A Biological Investigation of the Role of Omega-3 Fatty Acids on Psychopathology in Clinical High Risk of Psychosis

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A biological investigation of the role of omega-3 fatty acids on psychopathology in clinical high risk of psychosis

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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of Doctor of Philosophy

Supervisors:

Professor David R. Cotter and **Dr. Melanie Föcking**

March 2022

Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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List of Abbreviations

ACTH	Adreno-cortico-tropic hormone
ALA	Alpha linoleic acid
Apo	Apolipoprotein
APS	Attenuated psychotic symptoms
ARA	Arachidonic acid
ARMS	At-risk mental state
AUC	Area under the curve
BL	Baseline
BLIPS	Brief limited intermittent psychotic syndrome
BLIPS	Brief limited intermittent psychotic symptom
BMI	Body mass index
BPRS	Brief Psychiatric Rating Scale
C	Complement
CAARMS	Comprehensive assessment of at-risk mental states
CBCM	Cognitive behavioural case management
CFB	Complement Factor B
CHR	Clinically high risk
CV	Coefficient of variance
DHA	Docosahexaenoic acid
DSM	Diagnostic and statistical manual of mental disorders
DUP	duration of untreated psychosis
ELISA	Enzyme linked immune sorbent assay
EPA	Eicosapentaenoic acid
EU-GEI	European Network of National Schizophrenia Networks studying Gene-Environment Interactions
F5	Coagulation factor V
FA	Fatty Acid
FDR	False discovery rate
FEP	First episode of psychosis
FFQ	Food frequency questionnaire
GAF	Global assessment of functioning scale
GF: R	Global functioning role
GF: S	Global functioning social
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome wide association study
ICAM	Intercellular adhesion molecule
ICD	International classification for diseases

IGHG	Immunoglobulin heavy constant gamma chain
IL	Interleukin
INF	Interferon gamma
IQ	Intelligence Quotient
LA	Linoleic acid
MAC	Membrane attack complex
MADRS	Montgomery-Åsberg Depression Rating Scale
MHC	Major histocompatibility complex
MS	Mass Spectrometry
NAPLS	The North American Prodromal Longitudinal Studies
PASEF	Parallel accumulation serial fragmentation
PD	Psychotic disorder
PE	Phosphatidyl-ethanolamine
PORT	Program of Rehabilitation and Therapy
PSS	Positive symptoms summary
PUFA	Polyunsaturated fatty acids
RBC	Red Blood Cell
SANS	Scale of Assessment of Negative Symptoms
SD	Standard Deviation
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SIPS	Structured Interview for Psychosis-risk
SIPS	Structured Interviews for Psychosis-risk syndromes
SOFAS	Social and Occupational Functioning Assessment Scale
SPM	Specialized pro-resolving mediators
SZ	Schizophrenia
TCEP	tris (2-carboxyethyl) phosphine
TNF	Tumour necrosis factor
UHR	Ultra-high risk
VCAM	Vascular cell adhesion molecule
VHR	Vienna High risk study
VUL	vulnerable group with personality disorder
WHO	World health organization
YMRS	Youth Maniac Rating Scale

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Dedication

This thesis is dedicated to my parents

Fatima Mary S & **Susai Raj K**.

“See what no one else sees. See what everyone chooses not to see...
.. out of fear, conformity, or laziness.
See the whole world anew each day!”

~ Patch Adams ~

Summary

Background: Psychosis is a multifactorial disorder and one among the top 15 causes of disability worldwide. Availability of complex, expensive and incomplete treatment modalities for psychosis increases the need of more investigations in understanding the pathophysiology and prevent development of the disease at an early stage. The current thesis focuses on the therapeutic role of omega-3 fatty acids (FAs) and investigates the biological mechanism underpinning the omega-3 related clinical outcome at clinical high risk (CHR) of psychosis.

Methods: Initially, a systematic literature was performed to understand the existing evidence of omega-3 associated improvement in functional outcome in CHR participants. Then using baseline and follow-up plasma samples of the CHR subjects from the NEURAPRO clinical trial, the role of plasma immune markers and the plasma proteomic pathways on omega-3 associated improvement in clinical outcome was investigated. A total of 285 CHR participants aged 18.97 ± 4.49 years (mean ± SD) were included in this study. The molecular percentage of erythrocyte membrane FAs levels which are the markers of dietary omega-3 intake were quantified using the gas-chromatography. First the mediating role of plasma immune markers on omega-3 associated clinical outcomes was investigated. Secondly, using support vector machine learning techniques, we evaluated whether a combination of biological and clinical variables could predict future functional outcome in CHR individuals. Finally, using mass-spectrometry based proteomic analysis at baseline and 6-month follow-up plasma samples, we investigated the plasma proteomic pathways associated with omega-3 FAs and the mediating role of plasma proteins on omega-3 associated clinical improvement in psychosis.

Results: The systematic provided a mixed results regarding the association of omega-3 FAs with functional outcome in CHR state. In the NEURAPRO clinical trial, plasma immune markers expressed an inverse association with omega-3 FAs both in cross-sectional and longitudinal analysis. Although plasma immune markers did not provide any mediating effect on omega-3 associated clinical outcome in CHR participants. In the prediction models, baseline parameters of both clinical and biological markers did not predict the functional outcome and addition of biomarker data with clinical data did

not improve prediction of 12-month functional outcome compared to the model based on the baseline clinical data alone. Finally, 6-month change in okmegha-3 FAs associated significantly with plasma proteins of complement and coagulation pathways. Furthermore, the complement and coagulation pathway proteins showed a significant mediating effect on omega-3 associated reduction in psychotic symptoms and improvement in functional outcome and cognition in CHR participants.

Conclusion: Overall, the thesis has provided vital biological and clinical effects of omega-3 FAs in CHR state. The immune-assay results indicated a significant anti-inflammatory property of omega-3 FAs on plasma immune markers. Our proteomic analysis for the first time, has provided a relationship of complement and coagulation pathway proteins with functional outcome in CHR state. Furthermore, the mediation analysis indicated the involvement of complement and coagulation protein associated molecular mechanisms in omega-3 related clinical improvement in CHR state.

Publication details

List of publications considered for the thesis

S.No	Reference	Impact Factor	Quartile
1	**Susai SR**, Sabherwal S, Mongan D, Föcking M, Cotter DR. Omega-3 fatty acid in ultra-high-risk psychosis: A systematic review based on functional outcome. Early Interv Psychiatry. 2022 Jan;16(1):3-16. doi: 10.1111/eip.13133. Epub 2021 Mar 2. PMID: 33652502.	2.73	Q1
2	**Susai SR**, Mongan D, Healy C, Cannon M, Nelson B, Markulev C, Schäfer MR, Berger M, Mossaheb N, Schlögelhofer M, Smesny S, Hickie IB, Berger GE, Chen EYH, de Haan L, Nieman DH, Nordentoft M, Riecher-Rössler A, Verma S, Thompson A, Yung AR, McGorry PD, Föcking M, Cotter D, Amminger GP. The association of plasma inflammatory markers with omega-3 fatty acids and their mediating role in psychotic symptoms and functioning: An analysis of the NEURAPRO clinical trial. Brain Behav Immun. 2022 Jan;99:147-156. doi: 10.1016/j.bbi.2021.09.018. Epub 2021 Oct 5. PMID: 34624483.	7.21	Q1
3	**Susai SR***, Mongan D*, Healy C, Cannon M, Cagney G, Wynne K, Byrne J, Markulev C, Schäfer MR, Berger M, Mossaheb N, Schlögelhofer M, Smesny S, Hickie IB, Berger GE, Chen EYH, de Haan L, Nieman DH, Nordentoft M, Riecher-Rössler A, Verma S, Thompson A, Yung AR, Nelson B, McGorry PD, Föcking M, Cotter D, Amminger GP. Machine learning based prediction and the influence of complement - coagulation pathway proteins on clinical outcome: results from the NEURAPRO trial. Brain Behav Immun. Manuscript number: BBI-D-21-01432R1, DOI: https://doi.org/10.1016/j.bbi.2022.03.013 (Accepted for publication).	7.21	Q1
4.	**Susai SR**, Healy C, Mongan D, Heurich M, Byrne J, Cannon M, Cagney G, Wynne K, Markulev C, Schäfer MR, Berger M, Mossaheb N, Schlögelhofer M, Smesny S, Hickie IB, Berger GE, Chen EYH, de Haan L, Nieman DH, Nordentoft M, Riecher-Rössler A, Verma S, Thompson A, Yung AR, Nelson B, McGorry PD, Föcking M, Cotter D, Amminger GP. Evidence that complement and coagulation proteins mediating the clinical response to omega-3 fatty acids: A longitudinal mass spectrometry-based investigation in subjects at clinical high-risk psychosis. Translational Psychiatry. Manuscript number: 2022TP000306-T.	NA	NA

List of other relevant publications

S.No	Reference	Impact Factor	Quartile
1	Mongan D, Föcking M, Healy C, **Susai SR**, Heurich M, Wynne K, Nelson B, McGorry PD, Amminger GP, Nordentoft M, Krebs MO, Riecher-Rössler A, Bressan RA, Barrantes-Vidal N, Borgwardt S, Ruhrmann S, Sachs G, Pantelis C, van der Gaag M, de Haan L, Valmaggia L, Pollak TA, Kempton MJ, Rutten BPF, Whelan R, Cannon M, Zammit S, Cagney G, Cotter DR, McGuire P; European Network of National Schizophrenia Networks Studying Gene-Environment Interactions (EU-GEI) High Risk Study Group. Development of Proteomic Prediction Models for Transition to Psychotic Disorder in the Clinical High-Risk State and Psychotic Experiences in Adolescence. JAMA Psychiatry. 2021 Jan 1;78(1):77-90. doi: 10.1001/jamapsychiatry.2020.2459. PMID: 32857162; PMCID: PMC7450406.	21.59	Q1
2	Mongan D, Sabherwal S, **Susai SR**, Föcking M, Cannon M, Cotter DR. Peripheral complement proteins in schizophrenia: A systematic review and meta-analysis of serological studies. Schizophr Res. 2020 Aug;222:58-72. doi: 10.1016/j.schres.2020.05.036. Epub 2020 May 24. PMID: 32456884; PMCID: PMC7594643.	4.93	Q1
3.	Mongan D, **Susai SR**, Föcking M et al., Associations between plasma inflammatory markers and psychotic disorder, depressive disorder and generalised anxiety disorder in early adulthood: a nested case-control study (Under review)	NA	NA
4	**Susai SR***, Föcking M*, Mongan D et al., Response to treatment in first episode psychosis linked to levels of complement and coagulation proteins (Manuscript in preparation).	NA	NA

*Quartiles are based on Scrimago Journal Ranking (https://www.scimagojr.com/).

Conferences and presentations

S No	Conference details	Title of the presentation	Type of presentation
1	Schizophrenia International Research Society, 2022 Annual Congress, April 2022, Florence, Italy.	Upregulation of complement and coagulation proteins at baseline is associated with early response to amisulpride in first episode psychosis-Findings from the OPTiMiSE trial	Oral presentation
2	Schizophrenia International Research Society, 2022 Annual Congress, April 2022, Florence, Italy.	Machine learning based prediction and the influence of complement-coagulation pathway proteins on clinical outcome: Results from the NEURAPRO trial	Poster presentation
3	Schizophrenia International Research Society, 2022 Annual Congress, April 2022, Florence, Italy.	Do complement and coagulation pathways mediate the omega-3 fatty acids associated clinical response in early psychosis? A mass spectrometry-based exploration of plasma proteome from the NEURAPRO clinical trial	Poster presentation
4	Beaumont Hospital Translational Research Awards, December 2021	The association of plasma inflammatory markers with omega-3 fatty acids and their mediating role in psychotic symptoms and functioning: an analysis of the NEURAPRO clinical trial	Oral presentation
5	31st HUPO Human Brain Proteome Project Workshop, 18 & 19th May 2021	Complement and coagulation pathway proteins in first episode psychosis- An insight into the OPTiMiSE study	Oral presentation
6	Beaumont Hospital Translational Research Awards, October 2020	Complement proteins in first episode psychosis- A report from the OPTiMiSE study	Oral presentation
7	Neuroscience Ireland-Young Investigator Symposium, 6th November 2020	Relationship of omega-3 fatty acids with plasma immune markers in clinically high-risk psychosis: A study from the NEURAPRO clinical trial	Poster presentation
8	Schizophrenia International Research Society (SIRS)-Conference, 4-8 April 2020	Prediction of treatment response in first episode psychosis- Proteomic investigation from the OPTiMiSE study	Poster presentation

S No	Conference details	Title of the presentation	Type of presentation
9	Neuroinflammation Dublin Network Seminar Series, 26th February 2020, RCSI, Dublin.	Complement proteins in first episode psychosis- A preliminary report from the OPTiMiSE study	Oral presentation
10	British Neuroscience Association (BNA) - Conference, 14-17 April 2019, Dublin	The Effect of Omega-3 Fatty Acids on Functional Outcome of Pre-psychotic and Psychotic patients: A Systematic Review Protocol	Poster presentation

Award won

Shane O'Neill medal from Beaumont Hospital Translational Research Awards, December 2021

Chapter 1: Introduction

1.1. Psychosis- An introduction

The World Health Organization (WHO) has estimated that more than 16 % of the world's population are affected by mental or addictive disorders, equally involving both genders (1, 2). Despite the ongoing drive to predict and prevent psychiatric disorders, translation into real-world effects has been very slow (3). Schizophrenia (here after psychosis) is among the top 15 leading causes of disability worldwide (4). The WHO has estimated that the direct costs for psychosis account for 7% to 12% of the gross national product (5), despite its relatively low life-time prevalence of a median of 4.0 per 1,000 people (6). Psychosis is a multifactorial psychiatric disorder characterized by changes in thoughts, feelings, and behavior. Clinical symptoms of psychosis are classified into three major domains of symptoms: positive, negative, and cognitive. The positive symptoms such as delusion, hallucination and conceptual disorganization are the major reason for patients to seek help. In addition, psychosis also demonstrates negative symptoms such as apathy, anhedonia, blunted affect and poverty of speech (7). The symptoms of psychosis start usually in adolescence or early adulthood and in the majority of subjects continue to cause long-term functional impairment (8). Biological, behavioral and neuroimaging studies of patients with psychosis have demonstrated a broad array of differences from healthy controls (9-11). For instance, several inflammatory mediators that are involved in neurodevelopment such as Interleukin (IL)-1β, IL-6, IL-8 are found to be abnormal in psychosis patients compared to controls (12, 13). Neuroimaging studies have revealed decreased cortical thickness, reduced global gyrification and altered neural connectivity in psychosis patients compared to healthy controls (14). Currently, two widely established systems of classifications are used for the diagnosis of psychiatric disorders, the Diagnostic and Statistical Manual of Mental Disorders (DSM) of the American Psychiatric Association and the International classification for Diseases (ICD) published by WHO (15).

1.2. High risk state in Psychosis

The clinical stages of psychosis can be divided into four stages and all have their own relative risk of progressing to the next level in a critical timeline (16, 17). The first stage

is considered as the clinical high risk (CHR)/ Ultra High Risk (UHR) that has an 18-30% increased risk of progressing to the next level within the first 6 months according to different studies and systematic reviews (18, 19). The risk of progression almost doubles within the first three years of the CHR stage (18, 19). The term 'prodrome' is a retrospective term indicating the duration between the first observed change in a person's experience until the first diagnosis of psychosis (20). The current concept of prodromal phase encompasses the conditions such as 'at-risk mental state' (ARMS), UHR and CHR (21). The exact criteria for the CHR/UHR state generally comprise of a subgroup of help-seeking individuals aged between 14 to 35 years (19, 21). The CHR state is clinically assessed using different assessment scales, which include Comprehensive Assessment of At Risk Mental States (CAARMS), the Structured Interview for Psychosis-risk Syndrome and the Basel Screening Instrument for Psychosis (22). Although the definition of CHR slightly varies based on the tools, it generally comprises the following two subgroup of populations:

i) Subgroups subjects with positive psychotic symptoms that have reduced intensity and/or reduced duration, and
ii) Subgroup with a positive family history of psychosis or schizotypal personality disorder with functional impairment

The duration of untreated psychosis (DUP), the period between onset of symptoms until initiation of appropriate treatment by a healthcare professional, is one of the main determinants for successful treatment in psychosis (23-26). A shorter DUP is associated with clinical recovery and functional improvement and a longer DUP is associated with poor therapeutic response in psychosis (23-26).

The second stage, termed first episode of psychosis (FEP), is the stage where psychotic symptoms qualify for a psychosis and the first psychotic episode occurs (27). FEP most commonly occurs in late adolescence or early adulthood and the psychiatric disability at this stage significantly derails the patient from his normal trajectory of psychosocial development resulting in disturbance of the functional status of the patient (28). As the best response (almost 75%) to antipsychotics is achieved in this stage, FEP carries important clinical value (29, 30). Evidence from follow-up studies point to the existence of a narrow period during FEP that could determine the long-term outcome in the treatment of schizophrenia (31-35). In the last few decades

various models of early intervention programs have been initiated and showed some benefits in terms of remission rate, adherence, and retention in treatment (36-38). The third and fourth stages of psychosis are determined based on the treatment response to antipsychotics as remission and refractory phases, respectively (16, 17).

1.3. Functional impairment in early psychosis

Functional capacity is an objective measurement of an individual to perform various basic tasks that are needed for everyday life (39). In the field of psychosis, an increasing interest to develop more comprehensive models focuses on functional recovery (40-42) and evolved over the past number of years. Accumulating evidence suggests that functional recovery is influenced by the severity of positive psychotic symptoms as well as by other disease related aspects such as neurocognitive performance, and mood disorder and negative symptoms (43-47). Additionally, social, family, and environmental events contribute substantially to functional recovery beyond clinical manifestation of psychosis (43, 46, 48). Recent studies of CHR participants have increasingly moved beyond the focus on transition to psychosis, to explore the association of clinical symptoms to real-world functioning (49, 50) and other non-psychotic psychiatric outcomes (51, 52). A number of studies have focused on the presence of functional impairment in psychosis subjects compared to healthy individuals including those who convert to psychosis and those who did not (49, 50, 53-56). Cornblatt et al, reported that among social and role functioning, social functioning could be a potential marker of psychosis as it is not affected by anti-psychotic treatment (49). In first-episode non-affective psychosis patients, a five-year follow-up study indicated that normal social functioning at baseline could predict good treatment response to anti-psychotics (53). Another study reported that functioning status in the CHR state can be used as a diagnostic tool to reduce heterogeneity and to decrease false positive cases (50). In addition, in CHR participants' functioning status was demonstrated to be a reliable prognostic marker (53-56). For these reasons, it is important to understand beyond the concept of transition, and to additionally consider investigating other clinical outcome, and perhaps more importantly focus on functional outcome in the CHR state (40-43, 46, 48).

1.4. Importance of early intervention in psychosis

Treatment of schizophrenia is complex, expensive, and provides partial, limited improvement in two-thirds of sufferers (6). Treatment response is best for those in the early stage of psychosis, but unfortunately, due to treatment non-adherence, the majority of patients undergo relapse within a few years. With every new relapse, treatment resistance increases (57, 58). Thus, over its course, psychotic disorders have low overall functional recovery rates (34, 59), and remain among the leading causes of disability worldwide (60). A recent meta-analysis has estimated that even among CHR subjects who do not undergo transition to psychotic disorder (PD), less than half show remission of their symptoms over time (51). In addition to this, the observation of worse treatment outcomes in patients with long DUP led to the proposition that prevention of transition at an early stage might be substantially more beneficial than a standard treatment after the development of psychosis (51, 57, 61-65).

Early intervention strategies thus introduced to prevent psychosis are distinct from the standard approach by two major elements: First ‘the early detection’ which is defined as identification of a population who is likely to develop a psychotic disorder OR the identification of people who already have a psychotic disorder and have not yet received adequate treatment (57, 61). Second the ‘phase-specific treatment’, defined as psychological, social and physical interventions that are specifically targeted at people in the early stages of schizophrenia or first episode psychotic disorder (66). Early detection and phase-specific treatment can be provided separately or as a supplement to existing standard psychiatric care (67). Several early intervention strategies have been followed aiming to treat patients in the CHR phase of psychosis and preventing the development of psychosis. This includes nutritive supplements, alternative medicines, and psycho-social interventions (57). A Cochrane database systematic review recently evaluated the safety and efficacy of available early intervention strategies in the prodromal phase and found that the intervention with omega-3 FAs provided a low-quality effectiveness towards prevention of psychosis, whereas other intervention strategies did not provide any effective results (57).

1.5 Omega-3 fatty acids (FAs) and psychosis

1.5.1. Biological role of omega-3 FAs in the brain

Lipids are integral components of the brain and are necessary for the development and functioning of the neural network. The brain contains 60% lipids in its dry weight, and more than half of its lipids are polyunsaturated fatty acids (PUFAs) (68, 69). In humans, long chain PUFAs such as eicosapentaenoic acid [EPA, 20:5 (with 20 carbon chain and 5 double bonds)] and docosahexaenoic acid (DHA, 22:6) have to be either converted from short chain fatty acids (FAs) through a process called elongation in the liver or should be taken as long chain FAs in the diet (70). Preclinical studies have shown that EPAs and DHAs are vital for maintaining neuronal membrane integrity and exert neurotrophic activities in the brain (71). Notably, omega-3 FAs also participate in synaptic transmission and pruning (7, 72-74). Recent investigations also have identified possible anti-inflammatory properties of omega-3 FA metabolites (75, 76).

1.5.2. Omega-3 FAs in Psychiatric disorders

In healthy infants, the plasma levels of omega-3 FAs increase sharply from the last trimester of gestation to the first 6-10 months after birth (77-79). Clinical studies with omega-3 FA supplementation showed beneficial results in the early stage of development (80-86). Moreover, omega-3 FA supplementation has been shown to improve clinical symptoms of anxiety in a healthy population by blunting the plasma levels of epinephrine and adreno-cortico-tropic hormone (ACTH) (76, 87-91). Such beneficial results in healthy participants increased the interest in omega-3 FAs being used as potential therapeutic agents in psychiatric disorder especially in major depressive disorder and psychosis (92, 93). In relation to psychosis, a causal relationship between increased polyunsaturated fatty acids and decreased risk of psychosis was observed (94, 95). These observations led to the membrane phospholipid hypothesis where an imbalance in PUFAs was suspected to be a cause for the development of abnormal neurotransmission and thereby resulting in symptoms of psychosis (68, 96-108). The involvement of PUFAs in the regulation of synaptic pruning activity during the developmental stage provided preliminary evidence for a mechanistic involvement of PUFAs in the development of psychosis (109-111). In line with these observations, Madore et al. observed that maternal

omega-3 PUFA deficiency drives microglia associated synaptic pruning in rodents leading to cognitive impairment in the offspring (110). Also, they pointed out that the omega-3 FAs influence the synaptic pruning through modifying complement pathway proteins, the involvement of which is well known in psychosis (112-114). These observations further support the possibility of omega-3 associated therapeutic effects in the early stages of psychosis.

1.5.3. Omega-3 FAs in the prevention of psychosis

Omega-3 FAs supplementation is one of the many non-pharmacological interventions that have been studied in CHR individuals in the prevention of transition to psychosis (115). So far, only two clinical studies have been conducted in CHR subjects to evaluate the preventive role of omega-3 FAs. In the first clinical trial, the Vienna High risk study (VHR), 12 weeks of supplementation with 1.2g/d omega-3 FAs significantly reduced the transition rate in the omega-3 FA groups compared to controls (116). Furthermore, the same population showed a reduced risk of progression to psychiatric morbidity in long-term follow-up (117). In contrast, the results of the second omega-3 based clinical trial in CHR subjects (the NEURAPRO study) did not find the same beneficial effects (118, 119). These inconsistent results raise questions concerning the therapeutic effect of omega-3 FAs and point to a need to further understand potential biological mechanisms underlining this nutritional intervention in early psychosis.

1.6. Complement component proteins

1.6.1. Introduction

Complement proteins are membrane bound proteins produced mainly by the liver and play a central role in the innate immune response. Under physiological conditions complement proteins present as inactive zymogens and are sequentially cleaved and activated in a reaction cascade to maintain homeostasis. Complement proteins act through three distinct cascades of reactions called the classical, lectin and alternative pathways, which finally converge into one terminal pathway. This involves the complement protein 3 (C3), which is the most abundant complement protein found in blood. Activation of C3 leads to formation of C3a, C3b, C5a and the membrane attack complex (MAC) (105). Apart from this traditional, C3 dependant pathway, another peripheral pathway also has been identified that forms the MAC in a C3 independent

manner. Recently, a novel intracellular complement system called 'the complosome' has been discovered in cells such as lymphocytes, monocytes and endothelial cells (120). Through their inflammatory and non-inflammatory actions, the complement proteins seem to influence the brain and related activities (121). The extracellular and intracellular pathways of complement proteins are described in Figure 1.1.

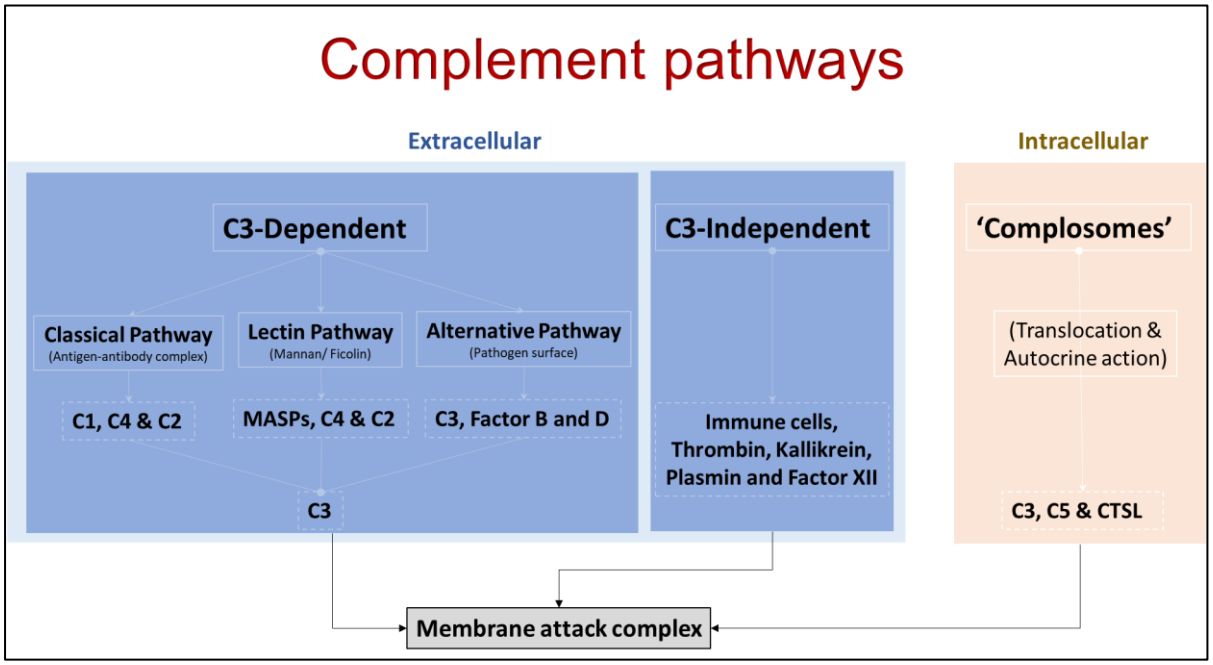

Figure 1.1. The extracellular and intracellular pathways of complement proteins. The flow chart depicts extracellular and intracellular pathways identified with complement proteins resulting in final activation of membrane attack complex (MAC). The extracellular pathway is further divided into two based on the involvement of Complement 3 proteins. The intracellular pathway mainly involves complement protein containing vesicle which in autocrine fashion activates MAC.

1.6.2. Complement proteins in the brain

Complement protein activities are noticed at various stages of brain development. For instance, localised expression of C1qR, C3, C5 and C3aR1 were recorded during the process of neurulation (122). Similarly, expression of C5aR1 and its precursor C5 was identified in human embryonic stem cell-derived progenitor cells (123). Cell culture studies have demonstrated that the complement proteins C3and C3aR1 control collective cell migration in neural crest cells (124). Knockdown mice lacking C3 and C3aR1 showed decreased neurogenesis in neocortical and cerebellar regions (125). Glial associated complement activity was also found to be involved in synaptic pruning activity, which is crucial for brain development and plasticity (126, 127). Furthermore, in preclinical studies, a toxicity specific neuroprotective activity of complement proteins was observed (128, 129). Recent evidence indicates a role for a C5a induced

upregulation of glutamate transporter-1 in microglia to decrease extracellular glutamate levels (130). Overall, complement pathway proteins were found to be involved in development, maintaining homeostasis, regeneration and repairing processes of neurons and glial cells. Hence, any abnormal expression of complement proteins could indicate or result in an abnormal development of neural structures.

1.6.3. Complement proteins in Schizophrenia

Epidemiological and clinical studies have indicated the association of altered immune activities with psychosis (131-137). In 2009, a Genome wide association study (GWAS) shed light on complement proteins in the pathophysiology of schizophrenia. Several reports from GWAS pointed to a strong association of the extended major histocompatibility complex (xMHC) with psychosis (138-146). Recently, fine mapping analyses further revealed the involvement of the complement component 4 region of the xMHC genome in relation to schizophrenia (113). Following genetic findings, a few hypothesis driven studies on peripheral complement proteins in psychosis patients provided mixed results. Most of the studies found inconsistent results regarding the peripheral C3 and C4 levels in schizophrenia patients (147-152). A few studies have investigated plasma C1q protein levels in psychosis and found increased expression of C1q and enhanced reactivity in schizophrenia patients (153-155). Provided that our knowledge regarding the neuropathology of the disease is limited, the quantification of peripheral complement proteins will be useful in understanding their part in the pathology of psychosis.

The rapid development in the field of proteomics provides new opportunities for understanding the role of multiple complement pathway proteins at various stages of schizophrenia. Our research group has recently reported that thirty-four proteins were differentially expressed in children who later developed psychotic experiences compared to healthy counterparts. Further, these studies pointed to an association of complement and coagulation pathways' dysregulation with the development of psychotic experiences (156). We have also reported an increased expression of complement and coagulation proteins in CHR subjects who transitioned to FEP and significantly predicted transition to disease (157)(Figure 1.2). As the complement and coagulation pathways have well recognized roles in inflammation, we view our findings

as reflecting a likely dysregulated inflammatory process in schizophrenia (13, 158, 159).

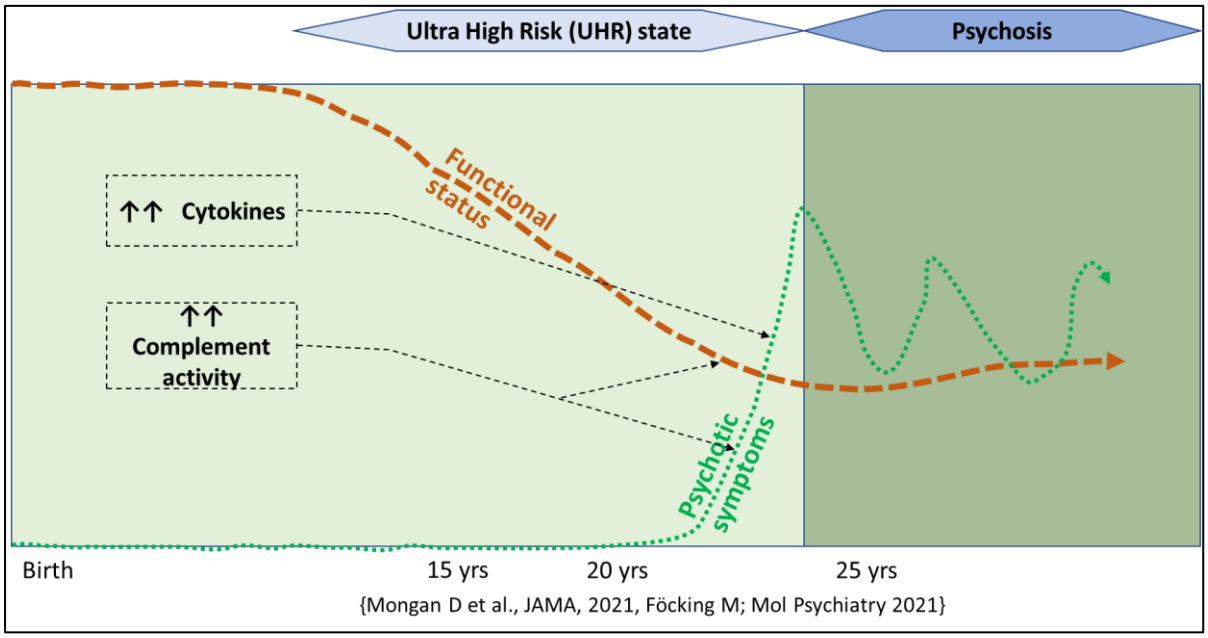

Figure 1.2. Schematic representation of clinical and immune markers at early stage of psychosis. The picture depicts the biological and clinical events happening during the early stage of psychosis. In high-risk of psychosis, decline in functioning happens which is followed by development of psychotic symptoms. The existing literature evidence indicate that alteration in immune markers such as cytokines and complement proteins at high-risk state predicted the future development of psychotic symptoms and functional deterioration.

1.7. Aim and objectives

The overall aim of this thesis is to investigate the relationship of omega-3 FA supplementation to biological parameters (such as plasma immune markers and plasma proteins) and to clinical parameters (such as psychotic symptoms, functional outcome and cognitive status) in CHR subjects.

Objective 1

To review the literature investigating the relationship of dietary omega-3 FAs treatment with functional status in CHR subjects. For objective 1, we hypothesised that high levels of omega-3 FAs will associate cross sectionally and longitudinally with functional improvement in the CHR population.

Objective 2

To estimate the immune association of omega-3 supplementation in CHR participants. For objective 2, we hypothesised that omega-3 FAs will exert anti-inflammatory properties by exerting an inverse relationship with plasma cytokines.

Objective 3

To investigate the mediating role of plasma immune markers on omega-3 associated functional improvement in CHR subjects. Here we hypothesised that plasma immune markers will, at least partially, mediate the relationship between omega-3 FAs and functional outcome.

Objective 4

To investigate the combined predictive ability of blood-based biological markers including inflammatory cytokines, erythrocyte membrane fatty acids and the plasma proteome on functional outcome. In this analysis, our hypothesis was that combining the baseline biomarkers relevant to psychosis with the clinical parameters will improve the performance of the machine learning based prediction model.

Objective 5

To explore the biological pathways that are substantially influenced by change in PUFAs (both omega-3 and omega-6). Since there is little literature available regarding the omega-3 FAs and associated plasma proteomic pathways, we conducted a hypothesis free approach using proteomics for this objective.

Objective 6

To identify whether the plasma proteins of complement and coagulation pathways mediate the relationship between change in omega-3 PUFAs and clinical outcomes. Based on the literature we hypothesised that complement proteins will mediate the relationship between omega-3 FAs and clinical outcome in CHR participants.

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Chapter 2

Thesis author's role:

Mr Subash Raj Susai was involved in all aspects of the study. He had full access to all the data in the study and was involved in the acquisition, analysis, and interpretation of data and drafting of the manuscript.

Reference:

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Title: Omega-3 Fatty acid in Ultra high-risk psychosis: A systematic review based on functional outcome.

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Abstract:

Introduction: Among different types of poly unsaturated fatty acids, omega-3 fatty acids (FAs) play a substantial role in brain development and functioning. This review was designed to evaluate and synthesise available evidence regarding omega-3FAs and functional outcome in the Ultra high risk (UHR) population.

Methodology: An electronic search in PubMed, EMBASE, PSYCINFO and COCHRANE search engines has been performed for all articles published until January 2019. The studies that have data regarding omega-3 FAs and functional outcome in UHR population were included.

Results: Out of 397 non-duplicate citations, 19 articles met selection criteria. These articles were from four different primary studies namely the Program of Rehabilitation and Therapy (PORT), the North American Prodromal Longitudinal Studies (NAPLS), Vienna High risk study (VHR) and the NEURAPRO. The data from the NAPLS study found a positive correlation between functional improvement and frequency of dietary intake omega-3 FA. Moreover, among the erythrocyte omegs-3 FA only eicosapentaenoic acid (EPA) showed a positive correlation with functional score. The VHR study found long term improvement in functional outcome in omega-3 group compared to control whereas such difference was noticed in the NEURAPRO. In the VHR study both omega-3 and omega-6 together predicted the functional improvement at 12 weeks.

Conclusion: The number of studies available remains insufficient and more studies with standardized outcome measures in a clinically comparable UHR population would be of more value to understand the clinical benefits of omega-3 FAs in the UHR population.

2.1. Rationale:

Lipids are an integral component of the brain and are necessary for the development and functioning of the neural network. The brain contains 60% lipids in its dry weight, and more than half of its lipids are polyunsaturated fatty acids (PUFA) (1, 2). Mammals lack the desaturase enzymes which are necessary for producing short chain PUFAs such as Linoleic acid (LA) and alpha linoleic acid (ALA), which are the precursors for the synthesis of long chain omega-6 and omega-3 PUFA, respectively (3-6). Evidence suggest that the efficiency of desaturase enzymes required to produce long chain PUFAs particularly that of omega-3 FA are relatively low in mammals (4, 6). This was noticed especially in children with inborn error of amino-acid metabolism who must consume a low protein diet deficient with very low long-chain PUFA content. In such children, intake of low levels of PUFA had little impact on synthesis of arachidonic acid (omega-6) but significantly decreased the production of plasma and erythrocyte DHA (omega-3) (7). Hence, long-chain omega-3 fatty acids such as DHA must be supplemented through the diet to maintain adequate levels (8). Various preclinical studies have shown that long chain FA such as eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are involved in maintaining membrane fluidity, enhancing the activity of peroxisome proliferator-activator receptors and in various neurotrophic activities within the brain (9). Notably, omega-3 FA participate in synaptic transmission and pruning functions which are proposed to be altered in neuropsychiatric disorders (10-13). Recent investigations have emphasized the possible anti-inflammatory and therapeutic properties of omega-3 FA metabolites such as eicosanoids, E- and D-series resolvins and neuroprotectin D1 in the field of psychiatry (14, 15).

Clinical evidence suggests the beneficial role of PUFA in the early stages of neurodevelopment. In healthy infants, the plasma levels of omega-3 FA increase sharply until the first 6-10 months after birth (16-19). Further studies on healthy subjects have reported clinically beneficial effects of dietary supplementation with omega-3 FA in early childhood. For instance, Jensen et al. reported that the children of mothers who had received omega-3 supplementation outperformed in tests for psychomotor activity, eye-hand coordination and visual acuity. Moreover, the same children performed better after 5 years on a test for sustained attention compared to

the placebo group (19, 20). Similarly, administering a DHA rich diet to the mothers improved mental processing scores, degree of stereopsis and stereo acuity in their children (21-25). Such beneficial results further increased the interest of a potential therapeutic role of omega-3 FA in neurological and psychiatric disorders. Omega-3 FA supplementation has been shown to improve the clinical symptoms of anxiety in a healthy population by blunting the plasma levels of epinephrine and adrenocorticotropic hormone (ACTH) (15, 26-28). Moreover, placebo-controlled trials in patients who had consumed EPA showed delayed onset and lower incidence of depression and clinical recovery from anxiety, depression, sleep and suicidal ideation (29, 30). These results support a possible clinical relevance of omega-3 FA in psychiatric disorders, especially in schizophrenia (31, 32).

Since early intervention is considered as a gold standard in schizophrenia, there is increasing focus on the preventive measures in ultra-high risk (UHR) subjects. The UHR status, otherwise known as clinical high risk or at-risk mental status, is defined as condition in which the subject has potential prodromal syndromes with a 30% increased risk of transition to psychotic disorder within 3 years (33-37). Several non-pharmacological interventions and nutritional options such as omega-3 supplements have been studied in UHR with the aim of preventing the development of psychosis (35). Therefore, in this review our aim was to evaluate and synthesise available evidence concerning the relationship between dietary and erythrocyte omega-3 fatty acids with functional outcome in the UHR population. Among various clinical outcomes, functional status has been preferred as it is a vital diagnostic component and is considered as a reliable prognostic marker in psychosis (38-46). Moreover, functional improvement is one of the essential objectives for any therapeutic intervention in the field of psychiatry.

2.2. Materials and methods

2.2.1. Protocol and registration

The protocol for this review is registered on the PROSPERO database (Registration number: CRD42019117423).

2.2.2. Eligibility criteria

We included all case-control, cohort and randomized controlled studies performed in UHR participants and studies published in English were selected. Since the erythrocyte membrane PUFA composition closely reflects that of neuronal membrane omega-3 composition and is a better indicator of dietary PUFA content than plasma omega-3 levels (47-50), the articles with dietary omega-3 PUFA measures or erythrocyte omega-3 levels for analysis were included. Studies without functional outcome assessment were excluded from the review.

2.2.3. Information sources and search strategy

We performed an electronic search in PubMed, EMBASE, PSYCINFO and COCHRANE search engines for articles published in English up until January 2019 (Complete search strategy: please see Appendix 2.10.1).

iv) Study selection process

Titles and abstracts were screened independently by two authors (SRS and SS). Full-text articles were obtained for the identified records. The articles were then segregated based on the type of study and divided into randomized and non-randomized trials. Studies containing data from the same study population were then clustered for data collection.

2.2.4. Data collection

Two investigators independently performed the data extraction. Details such as type of study, geographical location, corresponding authors and related reference articles were collected. Study-specific details such as aim, inclusion and exclusion criteria as well as study design were recorded, and data related to outcome measures such as that of omega-3 PUFA and functional outcomes at baseline and follow-up

stages were recorded (Table 2, 3 & 4). The risk of bias assessment was recorded using the Joanna Briggs Institute (JBI) Critical Appraisal Tool and the Cochrane Risk of Bias 2 Tool for cross sectional and randomized controlled trials respectively (51, 52) (Table 2.3.1).

2.3. Results:

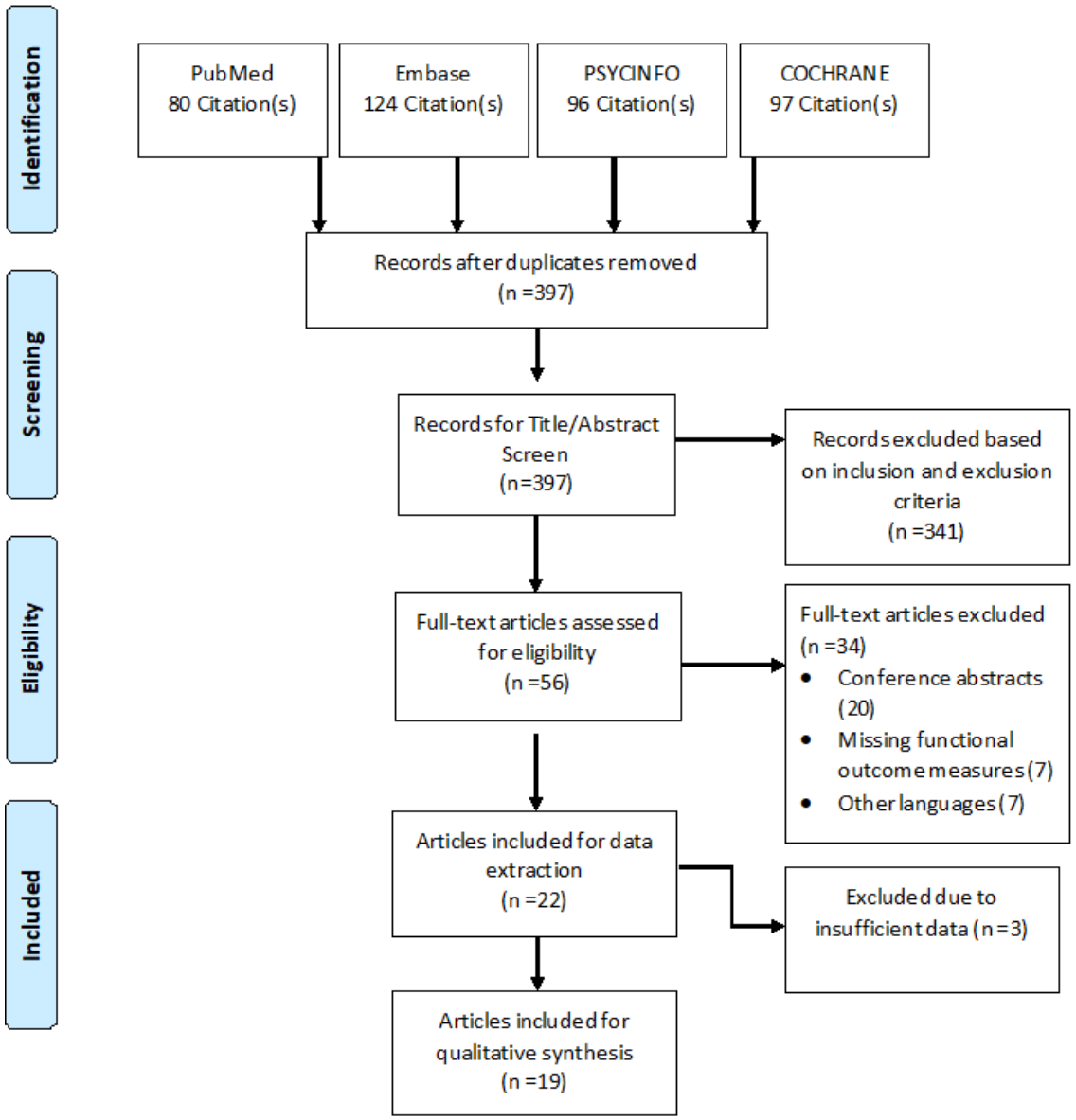

Figure 2.3.1: PRISMA flow diagram

Table: 2.3.1. Risk Assessment of studies

ROB 2 assessment for individual randomized trials		**VHR**	**NEURAPRO**
Domain 1	Risk of Bias arising from the randomization process	Low	Low
Domain 2	Risk of bias due to deviations from the intended interventions	Low	Low
Domain 3	Missing outcome data	Low	Some concerns
Domain 4	Risk of bias in measuring of the outcome	Low	Low
Domain 5	Risk of bias in selection of the reported results	Low	Low
Overall risk of bias		Low	Low
The Joanna Briggs Institute (JBI) Critical Appraisal Tool		**The PORT**	**The NAPLS**
Domain 1	Were the criteria for inclusion in the sample clearly defined?	Yes	Yes
Domain 2	Were the study subjects and the setting described in detail?	Yes	Yes
Domain 3	Was the exposure measured in a valid and reliable way?	Yes	Unclear
Domain 4	Were objective, standard criteria used for measurement of the condition?	Yes	Yes
Domain 5	Were confounding factors identified?	Yes	Yes
Domain 6	Were strategies to deal with confounding factors stated?	Yes	Yes
Domain 7	Were the outcomes measured in a valid and reliable way?	Yes	Yes
Domain 8	Was appropriate statistical analysis used?	Yes	Yes
Overall appraisal		Include	Include

Our search in PubMed. EMBASE, PSYCINFO, and COCHRANE yielded 397 non-duplicate citations altogether (Figure 2.3.1). We excluded 375 articles following the two-phased screening process and included 22 articles for data extraction. 19 articles were included in this review that had data for both, omega-3 FA and functional scores. These 19 articles were based on data from 4 different primary studies, namely the Program of Rehabilitation and Therapy (PORT), the North American Prodromal Longitudinal Studies (NAPLS), Vienna High risk study (VHR) and NEURAPRO (Table 2.3.2).

Table 2.3.2. Preliminary details of studies grouped under RCTs and Non-RCTs:
RCTs- Randomized controlled trials, VHR- Vienna High Risk, PORT, PORT - Program of Recognition and Therapy, NAPLS- North American Prodrome Longitudinal Study

Type of the study	Name/place of the study	Availability of data			References
		Assessment of Dietary omega-3 PUFA	Erythrocyte omega-3 measurement	Functional assessment	
RCTs	VHR, Vienna	-	Available	Available	(53-58)
	NEURAPRO, Multicenter	-	Available	Available	(59-66)
Non RCTs	PORT, Poland	Available	-	Available	(67, 68)
	NAPLS, United States	Available	Available	Available	(69)

Among these, PORT and NAPLS are non-randomized clinical trials (RCTs) containing cross-sectional/retrospective data, whereas VHR and NEURAPRO are RCTs with follow-up data. All studies except NEURAPRO are uni-centric studies conducted predominantly in the Caucasian population. All four studies together consist of data of a total of 560 participants between the age of 12 to 40 years. Each study used different criteria for recruiting UHR subjects, e.g. based on the Comprehensive Assessment of At-risk Mental Status (CAARMS), Structured Interview for Psychosis-risk syndromes (SIPS) and the Diagnostic and Statistical Manual of Mental Disorder (DSM). All these studies considered functional assessment as secondary outcome measure (Table 2.3.3).

i) Non-Randomized controlled trials:

Two non-RCT studies were found containing data for omega-3 FA and functioning scores of the UHR population: PORT and NAPLS (Table 2.3.3). The primary aim of the PORT study was to evaluate the role of dietary omega-3 and omega-6 FA levels on the transition to psychosis. The participants were between 16 and 30 years of age and were selected based on CAARMS scores, who fulfilled any one or more of the following three criteria: attenuated psychotic symptoms (APS), brief limited intermittent psychotic syndrome (BLIPS) or a vulnerable group with personality disorder in first degree relatives (VUL). In addition to the UHR individuals, the study included healthy

controls who had no previous history of psychosis. Dietary fatty acid content was recorded retrospectively using a semi-quantitative food frequency questionnaire (FFQ) and PUFA content of the diet was quantified using food composition tables. In the PORT study functional assessment was recorded using SOFAS which is a sub-requirement for CAARMS criteria (67, 68). The second study by Cadenhead et al. is a cross-sectional analysis of baseline characteristics from the NAPLS clinical trial. The study population included only UHR subjects between 12 to 29 years of age without any control group. Structured Interviews for Psychosis-risk syndromes (SIPS) were used to define the UHR status of the participants. Both studies have data of erythrocyte omega-3 composition measured using a capillary gas chromatography method. In contrast to the PORT study, the NAPLS study used a systematic checklist by Vilma Gabbay to evaluate the dietary omega-3 FA level and Global assessment scale (GAF) and Global functioning social & role (GF: S & R) scales were used to assess the functioning status of UHR subjects (69).

Table: 2.3.3. Study design of Non-Randomized Controlled trials with data PORT-Program of Rehabilitation and Therapy, NAPLS- North American Prodrome Longitudinal Study, PUFA-Poly Unsaturated Fatty Acids, CAARMS- Comprehensive Assessment of At-risk Mental Status, SIPS-Structured Interview for Psychiatric Risk Syndromes, DSM- Diagnostic and Statistical Manual of Mental Disorders, SOFAS- Social and Occupational Functioning Assessment Scale, GAF- The Global Assessment of Functioning, GF: S & R- Global Functioning Social and Role scale, EPA-Eicosapentaenoic acid, DHA- Docosahexaenoic acid, NC-Non converters, C-Converters, EPA-Eicosapentaenoic acid, DHA- Docosahexaenoic acid, SD-Standard Deviation, IQR- Inter Quartile Range, SOFAS- Social and Occupational Functional Assessment Scale, RBC- Red Blood Cell, GF-Global Functioning and GAF- The Global Assessment of Functioning.

Name of the study	PORT	NAPLS
Aim of the trial	To study the role of PUFA consumption on transition to psychosis	To investigate baseline cardio-metabolic, dietary omega-3 fatty acids and oxidative stress indices
Assessment tool used	CAARMS	SIPS
Age group (in years)	16-30	12-29
Key exclusion criteria	• diagnosed with mental retardation or organic brain disorder, • in whom symptoms occurred primarily due to drug or alcohol use and	• with schizophrenia spectrum disorder (DSM-IV), • history of antipsychotic medication in the previous month, • with concomitant medical or neurological illness, • Having IQ below 80,

Name of the study	PORT		NAPLS
	• those with consumed special diets or PUFA supplements in the past 12 months		• having active suicidal or homicidal ideation, • having allergies to seafood or seafood related products or no history of seafood consumption and • Pregnant and lactating mother
Total number of UHR participants	62		113
Details of control population	Healthy controls (n=33)		No control group
Definition of transition	Retrospective assessment of Frank psychotic symptoms occurring at least daily for one week or more (70)		-
Functional assessment scale(s) used	SOFAS		GAF and GF:S & R
Key results	• UHR population who had converted to psychosis showed higher consumption of n-6 fatty acids than non-converted population • Higher EPA and DHA consumption rate is associated with converted to psychosis Healthy controls showed higher consumption of omega-3 and lower consumption of n-6 than HR individuals		Metabolic parameters and a diet low in omega-3 rich foods were significantly associated with prodromal symptoms and poor functioning
References	(67, 68)		(69)
Groups (n)	NC (47)	C (15)	
Age [Mean ± SD]	19.0 ± 3.5	20.0 ± 3.4	18.7 ± 4.6
Sex (male/female)	23/24	8/7	65/48
Erythrocytes n-3 levels, % total [Mean ± SD]	-	-	0.4 ± 0.3 (EPA) 2.5 ± 1.0 (DHA) 4.8 ± 1.7 (omega-3 index)
Dietary n-3 intake,	(g/day) [Median, IQR]	(g/day) [Median, IQR]	Weekly frequency (Mean ± SD)

Name of the study	PORT		NAPLS
	2.9, 4.94 (Total) 0.04, 0.09 (EPA) 0.09, 0.16 (DHA)	3.14, 2.44 (Total) 0.05, 0.04 (EPA) 0.1, 0.11 (DHA)	13.9 ±10.7
SOFAS	[Mean ± SD] 59.85 ± 8.04	[Mean ± SD] 55.8 ± 6.29	Correlation Coefficient = -0.24 ($p<0.05$) vs RBC n-3 Index (EPA+DHA)
GF: Social	-	-	Not significant
GF: Role	-	-	Correlation Coefficient = 0.17 ($p=0.05 – 0.10$) vs RBC n-3 Index (EPA+DHA)
GAF	-	-	Correlation Coefficient = 0.37 ($p<0.01$) vs omega-3 FA diet Correlation Coefficient = 0.21 ($p<0.05$) vs RBC EPA

ii) Randomized Controlled Trials:

We included two RCTs with baseline and follow-up data at three different timepoints (Table 2.3.4). The VHR study is a double-blind placebo-controlled trial that was carried out at a psychosis detection unit at the Medical University of Vienna. In total 81 UHR subjects were recruited based on DSM-IV criteria. The active intervention group and the placebo group received a daily dosage of 1.2 g of omega-3 FA or coconut oil for 12-weeks, respectively. For this trial, follow-up data was recorded after 12 weeks, 12 months and 6.7 years from the time of the start of the intervention (53, 54, 56-58, 71). The NEURAPRO study is another double-blind placebo-controlled multi-centre clinical trial with 6 months of intervention. A total of 304 UHR participants were recruited based on the CAARMS protocol. Among them, 153 participants received a daily dosage of 1.4 g of omega-3 FA and the control group received the same amount of paraffin oil as a placebo. Irrespective of the group, all participants received cognitive behavioural case management (CBCM) as an adjunct therapy. In NEURAPRO the follow-up data were documented at 6 months, 12 months and 3.4 years from the beginning of the intervention. In the VHR study, the functional assessment was measured using the GAF scale whereas in the NEURAPRO trial SOFAS and GF: S & R scales were used. Even though both RCTs have different outcome measures, their primary objective was to evaluate the role of omega-3 FA in the transition to first episode psychosis (FEP) (59-66).

Table: 2.3.4. Study design of Randomized Controlled trials with baseline data

VHR- Vienna High Risk Study, PANSS- Positive and Negative Syndrome Scale, GRS- Global Rating Scale, CAARMS- Comprehensive Assessment of At-Risk Mental Status, SOFAS- Social and Occupational Functional Assessment Scale, GAF- The Global Assessment of Functioning, DSM- Diagnostic and Statistical Manual of Mental Disorders, IQ- Intelligence Quotient, EPA- Eicosapentaenoic acid, DHA- Docosahexaenoic acid and GF: S & R- Global Functioning Social and Role scale.

Name of the trial	VHR	NEURAPRO
Place	Austria	Multicenter
Aim of the trial	To determine the role of omega-3 on the rate of transition to first episode psychotic disorders	To determine the combined effect of omega-3 & CBCM on the rate of transition to first episode psychotic disorders
Age range (in years)	13-25	13-40
At risk population criteria	Attenuated Psychotic Symptoms Based on PANSS assessment	Attenuated positive psychotic symptoms group Based on GRS/CAARMS/SOFAS
	Transient Psychosis Based on PANSS assessment	Transient psychosis Based on GRS/CAARMS/SOFAS
	Trait Plus State Risk Factors Based on GAF score	Vulnerability group (trait and state risk factors) Based on SOFAS score
Key exclusion criteria	• history of a previous psychotic disorder or manic episode • substance-induced psychotic disorder • acute suicidal or aggressive behavior • a current DSM-IV diagnosis of substance dependence except cannabis dependence • other neurological disorders • premorbid IQ< 70 • Apparent MRI findings indicating structural brain changes	• a history of a treated or untreated psychotic episode of 1-week duration or longer • any physical illness with psychotropic effect, if not stabilized • current treatment with any mood stabilizer, or recreational use of • organic brain disease, for example, epilepsy, inflammatory brain disease • abnormal coagulation profile parameters or thyroid function test results >10%

Name of the trial	VHR	NEURAPRO
	• previous treatment with an antipsychotic or mood-stabilizing agent (>1week) • omega-3 supplementation within the last 8 weeks • laboratory values more than 10% outside the normal range for transaminases, thyroid hormones, C-reactive protein, or bleeding parameters; and	above or below the limits of the normal range • current treatment with any mood stabilizers/ recreational use of ketamine • past neuroleptic exposure equivalent to a total lifetime haloperidol dose of >50 mg • a diagnosis of a serious developmental disorder • premorbid IQ < 70 and a documented history of developmental delay or intellectual disability • current acute suicidality/self-harm or aggression/ dangerous behavior (indicated by a CAARMS severity score of 6 on items 7.3 and/ or5.4) • current pregnancy; • Greater than 4 weeks of regular omega-3 supplementation (>600 mg combined EPA/DHA) within the last 6 months.
Active intervention (daily dosage)	700 mg of EPA 480 mg of DHA 220 mg of other omega-3 FA 7.6 mg of Vitamin E	840 mg of EPA 560 mg of DHA 5 mg of Vitamin E
Placebo intervention (daily dosage)	Coconut oil 7.6 mg of Vitamin E 1% fish oil	Paraffin oil 5 mg of vitamin E 1% fish oil
Duration of intervention	12 weeks	6 months
Total number of participants	81	304

Name of the trial	VHR		NEURAPRO	
Functional assessment scale(s) used	GAF		SOFAS and GF: S & R	
Groups	Omega-3	Placebo	Omega-3	Placebo
Age in years [Mean ± SD]	16.8 ± 2.4	16.0 ± 1.7	19.4 ± 4.8	18.9 ± 4.3
Sex (male/female)	14/27	13/27	62/91	77/74
Erythrocytes n-3 levels, % total, [Mean ± SD]	5.6 ± 1.2 (Total) 0.5 ± 0.2 (EPA) 2.8 ± 0.8 (DHA)	5.3 ± 1.0 (Total) 0.5 ± 0.1 (EPA) 2.5 ± 0.6 (DHA)	3.00 ± 1.14 (n-3 index) 0.53 ± 0.18 (EPA) 2.48 ± 1.02 (DHA)	
Baseline Functional Data				
SOFAS [Mean ± SD]	-	-	53.2 (11.8)	53.5 (12.2)
GF: Social [Mean ± SD]	-	-	6.5 (1.2)	6.5 (1.3)
GF: Role [Mean ± SD]	-	-	6.0 (1.5)	5.9 (1.5)
GAF [Mean ± SD]	61.0 (12.0)	60.0 (13.1)	-	-
Post-intervention functional data				
SOFAS [Mean ± SD]	-	-	8.9 ± 16.5	12.6 ± 14.9
GF: Social [Mean ± SD]	-	-	0.5 ± 1.2	0.6 ± 1.4
GF: Role [Mean ± SD]	-	-	0.5 ± 1.7	0.9 ± 1.6
GAF [Mean ± SD]	61 ± 21.6	59.9 ± 3.2	-	-

The studies provide data with cross sectional and multiple follow-up analyses indicating varying effects of omega-3 FA on functional status. Given the heterogeneous study designs and diverse data presentation, we interpreted the results under three topics:

i) Evidence for cross-sectional associations between omega-3 FA and functional status in people at UHR

The NAPLS study's weekly frequency of omega-3 rich diet intake and erythrocyte EPA level showed a weak positive correlation with the baseline functional status. Moreover, the erythrocyte EPA level positively correlated with functional score at baseline (69). In contrast, in the NEURAPRO trial none of the omega-3 measures were found to be cross-sectionally associated with functional outcome (72). In the PORT study the direct relationship between omega-3 FA diet and functional outcome was not analyzed (68, 73).

ii) Evidence from intervention studies for associations between omega-3 FA and functional outcome in people at UHR

In the VHR study, the omega-3 group reported significantly higher functioning at 12 weeks, 12 months and long term follow-up (median of 6.7 years) (58, 74), however the omega-3 group of the NEURAPRO study showed no significant difference compared to the placebo group in follow-ups (75, 76) (Table 2.3.5).

iii) Evidence for omega-3 FA at baseline as a predictor of future functional outcome in people at UHR

In the VHR study, omega-3 FA along with omega-6 FA predicted the improvement of functioning at 12 weeks follow-up in both study arms (55).

Table: 2.3.5. Follow-up data of functional outcome from Randomized Controlled Trials- VHR- Vienna High Risk Study, SD- Standard Deviation, SOFAS- Social and Occupational Functional Assessment Scale, GF:S- Global Functioning Social, GF:R- Global Functioning Role and GAF- The Global Assessment of Functioning.

	The VHR-GAF		**NEURAPRO-SOFAS**		**NEURAPRO-GF:S**		**NEURAPRO-GF:R**	
Groups	n-3	Placebo	n-3	Placebo	n-3	Placebo	n-3	Placebo
Follow-up 1 [Mean ± SD]	12 months(change)		12 months(change)		12 months(change)		12 months(change)	
	17.7 ± 2.3	7.2 ± 2.3	14.7 ± 19.1	14.3 ± 16.8	0.5 ± 1.4	0.7 ± 1.6	0.9 ± 1.7	1.0 ± 2.0
Follow-up 2 [Mean ± SD]	6.7 years (change)		3.4 years (change)		3.4 years (change)		3.4 years (change)	
	7.7 ± 2.7	-0.8 ± 2.7	14.8 ± 18.3	15.3 ± 16.5	0.6 ± 1.4	0.8 ± 1.6	1.0 ± 1.6	1.3 ± 1.7

2.4. Discussion

To our knowledge, this is the first systematic literature review concerning the association between omega-3 FA and functioning in the ultra-high risk for psychosis population. Of the four primary studies included in this review, three (NAPLS, PORT and NEURAPRO) analysed the cross-sectional relationship between omega-3 FA and functional status and two (VHR and NEURAPRO) further analysed the longitudinal association of omega-3 FA supplementation with functional outcome on follow-up.

2.4.1. Evidence for cross-sectional associations between omega-3 FA and functional status in people at UHR

In the NAPLS study, the cross-sectional data showed a weak positive correlation between the weekly intake of omega-3 consumption (as measured by dietary questionnaire) and GAF functional scores at baseline. The authors also evaluated the correlation between fasting erythrocyte omega-3 FA composition with functional status. The total omega-3 FA level of the participants was found to be 4.8 % (SD1.7), of which the contribution of DHA is approximately 6 times higher than that of EPA (Table 2). In a correlation analysis, EPA showed a weak positive correlation with GAF general functioning score ($r=0.21$, $p<0.05$) but no significant correlations were found for other erythrocyte omega-3 FA measures. Moreover, the functional scores of Global social and role scales were associated neither with dietary omega-3 intake nor with erythrocyte omega-3 FA levels (69). Similarly, in the NEURAPRO study, none of the omega-3 FA measures were associated with functional status at baseline, although such an association was found for omega-6 FA (72).

In contrast to these findings, previous studies in psychiatric disorders have found a positive association of DHA with cognitive and behavioural functions. For instance, a decrease in the dietary DHA level and an altered erythrocyte FA ratio were commonly observed in patients with bipolar disease (77-79), (80). In both NAPLS and NEURAPRO, data regarding the erythrocyte omega-3 FA levels of UHR subjects in relation to healthy controls were not available. To our knowledge, only one study has reported the physiological difference in erythrocyte omega-3 FA levels between UHR patients and healthy subjects. According to Rice et al., erythrocyte levels of α-Linoleic acid and EPA in UHR subjects is lower than in the healthy population (81). Whereas in chronic schizophrenia patients, omega-3 FA levels have been found to be increased compared to the healthy control population (30, 82-84) and a meta-analysis by Hoen et al., has indicated a substantial decrease of DHA in schizophrenia patients compared to controls (85). Hence, this uncertainty has to be investigated further to understand the therapeutic role of omega-3 FA in psychosis. In the PORT study, the high-risk psychosis patients who transitioned to psychosis reported to have consumed less omega-3 FA than the non-converters. Although, no significant difference was noticed in their functional status between the groups (68).

2.4.2. Evidence from intervention studies for associations between omega-3 FA and functional outcome in people at UHR

The interventional studies included in this review (VHR and NEURAPRO) provided information regarding the relationship of omega-3 FA supplementation with functional outcome at different time points (Table 3). In the VHR study, those randomized to receive omega-3 FA supplementation showed a significantly improved functional score at 12 months follow-up compared to those who received placebo (effect size -0.72) (86). This improvement in functioning in the omega-3 FA group was consistently observed at both medium term (12 months) and long-term (median of 6.7 years) follow-up (87). For the biomarker analysis, the erythrocyte omega-3 FA level showed a significant increase compared to the omega-6 FA value in the active intervention group for 12 weeks intervention. Moreover, the change in the omega-6 to omega-3 FA ratio from baseline to 12 weeks was significantly associated with functional improvement (58).

On the other hand, the results of the NEURAPRO study contrasted that of the VHR study. In NEURAPRO, at six months follow-up, the functional scores of Global functioning Social & Role scales was found to be improved at the end of the intervention in all participants irrespective of study arms. A 96% increase in functional score was noticed during the intervention period and only 4 % increased after the intervention period until the medium-term follow-up in all participants (76). However, no statistical difference was found between the omega-3 FA and placebo group with respect to functional outcome. This may indicate that supplementation is ineffective in improving functioning in UHR. Such findings could be due to the relatively low levels of omega-3 FA in the erythrocyte membrane and a narrow range of variation before and after the intervention. The total omega-3 FA level, which was about 3% at baseline, increased by only 1% after omega-3 FA supplementation (75, 76). In addition to the low baseline omega-3 FA levels, the lack of compliance to omega-3 FA supplements and the overshadowing effects of effective co-intervention were reported to have an impact on the clinical outcome of omega-3 FA. From the assessment of study medication, Schlögelhofer et al. estimated that 57.9% of the participants of the omega-3 FA group were non-adherent to study medication (88). Furthermore, the non-adherent group had significantly lower baseline functional scores and a lower baseline erythrocyte omega-3 index compared to the adherent group (88). Similarly, the

additional beneficial effects of CBCM, which is an efficacious co-intervention given to both the groups, may have eclipsed the effect of omega-3 FA (76, 89).

2.4.3. Evidence for omega-3 FA at baseline as a predictor of future functional outcome in people at UHR

In the RCTs, multiple follow-up data enable analysis of whether omega-3 levels at baseline might predict future functional outcome in people at UHR. In the VHR study, none of the baseline erythrocyte membrane omega-3 FA individually predicted functional improvement. However, in a multivariate analysis, all FA of erythrocyte membrane together significantly predicted the improvement of functional status after 12-weeks in both, the intervention and placebo group (55). This finding further raises the question of a possible synergistic role of EPA and DHA acting in concert with other PUFAs to produce clinical improvement in UHR status.

In a recent analysis, Amminger et al. investigated the predictive nature of the change in omega-3 FA levels on various clinical outcomes in 218 participants in the NEURAPRO study. They reported that six months increase in omega-3 FA levels during the intervention successfully predicted the improvement of the functional score at 6 months and 12-months follow-up. According to this report, an increase in erythrocyte omega-3 FA levels significantly predicted an improvement of functional score by at least three points in UHR participants (90). This data further indicates that functional improvement could be achieved even in participants with low baseline omega-3 FA levels (90). Considering our limited knowledge on the underlying biological processes in psychosis, further studies to evaluate the dose dependent clinical effects of omega-3 FA would be helpful to understand the therapeutic role of omega-3 FA in psychosis.

2.4.4. Evidence in context of previous literature

Previous studies on animals support the positive influence of dietary omega-3 FA on biological and behavioural outcomes. For instance, intra-hippocampal infusion of DHA enhanced 5-HT levels and enrichment of rat brain with DHA caused an increase in synaptic dopamine and serotonin resulting in anti-depressant like behavioural changes (91, 92). Similarly, diet induced antidepressant effects of omega-3 FA were noticed by a few other studies in experimental animals (93-95). However, the results from clinical studies are not as convincing as the animal counterparts. In humans, the

functional outcome was estimated by various measures such as cognitive functioning, physiological and clinical symptoms. A recent meta-analysis on a young healthy population by Emery et al., did not find any beneficial effects of domain specific cognitive performances of omega-3 FA supplementation. Nevertheless, the meta-analysis indicated beneficial effects of EPA supplementation on some cognitive domains such as long-term memory, working memory and problem solving (96). On the other hand, findings from psychiatric patients revealed varying effects of dietary omega-3 FA levels on clinical symptoms (97-101). Such inconsistencies with clinical results are evident in the studies mentioned in our review. Clinical trials have started considering erythrocyte membrane omega-3 FA composition as a biological measure for biologically active omega-3 FA levels, since the FA composition of erythrocytes closely reflects that of neuronal membrane and easily influenced by FA content in the diet (102, 103). Even though, evidence regarding the influence of erythrocyte omega-3 FA levels on local environment in the brain is still not clearly understood and hence underlying biological activities should be investigated in order to appreciate the long-term effects of erythrocyte omega-3 FA levels on functional status (104, 105). Overall, however beneficial effects of an omega-3 FA diet on functional status was observed by cross-sectional and intervention studies (the NAPLS and the VHR respectively), more investigations are required to validate these results in UHR subjects to reach a definite conclusion.

2.5. Conclusion and future directions

To our knowledge, this is the first systematic review reporting the influence of omega-3 FA on functional outcome in UHR of psychosis. Cross-sectional data indicated a positive correlation between dietary omega-3 FA and functional status. Among various erythrocyte membrane omega-3 FA concentrations, the EPA associated positively, whereas omega-6/omega-3 FA ratio are inversely associated with functional improvement. Further, the combined concentrations of all baseline erythrocyte membrane FA successfully predicted functional enhancement.

2.6. Limitations and future directions

At the review level, the following limitations should be noticed. In the interest of asking a single focused research question, this review focused only on one clinical assessment (functional status) in a relatively rare population group (UHR). This

reduced the total number of available articles and substantially made it impossible to perform a meta-analysis.

At the individual study level, even though adequate sample sizes were reported for the analysis, the following limitations were noticed. Firstly, the co-interventions provided in clinical trials such as vitamin supplements and the frequency of CBCM which can influence the study outcomes were not controlled carefully in the analysis. The lack of uniform inclusion criteria across different trials, lack of standardized clinical assessments and low adherence to the study intervention make it difficult to compare the results to reach an informed conclusion.

In the future, we suggest the following strategies to enhance the retention and compliance of the clinical trials: i) explaining the expectations of the study at an early stage to the participants, ii) using digital options to motivate the participants to take their medication, iii) using a digital option to provide real-time feedback regarding adherence to the treatment, and iv) considering remote data collection procedures for data such as pills taken every day (106, 107). In addition, considering a unified study protocol for UHR subjects with standardized outcome assessments would increase the clinical validity of the data for understanding the role of omega-3 FA in psychosis. Since the number of available studies are insufficient and provided inconsistent results, it is too early to comment with confidence on the clinical effectiveness of omega-3 FA on functioning in the UHR population. However, our review stresses the need of more randomized controlled trials with additional measures to obtain meaningful results in terms of therapeutic role of omega-3 FA in UHR status.

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2.8. Disclosure of conflicts of interest

All authors declare no conflicts of interest in relation to this work.

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Chapter 3

Thesis author's role:

Mr Subash Raj Susai was involved in all aspects of the study. He had full access to all the data in the study and was involved in the acquisition, analysis, and interpretation of data and drafting of the manuscript.

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Title: The association of plasma inflammatory markers with omega-3 fatty acids and their mediating role in psychotic symptoms and functioning: an analysis of the NEURAPRO clinical trial

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Abstract

There is increasing evidence that dysregulation of polyunsaturated fatty acids (FAs) mediated membrane function plays a role in the pathophysiology of schizophrenia. Even though preclinical findings have supported the anti-inflammatory properties of omega-3 FAs on brain health, their biological roles as anti-inflammatory agents and their therapeutic role on clinical symptoms of psychosis risk are not well understood. In the current study, we investigated the relationship of erythrocyte omega-3 FAs with plasma immune markers in a clinical high risk for psychosis (CHR) sample. In addition, a mediation analysis was performed to examine whether previously reported associations between omega-3 FAs and clinical outcomes were mediated via plasma immune markers. Clinical outcomes for CHR participants in the NEURAPRO clinical trial were measured using the Brief Psychiatric Rating Scale (BPRS), Schedule for the Scale of Assessment of Negative Symptoms (SANS) and Social and Occupational Functioning Assessment Scale (SOFAS) scales. The erythrocyte omega-3 index [eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)] and plasma concentrations of inflammatory markers were quantified at baseline (n=268) and 6 month follow-up (n=146) by gas chromatography and multiplex immunoassay, respectively. In linear regression models, the baseline plasma concentrations of Interleukin (IL)-15, Intercellular adhesion molecule (ICAM)-1 and Vascular cell adhesion molecule (VCAM)-1 were negatively associated with baseline omega-3 index. In addition, 6-month change in IL-12p40 and TNF-α showed a negative association with change in omega-3 index. In longitudinal analyses, the baseline and 6 month change in omega-3 index was negatively associated with VCAM-1 and TNF-α respectively at follow-up. Mediation analyses provided little evidence for mediating effects of plasma immune markers on the relationship between omega-3 FAs and clinical outcomes (psychotic symptoms and functioning) in CHR participants. Our results indicate a predominantly anti-inflammatory relationship of omega-3 FAs on plasma inflammatory status in CHR individuals, but this did not appear to convey clinical benefits at 6 month and 12-month follow-up. Both immune and non-immune biological effects of omega-3 FAs would be resourceful in understanding the clinical benefits of omega-3 FAs in CHR papulation. Key words: Omega 3 Fatty Acid, n-3 Poly Unsaturated Fatty Acid, biological marker, immune markers and clinically high-risk.

3.1. Introduction

Schizophrenia is a mental disorder with a multifactorial etiology. Most patients with schizophrenia experience a prodromal phase of nonspecific psychiatric and subthreshold psychotic symptoms (1). The clinical high-risk (CHR) paradigm provides operational criteria to define a subpopulation at high risk of psychosis and offers the opportunity to intervene early to improve prognosis or even prevent transition to a psychotic disorder. CHR individuals have a 22% risk of developing psychosis in the first year after ascertainment and increases at least 38% after 16 years (2-6). A growing body of evidence has indicated the involvement of inflammation and oxidative stress from an early stage in psychotic disorders such as schizophrenia (7-15). Several inflammatory mediators that participate in neuronal development and synaptic pruning are consistently found to be involved in the early stages of psychosis, including Interleukin (IL)-1β, IL-6, IL-8, IL-12p40 and Tumor Necrosis Factor-α (TNF-α) (7, 8, 10, 16-19). Currently available antipsychotic medications still broadly target dopaminergic neurotransmission and are inadequate in treating negative and cognitive symptoms and can cause serious adverse events (20). Hence, there is a need for investigation of further interventions targeting alternative mechanistic pathways to delay or prevent the development of psychosis. In fact, neuroinflammation and antioxidative defense seem to represent potential candidates for those pathways.

Studies have identified several molecular mechanisms through which omega-3 fatty acids (FAs) produce health benefits in healthy and diseased states (21). The long chain omega-3 FAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are structurally and functionally vital for neuronal health (22). Both EPA and DHA are integral components of the neuronal membrane, which is necessary for maintaining membrane integrity and signal transduction in neurons (23). In addition, omega-3 FAs derived metabolites also known as specialized pro-resolving mediators (SPM), participate in the orchestration of the inflammatory response (24). SPM include compounds such as prostaglandins, thromboxanes, resolvins, protectins and maresins and are synthesized mainly from EPA and DHA (21, 24, 25). Serhan et al. first reported the anti-inflammatory and pro-resolutive properties of SPM in the peripheral endothelial cells and polymorphonuclear macrophages (26). Later, the receptors of SPM were identified in cells of both peripheral circulation and in the central

nervous system (16, 25, 27-38). Through these receptors, SPM fine-tune the intensity and duration of inflammation without compromising the quality of the immune response (24, 27, 29, 32, 34, 35, 37, 38). A common route by which omega-3 FAs achieve these pro-resolving activities is through concomitant regulation of inflammatory cytokines such as IL-6, TNF-α and IL-1β (39, 40).

In humans, the anti-inflammatory effects of omega-3 FAs have been observed in neurodegenerative disorders, cerebrovascular ischemic disorders, and psychiatric disorders such as depression and bipolar disorder (21, 41-43). However, in patients with established schizophrenia, clinical trials of omega-3 FA supplementation have not produced consistent results. For instance, five studies showed improved prognoses with greater efficacy in omega-3 group than the placebo whereas two studies found no difference and one study reported poor prognosis following omega-3 supplementation (44, 45). This may be due to factors such as late onset of omega-3 FA supplementation, confounding effects of age-related metabolic changes and varying regimens of anti-psychotics (44, 45). Since inflammation and disruption of membrane architecture (especially in neurons) have been noticed from an early stage of psychosis, intervening at an earlier phase with omega-3 FA has been suggested as an option for preventing or delaying the development of psychosis (7, 8, 44, 46, 47).

A previous clinical trial, the Vienna High Risk (VHR) study, found that omega-3 FA supplementation reduced the risk of transition to psychosis in CHR participants (48). The potential role of omega-3 FAs in modulating inflammation was investigated in a subsequent analysis (49). The authors analyzed the relationship of baseline erythrocyte omega-3 FA levels with three immune mediators including interleukin 6 (IL-6), the soluble alpha (Tac) subunit of the interleukin 2 receptor (sIL-2r), and the circulating soluble form of the intercellular adhesion molecule one (sICAM-1) and found no significant association with membrane omega-3 FAs at baseline (49). While previous animal and human studies have identified various molecular pathways of inflammatory reactions through which omega-3 FAs influence inflammatory cytokines, the clinical benefit in early psychosis is not yet clearly established (50-52).

The aims of the current study were to investigate the relationship of omega-3 FAs on plasma immune markers in CHR participants and to evaluate whether inflammatory cytokines mediated previously described relationships between omega-

3 FAs and clinical outcomes in CHR patients. We used plasma and erythrocyte samples from the NEURAPRO trial which is an omega-3 FA based randomized controlled trial aimed to prevent the onset of psychosis in CHR participants. The participants had little or no exposure to antipsychotic medication that can potentially influence inflammatory cytokines (53, 54). In this study we intend to answer the two questions: i) whether Omga-3 FAs exert any relationship with plasma inflammatory markers in CHR individuals measured at baseline and 6 month follow-up, and ii) whether plasma immune markers mediate the relationship between omega-3 FAs and clinical outcomes in particular psychotic symptoms and social functioning. Considering that in the NEURAPRO clinical trial no direct clinical association was observed with omega-3 FA supplementation (55), we mainly focus on the indirect effect through omega-3 FAs and the overall effect (Figure 1) of erythrocyte membrane omega-3 FAs level on psychopathology in CHR participants. Based on evidence from preclinical studies, we hypothesized that omega-3 FAs would be negatively associated with plasma inflammatory biomarkers in CHR participants and would mediate associations between omega-3 FAs and clinical outcomes.

3.2. Materials and Methods

3.2.1. The NEURAPRO clinical trial

The primary outcome of the NEURAPRO clinical trial was to assess the efficacy of omega-3 FA supplementation preventing transition to psychosis in CHR participants. The NEURAPRO clinical trial was registered with the Australian New Zealand Clinical Trial Registry as ACTRN 12608000475347. The study was conducted between March 2010 and the end of September 2014, in accordance with the Declaration of Helsinki and consistent with the International Council for Harmonization of Good Clinical Practice with appropriate ethical approval obtained from each site before the trial commenced. Ethical approval for the biomarker analysis presented in this study was obtained from the research ethics committee of the Royal College of Surgeons in Ireland [REC-No. 1699].

3.2.2. Study participants

A total of 285 out of 304 participants aged 18.97 ± 4.49 years (mean ± SD) who met CHR criteria and who had valid baseline and follow-up clinical data were

considered for the plasma biomarkers analysis study. The exclusion criteria were: history of psychotic episodes of seven days or longer; any current symptoms of intoxication, organic brain disease or developmental disorder; abnormal coagulation profile; thyroid abnormalities; physical illness with psychotropic effect, if not stabilized; current treatment with any mood stabilizers or recreational use of ketamine; past antipsychotic exposure equivalent to a total lifetime haloperidol dose of >50 mg; a diagnosis of a serious developmental disorder; premorbid IQ less than 70; current acute suicidality/self-harm or aggression/dangerous behavior; pregnancy; or intake of more than 4 weeks of supplementation with omega-3 FAs (57, 58).

3.2.3. Exposure: Erythrocyte omega-3 FAs measures

The study participants in the clinical trial were randomized to receive either omega-3 FA supplementation or placebo. The study medication contains a dose of four gelatin capsules taken daily throughout the 6-month treatment period. Participants were provided with dispensed bottles of capsules containing either omega-3 FAs or placebo and participants were instructed to take two capsules in the morning and two at night. The omega-3 group received ~2.8 g of marine fish oil containing approximately 840 mg of EPA and 560 mg of DHA per day, whereas the placebo group received an equivalent dose of paraffin oil per day for 6 months. In addition, all participants could receive up to 20 sessions of cognitive behavioral case management (CBCM) as a co-intervention. The full description of treatments participants received during the trail is provided by McGorry et al (59). Assessing adherence, McGorry et al., reported that nearly 58% of the participants were non-adherent to the study intervention in the NEURAPRO trial (60). To address this, we considered the erythrocyte omega-3 FA levels as a measure of exposure irrespective of the study arms (omega-3/placebo) as this is accepted to be an accurate biological marker for dietary intake of omega-3 FAs and closely reflects the omega-3 FA content of neuronal membranes (61-64). Fasting plasma samples were collected at baseline and 6 months following the intervention. The molecular percentage of total fatty acid levels of EPA, DHA and n3-index (EPA+DHA) were measured using gas chromatography (66). The Phosphatidyl-ethanolamine (PE) fraction was used to determine the omega-3 FA content, because of their high abundancy in the lipid raft (67, 68).

3.2.4. Plasma immune marker concentration

Peripheral blood samples were obtained from the participants at baseline and 6-month follow-up. Plasma levels of Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin (IL) -1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-15, Tumor necrosis factor-α (TNF-α), Interferon gamma (INF-γ), Inter-cellular Adhesion Molecule (ICAM)-1 and Vascular cell adhesion molecule (VCAM)-1 were measured using the Pro-inflammatory Panel 1, Cytokine Panel 1 and Vascular Injury Panel 2 v-PLEX® multiplex immunoassay kits (Mesoscale Discovery Systems) according to the manufacturer's instructions. A Sector Imager 2400 plate reader was used to quantify concentrations of each marker (Meso Scale Diagnostics).

3.2.5 Clinical outcome: psychotic symptoms and functioning

Positive psychotic symptoms of participants were measured the Brief Psychiatric Rating scale-psychotic score (BPRS-Psychotic) which comprises combined scores of suspiciousness, hallucination, bizarre thoughts & unusual thoughts. Negative symptoms were assessed using the Scale for the Assessment of Negative Symptoms (SANS). Functional status was measured using the Social and Occupational Functional Assessment Score (SOFAS) recorded at baseline, 6 month and 12 month follow-up.

3.2.6 Statistical Analysis

The erythrocyte membrane levels of EPA, DHA and the omega-3 index were calculated as percentage of total erythrocyte membrane fatty acid content. The plasma concentrations of inflammatory markers that were within the detection limits of the assay with a coefficient of variance (CV) of at most 20% were taken forward for analysis. Missing concentration of inflammatory markers were imputed with average values and converted to Z scores for the analysis.

Statistical analysis was performed using IBM® SPSS® statistics version 26 and STATA IC ® version 16. The association between erythrocyte omega-3 levels and plasma biomarkers were evaluated using linear regression models. The cross-sectional and longitudinal analyses were conducted for baseline, follow-up and for change of omega-3 values with corresponding inflammatory marker concentrations.

For cross-sectional models, the results were adjusted for age, sex, and body mass index (BMI) and for the longitudinal analysis the results were also adjusted for the baseline omega-3 FA levels. The inflammatory markers that showed significant association with change in omega-3 FA level were considered for the mediation analysis.

The mediation analysis was carried out to evaluate the direct effect of change in omega-3 index (exposure) on clinical outcome and the possible mediating role of plasma immune markers on an association between omega-3 index and clinical outcomes (indirect effect) at 6 and 12-month follow-up (69). Mediation analysis was performed in IBM® SPSS® using the PROCESS platform. Regression coefficients were constructed using conventional mediation analysis model (model 4) with a bootstrap sample size of 5000 and with 95% confidence interval (Figure 3.2.1). The mediation analysis was adjusted for age, sex, BMI and baseline omega-3 index levels. The results were corrected using a false discovery rate (FDR) of 5%, as described by Benjamini-Hochberg (70).

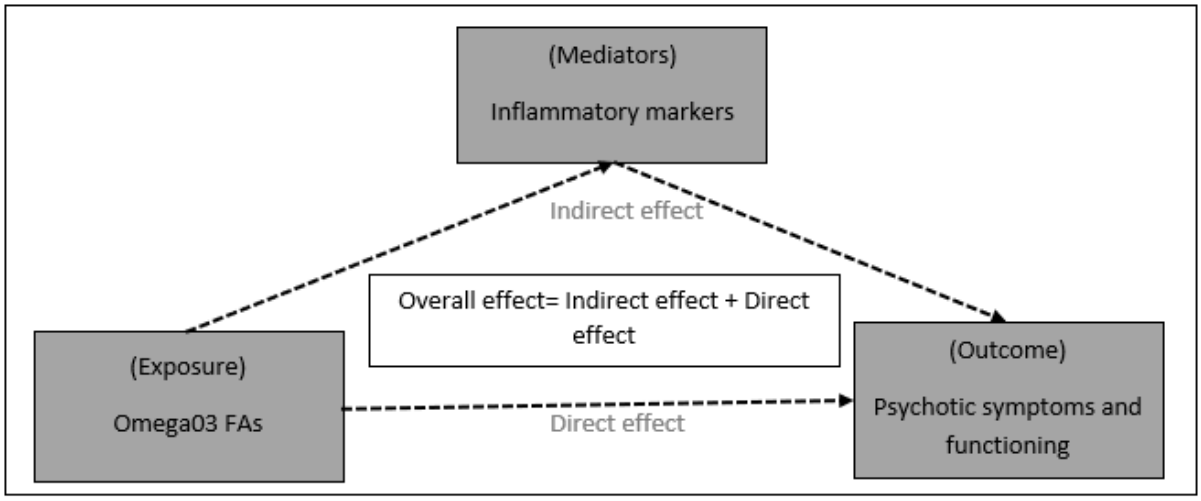

Figure 3.2.1. Mediation analysis model used for investigating the effect of omega-3 FAs on psychotic and functional outcome in CHR participants.

3.3. Results

3.3.1. Participant characteristics

Erythrocyte membrane FA and plasma immune marker concentrations were available for 268 participants at baseline and for 146 participants at both time-points (baseline and 6-month follow-up) (Table 3.3.1). Plasma levels of IL-6, IL-8, IL-10, IL-12p40, IL-15, TNF-α, ICAM-1 and VCAM-1 were selected as they were described in the literature to be associated with omega-3 FAs in animal and human studies (50, 51). The details of clinical symptoms of psychosis include BPRS-psychotic and SANS score with functional scores at baseline, 6- and 12-month follow-up are presented in Table 3.3.2.

Table 3.3.1. Demographic and biological details of the participants at baseline and 6-month follow-up. SD-Standard deviation, BMI-Body Mass Index, EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, ICAM-intercellular adhesion molecule and VCAM-Vascular cell adhesion molecule.

		Baseline (n=268)	**Follow-up (n=146)**
Gender	Male, n (%)	118 (44%)	57 (39%)
	Female, n (%)	150 (56%)	89 (61%)
Age in years (mean ± SD)		18.9 ± 4.4	18.22 ± 4.03
BMI in kg/m^2 (mean ± SD)		23.95 ± 5.46	24.39 ± 5.95
Erythrocyte membrane omega-3 percentage compositions			
EPA (%), (mean ± SD)		0.98 ± 0.34	1.76 ± 1.33
Erythrocyte DHA (%), (mean ± SD)		6.43 ± 1.62	7.10 ± 2.34
Omega-3 Index (%), (mean ± SD)		7.41 ± 1.78	8.86 ± 3.49
Concentrations of plasma immune marker			
IL-6 (pg/mL), (mean ± SD)		0.85 ± 2.17	0.97 ± 2.68
IL-8 (pg/mL), (mean ± SD)		4.41 ± 3.56	4.53 ± 3.75
IL-10 (pg/mL), (mean ± SD)		0.35 ± 0.27	0.37 ± 0.44
IL-12p40 (pg/mL), (mean ± SD)		164.01 ± 72.90	151.01 ± 69.13
IL-15 (pg/mL), (mean ± SD)		2.91 ± 0.69	2.55 ± 0.63
TNF-α (pg/mL), (mean ± SD)		2.30 ± 0.68	2.42 ± 0.81
ICAM-1 (pg/mL), (mean ± SD)		487544.35 ± 168040.96	534802.64 ± 191628.11
VCAM-1 (pg/mL), (mean ± SD)		496297.63 ± 149586.57	487405.13 ± 144755.91

Table 3.3.2. Symptomology and functional outcome of CHR participants at baseline, 6-month and 12-month follow-up. BPRS- Brief Psychiatric Rating Scale, SANS- Scale for the Assessment of Negative Symptoms, SOFAS- Social and Occupational Functioning Assessment Scale, SD-standard deviation.

Clinical outcome	Baseline (n=268)	6 month follow-up (n=146)	12 month follow-up (n=127)
BPRS-psychotic, (mean ± SD)	8.25 ± 2.80	5.92 ± 2.54	5.41 ± 2.08
SANS Total (mean ± SD)	18.23 ± 13.05	13.23 ± 13.00	10.69 ± 11.89
SOFAS Total (mean ± SD)	53.81 ± 12.23	66.86 ± 14.27	70.74 ± 16.09

3.3.2. Cross-sectional relationship between omega-3 FAs and analyses at baseline and at follow-up (adjusted for age, sex and BMI)

In a cross-sectional analysis at baseline, the omega-3 index was negatively associated with IL-15, ICAM-1 and VCAM-1 (β-coef = -0.31, -0.29 & -0.35; FDR-corrected p= 0.04, 0.04 & 0.02, respectively), whereas DHA was negatively associated with VCAM-1 (β-coef = -0.32; FDR-corrected p=0.03). At follow-up, no significant cross-sectional association was observed between omega-3 FAs and inflammatory cytokines (Table 3.3.3 & 3.3.4).

3.3.3. Relationship between 6-month change in omega-3 FAs and 6-month change in cytokine levels

In a linear regression model, omega-3 index changed inversely with changes in plasma levels of TNF-α such that those with increasing levels of TNF-α had decreasing levels of Omega-3 with a beta coefficient of 0.06 (FDR-corrected p= 0.032). A similar inverse association was noticed for EPA and DHA levels individually with plasma cytokines. An increase in DHA levels associated with decrease in plasma TNF-α and ICAM-1 (β-coef=-0.09 & 0.09; FDR-corrected p=0.02 & 0.02, respectively) at 6 months follow-up. Similarly, change in EPA showed an inverse association with change in plasma levels of IL-12p40, IL-15 and TNF-α (β-coef=-0.16, -0.12 & -0.14; FDR-corrected p= 0.048, 0.048 & 0.048, respectively) such that increasing EPA score was associated with decreasing plasma immune marker levels (Table 3.3.5).

3.3.4. Relationship of baseline omega-3 FAs and change in omega-3 measures with cytokine levels at follow-up (adjusted for age, sex, BMI, baseline omega-3 and baseline cytokine levels)

In the linear regression models, higher baseline omega-3 index and DHA levels in erythrocyte membrane was significantly associated with lower plasma VCAM-1 levels at 6 months follow-up (β-coef =-0.38 & -0.33; FDR-corrected p= 0.036 & 0.036, respectively) (Table 3.3.6). Moreover, 6 month increase in omega-3 index and DHA levels was significantly related with lower plasma levels of TNF-α at follow-up (β-coef =-0.06 & -0.09; FDR-corrected p= 0.036 & 0.023, respectively), whereas 6-month change in DHA was positively associated with ICAM-1 at follow-up after correction for multiple testing (β-coef =0.1; FDR-corrected p= 0.023) (Table 3.3.7).

Table 3.3.3. **Cross sectional association between erythrocyte omega-3 FA and inflammatory markers at baseline.** Results are presented from models adjusting for age, sex and BMI. B-H- Benjamini Hochberg correction (FDR <0.05%), EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, ICAM-intercellular adhesion molecule, VCAM-Vascular cell adhesion molecule.

	Omega-3 index				**DHA**				**EPA**			
Immune markers	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value
		Upper	Lower			Upper	Lower			Upper	Lower	
IL 6	**-0.003**	-0.21	-0.20	0.980	0.001	-0.19	0.19	0.988	-0.004	-0.04	0.03	0.841
IL 8	-0.029	-0.24	-0.18	0.891	-0.022	-0.21	0.17	0.988	-0.007	-0.04	0.03	0.829
IL 10	0.036	-0.18	-0.25	0.891	0.018	-0.18	0.21	0.988	0.018	-0.02	0.06	0.578
IL 12p40	-0.051	-0.27	0.17	0.891	-0.014	-0.21	0.18	0.988	-0.037	-0.07	0.00	0.259
IL 15	**-0.308**	**-0.53**	**-0.07**	**0.039**	-0.248	-0.45	-0.03	0.063	-0.061	-0.10	-0.01	0.054
TNF α	-0.090	-0.30	0.12	0.891	-0.077	-0.27	0.12	0.988	-0.013	-0.05	0.02	0.702
ICAM 1	**-0.287**	**-0.51**	**-0.06**	**0.039**	-0.256	-0.46	-0.05	0.063	-0.031	-0.07	0.01	0.286
VCAM 1	**-0.354**	**-0.58**	**-0.12**	**0.018**	**-0.319**	**-0.52**	**-0.11**	**0.027**	-0.035	-0.07	0.00	0.259

Table 3.3.4. Cross-sectional relationship between erythrocyte omega-3 FAs and inflammatory cytokines at follow-up. Results are presented from models adjusting for age, sex and BMI. B-H- Benjamini Hochberg correction (FDR =0.05%), EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, ICAM-intercellular adhesion molecule, VCAM-Vascular cell adhesion molecule.

	Omega-3 index				**DHA**				**EPA**			
Immune markers	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value
		Upper	Lower			Upper	Lower			Upper	Lower	
IL 6	0.267	-0.322	0.856	0.477	0.112	-0.284	0.508	0.649	0.155	-0.068	0.378	0.287
IL 8	0.729	0.125	1.333	0.086	0.553	0.150	0.956	0.068	0.176	-0.057	0.409	0.287
IL 10	-0.332	-0.930	0.265	0.477	-0.192	-0.594	0.209	0.621	-0.140	-0.367	0.088	0.291
IL 12p40	-0.299	-0.906	0.308	0.477	-0.146	-0.555	0.262	0.649	-0.153	-0.383	0.077	0.287
IL 15	-0.423	-1.106	0.260	0.477	-0.224	-0.684	0.236	0.621	-0.199	-0.458	0.060	0.287
TNF α	-0.861	-1.530	-0.191	0.086	-0.563	-1.013	-0.114	0.068	-0.297	-0.553	-0.041	0.207
ICAM 1	0.304	-0.307	0.914	0.477	0.319	-0.088	0.727	0.372	-0.015	-0.249	0.218	0.973
VCAM 1	-0.192	-0.819	0.436	0.615	0.022	-0.400	0.444	0.917	-0.214	-0.450	0.022	0.287

Table 3.3.5. **Cross-sectional relationship between change in erythrocyte omega-3 FAs and change in inflammatory cytokines.** Results are presented from models adjusting for age, sex, BMI and baseline omega-3 measures. The underlined markers were used for mediation analysis. B-H- Benjamini Hochberg correction (FDR =0.05%), EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, ICAM-intercellular adhesion molecule, VCAM-Vascular cell adhesion molecule.

	Omega-3 index				**DHA**				**EPA**			
Immune markers	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value
		Upper	Lower			Upper	Lower			Upper	Lower	
IL 6	-0.026	-0.075	0.023	0.395	-0.048	-0.120	0.024	0.252	-0.025	-0.155	0.104	0.702
IL 8	0.039	-0.002	0.080	0.122	0.061	0.001	0.121	0.106	0.072	-0.035	0.179	0.298
IL 10	-0.022	-0.072	0.029	0.456	-0.022	-0.097	0.052	0.557	-0.078	-0.210	0.055	0.329
IL 12p40	-0.052	-0.094	-0.009	0.072	-0.062	-0.125	0.001	0.106	**-0.155**	**-0.266**	**-0.045**	**0.048**
IL 15	-0.034	-0.074	0.005	0.142	-0.034	-0.093	0.024	0.281	**-0.123**	**-0.224**	**-0.021**	**0.048**
TNF α	**-0.062**	**-0.103**	**-0.021**	**0.032**	**-0.091**	**-0.152**	**-0.030**	**0.020**	**-0.135**	**-0.244**	**-0.027**	**0.048**
ICAM 1	0.047	0.002	0.092	0.112	**0.094**	**0.028**	**0.159**	**0.020**	0.024	-0.097	0.144	0.702
VCAM 1	0.015	-0.030	0.059	0.517	0.059	-0.005	0.123	0.114	-0.088	-0.203	0.026	0.260

Table 3.3.6. **Longitudinal relationship between baseline erythrocyte omega-3 FAs and inflammatory cytokines at follow-up.**
Results are presented from models adjusting for age, sex and BMI. B-H- Benjamini Hochberg correction (FDR =0.05%), EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, ICAM-intercellular adhesion molecule, VCAM-Vascular cell adhesion molecule.

	Omega-3 index				**DHA**				**EPA**			
Immune markers	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value
		Upper	Lower			Upper	Lower			Upper	Lower	
IL 6	0.054	-0.200	0.307	0.868	0.040	-0.184	0.265	0.811	0.013	-0.041	0.068	0.910
IL 8	0.029	-0.235	0.294	0.890	0.057	-0.176	0.291	0.811	-0.028	-0.085	0.029	0.893
IL 10	-0.094	-0.351	0.163	0.868	-0.085	-0.312	0.143	0.811	-0.010	-0.065	0.046	0.910
IL 12p40	-0.018	-0.279	0.243	0.890	-0.015	-0.246	0.216	0.897	-0.003	-0.060	0.053	0.910
IL 15	-0.119	-0.413	0.176	0.868	-0.140	-0.400	0.119	0.811	0.022	-0.042	0.086	0.893
TNF α	-0.064	-0.341	0.213	0.868	-0.059	-0.304	0.186	0.811	-0.005	-0.065	0.054	0.910
ICAM 1	-0.296	-0.553	-0.038	0.113	-0.247	-0.475	-0.019	0.153	-0.049	-0.105	0.007	0.486
VCAM 1	**-0.381**	**-0.639**	**-0.122**	**0.036**	**-0.334**	**-0.563**	**-0.106**	**0.036**	-0.047	-0.104	0.010	0.486

Table 3.3.7. **Longitudinal relationship between 6 month change in erythrocyte omega-3 FAs and inflammatory cytokines at follow-up.** Results are presented from models adjusting for age, sex and BMI. B-H- Benjamini Hochberg correction (FDR =0.05%), EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, ICAM-intercellular adhesion molecule, VCAM-Vascular cell adhesion molecule.

	Omega-3 index				**DHA**				**EPA**			
Immune markers	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value	β-Coef.	95% Conf. Interval		B-H p value
		Upper	Lower			Upper	Lower			Upper	Lower	
IL 6	-0.008	-0.024	0.007	0.444	-0.015	-0.038	0.008	0.284	-0.008	-0.049	0.033	0.790
IL 8	0.022	-0.001	0.044	0.137	0.034	0.001	0.067	0.119	0.040	-0.019	0.099	0.335
IL 10	-0.022	-0.075	0.030	0.513	-0.023	-0.100	0.054	0.557	-0.081	-0.218	0.057	0.371
IL 12p40	-0.046	-0.084	-0.008	0.081	-0.055	-0.111	0.001	0.119	-0.138	-0.237	-0.040	0.054
IL 15	-0.038	-0.082	0.006	0.160	-0.038	-0.103	0.027	0.316	-0.136	-0.249	-0.024	0.054
TNF α	**-0.064**	**-0.107**	**-0.021**	**0.036**	**-0.094**	**-0.156**	**-0.031**	**0.023**	-0.139	-0.251	-0.028	0.054
ICAM 1	0.051	0.002	0.100	0.126	**0.102**	**0.030**	**0.173**	**0.023**	0.026	-0.105	0.156	0.790
VCAM 1	0.016	-0.032	0.063	0.582	0.064	-0.005	0.133	0.128	-0.095	-0.219	0.028	0.293

3.3.5. Direct and indirect effects of omega-3 FAs on clinical outcome (mediation analysis)

Changes in the plasma inflammatory markers IL-12p40 and TNF-α were considered for the mediation analysis, as they were significantly associated with change in omega-3 index. However, mediation analyses did not provide evidence of significant direct or indirect effects (via plasma immune markers) on psychotic symptoms (BPRS-psychotic and SANS) or functional outcome (SOFAS) at 6-month (Table 3.3.8) or at 12-month (Table 3.3.9) follow-up.

Table 3.3.8. Mediating role of change in cytokines (IL-15, IL-12p40 and TNF-α) on the association between change omega-3 index and clinical outcome at 6th month (Covariates: Age, Sex, BMI and Baseline n-3 Index). Mediation analysis with regression co-efficient (95% confidence interval). EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, BPRS- Brief Psychiatric Rating Scale, SANS- Scale for the Assessment of Negative Symptoms, SOFAS- Social and Occupational Functioning Assessment Scale

Mediators	Outcome (6 month)	Mediation effects	Direct effect	Total effect
IL 12p40	BPRS-psychotic	0.00 (-0.03 to 0.03)	-0.9 (-0.23 to 0.04)	-0.10 (-0.23 to 0.04)
	SANS	-0.01 (-0.15 to 0.17)	-0.28 (-0.94 to 0.39)	-0.29 (-0.93 to 0.35)
	SOFAS	-0.04 (-0.33 to 0.13)	0.27 (-0.47 to 1.02)	0.23 (-0.49 to 0.96)
TNF-α	BPRS-psychotic	-0.12 (-0.06 to 0.01)	-0.08 (-0.22 to 0.06)	-0.10 (-0.23 to 0.04)
	SANS	-0.04 (-0.21 to 0.21)	-0.25 (-0.92 to 0.41)	-0.29 (-0.93 to 0.35)
	SOFAS	0.09 (-0.19 to 0.28)	0.15 (0.60 to 0.90)	0.23 (-0.49 to 096)

Table 3.3.9. Mediating role of change in cytokines (IL-15, IL-12p40 and TNF-α) on the association between change omega-3 index and clinical outcome at 12th month (Covariates: Age, Sex, BMI and Baseline n-3 Index). Mediation analysis with regression co-efficient (95% confidence interval). EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, BPRS- Brief Psychiatric Rating Scale, SANS- Scale for the Assessment of Negative Symptoms, SOFAS- Social and Occupational Functioning Assessment Scale.

Mediators	Outcome (12 month)	Mediation effects	Direct effect	Total effect
IL12p40	BPRS-psychotic	0.01 (-0.04 to 0.05)	-0.07 (-0.19 to 0.04)	-0.06 (-0.18 to 0.05)
	SANS	-0.06 (-0.27 to 0.10)	-0.37 (-1.00 to 0.27)	-0.43 (-1.04 to 0.18)
	SOFAS	-0.10 (-0.44 to 0.13)	0.49 (-0.34 to 1.38)	0.40 (-0.45 to 1.24)
TNF-α	BPRS-psychotic	-0.01(-0.07 to 0.02)	-0.05 (-0.16 to 0.07)	-0.06 (-0.18 to 0.05)
	SANS	-0.12 (-0.41 t 0.05)	-0.31 (-0.94 to 0.31)	-0.43 (-1.04 to 0.18)
	SOFAS	0.10 (-0.15 to 0.35)	0.30 (-0.58 to 1.18)	0.40 (-0.45 to 1.24)

3.4. Discussion

We examined the biological relationship of erythrocyte omega-3 FAs with plasma inflammation markers in a group of CHR participants from the NEURAPRO trial. We hypothesized that plasma inflammatory markers would be negatively associated with omega-3 FAs and at least partially mediate the association between omega-3 FAs and clinical outcomes. The results supported our first hypothesis as plasma inflammatory markers associated inversely with omega-3 index, but did not support our second hypothesis as they did not indicate any mediating effect on psychotic symptoms or functioning. The main results are as follows: Firstly, in a cross-sectional analysis, the omega-3 index at baseline was inversely associated with IL-15 and endothelial immune markers ICAM-1 and VCAM-1. Secondly, increase in omega-3 measures was significantly associated with decrease in TNF-α over the 6-month interval. Thirdly, in the longitudinal assessments, higher baseline omega-3 index and DHA predicted lower plasma levels of VCAM-1 at follow-up. Finally, 6-month change in omega-3 index expressed similar inverse association with TNF-α at follow-up. In the mediation analysis, omega-3 FA associated changes in plasma inflammatory markers did not exert any significant mediation role on psychotic or functional outcome of CHR participants.

At baseline, the vascular endothelial markers VCAM-1 displayed a strong negative association with omega-3 index and DHA levels both cross-sectionally and longitudinally. In addition, ICAM-1 showed a negative association with erythrocyte omega-3 index cross-sectionally among baseline samples. The vascular adhesion molecules ICAM-1 and VCAM-1 belong to the immunoglobulin super family that are synthesized chiefly by leukocytes and endothelial cells (71). Studies have found varying levels of endothelial immune markers in schizophrenia patients compared to healthy controls (72-77) . To understand the relative contribution of these endothelial markers in schizophrenia patients, Nguyen et al. developed a composite measure called “Vascular endothelial index” (VEI). The VEI was based on the linear combination of endothelial markers that differed most between the groups, and VEI was found to be increased in schizophrenia patients compared to healthy controls (72, 78). Our findings of an inverse association between endothelial markers and omega-3 FAs were supported by Baker et al (2018). In this review, the authors identified

cellular mechanisms through which EPA and DHA inhibit the synthesis of endothelial markers at various levels. At the molecular level, omega-3 FAs decrease the expression of messenger RNAs responsible for coding endothelial cytokine synthesis (but increase the level of arachidonic acid as the main precursor of cytokines such as prostaglandins). At the cellular level, omega-3 FAs inhibit the adhesion and migration of leukocytes across the endothelium (79). Apart from such immune roles, endothelial markers are also involved in mechanisms such as disruption of the blood brain barrier (BBB), neuronal apoptosis and age-related impairments in neuronal precursor cells (80, 81). Considering that these mechanisms have already been related to the pathophysiology of schizophrenia (82-84), our results of negative associations between omega-3 FAs and endothelial immune markers provide preliminary evidence of an immune modulating effect of omega-3 FAs in early psychosis (CHR).

Our study also identified an inverse relationship between EPA and IL-12p40 which is a common subunit of cytokines IL-12 and IL-23 and exerts a pivotal agonistic role in early inflammatory reaction (85-87). In psychosis, a meta-analysis showed elevated levels of the pro-inflammatory cytokine IL-12 in schizophrenia patients compared to healthy controls (88). Moreover, our group previously observed increased plasma levels of IL-12p40 distinguishing CHR subjects who transitioned to psychotic disorder from who did not (89). A similar strong negative association was found for omega-3 FAs with pro-inflammatory cytokines IL-15 and TNF-α. The biological evidence relating the acute phase inflammatory state cytokine TNF-α with omega-3 FAs has been extensively reviewed and altered regulation of TNF-α and IL-15 have been consistently observed in psychosis (24, 90-93). Here, for the first time, we report an association of omega-3 FAs with TNF-α in those potentially in as early stage of a psychotic disorder. In a placebo-controlled randomized trial, a decrease in the omega-3:omega-6 ratio showed a positive association with IL-6 and TNF-α production, suggesting an anti-inflammatory role of omega-3 FAs on peripheral cytokines (94). The same research group also observed anti-inflammatory properties of omega -3 FAs rich diet in an adult population (95). Moreover, an extensive interaction between TNF-α and IL-15 has been observed at the blood brain barrier (BBB) since TNF-α enhances IL-15 synthesis and IL-15 in turn regulates TNF-α signaling at the level of the BBB (96, 97). Although the current study did not consider the interactions of these pro-

inflammatory mediators, the findings of significant associations between omega-3 FAs and these cytokines indicate a possible anti-inflammatory role on BBB by omega-3 FAs in CHR participants.

In the NEURAPRO clinical trial, although omega-3 FA supplementation was not found to be effective in the prevention of transition to psychosis, baseline erythrocyte omega-3 FAs levels have been shown to be associated with improvement of clinical symptoms in CHR individuals. At baseline, the n-3 index (EPA+DHA) was negatively correlated with general psychopathology, psychotic, depressive and manic symptoms, while the n-6/3 PUFA ratio was positively correlated with general psychopathology and depressive symptoms (55). In addition, 6-month increase in omega-3 FAs levels predicted less severe psychopathology and better functioning at 6-month and 12-month follow-up (98). While these results suggested possible therapeutic effects of omega-3 FAs in CHR, the current study, which investigates the mechanistic (rather than the predictive) role of omega-3 FAs, found no clinical effect. We suspect that the absence of an association found between omega-3 FAs and clinical outcome (directly or indirectly through inflammatory mediators) could be due to the following reasons: (i) sampling bias which occurred due to the drop in sample numbers at follow-up as comparison of baseline characteristics of samples with and without follow-up indicated significant difference in biological and clinical parameters (Appendix 3.8.1), (ii) consideration of a different biomarker for the omega-3 index which was derived from gas chromatography utilizing a specific phospholipid fraction, which has a 3-4 fold higher magnitude compared to the mass spectrometric measure of total membrane fatty acids used in the previous analysis, or (iii) it could be a real effect with no association between omega-3 FAs and clinical outcomes in UHR state as observed previously (99).

The strengths of our study include well-characterized CHR participants and the availability of baseline and follow-up biological and clinical data. This enabled us to understand the long-term influence of omega-3 FAs on immune status of the CHR participants in a unique manner. The multiplex assay provided the opportunity to analyze the biological effects of omega-3 FAs on a broad array of plasma immune markers in CHR participants.

Our study also has limitations. Firstly, the participants of the clinical trial displayed a low adherence to study intervention, which limited our ability to evaluate group difference between the omega-3 FA and placebo study arms (60). We overcame this limitation by considering the erythrocyte omega-3 FA levels. Secondly, the number of follow-up samples was only 55% of the baseline sample size. This drop-out may have resulted in some attritional bias as this affected the statistical power. Thirdly, the interaction within different immune mediators was not considered in the mediation analysis. Finally, the multiplex assay performed to study a broad array of immune biomarkers simultaneously, comes with its own limitations of a possible cross-reactivity within the assays (100).

In conclusion, our results showed an inverse relationship between omega-3 FAs and plasma immune markers that are involved in the pathophysiology of schizophrenia in this CHR sample. In the cross-sectional analysis, erythrocyte membrane omega-3 FAs were inversely associated with pro-inflammatory cytokines IL-15, IL-12p40, TNF-α, endothelial markers ICAM-1 and VCAM-1 and in the longitudinal analysis a similar negative association was found with TNF-α and VCAM-1. The predominant negative associations observed with several pro-inflammatory mediators are in keeping with known immune actions of omega-3 FAs and suggest that omega-3 FAs may reduce inflammatory load in CHR individuals. While the existence of an inflammatory subtype of schizophrenia is still under investigation, we speculate that omega-3 FAs could be more clinically beneficial in those who have high inflammatory load at baseline. However, no overall clinical benefits of omega-3 FAs, related to cytokine measures, were observed after 6-month of follow-up in CHR individuals. This raises the possibility that non-immune function of omega-3 FAs, such as recovering synaptic membrane activity in the brain, modulation of microbiota-gut-brain axis or production neuroprotective metabolites may impact on clinical outcome in early psychosis (44, 101) Future randomized control trials with multiple follow-up time points would be beneficial in understanding the possible long-term biological benefits of omega-3 in (those at risk for) psychosis (102).

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Chapter 4

Thesis author's role:

Mr Subash Raj Susai was involved in all aspects of the study. He had full access to all the data in the study and was involved in the acquisition, linear regression analysis of complement proteins, and interpretation of data and drafting of the manuscript. Machine learning analysis and interpretation of results were performed by David Mongan.

Reference:

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Title: Machine learning based prediction and the influence of complement - coagulation pathway proteins on clinical outcome: results from the NEURAPRO trial

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Abstract

Background: Functional outcomes are important measures in the overall clinical course of psychosis and individuals at clinical high-risk (CHR), however, prediction of functional outcome remains difficult based on clinical information alone. In the first part of this study, we evaluated whether a combination of biological and clinical variables could predict future functional outcome in CHR individuals. The complement and coagulation pathways have previously been identified as being of relevance to the pathophysiology of psychosis and have been found to contribute to the prediction of clinical outcome in CHR participants. Hence, in the second part we extended the analysis to evaluate specifically the relationship of complement and coagulation proteins with psychotic symptoms and functional outcome in CHR.

Materials and methods: We carried out plasma proteomics and measured plasma cytokine levels, and erythrocyte membrane fatty acid levels in a sub-sample (n=158) from the NEURAPRO clinical trial at baseline and 6 months follow up. Firstly, we used support vector machine learning techniques to develop predictive models for functional outcome at 12 months. Secondly, we developed linear regression models to understand the association between 6-month follow-up levels of complement and coagulation proteins with 6-month follow-up measures of positive symptoms summary (PSS) scores and functional outcome.

Results and conclusion: A prediction model based on clinical and biological data including the plasma proteome, erythrocyte fatty acids and cytokines, poorly predicted functional outcome at 12 months follow-up in CHR participants. In linear regression models, four complement and coagulation proteins (coagulation protein X, Complement C1r subcomponent like protein, Complement C4A & Complement C5) indicated a significant association with functional outcome; and two proteins (coagulation factor IX and complement C5) positively associated with the PSS score. Our study does not provide support for the utility of cytokines, proteomic or fatty acid data for prediction of functional outcomes in individuals at high-risk for psychosis. However, the association of complement protein levels with clinical outcome suggests a role for the complement system and the activity of its related pathway in the functional impairment and positive symptom severity of CHR patients.

Key words: clinical high risk, functional outcome, prediction models, schizophrenia, psychosis.

4.1. Introduction

Psychosis research is increasingly focusing on those in the clinical high risk (CHR) population who experience early signs of emerging psychosis (1). The CHR criteria comprise of the attenuated psychotic symptom (APS) criterion, the brief limited intermittent psychotic symptom (BLIPS) criterion, and the genetic risk and functional decline criterion (2). The functional impairment of CHR participants substantially impacts personal, familial and social well-being (3-5) and responds poorly to currently available treatments (6-8). The association of early functional deterioration with the development of psychotic symptoms indicates that functional measures could be used to improve early intervention strategies in psychosis (9-17).

Previous studies involving CHR participants have investigated baseline predictors of transition to psychosis and found that factors such as social dysfunction, neurocognitive measures, duration of untreated psychosis and severity of attenuated psychotic symptoms predict later transition to psychosis (18-37). The biological aspects of psychosis have been increasingly studied in relation to the clinical symptoms in the CHR state. Thus far, biological parameters such as neuroimaging data and electrophysiological indicators have provided some valuable prediction of functional outcome and transition to psychosis in CHR populations (26, 38-42). Studies of immune markers (43, 44) and membrane phospholipids (45-47) have also been undertaken in CHR participants. Although some alterations have been found to be associated with the development of psychosis (43, 44, 48-57), the clinical implication of these findings in terms of prediction of functional outcome in CHR individuals has not been specifically studied (52, 58).

Blood based biological marker studies have focused on predicting the development of psychosis in CHR participants (59, 60). Mongan et al. used mass spectrometry based proteomic data to predict clinical outcomes in a longitudinal CHR study (61). Combined clinical and proteomic data predicted the development of psychosis better than clinical data alone (61). In addition to the prediction of psychosis, the proteomic variables also predicted functional outcome with an AUC of 0.76 at two years follow-up in 133 CHR participants (61). In this prediction model the most abundant proteins that significantly predicted functional outcome were complement and coagulation proteins. (62).

Similarly, other studies have found clinical and demographic features such as duration of treatment or untreated psychosis and poor cognition to be the predictors of later functional decline in the CHR (10, 13, 14, 17, 39, 63-68).

In the current study we attempted to investigate the combined predictive ability of blood based biological markers including inflammatory cytokines, erythrocyte membrane fatty acids and the plasma proteome on functional outcome. Using machine learning we sought to develop two prediction models, one using baseline clinical data alone and another using both baseline clinical and biological data. We developed these models in a subsample of the NEURAPRO clinical trial, which tested the potential preventive role of omega-3 fatty acids in CHR participants (69). Our team has previously reported dysregulation of complement and coagulation pathway proteins in relation to development of psychotic symptoms and functional decline in high-risk population (49, 61). These results supported the findings of Sekar et al., suggesting that the complement related activity might be involved in the development of clinical symptoms in the early stage of schizophrenia (70, 71). Hence, in the current study we extended our analysis to explore the individual relationship of complement associated proteins with positive symptoms and functional status. Based on our previous findings, we hypothesized that baseline biological data along with clinical parameters would predict functional improvement in CHR participants better than the clinical model alone. In addition, we also hypothesised that higher complement and coagulation proteins would associate with poor clinical outcomes.

4.2. Materials Methods

4.2.1. The NEURAPRO clinical trial

The NEURAPRO clinical trial was registered with the Australian New Zealand Clinical Trial Registry as ACTRN 12608000475347. The trial aimed to investigate the role of omega-3 fatty acids (FAs) on prevention of psychosis in CHR participants (72). The study was conducted between March 2010 and the end of September 2014, in accordance with the Declaration of Helsinki and consistent with the International Council for Harmonization of Good Clinical Practice with appropriate ethical approval obtained from each site before the trial commenced. Ethical approval for the plasma

biomarker analysis presented in this study was obtained from the research ethics committee of the Royal College of Surgeons in Ireland [REC-No. 1699].

The inclusion criteria include participants aged between 13 and 40 years who fulfilled one of the criteria for at-risk state defined by the Comprehensive Assessment of At-Risk Metal State (CAARMS)(2). The exclusion criteria were: history of psychotic episodes of seven days or longer; any current symptoms of organic brain disease or developmental disorder; abnormal coagulation profile; thyroid abnormalities; physical illness with psychotropic effect, if not stabilized; current treatment with any mood stabilizers or recreational use of ketamine; past neuroleptic exposure equivalent to a total lifetime haloperidol dose of >50 mg; a diagnosis of a serious developmental disorder; premorbid IQ less than 70; current acute suicidality /self-harm or aggression/dangerous behavior; pregnancy; or intake of more than 4 weeks of supplementation with omega-3 FAs (73).

4.2.2. Participants

A total of 170 CHR participants who provided baseline and 12-month follow-up plasma samples and who had clinical outcome data available at 12 months were considered for this plasma biomarker analysis study.

4.2.3. Clinical measures

Baseline psychopathological scores of CHR participants were measured using the Brief Psychiatric Rating scale (BPRS) (74), Scale for the Assessment of Negative Symptoms (SANS) (75), Youth Maniac Rating Scale (YMRS) (76), Montgomery-Åsberg Depression Rating Scale (MADRS) (77), Social and Occupational Functional assessment Score (SOFAS) (78), Global functioning Social (GF:S) (79) and Global functioning Role (GF:R) (80) were used for machine learning.

4.2.4. Gas-chromatography based Erythrocyte membrane fatty acid measures

Fasting plasma samples were collected at baseline and 6 months follow-up. The erythrocytes were separated from the plasma using an automated high-throughput method described in (81). The molecular percentage of Total omega-3 FAs, total omega-6 FAs, docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), linoleic

acid (LA), arachidonic acid (ARA), omega-3 index (EPA+DHA), omega-6:omega-3 ratio, Alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) were measured using gas chromatography (73, 82). The Phosphatidyl-ethanolamine (PE) fraction was used to determine the omega-3 FA content, because of their high abundancy in the lipid raft (56, 83).

4.2.5. Mass spectrometry based proteomic measures

Plasma samples of baseline and follow-up time points were processed according to the manufacturer's instructions (PreOmics iST kit, no.iST 96x). Briefly, 4 µl of individual samples were solubilized in 50 µL of "Lyse" buffer (containing Tris-HCl, sodium deoxycholate (SDC), 0.1% sodium dodecyl sulfate (SDS), tris (2-carboxyethyl) phosphine (TCEP), and 2-chloroacetamide and heated to 95 °C for 10 min. 50 µL of the resulting denatured, reduced, and alkylated solution was transferred to the reaction tube. Enzyme (LysC and trypsin) was added, and samples were hydrolysed at 37°C for 1.5 hours. The resulting peptide mixture was washed and eluted as per the manufacturer's instructions. The eluted peptides were vacuum-dried and dissolved in 100 µl of LC Load buffer. The reconstituted digested peptide mixture [200 ng/ µl] was then eluted using Evotips and injected using Evosep One (Evosep, Odense, Denmark (84). The digested samples were run on a Bruker timeTof Pro mass spectrometer connected to a Evosep One liquid chromatography system. The mass spectrometry was operated in positive ion mode with a capillary voltage of 1500 V, dry gas flow of 3 l/min and a dry temperature of 180^{o}C. Trapped ions were selected for ms/ms using parallel accumulation serial fragmentation (PASEF). A scan range of (100-1700 m/z) was performed at a rate of 10 PASEF MS/MS frames to 1 MS scan with a cycle time of 1.15s (85, 86). The MS raw files were then processed with MaxQuant (87) version 1.6.17.0 as described in (86) and the peptide data were further annotated and interpreted using the Perseus platform (V 1.6.7, www.maxquant.net/perseus/) (88). FDR was set at 0.01 to global protein identification level. Proteins that were identified in less than 70% of the total samples were not taken forward for analysis. Log_2 transformed values of LFQ intensities were used for statistical analysis. Missing values of mass spectrometry based proteomic data (corresponding to values below the level of detection) were imputed with minimum values.

4.2.6. Multi-plex assay-based estimation of plasma immune markers

Plasma levels of Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin (IL) -1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-15, Tumor necrosis factor-α (TNF-α), Interferon gamma (INF-γ), Inter-cellular Adhesion Molecule (ICAM)-1 and Vascular cell adhesion molecule (VCAM)-1 were measured at baseline using the Pro-inflammatory Panel 1, Cytokine Panel 1 and Vascular Injury Panel 2 v-PLEX® multiplex immunoassay kits (Mesoscale Discovery Systems) according to the manufacturer's instructions. A Sector Imager 2400 plate reader was used to quantify concentrations of each marker (Meso Scale Diagnostics). The concentrations of plasma markers that were expressed in at least 70% of all samples with a coefficient of variation of a maximum of 20% were taken forward. These included: IL12p40, IL15, IFN-gamma, IL6, IL8, IL10, TNF-alpha, CRP, sVCAM-1 and sICAM-1. Log2-transformed concentrations for these cytokines were used for the statistical analysis.

4.2.7. Clinical outcome measure

Functional outcome was assessed using the SOFAS scale at baseline, 6-month and 12-month follow-up. For the machine learning model, we investigated prediction of SOFAS score as a continuous variable with a minimum possible score of 0 points and a maximum possible score of 100 points. For the linear regression model a positive symptom summary (PSS) score was used along with the SOFAS score. The PSS score was derived from CAARMS symptom severity score by summing up the product of global rating scale score (0-6) and frequency (0-6) of the four subscales (89).

4.2.8. Statistical analysis

Machine learning models

Samples with clinical and biological data available at baseline and SOFAS outcome data available at 12-month follow-up were used for the machine learning analysis. Missing values of mass spectrometry based proteomic data were imputed with minimum values. Missing values of the remaining data were imputed using k nearest neighbours' imputation (k=7). All continuous measures were standardised to z scores and winsorised within +/- 4z. Models were developed using a support vector machine

(SVM) approach based on the LIBSVM algorithm. SVM is a computationally efficient form of supervised machine learning that has been used previously in multiple different contexts within psychiatry (90-92). SVM methods can integrate hyperparameter optimization to reduce propensity for over-fitting. Neurominer version 1.0 (https://github.com/neurominer-git) for MatLab 2018a (Math Works Inc) was used to develop SVM models with nested cross-validation. For a detailed description of nested cross-validation, see (93). The data were first divided into 5 random folds in the 'outer loop'. For each cycle of cross-validation, data from each fold were held out and the rest of the data moved into the 'inner loop'. Within the inner loop, we used 5 non-overlapping folds with iterative training-test cycles. Models were trained and tested with a range of values for the regularisation parameter and the best-performing models tested against the held-out data in the outer loop to derive the optimal model.

Model 1: clinical predictors

Firstly, we generated a model for prediction of 12-month SOFAS score based on data for the 11 clinical predictors. We used the LIBSVM algorithm with a linear kernel where mean squared error was used as the performance criterion. The regularisation parameter was applied across a range of 7 values (0.015625, 0.03125, 0.0625, 0.125, 0.25, 0.5,1 1) and the epsilon parameter across a range of 6 values (0.05, 0.075, 0.1, 0.125, 0.15, 0.2).

Model 2: biomarker predictors

Secondly, we generated a model for prediction of 12-month SOFAS score based on data for the 177 potential biomarkers. This model was also developed using the LIBSVM algorithm with linear kernel, mean squared error as the performance criterion, and regularisation parameter optimisation as for Model 1.

Model 3: clinical and biomarker predictors

Thirdly, we generated a model for prediction of 12-month SOFAS score based on data for the 11 clinical predictors plus data for the 177 potential biomarkers. This model was also developed using the LIBSVM algorithm with linear kernel, mean squared error as the performance criterion, and regularisation parameter optimisation as for

Model 1. For all three models, random-label permutation analysis with 1000 permutations was used to derive *p*-values for model significance and mean feature weights. Presented performance metrics include the mean squared error, Pearson's r, coefficient of determination, mean absolute error and normalised root mean square deviation. We also present classification-based performance metrics (such as sensitivity and specificity) for each model based on a SOFAS threshold of 70 points (70 points and below reflects some, moderate or major functional impairment, whereas 71 points and above reflects no more than slight functional impairment).

Linear regression models:

The participants who had proteomic data available at baseline and 6 month follow-up along with SOFAS and CAARMS score were considered for the secondary analysis. Linear regression models were developed to assess the relationship between protein levels at 6-month follow up with clinical scores (positive symptom summery score and functional score) at 6-month follow-up and the model was adjusted for age, sex, BMI along with corresponding baseline complement protein levels and baseline clinical scores. The level of significance was set to 0.05.

4.3. Results

4.3.1. Sample characteristics

Out of 170 participants, 158 participants have baseline clinical and biological measures and functional outcome at 12 months follow-up. The mean age of the study sample participants was 18 years (SD 4) with an average BMI of 24 kg/m^2 (SD 6). 58% of the study participants were females. The baseline demographic, clinical and biological characteristics of the study participants are given in Table 4.3.1.

Table 4.3.1. Baseline Characteristics of the participants

	Individuals with baseline and follow-up data		**Individuals with only baseline data**		**Total**	
N	158		146		304	
	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Age in years	16.32	(±3.12)	19.67	(±4.5)	18.98	(±4.49)
BMI in Kg/m^2	24.33	(±4.77)	23.92	(±4.03)	23.97	(±5.46)
Sex-Females (%)	80 (50.6 %)		76 (50 %)		156 (46.8%)	
Clinical Characteristics						
BPRS Total	41.95	(±10.32)	37.11	(±9.64)	41.07	(±9.72)
SANS Total	19.53	(±15.33)	13.11	(±11.1)	17.98	(±12.8)
YMRS Total	3.32	(±2.38)	1.89	(±2.47)	3.25	(±3.02)
MADRS Total	19.63	(±10.42)	13.67	(±11.7)	19.27	(±8.97)
SOFAS	53.53	(±10.72)	65.00	(±15.8)	53.51	(±11.9)
Global Functioning - Social	6.11	(±1.24)	7.33	(±1.22)	6.51	(±1.21)
Global Functioning - Role	5.58	(±1.83)	6.89	(±1.36)	5.96	(±1.54)
Omega-3 Fatty acid levels						
EPA in %	0.98	(±0.31)	0.97	(±0.35)	0.98	(±0.33)
DHA in %	6.23	(±1.25)	6.64	(±1.78)	6.44	(±1.61)
Omega-3 INDEX in %	7.22	(±1.38)	7.62	(±1.97)	7.41	(±1.77)
Total Omega-3 in %	12.01	(±1.66)	12.10	(±2.11)	12.03	(±2.01)

4.3.2. Predictive models

The clinical predictor pool comprised 11 features in total (4 demographic variables including sex, age, smoking status, BMI; and 7 symptom scale scores). The biomarker predictor pool comprised 177 features in total (10 cytokines; 157 proteomic markers; and 10 fatty acid markers). The full list of features is provided in Supplementary Table 4.8.1.

Model 1: Clinical predictors

Model 1 demonstrated poor predictive performance with mean squared error of 239.00 (p<0.001 on permutation analysis). Pearson's r was 0. 30 (95% confidence interval

0.13 – 0.45), coefficient of determination 8.9%, mean absolute error 13.0 and normalised root mean square deviation 22.4. Further performance metrics, including classification performance based on a threshold of 70 points, are presented in Table 4.3.2. Observed versus predicted SOFAS values are plotted in Figure 4.3.1. Features are ranked by mean feature weight in Table 4.3.3.

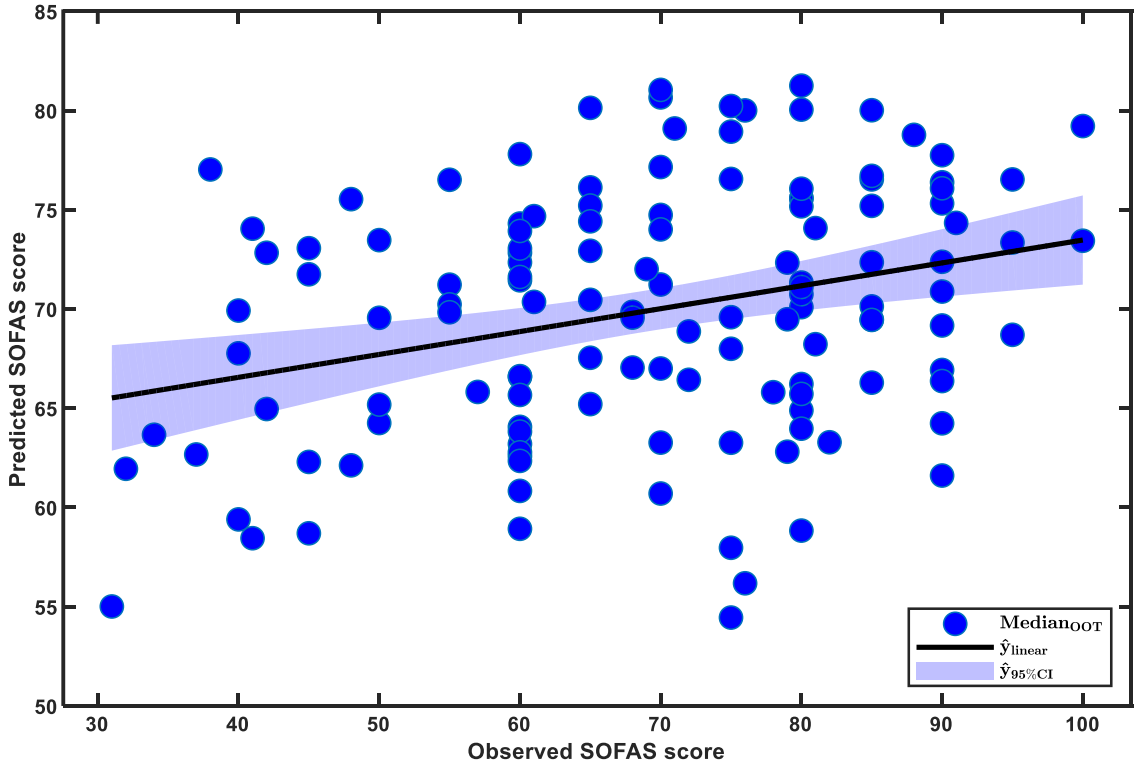

Figure 4.3.1. Observed vs. predicted SOFAS score for Model 1. SOFAS: Social and Occuptional Functioning Scale

Model 2: Biomarker predictors

Model 2 demonstrated poor predictive performance with mean squared error of 256.2 ($p<0.001$ on permutation analysis). Pearson's r was 0.25 (95% confidence interval 0.08 – 0.40), coefficient of determination 6.2%, mean absolute error 13.4 and normalised root mean square deviation 23.2. Further performance metrics, including classification performance based on a threshold of 70 points, are presented in Table 4.3.2. Observed versus predicted SOFAS values are plotted in Figure 4.3.2. The highest-weighted 10% of predictors ranked by mean feature weight are provided in Table 4.3.3.

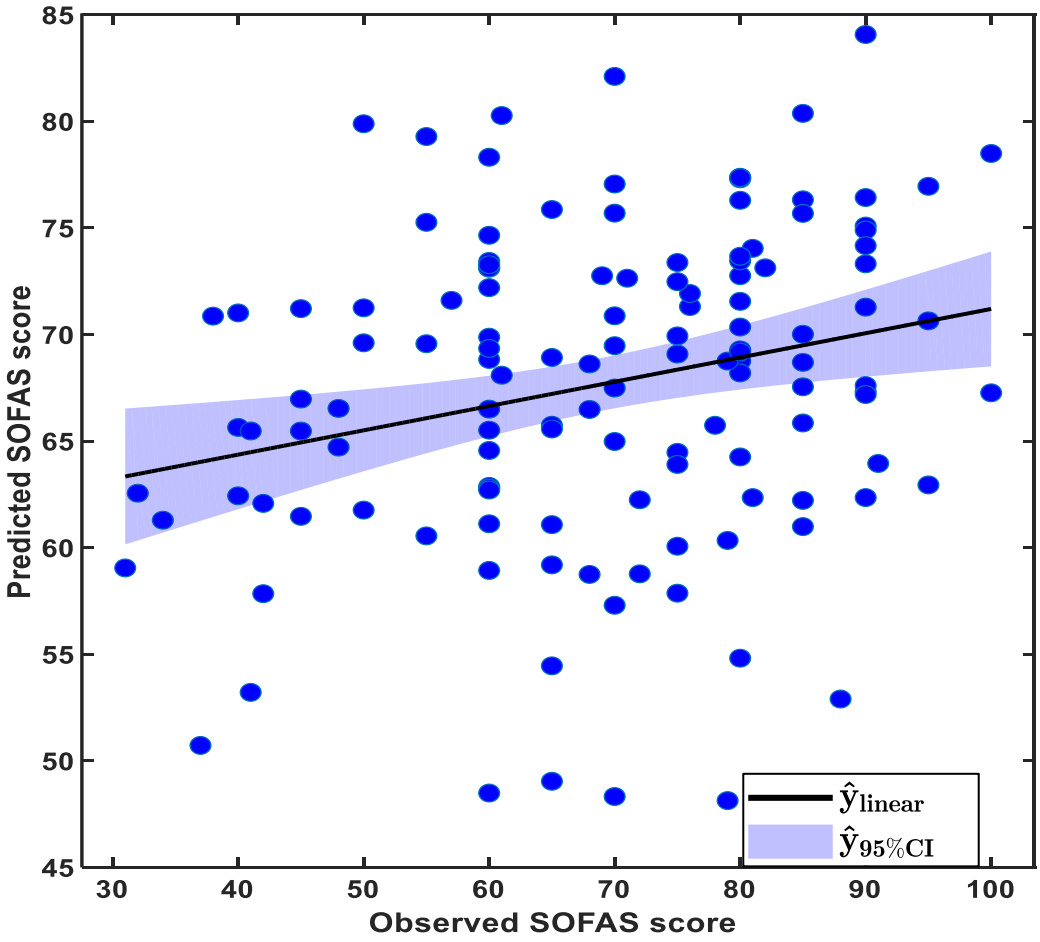

Figure 4.3.2. Observed vs. predicted SOFAS score for Model 2 (biomarker predictors). SOFAS: Social and Occupational Functioning Scale

Model 3: Clinical and biomarker predictors

Model 3 demonstrated poor predictive performance with mean squared error of 250.0 (*p*=0.023 on permutation analysis). Pearson's r was 0.22 (95% confidence interval 0.05 – 0.38), coefficient of determination 5.0%, mean absolute error 13.4 and normalised root mean square deviation 22.9. Further performance metrics, including classification performance based on a threshold of 70 points, are presented in Table 4.3.2. Observed versus predicted SOFAS values are plotted in Figure 4.3.3. The highest-weighted 10% of predictors ranked by mean feature weight are provided in Table 4.3.3.

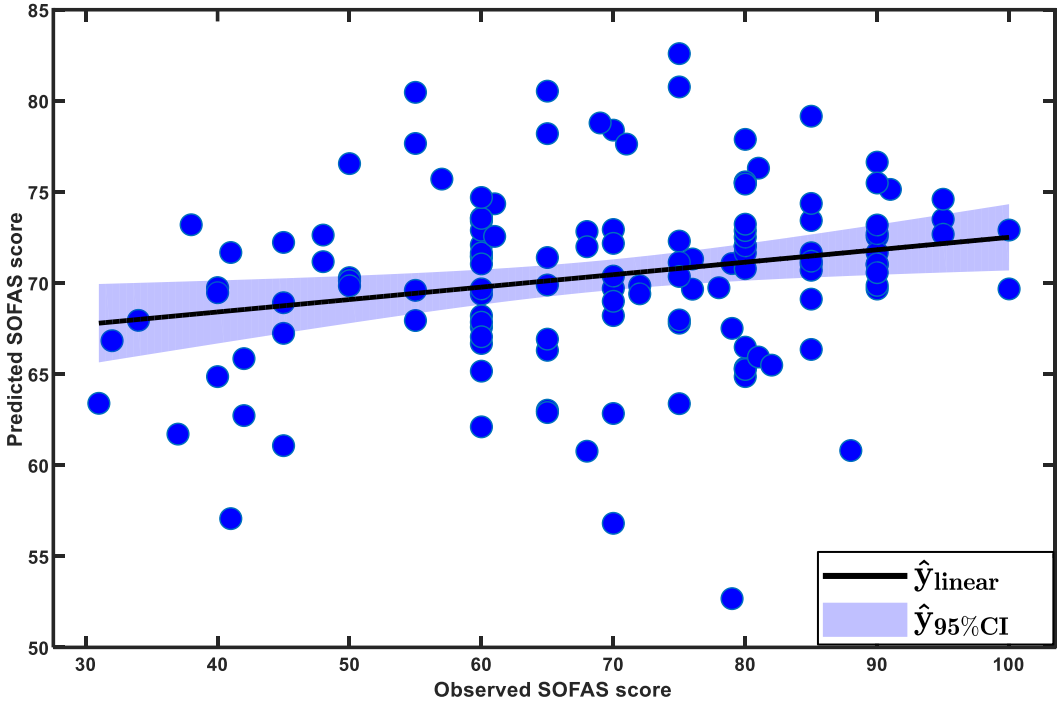

Figure 4.3.3. Class predictions based on mean algorithm score for Model 3 (clinical and biomarker predictors). SOFAS: Social and Occupational Functioning Scale.

Table 4.3.2. Performance metrics for Model 1 (clinical predictors), Model 2 (biomarker predictors) and Model 3 (clinical and biomarker predictors)

Model parameters	Model 1	Model 2	Model 3
Regression performance metrics			
Coefficient of determination (R^2), %	8.9	6.2	5.0
Pearson's r	0.30	0.25	0.22
Mean absolute error	13.0	13.4	13.4
Mean squared error	239.0	256.2	250.0
Normalised root mean square deviation	22.4	23.2	22.9
Classification performance metrics (≤70 vs > 70 points)			
True positives, n	41	34	45
True negatives, n	33	42	31

False positives, n	27	18	29
False negatives, n	30	37	26
Sensitivity, %	57.7	47.9	63.4
Specificity, %	55.0	70.0	51.7
Balanced accuracy, %	56.4	58.9	57.5
Area under the curve	0.63	0.62	0.58
Positive predictive value	60.3	65.4	60.8
Negative predictive value	52.4	53.2	54.4
Positive likelihood ratio	1.3	1.6	1.3
Negative likelihood ratio	0.8	0.7	0.7

Table 4.3.3. Mean feature weighting in each model (top 10% of features shown for Models 2 and 3)

Model 1		**Model 2 (top 10% features)**		**Model 3 (top 10% features)**	
Feature	**Mean weight**	**Feature**	**Mean weight**	**Feature**	**Mean weight**
Log BMI	-0.53	Immunoglobulin heavy constant delta	0.20	GFS score	-0.24
Log Age	0.40	Beta-Ala-His dipeptidase	-0.17	SOFAS score	-0.17
MADRS score	-0.38	Biotinidase	-0.15	Immunoglobulin heavy variable 2-26	-0.17
GFS score	0.36	Actin, cytoplasmic 1	0.15	Monocyte differentiation antigen CD14	0.16
SANS score	0.21	Platelet factor 4 variant	-0.14	Biotinidase	0.15
YMRS score	-0.18	Monocyte differentiation antigen CD14	-0.14	MADRS score	0.14
Male sex	-0.16	Complement C4-A	0.14	Linoelic acid	-0.13
SOFAS score	0.16	Immunoglobulin heavy variable 3-15	-0.14	Immunoglobulin lambda variable 3-21	0.13

Model 1		Model 2 (top 10% features)		Model 3 (top 10% features)	
BPRS score	-0.07	Total Omega-6	0.13	Serum paraoxonase/arylesterase 1	-0.12
GFR score	0.07	TNF-alpha	-0.13	Complement C1r subcomponent-like protein	-0.12
Smoking	-0.07	Secretoglobin family 3A member 1	0.13	Immunoglobulin heavy variable 3-9	0.12
		Prothrombin	-0.13	Immunoglobulin lambda variable 1-36	0.12
		Actin, alpha skeletal muscle	0.12	Apolipoprotein E	0.12
		Filamin A-interacting protein 1-like	0.12	Immunoglobulin heavy constant mu	-0.12
		Immunoglobulin heavy constant alpha 1	-0.12	Immunoglobulin heavy variable 3-15	0.12
		Alpha-2-macroglobulin	0.11	Log Age	-0.11
		Complement C4-B	0.11	Transthyretin	0.11
				sICAM1	0.10

4.3.3. Associations between complement proteins and clinical outcome

A total of 114 participants had proteomic and functional data at baseline and 6 months follow-up. In a linear regression analysis using 6-month SOFAS score, Coagulation protein Factor X at 6-month follow-up showed a positive association with functional outcome [β coef (95% CI)= 2.6(0.1to5.2), p value= 0.04], whereas complement proteins Complement C1r subcomponent like protein, C4A and C5 expressed an inverse association with functional outcome [β coef (95% CI)= -2.7 (-5.3to-0.2), -3.1(-5.8to-0.5) & -2.9(-5.6to-0.3), p value= 0.04, 0.02 & 0.03, respectively] (Table 4.3.4). The complement C5 and coagulation factor IX associated positively with the positive symptom score after adjusting for age, sex, BMI and baseline clinical score [β coef

(95% CI) =2.6(0.2to5.0) and 2.6(0.1to5.0); p value= 0.034 and 0.043, respectively] (Figure 4.3.5).

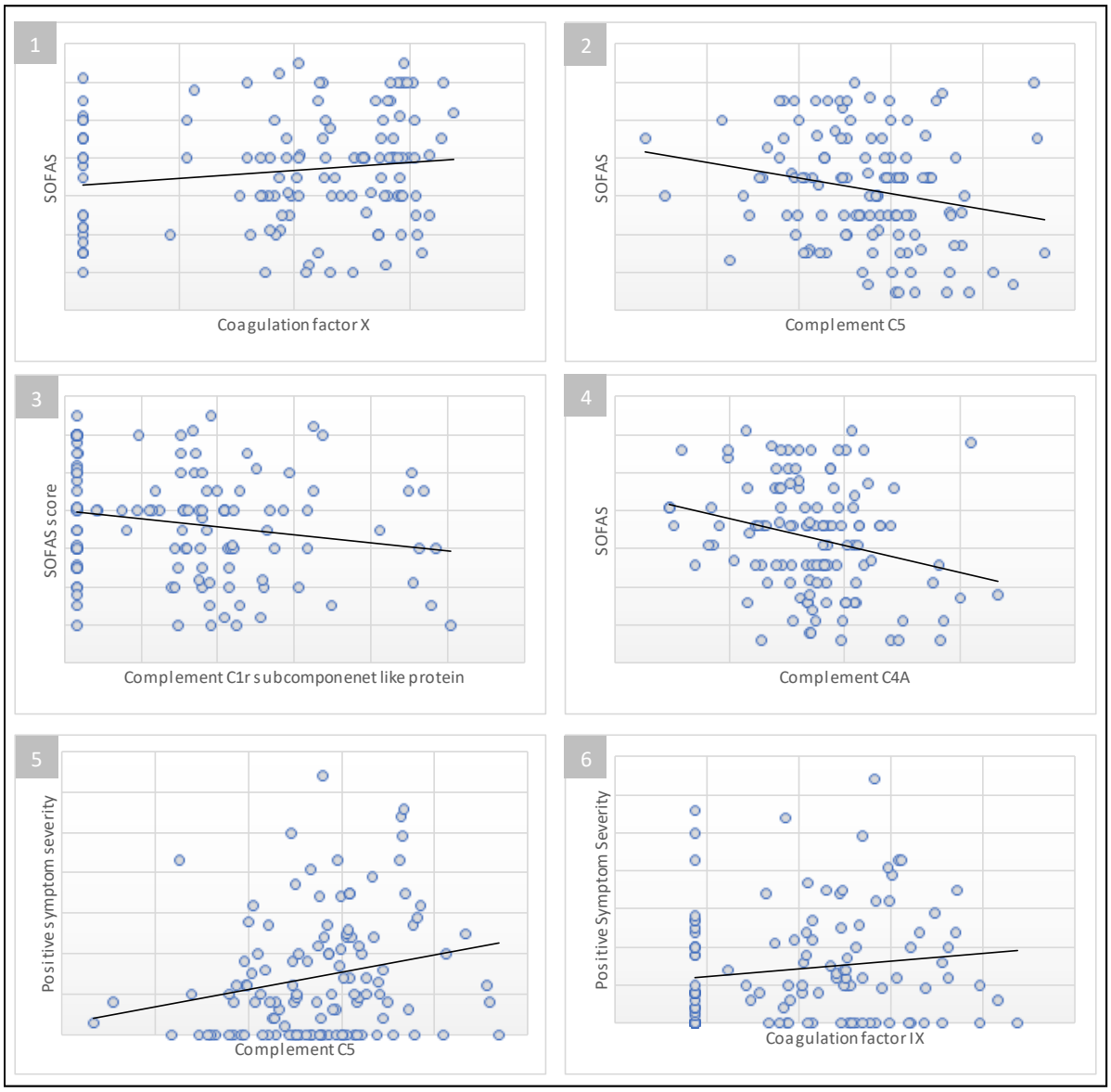

Figure 4.3.4. Scatter plot depicting the relationship of complement and coagulation proteins with clinical outcomes at follow-up (SOFAS and PSS). SOFAS- Social and Occupational Functioning Assessment Scale, PSS- positive symptom severity scale

Table 4.3.4. The results of linear regression analysis between follow-up complement and coagulation proteins and follow-up SOFAS score adjusting for age, sex, baseline protein levels and baseline SOFAS score

Protein Names	Coef.	p value	[95% Conf.	Interval]
Clusterin	2.087	0.112	-0.493	4.668
Coagulation factor IX	-2.264	0.089	-4.879	0.351
Coagulation factor V	1.624	0.230	-1.040	4.287
Coagulation factor X	**2.634**	**0.042**	**0.101**	**5.168**
Coagulation factor XII	0.598	0.682	-2.284	3.480
Coagulation factor XIII A chain	-1.348	0.311	-3.972	1.276
Coagulation factor XIII B chain	1.873	0.169	-0.809	4.556
Complement C1q subcomponent subunit B	0.997	0.468	-1.714	3.709
Complement C1q subcomponent subunit C	1.326	0.321	-1.309	3.962
Complement C1r subcomponent	-1.283	0.334	-3.903	1.338
Complement C1r subcomponent like protein	**-2.720**	**0.036**	**-5.263**	**-0.177**
Complement C1s subcomponent	-0.299	0.824	-2.949	2.352
Complement C2	0.038	0.977	-2.574	2.649
Complement C3	-1.637	0.227	-4.307	1.034
Complement C4A	**-3.132**	**0.020**	**-5.753**	**-0.511**
Complement C4B	-1.251	0.404	-4.211	1.709
Complement C5	**-2.936**	**0.030**	**-5.580**	**-0.291**
Complement component C6	-0.522	0.693	-3.131	2.088
Complement component C7	-0.344	0.806	-3.110	2.421
Complement component C8 alpha chain	0.158	0.907	-2.521	2.837
Complement component C8 beta chain	-1.756	0.197	-4.437	0.924
Complement component C8 gamma chain	0.521	0.694	-2.100	3.142
Complement component C9	-0.715	0.600	-3.408	1.977
Complement factor B	-1.115	0.428	-3.893	1.663
Complement factor H	-1.086	0.429	-3.797	1.624
Fibrinogen alpha chain	-1.496	0.251	-4.065	1.073
Fibrinogen beta chain B	-1.942	0.138	-4.516	0.631
Fibrinogen beta chain C	-0.836	0.525	-3.437	1.764
Fibrinogen gamma chain	-2.030	0.119	-4.593	0.533
Ficolin3	2.723	0.051	-0.014	5.460
Heparin co factor 2	-1.597	0.232	-4.233	1.039
Protein Z dependent protease inhibitor	0.051	0.970	-2.608	2.710
Prothrombin	1.131	0.429	-1.695	3.957
Vitronectin	1.304	0.379	-1.622	4.230

Table 4.3.5. The results of linear regression analysis between follow-up complement and coagulation proteins and follow-up Positive symptom summery score

Protein Names	Coef.	P value	[95% Conf.	Interval]
Clusterin	-0.022	0.987	-2.646	2.603
Coagulation factor IX	**2.559**	**0.043**	**0.086**	**5.031**
Coagulation factor V	-0.881	0.504	-3.489	1.727
Coagulation factor X	-0.426	0.741	-2.985	2.132
Coagulation factor XII	0.424	0.761	-2.332	3.179
Coagulation factor XIII A chain	0.632	0.608	-1.803	3.068
Coagulation factor XIII B chain	-0.609	0.642	-3.203	1.984
Complement C1q subcomponent subunit B	1.011	0.421	-1.475	3.497
Complement C1q subcomponent subunit C	0.067	0.955	-2.300	2.434
Complement C1r subcomponent	0.165	0.894	-2.281	2.611
Complement C1r subcomponent like protein	-0.993	0.429	-3.476	1.490
Complement C1s subcomponent	1.479	0.248	-1.047	4.004
Complement C2	-0.576	0.623	-2.899	1.746
Complement C3	0.354	0.782	-2.180	2.889
Complement C4A	-0.092	0.942	-2.615	2.431
Complement C4B	-0.654	0.649	-3.495	2.186
Complement C5	**2.610**	**0.034**	**0.199**	**5.020**
Complement component C6	0.406	0.736	-1.982	2.790
Complement component C7	-0.319	0.806	-2.890	2.252
Complement component C8 alpha chain	0.825	0.511	-1.655	3.305
Complement component C8 beta chain	0.860	0.492	-1.614	3.333
Complement component C8 gamma chain	0.593	0.635	-1.877	3.063
Complement component C9	0.128	0.921	-2.428	2.684
Complement factor B	-1.182	0.427	-4.124	1.760
Complement facto H	-0.727	0.570	-3.255	1.801
Fibrinogen alpha chain A	-0.323	0.802	-2.873	2.227
Fibrinogen beta chain B	-0.479	0.706	-2.991	2.032
Fibrinogen beta chain C	-0.504	0.679	-2.915	1.907
Fibrinogen gamma chain	-0.088	0.946	-2.666	2.490
Ficolin 3	-0.330	0.806	-2.995	2.335
Heparin co factor 2	0.269	0.837	-2.310	2.847
Protein Z dependent protease inhibitor	2.139	0.092	-0.352	4.630
Prothrombin	1.399	0.302	-1.278	4.075
Vitronectin	-0.147	0.914	-2.845	2.550

4.4. Discussion

In this study, we attempted to develop a machine learning model to predict 12-month functional outcome in a CHR population using baseline clinical data (symptom and sociodemographic measures) and baseline levels of plasma biomarkers (fatty acids, immune markers and proteomic measures). We hypothesized that baseline biological and clinical measures would collectively show better prediction of functional outcome than clinical measures alone. The clinical model (Model 1) had poor predictive performance in relation to functional outcome at 12 months follow-up with mean squared error of 239.0 (Area Under the receiver-operating characteristic Curve [AUC] 0.63). A model based on biomarker data from several modalities (Model 2) showed poor predictive performance with mean squared error of 256.2 (AUC 0.62). A model based on combined clinical and biomarker data (Model 3) also showed poor predictive performance (mean squared error 250.0, AUC 0.58). Hence our results did not support the hypothesis that biomarkers would improve prediction of functional outcome at 12-month follow-up. However, in regression analysis, several complement and coagulation proteins at 6-month follow-up associated with psychotic symptoms and functional outcome at follow-up. In particular, an increased level of complement C5 and coagulation protein factor IX at 6-month follow-up associated with high positive symptoms at 6-month follow-up after adjusting for their corresponding baseline clinical and proteomic measures. Similarly, an increase in complement proteins C1r subcomponent like protein, C4A and C5 associated with decrease in functional outcome, while coagulation protein Factor X associated inversely with functional outcome.

Previous studies of CHR individuals have investigated the role of plasma-based biological markers in the prediction of transition to psychosis in the CHR population. In the North American Prodrome Longitudinal Study, 15 selected plasma analytes not only distinguished the CHR participants from healthy controls, but also successfully differentiated CHR participants who developed psychosis from those who did not (59). Similarly, a combination of 26 plasma biomarkers which were found to be differentially expressed in schizophrenia patients compared to controls, predicted the development of psychosis within two years follow-up. In this model, addition of clinical parameters

increased the performance of this prediction model (60). However, the ability of these models to predict functional outcome in CHR was not evaluated.

A recent study from our team investigated the predictive ability of plasma proteome on the development of psychotic disorder among the CHR (61). The proteomic data successfully predicted the development of psychosis with and without clinical parameters. Moreover the baseline proteomic data along with clinical variables also predicted functional outcome at 2 years follow-up in CHR participants, albeit more weakly than models predicting transition outcome (61). In contrast, the current study did not predict the functional outcome at short term follow-up (12 months) using biological and clinical markers together. The current study investigated a wider array of biological predictors including plasma inflammatory markers measured using multiplex assays and erythrocyte membrane fatty acid assessed by gas chromatography levels but found no evidence of significant predictive performance. This finding could be due to the presence of masking effects of multiple biological variables such as plasma proteins that are not directly related to the functional outcome.

The membrane phospholipid hypothesis has specified the potential involvement of fatty acid imbalance in the development of psychosis (43, 44, 50, 51, 53, 94-97). However, very few clinical studies have investigated the biological relationship of omega-3 FAs with functional outcomes such as social, role functioning and occupational functioning in CHR participants (58, 98-101). These studies suggest that there is a weak cross-sectional association between omega-3 FAs and functional outcome, and longitudinal analyses in the same samples have not shown evidence for strong relationships (58). Considering the limited knowledge of omega-3 FAs and plasma immune markers with functional outcome, the negative results of our study may suggest that more investigations are required to understand the therapeutic and prognostic ability of these fatty acid biomarkers in terms of functional status to consider them in the prediction models. For instance, a recent study has indicated that plasma levels of docosahexaenoic acid were associated cross-sectionally and longitudinally with psychotic disorder in early adulthood in the general population (54) but whether this extends to general functioning in the wider population is not yet known.

Apart from biological markers, previous studies have identified demographic, clinical and neuro-anatomical markers as reliable predictors of functional outcome in CHR psychosis. Another combined machine learning approach in CHR participants by Koutsouleris et al., revealed that social functioning impairment can be predicted using both clinical and neuro-anatomical measures (38). In this latter study, the authors also showed that the combination of neuroimaging models with clinical prediction models increased the performance by 1.9 fold compared to models based on the clinical measures alone (38). Moreover, among the clinical measures, neurocognition and functioning at baseline provided a strong link with functional outcome and provided a basis for domain-wise prediction in functional outcome (10, 13, 14, 17, 63-68). For instance, baseline processing speed and social functioning predicted social functioning at follow-up whereas baseline verbal memory and role functioning predicted role functioning at follow-up (102). In contrast, in our current study, blood-based biomarkers were not able to match the predictive ability of neuroanatomical parameters and domain specific cognitive measures (38).

The results of linear regression analyses revealed an important relationship between complement and coagulation proteins and functional outcome. Increased complement proteins at follow-up were associated cross-sectionally with increased positive symptoms and decreased functional symptoms at follow-up in CHR participants. Several complement pathway proteins are involved in synaptic pruning at early developmental stages and previous genetic and preclinical investigations have revealed the importance of complement related activity in relation to schizophrenia (51, 52, 71, 103, 104). In a population-based study, Föcking et al, (2021) reported an increase in complement and related proteins significantly associated with future development of psychotic symptoms. In this study the authors reported an upregulation of six proteins including C1r subcomponent like protein, and C5 associated with future psychotic symptoms (50). Furthermore, Mongan et al. also observed that the complement proteins were among the top weighted predictive features of functional outcome and transition to psychosis in machine learning models (105). In line with our previous findings in the current study we evaluated the association of individual complement proteins with functional status and found that higher C1r subcomponent like protein, C4A and C5 complement proteins at follow-up

were cross sectionally associated with poor clinical outcomes such as high positive symptoms and poor functional outcome. This supports an inflammatory association with clinical outcome among participants with early psychosis and adds further support to the importance of complement associated changes in the pathophysiology of schizophrenia. Our proteomic findings are in line with genetic results of GWAS study which found that an association of increased risk of schizophrenia with an increased expression C4A gene (70, 71). These findings together suggest that an increase in baseline complement pathway protein levels predispose towards pathological changes on functioning at follow-up during the early stage of psychosis. The results also indicate a group level association of a few complement proteins with functional status even though individualized prediction was not achieved in machine learning based approach. The findings open up new avenues for understanding the molecular mechanisms through which complement and coagulation proteins might influence functional outcome among subjects in the CHR. Furthermore, it is possible that a subgroup of individuals vulnerable to psychosis with dysregulated complement activity may benefit from modulation of the complement pathway (for example through pharmacological interventions targeting complement activity) but this hypothesis would require extensive preclinical testing before human trials.

4.5. Strengths and limitations

Our study has several strengths. First our study utilised unique and in-depth biological data which included proteomic, inflammatory cytokine, membrane FA measures and in-depth clinical measures from a valuable CHR population. Secondly, the analysis focused on functional outcome among the CHR. This is an area that has been under investigated in the past. Thirdly our study is unique in quantifying erythrocyte omega-3 markers and plasma complement protein levels at both baseline and follow-up time points in CHR trajectory. Furthermore, we were able to adjust the linear models for potential confounders. This statistical approach allowed us to take inter-individual heterogeneity into account. The limitations of our study include: i) use of relative quantification methods such as discovery proteomics and semi-quantitative biological assays such as multi-plex ELISA assays; ii) absence of some potentially relevant measures such as neuro-imaging data that has successfully predicted the functional outcome in CHR participants in the past; iii) a relatively small number of samples

(n=158) compared to those who contributed to the NEURAPRO clinical trial as a whole (n=304), due to non-participation in some aspects of the study; iv) the large number of predictors relative to the sample size may give rise to concern regarding overfitting and v) in linear regression analyses, considering the use of discovery based proteomic data and clinically homogenous samples, we did not adjust the results for multiple correction.

4.6. Conclusion

Our study suggests that in CHR participants, addition of baseline plasma biomarker data involving proteomic markers, erythrocyte membrane FA levels and plasma cytokine levels did not improve prediction of 12-month functional outcome beyond baseline clinical data alone. However statistical analysis found an association between increased complement pathway proteins and worsening of clinical outcome such as increased positive symptoms and poor functional outcome in CHR participants. These findings point to a need of further studies exploring and validating the association of complement and related pathway activity with clinical outcome in psychosis. Furthermore, the machine learning models point to a need for a deeper understanding contribution of other types of biological and clinical markers to improve prognostication in CHR individuals.

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Chapter 5

Thesis author's role:

Mr Subash Raj Susai was involved in all aspects of the study. He had full access to all the data in the study and was involved in the acquisition, analysis, and interpretation of data and drafting of the manuscript.

Reference:

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Title: Evidence that complement and coagulation proteins mediating the clinical response to omega-3 fatty acids: A longitudinal mass spectrometry-based investigation in subjects at clinical high-risk psychosis

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Abstract

Preliminary evidence indicates beneficial effects of omega-3 polyunsaturated fatty acids (PUFAs) in early psychosis. The present study investigates the molecular mechanism of omega-3 associated therapeutic effects in clinically high-risk (CHR) participants. Plasma samples of 126 CHR psychosis participants at baseline and 6-months follow-up were included. Plasma protein levels were quantified using mass spectrometry and erythrocyte omega-3 PUFA levels were quantified using gas chromatography. We examined the relationship between change in polyunsaturated PUFAs and plasma proteins and using mediation analysis, we investigated whether plasma proteins mediated the relationship between change in omega-3 PUFAs and clinical outcome. A 6-months change in omega-3 PUFAs was associated with 24 plasma proteins at follow-up. Pathway analysis revealed the complement and coagulation pathway to be the main biological pathway to be associated with change in omega-3 PUFAs and mediated the relationship between change in omega-3 PUFAs and the clinical outcomes at follow-up. The inflammatory protein complement C5 and protein S100A9 negatively mediated the relationship between change in omega-3 PUFAs and positive symptom severity, while C5 positively mediated the relationship between change in omega-3 and functional outcome. The relationship between change in omega-3 PUFAs and cognition was positively mediated through coagulation factor V and complement C1QB. Our findings provide first evidence for a longitudinal association of omega-3 PUFAs with complement and coagulation protein changes in the blood. Further, the results suggest that increase in omega-3 PUFAs decrease symptom severity and improve cognition in the CHR state through modulating effects of complement and coagulation proteins.

5.1. Introduction

The brain is a lipid rich organ and 60% of its total membrane is composed of phospholipids (1). Polyunsaturated Fatty acids (PUFAs) are a vital component of neuronal membrane phospholipids. Omega-3 and omega-6 fatty acids are two major classes of PUFAs present in the brain, among which omega-3 PUFAs have superior health benefits in humans (2-6). Pre-clinical investigations have identified several mechanisms in which omega-3 PUFAs play an important role, such as maintenance of cell membrane integrity (7, 8), release of specialized pro-resolving mediators (9-12), and modification of gut microbiome (13) and regulation of synaptic pruning activity in the brain (14-16).

In psychotic disorder, insufficient consumption of omega-3 PUFAs (17-19) or abnormal fat metabolism (20-27) were found to be associated with disease pathology. The membrane phospholipid hypothesis of schizophrenia proposes a possible link between PUFA abnormalities and psychosis and proposed a potential therapeutic role of omega-3 PUFAs in the treatment of schizophrenia and related disorder at an early stage(20, 28-40). To date, the evidence regarding the therapeutic role of omega-3 PUFAs in Clinically High Risk (CHR) population appears inconclusive. The first omega-3 fatty acid placebo-controlled randomized UHR trial [the Vienna High Risk (VHR) study] found a large preventive effect on transition rate(41) while consecutive multicentre replication study (the NEURAPRO trial) was not able to confirm the latter finding(42),(43). However, both studies have provided preliminary biological evidence for altered PUFA metabolism in emerging psychiatric population. The VHR trial found a reduction in phospholipase A2 (PLA2) activity in relation to omega-3 PUFAs after 12 weeks follow-up (44). The PLA2 enzymes are vital for regulation of phospholipid metabolism, membrane integrity, synaptic integrity and neurotransmission and in schizophrenia the calcium dependant PLA2 activity found to be increased (45). Furthermore, the VHR study indicates an omega-3 PUFA related increase in soluble intercellular adhesion molecule-1 (sICAM-1), whilst no such association was found with plasma cytokines such as Interleukin (IL)-6 and IL-2 (46). The NEURAPRO study found an inverse relationship between omega-3 PUFAs and plasma pro-inflammatory cytokines, although the results did not indicate any association with clinical symptoms in CHR state (47). Knowledge of the underlying mechanism of omega-3 PUFAs will

provide an insight into the biological role of omega-3 PUFAs in the pathophysiology of psychosis and help in designing early intervention strategies (41, 48, 49).

The current study investigates the relationship of PUFAs (omega-3 and omega-6) with plasma proteomic pathways in a clinical CHR population. We performed mass spectrometry-based analysis in plasma samples at baseline and follow-up, to investigate the longitudinal association between PUFAs and the plasma proteome. Furthermore, we evaluated the proteomic pathways through which omega-3 PUFAs may influence psychopathology in CHR participants. We addressed the following research questions:

i) Is there any association between changes in omega-3 PUFAs with plasma proteins at follow-up in CHR participants?

ii) Which biological pathways are most substantially influenced by change in PUFAs (both omega-3 and omega-6 PUFAs)?

iii) Do the identified plasma proteins mediate the relationship between change in omega-3 PUFAs and clinical outcomes?

5.2. Materials and Methods

5.2.1. Study participants

The NEURAPRO study is a multicentre randomized placebo-controlled clinical trial registered with the Australian New Zealand Clinical Trial Registry as ACTRN 12608000475347. The study was performed abiding with the Declaration of Helsinki (50) and adhering to the National Health and Medical Research Council of the Australia National Statement on Human Research. The trial aimed to evaluate the therapeutic role of omega-3 PUFAs in preventing the development of psychosis in CHR patients. Informed consent was obtained from all the participants or from their parents/guardians if they were younger than 17 years. The inclusion and exclusion criteria of the participants of the study are provided in (51).

The participants received either omega-3 PUFAs (840 mg eicosapentaenoic acid [EPA] and 560 mg docosahexaenoic acid [DHA] per day) or placebo (equivalent dose

of paraffin oil) for 6 months (43). The adherence to the study interventions was assessed monthly. At the end of 6 months a low adherence of 43% to the omega-3 intervention and a 41% to the placebo was reported (52).

5.2.2. Measurement of omega-3 PUFAs

Fasting blood samples were collected at baseline and 6-month follow-up. The molecular percentage of the total sum of the omega-3 and omega-6 fatty acid series in erythrocyte membrane rafts were measured based on the phosphatidyl-ethanolamine fraction using gas chromatography (53). Total omega-3 PUFAs comprise of alpha linolenic acid (18:3), eicosapentaenoic acid (20:5), docosapentaenoic acid (22:5) and docosahexaenoic acid (22:6). Total omega-6 PUFAs include linoleic acid (18:2), gamma-linoleic acid (18:3), eicosadienoic acid (20:2), dihomo gamma-linoleic acid (20:3), arachidonic acid (20:4) and adrenic acid (22:4). Since a poor adherence to the study intervention was observed in both study arms, the erythrocyte membrane levels were used as objective measure of dietary intake of PUFAs (exposure variable)(54, 55).

5.2.3. Quantification of plasma proteome

Plasma samples of baseline and follow-up time points were used for discovery-based data-dependant acquisition mass spectrometry. For sample preparation steps and mass spectrometry data acquisition protocols refer to supplementary methods.

5.2.4. Clinical outcome measures

The clinical outcomes of psychotic symptom severity (PSS), functional status and cognitive status at 6-months follow-up were considered for the analyses. The PSS was assessed using the Comprehensive Assessment of At-Risk Mental State (CAARMS) scale (56). The subscales of positive symptoms from the CAARMS assessment (unusual thought content, non-bizarre ideas, perceptual abnormalities and disorganized speech) were used for the calculation of the PSS score. The summed scores of the product of global rating scale score (0-6) and frequency (0-6) of these subscales were calculated, as per previous research (57, 58). Functional outcome was measured using the Social and Occupational Functional assessment Scale (SOFAS)

and cognitive outcome using the Brief Assessment of Cognition in Schizophrenia (BACS), both at 6-months follow-up (59, 60).

5.2.5. Statistical analysis

Statistical analysis was performed using IBM® SPSS® statistics version 26 and STATA IC® version 16.

Analysis 1- Identification of proteins and pathways associated with change in PUFAs

Linear regression models were used to assess longitudinal associations between 6-month change in erythrocyte PUFAs (total omega-3 or total omega-6 PUFAs) and plasma proteins at follow-up. Models were adjusted for age and sex. Proteins that were significantly associated ($p<0.05$) with change in total omega-3 and omega-6 PUFAs were then taken forward for pathway analysis. Pathway analysis was conducted using the Reactome Pathway Knowledgebase Enrichment Analysis and a probability factor (*p*-value) was generated for each pathway based on the protein representations (61). A list of biological pathways based on the p-values after Benjamini-Hochberg correction for multiple tests (FDR 5%) was generated. The UNIPROT entities that were associated with total omega-3 PUFAs were considered for further analysis.

Analysis 2- Relationship of total omega-3 associated proteins and clinical outcome

The relationship of total omega-3 PUFAs associated proteins (from analysis 1) with clinical outcomes at 6-month follow-up were assessed using a linear regression model. The PSS, SOFAS and BACS scores were used for the analysis. The models were adjusted for age, sex and corresponding baseline protein levels.

Analysis 3: Univariate mediation model

Mediation analysis was performed to evaluate the potential mediating role of plasma proteins in the relationship between total omega-3 PUFAs and clinical outcomes (62). Regression-based mediation analysis was performed in IBM® SPSS® using the PROCESS platform. Regression beta coefficients were constructed using a conventional mediation analysis model with a bootstrap sample size of 5000 and with

95% confidence interval. In the mediation model the change in total omega-3 levels were used as exposure variable, the protein measures at follow-up were used as mediators and the clinical and neurocognitive outcomes (PSS/SOFAS and BACS) at follow-up were used as the outcome measures. The mediation analysis was adjusted for age, sex and corresponding baseline plasma protein levels (Figure 5.3.1). The role of baseline total omega-3 PUFAs on the mediation model was then assessed by repeating the model with baseline total omega-3 PUFAs levels as an additional covariate.

5.3. Results

From a total of 285 CHR participants in the NEURAPRO trial, 146 participants provided plasma samples at both time-points, baseline and 6-month follow-up. Out of these, 128 participants had erythrocyte omega-3 PUFA levels and proteomic measurements at both time-points. These 128 participants were considered for the statistical analysis and the baseline characteristics of these participants are given in Table 5.3.1. A total of 165 proteins from discovery proteomics that passed quality control were eligible for analysis (Table 5.3.1.).

Table 5.3.1. Participants' demographic, anthropometric, PUFA and clinical characteristics at baseline and follow-up. SD- Standard deviation

Variable names		Baseline	6-month follow-up
Demographic details			
Age in years, mean ± SD		18 ± 4	-
BMI in kg/m^2, mean ± SD		24.20 ± 5.43	-
Gender, n (%)	Female	81 (63%)	-
	Male	47 (37%)	-
Biological and clinical measures			
Erythrocyte membrane fatty acid levels in %, mean ± SD	Total omega-3 fatty acids	11.94 ± 1.68	13.34 ± 4.40
	Total omega-6 fatty acids	35.56 ± 1.73	31.47 ± 4.12
Positive symptom severity (PSS) score, mean ± SD		25 ± 22	14 ± 15
Social and Occupational Functional Assessment Scale score,		55 ± 10	67 ± 15

Variable names	Baseline	6-month follow-up
mean ± SD		
Brief Assessment of Cognition in Schizophrenia- composite score, mean ± SD	25 ± 22	51 ± 13

5.3.1. Results from analysis 1: The longitudinal association between change in PUFAs and plasma proteins

In a linear regression model, 6-month change in total omega-3 PUFAs was associated with 24 plasma proteins at follow-up after adjusting for age, sex, and baseline total omega-3 levels. Using pathway analysis, these 24 proteins represented three major biological pathways, namely i) the immune system, ii) hemostasis (coagulation), and iii) vesicle mediated transport. The complement system and sub-pathways were the top pathways denoted by the change in total omega-3 PUFAs associated proteins (Table 5.3.2, Appendix 5.8.2 and Figure 5.3.2). Under the coagulation cascade, the plasma proteins associated with change in omega-3 PUFAs (hereafter omega-3 related proteins) associated with platelet activation and clotting cascade related mechanisms (Appendix 5.8.2 and Table 5.3.4). Change in omega-6 PUFAs associated with six plasma proteins (out of 165 proteins) and did not associate with any major pathways (Appendix 5.8.2) (Appendix 5.8.3).

Table 5.3.2. Pathways significantly associated with 6-month change in total omega-3 PUFAs. The table shows the lists of pathways that were significantly represented by total omega-3 associated plasma proteins. The names of pathways are given in the order of p values from low to high.

Pathway name	Entities found	Reactions found	Interactors found	Entities FDR
Regulation of Complement cascade	10	31	6	<0.001
Complement cascade	10	49	6	<0.001
Initial triggering of complement	6	11	0	<0.001
Classical antibody-mediated complement activation	5	2	0	<0.001
Creation of C4 and C2 activators	5	2	0	<0.001
Platelet degranulation	5	2	0	<0.001
Innate Immune System	14	114	8	<0.001
Binding and Uptake of Ligands by Scavenger Receptors	5	9	1	<0.001
Post-translational protein phosphorylation	4	1	0	<0.001
Response to elevated platelet cytosolic Ca2+	5	2	0	<0.001

Pathway name	Entities found	Reactions found	Interactors found	Entities FDR
Scavenging of heme from plasma	4	6	1	<0.001
FCGR activation	4	6	0	<0.001
Terminal pathway of complement	2	5	1	0.002
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	4	1	0	0.009
Role of phospholipids in phagocytosis	4	6	1	0.012
Plasma lipoprotein assembly	3	6	1	0.013
Transport of gamma-carboxylated protein precursors from the endoplasmic reticulum to the Golgi apparatus	2	2	0	0.013
FCGR3A-mediated IL10 synthesis	4	10	1	0.013
Parasite infection	4	14	1	0.013
FCGR3A-mediated phagocytosis	4	14	1	0.013
Leishmania phagocytosis	4	14	1	0.013
Gamma-carboxylation of protein precursors	2	2	0	0.016
Formation of Fibrin Clot (Clotting Cascade)	3	14	0	0.016
Removal of aminoterminal propeptides from gamma-carboxylated proteins	2	2	0	0.017
Defective F9 secretion	1	1	0	0.021
Activation of C3 and C5	2	2	1	0.021
Gamma-carboxylation, transport, and amino-terminal cleavage of proteins	2	6	0	0.021
Regulation of actin dynamics for phagocytic cup formation	4	7	1	0.025
Chylomicron assembly	2	2	1	0.039
Intrinsic Pathway of Fibrin Clot Formation	2	5	0	0.039
Neutrophil degranulation	4	4	0	0.044
Leishmania parasite growth and survival	4	10	1	0.044
Anti-inflammatory response favouring Leishmania parasite infection	4	10	1	0.044
Fcgamma receptor (FCGR) dependent phagocytosis	4	19	1	0.048

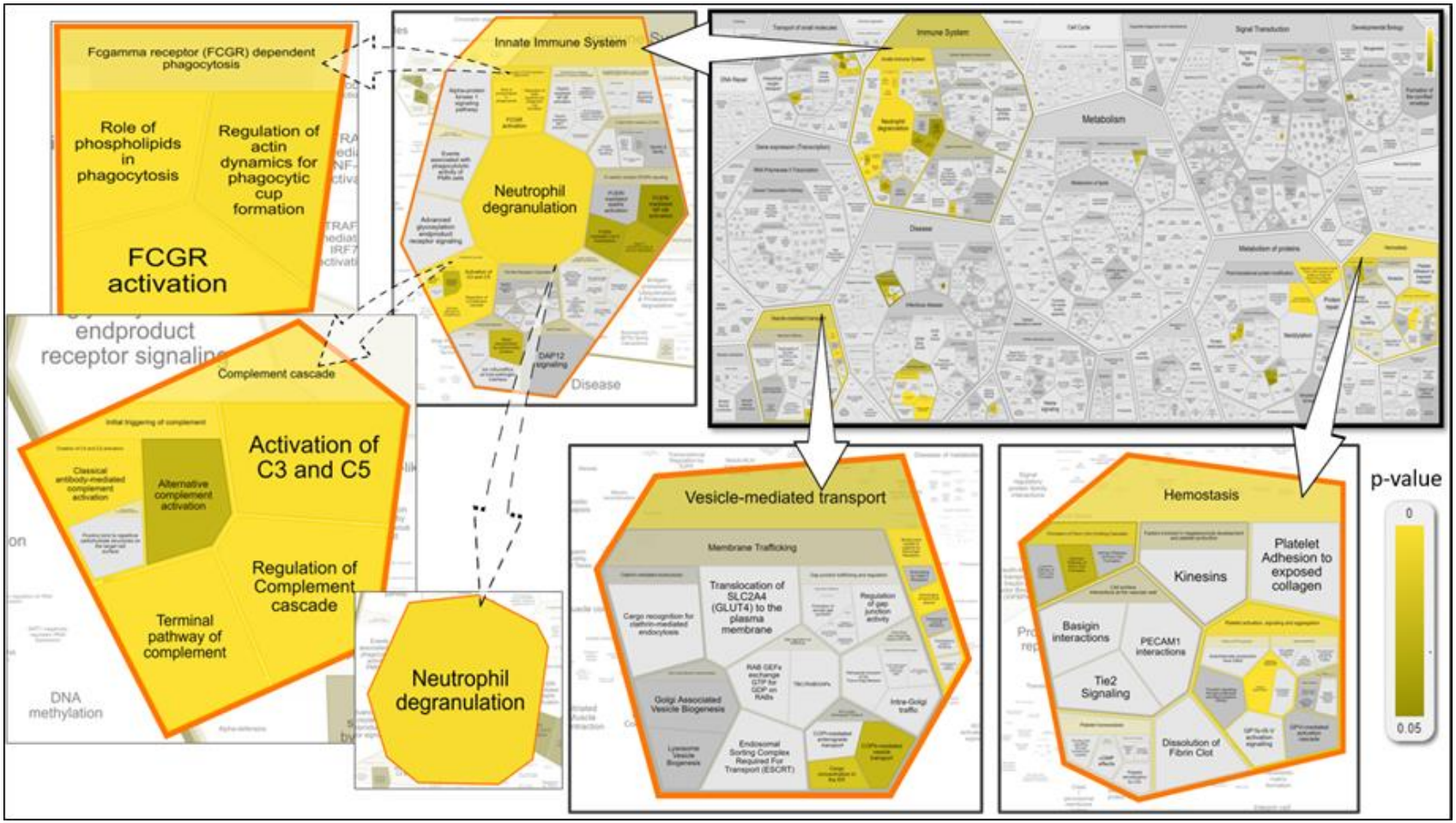

Figure 5.3.1. Results of pathway analysis using reactome.org (https://reactome.org/) depicting the protein pathways associated with 6-months change in total omega-3 PUFA

5.3.2. Results from analysis 2: The association between omega-3 related plasma proteins and clinical outcome at 6-month follow-up

i) Association with positive symptom severity

In linear regression models, three plasma proteins at follow-up associated cross-sectionally with PSS score at follow-up: Complement component 5 (C5), and protein S100A-9 showed a positive association (β coef = 3.54, CI 95ile: 0.79 to 6.30, p-value= 0.01* & 3.40, CI 95ile:0.27 to 6.52; p-value= 0.03*, respectively), while Immunoglobulin heavy constant gamma chain-4 (IGHG-4) showed an inverse association with the PSS score (β coef = -3.13, CI95%ile: -5.79 to -0.47 & p-value= 0.02*) (Table 5.3.3).

ii)Association with functional outcome

Complement C5 associated inversely with SOFAS score at follow-up (β coef = -3.23, CI95%ile= -5.87 to -0.59 & p-value= 0.02*). Apolipoprotein D (Apo D) at follow-up indicated a positive association with SOFAS score with a β coefficient of 2.77 (CI 955ile: 0.13 to 5.41, p-value= 0.04*) (Table 5.3.3).

iii)Association with cognition

In linear regression models, six proteins that are involved with the complement -coagulation cascade and lipid transport pathways expressed association with cognition. Among these Complement Factor B (CFB) inversely associated with BACS score at follow-up (β coef = -3.18, CI 95%ile: 5.72 to -0.63 & p value= 0.02) whereas Complement C1q subcomponent-B (C1QB) and coagulation factor V (F5) were positively associated with BACS score at follow-up (β coef = 3.93, CI95%ile: 1.58 to 6.28, p-value=0.001* & β coef= 3.67, CI95%ile= 1.22 to 6.11; p-value= 0.004*). From the proteins involved in lipid transport mechanism, Apolipoprotein E, C-III and D were positively associated with BACS score (β coef = 3.09, 2.85 & 3.87; CI95%ile= (0.71 to 5.48), (0.36 to 5.34) & (1.25 to 6.50), p value= 0.01*, 0.03* & 0.004*) (Table 5.3.3).

Table 5.3.3. Results of linear regression model II, showing association between total omega-3 related proteins with clinical outcomes at 6 months follow-up. The table shows the results of linear regression models between plasma proteins at follow-up with clinical outcomes at follow-up. The models were adjusted for age, sex, and corresponding baseline protein levels. PSS- Positive Symptom Severity score (based on the CAARMS assessment), SOFAS- Social and Occupational Functional Assessment scale, BACS- composite score of Brief Assessment of Cognitive Function & *significant findings.

Clinical outcomes	**PSS**			**SOFAS**			**BACS**		
Protein Names	Coef.	P value	95% Conf. Interval	Coef.	P value	95% Conf. Interval	Coef.	P value	95% Conf. Interval
Alpha-1-antitrypsin	0.41	0.78	-2.44 to 3.27	0.02	0.99	-2.73 to 2.77	-0.77	0.55	-3.32 to 1.77
Alpha-1B-glycoprotein	0.79	0.57	-1.94 to 3.53	0.05	0.97	-2.59 to 2.69	-0.50	0.7	-3.08 to 2.08
Apolipoprotein C-I	1.03	0.47	-1.80 to 3.86	-1.22	0.37	-3.94 to 1.49	-1.16	0.36	-3.68 to 1.35
Apolipoprotein C-III	-0.98	0.5	-3.82 to 1.87	1.30	0.34	-1.40 to 4.01	2.85	0.03*	0.36 to 5.34
Apolipoprotein D	1.17	0.41	-1.62 to 3.96	2.77	0.04*	0.13 to 5.41	3.87	0.00*	1.25 to 6.50
Apolipoprotein E	-2.15	0.12	-4.87 to 0.58	1.37	0.3	-1.24 to 3.97	3.09	0.01*	0.71 to 5.48
Apolipoprotein L1	1.23	0.38	-1.52 to 3.99	1.13	0.4	-1.51 to 3.78	1.96	0.12	-0.49 to 4.42
Caspase-14	1.84	0.19	-0.95 to 4.62	1.08	0.43	-1.60 to 3.75	-1.29	0.31	-3.81 to 1.24
Coagulation factor V	-0.05	0.97	-2.86 to 2.76	1.48	0.28	-1.20 to 4.16	3.67	0.00*	1.22 to 6.11
Complement C1q subcomponent subunit B	-0.01	0.99	-2.83 to 2.80	0.95	0.49	-1.75 to 3.64	3.93	0.00*	1.58 to 6.28
Complement C5	3.54	0.01*	0.79 to 6.30	-3.23	0.02*	-5.87 to -0.59	-0.88	0.49	-3.38 to 1.63
Complement component C7	0.16	0.91	-2.66 to 2.98	0.06	0.97	-2.62 to 2.74	2.06	0.1	-0.39 to 4.51
Complement factor B	0.78	0.58	-2.01 to 3.58	-1.98	0.15	-4.66 to 0.71	-3.18	0.02*	-5.72 to -0.63
Complement factor I	2.49	0.07	-0.22 to 5.21	-2.36	0.08	-4.97 to 0.25	-0.30	0.81	-2.77 to 2.16
Filamin A-interacting protein 1-like protein	0.18	0.9	-2.56 to 2.92	0.84	0.54	-1.84 to 3.53	1.20	0.34	-1.28 to 3.67
Galectin-3-binding protein	0.24	0.87	-2.67 to 3.16	-0.89	0.53	-3.68 to 1.91	0.27	0.85	-2.46 to 2.99
Haptoglobin	1.07	0.45	-1.72 to 3.85	-1.34	0.32	-4.01 to 1.33	-0.58	0.65	-3.11 to 1.95
Immunoglobulin heavy constant gamma 2	-1.83	0.19	-4.56 to 0.89	0.81	0.54	-1.80 to 3.42	-0.85	0.5	-3.32 to 1.62

Clinical outcomes	PSS			SOFAS			BACS		
Immunoglobulin heavy constant gamma 4	-3.13	0.02*	-5.79 to -0.47	-0.22	0.87	-2.80 to 2.36	1.98	0.11	-0.46 to 4.42
Immunoglobulin heavy variable 1-18	0.2	0.89	-2.59 to 3.00	-0.05	0.97	-2.74 to 2.65	0.75	0.56	-1.79 to 3.29
Immunoglobulin heavy variable 3-7	-0.29	0.84	-3.05 to 2.48	0.27	0.84	-2.38 to 2.91	1.57	0.21	-0.87 to 4.01
Immunoglobulin kappa variable 3-20	-0.25	0.86	-3.08 to 2.57	0.28	0.84	-2.41 to 2.97	2.27	0.08	-0.24 to 4.79
Protein S100-A9	3.4	0.03*	0.27 to 6.52	-1.48	0.34	-4.56 to 1.60	-0.63	0.66	-3.50 to 2.23
Talin-1	1.02	0.47	-1.74 to 3.78	-1.95	0.14	-4.58 to 0.68	0.40	0.75	-2.04 to 2.83

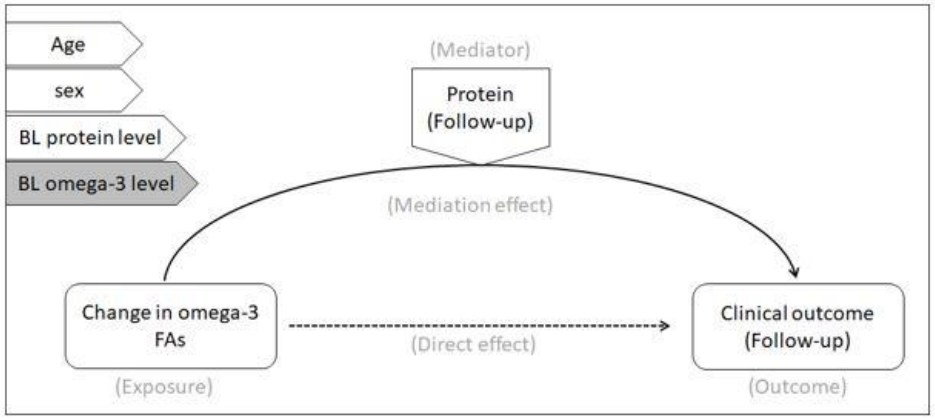

Figure 5.3.2: Mediation model. The picture depicts the structure of mediation model used for the analysis. The model was adjusted for covariates which include age, sex and baseline protein levels.

5.3.3. Results of analysis 3: Univariate mediation analysis

i) Positive symptom severity

In the uni-variate mediation model of C5, IGHG-4 and S100A49, total omega-3 did not exert any direct or total effect on PSS score at follow-up. However, C5 and S100A49 exerted a significant negative indirect effect (mediation effect) on the relationship between change in total omega-3 PUFAs and PSS score at follow-up [β coef= -0.21 & -0.18; 95%ile CI= (-0.46 to -0.03) & (-0.42 to -0.01)] (Table 5.3.4) (Figure 5.3.3).

ii) Functional outcome

For SOFAS score at follow-up, no direct or total effect was observed for total omega-3 PUFAs. However, complement C5 showed a significant positive mediation effect on the relationship of change in total omega-3 PUFAs on SOFAS score at follow-up [β coef=0.19; 95%CI= (0.01 to 0.42)] (Table 5.3.4) (Figure 5.3.3).

iii) Cognitive outcome

Univariate mediation analysis was developed for six plasma proteins (FB, C1QB, F5, Apo E, Apo CIII & Apo D). A significant positive total effect was observed for total omega-3 PUFAs associated with cognitive outcome. C1QB and F5 exerted a significant positive mediation effect on total omega-3 PUFAs related cognitive improvement [β coef=0.24 & 0.18; 95%CI= (0.05 to 0.54) & (0.02 to 0.38)] (Table 5.3.4). This mediation effect of C1QB and F5 was found to be 39% and 27% of the total effect of total omega-3 PUFAs on cognition, respectively (Figure 5.3.3).

iv) Role of baseline total omega-3 PUFAs on the mediation effect

In this model, no total effect was observed for change in total omega-3 PUFAs on any of the clinical outcomes. However, the mediation effect of complement and coagulation proteins on total omega-3 associated clinical outcome remained significant after adjusting the models for baseline total omega-3 PUFAs. (Appendix 5.8.4) (Figure 3).

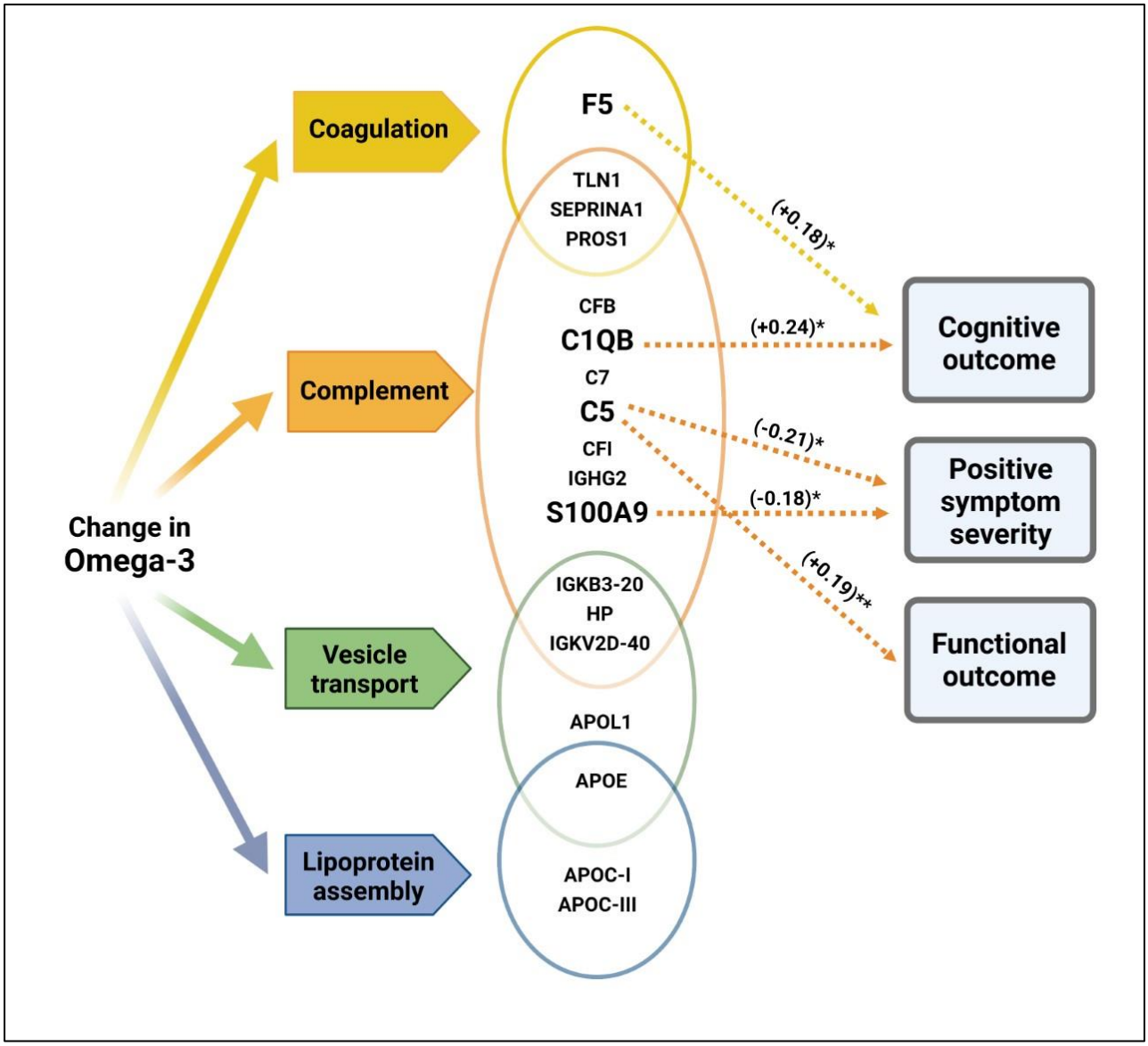

Figure 5.3.3: Schematic representation of key results depicting the relationship of total omega-3 PUFAs, key plasma proteins/pathways and clinical outcome in clinically high-risk population.

Table 5.3.4. Results of univariate mediation analysis of omega-3 fatty acid associated plasma proteins on clinical outcome at follow-up. The table shows the results of mediation analysis using change in omega-3 PUFAs, plasma proteins and clinical outcomes as exposure, mediator and outcome variables, respectively. The model is adjusted for age, sex and baseline total omega-3 levels.CI- confidence interval, PSS- Positive Symptom Severity score, SOFAS- Social and Occupational Functional Assessment scale, BACS- Brief Assessment of Cognitive Function & *significant findings

Outcome	Mediator	Mediation effect β coef (95%ile CI)	Direct effect β coef (95%ile CI)	Total effect β coef (95%ile CI)
PSS	**Complement C5**	**-0.21*** **(-0.46 to -0.03)**	-0.06 (0.85 to -0.70)	-0.27 (0.39 to -0.90)
	Protein S100-A9	**-0.18*** **(-0.42 to -0.01)**	-0.09 (-0.78 to 0.73)	-0.27 (0.38 to -0.90)
	Immunoglobulin heavy constant gamma 4	-0.17 (-0.46 to 0.029)	-0.09 (-0.72 to 0.54)	-0.26 (-0.88 to0.37)
SOFAS	**Complement C5**	**0.19*** **(0.006 to 0.42)**	0.11 (-0.51 to 0.73)	0.30 (-0.30 to 0.90)
	Apolipoprotein D	0.15 (-0.004 to 0.34)	0.20 (-0.42 to 0.82)	0.34 (-0.25 to 0.95)
	Complement factor B	0.11 (-0.02 to 0.31)	0.54 (-0.04 to 1.13)	0.65* (-0.07 to 1.24)
BACS	**Complement C1q subcomponent subunit B**	**0.24*** **(0.05 to 0.54)**	0.38 (-0.19 to 0.95)	**0.62*** **(0.06 to 1.18)**
	Coagulation factor V	**0.18*** **(0.02 to 0.38)**	0.47 (-0.10 to 1.05)	**0.66*** **(0.09 to 1.23)**
	Apolipoprotein E	0.16 (-0.01 to 0.43)	0.46 (-0.11 to 1.03)	**0.62*** **(0.06 to 1.18)**
	Apolipoprotein C-III	0.14 (-0.02 to 0.39)	0.52 (-0.06 to 1.10)	0.66 (-0.10 to 1.23)
	Apolipoprotein D	0.15 (-0.02 to 0.33)	0.51 (0.08 to -0.06)	0.67 (0.10 to 1.24)

5.4. Discussion

The current study investigated both the biological and clinical effect of PUFAs in a clinical high-risk population for a first psychotic episode. The mass-spectrometry based exploration of the plasma proteome at baseline and follow-on time points enabled us to study the longitudinal relationship of total omega-3 PUFAs on various biological mechanisms associated with psychopathology of CHR participants. First, change in total omega-3 PUFAs was associated with plasma proteins that represent immune, clotting and vesicle mediated transport mechanisms in CHR participants. Secondly, among the omega-3 PUFA associated proteins, those participating in immune pathways of the complement system (C5, CFB, C1QB & S100A9), the coagulation pathway (F5) and lipid transport pathways (Apo E, Apo CIII and Apo D) were significantly associated with clinical outcomes. Thirdly, the results of the mediation analysis demonstrated that omega-3 PUFAs may exert a beneficial clinical response through immune pathway proteins (mainly the complement and coagulation cascade). There was evidence that C5 and S100A9 mediated the association of change in total omega-3 PUFAs with reduction in positive symptom severity and improvement in functioning. Furthermore, the association between change in total omega-3 PUFAs and cognitive improvement at follow-up was mediated by the proteins F5 and C1QB.

The current study is the first to observe that the complement cascade as the top biological pathway to be related with change in omega-3 PUFAs in CHR population. These observations provide vital evidence in omega-3 based treatment response in psychosis for the following reasons: i) Imbalances in PUFAs in individuals with psychosis have previously been suspected as genetic studies have reported evidence of a potentially causal relationship between increased long chain PUFA concentrations and lowered risk of psychosis (63, 64); ii) On the other hand, complement related immune activity has been found to be involved in the pathophysiology of schizophrenia(65-69); and iii) whereas in rodents, Madore. et al observed that maternal omega-3 PUFA deficiency drives microglia associated synaptic pruning and associated cognitive impairment in off-spring (15). Previous studies have observed beneficial effects of omega-3 PUFAs including anti-thrombotic, anti-inflammatory and lipid lowering properties in various inflammatory and metabolic conditions (14, 15, 70-

73). A recent study investigated the role of dietary intake of marine omega-3 on the plasma proteome in patients with fatty liver disease. This study has reported the influence of marine omega-3 PUFAs supplements on the coagulation pathway in pooled plasma samples (74). Unlike the previous study, in the current study the inter-individual heterogeneity was taken into account by quantifying the proteins in each sample and by using erythrocyte omega-3 PUFAs, which is a more reliable marker for dietary omega-3 PUFAs and neuronal membrane omega-3 PUFAs (54, 55, 75, 76).

The second important finding of the present study is the association between the omega-3 related proteins and clinical outcomes. Complement and coagulation pathway proteins (that were associated with omega-3 PUFAs) indicated a relationship with psychotic symptoms (PSS), functioning status (SOFAS) and cognitive symptoms (BACS), whereas proteins from lipoprotein assembly associated with cognition and functional outcome. In line with our findings, existing evidence indicates consistent associations of complement dysregulation with psychotic symptoms in early psychosis although not in established schizophrenia (77-83). For instance, the upregulation of complement proteins and apolipoprotein-E were found to be associated with future development and persistence of psychotic symptoms in adolescence (80, 81).

Finally, the mediation analysis revealed a potential molecular mechanism through which total omega-3 PUFAs could influence clinical outcomes as measured by PSS (positive symptom score calculated based on CAARMS score), SOFAS (functioning) and BACS scales (cognition) (Figure 5.3.3). Complement protein (C5) mediated the association between change in total omega-3 PUFAs with reduction in positive symptoms and improvement in functional outcome in CHR. S100A9 which can regulate the expression of C3 (84, 85) mediated the omega-3 PUFA associated reduction in PSS. In the mediation models no direct association (no direct effect) was noticed for change in total omega-3 PUFAs (exposure) with clinical outcomes which include functional outcome and PSS score. However, a significant indirect association (indirect effect) was observed for change-in omega-3 PUFAs with clinical outcome through the mediators (C5 and S100A9 for PSS; C5 for SOFAS score). Such type of mediation without direct effect is called as indirect-only mediation (86). In contrast, in mediation models of cognitive outcome, a significant direct and indirect effect was observed. Importantly, an increase in omega-3 PUFAs significantly associated with a

high cognitive score at follow-up in which complement and coagulation proteins (C1QB and F5) exerted a partial mediation effect. Such mediation is termed ‘complementary mediation’, where the mediators (plasma proteins) complement the association of omega-3 PUFA on cognitive outcome (86). This partial mediation of C1QB and F5 contributed approximately 33% and 27% of the total effect of change in total omega-3 PUFAs on cognition. Latter mediation effect did not change even after adjusting the model for baseline omega-3 FAs. Previous investigations in the same cohort have reported significant cross-sectional and longitudinal associations of omega-3 PUFAs (EPA and DHA) on plasma immune markers, although these associations did not indicate any clinical significance (47). Hence the results from the mediation analysis suggest that omega-3 PUFAs associated changes in complement and coagulation proteins (F5, C1QB, C5 and S100A9) partially mediate clinical response in CHR state.

The key proteins that indicated a mediation effect were previously found to be involved with neuronal development and functioning (87, 88). The activated product of C5, namely C5a, and S100A9, are pro-inflammatory in nature and play crucial roles in neuronal progenitor cell proliferation (89-91). In our study total omega-3 PUFAs demonstrated inverse association with C5 and S100A9 and in turn, the high levels of these proteins showed a positive relationship with positive symptom severity. Overall, the mediation analysis suggests that an increase in total omega-3 PUFAs leads to symptomatic improvement by reducing the potentially pro-inflammatory components (C5 & S100A9). Similarly, members of the C1Q protein family are involved in the synaptic pruning process, which is responsible for systematic elimination of unwanted synapses during development and aging (92, 93). A positive association was observed between total omega-3 PUFAs with C1QB and between C1QB and cognition. The mediation analysis hence suggested that total omega-3 PUFAs improve cognition by increasing proteins that are involved in synaptic pruning processes (C1QB). The animal study by Madore et al. provided a similar relationship of omega-3 PUFAs-C1Q-cognition axis. Madore et al.’s study reported that C1Q-receptor level was reduced in omega-3 deficient animals resulting in cognitive impairment (15). Such symptom-specific complement alterations in a psychiatric population unfolds novel therapeutic opportunities to consider complement targeted medicine in the early intervention of psychosis. These findings provide further evidence for the possible influence of peripheral (plasma) complement proteins on the central nervous system.

5.5. Strengths and limitations

Our study has several strengths: i) a state-of-the-art discovery proteomic approach allowed us to investigate a wide range of molecular mechanisms from plasma samples, ii) the availability of biological and clinical data of the NEURPRO clinical trial enabled us to look at both the biological and clinical relationship of omega-3 PUFAs at the same time, iii) the exposure variable erythrocyte membrane total omega-3 PUFA levels provided a reliable measure of dietary omega-3 PUFAs and neuronal membrane omega-3 PUFAs(94), iv) the use of a unique study population (CHR) with mild psychotic symptoms, functional decline and cognitive impairment with no exposure to anti-psychotic medication(51), v) the availability of both erythrocyte total omega-3 PUFAs and plasma proteome data at two time points provided the possibility of analysing the longitudinal biological effects in the study population and vi) the findings have important clinical implications to early intervention strategies in psychosis. Our study is not without limitations. Firstly, in the statistical analysis, the results were not adjusted for multiple correction mainly due to the exploratory nature of the analysis and the nature of the mass spectrometry which is data-dependant acquisition (DDA) based discovery approach. Secondly, in the statistical analysis we adjusted the models for age and sex. The association of other covariates such as BMI and exposure to anti-depressants on both biological and clinical variables is not clearly understood and hence did not consider in the analysis. Finally, the absence of a direct effect in the mediation analysis limited us from understanding the percentage contribution of mediation in the overall effect (86, 95).

5.6. Conclusion

In conclusion, our findings provide novel insights into omega-3 PUFA related protein mechanisms in the psychopathology of CHR participants. Pathway analysis indicated that the complement cascade showed the strongest association with change in omega-3 PUFAs. Furthermore, current findings suggest that the impact of omega-3 PUFA on clinical symptoms in psychosis is mediated, at least in part, through complement and coagulation pathway proteins. For positive symptom and functional outcome, the complement cascade proteins (C5 and S100A9) exerted an ‘indirect-only mediation’ effect. Whereas for cognitive outcome complement pathway and coagulation pathway proteins (C1QB and F5) expressed a ‘complementary mediation’

effect. We speculate that omega-3 PUFAs may improve psychotic symptom severity and functional status through anti-inflammatory property and enhances cognition by modifying C1Q mediated synaptic pruning. Our study opens future opportunities to investigate the immune associated intervention strategies in psychosis mainly targeting complement pathway proteins.

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5.8. Disclosure of conflicts of interest

All authors declare no conflicts of interest in relation to this work.

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Chapter 6: General discussion

Chapter overview

This chapter summarizes and discusses the important findings, related literature evidence and their clinical implications of the findings. In addition, the implications of the results in future studies along with the overall strengths and limitation of the methodological approach of the thesis are discussed.

The aim of the thesis was to improve the understanding of the therapeutical effects of omega-3 supplementation in the clinically high-risk (CHR) population. Initially a systematic review was performed to summarize and analyze the evidence from the existing literature related to omega-3 based functional improvement in CHR participants. Then we investigated the biological and clinical effects of omega-3 supplementation in a cohort of CHR subjects from the NEURAPRO clinical trial. In addition, the effect of omega-3 FAs on complement and coagulation pathway proteins and their mediating effect on clinical outcome were studied in the same cohort (Table 6.1).

Table 6.1: Overview of studies included in the thesis with their main findings

Studies included	Study population	Investigations performed	Main findings
Chaper 2: Omega-3 fatty acid in ultra-high-risk psychosis: A systematic review based on functional outcome.	CHR subjects from the NAPLS, the PORT, the VHR and the NEURAPRO studies	N/A	Cross sectional relationships: • In the NAPLS study, a weak positive correlation of the weekly intake of omega-3 consumption and erythrocyte EPA levels with functional scores at baseline was found • In the NEURAPRO study, no association was found Longitudinal relationship: • In the VHR study, the omega-3 treatment group showed short term and long-term improvement in functioning compared to placebo • In the NEURAPRO study, no such association was found
Chaper 3: The association of plasma inflammatory markers with omega-3 fatty acids and their mediating role in psychotic symptoms and functioning: An analysis of the NEURAPRO clinical trial.	CHR subjects from the NEURAPRO clinical trial	Multi-plex ELISA	• Omega-3 FAs expressed an inverse association with plasma immune markers • The immune association of omega-3 did not indicate any mediating effect on clinical outcome.
Chaper 4: Machine learning based prediction and the influence of complement - coagulation pathway proteins on clinical outcome: results from the NEURAPRO trial.		Multi-plex ELISA and mass spectrometry	• Biological and clinical markers separately did not predict the functional improvement of machine learning based prediction model. • Addition of biomarkers did improve the performance of the prediction model of clinical parameters

Studies included	Study population	Investigations performed	Main findings
			• Complement and coagulation pathway proteins indicated a cross-sectional association with psychotic symptoms and functional outcome at follow-up.
Chaper 5: Evidence that complement and coagulation proteins mediating the clinical response to omega-3 fatty acids: A longitudinal mass spectrometry-based investigation in subjects at clinical high-risk psychosis.		Multi-plex ELISA and mass spectrometry	• 6 months change in omega-3 FAs associated with levels of complement and coagulation pathway proteins • Complement and coagulation pathway proteins mediated omega-3 associated improvement of psychotic symptoms, functioning and cognition

6.1 Overview of findings

This thesis describes four different investigations focusing on the immunological and clinical role of omega-3 FAs on functional outcome of CHR of psychosis subjects.

6.1.1. Results of a systematic review focussing on the effects of omega-3 FAs supplementation on functional outcome in the CHR state

The review (Chapter 2) evaluated evidence from the literature concerning the association between omega-3 FAs and functional outcome in a CHR population. Of the four studies included in the review, the NAPLS study reported a weak positive correlation between the incidence of weekly consumption of an omega-3 enriched diet with functional outcome in CHR participants. A similar weak positive correlation between fasting erythrocyte omega-3 levels and functional status was observed in the same participants. Whereas no similar cross-sectional association was observed between omega-3 FAs with functioning in the NEURAPRO and the PORT study. Among the clinical trials that have follow up omega-3 levels, the VHR study reported a significant improvement in functioning in the omega-3 group compared to controls. Furthermore, the omega-6 to omega-3 ratio after 12 weeks also significantly associated with functional improvement in CHR state. No such longitudinal association

was noticed in the NEURAPRO study. Overall, the systematic review revealed an inconclusive result regarding the relationship of omega-3 FAs and their effect on functional status in CHR subjects.

6.1.2. Role of plasma immune markers on omega-3 FAs associated changes in the functional outcome in CHR participants

Chapter 3 aimed to investigate the relationship of the omega-3 index (EPA+DHA) with plasma immune markers and further evaluated the mediating role of immune markers on omega-3 associated functional outcome in CHR participants of the NEURAPRO clinical trial. We hypothesised that omega-3 FAs will be negatively associated with plasma immune markers and that this immune association would at least partially mediate the omega-3 associated improvement in clinical outcome in CHR subjects. In a cross-sectional analysis, a high omega-3 index at baseline was associated with low baseline levels of IL-15, ICAM-1 and VCAM-1. Similarly, an increase in the omega-3 index was associated with a decrease in TNF-α over the 6-month interval. In the longitudinal assessments, a higher baseline omega-3 index and DHA predicted lower plasma levels of VCAM-1 at follow-up. Finally, 6-month increase in omega-3 index associated with lower TNF-α at follow-up. In the mediation analysis, omega-3 FA associated changes in plasma inflammatory markers did not exert any significant mediation role on psychotic or functional outcome of CHR participants. Overall, the results supported our initial hypothesis as plasma inflammatory markers associated inversely with the omega-3 index but did not support our second hypothesis as no mediating effect of plasma immune markers was found on omega-3 related changes in psychotic symptoms or functioning.

6.1.3. Biological and clinical predictors of functional outcome in CHR state

The main objective of the study presented in Chapter 4 was to use machine learning algorithms to evaluate the predictive role of biological as well as clinical parameters at baseline. We hypothesized that baseline biological and clinical measures would collectively show better prediction of functional outcome than clinical measures alone. However, all three models of clinical data (Model 1), biomarker data (Model 2) and combined data (Model 3) did not provide a clinically sufficient performance of prediction of functional outcome at 12 months in the CHR state. The mean squared

error of Models 1, 2 and 3 were 239.0 (AUC 0.63), 256.2 (AUC 0.62) and 250.0 (AUC 0.58), respectively. Hence, our results could not support the hypothesis that biomarkers would improve prediction of functional outcome at 12-month follow-up.

6.1.4. Omega-3 FA associated changes in plasma proteome and their influence in improvement in psychopathology and cognition in CHR

The study in Chapter 5 investigated proteomic changes associated with PUFAs and evaluated the mediating role of the plasma proteome in omega-3 associated improvement in clinical outcome of CHR subjects. The mass-spectrometry based exploration of the plasma proteome at baseline and follow-up time points enabled us to study the longitudinal relationship of total omega-3 PUFAs on various proteomic mechanisms in CHR participants. The results showed that change in total omega-3 PUFAs was associated with plasma proteins that represent immune, clotting and vesicle mediated transport mechanisms in CHR participants. Among the omega-3 PUFA associated proteins, those participating in immune pathways of the complement system, the coagulation pathway and lipid transport pathways were significantly associated with clinical outcomes. The results of the mediation analysis demonstrated that omega-3 PUFAs may exert a beneficial clinical response through immune pathway proteins (mainly the complement and coagulation cascades). There was evidence that C5 and S100A9 mediated the association of change in total omega-3 PUFAs with reduction in positive symptom severity and improvement in functioning. Furthermore, the association between change in total omega-3 PUFAs and cognitive improvement at follow-up was mediated by the proteins F5 and C1QB.

6.2 Literature evidence and implications related to findings

In the following sections, the evidence and associated implication of the results of four major themes that evolved from the studies are discussed.

6.2.1. Chapter 2: The association of omega-3 FAs and functional outcome

The systematic review we undertook noticed preliminary evidence linking omega-3 FAs with functional outcome. However, the results were not consistent in all clinical studies. For example, in the VHR study, none of the baseline erythrocyte membrane omega-3 FA individually associated with functional improvement at 12 weeks follow-up. Nevertheless, in a multivariate analysis, all poly-unsaturated FAs of erythrocyte membrane together predicted an improvement of functional status after 12-weeks in both, the intervention and placebo group. This further supports a synergistic role of EPA and DHA acting in concert with other PUFAs to produce clinical improvement in UHR status(1). In the NEURAPRO study the functional scores of the participants improved irrespective of the study arms after 6 months and no statistical difference was noticed between the groups (2). We speculate that such findings could be due to the relatively low levels of omega-3 FA in the erythrocyte membrane and a narrow range of variation before and after the intervention. For instance, the total omega-3 FA level, which was about 3% of fatty acid content at baseline, increased by only 1% after omega-3 FA supplementation (2, 3). In addition to the low baseline omega-3 FA levels, the lack of compliance to omega-3 FA supplementation and the overshadowing effects of an effective co-intervention (cognitive behavioural therapy) were reported to have an impact on the clinical outcome of omega-3 FA. From the assessment compliance, Schlögelhofer et al. estimated that 57.9% of the participants of the omega-3 FA group were non-adherent to study medication(4).

In the NEURAPRO clinical trial, omega-3 FA supplementation was not found to be effective in the prevention of transition to psychosis. At baseline, the n-3 index (EPA + DHA) was negatively correlated with general psychopathology, psychotic, depressive and manic symptoms, while the n-6/3 PUFA ratio was positively correlated with general psychopathology and depressive symptoms (5). In addition, a 6-month increase in omega-3 FAs levels predicted less severe psychopathology and better functioning at 6-month and 12-month follow-up (6). While these results suggested possible therapeutic effects of omega-3 FAs in CHR, the current study, which investigates the mechanistic (rather than the predictive) role of omega-3 FAs, found no mediating role of plasma immune markers on positive symptoms and functioning in CHR subjects.

Previous studies on animals have investigated the effects of various omega-3 FAs on biological and behavioural outcomes. Intra-hippocampal infusion of DHA enhanced 5-HT levels and enrichment of rat brain with DHA caused an increase in synaptic dopamine and serotonin resulting in anti-depressant like behavioural changes (7),(8). Similarly, diet induced antidepressant effects of omega-3 FA were noticed in experimental animals (9-11). However, the results from clinical studies are not as convincing as the animal counterparts. In humans, the functional outcome was estimated by various measures such as cognitive functioning, physiological and clinical symptoms. A recent meta-analysis on a young healthy population by Emery et al. did not find any beneficial effects of overall cognitive performances after supplementation with omega-3 FAs. Nevertheless, their meta-analysis indicated beneficial effects of EPA supplementation on some cognitive domains such as long-term memory, working memory and problem solving (12). A recent systematic review pointed out that an omega-3 related positive effect on cognitive function was more likely when there is daily supplementation of >450 mg/day of EPA+DHA and >6% increase in the omega-3 index (13). In addition, studies in healthy subjects indicated an association of omega-3 intake with neuronal functional connectivity within the brain (14),(15). However, studies failed to provide consistent results about the therapeutic effects of omega-3 FA levels in other psychiatric patients such as depression and other mood disorders (13, 16-28). Similar inconsistencies with clinical results are evident in other studies discussed in our review. The influence of erythrocyte membrane omega-3 FA levels on the local environment in the brain is still not clearly understood. Hence it is important to investigate the underlying biological connection between erythrocyte omega-3 FAs and the brain to understand the long-term effects of erythrocyte omega-3 FA levels on functional status in general(29, 30). Overall, although beneficial effects of an omega-3 FA diet on functional status were observed by cross-sectional and intervention studies (the NAPLS and the VHR studies, respectively), further investigations are required to validate these results in UHR subjects to reach a definite conclusion.

6.2.2. Chapter 3: Role of plasma immune markers on omega-3 associated clinical outcome

The study described in Chapter 3 investigated the biological and clinical effects of omega-3 FAs supplementation on the participants of the NEURAPRO clinical trial and found an inverse association between omega-3 FAs and plasma immune markers. The results of this study add to the existing literature regarding the role of omega-3 based therapeutic effects in several ways. At baseline, the vascular endothelial markers VCAM-1 displayed a strong negative association with omega-3 index and DHA levels, both cross-sectionally and longitudinally. In addition, ICAM-1 showed a negative association with the erythrocyte omega-3 index cross-sectionally among baseline samples. Previous studies have found varying levels of endothelial immune markers in schizophrenia patients compared to healthy controls (31),(32-35). The vascular adhesion molecules ICAM-1 and VCAM-1 belong to the immunoglobulin super family that are synthesized mainly by leukocytes and endothelial cells (36). To understand the relative contribution of these endothelial markers in schizophrenia patients, Nguyen et al. developed a composite measure called “Vascular endothelial index” (VEI). The VEI was based on the linear combination of endothelial markers that differed most between the groups, and the VEI was found to be increased in schizophrenia patients compared to healthy controls (31). Our findings of an inverse association between endothelial markers and omega-3 FAs were supported by Baker et al (37), in which the authors have identified a molecular level relationship between omega-3 and endothelial markers.

Our study also identified an inverse relationship between EPA and IL-12p40, a common subunit of cytokines IL-12 and IL-23 that exerts a pivotal agonistic role in early inflammatory reactions (38-40). In psychosis, a meta-analysis showed elevated levels of the pro-inflammatory cytokine IL-12 in schizophrenia patients compared to healthy controls (41). Moreover, our group previously observed increased plasma levels of IL-12p40 distinguishing CHR subjects who transitioned to psychotic disorder from those who did not (42). The biological evidence relating the acute phase inflammatory cytokine TNF-α with omega-3 FAs has been extensively reviewed and an altered regulation of TNF-α and IL-15 has been consistently observed in psychosis (43-47). Here, for the first time, we report an association of omega-3 FAs with TNF-α

in the CHR. In a placebo-controlled randomized trial, a decrease in the omega-3:omega-6 ratio showed a positive association with IL-6 and TNF-α production, suggesting an anti-inflammatory role of omega-3 FAs on peripheral cytokines (48). The same research group also observed anti-inflammatory properties of an omega−3 FAs rich diet in an adult population (49). Moreover, an extensive interaction between TNF- α and lL-15 has been observed at the blood brain barrier (BBB) since TNF-α enhances IL-15 synthesis and IL-15 in turn regulates TNF-α signalling at the level of the BBB (50, 51). Overall, our findings provided vital evidence that in the CHR state omega-3 FAs could decrease the immune markers that are known to be associated with the pathophysiology of psychosis. Nevertheless, the results did not indicate any related clinical benefits in the CHR participants. This needs to be investigated in future studies.

6.2.3. Chapter 4: Predictive role of biological and clinical markers on functional outcome in omega-3 based interventions in CHR subjects

Chapter 4 investigated a machine learning-based predictive role of biological and clinical parameters on functional outcome in the NEURAPRO clinical trial. The results did not find any improvement in prediction of functional outcome at 12 months follow-up after adding the baseline biological predictors to the existing model of baseline clinical predictors. Previous studies of CHR individuals have investigated the role of plasma-based biological markers in the prediction of transition to psychosis in the CHR population. In the North American Prodrome Longitudinal Study (NAPLS), 15 plasma analytes distinguished the CHR participants from healthy controls and successfully predicted transition to psychosis at baseline(52). In this latter model, the addition of clinical parameters increased the performance of this prediction model (53). Unlike in Chapter 4, the ability of these models to predict functional outcome in CHR was not evaluated in the NAPLS study(52, 53). Our team investigated the predictive ability of the plasma proteome on the development of psychotic disorder among the CHR in the European Network of National Schizophrenia Networks Studying Gene-Environment Interactions (EU-GEI) study (54). The proteomic data successfully predicted the development of psychotic symptoms with and without clinical parameters. Moreover the baseline proteomic data along with clinical variables also predicted functional outcome at 2 years follow-up in CHR participants, albeit more weakly than models

predicting transition outcome (54). The current study investigated a wider array of biological predictors including plasma inflammatory markers measured using multiplex assays and erythrocyte membrane fatty acids assessed by gas chromatography levels but found no evidence of a significant predictive performance. This could possibly be due to the masking effects of multiple biological variables including plasma proteins and erythrocyte membrane omega-3 levels that are not directly related to the functional outcome. Furthermore, we suspect factors such as the relatively small number of participants and lower incidence of transition among the participants after 12 months compared to the EU-GEI study might have influenced the results.

The membrane phospholipid hypothesis has specified the potential involvement of fatty acid imbalance in the development of psychosis (55-63). However, only a few clinical studies have investigated the biological relationship of omega-3 FAs with functional outcomes such as social, role functioning and occupational functioning in CHR participants (64-68). These studies suggest that there is a weak cross-sectional association between omega-3 FAs and functional outcome, and longitudinal analyses in the same samples have not been able to show evidence for strong relationships (68). The negative results of prediction models suggest the need for a deeper understanding of the role of omega-3 FAs and plasma immune markers in relation to functional outcome in psychosis.

Apart from biological markers, the predictive role of demographic, clinical and neuro-anatomical markers on functional outcome in CHR psychosis has been investigated by other studies. A combined machine learning approach in CHR participants by Koutsouleris et al., revealed that an impairment in social functioning can be predicted using both clinical and neuro-anatomical measures(69). In this study, the authors showed that the combination of neuroimaging models with clinical prediction models increased the performance by 1.9-fold compared to models based on the clinical measures alone(69). Among the clinical measures, neurocognition and functioning at baseline provided a strong link with functional outcome and provided a basis for domain-wise prediction of functional outcome(70-79). For instance, baseline processing speed and social functioning predicted social functioning at follow-up whereas baseline verbal memory and role functioning predicted role functioning at follow-up (80). In contrast, in Chapter 4, blood-based biomarkers were not able to be

matched with the predictive ability of neuroanatomical parameters and domain specific cognitive measures (69).

6.2.4. Chapter 5: The association of change in omega-3 FAs with plasma proteomic pathways at follow-up and related influence on improvement in psychopathology in CHR participants

The results from Chapter 5 demonstrated the importance of complement and coagulation pathway proteins in omega-3 FA associated clinical improvement in early psychosis. The current study is the first to observe that the complement cascade as the top biological pathway is related to a change in omega-3 PUFAs in a CHR population. Imbalances in PUFAs in individuals with psychosis have previously been suspected after genetic studies have reported evidence of a potentially causal relationship between increased long chain PUFA concentrations and lowered risk of psychosis(81, 82). In rodents, Madore et al. observed that maternal omega-3 PUFA deficiency drives microglia associated synaptic pruning and this associated with cognitive impairment in the off-spring(83). These findings along with the known complement associated immune activity in psychosis increases the possibilities of using omega-3 FAs as a potential therapeutic agent in psychosis. They also raise the possibility that one mechanism of action of omega-3 FAs is through modulation of complement pathway activity, in addition to proposed direct effects on membrane integrity and plasticity (83).

The second important finding of the present study is the presence of a mediation effect for complement and coagulation pathway proteins on omega-3 associated clinical outcome. The clinical outcomes include the positive symptom score (PSS, calculated based on CAARMS score), functioning (measured using SOFAS) and cognition (by BACS scales). Complement protein 5 (C5) mediated the association between change in total omega-3 PUFAs with reduction in positive symptoms and improvement in functional outcome in CHR. S100A9, which can regulate the expression of C3(84, 85), mediated the omega-3 PUFA associated reduction in PSS. In the mediation models, no direct association (no direct effect) was noticed for change in total omega-3 PUFAs (exposure) with clinical outcomes which include functional outcome and PSS score. However, a significant indirect association (indirect effect) was observed for change-in omega-3 PUFAs with clinical outcome through the mediators (C5 and S100A9 for

PSS; C5 for SOFAS score)(86). In contrast, in mediation models of cognitive outcome, a significant direct and indirect effect was observed. Importantly, an increase in omega-3 PUFAs significantly associated with a high cognitive score at follow-up in which complement and coagulation proteins (C1QB and F5) exerted a partial mediation effect(86). This partial mediation of C1QB and F5 contributed to approximately 33% and 27% of the total effect of change in total omega-3 PUFAs on cognition. Overall, the results from the mediation analysis suggest that omega-3 PUFAs associated changes in complement and coagulation proteins (F5, C1QB, C5 and S100A9) partially mediate clinical response in CHR state.

The key proteins that indicated a mediation effect were previously found to be involved with neuronal development and functioning(61, 87). The activated product of C5, namely C5a, and S100A9, are pro-inflammatory in nature and play crucial roles in neuronal progenitor cell proliferation(88-90). In our study total omega-3 PUFAs demonstrated an inverse association with C5 and S100A9 and in turn, the high levels of these proteins showed a positive relationship with positive symptom severity. Overall, the mediation analysis suggests that an increase in total omega-3 PUFAs leads to symptomatic improvement by reducing the potentially pro-inflammatory components (C5 & S100A9). Similarly, members of the C1Q protein family are involved in the synaptic pruning process, which is responsible for systematic elimination of unwanted synapses during development and aging(91, 92). A positive association was observed between total omega-3 PUFAs with C1QB and between C1QB and cognition. The mediation analysis hence suggested that total omega-3 PUFAs improve cognition by increasing proteins that are involved in synaptic pruning processes (C1QB). The animal study by Madore et al. provided a similar relationship of omega-3 PUFAs-C1Q-cognition axis. Madore et al.'s study reported that C1Q-receptor level was reduced in omega-3 deficient animals resulting in cognitive impairment(83). Such symptom-specific complement alterations in a psychiatric population unfolds novel therapeutic opportunities to consider complement protein targeted medication development in the early intervention of psychosis. These findings provide further evidence for the possible influence of peripheral (plasma) complement proteins on the central nervous system.

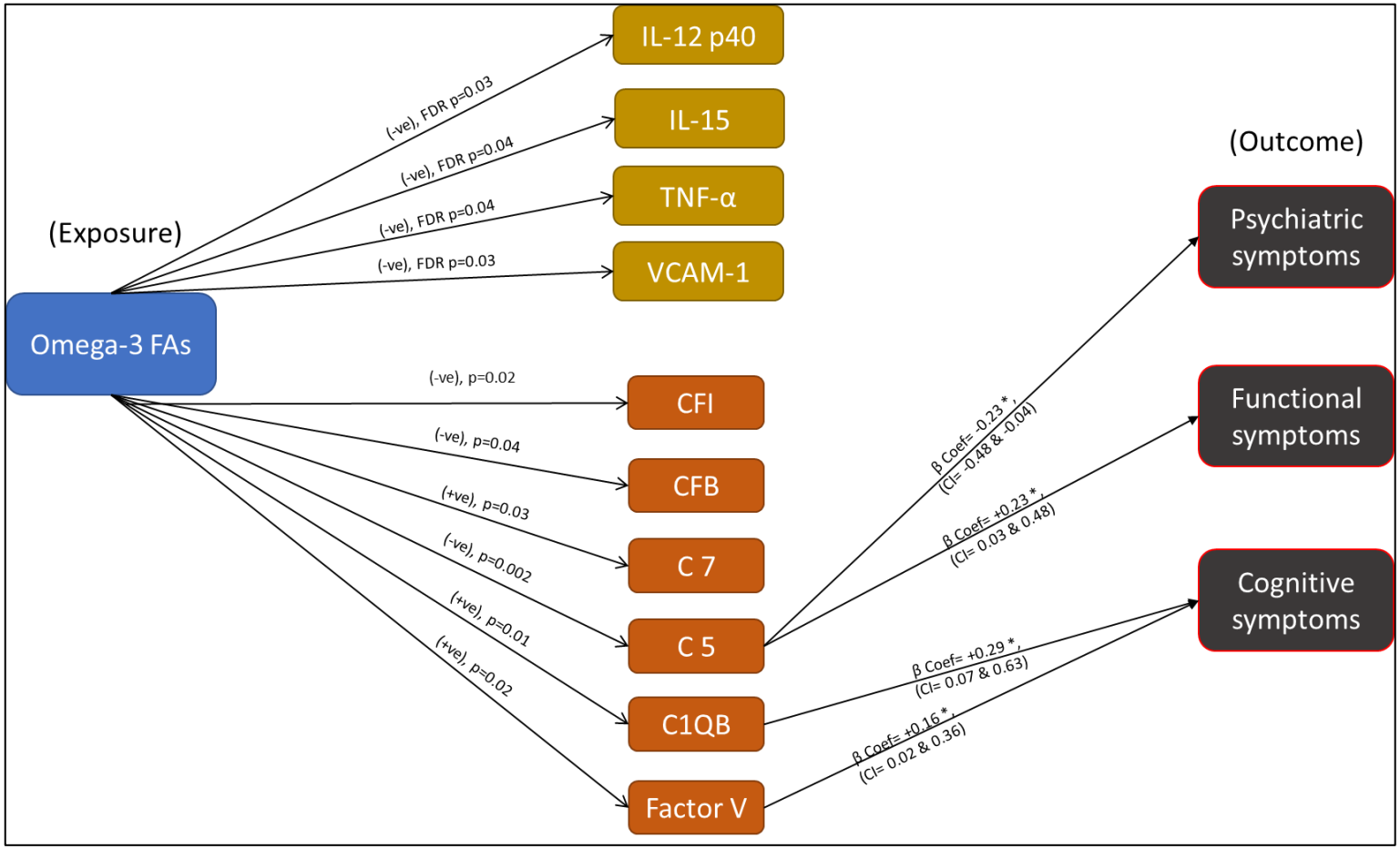

Figure 6.1: The schematic representation of relationship of plasma cytokines, complement and coagulation proteins with omega-3 FAs and clinical outcome in CHR participants. Figure represents the mediating role of plasma cytokines and proteins of complement and coagulation pathway proteins on clinical outcome in CHR subjects. FAs-fatty acids, IL-Interleukin, TNF-Tumour necrosis factor, VCAM- vascular endothelial adhesion molecule, CFI-Complement factor I, CFB-Complement factor B, C-Complement, and Factor V- coagulation factor V.

6.3. Strengths and limitations of the thesis

The following strengths and limitations were observed in the studies

6.3.1. Strengths

All four studies of the thesis used data from well-characterized CHR participants at baseline and follow-up (single or multiple) time points. This enabled us to understand the cross-sectional and longitudinal relationship of biological and clinical parameters in the CHR state. In addition, the following strengths were noticed in the individual studies.

The systematic review in Chapter 2 followed the recommended methodological steps to analyse the association between omega-3 FAs and functional outcome in CHR participants. This is the first systematic review of its kind that included all different forms of omega-3 fatty acids (such as dietary, plasma levels, erythrocyte membrane levels of omega-3 FAs).

In Chapter 3, the use of a multiplex ELISA assay provided the opportunity to analyse the biological effects of omega-3 FAs on a broad array of plasma immune markers in CHR participants simultaneously.

In Chapter 4 our study utilised unique and in-depth biological data which included proteomic, inflammatory cytokine, membrane FA measures as well as multiple clinical measures from a valuable CHR population. In addition, a machine learning based statistical approach allowed us to investigate the group level associations within different parameters for clinical use.

Chapter 5 has several strengths: As in Chapter 4, the availability of biological and clinical data from the NEURAPRO clinical trial enabled us to look at both the biological and clinical relationship of omega-3 PUFAs at the same time. The findings of chapter 5 provided key evidence for clinical implications of omega-3 FAs in early intervention strategies in psychosis.

6.3.2. Limitations

Chapter 2 has the following limitations. First, in the interest of asking a single research question, my systematic review focused only on one clinical assessment (functional status) in a relatively rare population group (CHR). This reduced the total number of available articles and made it impossible to perform a meta-analysis. Secondly, the individual studies included in the systematic review had weaknesses. For example, the co-interventions provided in clinical trials such as vitamin supplements and the frequency of CBCM, which can influence the study outcomes, were not carefully controlled for in the analysis. Further, the lack of uniform inclusion criteria across different trials, lack of standardized clinical assessments and low adherence to the study intervention made it challenging to compare the results.

In Chapter 3, the participants of the clinical trial displayed a low adherence to study intervention, which limited our ability to evaluate group difference between the omega-3 FA and placebo study arms(4). We overcame this limitation by considering the erythrocyte omega-3 FA levels. In addition, the number of follow-up samples was only 55% of the baseline sample size. This drop-out may have resulted in some attrition bias as this affected the statistical power. Thirdly, the interaction within different immune mediators was not considered in the mediation analysis. Finally, the multiplex assay performed to study a broad array of immune biomarkers simultaneously, comes with its own limitations of a possible cross reactivity within the assays.

Chapter 4 also has limitations and these are as follow; Firstly, the use of relative quantification methods such as discovery proteomics and semi-quantitative biological assays such as multi-plex ELISA assays; Secondly, the absence of some potentially relevant measures such as neuro-imaging data that has successfully predicted the functional outcome in CHR participants in the past; Thirdly, a relatively small number of samples (n=158) compared to those who contributed to the NEURAPRO clinical trial (n=304) at follow-up. Fourthly, the large number of predictors relative to the sample size may give rise to concern regarding overfitting.

Our study in Chapter 5 observed the following limitations: In the statistical analysis, the results were not adjusted for multiple corrections mainly due to the exploratory nature of the analysis and the nature of the mass spectrometry, which is data-

dependant acquisition (DDA), a discovery based approach. The association of other covariates such as BMI and exposure to anti-depressants on both biological and clinical variables is not clearly understood and hence, we did not consider this in the analysis. Finally, the absence of a direct effect in the mediation analysis limited us from understanding the percentage contribution of mediation in the overall effect(86, 93).

6.4. Conclusion

Overall, the thesis provided valuable insights into the biological and clinical role of omega-3 fatty acids in the early intervention of psychosis. Chapter 2 (systematic review) found mixed evidence regarding the relationship between omega-3 FAs and functional outcome in CHR from the available studies. Cross-sectional data from the literature indicated a positive correlation between dietary omega-3 FA and functional status. Among various erythrocyte membrane omega-3 FA concentrations, the EPA associated positively, whereas the omega-6/omega-3 FA ratio was inversely associated with functional improvement. Further, the combined concentrations of all baseline erythrocyte membrane FA successfully predicted functional enhancement. However, these associations were not consistent in all studies. In Chapter 3 the results from the NEURAPRO clinical study showed an inverse relationship between omega-3 FAs and plasma immune markers that are involved in the pathophysiology of schizophrenia in this CHR cohort. In the cross-sectional analysis, erythrocyte membrane omega-3 FAs were inversely associated with the pro-inflammatory cytokines IL-15, IL-12p40, TNF-α, endothelial markers ICAM-1 and VCAM-1 and in the longitudinal analysis a similar negative association was found with TNF-α and VCAM-1. The predominant negative associations observed with several pro-inflammatory mediators are in keeping with known immune actions of omega-3 FAs, suggesting that omega-3 FAs may reduce the inflammatory load in CHR individuals. However, the immune relationship of omega-3 FAs was found to be clinically ineffective as no mediation effect of immune markers was noticed between omega-3 FAs and functional outcome. In Chapter 4, the results of the machine learning based prediction models of the NEURAPRO study suggested that in CHR participants, baseline plasma biomarker data involving proteomic markers, erythrocyte membrane FA levels and plasma cytokine levels did not improve prediction of 12-month functional

outcome beyond baseline clinical data alone. Finally in chapter 5, the complement and coagulation pathway proteins not only showed an association with change in omega-3 FAs but also provided evidence of mediating the omega-3 related improvement in psychopathology and cognition in CHR individuals. These findings provide novel insights into omega-3 PUFA related protein mechanisms in the psychopathology of CHR participants.

In summary, the thesis has investigated the therapeutic role of omega-3 supplementation in preclinical psychosis and identified novel molecular mechanisms involving plasma complement and coagulation pathway proteins that are involved with omega-3 related clinical recovery in CHR subjects. These findings open future opportunities to investigate the immune associated intervention strategies in psychosis mainly targeting complement pathway proteins.

6.5. Future directions

The results of my thesis have raised further research questions which will be the basis for future investigations.

6.5.1. Biological perspective

- In the NEURAPRO clinical trial (Chapter 3), the immune association of omega-3 FAs did not indicate any beneficial clinical effect of the supplementation after 6 months follow-up of participants (6). This raises the possibility that a potential non-immune function of omega-3 FAs, such as recovering synaptic membrane activity in the brain, modulation of the microbiota-gut-brain axis or production of neuroprotective metabolites may impact on the clinical outcome in early psychosis (94). Future randomized controlled trials with more biological markers measured at multiple time points would be beneficial in understanding the possible long-term biological effects of omega-3 in psychosis.
- In the machine learning prediction model (chapter 4), incorporation of biological parameters such as plasma proteome, erythrocyte omega-3 FA levels did not improve the performance of the algorithm. The overfitting of data due to the large number of predictor variables relative to the sample size was suspected to be one of the reasons for the underperformance of this model. This suggests a need for a deeper understanding regarding the contribution of different types

of biological markers to be considered for the use of the algorithm in order to improve prediction in CHR individuals**.** Furthermore, future studies with higher numbers of participants and a greater number of subjects that transition to psychosis will be helpful in developing a more reliable prediction algorithm for clinical improvement.

6.5.2. Clinical perspective

- In Chapter 2, we found very few omega-3 related clinical trials in CHR subjects and we reported a lack of a uniform protocol across the clinical trials which limited us from reaching an informed conclusion. Hence, in the future, we suggest that more clinical trials are required with a uniform selection protocol and with standardised clinical assessments in CHR. Moreover, we suggest the following strategies to improve the adherence to study medication, which has been reported to be a common issue in clinical trials with nutritional supplements. This includes; i) strategies such as explaining the expectations of the study at an early stage to the participants, ii) using digital options to motivate the participants to take their medication, iii) using a digital option to provide real-time feedback regarding adherence to the treatment, and iv) considering remote data collection procedures for data such as pills taken every day. In addition, considering a unified study protocol for CHR subjects with standardized outcome assessments would increase the clinical validity of the data for understanding the role of omega-3 FA in psychosis.
- The mediating role of complement and coagulation pathway proteins in association with omega-3 related clinical improvement provides novel insight into omega-3 PUFA related protein mechanisms in the psychopathology of CHR. From our findings, we suggest that further proteomic investigations using data independent acquisition would provide deeper understanding about the absolute distribution of complement and coagulation proteins in psychosis. These investigations not only will validate the current findings but will also provide a vital platform for immune targeted intervention strategies in psychosis.

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Chapter 7: Appendix

7.1. Appendix Chapter 2

7.1.1. Search terms used for each database

MEDLINE PubMed		
1	"Fatty Acids, Omega-3"[Mesh] OR "Fatty Acids, Unsaturated"[Mesh] OR "polyunsaturated fatty acids"[Title/Abstract] OR "unsaturated fatty acids"[Title/Abstract] OR "omega 3"[Title/Abstract] OR "omega 6"[Title/Abstract]	233,940
2	"Schizophrenia"[Mesh] OR "Schizophrenia Spectrum and Other Psychotic Disorders"[Mesh] OR schizophrenia[Title/Abstract] OR first episode psychosis[Title/Abstract] OR "Psychotic Disorders"[Mesh] OR psychosis[Title/Abstract] OR psychotic[Title/Abstract]	189,673
3	"at risk"[Title/Abstract] OR "high risk"[Title/Abstract] OR "ultra high"[Title/Abstract]	400,747
4	"Fatty Acids, Omega-3"[Mesh] OR "Fatty Acids, Unsaturated"[Mesh] OR "polyunsaturated fatty acids"[Title/Abstract] OR "unsaturated fatty acids"[Title/Abstract] OR "omega 3"[Title/Abstract] OR "omega 6"[Title/Abstract] AND "Schizophrenia"[Mesh] OR "Schizophrenia Spectrum and Other Psychotic Disorders"[Mesh] OR schizophrenia[Title/Abstract] OR first episode psychosis[Title/Abstract] OR "Psychotic Disorders"[Mesh] OR psychosis[Title/Abstract] OR psychotic[Title/Abstract] AND "at risk"[Title/Abstract] OR "high risk"[Title/Abstract] OR "ultra high"[Title/Abstract]	80
EMBASE Elsevier 31/1/2019		
1	'polyunsaturated fatty acid'/exp OR 'unsaturated fatty acid'/exp OR 'omega 3 fatty acid'/exp OR 'omega 6 fatty acid'/exp OR ((unsaturated NEXT/1 fatty NEXT/1 acid):ti,ab) OR ((saturated NEXT/1 fatty NEXT/1 acid):ti,ab) OR ((omega NEXT/1 3):ti,ab) OR ((omega NEXT/1 6):ti,ab)	147,668
2	'psychosis'/exp OR psychosis:ti,ab OR psychotic:ti,ab OR 'schizophrenia'/exp OR schizophrenia:ti,ab,de OR (first NEAR/2 episode NEAR/2 psychosis)	311,530

3	((at NEXT/1 risk):de,ti,ab) OR ((high NEXT/1 risk):de,ti,ab) OR ((ultra NEXT/1 high):de,ti,ab)	23,449
4	1 AND 2 AND 3	244
EMBASE Elsevier 31/1/2019		
1	'polyunsaturated fatty acid'/exp OR 'unsaturated fatty acid'/exp OR 'omega 3 fatty acid'/exp OR 'omega 6 fatty acid'/exp OR ((unsaturated NEXT/1 fatty NEXT/1 acid):ti,ab) OR ((saturated NEXT/1 fatty NEXT/1 acid):ti,ab) OR ((omega NEXT/1 3):ti,ab) OR ((omega NEXT/1 6):ti,ab)	147,668
2	'psychosis'/exp OR psychosis:ti,ab OR psychotic:ti,ab OR 'schizophrenia'/exp OR schizophrenia:ti,ab,de OR (first NEAR/2 episode NEAR/2 psychosis)	311,530
3	((at NEXT/1 risk):de,ti,ab) OR ((high NEXT/1 risk):de,ti,ab) OR ((ultra NEXT/1 high):de,ti,ab)	723,449
4	1 AND 2 AND 3	244
COCHRANE LIBRARY & CENTRAL REGISTER OF CLINCAL TRIALS Wiley		
1	MeSH descriptor: [Fatty Acids, Unsaturated] explode all trees OR unsaturated near/1 fatty OR polyunsaturated near/1 fatty OR omega near/1 3 OR omega near/1 6	24,534
2	MeSH descriptor: [Schizophrenia] explode all trees OR (schizophrenia):ti,ab,kw OR "first episode psychosis" OR (psychotic OR psychosis):ti,ab,kw OR MeSH descriptor: [Psychotic Disorders] explode all trees	16,987
3	risk:ti,ab,kw OR at NEAR/1 risk OR high NEAR/1 risk OR ultra NEAR/1 high	177,022
4	1 AND 2 AND 3 (Protocols = 1; Trials = 81; Reviews = 15)	97

7.1.2. PRISMA checklist for systematic review

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5

Section/topic	#	Checklist item	Reported on page #
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	23-24
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	N/A
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	N/A
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	N/A
Section/topic	**#**	**Checklist item**	**Reported on page #**
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	24
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	24

Section/topic	#	Checklist item	Reported on page #
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6 & 16
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	7-8, 17,18 & 19
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment.	24
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	20-22
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	N/A
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies.	N/A
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	14
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	13

Section/topic	#	Checklist item	Reported on page #
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	14
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	N/A

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**

7.2. Appendix- Chapter 3

7.2.1. Comparison of baseline details of participants with and without 6-month follow-up data.

SD-Standard deviation, BMI-Body Mass Index, EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, IL-Interleukin, TNF-Tumour necrosis factor, ICAM-intercellular adhesion molecule, VCAM-Vascular cell adhesion molecule, BPRS- Brief Psychiatric Rating Scale, SANS-Scale for the Assessment of Negative Symptoms, SOFAS- Social and Occupational Functioning Assessment Scale, SD-standard deviation.

Variable names	Samples with follow up measures	Samples without follow up measures	P value
N	146	122	
Gender			
Male, n (%)	57 (39 %)	61 (50 %)	.071
Female, n (%)	89 (61 %)	61 (50 %)	
Age in years (mean ± SD)	18.2 ± 4.0	19.8 ± 4.7	.004
BMI in kg/m^2 (mean ± SD)	24.4 ± 6	23.4 ± 4.8	.163
EPA (%), (mean ± SD)	0.98 ±0.34	0.96 ± 0.34	.709
Erythrocyte DHA (%), (mean ± SD)	6.3 ± 1.3	6.6 ± 1.9	.085
Omega-3 Index (%), (mean ± SD)	7.3 ± 1.5	7.6 ± 2.1	.136
IL-6 (pg/mL), (mean ± SD)	1.04 ± 2.9	0.64 ± 0.46	.137
IL-8 (pg/mL), (mean ± SD)	4.66 ± 4.45	4.11 ± 2.00	.202
IL-10 (pg/mL), (mean ± SD)	0.35 ± 0.21	0.36 ± 0.33	.635
IL-12p40 (pg/mL), (mean ± SD)	169.87 ± 80.29	156.99 ± 62.53	.150
IL-15 (pg/mL), (mean ± SD)	2.94 ± 0.69	2.88 ± 0.69	.473
TNF-α (pg/mL), (mean ± SD)	2.39 ± 0.73	2.19 ± 0.60	.014
ICAM-1 (pg/mL), (mean ± SD)	516922.24 ± 162657.39	452387.20 ± 168276.49	.002
VCAM-1 (pg/mL), (mean ± SD)	510857.34 ± 139979.34	478873.71 ± 159163.10	.081
BPRS-Psychotic (mean ± SD)	8.6 ± 2.78	7.81 ± 2.06	.023
SANS (mean ± SD)	19.68 ± 13.48	19.39 ± 12.30	.042
SOFAS (mean ± SD)	54.02 ± 10.86	53.55 ± 13.80	.757

7.3. Appendix- Chapter 4

7.3.1. list of 187 predictors

Sex	log2_B_O147912	log2_B_P01859	log2_B_P51884
Smoking_Status	log2_B_O95445	log2_B_P01860	log2_B_P02753
log_age	log2_B_P25311	log2_B_P01861	log2_B_O75460
log_BMI	log2_B_P00736	log2_B_P01871	log2_B_Q08380
B_bprst	log2_B_P09871	log2_B_P01834	log2_B_P07225
B_sanst	log2_B_P06681	log2_B_P01615	log2_B_P35542
B_ymrst	log2_B_P01024	log2_B_P0DOY3	log2_B_P51451
B_madrst	log2_B_P0C0L5	log2_B_B9A064	log2_B_P07360
B_sofas	log2_B_P01031	log2_B_P19827	log2_B_Q14520
B_gf_s	log2_B_P13671	log2_B_P19823	log2_B_A0A0B4J1V2
B_gf_r	log2_B_P07357	log2_B_Q14624	log2_B_Q06033
logIL12p40_bl_nooutliersCV20	log2_B_P07358	log2_B_P03952	log2_B_P0C0L4
logIL15_bl_nooutliersCV20	log2_B_P00751	log2_B_P01042	log2_B_P02746
logIFNy_bl_nooutliersCV20	log2_B_P08603	log2_B_P02750	log2_B_O75636
logIL6_bl_nooutliersCV20	log2_B_P05156	log2_B_P02763	log2_B_P02748
logIL8_bl_nooutliersCV20	log2_B_P10909	log2_B_P19652	log2_B_Q9Y490
logIL10_bl_nooutliersCV20	log2_B_P00450	log2_B_P00747	log2_B_P06702
logTNFa_bl_nooutliersCV20	log2_B_P00748	log2_B_P27169	log2_B_P01764
logCRP_bl_nooutliersCV20	log2_B_P00488	log2_B_P01009	log2_B_P23142
logsVCAM1_bl_nooutliersCV20	log2_B_P00734	log2_B_P01011	log2_B_P06312
logsICAM1_bl_nooutliersCV20	log2_B_P02671	log2_B_P29622	log2_B_Q96PD5
log2_B_P04217	log2_B_P02675	log2_B_P08185	log2_B_Q92820
log2_B_P01023	log2_B_P02679	log2_B_P05543	log2_B_P02749
log2_B_P43652	log2_B_Q4L180	log2_B_P01008	log2_B_P01619
log2_B_P01019	log2_B_P0275114	log2_B_P05546	log2_B_P01782
log2_B_P02765	log2_B_P02774	log2_B_P36955	log2_B_Q96QR1
log2_B_P02768	log2_B_P06396	log2_B_P08697	log2_B_Q15582
log2_B_P02760	log2_B_P69905	log2_B_P05155	log2_B_P08571
log2_B_P02743	log2_B_P68871	log2_B_P02787	log2_B_P18428
log2_B_P02647	log2_B_P00738	log2_B_P02766	log2_B_Q04756
log2_B_P02652	log2_B_P00739	log2_B_P04004	log2_B_Q15848
log2_B_P06727	log2_B_P02790	log2_B_P60709	log2_B_P01591

log2_B_P04114	log2_B_P04196	log2_B_P02654	log2_B_P31944
log2_B_P02655	log2_B_P35858	log2_B_P05090	log2_B_P01880
log2_B_P02656	log2_B_P01876	log2_B_P10643	log2_B_P01817
log2_B_P02649	log2_B_P01857	log2_B_P15169	log2_B_Q16610
log2_B_P80748	log2_B_A0A0B4J1U3	log2_B_P43251	log2_B_P00742
log2_B_P01614	log2_B_P02747	log2_B_P26927	log2_B_A0A0C4DH38
log2_B_Q6EMK4	log2_B_A0A0B4J1V0	log2_B_Q96KN2	log2_B_P01780
log2_B_P02775	log2_B_P04003	log2_B_P12259	log2_B_A0A0C4DH33
log2_B_A0A0B4J1X5	log2_B_P10720	log2_B_P231424	log2_B_Q9NZP8
log2_B_P20742	log2_B_P22792	log2_B_Q9UK55	ARA_t1
log2_B_P04406	log2_B_A0A0C4DH31	log2_B_P0DJI8	Omega_3_index_t1
log2_B_P68133	log2_B_P00740	Total_Omega_3_t1	Omega_6_3_ratio_t1
log2_B_A0A0B4J1Y9	log2_B_Q9H4B7	DPA_t1	logALA_t1
log2_B_P21333	log2_B_P16070	DHA_t1	logEPA_t1
log2_B_P05160	log2_B_P35579	Total_Omega_6_t1	LA_t1

7.3.2. Mean and standard deviation of biological predictors at baseline

Biological Measures	
C Reactive Protein in pg/ml (Mean±SD)	9223.4 ± 10146861.6
Iterferon γ in pg/ml (Mean±SD)	6.0 ± 18.3
Inter leukin-10 in pg/ml (Mean±SD)	0.4 ± 0.2
Inter leukin-12p40 in pg/ml (Mean±SD)	166.2 ± 75.3
Inter leukin-6 in pg/ml (Mean±SD)	1.0 ± 2.8
Inter leukin-8 in pg/ml (Mean±SD)	4.5 ± 4.3
Intercellular adhasion molecule-1 in pg/ml (Mean±SD)	9223.4 ± 9223.4
Vascular cell adhesion molecue-1 in pg/ml (Mean±SD)	9223.4 ± 9223.4
Tumor necrosis factor-α in pg/ml (Mean±SD)	2.3 ± 0.7
Eicosapentaenoic acid (20:5) in %	1.0 ± 0.3
Docosahexaenoic acid (22:5) in %	6.3 ± 1.3
Total Omega-3 fatty acids in %	11.9 ± 1.7
Palmitic acid (16:0) in %	32.6 ± 2.4

Margeric acid (17:0) in %	1.3 ± 0.2
Stearic acid (18:0) in %	12.9 ± 1.5
Oleic acid (18:1) in %	16.6 ± 2.1
Linoleic acid (18:2) in %	12.0 ± 2.1
Dihomo-γ-linolenic acid (20:3) in %	0.8 ± 0.3
Arachidonic acid (20:4) in %	8.4 ± 2.6
Eicosapentaenoic acid (20:5) in %	1.0 ± 0.3
Docosatetraenoic acid (22:4) in %	0.4 ± 0.3
Docosahexaenoic acid (22:5) in %	6.3 ± 1.3
Cervonic acid (22:6) in %	2.3 ± 1.0
Lignoceric acid (24:0) in %	4.5 ± 2.2
Nervonic acid (24:1) in %	6.4 ± 3.3
P04217, LFQ (Mean ± SD)	926379.2 ± 211751.5
P01023, LFQ (Mean ± SD)	5180773.4 ± 1556636.7
P43652, LFQ (Mean ± SD)	69980.1 ± 27880.8
P01019, LFQ (Mean ± SD)	422425.4 ± 210805.5
P02765, LFQ (Mean ± SD)	3290935.6 ± 1367534.3
P02768, LFQ (Mean ± SD)	50363651.9 ± 9957066.2
P02760, LFQ (Mean ± SD)	484019.4 ± 300487.7
P02743, LFQ (Mean ± SD)	240745.6 ± 105269.4
P02647, LFQ (Mean ± SD)	5971527.8 ± 1420031.2
P02652, LFQ (Mean ± SD)	501833.8 ± 694358.5
P06727, LFQ (Mean ± SD)	874553.5 ± 307956.5
P04114, LFQ (Mean ± SD)	1200241.3 ± 333413.4
P02655, LFQ (Mean ± SD)	505803.3 ± 237060.2
P02656, LFQ (Mean ± SD)	967881.2 ± 550408.9
P02649, LFQ (Mean ± SD)	111951.5 ± 44456.8
O14791, LFQ (Mean ± SD)	246066.4 ± 108181.4
O95445, LFQ (Mean ± SD)	272500.1 ± 126474.8
P25311, LFQ (Mean ± SD)	286765.8 ± 129124.0
P00736, LFQ (Mean ± SD)	138054.1 ± 48333.9
P09871, LFQ (Mean ± SD)	133487.7 ± 43612.9
P06681, LFQ (Mean ± SD)	86331.4 ± 20472.8
P01024, LFQ (Mean ± SD)	3126393.0 ± 736850.6
P0C0L5, LFQ (Mean ± SD)	1674898.2 ± 473435.8
P01031, LFQ (Mean ± SD)	611807.3 ± 159410.2
P13671, LFQ (Mean ± SD)	229613.6 ± 70526.4
P07357, LFQ (Mean ± SD)	312692.5 ± 92151.8
P07358, LFQ (Mean ± SD)	92111.0 ± 30765.1

P00751, LFQ (Mean ± SD)	698053.2 ± 203160.8
P08603, LFQ (Mean ± SD)	828811.1 ± 179926.5
P05156, LFQ (Mean ± SD)	156576.4 ± 53584.8
P10909, LFQ (Mean ± SD)	790484.4 ± 215506.2
P00450, LFQ (Mean ± SD)	2260714.4 ± 593770.3
P00748, LFQ (Mean ± SD)	255915.9 ± 104593.8
P00488, LFQ (Mean ± SD)	99887.5 ± 127954.9
P00734, LFQ (Mean ± SD)	218670.1 ± 106638.9
P02671, LFQ (Mean ± SD)	7868638.0 ± 4554649.4
P02675, LFQ (Mean ± SD)	6377647.5 ± 3641791.0
P02679, LFQ (Mean ± SD)	5895613.3 ± 3165533.7
Q4L180, LFQ (Mean ± SD)	107156.0 ± 52011.2
P027511, LFQ (Mean ± SD)	757063.8 ± 1125560.6
P02774, LFQ (Mean ± SD)	759986.5 ± 346360.4
P06396, LFQ (Mean ± SD)	612246.4 ± 194166.0
P69905, LFQ (Mean ± SD)	2996032.7 ± 1534224.3
P68871, LFQ (Mean ± SD)	2346903.7 ± 1182810.7
P00738, LFQ (Mean ± SD)	4505029.4 ± 1791915.5
P00739, LFQ (Mean ± SD)	364916.9 ± 235317.1
P02790, LFQ (Mean ± SD)	2140823.4 ± 553552.2
P04196, LFQ (Mean ± SD)	351694.1 ± 137271.4
P35858, LFQ (Mean ± SD)	207829.0 ± 56511.6
P01876, LFQ (Mean ± SD)	609347.8 ± 437131.3
P01857, LFQ (Mean ± SD)	25562246.8 ± 9716938.8
P01859, LFQ (Mean ± SD)	13778848.1 ± 7978493.8
P01860, LFQ (Mean ± SD)	8118640.5 ± 3032653.0
P01861, LFQ (Mean ± SD)	452901.9 ± 397949.0
P01871, LFQ (Mean ± SD)	2588777.5 ± 1387710.2
P01834, LFQ (Mean ± SD)	12143191.1 ± 3168508.4
P01615, LFQ (Mean ± SD)	440919.1 ± 260914.2
P0DOY3, LFQ (Mean ± SD)	1111800.3 ± 1744134.9
B9A064, LFQ (Mean ± SD)	613847.3 ± 290571.4
P19827, LFQ (Mean ± SD)	646335.3 ± 258491.7
P19823, LFQ (Mean ± SD)	1278065.5 ± 284617.1
Q14624, LFQ (Mean ± SD)	827661.2 ± 170778.7
P03952, LFQ (Mean ± SD)	112914.3 ± 34709.4
P01042, LFQ (Mean ± SD)	1365858.1 ± 272388.5
P02750, LFQ (Mean ± SD)	502177.8 ± 211514.2
P02763, LFQ (Mean ± SD)	7349527.2 ± 2174441.9

P19652, LFQ (Mean ± SD)	887286.0 ± 258657.5
P00747, LFQ (Mean ± SD)	774582.7 ± 169004.5
P27169, LFQ (Mean ± SD)	627053.2 ± 210113.2
P01009, LFQ (Mean ± SD)	9887256.3 ± 1951893.3
P01011, LFQ (Mean ± SD)	1019439.2 ± 259491.4
P29622, LFQ (Mean ± SD)	82565.4 ± 32204.3
P08185, LFQ (Mean ± SD)	369387.5 ± 125087.6
P05543, LFQ (Mean ± SD)	58999.0 ± 47301.5
P01008, LFQ (Mean ± SD)	1076794.7 ± 191550.8
P05546, LFQ (Mean ± SD)	228000.2 ± 82784.6
P36955, LFQ (Mean ± SD)	292096.0 ± 91292.7
P08697, LFQ (Mean ± SD)	1205121.2 ± 256794.4
P05155, LFQ (Mean ± SD)	1859243.5 ± 834119.1
P02787, LFQ (Mean ± SD)	4636338.6 ± 869870.0
P02766, LFQ (Mean ± SD)	4429880.1 ± 1105753.7
P04004, LFQ (Mean ± SD)	552299.9 ± 116630.4
P15636, LFQ (Mean ± SD)	2803067.6 ± 2575238.4
P60709, LFQ (Mean ± SD)	355878.7 ± 442117.2
P02654, LFQ (Mean ± SD)	95730.0 ± 91909.7
P05090, LFQ (Mean ± SD)	146637.1 ± 79555.4
P10643, LFQ (Mean ± SD)	226166.3 ± 128551.1
P15169, LFQ (Mean ± SD)	64560.3 ± 31863.4
P51884, LFQ (Mean ± SD)	86791.9 ± 39340.8
P02753, LFQ (Mean ± SD)	121092.5 ± 65430.3
O75460, LFQ (Mean ± SD)	487720.3 ± 241985.1
Q08380, LFQ (Mean ± SD)	153232.9 ± 69192.8
P07225, LFQ (Mean ± SD)	133708.0 ± 66554.4
P35542, LFQ (Mean ± SD)	152378.2 ± 96834.2
P51451, LFQ (Mean ± SD)	1246495.6 ± 719864.7
P07360, LFQ (Mean ± SD)	201121.3 ± 108212.7
Q14520, LFQ (Mean ± SD)	49573.6 ± 15528.6
A0A0B4J1V2, LFQ (Mean ± SD)	92695.8 ± 53465.4
Q06033, LFQ (Mean ± SD)	69707.0 ± 36693.6
P0C0L4, LFQ (Mean ± SD)	259380.0 ± 213745.6
P02746, LFQ (Mean ± SD)	753308.6 ± 1347829.9
O75636, LFQ (Mean ± SD)	46782.9 ± 22892.4
P02748, LFQ (Mean ± SD)	112312.2 ± 142523.7
Q9Y490, LFQ (Mean ± SD)	21745.6 ± 11162.7
P06702, LFQ (Mean ± SD)	88699.3 ± 644187.6

P01764, LFQ (Mean ± SD)	120193.9 ± 118078.1
P23142, LFQ (Mean ± SD)	28387.2 ± 20581.2
P06312, LFQ (Mean ± SD)	112357.3 ± 165125.5
Q96PD5, LFQ (Mean ± SD)	44333.3 ± 27338.2
Q92820, LFQ (Mean ± SD)	105851.0 ± 76675.7
P02749, LFQ (Mean ± SD)	97265.9 ± 91479.2
P01619, LFQ (Mean ± SD)	264531.3 ± 304933.2
P01782, LFQ (Mean ± SD)	68173.7 ± 74239.0
Q96QR1, LFQ (Mean ± SD)	41220.2 ± 32644.5
Q15582, LFQ (Mean ± SD)	20837.2 ± 14092.2
P08571, LFQ (Mean ± SD)	17145.2 ± 11300.2
P18428, LFQ (Mean ± SD)	17218.5 ± 16056.4
Q04756, LFQ (Mean ± SD)	47221.5 ± 35456.9
Q15848, LFQ (Mean ± SD)	33871.3 ± 23503.3
P01591, LFQ (Mean ± SD)	106243.8 ± 133385.4
P31944, LFQ (Mean ± SD)	50632.3 ± 71177.8
P01880, LFQ (Mean ± SD)	93563.3 ± 135165.9
P02676, LFQ (Mean ± SD)	77939.0 ± 120292.0
P01817, LFQ (Mean ± SD)	83955.1 ± 71659.8
Q16610, LFQ (Mean ± SD)	41353.9 ± 84882.6
P00742, LFQ (Mean ± SD)	28964.0 ± 24339.7
P80748, LFQ (Mean ± SD)	153427.4 ± 156940.0
P01614, LFQ (Mean ± SD)	53768.5 ± 50380.3
Q6EMK4, LFQ (Mean ± SD)	16083.3 ± 11734.5
P02775, LFQ (Mean ± SD)	66113.3 ± 63716.3
A0A0B4J1X5, LFQ (Mean ± SD)	19783.8 ± 24511.9
P20742, LFQ (Mean ± SD)	193488.8 ± 507297.4
A0A0B4J1U3, LFQ (Mean ± SD)	48484.2 ± 50882.7
P02747, LFQ (Mean ± SD)	36434.6 ± 30286.5
A0A0B4J1V0, LFQ (Mean ± SD)	40782.7 ± 34775.1
P04003, LFQ (Mean ± SD)	42664.8 ± 41170.1
P10720, LFQ (Mean ± SD)	32531.7 ± 34941.1
P22792, LFQ (Mean ± SD)	42792.0 ± 60629.0
P43251, LFQ (Mean ± SD)	14252.0 ± 14373.0
P26927, LFQ (Mean ± SD)	9320.5 ± 7766.1
Q96KN2, LFQ (Mean ± SD)	16516.7 ± 14285.7
P12259, LFQ (Mean ± SD)	50112.7 ± 42420.0
P231424, LFQ (Mean ± SD)	23006.2 ± 18314.4
Q9UK55, LFQ (Mean ± SD)	13918.7 ± 16722.8

A0A0C4DH38, LFQ (Mean ± SD)	34472.4 ± 39744.1
P01780, LFQ (Mean ± SD)	151323.9 ± 134699.9
A0A0C4DH33, LFQ (Mean ± SD)	14261.1 ± 27913.7
Q9NZP8, LFQ (Mean ± SD)	35350.7 ± 43068.8
P04406, LFQ (Mean ± SD)	14004.1 ± 24652.0
P68133, LFQ (Mean ± SD)	70577.0 ± 98559.6
A0A0B4J1Y9, LFQ (Mean ± SD)	13187.2 ± 15818.0
P21333, LFQ (Mean ± SD)	27941.8 ± 65925.6
P05160, LFQ (Mean ± SD)	27430.3 ± 58784.5
A0A0C4DH31, LFQ (Mean ± SD)	34929.5 ± 36954.7
P00740, LFQ (Mean ± SD)	10425.1 ± 16104.1
Q9H4B7, LFQ (Mean ± SD)	2758543.1 ± 2776277.6
P16070, LFQ (Mean ± SD)	5348.0 ± 4828.8
P35579, LFQ (Mean ± SD)	25449.8 ± 50149.2
P0DJI8, LFQ (Mean ± SD)	32355.5 ± 86921.2

7.3.3: Details of percentage of missing values

Variable	n missing	% missing
M12_sofas	0	0.0
male	0	0.0
Smoking_Status	0	0.0
log_age	0	0.0
log_BMI	9	6.9
B_bprst	0	0.0
B_sanst	0	0.0
B_ymrst	0	0.0
B_madrst	0	0.0
B_sofas	0	0.0
B_gf_s	0	0.0
B_gf_r	0	0.0
logIL12p40_bl_nooutliersCV20	3	2.3
logIL15_bl_nooutliersCV20	3	2.3
logIFNy_bl_nooutliersCV20	3	2.3
logIL6_bl_nooutliersCV20	2	1.5
logIL8_bl_nooutliersCV20	2	1.5
logIL10_bl_nooutliersCV20	0	0.0
logTNFa_bl_nooutliersCV20	3	2.3
logCRP_bl_nooutliersCV20	0	0.0
logsVCAM1_bl_nooutliersCV20	1	0.8
logsICAM1_bl_nooutliersCV20	2	1.5
Total_Omega_3_t1	2	1.5
DPA_t1	2	1.5
DHA_t1	2	1.5
Total_Omega_6_t1	2	1.5
LA_t1	2	1.5
ARA_t1	2	1.5
Omega_3_index_t1	2	1.5
Omega_6_3_ratio_t1	2	1.5
logALA_t1	5	3.8
logEPA_t1	2	1.5

7.4. Appendix- Chapter 5

7.4.1. Results showing the list of plasma proteins associated significantly with change in omeha-3 PUFAs (adjusted for age, sex and baseline total omega-3 levels).

The table shows the results of linear regression models between change in total omega-3 PUFAs and plasma proteins at follow-up. The models were adjusted for age, sex and baseline total omega-3 levels.

Protein Names	Coef.	P value	[95% Conf.	Interval]
Alpha-1-antitrypsin	-1.05	0.01	-1.82	-0.27
Alpha-1B-glycoprotein	-1.06	0.01	-1.83	-0.3
Apolipoprotein C-I	-0.88	0.02	-1.64	-0.12
Apolipoprotein C-III	1.36	0	0.61	2.12
Apolipoprotein D	0.9	0.03	0.1	1.7
Apolipoprotein E	1.03	0.01	0.28	1.77
Apolipoprotein L1	**-1.45**	**0**	**-2.18**	**-0.72**
Caspase-14	-0.89	0.02	-1.65	-0.12
Coagulation factor V	0.9	0.02	0.14	1.66
Complement C1q subcomponent subunit B	1.18	0	0.44	1.91
Complement C5	-1.13	0	-1.88	-0.37
Complement component C7	0.83	0.03	0.07	1.6
Complement factor B	-0.87	0.02	-1.62	-0.12
Complement factor I	-0.78	0.04	-1.54	-0.03
Filamin A-interacting protein 1-like	-0.96	0.01	-1.73	-0.2
Galectin-3-binding protein	-1.28	0	-2.05	-0.52
Haptoglobin	-0.97	0.01	-1.72	-0.22
Immunoglobulin heavy constant gamma 2	0.93	0.02	0.17	1.7
Immunoglobulin heavy constant gamma 4	0.98	0.01	0.23	1.72
Immunoglobulin heavy variable 1-18	0.82	0.03	0.07	1.58
Immunoglobulin heavy variable 3-7	-0.8	0.04	-1.55	-0.05
Immunoglobulin kappa variable 3-20	1.05	0.01	0.3	1.81
Protein S100-A9	-1.03	0.01	-1.78	-0.29
Talin-1	-0.92	0.02	-1.68	-0.17

7.4.2. Results of linear regression model showing the list of plasma proteins associated significantly with change in omeha-6 PUFAs (adjusted for age, sex and baseline omega-6 levels).

The table shows the results of linear regression models between change in omega-6 PUFAs and plasma proteins at follow-up. The models were adjusted for age, sex and baseline omega-6 levels

Protein names	Coef.	P value	[95% Conf.	Interval]
Apolipoprotein C-I	0.8	0.02	0.12	1.48
Apolipoprotein L1	**1.05**	**0.00**	**0.39**	**1.72**
Hemoglobin subunit beta	0.79	0.03	0.08	1.49
Immunoglobulin lambda variable 1-36	-0.82	0.02	-1.5	-0.14
Protein S100-A9	0.78	0.02	0.1	1.46
Vitamin D-binding protein	-0.84	0.02	-1.53	-0.15

7.4.3. Pathways significantly associated with 6-month change in omega-6 PUFAs

Pathway name	#Entities found	#Interactors found	#Reactions found	Entities pValue	Entities FDR
Scavenging of heme from plasma	2	0	5	0.00	0.06
Binding and Uptake of Ligands by Scavenger Receptors	2	0	5	0.00	0.06
Inhibition of nitric oxide production	0	1	1	0.00	0.06
VLDL clearance	1	0	3	0.00	0.06
Metal sequestration by antimicrobial proteins	1	0	2	0.00	0.06
Erythrocytes take up oxygen and release carbon dioxide	1	0	1	0.00	0.06
Erythrocytes take up carbon dioxide and release oxygen	1	0	2	0.01	0.06
O2/CO2 exchange in erythrocytes	1	0	3	0.01	0.06
IRAK4 deficiency (TLR2/4)	1	0	2	0.01	0.09
Neutrophil degranulation	2	0	3	0.01	0.09
MyD88 deficiency (TLR2/4)	1	0	2	0.01	0.10
Transport of small molecules	2	1	11	0.01	0.10
Regulation of TLR by endogenous ligand	1	0	1	0.01	0.10
VLDL assembly	1	0	1	0.02	0.10
Vesicle-mediated transport	2	1	9	0.02	0.12
Plasma lipoprotein clearance	1	0	3	0.03	0.12

Pathway name	#Entities found	#Interactors found	#Reactions found	Entities pValue	Entities FDR
Thrombin signalling through proteinase activated receptors (PARs)	0	1	2	0.03	0.12
Autophagy	1	1	13	0.03	0.12
Late endosomal microautophagy	1	0	3	0.03	0.12
Plasma lipoprotein assembly	1	0	1	0.03	0.12
Post-translational protein phosphorylation	1	0	1	0.03	0.12
Mitochondrial Fatty Acid Beta-Oxidation	0	1	1	0.04	0.12
Defective CFTR causes cystic fibrosis	0	1	2	0.04	0.12
Antimicrobial peptides	1	0	2	0.04	0.12
ABC transporter disorders	0	1	2	0.04	0.12
WNT5A-dependent internalization of FZD4	0	1	2	0.05	0.12
PINK1-PRKN Mediated Mitophagy	0	1	2	0.05	0.12

7.4.4. Results of mediation analysis adjusted for baseline total omega-3 PUFAs in addition to age, sex and baseline protein levels.

The table shows the results of mediation analysis using change in total omega-3 PUFAs, plasma proteins and clinical outcomes as exposure, mediator and outcome variables, respectively. The model is adjusted for age, sex, baseline total omega-3 levels and baseline total omega-3 PUFA levels.CI- confidence interval, PSS- Positive Symptom Severity score, SOFAS- Social and Occupational Functional Assessment scale, BACS- Brief Assessment of Cognitive Function & *significant findings

Outcome	Mediator	Mediation effect	Direct effect	Total effect
SOFAS	C5	0.23 * (0.03 to 0.50)	-0.06 (-0.68 to 0.56)	0.17 (-0.44 to 0.78)
	APOD	0.09 (-0.04 to 0.25)	0.12 (-0.50 to 0.74)	0.21 (-0.40 to 0.82)
BACS	CFB	0.10 (-0.02 to 0.31)	0.47 (-0.14 to 1.07)	0.57 (-0.04 to 1.17)
	C1QB	0.29 * (0.06 to 0.63)	0.24 (-0.35 to 0.83)	0.53 (-0.05 to 1.11)
	Factor V	0.16 * (0.02 to 0.35)	0.42 (-0.17 to 1.00)	0.58 (-0.04 to 1.17)
	APOE	0.20 (-0.00 to 0.48)	0.37 (-0.22 to 0.97)	0.57 (-0.01 to 1.15)

Outcome	Mediator	Mediation effect	Direct effect	Total effect
PSS	APOC3	0.18 (-0.02 to 0.43)	0.40 (-0.21 to 1.00)	0.58 (-0.001 to 1.16)
	APOD	0.10 (-0.01 to 0.26)	0.48 (-0.10 to 1.07)	0.59 (-0.001 to 1.17)
	C5	0.23 * (-0.49 to -0.36)	-0.08 (-0.74 to 0.58)	-0.31 (-0.96 to 0.34)
	S100A9	0.18 * (-0.44 to -0.01)	-0.11 (-0.77 to 0.55)	-0.29 (-0.93 to 0.35)
	IGHG4	-0.19 (-0.49 to 0.04)	-0.12 (-0.72 to 0.77)	-0.30 (-0.94 to0.34)

7.4.5. Supplementary methods

Gas chromatography-based quantification of PUFA levels

Fasting blood samples were collected at baseline and 6-month follow-up. The molecular percentage of the total sum of the omega-3 and omega-6 fatty acid series in erythrocyte membrane rafts were measured based on the phosphatidyl-ethanolamine fraction using gas chromatography[95]. Total omega-3 PUFAs comprise alpha linolenic acid (18:3), eicosapentaenoic acid (20:5), docosapentaenoic acid (22:5) and docosahexaenoic acid (22:6). Total omega-6 PUFAs include linoleic acid (18:2), gamma-linoleic acid (18:3), eicosadienoic acid (20:2), dihomo gamma-linoleic acid (20:3), arachidonic acid (20:4) and adrenic acid (22:4).

Mass spectrometry based proteomic measures

Plasma samples of baseline and follow-up time points were processed according to the manufacturer's instructions (PreOmics iST kit, no.iST 96x). Briefly, 4 µl of individual samples were solubilized in 50 µL of "Lyse" buffer (containing Tris-HCl, sodium deoxycholate (SDC), 0.1% sodium dodecyl sulfate (SDS), tris (2-carboxyethyl) phosphine (TCEP), and 2-chloroacetamide and heated to 95 °C for 10 min. 50 µL of the resulting denatured, reduced, and alkylated solution was transferred to the reaction tube. Enzyme (LysC and trypsin) was added, and samples were hydrolysed at 37°C for 1.5 hours. The resulting peptide mixture was washed and eluted as per the manufacturer's instructions. The eluted peptides were vacuum-dried and dissolved in 100 µl of LC Load buffer. The reconstituted digested peptide

mixture [200 ng/ µl] was then eluted using Evotips and injected using Evosep One (Evosep, Odense, Denmark [96]. The digested samples were run on a Bruker timeTof Pro mass spectrometer connected to a Evosep One liquid chromatography system. The mass spectrometry was operated in positive ion mode with a capillary voltage of 1500 V, dry gas flow of 3 l/min and a dry temperature of 180^{o}C. Trapped ions were selected for ms/ms using parallel accumulation serial fragmentation (PASEF). A scan range of (100-1700 m/z) was performed at a rate of 10 PASEF MS/MS frames to 1 MS scan with a cycle time of 1.15s [97,98]. The MS raw files were then processed with MaxQuant [99] version 1.6.17.0 as described in [98] and the peptide data were further annotated and interpreted using the Perseus platform (V 1.6.7, www.maxquant.net/perseus/) [100]. FDR was set at 0.01 to global protein identification level. Proteins that were identified in less than 70% of the total samples were not taken forward for analysis. Log_2 transformed values of LFQ intensities were used for statistical analysis. Missing values of mass spectrometry based proteomic data (corresponding to values below the level of detection) were imputed with minimum values.