iron	Children with iron deficiency will be offered iron supplements and postponed entry into the trial after 3 months. Children will not be retested. A child will be iron deficient when 2 of the following pools are abnormal: Red Cell Pool (haemoglobin < 111gr/L, red blood cell distribution width >14%), transport iron (iron saturation < 16%) and/or storage iron (serum ferritin ≤ 15 μg/L)*. Criteria for treatment will be according to the New Zealand Ministry of Health guidelines**.
Vitamin B_{12}	Children with serum levels <110 pmol/L will be offered the option of prescribed supplements or dietary advice to improve status

*New Zealand Ministry of Health 2015 [73]
**Retrieved from https://www.starship.org.nz/for-health-professionals/starship-clinical-guidelines/i/iron-deficiency/ on 5th of March 2015

Adverse events

Compliance and gastrointestinal symptoms diaries will be monitored on a weekly basis and all side effects will be recorded in the adverse events log to keep a track of the child's general health and behavioural reactions. In the case of any adverse events, the child's health will be monitored more closely for 3-4 consecutive weeks, and if the adverse event persists, the reports will be referred to the trial paediatrician and senior investigator for further investigation.

Table 3: Total daily intake of vitamin D and omega-3 LCPUFA and contents of each capsule, vitamin D, omega-3 LCPUFA and placebo.

Treatment groups	Daily intake
Vitamin D	2 x 750 mg capsules of olive oil, 2 x 1000 IU capsules of vitamin D_3 in medium-chain triglycerides (MCT), alpha tocopherol
Omega-3 LCPUFA	2 x 750 mg capsules of olive oil, 2 x high DHA triglyceride fish oil capsules (total DHA dose = 722 mg/day), alpha tocopherol
Vitamin D and omega-3 LCPUFA	2 x 1000 IU capsules of vitamin D_3 in 750mg MCT, 2 x high DHA triglyceride fish oil capsules (total DHA dose = 722 mg/day), alpha tocopherol
Placebo	750 mg capsules of olive oil plus alpha tocopherol (antioxidant)

Dissemination of results

Following the receipt and analysis of the food record and the completion of the biochemical assays at screening, each participant will receive a feedback form. Anthropometric measurements and blood results (iron studies, B_{12}, folate and full blood count) will be also included. Once recruitment into the trial is completed, participants who have not proceeded into the trial will receive notification of their vitamin D and RBC fatty acid levels.

On completion of the trial, participants will be informed of their baseline and end vitamin D and RBC fatty acid status, and whether they were taking the active or placebo dose. They will also receive a summary of psychological assessment outcomes of SRS-2 and SPM.

Participants and other stakeholders (such as health professionals, district health boards, primary health organisations, ASD support groups) will be given access to the study's findings. Results will be presented at scientific conferences both nationally and internationally, prepared for publication in peer-reviewed journals, and circulated to the media.

Data handling and statistical analysis

Name and address details will be maintained in Microsoft Excel. Check boxes will record the progress of a participant through the study. All other data will be entered into a single Microsoft Excel spreadsheet with participants identified only by their unique Subject Number. Scorings of the questionnaires will be double checked by the psychologist and the researcher. All entries will be double checked by another member of the research team. All documents will be stored safely under confidential conditions and archived for five years.

Statistical analysis will be performed using IBM SPSS version 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows Version 21.0. Armonk, NY. IBM Corp.). Before commencement of statistical analysis the data will be cleaned and checked for coding errors. The data will be checked for plausibility by randomly checking the accuracy and completeness and verifying against source data. The variables will be tested for normality using the Kolmogorov-Smirnov, Shapiro-Wilk tests and normality plots. Non-normally distributed data will be transformed into approximate normal distributions by logarithmic transformations. The data will be reported appropriately as mean (standard deviation) for normally distributed data; transformed data will be back transformed from summary statistics into geometric mean (95% CI), non-normally distributed data will be described as median (25, 75 percentiles) and categorical data as frequencies.

Baseline characteristics of participants will be compared among groups using analysis of variance (ANOVA) for parametric data and the Kruskal-Wallis test for non-parametric data. The primary analysis, comparing the effects of treatment on symptoms of autism over 12-months, will be conducted using a general linear mixed models procedure. Treatments and time will be included as fixed effects and the interactions between interventions and time will be tested. If significant main effects or interaction effects are observed, post-hoc analysis with Bonferroni adjustments will be performed. Potential confounding factors and effect modifiers (e.g. baseline 25(OH)D and RBC fatty acids, symptoms of autism at baseline, age and sex) will be investigated within the model.

The secondary analysis comparing the effects of treatment on problem behaviours over 12 months, will be conducted using the same procedure. Potential confounding factors and effect modifiers (e.g. baseline symptoms and medication/supplement use) will be investigated within the model[15].

[15] Because the validation of DICE was not considered at the time of writing this manuscript, details regarding the data handling and statistical analysis are not included in this manuscript. The relative validity of the DICE total score compared with the 4DFR total score was assessed using Pearson correlation coefficients, cross-classification, and the weighted kappa (κ) statistic. Also, the Wilcoxon

Differences between participants who complete and withdraw from the trial will be analysed using independent t test or Mann-Whitney tests for continuous variables (e.g. age) and chi-square for categorical variables (e.g. sex).

Both intention-to-treat and per protocol analysis will be utilised, though the primary method of analysis will be intention-to-treat. Statistical significance will be based on two-tailed tests, with $P<0.05$ considered significant.

Discussion

Autism Spectrum Disorder is a life-long, disabling condition that is associated with deficits in social-communicative functioning, stereotypic behaviour and many behavioural and medical conditions including gastrointestinal symptoms [1,2,5-7]. The main purpose of this study is to measure the effect of vitamin D, omega-3 LCPUFA or a combination of both on the symptoms of ASD in affected children. There is a widespread interest in the mechanistic role of vitamin D and omega-3 LCPUFA in the brain development and function, with some supportive clinical and epidemiologic studies. However, the effect of supplementing these nutrients on ASD pathogenesis and progression is not known. We anticipate that this trial will provide important insights into this causality of reported associations. As far as we are aware, no other randomised, placebo-controlled, double-blind trial has investigated the effects of vitamin D on symptoms of ASD, and the few trials that have been conducted with omega-3 LCPUFA [38-41] have been limited by small samples sizes, short trial duration and have shown conflicting results.

The strength of this project lies in its design: part one has been designed to provide insight into the nutritional status of children with ASD in New Zealand. Part two has been designed using a "Criterion Standard" approach (randomised, double-blind, placebo-controlled trial) to investigate the effect of supplementation. The design minimises the effect of potential confounding factors by correcting some nutritional deficiencies prior to the trial entry and taking into account the effect of confounders and covariates on ASD symptoms over time. Our sample size and trial duration will also ensure an adequate power to detect clinically and statistically significant results.

signed rank test and Spearman correlation coefficients were used to compare the sub-scores of DICE for each component against the same component sub-scores from the 4DFR. Construct validity was assessed by general linear modelling in order to investigate the relationship between the tertile of DICE total score distribution and energy and nutrient intakes calculated from 4DFRs.

If this trial is able to identify nutritional interventions that can make even a small difference to the lives of children with ASD by reducing the symptoms, the benefits will be considerable in terms of social and emotional well-being and educational achievements. Furthermore, it will also reduce the emotional, physical and financial strains among families or caregivers of children with autism and the wider societal networks. The potential benefits of the current study go beyond New Zealand and will affect all regions where ASD exists, and both vitamin D and omega-3 status below the optimal level is highly prevalent – the general trend of the most regions worldwide.

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hajar Mazahery

Name/Title of Principal Supervisor: Assoc. Prof. Pamela von Hurst

Name of Published Research Output and full reference:

Mazahery, H., C. Conlon, K.L. Beck, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., B.J. Meyer, B. Tsang, O. Mugridge, and P.R. von Hurst, Vitamin D and omega-3 fatty acid supplements in children with Autism Spectrum Disorder: A study protocol for a factorial randomised, double-blind, placebo-controlled trial. Trials, 2016. 17(1): p. 295.

In which Chapter is the Published Work: Chapter 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Responsible for most aspects of the manuscripts including: design of manuscript, searching the literature, drafting manuscript, and manuscript submission

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Chapter 4: Dietary Adequacy and Nutritional Status of New Zealand Children with Autism Spectrum Disorder and Validation of a Dietary Index of Children's Eating (Paper IV)

This chapter reports the secondary findings of the VIDOMA trial and should be presented after the next chapter which reports the primary outcome of the trial, the effect of intervention on core symptoms of ASD. However, to follow the order of events in the trial, this chapter is presented first.

As an inclusion criterion for trial entry (reported in previous chapter), the dietary adequacy and nutritional status of children with ASD living in New Zealand was investigated at the screening stage. This investigation provided valuable information on dietary adequacy/nutritional status as well as other dietary, health and life style factors of these children. Furthermore, the collection of data regarding different aspects of the diet using different tools – food records and diet quality assessment tools – provided an opportunity to validate the Dietary Index of Children's Eating (DICE) questionnaire against 4 day estimated food record (4DFR). This is the first descriptive study of this nature to be conducted in this population in New Zealand.

This report is presented in manuscript format but has not been published yet.

Abstract

Background: Children with Autism Spectrum Disorder (ASD) are susceptible to nutritional issues and poor diet quality due to sensory, behavioural and gastrointestinal issues associated with the condition, though no information regarding these children's nutritional status is available in New Zealand. Determining nutritional quality of young children especially children with ASD through blood tests and food records is associated with a huge burden for both parents and health professionals, and no validated nutritional quality assessment tools is available for this population.

Objectives: To investigate nutritional status/dietary adequacy of children with ASD and to validate a Dietary Index of Children's Eating (DICE) questionnaire against 4-day estimated food record (4DFR).

Methods: New Zealand children aged 2.5-8.0 years with confirmed medical diagnosis of ASD were recruited in a vitamin D and omega-3 intervention trial (VIDOMA) and their dietary adequacy and nutritional status was assessed at baseline. Children's dietary adequacy and nutritional status were assessed by 4DFR, DICE questionnaire (designed based on New Zealand Ministry of Health Food and Nutrition guidelines), and nutritional biomarkers (25(OH)D, iron, calcium, albumin, vitamin B_{12}, and folate). Data regarding dietary supplement use and special/exclusion diet, demographics and anthropometrics (height and weight) were also collected.

Results: Eighty-six families completed food records, Out of 86 children, three children (4%) were underweight and approximately one third of children (33%) were overweight or obese. Approximately half of children (39/86) reported taking dietary supplement and 15% (13/86) were on a special/exclusion diet. A large proportion of children had dietary intake for vitamin D below the Adequate Intake (AI, 96%), protein below the Average Macronutrient Distribution Range (AMDR, 65%), and iodine below the Estimated Average Requirement (EAR, 54%). Dietary intake of fibre (43%) and vitamin E (37%) was also below the AI by at least one third of children. All or most children exceeded the recommendations for sodium (100%), total saturated fat (80%) and sugar (52%). Approximately one third of children had serum 25(OH)D <50 nmol/L or omega-3 <4%. There was a significant and positive correlation (r=0.7; P<0.001) and good agreement (κ=0.6) between total scores from DICE (64±16) and 4DFR (58±11). Participants in the highest tertile of DICE had higher intakes of magnesium (P=0.02), vitamin A (P=0.03) and fibre (P=0.06).

Conclusion: The present study confirmed nutritional issues and poor diet quality in children with ASD. Given the importance of nutrition in growth and development and in the management of ASD, screening of the nutritional status of children with ASD for nutrient adequacy to reduce under- or over-consumption of nutrients is recommended. DICE is a a relatively valid tool for the assessment of diet quality in children with ASD living in New Zealand.

Introduction

Autism Spectrum Disorder (ASD) is believed to affect 1% of the New Zealand population [1]. ASD is associated with impairments in social and communicative functioning and restricted interests and behaviour [1]. In addition, ASD is linked to a variety of cognitive, behavioural and medical conditions such as sensory problems [2,3], unusual eating habits/mealtime problems [4,5] and gastrointestinal issues [6-8], all having potentially serious implications for the children's nutritional status and diet quality.

Although little is known about overweight and obesity in children with ASD [9], a few studies suggest that children with ASD are at increased risk for obesity. Hill *et al.* (2015) reported 37% and 18% of US children with ASD being overweight and obese, respectively [10]. Among young children (2-5 years) and adolescents (12-17 years) with ASD, the prevalence of overweight and obesity was higher than that reported in general US population [10]. Children with ASD have also consistently been shown to have inferior diet quality than healthy controls and to a have high prevalence of nutritional issues [5,6,11]. Several reports from different countries refer to inadequate intakes of certain nutrients in children with ASD (**Appendix 1**). For example, researchers from Egypt [12], the US [13], and China [6] report that a large proportion of children with ASD have inadequate or borderline intakes of at least one of the following key nutrients: vitamins A, D and E, B vitamins (including B_1, B_6, and folic acid), calcium, magnesium, iron, iodine, selenium, and fluoride [6,11-13]. Furthermore, compared to healthy controls, children with ASD have lower intakes of protein, vitamins D, C and B (including B_1, B_2, B_3, B_6, and folate) and calcium [6,11-13]. Iron deficiency has also been suggested to be highly prevalent in children with ASD [14], and this high prevalence has been attributed to inadequate dietary iron intake and malabsorption in these children [15]. Overall, the results are mixed, and a broad picture of dietary adequacy/nutritional status of children with ASD in New Zealand is lacking.

Despite the high prevalence of poor diet quality and nutritional issues in children with ASD, dietitians and nutritionists are not equipped with a practical and efficient tool to assess diet quality of these children. Most nutrient intake studies in ASD use food records (mostly 3-day

food record, 3DFR). Food records can be a burden for parents, particularly those of children with ASD who may already experiencing a stressful life. Furthermore, analysis of food records is often problematic due to the amount of detail and time required. Diet quality assessment tools (including dietary indices which are typically developed according to dietary guidelines or a particularly dietary pattern), on the other hand, are simple and efficient way of evaluating diet quality. These tools can be used to identify at risk individuals for further detailed investigation and timely interventions. A Dietary Index of Children's Eating (DICE) has been developed for the assessment of diet quality of 2-8-year-old New Zealand children. This tool was developed according to the 'New Zealand Food and Nutrition guidelines for healthy children'. The relative validity and reliability of DICE has been documented in typically developing children [16], but its relative validity in children with ASD has not been determined.

Therefore, the present study aimed to investigate dietary adequacy and nutritional status of children with ASD, and to validate the DICE questionnaire against a 4-day estimated food record (4DFR).

Subjects and Methods

This study was part of a double-blind, placebo-controlled trial that examined the effect of vitamin D and omega-3 long chain polyunsaturated fatty acid on ASD symptoms (VIDOMA). Study design and data collection methods for the earlier trial is reported elsewhere [17] and study procedure and baseline assessment methods relevant to the present study are briefly described here.

The study was registered with the Australian New Zealand Clinical Trial Registry, ACTRN12615000144516. Ethical approval was granted by Health and Disability Ethics Committees, NZ, Reference NO. 14/NTA/113. All parents/caregivers were provided with an information sheet explaining the study protocol in detail and signed an informed consent form.

Study Participants

New Zealand children were included if they were between 2.5 and 8.0 years, had a medical diagnosis of ASD confirmed by a developmental paediatrician in accordance with the criteria listed in the *Diagnostic and Statistical Manual of Mental Disorders, version five* (DSM-5) [18], and onset of symptoms after 18 months of age. Children were excluded if they were diagnosed as having developmental delay since birth.

Data Collection

Parents/caregivers were considered as proxy reporters of their children's dietary intake. Parents/caregivers completed a demographic and medical history questionnaire, and a dietary supplement and special/exclusion diet questionnaire. The dietary supplement and special/exclusion diet questionnaire was designed for this study. Parents/caregivers were asked to give information on any dietary supplements their child was on, and to report if their child was on any special dietary regimen (e.g. gluten free, casein free, vegetarian).

Dietary adequacy and nutritional status were assessed using a 4DFR (**Appendix 2**), the DICE questionnaire (SurveyMonkey link, **Appendix 3**), and nutritional biochemical markers [25(OH)D, iron studies (iron, total iron binding capacity, transferrin saturation, ferritin, and full blood count), calcium, albumin, vitamin B_{12}, and folate]. For the detailed information on how the dietary intake data were collected, analysed and scored refer to **Appendix 4**. Children's weight and height were measured to calculate BMI. Weight and height were assessed using a portable scale (light clothing) and a standard stadiometer (bare foot). Weight status was determined using the NZ MoH BMI calculator (NZ-specific percentiles) [16].

4-Day Estimated Food Record (4DFR)

Dietary intake data were collected by 4DFR, including one weekend day. A 4DFR was chosen because of the high respondent burden and time-consuming characteristics of longer food records such as a 7-day food record (7DFR). Because of the high within-person variation in nutrient intakes, it is recommended to record dietary intakes over a longer period of time to have a highly accurate estimate of intake. However, if a 4DFR covers different days randomly, it can provide accurate estimates of dietary intake [19].

Instructions on how to accurately complete the food record were provided with the food record (**Appendix 2**). Participants were given a free-post, pre-addressed envelope for the return of the food record. The data were checked and entered the FoodWorks Professional Edition 7 (Xryis Software, Brisbane, QLD, Australia, 2013) by four trained nutritionists and dietitians for further analyses (the detailed information is available in **Appendix 4)**.

Mean intake of energy, macro and micronutrients were assessed over four reported days excluding supplements. Supplements were excluded because detailed information (brand and dose) about supplement used by children was not available. The study used the Nutrient

[16] Retrieved from https://www.health.govt.nz/your-health/healthy-living/food-activity-and-sleep/healthy-weight/healthy-weight-bmi-calculator on 20th June 2018.

Reference Values (NRV), including the acceptable macronutrient distribution range (AMDR), estimated average requirement (EAR) and recommended dietary intake (RDI) or adequate intakes (AI) recommended for Australian and New Zealand children, as norms for individual nutrient intake [20]. Results were converted to percentage of age specific-EAR, -RDI or -AI (when EAR is not available) for micronutrients. In this study, 'inadequate intake' is defined as <100% of the EAR. Regarding those nutrients where EAR is not available and AI is used (vitamins D and E, fibre and sodium), no evaluation can be made of the probability of an inadequate intake when the intake falls below the AI (both at individual and group population level) because the requirement is unknown. The AI is the average daily nutrient intake level and is based on observed or experimentally derived approximations or estimates of the nutrient intake by a group of apparently healthy people – these observed intakes are assumed to be adequate and are used only as a 'goal'. Accordingly, when the dietary intake falls below the AI for these nutrients, the only statement that can be made is that the individual/group should be encouraged to increase their intakes to meet the AIs. The present study considered nutrients that have been frequently reported to be low or high in studies of children with ASD (**Appendix 1**).

For imputation to the DICE, average daily amount of foods and beverages, number of different colours of fruits and vegetables, number of days wholegrain products was consumed, and amount of fluid, sugar, fat and salt obtained from 4DFRs were converted to dietary guideline servings using methods described elsewhere ([16] and **Appendix 4**).

Dietary Index of Children's Eating (DICE)

The development and validation of DICE in healthy typically developing children have been described in detail elsewhere [16]. The questionnaire is shown in **Appendix 3** and the cut-offs, scoring, and details of each index component are presented in **Appendix 4**. In summary, the DICE is based on the NZ Ministry of Health (MoH) Food and Nutrition guidelines for healthy children and young people (aged 2-18 years) [20] and is used to calculate the overall dietary adequacy and quality. It consists of 17 questions assessing the usual dietary intake of foods and drinks. The questionnaire comprises the following components: servings of main food groups (including fruits, vegetables, milk products, meat/alternatives, bread and cereals), variety of fruits and vegetables, frequency of main meal and snack consumption and consumption of whole grain products, fluids, low fat milk and low fat/sugar/salt meals and snacks. A score of up to 100 is possible where higher scores reflect higher diet quality.

Biochemical Markers

Nutritional biomarkers were assayed from a non-fasted venous blood sample. These included the following: full blood count, erythrocyte fatty acids, and plasma (or serum) was measured for 25(OH)D, calcium, albumin, iron studies (iron, iron binding capacity, ferritin, and transferrin saturation), vitamin B_{12} and folate. With the exception of erythrocyte fatty acids, all other biomarkers were analysed at North Shore Hospital (IANZ accredited) and the laboratory reference range was used for each biochemical marker. Erythrocyte fatty acids were analysed at the University of Wollongong, Australia [17]. Because there are no established reference values for erythrocyte fatty acids, reference values from different sources were used. These were total SFA + trans fatty acids (major saturated fatty acids in erythrocytes are palmitic acid and stearic acid) [21], monounsaturated fatty acids (MUFA, the primary fatty acid in this category is oleic acid) [21], omega-6 fatty acids [primarily composed of linoleic acid (LA) and arachidonic acid (ARA)] [21], omega-3 fatty acids [eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), alpha-linolenic acid (ALA), and docosapentaenoic acid (DPA)] [21], omega-3 index (EPA + DHA) [22], omega-6 to omega-3 [21] and ARA to EPA [23] ratios.

Statistical Analysis

Statistical analysis was conducted using SPSS version 24.0 (IBM corporation, New York, USA). Normality of distribution was evaluated using normality plots and Kolmogorov-Smirnov tests. The population was described using mean ± standard deviation (SD) for normally distributed data; median (25^{th}, 75^{th} percentile) for non-normally distributed data; or frequency summary statistics for categorised data.

The relative validity of the DICE total score compared with the 4DFR total score was assessed using Pearson correlation coefficients, cross-classification, and the weighted kappa *(κ)* statistic. Cross-classification was used to determine whether participants were correctly categorised into the same tertile or grossly misclassified into opposite tertiles. Participants were correctly classified when more than 50% were allocated to the same tertile; when >10% were allocated into opposite tertiles then misclassification was deemed to have occurred [24]. In order to overcome the effect of chance, the two dietary assessment methods were compared using the weighted κ statistic. A weighting of one was used for participants classified into the same tertile, 0.5 for adjacent tertiles and zero for opposite tertiles. A weighted *κ* statistic of >0.80, 0.6-0.8, 0.4-0.6, 0.2-0.4, and <0.2 was considered to show very good, good, moderate, fair, and poor agreement, respectively [25].

The Wilcoxon signed rank test and Spearman correlation coefficients were used to compare the sub-scores of DICE for each component against the same component sub-scores from the 4DFR. The results of Spearman correlation was described as below; almost perfect (0.9-1); very high (0.7-0.9); high (0.5-0.7); moderate (0.3-0.5); low (0.1-0.3), and insubstantial (0-0.1) [26].

Construct validity was assessed by general linear modelling in order to investigate the relationship between the tertile of DICE total score distribution and energy and nutrient intakes calculated from 4DFRs. Linear contrast analysis was used to compare nutrient intakes across the tertiles of DICE score. Polynomial contrast for nutrient intakes was used in order to calculate the *P*-value for the linear trend.

Results

Out of 119 participants, all completed the DICE questionnaire and 86 completed the 4DFR. Demographics and anthropometric characteristics of participants who completed all assessments and those who did not were not significantly different. Demographic characteristics of those participants who completed both DICE and 4DFR assessments (*n*=86) are reported here. **Table 1** summarises the characteristics of these children. Three children out of 86 (4%) were underweight and approximately one third of children (27/86) were overweight (16%) or obese (17%).

Dietary Adequacy and Nutritional Status

Thirty-nine children (45%) were reported as taking dietary supplements and 15% as being on a special/exclusion diet (**Table 2**). Multivitamins (21%), followed by probiotics and fish oil/omega-3 LCPUFA (13%) and vitamin C (11%) were the most reported dietary supplements. Dairy-free (9%) and/or gluten-free (8%) diets were the most reported special/exclusion diets. Out of 86 children, fish (of any type) was consumed by 22 (26%) children, of whom eight (9%) consumed oily fish.

Table 1: Baseline characteristics of children with Autism Spectrum Disorder (*n*=86)

Characteristics	**Mean±SD or n (%)**
Age (years)	5.1±1.4
Sex	
Male	72 (84)
Female	14 (16)
Ethnicity	
New Zealand European	42 (51)
Māori	10 (12)
Pacifica	9 (11)
Asian	9 (11)
Others	13 (16)
Socioeconomic status, parent's household income (n=76)	
<60,000 NZ$	18 (24)
60,000 to 120,000 NZ$	43 (57)
>120,000 NZ$	15 (20)
Anthropometrics (*n*=84)	
Height (cm)	112±11
Weight (kg)	22±8
BMI percentile for age	
Underweight (<5th %ile)	3 (4)
Normal weight ((≥5 – 85th %ile)	54 (64)
Overweight (≥85 – 95th %ile)	13 (16)
Obese (≥95 %ile)	14 (17)
ASD severity diagnosis*	
Mild	34 (40)
Moderate	35 (41)
Severe	17 (20)

ASD, Autism Spectrum Disorder.
* Based on confirmed clinical diagnosis

Table 2: Dietary supplement use and special/exclusion diet in children with Autism Spectrum Disorder (*n*=86)

Dietary supplement use and especial/exclusion diet*	**n (%)**
Supplement of any type**	39 (45)
Probiotics	11 (13)
Fish oil/omega-3	11 (13)
Multivitamin	18 (21)
Iron	4 (5)
Zinc	8 (9)
Magnesium	7 (8)
Calcium	2 (2)
Vitamin D	8 (9)
Vitamin B_6	1 (1)
Vitamin B_{12}	5 (6)
Folic acid	1 (1)
Vitamin C	9 (11)
Special/exclusion diet of any type	13 (15)
Vegetarian	2 (2)
Gluten-free	7 (8)
Casein-free	3 (4)
Dairy-free	8 (9)
Sugar-free	2 (2)
Preservative/additive-free	2 (2)
Caffeine-free	2 (2)
Low oxalate	2 (2)

* Based on parents/caregivers' reports; also confirmed by careful investigation of 4 day estimated food records (4DFR). ** Out of 39 children on supplement of any type, 21 (54%) were single supplement users and others (46%) were 2 or more supplement users.

The mean and standard deviation (SD) or median (25^{th}, 75^{th} percentiles) of intake of energy, macronutrients and micronutrients are presented in **Table 3**. Average macronutrient distribution range for protein, carbohydrate, and fat was not met by 65, 25, and 9% of children. Dietary intake of fat and carbohydrate exceeded the AMDR by 34 and 2% of children, respectively. Dietary intake of vitamin D, fibre, and vitamin E was below the AI in 96%, 43%, and 37% of children, respectively. Also, 54%, 22%, and 21% of children did not meet the EAR for iodine, calcium and vitamin C, respectively. Approximately 97, 13, 6 and 2% of children exceeded the Tolerable Upper Intake Level (UL) for niacin, zinc, iodine and iron, respectively. All children exceeded the recommendations for dietary sodium intake, 80% exceeded the recommendation for SFA intake (≤10% of total energy [27]), and approximately half of children exceeded the recommendation for sugar intake (<10% of total energy [28]).

The mean and SD or median (25^{th}, 75^{th} percentiles) of nutritional biochemical markers are presented in **Table 4**. The means of all biochemical markers are within the hospital-based clinical laboratory reference range. However, However, three children (4%) were identified as having iron deficiency (defined as having two of the following pools in the abnormal range: red cell pool (haemoglobin <111 and <115 g/L for 2-5 and 5-8 years old children, respectively), transport iron (iron saturation <12% and <14% for 2-5 and 5-8 years old children, respectively) and/or storage iron (serum ferritin ≤10 and ≤12 μg/L μg/L for 2-5 and 5-8 years old children, respectively) [29,30]]). Also, 37% of children had serum 25(OH)D <50 nmol/L and approximately one third of children had omega-3 index <4% and only five children (6%) had levels ≥8%. Furthermore, all children exceeded the reference range for MUFA fraction, 33% the ARA to EPA ratio, and 20% exceeded total SFA plus trans fatty acids fraction of RBC.

Serum 25(OH)D concentration was higher in vitamin D containing supplements (including multivitamin) users (n=8) than non-users (n=78), 71±25 nmol/L *vs.* 58±24 nmol/L, *P*=0.04 (adjusted for season of enrolment). Children who were reported taking fish oil/omega-3 LCPUFA supplements (n=11) had higher DHA [5.4 (4.5, 5,9) *vs.* 4.0 (3.7, 4.3), *P*=0.008] and omega-3 index [5.9 (5.1, 7.1) *vs.* 4.4 (4.1, 4.9), *P*=0.007], and lower ARA to DHA ratio [0.4 (0.2, 0.5) *vs.* 0.6 (0.5, 0.7), *P*=0.02] and omega-6 to omega-3 LCPUFA ratio (3.4±0.7 *vs.* 4.1±1.1, *P*=0.04) than those who did not (n=75). Also, children consuming oily fish (n=7) had a higher omega-3 index [5.5 (5.3, 5.8) *vs.* 4.3 (4.1, 4.8), *P*=0.02] and DHA [5.0 (4.7, 5.2) *vs.* 3.9 (3.6, 4.2), *P*=0.02], and lower ARA [2.1 (2.0, 2.2) *vs.* 2.4 (2.3, 2.5), *P*=0.04] and ARA to DHA ratio [2.6 (2.5 *vs.* 2.7) *vs.* 3.7 (3.2, 3.9), *P*=0.009] than those not consuming (n=79).

Relative Validity of DICE

The mean and standard deviation of DICE score was 64±16 (range, 15, 99) and the 4DFR was 58±11 (range, 31, 84). Less than half of participants scored more than 70 points according to DICE (44%) and the 4DFR (18%). A significant positive correlation was observed between the total scores for DICE and the 4DFR (r=0.7; P<0.001). Results from cross-classification showed 55% of participants were correctly categorised into the same tertile from DICE and the 4DFR, and 4% were misclassified into opposite tertiles. The weighted κ-statistic demonstrated good agreement (κ=0.63) between DICE and the 4DFR.

Sub-component scores from DICE and the 4DFR are shown in **Table 5** and **Figure 1**. Spearman's correlation coefficients showed significant positive correlations between the DICE and 4DFR for servings of fruit (r=0.8), variety of fruits (r=0.6), servings of vegetables (r=0.5), variety of vegetables (r=0.4), servings of bread and cereals (r=0.4), consumption of wholegrain products (r=0.3), servings of milk and milk products (r=0.6), consumption of low fat milk and milk products (r=0.9), servings of meat and its alternatives (r=0.4), and fluid consumption (r=0.1). ‘

Table 3: Nutrient intake and adequacy of nutrient intake in children with Autism Spectrum Disorder (ASD) (*n*=86)

Nutrients	NRV*			Mean±SD or Median (25^{th}, 75^{th} percentiles)	Range**	Not meeting NRV n (%)
	NRV used	2-3 years	4-8 years			
Energy	-	-	-			-
Kcal				1646±387	998, 2980	
KJ				6913±1625	4192, 12516	
Carbohydrate (%TE intake)	AMDR	45-65	45-65	49±9	12, 68	21 (25)
Sugar (%TE intake)	***	<10	<10	11±5.8	1.5, 29	NA
Protein (%TE intake)	AMDR	15-25	15-25	14±3	4, 20	56 (65)
Total fat (%TE intake)	AMDR	20-35	20-35	32±8	6, 47	7 (9)
SFA (%TE intake)	****	≤10	≤10	13±4	3, 23	NA
Fibre (g)	AI	14	18	18 (16, 20)	5, 66	37 (43)
Vitamin A (µg RAE)	EAR/RDI	210/300	275/400	438 (376, 499)	50, 2260	14 (16) / 32 (37)
Vitamin D (µg)	AI	5	5	1.1 (0.8, 1.4)	0, 9	82 (96)
Vitamin E (mg α-TE)	AI	5	6	6.2 (5.8, 7.0)	2.0, 21	32 (37)
Vitamin C (mg)	EAR/RDI	25/30	25/35	59 (45, 76)	3, 345	18 (21) / 27 (32)
Thiamine (mg)	EAR/RDI	0.4/0.5	0.5/0.6	1.4 (1.3, 1.6)	0.4, 5.2	0 (0) / 2 (2)
Riboflavin (mg)	EAR/RDI	0.4/0.5	0.5/0.6	1.8 (1.6, 2.0)	0.7, 3.6	0 (0) / 0 (0)
Niacin (mg NE)	EAR/RDI	5/6	6/8	24±6.9	9.1, 46	0 (0) / 0 (0)
Vitamin B_6 (mg)	EAR/RDI	0.4/0.5	0.5/0.6	1.5±0.7	0.4, 3.8	3 (4) / 5 (6)
Vitamin B_{12} (mg)	EAR/RDI	0.7/0.9	1.0/1.2	2.4 (2.1, 2.8)	0, 40	6 (7) / 10 (12)
Folic acid (µg DFE)	EAR/RDI	120/150	160/200	312±139	52, 673	7 (8) / 14 (16)
Iron (mg)	EAR/RDI	4.0/9.0	4.0/10	11±4	3, 24	4 (5) / 38 (44)
Zinc (mg)	EAR/RDI	2.5/3.0	3.0/4.0	7.5±2.3	3.9, 16	0 (0) / 0 (0)
Magnesium (mg)	EAR/RDI	65/80	110/130	235±65	123, 416	0 (0) / 2 (2)
Calcium (mg)	EAR/RDI	360/500	520/700	671 (613, 735)	190, 2066	19 (22) /42 (49)
Phosphorus (mg)	EAR/RDI	380/460	405/500	1045±246	582, 1763	0 (0) / 0 (0)
Iodine (µg)	EAR/RDI	65/90	65/90	47 (42, 63)	12, 838	54 (63) / 69 (80)
Sodium (mg)	AI	200-400	300-600	2064 (1935, 2177)	813, 5234	0 (0)

AI, Adequate Intake; AMDR, Acceptable Macronutrient Distribution Range; DFE, Dietary Folate Equivalent; NA, Not Applicable; NE, Niacin Equivalent; NRV, Nutrient Reference Values; RAE, Retinol Activity Equivalent; EAR, Estimated Average Requirement; RDI, Recommended Dietary Intake; TE, Tocopherol Equivalent; TE, total energy

* Adapted from [20] unless otherwise stated. ** Minimum, maximum.***Adopted from [28].**** Adopted from [27].

Table 4: Biochemical markers in children with Autism Spectrum Disorder (*n*=86)

Biochemical markers	Reference range ☨	Mean±SD or Median (25^{th}, 75^{th} percentiles)	Range *	<Reference range n (%)	>Reference range n (%)
Iron studies					
Haemoglobin (g/L)	105-140	129±7.2	108, 150	0 (0)	4 (5)
Iron (umol/L)	5-23	13±5.6	3.6, 29	6 (7)	3 (3)
Iron binding capacity (umol/L	38-76	66 (63, 68)	3.1, 90	1 (1)	13 (15)
Iron saturation (%)	0.1-0.4	0.2±0.1	0.1, 0.4	11 (13)	0 (0)
Ferritin (µ/L)	15-150	24 (22, 29)	3.0, 160	16 (19)	1 (1)
Other biochemical markers					
B_{12} (pmol/L)	140-800	473 (428, 531)	115, 1438	1 (1)	8 (9)
Folate (nmol/L)	7-45	43 (40, 45)	9.4, 45	0 (0)	0 (0)
Calcium (mmo/L)	2.1-2.6	2.4 (2.4, 2.4)	2.2, 2.6	0 (0)	0 (0)
Albumin (g/L)	32-48	40 (40, 41)	27, 46	1 (1)	0 (0)
25(OH)D (nmol/L)	50-250	61±25	9, 121	32 (37)	0 (0)
Erythrocyte fatty acid profile (% fatty acids), *n*=79					
Total SFA + trans fatty acids **	37.8-45.5	45 (45, 45)	42, 48	0 (0)	16 (20)
Total MUFA **	12-18	21±1.0	18, 23	0 (0)	86 (100)
Total PUFA	-	34±1.1	32, 38	-	-
Total omega-6 **	27-34	27 (27, 27)	20, 31	24 (30)	0 (0)
Total omega-3 **	5-12	6.9 (6.7, 7.4)	4, 12	4 (5)	0 (0)
Omega-3 index †	<4, ≥8	4.7 (4.3, 5.1)	2.5, 10	23 (29)	5 (6)
ARA	-	2.4 (2.2, 2.4)	0.8, 3.2	-	-
EPA	-	0.6 (0.5, 0.6)	0.1, 1.9	-	-
DHA	-	4.1 (3.9, 4.5)	2.3, 8.7	-	-
Omega-6 to omega-3 ratio **	1.9-5.7	4.0±1.0	1.7, 7.7	2 (3)	4 (5)
ARA to EPA ratio ††	1.5-<5.0	4.2 (4.0, 4.9)	0.5, 25	7 (9)	29 (33)
ARA to DHA ratio	-	0.6±0.2	0.1, 1.3	-	-

25 (OH)D, 25-hydroxyvitamin D; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

† All are hospital-based clinical laboratory reference range, unless otherwise stated. * Minimum, maximum. ** Adopted from [21]. †Omega-3 index <4% is considered a risk factor for coronary heart disease and omega-3 index ≥8% is considered optimal [22]. †† Ratios <1.5 are considered to be suboptimal and if fish oil is taken lowering the dose should be considered. Nine children (11%) had a ratio above 10 which is associated with a high risk for inflammation [23].

Table 5. Comparison of sub-scores for each component and Spearman correlation coefficients and agreement between each component of DICE with the same component from 4DFR (n=86)

DICE components	Dietary Intakes			Scores			Wilcoxon test	Correlation coefficients	
	DICE * (serves/d)	4DFR* (serves/d)	Difference DICE-4DFR (95% CI)	DICE *	4DFR *	Difference DICE-4DFR (95% CI)	*P*-value	*r*	*P*-value
Serves of fruit	2.4 ± 1.4	1.4 ± 1.2	1.0 (0.7, 1.2)	6.0 ± 3.5	6.0 ± 3.6	0.1 (-0.4, 0.6)	0.77	0.76	<0.001
Variety of fruit	-	-	-	3.4 ± 1.7	1.5 ± 1.0	1.9 (1.6, 2.2)	<0.001	0.60	<0.001
Serves of vegetables	1.7 ± 1.2	1.2 ± 0.7	0.5 (0.3, 0.8)	4.4 ± 3.1	5.9 ± 2.9	-1.4 (-2.1, -0.8)	<0.001	0.46	<0.001
Variety of vegetables	-	-		3.0 ± 1.4	1.6 ± 0.8	1.3 (1.1, 1.6)	<0.001	0.43	<0.001
Serves of bread and cereals	3.1 ± 0.9	4.1 ± 1.4	-1.0 (-1.6, -0.7)	7.9 ± 2.3	8.2 ± 2.3	-0.3 (-0.9, 0.2)	0.18	0.37	<0.001
Serves of wholegrain products	-	-	-	2.9 ± 2.0	3.7 ± 2.0	-0.7 (-1.2, -0.2)	<0.01	0.27	<0.05
Serves of milk and milk products	2.7 ± 1.2	1.6 ± 1.0	1.7 (1.4, 2.0)	6.8 ± 3.1	6.8 ± 3.2	0.0 (-0.5, 0.6)	0.92	0.57	<0.001
Serves of low fat milk and milk products	-	-	-	1.2 ± 1.8	1.1 ± 1.8	0.1 (0.0, 0.3)	0.10	0.89	<0.001
Serves of meat and its alternatives	1.4 ± 0.6	1.3 ± 0.7	0.2 (0.0, 0.4)	7.3 ± 3.1	8.6 ±2.7	-1.2 (-1.9, -0.6)	<0.01	0.37	<0.001
Number of meals and snacks	-	-	-	4.9 ± 0.3	5.0 ± 0.0	-0.1 (-0.2, 0.0)	0.02	-	-
Frequency of consuming low-fat foods, snacks, and drinks	-	-	-	2.2 ± 2.5	0.8 ± 1.9	1.4 (0.7, 2.0)	<0.001	0.07	0.50
Frequency of consuming low-salt foods, snacks, and drinks	-	-	-	3.0 ± 2.4	0.0 ± 0.0	3.1 (2.5, 3.6)	< 0.001	-	-
Frequency of consuming low-sugar foods, snacks, and drinks	-	-	-	3.9 ± 2.0	2.5 ± 2.5	1.5 (0.8, 2.2)	< 0.001	0.06	0.60
Fluid consumption	-	-	-	6.1 ± 4.1	6.1 ± 4.1	0	1.0	1.0	<0.001

DICE, Dietary Index of Children's Eating; 4DFR, 4-day estimated food record
* Presented as mean±SD. **Presented as mean (95% CI).

Linear contrast analysis showed that higher intake of magnesium (*P*=0.02) and vitamin A (*P*=0.03) was associated with increasing tertiles of DICE total score (**Table 6**). A trend for an association between fibre (*P*=0.06) and vitamin D (*P*=0.07) intake and an increase in tertiles of DICE total score was observed.

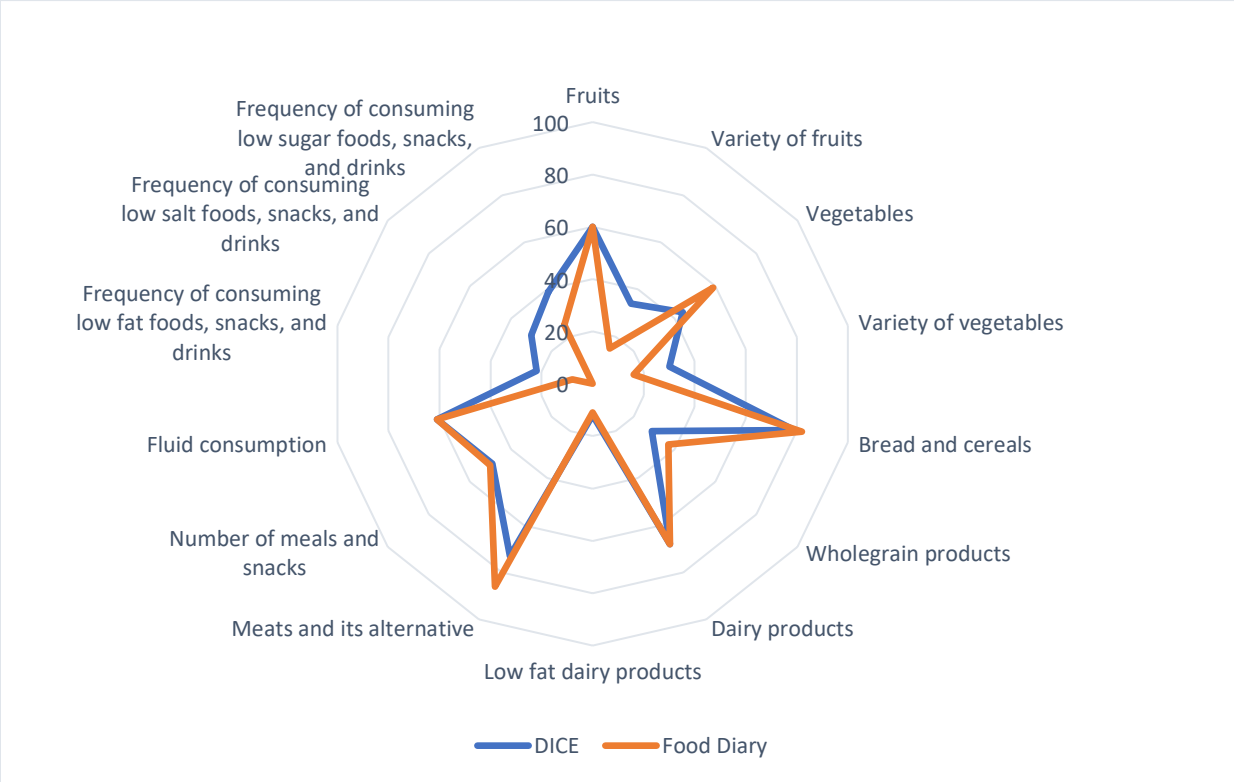

Figure 1: Radar diagram of DICE and 4DFR scores

Discussion

Results of this study reveal that children with ASD have diets adequate in carbohydrate and fat, however, protein is under consumed. The dietary intakes of vitamins D, E and C, and calcium, iodine and fibre are below the NRVs. On the other hand, dietary intake of fat, SFA, sugar and sodium exceeds the recommendations in a large number of children. The present study also demonstrated that the DICE has good relative validity with 4DFR total scores. The wide range in total DICE scores reflects the ability of the tool to determine a range of adherence to current guidelines. Construct validity results showed that participants in the highest tertile of DICE had higher intakes of magnesium, vitamin A, and fibre.

Table 6: 4DFR dietary intakes categorised by tetiles of the DICE scores (*n*=86)

Nutrients	Tertiles of DICE scores			*P*-value
	1 (*n*=29)	2 (*n*=28)	3 (*n*=29)	
Energy (kcal)	1808±757	1639±456	1877±1131	0.75
Carbohydrate (g)	207 ± 53	216±66	198± 3.1	0.54
Protein (g)	57±14	57± 14	60±13	0.35
Total fat (g)	64±23	54±19	60± 6.2	0.39
Saturated fat (g)	26±8.9	22±11	22±6.2	0.16
Sugar (g)	89±47	98 ±47	82±36	0.54
Dietary fibre (g)	17±9.3	20±7.2	22±9.8	0.06
Vitamin A (µg)	432±284	523±277	618±431	0.03
Vitamin C (mg)	79±73	73± 64	69±50	0.54
Vitamin D (µg)	1.4±1.4	1.4± 1.1	2.1±2.2	0.07
Vitamin B_6	1.4±0.7	1.5±0.7	1.6± .6	0.40
Vitamin B_{12}	4.4±7.9	4.3±7.0	2.8 ± 1.6	0.33
Folate (µg)	309±165	308±145	318±105	0.80
Magnesium (mg)	213±61	241±65	252 ±63	0.02
Calcium (mg)	745±305	794±390	644±263	0.23
Iron (mg)	10.2±4.2	11±4.7	11±2.8	0.69
Zinc (mg)	7.5±2.5	7.1±2.5	8.0 ±1.8	0.46
Sodium (mg)	2214±878	2004±837	2307± 805	0.67
Iodine (µg)	110± 80	120±251	72 ± 105	0.44

DICE, Dietary Index of Children's Eating; 4DFR, 4-day estimated food record

Dietary Adequacy and Nutritional Status

The maximum score of 100 for DICE would represent meeting all the recommendations for children from the NZ MoH Food and Nutrition guidelines. Therefore, the higher the score on DICE the greater adherence is to current guidelines in NZ. The range of DICE total scores in this study was 15 to 99, with a mean total score of 64, and only 44% of children scored more than 70 points. These results show that the adherence of children with ASD to the New Zealand Ministry of Health Food and Nutrition guidelines was low to moderate [20], and was lower than that of 2-8 year old New Zealand typically developing children (who using the same dietary index had a mean score of 78 and scores ranging from 45 to 100 [16]). Only 11, 30 and 40% of children in the present study met the recommendations for vegetables, fruits and milk/milk products, respectively, as compared with ≥40, ≥69 and 68% of typically developing children [16,31]. Other researchers also reported milk and milk products being the most reported food groups consumed in inadequate amounts in children with ASD [5,32,33]. Furthermore, a lower proportion of children with ASD achieved the maximum scores for variety of vegetables (15% *vs.* 48%) and variety of fruits (44% *vs.* 86%), compared with typically developing children [16]. Children with ASD have frequently been reported to have a less varied diet [33,34] and low intake of different food groups [35]. Nearly half of children with ASD ate brown/whole meal/wholegrain bread most of the time whereas 68% of typically developing children consumed these food types most of the time [16].

A large proportion of children in the present study (65%) had a protein intake below the AMDR. In line with this, several previous studies reported lower protein intake in children with ASD than typically developing controls [11-13, 36-38]. Although average intake for fat was within the AMDR in the present study, some individual differences are worth noting. Twenty-eight children (33%) exceeded the AMDR, consuming more than 35% of energy from fat. Similarly, Moore *et al.* (2012) [39] and Raiten and Massaro (1986) [40] reported increased fat intake among children with ASD. Also, the average intakes for sugar and SFA exceeded the recommendations, with 51% and 80% of children consuming more than 10% of energy from sugar and SFA, respectively. These findings indicate that (1) a large proportion of carbohydrate intake was from sugar (not from milk and fruit), (2) a large proportion of fat was from saturated fat (probably margarine, snacks, and processed foods), and (3) high intake of fat was at the expense of protein intake in a large proportion of children in the present study. Furthermore, 17% of children in the present study were obese, which is higher than the prevalence rate of 12% reported in children in the general population [41]. Similarly, Curtin *et al.* reported that the prevalence of obesity was higher among children with ASD than healthy controls (by approximately 6%) [42]. These findings highlight the importance of ongoing nutritional assessment in children with ASD in order to implement early dietary interventions to prevent obesity and related health problems.

The present study showed that the average intake for vitamin D is below the AI (22% of AI) with 96% of children not meeting the recommendation, a finding confirmed by others [43-45]. It is important to note that based on the AI, no assumption about the prevalence of inadequate intake of vitamin D can be made. However, 37% of children in the present study had serum 25(OH)D concentrations <50 nmol/L. These findings highlight the lack of vitamin D in the NZ food supply (no mandatory fortification) and important role of other sources of vitamin D (e.g. sun exposure) in vitamin D status.

The average intake of iodine (47 µg, 72% of EAR) in the present study was below the mean intake of 65 µg (from food only) and 101 µg (from food + iodised salt) among New Zealand school-aged children (8-10 years old) [46]. It is important to note that we did not investigate the contribution of discretionary iodised salt, however, one can argue that these children may have inadequate iodine intake because a large proportion of children did not meet the recommendation for servings of bread and cereals. In New Zealand, fortification of bread with iodised salt is mandatory, and bread has been reported to contribute half of total iodine intake (from food only) [44]. In line with this, the present study also found a significant positive correlation between sub-score component of servings of bread and cereals and iodine intake (r=0.7, P=0.01, large effect size).

Although the present study did not investigate biochemical indices of iodine status in children, others have demonstrated that children with ASD have lower iodine status and thyroid hormones than healthy controls and low iodine status has been associated with autism symptomatology [46,47]. Low intake of vitamin D and iodine may warrant further investigations since recent reports suggest that deficiencies in vitamin D and iodine may be related to the pathophysiology of autism [47-49].

Furthermore, more than one third of children in the present study had intakes below the AIs for fibre and vitamin E, a finding confirmed by others [39,45,50]. Although no assumptions can be made regarding the dietary adequacy of these nutrients, they are important factors to consider and address when assessing the nutritional status of this population. Twenty three percent of children in the present study had complained about constipation, accordingly adequate fibre intake may alleviate this complaint and should be emphasised when giving nutritional recommendations to parents/caregivers of children with ASD.

In addition to the above-mentioned nutrients, considering the RDI for nutrients, 20-30% of children had intakes <80% of RDI for calcium (29%), iron (21%), vitamins A (21%), and vitamin C (21%). It is important to note that the RDI for a nutrient is the intake that meets the nutrient need of almost all (97-98%) individuals in a group of people, which is not intended to be used to assess the diets of either individuals or groups or to plan diets for groups. However, a usual intake that is well below the RDI may be an indication of the need for further assessment of nutritional status by biochemical tests or clinical examination.

Little has been published on the health and behaviour effects of long-term excessive intake of micronutrients. The present study found intakes averaged more than 100% of NRV for most micronutrients. Other researchers have also reported increased intakes of some micronutrients (e.g. B vitamins and magnesium) [39,40,45,51]. This high intake of nutrients from food only is of concern because supplemental sources of nutrients were not included in the analysis. The excess intakes in children with ASD may reflect their more limited and repetitive eating patterns, consumption of fortified foods such as cereals and grains and fruits juices or being on exclusion/special diet. For example, increased intake of magnesium could be due to higher intake of gluten and milk substitutes, such as soy-based foods, nuts or nut-based milk, in children on gluten-free, casein-free diet.

Approximately, 90% of children in the present study consumed more than the maximum recommended amounts set for sodium for their age group (1000 and 1400 mg for 2-3 and 4-8 age group, respectively). It is important to note that dietary assessment of sodium intake is

inaccurate due to difficulties in quantifying discretionary salt use and considerable variation in in the sodium content of processed foods which are the major sodium contributing foods. However, based on 24-hour urinary sodium, daily sodium intake has been estimated to exceed 3000 mg in adults [20]. As no accurate data is available for the consumption levels of children, further comparisons between children with ASD and typically developing children cannot be made. However, excess salt consumption in children warrants further studies and interventions as increased blood pressure due to excess consumption can be a risk factor for cardiovascular disease in adulthood [52].

Based on biochemical markers, the prevalence of low ferritin (defined as ≤10 and ≤12 μg/L μg/L for 2-5 and 5-8 years old children, respectively 10 vs. <4%) and low iron saturation (defined as <12% and <14% for 2-5 and 5-8 years old children, respectively; 23 vs. ≤11%) as well as iron deficiency (defined as having two of the iron pools in the abnormal range; 4 vs. <1%) appeared to be higher in children with ASD than 5-10-year-old children from NZ general population [29]. Taking season of enrolment into consideration, vitamin D deficiency (<50 nmol/L) was more prevalent in our study population than NZ typically developing preschool children (37 out of 86 children were enrolled during winter and spring months; 68 vs. 55%) [53]. Also, children of our study apparently had lower omega-3 index than Australian typically developing children (a mean of 4.7 vs. 6.5%, respectively) [54].

Relative Validity of DICE

To our knowledge, there is no previous study that evaluated the relative validity of a diet quality index in populations with ASD and therefore the results of the present study are compared with the reports on healthy populations. The DICE total score showed a high correlation with the 4DFR (r=0.7; P<0.001) and good agreement (κ=0.6). Furthermore, approximately 55% of children with ASD were correctly categorised into the same tertile. Similarly, Delshad *et al.* reported a high correlation but moderate agreement between DICE and 4DFR in healthy children aged 2-8 years in New Zealand [16]. Because problems with eating are frequently reported in children with ASD (e.g. limited food variety, selective eating, and food refusal) [34], parents/caregivers of these children might be more aware of their child's dietary intake due to concerns about their dietary behaviour [50]. Therefore, errors associated with dietary measurements using two different methods might be lower in this population, resulting in a higher agreement between two assessment methods.

With the exception of the sub-score components on the frequency of consuming low fat, low salt, and low sugar foods/snacks/drinks, all other sub-score components had significant, positive and small to high correlations (ranging from 0.3 for servings of wholegrain products

to 1.0 for fluid consumption) with relevant scores obtained from 4DFR. These findings are consistent with other researchers reporting significant positive associations for most recommended food items/categories from DICE and 4DFRs in healthy New Zealand children [16], from food frequency questionnaires (FFQs) and 4DFRs in healthy New Zealand adolescents [55], and from Australian Recommended Food Scores for Preschoolers (ARFS-P) and FFQs in healthy Australian preschool children [56]. Burrows *et al.* (2014) [57] reported a negative relationship between sub-scores for non-recommended food items from ARFS-P and FFQ, however Delshad *et al.* [16] and the current study found no relationship between low fat, low salt, low sugar foods/snack/drinks subs-score components from DICE and 4DFR.

One can propose several explanations for the lack of agreement for these components; (1) the recommendations assume that children are eating processed foods and if they do not eat such foods they are less likely to choose low fat, salt, and sugar foods/snacks/drinks, (2) parents/caregivers may underreport high fat, salt and sugar foods/snacks/drinks, (3) parents/caregivers may have limited awareness of foods/snacks/drinks that are low in fat, salt and sugar; (4) due to limitations to the design of relevant questions, parents/caregivers may have incorrect estimates of children's low fat, salt and sugar foods/snacks/drinks consumption, and finally (5) this lack of agreement could be due to differences in the analysis of these components between DICE and 4DFR. Because these items were found to be the "problem components" in both children with and without ASD, the design of these questions is recommended to be revised (e.g. to include more clarification). Also, a possibility remains that parents/caregivers do not clearly understand the dietary guidelines and therefore it is recommended to modify the guidelines so that the message regarding these components is clearly delivered to both consumers and food industry.

The present study demonstrated a positive relationship between higher DICE score and intake of a limited number of nutrients (including magnesium, vitamin A, and fibre), which is in agreement with Delshad *et al.* for two nutrients, vitamin A and fibre [16]. In addition to these nutrients, total DICE score was associated with higher intake of magnesium in children with ASD and with vitamin C, folate, calcium and vitamin D in healthy children. In the present study, the relationship between DICE total score and vitamin A could be partly explained by servings of vegetables, fibre by servings of fruits and vegetables, and magnesium by servings of fruits, vegetables, and meat and meat alternatives components.

Strengths and Limitations

The current study is the first to investigate dietary adequacy and nutritional status of children with ASD in NZ and to evaluate a novel, stand-alone index of children's diet quality, the DICE, in this population.

One possible weakness of the present study is that it was a cross-sectional study and lacked a typically developing control group for comparison. Furthermore, nutritional data are only as good as the accuracy of the food records and the completeness of food databases. Calculations of nutrient intake from 4DFR supplied by parents/caregivers (based on estimation of portion sizes) could be less accurate than using weighed records of foods consumed. Accordingly, the agreement between DICE and 4DFR may reflect similar errors associated with the estimation of portion sizes in these methods. Also, there was a lack of detailed information on supplements and the estimates are based on dietary intake of nutrients from food only. Accordingly, nutrient intakes might be underestimated. A further limitation is that volunteers were predominantly children with mild to moderate ASD and of New Zealand European ethnicity. Whether these findings are applicable to children with severe ASD or of other ethnicities is unknown and warrants further investigation. Finally, DICE may not have best captured the dietary implications of selective eating and restricted food choices among children with ASD, through inability to detect low food variety within each food group (with the exception of fruits and vegetables).

Conclusions

The present study confirmed nutritional issues and low diet quality in children with ASD. Given the importance of nutrition in growth and development and in the management of ASD, careful attention should be given to the nutritional status of these children. In addition, this study demonstrated that DICE is a relatively valid tool for the assessment of 2 to 8 year old children's adherence to a healthy diet, as recommended in the NZ MoH Food and Nutrition guidelines. Further research should be undertaken to refine the content and apply the DICE questionnaire to a more symptomatically and ethnically diverse population with ASD.

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Chapter 5: A Randomised Controlled Trial of Vitamin D and Omega-3 Long Chain Polyunsaturated Fatty Acids in the Treatment of Core Symptoms of Autism Spectrum Disorder in Children (Paper V)

Once children with ASD were screened for major nutritional deficiencies and met the inclusion criteria, they entered the intervention.

Autism Spectrum Disorder is associated with an impairment in social and communicative functioning and repetitive/stereotypic interests and behaviours. Because very little is known about the efficacy of vitamin D and omega-3 LCPUFA, each individually or together, on the management of core symptoms of ASD, this study aimed to investigate this by using a randomised controlled trial. This chapter reports the findings relevant to the core symptoms of ASD (primary outcome of trial).

This chapter is presented in manuscript format and has been published in the "Journal of Autism and Developmental Disorder".

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Abstract

We evaluated the efficacy of vitamin D (VID), omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFA, OM), or both (VIDOM) on core symptoms of ASD in children. New Zealand children with ASD (n=73; aged 2.5-8.0 years) randomly received daily 2000IU vitamin D_3, 722mg docosahexaenoic acid, both supplements, or placebo. Primary outcome measures were Social Responsiveness Scale (SRS) and Sensory Processing Measure (SPM). Compared to placebo, of the 42 outcome measure comparisons, two showed statistically significant improvements and four showed trends for greater improvements in the intervention groups: SRS-social awareness for OM (*P*=0.03) and VIDOM (*P*=0.03); SRS-social communicative functioning for VIDOM (*P*=0.07); SRS-total for OM (*P*=0.08); SPM-taste and smell for VIDOM (*P*=0.06). and SPM-balance and motion for OM (*P*=0.09) Omega-3 LCPUFA alone or in combination with vitamin D may improve some core symptoms of ASD but no definitive conclusions can be drawn. Large trials with both vitamin D and omega-3 LCPUFA are warranted.

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder encompassing impairment in social and communicative functioning and repetitive/stereotypic interests and behaviours (RRB), with a wide range of variety of symptoms [1]. Sensory issues are considered as a distinctive criterion for RRB [1]. The symptoms clinically cause significant impairment in social, occupational and other functioning [1]. It is generally agreed that ASD can be caused by a combination of genetic and environmental factors, although specific causes are not clearly understood. Accordingly, there are no well-documented, mechanism-based pharmacological treatment agents, and those prescribed focus mainly on co-occurring problem behaviours (e.g. irritability and hyperactivity) rather than core symptoms of ASD (e.g. social communication and RRB) [2,3]. Therefore, many parents/caregivers of children with ASD turn towards complementary and alternative treatments including vitamin D and omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFA), because they are dietary supplements and their administration, in appropriate doses, to children are considered to be safe and well-tolerated [4,5], and may increase the efficacy of pharmacological medications [6].

There are accumulating data to support that vitamin D and omega-3 LCPUFA (especially eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA) are important for brain function and structure and for neurotransmitter and glutamatergic systems, and both have

immunomodulatory, anti-inflammatory and anti-oxidant properties [5,7-10], making these nutrients powerful candidates, either alone or in combination, for the management of ASD.

Several reports suggest children with ASD have inadequate intakes of vitamin D and omega-3 LCPUFA [11-15], as is reflected in significantly lower vitamin D and/or omega-3 LCPUFA status than their healthy counterparts [5,16].

Evidence to support the use of vitamin D, omega-3 LCPUFA, or both for the treatment of core symptoms of ASD is either inconclusive, inadequate or lacking [5,7]. A recent review of evidence linking vitamin D and ASD identified six intervention trials with inconsistent findings [7]; while some of the identified studies showed positive effects of vitamin D on core symptoms [17-20], others failed to confirm such effects [21,22]. Two more randomised controlled trials have been published since this review, and again the results are mixed [4,23]. Similarly, the results of omega-3 LCPUFA intervention trials in ASD have been somewhat variable; while all open label intervention trials (including one case study) along with three RCTs showed beneficial effects on some core symptoms of ASD [24-32], others failed to replicate those findings [33-35]. Small sample size has been suggested to be the major limitation of these studies. The pooled analyses of RCTs have demonstrated that omega-3 LCPUFA might improve core symptoms of ASD (lethargy and repetitive behaviours) [5,36,37], though the authors of these meta-analyses precluded any definitive conclusions due to the limited number of studies, and small sample size and overall effects. To our knowledge there are no studies that have tested the efficacy of a combination of vitamin D and omega-3 LCPUFA on ASD symptoms.

The primary objective of the study was to test the hypothesis that supplementation with vitamin D (VID), omega-3 LCPUFA (OM) or both (VIDOM) improves core symptoms of ASD in children living in New Zealand. The secondary objective of the study was to test the relationship between change in biomarkers of vitamin D (serum 25(OH)D) and omega-3 LCPUFA (omega-3 index) and the change in core symptoms of ASD.

Subjects and Methods

Study design and data collection methods for this study are described briefly below, with further details reported elsewhere [38]. The study was registered with the Australian New Zealand Clinical Trial Registry, ACTRN12615000144516. Ethical approval was granted by Health and Disability Ethics Committees, NZ, Reference NO. 14/NTA/113. All parents/caregivers were provided with an information sheet explaining the study protocol in detail and signed an informed consent form.

Study Participants

New Zealand children were included if they were between 2.5 and 8 years, had a medical diagnosis of ASD confirmed by a developmental paediatrician in accordance with the criteria listed in the *Diagnostic and Statistical Manual of Mental Disorders, version five* (DSM-5) [1], and onset of symptoms after 18 months of age. Children were excluded if they were diagnosed as having developmental delay since birth; if they failed to take corrective action for nutritional deficiencies; serum 25(OH)D ≥75 + 10 nmol/L (≥85 nmol/L) if they entered the trial in winter and ≥105 nmol/L + 10 nmol/L (≥115 nmol/L) if they entered the trial in summer. There is a consensus between the Ministry of Health and Cancer Society of New Zealand [39] and Institute of Medicine [40] that the minimum 25(OH)D concentrations for a better health outcome are at least 50 nmol/L. However, the Endocrine Society in the US propose concentrations of 75 nmol/L or more for multiple clinical outcomes [41]. Two different cut-off points for exclusion were applied due to a large seasonal variation in serum 25(OH)D concentrations in New Zealand ranging from 30 nmol/L [42] to 44 nmol/L [43]. A 10 nmol/L variation was chosen because of the potential assay variability [44].

Study Design

A 12- month randomised placebo controlled double-blind study design was used (**Figure 1**). Children who met the initial inclusion criteria had a blood draw and were screened for nutritional deficiencies (including vitamin D, iron and vitamin B_{12} deficiencies). Prior to randomisation, those deficiencies were addressed. Refer to VIDOMA study protocol [38] and reference [45] for a list of nutritional deficiencies and the management strategies applied in this trial. Children were then randomly assigned to one of four treatment groups, each consuming four capsules per day for 12 months; vitamin D_3 (2000 IU/day), DHA (722 mg/day), both, or placebo. The treatment materials were delivered in 750 mg gel capsules with a tear-off nozzle manufactured and supplied by Douglas Nutrition Ltd, Auckland, NZ. The treatment materials were dispensed every four months. All study capsules were identical in appearance and were tasteless and colourless. A third party not involved in any aspect of the study was responsible for generating the randomisation sequence. Randomisation was stratified by age (2.5-5.0 years old and 5-8.0 years old) and severity of ASD (mild, moderate, and severe) and the sequence was generated using the Website Randomisation.com (http://www.randomization.com/) and random block design in blocks of 4 and 8. Researchers, children, and caregivers were blinded to treatment allocations until after data analysis.

Data were collected during participants' visit to the Massey University Human Nutrition Research Unit (HNRU), NZ. Demographic and medical history were recorded at baseline. ASD related outcomes were assessed and height and weight were measured at baseline and after 12 months. Non-fasting venous blood samples were collected at the North Shore or Waitakere Hospitals in Auckland, NZ, on three occasions, baseline, 6 months and 12 months (endpoint). Caregivers completed weekly online surveys to collect information regarding adverse events, supplement and medication use, behavioural therapies and compliance. Compliance was calculated using cumulative pill counts at the end of the study, and adherence was measured as a percentage; (number of pills supplied minus number of pills not taken)/number of pills supplied × 100. Compliance was also confirmed by biomarker analysis (serum 25(OH)D and omega-3 index).

Data Collection

Standardised psychological tests were used to assess the core symptoms of ASD. Standardised instructions were given to all caregivers on how to complete the questionnaires during their visit to the HNRU.

Social Responsiveness Scale™, Second Edition (SRS-2) [46]: The SRS–2 is a quantitative approach to screen and diagnose social impairment and repetitive behaviour as a single trait in ASD. SRS provides scores for five treatment subscales (social awareness, social cognition, social communication, social motivation, and RRB) as well as scores for two DSM-5 compatible subscales, social communicative functioning (the composite score of social awareness, social cognition, social communication and social motivation) and RRB. The SRS has been found to discriminate children with ASD from typically developing children [47], from children with oppositional defiant/conduct disorder [47], and from other psychiatric patients with/without pervasive developmental disorders [47]. In a cross-cultural validity study of the SRS, Bolte *et al.* (2008) reported a good internal consistency, test–retest reliability and inter-rater reliability, and a good convergent validity with other ASD diagnostic tools [48].

Sensory Processing Measures™ (SPM) [49]: The SPM has been developed according to the sensory integration theory – that sensory issues affect higher levels of functioning such as social participation and planning and ideas (praxis) [49]. The SPM assesses sensory processing in relation to vision, hearing, touch, body awareness (proprioception), and balance and motion (vestibular function) in children. The Home Form has been standardized on a demographically representative sample of 1,051 typically developing children [49]. The internal consistency and test–retest reliability of SPM has been documented [49, 50]. The

SPM has been also validated in a group of children with additional needs [51], and its convergent validity with other tools assessing sensory features in children with ASD has been documented [52].

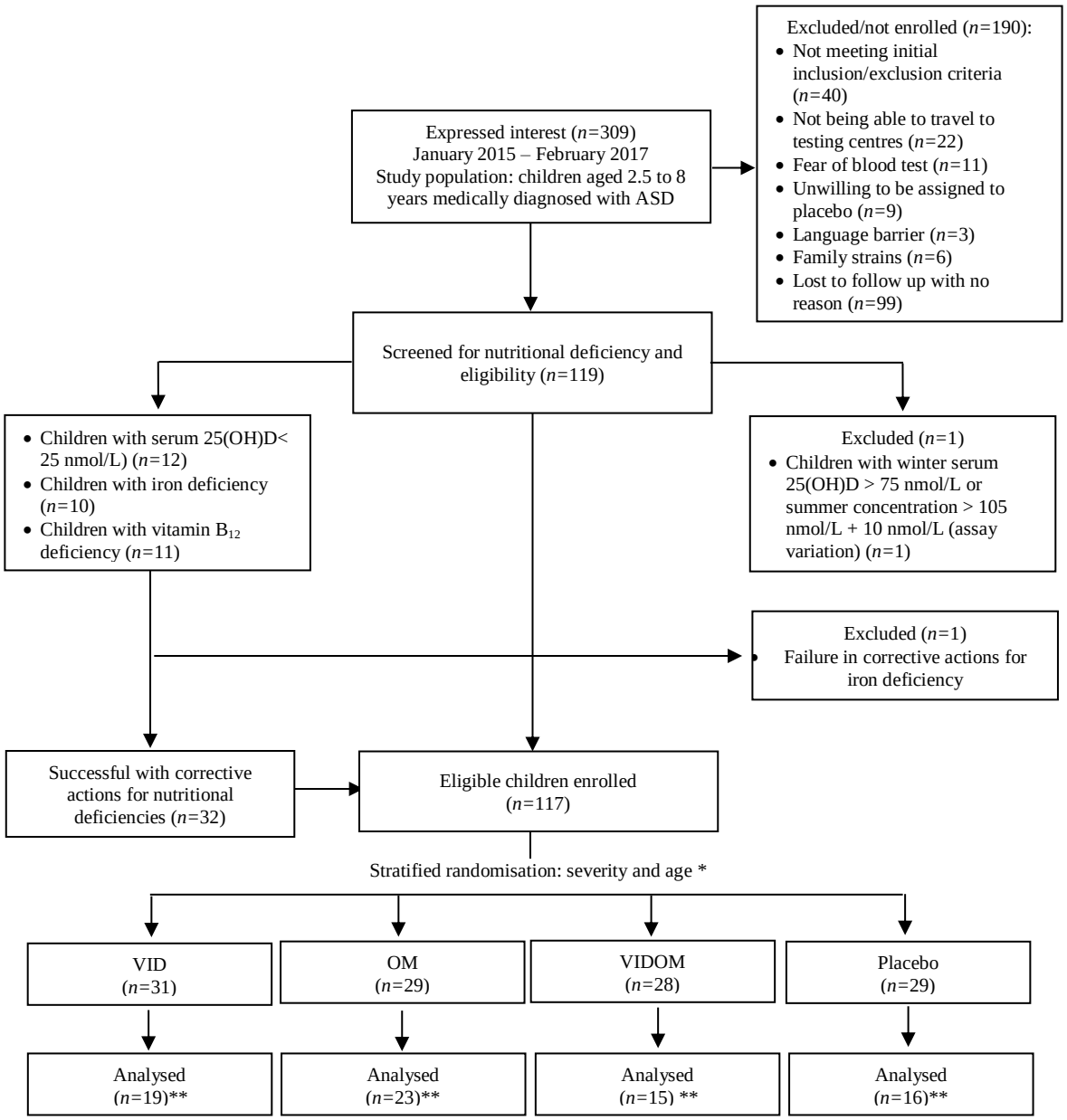

Figure 1: Schematic diagram of study design and flow of participants through the study. VID (vitamin D), OM (omega-3 LCPUFA), VIDOM (vitamin D+omega-3 LCPUFA). * Corrective action was taken for nutritional deficiencies before randomisation. ** The reasons for drop out were parents/caregivers' concerns about side effects and having a blood sample taken (VID 2; OM 1; VIDOM 0; placebo 1), family and time constraints (VID 3; OM 2; VIDOM 4; placebo 3), child disliking the supplement (VID 1; OM 0; VIDOM 4; placebo 2), not seeing any benefits (VID 1; OM 0; VIDOM 0; placebo 1), moving overseas (VID 2; OM 0; VIDOM 0; placebo 0), child being diagnosed with other medical conditions during the study period (VID 1; OM 0; VIDOM 0; placebo 0), and no reason (VID 3; OM 2; VIDOM 5; placebo 6).

Biochemical analysis

Nutritional biomarkers were assayed from a non-fasted venous blood sample. These included the following: full blood count, erythrocyte fatty acids, and 25(OH)D, calcium, albumin, iron studies (iron, iron binding capacity, ferritin, and transferrin saturation), vitamin B_{12} and folate. With the exception of erythrocyte fatty acids, all other biomarkers were analysed at North Shore Hospital (IANZ accredited). Erythrocyte fatty acids were analysed at the University of Wollongong, Australia [38]. Omega-3 index is defined as the combined percentage of two omega-3 LCPUFA, EPA and DHA, in red blood cells [53]. Although an omega-3 index for neurodevelopmental disorders has yet to be determined, levels <4% are associated with greater risk of mortality from cardiovascular disease while 8-12% are shown to be protective [53]. The omega-3 index has been reported to be 1.4, 3.2, and 6.5% in children with ASD, ADHD, and typical development, respectively, indicating that levels under 4% could also be considered as a risk factor for neuropsychological disorders [54]. Cut-offs of >225 nmol/L and >2.7 mmol/L were used to define hypervitaminosis D and hypercalcaemia, respectively [55].

Statistical Analysis

It was calculated that a total of 168 children (42 per treatment group) would be required to demonstrate a clinically significant difference at 80 % power and 5 % statistical significance. Sample size was calculated using a potential dropout rate of 20% and the following formula [56]: $N=2\alpha^2K/(\mu_2-\mu_1)^2$ [*N*, sample size; α, SD SRS-total (primary outcome), 24.7 [from our 2015 pilot study, unpublished]; *K*, constant [7.9]; $\mu_2-\mu_1$, clinically significant difference in SRS-total means, 17 units [57]].

Statistical analysis was performed using IBM SPSS version 24.0 (Armonk, NY). A *P*-value 0.05 was considered significant and a *P*-value of <0.1 as a trend for a relationship (due to large drop-out and potential loss of statistical power). The variables were tested for normality using the Kolmogorov-Smirnov, Shapiro-Wilk tests and normality plots. Non-normally distributed data were transformed into approximate normal distributions by logarithmic transformations. The data were reported appropriately as mean±SD for normally distributed data and as median (25, 75 percentiles) for non-normally distributed data and as frequencies for categorical data. Baseline between group differences were examined using analysis of variance (ANOVA) for parametric data, the Kruskal-Wallis test for non-parametric data and X^2-test for categorical data. A Bonferroni correction was applied for multiple comparisons (0.05/number of comparisons).

The primary analysis, comparing the effects of treatment on core symptoms of autism over 12-months, was conducted using a pair-wise mixed effects longitudinal models general linear mixed model design (variance components structure). Treatment (VID *vs.* placebo, OM *vs.* placebo, and VIDOM *vs.* placebo) and time (baseline and endpoint) were included as fixed effects, and participant was included as a random effect to account for the repeated measures within individuals. The interactions between treatments and time were tested with and without considering the potential effect of confounders. Potential confounding factors and effect modifiers investigated within the models were compliance, medication use, and therapy. Analysis was conducted on completers for each outcome measure using data from those who completed assessments at both time points (baseline and endpoint). No adjustments for multiple testing were made in order to avoid missing potentially important cause and effect relationships due to an inflation of the type II error for our final model [58]. This approach has been used by others [31].

As an exploratory analysis, treatment groups were parameterised differently (all receiving vitamin D, including VID and VIDOM, *vs.* others and all receiving omega-3 LCPUFA, including OM and VIDOM, *vs.* others), and the data was reanalysed using the above-mentioned statistical analysis. The second parameterisation method was performed to investigate the effect of treatments or any synergy/antagonism between VID and OM (or any additive effect of both) on primary outcome measures. The goal of latter parameterisation was to answer the research question much more clearly. However, due to not being the pre-defined statistical method of the present trial, the findings of the latter analysis are considered exploratory findings.

The relationship between biochemical markers (25(OH)D and omega-3 index) (as continuous variables) and change in outcome measures were assessed using Pearson for normally distributed variables and Spearman correlations for non-normally distributed variables. Also, the difference in change in outcome measures between low and high levels of biomarkers were assessed using independent samples t-test and Mann Whitney U test for normally and non-normally distributed variables, respectively.

Results

Baseline Socio-Demographic and Behavioural Characteristics

The flow of participants through the study are summarised in **Figure 1**. Over the 25-month period of recruitment (from January 2015 to February 2017), 309 families registered their interest in the study, of whom 190 families were either excluded or not enrolled. The children

of remaining families (*n*=119) were screened for nutritional deficiencies and high serum 25(OH)D concentrations. Out of 119 children, 33 were treated for nutritional deficiencies, of whom one was excluded due to failure to take corrective action for iron deficiency. A total of 117 children were randomised to treatment groups. Seventy-three children completed the study out of 117 who completed the baseline assessments, with a median follow-up of 54 (53, 55) weeks and compliance rate of 95% (92%, 96%). Out of 44 children who did not complete the study, 16 were lost to follow up and 28 dropped out. The reasons for dropping out were parents/caregivers' concerns about side effects and having a blood sample taken (*n*=4), family and time constraints (*n*=12), child disliking the supplement (*n*=7), not seeing any benefits (*n*=2), moving overseas (*n*=2), and child being diagnosed with other medical conditions during the study period (*n*=1). The reasons for drop out were related to study supplements in 13-14% of drop outs in VID, VIDOM, and placebo groups, as compared with 3% in OM group, *P*=0.48 (**Figure 1**).

Baseline characteristics of children randomised (*n*=117) did not differ significantly across treatment groups (**Table 1)**. After exclusion of children who did not complete the study, the baseline characteristics were equally distributed across treatment groups (**Appendix 1**). For a list of types of behavioural interventions and medications used by children over the study period refer to **Appendix 2**.

Treatment Efficacy

The baseline, endpoint (after 12 months) and the changes in SRS-total behavioural scores and SRS domains of study populations across treatment groups are presented in **Table 2**. Both unadjusted and adjusted analysis of the scores on SRS-total and SRS domains revealed a significant effect of time. The analysis of the scores on SRS-total showed a trend for a greater improvement in OM group (*P*=0.09 unadjusted for covariates and *P*=0.08 adjusted for covariates) than placebo **(Figure 2A)**. The rate of positive response (at least a 30% improvement in SRS-total score) was 20-22% in active treatment groups (4/19, 5/23, and 3/15 children in VID, OM and VIDOM groups, respectively) and nil in the placebo group. Furthermore, while there was a decrease in the proportion of children with severe autism (level 3) after 12 months in all active treatment groups (VID, -13%; OM, -26%; and VIDOM, -14%), the proportion increased by 6% in the placebo group (*P*=0.02).

The unadjusted analysis of the scores on SRS-social communicative functioning revealed no effect of active treatments. However, when the analysis was adjusted for covariates a trend for a greater improvement with VIDOM was identified than with placebo (*P*=0.07) (**Figure 2B).** Of all four SRS-social communicative functioning subdomains (social awareness,

cognition, communication, and motivation), a significant effect of treatment groups was identified only for social awareness (OM *vs.* placebo: *P*=0.06 unadjusted for covariates and *P*=0.03 adjusted for covariates; and VIDOM *vs.* placebo: *P*=0.04 unadjusted for covariates and *P*=0.03 adjusted for covariates) and a trend for an effect of treatment groups for social motivation [OM *vs.* placebo: *P*=0.06 unadjusted for covariates and *P*=0.1 adjusted for covariates) subdomains (**Figures 2C and 2D**).

The baseline, endpoint and the changes in SPM-total and subdomain scores of study populations across treatment groups are presented in **Table 3**. With the exception of SPM-taste and smell and SPM-balance and motion subdomains, the slopes of change over time for the SPM composite score as well as four other SPM subdomains (vision, hearing, touch, and body awareness) did not differ between active treatment and placebo groups. There was a possible relationship between VIDOM and SPM-taste and smell [F (1, 74) = 3.8, P=0.06 (unadjusted) and F (1, 69) = 3.7, P=0.06 (adjusted for covariates)] and between OM and SPM-balance and motion, which became weaker when the analysis was adjusted for covariates [F (1, 74) = 3.3, P=0.07 (unadjusted) and F (1, 69) = 3.0, P=0.09 (adjusted for covariates)].
In an exploratory fashion, the effect of treatments on the social participation domain of SPM was assessed (**Table 3**). The analysis of the scores revealed a greater improvement with VID (P=0.06) and VIDOM (P=0.08) than placebo.

Also, when other parameterisation method was used the relationship between OM and SRS-total (P=0.06), SRS-social communicative functioning (P=0.04), SRS-awareness (P=0.008) and SRS-communication (P=0.01), and the relationship between VID and SPM-social participation (P=0.03) became stronger (based on P-value adjusted for covariates).

The analyses of serum 25(OH)D concentrations and omega-3 index showed a significant effect of time of follow-up (P<0.01) and the interaction between time point and treatment groups (P<0.01) (**Figures 3A and 3B).** Significant increases were seen in mean serum 25(OH)D concentration in the VID and VIDOM groups and in the median omega-3 index in the OM and VIDOM groups. The proportion of children having serum 25(OH)D ≥75 nmol/L in the VID, VIDOM, OM, and placebo groups increased from 29, 33, 30, and 14% at baseline to 71, 100, 35, and 21% at endpoint, respectively.

As the secondary findings, no relationship was observed between change in biochemical markers and change in any outcome measure parameters (r values ≤0.1 and P values >0.1). However, a greater improvement in SRS-social awareness (-1.5±2.9 *vs.* -0.3±3.0, P=0.09)

and SPM-total (-15±19 *vs.* -7.4±19, *P*=0.03) was seen in children with initial serum 25(OH)D concentration <50 nmol/L (*n*=26) than ≥50 nmol/L (*n*=46), respectively. Greater improvement in SRS-total (-23±16 *vs.* -9.9±18, *P*=0.05), SRS-social communicative functioning (-19±14 *vs.* -9.4±18, *P*=0.03), SRS-communication (-11±8.3 *vs.* -3.2±7.7, *P*=0.01), and SPM-social participation (-3.2±2.8 *vs.* -0.2±4.6, *P*=0.05) was observed in children with endpoint serum 25(OH)D >100 nmol/L (*n*=9, all on vitamin D and VIDOM supplements) than ≤100 nmol/L (*n*=49).

A single possible relationship was noted between endpoint omega-3 index and change in SPM-total (r_s = -0.2, *P*=0.08). Further analysis showed children with an endpoint omega-3 index >8% (*n*=21, all from OM and VIDOM groups) had a trend for larger improvement in SPM-total score than those with endpoint omega-3 index ≤8% (*n*=33), -16±20 *vs.* -8.4±20, respectively, *P*=0.07.

Safety and Adverse Events

Among all the study groups, there were no significant changes in the levels of serum calcium, and there were no reports of serum calcium (corrected for albumin) ⩾ 2.7 mmol/L (hypercalcaemia) and serum-25(OH)D > 225 nmol/L (hypervitaminosis D). A list of adverse events reported by parents/caregivers over the study period is presented in **Appendix 4**. All the gastrointestinal symptoms appeared to be unrelated to treatments because they were equally distributed across all treatment groups. Six parents/caregivers (OM, 5 and VIDOM, 1) reported allergic reactions. The allergic reactions included rash (*n*=4), facial pimple (*n*=1) and red ear (*n*=1) and were reported on a few occasions over the study period (occurred for more than four weeks but at irregular intervals).

Adequacy of Blinding

All parents/caregivers who completed the study responded to the question, ‘‘which study supplements do you think your child was consuming over the study period?’’ at the end of the study. The proportion of parents who incorrectly guessed the treatment groups was 62% (45/73). Assigned groups were incorrectly guessed by 52% (12/23) in OM, followed by 60% (9/15) in VIDOM, 68% (13/19) in VID and 69% (11/16) in placebo groups, *P*=0.66.

Table 1: Baseline socio-demographic and behavioural characteristics of children who were randomised to treatment groups (*n*=117)

Characteristics *	**Total (*n*=117)**	**VID (*n*=31)**	**OM (*n*=29)**	**VIDOM (*n*=28)**	**Placebo (*n*=29)**	**P value†**
Socio-demographic characteristics						
Age, years (mean±SD)	5.2±1.4	5.2±1.5	5.0±1.5	5.2±1.5	5.5±1.3	0.56
Sex, *n* (%)**						0.50
Male	100 (85)	28 (90)	24 (83)	25 (89)	23 (79)	
Female	17 (15)	3 (10)	5 (17)	3 (11)	6 (21)	
Ethnicity (*n*=116), *n* (%)						0.41
NZ European	57 (49)	18 (58)	12 (43)	16 (55)	11 (38)	
Māori	15 (13)	4 (13)	6 (21)	3 (10)	2 (7)	
Pacific Island	13 (11)	2 (6)	1 (4)	4 (14)	6 (21)	
Asian	13 (11)	3 (10)	5 (18)	1 (3)	4 (14)	
Others	18 (16)	4 (13)	4 (14)	4 (14)	6 (21)	
Country of birth (*n*=110), *n* (%)						0.09
New Zealand	99 (90)	28 (97)	25 (89)	24 (96)	22 (79)	
Others ***	11 (10)	1 (3)	3 (10)	1 (4)	6 (21)	
Season of birth (*n*=110), *n* (%)						0.41
Summer	31 (28)	9 (31)	8 (28)	5 (20)	9 (32)	
Autumn	21 (19)	5 (17)	3 (11)	7 (28)	6 (21)	
Winter	34 (31)	9 (31)	9 (32)	5 (20)	11 (39)	
Spring	24 (22)	6 (21)	8 (28)	8 (32)	2 (7)	
Socioeconomic status, parent's household income (*n*=109), *n* (%)						0.22
<60,000 NZ$	31 (28)	8 (27)	8 (30)	4 (16)	11 (41)	
60,000 to 120,000 NZ$	61 (56)	16 (53)	16 (59)	14 (56)	15 (56)	
>120,000 NZ$	17 (16)	6 (20)	3 (11)	7 (28)	1 (4)	
Season of enrolment, *n* (%)						0.70
Summer	22 (19)	7 (23)	7 (24)	5 (18)	3 (10)	
Autumn	35 (30)	8 (26)	10 (34)	7 (25)	10 (35)	
Winter	32 (27)	6 (19)	6 (21)	11 (39)	9 (31)	
Spring	28 (24)	10 (32)	6 (21)	5 (18)	7 (24)	

Table 1: *Cont.*

Characteristics *	**Total (*n*=117)**	**VID (*n*=31)**	**OM (*n*=29)**	**VIDOM (*n*=28)**	**Placebo (*n*=29)**	**P value†**
BMI–for–age categories, *n* (%)						0.42
Underweight (<5th %ile)	6 (5)	2 (7)	1 (3)	2 (7)	1 (3)	
Normal weight (≥5 – 85th %ile)	68 (58)	19 (61)	21 (72)	17 (61)	11 (38)	
Overweight (≥85 – 95th %ile)	20 (17)	5 (16)	3 (10)	4 (14)	8 (28)	
Obese (≥95 %ile)	23 (20)	5 (16)	4 (14)	5 (18)	9 (31)	
ASD behavioural characteristics						
ASD severity (clinical diagnosis), *n* (%)						0.80
Mild (level 1)	47 (40)	13 (42)	14 (48)	10 (36)	10 (35)	
Moderate (level 2)	49 (42)	11 (35)	13 (45)	13 (46)	12 (41)	
Severe (level 3)	21 (18)	7 (23)	2 (7)	5 (18)	7 (24)	
Social Responsiveness Scale (SRS) (mean±SD)						
Total	118±27	110±25	100±25	108±29	116±29	0.16
Social-communicative functioning	87±22	88±20	81±20	86±24	94±24	0.16
Social awareness	14±3.9	14±3.3	13±3.4	15±4.3	15±4.7	0.49
Social cognition	21±5.5	21±5.7	20±5.4	21±5.8	22±5.1	0.46
Social communication	46±10	36±8.8	32±9.3	36±11	39±10	0.05
Social motivation	16±6.7	17±8.0	15±5.4	14±6.7	17±6.3	0.33
RRB	21±6.5	23±6.3	19±6.2	21±6.1	23±6.7	0.06
Sensory Processing Measure (SPM) (mean±SD)						
Total	118±28	122±30	112±25	119±29	120±29	0.55
Vision	22±7.0	23±7.4	22±6.1	23±7.2	24±7.4	0.83
Hearing	19±6.6	20±7.4	18±6.2	18±6.1	19±6.8	0.58
Touch	27±9.1	29±9.2	25±8.5	27±9.2	27±9.6	0.52
Taste and smell	9.5±3.2	10±3.6	9.0±2.5	10±3.9	9±2.7	0.67
Body awareness	20±5.9	21±5.9	19±5.4	21±6.0	21±6.3	0.30
Balance and motion	19±5.3	19±4.6	19±5.5	19±4.6	19±6.7	0.95
Social participation	25±5.8	25±4.5	23±6.0	26±5.6	27±6.7	0.11

Table 1: Cont.

Characteristics *	**Total (*n*=117)**	**VID (*n*=31)**	**OM (*n*=29)**	**VIDOM (*n*=28)**	**Placebo (*n*=29)**	**P value†**
Biochemical markers						
Serum 25(OH)D, nmol/L (mean±SD)	59±26	63±27	60±25	56±26	56±27	0.65
Omega-3 index, % [median (25^{th}, 75^{th} percentiles)]	4.5 (4.3, 4.9)	5.1 (4.1, 5.5)	4.4 (4.2, 5.1)	4.3 (4.2, 5.1)	4.0 (3.7, 4.8)	0.44

VID, vitamin D; OM, omega-3; VIDOM, vitamin D+omega-3, BMI, body mass index (kg/m^2), RRB, Repetitive and Restricted Interests and Behaviour. RBC, red blood fatty acid

† X^2 tests for categorical data and analysis of variance (ANOVA) for continues data (Bonferroni correction was applied for multiple comparisons). * Where n (%) is reported, the percentage within each treatment group is reported. ** A female to male ratio is approximately 1: 6. *** Others include Russia, United Arab Emirates, Japan, Philippines, Singapore, England, United States of America, and Brazil (*n*=1 each) and Australia (*n*=2).

Table 2: Core symptoms of ASD (assessed using Social Responsiveness Scale, SRS) among children who completed the study across treatment groups (n=73)

Outcome variables	Study groups			
	VID (n=19)	**OM (n=23)**	**VIDOM (n=15)**	**Placebo (n=16)**
Total				
Baseline	101±24	99±25	96±29	108±27
Endpoint	90±30	82±29	84±33	102±24
Change	-11±25	-17±18	-13±21	-5.8±12
Difference in change*	-6.3 (-31, 8.2)	-13 (-27, 1.0)	-12 (-28, 4.2)	
P-value†	0.39	0.08	0.14	
Social communicative functioning				
Baseline	82±19	80±20	78±23	88±24
Endpoint	73±24	67±24	62±28	82±19
Change	-8.8±19	-13±16	-16±24	-5.6±10
Difference in change*	-3.9 (-17. 9.1)	-9.3 (-22, 3.0)	-14 (-29, 0.2)	
P-value[1]	0.55	0.14	0.07	
Social awareness				
Baseline	13±3.0	13±3.8	13±4.9	13±4.9
Endpoint	13±3.2	12±4.1	11±5.1	14±4.2
Change	-0.2±3.2	-1.4±2.3	-1.7±3.5	0.4±2.9
Difference in change*	-0.8 (-2.9, 1.3)	-2.4 (-4.4, -0.4)	-2.9 (-5.3, -0.6)	
P-value†	0.44	0.03	0.03	
Social cognition				
Baseline	20±5.4	20±5.5	20±6.6	22.±5.1
Endpoint	18±7.2	19±13	17±7.0	20±5.5
Change	-1.7±4.5	-1.0±11	-2.3±4.1	-2.3±2.8
Difference in change*	0.6 (-4.6, 5.8)	1.2 (-3.8, 6.2)	0.3 (-5.5, 6.2)	
P-value†	0.80	0.63	0.91	
Communication				
Baseline	34±9.3	32±9.4	33±11.7	37±11
Endpoint	29±11	26±11	28±12	34±9.9
Change[1]	-4.6±9.9	-5.2±8.0	-5.3±9.8	-2.4±7.1
Difference in change*	-3.3 (-9.5, 2.9)	-4.2 (-10, 1.7)	-6.0 (-13, 1.2)	
P-value†	0.29	0.16	0.11	
Social motivation;				
Baseline	15±5.5	15±5.4	13±5.3	16±6.2
Endpoint	13±5.5	11±5.3	11±5.6	14.3±4.0
Change	-2.2±4.8	-3.9±4.2	-2.0±4.5	-1.2±4.1
Difference in change*	-0.8 (-4.1, 2.5)	-2.6 (-5.7, 0.5)	-1.0 (-4.7, 2.6)	
P-value†	0.63	0.10	0.58	
Repetitive and restricted interest and behaviour				
Baseline	21±6.3	19±6.4	18±5.6	22±5.7
Endpoint	17±7.2	16±7.5	17±7.0	20±5.5
Change	-3.5±5.2	-3.2±4.2	-1.4±4.9	-1.8±5.4
Difference in change*	-1.9 (-5.4, 1.6)	-1.8 (-5.1, 1.6)	-0.4 (-4.3, 3.6)	
P-value†	0.29	0.16	0.86	

VID, vitamin D; OM, omega-3; VIDOM, vitamin D+omega-3; SRS, Social Responsiveness Scale; CI, confidence intervals.

Values are reported as mean±SD, unless otherwise stated.

*Adjusted difference (95% confidence intervals) in means (active treatments vs. placebo). The analyses were adjusted for therapy, medication and compliance over the study period.

†Pair-wise mixed effects longitudinal models.

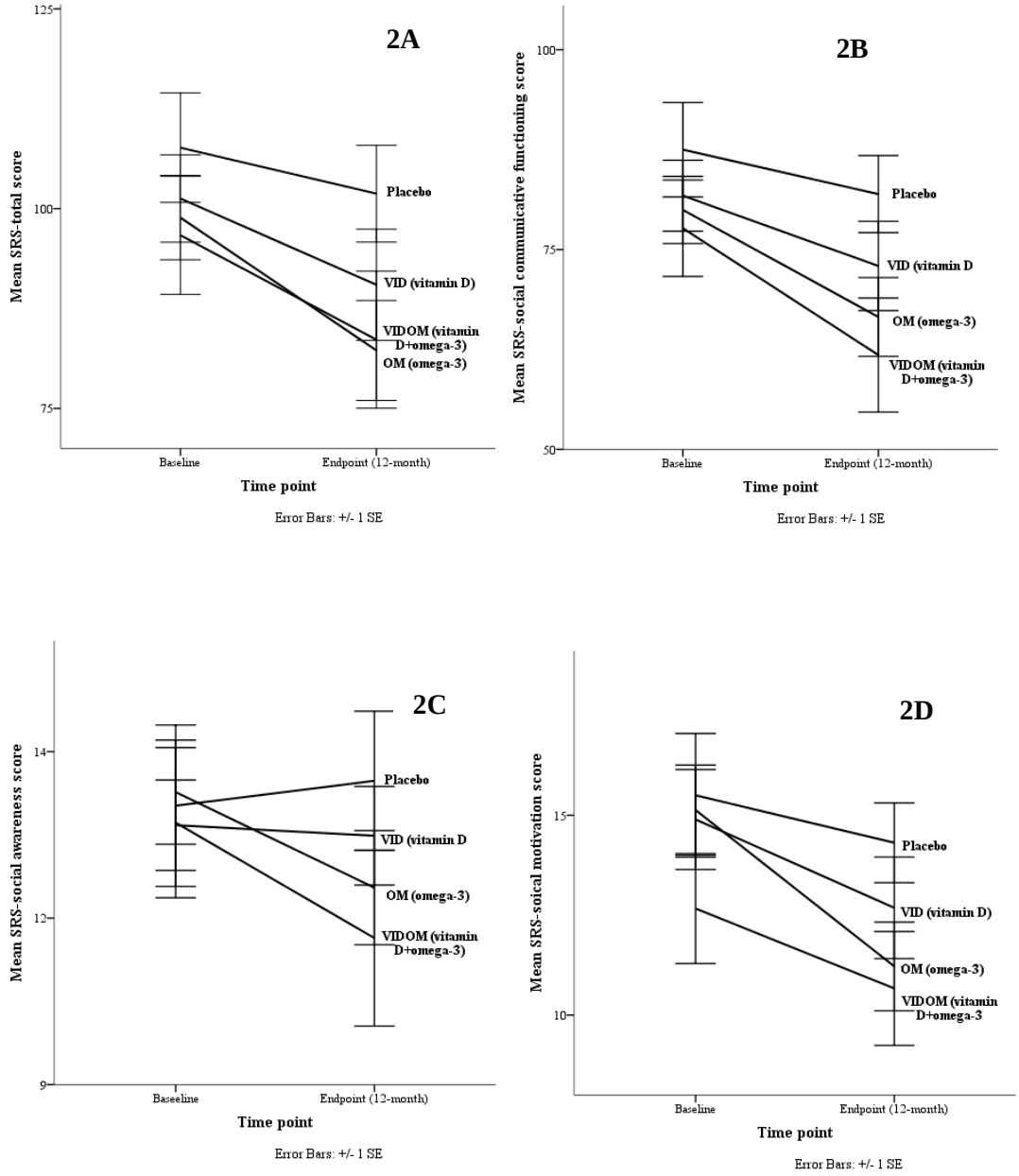

Figure 2: Graphical presentation of the pattern of change in SRS-total score **(2A),** SRS-social communicative functioning **(2B),** SRS-social awareness **(2C),** and SRS-social motivation **(2D)** over the study period (baseline and endpoint) within each of the treatment groups. **(2A)** Significant effect of time point, F (1, 73) = 14, P<0.001 (unadjusted), and a trend for a greater improvement with omega-3 (OM) than placebo, F (1, 69) = 3.1, P=0.08 (adjusted for therapy, medication and compliance). **(2B)** Significant effect of time point, F (1, 73) = 16, P<0.001 and a trend for a greater improvement with vitamin D+omega-3 (VIDOM), F (1, 69) = 3.4, P=0.07 (adjusted for covariates) than placebo. (**2C)** Significant effect of time point, F (1, 73) = 8.0, P<0.01, and a greater improvement with OM, F (1, 69) = 5.0, P=0.03 (adjusted for covariates)] and VIDOM, F (1, 69) = 5.0, P=0.03 (adjusted for covariates) than placebo. (2D) Significant effect of time point, F (1, 69) = 2.6, P<0.01, and a trend for a greater improvement with OM, F (1, 69) = 2.6, P=0.1 (adjusted for covariates) than placebo.

Table 3: Sensory profile and social participation (assessed using Sensory Processing Measure, SPM) in children with ASD across treatment groups and different time points (baseline and endpoint) and the change over time

Outcome variables	Study groups			
	VID (n=19)	**OM (n=23)**	**VIDOM (n=15)**	**Placebo (n=16)**
Total				
Baseline	116±31	112±27	109±21	112±26
Endpoint	109±29	98±29	100±26	104±27
Change	-7.5±23	-14±20	-8.9±12	-8.9±18
Difference in change*	1.7 (-13, 16)	-5.1 (-19, 8.4)	1.0 (-15, 17)	
P-value†	0.81	0.45	0.90	
Vision				
Baseline	22±7.6	23±6.6	20±5.0	22±7.0
Endpoint	20±6.4	20±6.8	19±6.0	19±5.9
Change	-1.8±5.5	-3.0±4.6	-0.7±2.3	-2.6±5.3
Difference in change*	1.5 (-1.8, 4.8)	-0.5 (-3.6, 2.7)	2.2 (-1.5, 6.0)	
P-value†	0.37	0.78	0.24	
Hearing				
Baseline	19±7.4	17±6.2	16±4.1	18±6.5
Endpoint	18±7.1	16±6.1	15±5.5	17±6.9
Change	-1.7±6.1	-0.7±4.9	-0.8±3.6	-1.8±3.9
Difference in change*	-0.2 (-3.7, 3.3)	1.0 (-2.3, 4.3)	0.8 (-3.1, 4.7)	
P-value†	0.92	0.54	0.68	
Touch				
Baseline	26±9.3	26±8.8	25±7.8	25±9.8
Endpoint	25±8.6	21±7.6	22±7.5	22±7.9
Change	-1.1±5.7	-5.1±6.7	-3.3±6.5	-3.0±8.5
Difference in change*	2.6 (-2.4, 7.6)	1.5 (-6.3, 3.2)	1.3 (-4.3, 6.9)	
P-value†	0.30	0.52	0.65	
Taste and smell				
Baseline	8.9±2.9	9.5±2.5	10±4.4	8.6±2.3
Endpoint	7.9±3.9	8.4±3.4	7.7±3.9	8.3±2.1
Change	-1.0±3.3	-1.0±3.2	-2.5±4.3	-0.3±1.7
Difference in change*	-0.8 (-3.1, 1.5)	-0.9 (-3.2, 1.3)	-2.3 (-4.7, 0.1)	
P-value†	0.49	0.39	0.06	
Body awareness				
Baseline	20±6.4	18±5.6	20±6.1	20±6.2
Endpoint	19±5.7	17±4.9	18±5.3	19±5.6
Change	-1.1±5.5	-1.6±5.1	-2.1±5.0	-1.1±5.8
Difference in change[1]	-0.3 (-4.2, 3.7)	-0.5 (-4.3, 3.3)	-1.4 (-5.8, 3.1)	
P-value†	0.34	0.78	0.54	
Balance and motion				
Baseline	20±5.3	19±6.9	18±4.2	19±5.7
Endpoint	19±5.0	17±5.5	18±4.7	19±5.0
Change	-0.8±4.0	-2.6±4.3	0.6±3.9	-0.1±4.7
Difference in change*	-1.2 (-4.3, 2.0)	-2.8 (-6.0, 0.4)	-0.3 (-3.6, 3.5)	
P-value†	0.46	0.09	0.99	
Social participation				
Baseline	24±4.3	23±6.4	24±5.3	26±6.4
Endpoint	22±5.4	22±6.0	22±7.0	26±5.9
Change	-2.3±3.7	-0.6±4.9	-2.2±4.1	0.6±5.1
Difference in change*	-3.7 (-6.8, 0.1)	-2.0 (-4.9, 1.0)	-3.7 (-8.0, 0.6)	
P-value†	0.06	0.19	0.08	

SPM, Sensory Processing Measure; VID, vitamin D; OM, omega-3; VIDOM, vitamin D+omega-3
Values are reported as mean±SD, unless otherwise stated.
*Adjusted difference (95% confidence intervals) in means (active treatments vs. placebo). The analyses were adjusted for therapy, medication and compliance over the study period.
† Pair-wise mixed effects longitudinal models.

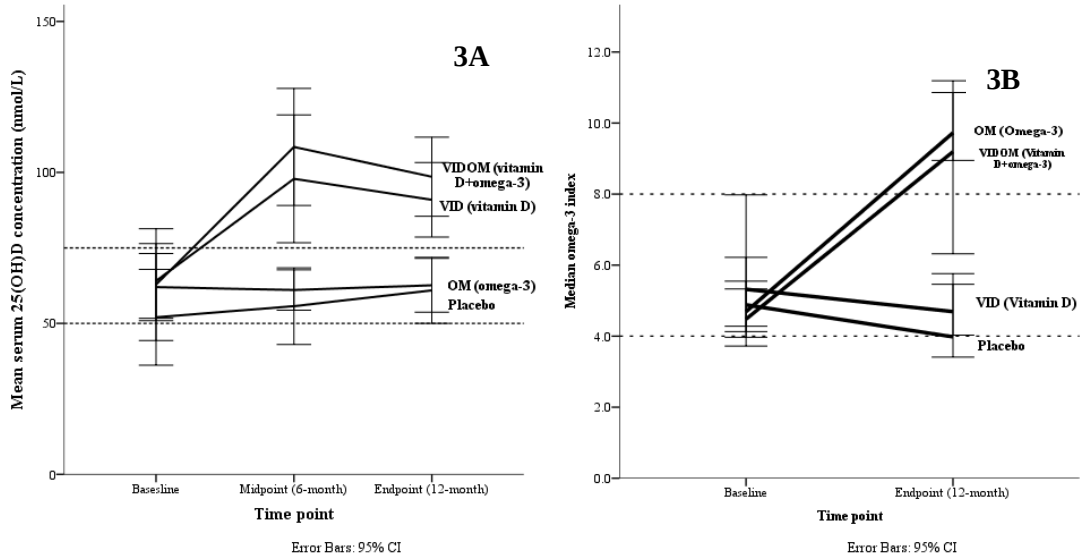

Figure 3: Graphical presentation of the pattern of change in serum 25(OH)D concentration (**3A**) and omega-3 index **(3B**) over the study period (baseline, six months, and endpoint) for serum 25(OH)D concentration and baseline and endpoint for omega-3 index) within each of the treatment groups. **(3A)** Reference lines at 50 and 75 nmol/l (dotted lines) were added for clarification. Significant effect of time of the follow-up, F (2, 42) = 27, P<0.001, effect size = 0.6 large effect size, and the interaction between time point and treatment groups, F (6, 82) = 4.4, P=0.001 [from 64±25, 63±28, 62±24 and 52±28 nmol/L at baseline to 98±38, 99±17, 63±20 and 61±19 nmol/L at endpoint in the VID (n=14), VIDOM (n=9), OM (n=20), and placebo (n=14) groups, respectively]. **(3B)** Reference lines at 4% and 8% (dotted lines) were added for clarification. Significant effect of time of the follow-up, F (1, 62) = 62, P<0.0001, and the interaction between time point and treatment groups, F (3, 74) = 13, P<0.0001 [from 4.7 (4.4, 5.6), 4.5 (4.0, 6.2), 5.3 (4.7, 6.3) and 4.8 (3.7, 8.0) at baseline to 9.6 (9.0, 11), 9.2 (7.3, 10), 4.7 (4.4, 5.5) and 4.0 (3.4, 5.8) at endpoint in OM (n=19), VIDOM (n=8), VID (n=13) and placebo (n=13) groups, respectively].

Discussion

This work suggests the possible efficacy of omega-3 LCPUFA alone or in combination with vitamin D in managing some core symptoms of ASD, including social-communicative functioning and some domains of sensory issues. However, treatment with vitamin D alone did not lead to a statistically significant improvement in core symptoms of ASD. Although not a specific goal of this study, vitamin D was associated with a significantly greater improvement in social participation measured by SPM. Furthermore, both omega-3 index and vitamin D status significantly increased in response to omega-3 LCPUFA and vitamin D supplementation, a finding suggestive of a good compliance.

Evidence suggests that supplementation with omega-3 LCPUFA can positively affect neurodevelopment, modulate inflammatory responses, promote homeostasis, and have a positive effect on behaviours associated with mental disorders including ASD in animal models [59]. The findings of the present trial is in accordance with one previous report where omega-3 LCPUFA might be associated with a greater improvement in social interaction and

communication domains [32] but was associated with no change in sensory profile [34,60]. However, the findings are inconsistent with those of other trials, where no beneficial effect of omega-3 LCPUFA on symptoms of ASD was reported [30, 31, 33-35].

The inconsistent findings of omega-3 LCPUFA ASD RCTs and those of the present trial could be attributed to the study duration (12 months *vs.* 16-24 weeks), dose of omega-3 LCPUFA (722 mg/day *vs.* 200-700 mg/day, with some previous reports using a combination of EPA and DHA [30,34]), and outcome measures used (each having unique psychometric properties). Evidence suggests that PUFA erythrocyte membrane reaches a steady state after 6 months [61] and longer study periods of one year might be needed to demonstrate behavioural changes in response to omega-3 LCPUFA supplementation [62]. It is also well documented that erythrocyte omega-3 index increases in a dose-dependent manner in response to DHA and EPA supplementation [63], with DHA having a greater impact [64].

Many repetitive behaviours seen in populations with ASD are related to seeking or avoiding sensory inputs and because of that sensory issues are considered as a distinct criterion under RRB domain in ASD diagnosis [6]. Sensory issues and sensory overload have been shown to be affected by omega-3 LCPUFA treatment in animal models [65, 66]. In line with Boone *et al.* (2017) [60], the present trial found a trend for a greater improvement in two behaviours associated with sensory issues with omega-3 LCPUFA (alone or in combination with vitamin D) in children with ASD. However, Mankad *et al.* (2015) failed to confirm those findings [34].

Vitamin D has been recently proposed to be an important component of ASD aetiology and symptoms management [7]. However, only a few intervention trials have examined its efficacy in ASD, and the results from these trials are inconclusive. The findings of the current trial support those of one previous randomised controlled placebo trial where vitamin D was found to be ineffective in improving core symptoms of ASD [23], though are inconsistent with the findings of others [4]. Compared with the latter trial [4], the present and Kerley's *et al.*'s trials (2017) [23] used lower doses of vitamin D (2000 IU/day *vs.* 300 IU/kg/day not to exceed 5,000 IU/day), included both vitamin D deficient and sufficient children (*vs.* vitamin D sufficient children), had larger standard deviations for SRS-total (25-27 units *vs.* <5 units), and had smaller sample sizes (<20 *vs.* 55 children in vitamin D group analysed). Serum 25(OH)D has been shown to increase in a dose-dependent manner in response to vitamin D supplementation, with higher doses resulting in higher long-run concentrations in adults and children [67,68], which is consistent with well-being in adults and better neurodevelopmental outcomes in children [69,70]. Although the daily dose of 2000 IU for 12 months was adequate

to increase serum 25(OH)D concentration to levels at or above 75 nmol/L (that are considered sufficient) in most children, higher serum 25(OH)D concentrations and accordingly higher doses might be required to see beneficial effect on behavioural symptoms in children with ASD (Saad et al. 2018). Also, baseline vitamin D status is a significant predictor of circulating 25(OH)D in response to vitamin D supplementation [71], and may modify the relationship between vitamin D supplementation and health outcomes. Furthermore, the small SD observed in Saad *et al.*'s trial [4] indicates there is a small amount of variance in ASD symptomatology and the study population is more homogenous (and likely to respond similarly to an intervention) than those of the present and Kerley *et al.*'s trials [23]. Finally, inconsistent with Kerley *et al.* and Saad *et al.*, mean serum 25(OH)D concentration in the current trial increased in some children on placebo (n=8; +27±12 nmol/L). This increase might have masked the difference in outcome measures between vitamin D and placebo.

There are speculations that vitamin D and omega-3 LCPUFAs may improve ASD symptoms because of their shared and complementary nutrient functions [8]. However, no ASD intervention trials have examined the effect of both treatments and therefore no comparison can be made. The present trial found a non-significant positive effect of combined treatment on composite score of social interaction and communication, which was mainly attributed to the improvement in social awareness domain. Vitamin D and omega-3 LCPUFA supplementation was not associated with any serious adverse events in this study, and others have demonstrated safety with larger doses of these supplements than those used here [4,72].

This is the first randomised controlled trial to our knowledge to study the efficacy of vitamin D, omega-3 LCPUFA, or both in children with confirmed clinical diagnosis of ASD. Previous studies have evaluated either vitamin D or omega-3 LCPUFA alone and no studies considered the efficacy of both on ASD symptoms. Moreover, major nutritional deficiencies were addressed prior to the trial entry to limit their potential confounding effects on outcome measure parameters. Also, the four treatment groups were well matched at baseline with no significant differences in demographics, behavioural characteristics and blood biomarkers of vitamin D and omega-3 LCPUFA. Furthermore, the study has the longest study duration (12 months), allowing us to examine effects on functional outcomes and accounting for seasonal variation. The good compliance rate as confirmed by increases in biomarkers is another strength of the present trial. Finally, children had low initial omega-3 index, and possibly a greater room for improvement.

This study is limited by a smaller sample than was originally proposed and the relatively high attrition rate. Although the study was designed to enrol more children and despite an intensive

approximately 25-month recruitment, the present trial was only able to enrol 117 children (28-31 children per group) and retain 15-23 children per group at endpoint (an attrition rate of approximately 38%). Researchers often find recruitment and retention of participants in studies involving children and adolescents, particularly those with chronic medical conditions and neuropsychological disorders, challenging [73, 74]. A recent report shows the attrition rate in paediatric intervention trials ranges between 0 and 54% [73], and the presence of psycho-behavioural concerns is associated with the greater drop-out rate [75]. Similarly, the average attrition rate in omega-3 LCPUFA paediatric intervention trials has been reported to vary between 0 and 58% (35% in Australia) [74], with studies involving participants with health conditions having higher attrition rate.

High attrition rate may compromise the internal, external, or statistical validity of randomised controlled trials [76]. Although the high attrition rate in the current study did not alter the random composition of groups and their equivalence in relation to demographic and behavioural characteristic, the possibility remains that some children might drop out due to the worse variation in their symptoms over time (natural variability). Furthermore, the external validity of the current study might be compromised because a larger proportion of non-completers were of Pacific ethnicity or were more likely to have severe autism and lower omega-3 index than completers ($P<0.001$). Finally, statistical validity of the current study might have been compromised due to the reduced sample size and power. However, despite the small sample size, the present trial found a positive effect of omega-3 LCPUFA on ASD core symptoms. It is important to note that some patients do not adhere to medications/dietary supplements in the real life usual care, despite health professionals' advice.

Conclusions

Our findings suggest the possible efficacy of supplementary omega-3 LCPUFA alone or in combination with vitamin D in the management of core symptoms of ASD in children. However, large attrition rates and resultant loss of statistical power preclude any definitive conclusion. Also, the interpretation of findings regarding vitamin D alone is difficult. Taking the adequate length and good compliance rate of the present trial into consideration, and in the light of well-documented, biological values of both nutrients and the extent of deficiencies in these nutrients in children with ASD, large and preferably more ASD friendly trials (e.g. using less invasive approaches figure prick for blood testing) with both vitamin D (but larger doses and in children who have lower vitamin D status) and omega-3 LCPUFA are warranted.

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DRC 16

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hajar Mazahery

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Name of Published Research Output and full reference:

Mazahery, H., C. Conlon, K.L. Beck, O. Mugridge, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., B.J. Meyer, B. Tsang, B. Jones, and P.R. von Hurst, A randomised controlled trial of vitamin D and omega-3 long chain polyunsaturated fatty acids in the treatment of core symptoms of Autism Spectrum Disorder in children (Submitted to Journal of Autism and Developmental Disorders)]

In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Responsible for all aspects of the manuscripts including: conceptualisation and design of manuscript, searching the literature, data extraction, data analysis, drafting manuscript, and manuscript submission

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Chapter 6: A Randomised Controlled Trial of Vitamin D and Omega-3 Long Chain Polyunsaturated Fatty Acids in the Treatment of Irritability and Hyperactivity among Children with Autism Spectrum Disorder (Paper VI)

Irritability and hyperactivity are common in children with ASD and co-occurrence of problem behaviours and ASD is associated with more adverse outcomes. This chapter presents the secondary findings of the trial, the efficacy of vitamin D, omega-3 LCPUFA, or both on irritability and hyperactivity.

This report is presented in manuscript format and has been published in "Journal of Steroid Biochemistry and Molecular Biology".

Mazahery, H., C. Conlon, K.L. Beck, O. Mugridge, M.C. Kruger, W. Stonehouse, C.A. Camargo, Jr., B.J. Meyer, B. Jones, and P.R. von Hurst, A randomised controlled trial of vitamin D and omega-3 long chain polyunsaturated fatty acids in the treatment of irritability and hyperactivity among children with Autism Spectrum Disorder. J. Steroid Biochem. Mol. Biol., 2018 (online)

Abstract

Irritability and hyperactivity are common in children with Autism Spectrum Disorder (ASD). Because pharmacological treatments may have adverse effects, and despite limited evidence, caregivers/parents often use dietary supplements such as vitamin D and omega-3 fatty acids to address these behavioural symptoms. As a secondary objective of the VIDOMA (Vitamin D and Omega-3 in ASD) trial, we evaluated the efficacy of vitamin D, omega-3 long chain polyunsaturated fatty acid [omega-3 LCPUFA; docosahexaenoic acid (DHA)], or both on irritability and hyperactivity. New Zealand children with ASD (aged 2.5-8.0 years) participated in a 12-month randomised, double-blind, placebo-controlled trial of vitamin D (2000 IU/day, VID), omega-3 LCPUFA (722 mg/day DHA, OM), or both (2000 IU/day vitamin D+722 mg/day DHA, VIDOM). The primary outcomes were the Aberrant Behaviour Checklist (ABC) domains of irritability and hyperactivity. Biomarkers (serum 25-hydroxyvitamin D [25(OH)D] and omega-3 index) and primary outcomes were measured at baseline and 12-months. Out of 111 children who completed baseline data collection, 66% completed the study (VID=19, OM=23, VIDOM=15, placebo=16). After 12 months, children receiving OM (-5.0±5.0, P=0.001) and VID (-4.0±4.9, P=0.01) had greater reduction in irritability than placebo (0.8±6.1). Compared to placebo, children on VID also had greater reduction in hyperactivity (-5.2±6.3 *vs.* -0.8±5.6, P=0.047). A greater decrease in symptoms of irritability was seen in children with endpoint omega-3 index >4% (n=41) as compared with ≤4% (n=13) (-3.8±5.6 *vs.* 0.3±6.5, P=0.03), and of hyperactivity in those with endpoint serum-25(OH)D ≥75 nmol/L (n=29) as compared with <75 nmol/L (n=29) (-5.2±6.6 *vs.* -1.0±6.4, P=0.02). The results indicate that vitamin D and omega-3 LCPUFA reduced irritability symptoms in children with ASD. Vitamin D also reduced hyperactivity symptoms in these children.

Introduction

The core symptoms of Autism Spectrum Disorder (ASD) include impaired social and communicative functioning and repetitive/stereotypic interests and behaviours. In addition to these, children with ASD may often have problem behaviours, such as irritability (tantrums, aggression and self-injuries) and/or other neurodevelopmental disorders, such as attention deficit hyperactivity disorder (ADHD) [1,2]. Approximately 20% of people with ASD exhibit irritability and aggression at moderate to severe levels [2], and 67% have clinical comorbidity with ADHD [1]. Evidence suggests that co-occurrence of problem behaviours and ASD is associated with a lower quality of life, poorer adaptive functioning, higher psychiatric medication use, and lower responsiveness to standard treatments as compared to children with one disorder only [3-6].

There are several pharmacological agents (including atypical antipsychotics, antidepressants, oxytocin and modulators of glutamate) for the treatment of ASD symptoms, mostly targeted at problem behaviours [7]. Although the effect of pharmacological agents on core symptoms has been investigated and their efficacy in the management of some core symptoms has been shown, due to very limited evidence their routine use is not recommended [7]. Atypical antipsychotics, risperidone (age ≥ 5 years and ≥ 20 pounds) and aripiprazole (age ≥6 years), are the only FDA-approved medications for ASD which are used for the treatment of problem behaviours [7]. Although they are considered effective, they do not work in all patients. Moreover, their efficacy before the age of five has not been investigated and their use after the age of five are associated with both short- and long-term adverse effects [8-10]. Accordingly, they are recommended only when other treatment approaches have failed [7].

However, molecular targets of these pharmacological agents can be used in the search for safer alternative treatments that can be also used before the age of five years. Interventions that begin during this period may have dramatic impact because ASD symptoms start emerging and brain plasticity is at its peak during this period of life [11]. Neurotransmission (including serotonergic, dopaminergic and oxytocinergic systems) and glutamatergic systems are the molecular targets of these pharmacological agents [12-14]. On the other hand, multiple lines of evidence suggest that vitamin D and omega-3 long chain polyunsaturated fatty acid (omega-3 LCPUFA) have important roles in neurotransmission and glutamatergic systems [15-24].

A few randomised controlled trials have investigated the effect of vitamin D and omega-3 LCPUFA, each individually, on core symptoms and problem behaviours in children with ASD, however, the findings are mixed [25-27]. In view of limited and inconclusive evidence and high use of vitamin D and omega-3 LCPUFA/fish oil supplements among populations with ASD [28, 29], we tested the efficacy of vitamin D and omega-3 for treatment of core symptoms of ASD in children and found inconclusive results (VIDOMA trial, vitamin D and omega-3 in ASD; under review). As the secondary objective, we tested the hypothesis that dietary vitamin D, omega-3 LCPUFA or both together are effective in reducing symptoms of irritability and hyperactivity in children with ASD, using a randomised, double-blind, placebo-controlled design. We also investigated the impact of changes in biomarkers of vitamin D (serum 25(OH)D) or omega-3 LCPUFA (omega-3 index) on treatment response.

Material and Methods

The study design and data collection methods for this study are described briefly below, with further details reported elsewhere [30].

Study Participants

New Zealand children were included if they were between 2.5 and 8.0 years, had a medical diagnosis of ASD confirmed by a developmental paediatrician in accordance with the criteria listed in the *Diagnostic and Statistical Manual of Mental Disorders, version five* (DSM-5) [31], and onset of symptoms after 18 months of age. The lower limit of 2.5 years has been chosen based on the age criteria of the psychological assessment tools, and the upper limit of 8 years has been chosen to avoid the confounding effect of behavioural changes associated with pubertal stage. Children were excluded if they were diagnosed as having developmental delay since birth. Children were also excluded if they failed to take corrective action for nutritional deficiencies identified at recruitment. Additional exclusion criteria were serum 25(OH)D ≥75 + 10 nmol/L (≥85 nmol/L) if children entered the trial in winter and ≥105 nmol/L + 10 nmol/L (≥115 nmol/L) if children entered the trial in summer. According to the Endocrine Society's recommendation, concentrations of 75 nmol/L or more are proposed for multiple clinical outcomes [32]. Two different cut-off points for exclusion were applied due to a large seasonal variation in serum 25(OH)D concentrations in New Zealand [33], and 10 nmol/L variation was chosen because of the potential assay variability [34].

Study Design and Data Collection

A 12-month randomised double-blind, placebo-controlled study design was used (**Figure 1**). Children who met the initial inclusion criteria had a blood draw and were screened for nutritional deficiencies (including vitamin D, iron and vitamin B_{12} deficiencies). Prior to randomisation, those deficiencies were addressed (refer to reference [30] for the list of deficiencies and the strategies used to address them). Children were then randomly assigned to one of four treatment groups, each consuming four capsules per day for 12 months; vitamin D_3 (2000 IU/day), omega-3 LCPUFA (722 mg docosahexaenoic acid (DHA)/day), both, or placebo. The dosing regimens used in the current trial are based on their safety, adequacy and practicality [30]. The treatment materials were delivered in 750 mg gel capsules with a tear-off nozzle manufactured and supplied by Douglas Nutrition Ltd (Auckland, NZ). All study capsules were identical in appearance and were tasteless and colourless. A third party not involved in any aspects of the study was responsible for generating the randomisation sequence using the Website Randomisation.com (http://www.randomization.com/) and random block design in blocks of 4 and 8. Randomisation was stratified by age (2.5-5.0 and

5.0-8.0 years old age groups) and severity of ASD (mild, moderate, severe). Researchers, children, and caregivers were blinded to treatment allocations until after data analysis.

Data were collected during participants' visit to the Massey University Human Nutrition Research Unit (HNRU), NZ at baseline and after 12 months (**Figure 1**). The Aberrant Behaviour Checklist (ABC), a standardised psychological test was used to assess irritability and hyperactivity [35].

The ABC is a symptom checklist that measures psychiatric symptoms and problem behaviours across five domains of irritability/agitation, lethargy/social withdrawal, stereotypic behaviour, hyperactivity/noncompliance and inappropriate speech. Due to high prevalence of irritability and hyperactivity in children with ASD [1, 2] these domains were the pre-specified outcomes of the present trial and other domains were the exploratory outcomes. Having irritability symptoms at high levels is defined as scores above 17 units (out of 45 units) [36], and having hyperactivity symptoms at levels above 20 units (out of 48 units) [37].

The validity and reliability of the ABC has been demonstrated by the initial examination of its clinical utility [35]. The ABC has been also been shown to have high internal consistency, excellent test-retest reliability, moderate correlation with adaptive behaviour and robust factor structure [38, 39], and has been validated in children with ASD [38] and commonly used in ASD intervention trials [40, 41].

Caregivers also completed weekly online surveys to collect information regarding adverse events, supplement and medication use, behavioural therapies and compliance. Compliance was calculated using cumulative pill counts at the end of the study, and adherence was measured as a percentage; (number of pills supplied minus number of pills not taken)/number of pills supplied × 100. Compliance was also confirmed by biomarker analysis (serum 25(OH)D and omega-3 index).

The study was registered with the Australian New Zealand Clinical Trial Registry, ACTRN12615000144516. Ethical approval was granted by Health and Disability Ethics Committees, NZ, Reference NO. 14/NTA/113.

Biochemical Analysis

Nutritional biomarkers were assayed from non-fasted venous blood samples at baseline and one year (end of trial). These biomarkers included full blood count, erythrocyte fatty acids, and plasma (or serum) was measured for 25(OH)D, calcium, albumin, iron studies (iron, iron binding capacity, ferritin, and transferrin saturation), vitamin B_{12} and folate. Serum 25(OH)D was analysed using ADVIA Centaur Vitamin D total assay which is standardised using internal standards (traceable to LC/MS/MS). However, the assay was not certified by the Vitamin D External Quality Assurance Standards (DEQAS). With the exception of erythrocyte fatty acids, all other biomarkers were analysed at North Shore Hospital (IANZ accredited). Erythrocyte fatty acids were analysed at the University of Wollongong, Australia [30]. Omega-3 index then was defined as the combined percentage of two omega-3 LCPUFA, eicosapentaenoic acid (EPA) and DHA, in the red blood cell membrane [42].

Statistical Analysis

Because this analysis was an a priori secondary objective of the VIDOMA trial [30], sample size calculation was not based on either irritability or hyperactivity. In brief, it was calculated that 42 participants were required for each arm of the trial to demonstrate a clinically significant difference at 80% power and 5% statistical significance. Power calculations were based on a 17-unit difference between supplemented groups and placebo in change from baseline to endpoint on the Social Responsiveness Scale (SRS) total score, on a mean SRS and standard deviation of 105 and 25 units in untreated children with ASD, respectively [30].

Statistical analysis was performed using IBM SPSS version 24.0 (Armonk, NY). A two-sided $P<0.05$ was considered statistically significant. Variables were tested for normality using the Kolmogorov-Smirnov, Shapiro-Wilk tests and normality plots. Non-normally distributed data were transformed into approximate normal distributions by logarithmic transformations. The data were reported as mean±SD for normally distributed data, as median (25^{th}, 75^{th} percentiles) for non-normally distributed data, and as frequencies for categorical data. Baseline between-group differences were examined using analysis of variance (ANOVA) for parametric data, the Kruskal-Wallis test for non-parametric data and X^2-test for categorical data.

The primary analysis, comparing the effects of treatment on irritability and hyperactivity as well as other ABC domains (as an exploratory analysis) over 12-months, was conducted using pair-wise mixed effects longitudinal models. Treatment (VID *vs.* placebo, OM *vs.* placebo, and VIDOM *vs.* placebo) and time (baseline and endpoint) were included as fixed

effects, and participant was included as a random effect to account for the repeated measures within individuals. Thus interactions between treatments and time indicate differences in efficacy. These were tested with and without considering the potential effect of confounders. Potential confounding factors and effect modifiers investigated within the models were compliance, medication use, and therapy. Analysis was conducted on completers for each outcome measure using data from those who completed assessments at both time points (baseline and endpoint).

The relationship between biochemical markers (25(OH)D and omega-3 index) (as continuous variables) and change in outcome measures were assessed using Pearson correlations for normally distributed variables and Spearman correlations for non-normally distributed variables. Also, the difference in change in outcome measures between low and high levels of biomarkers were assessed using the independent samples t-test and Mann Whitney U test for normally and non-normally distributed variables, respectively.

Results

A flow diagram of the study design is presented in **Figure 1**. Out of 117 families who were enrolled and were eligible for trial entry, 111 families completed the baseline assessment of problem behaviours (the baseline characteristics of these children are presented in **Appendix 1**). Out of 111 children, 73 completed the study, with the median follow-up of 54 (53, 55) weeks. The characteristics of these children are presented in **Table 1**. The baseline demographic characteristics did not differ between completers and non-completers, however non-completers had significantly higher scores on irritability (P<0.001) and hyperactivity (P<0.001) as well as other ABC domains and lower omega-3 index than completers (**Appendix 1**).

Out of 73 children, 26 (36%) were characterised as having irritability symptoms at high levels [36] and 33 (45%) having hyperactivity symptoms at high levels [37]. The four groups were similar at baseline with respect to a range of demographic and clinical characteristics, including ASD severity and mean scores on the ABC domains.

Over the one-year study period, 21% (15/73) of parents reported their children taking medications for coexisting problem behaviours (3 children on risperidone, 2 on fluoxetine, 2 on clonidine, and 1 on methylphenidate) and sleep disturbances (13 on melatonin, 13 children). Medication use did not differ significantly across treatment groups.

Serum 25(OH)D concentration (nmol/L) increased by 27±14 in VID and by 36±17 in VIDOM groups (P<0.0001), and omega-3 index by 4.4 (3.3, 5.9) in OM and by 4.0 (2.0, 6.0) in VIDOM groups (P<0.0001), indicating a good compliance rate (confirming the capsule count). However, regarding the vitamin D group, the possibility of cross-contamination or change in sun exposure habits (over the one year study period) remains because serum 25(OH)D concentration increased by 38% (-16, 61) in the placebo group.

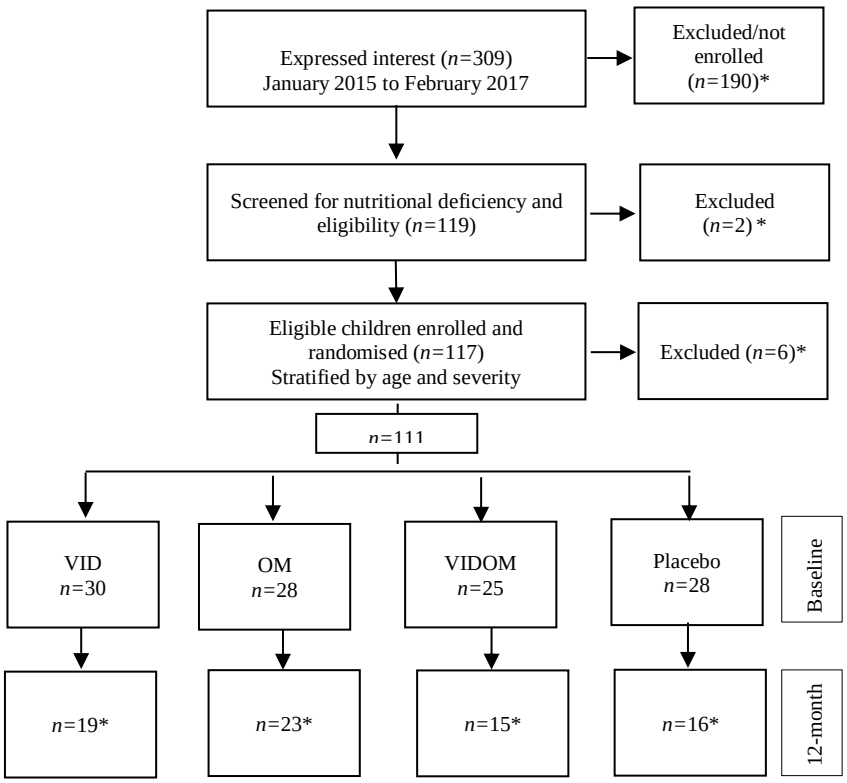


Figure 1: Schematic diagram of study design (2015 – 2017). VID, vitamin D; OM, omega-3; VIDOM, vitamin D+omega-3. * For a list of reasons for not being enrolled, being excluded, or drop out refer to **Appendix 2**.

Table 1: Characteristics of children who completed the study (*n*=73) across treatment groups

Characteristics *	**VID (*n*=19)**	**OM (*n*=23)**	**VIDOM (*n*=15)**	**Placebo (*n*=16)**	***P*-values**
Age, years, mean±SD	5.3±1.5	4.8±1.5	5.4±1.3	5.7±1.0	0.25
Sex, *n* (%)					0.92
Male	16 (84)	18 (78)	13 (87)	13 (81)	
Female	3 (16)	5 (22)	2 (13)	3 (19)	
Ethnicity, *n* (%)					0.28
NZ European	11 (58)	9 (41)	10 (67)	6 (37)	
Others	8 (42)	13 (59)5	5 (43)	12 (63)	
Season of enrolment, n (%)					0.72
Summer	5 (26)	6 (26)	1 (7)	2 (13)	
Autumn	5 (26)	9 (39)	4 (27)	6 (38)	
Winter	5 (26)	5 (22)	6 (40)	6 (38)	
Spring	4 (21)	3 (13)	4 (27)	2 (13)	
BMI–for–age categories, *n* (%)					0.28
< 85th %ile (underweight or normal)	15 (79)	17 (74)	10 (67)	8 (50)	
≥85th %ile (overweight or obese)	4 (22)	6 (26)	5 (33)	8 (50)	
ASD severity (clinical diagnosis), *n* (%)					0.93
Mild	9 (47)	11 (48)	7 (47)	5 (31)	
Moderate	7 (37)	10 (44)	6 (40)	8 (50)	
Severe	3 (16)	2 (9)	2 (13)	3 (19)	
Having symptoms of irritability, *n* (%)					0.88
Scores >17	8 (42)	7 (30)	5 (33)	6 (38)	
Having symptoms of hyperactivity, *n* (%)					0.58
Scores >20	11 (58)	9 (39)	7 (47)	6 (38)	
Time on study, weeks, median (25th, 75th percentiles)	55 (53, 57)	53 (52, 57)	54 (53, 57)	54 (53, 57)	0.59
Compliance, %, median (25th, 75th percentiles)	95 (92, 96)	95 (90, 97)	90 (77, 94)	94 (92, 97)	0.09
Therapy, *n* (%)					0.58
No	9 (48)	13 (55)	6 (36)	10 (60)	
Yes	10 (52)	10 (45)	9 (54)	6 40)	

Table 1: *Cont.*

Characteristics *	**VID (*n*=19)**	**OM (*n*=23)**	**VIDOM (*n*=15)**	**Placebo (*n*=16)**	***P*-values**
Medication use, *n* (%)					0.26
No	14 (74)	19 (83)	10 (67)	15 (94)	
Yes	5 (26)	4 (17)	5 (33)	1 (6)	
Biochemical markers					
Serum 25(OH)D, nmol/L, mean±SD	68±21	63±24	60±24	55±27	0.47
Erythrocyte omega-3 index, %, median (25^{th}, 75^{th} percentiles)	5.3 (4.3, 5.5)	4.7 (4.3, 5.3)	4.5 (4.3, 5.3)	4.9 (3.9, 6.6)	0.77

BMI, body mass index (kg/m^2); OM, omega-3; VID, vitamin D; VIDOM; vitamin D+omega-3

* Where n (%) is reported, the percentage within each treatment group is reported.

The baseline, endpoint (after 12 months) and the changes in ABC-irritability and hyperactivity scores as well as other ABC domains of study populations across treatment groups are presented in **Table 2**. The analysis of scores on irritability and hyperactivity showed a significant effect of time (the difference between baseline and endpoint, $P<0.001$). Compared with placebo, both unadjusted and adjusted analysis of the scores showed a greater reduction with VID ($P=0.007$ unadjusted for covariates and $P=0.01$ adjusted for covariates) and with OM ($P=0.001$ for both adjusted and unadjusted for covariates) and a trend for a greater reduction with VIDOM ($P=0.06$ unadjusted for covariates and $P=0.09$ adjusted for covariates) (**Figure 2A**). The rate of positive response (at least a 25% reduction in ABC-irritability score) was 63% (12/19, $P=0.02$), 74% (17/23, $P=0.003$), and 53% (8/15, $P=0.11$) in VID, OM, and VIDOM groups, respectively, as compared with 25% (4/16) in the placebo group ($P=0.02$) (**Figure 3A).**

Both unadjusted and adjusted analysis of the scores on hyperactivity revealed a greater reduction with VID than placebo ($P=0.04$ unadjusted for covariates and $P=0.047$ adjusted for covariates) (**Figure 2B**). The rate of positive response (at least a 25% reduction in ABC-hyperactivity score) was 68% (13/19, $P=0.03$), 48% (11/23, $P=0.30$), and 47% (7/15, =0.38) in VID, OM, and VIDOM groups, respectively, as compared to 31% (5/16) in the placebo group ($P=0.18$) (**Figure 3B).**

Table 2: Problem behaviours (assessed using Aberrant Behaviour Checklist, ABC) in children with ASD across treatment groups and different time points and the change over time.

ABC-domains	Study groups				*P*-values*
	VID (*n*=19)	**OM (*n*=23)**	**VIDOM (*n*=15)**	**Placebo (*n*=16)**	
Irritability					
Baseline	17±7.8	14±8.0	13±8.2	13±8.7	VID *vs.* placebo (*P*=0.01)
Endpoint	13±7.7	9.1±7.4	11±8.4	14±10	OM *vs.* placebo (*P*=0.001)
Change	-4.0±4.9	-5.0±5.1	-2.7±5.1	0.8±6.1	VIDOM *vs.* placebo (*P*=0.09)
Hyperactivity					
Baseline	22±9.6	17±9.2	21±12	20±11	VID *vs.* placebo (*P*=0.047)
Endpoint	17±11	14±8.8	16±11	19±12	
Change	-5.2±6.3	-3.4±7.3	-4.5±6.3	-0.8±5.6	
Lethargy					
Baseline	9.2±8.2	9.0±8.3	7.7±5.3	12±8.3	OM *vs.* placebo (*P*=0.02)
Endpoint	7.4±8.2	5.7±5.9	6.3±5.3	12±9.0	
Change	-1.8±4.3	-3.4±4.8	-1.4±3.5	0.0±6.4	
Stereotypy					
Baseline	5.1±4.3	3.7±3.19	3.0±2.8	4.8±3.9	All active treatments *vs.* placebo (*P*>0.1)
Endpoint	4.4±5.8	3.1±4.1	2.7±3.1	3.7±2.9	
Change	-0.7±3.3	-0.7±3.3	-0.5±2.2	-1.3±2.4	
Inappropriate speech					
Baseline	4.3±3.2	3.7±2.3	3.2±2.8	3.8±2.9	All active treatments *vs.* placebo (*P*>0.1)
Endpoint	3.5±3.4	2.9±2.2	2.5±2.6	3.9±2.1	
Change	-0.8±1.7	-0.8±1.9	-0.7±2.4	0.1±2.6	

ABC, Aberrant Behaviour Checklist; VID, vitamin D, VID; OM, omega-3; VI OM; vitamin D+omega-3
*Pair-wise mixed effects longitudinal models. The analyses were adjusted for therapy, medication and compliance over the study period.

As an explanatory analysis, ABC-lethargy, -stereotypy and -inappropriate speech domains of study populations were also analysed (**Table 2)**. The analysis of scores revealed an effect of OM on lethargy, with OM group having a greater reduction than placebo (*P*=0.03 unadjusted for covariates and *P*=0.02 adjusted for covariates).

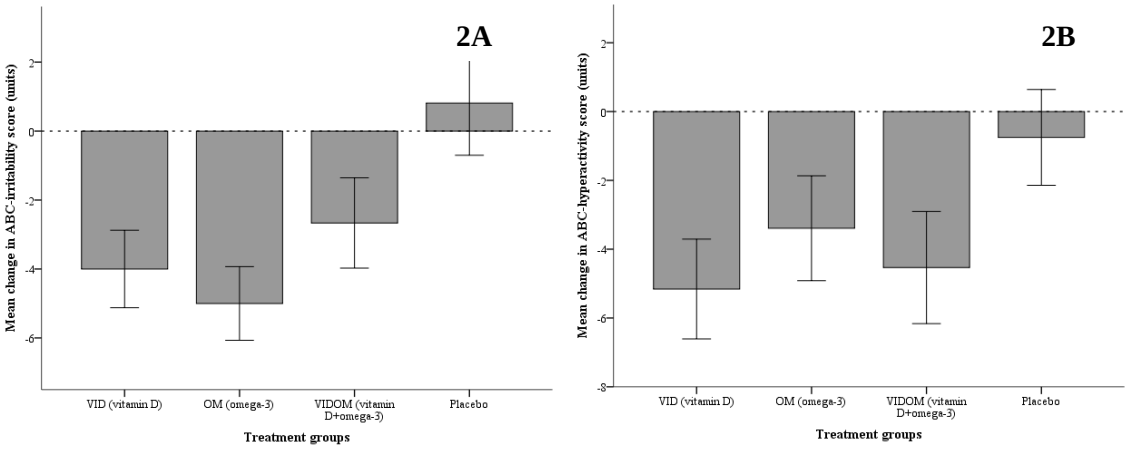
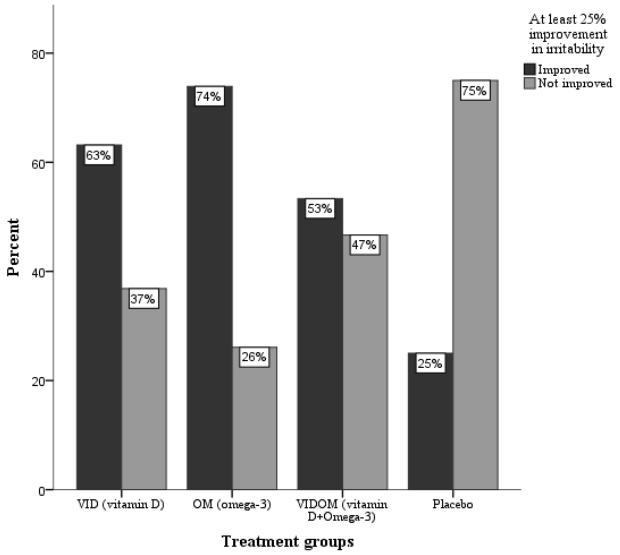
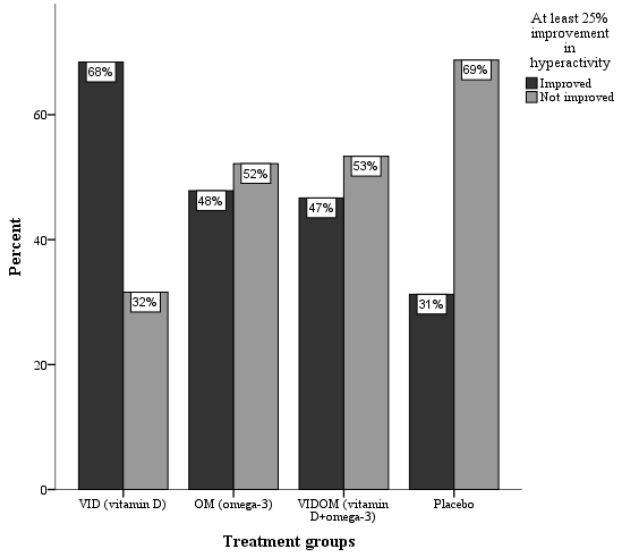


Figure 2: Vitamin D and omega-3 long chain polyunsaturated fatty acids reduce symptoms of irritability and hyperactivity. (2A) Pattern of change (mean ± standard error) in ABC-irritability: greater reduction with VID [*F* (1, 73) = 7.7, *P*=0.007 (unadjusted) and *F* (1, 69) = 6.9, *P*=0.01 (adjusted for covariates)] and OM [*F* (1, 73) = 12, *P*=0.001 (unadjusted), *F* (1, 69) = 13, *P*=0.001 (adjusted for covariates)] and a trend for a greater reduction with VIDOM [*F* (1, 73) = 3.6, *P*=0.06 (unadjusted) and *F* (1, 69) = 3.0, *P*=0.09 (adjusted for covariates)] than placebo. (2B) Pattern of change (mean ± standard error) in ABC-hyperactivity: a greater reduction with VID than placebo, *F* (1, 73) = 4.2, *P*=0.04 (unadjusted for covariates) and *F* (1, 69) = 4.1, *P*=0.047 (adjusted for covariates). VID, vitamin D; OM, omega-3; VIDOM, vitamin D+omega-3; placebo.

As the secondary analysis, no relationship was observed between change in serum 25(OH)D concentration or omega-3 index and change in two outcomes (both *r* values ≤0.1 and *P* values >0.10). However, children with endpoint omega-3 index >4% (*n*=41) had a larger reduction in ABC-irritability score than those with endpoint omega-3 index ≤4% (*n*=13, all from VID and placebo groups), -3.8±5.6 *vs.* 0.3±6.52, *P*=0.03. Serum 25(OH)D concentration at endpoint was correlated with change in ABC-hyperactivity (*r*= -0.3, *P*=0.03). Further analysis showed that children with serum 25(OH)D concentration ≥75 nmol/L (*n*=29) had a greater reduction in ABC-hyperactivity score than those with endpoint serum 25(OH)D of <75 nmol/ L (*n*=29) (-5.2±6.6 *vs.* -1.0±6.4, *P*=0.016).



3A



3B

Figure 3: Vitamin D and omega-3 long chain polyunsaturated fatty acids groups have higher proportion of responders in relation to irritability and hyperactivity. (**3A**) In relation to irritability: VID vs. placebo*:* x^2=5.1, *P*=0.02; OM vs. placebo: x^2=9.1, *P*=0.003; VIDOM vs. placebo: x^2=2.6, *P*=0.11. **(3B**) In relation to hyperactivity: VID vs. placebo: x^2=4.8, *P*=0.03; OM vs. placebo: x^2=1.1, *P*=0.30; VIDOM vs. placebo: x^2=0.8, *P*=0.38. Responders are defined as those who had ≥25% reduction in baseline irritability or hyperactivity scores. VID, vitamin D; OM, omega-3; VIDOM, vitamin D+omega-3; placebo.

Discussion

The present trial provides clear evidence for the efficacy of long-term treatment of irritability with omega-3 LCPUFA and vitamin D supplements in children with ASD. Vitamin D was also efficacious in the treatment of hyperactivity symptoms in these children. Although not a specific goal of this study, omega-3 LCPUFA was associated with a greater reduction in the lethargy subdomain of ABC than placebo.

Regarding vitamin D, the findings of present trial confirm those of Saad *et al.* [26], but are inconsistent with those of Kerley *et al.* [25]. Saad *et al.* (2018) randomly assigned 109 children with ASD to either vitamin D (300 IU/kg/day not exceeding 5000 IU/day) or placebo for four months, and demonstrated a greater reduction in irritability (-14 points) and hyperactivity (-9 points) than those observed in the current study (-4 and -5 points, respectively) [26]. The larger reduction in Saad *et al.*'s study could be due to their use of a larger dose of vitamin D supplement. Despite using the same dose of vitamin D as the present trial and having comparable baseline irritability and hyperactivity scores in their 38 participants, Kerley *et al.* (2017) did not find an effect of vitamin D on irritability and hyperactivity symptoms [25], perhaps due to the shorter trial duration (20 weeks) and lack of accumulative dose over time. Vitamin D supplementation has also been shown to reduce hyperactivity symptoms in populations with ADHD [43, 44], providing further evidence for a role of vitamin D in hyperactivity.

A recent meta-analysis of four omega-3 LCPUFA randomised controlled trials in ASD (total n=107) found no effect of treatment on the symptoms of irritability, but a greater reduction, though not significant, in the symptoms of hyperactivity [27]. The difference between omega-3 LCPUFA and placebo in change from baseline in hyperactivity score of the present trial was comparable to that reported by the meta-analysis (-2.6 and -2.1 points, respectively) [27]. Inconsistent with the findings of this meta-analysis, the present trial found a greater reduction in irritability symptoms with omega-3 LCPUFA than placebo. The longer study duration (one year) in the current trial could explain positive findings. The study length of previous reports included in that meta-analysis ranged from 6 to 26 weeks [36]. The beneficial effect of omega-3 LCPUFA on irritability has been reported in populations with other psychological and behavioural disorders (e.g. patients with bipolar disorder and persistent irritability [45] and patients with recurrent self-harm [46]).

Although taking both supplements (VID and OM) resulted in a seemingly greater reduction in irritability and hyperactivity than placebo**,** the observed differences did not reach statistical significance, probably due to low statistical power (only 15 participants). To our knowledge, no ASD intervention trials with these two nutrients is available and therefore comparisons with other studies is not possible.

Previous reports suggest correlations between changes in serum 25(OH)D or omega-3 index and changes in cognitive and behavioural outcomes in neurodevelopment disorders including ASD and ADHD [40, 47, 48], which the present study could not confirm despite a significant increase in both biomarkers.

The positive findings of the current study are supported by mechanistic evidence. Firstly, vitamin D receptors and 1α-hydroxylase have been identified in different regions of the brain and sensing neurons [49, 50]. The active form of vitamin D has been shown to have an important role in the neuronal differentiation, structure, function and connectivity of the developing brain [51]. Also, omega-3 LCPUFAs, mainly DHA, are necessary for normal development and functioning of the brain [52]. Long-term DHA depletion results in significant losses in brain DHA with consequent loss in brain function [53]. Furthermore, vitamin D and omega-3 LCPUFA have been shown to have important roles in neurotransmission systems which are reported to be disrupted, at least, in a subset of individuals with ASD [27, 54]. Finally, ASD is considered a disease of inflammation and oxidative stress, and there is strong evidence to suggest that vitamin D and omega-3 LCPUFA can exert their roles in ASD through their anti-inflammatory, anti-oxidative, and immunomodulatory properties [27, 54].

To our knowledge, this is the first trial using the gold standard study design (randomised controlled placebo trial) to investigate the efficacy of three interventions; vitamin D, omega-3 LCPUFA, or both in children with ASD. Furthermore, major nutritional deficiencies were addressed prior to trial entry to control for their potential confounding effects. Also, the length of our study (one year) is worth noting; one year was long enough to detect any potential beneficial effect of treatments on behaviours and allowed us to control for seasonal differences in vitamin D status and behaviour. Finally, the good compliance rate in those completing the trial indicate that intervention was well-tolerated and accepted. However, this study is limited by its relatively small sample size and inadequate generalisability. Children who did not complete the trial had higher scores on irritability and hyperactivity and lower omega-3 index and whether the findings are applicable to these children is not clear. It is plausible to suggest that these children would benefit more from the intervention and if they were able to complete the trial it would be possible to observe stronger relationship between treatment and outcomes.

Conclusion

Our results support the use of vitamin D and omega-3 LCPUFA supplements for treating irritability symptoms in children with ASD. Vitamin D also has significant beneficial impact on hyperactivity in these children. Future studies with a larger sample size are warranted to investigate the impact of both nutrients on problem behaviours associated with ASD, to replicate the findings of the current study, and to explore the mechanistic pathways involved.

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Hajar Mazahery

Name/Title of Principal Supervisor: Assoc. Prof. Pamela von Hurst

Name of Published Research Output and full reference:

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In which Chapter is the Published Work: Chapter 6

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Responsible for all aspects of the manuscript including: conceptualisation and design of manuscript, searching the literature, data extraction, data analysis, drafting manuscript, and manuscript submission

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Chapter 7: Discussion and Conclusions

This chapter provides an overview of findings of this study and discusses the implications of those findings and concludes accordingly. The chapter then ends with an outline of directions for future research.

Overview of Main Findings and Discussion

Thirteen studies reporting vitamin D status in ASD cases and typically developing controls (Chapter 2 – Section 2) and fifteen reporting LCPUFA status in ASD cases and typically developing controls (Chapter 2 – Section 3) were identified for inclusion in the vitamin D-ASD review and omega-3 LCPUFA-ASD meta-analysis, respectively. Most included studies in both reviews reported populations with ASD having lower 25(OH)D and omega-3 LCPUFA status than typically developing controls. Since the publication of these reviews, a meta-analysis of case-control studies of vitamin D status (including 11 studies) [1] and a case-control study of omega-3 LCPUFA [2] in ASD children have been published. Wang *et al.* (2016) [1] reached the same conclusion as ours and demonstrated that children with ASD had lower 25(OH)D concentrations than typically developing children. However, Howsmon *et al.* (2018) [2], did not confirm our findings regarding the lower status of omega-3 LCPUFA among populations with ASD than typically developing counterparts. Although cases and controls were matched by age and sex, both children and adolescents were included in the study, and the inclusion of a wide age range may have resulted in a lack of difference across groups.

One RCT of vitamin D and four RCTs of omega-3 LCPUFA were identified for inclusion in the vitamin D-ASD review and omega-3 LCPUFA meta-analysis, respectively (Chapter 2 – Sections 2 and 3). Since the publication of these reviews, two more RCTs of vitamin D [3,4] and none of omega-3 LCPUFA in populations with ASD have been published. The newly published vitamin D RCTs have been discussed in Chapters 5 and 6. The findings of three RCTs of vitamin D in ASD are mixed; while one reported beneficial effect, others failed to replicate such findings. With reference to omega-3 LCPUFA RCTs, although interventions appeared to be beneficial, due to small number of studies included, definitive conclusions and recommendations could not be made. Both nutritional supplements were well-tolerated and accepted. Formulations and doses of vitamin D and omega-3 LCPUFA as well as treatment periods and outcome measures varied across studies.

The investigation of the dietary adequacy and nutritional status of participating children (n=86) at baseline (Chapter 4) suggested that overweight and obesity, the use of dietary supplements, nutritional issues, poor diet quality, and insufficient serum 25(OH)D and omega-3 index were common in this cohort of children. Approximately, half of children were on dietary supplements. On the other hand, half of children or more had consumption of protein, vitamin D, iodine and fibre below the Nutritional Reference Values (NRVs), whilst all or most children exceeded the recommendations for sodium, saturated fats (SFA), and sugar. B vitamins were other nutrients over consumed by these children. Because detailed

information on dietary supplement was not collected and supplement data were not included in the analysis, children in this cohort might have had excessive intake of B vitamins and other nutrients. However, the effect of vitamin and mineral supplementation has previously been examined in children with ASD, revealing that supplementation can lead to excessive intake but does not correct for micronutrient inadequacies (e.g. vitamin D) [5]. The high prevalence of overweight and obesity along with excessive intake of SFA, sugars and salt in this cohort of children is of serious concern. The prevalence of obesity in children with ASD is at least as high if not higher than their typically developing counterparts [6]. It is well established that obesity and a poor diet (including high in fats, sugars and salt) are associated with several metabolic abnormalities (including diabetes and cardiovascular diseases) and increased risk of some cancers (stomach and colorectal) [7]. An early and timely intervention considering challenges these children face for the prevention of obesity and noncommunicable diseases is warranted. Mealtime/feeding issue, selective eating, difficulties with motor skills to participate in physical activity, heterogeneous nature of the condition, insistence on sameness and avoiding new foods/activities are among factors that need to be considered when designing interventions for children with ASD.

One of the most novel aspects of this study was the inclusion of a measure of diet quality (DICE), which was also validated in the same cohort, and subsequently the identification of diet components that require careful attention (e.g. consumption of fruits, vegetables, milk and milk products as well as variety of fruits and vegetables). The poor diet quality and under- or over-consumption of nutrients may indicate sensory problems, unusual eating habits/meal time problems, and/or gastrointestinal issues in these children, so warrants further research to confirm the reasons and perhaps manipulate or treat those behaviours/conditions to promote positive dietary change.

The prevalence of vitamin D deficiency (defined as serum 25(OH)D <50 nmol/L) in our study population (approximately 40%) appeared to be lower than typically developing preschool children (55%) [8]. However, when the season of enrolment was taken into consideration, the prevalence in our study population was higher than New Zealand preschool children, 81% (45 children were enrolled during winter and spring months and 37 children were vitamin D deficient) *vs.* 55%. It is difficult to make comparisons due to different study protocols and assay variations, however the findings can provide further evidence for the lower vitamin D status of populations with ASD as compared with typically developing populations ([1] and Chapter 2 – Sectiton 2). With reference to omega-3 LCPUFA status, the findings of our study cannot be compared with typically developing children in New Zealand due to lack of evidence. However, our study population had a lower mean omega-3 index

than typically developing Australian children (5.0% in all children and 4.7% in the subset of children providing 4DFR *vs.* 6.5% in Australia) [9].

Building on the evidence of inadequate dietary intakes of vitamin D and omega-3 LCPUFA rich foods and subsequently low vitamin D and omega-3 LCPUFA status from the baseline assessment of nutritional status (Chapter 4), the intervention trial (Chapters 5 and 6) highlighted that active treatments were associated with greater improvement in multiple outcomes compared with placebo; omega-3 LCPUFA alone and in combination with vitamin D were effective in improving SRS-social awareness, omega-3 LCPUFA in combination with vitamin D in SRS-social communicative functioning, omega-3 LCPUFA alone in SRS-total, vitamin D and omega-3 LCPUFA, each individually, in ABC-irritability, and vitamin D alone in ABC-hyperactivity. Additionally, the active treatments led to increases in mean serum 25(OH)D concentrations and median omega-3 index, indicating a good compliance with the treatment in these children (Chapters 5 and 6). However, the change in these biomarkers did not correlate with the change in behavioural outcome measures.

In view of strong mechanistic evidence for vitamin D and omega-3 LCPUFA in ASD (Chapter 2 – Sections 2 and 3), it is important to discuss the causes for mixed and inconclusive findings, particularly in relation to ASD core symptoms. Treatment response in ASD clinical trials has been consistently reported to be affected by variances in placebo and active treatment responses. Placebo response has been shown to have a moderate effect and to be predicted by several factors (e.g. adjunctive therapy and outcome-related measures) [10]. King et al (2013), in their multi-site RCT in children with ASD, reported a placebo response effect rate of 22-50% [11]. In the current trial, a decrease of 6-7% in SRS-total and -social communicative functioning with standard deviations of 10 and 12 units, respectively, in the placebo group may explain some of variability in treatment response.

Similarly, the large variability in active treatment response is a challenge in ASD clinical trials and has raised concerns about refuting the efficacy of a treatment solely based on group-level analysis [12]. As previously discussed (Chapter 2 – Section 1), the aetiology and clinical presentation of ASD is heterogeneous and therefore not all children respond comparably to the same treatment (vitamin D, omega-3 LCPUFA or both). Our analyses demonstrated that within the active treatments, 20-22%, 53-74%, and 45-68% of children were positive responders for SRS-total, ABC-irritability and ABC-hyperactivity, respectively. The main question here is that why some children respond to a treatment while others do not.

Baseline severity has been reported to moderate the response to treatment in ASD [13] and ADHD [14] clinical trials. The general pattern is that greater behavioural improvement is reported in those with higher than lower baseline severity [13,14]. The effect of baseline severity is in line with the "Law of Initial Value" hypothesis, stating that a higher chance of response is associated with greater baseline severity [15]. We successfully distributed the severity across treatment groups even after the exclusion of those who did not complete the study. However, it is important to note that only 18% of enrolled children had severe ASD (level 3), of whom more than 50% did not complete the study leaving only 2-3 children with severe ASD within each treatment group. It is plausible to suggest that a clear picture of the efficacy of intervention would be available if more children with severe ASD were enrolled and kept in the trial. One can suggest that families of children on the severe end of spectrum may be less motivated to participate in such trials probably due to loss of hope for any intervention to work or because they may experience more difficulties in caring for a child with severe ASD and have fewer resources (e.g. time) to invest in research projects.

Age is another factor that may explain the variability in treatment response [16]. Evidence suggests that because several regions of the brain are not completely mature until adolescence [17] and the brain plasticity is higher in children [18], interventions given during the sensitive period of life may affect neurodevelopmental trajectories and have profound effects later in life [19-21]. The longitudinal course of ASD is another rationale for considering age in ASD clinical trials. Literature investigating the developmental course of ASD symptomatology report age-related differences or changes in the severity of autism, with a general pattern of improvement reported with aging [22,23]. Age-related differences in body weight/size can also partly explain why age is a contributing factor. Early childhood is associated with rapid growth, and because older children weigh more and have larger body size than younger children the dose of treatment might be too high or too low. A possibility remains that a high dose may result in more adverse effects and consequently lower compliance rate (in younger children or in those with lower bodyweight or smaller body size) or conversely, a low dose may be associated with lack of positive effect and consequently higher withdrawal rate (in older children or in those with higher bodyweight or larger body size). To account for age, we narrowed our age range to 2.5 to 8.0 years (pre-adolescent period) and distributed different age groups equally across treatment groups (randomisation stratified by age). However, it should be noted that we used a "one dose fits all" approach and this might be a drawback of our trial.

Furthermore, our trial demonstrated that more than one third of children were overweight/obese and the doses used might be inadequate for these children. The Clinical

Practice Guidelines by the Endocrine Society recommends participants with obesity be given two to three times more vitamin D to satisfy their body's vitamin D requirement, although there are no studies that clearly justify this [24]. Similarly, omega-3 LCPUFA dose has been recommended to be made on the basis of body weight as individuals with lower body weight tend to have a greater response to a given dose [25].

In addition, concurrent treatment and adherence have been shown to predict treatment response in mental health clinical trials [13,26]. Fraizer et al (2010) demonstrated that children with ASD who received intensive behavioural intervention plus medication had a greater improvement in aggression than those on intensive behavioural intervention alone [26]. Scahill et al (2012) also showed that medication plus parental training regarding children's behaviour had modest additional benefit over medication alone in adaptive functioning in children with Pervasive Developmental Disorder (PDD) and serious problem behaviours [27]. Similar findings have been reported for concurrent treatment with dietary supplement and medication; vitamin D plus fluoxetine and omega-3 LCPUFA plus antidepressants have been shown to be superior over medication only in improving symptoms of depression [28,29]. It is important to note that although these studies did not investigate symptoms of ASD, they provide an indication for a role of concurrent treatment in treatment response. In our trial, we allowed parents/caregivers to have their choice of medication or behavioural therapy because we thought that it was unethical and impractical to prevent parents/caregivers from using other interventions over a period of one year. Given the high rate of behavioural therapy and medication use in our children, we adjusted all primary analyses for medication use and therapy.

"Adherence in a clinical trial is defined as the extent to which the clinical trial participant's behaviour coincides with the trial protocol in terms of keeping clinic appointments, taking medication, and following the diet or executing life-style changes" [30]. As previously discussed (Chapter 5 – Discussion), it was not easy to recruit patients into the trial and to keep families in the trial for one year, with many families withdrawing or being lost to follow up during early stages of trial. To note, we employed different strategies including making monthly phone calls, publishing quarterly newsletters including quizzes for parents/caregivers, and arranging competitions for children and prize giving. These experiences suggest that it is initial motivation that may be crucial for the recruitment and improving retention. The recruitment strategies should perhaps focus on awareness raising activities so that the importance of research in our ability to better understand the disorder and to develop scientific-based interventions is emphasized.

Data collection regarding nutritional status and blood tests (at 6 and 12 months) were other protocols that many families/caregivers did not adhere to, resulting in a limited number of 4DFRs at baseline and 25(OH)D and omega-3 index measurements at the follow up visits. Although only 86 (out of 117) families/caregivers provided 4DFRs, this number was comparable to that reported in other studies (Chapter 4). Food records have consistently been reported to be associated with participant burden and to address this we confirmed the validity of DICE for assessing diet quality for future research. With reference to biomarker measurements at 6 and 12 months, many parents/caregivers were unwilling to have further blood tests due to heightened anxiety levels in children (probably why many ASD clinical intervention trials do not measure blood biomarkers), suggesting that less invasive blood sampling techniques would be required to address this issue.

Adherence to intervention has also been shown to predict the treatment response [13], with better response reported in those with higher versus lower compliance rates. In our trial, the compliance rate was high (as was confirmed by both capsule counts and biomarkers), suggesting that once parents/caregivers of children with ASD got through the initial stages of the trial (recruitment and stage 1 of the intervention), the adherence to intervention, here vitamin D and omega-3 LCPUFA, was less of an issue. Both vitamin D and omega-3 LCPUFA were well tolerated and safe, and this could partly explain why the adherence rate was high in our trial.

Non-adherence, in general, may compromise the internal, external and statistical validity of a study. Fortunately, despite the relatively high attrition rate in the current trial, the internal validity was not affected because the random composition of groups and their equivalence was not altered. However, the external validity of the current study might be compromised because (1) a larger proportion of non-completers were of Pacific ethnicity, (2) non-completers had higher scores on core and co-existing symptoms of ASD (more severe), and (3) non-completers had a lower omega-3 index than completers ($P<0.001$). In view of the importance of baseline severity in treatment response, one can postulate that if these children were kept in the trial, a stronger relationship between active treatments and outcome measures would be found as these children would be more responsive to treatment.

The statistical validity of our trial might be compromised because the number of children completing the trial and providing blood biomarker measurements was well below the calculated initial sample size. We found no relationship between change in biomarkers and change in outcome measures and found many treatment-outcome relationships with *P*-values

ranging between 0.05 and 0.1, indicating that a larger sample size would have probably resulted in more promising and conclusive findings.

Finally, outcome measure is another predictor of treatment response. There is a large variability in the outcome measures used in ASD clinical trials, with approximately 290 unique outcome measures across behavioural, pharmacological and complementary/alternative medicine studies [31]. Masi et al (2017) reported large effect sizes for pharmacological and dietary supplement treatments in ASD trials where the following outcome measures were used: Clinical Global Impression (CGI-total score), Childhood Autism Rating Scale (CARS-total score), and Aberrant Behaviour Checklist (ABC-irritability and ABC-hyperactivity subscales) [16]. This was clearly shown in our trial (Chapters 5 and 6) where we demonstrated that when the behaviour was assessed using SRS, a trend for an effect of omega-3 LCPUFA on social interaction was found, but when ABC-lethargy and SPM-social participation subscales were considered, omega-3 LCPUFA and vitamin D respectively, were found to be the effective treatments (with *P*-values <0.05). This discrepancy could be explained by psychometric properties of the outcome measure [16]. It is also important to note that most ASD outcome measures have mainly been designed and developed for diagnostic purposes, but not for capturing slight changes in behaviour in response to treatment. Of the outcome measures used in our trial, ABC has been the most widely used to assess behavioural change in response to both pharmacological and dietary supplement treatments.

Within the context of outcome measures, it is of relevance to discuss the role of parents/caregivers rating the behaviours in treatment response. Parents/caregivers' education, socioeconomic status, anxiety/stress, and perception of medication benefits have all been associated with treatment response [13,32,33]. More educated families, families of higher socio-economic status, families with lower stress/anxiety (though inconsistently), and families with higher perception of medication benefits have been reported to report greater behavioural improvements with interventions in their children [13,32,33]. Cultural variables such as values/beliefs and language may also affect the treatment response. Populations from different cultures may have systematically different test profiles due to having different languages and beliefs, values and perception regarding discipline, aggression, education, and fairness towards others [34,35]. Finally, ethnic differences in treatment response have been reported in the pharmacological treatment of schizophrenia [36], and ADHD [37]. Most studies have found that differences in socioeconomic, clinical characteristics (e.g. comorbidities) and adherence to medication may largely account for these ethnic differences [37-39]. Biological factors (e.g. genetic) may also explain some of these ethnic differences

[38,40]. Clear evidence of health disparity among children of different ethnic groups was shown in our study (**Appendix 1,** conference presentation II) where children of Māori or Pacific ethnicities were shown to have significantly lower 25(OH)D levels. This ethnic difference might have important implications for treatment response. For example, we demonstrated a greater improvement in SRS-social awareness and SPM-total in children with initial serum 25(OH)D concentration <50 nmol/L than ≥50 nmol/L, a finding suggesting that inclusion of children of ethnic groups with documented high prevalence of vitamin D deficiency, for example Māori or Pacific children may benefit more from treatment. It is important to note that a small proportion of our study population were from Māori (13%), Pacific (11%) and other ethnic minorities (11-16%), yet more than half of Māori and approximately 85% of Pacific children did not complete the trial *vs.* 0-37% of children of other ethnic groups. These findings may reflect ethnic differences in the recognition and diagnosis of ASD and parents/caregivers' availability of adequate resources to engage in research projects [41]. To overcome this issue several solutions can be proposed: opening trials within community sites and traveling of research staff to participants to overcome logistic problems and building relationships with health care providers working with particular ethnicities to encourage referrals from those ethnic groups.

The findings of the VIDOMA trial make an important contribution to the current body of knowledge about the role of vitamin D and omega-3 LCPUFA, each individually or in combination, in the management of ASD symptoms in children. This is particularly true for omega-3 LCPUFA because it was found to be effective in improving both autism severity (a trend) and social motivation as well as irritability. Regarding vitamin D alone, with only four RCTs, all with different study designs (dose, study length, and population characteristics) and three of which (including ours) showing no positive effect on core symptoms, definitive conclusions cannot be made. However, vitamin D was clearly shown to be effective in improving both irritability and hyperactivity in children with ASD. Finally, with regard to both nutrients given together, we could not show a synergistic effect on all outcome measures. The synergy between these two nutrients was observed only where core symptoms were assessed. Possible explanations for inconsistent findings could be that our population was heterogenous (particularly in terms of co-existing problem behaviours) and our trial had low statistical power to find a positive effect for all outcome measures. In view of the following; (1) lack of evidence for antagonistic effects of these nutrients on biological and disease outcomes, (2) potential synergy between these nutrients (discussed in Chapter 2 – Section 4), (3) the greater improvement in vitamin D status with both nutrients than a single nutrient (**Appendix 2**, conference presentation IV), and (4) comparable change in outcome measures (e.g. hyperactivity) with vitamin D with and without omega-3 LCPUFA but not

reaching statistical significance in the combined group, further research with both nutrients is warranted.

Conclusions

Despite the increasing interest in the role of nutrients in the pathophysiology of ASD, vitamin D and omega-3 LCPUFA remain under-researched nutrients. Investigating the role of both nutrients, each individually and together, VIDOMA is the first and only randomised controlled trial in children with ASD. It is also the only RCT that corrected major nutritional deficiencies prior to trial entry and investigated the impact of intervention for one year. It is also one of only a few trials that have investigated the impact of intervention on behavioural outcomes together with biochemical measures of vitamin D and omega-3 LCPUFA status.

The primary objective of this study was to investigate the effect of supplementation with vitamin D, omega-3 LCPUFA or both on core symptoms of ASD. An improvement in social awareness with omega-3 LCPUFA alone and in combination with vitamin D and a trend for an improvement in social communicative functioning with both supplements and in autism severity with omega-3 LCPUFA was seen, demonstrating the importance of adequate omega-3 LCPUFA and vitamin D status on core symptoms of ASD. This trial also demonstrated an improvement in irritability with vitamin D and omega-3 LCPUFA, each individually, and in hyperactivity with vitamin D in children with ASD. The prevalence of irritability and hyperactivity was high in enrolled children, and it is possible that suboptimal vitamin D status and omega-3 index contributed to these. Based on mechanistic evidence, the actions of vitamin D and omega-3 LCPUFA in the management of behavioural symptoms of ASD are many and varied. Consequently, the response to vitamin D and omega-3 LCPUFA supplementation may depend on the involved biological factors that differ between individuals with ASD.

The results of the VIDOMA trial show nutritional issues and suboptimal vitamin D and omega-3 LCPUFA status in children with ASD living in New Zealand. These results confirm previous reports. Also, vitamin D and omega-3 LCPUFA are shown to be effective in improving some symptoms of ASD and to be well-tolerated and accepted (up to one year). Accordingly, it seems prudent to recommend health care providers and researchers to screen for insufficiencies in vitamin D and omega-3 LCPUFA status and to consider these nutrients as the disease-modifying measures for ASD.

Future Directions

1. Nutritional issues were identified in children with ASD. Investigation of reasons (including gastrointestinal issues, sensory issues and unusual eating habits) for such issues to develop ASD-appropriate recommendations is required.

2. Many children were identified having suboptimal vitamin D status and omega-3 index. Further investigation of vitamin D and omega-3 index status of children with ASD and comparing these children with typically developing children in New Zealand is required to see if routine screening of vitamin D and omega-3 index for children with ASD should be recommended.

3. The optimum levels of serum 25(OH)D and omega-3 index for optimum behavioural outcomes needs to be determined.

4. A large randomised controlled trial with both vitamin D and omega-3 LCPUFA supplementation in children with ASD with low vitamin D status and omega-3 index is required to investigate the effect of improving vitamin D status and omega-3 index on core symptoms of ASD. This trial should exclude children on vitamin D and omega-3 LCPUFA containing supplements, and include children with a range of baseline ASD severity ranging from mild to severe.. Such a study would consider weight-adjusted doses of vitamin D and omega-3 LCPUFA. The study protocol would include more ASD friendly; e.g. less invasive blood testing (such as finger prick) and researchers traveling to participants.

5. Some children were identified to have high levels of problem behaviours (irritability and hyperactivity). A large randomised controlled trial with vitamin D and omega-3 LCPUFA supplementation, either individually or together, in children with autism with and without problem behaviour is required to determine if these nutrients improve problem behaviours in both population groups.

6. An investigation of potential biochemical markers that link vitamin D and omega-3 LCPUFA to ASD pathophysiology is needed to explain why every child with ASD exhibits different symptoms and to help identify children who would most benefit from supplementation.

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Appendices

Chapter 2

Section 1

Chapter 2 – Section 1 – Appendix 1: ASD diagnosis based on DSM-5

DSM-5 Behavioural domain	Behavioural criteria	Examples
A. Persistent deficit in social communication and social interaction across multiple context All three should be met	Deficits in social-emotional reciprocity, ranging	• Abnormal social approach and • Failure of normal back-and-forth conversation • Reduced sharing of interests, emotions, or affect • Failure to initiate or respond to social interactions
	Deficits in nonverbal communicative behaviours used for social interaction, ranging	• Poorly integrated verbal and nonverbal communication • Abnormalities in eye contact and body language or • Deficits in understanding and use of gestures • Total lack of facial expressions and nonverbal communication
	Deficits in developing, maintaining, and understanding relationships	• Difficulties adjusting behaviour to suit various social contexts • Difficulties in sharing imaginative play • Difficulties in making friends • Absence of interest in peers.
B. Restricted, repetitive patterns of behaviour, interests, or activities, as manifested by at least two of the following, currently or by history At least two criteria should be met	Stereotyped or repetitive motor movements, use of objects, or speech	• Simple motor stereotypies • Lining up toys • Flipping objects • Echolalia • Idiosyncratic phrases

		Insistence on sameness, inflexible adherence to routines, or ritualized patterns or verbal nonverbal behaviour	• Extreme distress at small changes • Difficulties with transitions, • Rigid thinking patterns, • Greeting rituals, need to take same route or eat food every day
		Highly restricted, fixated interests that are abnormal in intensity or focus	• Strong attachment to or preoccupation with unusual objects, • Excessively circumscribed or perseverative interest
		Hyper- or hypo-reactivity to sensory input or unusual interests in sensory aspects of the environment	• Apparent indifference to pain/temperature • Adverse response to specific sounds or textures, excessive smelling or touching of objects, • Visual fascination with lights or movement
C.	Symptoms must be present in the early developmental period		
D.	Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning		
E.	These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay**		

DSM-5, Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

* The severity is based on social communication impairments and restricted, repetitive patterns of behaviour. **Intellectual disability and ASD frequently co-occur and to make comorbid diagnoses of ASD and intellectual disability, social communication should be below that expected for general developmental level

Chapter 2 – Section 1 – Appendix 2: Severity level for ASD based on DSM-5

Severity	Social communication	Restricted, repetitive behaviours
Level 1 Requiring support	Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful response to social overtures of others. May appear to have decreased interest in social interactions. For example, a person who is able to speak in full sentences and engages in communication but whose to- and-from conversation with others fails, and whose attempts to make friends are odd and typically unsuccessful.	Inflexibility of behaviour causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence.
Level 2 Requiring substantial support	Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others. For example, a person with few words of intelligible speech who rarely initiates interaction and, when he or she does, makes unusual approaches to meet needs only and responds to only very direct social approaches.	Inflexibility of behaviour, difficulty coping with change, or other restricted/repetitive behaviours appear frequently enough to be obvious to the casual observer and interfere with functioning in a variety of contexts. Distress and/or difficulty changing focus or action.
Level 3 Requiring very substantial support	Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others. For example, a person with few words of intelligible speech who rarely initiates interaction and, when he or she does, makes unusual approaches to meet needs only and responds to only very direct social approaches.	Inflexibility of behaviour, extreme difficulty coping with change, or other restricted/repetitive behaviours markedly interfere with functioning in all spheres. Great distress/difficulty changing focus or action.

DSM-5, Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

Section 3

Chapter 2 – Section 3 – Appendix 1: Study characteristics of open label trials and case-studies excluded from systematic literature review

Open label trials (*n*=6)							
Reference and setting	**Age (years)**	**Sex distribution (M, F)**	**Sample size**	**Intervention**	**Duration**	**Outcome measure**	**Outcome**
Johnson (2010) [29] US	1-5	NR	*n*=10 (Intervention group), *n*=13 (Healthy diet control group)	0.4 g DHA	3 months	CBCL DBOM Mullen's Scales of Early Learning	Significant improvement in externalising subscale of CBCL was reported in children in the DHA group. Significant improvement in affective subscale of CBCL was reported in children on the healthy diet. No other differences were reported. Well tolerated.
Meguid (2008) [30] Egypt	3-11	18M, 12F	*n*=30	0.03 g EPA 0.01 g DHA 0.02 g LA 0.01 g AA	3 months	CARS	Significant improvement in whole blood omega-3 and -6 levels Significant improvement in autistic behaviour (concentration, eye contact, language development, motor skills) in 20 children DHA levels correlated negatively with CARS in 10 non-respondent children.
Meiri (2009) [31] Israel	4-7	NR	*n*=10, 1 drop out	0.4 g EPA 0.2 g DHA	12 weeks	CGI ATEC CPRS	8/9 children showed an average improvement of 33% as measured by ATEC and one child did not respond at all. No adverse effect.

Chapter 2 – Section 3 – Appendix 1: *Cont.*

Open label trials (*n*=6)							
Reference and setting	**Age (years)**	**Sex distribution (M, F)**	**Sample size**	**Intervention**	**Duration**	**Outcome measure**	**Outcome**
Ooi (2015) [32] Singapore	7-18	36M, 5F	*n*=41	0.2 g EPA 0.8 g DHA 0.1 g LA 0.07 g AA	12 weeks	SRS CBCL	Significant increase in percentage of EPA and DHA and significant decrease in AA:EPA ratio. Significant improvement in total (-21 units) and all subscales of SRS (2-7 units, *P*<0.01 for all and medium to large effect sizes) and in social problem subscale of CBCL (*P*=0.02 and medium effect size). Change in RBC fatty acids correlated negatively with autism mannerism severity and higher baseline EPA was associated with a better response. Well tolerated.
Patrick (2005) [33] NR	3-10	NR	*n*=22, 4 drop outs	0.3 g omega-3 0.04 g omega-6	90 days	ABBLS	Significant increase in language development and learning skills.
Politi (2008) [34] Italy	18-40	15M, 4F	*n*=19	0.9 g EPA + DHA	6 weeks	RBC	No significant improvement in problem behaviours and their severity.

Chapter 2 – Section 3 – Appendix 1: Cont.

Case study (*n*=1)							
Reference and setting	**Age (years)**	**Sex distribution (M, F)**	**Sample size**	**Intervention**	**Duration**	**Outcome measure**	**Outcome**
Johnson (2003) [35] US	11	1M	*n*=1 case-study	3.0 g omega-3 (0.5 EPA)*	4 weeks	Clinical observation	Significant improvement in anxiety, agitation and quality of life

AA, arachidonic acid; ABBLS, Assessment of Basic Language and Learning Skills; ATEC, Autism Treatment Evaluation Checklist; CARS, Childhood Autism Rating Scale; CBCL, Childhood Behaviour Checklist; CPRS, Children's Psychiatric Rating Scale; DBOM, direct behaviour observation measure; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; F, Female; LA, Linoleic acid; M, Male; n, Number; NR, not reported; RBC, Rossago Behavioural Checklist; US, the United States
*The amount of other omega-3 fatty acids was not reported

Chapter 2 – Section 3 – Appendix 2: Quality appraisal of included case-control studies†

Reference	Inclusion & exclusion	Attrition		Exposure		Health outcome		Blinding	Comparability of study groups	Statistical significance of trend	Confounders		Total	Confounders not controlled for
		Reported	Reasons	Methodology	Repeated measurement*	Methodology	Verification				Participants demographics	Other risk factors		
Al-Farsi (2013) [89]	1	0	0	1	0	0	0	1	1	1	1	0	6	Cases had lower frequency intake of high DHA foods, lower intake of ALA, lower energy intake, and shorter duration of breastfeeding (all were not considered in statistical analysis), medication use (NR)
Bell (2004) [88]	0	0	0	1	0	0	0	1	1	1	0	0	4	Age, sex, intake of LCPUFA, medication and supplement use (all NR)
Bell (2010) [27]	1	1	1	1	0	1	0	1	1	1	1	0	9	Dietary intake of LCPUFA (NR)

Chapter 2 – Section 3 – Appendix 2: *Cont.*

Reference	Inclusion & exclusion	Attrition		Exposure		Health outcome		Blinding	Comparability of study groups	Statistical significance of trend	Confounders		Total	Confounders not controlled for
		Reported	Reasons	Methodology	Repeated measurement*	Methodology	Verification				Participants demographics	Other risk factors		
Brigandi (2015) [24]	1	0	0	1	0	1	0	1	0	1	0	0	5	Age, sex, dietary intake of LCPUFA, supplement and medication use
Bu (2006) [28]	1	0	0	1	0	1	1	1	1	1	1	0	8	Dietary intake of LCPUFA, medication use
El-Ansari (2011a) [73]	1	0	0	1	0	1	1	1	1	1	0	0	7	Sex, dietary intake of LCPUFA, medication and supplement use
El-Ansari (2011b) [87]	1	0	0	1	0	1	1	1	1	1	0	0	7	Sex, dietary intake of LCPUFA, medication and supplement use
Ghezzo (2013) [14]	1	1	1	1	0	0	1	1	1	1	1	0	9	Dietary intake of LCPUFA, medication use (NR)
Jory (2016) [74]	1	1	1	1	0	1	0	1	0	1	0	0	7	Sex, dietary intake of LCPUFA

Chapter 2 – Section 3 – Appendix 2: *Cont.*

Reference	Inclusion & exclusion	Attrition		Exposure		Health outcome		Blinding	Comparability of study groups	Statistical significance of trend	Confounders		Total	Confounders not controlled for
		Reported	Reasons	Methodology	Repeated measurement*	Methodology	Verification				Participants demographics	Other risk factors		
Meguid (2008) [30]	1	0	0	1	0	1	1	1	1	1	1	0	7	Dietary intake of LCPUFA, medication and supplement use
Mostafa (2015) [26]	1	0	0	1	0	1	1	1	1	1	1	0	8	Dietary intake of LCPUFA
Parletta (2016) [53]	1	1	1	1	0	1	1	1	1	1	0	0	9	Age, dietary intake of LCPUFA
Sliwinski (2006) [72]	1	0	0	1	0	1	1	1	1	1	1	0	8	Supplement use, dietary intake of LCPUFA
Tostes (2013) [90]	1	1	0	1	0	1	0	1	1	1	1	0	8	Medication use, dietary intake of LCPUFA
Yui (2016) [75]	1	0	0	1	0	1	1	1	1	1	1	1	9	-

LCPUFA, long chain polyunsaturated fatty acids; NR, not reported.
† Health Canada Quality Appraisal Tool for Observational Studies; A quality score of ≥ 7 was considered higher quality [70]. * Measuring the exposure in duplicate or more is of no relevance for case-control studies and therefore all studies received a score of "0" for this criterion. Accordingly, a total score of 11 has been employed for this review instead of using a total score of 12.

Chapter 2 – Section 3 – Appendix 3: Quality appraisal of included RCTs†

Reference	Inclusion and exclusion	Group allocation				Blinding		Attrition		Intervention		Methodology to measure the health effect	Statistical analysis		Potential confounders	Total	Confounders not controlled for
		Described as randomised	Randomisation method	Randomisation	Randomisation	Participants	Researchers	Reported numerically	Reasons	Type described	Amount described		Between	Intention to treat			
Amminger (2007) [36]	1	1	0	0	0	1	1	1	1	1	1	1	1	0	0	10	Age, dietary intake or LCPUFA status, higher hyperactivity in omega-3 group, compliance (NR)
Bent (2011) [91]	1	1	1	1	1	1	1	1	1	1	0*	1	1	1	0	13	Medical regimen
Bent (2014) [92]	1	1	1	1	1	1	1	1	1	1	0*	1	1	1	0	13	Sex (the distribution across groups NR), medical regimen, dietary intake or LCPUFA status
Mankad (2015) [37]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	14	Gastrointestinal distress

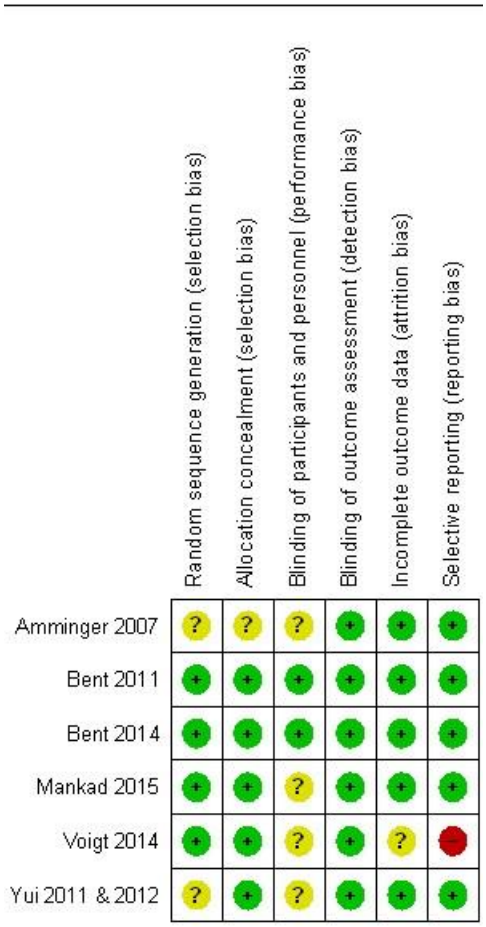
Chapter 2 – Section 3 – Appendix 3: *Cont.*

Reference	Inclusion and exclusion	Group allocation				Blinding		Attrition		Intervention		Methodology to measure the health effect	Statistical analysis		Potential confounders	Total	Confounders not controlled for
		Described as randomised	Randomisation method	Randomisation	Randomisation	Participants	Researchers	Reported numerically	Reasons	Type described	Amount described		Between	Intention to treat			
Voigt (2014) [77]	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	13	Sex (the distribution across groups NR), medical regimen, compliance raw data (NR)
Yui (2011 & 2012)* [76, 79]	1	1	1	0	1	1	0	1	1	1	1	1	1	0	0	11	Age, compliance (NR)

LCPUFA, long chain polyunsaturated fatty acids; NR, not reported; RCTs, randomised controlled trials

† Health Canada Quality Appraisal Tool for Experimental Studies; A quality score of >7was considered higher quality [70]. * Different outcomes were reported in two different papers. *The intervention material was delivered in a pudding form – no information regarding the pudding ingredients is provided.

Chapter 2 – Section 3 – Appendix 4: Risk of bias



Appendix 4: Risk of bias table showing judgments on each risk factor for each primary study included in both meta-analyses and overall interpretation. + = low risk (green), ? = unclear risk (yellow), - = high risk (red)

Chapter 3

Chapter 3 – Appendix 1 – Information Sheet

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The VIDOMA Study

A nutrition intervention in children with ASD

Information sheet for study participants

This information sheet provides you with the background to the research and other important details about what is involved, so please read carefully before deciding whether or not to participate.

We are currently recruiting children with Autism Spectrum Disorder (ASD) aged 2.5 - less than 8 years old to take part in this research. The study is nicknamed the VIDOMA study, short for **VI**tamin **D** and **OM**ega-3 in **A**utism. As a parent/guardian, it is important for you to understand why we doing this research and what it will involve for you if you decide that your child can participate. This information sheet tells you about the purpose of the study and what will happen to if you choose for your child to take part. Please take time to read it carefully and discuss it with others if you wish. Please ask if anything is not clear, or if you would like more information.

Introducing the researchers

This study involves a team of researchers from Massey University and from Waitemata District Health Board (WDHB).

The principal investigator is Dr Pamela von Hurst, Co-director of Massey's Vitamin D Research Centre. The study manager is Mr Owen Mugridge whose contact details are at the end of this information sheet. The psychologist on the team is Ms Lindy Thomas and the Paediatrician is Dr Bobby Tsang from WDHB. A number of other staff and students from the Division of Human Nutrition and Dietetics are also involved.

What is the purpose of this study?

Children with ASD often have deficiencies in their diet due to physical and behavioural issues related to the condition. Some of the nutrients which can be deficient in children with ASD are known to affect brain development and function. The two nutrients that this study is going to investigate are vitamin D and omega-3. Recent studies have shown that there are a variety of ways by which both these nutrients can affect the function and connectivity of the developing brain.

If shown to be effective, increasing the vitamin D and omega-3 status of children with ASD may be a powerful, non-invasive and low cost strategy for improving some of the symptoms of ASD and improving the quality of life for children and their families.

Why have I been chosen?

You have been invited to participate because your child has been diagnosed with Autism Spectrum Disorder.

Does my child have to take part?

No, it is completely up to you and your child to decide whether or not to take part. If you do decide to enter your child into the study you will be asked to sign a consent form. You are free to withdraw your child at any time without giving a reason. Your child will also be given a sheet describing what will happen (very simply) so that he or she has the opportunity to ask questions and agree to take part.

If you decide to withdraw your child from the study, data obtained to date may be kept and used to contribute to the overall results. However, if you request that your child's data and other information relating to your child are destroyed. Taking part in the study does not affect any aspect of the routine care your child received, or could receive from the local DHB.

What do I have to do?

You and your child will attend 5 appointments during the 12 month period. Your first visit will be at North Shore Hospital or Waitakere Hospital where your child will have a blood test. The blood test will look at vitamin D, iron, vitamin B12, folate, and magnesium. If your child is deficient in iron, vitamin B12 or magnesium, the study doctor will prescribe supplements to correct this, then your child can continue with the study. If your child has especially high vitamin D, he or she will not be able to continue with the study. However, we would like to continue with the collection of their dietary information which we will analyse and report back to you.

The second visit will be at Massey University, Albany. This visit will last for approximately 1.5 hours. Before you come, we will send you a "Preparation Kit" to prepare your child for all aspects of the study visit, to help familiarise them with the new surroundings and people. We will also send you a food diary to record 4 days of your child's total food intake. At this visit you will be asked to complete some questionnaires about the symptoms of your child's condition such as behaviour, sensory issues and socialisation. There will also be some questions regarding sun exposure habits of you and your child. We will measure the height and weight of your child and they will have time to play with some toys until you are finished.

As part of the study your child will have to take supplements. The supplements are in the form of a tasteless, colourless oil, and can be mixed in with your child's food or drink, or administered with a medicine syringe. Your child will be randomised into a study group and you will be given the supplements that your child will take for the next 12 months. The supplements will either be vitamin D, Omega-3, a combination of vitamin D and Omega-3 or a placebo. The study is double-blinded meaning that neither you nor the researchers will know what your child is taking until the end of the study.

Six months after starting the study, another blood test will be required, at either North Shore Hospital or Waitakere Hospital. We will send you all the instructions for this at the time. Similarly, at the end of the 12 months there will be another blood test, and a visit to Massey University (and North Shore or Waitakere Hospital) for the same tests as were done at the beginning.

Altogether, you will make 5 trips to either the hospital and/or Massey University over the year of your child's participation in the study. You are welcome to bring supporting friends or Whanau with you to any of the appointments.

Your child will have three blood tests during this period, and will have to take the supplement every day for 12 months.

There will also be questionnaires for you to complete at the beginning and end of the study.

What will happen to the blood samples?

Nearly all of the blood samples will be processed immediately at the North Shore Hospital Laboratory. A small sample of red cells will be saved and frozen for later analysis of red cell fatty acids. This analysis will be completed in a laboratory at the **University of Wollongong in Australia**. Some of the samples may not be analysed immediately after the study and will

remain stored at the Massey Nutrition Laboratory in the meantime. These samples may be used for further analysis, including genetic analysis. There is a separate information sheet and consent form relating to these samples. If you are not comfortable with these aspects of the study, you do not have to consent to them. This will in no way affect your child's participation in the rest of the study.

Will I be reimbursed for my time?

You will not receive reimbursement for your time. However, we will support you with travel for the trips made during the study, if requested.

What are the possible risks and disadvantages of taking part?

There are no reported risks of taking either vitamin D or Omega-3 at the dose we will be using. There is a very small risk that vitamin D supplementation will increase calcium levels in the blood. A safety check blood test will be carried out at the 6 month stage to ensure that all is going well with blood levels of vitamin D, Omega-3 and calcium.

It is quite possible that the blood test will cause your child (and you) some distress. The hospital staff are very experienced at conducting blood tests, and the Paediatric Registrar will be on hand if any further help is required. If you know that your child gets very distressed by blood tests, let us know and we can discuss with you the option of some light sedation which will be administered by the Paediatrician.

What are the advantages to taking part in the study?

As a participant in this study, your child will have a number of assessments which are not usually available through standard care from the District Health Board. Any nutritional deficiencies will be identified at the beginning of the study and addressed. You will receive a wealth of information about your child's responses to the psychological assessments which are normally valued at approximately $400. You will also be helping with research which, if successful, could make an important difference for many other children and families like yours.

What will happen if anything goes wrong?

The risks involved in this study are very small and all of the measurements are routinely made. If you have any concerns during the study you can discuss these with a member of the study team.

If your child has any other problems, illnesses or concerns you should discuss these with a member of the study team.

Any complaints you have will be fully investigated. If you have a concern about any aspect of this study, you should speak to a member of the study team who will do their best to answer your questions.

Will my taking part in the study be kept confidential?

Yes. All information collected about you and your child during the study will be kept strictly confidential. Information will be entered into a protected database at Massey University. Massey University code all data so that your names and address are kept separate from any other information about your child.

Information collected about you will be kept strictly confidential and secure in locked filing cabinets and/or electronic files on computers with passwords and restricted access. Each participant is assigned a unique code which is used on all data collected. Only the specified research team will have access to personal identifying information.

Massey University maintains a central record of all research projects but this does not include any personal information about participants. We will store the data for 10 years, at which point it will be destroyed.

What will happen to the results?

You will receive all the results that apply to your child. At the end of the study, we will also be in touch to let you know the results of the study. The overall results may be presented at scientific meetings or published in scientific journals to ensure that the wider community including health professionals know about the findings. The findings may also be featured in the media. Your child will not be identified in any of these presentations or publications.

Who is organising and funding the study?

This study is being co-ordinated by Massey University's College of Health in collaboration with Waitemata District Health Board. The Principal Investigator is Dr Pamela von Hurst. The study is funded from a number of sources including Douglas Nutrition Ltd who provided the supplements free of charge, and Massey University.

Who has reviewed the study?

This project has been reviewed and approved by the Health and Disability Ethics Committee: 14/NTA/113.

Contact for further information:

If you have any further questions or if you have any concerns whilst participating in the study then please contact Owen Mugridge – 09 213 6650.

Massey University, Albany
College of Health
Gate 4 – Building 80
Turitea Place
Albany 0632
Auckland
09 414 0800 Ext 43650

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

Cultural Support

If you require Māori cultural support, talk to your whanau in the first instance. Alternatively you may contact the administrator for He Kamaka Waiora (Māori Health Team) by telephoning 09 486 8324 ext 2324

If you have any questions or complaints about the study you may contact the Auckland and Waitemata District Health Board's Māori Research Committee or Māori Research Advisor by telephoning 09 486 8920 ext 3204

Chapter 3 – Appendix 2 – Consent Form (General)

The VIDOMA Study

PARTICIPANT CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree for my child to participate in this study under the conditions set out in the Information Sheet.

Signature: ..... **Date:** .....

Full Name of Parent/Legal Caregiver Please print .....

Full Name of child Please print .....

Chapter 3 – Appendix 3 – Consent Form (Storage of Blood Samples)

The VIDOMA Study

Storage of blood samples for future analysis
PARTICIPANT CONSENT FORM

I have read the Information Sheet regarding the storage of blood samples for future analysis, including genetic analysis, and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

Please select one of the following:

- ☐ Yes, I agree to having part of my child's blood sample held in storage for future analysis including genetic analysis under the conditions set out in the Information Sheet.

- ☐ No, please do not hold samples of my child's blood for any future analysis, including genetic analysis.

Signature: **Date:**

Full Name of Parent/Legal Caregiver Please print _____

Full Name of Child Please print _____

Chapter 4

Chapter 4 – Appendix 1 – Dietary intake of children with Autism Spectrum Disorder across different studies

Reference	Year	Country	Study type	Age range (years)	Sample size	Dietary assessment tools	ASD *vs.* TD		All study population	
							Higher intake	Lower intake	In excess	Inadequacy
[33] Shearer *et al.*	1982	US	Case-control	Mean: 8	12 ASD 12 TD	3DFR		Ca, B_2, serving of milk		
[40] Raiten *et al.*	1986	US	Case-control	Mean: 11 and 9	40 ASD 34 TD	7DFR	Energy, Pro, CHO, Ca, P, Fe, vits B_1, B_2, B_3,			
[35] Ho *et al.*	1997	Canada	Cross-sectional	Mean: 13	54 ASD	3DFR	NA	NA	CHO	Servings for each food group, fat
[43] Cornish	1998	UK	Cross-sectional	4-10	17 ASD	3DFR, FFQ	NA	NA	Ca	Ca, Fe, Zn, vits D, B_2, B_3, B_6
[56] Levy *et al.*	2007	US	Cross-sectional	3-8	62 ASD	3DFR	NA	NA	Pro	
[50] Lockner *et al.*	2008	US	Case-control	3-5	20 ASD 20 TD	3DFR	Supplement use	Ca, vit A		Fibre, Ca, vits A and E
[32] Herndon *et al.*	2009	US	Case-control	Mean: 5	46 ASD 31 TD	3DFR	Non-dairy Pro, vit E, B_6	Ca, dairy		Fibre, Ca, Fe, vits D and E
[59] Bandini *et al.*	2010	US	Case-control	3-11	53 ASD 58 TD	3DFR, FFQ		Ca, vits A and D		Fibre, Ca, vits D and E

Chapter 4 – Appendix 1: Cont.

Reference	Year	Country	Study type	Age range (years)	Sample size	Dietary assessment tools	ASD *vs.* TD		All study population	
							Higher intake	Lower intake	In excess	Inadequacy
[51] Xia *et al.*	2010	China	Cross-sectional	2-9	111 ASD	3DFR	NA	NA	Vits E and B_3	Fat, Mg*, Fe*, vits B_1* and B_2*, Ca**, Zn**, vits A**, B_6**, C** and B_9**
[36] Hyman *et al.*	2012	US	Case-control	2-11	252 ASD NHANES TD	3DFR	CHO $^{\delta}$, vits K $^{\delta\delta}$ and E $^{\delta\delta}$ & $^{\delta\delta\delta}$	Energy$^{\delta}$ Pro$^{\delta\delta}$, Zn$^{\delta}$, P$^{\delta\delta\delta}$, vits A$^{\delta}$ and C$^{\delta}$		Fibre, choline, Ca, K, D, Vit K
[39] Moore *et al.*	2012	US	Cross-sectional	2-8	54 ASD	FFQ	NA	NA	Vits A, B_1, B_2, B_6, C	Fibre, Ca, vits K, E and D
[60] Soden *et al.*	2012	US	Cross-sectional	10-18	26 ASD	3DFR	NA	NA		
[37] Zimmer *et al.*	2012	US	Case-control	Mean: 8	22 ASD 22 TD	FFQ	Mg	Pro, Ca, vits D and B_1		Fibre, Ca, K, Zn, vits A, D, E, K, B_3 and B_9
[5] Graf-Myles *et al.*	2013	US	Case-control	1-6	69 ASD 14 DD 37 TD	3DFR, HEI		Ca, dairy		Fibre, vit D
[6] Sun *et al.*	2013	China	Case-control	4-6	53 ASD 53 TD	3DFR		Ca, vit C		Ca, Zinc, vits B6 and A
[11] Mari-Bauset et al	2015	Spain	Case-control	6-9	40 ASD 111 TD	3DFR, HEI	Vit E	Pro, F, Ca, Fe, vit C	Fat, Cholesterol	CHO, fibre
[44] Meguid *et al.*	2015	Egypt	Cross-sectional	3-9	80 ASD	3DFR	NA	NA		Ca, Mg, P, Zn, Fe vits D, C, and B_9

Chapter 4 – Appendix 1: Cont.

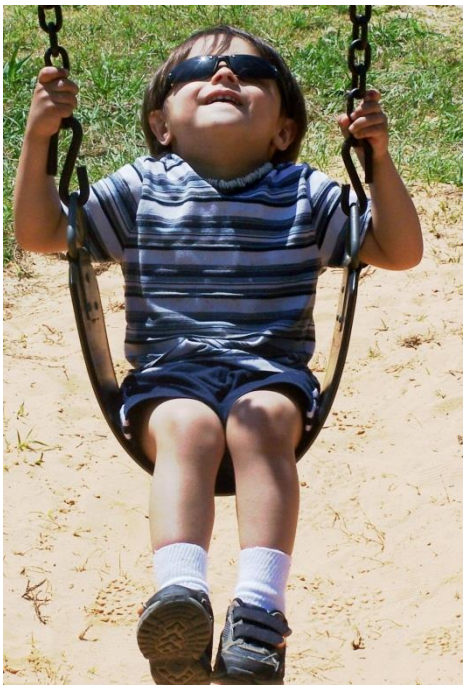
Reference	Year	Country	Study type	Age range (years)	Sample size	Dietary assessment tools	ASD *vs.* TD		All study population	
							Higher intake	Lower intake	In excess	Inadequacy
[45] Stewart *et al.*	2015	US	Cross-sectional	2-11	288 ASD	3DFR	NA	NA	Cu†, Mn†, Zn††, vits A††, and B$_9$††,	Ca, K, Choline, vits D and E
[61] Liu *et al.*	2016	China	Case-control	Mean: 5	154 ASD 73 TD	24HFWR, 2DFR		Energy, CHO, Pro, fat		Vit A
[12] Meguid *et al.*	2017	Egypt	Case-control	4-6	80 ASD 80 TD	3DFR	Fibre, K, vits C and B$_6$	Pro, Ca, Fe, Mg		Zn
[13] Barnhill *et al.*	2018	US	Case-control	2-8	86 ASD 57 TD	3DFR		Pro, Ca, vits B$_1$, B$_2$, B$_3$, B$_6$, and B$_9$		Ca, vits A, D, E, and B$_9$
[38] Neumeyer *et al.*	2018	US	Case-control	8-17	25 ASD 24 TD	3DFR	CHO	Pro, animal Pro, fat, P, Ca, Fe, Se, vits B$_6$, B$_{12}$, and B$_9$		

ASD, Autism Spectrum Disorder; Ca, calcium; CHO, carbohydrate; DD, developmentally delayed; FFQ, food frequency questionnaire; F, fluoride; Fe, iron; HEI, Healthy Eating Index; K, potassium; Mg, magnesium; Mn, manganese; P, phosphorus; Pro, protein; Se, selenium; TD, typically developing; vit, vitamin; Zn, zinc; 2DFR, 2-day estimated food record; 24HFWR, 24-hour food weighed record; 3DFR, 3-day estimated food record; 7DFR, 7-day estimated food record;
* 80-90% of NRV (Recommended Dietary Intake or Adequate Intake). ** <80% of NRV (Recommended Dietary Intake or Adequate Intake). $^{\delta}$ In 4-8-year-old age group. $^{\delta\delta}$ In 1-8-year-old age group. $^{\delta\delta\delta}$ In 9-11-year-old age group. † In 4-8-year-old age group. †† In 2-3-year-old age group as well as in supplement users

Chapter 4 – Appendix 2 – Food Diary (4-day estimated food record)

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The VIDOMA Study



4 Day Food Record

Thank you very much for taking part in this study. We are extremely grateful for your time, effort and commitment

If you have any questions, please contact Owen Mugridge on 09 414 0800 Ext. 43650

All information in this diary will be treated with the strictest confidence. No one outside the study will have access to this.

Please bring the food diary with you when you bring your child in for assessment at Massey University.

4 day food diary - what to do?

- Record all of the food and supplements that your child eats and drinks on the following dates.

- **Please complete the diary on consecutive days for 1 weekend day and 3 week days. For example, Sunday, Monday, Tuesday and Wednesday <u>OR</u> Wednesday, Thursday, Friday and Saturday.**

- If possible record food at the time of eating or just after – try to avoid doing it from memory at the end of the day.

- Include all meals, snacks, and drinks, even tap water.

- Include anything you have added to foods such as sauces, gravies, spreads, dressings, etc.

- Write down any information that might indicate size or weight of the food to identify the portion size eaten.

- Include all supplements and write down brand and dose of the supplements.

- Use a new line for each food and drink. You can use more than one line for a food or drink. See the examples given.

- Use as many pages of the booklet as you need.

- You can also save any packets such as muesli bar wrappers and bring them in with your child's food diary

Describing Food and Drink

- Provide as much detail as possible about the type of food eaten. For example **brand names and varieties / types** of food.

General description	Food record description
Breakfast example – cereal, milk, sugar	2 Weetbix (Homebrand) 1 cup Pam's whole milk 1 tsp Chelsea white sugar
Lunch – Ham sandwich	2 slices of wholegrain bread (Vogels) 1 slice ham 2 slices edam cheese 2 tsp flora margarine Water 1 cup to drink
Dinner – Spaghetti Bolognese	½ cup mince sauce (see attached recipe) 1 cup spaghetti pasta (Homebrand) Milk 1 cup Pam's whole milk
Snacks	Flemmings apricot chocolate chip muesli bar (35g) 1 small banana 2 Salada crackers with 1 tsp peanut butter Small packet of Bluebird salt and vinegar chips

- Give details of all the **cooking methods** used. For example, fried, grilled, baked, poached, boiled…

General description	Food record description
2 eggs	2 size 7 eggs fried in 2tsp canola oil 2 size 6 eggs (soft boiled)
Fish	100g white fish pan-fried

- When using foods that are cooked (eg. pasta, rice, meat, vegetables, etc), please record the **cooked portion** of food.

General description	**Food record description**
Rice	1 cup cooked Jasmine rice (cooked on stove top)
Meat	½ cup of casserole beef or 5 chicken nibbles in honey soy marinade
Vegetables	½ cup cooked mixed vegetables (Wattie's peas, corn, carrots)

- Please specify the **actual amount of food eaten** (eg. for leftovers, foods where there is waste)

General description	**Food record description**
Apple	1 x 120g Granny Smith Apple (peeled, core not eaten – core equated to ¼ of the apple)
Fried chicken drumstick	100g chicken drumstick (100g includes skin and bone); fried in 3 Tbsp Fern leaf semi-soft butter

General description	**Food record description**
Milo	I x cup Milo made with Milo powder and 150mls Calci-trim milk, 100 ml hot water. No sugar

- **Record recipes** of home prepared dishes where possible and the proportion of the dish your child ate. There are blank pages for you to add recipes or additional information.

Recording the amounts of food your child eats

It is important to also record the quantity of each food and drink consumed. This can be done in several ways.

- By using household measures – for example, cups, teaspoons and tablespoons. Eg. 1 cup frozen peas, 1 heaped teaspoon of sugar.

- By weight marked on the packages – e.g. a 425g tin of baked beans, a 32g cereal bar,

- Weighing the food – this is an ideal way to get an accurate idea of the quantity of food eaten, in particular for foods such as meat, fruits, vegetables and cheese.

- For bread – describe the size of the slices of bread (e.g. sandwich, medium, toast) – also include brand and variety.

- Using comparisons – e.g. Meat equal to the size of a pack of cards, a scoop of ice cream equal to the size of a hen's egg.

- Use the food record instructions provided to help describe portion sizes.

General description	**Food record description**
Cheese	1 heaped tablespoon of grated edam cheese 1 slice cheese edam (8.5 x 2.5 x 2mm) 1 cube edam cheese, match box size

- If you go out for meals, describe the food eaten in as much detail as possible.

- ***Please try to have your child eat as normally as possible – ie. Don't adjust what he/she normally eats just because you are keeping a diet record and be honest! This record will give us important information about your child's diet, and help us identify any possible deficiencies which we can then help you correct.***

Example day

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed (units, measures, weight)
Example7:55am	Sanitarium Weetbix	2 weetbix
" "	Anchor Blue Top milk	150ml
" "	Chelsea white sugar	2 heaped teaspoons
" "	Orange juice (Citrus Tree with added calcium – nutrition label attached)	1 glass (275 ml)
10.00am	Raw Apple (gala)	Ate all of apple except the core, whole apple was 125g (core was ¼ of whole apple)
12.00pm	Home made pizza (recipe attached)	1 slice (similar size to 1 slice of sandwich bread, 2 Tbsp tomato paste, 4 olives, 2 rashers bacon (fat removed), 1 Tbsp chopped spring onion, 3 Tbsp mozzarella cheese)
1.00pm	Water	500ml plain tap water
" "	Multivitamin & mineral	1 gummy bear (Yami Bears multivitamin & mineral)
3.00pm	Biscuits	6 x chocolate covered Girl Guide biscuits (standard size)
6.00pm	Lasagne	½ cup cooked mince, 1 cup cooked Budget lasagne shaped pasta , ½ cup Wattie's creamy mushroom and herb pasta sauce, ½ cup mixed vegetables (Pam's carrots, peas and corn), 4 Tbsp grated Edam cheese
6.30pm	Banana cake with chocolate icing (homemade, recipe attached)	1/8 of a cake (22cm diameter, 8 cm high), 2 Tbsp chocolate icing
" "	Tip Top Cookies and Cream ice cream	1/2cup (g) (125g)

Study ID: VID_____

Date_____ DAY 1

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Study ID: VID_____

Date_____ DAY 1 continued

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Study ID: VID_____

Recipes (DAY 1)

Study ID: VID_____

Date_____ DAY 2

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Study ID: VID_____

Date_____ DAY 2 continued

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Study ID: VID_____

Recipes (DAY 2)

Study ID: VID_____

Date_____ DAY 3

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Study ID: VID_____

Date_____ DAY 3 continued

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Study ID: VID_____

Recipes (DAY 3)

Study ID: VID_____

Date_____ DAY 4

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Study ID: VID_____

Date_____ DAY 4 continued

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Study ID: VID_____

Recipes (DAY 4)

Chapter 4 – Appendix 3 – Dietary Index of Children's Eating (DICE)

The aim of this questionnaire is to find out about your child's diet.

There are no right or wrong answers. You do not have to answer any questions you are uncomfortable with, and you can opt out of the questionnaire at any time. The questionnaire will take less than 10 minutes to complete. Only completed questionnaires will be included in the study.

Thank you for accessing this online questionnaire.

Autism Study Team

1. Study ID number (this number can be found on the front of your study diary)

2. Child's date of birth

DD….MM…..YYYY

What your child eats?

We want to find out about the number of servings of different foods your child eats. Below is a guide as to what a serving looks like.

Your child may not consume an entire serving size in one sitting - eg: a serving of milk is a 250ml glass but they may have 125ml portion of milk at breakfast and another portion again at morning tea. This counts as one serving. Please see the serving size examples above for examples of serving sizes.

3. How many servings of fruit does your child eat each day?

Examples of servings are:

- **1 apple, pear, banana or orange**
- **2 small apricots or plums**
- **½ cup of fresh fruit salad**
- **½ cup of stewed or canned fruit**

◯ None

◯ ½ serving

◯ 1 serving

◯ 1 and ½ servings

◯ 2 servings or more

4. How many servings of vegetables does your child eat each day?

Examples of servings are:

- **1 medium potato, taro or kūmara**
- **½ cup of cooked vegetables eg: peas, carrots, broccoli**
- **½ cup of salad eg: lettuce, cucumber**
- **1 tomato**

◯ None

◯ ½ serving

◯ 1 serving

◯ 1 and ½ servings

◯ 2 servings or more

5. Fruits come in many different colours. How many different types does your child eat from the following colour groups?

	0	1	2	3	4	5 or more types
Red fruits (eg: red apples, cherries, red grapes, strawberries, watermelon)	○	○	○	○	○	○
Orange/yellow fruits (eg: yellow apples, apricots, oranges, mandarins, peaches)	○	○	○	○	○	○
Green fruits (eg: green apples, green grapes, kiwi fruit)	○	○	○	○	○	○
Blue/purple fruits (eg: blackberries, blueberries, plums, purple grapes, raisins)	○	○	○	○	○	○
White fruits (eg: bananas, brown pears, nashi pears)	○	○	○	○	○	○

6. Vegetables come in many different colours. How many different types does your child eat from the following colour groups?

	0	1	2	3	4	5 or more types
Red vegetables (eg:red potatoes, red capsicums, tomatoes, yams)	○	○	○	○	○	○
Orange and yellow vegetables (eg: carrots, pumpkin, sweet corn, gold or orange kumara)	○	○	○	○	○	○
Green vegetables (eg: avocados, green beans, broccoli, green cabbage, cucumber, peas, spinach, puha, watercress, kamokamo, bok choi)	○	○	○	○	○	○
Blue/purple vegetables (eg: eggplant, red cabbage)	○	○	○	○	○	○
White vegetables (eg: cauliflower, mushrooms, onions, parsnips, potatoes, taro, cassava, breadfruit)	○	○	○	○	○	○

7. How many servings of bread and cereals does your child eat each day?

Examples of servings are:

- **1 roll**
- **1 medium slice of bread**
- **1 medium slice of rēwena bread**
- **1 pita pocket or tortilla**
- **4 crackers**
- **½ cup ofmuesli**
- **1 cup of cornflakes or rice bubbles or 2 breakfast wheat biscuits**
- **½ cup of cooked cereal (eg: porridge)**
- **1 cup of cooked pasta, noodles or rice**
- **1 cup of cassava or tapioca**
- **2 plain sweetbiscuits**

◯ None

◯ 1 serving

◯ 2 servings

◯ 3 servings

◯ 4 servings or more

8. How often does your child eat wholegrain versions of these foods?

◯ Never

◯ Rarely

◯ Some days

◯ Most days

◯ Every day

9. How many servings of milk, milk products and calcium-fortified milk alternatives does your child eat each day?

Examples of servings are:

- **1 cup ofmilk**
- **1 pottle ofyoghurt**
- **2 slices or ½ cup of grated cheese**

◯ None

◯ ½ serving

◯ 1 serving

◯ 1 and ½ servings

◯ 2 servings or more

10. How often does your child eat reduced or low fat versions of these foods?

◯ Never

◯ Rarely

◯ Some days

◯ Most days

◯ Every day

11. How many servings of meats, chicken, seafood, eggs, legumes, nuts and seeds does your child eat each day?

Examples of servings are:

- **2 slices of cooked lean meat (eg: lamb, chicken, beef or pork)**
- **¾ cup of mince or casserole**
- **1 medium fillet of fish**
- **2 chicken drumsticks or 1 chicken leg**
- **1 medium pāua or kina**
- **1 egg**
- **¾ cup of cooked dried beans (eg: baked beans)**
- **⅓ cup of nuts or seeds**
- **¾ cup of tofu**
- **½ cup canned tuna or salmon**

◯ None

◯ ½ serving

◯ 1 serving or more

12. Please tell us about any cultural or traditional foods which are important to your child

13. Does your child eat

	Never	Rarely	Some days	Most days	Every day
Breakfast	○	○	○	○	○
Mid morning snack	○	○	○	○	○
Lunch	○	○	○	○	○
Mid afternoon snack	○	○	○	○	○
Dinner	○	○	○	○	○

14. How often do you prepare or choose low fat food, snack and/or drink options for your child?

○ Never

○ Rarely

○ Some days

○ Most days

○ Every day

15. How often do you choose low sugar food, snack and/or drink options for your child?

○ Never

○ Rarely

○ Some days

○ Most days

○ Every day

16. Is your child

	Yes	No
Vegetarian	○	○
Vegan	○	○

17. How many cups/glasses of the following options does your child usually drink each day?

	None	1	2	3	4 or more
Water	○	○	○	○	○
Cow's milk (or alternative)	○	○	○	○	○
Flavoured milk based drinks	○	○	○	○	○
Fruit juice	○	○	○	○	○
Fizzy drinks (including diet drinks)	○	○	○	○	○
Cordial or fruit drinks	○	○	○	○	○
Tea or coffee	○	○	○	○	○
Sports or energy drinks	○	○	○	○	○
Others (please specify)	○	○	○	○	○

Chapter 4 – Appendix 4 – Standard Operating Procedure

Dietary assessment of children with Autism Spectrum Disorder using 4-day estimated food record (4DFR) and Dietary Index of Children's Eating (DICE) (VIDOMA trial)

Scope

This standard operating procedure applies to the VIDOMA (Vitamin D and Omega-3 in Autism) research members who are responsible for the assessment of diet (collection, entry and scoring of 4DFR and DICE) in children with Autism Spectrum Disorder (ASD) at Massey University.

Objectives

The objective is to describe the procedure of training the VIDOMA research team or the delegated persons who are are involved in the collection, entry and scoring of the 4DFR and DICE questionnaire throughout the study period.

It is important that the VIDOMA team members or delegated persons who collect, enter and score the 4DFRs and DICE use the same procedure to ensure continuity and consistency in dietary assessments.

4DFR and DICE are administered to determine the participants' (children with ASD) dietary adequacy and nutritional status at the baseline, and to validate the DICE against 4DFR (as the reference method).

Definitions

- **4-day estimated food record (4DFR):** The 4DFR is a self-reported account of all foods and beverages (and possibly, dietary supplements) consumed by a respondent (children with ASD) over 4 days.

- **Dietary Index of Children's Eating (DICE):** The DICE is an assessment tool that evaluates the quality of diet and consists of 17 questions. The DICE components, scoring, and cut-off points are based on the NZ Ministry of Health (MoH) Food and Nutrition guidelines for 2-18 years old [1].

- **Foodworks software:** Foodworks is a nutritional analysis software. Foodworks Professional Edition 7 (Xryis Software, Brisbane, QLD, Australia, 2013) is used for the purpose of this study.

- **Food composition database:** Food composition database provides information regarding the nutrient content of foods. New Zealand FOODfiles 2016 is used for the purpose of this study.

- **New Zealand FOODfiles:** New Zealand FOODFiles provide the information regarding the nutritional composition of foods commonly prepared and consumed in New Zealand. For the purpose of this research the New Zealand FOODfiles 2016 (data is presented for 2,631 foods) is used. To note, some records do not contain values for every nutritional component for every food.

- **Weight yield:** Weight yield refers to how much of a food remains after cooking/processing. Reliable sources (e.g. USDA, FAO) providing weight yield tables are used for the purpose of this study.

- **Retention factors:** Retention factors are a way of calculating the amount of nutrients in foods that may have been lost or gained during the process of cooking and preparation. The retention factors provided by foodworks software are used for the purpose of this study.

Responsibilities

- The members of the VIDOMA team and designated persons are responsible for the following SOP.

- The lead researcher is responsible for ensuring that this SOP is followed.

Procedure

Prior to data collection

- Ask the VIDOMA team members who are involved in dietary assessments to read this SOP.

- Highlight the importance of complete 4DFR and DICE assessments.

- Train the VIDOMA team to consider the following points when checking 4DFRs (two trained dietitian and two trained nutritionists):
    - Time food eaten
    - Type of food eaten
    - Amount of food eaten (preferably weighed otherwise estimated)

- Brand name of foods eaten or ingredients used in recipes.
- Recipe (ingredients of mixed dishes) and food preparation methods of all foods and beverages, the number of portions, and how much of that portion was eaten by children.
- Type and brand of supplements (if used by a child).

- Provide the assumptions record booklet. Ask the researchers to record all assumptions they make while entering the foods in foodworks software.

- Ask the researchers to use only one food composition database; New Zealand FOODfile 2016.

- Ask the researchers to consider weight yield using reliable sources [2-4] when entering recipes.

- Train the researchers to consider retention factors provided by foodworks software when entering recipes.

- Familiarise the researchers with the DICE and the format of questions and why it is used.

Data collection

- Send the 4DFR to participants (parents/caregivers of children with ASD) prior to their visit at Massey University.

- Arrange a visit at Massey University for parents/caregivers and their children within one to four weeks of their receipt of 4DFR.

- Give instructions on the phone and accompany the written instructions with the 4DFR. In order to simplify estimates of portion size, caregivers to be asked to use household measures (e.g. cups, teaspoons, tablespoons), weights marked on food packages, and comparisons (e.g. a scoop of ice cream equal to the size of a hen's egg).

- Ask participants (parents/caregivers of children with ASD) to complete the 4DFR considering 4 consecutive days (3 working days and one weekend day; e.g. Wednesday, Thursday, Friday and Saturday OR Sunday, Monday, Tuesday, and Wednesday).

- Explain the importance of precise information and following normal dietary habits during the study to parents/caregivers.

- Ask parents/caregivers to call or email you if they have any question or if they are unsure how to complete the food record.

- Ask parents/caregivers to bring their children completed 4DFR to their appointment with the VIDOMA team at Massey University.

- Check the 4DFR during parents/caregivers' visit and ask for more information or clarification if needed. Check the information regarding time, type and amount of food eaten, brand name and if possible, the pocket of product if it is not home-made. Check the recipes, ingredients, the brand and type of ingredients, the number of portions made with that recipe and how much of that portion was eaten by child. Ask parents/caregivers if the child is on any dietary supplement (brand name and type).

- If any extra information is provided by email or a phone call, copy the email or record the phone call on paper and attach it to the 4DFR.

- Make sure the child was not sick during the 4-day food records.

- Ask parents/caregivers to complete the DICE (on the computer) during their visit at Massey University, and clarify any points if is needed.

Data entry

- Use the Foodworks software (Foodworks Professional Edition 7 (Xryis Software, Brisbane, QLD, Australia, 2013).

- Use the New Zealand FOODfiles, 2016.

- Find the foods in the database. Consider similar foods if a food is not included in the database.

- Enter recipes and consider weight yield and retention factors (from sources reported above).

- If any assumptions are made, record them in the assumption record booklet so that other researchers have access to those assumptions (for the sake of consistency).

- Two other researchers (two trained nutritionists) check the data entry through careful investigation of foods and quantities entered and look for unusual values when the data is exported to Excel spreadsheet. In case of finding any unusual values (very high or very low values; ±2SD) double check 4DFR and foodworks for the unusal case to see if foods have been entered accurately.

- Check for under-reporters using the following method [5]. If the energy intake is under-reported, exclude that case.
    - Determine the energy requirements based on Nutrient Reference values for Australia and New Zealand children. These are estimated for each child using the following calculation: multiple basal metabolic rate (BMR, the Schofield equations based on height and weight) by physical activity level (PAL, sedentary)
    - Calculate the ratio of measured energy intake (from 4DFR) to estimated requirement (BMR*PAL)
    - Use the 95 per cent cut-off point as under reporters.

- Check the DICE for completeness and ask for clarification if needed.

Scoring

- Prior to scoring, the following information should be extracted from 4DFRs:
    - Number of serves of fruit; averaged over four days.
    - Number of serves of vegetables; averaged over four days.
    - Variety of fruits (5 different colours): the number of different colours over four days.
    - Variety of vegetables (5 different colours); the number of different colours over four days.
    - Number of serves of bread/cereals; averaged over four days.
    - Consumption of wholegrain bread/cereals; the number of times wholegrain options consumed over four days.
    - Number of serves of milk/milk products; averaged over four days
    - Consumption of low fat milk/milk products; the number of times low fat milk/milk products consumed over four days.
    - Number of serves of meat/alternatives; averaged over four days.

- Consumption of fluid; all beverages consumed over the four days.
- Number of meals and snacks; averaged over four days.
- Consumption of low sugar foods/snacks/drinks; averaged over four days and to be obtained from the Foodworks analysis of the 4DFR. The World Health Organisation (WHO) recommends limiting sugar to <10% of total energy intake [6].
- Consumption of low fat foods/snacks/drinks: averaged over four days and to be obtained from the Foodworks analysis of the 4DFR. The NZ MoH Food and Nutrition guidelines recommend that <10% of total energy should be provided from saturated fatty acids [1].
- Consumption of low sodium foods/snacks/drinks: averaged over four days and to be obtained from the Foodworks analysis of the 4DFR. According to the guidelines, the adequate intake of sodium for children 2-3 years old is 200-400 mg and for 4-8 years old is 300-600 mg [1].

- Table 1 provides the scoring systems for both DICE and 4DFR. Use the table below to score both dietary assessments and record all scores obtained from the DICE and 4DFR in an Excel spreadsheet.

Precautions

- Participant's confidentiality: make sure that all information provided by parents/caregivers are confidential and assure parents/caregivers of that.

- Keep all food records in a secured cabinet.

- Participant's burden: Participants' comfort is of high importance to us. Try not to increase parents/caregivers' burden by too many contact/phones/emails. Ask for information on three occasions and if there is no response don't follow up.

- Keep children entertained while parents/caregivers completing the DICE during their visit at Massey University.

- Ensure the dietary assessments are complete and the child was not sick during the 4-day food records.

References

1. Ministry of Health (MoH), Food and nutrition guidelines for healthy children and young people (aged 2–18 years): A background paper. 2012.
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5. Goldberg, G.R., *et al.*, Critical evaluation of energy intake data using fundamental principles of energy physiology: 1. Derivation of cut-off limits to identify under-recording. Eur. J. Clin. Nutr., 1991. **45**(12): p. 569-81.
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Table 1: Components of the DICE and 4DFR and their scoring system

Components	Dietary guideline for NZ	DICE & 4DFRs	DICE			4DFR		
		Score range	Criteria for minimum score	Criteria for intermediate range scores	Criteria for maximum score	Criteria for minimum score	Criteria for intermediate range scores	Criteria for maximum score
Serves of fruits	Include all fruits fresh, frozen canned, no-sugar-added fruit juice or dried fruit	0-10	0 serve/d=0	0.5 serve/d=2.5 1 serve/d=5 1.5 serves/d=7.5	≥ 2 serves/d=10	0 serve/d=0	0.5 serve/d=2.5 1 serve/d=5 1.5 serves/d=7.5	≥ 2 serves/d=10
Serves of vegetables	Include all vegetables fresh, frozen or canned	0-10	0 serves/d	0.5 serve/d=2.5 1serve/d=5 1.5 serves/d=7.5	≥ 2 serves/d	0 serves/d	0.5 serve/d=2.5 1serve/d=5 1.5 serves/d=7.5	≥ 2 serves/d
Variety of fruits	How many different colours of all fruits fresh, frozen canned, no-sugar-added fruit juice or dried fruit	0-5	Not consuming any colour=0	1 colour=1 2 colours=2 3 colours=3 4 colours=4	5 different colours=5	Not consuming any colour=0	1 colour=1 2 colours=2 3 colours=3 4 colours=4	5 different colours=5
Variety of vegetables	How many different colours of all vegetables- fresh, frozen or canned	0-5	Not consuming any colour=0	1 colour=1 2 colours=2 3 colours=3 4 colours=4	5 different colours=5	Not consuming any colour=0	1 colour=1 2 colours=2 3 colours=3 4 colours=4	5 different colours=5
Serves of bread and cereals	Include breads, cereals, rice, and pasta	0-10	0 serve/d=0	1 serve/d=2.5 2 serves/d=5 3 serves/d=7.5	≥ 4 serves/d=10	0 serve/d=0	1 serve/d=2.5 2 serves/d=5 3 serves/d=7.5	≥ 4 serves/d=10
Wholegrain products*	Include wholegrain versions of breads, cereals, rice, and pasta	0-5	Never & rarely=0	Some days=2.5	Most days & Every day=5	Never & rarely	Some days=2.5	Most days & Every day=5

Table 1: *Cont.*

Components	Dietary guideline for NZ	DICE & 4DFRs	DICE			4DFR		
		Score range	Criteria for minimum score	Criteria for intermediate range scores	Criteria for maximum score	Criteria for minimum score	Criteria for intermediate range scores	Criteria for maximum score
Serves of dairy products	**Include milks, yoghurts, cheeses and/or alternatives**	**0-10**	**0 serve/d=0**	**1.5 serves/d=5**	**≥ 3 serves/d=10**	**0 serve/d=0**	**1.5 serves/d=5**	**≥ 3 serves/d=10**
Serves of low fat dairy product	Include low fat milks, yoghurts, cheeses and/or alternatives	0-5	Never & rarely=0	Some days=2.5	Most days & Every day=5	Never & rarely=0	Some days=2.5	Most days & Every day=5
Serves of lean meat, poultry, seafood, eggs, legumes, nuts and seeds	Include lean meat, poultry, seafood, eggs, legumes (e.g. peas, beans, lentils), nuts and seeds (limit processed meats)	0-10	0 serve/d=0	-	≥ 1 serve/d=10	0 serve/d=0	-	≥ 1 serve/d=10
Number of meals and snacks per day	Include breakfast, morning snacks, lunch, afternoon snacks, and dinner	0-5	< 3 meals or/and snacks/d=0	-	≥ 3 meals or/and snack/d=5	< 3 meals or/and snacks/d=0	-	≥ 3 meals or/and snack/d=5

Table 1: *Cont.*

Components	Dietary guideline for NZ	DICE & 4DFRs	DICE			4DFR		
		Score range	Criteria for minimum score	Criteria for intermediate range scores	Criteria for maximum score	Criteria for minimum score	Criteria for intermediate range scores	Criteria for maximum score
Fluid and other drinks	Include water and reduced or low fat milk Limit fruit juice or/and flavoured milk	0-10	Other drinks**=0	-	Only water & milk ≥ 1 serve/d=10	Other drinks**=0	-	Only water & milk ≥ 1 serve/d=10
Prepare low fat foods, snacks, and drinks, especially saturated fat	Include low fat foods, snacks and drink	0-5	Never & rarely=0	Some days=2.5	Every day & most days=5	SFAs ≥ 10%=0	-	SFAs < 10% of energy=5
Prepare low salt foods, snacks, and drinks	Include low salt foods, snacks and drink	0-5	Never & rarely=0	Some days=2.5	Every day & most days=5	Sodium >200-400 mg/d (2-4 years old) & >300-600 mg/d (4-8 years old)=0	-	Sodium <200-400 mg/d (2-4 years old) & <300-600 mg/d (4-8 years old)=5
Prepare low sugar foods, snacks, and drinks	Include low sugar foods, snacks and drink	0-5	Never & rarely=0	Some days=2.5	Every day & most days=5	Sugar† ≥ 10% of energy=0	-	Sugar † < 10% of energy=5

DICE, Dietary Index of Children's Eating, 4DFR, 4-day estimated food record; SFAs, saturated fatty acids.

**Whole wheat, whole-wheat flour, wheat flakes, bulgur wheat, whole and rolled oats, oatmeal, oat flakes, brown rice, whole rye and rye flour, whole barley and popcorn are considered as wholegrain component. * Energy drinks, cordial, fizzy drinks, sport drinks, sports water, tea, coffee; †Sugar comprises of glucose, sucrose and maltose (fructose and lactose were not included because they are considered intrinsic sugars).

Chapter 5

Chapter 5 – Appendix 1: Characteristics of children who completed the study (*n*=73) across treatment groups

Characteristics *	**VID (*n*=19)**	**OM (*n*=23)**	**VIDOM (*n*=15)**	**Placebo (*n*=16)**	***P*-value†**
Age, years	5.3±1.5	4.8±1.5	5.4±1.3	5.7±1.0	0.25
Sex, *n* (%)					0.92
Male	16 (84)	18 (78)	13 (87)	13 (81)	
Female	3 (16)	5 (22)	2 (13)	3 (19)	
BMI–for–age categories, *n* (%)					0.57
Underweight (<5th %ile)	2 (11)	0 (0)	1 (7)	0 (0)	
Normal weight (≥5th – 85th %ile)	13 (68)	17 (74)	9 (60)	8 (50)	
Overweight (≥85th – 95th %ile)	2 (11)	3 (13)	3 (20)	5 (31)	
Obese (≥95 %ile)	2 (11)	3 (13)	2 (13)	3 (19)	
Ethnicity, *n* (%)					0.17
NZ European	11 (58)	9 (41)	10 (67)	6 (37)	
Māori	3 (16)	5 (23)	1 (7)	0 (0)	
Pacific Island	0 (0)	0 (0)	0 (0)	2 (13)	
Asian	3 (16)	5 (23)	1 (7)	4 (25)	
Others	2 (11)	3 (14)	3 (20)	4 (25)	
Country of birth, *n* (%)					0.09
New Zealand	18 (95)	21 (91)	15 (100)	12 (75)	
Others **	1 (5)	2 (9)	0 (0)	4 (25)	
Season of birth, *n* (%)					0.92
Summer	5 (26)	6 (26)	5 (33)	6 (38)	
Autumn	4 (21)	3 (13)	3 (20)	3 (19)	
Winter	5 (26)	7 (30)	2 (13)	5 (31)	
Spring	5 (26)	7 (30)	5 (33)	2 (13)	

Chapter 5 – Appendix 1: *Cont.*

Characteristics *	**VID (*n*=19)**	**OM (*n*=23)**	**VIDOM (*n*=15)**	**Placebo (*n*=16)**	***P*-value†**
Socioeconomic status, parent's household income, *n* (%)					0.33
<60,000 NZ$	3 (17)	5 (26)	3 (21)	5 (33)	
60,000 to 120,000 NZ$	9 (50)	12 (63)	6 (43)	9 (60)	
>120,000 NZ$	6 (33)	2 (11)	5 (36)	1 (7)	
Season of enrolment, *n* (%)					0.72
Summer	5 (26)	6 (26)	1 (7)	2 (13)	
Autumn	5 (26)	9 (39)	4 (27)	6 (38)	
Winter	5 (26)	5 (22)	6 (40)	6 (38)	
Spring	4 (21)	3 (13)	4 (27)	2 (13)	
ASD severity (clinical diagnosis), *n* (%)					0.93
Mild	9 (47)	11 (48)	7 (47)	5 (31)	
Moderate	7 (37)	10 (44)	6 (40)	8 (50)	
Severe	3 (16)	2 (9)	2 (13)	3 (19)	
Period on study, weeks	55 (53, 57)	53 (52, 57)	54 (53, 57)	54 (53, 57)	0.59
Compliance, %	95 (92, 96)	95 (90, 97)	90 (77, 94)	94 (92, 97)	0.09
Therapy, *n* (%)					0.46
No therapy	9 (48)	13 (55)	6 (36)	10 (60)	
≤ 6 months	5 (26)	4 (18)	3 (21)	5 (33)	
>6 months to ≤12 months	5 (26)	6 (27)	6 (43)	1 (7)	
Medication use, *n* (%)					0.16
No	14 (74)	19 (83)	10 (67)	15 (94)	
Yes	5 (26)	4 (17)	5 (33)	1 (6)	

VID, vitamin D; OM, omega-3; VIDOMO, vitamin D+omega-3; BMI, body mass index (kg/m^2)9

†X^2 test for categorical data and analysis of variance (ANOVA) and Kruskal Wallis tests for normally and not normally distributed data, respectively. * Where n (%) is reported, the percentage within each treatment group is reported.

Chapter 5 – Appendix 2: Types of behavioural interventions and medications reported by parents over the study period.

Types of Behavioural interventions	Number of children*	Types of medications	Number of children**
Speech and language therapy	14	Melatonin	13
Occupational therapy	11	Risperidone	3
Applied Behavioural Analysis (ABA)	7	Fluoxetine	2
Transdisciplinary based intervention	9	Clonidine	2
Music therapy	2	Methylphenidate	One
Riding for disabled	1		
Physiotherapy	1		
Gym therapy	1		
Intervention based on investigation of neurological disorganisation	1		
Homeopathy	1		
Emotional regulation	1		
Sensory intervention	1		

*Some children were reported having a combination of the mentioned behavioural interventions (e.g. a combination of speech and language therapy and occupational therapy). The duration of therapy: speech and language therapy (9-12 months, 3; 6-9 months, 2; 3-6 months, 4; less than 3 months, 5); occupational therapy (9-12 months, 2; 6-9 months, 2; 3-6 months, 3; less than 3 months, 4); ABA (9-12 months, 2; 6-9 months, 2; 3-6 months, 1; less than 3 months, 3); transdisciplinary based intervention (9-12 months, 1; 6-9 months, 2; 3-6 months, 1; less than 3 months, 5); music therapy (6-9 months, 1; 3-6 months, 1); riding for disabled (9-12 months, 1); physiotherapy (9-12 months, 1); gym therapy (9-12 months, 1); intervention based on investigation of neurological disorganisation (9-12 months, 1); homeopathy (less than 3 months, 1); emotional regulation (9-12 months, 1); sensory intervention (9-12 months, 1)

**Some children were reported taking a combination of the mentioned medications mostly melatonin with other medications (n=5) and two children clonidine with other medications.

Chapter 5 – Appendix 3: Baseline socio-demographic and behavioural characteristics of children who completed the study (*n*=73) and those who were lost to follow up (*n*=44)

Variables	Completers	Non-completers	*P*-value†
Treatment groups, *n* (%)			0.16
VID	19 (61)	12 (39)	
OM	23 (79)	6 (21)	
VIDOM	15 (54)	13 (46)	
Placebo	16 (55)	13 (45)	
Socio-demographic characteristics			
Age, years	5.28±1.40	5.19±1.49	0.76
Sex, *n* (%)			0.20
Male	60 (60)	40 (40)	
Female	13 (76)	4 (24)	
BMI–for–age categories, *n* (%)			0.15
Underweight (<5th %ile)	3 (50)	3 (50)	
Normal weight (≥5th – 85th %ile)	47 (69)	21 (31)	
Overweight (≥85th – 95th %ile)	13 (65)	7 (35)	
Obese (≥95 %ile)	10 (44)	13 (56)	
Socioeconomic status, parent's household income, *n* (%)			0.11
<60,000 NZ $	16 (52)	15 (48)	
60,000 to 120,000 NZ $	36 (61)	23 (39)	
>120,000 NZ $	14 (82)	3 (18)	
Ethnicity, *n* (%)			<0.0001
NZ European	36 (63)	21(34)	
Māori	9 (60)	6 (40)	
Pacific Island	2 (15)	11(85)	
Asian	13 (100)	0 (0)	
Others	12 (67)	6 (33)	
Behavioural characteristics			
ASD severity (clinical diagnosis), *n* (%)			0.27
Mild	32 (68)	15 (32)	
Moderate	31 (63)	18 (37)	
Severe	10 (48)	11 (52)	
Social Responsiveness Scale (SRS)			
Total	101±26	120±25	<0.0001
Social-communicative functioning	82±21	96±21	<0.0001
Social awareness	13±3.9	15±3.5	0.002
Social cognition	20±5.6	23±5.0	0.04*
Communication	34±10	39±8.9	0.004*
Social motivation	15±5.6	19±7.7	0.001
RRB	20±6.1	24±6.4	0.001*
Sensory Processing Measure (SPM)			
Total	113±26	127±29	0.006
Vision	22±6.6	26±6.7	0.001
Hearing	18±6.2	20±7.0	0.05
Touch	26±8.8	30±9.1	0.02*
Taste and smell	9.8±3.4	9.2±3.0	0.51
Body awareness	20±36	22±5.6	0.04*
Balance and motion	19±5.3	20±5.4	0.64*
Biochemical markers			
Serum 25(OH)D (nmol/L)	62±24	53±28	0.09
Omega-3 index (%)**	4.8 (4.4, 5.3)	4.0 (3.8, 4.3)	0.001

VID, Vitamin D; OM, Omega-3; VIDOM, Vitamin D+Omega-3; RRB, repetitive and restricted interests and behaviours; 25(OH)D, 25-hydroxyvitamin D

Values are reported as mean±SD, unless otherwise stated. †X^2 tests for categorical data and independent samples t-test and Mann-Whitney U test for normally and not normally distributed continuous data, respectively. * Not normally distributed but for the sake of consistency mean±SD reported. ** Median (25th, 75th percentile) and non-parametric test was used to compare groups.

Chapter 5 – Appendix 4: Gastrointestinal symptoms and other adverse events reported over the study period across treatment groups (*n*=73)

Adverse events reported by parents/caregivers	Treatment groups				Total
	VID	OM	VIDOM	Placebo	
Gastrointestinal symptoms *					
Diarrhoea	2	4	3	1	10
Constipation	0	3	1	4	8
Nausea/vomiting	1	0	0	0	1
Abdominal pain	6	2	2	3	13
Excessive wind	2	1	3	0	6
Bloating	1	0	0	0	1
Other adverse events					
Allergic reaction	0	5	1	0	6
Headache	0	2	1	0	3
Increased urination/bedwetting	1	3	1	2	7
Hyperactivity	1	1	1	1	4
Nail problems	0	1	1	0	2
Nose-bleeds	1***	1	0	0	2

VID, Vitamin D; OM, Omega-3; VIDOM, Vitamin D+omega-3
*Number of children reported here if their parents reported a gastrointestinal symptom for more than four weeks, though at irregular intervals. **Nail problems included white lines on nail, nail picking. ***The child had nose-bleeds for two weeks toward the end of study.

Chapter 6

Chapter 6 – Appendix 1: Baseline characteristics of children who completed the study (*n*=73) and those who were lost to follow up (*n*=38)

Variables	**All children (*n*=111)**	**Completers (*n*=73)**	**Non-completers (*n*=38)**	***P*-value***
Treatment groups, *n* (%)				0.20
VID	30 (27)	19 (63)	11 (37)	
OM	28 (25)	23 (82)	5 (18)	
VIDOM	25 (23)	15 (60)	10 (40)	
Placebo	28 (25)	16 (57)	12 (43)	
Socio-demographic characteristics				
Age, years	5.3±1.4	5.3±1.4	5.3±1.5	0.95
Sex, *n* (%)				0.16
Male	95 (86)	60 (63)	35 (37)	
Female	16 (14)	13 (81)	3 (19)	
Ethnicity, *n* (%)				0.60
NZ European	53 (48)	36 (68)	17 (32)	
Others	57 (52)	36 (63)	21 (37)	
BMI–for–age categories, *n* (%)				0.17
< 85th %ile (underweight or normal)	71 (64)	50 (70)	21 (30)	
≥85th – 95th %ile (overweight or obese)	40 (36)	23 (56)	17 (43)	
Behavioural characteristics				
ASD severity (clinical diagnosis), *n* (%)				0.36
Mild	45 (41)	32 (71)	13 (29)	
Moderate	47 (42)	31 (66)	16 (34)	
Severe	19 (17)	10 (53)	9 (47)	

Chapter 6 – Appendix 1: Cont.

Variables	All children (*n*=111)	Completers (*n*=73)	Non-completers (*n*=38)	*P*-value*
Aberrant Behaviour Checklist (ABC)				
Irritability	17±8.6	14±8.1	21±8.1	<0.001
Hyperactivity	23±11	20±11	29±8.9	<0.001
Lethargy	12±8.6	9.6±7.3	16±9.2	<0.001
Stereotypy	5.3±4.4	4.2±3.6	7.4±5.0	<0.001
Inappropriate speech	4.5±3.2	3.8±2.8	5.9±3.6	<0.01
Biochemical markers				
Serum 25(OH)D (nmol/L)	59±26	62±24	54±28	0.12
Omega-3 index (%) **	4.6 (4.3, 4.9)	4.7 (4.4, 5.3)	4.0 (3.7, 4.3)	<0.01

VID, Vitamin D; OM, Omega-3; VIDOM, Vitamin D+Omega-3; RRB, repetitive and restricted interests and behaviours; 25(OH)D, 25-hydroxyvitamin D
Values are reported as mean±SD, unless otherwise stated. * Comparison of characteristics between completers and non-completers; X^2 tests for categorical data and independent samples t-test and Mann-Whitney U test for normally and not normally distributed continuous data, respectively. ** Median (25^{th}, 75^{th} percentile) and non-parametric test was used to compare groups.

Chapter 6 – Appendix 2: Reasons for exclusion/drop-outs

Reasons for exclusion	**Number**	**Number within each treatment group**
Reasons for exclusion/not being enrolled before screening for nutritional deficiencies	190	NA
Not meeting initial inclusion/exclusion criteria (*n*=40)	40	NA
Not being able to travel to testing centres (*n*=22)	22	NA
Fear of blood test (*n*=11)	11	NA
Unwilling to be assigned to placebo (*n*=9)	9	NA
Language barrier (*n*=3)	3	NA
Family strains (*n*=6)	6	NA
Lost to follow up with no reason (*n*=99)	99	NA
Reasons for exclusion after screening for nutritional deficiencies	2	NA
High serum 25(OH)D concentration	1	NA
Unsuccessful to take corrective actions for nutritional deficiencies (iron deficiency)	1	NA
Reasons for exclusion/drop-out after randomisation	44	
Not completing baseline assessments	6	VID 1; OM 1; VIDOM 3; placebo 1
Concerns about side effects and having a blood sample taken	4	VID 2; OM 1; VIDOM 0; placebo 1
Family and time constraints	12	VID 3; OM 2; VIDOM 4; placebo 3
Child disliking the supplement	7	VID 1; OM 0; VIDOM 4; placebo 2
Not seeing any benefits	2	VID 1; OM 0; VIDOM 0; placebo 1
Moving overseas	2	VID 2; OM 0; VIDOM 0; placebo 0
Child being diagnosed with other medical conditions during the study period	1	VID 1; OM 0; VIDOM 0; placebo 0
No reason	16	VID 3; OM 2; VIDOM 5; placebo 6

OM, omega-3; NA, not applicable; VID, vitamin D; VIDOM, vitamin D+omega-3

Chapter 7

Chapter 7 – Appendix 1: Conference presentation II (Poster)

MASSEY UNIVERSITY TE KUNENGA KI PŪREHUROA UNIVERSITY OF NEW ZEALAND — THE ENGINE OF THE NEW NEW ZEALAND

VITAMIN D STATUS AND ITS PREDICTORS IN CHILDREN WITH AUTISM SPECTRUM DISORDER

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INTRODUCTION

Low vitamin D status in utero, postnatal and in early childhood has been hypothesized as a risk factor for neurodevelopmental disorders, specifically ASD, and a large number of observational studies report ASD children having low vitamin D status [1]. Children with ASD are likely to have medical conditions and use medications – that affect vitamin D metabolism or absorption – or to have inadequate intake of vitamin D rich foods. However, the role of socio-demographic factors in vitamin D status of children with ASD has not been well investigated. In New Zealand, there is no research reporting vitamin D status and its predictors in children with ASD.

AIMs

Using the baseline data from the Vitamin D and Omega-3 in ASD intervention trial (VIDOMA) [2], we aimed to investigate vitamin D status and predictors of serum 25-hydroxyvitamin D [25(OH)D] concentrations in children with ASD living in New Zealand..

METHOD

Serum 25(OH)D concentrations were measured in ASD children aged 2.5 to 8 years old (*n*=117). Parents/caregivers completed questionnaires including socio-demographic, medication use, and supplement use. Children's intake of vitamin D rich foods was assessed using 4-day food diary and their weight and height were measured to calculate BMI (percentiles calculated for age and sex). We examined predictors of serum 25(OH)D concentrations using a general linear mixed model.

RESULTS

CHILDREN'S DEMOGRAPHICS

Children's demographic characteristics are presented in table 1.

TABLE 1: Children's demographics (n=117)

Age (mean±SD)	5.2±1.4 years
Male/female ratio	5.6 to 1
Overweight/obesity (≥85th percentiles).(n=114), n (%)	39 (34)
Season of enrolment , n (%) Summer and autumn Winter and spring	 58 (49.6) 59 (50.4)
Ethnicity, (n=115), n (%) New Zealand European Others Asian Maori Pacific	 58 (50.4) 18 (15.7) 13 (11.3) 14 (12.2) 12 (10.4)
Vitamin D supplement (over the counter) use, n (%)	12 (10.3)
Multivitamin containing vitamin D use, n (%)	18 (15.4)
Oily fish consumption ≥1 serving (n=90), n (%)	9 (10);
Fortified milk (n=90), n (%)	0 (0)

VITAMIN D STATUS OF CHILDREN WITH AUTISM SPECTRUM DISORDER

Children had a mean±SD serum 25(OH)D concentration of 59.2±26.8 nmol/L. Most (73%) of children had serum 25(OH)D concentrations <75 nmol/L (<50 nmol/L, 41%; <25 nmol/L, 10%).

PREDICTORS OF SERUM 25(OH)D CONCENTRATION

- Season of blood collection (*B*-coefficient ±s.e; -26.7±4.0, P<0.001): serum 25(OH)D is expected to be lower by 26.7 nmol/L if the blood collection is done in the winter and spring months (Figure 1).
- Vitamin D supplements (21.4±6.6, P<0.01): serum 25(OH)D is expected to be lower by 21.4 nmol/L in children not taking vitamin D supplements purchased over the counter (Figure 2).
- Vitamin D containing multivitamins (13.9±5.5, P=0.01): serum 25(OH)D is expected to be lower by 13.9 nmol/L in children not taking vitamin D containing multivitamins (Figure 3).
- Ethnicity (Pacific, -20.1±6.7, P<0.01; others, -15.0±6.5, P,0.01; Asian, -16.0±5.7, P=0.02; Maori, -12.0±6.2, P=0.06): serum 25(OH)D is expected to be lower by 20.1 nmol/L in Pacific children, by 15.0 nmol/L in children of other ethnicities, by 16.0 nmol/L in Asian children, and by 12.0 nmol/L in Maori children compared to New Zealand European children (Figure 1).

FIGURE 1: The effect of ethnicity stratified by season of enrolment on serum 25(OH)D concentrations

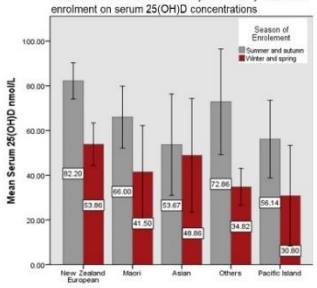


FIGURE 2: The effect of over the counter vitamin D supplements on serum 25(OH)D concentrations

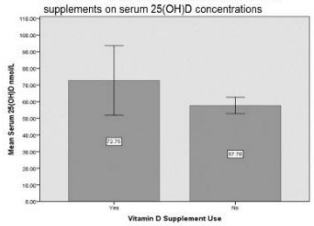
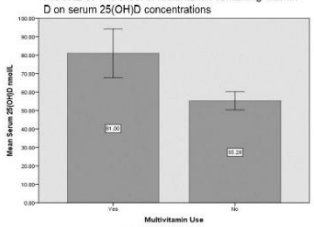


FIGURE 3: The effect of multivitamin containing vitamin D on serum 25(OH)D concentrations



DISCUSSION/CONCLUSION

The proportion of children with serum 25(OH)D concentrations <75 nmol/L appears high but cannot be compared to normal developing children due to lack of NZ data. However, it appears to be lower than normal developing preschool children and comparable to school aged children (5-14 years old) [3, 4]. It is important to note that this indirect comparisons should be interpreted with cautious because participants in different conditions should be matched in terms of any important characteristics that might affect serum 25(OH)D concentrations (e.g. age, sex, and ethnicity). Predictors of lower serum 25(OH)D concentrations were consistent with those identified in normal developing New Zealand children. However, neither age and sex nor the consumption of vitamin D rich foods was associated with serum 25(OH)D concentration. It is worth noting that food sources of vitamin D are limited unless they are fortified with vitamin D, which is not compulsory in New Zealand (e.g. fortification of dairy products). The future findings of the VIDOMA trial should clarify the optimum vitamin D status for children with ASD, however, evidence to date suggests that this target is ≥75 nmol/L. Meanwhile, given the findings of this study, it would seem prudent for paediatricians to include 25(OH)D in their biochemical assessments of children with ASD, and to be prepared to prescribe adequate vitamin D supplementation to achieve concentrations ≥75 nmol/L.

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Ethical approval was granted by Health and Disability Ethics Committees, NZ, Reference No. 14/NTA/113.

Chapter 7 – Appendix 2: Conference presentation IV (Poster)

UNIVERSITY OF NEW ZEALAND

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OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS MAY MODULATE THE EFFECT OF VITAMIN D SUPPLEMENTATION ON VITAMIN D STATUS IN CHILDREN WITH AUTISM SPECTRUM DISORDER

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INTRODUCTION

Because vitamin D is a fat-soluble vitamin, dietary fat is required to enhance its absorption. However, certain fatty acids (such as n-3 long chain polyunsaturated fatty acids) may interfere with vitamin D absorption..

AIMs

As an exploratory analysis of the VIDOMA trial (Vitamin D and Omega-3 in Autism Spectrum Disorder [ASD]), we investigated the hypothesis that long-term (12-month) docosahexaenoic acid (DHA) supplementation may reduce the effect of vitamin D supplementation on serum 25-hydroxyvitamin D [serum-25(OH)D] in children with ASD

METHOD

New Zealand children with ASD (aged 2.5-8 years) participated in a 12-month randomized, double-blind, placebo-controlled, 2x2 factorial trial of vitamin D (2000 IU/day), DHA (722 mg/day), both supplements, or placebo. The outcome measure was serum-25(OH)D concentration. Venous blood samples were collected at baseline, 6 months and 12 months. Ethical approval was granted by Health and Disability Ethics Committees, NZ, Reference No. 14/NTA/113.

RESULTS

Children's demographics

Out of 117 enrolled children, Serum 25(OH)D for both baseline and 12-month visits were available for 57 children (vitamin D 14; DHA 20; both 9; placebo 14). Children's demographic characteristics across treatment groups are presented in **Table 1**..

TABLE 1: Children's demographics (n=57)*

	Vitamin D	DHA	Both	Placebo
Age (years), mean±SD	5.3±1.5	5.0±1.6	5.6±1.2	5.9±1.0
Male/female, %	93/7	75/25	100/0	79/21
BMI (kg/m), mean±SD	22.1±5,3	20.0±4.3	23.3±5.0	24.1±5.3
Season of enrolment , n (%)				
Summer and autumn	8 (57)	14 (70)	2 (22)	7 (50)
Winter and spring	6 (43)	6 (30)	7 (78)	7 (50)
Ethnicity, n (%)				
New Zealand European	9 (64)	9 (47)	6 (67)	5 (36)
Others	5 (36)	10 (53)	3 (33)	9 (64)
Compliance, mean±SD	92.1±8.1	91.3±8.6	85.2±9.8	94.4±4.7

*Baseline characteristics and compliance were equally distributed across treatment groups (P>0.05).

Change in serum 25(OH)D concentration over the 12 months period

- Mean serum 25(OH)D increased by 27±14, 1±14, 36±17, and 9±23 nmol/L at the 12-month visit in vitamin D, DHA, both, and placebo groups, respectively **(Figure 1)**.
- The proportion of children with 25(OH)D ≥75 nmol/L in vitamin D and both groups increased from 29% and 9% at baseline to 71% and 100% at the 12-month visit, respectively. In DHA and placebo groups, the proportion increased from 30% and 14% at baseline to only 35% and 21% at the 12-month visit, respectively (P<0.001).

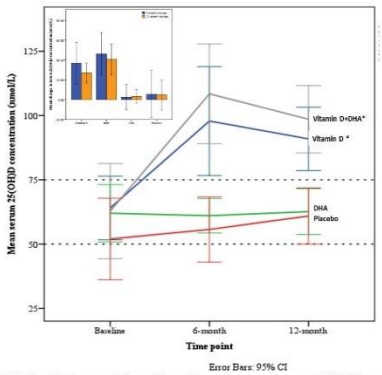

FIGURE 1: Graphical presentation of the pattern of change in serum 25(OH)D concentration over the study period (baseline, 6-months and 12 month) within each of the treatment groups. Reference lines at 50 and 75 nmol/l (dotted lines) were added for clarification.
* Significantly different from placebo at 6-month (vitamin D+DHA, P<0.01 and vitamin D, P=0.02) and 12-month visits (vitamin D+DHA, P<0.01 and vitamin D, P=0.01).

Predictors of change in serum 25(OH)D concentration after 12 months

Predictors of change in serum 25(OH)D concentration after 12 months are presented in Table 2. Together, the variables (treatment groups, baseline serum 25(OH)D and compliance) accounted for 66% of variance.

TABLE 2: Predictors of change in serum 25(OH)D concentration after 12 months †

Change in serum 25(OH)D concentration	Coefficient (B)	Standard Error (B)	95% CI B	Standardized B	R^2	P-value
Model					0.66*	<0.0001
Intercept	0.55	26.28	-52.29, 53.39			
Group, DHA	-26.46	4.87	-36.26, -16.66	-0.60		<0.0001
Baseline serum 25(OH)D	-0.44	0.08	-0.60, -0.29	-0.49		<0.0001
Group, placebo	-24.07	5.72	-35.57, -12.57	-0.48		<0.0001
Group, both	13.75	6.23	1.23, 26.27	0.23		0.03
Compliance	0.54	0.24	0.06, 1.03	0.21		0.03

†Enter technique. In the model, treatment groups (3 dummy variables; 1 placebo 0 otherwise; 1 DHA, 0 otherwise; 1 both, 0 otherwise), baseline serum 25(OH)D, compliance, baseline BMI, age, and sex were included.
*F (8, 56) = 11.83, P<0.0001

- Receiving DHA was associated with a lower change of 26.46 nmol/L (as compared with vitamin D) in serum 25(OH)D concentration after 12 months
- Each unit (nmol/L) decrease in baseline serum 25(OH)D was associated with an increase of 0.44 nmol/L in serum25(OH)D concentration after 12 months.
- Receiving placebo was associated with a lower change of 24.07 nmol/L (as compared with vitamin D) in serum 25(OH)D concentration after 12 months
- Receiving both treatments was associated with a greater increase of 13.75 nmol/L (as compared with vitamin D) in serum 25(OH)D concentration after 12 months.
- Each unit (%) increase in compliance was associated with an increase of 0.54 unit in serum 25(OH)D concentration after 12 months

DISCUSSION/CONCLUSION

It is postulated that fat stimulates the secretion of bile and promotes the formation of micelles and therefore may enhance vitamin D absorption. However, fatty acid composition of foods may have differential effects; while some are considered to be enhancers others to have inhibitory effects. Polyunsaturated fatty acid, linoleic and linolenic acids have been reported to decrease absorption of vitamin D. It has been suggested that these fatty acids may increase the solubility of vitamin D in the micelles and change the partition coefficient such that the vitamin D stays in the micelle. Alternatively, they may increase the size of the micelle and thereby reduce its diffusion rate and increase its difficulty in crossing the unstirred water layer lining the intestinal mucosa.

Herein, however, the analysis showed, in children with ASD, that **the addition of DHA to vitamin D supplementation did not dampen the serum 25(OH)D response to vitamin D supplementation but was instead associated with a greater response.** The basis for the discrepancy is unknown, but one can suggest that the long-term supplementation with omega-3 LCPUFA might;

1. enhance vitamin D absorption/uptake from supplements
2. upregulate vitamin D receptors
3. favourably alter the gut microbiota composition, which is altered in ASD. Gut microbiota composition, in turn, is associated with increased circulating 25(OH)D concentration.

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