

Investigating the role of the endocannabinoid system and gut-microbiome in psychotic and severe mental illness: a focus on negative symptoms

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Abstract

Negative symptoms, such as anhedonia and amotivation, represent unmet therapeutic needs and key determinants of functional loss in severe mental illness. Albeit traditionally considered a unique feature of schizophrenia, anhedonia and amotivation manifest outside the psychotic spectrum, where they are equally debilitating and difficult to treat. A number of clinical trials using newly developed compounds or re-repurposing existing drugs have tried to treat negative symptoms without success. There is therefore urgent need of clarifying the mechanisms underlying negative symptoms, before engaging in further trials. The aim of my DPhil was to investigate the relevance for negative symptoms of two recently discovered biological systems, the gut-microbiome and the endocannabinoid systems. The rationale was based on emerging evidence showing an independent contribution of these two systems to the pathophysiology of severe mental illness and their proven interplay in other fields of medicine. I initially explored the independent contribution of the endocannabinoid system and the gut microbiome to schizophrenia and severe mental illness through two systematic reviews and meta-analyses. The first meta-analysis aimed at investigating disturbance of the endocannabinoid system in psychotic illness. Pooled results from 18 studies including 442 patients and 590 controls showed that anandamide, the main agonist of the endocannabinoid system, was increased in blood and cerebrospinal fluid of patients with psychotic illness, at any stage (including the prodrome) and independent of medication status. Across the included studies the increase in anandamide was inversely related to the severity of negative symptoms, suggesting endocannabinoids are protective towards illness mechanisms and severity of clinical features. The second meta-analysis aimed at investigating blood biomarkers of reduced microbial diversity (gut dysbiosis) in severe mental illnesses (schizophrenia, depression, and bipolar disorder) and chronic fatigue. Pooled results from 33 studies including 2,761 patients and 1,847 controls showed that blood biomarkers of gut dysbiosis were increased and positively associated with severity of negative symptoms in patients vs controls, independent of medication status and across diagnostic boundaries. I then brought the gut-microbiome and the endocannabinoid system together and explored the relevance of their interplay for negative symptoms in a general population cohort (TwinsUK). Data from 786 individuals showed that the endocannabinoid system mediated the association between gut dysbiosis and the severity of negative symptoms. In particular, gut dysbiosis was associated with increased faecal excretion of endocannabinoids (protective for mental health), which in turn was associated with more severe symptoms. These findings advocate for the existence of a gut-microbiome-endocannabinoid axis, relevant for negative symptoms and a putative novel target for intervention.

I therefore explored if existing molecules targeting the first node of this axis, the gut-microbiome, could palliate negative symptoms in severe mental illness using two systematic reviews and meta-analyses. The first meta-analysis investigated the efficacy of already known gut-microbiome-targeted therapeutics (antibiotics, antimicrobials, pre and probiotics) for the treatment of negative symptoms in psychotic illness. Pooled results from 28 eligible randomised controlled trials showed that none of the already known gut-microbiome based therapeutics were effective for treating negative symptoms of psychotic illness. Results were mainly led by antibiotics and antimicrobials (25 trials), with paucity of evidence on pre and probiotics. The second meta-analysis investigated the efficacy of metformin, which novel evidence showed to have a specific action on the gut-microbiome (i.e., increase in the relative abundance of butyrate-producing bacteria, beneficial for the host's mental health). Analyses on the TwinsUK cohort showed that the relative abundance of these bacteria is associated with fecal levels of endocannabinoid metabolites. Pooled results from 5 eligible randomised controlled trials showed that metformin has pro-cognitive effects, with preliminary evidence showing efficacy on negative symptoms. Altogether these findings suggest that aspecific approaches targeting the gut-microbiome are not effective for the treatment of negative symptoms, while more defined approaches targeting a specific biological axis might translate into clinically meaningful results.

In conclusion, findings from my DPhil advocate for the existence of a gut microbiome-endocannabinoid axis, which might be relevant for negative symptoms across severe mental illness and beyond, such as in chronic fatigue and in general population. Future studies should validate findings in a well-powered clinical sample of patients with severe mental illness and test the efficacy of compounds targeting this axis.

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Declaration

- The following parts of this thesis are available in publication

Chapter 2

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Chapter 5

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Chapter 5

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Chapter 1. Introduction

1.1 Overview

Negative symptoms are an unmet therapeutic need in severe mental illness, where they represent a key predictor of functional loss for patients. Research aimed at understanding the biological underpinnings of negative symptoms will facilitate the development of treatment breakthroughs in an area of unmet need.

During my DPhil, I wanted to shed light on the relationship between two recently discovered biological systems (the gut microbiome and the endocannabinoid system) and negative symptoms.

Given its modifiable nature and putative link with brain development and functionality, the gut-microbiome has been receiving mounting attention as a novel and safe target of intervention for a number of neurological and psychiatric disorders. However, while a multitude of gut-to-brain paths have been explored, there is no clear evidence to date as to whether and how perturbations of the gut microbiome can manifest with mental health features. Based on evidence from other medical fields, my hypothesis was that the endocannabinoid system would play a key role in the crosstalk between the gut-microbiome and negative symptoms.

In this thesis, I will present data aimed at addressing questions related to the biological underpinnings of negative symptoms (Chapter 1, 2, 3, and 4) and to the therapeutics implications of findings (Chapter 5).

In particular,

- In Chapter 1, I will provide a narrative review on negative symptoms, highlighting benefits and pitfalls of the current research approaches and key gaps in knowledge.
- In Chapter 2, I will present data from a systematic review and meta-analysis investigating abnormalities of the endocannabinoid system in psychotic illness and their association with severity of symptoms (including negative symptoms).
- In Chapter 3, I will present data from a systematic review and meta-analysis investigating proxy biomarkers of gut microbiome dysbiosis in patients with severe mental illness and chronic fatigue. A specific focus will be given to negative symptoms and their trans-diagnostic association with gut dysbiosis biomarkers.

- In Chapter 4, I will test the hypothesis that the association between gut dysbiosis and negative symptoms is mediated by the endocannabinoid system by using a multilevel mediation model in a general population cohort (TwinsUK).
- In Chapter 5, I will discuss implications of findings from previous chapters for therapeutics. I will present data from two systematic reviews and meta-analyses investigating the efficacy and tolerability of add-on gut microbiome targeted interventions for the treatment of negative symptoms.
- In Chapter 6, I will draw general conclusions and discuss future directions.

1.2 Negative symptoms – current challenges

1.2.1 Definition

The term “negative symptoms” refers to a “gap” in experience that patients typically display in the domains of volition, affectivity, enjoyment, sociality, and expressivity[1]. Based on clinicians’ descriptions of these common features, diagnostic manuals and international classification systems have agreed upon a list of five negative symptoms (avolition, blunted or flat affect, anhedonia, asociality, alogia)[2].

Table 1. Definition of negative symptoms: bifactorial and five-items structure

Bifactorial	Five-items	Definition
Experiential	<i>Avolition</i>	Reduction in the initiation of and persistence in activities
	<i>Anhedonia</i>	Impairments in the intensity of pleasure (anticipated and/or experienced during the act) from activities usually perceived as pleasurable. This might be associated with reduced engagement in activities
	<i>Asociality</i>	Reduced interaction with others and diminished will to form social bonds
Expressive	<i>Blunted or flat affect</i>	Decrease in the outward expression of emotion
	<i>Alogia</i>	

		Reduction in the quantity of speech and amount of spontaneous elaboration
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Experiential negative symptoms are listed in the DSM-V as descriptors for major depressive disorder and represent diagnostic criteria (or associated features) for bipolar disorder, post-traumatic stress disorder, social anxiety disorder, and neurocognitive disorder (including Alzheimer's disease, frontotemporal dementia, multiple sclerosis, Parkinson's disease). Otherwise healthy subjects, with no history of mental illness might also display negative symptoms.

Traditionally, negative symptoms were considered a unique feature of schizophrenia, a chronic and highly debilitating disorder, where they manifest in 60 to 90% of patients[2]. The typical course of schizophrenia has an insidious onset characterised by the predominance of negative symptoms (progressive social withdrawal and loss of drive for daily-life activities)[3]. This period ("prodrome") has a variable duration ranging from months to years[3]. During the prodrome, patients rarely present to clinical services[4, 5]. Presentation to services often occurs when the illness manifests with delusions and hallucinations ("positive symptoms"), which are considered by many the hallmark of the disorder[4, 5]. The onset of positive symptoms, which peaks around 15-25 years, is very distressing for patients and carers. However, positive symptoms respond very well to antipsychotics, the cornerstone of schizophrenia's treatment[3]. Contrarily, negative symptoms tend to become more stable throughout illness course, to persist beyond relapse and remissions of positive symptoms, and not respond to medications[1].

Early presentation, long-term consequences, and clinical challenges, have made research on these symptoms a priority.

Most scientific literature suggests that negative symptoms can be clustered into two categories: experiential (avolition, anhedonia, asociality) and expressive (blunted or flat affect, alogia)[6-8]. This bifactorial structure has been proven to be more clinically relevant than the five-items structure (**Table 1**), as experiential negative symptoms are greatly informative towards illness trajectories[9]. Experiential, but not expressive, negative symptoms, are associated with loss in functioning, poor response to treatment, and long-term detrimental outcomes in mental illness[10, 11]. The severity and persistence of negative symptoms in some patients (~20%) has led some authors to hypothesise the existence of a distinct form of schizophrenia ("deficit schizophrenia"), having a more severe illness course and specific underlying etiopathogenetic mechanisms[12].

1.2.2 Primary vs secondary negative symptoms

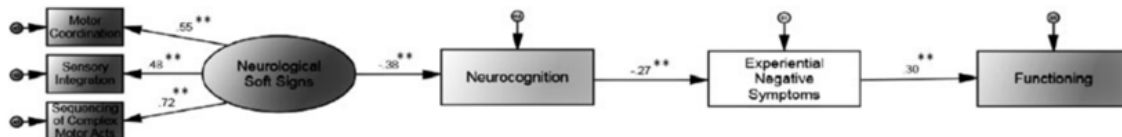
The specific and tight link with illness mechanisms laid the basis for the proposed dichotomy between “primary” and “secondary” negative symptoms, which often recurs in the scientific literature on the topic[13]. In 2005, the NIMH-MATRICES consensus statement on negative symptoms referred to primary negative symptoms as manifestations of processes tightly related to the pathophysiology of schizophrenia[14]. Secondary negative symptoms were defined as “secondary” to pathophysiological processes frequently associated with schizophrenia, but not related to core illness mechanisms, such as depression or movement disorders[14]. The primary vs secondary distinction had some clinical rationale because secondary, but not primary, symptoms were believed to improve with treatments targeting a known underlying conditions (e.g., antidepressants targeting depression thus resulting in improved anhedonia/amotivation, titration of antipsychotic aimed at ameliorating movement disorders) [13]. However, in recent years accumulating evidence has challenged this distinction[15, 16], as: 1) the “primary” nature of symptoms is misleading, as the etiopathogenesis of schizophrenia is unclear; and 2) conditions underlying secondary negative symptoms are not always easy to treat. To further challenge the clinical argument for a distinction between primary and secondary negative symptoms, there is now consistent evidence that negative symptoms manifest in non-psychotic illness, such as in depressive and neurocognitive disorders[15, 16], and in chronic fatigue[17]. Outside the psychosis spectrum, negative symptoms might be as difficult to treat as in schizophrenia (e.g., residual depressive symptoms, treatment-resistant depression, fatigue, etc.) and lead to similar detrimental outcomes[10, 11, 17]. **Figure 1** summarises the main findings from two studies showing how negative symptoms (in particular experiential negative symptoms) are associated with poor functional outcomes both in psychotic (**Fig.1B**) [18]and non-psychotic disorders (**Fig.1A**)[11]. These two studies report results of path analyses based on the same theoretical ground, i.e., early neurodevelopmental abnormalities triggering neurocognitive deficits, leading in turn to experiential negative symptoms and loss of functioning[11, 18].

The existence of common paths leading to the same psychopathology latent factor (in this case: experiential negative symptoms) across different diagnoses is the key assumption of large-scale initiatives such as the Research Domain Criteria (RDoC) and the Hierarchical Taxonomy of Psychopathology (HiTOP). These initiatives aim to address one of the main limitations of the current diagnostic approach in psychiatry, i.e., relying on cluster of symptoms (“diagnostic categories”) rather than on spectra of symptoms (“dimensional approach”)[19]. The latter approach, being more tailored to the individual (rather than on the diagnostic category) need, held the promise to unveil more precise pathophysiological

mechanisms and new treatment targets. Based on these assumptions, the RDoC and the HiTOP initiatives advocate for integrating the evidence on psychopathological latent factors from both clinical and non-clinical (general population) cohorts as a mean to consolidate findings on dimensional spectra[19, 20].

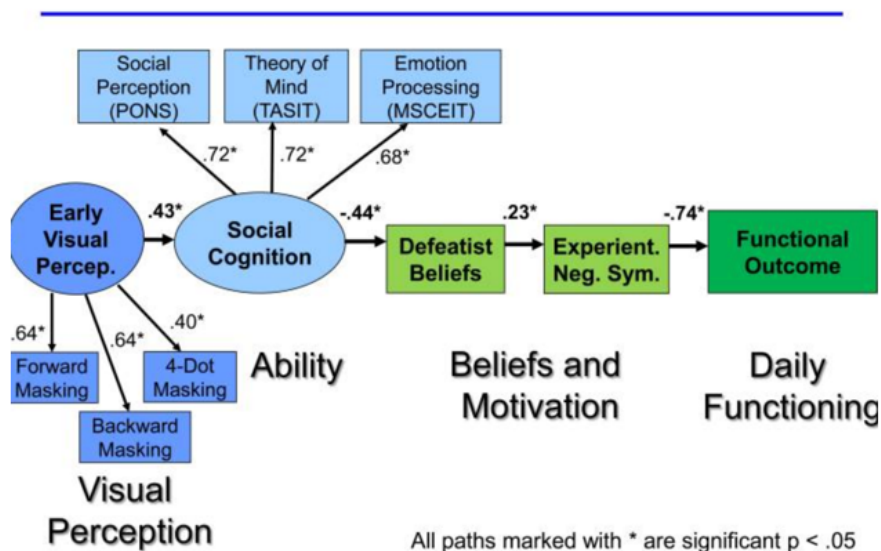
Figure 1. Path analyses investigating shared paths leading to detrimental functional outcomes in psychotic and non-psychotic disorders.

A) Theoretical model showing the path leading from early neurodevelopmental processes to detrimental functional outcomes via experiential negative symptoms in non-psychotic disorders



Taken from: Minichino et al., 2016. From neurological soft signs to functional outcome in young individuals in treatment with secondary services for non-psychotic disorders: a path analysis. Psychological Medicine.

B) Theoretical model showing the path leading from early neurodevelopmental processes to detrimental functional outcomes via experiential negative symptoms in psychotic illness



Taken from: Green et al., 2012. From Perception to Functional Outcome in Schizophrenia: Modelling the Role of Ability and Motivation. JAMA Psychiatry.

The figure shows that experiential negative symptoms represent a key, shared, node leading to detrimental functional outcomes in both psychotic and non-psychotic illness.

1A. Longitudinal path analysis on 138 non-psychotic patients suffering with severe mental illness. Data supported a model where early neurodevelopmental abnormalities led to more severe cognitive deficits and experiential negative symptoms, which in turn led to poor functional outcomes.

1B. Cross-sectional path analysis on 191 patients with schizophrenia. Similar to fig.1A, data supported a model where neurodevelopmental abnormalities led to more severe cognitive deficits and experiential negative symptoms, which in turn led to poor functional outcomes.

1.2.3 Brain mechanisms underlying negative symptoms

To date, the pathophysiology of negative symptoms remains unclear.

Current pathophysiological models suggest that negative symptoms result from dysfunctions of a specific brain network composed of subcortical and higher-order areas[1, 2].

Subcortical structures, such as the ventral tegmental area and the ventral striatum, are responsible for the central processing of rewarding cues[21]. There is consistent evidence of an association between the reduced activation of these subcortical structures to rewarding cues (e.g., monetary tasks) and the severity of experiential negative symptoms across psychiatric diagnoses[16]. The reduced response to rewarding cues would explain the hedonic deficit (anhedonia) that characterises experiential negative symptoms.

Alongside subcortical areas, high-order brain regions are also at play. The anterior cingulate and dorsolateral prefrontal cortices integrate rewarding cues from subcortical areas with effort/cost (e.g., value of the reward, likelihood of success) estimation information[22]. This, in turn provides an internal representation of potential outcomes to rewards that, when impaired, could manifest with amotivational behaviours[22, 23].

Therefore, experiential negative symptoms in severe mental illness might manifest as the result of either hedonic or cognitive deficits, or a combination of the two.

1.3 Negative symptoms – the RDoC initiative

The RDoC initiative was launched in 2010 by the National Institute of Mental Health (NIMH) in the United States[19]. One of the RDoC's dimensional constructs, the Positive Valence system, is often mentioned in trans-diagnostic dimensional investigations of experiential negative symptoms (**Table 1**) [23, 24].

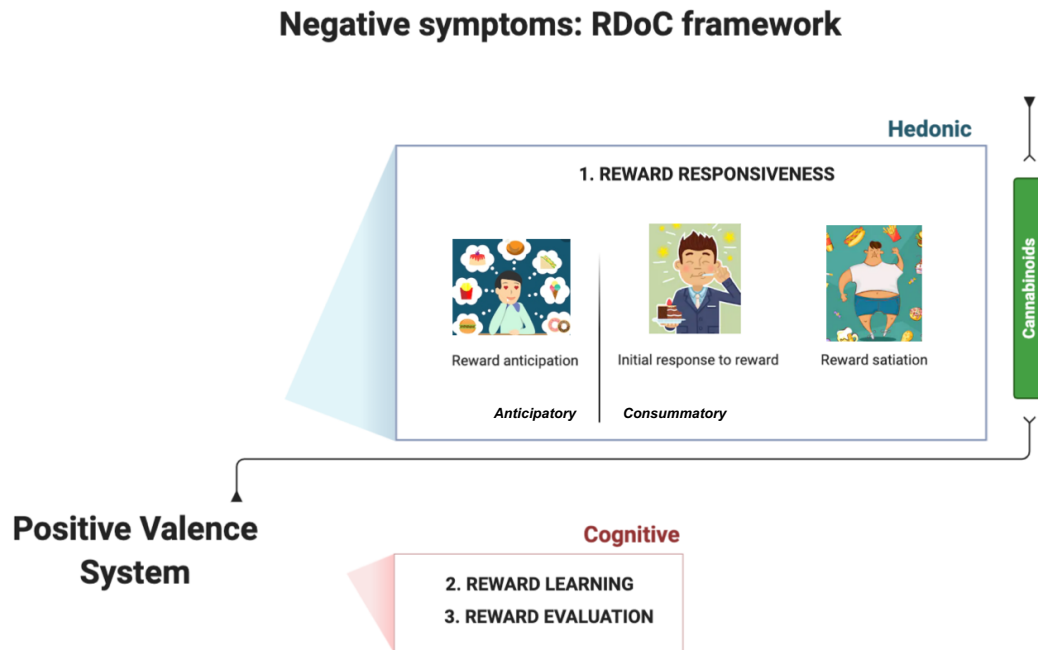
Based on the considerations outlined in 1.2.3, the RDoC Positive Valence system identifies a hedonic and a cognitive pathway. The impairments of either one or both pathways is believed to manifest with experiential negative symptoms[23, 24].

According to the RDoC initiative, the hedonic pathway encompasses one domain (reward responsiveness) and the cognitive pathway two domains (reward learning and reward

evaluation)[19]. The reward responsiveness domain can be further deconstructed into three subdomains: reward anticipation, initial response to reward, and reward satiation. In brief: reward anticipation refers to the anticipatory response to a positive cue (e.g., excitement thinking about a cake – **Fig. 2**); while initial response to reward and reward satiation are involved in the consummatory aspect of the response to a positive cue (e.g., excitement while eating the cake, then satiation – **Fig.2**).

Differentiating between anticipatory vs consummatory responses to rewards is considered relevant by some authors, as preliminary evidence suggests altered anticipatory, but not consummatory, responses are impaired in schizophrenia[25]. The lack of “consummatory” impairment to rewarding cues has led some authors to hypothesise that experiential negative symptoms manifest as a consequence of impairments in the cognitive, rather than the hedonic, path in schizophrenia[1]. According to this hypothesis, in schizophrenia, amotivation, anhedonia, and asociality would result from the loss of higher-order cognitive functions (e.g., reward learning and evaluation) rather than from the lack of pleasure experienced during rewarding activities[1]. In contrast, this latter impairment would be prominent in disorders where cognitive deficits are less pronounced, such as in depression[16]. However, the evidence supporting this hypothesis is mixed and inconclusive[25]. New clinical tools, such as the Brief Negative Symptoms Scale (BNSS), which takes into account anticipatory and consummatory aspects of rewarding experiences, have been developed with the purpose of providing more definitive answers [26].

Figure 2. Negative symptoms in the RDoC framework



Experiential negative symptoms are represented by the Positive Valence System in the RDoC Matrix. The Positive Valence System encompasses three main domains (reward responsiveness, reward learning, reward evaluation) that can be grouped in two broader categories: hedonic and cognitive aspects of the rewarding experience. The hedonic path of the Positive Valence System comprehends processes related to the anticipation and consummation of positive cues (in the picture represented as food-related experiences).

1.4 Negative symptoms – the search for therapeutic targets

Hedonic and cognitive deficits underlying experiential negative symptoms have been traditionally related to dysfunctional dopaminergic, glutamatergic, and cholinergic neurotransmission[2]. However, treatment efforts aimed at palliating negative symptoms based on these neurotransmitter systems (NMDA receptor enhancers, mGluR2/3 positive allosteric modulators, muscarinic acetylcholine agonists, amphetamine based compounds) have had limited success[27].

Therefore, alternative biological routes have been explored.

1.4.1 The inflammatory system

Over the past two decades, inflammation has been the most widely investigated biological correlate of negative symptoms in severe mental illness. The interest of the scientific community towards inflammation is justified by consistent associative findings between immune genes (the major histocompatibility complex region on chromosome 6) and

schizophrenia[28] and by meta-analytic evidence of increased circulating cytokines[29] as well as increased auto-immune comorbidity in patients with severe mental illness vs controls[30].

Increasing levels of pro-inflammatory cytokines (e.g., interleukin-6, tumor necrosis factor alfa, etc.) have been associated with the severity of experiential negative symptoms, both in mood disorders[31] and in schizophrenia[32]. However, the specificity of this association has been questioned as increased pro-inflammatory cytokines have been also associated with increased severity of positive symptoms, poor illness course, and chronicity-related mechanisms (e.g., increased cardiometabolic comorbidities) [32].

Despite this latter consideration, a number of anti-inflammatory compounds (inhibitor of cyclooxygenase type-2[33], non-steroidal anti-inflammatory drugs[33], minocycline[34], methotrexate[35]) have been tested as add-on therapeutics with the aim of reducing inflammation and therefore palliating negative symptoms. Findings were mainly negative. Two explanations have been proposed for the lack of efficacy of anti-inflammatory compounds in treating negative symptoms in severe mental illness.

The first proposed explanation was the heterogeneity of the investigated clinical conditions. For instance, in schizophrenia, some studies have shown a beneficial effect of anti-inflammatory compounds in early-stage, highly-inflamed, patients[34]. This has led some authors to hypothesise the existence of a separate form of schizophrenia, characterised by increased inflammation and a higher likelihood to respond to anti-inflammatory compounds, especially if these were delivered early in the course of illness[36]. However, this hypothesis was disconfirmed by meta-analytic evidence and by well-powered clinical trials showing inefficacy of anti-inflammatory molecules even in highly-inflamed, early-stage, psychosis individuals[35, 37].

The second proposed explanation is the lack of a clear mechanism explaining how increased inflammation might manifest with experiential negative symptoms, and more generally with central brain dysfunctions. For instance, the majority of evidence linking inflammation to mental illness relies on peripheral measures of relatively large molecules (C-reactive proteins and cytokines), which do not freely cross the blood-brain barrier[38]. Other hypotheses, such as the involvement of an hyperactive complement system in synaptic pruning, and microglia dysfunctions, can better explain the relationship with central brain mechanisms[39], but did not find confirmation in large prospective studies in clinical cohorts, yet.

The search for new targets for the treatment of negative symptoms has continued and expanded beyond inflammation.

The relatively recent discovery of two new biological systems, the gut microbiome and the endocannabinoid system, has attracted growing attention from the mental health scientific community. This is due to their modifiable nature and the putative relevance for illness mechanisms.

1.4.2 The gut microbiome

The gut-microbiome is a complex and dynamic ecosystem of micro-organisms present in the intestinal tract[40]. Over the last decade, consistent evidence has shown that the gut microbiome is involved in a number of physiological processes of key relevance for the host's health[41].

Reduced gut microbial diversity ("gut dysbiosis") has been associated with an anhedonic/amotivational syndrome ("sickness behaviour") that manifests across severe mental disorders and represents the key clinical feature of chronic fatigue[17, 42]. As outlined in more detail in Chapter 3, symptoms of sickness behaviour clearly overlap with experiential negative symptoms and the RDoC Positive Valence System.

As reported in **Table 2**, there are a number of gut-microbiome-brain paths that can underlie symptoms of severe mental illness.

Table 2. Interplay between the gut-microbiome and biological systems of relevance for severe mental illness.

Biological system	Metabolites/molecules	Relevance for severe mental illness
Immune system [43]	Cytokines; Antibodies against neural surface antigens (e.g., Anti-NMDAR); Complement component 4 (C4)	Reduced gut-microbiome diversity has been related to altered immune development by enhancing the sensitivity to both internal and external antigens. In the host, this translates in the activation of a pro-inflammatory response, which includes: (i) the release of pro-inflammatory cytokines (IL-6, IFN-γ and TNF-α); [44] the activation of the complement system: and (iii) molecular mimicry mechanisms underlying autoimmunity phenomena (antibodies against antigens of the neural surface). Meta-analytic evidence and GWAS analyses advocate for a key role of the immune system in severe mental illness. Both direct (C4 and synaptic pruning; NMDAR antagonism) and indirect (increased blood-brain barrier permeability) mechanisms might be at play.

Indole and tryptophan metabolism [45]	Kynurenate: tryptophan ratio	Production of kynurenate, a N-Methyl-D-Aspartate Receptor (NMDAR) antagonist, is influenced by certain bacterial species. The activity of Indoleamine-pyrrole 2,3-dioxygenase (IDO; a key enzyme on the conversion pathway from tryptophan to kynurenate) is upregulated by cytokines such as IFN- γ and TNF- α , of which levels are influenced by the gut microbiome. NMDA hypofunction in cortical and hippocampal areas is central for pathophysiological models of severe mental illness.
Biological barriers [46]	Short chain fatty acid (SCFA)	SCFA (acetate, butyrate, propionate) are primary products of the breakdown of non-digestible carbohydrates by gut bacteria. SCFA facilitate the tight-junction assembly in intestinal (gut-barrier) and endothelial (blood-brain barrier) cells. In pathophysiological models of severe mental illness, increased permeability of biological barriers is believed to facilitate the leakage of neuroactive material (e.g., kynurenate, antibodies, cytokines) in the brain.
Endocannabinoid system [47]	Anandamide (AEA) 2-Arachidonoylglycerol (2-AG)	In the gastrointestinal tract, the endocannabinoid system has “gate-keeper” functions and is activated upon “gate-opener” stimuli, such as reduced gut-microbial diversity and loosening of tight junctions. The main endocannabinoid mediators are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The relevance of endocannabinoids for severe mental illness is through the modulation of key neurotransmitter systems (glutamate, dopamine, GABA) and NMDAR expression.

Legend: GABA: γ – aminobutyric acid; GWAS: Genome wide association analysis; IFN- γ : Interferon- γ ; Interleukin-6: IL-6; NMDAR: N-methyl-D-aspartate receptor; TNF- α : Tumor Necrosis Factor- α

However, to date there is no convincing evidence from well-powered human studies confirming that any of these paths can manifest with mental health features. This uncertainty has resulted in a number of failed clinical trials that targeted the gut-microbiome with aspecific interventions in the attempt of improving symptoms of severe mental illness[34]. The lesson learned from the inflammation story outlined in 1.4.1, is that we need to identify precise mechanisms before engaging in further trials.

One of the gut-microbiome-to-brain pathways highlighted in **Table 2** crosses the endocannabinoid system. Of note, endocannabinoids are listed among the molecular targets of the RDoC Positive Valence System[48]. This suggests that gut-microbiome alterations may contribute to negative symptoms via a specific biological axis: the one that involves the endocannabinoid system.

1.4.3 Endocannabinoids

As outlined in detail in Chapter 2, the endocannabinoid system is a key modulator of central neurotransmission[49]. Endocannabinoids provide regulatory/inhibitory feedback for the release of dopamine, glutamate and gamma-amino-butyric acid (GABA) in brain areas of key relevance for negative symptoms (ventral striatum, the anterior cingulate and dorsolateral prefrontal cortices) [50]. Delta-9-Tetrahydrocannabinol (THC), an exogenous cannabinoid and the main psychoactive component of cannabis, has been shown to deplete dopamine in the ventral striatum[51]. This, in turn, would dampen striatal dopaminergic release in response to rewarding cues[52] and would explain why cannabis consumption can induce an amotivational syndrome in otherwise healthy subjects[53].

The effect of THC on the reward system can be prevented by another exogenous cannabinoid, cannabidiol (CBD) [54, 55]. Contrarily to THC, CBD enhances endocannabinoid signalling[56]. This in turn would prevent excessive release of dopamine and its depletion[54, 55]. Based on these considerations, the RDoC matrix suggests that cannabinoids are at play in the hedonic path of the Positive Valence System (**Figure 2**). More recent evidence has shown that both exogenous and endogenous cannabinoids modulate higher cognitive functions by acting on glutamate and GABA-ergic signalling in the anterior cingulate and dorsolateral prefrontal cortices[57, 58].

Being involved in both the hedonic and cognitive paths, the endocannabinoids represent a perfect molecular target for investigations on experiential negative symptoms. Endogenous cannabinoids can be measured both centrally (cerebrospinal fluid) and peripherally (blood, faeces)[59]. Peripheral measures can be obtained with non-expensive and non-invasive measures, characteristics that can facilitate replicability and real-world applications. As outlined in more detail in Chapter 2, evidence suggests that peripheral cannabinoids might be informative for central brain processes related to negative symptoms.

1.4.4 The gut-microbiome-endocannabinoid axis

Growing evidence from other medical fields, such as those related to metabolism and inflammation, suggests the existence of a close interplay between the gut-microbiome and the endocannabinoid system[47]. A number of studies on obesity[60], type 2 diabetes[61], inflammatory bowel disorders[62], and multiple sclerosis[63] (all highly comorbid with

severe mental illness) advocate for the existence of a dysfunctional gut-microbiome-endocannabinoid axis. A better understanding of the functionality of this axis, which is strategically positioned between the environment and key physiological bodily functions, holds the promise of unveiling novel biomarkers for prognostic stratification and new therapeutic targets for intervention.

Despite the mounting attention that the gut-microbiome and endocannabinoids have received from the mental health scientific community, all studies to date have considered these two biological systems as independent compartments.

In this thesis, I will provide the first investigation on the interplay between these two systems and their relevance for negative symptoms.

Chapter 2. The endocannabinoid system in psychotic illness: a systematic review and meta-analysis

2.1 Introduction

2.1.1 *The endocannabinoid system*

The endocannabinoid system is an endogenous lipid-based signaling system (**Figure 3**)[49]. It regulates key physiological functions in the body via two main receptors, CB1R and CB2R, G-protein coupled membrane receptors[64, 65]. When initially discovered, CB1R was believed to be uniquely expressed in brain cells and peripheral nerve terminals, while CB2R solely in immune cells[64, 65]. Over the last decade, it has become clear that these receptors have ubiquitous distribution, although the proposed initial differences still stand in terms of preferential tissue expression[66]. In the periphery, CB1R, CB2R, and their agonists, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), can be found in the gastrointestinal tract, immune cells, liver, adipose tissue, and pancreas, where they regulate key functions such as, inflammatory responses, gastrointestinal mobility and permeability, adipose storage, and energy metabolism[47]. Brain cannabinoid receptors are mainly located on dopaminergic presynaptic terminals, cholecystokinin(CCK)-positive interneurons (CB1R), and on microglia and astrocytes (CB2R)[67]. As suggested by the presynaptic and inter-neural location of CB1R, the central functions of the endocannabinoid system are mainly modulatory[64, 65]. Endocannabinoid agonists, AEA and 2-AG, are synthesised and released by dopaminergic post-synaptic terminals upon neurotransmitter release and provide retrograde inhibitory feedback on the pre-synaptic terminal via CB1R activation[68]. On CCK interneurons, CB1R modulates the synchronised activation of pyramidal cells, which is key for the generation of brain-rhythms, such as gamma oscillation, believed to underlie higher-order cognitive functions[69, 70]. CB2R is expressed on microglia and astrocyte following brain injuries or pro-inflammatory stimuli[71]. When activated, CB2R can reduce (neuro)inflammatory and (neuro)oxidative damages resulting from external or internal (e.g., autoimmune) insults[71]. Alongside the two main endocannabinoid mediators (AEA and 2-AG), which can directly bind cannabinoid receptors, there are other molecules that constitute the wider “endocannabinoidome”[72]. These molecules are often referred to as endocannabinoid analogues. Endocannabinoid analogues are structurally related to AEA and 2-AG and can enhance the activity of the system by modulating the expression of synthesising and degrading enzymes. The most investigated endocannabinoid analogue is

palmitoylethanolamide (PEA)[73]. PEA is an endogenous enhancer of the endocannabinoid system and acts by inhibiting the fatty acid amid hydrolase (FAAH), the main catabolic enzyme of AEA and 2-AG[73].

2.1.2 Endocannabinoids: relevance for pathophysiology of psychotic and severe mental illness

The evidence to date suggests that centrally the endocannabinoid system regulates the activity of three main neurotransmitter networks: dopaminergic, glutamatergic, and γ -aminobutyric acid (GABA)ergic[49, 58, 66, 74]. These brain networks are of key relevance for pathophysiological models of severe mental illness[3].

Traditional models of psychotic illness suggest that dopaminergic dysfunctions underlie its main clinical features. According to these models, enhanced meso-limbic and reduced meso-cortical dopaminergic signalling would manifest with positive symptoms and negative symptoms/cognitive deficits, respectively[3]. More recently, the “dopaminergic theory” of psychotic illness has been revisited by integrating new evidence highlighting dysfunctions in glutamatergic networks. The revisited psychopathological model gives a central role to N-methyl-D-aspartate (NMDA) receptors[3]. The hypofunction of these receptors, strategically located on parvalbumin-positive interneurons in key brain areas, is proposed to reduce the activity of glutamate efferent neurons synapsing with basal ganglia interneurons. This leads to overactive meso-limbic dopaminergic signalling, which in turn manifests as positive symptoms. The NMDA-related reduced activity of glutamate outputs from the cortex directly to dopamine neurons in the VTA, leads to decreased meso-cortical signalling, which is thought to underlie cognitive deficits and negative symptoms[3].

Abnormalities of the endocannabinoid system would nicely fit in both classical and revisited pathophysiological models of psychotic illness. Given its role in modulating the pre-synaptic release of dopamine, the endocannabinoid system is likely to be involved in both meso-limbic and meso-cortical dopaminergic dysfunctions. Furthermore, CB1R are co-expressed with NMDA receptors on the neural surface of interneurons, where they regulate NMDA functionality[58].

Finally, in the last two decades a number of studies have advocated a strong link between psychotic illness and neuroinflammation. Another link between alternated endocannabinoid system and psychotic illness is impaired in CB2R activation, failing to suppress neuroinflammation[71].

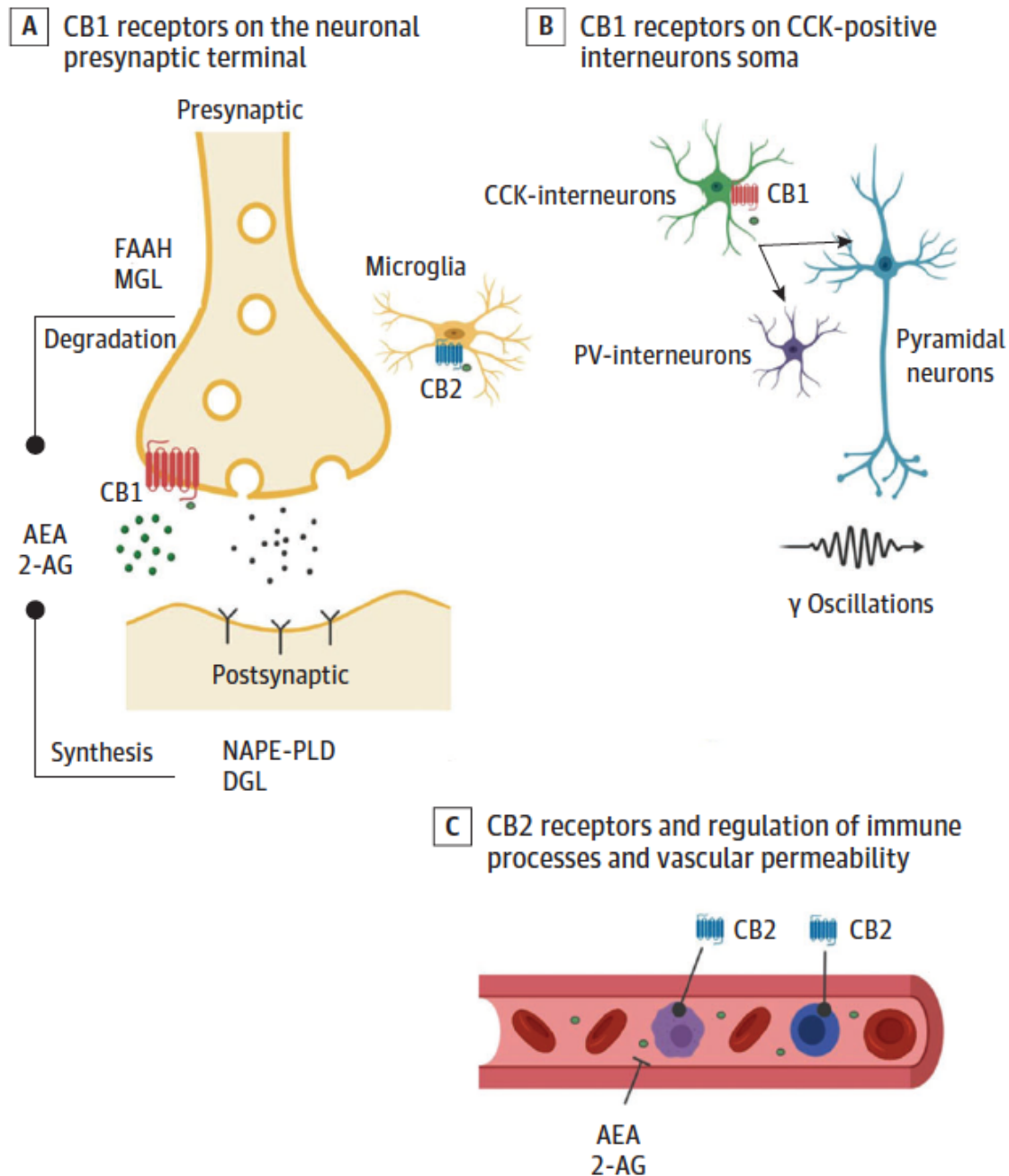
2.1.2 Exogenous cannabinoids: relevance for pathophysiology of psychotic and severe mental illness

The endogenous activity of the endocannabinoid system is affected by exogenous cannabinoids, such as delta-9-tetrahydrocannabinol (Δ 9-THC - the main psychoactive components of cannabis) and cannabidiol (CBD)[53, 72]. Impairment of the endocannabinoid system through cannabis use has been related to an increased risk of psychosis[75, 76]. With relevance to negative symptoms, acute and chronic consumption of Δ 9-THC have been shown to cause hedonic (altered glutamatergic and dopaminergic transmission in the reward system)[52] and cognitive (reduced gamma-oscillations) deficits in otherwise healthy controls[69, 70]. These deficits manifest with an anhedonic/amotivational syndrome that can be prevented by CBD administration[54, 55].

2.1.3 Endocannabinoids: RDoC Positive Valence System

Based on the aforementioned considerations, it is likely that the cannabinoid system may be at play in both hedonic (reward responsiveness) and cognitive (reward learning and evaluation) aspects of the RDoC Positive Valence System. In support of this hypothesis, recent evidence showed that CBD can modulate the activity of neural networks associated with experiential negative symptoms in both depressive and psychotic disorders.

Figure 3. The endocannabinoid system



From: Minichino et al., Measuring Disturbance of the Endocannabinoid System in Psychosis: A Systematic Review and Meta-analysis. JAMA Psychiatry. 2019 Jun 5;76(9):914–23.

A. Type 1 cannabinoid (CB1) receptors are mainly localised on the neuronal presynaptic terminal, possibly mediating retrograde inhibitory feedback and neurotransmitter release. **B.** CB1 receptors are also localised on the soma of cholecystokinin (CCK)-positive interneurons, contributing to the synchronisation of pyramidal cells cortical firing, which is involved in the genesis of γ oscillations. **C.** Given their high expression on immune cells, type 2 cannabinoid (CB2) receptors are mainly associated with the peripheral functions of the endocannabinoid system, particularly the regulation of immune processes and vascular permeability. However, CB2 receptors are expressed in the human brain (microglia and blood vessels).

Legend: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; DGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; MGL, monoacylglycerol lipase; NAPE-PLD, N-acyl-phosphatidylethanolamine phospholipase D; PV, parvalbumin.

2.2 Aims

The aim of this chapter is to clarify if measures of the activity of the endocannabinoid system are clinically informative for psychotic illness, and the nature of their relationship with negative symptoms.

2.3 Methods

Blood and cerebrospinal fluid (CSF) markers of endocannabinoid system activity in psychotic illness were explored through a systematic review and meta-analysis.

The systematic review and meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines[77].

The original protocol was registered in PROSPERO (registration number: CRD42018099863).

The search for studies was conducted from inception to June 13, 2018.

I included peer-reviewed original studies that reported data on blood and/or CSF-based endocannabinoid markers in patients with psychotic illness. Psychotic illness included schizophrenia, schizoaffective disorders, first episode psychosis, and prodromal psychosis. No restrictions were set in terms of duration of illness, illness phase (acute vs stable/remitted), medications status.

The entire search process was independently conducted by two collaborators (see Contributions). I extracted data from each included study. Data extraction was independently checked by another collaborator. The adapted version for observational studies of the Newcastle-Ottawa scale was used to assess quality of included studies (see Appendix).

The clinical relevance of endocannabinoid system modifications in psychosis was investigated by: (i) comparing differences in blood and CSF endocannabinoid markers between patients and healthy controls; [44] reporting data on correlation analysis between severity of positive and negative symptoms and blood and CSF endocannabinoid markers in patients; (iii) reporting data on stage-related modifications of blood and CSF endocannabinoid markers in patients vs healthy controls; and (iv) reporting data on the relationship between modifications of blood and CSF endocannabinoid markers and response to treatment.

2.3.1 Statistical analysis

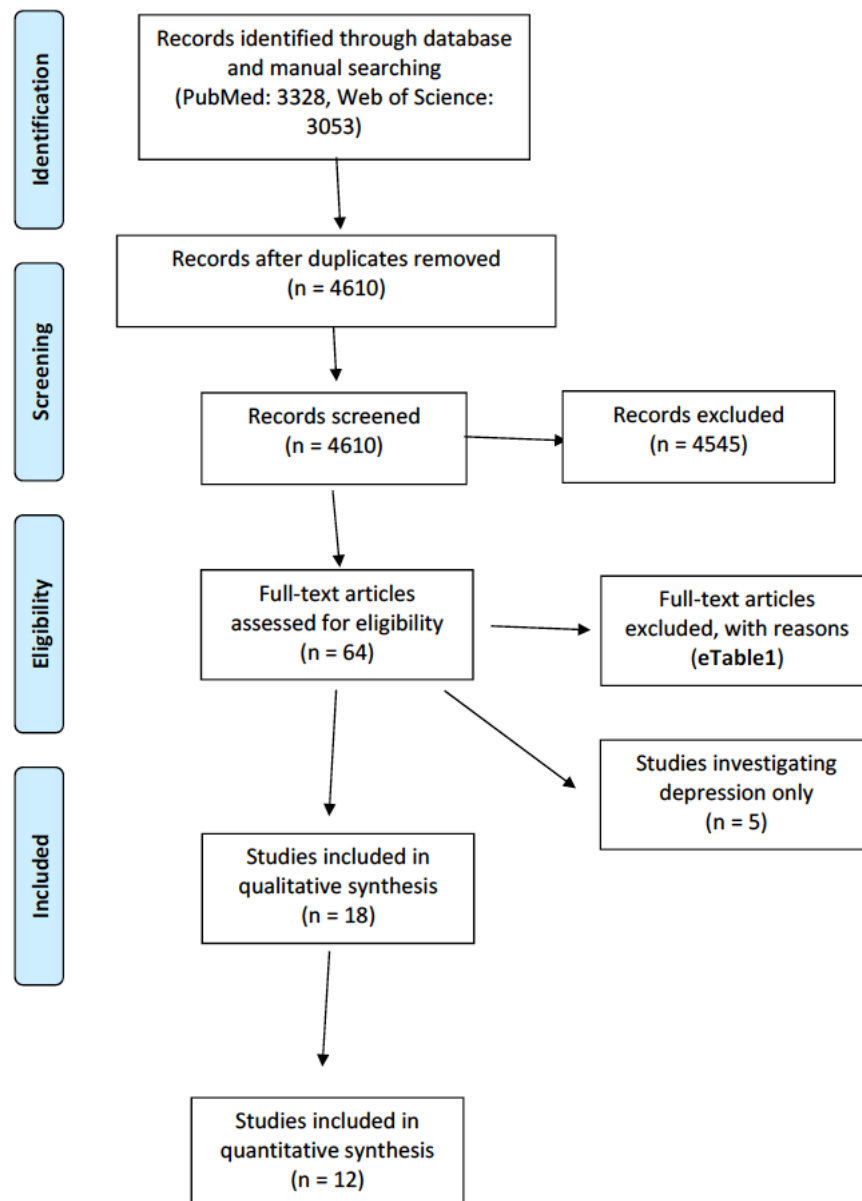
Meta-analytic pooled estimates of the differences in biomarkers between patients and controls were provided when data from two or more studies were available for a certain biomarker. All meta-analyses were conducted in Stata, version 13.0 (StatCorp LCC), using random-effect models. Standardised mean difference (SMD) was used as summary statistics; the Higgins I^2 was used to assess between-study heterogeneity. Sensitivity analyses were carried out by sequentially removing single studies and re-running the analyses[78].

When feasible, I performed additional descriptive analyses to explore the putative influence of *a-priori* identified clinical subgroups on findings. Clinical subgroups were defined as follows: 1) Illness stage, with three subgroups: prodromal, first-episode, multi-episode; 2) illness phase, with two subgroups: acutely ill (inpatients or outpatients defined as acutely ill) and clinically stable (i.e., outpatients not defined as acutely ill); 3) Antipsychotic use, with two subgroups: antipsychotic-free/antipsychotic-naïve and antipsychotic-treated; 4) Cannabis use: with two subgroups: current users (patients included in studies where current cannabis use was not an exclusion criterion) and not-current users (no previous use or past use).

2.4 Results

As outlined in **Figure 4** (PRISMA flow-chart), eighteen studies met the inclusion criteria[56, 79-95] and of these, twelve provided data for a quantitative synthesis. Across the included studies, the following endocannabinoid markers were investigated: anandamide (both in blood and CSF); CB1R and CB2R (in blood - peripheral immune cells); the endocannabinoid synthesising enzyme N-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipase (DLG) (in blood - peripheral immune cells); the endocannabinoid degrading enzyme fatty acid amid hydrolase (FAAH) and monoacylglycerol lipase (MGL).

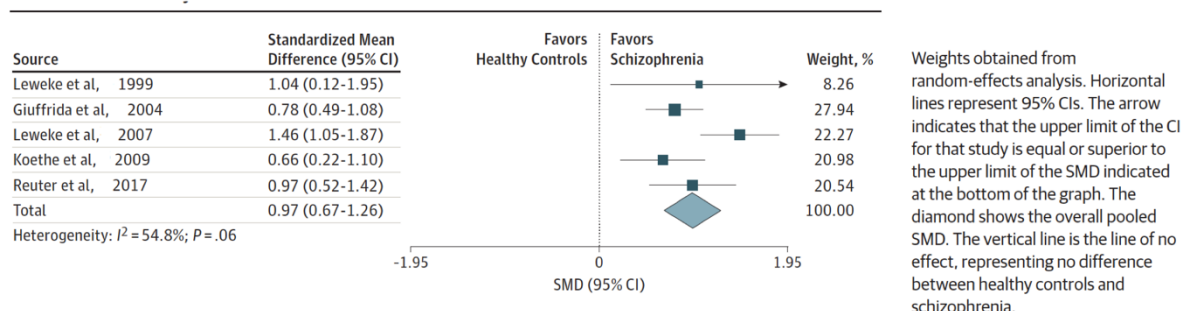
Figure 4. PRISMA flow-chart: endocannabinoids in psychotic illness



Five studies reported data on CSF levels of anandamide[81-83, 85, 89]. Pooled data from 226 patients and 385 controls showed that anandamide was significantly increased in the CSF of patients vs controls (SMD,0.97; 95%CI,0.67-1.26;P < 0.001) (**Figure 5**). The heterogeneity was moderate (I^2 : 54.7%); the exclusion of one outlying study[82] consistently reduced heterogeneity and still yielded significant results (SMD, 0.81; 95% CI, 0.60-1.02;P <0.001; I^2 = 0%). Findings of increased anandamide in the CSF of patients with psychotic illness were

independent of illness stage, cannabis use, and antipsychotic medication (Appendix). None of the studies investigating CSF anandamide levels included clinically stable participants.

Figure 5. Forest plot of standardised mean difference (SMD) in cerebrospinal fluid anandamide levels in patients and healthy controls

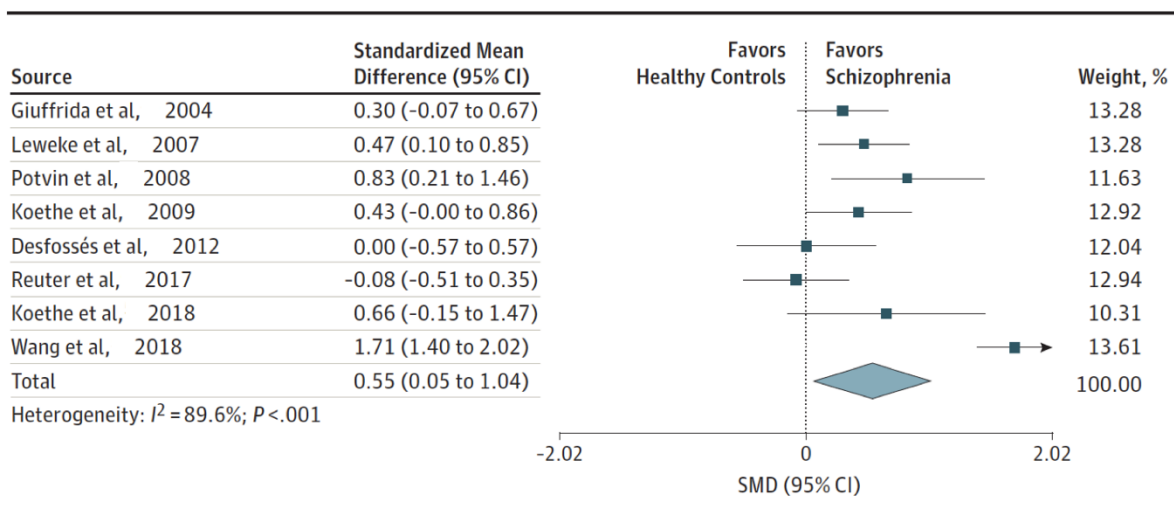


From: Minichino et al., Measuring Disturbance of the Endocannabinoid System in Psychosis: A Systematic Review and Meta-analysis. JAMA Psychiatry. 2019 Jun 5;76(9):914–23.

Nine studies reported data on circulating levels of anandamide[80-83, 85, 91, 92, 94], but only eight studies were considered for meta-analytic synthesis. One study was excluded because it measured anandamide levels in whole blood[90], a method believed to produce heterogeneous results when compared to the more commonly used plasma and serum extraction.

Pooled estimates on eight studies, including 344 patients and 411 controls, showed a significant increase of circulating anandamide in psychotic illness (SMD, 0.55; 95%CI, 0.05-1.04; $P = 0.03$) (**Figure 6**).

Figure 6. Forest plot of standardised mean difference (SMD) in blood anandamide levels in patients and healthy controls



There was evidence of high heterogeneity (I^2 : 89.6%). However, by excluding single studies and re-running the analysis, heterogeneity dropped to low (SMD, 0.33; 95% CI, 0.11-0.55; $P = 0.03$; I^2 : 31.1%) with results remaining still statistically significant.

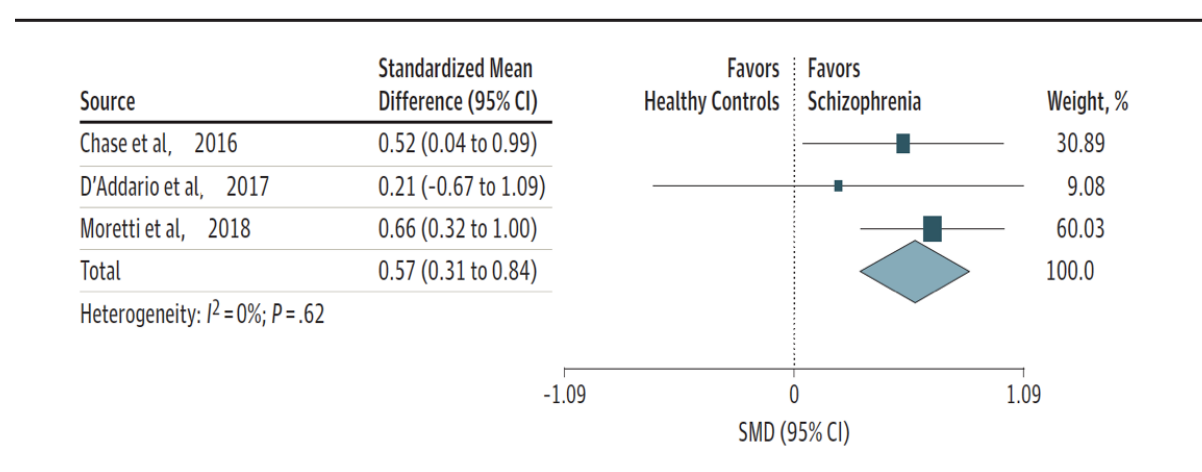
Results from the excluded study[90], which investigated anandamide in whole blood, pointed in the same direction of the pooled estimate.

None of the clinical subgroups: stage, phase, cannabis use and medication, substantially contributed to differences in circulating levels of anandamide between patients and controls. However, differences between acutely ill patients, multi-episode patients and controls showed larger effect sizes, suggesting that results obtained in these two clinical groups could drive the results observed in the pooled estimate.

Of note, levels of anandamide in CSF and blood did not correlate in any of the studies that reported data on both these fluids[81-83, 85].

Five studies reported data on the expression of CB1R in peripheral immune cells in patients vs controls[79, 84, 86, 87, 95]. It was only possible to provide a pooled estimate using data from three of these five studies[79, 86, 95], due to lack of extractable data in two studies[84, 87]. The pooled estimate showed increased CB1R levels in peripheral immune cells of patients compared to controls (SMD, 0.57; 95%CI, 0.31-0.84; $P < 0.001$; $I^2 = 0\%$) (**Figure 7**).

Figure 7. Forest plot of standardised mean difference (SMD) in type 1 cannabinoid receptor expression in blood of patients and healthy controls



Data was obtained from multi-episode patients and from mixed sample of medicated/unmedicated patients, both acutely ill and stable, current and not-current cannabis users. These characteristics did not allow any consideration on the clinical subgroups of interest. The two studies that investigated CB1R in psychotic illness not included in the quantitative synthesis reported mixed findings: one study showed increased expression of CB1R in peripheral immune cells in patients vs controls[84], while the other showed no differences[87].

Five studies reported data on the expression of CB2R on peripheral immune cells of patients and controls[84, 86, 87, 93, 95]. However, due to the lack of extractable data, it was not possible to provide a quantitative synthesis of findings. Two of these five studies reported increased expression of CB2R in patients vs controls[86, 87], while three reported no differences[84, 93, 95].

Three studies reported data on degrading and synthesising endocannabinoid enzymes on peripheral immune cells of patients vs controls[90, 93, 95]. Two studies showed increased expression of degrading endocannabinoid enzymes on peripheral immune cells of patients vs controls[90, 93], while another study showed no differences[95]. One study showed reduced expression of synthesising endocannabinoid enzymes on peripheral immune cells of patients vs controls[88], while another study reported no differences[95].

The qualitative summary of the included studies is reported in **Table 3**.

Seven studies reported data on the association between endocannabinoid markers and severity of symptoms in patients with psychotic illness[81, 83, 85-88, 92, 93]. Severity of positive, negative, and cognitive symptoms was associated with: 1) decreased anandamide levels in CSF and blood; and 2) increased expression of CB1R and CB2R in peripheral immune cells. Severity of cognitive symptoms was further associated with reduced expression of endocannabinoid synthesising enzymes and increased expression of endocannabinoid degrading enzymes.

Finally, only two studies investigated the association between disturbance of the endocannabinoid system and treatment outcomes in psychotic illness[56, 90]. One study showed that patients that remitted after three months of treatment as usual (TAU) showed a significant reduction in circulating levels of anandamide compared to not remitted patients[90]. The other study, a randomised control trial investigating the efficacy of CBD augmentation on TAU, showed that a post-treatment increase in circulating levels of anandamide was associated with significant improvements in positive symptoms[56].

2.5 Discussion

This is the first systematic review and meta-analysis investigating disturbance of the endocannabinoid system in psychotic illness. The main finding was of increased endocannabinoid system activity in patients compared to controls, as shown by increased levels of anandamide in both CSF and blood and increased CB1R expression in peripheral immune cells. This increase was independent of antipsychotics and cannabis use. It was observed at any stage of illness (including the prodrome), and it was inversely associated with severity of symptoms (including negative symptoms).

The pooled estimate of anandamide in CSF showed increased levels in patients vs controls. This increase, which was observed from prodromal to longstanding illness, was independent of cannabis use and antipsychotic medications and was inversely associated with severity of symptoms. Taken together, these findings suggest that anandamide levels in CSF could represent a biomarker of psychotic illness and that engagement of the endocannabinoid system in the brain could be protective towards disease mechanisms[49]. Anandamide provides regulatory inhibitory feedback at the synaptic level[96]. In the context of psychotic illness, where positive symptoms are believed to arise from increased meso-limbic dopaminergic signalling, anandamide could work as a brake against further dopamine release[68]. This would explain why patients with higher CSF levels of anandamide showed less severe positive symptoms. In support of this hypothesis, efficacy of add-on CBD (an inhibitor of anandamide degradation) on positive symptoms was related to an increase in anandamide levels[56]. The inverse association between CSF anandamide levels, negative symptoms and cognitive functions can be interpreted in the light of the modulatory action of endocannabinoids on NMDA receptors[58] and CCK-interneurons[69, 70]. In the brain, anandamide enhances NMDA expression[58] and regulates the genesis of gamma-oscillation[69, 70]. Based on glutamatergic models of psychotic illness, these two complementary actions of anandamide would explain why patients with greater levels of endocannabinoids in the brain have less severe negative symptoms and better cognitive performances (**Figure 1**).

In the blood, anandamide levels were elevated in patients compared to controls. There were no significant differences in circulating levels of anandamide between early-stage vs multi-episode patients, antipsychotic free/naïve vs medicated patients, or current cannabis

users vs non users. However, differences between multi-episode and acutely ill patients vs controls showed the largest effect sizes, suggesting that longstanding illness and relapses might have an influence on peripheral levels of anandamide. Peripherally, the endocannabinoid system can be activated by a number of different factors, such as inflammation[63], increased fat storage[97], or modifications of the gut-microbiome[63]. These factors are frequently associated with chronicity and illness relapses, which would explain why higher peripheral anandamide levels were observed in multi-episode and acutely ill patients. The existence of peripheral triggers for the activation of the endocannabinoid system would also explain why four studies did not find an association between peripheral and central levels of anandamide.

However, results from correlation analyses across the included studies showed that circulating biomarkers of endocannabinoid system activity may still be informative on the disease mechanisms underlying psychotic illness. Reduced levels of circulating anandamide and endocannabinoid synthesising enzymes, increased expression of cannabinoid receptors and increased expression of degrading endocannabinoid enzymes, were all associated with more severe negative symptoms and cognitive deficits. A possible explanation for these findings is provided by Reuter and colleagues[83], who suggest that the association between peripheral endocannabinoid markers and negative/cognitive features of psychotic illness might be the result of these molecules directly draining to the circulation from frontal regions of the brain through the dural venous sinuses.

The meta-analysis also showed increase CB1R in peripheral immune cells of patients vs controls. This increase might be explained by a number of different factors. In light of the anti-inflammatory properties of the endocannabinoid system, one hypothesis is that increased CB1R expression would represent a compensatory mechanism towards increased inflammation, a common finding in psychotic illness[98]. Similarly, altered gut-microbiome composition has been shown to trigger the expression of peripheral CB1R as a means to restore gut-barrier permeability[47]. As outlined in the next chapter, modifications in gut-microbiome taxonomy have been associated with a number of detrimental health outcomes, including psychotic illness. Finally, increased CB1R might be a compensatory mechanism associated with reduced circulating levels of the mediator anandamide. This hypothesis is consistent with findings showing that reduced anandamide, but increased CB1R, are associated with more severe symptoms of psychotic illness.

Results showing altered CB2R and endocannabinoid enzymes in patients vs controls were limited. However, the available evidence was consistent with the meta-analytic findings, showing increased endocannabinoid system activity in psychosis and an inverse association with more severe illness features.

2.5.1 Limitations

Limitations of this systematic review and meta-analysis were: 1) considerations on clinical subgroups were based on small group of studies; in particular, the definition of cannabis use was heterogeneous across studies and the subgroup “non-current use” included participants with past cannabis use, a variable known to influence endocannabinoid system activity; 2) studies investigating CSF levels of anandamide were all from the same catchment areas, limiting generalisability of findings; 3) despite efforts in trying to retrieve data from all included studies, it was not possible to provide a quantitative synthesis on CB2R and endocannabinoid enzymes.

2.5.2 Conclusion

This systematic review and meta-analysis showed that the endocannabinoid system is abnormal in psychotic illness, from the prodromal phase to longstanding illness, independent of medications and cannabis use. Peripheral biomarkers of endocannabinoid system activity might be informative for mechanisms associated with negative symptoms and cognitive deficits. In particular, increased peripheral endocannabinoid system tone (i.e., increased levels of mediators, reduced levels of cannabinoid receptors, increased levels of synthesising enzymes, reduced levels of degrading enzymes) could represent a novel biomarker and a putative therapeutic target for negative symptoms in psychotic illness. Future studies should clarify the diagnostic specificity of these abnormalities, both within psychotic illness and trans-diagnostically.

Table 3. Qualitative summary – endocannabinoids in psychotic illness

Study	Participants	Gender (% of Females)	Tissue-ECS	Medication	Stage	Phase	Cannabis use	Main findings
<i>Leweke 1999 [89]</i>	N=10 SCZ N=11 HS	SCZ: 30.1% F HS: not reported	- CSF	N=3 on APs N=7 APs-free	SCZ	Acutely ill “BPRS total scores 49-73”	a. Subjects with a comorbid substance abuse/dependence were excluded. b. N= 3 SCZ reported intermittent use of cannabis c. No correlation between cannabis use and AEA levels	1. ↑ AEA in CSF of SCZ vs HS
<i>De Marchi 2003 [90]</i>	N=12 SCZ N=20 HS	SCZ: 25.0% F HS: not reported	- Whole blood - PBMCs	N=12 AP-free (Baseline) N=5 Olanzapine (remitted patients) *Remission: at least 50% pre-post reduction in BPRS score	SCZ	Acutely ill “Acute phase of their schizophrenic illness”	a. Subjects reporting cannabis use in the month preceding the study were excluded	1. ↑ AEA in blood of SCZ vs HS 2. ↑ FAAH in PBMCs of SCZ vs HS 3. Remission* of psychotic symptoms associated with ↓ AEA in whole blood; ↓ CB2 and ↓ FAAH in PBMCs *Remission: at least 50% pre-post reduction in BPRS score
<i>Giuffrida 2004</i>	N=47 FEP	FEP: 27.7% F SCZ:	- Serum - CSF	Among FEP/SCZ: N=47 APs-free	FEP/SCZ	Acutely ill “Inpatients”	a. Subjects positive to urine drug screen (including cannabis) were excluded	1. ↑ AEA in CSF of AP-naïve FEP vs HS

[85]	N= 71 SCZ N=84 HS	19.7% F HS: 62.4%		N=37 typical AP N=35 atypical AP			b. Measured self-reported life time cannabis use c. No effect of cannabis use on primary endpoints of the study	2. ↑ AEA in CSF of SCZ treated with atypical AP vs HS 3. = AEA in CSF of SCZ treated with typical AP vs HS 4. = AEA in serum of FEP/SCZ vs HS 5. Inverse correlation between levels of AEA in CSF and negative symptoms in FEP/SCZ
Leweke 2007 [82]	N= 44 FEP N=81 HS.	FEP: 39.1% F HS: 44.4% F	- Serum - CSF	All FEP were AP-naive.	FEP	Acutely ill “Inpatients”	a. Subjects were divided in groups based on life-time cannabis use: HF-FEP=19 LF-FEP=25 LF-HC: n=55 HF-HC: n=26 HF: High Frequency life-time cannabis use (more than 20 life-times) LF: Low Frequency life-time cannabis use (less/equal 5 life-times) b. Subjects with a comorbid substance abuse/dependence were excluded.	1. ↑ AEA in CSF of LF-FEP vs HS 2. = AEA in CSF of HF-FEP vs HS 3. Inverse correlation between levels of AEA in CSF and negative symptoms in HF-FEP and LF-FEP 4. Inverse correlation between levels of AEA in CSF and positive symptoms in LF-FEP 5. =AEA in serum of FEP vs HS

							c. No effect of cannabis use on primary endpoints of the study	
<i>Potvin 2008</i> [91]	N= 29 SCZ+SUD* N=17 HS *Substance abuse disorder [99]	SCZ-SUD: 24.0% F HS: 16.0% F	-Plasma	Mixed typical and atypical APs	SCZ	Clinically stable “Not acutely ill/not hospitalized”	a. All patients had a comorbid SUD*. N=18/29 Cannabis SUD* *Substance abuse disorder [99]	1. ↑ AEA in plasma in SCZ+SUD vs HS
<i>Koethe 2009</i> [81]	N=27 Prodromal SCZ N=81 HS	Prodromal SCZ: 30.2% HS: 44.3% F	- Serum - CSF	APs-free	Prodromal SCZ	Not applicable	a. No cannabis use within 6 weeks prior to the study b. Subjects positive to urine drug screen were excluded c. Life-time cannabis use was considered HF-Prodromal=16 LF-Prodromal=11 (High Frequency-HF vs Low Frequency-LF, more than/less than 20 life-times use). d. No effect of cannabis use on primary endpoints of the study	1. ↑ AEA in CSF of Prodromal SCZ vs HC 2. =AEA in serum of Prodromal SCZ vs HC 3. Prodromal SCZ with high values of AEA in CSF (split on median value) had delayed psychotic onset. 4. In prodromal SCZ, inverse correlation between levels of AEA in CSF and “cognitive factor” as measured by the PANSS.

<i>Desfosses 2012 [94]</i>	N=23 SCZ N=38 SUD* N=27 HS *Substance abuse disorder [99]	SCZ: 32.0% F SUD: 34.2% HS: 40.7% F	- Plasma	Among FEP/SCZ: N=4 APs-free N=14 atypical APs N=5 typical APs	SCZ	Clinically stable “Patients excluded if total score on PANSS<65; Inpatients excluded”	a. In SCZ: exclusion of any SUD (including cannabis) b. In SUD: 20 had a cannabis abuse disorder	1. ↑ AEA in plasma of SUD vs HS 2. =AEA in plasma of SCZ vs HS
<i>Leweke 2012 [56]</i>	N= 42 SCZ	SCZ: 31.2% F	- Serum	Randomized controlled trial: Amisulpride vs Cannabidiol	SCZ	Acutely ill “Brief Psychiatric Rating Scale >36; and BPRS- thought content factor≥12”.	a. Subjects with a comorbid substance abuse/dependence were excluded. b. Patients positive to drug screening excluded.	1. Treatment with Cannabidiol ↑ AEA in Plasma of SCZ 2. Post-treatment ↑ AEA in Plasma of SCZ was positively associated with improvement in psychotic symptoms

<i>Bioque 2013</i> [93]	N=95 FEP N=90 HS	FEP: 28.0% HS: 28.1%	- PBMCs	N=18 APs-naïve N=77 on APs (typical and atypical; mean CPZ equivalents = 357.33 mg/day)	FEP	Mixed - Not clearly stated	a. Comorbid diagnosis of substance abuse/dependence was used to divide the sample in: FEP CAN+=46 FEP CAN-=49 CAN+=comorbid diagnosis of cannabis abuse/dependence over at least 12 consecutive months during their life b. Among FEP, FEP-CAN+ showed more severe ECS abnormalities	1. In FEP vs HS: ↓ CB2*; ↓ NAPE, ↓ DGL (synthetizing enzymes); and ↑ MGL, ↑ FAAH (degrading enzymes) 2. In FEP, ↑ FAAH in males vs females *No longer significant after adjusting for confounding factors
<i>Ferretjans 2014</i> [87]	N=53 SCZ N=22 HS	SCZ: 45.3% F HS: 57.1 % F	- PBMCs	Mixed typical and atypical APs (mean CPZ equivalents= 344.22 mg/day)	FEP/ SCZ	Clinically stable “PANSS positive score of 19 or less (and less than 4 in any item of the positive scale)”	a. Subjects with self-report of no cannabis use in the month preceding the start of study were excluded	1. ↓ CB2 in SCZ vs HS* 2. = CB1 in SCZ vs HS 3. In SCZ, inverse association between CB1, CB2 expression and overall cognitive performances *No longer significant after adjusting for confounding factors
<i>Bioque 2016</i>	N=73 FEP N=67 HS	FEP: 28.8% F	- PBMCs	N=16 APs-naïve	FEP	Mixed – Not clearly stated	a. Comorbid diagnosis of substance abuse/dependence	1. In FEP, decrease expression of ECS synthetizing enzymes and increased expression of

[88]		HS: 31.4% F		N=57 on APs (typical and atypical)			was used to divide the sample in: FEP CAN+=33 FEP CAN-=35 CAN+=comorbid diagnosis of cannabis abuse/dependence over at least 12 consecutive months during their life b. Correlation analyses corrected for cannabis use c. No effect of cannabis use on primary endpoints of the study	degrading enzymes associated with poorer cognitive performances.
<i>Chase 2016</i> [86]	N=35 SCZ N=35 HS	SCZ: 49.0% F HS: 51.1% F	- PBMCs	N=3 drug-free N=3 typical APs N=29 atypical APs	SCZ	Mixed N=15 Inpatients N=20 Outpatients	a. Participants with comorbid cannabis abuse/dependence or cannabis use in the last 12 months were excluded	1. ↑ CB1, ↑ CB2 in SCZ vs HS 2. In SCZ, inverse correlation between CB1 and CB2 expression and performances specific cognitive domains (attention, processing speed, working memory)
<i>De Campos-Carli 2017</i> [84]	N=55 SCZ N=48 HS	SCZ: 50.0% F HS: 46.0% F	- PBMCs	N=16 atypical APs N=39 typical APs (mean CPZ equivalents =	SCZ	Clinically stable Outpatients	a. Subjects with self-report of no cannabis use in the month preceding the start of study were excluded	1. ↑ CB1, ↑ CB2 in SCZ vs HS

				358.00 mg/day)				
<i>D'Addario 2017 [95]</i>	N=25 SCZ N=34 HS	SCZ: 44.1% F HS: 55.9%	- PBMCs	Among SCZ N=14 atypical APs N=11 typical APs (mean CPZ equivalents = 506.12 mg/die)	SCZ	Mixed	a. Patients with a comorbid substance abuse in the last 2 months were excluded.	1. ↑ CB1 in SCZ vs HS 2. = CB2; = NAPE and DGL; = FAAH and MGL in SCZ vs HS
<i>Reuter 2017 [83]</i>	N=28 FEP N=81 HS	FEP: 21.4% F HS: 45.6% F	- Serum - CSF	All patients were AP-naïve	FEP	Acutely ill “Inpatients only”	a. Investigated life-time cannabis use b. No effect of cannabis use on primary endpoints of the study	1. ↑ AEA in CSF of FEP vs HS 2. In FEP, positive association between serum levels of AEA and cognitive performances (measure of higher visual information processing) 3. In FEP, no correlation between AEA in CSF and serum 4. =AEA in FEP and serum
<i>Koethe 2018 [92]</i>	Twin pairs discordant for disease status	SCZ: 44.0% F BD: 85.7% F	- Plasma	Among SCZ Mixed typical and atypical APs	SCZ	Clinically stable	a. N= 13 twin pairs reported use of cannabis in past, none met DSM-III-R criteria for abuse or dependence	1. ↑ AEA in plasma of SCZ twins vs HS

	N=50 twins SCZ N=14 twins BD N= 16 twins HS	HS: 37.5% F		Not reported in details.			b. Patients positive to urine drug screening excluded.	2. =AEA in plasma of affected vs non-affected SCZ twins 3. ↑ AEA in plasma of BD twins vs HS 4. ↓ AEA in plasma of non- affected SCZ twins who later will develop the disorder.
<i>Moretti 2018 [79]</i>	N=162 SCZ N=94 HS	SCZ: 35.9% F HS: 43.6% F	- PBMCs	Mixed typical and atypical APs Not reported in details.	SCZ	Clinically stable “Chronic outpatients”	Not reported	1. ↑ CB1 in SCZ vs HS
<i>Wang 2018 [80]</i>	N=115 SCZ N=88 HS	SCZ: 55.7% F HS: 65.7% F	- Serum	N=27 APs- naïve N= 128 APs- free	FEP and SCZ	Acutely ill “Inpatient”	Not reported	1. ↑ AEA in serum of SCZ vs HS 2. =AEA in serum of FEP vs SCZ 3. ↓ AEA in serum of SCZ after 8 weeks of TAU

Legend. AEA: Anandamide; APs: Antipsychotics; DGL: diacylglycerol lipase; FAAH: Fatty Acid Amid Hydrolase; FEP: First Episode Psychosis; HS = Healthy Subjects; NAPE: N-acyl phosphatidylethanolamine phospholipase; PBMCs: Peripheral blood mononuclear cells; Prodromal: Prodromal psychosis; SCZ: Schizophrenia; SUD= Substance Use Disorder
From: Minichino et al. Measuring Disturbance of the Endocannabinoid System in Psychosis: A Systematic Review and Meta-analysis. JAMA Psychiatry. 2019;76(9):914–923.

Chapter 3. Trans-diagnostic relevance of gut dysbiosis and sickness behaviour in severe mental illness and chronic fatigue: a systematic review and meta-analysis

3.1 Introduction

The gut microbiome, an ecosystem of microbial organisms inhabiting the human gut, has been receiving mounting attention as a key regulator of the host's physical and mental health[41]. The genetic repertoire of the gut microbiome is much larger than the human genome and, unlike the latter, it can be modified[40, 100]. This has generated an understandable enthusiasm in the scientific community since the investigation of gut microbe taxonomy and functionality could provide novel therapeutic targets in areas of unmet need. Large-scale initiatives, such as the Human Microbiome Project (HMP)[40], the Flemish gut-flora[101], and the British TwinsUK[102, 103] have characterised typical gut microbiome composition and function in general population cohorts. These data repositories have clarified that each individual's gut microbiome has unique characteristics. However, high level taxonomic features (i.e., microbes composition) tend to be relatively stable over time within participants, with the large majority (~90%) of bacteria belonging to three main phyla: Firmicutes, Bacteroidetes, and Actinobacteria[104].

Research on the bidirectional interplay between the gut microbiome and the host's health has recently demonstrated that the diversity and the balanced representation of microbes within each individual's gut is more relevant for health outcomes than the presence/absence of specific microbes[100]. Reduced microbial diversity ("gut dysbiosis") has been associated with a wide range of detrimental outcomes, including severe mental illness[42]. Based on this evidence, microbial diversity is considered by many as the hallmark of a "healthy" microbiome[105].

Gut dysbiosis can trigger a cascade of events that can influence the host's mental health via a number of biological paths, such as pro-inflammatory responses[43], engagement of the endocannabinoid system (main hypothesis of this thesis) [47], activation of the hypothalamic-pituitary-adrenal axis, and the modulation vagal nerve activity between the brain and the gut[45].

A putative shared starting point for each of these paths is the loosening of gut barrier integrity[46, 106, 107], which has been shown to follow faecal transplant of dysbiotic gut microbiome in germ-free mice[46, 106, 107]. Breakdown of the gut barrier leads to the leakage of bacterial endotoxin (e.g., lipopolysaccharide – LPS) from the gut lumen to the bloodstream (“endotoxemia”)[108]. Experimental models conducted both in animals and in otherwise healthy controls show that endotoxemia manifests with flu-like symptoms, which most authors refer to as “sickness behaviour”[109].

As outlined in **Table 4**, symptoms of sickness behaviour can be considered clinical features of severe mental illness, including schizophrenia (negative symptoms), mood disorders (depressive symptoms) and other presentations, such as chronic fatigue.

Consistent with the trans-diagnostic considerations on negative symptoms outlined in Chapter 1, a recent review nicely summarised findings from neuroimaging and electrophysiological studies investigating symptoms of sickness behaviour (in particular, anhedonia, amotivation and fatigue) in severe mental illness[15]. Diagnostic terminology aside, the evidence clearly points towards a shared neurological vulnerability underlying negative symptoms[110]. The influence of the periphery should also be considered. Meta-analytic evidence shows increased levels of pro-inflammatory cytokines in patients with depression[111], bipolar disorder[112], schizophrenia[29], and chronic fatigue[113] compared to controls. Severity of negative symptoms (in particular the experiential component) has been consistently associated with an increase pro-inflammatory status across severe mental illness[38]. The existence of a shared central and peripheral vulnerability cutting across traditional diagnostic boundaries is not surprising considering the common genetic background highlighted by genome-wide association studies of severe mental illness[114]. These considerations are in line with trans-diagnostic initiative, such as the RDoC.

Based on the aforementioned considerations, in this chapter I present data from a systematic review and meta-analysis aimed at summarising the evidence of gut-dysbiosis in severe mental illness and in chronic fatigue. My hypothesis is that gut dysbiosis manifests with symptoms of sickness behaviour (**Table 4**) trans-diagnostically.

Table 4. Symptoms of sickness behaviour in schizophrenia (negative symptoms), depression, bipolar disorder (depressive symptoms), and chronic fatigue

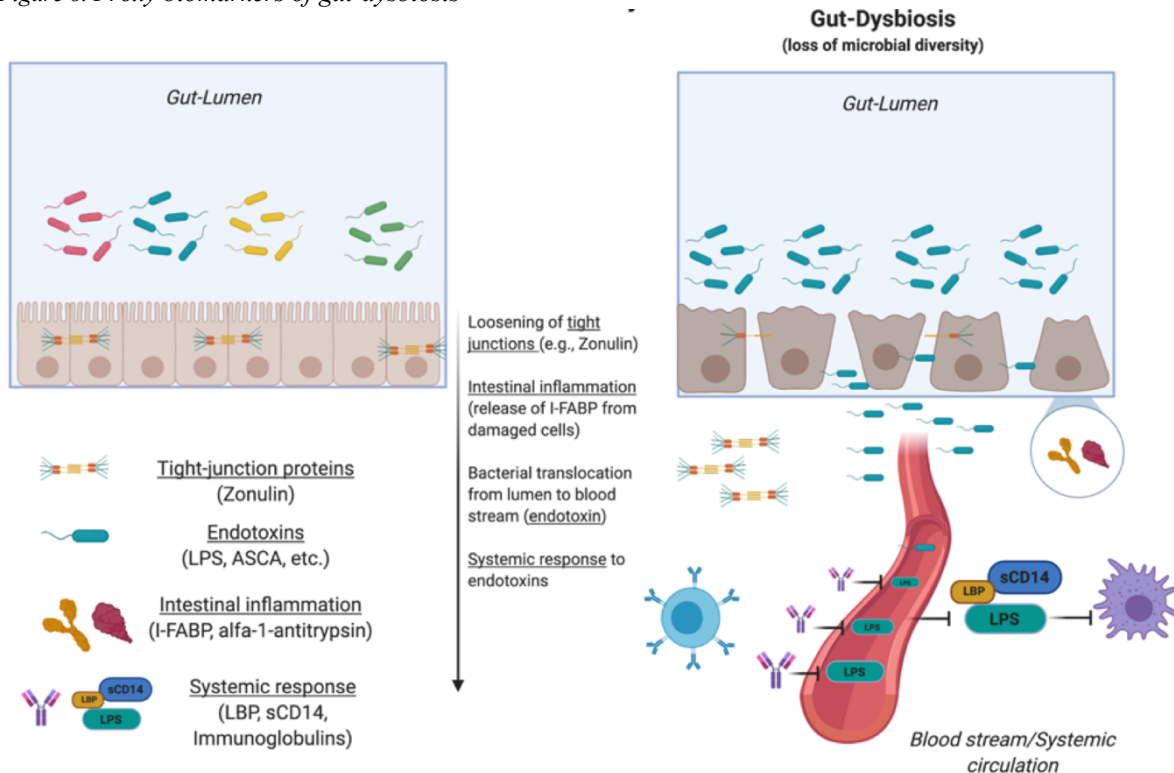
	Sickness Behaviour	Schizophrenia (Negative Symptoms)	Depression and Bipolar disorder (Depressive Symptoms)	Chronic Fatigue Syndrome
Fatigue	X	X	X	X
Impaired concentration	X	X	X	X
Curbing of Interests	X	X	X	X
Diminished social drive	X	X	X	X
Anhedonia	X	X	X	
Psychomotor retardation or agitation	X	X	X	

3.1.1 Biomarkers of gut-dysbiosis

A direct measure of gut dysbiosis can be obtained by investigating the gut microbiome communities in patients compared to controls. However, despite the growing number of studies providing data on gut microbial features in severe mental illness, the evidence is still limited. Furthermore, given the large variability in the research approaches (both experimental and analytical) used to characterise the gut microbiome, it is not currently possible to provide reliable comparisons within and across diseases to identify common microbial effects[101].

To overcome these difficulties, I decided to rely on a well-validated battery of proxy biomarkers of gut-dysbiosis to compare patients and controls within and across severe mental illnesses and in chronic fatigue (**Figure 8**).

Figure 8. Proxy biomarkers of gut-dysbiosis



Reduced microbial diversity (“gut-dysbiosis”) triggers a cascade of events, including loosening of gut-barrier permeability, intestinal inflammation, translocation of bacterial antigens from the gut-lumen to the bloodstream, and a systemic response to endotoxins. Each of these events can be measured with specific biomarkers. In order: tight junction proteins, intestinal fatty acid binding protein (I-FABP), alfa-1-antitrypsin, endotoxins (lipopolysaccharide – LPS; saccharomyces cerevisiae antigens – ASCA), lipopolysaccharide binding protein (LBP), soluble CD14 (sCD14), and immunoglobulins against bacterial endotoxins.

From: Safadi, J.M., Quinton, A.M.G., Lennox, B.R. *et al.* Gut dysbiosis in severe mental illness and chronic fatigue: a novel trans-diagnostic construct? A systematic review and meta-analysis. *Mol Psychiatry* (2021).

3.2 Aims

The aims of this chapter are: (i) to compare differences in proxy biomarkers of gut dysbiosis between patients with severe mental illnesses and chronic fatigue vs controls; and [44] to explored the trans-diagnostic association between these biomarkers and the severity of “sickness behaviour”.

3.3 Methods

In this chapter, I summarise the available evidence on proxy biomarkers of gut dysbiosis in severe mental illness and chronic fatigue through a systematic review and meta-analysis.

The systematic review and meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines [77]. The original protocol was registered in PROSPERO (registration number: CRD42019135329). The search for studies was conducted from inception to April 1, 2020 in Web of Science and PubMed. Inclusion

criteria for full-text analysis were as follows: (i) case-control studies reporting data on proxy markers of gut dysbiosis in patients vs controls; and [44] studies investigating the association of these biomarkers with symptom severity and treatment response. Clinical conditions of interest were: schizophrenia, depression, bipolar disorder, and chronic fatigue. No restrictions were set in terms of duration of illness, illness phase (acute vs stable/remitted), medication status.

The full list of proxy biomarkers of gut dysbiosis was agreed with an expert (Phil Burnet) after a preliminary search of the literature. The list included: tight junction proteins (zonulin, claudin, E-cadherin); intestinal inflammation markers (alfa-1-antitripsin, intestinal fatty acid binding protein – I-FABP; calprotectin); endotoxins; proteins related to the immune response to bacterial antigens (Lipopolysaccharide binding protein – LBP and sCD14); antibodies against bacterial endotoxins.

The entire search process was independently conducted by two collaborators, who also independently extracted data from each included study.

The primary outcome was to compare differences in proxy biomarkers of gut dysbiosis in patients vs controls. The secondary outcome was to explore the association between biomarkers of gut dysbiosis and severity of sickness behaviour across diagnosis (as outlined in **Table 4**).

3.3.1 Statistical analysis

Meta-analytic pooled estimates of the differences in biomarkers between patients and controls were provided when data from two or more studies were available for certain biomarkers. When more than one type of immunoglobulin was reported in one study, we selected for the analysis the immunoglobulin type with the highest affinity for the antigens (IgG > IgA > IgM).

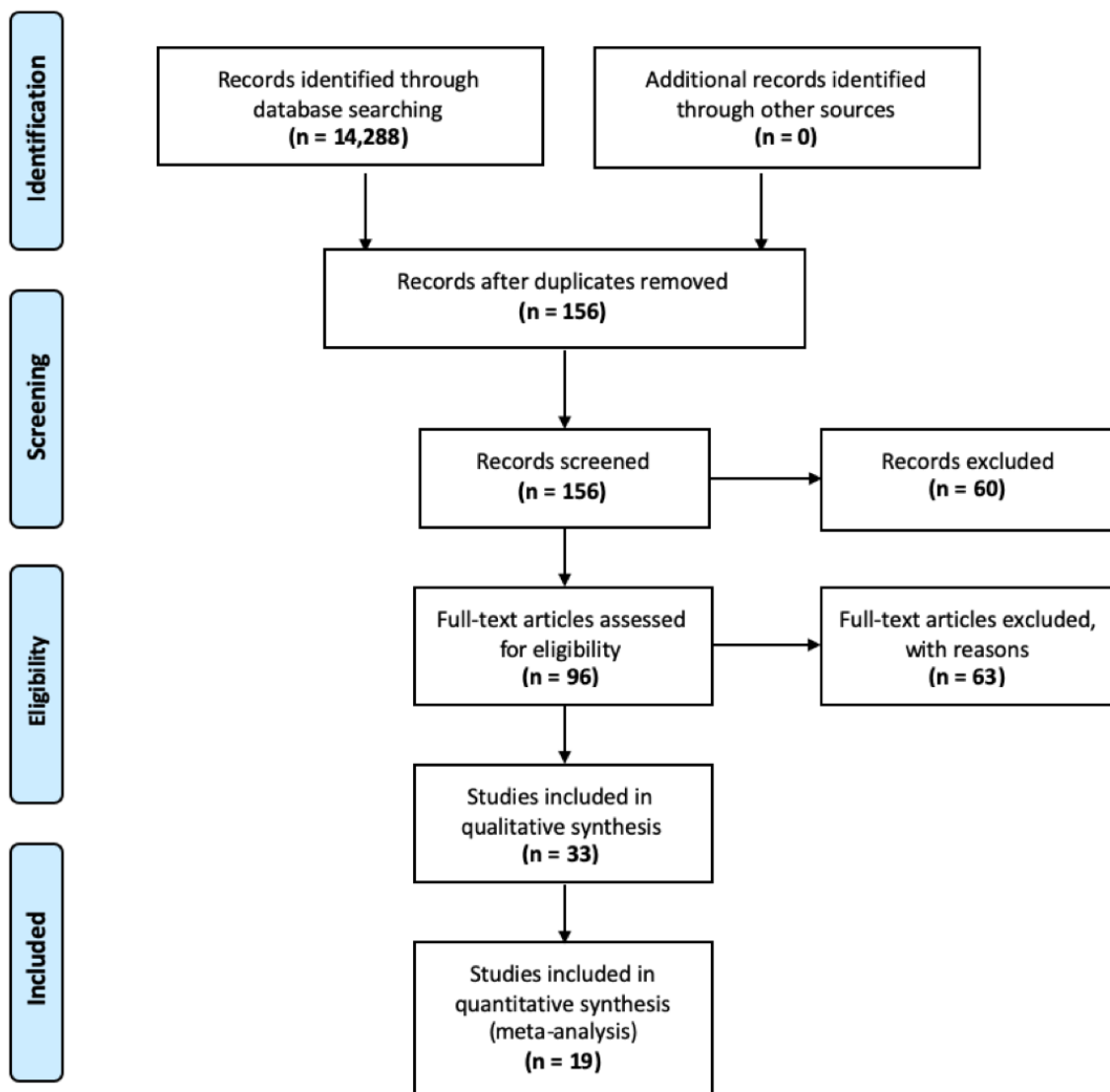
All meta-analyses were conducted in R, version 1.2.5, using random-effect models. Standardised mean difference (SMD) was used as summary statistics; the Higgins I^2 was used to assess between-study heterogeneity. Sensitivity analyses were carried out by sequentially removing single studies and re-running the analyses[78]. When feasible, the effect medication status on meta-analytic findings was explored by comparing differences in medicated and un-medicated patients. The effect of body mass index and smoking status on findings was also investigated. Finally, all the analyses were re-run by removing studies

investigating chronic fatigue, to explore if findings were specific for severe mental illness only.

3.4 Results

As outlined in **Figure 9** (PRISMA flow-chart), thirty-three studies met the inclusion criteria[98, 99, 115-144]. Of these, nineteen provided data for a quantitative synthesis (N=2765 patients and N=1847 healthy controls) on the following biomarkers: zonulin, LPS, LBP, sCD14, antibodies against endotoxins, alfa-1-antitripsin, and I-FABP.

Figure 9. PRISMA flow-chart: gut-dysbiosis and sickness behaviour



3.4.1 Gut dysbiosis biomarkers in patients vs controls

Four studies reported data on circulating levels of zonulin (N=3 investigated depression, N=1 bipolar disorder)[115, 123, 135, 141]. Pooled data from 98 patients and 100 controls showed that zonulin was significantly increased in the blood of patients vs controls (SMD = 0.97; 95% CI = 0.10 to 1.85; $P = 0.03$), with evidence of high heterogeneity ($I^2 = 86.61\%$) (**Figure 10a**). The high heterogeneity was explained by one study that included patients with comorbid anxiety and depression[141]. Removing this study from the analysis resulted in reduced heterogeneity (low: $I^2 = 32.48\%$) and still significant results (SMD = 0.55; 95% CI = -0.12 – 0.97; $P = 0.01$).

Two studies reported data on circulating levels of LPS (N=1 investigated depression, N=1 chronic fatigue)[119, 141]. Pooled data from 71 patients and 66 controls showed increased levels of LPS in patients vs controls (SMD = 0.77; 95% CI = 0.42 to 1.12; $P < 0.01$; $I^2 = 0\%$) (**Figure 10b**). A third study measured LPS in the root canal of patients with depression vs controls and it was excluded from the analysis because of the different measurement approach; results were consistent with the pooled estimate on LPS, showing increased levels of this endotoxin in patients vs controls[120].

Seven studies reported data on circulating levels of antibodies against endotoxins (N=1 investigated a mixed sample of bipolar disorder and depression; N=1 chronic fatigue; N=1 depression; N=1 bipolar disorder; N=3 schizophrenia)[116, 124, 125, 128, 137, 139, 140]. Pooled data from 1104 patients and 603 controls showed increased levels of antibodies against endotoxins in patients vs controls (SMD = 0.99; 95% CI = 0.28 - 1.71; $P < 0.01$), with evidence of high heterogeneity ($I^2 = 97.14\%$) (**Fig 10c**). The high heterogeneity was explained by removing one study[137], with results remaining statistically significant (SMD = 0.67; 95% CI 0.44 - 0.90; $p < 0.01$; $I^2 = 56.11\%$). Three studies (N=2 investigated chronic fatigue; N=1 mixed sample of patients with bipolar disorder and unipolar depression) were not included in the quantitative synthesis on circulating levels of antibodies against bacterial endotoxins because they did not provide data[127, 128, 130]. These three studies all reported findings of increased levels of antibodies against bacterial endotoxins in patients vs controls, in line with the pooled estimate.

Six studies reported data on circulating levels of sCD14 (N=2 investigated a mixed sample of bipolar and schizophrenia patients; N=1 chronic fatigue; N=1 depression; N=1 schizophrenia; N=1 bipolar disorder)[99, 119, 121, 134, 135, 138]. Pooled data from 1234 patients and 962 controls showed increased levels of sCD14 in patients vs controls (SMD = 0.54; 95% CI 0.16

to 0.81; $P < 0.01$), with evidence of high heterogeneity ($I^2 = 90.68\%$) (**Figure 10d**). Removal of single studies from the analysis did not improve heterogeneity.

Two studies reported data on circulating levels of LBP (N=1 investigated depression; N=1 chronic fatigue)[115, 119]. Pooled data from 71 patients and 51 controls showed increased levels of LBP in patients vs controls (SMD = 0.87; 95% CI = 0.25 to 1.48; $P < 0.01$; $I^2 = 56.80\%$) (**Figure 10e**). Two studies investigated LBP levels in patients in prodromal psychosis (N=1) and in bipolar patients (N=1) but were not included in the quantitative synthesis because of lack of extractable data[137, 142]. These two studies reported mixed findings: increased levels of LBP in patients with bipolar disorder[137] and no differences in prodromal psychosis[142], when compared to controls.

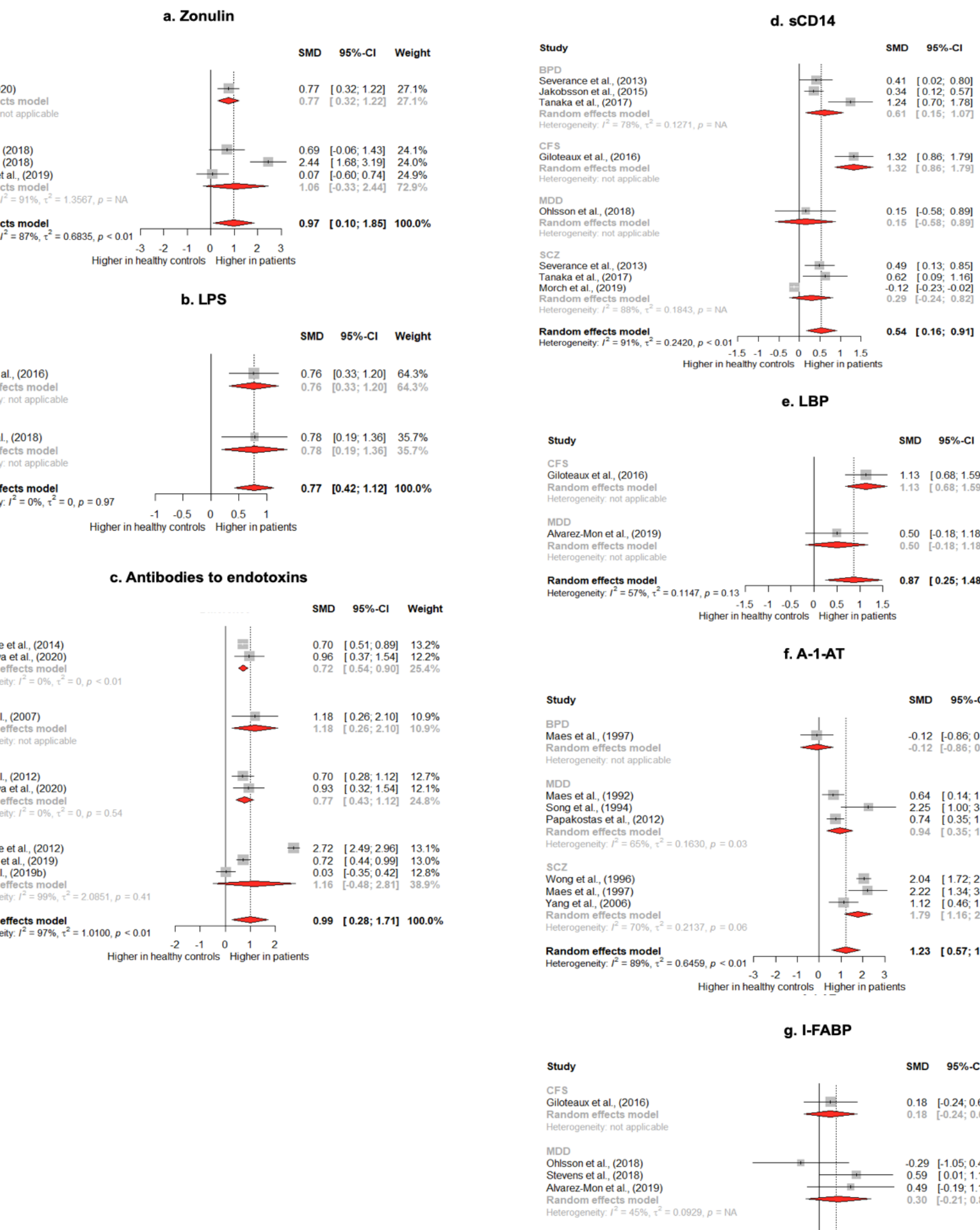
Six studies reported data on circulating levels of alfa-1-antitripsin (N=1 investigated a mixed sample of patients with bipolar disorder and schizophrenia, N=2 schizophrenia; N=3 depression)[123, 129, 136, 143, 144]. Pooled data from 342 patients and 209 controls showed increased levels of alfa-1-antitripsin in patients vs controls (SMD = 1.23; 95% CI = 0.57 to 1.88; $P < 0.01$), with evidence of high heterogeneity ($I^2 = 89.25\%$) (**Figure 10f**). Sensitivity analysis did not improve heterogeneity.

Four studies reported data on circulating levels of I-FABP (N=1 investigated chronic fatigue; N=3 depression)[115, 119, 135, 141]. Pooled data from 101 patients and 92 controls showed a not significant increase in I-FABP between patient vs controls (SMD: 0.27; 95% CI -0.07 to 0.60; $P = 0.12$; $I^2 = 23.05\%$) (**Figure 10g**).

In summary, the pooled estimates showed significantly increased levels of all proxy biomarkers of gut-dysbiosis but I-FABP in patients vs controls. In particular the evidence showed increased levels of tight junction proteins (zonulin, 4 studies); bacterial endotoxins (LPS, 2 studies); intestinal inflammation (alfa-1-antitripsin, 7 studies); systemic response to bacterial endotoxins (LBP, 2 studies; sCD14, 8 studies; antibodies against endotoxins, 8 studies). Sensitivity analyses excluding data obtained from patients with chronic fatigue showed no significant differences with the overall pooled estimates.

Medication status did not influence findings. The majority of studies corrected their findings for BMI and smoking status.

Forest plot of standardised mean difference (SMD) in biomarkers of gut dysbiosis in patients vs controls



3.4.2 Association between gut dysbiosis biomarkers and sickness behaviour

Fourteen studies reported data on the association between biomarkers of gut dysbiosis and symptoms of sickness behaviour[98, 99, 120, 124, 125, 128-132, 134, 135, 139, 140]. Of these, seven studies investigated the association with negative symptoms of schizophrenia[98, 99, 124, 130-132, 134], five investigated the association with depressive symptoms in depression[120, 129, 130, 135, 140], four investigated the association with depressive symptoms in bipolar disorder[99, 130, 139, 140] and two investigated the association with fatigue in chronic fatigue[128, 133].

As summarised in **Table 5**, four studies reported a significant association between greater levels of gut dysbiosis biomarkers (in particular, tight junction proteins, endotoxins, antibodies against endotoxins) and severe and persistent negative symptoms in schizophrenia (deficit schizophrenia)[124, 131, 132, 140]; this association was not found in three studies[98, 99, 134], where patients displayed less severe and not persistent symptoms. The large majority of studies investigating mood disorders (six out of seven) reported a significant association between greater levels of gut-dysbiosis biomarkers (in particular, intestinal inflammation markers, endotoxins, antibodies against endotoxins) and severity of depressive symptoms[99, 120, 129, 130, 135, 139, 140].

The only two studies investigating chronic fatigue both found a significant association between greater levels of gut dysbiosis biomarkers (in particular, antibodies against endotoxins) and severity of fatigue[128, 133].

Table 5. Association between proxy markers of gut dysbiosis and severity of sickness behaviour in of schizophrenia, bipolar disorder, major depression and chronic fatigue.

Markers	Chronic fatigue (fatigue)	Schizophrenia (negative symptoms)	Bipolar disorder (depressive symptoms)	Depression (depressive symptoms)
Tight junction proteins		++ [131, 132]		
Circulating endotoxin				+ [120]
sCD14		= = [99, 134]	= [99]	
Antibodies against bacterial endotoxins	++ [128, 133]	= [98] +++ [124, 132, 140]	= [140] ++ [130, 139]	++ [130, 140]
Intestinal inflammation				++ [129, 135]

The association between higher levels of gut-dysbiosis biomarkers severity of symptoms was significant (+) or not significant (=). The colour green highlights instances where the majority of the evidence is in favour of a significant association.

From: Safadi, J.M., Quinton, A.M.G., Lennox, B.R. *et al.* Gut dysbiosis in severe mental illness and chronic fatigue: a novel trans-diagnostic construct? A systematic review and meta-analysis. *Mol Psychiatry* (2021).

A qualitative summary of the included studies is reported in **Table 6**.

3.5 Discussion

Data reported in this chapter represents the first attempt to summarise findings of gut dysbiosis across severe mental illness and chronic fatigue. The main finding was of increased gut dysbiosis biomarkers in severe mental illness and chronic fatigue, independent of medication status. The increase in gut dysbiosis biomarkers was associated with more severe symptoms of sickness behaviour in schizophrenia (negative symptoms), bipolar disorder and depression (depressive symptoms), and chronic fatigue (fatigue).

The first pooled estimate showed increased circulating levels of zonulin in patients vs controls (**Fig. 10a**). Zonulin is a tight-junction protein, and its circulating levels can rise as a consequence of loss of integrity of the gut barrier, a phenomenon shown to be triggered by gut-dysbiosis[145]. Increased circulating zonulin is also considered by some authors as a proxy of increased permeability of all biological barriers, including the blood brain barrier[146, 147]. In either case, the loss of the protective function of any physical barrier would allow the passage of molecules with direct (e.g., neurotransmitter derivatives) or indirect (e.g., pro-inflammatory triggers) neural effects, with downstream consequences on the host's mental health[146, 147].

In line with these considerations, the endotoxin LPS was increased in patients vs controls. Endotoxins such as LPS are key cell wall components of pathogenic bacteria and under normal physiological conditions are confined within the gut lumen. Increased circulating levels of LPS are considered a sign of both gut dysbiosis (predominance of gram-negative bacteria among gut-microbes) and gut leakage (translocation of bacterial material from the gut to the blood stream)[147]. In the blood, LPS induces a peripheral pro-inflammatory response, which is a consistent finding across severe mental illness and chronic fatigue[147]. The activation of a pro-inflammatory response to bacterial endotoxins in these disorders is clearly demonstrated by the pooled estimates on LBP, sCD14, and immunoglobulins against endotoxins. All the biomarkers investigating the systemic response to gut dysbiosis were

increased in patients vs controls. The LBP/sCD14 complex is released by monocytes as part of the innate immune system's response to LPS/endotoxin stimuli (**Fig. 8**)[148]. When LPS binds to LBP/sCD14 complexes on the monocyte surface, this leads to the release of proinflammatory cytokines[148], which meta-analyses have shown to be increased in severe mental illness and chronic fatigue[32, 111]. The increase in circulating levels of immunoglobulin against endotoxin in patients compared to controls suggests an engagement of the adaptive immune system. The gut microbiome is involved in the maturation of the immune system and in the development of tolerance mechanisms (self vs non-self antigens recognition)[149]. Gut dysbiosis can trigger dysfunctional mechanisms related to molecular mimicry and cross-reactivity towards neural targets, as hypothesised by a number of authors and supported by consistent evidence[150].

The pooled estimate on intestinal inflammation markers showed conflicting findings, with increased levels of alfa-1-antitripsin, but not I-FABP, in patients compared to controls. In contrast to alfa-1-antitripsin, I-FABP is uniquely localised in the gut[44, 151]. It is possible that these findings reflect a systemic, rather than a local (gastro-intestinal), engagement of the immune system. This hypothesis is in line with the pooled estimate on LBP, sCD14 and immunoglobulins.

Finally, the current investigation demonstrated that biomarkers of gut dysbiosis were associated with symptoms of sickness behaviour in severe mental illness and chronic fatigue. This finding is consistent with my hypothesis, which was based on experimental models of endotoxemia[109]. In these models the chronic pro-inflammatory status that follows bacterial translocation results in a whole-body response that manifests with sickness behaviour in the attempt to conserve energy and recover[109]. Symptoms of sickness behaviour (**Table 3**) manifest in severe mental illness and receive a different label (negative symptoms; depressive symptoms; fatigue) depending on the diagnostic category where they present in (schizophrenia, mood disorders, chronic fatigue). Gut dysbiosis might therefore underlie these symptoms and represent a putative target for trans-diagnostic interventions.

3.5.1 Limitations

Limitations of this systematic review and meta-analysis were: 1) the use of indirect markers of gut dysbiosis; reasons for this approach are outlined in section 3.1; 2) for a number of biomarkers, but sCD14 and immunoglobulins, data were available for only two or three diagnostic categories, therefore, trans-diagnostic considerations on these biomarkers need

replication; 3) none of the studies provided a direct measure of “sickness behaviour”; differences in clinical scales used to measure negative symptoms, depression, and fatigue could explain the high heterogeneity observed in some of the pooled estimates; 4) recent evidence has put into question the reliability of zonulin as a biomarker of intestinal permeability because of inadequacy of commercially available enzyme assays. However, our results rely on multiple biomarkers of gut dysbiosis, reducing the possibility of biased interpretations.

3.5.2 Conclusion

Biomarkers of gut dysbiosis are increased in severe mental illness and chronic fatigue, independent of medication status. This increase was positively associated with symptoms of sickness behaviour across diagnostic categories. Diagnostic terminology aside, symptoms of sickness behaviour clearly overlap with descriptions of negative symptoms in schizophrenia, depressive symptoms in bipolar disorder and depression, and fatigue in chronic fatigue. Independent of the diagnostic categories, these symptoms clearly fall within the scope of the RDoC Positive Valence System, as described in Chapter 1.

Based on the evidence presented in this chapter, it is possible to hypothesise that gut dysbiosis might underlie symptoms related to the RDoC Positive Valence System and represent a new target for trans-diagnostic interventions in an area of unmet need.

Table 6. Qualitative summary of findings by diagnostic category – gut-dysbiosis and sickness behaviour

Study	Subjects	Gender (%F)	Markers	Stage	Phase	Main Findings
Schizophrenia						
<i>Wong, CT</i> 1996 [143]	SCZ (Cohort 1) = 98 SCZ (Cohort 2) = 50 HC = 41	SCZ: 0% HC: 0%	1. A-1-AT (Blood)	Mixed	Mixed	1. ↑ A-1-AT in SCZ vs. HC
<i>Maes, M</i> 1997 [123]	SCZ = 27 HC = 21	Not reported	1. A-1-AT (Blood)	Mixed/Unclear	Mixed/Unclear	1. = A-1-AT in SCZ vs. HC
<i>Yang, Y</i> 2006 [144]	SCZ = 22 HC = 20	SCZ: 0% HC: 0%	1. A-1-AT (Blood)	Mixed/Unclear	Mixed/Unclear	1. ↑ A-1-AT in SCZ vs. HC 2. No change in patient levels after 2 months of treatment with Clozapine

<i>Severance, EG</i> 2012 [137]	SCZ = 363 HC = 207	SCZ: 32.7% HC: 72.9%	1. ASCA (Blood)	Mixed	Mixed	1. ↑ ASCA in SCZ vs. HC
<i>Severance, EG</i> 2013 [138]	SCZ = 141 HC = 78	SCZ: 39.7% HC: 71.8%	1. sCD14 (Blood) 2. LBP (Blood)	Unclear	Unclear	1. ↑ sCD14 in SCZ vs. HC 2. = LBP in SCZ vs. HC
<i>Dickerson, F</i> 2016 [98]	SCZ = 249 FEP = 79 HC = 260	SCZ: 31% FEP: 34% HC: 61%	1. ASCA (Blood)	Multi-episode SCZ and FEP	Mixed	1. ↑ ASCA in SCZ vs. HC 2. ↓ ASCA in FEP vs. HC
<i>Dickerson, F</i> 2017 [118]	Patients = 210 [SCZ = 90 BPD = 72 MDD = 48] HC = 72	Patients: 46.6% HC: 64%	1. IgA-ASCA (Blood) 2. IgA-LPS (Blood)	Mixed/Unclear	Mixed/Unclear	1. ↑ ASCA-IgG in rSA vs. HC 2. ↑ LPS-IgA in rSA v. HC

<i>Weber, NS</i> 2018 [142]	SCZ = 80 HC = 80	SCZ: 15% HC: 15%	1. sCD14 (Blood) 2. LBP (Blood)	“Early stages”	Outpatient	1. ↑ sCD14 in SCZ v. HC 2. = LBP in SCZ v. HC
<i>Delaney, S</i> 2019 [117]	SCZ = 42 CHR = 17 HC = 33	SCZ: 36% CHR:35% HC: 57.5%	1. IgA to LPS (Blood) 2. IgG to LPS (Blood) 3. IgM to LPS (Blood)	Mixed/Unclear	Mixed/Unclear	1. = IgA, IgG, and IgM in SCZ v. HC 2. = IgA, IgG, and IgM in CHR v. HC
<i>Ciháková, D</i> 2019 [116]	SCZ = 160 HC = 80	SCZ: 38% HC: 36%	1. ASCA IgA (Blood) 2. ASCA IgG (Blood)	Mixed/Unclear	Mixed/Unclear	1. ↑ ASCA IgG in SCZ v. HC
<i>Mørch, RH</i> 2019 [134]	SCZ = 675 HC = 647	SCZ: 39.85% HC: 46.83%	2. sCD14 (Blood)	Mixed/Unclear	Mixed	1. ↓ sCD14 in SCZ v. HC
<i>Maes, M</i> 2019a [124]	SCZ = 80 [n = 40 deficit SCZ] HC = 38	Not Reported	1. IgA gram-negative bacteria 2. IgM gram-negative bacteria	Mixed/Unclear	Outpatients	1. ↑ IgA to 4 gram-negative bacteria in Deficit SCZ v. Non-Deficit SCZ and HC

						2. ↑ IgM to 5 gram-negative bacteria in deficit SCZ v. Non-deficit SCZ and vs HC
<i>Maes, M 2019b [132]</i>	SCZ = 78 HC = 40	SCZ: 46.8% HC: 75%	1. IgM zonulin 2. IgM occludin 3. IgM e-cadherin 4. IgA gram-negative bacteria	Multi-episode	Mixed/Unclear	1. ↑ IgM to zonulin in SCZ vs. HC 2. ↑ IgM to occludin in deficit vs. non-deficit SCZ and HC
<i>Maes, M 2019c [131]</i>	SCZ = 79 HC = 40	PT: 46.8% HC: 75%	1. IgA Tight Junctions (TJ) + Adherence Junctions (AJ)(Blood) 2. e-Cadherin, occludin, claudin-5, beta-catenin] 3. IgA gram-negative (blood)	Mixed/Unclear “stabilized phase of illness”	Mixed/Unclear	1. ↑ IgA to TJ/AJs in deficit vs. non-deficit SCZ and HC 2. ↑ IgA to gram-negative bacteria in deficit vs. non-deficit SCZ 3. ↑ IgA occludin in deficit vs. non-deficit SCZ

Bipolar Disorder						
<i>Maes, M</i> 1997 [123]	BPD = 23 HC = 21	Not reported	1. A-1-AT (blood)	Mixed/Unclear	Mixed/Unclear	1. = levels of A-1-AT in BPD vs. HC
<i>Severance, EG</i> 2013 [138]	BPD = 75 HC = 78	BPD: 69.3% HC: 71.8%	1. sCD14 (Blood) 2. LBP (Blood)	Unclear	Unclear	1. ↑ sCD14 in BPD vs. HC 2. = LBP in BPD vs. HC 3. ↑ LBP in BPD vs. SCZ
<i>Severance, EG</i> 2014 [139]	BPD = 264 HC = 207	BPD: 69.7% HC: 72.9%	1. IgG-ASCA (Blood)	Mixed/Unclear	Outpatients	1. ↑ ASCA IgG in BPD vs. HC
<i>Jakobsson, J</i> 2015 [121]	BPD = 221 HC = 112	BPD: 62% HC: 55%	1. sCD14 (blood)	Mixed/Unclear	Outpatients	1. ↑ sCD14 in BPD vs. HC
<i>Tanaka, T</i> 2017 [99]	BPD=32 HC = 32	BPD: 56% HC: 56%	1. sCD14 (blood)	Mixed/Unclear	Outpatients	1. ↑ sCD14 in BPD vs. HC

<i>Kılıç, F.</i> 2020 [122]	BPD = 41 HC = 41	BPD: 56.1% HC: 48.7%	1. Zonulin (Blood) 2. Claudin-5 (Blood)	Mixed/Unclear	Mixed/Unclear	1. ↑ Zonulin in BPD v. HC 2. ↑ Claudin-5 in BPD v. HC
Depression						
<i>Maes, M</i> 1992 [129]	MDD = 42 HC = 26	Not reported	1. A-1-AT (Blood)	Multi-episode	Mixed/Unclear	1. ↑ A-1-AT in MDD vs HC

Maes, M 1997 [123]	MDD = 29 HC = 21	Not Reported	1. A-1-AT (blood)	Mixed/Unclear	Mixed/Unclear	1. = levels of A-1-AT in MDD vs. HC
Papakostas, GI 2013 [136]	<u>Pilot Study</u> MDD = 36 HC = 43 <u>Replication Study</u> MDD = 34 HC = 43	<u>Pilot Study:</u> MDD: 36.1% HC: 67.4% <u>Replication Study:</u> MDD: 55.8% HC: 67.4%	1. A-1-AT (Blood)	Multi-episode	Mixed/Unclear	1. ↑ A-1-AT in MDD patients v. HC (both pilot and replication study)

<i>Maes, M</i> 2013 [125]	MDD = 113 HC = 28	MDD: 52.2% HC: 67.9%	1. IgM to LPS (Blood) 2. IgA to LPS (Blood)	Mixed/Unclear	Outpatients	1. ↑ IgM and IgA to LPS in MDD v. HC
<i>Stevens, BR</i> 2018 [141]	MDD/ANX = 22 HC = 28	Not reported	1. LPS (Blood) 2. Zonulin (Blood) 3. FABP2 (Blood)	Mixed/Unclear	Mixed/Unclear	1. ↑ LPS in MDD/ ANX vs. HC 2. ↑ Zonulin in MDD/ ANX vs. HC 3. ↑ FABP2 in MDD/ ANX vs. HC
<i>Gomes, C</i> 2018	MDD = 24	Not reported	1. LPS (root canal)	Mixed/ Unclear	Outpatients	1. ↑ root canal LPS MDD vs. HCC

[120]	HC = 23					2. ↑ root canal LPS associated with depression symptom severity
Ohlsson, L 2019 [135]	MDD = 13 Mixed (recent Suicide Attempt - SA) = 54 HC = 17	noSA MDD: 53.8% rSA: 55.5% HC: 47.1%	1. Zonulin (Blood) 2. I-FABP (Blood) 3. sCD14 (Blood)	Mixed/Unclear	Mixed/Unclear	1. ↓ Zonulin in rSA v. HC 2. ↑ I-FABP in rSA v. HC 3. = Zonulin, I-FABP, and sCD14 in MDD v. HC
Alvarez-Mon, 2019 [115]	MDD = 22 HC = 14	MDD: 59.1% HC: 57.1%	1. LBP (Blood) 2. Zonulin (Blood) 3. I-FABP (Blood)	Mixed/Unclear	1. Outpatient	1. ↑ LBP in MDD v. HC 2. ↑ I-FABP in MDD v. HC 3. = Zonulin in MDD v. HC
Maes, M 2019d [130]	MOOD = 96 [27 BPD1, 25 BPD2, 44 MDD] HC = 22	MOOD: 46.8% HC: 36.3%	1. IgM/IgA gram-negative bacteria (Blood)	Multi-episode	Outpatient	1. ↑ IgM/IgA to gram-negative bacteria in MOOD patients vs. HC

<i>Simeonova, D.</i> 2020 [140]	MDD = 44 HC = 30	MDD: 47.7% HC: 66.6%	1. IgM/IgA to gram-negative bacteria (Blood)	Multi-episode	Mixed/Unclear	1. ↑ IgM/IgA in MDD v. HC
Chronic Fatigue						
<i>Maes, M</i> 2007 [128]	CFS = 15 HC = 11	CFS: 66.7% HC: 72.7%	1. IgM to LPS* (Blood) 2. IgA to LPS* (Blood) *LPS from 7 different bacterial strains	Not reported	Outpatient	1. ↑ IgA LPS in all 7 bacteria in CFS v. HC 2. ↑ IgM LPS in 3/7 bacteria in CFS v. HC
<i>Maes, M</i> 2008 [126]	CFS = 41	CFS: 82.9%	1. IgM LPS (Blood) 2. IgA LPS (Blood)	Not reported	Outpatient	1. ↓ in IgM and IgA to LPS post- NAIOS treatment 2. ↓ in FFS symptoms post- NAIOS

						treatment, especially in younger patients with shorter DOI
<i>Maes, M</i> 2012 [133]	CFS = 90 CF, un-diagnosed = 31	CFS = 83.3% CFS = 71%	1. IgA LPS (Blood) 2. IgM LPS (Blood)	N/A	Mixed/Unclear	1. ↑ IgM and IgA LPS in CFS v. CF
<i>Maes, M</i> 2014 [127]	CFS = 90 CF, un-diagnosed = 31	CFS: 80.9% CF: 73.5%	1. IgM LPS (Blood) 2. IgA LPS (Blood)	N/A	Mixed/Unclear	1. ↑ levels of IgM for LPS in CFS patients v. CF patients 2. ↑ levels of IgA for LPS in patients v. CF patients
<i>Giloteaux, L</i> 2016 [119]	CFS = 49 HC = 39	CFS: 77.6% HC: 76.9%	1. LPS (Blood) 2. I-FABP (Blood) 3. sCD14 (Blood) 4. LBP (Blood)	N/A	Mixed/Unclear	1. ↑ LPS in CFS vs. HC 2. = I-FABP in CFS v. HC 3. ↑ levels of sCD14 in CFS v. HC 4. ↑ levels of LBP in CFS v. HC

Legend: ↑, significantly higher; ↓, significantly lower; =, no significant difference; A-1-AT, alpha-1-antitrypsin; ANX, anxiety disorder; BPD, bipolar disorder; CF, formally undiagnosed chronic fatigue; CFS, chronic fatigue syndrome; FEP, First Episode Psychosis; HC, healthy controls; I-FABP, intestinal fatty-acid binding protein; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; MDD, major depressive disorder; noSA, no suicide attempt; PT, patients; rSA, recent suicide attempt; SCZ, schizophrenia
From: Safadi, J.M., Quinton, A.M.G., Lennox, B.R. *et al.* Gut dysbiosis in severe mental illness and chronic fatigue: a novel trans-diagnostic construct? A systematic review and meta-analysis. *Mol Psychiatry* (2021).

Chapter 4. The gut-microbiome-endocannabinoid axis and negative symptoms: results from a general population study

4.1 Introduction

The meta-analytic evidence presented in Chapter 2 and 3 advocates for the existence of endocannabinoid system abnormalities and gut dysbiosis in severe mental illness. It also suggested that reduced circulating endocannabinoids and increased levels of gut dysbiosis biomarkers were associated with more severe negative symptoms across diagnostic boundaries. In this chapter I will now aim to bring these two systems together and explore the interaction between the two, and relevance for mental health.

4.1.1 The gut microbiome-endocannabinoid axis

The gut microbiome and the endocannabinoid system are not isolated systems[47]. Recent evidence on gastrointestinal and cardiometabolic illness, highly comorbid with mental health disorders, has shown that gut dysbiosis is one of the strongest triggers for the activation of the endocannabinoid system[60, 61, 63].

Around 10% of the intestinal transcriptome is regulated by the gut microbiome[152]. Among the genes that are mostly affected by the gut microbiome are *cnr1* and *cnr2*, which codify for the cannabinoid receptors 1 (CB1R) and 2 (CB2R), respectively[47]. In the gastrointestinal tract, the endocannabinoid system has a “gate-keeper” function and its activation is believed to provide regulatory feedback to “gate-opener” stimuli[47].

Increased expression of CB1R receptors in the colon has been shown to follow antibiotic-induced gut dysbiosis and to reverse abnormalities in gut-dysbiosis biomarkers (i.e., to restore the localisation of tight-junction proteins and to reduce circulating levels of lipopolysaccharide)[153]. The endocannabinoid mediators anandamide (AEA) and 2-arachydonoilglycerol (2-AG) are released upon gut dysbiotic triggers and have been shown to regulate gut barrier functions in a CB1R-dependent manner[154].

Despite the growing amount of separate investigations on the gut-microbiome and the endocannabinoid system in mental health, there is a key gap in knowledge about their interaction.

As outlined in Chapter 2 and 3, gut dysbiosis and a dysfunctional endocannabinoid system were independently associated with severity of negative symptoms in psychotic and severe mental illness. This is not surprising considering that: (i) delta-9-tetrahydrocannabinol (THC) can cause an anhedonic/amotivational syndrome in consumers, which can be prevented by cannabidiol (CBD)[54, 55]; and gut dysbiosis can cause similar features (flu-like/sickness behaviour symptoms)[109].

CBD inhibits the degradation of the main endogenous cannabinoid agonists, AEA and 2-AG, which have well-known anti-(neuro)inflammatory and anti-(neuro)oxidative central properties[54, 56]. Endocannabinoids are mentioned among the molecular targets of the RDoC Positive Valence System as they are believed to play a central role in the pathogenesis of anhedonia/amotivation[48].

Palmitoylethanolamide (PEA) is considered the endogenous equivalent of CBD, as it shares a similar pharmacodynamic (i.e., inhibition of endocannabinoid mediators degradation)[155].

A recent randomised trial in patients with depression showed that the oral supplementation of PEA have a beneficial effect on anhedonia and amotivation[156]. Increased circulating levels of PEA are believed to have anti-depressant effect by enhancing the activity of the endogenous endocannabinoid mediators[157, 158]. Contrarily, increased stool PEA levels suggest increased excretion, with loss of the enhancing protective mechanisms via endocannabinoid mediators.

Blood and stool levels of PEA are influenced by the gut microbiome composition and this might have a downstream effect on the host's mental health[159, 160].

4.1.2 Hypothesis and theoretical model

In this chapter, I present the results of a data investigation on a large population cohort (TwinsUK). The TwinsUK cohort collected longitudinal measures of gut microbiome composition, serum and stool levels of PEA and measures of anhedonia/amotivation from a large sample of volunteer twins.

Using this well-validated cohort[102, 103], I tested the hypothesis that altered gut microbiome composition leads to anhedonia/amotivation via the endocannabinoid system.

In particular, based on the aforementioned considerations, I expected that reduced gut microbiome diversity (gut dysbiosis) would lead to either reduced circulating levels of PEA

or increased stool levels of PEA, which in turn would lead to more severe anhedonia/amotivation.

I then conducted exploratory analyses to test if any specific gut microbiome taxa, net of gut microbial diversity, could explain these associations.

4.1.2 Negative symptoms in the general population

Anhedonia and amotivation are debilitating symptoms, highly prevalent in the general population (prevalence rates ranging from 15 to 20%)[161]. As outlined in Chapter 1 and 3, anhedonia and amotivation represent key and early signs of a number of clinical conditions (psychosis, depression, substance misuse, chronic fatigue, and dementia).

Therefore, investigation of biological correlates of these symptoms in the general population might offer unique insight to inform on treatment breakthroughs for a range of clinical conditions.

4.2 Aims

In this chapter I wanted to investigate the relationship between gut dysbiosis, endocannabinoid system, and negative symptoms in a general population cohort. In particular, I wanted to test the hypothesis that the endocannabinoid system mediates the association between gut-dysbiosis and negative symptoms.

4.3 Methods

4.3.1 Study population: TwinsUK registry

To test my hypothesis I used data from the TwinsUK cohort.

TwinsUK is a British registry that has collected health-related data from over 14,000 volunteer twins (55% monozygotic and 45% dizygotic) over the past twenty years. A large proportion of the TwinsUK sample is represented by middle-aged women. This reflects the original aim of the registry, i.e., to investigate osteoarthritis and osteoporosis. However, over the past decade the registry expanded its interest to a wider range of health outcomes, including mental health. Volunteer twins are asked to provide biological samples, which has allowed the collection of gut microbiome data as well as serum and faecal metabolites, including the endocannabinoid analogue PEA.

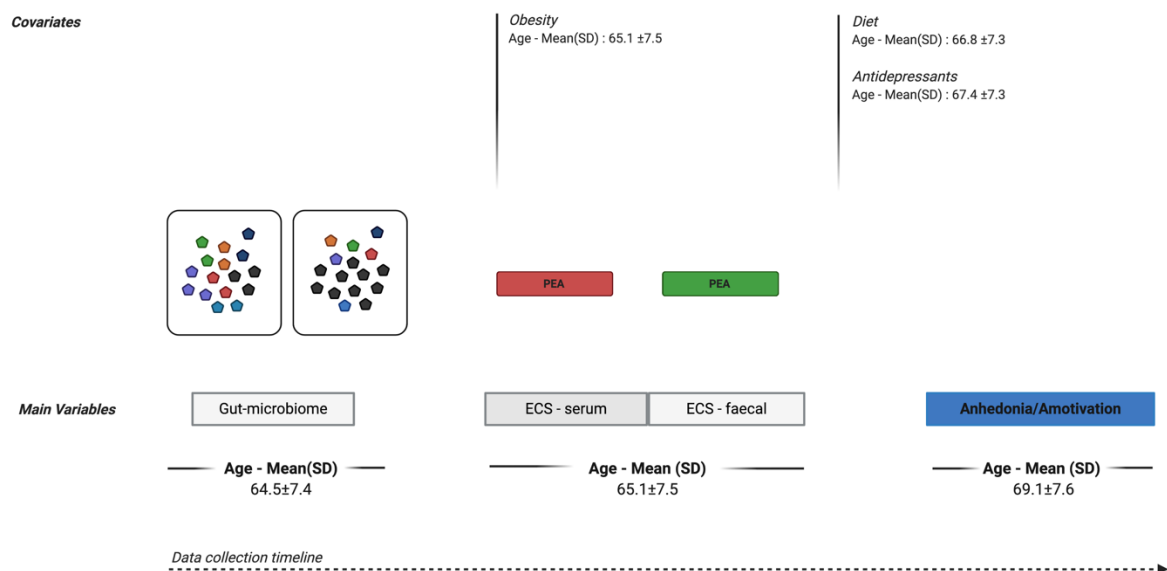
The study analytic sample consisted of 786 twins, which was based on the availability of PEA.

TwinsUK received ethics approval and all participants provided informed consent (REC Reference No.: EC04/015).

4.3.2 Measures

The TwinsUK registry collected single-time measures of gut microbiome, serum and faecal PEA, and anhedonia/amotivation. As outlined in **Figure 11**, data collection was spread over the course of about five years.

Figure 11. Data collection timeline in Twins UK



Single measures of gut-microbiome were collected first, followed by measures of endocannabinoid metabolites, followed by measures of anhedonia/amotivation. Single measures of covariates were also collected over time, as outlined in the figure.

From : Minichino A, Jackson M, Francesconi M, Steves C, Menni C, Burnet PWJ, Lennox BR. The endocannabinoid system mediates the association between gut microbiome diversity and anhedonia/amotivation in a general population cohort. *Molecular Psychiatry*. Accepted, in Press

Gut microbiome data was obtained from stool samples, which were collected at home by each participant. These samples were then either shipped by mail or brought in person to the main research facility (Department of Twins Research and Genetic Epidemiology, King's

College London), where they were stored frozen at -80°C. After storage, stool samples were sent to Cornell University for analysis. The gut microbiome analytic pipeline followed a standard procedure: 1) bacterial DNA extraction from stool samples; 2) targeted amplification of the V4 region of the 16S rRNA gene (see **Table 7** for glossary and definitions); 3) sequencing using the Illumina MiSeq platform[162, 163]. Sequencing data were screened by TwinsUK bioinformatics to remove chimeric sequences generated during library preparation before carrying out de novo clustering of remaining reads into operational taxonomic units (OTUs) at 97% identity.

Table 7. Definition of common terms related to microbiome research

Term	Definition
Microbiome	Refers to the taxonomic and functional properties of the microbial ecosystem
Microbiota	Refers to the taxonomic properties of the microbial ecosystem
16S	Refers to 16S rRNA, a gene containing highly conserved regions suitable for PCR primer design
V4 region	Highly conserved region of the 16S rRNA used for PCR amplification and taxonomic classification
OTU	Refers to any analytical unit (Operational Taxonomic Unit) created by grouping genetically related microbes. It is used to group 16S rRNA gene sequencing reads into taxonomic unit to reduce data dimensionality. An OTU is conventionally defined as containing sequences that are no more than 3% different from each other
Alpha Diversity	Refers to the ecological diversity within a microbiome sample. It gives an indication on how many different taxa are observed (richness) and how comparable their abundances are (evenness)
Beta Diversity	Refers to the ecological diversity between two microbiome samples. It is usually quantified using principle co-ordinate analyses, which allow to identify clustering of multiple microbiome samples
Taxonomy (microbiome)	Hierarchical classification of microbes based on 16S rRNA sequence similarity. Phylum (sequence similarity greater $\geq$ 80%) ↓ Family

	(sequence similarity $\geq 90\%$) ↓ Genus (sequence similarity $\geq 95\%$) ↓ Species/OTU (sequence similarity $\geq 97\%$)
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Starting from the raw OTU counts, I calculated the Shannon index, a measure of within-individual diversity (alpha diversity), and I extracted the first five axes of principal coordinate analysis of the beta diversity (between sample distances) (Weighted and Unweighted UniFrac metrics) to represent between-individual differences in overall microbiome composition. OTU taxonomy was assigned by alignment to the Greengenes database (v13_8) and log transformed (following addition of a pseudo count of 10^{-6}) relative abundances of aggregated counts were used when modelling individual taxa.

PEA concentrations in faeces and serum were obtained using an untargeted LC/MS platform by Metabolon Inc[164].

Anhedonia/amotivation was measured as the sum of a subgroup of seven items from the the Hospital Anxiety and Depression Scale (HADS) (**Table 8**), a 14 items self-reported questionnaire sent to the TwinsUK cohort in 2017. The use of these seven items as a measure of anhedonia/amotivation has been validated by previous factor analyses[165]. The sum obtained from the other 7 items of the HADS is a well-validated measure of anxiety[165].

Table 8. List of items used to assess anhedonia/amotivation – Hospital Anxiety Depression Scale (HADS)

HADS Items	
Item 2	I still enjoy the things I used to enjoy/ past week
Item 4	I can laugh and see the funny side of things/ past week
Item 6	I feel cheerful/ past week
Item 8	I feel as if I am slowed down/ past week
Item 10	I have lost interest in my appearance/ past week
Item 12	I look forward with enjoyment to things/ past week
Item 14	I can enjoy a good book or radio or TV programme/ past week

Based on previous evidence that showed an impact on primary measures (gut microbiome, endocannabinoids, anhedonia/amotivation), I selected the following covariates: age, gender, obesity, unhealthy diet, use of antidepressants, and technical confounders (microbiome collection method: post or visit, operator for microbial pre-processing, storage time of

samples for PEA analysis in fridge and freezer).

A BMI ≥ 30 indicated obesity. Unhealthy diet was measured with a validated index (healthy eating index, HEI). A HEI < 60 is considered as unhealthy diet[166].

4.3.3 Statistical analysis

Analyses were performed using both STATA 16.0 and the R package.

I initially explored the relationship between alpha and beta-diversity measures, serum and faecal PEA, and severity of anhedonia/amotivation using Pearson's correlation analyses.

Based on the results of the correlation matrix, I used a multilevel mediation model with family structure as random intercept, to test the main hypothesis. The model was adjusted for the full list of covariates.

Then I explored the relationship between taxonomic units collapsed at the genus level, serum and faecal PEA, and anhedonia/amotivation using linear mixed models setting family structure as random intercept and Shannon index, age, BMI, and technical covariates as fixed effect. P values were adjusted for multiple testing using the false discovery rate method.

When the association between the relative abundance of taxonomic units, PEA and anhedonia/amotivation was significant, the relationship between these three variables was further investigated with a multilevel mediation model using family structure as random intercept, adjusted for the full list of covariates. Mediation models were tested using microbial features as predictor, PEA as mediator, and anhedonia/amotivation as outcome.

When models revealed significant mediation, I re-tested them using anxiety as an outcome to investigate specificity of findings for anhedonia/amotivation. In each of these analyses PEA levels were log-transformed and scaled to a mean of 0 and standard deviation of 1 (z scores). PEA levels were available for all twins, while gut microbiome and HADS data were missing at 12.2% and 32.7%, respectively. Missingness among covariates ranged from 12.7% and 46.3%. Missing data were imputed (20 imputed datasets) using multiple imputation by chained equations. To predict missing data, we used all variables selected for analysis models. Among microbial features, only alpha diversity measures were imputed, as beta diversity metrics were the result of principal coordinate analyses.

4.4 Results

Descriptive analysis

The main characteristics of the sample are reported in **Table 9**. The large majority of twins were middle-aged/elder women, in line with the original aim of the TwinsUK registry. Around 1/3 of the sample was obese and 4.5% were using antidepressants.

Table 9. Sample characteristics

	N	mean(SD)	Proportion
Socio-demographics			
Age	786	65.2(7.6)	
Gender (Females)	734		93.4%
Ethnicity	780		99.2%
Anhedonia			
Anhedonia	601	2.6(2.8)	
Endocannabinoids			
PEA serum	786	1.0 (0.6) mmol/L	
PEA faecal	786	1.9(3.3) mmol/Kg	
Covariates			
Obesity	218		27.4%
Unhealthy diet	307		39.6%
Antidepressants	22		4.5%

Exploratory analyses: microbial diversity

I initially explored the relationship between alpha and beta diversity, serum and faecal PEA, and anhedonia/amotivation.

Alpha diversity, measured with the Shannon index, was significantly associated with both faecal PEA levels ($\beta=-0.31$; $P<0.001$) and severity of anhedonia/amotivation ($\beta=-0.10$; $P=0.02$). The directions of the associations suggested that reduced alpha diversity corresponded to increased faecal excretion and to more severe anhedonia/amotivation. Faecal PEA was significantly associated with anhedonia/amotivation ($\beta=0.13$; $P<0.01$), such that to increased faecal excretion corresponded more severe anhedonia/amotivation. Serum PEA was not associated with either gut microbiome diversity metrics or anhedonia/amotivation. Some of the beta diversity components were independently associated with either faecal PEA levels or anhedonia/amotivation (Appendix). None of the

beta diversity components was associated with serum PEA.

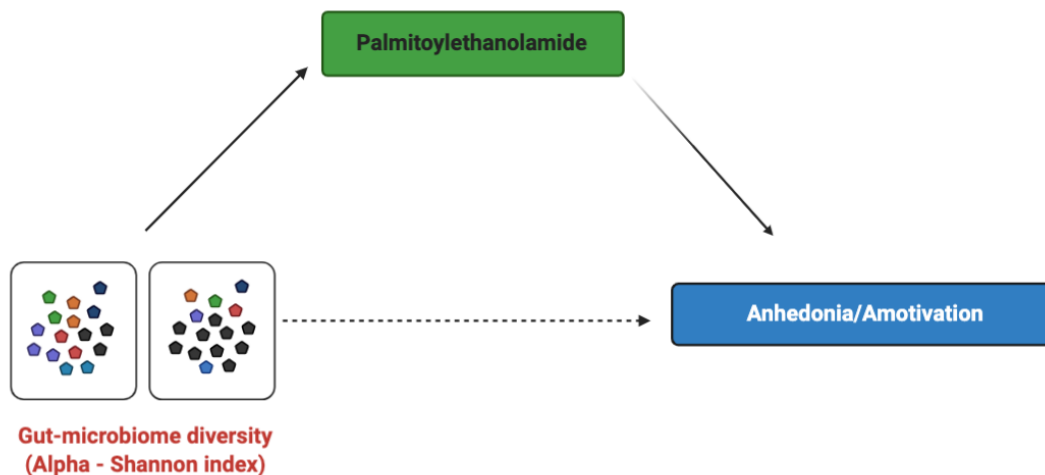
Mediation model 1: microbial diversity as predictor

These initial findings laid the basis for testing our primary hypothesis. I tested a mediation model using alpha diversity as predictor, faecal PEA as mediator, and severity of anhedonia/amotivation as an outcome.

There was a significant direct association between alpha diversity and anhedonia/amotivation ($\beta=-0.37$; 95%CI: -0.71 to -0.03; $P=0.03$). As reported in **Figure 12**, PEA completely mediated the association between alpha diversity and anhedonia/amotivation. In the unadjusted model, the indirect effect was significant ($\beta=-0.13$; 95%CI: -0.24 to -0.01; $P=0.03$), as was the total effect ($\beta=-0.38$; 95%CI: -0.72 to -0.04; $P=0.03$), whereas the direct effect of predictor on the outcome was attenuated fully. The same results were obtained when the model was adjusted for the full set of covariates.

Figure 12. Unadjusted and adjusted coefficients of mediation model

Theoretical model: Palmitoylethanolamide (PEA) mediates the association between microbial diversity and anhedonia/amotivation



Coefficients in unadjusted and adjusted models

Path	Unadjusted Model		Model A ^a		Model B ^b	
	<i>Coeff</i> (<i>N</i> =786)	<i>P</i>	<i>Coeff</i> (<i>N</i> =786)	<i>P</i>	<i>Coeff</i> (<i>N</i> =786)	<i>P</i>
<i>Shannon</i> → <i>Anhedonia/Amotivation</i>	-0.25 (0.19)	0.18	-0.28 (0.19)	0.14	-0.25 (0.19)	0.20
<i>Shannon</i> → <i>PEA</i>	-0.36 (0.05)	0.00	-0.35 (0.05)	0.00	-0.37 (0.05)	0.00

<i>PEA → Anhedonia/Amotivation</i>	0.43 (0.14)	0.00	0.48 (0.14)	0.00	0.50 (0.14)	0.00
Total effect	-0.41 (0.18)	0.02	-0.45 (0.18)	0.01	-0.43 (0.19)	0.02

Predictor: Alpha diversity (Shannon index); *Mediator:* Faecal PEA; *Outcome:* Anhedonia/amotivation;

Note: PEA values were log-transformed and scaled to a mean of 0 and standard deviation of 1

^a**Model A:** Adjusted for technical confounders: storage time (freezer) – PEA; storage time (fridge) – PEA; microbiome operator; microbiome collection method (post or visit) AND for basic demographics (age, gender, ethnicity)

^b**Model B:** Model A + obesity, unhealthy diet, antidepressants

Results on the complete case dataset are reported in the Appendix

From : Minichino A, Jackson M, Francesconi M, Steves C, Menni C, Burnet PWJ, Lennox BR. The endocannabinoid system mediates the association between gut microbiome diversity and anhedonia/amotivation in a general population cohort. *Molecular Psychiatry*. Accepted, in Press

Contrarily, when anxiety was set as the outcome instead of anhedonia/amotivation, there was no evidence of mediation (indirect effect: $\beta=-0.05$; 95%CI: -0.13 to 0.14; $P=0.94$).

Exploratory analyses on taxonomic features (genera)

I then explored if specific taxonomic features (collapsed at the genus level), net of microbial diversity, contributed to anhedonia/amotivation via PEA. Exploratory analyses showed that the relative abundances of *Blautia* and *Dorea* were significantly associated with both faecal PEA and anhedonia/amotivation.

Mediation model 2: genera as predictors

Based on these initial findings, I used the relative abundance of *Blautia* and *Dorea* as predictor in two separate mediation models, with faecal PEA as mediator, and severity of anhedonia/amotivation as an outcome. Neither of the two models showed statistically significant mediation. However, the mediation model using the relative abundance of *Blautia* as predictor showed a significant total effect (unadjusted model: $N=529$; $\beta=0.49$; 95%CI: 0.25 to 0.72; $P<0.001$; fully adjusted model: $N=321$; ; $\beta=0.40$; 95%CI: 0.07 to 0.73; $P=0.02$); and a statistic trend for the indirect effect (unadjusted model: $N=529$; $\beta=0.05$; 95%CI: -0.01 to 0.11; $P=0.06$; fully adjusted model: $N=321$; ; $\beta=0.07$; 95%CI: -0.01 to 0.15; $P=0.07$). The direct effect of *Blautia* remained significant in both the unadjusted and fully adjusted models. These results might suggest a partial mediation of PEA on the association between the relative abundance of *Blautia* and severity of anhedonia/amotivation.

4.5 Discussion

The results presented in this chapter support the hypothesis that the endocannabinoid system mediates the association between gut dysbiosis (reduced within-individual microbial diversity) and anhedonia/amotivation. Findings reconcile the evidence presented in Chapter 2 and 3, where endocannabinoid system abnormalities and gut dysbiosis were found to be independently associated with severity of negative symptoms in psychotic and severe mental illness.

In the TwinsUK cohort, reduced alpha diversity (within-individual microbial diversity) was associated with more severe symptoms of anhedonia/amotivation. This finding is in line with the view that high gut microbial diversity is a hallmark of good mental health[41]. However, case-control studies investigating gut microbiome diversity in clinical conditions that manifest with anhedonia/amotivation (e.g., schizophrenia and depression) do not always report consistent findings[167, 168]. This might be due to the high heterogeneity of the clinical presentation of these disorders and to the limitation of the diagnostic categorical approach[110]. If reduced gut microbial diversity is associated with only certain features of the disorder (e.g., sickness behaviour; anhedonia/amotivation), case-control studies focused on traditional diagnostic criteria can easily fail to find differences (e.g., when they include patients who do not manifest those features). These considerations might also explain the high heterogeneity found in some of the pooled estimates investigating gut dysbiosis biomarkers presented in Chapter 3. As suggested by the RDoC initiative, research targeting a specific psychopathological latent factor might have more chance identifying underlying mechanisms compared to the traditional categorical approach. In line with these considerations and with the evidence on the association between gut dysbiosis biomarkers and severity of sickness behaviour presented in Chapter 3, studies on depression and schizophrenia have shown a more consistent association between reduced gut microbial and severity of anhedonic/amotivation features, rather than with the diagnoses *per se*[169]. Some measures of between-individual (beta) diversity were associated with more severe anhedonia/amotivation. However, this association was not mediated by the endocannabinoid system. There are a number of alternative gut-brain pathways and metabolites that could explain this association (**Table 2** in Chapter 1). Another possibility is that these wide-scale compositional changes may reflect shifts in the microbiome in response to disease itself or lifestyle changes associated with anhedonia/amotivation.

Faecal, but not serum, levels of PEA were associated with more severe anhedonia/amotivation. The pharmacodynamic of PEA is very similar to that of cannabidiol (CBD)[73, 155], an exogenous cannabinoid considered protective for mental health and in particular towards anhedonia/amotivation (see Chapter 1 and 2). Both CBD and PEA are inhibitors of the fatty acid amid hydrolase (FAAH), the main catabolic enzyme of the endocannabinoid mediators anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [73, 155]. As outlined in Chapter 2, the endocannabinoid system is believed to provide protection against central and peripheral noxious stimuli[49]. Increased faecal levels of PEA suggest increased excretion. The increased excretion of PEA would translate in reduced inhibition of FAAH, with consequent increased degradation of endocannabinoid mediators. The reduced availability of endocannabinoid mediators has a detrimental effect on mental health (see Chapter 2)[59] and would explain the finding of an inverse association between faecal levels of PEA and severity of anhedonia/amotivation. These considerations are line with the results of a recent clinical trial showing anti-depressant effects of PEA augmentation in patients with major depressive disorder[156]. The engagement of the endocannabinoid system in anhedonia/amotivation is in line with RDoC recommendations, which suggest that endocannabinoids are among the molecular targets of the Positive Valence System (see Chapter 1)[48]. PEA is also an immunomodulator, as it can block the peroxisome proliferator-activated receptors γ and exerts other anti-inflammatory actions[155]. Given the well-known link between inflammation and negative symptoms[38], it is possible that the effect of PEA on anhedonia/amotivation might follow a complementary biological path to the endocannabinoid system.

Faecal metabolites provide an overview of function at the intersection of host, diet, and microbiome level, and they are considered a more accurate readout of the gut microbiome than serum metabolites[164]. In fact, levels of serum metabolites are under the influence of multiple peripheral sources (e.g., metabolism, inflammation, etc.), resulting in greater variability. This could explain the finding of lack of association between serum PEA and anhedonia/amotivation.

Findings on the association between faecal PEA and gut microbiome diversity were not surprising, considering the impact that the gut microbiome has on the endocannabinoid system[47, 60, 61, 108]. Endocannabinoids, including PEA, have a gatekeeper functions and

they are released upon gut dysbiotic stimuli, such as increased gut permeability and lipopolysaccharide leakage (endotoxemia).

Increased excretion of PEA mediated the association between reduced gut microbial diversity and anhedonia/amotivation. This finding represents a key missing piece in knowledge that reconciles the evidence on gut-microbiome and endocannabinoid system abnormalities outlined in Chapter 2 and 3.

Even though they were obtained from a general population cohort, results from this chapter highlight the existence of a biological pathway that is potentially modifiable and that underlies the debilitating symptoms that often predate the onset of a wide range of severe clinical conditions.

4.5.1 Limitations

Limitations include: 1) The impossibility of excluding reverse causality in the mediation model. Even if theoretical assumptions were based on strong ground, it was not possible to test (and therefore exclude) reverse causality: the variables of interest were collected over time (microbiome first – PEA second – anhedonia/amotivation last), so the one presented here was the only model that was possible to test 2) Predictors, mediators and outcome were measured at a single time point. The longitudinal nature of the study partially corrected for this bias; 3) A subgroup of items of the HADS was used as a measure of anhedonia/amotivation. The HADS was not originally designed for the assessment of these symptoms. However, subsequent meta-analytic evidence and factorial analyses showed that the selected subgroup of items used to measure our outcome did provide a reliable measure of anhedonia/amotivation; 4) The use of a general population cohort to test our model. Future studies should validate findings in clinical samples; 5) Missingness in some covariates data led to loss of power in complete case analysis (see Appendix); however, the chained data imputation partially corrected for this bias.

The main strengths are the large sample size, the longitudinal design and the use of a well-known data registry (TwinsUK). To protect against multiple testing, the TwinsUK registry provide data only upon hypothesis-based request. Therefore pre-registration was not required, as our hypothesis was outlined in the data request form (TwinsUK E1019).

Finally, findings on gut microbial taxonomic features are in line with a growing body of evidence showing that the overall composition (and functional readout) of the gut microbiome, rather than specific taxonomic features, have implications for the host's health[170]. In fact, none of the bacterial species, with the exception of *Blautia*, seemed to contribute to the interplay between PEA and anhedonia/amotivation. Findings on *Blautia* are interesting, as they are in line with previous evidence suggesting a role in depression[171], however these are limited to non-significant statistical trends and need replication.

4.5.2 Conclusion

The results presented in this chapter suggest that gut microbial diversity contributes to anhedonia/amotivation via the endocannabinoid system. These findings shed light on the biological underpinnings of anhedonia/amotivation and suggest the gut microbiota-endocannabinoid axis is a promising therapeutic target in an area of unmet clinical need.

Chapter 5. The gut microbiome as a therapeutic target for negative symptoms

5.1 Introduction

Taken together, the evidence presented in the previous chapters advocates for the existence of a gut microbiome-endocannabinoid axis, the abnormalities of which can manifest as negative symptoms both in severe mental illness (Chapter 2 and 3) and in the general population (Chapter 4). In particular, the mediation model presented in Chapter 4 suggests that the interplay between the gut microbiome and the endocannabinoid system might underlie experiential negative symptoms, which are the subgroup of negative symptoms more tightly related to detrimental functional outcomes in severe mental illness.

As outlined in detail in Chapter 1, negative symptoms are an unmet therapeutic need across diagnostic boundaries[15, 110] and the gut microbiome could represent a novel, modifiable, target for intervention. The efficacy of interventions based on the gut microbiome is likely to depend on the downstream activation of specific “mediating systems”, i.e., putative biobehavioural pathways that underlies negative symptoms. During my DPhil, I focused on one of these systems (endocannabinoids), but there are others that might be relevant (see Table 2 - Chapter 1).

Despite the growing body of evidence showing that the interplay between the gut microbiome and the host physiology could be key for mental health[100, 172, 173], there are only a few studies that have investigated the therapeutic implications of these findings.

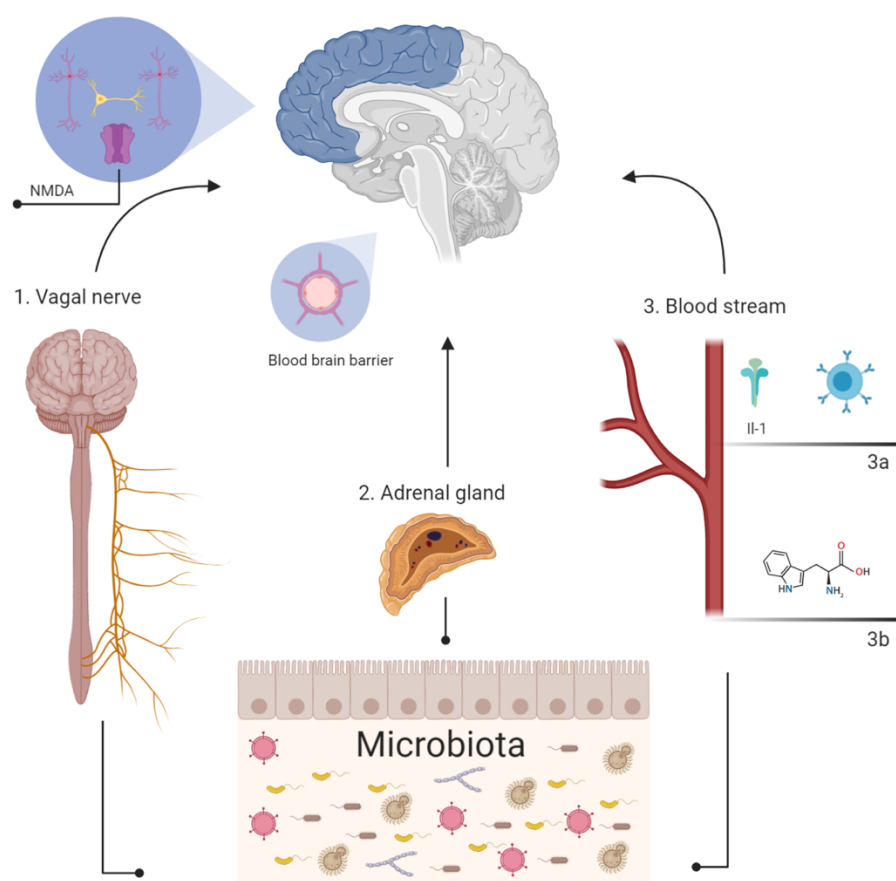
In this chapter, I will present the results of two systematic reviews and meta-analyses that investigated the therapeutic potential of gut microbiome targeted interventions for negative symptoms. The first systematic review and meta-analysis is focused on already known modifiers of the gut microbiome, such as antibiotics, antimicrobics, and pre/probiotics and their efficacy for the treatment of negative symptoms in schizophrenia. The second systematic review and meta-analysis is focused on a compound, metformin, which has been

recently found to affect the gut-microbiome and that has shown promising pro-cognitive and antidepressant activity.

5.2 Antibiotics, antimicrobics, pre- and pro-biotics

Only a few clinical trials have tested the efficacy of add-on therapeutics with the *a-priori* rationale of targeting the gut-microbiome for treating negative symptoms in schizophrenia (**Fig. 13**). These studies mainly used add-on pre- and pro-biotic compounds. However, there are a number of studies that have used add-on compounds, such as antibiotics and antimicrobials, that are well known modifiers of the gut microbiome. Combining and interpreting data from these trials will help in clarifying the therapeutic potential of gut microbiome based interventions for negative symptoms in schizophrenia.

Figure 13. The gut microbiome-brain axis: implications for negative symptoms in schizophrenia



Gut microbiota-brain pathways include: **1.** Vagal and sympathetic nerve activation; **2.** Hypothalamic-pituitary-adrenal axis activation; **3a.** Modulation of the inflammatory response and **3b.** Synthesis and release of modulators of central neurotransmissions in the blood stream. The hypofunction of N-methyl-D-aspartate (NMDA) in the brain is believed to play a central role in the pathophysiology of negative symptoms in schizophrenia. Perturbations in the gut microbiota (“gut dysbiosis”) result in increased synthesis and release in the blood stream of a series of molecules that have the potential to

reduce central N-methyl-D-aspartate (NMDA) receptor activity, including pro-inflammatory molecules (e.g., Interleukin-1, Il-1) and neurotransmitters-derivatives (kynurenate). The passage of these molecules from the blood stream to the central nervous system seems to be facilitated by the increased permeability of biological barriers – a phenomenon related to gut dysbiosis.

From: Minichino A, Brondino N, Solmi M, Del Giovane C, Fusar-Poli P, Burnet PWJ, Cipriani A, Lennox BR. The gut-microbiome as a target for the treatment of schizophrenia: A systematic review and meta-analysis of randomised controlled trials of add-on strategies. *Schizophr Res.* 2020 Apr 12:S0920-9964(20)30101-8.

In this systematic review and meta-analysis, I summarise the evidence of the efficacy and tolerability of add-on antibiotics, antimicrobics, pre- and pro-biotics for the treatment of schizophrenia. A summary of the included compounds can be found in **Table 10**, with a description of their putative mechanisms of actions.

Table 10. List of add-on compounds included in the systematic review: putative mechanisms of actions on the gut-microbiome-brain axis

Compound	Cross BBB	Effect on the central nervous system	Effect on the gut-microbiome
<i>Pre/Probiotics</i>			
Prebiotics (nutrients for “beneficial” bacteria, such as galacto/fructo-oligosaccharides) Probiotics (live “beneficial” bacterial, such as Bifidobacterium and Lactobacillus families)	No	Indirect effect Modulates expression of NMDA-R and GABA-R in hippocampus and frontal cortex [174]	↑ Bifidobacterium and Lactobacillus families [174]
<i>Antibiotics</i>			
Azithromycin	Yes	No direct effect	↓Species richness ↓Bifidobacterium species ↓Butyrate producers [175]
Trimethoprim	Yes	No direct effect	↓Species richness ↓Bifidobacterium species ↓Butyrate producers [175]
Minocycline	Yes	Unclear Proposed direct effect: inhibition of microglia activation AND inhibition of NOS	↓Species richness ↓Bifidobacterium species ↓Butyrate producers [175]

		[176]	
D-Cycloserine	Yes	Direct effect NMDA-R partial agonist [177]	↓Species richness ↓Bifidobacterium species ↓Butyrate producers [175]
Antimicrobials			
Sodium benzoate	Yes	Direct D-amino acids oxidase (DAAO) inhibitor – modulates NMDA-R activity [178]	↓Bifidobacterium Longum and Lactobacillus Paracasei [179]
Artemisinin/Artemether	Yes	Direct (Artemether only) ↑ NMDA-R expression [180]	Anti-parasitic activity [181]

Legend. ↑:increase; ↓:decrease; BBB: Blood Brain Barrier; GABA: Gamma-Aminobutyric acid; NMDA-R: N-Methyl-D-Aspartic acid receptors; NOS: Nitric Oxide Synthase

From: Minichino A, Brondino N, Solmi M, Del Giovane C, Fusar-Poli P, Burnet PWJ, Cipriani A, Lennox BR. The gut-microbiome as a target for the treatment of schizophrenia: A systematic review and meta-analysis of randomised controlled trials of add-on strategies. *Schizophr Res.* 2020 Apr 12:S0920-9964(20)30101-8.

5.2.1 Methods

I led a systematic review and meta-analysis of randomised double-blind controlled trials (RCTs-DB) comparing antibiotics, antimicrobials, and pre/probiotics vs placebo as add-on treatment of schizophrenia and related psychosis. I followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines [77]. The original protocol was registered in PROSPERO (CRD42019137715). The search for studies was conducted in Web of Science and PubMed. For full text analysis, I included double-blind randomised controlled trials comparing antibiotics, antimicrobials, pre/probiotics vs placebo as add-on to treatment as usual in schizophrenia and related psychosis (ICD and/or DSM-codified diagnosis of schizophrenia, schizoaffective disorder, schizophreniform disorder, first-psychotic episode). Based on a preliminary search of the literature, the following list of molecules were included in the search: d-cycloserine, minocycline, sodium benzoate, lactobacillus, bifidobacterium, azithromycin, artemisinin, artemether, trimethoprim. This list was agreed with an expert (Phil Burnet). Trials with cluster randomisation and trials with fewer than 5 participants per arm

were excluded. Trials with a cross-over design were included, with priority given to data from the first leg (prior to cross-over). No restrictions were applied in terms of age, duration of illness, previous treatments, in- or out-patient setting, symptom profile.

The entire search process was independently conducted by two collaborators, who also independently extracted data from each included study.

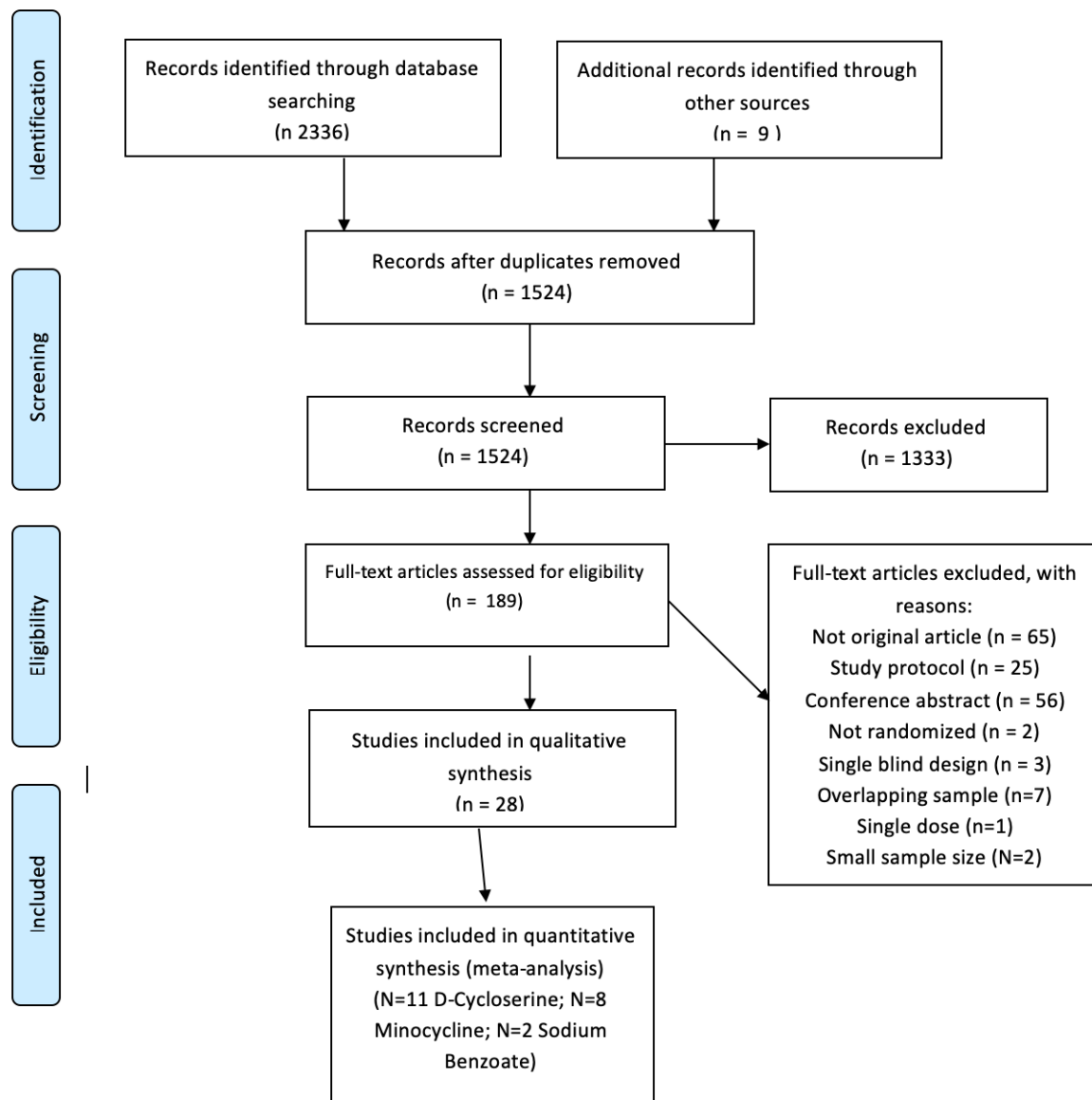
The primary outcome was severity of negative symptoms at the end of follow-up. When the end score was not available, the change score was taken into account. Secondary outcomes included: a) acceptability of treatment, measured as % of patients dropping from the trial for any cause; b) relationship of negative symptoms outcomes with clinical subgroup of interests (first-episode vs multi-episode; clozapine vs other treatment; greater severity of symptoms at baseline); c) severity of positive, total, and cognitive symptoms at end of follow (or change score, when end scores were not available).

Meta-analytic pooled estimates were provided when two or more studies per add-on compounds were available. All meta-analyses were conducted in STATA, version 13.0, using random-effect models. Standardised mean difference (SMD) was used as summary statistics for continuous outcomes and Risk Ratio (RR) for dichotomous outcomes. The Higgins I^2 was used to assess between-study heterogeneity. The Cochrane Risk of Bias Tool was used to assess risk of bias[78]. When ten or more studies were included in the analysis, funnel plots and Egger's test were used to assess the possibility of publication bias[182]. Sensitivity analyses were carried out by sequentially removing single studies and re-running the analyses and by excluding cross-over trials, when any.

5.2.2 Results

As outlined in **Figure 14** (PRISMA flow-chart), twenty-eight studies met the inclusion criteria[37, 177, 183-208]. Of these, twenty-one compared add-on antibiotics to placebo (N=11 D-Cycloserine; N=8 Minocycline[37, 185, 186, 199, 200, 202, 204, 208]; N=1 Azithromycin[189]; N=1 Trimethoprim[205]), four compared add-on antimicrobial to placebo (N=2 Sodium Benzoate; N=1 Artemisinin[187]; N=1 Artemether[209]); three compared add-on pre/probiotics vs placebo (N=2 probiotics; N=1 prebiotics[198]). See **Table 11** for a qualitative summary of the included studies.

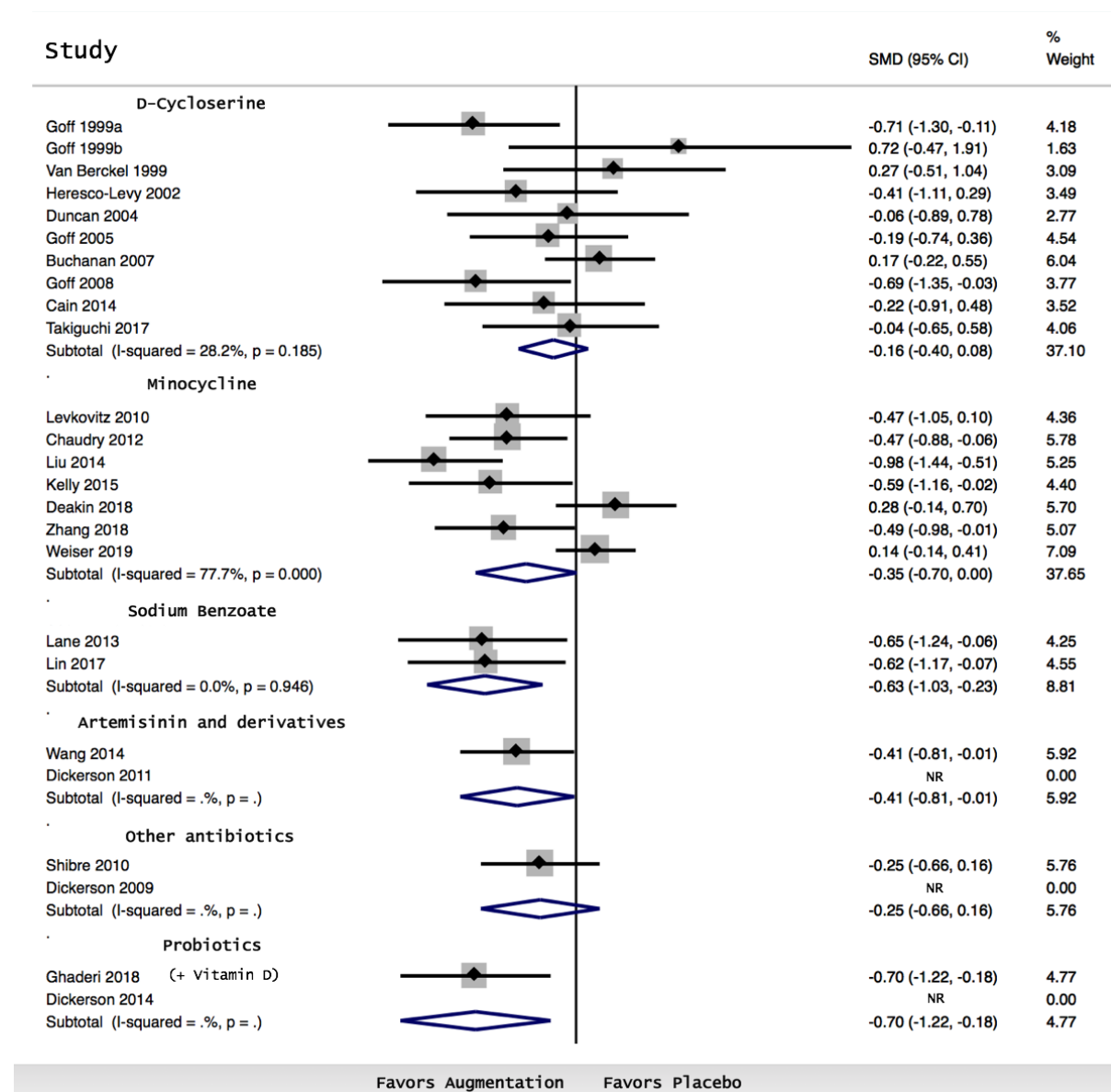
Figure 14. PRISMA flow-chart: add-on gut-microbiome based intervention in schizophrenia



Negative symptoms

For the primary outcome (negative symptoms), ten studies reported data on the antibiotic D-Cycloserine, seven on the antibiotic Minocycline and two on the antimicrobial sodium benzoate. The pooled estimates showed that neither add-on Minocycline (SMD: -0.35; 95% CI -0.70, 0.00; $P=0.05$, $I^2:77.7\%$) or D-Cycloserine (SMD, -0.16; 95% CI -0.40, 0.08; $P=0.20$; $I^2: 28.2\%$) were superior to placebo for treating negative symptoms of schizophrenia. In contrast, Sodium benzoate was found to be effective when compared to placebo (SMD, -0.63; 95%CI -1.03, -0.23; $P<0.001$; $I^2:0\%$) (**Figure 15**).

Figure 15. Forest plot of standardised mean difference in end-point negative symptoms scores in patients treated with add-on gut-microbiome-target intervention vs placebo



In this forest plot are reported all the included trials that assessed negative symptoms at end of follow-up. NR: Data was not reported AND not retrievable (i.e., no replies from authors).

From: Minichino A, Brondino N, Solmi M, Del Giovane C, Fusar-Poli P, Burnet PWJ, Cipriani A, Lennox BR. The gut-microbiome as a target for the treatment of schizophrenia: A systematic review and meta-analysis of randomised controlled trials of add-on strategies. *Schizophr Res.* 2020 Apr 12:S0920-9964(20)30101-8.

The pooled estimate on Minocycline did not take into account two studies: one that did not assess negative symptoms[195] and another that reported outlying results and was at high risk of bias[200]. Due to lack of extractable data, it was not possible to provide pool estimates for Azithromycin, Trimethoprim, Artemisinin, Artemether, pre/probiotics.

The two trials that investigated Azithromycin and Trimethoprim did not report any beneficial effect for negative symptoms over placebo[189, 205]. Similarly, the trial on Artemisinin

yielded negative results[187], while the trial on Artemether showed efficacy over placebo[209]. The two trials on add-on probiotics reported mixed findings. One trial combined probiotics to vitamin D, showing greater efficacy over placebo[191]. The other study used probiotics alone and showed no beneficial effect on negative symptoms than placebo[188]. The only study that investigated prebiotics did not assess negative symptoms[198].

Negative symptoms: influence of clinical subgroups on outcomes

It was possible to evaluate the influence of illness stage on negative symptoms outcomes only for Minocycline; the lack of efficacy over placebo was confirmed even when studies were grouped based on illness stage.

It was possible to evaluate the influence of baseline severity of negative symptoms on outcomes of negative symptoms only for Minocycline and D-Cycloserine. Add-on Minocycline, but not D-Cycloserine, was found to be effective in patients with more severe negative symptoms at baseline, in particular when at an early stage of illness (Appendix). Clozapine was used in three studies, one investigated add-on D-Cycloserine[177], one add-on Minocycline[199], and one add-on Sodium Benzoate[203]. Compared to placebo, D-Cycloserine worsened negative symptoms when added to clozapine, Minocycline did not have any effect, while Sodium Benzoate was more effective for treating negative symptoms.

Acceptability of treatment

It was possible to provide a quantitative summary on acceptability of treatment for the antibiotics D-Cycloserine (11 studies; RR, 0.91; 95% CI, 0.64-1.30; I^2 :0%; P =0.62), the antibiotic Minocycline (8 studies[37, 185, 186, 199, 200, 202, 204, 208]; RR, 1.15; 95% CI, 0.96-1.38; P =0.13; I^2 :0%; P =0.13), and the antimicrobial Sodium Benzoate (2 studies; RR, 0.23; 95% CI, 0.04-1.36; I^2 :0%; P =0.10).

None of these compounds differed from placebo in terms of acceptability. Similar results were reported by the trials on Azithromycin[189], Trimethoprim[205], Artemether[209], Artemisinin[187], probiotics alone[188], probiotics augmented with Vitamin D[191].

Positive, total, and cognitive symptoms

It was possible to provide a quantitative summary on the efficacy of D-Cycloserine, Minocycline and Sodium Benzoate for the treatment of positive, total and cognitive symptoms in schizophrenia. D-Cycloserine and Minocycline were not superior to placebo in

any of the investigated domains (see Appendix). Sodium Benzoate was superior to placebo in treating positive symptoms (2 studies, SMD, -0.94; 95%CI -1.35, -0.53; $P < 0.001$; $I^2: 0\%$) and total symptoms (2 studies, SMD, -0.68; 95%CI -1.08, -0.28; $P = 0.001$; $I^2: 0\%$), but not cognition.

Neither Azithromycin[189] or Trimethoprim[205] were found to be superior to placebo in improving positive, total, and cognitive symptoms. Probiotics alone were not superior to placebo for the treatment of positive and total symptoms, cognition was not investigated[188]. The augmentation of probiotics with vitamin D resulted in improved total, but not positive, symptoms compared to placebo; cognition was not investigated[191]. Prebiotics showed a pro-cognitive effect when compared to placebo; positive and total symptoms were not investigated[198].

Methodological considerations

No differences were found in any of the investigated outcomes when cross-over trials were excluded from the analyses. No publication bias was detected with funnel plots or Egger's test for small study effects.

5.3 Metformin

Metformin, the most widely used treatment for type 2 diabetes mellitus, has shown pro-cognitive and antidepressant efficacy in preclinical studies[210, 211].

In the periphery, metformin is believed to improve glucose tolerance by suppressing hepatic gluconeogenesis through the activation of the AMP-activated protein kinase (AMPK)[212]. As it can cross the blood-brain barrier, metformin has also a central effect[213]. In the brain, metformin exerts anti-(neuro)inflammatory and anti-(neuro)oxidative actions and improves central insulin sensitivity[213]. There is increasing evidence suggesting that at least some of the biological effects of metformin pass through the gastrointestinal system[214-216].

Metformin is more effective when administered orally rather than injected, an effect that seems to be mediated by the gut microbiome[217].

Preclinical studies have shown that metformin increases the abundance of Bacteroidetes, reduces the abundance of Firmicutes, and enriches several short chain fatty acid (SCFA)-producing bacteria[214-216]. These findings have translational relevance, as observational studies in clinical populations have shown that a high Bacteroidetes: Firmicutes ratio and an increased abundance of SCFA-producing bacteria are associated with reduced

cardiometabolic risk[218]. Based on these initial findings, a recent randomised controlled trial tested the hypothesis that metformin antidiabetic action was mediated by the gut-microbiome[216]. The authors transferred faecal samples, collected at baseline and at the end of trial, of metformin-treated donors in germ-free mice. Results showed that metformin improved glucose tolerance by increasing the abundance of SCFA-producing bacteria, including *Blautia* and *Akkermansia Muncipihila*[216].

The increased abundance of SCFA-producing bacteria is believed to be beneficial for mental health, with preclinical studies showing that SCFA can enhance NMDAR-glutamatergic transmission in the brain (hypofunctioning in severe mental illness)[174]. Furthermore, findings from chapter 4 showed that reduced relative abundance of *Blautia* and *Akkermansia Muncipihila* was significantly associated with increased endocannabinoid excretion. By increasing the relative abundance of these two bacterial species, it is possible that metformin beneficial effects for mental health would also pass through the endocannabinoid system. Given its safety profile and its putative peripheral and central targets, there is potential for metformin to be repurposed for the treatment of cognitive and negative symptoms in severe mental illness. Some clinical trials have been already conducted in this regard, producing mixed evidence.

Here, I present the results of the first systematic review and meta-analysis of randomised controlled trials investigating the efficacy of metformin for the treatment of cognitive deficits and negative symptoms in severe psychiatric and neurological disorders.

Based on the considerations outlined in Chapter 1 and Chapter 3, the term “negative symptoms” here is used as a correlate of the RDoC Positive Valence construct and therefore extends to depressive symptoms.

5.3.1 Methods

I led a systematic review and meta-analysis investigating the efficacy of metformin vs placebo on cognitive functions and “negative symptoms” (this term includes: negative symptoms as defined in schizophrenia; and depressive symptoms as defined in major depressive disorder and bipolar disorder). The systematic review and meta-analysis was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines[77]. The protocol was registered with PROSPERO (CRD42020184547).

The search for studies was conducted in Web of Science and PubMed from inception to May 6, 2020. Inclusion criteria were: (i) any double-blind randomised controlled trial investigating the efficacy of metformin for cognition and “negative symptoms”; [44] age of participants ≥ 18 years. No restriction was applied in terms of clinical population (i.e., any underlying condition), severity of symptoms, sex, type or duration of previous treatments. The entire search process was independently conducted by two collaborators, who also independently extracted data from each included study.

The primary outcome was severity of cognitive deficits, measured as change score (baseline to end-of-treatment), in patients with any underlying condition treated with metformin vs placebo. When multiple measures of cognition were reported, composite scores were used first, followed by proxy scores for domains (e.g., executive function, attention, working memory etc.).

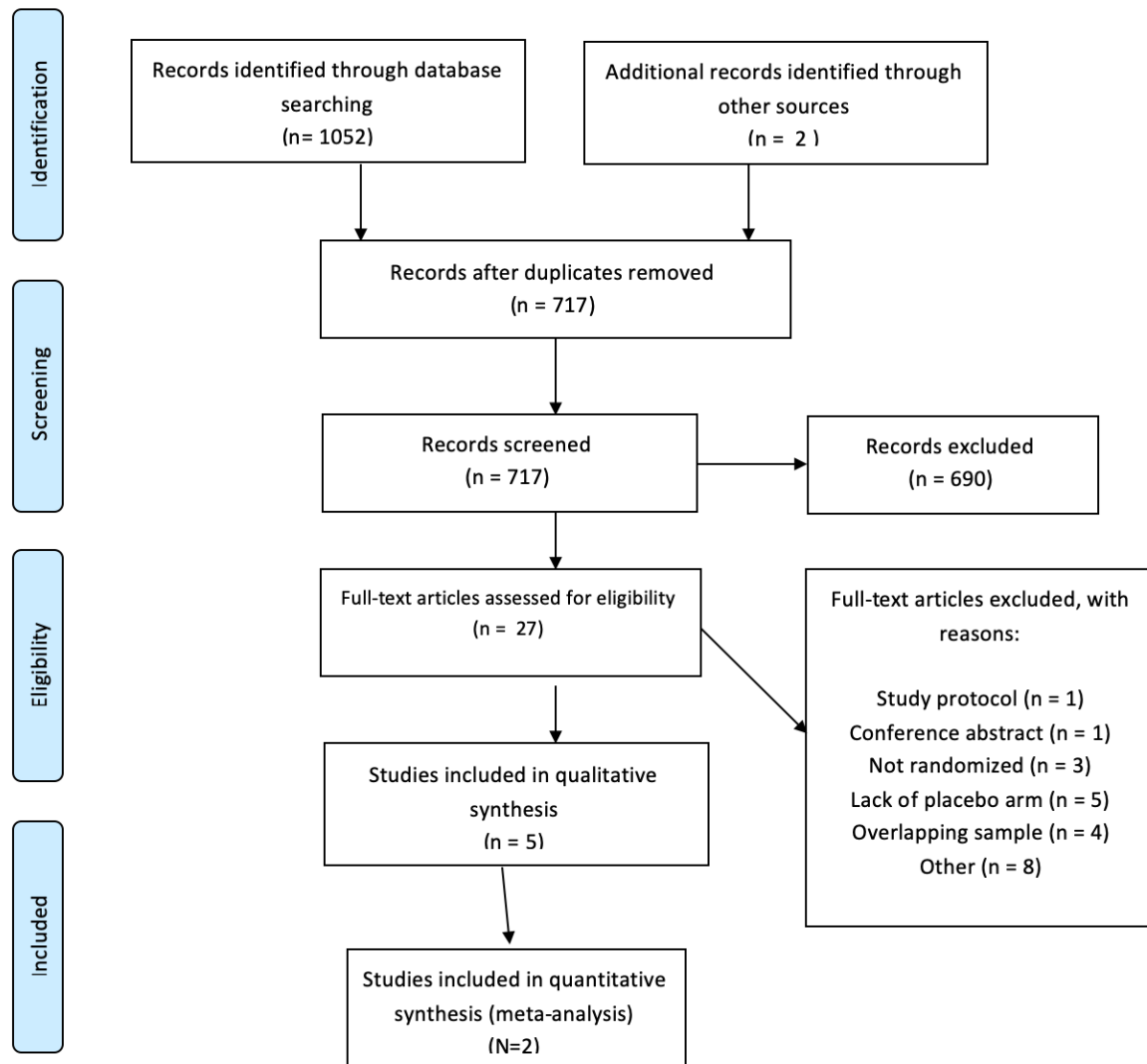
The secondary outcome was severity of “negative symptoms”, measured as change score (baseline to end-of-treatment) in patients with any underlying condition treated with metformin vs placebo.

Meta-analytic pooled estimates were provided when data from two or more studies per outcome were available. All meta-analyses were conducted in STATA, version 13.0, using random-effect models. Standardised mean difference (SMDs) was used as summary statistics. The Higgins I^2 was used to assess between-study heterogeneity. The Cochrane Risk of Bias Tool was used to assess risk of bias[78]. Sensitivity analyses were carried out by sequentially removing single studies and re-running the analyses and by excluding cross-over trials, when any. Given the wide inclusion criteria, when feasible, studies were grouped based on the underlying clinical conditions of the included participants and its association with the outcome of interest (i.e., for the cognitive outcome: conditions manifesting with cognitive impairments vs not, and for the depression outcome: conditions manifesting with depressive symptoms vs not).

5.3.1 Results

Five studies met inclusion criteria (**Figure 16**)[219-223]. Of these, three studies investigated cognitive deficits[221-223], one study investigated both cognitive deficits and “negative symptoms” (depressive symptoms)[220], one study investigated “negative symptoms” only (depressive symptoms)[219]. None of the included studies investigated negative symptoms, as defined in schizophrenia diagnostic criteria.

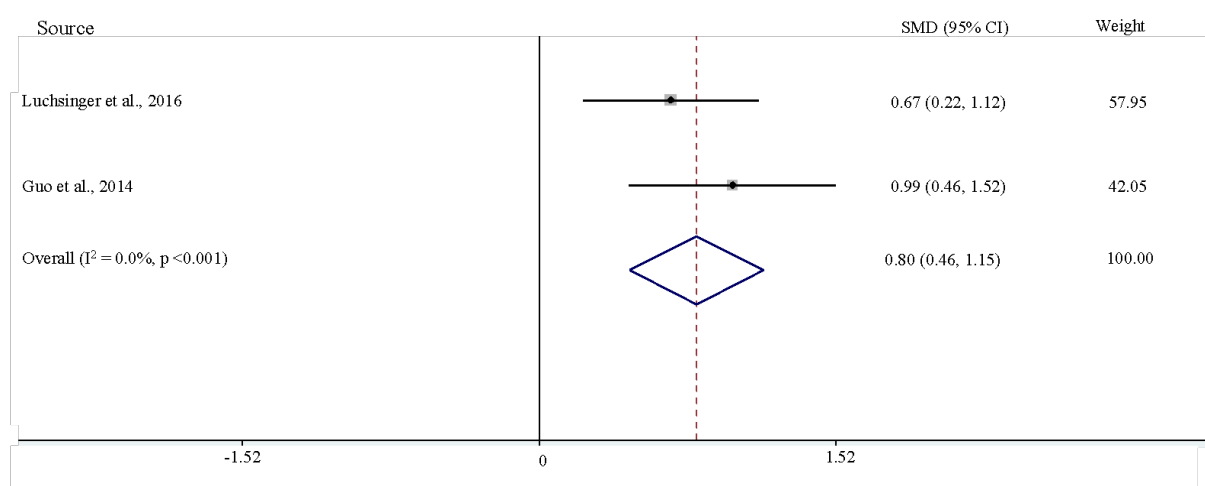
Figure 16. PRISMA flow-chart: metformin for the treatment of cognitive deficits and “negative symptoms”



Of the four trials that reported outcomes on cognitive deficits, three were conducted in cognitively impaired cohorts, i.e., mild cognitive impairment (N=2 studies)[222, 223], and major depressive disorder (N=1 study)[220], while one study was conducted in overweight breast cancer survivors, a condition not typically associated with cognitive impairments[221]. This latter study did not find differences between metformin and placebo on cognitive

outcomes[221]. Contrarily, the three trials conducted in cognitively impaired cohorts consistently reported improved cognitive functions in at least one cognitive domain in patients treated with metformin vs placebo (see **Table 12** for a qualitative summary of findings)[220, 222, 223]. One of these three studies did not provide data for a quantitative synthesis[222], so it was possible to provide a meta-analytic pooled estimate based on only two studies. The pooled estimate, which combined data from trials conducted on major depressive disorder and mild cognitive impairment, showed that metformin was superior to placebo in treating cognitive deficits in patients suffering with conditions typically associated with cognitive impairments (SMD: 0.80; 95%CI: 0.46 to 1.15; $p<0.001$; $N=2$ studies; $I^2 = 0.0\%$) (**Figure 17**).

Figure 17. Forest plot of standardised mean difference (SMD) in cognitive function in metformin group compared to placebo



Of the two studies that investigated depressive symptoms[219, 220], one was conducted in patients with major depressive disorders[220], the other in patients with type 2 diabetes[219]. The first study, conducted on a clinical population with severe symptoms of depression, showed that metformin had anti-depressant effects that were superior to placebo[220]. The second study, conducted on patients with type 2 diabetes and low depression scores at baseline, found no differences between metformin and placebo on depression outcomes[219].

In summary, metformin was found to have pro-cognitive and antidepressant effects in clinical conditions associated with cognitive impairments and depressive symptoms.

5.4 Discussion

In this chapter, I presented the results of two systematic reviews and meta-analyses aimed at investigating the therapeutic potential of targeting the gut microbiome for the treatment of negative symptoms.

The first systematic review and meta-analysis aimed at investigating the efficacy of compounds with a well-known effect on the gut microbiome for the treatment of negative symptoms of schizophrenia. Results showed that none of the included antibiotics were superior to placebo, while the evidence on pre and probiotics was limited.

The second systematic review and meta-analysis aimed at investigating the efficacy of metformin for the treatment of “negative symptoms” (include: negative symptoms as defined in schizophrenia as well as depressive symptoms) and cognitive deficits in severe mental illness. The evidence was limited to few studies, but results showed promising pro-cognitive and anti-depressant efficacy of metformin compared to placebo.

Antibiotics have a strong impact on the gut microbiome[175]. Animals and human studies have consistently shown that wide-spectrum antibiotics, such as D-Cycloserine, Minocycline, Azithromycin, and Trimethoprim, reduce gut microbiome diversity and the relative abundance of SCFA-producing bacteria[175]. These two factors are both associated with poor mental health outcomes. D-Cycloserine and Minocycline have putative central effects (**Table 10**), which is the reason why they were initially tested as add-on treatments in schizophrenia. It is possible that their peripheral detrimental action on the gut-microbiome “counteracted” their central effect, explaining the lack of findings in the overall pooled estimates. In support of this hypothesis, the antimicrobial Sodium Benzoate, which is a bland antimicrobial (used mainly as a food preservative)[179] with well-defined central action (NMDAR enhancer)[201], was found to be effective for the treatment of negative symptoms in schizophrenia. Similarly, Artemether, but not Artemisinin, was found to be effective for treating negative symptoms of schizophrenia. These two compounds share similar peripheral properties, but different neuroactive potential (**Table 10**). Artemether can cross the blood-brain barrier and increase the expression of NMDAR in the brain[180]. These “central” properties explain why Artemether, but not Artemisinin, is licensed for the treatment of neural complications of malaria.

On the same line are findings on probiotics. A beneficial effect of probiotics on negative symptoms was observed only in combination with Vitamin D, which has well-known central

pro-cognitive and anti-depressant effects[224]. Probiotics alone were not found to be effective for treating negative symptoms. Animal studies are starting to clarify that probiotics do not alter the composition of the gut-microbiome, but they have indirect aspecific effects on the host's health, likely via the activation of the immune system[174].

The only trials conducted on prebiotics found them to be beneficial for cognitive functions, however the authors did not assess negative symptoms[198]. Prebiotics, such as the galacto-oligosaccharide (B-GOS), increased the circulating levels of SCFA [198], similar to what has been observed with oral administration to metformin[215].

In summary, the majority of compounds with a well-known effect on the gut-microbiome were ineffective for the treatment of negative symptoms of schizophrenia. Efficacy, where found, was supported by limited evidence and was likely related to a central, rather than to a gut microbiome based mechanism of action. The lack of efficacy observed on the majority of trials likely resulted from the lack of specificity of the proposed interventions, which can be seen as a consequence of the gap in knowledge about the relationship between the gut microbiome and the host's mental health.

Albeit limited to one study, findings from the trial of prebiotics are encouraging and suggest that manipulation of the gut-microbiota can effectively lead to better therapeutic outcomes[198]. Among the compounds included in the first systematic review and meta-analysis, prebiotics are the only one known to act via a specific gut-microbiome-brain pathway (SCFA, see Table 2 in Chapter 1). Findings on prebiotics reconcile with those on metformin, which also has a specific beneficial action on the gut-microbiome, increasing the relative abundance of SCFA-producing bacteria[214-216]. The pooled estimate on metformin shows pro-cognitive effects in mild cognitive impairment and depression. There was also evidence, limited to one study, of an antidepressant effect.

It is possible that the observed beneficial effect of metformin on mental health might be due to improved glucose tolerance, which is known to influence cognitive function through cerebrovascular and neurodegenerative mechanisms[225]. However, among the three studies that showed a pro-cognitive effect of metformin[220, 222, 223], only one (Guo et al., 2014) reported a statistically significant decrease in fasting blood glucose in the metformin arm. Therefore, additional pro-cognitive mechanisms, such as a gut microbiome based action, may be at play. These mechanisms might involve different and likely interrelated gut-brain pathways. For instance, other than the already mentioned SCFA pathway, metformin

influences the abundance of bacterial species found to be associated with peripheral endocannabinoid activation.

An antidepressant action of metformin was supported by only one study[220], which requires replication. It is not possible to exclude the possibility that the antidepressant action of metformin was secondary to its pro-cognitive effect. As the authors showed, depressive symptoms and cognitive functions were negatively correlated. Therefore, it is not possible to disentangle the specific and differential contribution of metformin to cognition and depression.

From an RDoC perspective, the pro-cognitive and antidepressant effects of metformin could be potentially used for targeting the cognitive and hedonic components of the positive valence system. The results presented here provide a strong rationale for future randomised controlled trials to directly address this question. These studies could use a direct trans-diagnostic approach by including patients diagnosed with severe mental illness, which shared cognitive and hedonic deficits despite falling into different diagnostic categories.

5.4.3 Limitations

The main limitation of the results presented in this chapter is that none of the included trials provided direct measures of the gut microbiome. Therefore, all the considerations on the potential mechanisms of action were inferred from indirect evidence. Another important limitation is that none of the trials investigating metformin vs placebo provided measures of the “negative symptoms” construct, other than depressive symptoms. Trans-diagnostic considerations are therefore only speculative.

5.4.2 Conclusion

Findings from the two systematic reviews and meta-analyses suggest that specific approaches targeting the gut microbiome are not effective for the treatment of negative symptoms. Future trials should carefully consider the impact of gut microbiome perturbations on specific gut-brain pathways, such as the ones crossing SCFA or the endocannabinoid system. Even if the evidence is limited, molecules such as metformin and prebiotics might have the potential to be repurposed for the treatment of negative symptoms.

a schizophrenia: qualitative summary

Gender (%) - emales	Inclusion criteria <i>Clinically-based</i>	Duration	Add-on	Add-on dose	TAU	N add- on	N placeb o	Negative Symptoms Scale (main)	NS Baseli e scor <i>Mean (SD)</i>
2 (17.4)	1.Primary Deficit Syndrome (Kirkpatrick 1989) 2. SANS $\geq$ 30 3. SAS < 8 4. HAM-D 18 < 25	8 weeks	D-Cycloserine	50 mg once daily	Typical APs	23	24	SANS	52.6 (13.8)
(28.5)	1. SANS > 30	6 weeks	D-Cycloserine	50 mg once daily	Clozapine	17	17	SANS	49.5 (NA)
(15.4)	1. At least "moderate" scores on two items of the PANSS Negative	8 weeks	D-Cycloserine	100 mg daily	Typical APs	13	13	PANSS Negative	25.2 (4.7)
(37.5)	1. AP-resistance	6 weeks	D-Cycloserine	50 mg once daily	Mixed typical and atypical APs	8	8	PANSS Negative	31.5 (4.7)

(0)	1. Primary Deficit Syndrome (Kirkpatrick 1989) 2. SANS $\geq$ 30	4 weeks	D-Cycloserine	50 mg once daily	Mixed typical and atypical APs	10	12	SANS	69.1 (16.7)
1 (20.0)	1. SANS $\geq$ 40	6 months	D-Cycloserine	50 mg once daily	Typical APs	27	28	SANS	50 (11.6)
R	1. SANS $\geq$ 20 OR SANS Affective flattening $\geq$ 3 OR SANS Alogia $\geq$ 3 2. BPRS Positive $\leq$ 18	16 weeks	D-Cycloserine	50 mg once daily	Mixed typical and atypical APs	53	52	SANS-Modified (see original work)	2.3 (0.6) * *SANS total score divided by the

	3. BPRS Anxiety/depression factor ≤ 14 4. Simpson-Angus ≤ 8								number of items
5 (39.4)	None	8 weeks	D-Cycloserine	50 mg once weekly	Mixed typical and atypical APs	19	19	SANS- Modified (SANS total minus Attention subscale)	25.2 (10.1)
(35)	1. SAPS ≥ 3 "on the global measure of delusional severity"	2 weeks	D-Cycloserine	50 mg once weekly	Mixed typical APs	11	9	Not measured	NA
(13.9)	None	8 weeks	D-Cycloserine	50 mg once weekly	Mixed typical and atypical APs	18	18	SANS- Modified (SANS total minus Attention subscale)	25.2 (12.1)

4 (38.9)	None	6 weeks	D-Cycloserine	50 mg once daily	Mixed typical APs	17	19	SANS	50.5 (21.3)

4 (25.9)	1. PANSS Total $\geq$ 60	6 months	Minocycline	200mg once daily	Mixed typical and atypical APs	36	18	SANS	42.9 (18.5)
8 (40.3)		12 months	Minocycline	200mg once daily	Mixed typical and atypical APs	71	73	PANSS Negative	21.9 (7.3)

0 (38.0)		4 months	Minocycline	200mg once daily	Risperidone only	39	40	SANS	60.3 (14.7)
1 (27.5)	1. HDRS-17 < 14 2. PANSS Depression item < 4	2 months	Minocycline	200mg once daily	Risperidone only	20	20	PANSS negative	17.3 (2.6)
3 (25.4)	1. BPRS Total ≥ 45 2. BPRS Positive ≥ 8 with a score ≥ 4 on at least one individual item	2.5 months	Minocycline	100 mg twice daily	Clozapine	29	23	SANS-Modified (SANS total score minus "global items,	29.0 (12.9)

								inappropriate affect, poverty of content of speech, attention")	
7 (27.5)	1. PANSS Delusions ≥ 2 <i>or</i> 2. PANSS Conceptual disorganisation ≥ 2 <i>or</i> 3. PANSS Hallucinatory behaviour ≥ 2 <i>or</i> 4. PANSS Suspiciousness ≥ 2	12 months	Minocycline	100mg twice daily for 2 weeks, 100mg three times daily for the remaining 12 months	Mixed typical and atypical APs	104	103	PANSS negative	17.2 (5.7)
8 (50-6)	1. PANSS negative > 20 2. PANSS Positive > PANSS Negative	3 months	Minocycline	1. 100mg once daily 2. 100mg twice daily	Risperidone only	1. 25 (100mg dose) 2. 25 (200mg dose)	25	SANS	64.2 (5.6)

3.4 (10.1)	1. PANSS Delusions ≥ 4 <i>or</i> 2. PANSS Conceptual disorganisation ≥ 4 <i>or</i> 3. PANSS Hallucinatory behaviour ≥ 4 <i>or</i> 4. PANSS Suspiciousness ≥ 4 <i>or</i> 5. PANSS Negative ≥ 18	4 months	Minocycline	100 mg twice daily	Mixed typical and atypical APs	100	100	PANSS Negative	25.1 (5.5)
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5 (54.0)	1. At least "moderately-severe symptoms" based on PANSS Total score 2. Detectable IgG class antibodies to T. gondii	16 weeks	Azithromycin	600mg once daily for the first two weeks; then once weekly for the remaining 14 weeks	Mixed typical and atypical APs	13	15	PANSS Negative	Not reported
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(0)	1. PANSS Total ≥ 60	6 months	Trimethoprim	200mg once daily	Mixed typical and atypical APs	46	45	PANSS Negative	35.7 (9.6)
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8 (42.0)	1. "Psychotic symptoms which were at least moderately severe" based on PANSS Positive <i>and/or</i> 2. PANSS Negative ≥ 4 <i>or</i> 3. PANSS Total ≥ 50 "containing at least three positive or negative items with scores ≥ 3 "	10 weeks	Artemisinin	100 mg twice daily	Mixed typical and atypical APs	33	33	PANSS Negative	19.4 (4.0)
3 (53.0)	1. PANSS Total ≥ 60 2. T. gondii IgG or IgM seropositivity	8 weeks	Artemether	80 mg once daily at week 2 and week 4	Risperidone only	50	50	PANSS Negative	18.7 (5.7)
6 (50.0)	1. PANSS Total ≥ 60	6 weeks	Sodium Benzoate	1g once daily	Mixed typical and atypical APs	25	27	SANS	57 (13.6)

9 (31.7)	1. SANS-20 ≥ 40 2. PANSS total ≥ 70 3. AP-resistance 4. Clozapine-resistance (12 weeks of treatment without satisfactory response)	6 weeks	Sodium Benzoate	1. 1g once daily 2. 2 g once daily	Clozapine	1. 20 (1g dose) 2. 20 (2g dose)	20	SANS-20	59.5 (12.4)
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3 (35.4)	1. PANSS Positive ≥ 1 <i>or</i> 2. PANSS Negatives ≥ 4 <i>or</i> 3. PANSS Total ≥ 50 , “containing at least 3 positive or negative items with scores ≥ 3 ”	14 weeks	Probiotics* * <i>Lactobacillus rhamnosus</i> strain GG and <i>Bifidobacterium animalis</i> subsp. lact is strain Bb12	10 ⁹ CFU <i>Lactobacillus</i> and 10 ⁹ CFU <i>Bifidobacterium</i> once daily	Mixed typical and atypical APs	33	32	PANSS Negative	20.0 (3.6)
(6.7)	1. PANSS total ≥ 55 2. HDRS <14 OR PANSS depression < 4	12 weeks	Probiotics*+ Vitamin D * <i>Lactobacillus acidophilus</i> , <i>Bifidobacterium bifidum</i> , <i>Lactobacillus reuteri</i> , <i>Lactobacillus</i>	8×10 ⁹ CFU once daily probiotics and 50,000 IU Vitamin D every two weeks	Mixed typical and atypical APs	30	30	PANSS negative	28.4 (4.1)

			<i>fermentum</i>						
	1. Global cognitive score below “0.5 SD the healthy average”	24 weeks	B-GOS	Once daily	Mixed typical and atypical APs	21	18	None	

ipsychotics; B-GOS: galacto-oligosaccharide prebiotic; CFU: colony forming unit; HDRS: Hamilton Depression Rating Scale; NIH: National Institutes of Health; PANSS: Positive and Negative Syndrome Scale; PHS Public Health Service; RCDBT: Randomised-Controlled Trial; WHO-UCLA AVLT: World Health Organization - University of California - Los Angeles Auditory Verbal Learning Test; TMT: Trail Making Test; WHO-UCLA AVLT: World Health Organization - University of California - Los Angeles Auditory Verbal Learning Test

oli P, Burnet PWJ, Cipriani A, Lennox BR. The gut-microbiome as a target for the treatment of schizophrenia: A systematic review and meta-analysis of randomised controlled trials of a

ficits and “negative symptoms”: qualitative summary

Conditions	No. patients (Female%)	Participants	Study duration	Metformin Dose	Outcome Measure	Meta-analysis
Amnesic Mild Cognitive Impairment	58 (38%)	Metformin n=29 Placebo n=29	24 weeks	2000 mg/d	Cognition and Depression measured with WMS-R and MADRS	Meta-analysis of placebo-controlled trials
Obesity and Type 2 Diabetes	333 (100%)	Metformin n=84; Placebo n=83; Weight loss + placebo n=83; Weight loss + metformin n=83	24 weeks	1500 mg/d	Cognition measured with NeuroTrax	Trail Making Test; TMT: Trail Making Test
Alzheimer's Disease	20 (45%)	Metformin n=10 Placebo n=10	8 weeks)	2000 mg/d	Cognition measured with CANTAB and ADAS-Cog	Meta-analysis of placebo-controlled trials
Depression	80 (50%)	Metformin n=40 Placebo n=40	52 weeks	1000 mg/d	Cognition measured with SRT	Trail Making Test; TMT: Trail Making Test
Adults with high risk of developing schizophrenia	1,985 (68%)	Metformin n=992 Placebo n=993	52 weeks	1700 mg/d	Depression measured with BDI	Beck's Depression Inventory; BDI: Beck's Depression Inventory

mmers Scale-Cognitive Subscale; AMCI: Amnesic Mild Cognitive Impairment; BDI: Beck's Depression Inventory; CANTAB: Cambridge Neuropsychological Test; TMT: Trail Making Test; WHO-UCLA AVLT: World Health Organization - University of California - Los Angeles Auditory Verbal Learning Test

Chapter 6. General Discussion

6.1 Summary

6.1.1 Rationale in brief

Negative symptoms are traditionally described as key clinical features of psychotic illness[1]. However, a growing amount of evidence supported by large-scale initiatives, such as the RDoC, suggests that negative symptoms manifest across traditional diagnostic boundaries[15, 110]. Negative symptoms can be found in major mood disorders (depressive symptoms) in schizophrenia (negative symptoms) and in chronic fatigue (fatigue). Within the RDoC matrix, the RDoC Positive Valence System, which includes a hedonic and a cognitive component, is the one that more closely describes negative symptoms[48, 226]. Negative symptoms are often hard to treat and represent one of the main barriers for the functional recovery of patients suffering with severe mental illness[1, 14, 38]. Investigations aimed at understanding the pathophysiological underpinnings of negative symptoms are therefore a key clinical and research priority.

A consistent body of evidence has shown that modifications of the gut-microbiome can manifest with negative symptoms[119, 173, 227, 228]. This is of great relevance because the gut microbiome represents a potential modifiable target for interventions. However, translational evidence is limited, with a number of clinical trials using gut microbiome based therapeutics for the treatment of severe mental illness that reported negative results[34]. This might be due to the lack of specificity of the proposed interventions, which is the direct consequence of the gap in knowledge of the precise mechanisms linking gut microbiota abnormalities to central brain functions. Consistent evidence from other fields of medicine, such as cardiometabolic and gastrointestinal conditions, has shown that modifications of the gut microbiome impact the activity of the endocannabinoid system[47, 60, 108].

The RDoC initiative suggests that endocannabinoids might be at play for the Positive Valence System[48]. This is consistent with evidence showing that exogenous cannabinoids, such as THC, can induce an anhedonic/amotivation syndrome in sufferers, which can be prevented by another cannabinoid (CBD) [54, 55].

Based on these considerations, during my DPhil I decided to explore if the interplay between the gut microbiome and the endocannabinoid system would be relevant for negative symptoms.

With this aim in mind, I conducted four systematic reviews and meta-analyses and a general population study.

6.1.2 Summary of results

I initially explored the independent contribution of the endocannabinoid system and the gut microbiome to psychotic and severe mental illness through two systematic reviews and meta-analyses.

The first meta-analysis aimed at investigating disturbance of the endocannabinoid system in psychotic illness. Pooled results from 18 studies including 442 patients and 590 controls showed that anandamide, the main agonist of the endocannabinoid system, is increased in blood and cerebrospinal fluid of individuals suffering with psychotic illness. This increase was found at an early stage of illness, in antipsychotic-naïve individuals, had an inverse association with symptom severity (including negative symptoms) and was normalised after successful treatment. In brief, these findings showed that: (i) the endocannabinoid system is altered in psychotic illness; and (ii) its activation might be protective towards illness mechanisms and severity of clinical features.

The second meta-analysis aimed at investigating blood biomarkers of reduced microbial diversity (gut dysbiosis) in severe mental illnesses (schizophrenia, depression, and bipolar disorder) and chronic fatigue. Pooled results from 33 studies including 2,761 patients and 1,847 controls showed that blood biomarkers of gut-dysbiosis were increased and positively associated with severity of negative symptoms in patients vs controls independent of medication status and across diagnostic boundaries, including chronic fatigue. These findings provide indirect evidence of gut dysbiosis in a number of clinical conditions that manifest with symptoms related to the RDoC Positive Valence System.

I then brought the gut-microbiome and the endocannabinoid system together and explored the relevance of their interplay for negative symptoms in a general population cohort (TwinsUK).

To facilitate treatment breakthroughs, the RDoC strongly advocates for investigations on the transdiagnostic relevance of negative symptoms, including general population studies. Therefore, I conducted a multilevel mediation analysis on 786 twins (Twins UK cohort: general population) that showed that faecal levels of the endocannabinoid analogue palmitoylethanolamide (PEA) mediated the association between severity of experiential negative symptoms (anhedonia/amotivation) and gut microbial diversity. In particular, reduced microbial diversity was associated with increased faecal PEA excretion, which in turn was associated with more severe experiential negative symptoms. The analysis was adjusted for a number of clinical, sociodemographic and technical covariates known for their role on predictor, mediator, and outcome.

The mediation operated by PEA on the association between microbial diversity and experiential negative symptoms fills a gap in knowledge in the scientific literature on the topic. PEA is considered by most literature as the endogenous equivalent of cannabidiol, an inhibitor of the Fatty Acid Amid Hydrolase (FAAH), which is the main enzyme involved in the catabolism of anandamide. Increased PEA excretion might translate in an increased catabolic rate of anandamide, the latter being protective towards experiential negative symptoms. These findings advocate for the existence of a gut-microbiome-endocannabinoid axis, relevant for negative symptoms and a putative novel target for intervention.

I therefore explored if existing molecules targeting the first node of this axis, the gut-microbiome, could palliate negative symptoms in severe mental illness using two systematic reviews and meta-analyses.

The first meta-analysis investigated the efficacy of already known gut-microbiome-targeted therapeutics (antibiotics, antimicrobials, pre and probiotics) for the treatment of negative symptoms in psychotic illness.

Pooled results from 28 eligible randomised controlled trials showed that none of the already known gut-microbiome based therapeutics were effective for treating negative symptoms of psychotic illness. Results were mainly led by antibiotics and antimicrobials (25 trials), with paucity of evidence on pro and prebiotics.

The second meta-analysis investigated the efficacy of metformin, which novel evidence showed to have a specific action on the gut-microbiome. Pooled results from 5 eligible randomised controlled trials showed that metformin has pro-cognitive effects, with preliminary evidence showing efficacy on negative symptoms. Preclinical studies and a recent randomised controlled trial showed that metformin action is mediated by specific modification of the gut-microbiome, such as increasing the abundance of SCFA-producing bacteria. The relative abundance of these bacteria has also been related to the engagement of the endocannabinoid system.

6.2 Discussion

6.2.1 Implications of findings

Findings presented in this thesis advocate for the existence of a gut-microbiome endocannabinoid axis, relevant for negative symptoms across diagnostic boundaries.

In particular, results suggested that: a) reduced gut microbiome diversity is associated with more

severe negative symptoms; b) reduced engagement of the endocannabinoid system is associated with more severe negative symptoms; c) reduced engagement of the endocannabinoid system mediates the association between reduced gut microbiome and severity of negative symptoms.

Endocannabinoid agonists, such as anandamide (AEA) and 2-acilglycerol (2-AG) are protective for mental health[49]. Because of anatomical considerations (frontal brain regions draining directly into the bloodstream), circulating endocannabinoids are believed to be relevant for higher-order cognitive processes[83], which are at play in both the hedonic and cognitive components of the RDoC Positive Valence System. The endocannabinoid analogue PEA regulates the circulating levels of the two main endocannabinoid agonists (PEA is an inhibitor of their degradation)[73, 155]. Increased excretion of PEA may result in reduced circulating levels of endocannabinoid agonists, which in turn would lead to more severe negative symptoms. This would explain the direction of findings of the mediation model presented in Chapter 4.

The existence of a specific biological axis underlying negative symptoms would explain the lack of efficacy for treating negative symptoms of antibiotics and antimicrobials.

Antibiotics and antimicrobials lead to large-scale modifications of the gut-microbiome. These modifications do not necessarily cross the host's biological pathways that are relevant for negative symptoms. The aspecificity of these interventions might explain their lack of efficacy.

Contrarily, compounds that have a specific action on gut microbiome-brain pathways have the potential to represent a treatment breakthrough in an area of unmet need. This might be the case of metformin. As highlighted in Chapter 5 metformin acts on a specific gut microbiome path (increasing the abundance of butyrate-producing bacteria), which has been shown to have beneficial effects on the host mental health.

6.2.1 Limitations and directions for future studies

Results on the mediation model presented in Chapter 4 were obtained from a general population cohort and they need replication in a clinical population.

Future well-powered longitudinal studies should investigate the relationship between the gut microbiome and the endocannabinoid system in severe mental illness using specific accurate measures of the negative symptoms construct (e.g., the newly developed brief assessment scale for negative symptoms, BNSS). Furthermore, direct measures of the endocannabinoid agonists (AEA, 2-AG) were not available in the TwinsUK dataset. Future studies should therefore clarify if the

mediation operated by PEA was obtained via endocannabinoid agonists, as I hypothesised, or through other paths (e.g., inflammation).

The meta-analysis approach that was used to investigate gut dysbiosis was limited by the heterogeneity of the included studies. Future trans-diagnostic investigations should focus on specific subgroups of patients that predominantly/solely manifest negative symptoms, such as deficit schizophrenia and treatment-resistant depression. These investigations would also clarify the specific contribution of the gut-microbiome-endocannabinoid axis to negative symptoms.

It is in fact possible that abnormalities on this axis could manifest with a wide range of mental health phenotypes. Findings of gut dysbiosis in chronic fatigue, which differently from other conditions manifests exclusively with symptoms related to the RDoC Positive Valence System, suggest that reduced gut microbiome diversity might be specifically associated with negative symptoms. However, the evidence was limited and, as acknowledged in Chapter 3, indirect. Similarly, findings on the association between endocannabinoid system abnormalities and negative symptoms might be not specific and just suggest a more general relationship with mental well-being. In the general population study presented in Chapter 4, I tried to account for this potential bias using another psychopathological measure (anxiety) as outcome of the mediation model. Results were negative. The RDoC suggests the endocannabinoid system to be mainly related to the Positive Valence System and not to other components of the RDoC Matrix[19, 48, 226]. However, THC and the reduced engagement of the endocannabinoid system can also induce positive symptoms of psychotic illness[75]. Aspecificity might be therefore a possibility and future studies should investigate this further.

The meta-analytic findings on metformin were limited by the small number of studies, the lack of direct measures of the gut-microbiome, and the absence of data on negative symptoms in conditions other than depression. Future studies should address these limitations and clarify mechanisms of action.

6.2.2 Conclusions

The gut microbiome-endocannabinoid axis might represent a novel trans-diagnostic marker and trans-therapeutic target for negative symptoms. The gut microbiome is modifiable and targeted interventions might result in treatment breakthrough in an area of unmet need.

Results from the last chapter of this thesis underlie the importance of understanding the contribution of the gut microbiome to specific gut-to-brain pathways for therapeutics. Gut-microbiome based

interventions that target specific biological pathways, such metformin, might find use in the treatment of a number of clinical conditions manifesting with negative symptoms.

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Appendix

Chapter 2. Endocannabinoids in psychotic illness

2.1 Risk of Bias

Risk of Bias – adapted version of Newcastle-Ottawa quality assessment Scale (NOS)

SELECTION (range from 0 to 4)

- 1) **Is the case definition adequate?** *Yes, with independent validation (1 point)*
- 2) **Representativeness of the cases** *Consecutive or obviously representative series of cases (1 point)*
- 3) **Selection of Controls** *Community controls (1 point)*
- 4) **Definition of Controls** *No history of disease (1 point)*

COMPARABILITY

- 1) **Comparability of cases and controls based on the design or analysis (range from 0 to 2)**
 - a) *study controls for age (1 point)*
 - b) *study controls for cannabis use (1 point)*

EXPOSURE

- 1) **Ascertainment of exposure**
 - a) *secure record (1 point)*
- 2) **Same method of ascertainment for cases and controls**
 - a) *yes (1 point)*

Risk of Bias – adapted version of Newcastle-Ottawa quality assessment Scale (NOS)

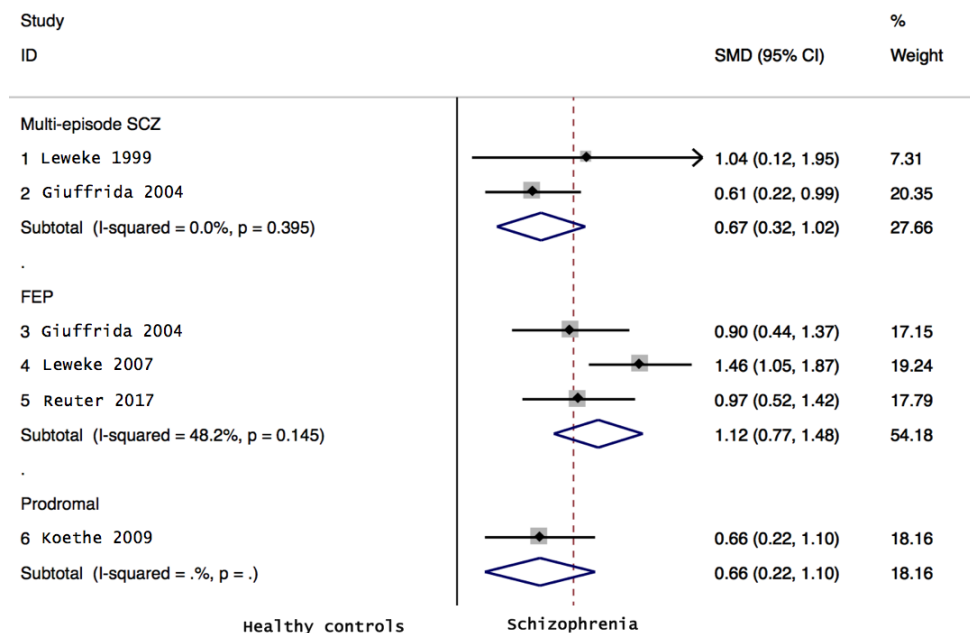
Study	Selection	Comparability	Exposure	Overall Score
Leweke et al. 1999	4	2	2	8
De Marchi et al. 2003	4	1	2	7
Giuffrida et al., 2004	4	2	2	8
Leweke et al., 2007	4	2	2	8
Hill et al., 2008	4	1	2	7
Potvin et al., 2008	4	2	2	8
Hill et al., 2009	4	1	2	8
Koethe et al., 2009	4	2	2	8
Desfosses et al., 2012	4	2	2	8
Leweke et al., 2012	4	2	2	8
Bioque et al., 2013	4	2	2	8
Ferretjans et al., 2014	4	1	2	7
Ogawa et al., 2015	4	1	2	7
Bioque et al., 2016	4	2	2	8

Chase et al., 2016	4	1	2	7
D'Addario et al., 2017	4	1	2	8
Kranaster et al., 2017	4	1	2	7
Reuter et al., 2017	4	2	2	8
Koethe et al., 2018	4	1	2	7
Moretti et al., 2018	4	1	2	7
Wang et al., 2018	4	1	2	7

A NOS score of 6/7 can be considered a “good” study

2.2 Subgroup analyses

AEA levels in the CSF – Stage



AEA levels in the CSF – Antipsychotic medications

Antipsychotic free/naïve: SMD 1.12; 95 % CI (0.77-1.48); I²: 48.2%

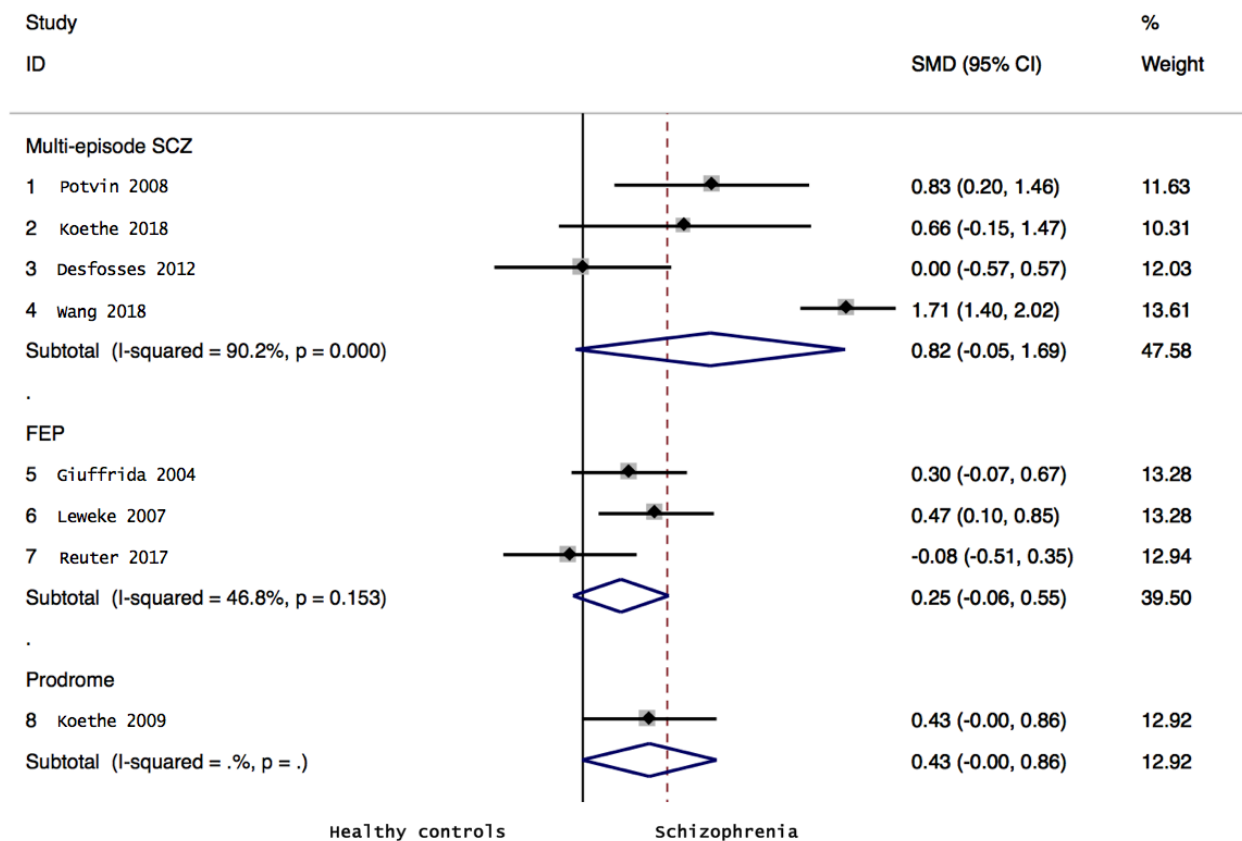
Antipsychotic treatment: SMD 0.61; 95 % CI (0.22-0.99); I²: NA

AEA levels in the CSF – Cannabis use

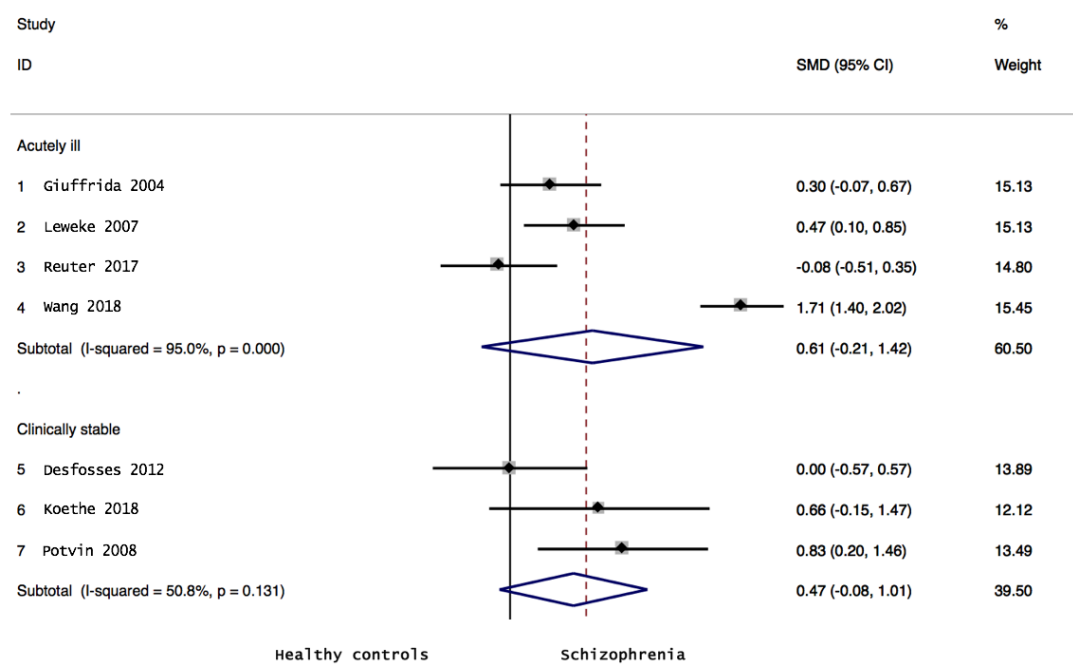
Current use: SMD 0.84; 95 % CI (0.60-1.08); I²: 0%

No current use: SMD 1.46; 95 % CI (1.05-1.87); I²: NA

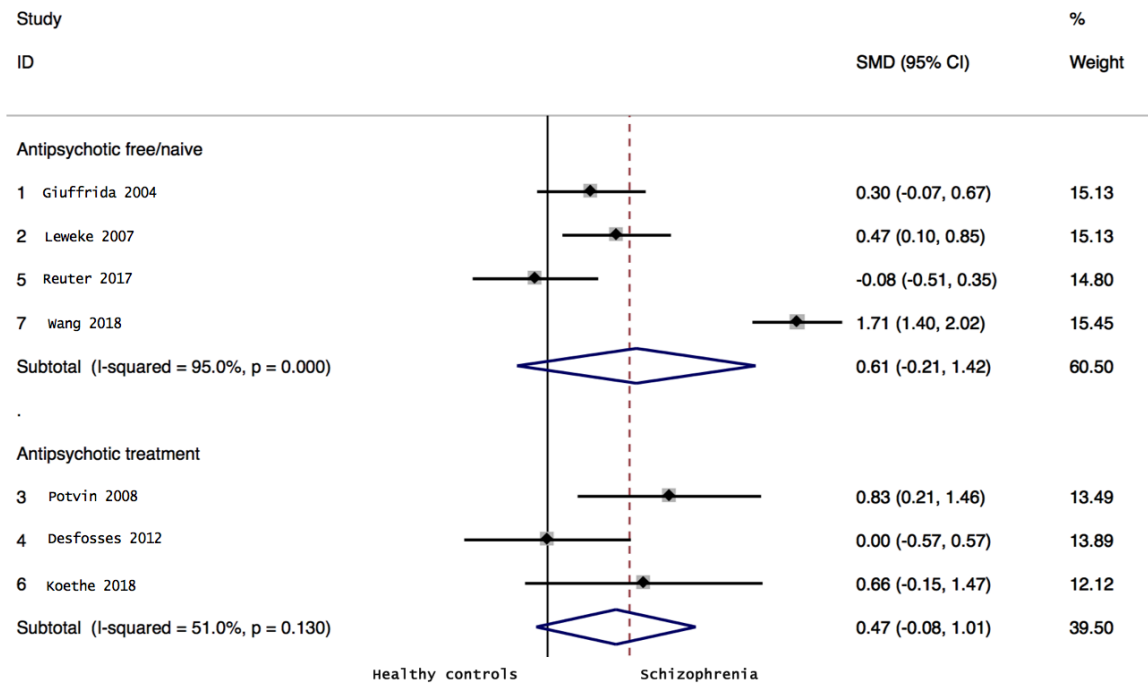
AEA levels in the blood – Stage



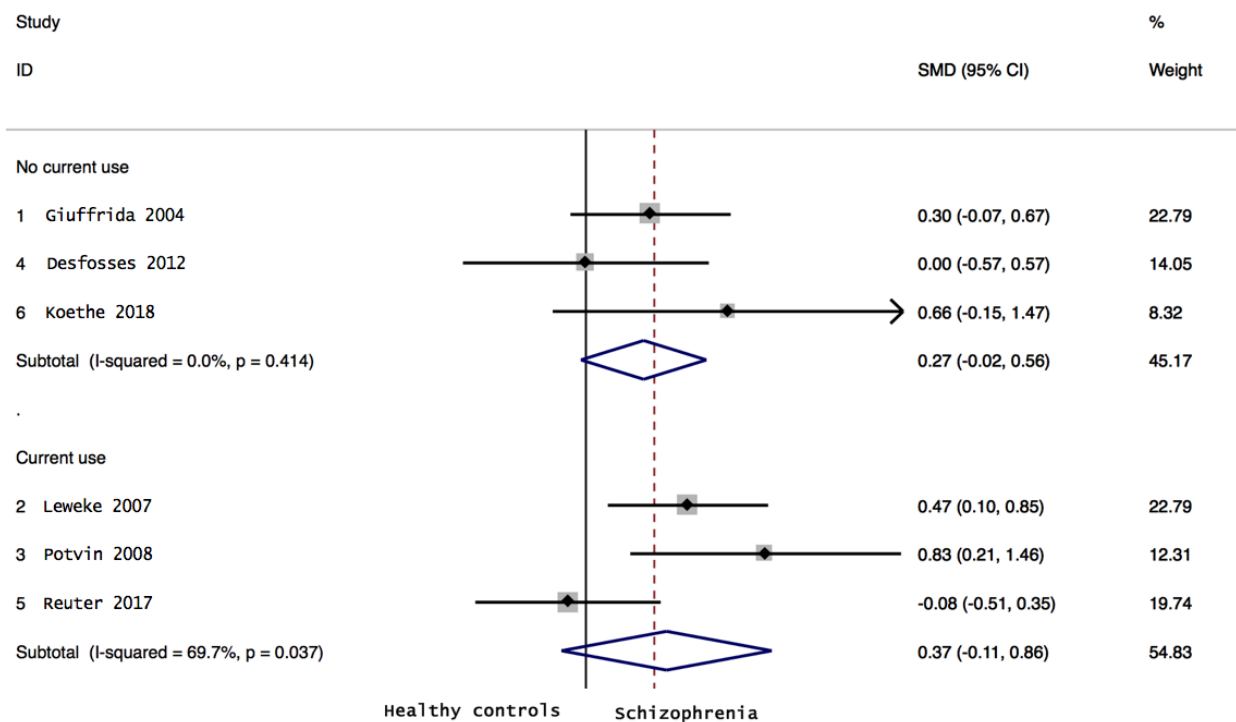
AEA levels in the blood – Phase



AEA levels in the blood – Antipsychotic treatment



AEA levels in the blood – Current cannabis use



Chapter 4. Endocannabinoids mediate the association between gut microbiome diversity and anhedonia/amotivation

4.1 Beta diversity metrics associated with both Anhedonia and faecal PEA: mixed linear regression models (random intercept: family structure)

Outcome (DV)	Exposure (IV)	Coeff.	SE	P value
Anhedonia	Unweighted Unifrac Axis 1	-3.16 ^a	1.12 ^a	0.005^a
		-2.51 ^b	1.46 ^b	0.085 ^b
Anhedonia	Weighted Unifrac Axis 4	-7.10 ^a	1.87 ^a	0.000^a
		-7.34 ^b	2.32 ^b	0.002^b
PEA faecal	Unweighted Unifrac Axis 1	-2.55 ^a	0.28 ^a	0.000^a
		-2.69 ^b	0.37 ^b	0.000^b
PEA faecal	Weighted Unifrac Axis 4	-3.73 ^a	0.48 ^a	0.000^a
		-4.13 ^b	0.61 ^b	0.000^b

^aAdjusted for technical confounders (microbiome operator, microbiome collection method, storage time) and basic demographics (age, gender, ethnicity)

^bFully adjusted: technical confounders (microbiome operator, microbiome collection method, storage time), basic demographics (age, gender, ethnicity), and obesity, antidepressants, unhealthy diet

Mixed linear regressions showed a significant association of a number of beta-diversity measures with Anhedonia and faecal PEAs both in unadjusted and fully adjusted models. However, unadjusted models for complete cases **did not** show a mediation effect of PEA. This suggests that beta-diversity measures might affect Anhedonia with pathways independent of PEA.

4.2 Complete case analysis

	Unadjusted Model		Model A ^b				Model B ^b			
	Complete cases		Complete cases		Imputed dataset		Complete cases		Imputed dataset	
	Coef.	p	Coef.	p	Coef.	p	Coef.	p	Coef.	p
	(n=529)		(n=408)		(n=786)		(n=321)		(n=786)	
Direct effect	-0.25 (0.18)	0.16	-0.25 (0.20)	0.21	-0.28 (0.19)	0.14	-0.66 (0.28)	0.51	-0.24 (0.19)	0.21
Indirect effect	-0.13 (0.06)	0.03	-0.13 (0.07)	0.04	-0.17 (0.06)	0.00	-0.18 (0.09)	0.04	-0.18 (0.06)	0.00
Total effect	-0.38 (0.17)	0.03	-0.39 (0.19)	0.04	-0.45 (0.18)	0.01	-0.34 (0.24)	0.14	-0.43 (0.19)	0.02

Predictor: Alpha diversity (Shannon index); *Mediator:* Faecal PEA; *Outcome:*

Anhedonia/amotivation;

Note: PEA values were log-transformed and scaled to a mean of 0 and standard deviation of 1

^a**Model A:** Adjusted for technical confounders: storage time (freezer) – PEA; storage time (fridge) – PEA; microbiome operator; microbiome collection method (post or visit) AND for basic demographics (age, gender, ethnicity)

^b**Model B:** Model A + obesity, unhealthy diet, antidepressants

When the model was fully adjusted, (Model B), the total effect was no longer significant in the complete case analysis. However, the total effect was significant in the imputed dataset, suggesting that the lack of significance in the complete case analysis was due to loss of power rather than to an effect of the covariates on the included variables

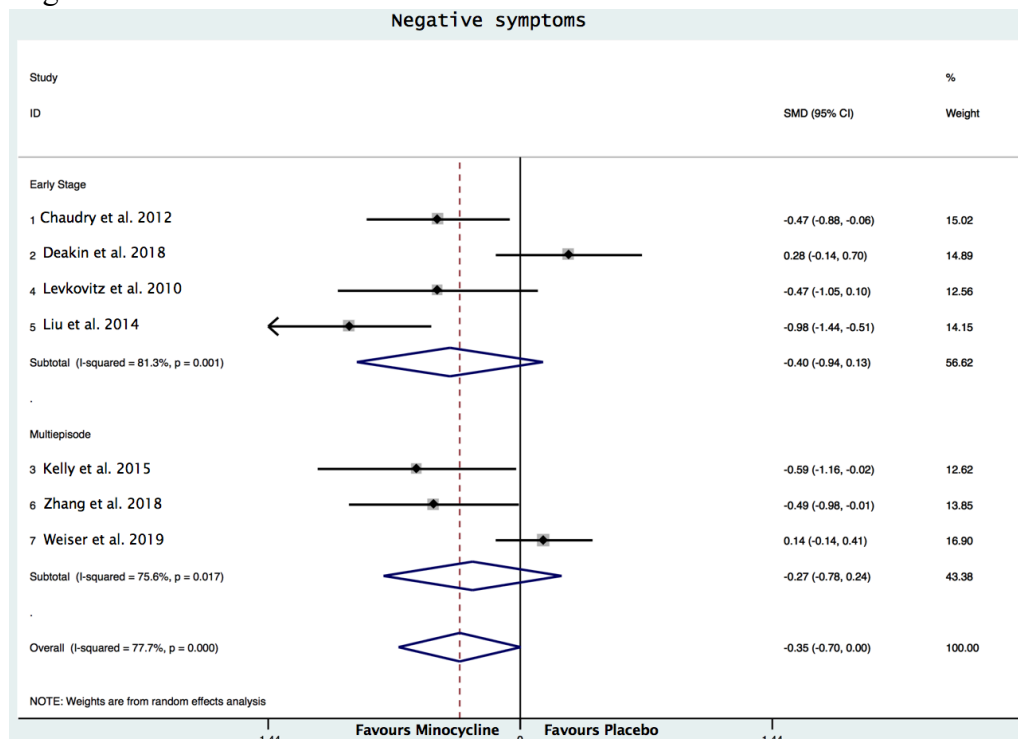
Chapter 5. Gut microbiome-based therapeutics

5.1 Categorization of neurocognitive tasks used in the meta-analysis

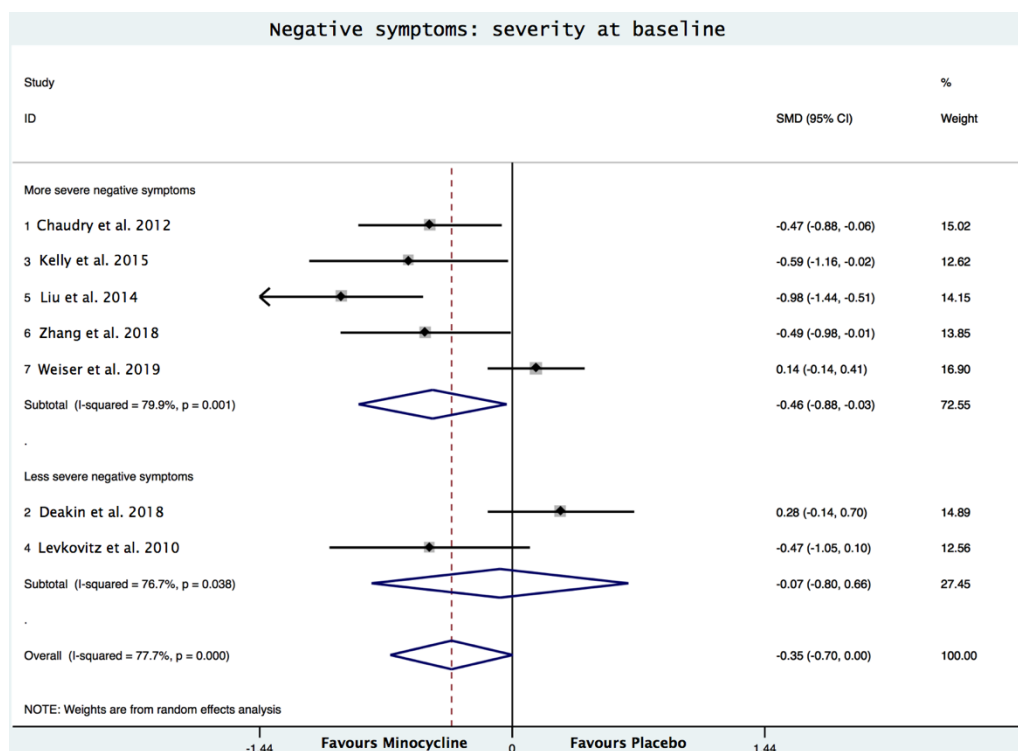
Cognitive Domain	Neurocognitive tasks
Speed of Processing	Digit-Symbol Coding (DS) Trail Making Task part A (TMT-A)
Attention / Vigilance	Continuous Performance Test (CPT) Rapid Visual Information Processing (which is a form of CPT)
Working Memory	Digit Span – Total (preferred) Digit Span – Forward (DSB) Digit Span – Backwards (DSF) Digit sequencing Wechsler Memory Scale (WMS) - Spatial Span (SS) Sternberg Short Term Memory Scanning Test Letter-Number Sequencing with reordering Spatial Working Memory (SWM) task
Verbal Learning and Memory	Hopkins Verbal Learning Test (HVLT) Logical Memory subtest of WMS-III (LM) Miller-Selfridge Test Rey Auditory Verbal Learning Test
Visual Learning and Memory	Face I and II of WMS-III Pattern Recognition Memory Spatial Recognition Memory Brief Visuospatial Memory Test (BVMt)
Executive functions/Reasoning and Problem Solving	Stroop Task – Interference Trail Making Test Part B (TMT-B) Trail Making Test B minus A WCST- perseverative errors WCST- conceptual level of response Tower of London Mazes
Motor speed	Finger tapping Pegboard Token movement
Verbal fluency	Phonemic Fluency (F,A, S) Categories (animal)

5.2 Subgroup analyses

Add-on Minocycline vs placebo for the treatment of negative in schizophrenia: subgroup analysis – stage

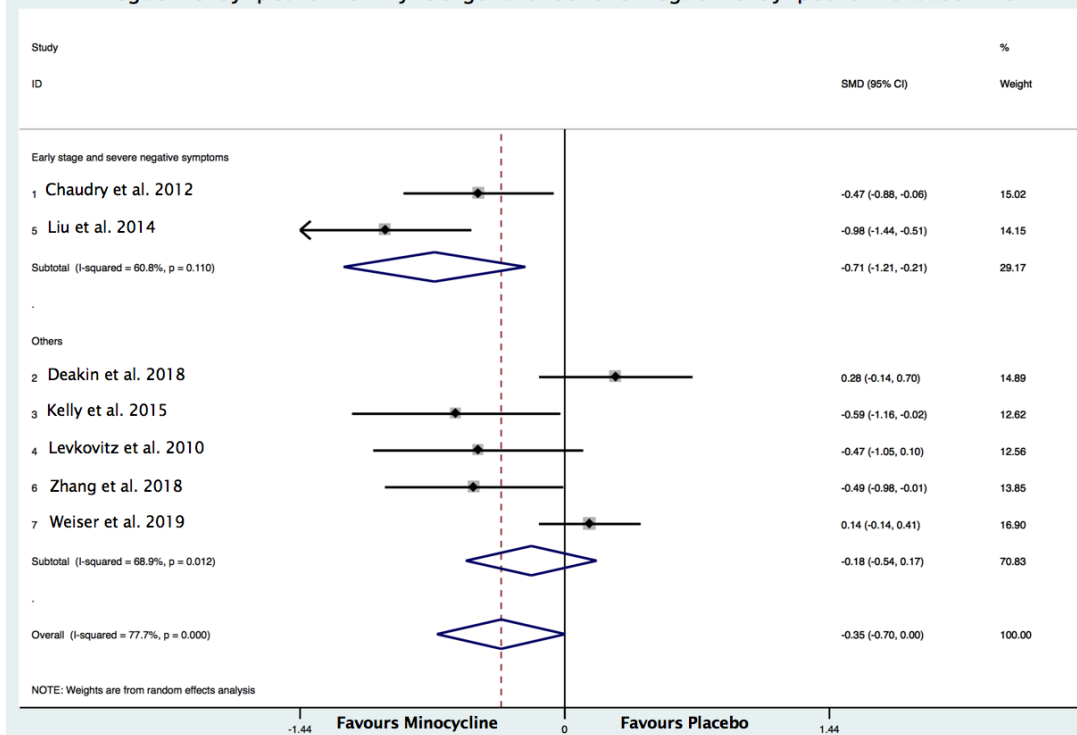


Add-on Minocycline vs placebo for the treatment of negative in schizophrenia: subgroup analysis – severity of negative symptoms at baseline



Severity of negative symptoms at baseline. The “more severe” subgroup includes studies that recruited participants with a score higher than the median value for PANSS negative or SANS across the included add-on Minocycline RCTs.

Negative symptoms: early stage and severe negative symptoms at baseline



Severity of negative symptoms at baseline. The “more severe” subgroup includes studies that recruited participants with a score higher than the median value for PANSS negative or SANS across the included add-on Minocycline RCTs. Others: RCTs investigating add-on Minocycline vs placebo with included participants being at a multi-episode stage of illness AND/OR having a PANSS negative/SANS score below the median value.

5.3 Positive, Total and Cognitive symptoms

Positive symptoms

D-Cycloserine (7 studies; SMD, -0.19; 95% CI, -0.54, 0.16; P=0.29; I²:49.6%)

Minocycline (8 studies; SMD, -0.14; 95% CI -0.27, 0.02; P=0.09; I²:0%)

Total symptoms

D-Cycloserine (4 studies; SMD, 0.01; 95% CI, -1.35, 1.37; P=0.99; I²:92.3%)

Minocycline (8 studies; SMD, -0.36; 95% CI -0.72, 0.00; P=0.05; I²:78.0%);

Cognition

D-Cycloserine

Composite score (3 studies; SMD, 0.13; 95% CI, -0.32, 0.58; P=0.56; I²: 42.0%)

Attention (N=3 studies; SMD, -0.21; 95% CI, -0.66, 0.23; P=0.34; I²: 44.4%)

Executive functions (N=3 studies; SMD, -0.30; 95% CI, -0.62, 0.02; P=0.06; I²: 0%)

Working memory (N=4 studies; SMD, -0.17; 95% CI, -0.49, 0.14; P=0.27; I²: 0%)

Attention (3 studies; SMD, 0.00; 95% CI -0.53, 0.54; P=1.00; I²: 57.6%)
Executive functions (3 studies; SMD, 0.18; 95% CI -0.51, 0.88; P=0.61; I²: 73.7%)
Working memory (4 studies; SMD, -0.08; 95% CI -0.52, 0.35; P=0.71; I²: 57.9%)
Verbal Memory (2 studies; SMD, -0.13; 95% CI, -0.62, 0.35; P=0.59; I²: 46.9%)
Visual memory (2 studies; SMD, -0.04; 95% CI -0.54, 0.09; P=0.88; I²: 52.5%)
Processing speed (3 studies; SMD, -0.18, 95% CI -0.45, 0.09; P=0.19; I²: 0%)
Motor speed (2 studies; SMD, 0.25, 95% CI, -0.43, 0.93; P=0.47, I²:50.0%)

Sodium Benzoate

Composite score (2 studies: SMD, 0.10; 95% CI -0.74, 0.95; P=0.81; I²: 75.6%)
Attention (2 studies; SMD, 0.09; 95% CI -0.31, 0.48; P=0.66; I²: 0%)
Executive functions (2 studies; SMD, 0.06; 95% CI -0.33, 0.45; P=0.76; I²: 0%)
Working memory (2 studies; SMD, 0.18; 95% CI -0.60, 0.96; P=0.65; I²: 72.6%)
Verbal Memory (2 studies; SMD, 0.07; 95% CI, -0.75, 0.89; P=0.87; I²: 76.1%)
Visual memory (2 studies; SMD, 0.11; 95% CI -0.98, 1.21; P=0.84; I²: 86.2%)
Processing speed (2 studies; SMD, 0.10, 95% CI -0.53, 0.72; P=0.76; I²: 58.3%)

*Both trials on sodium benzoate used the same modified version of the MATRICS