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Investigating cognition in chronic opioid use: Potential role for the gut microbiota

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Investigating cognition in chronic opioid use: Potential role for the gut microbiota.

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Dr Katrina Green, Dr Yinghua Yu

This thesis is presented as part of the requirement for the conferral of the degree:

Masters of Research (Medicine)

This degree included coursework and a 1 year research component, which is represented in the present thesis.

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School of Medicine

November 2021

Certification

I, Mayank Devsegran Nair declare that this thesis submitted in fulfilment of the requirements for the conferral of the degree Masters of Research (Medicine)*, from the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.*

_____

Mayank Devsegran Nair

06 November 2021

Abstract

Impairment in cognitive functioning is a core component of opioid dependence due to its importance in the course of addiction and its role in treatment, but the effect of opioid use on cognition in individuals undergoing early stages of treatment is under examined, particularly in the Australian population. Although existing pharmacological options have demonstrated some efficacy in treating opioid dependence, they are limited in their ability to treat the cognitive dysfunction present in opioid dependent individuals. Hence, there is a need for novel treatment options that address these limitations. The commensal gut microbiota can engage in bidirectional communication with the brain and thus influence brain function, including cognition. Dysbiosis of the microbiota has been reported in several areas of addiction and concomitant cognitive impairment, and may serve as a target for potential future novel treatments. The effect of opioid use on the gut microbiota is inconclusive, however. The present thesis aimed to: a) investigate cognition in individuals with a history of chronic opioid use during the early stages of rehabilitation treatment in an Australian setting; b) examine the effect of opioid use on the gut microbiota, and; c) outline the functional potential of the gut microbiota in opioid use and how it may relate to key signalling pathways of the microbiota-gut-brain axis. In Chapter 2, Australian participants at early stages of community-based rehabilitative treatment (including treatment with methadone or buprenorphine-naloxone, BNX) underwent neurocognitive testing. Results demonstrated impaired cognitive functioning compared to the general population, but no significant differences between performance in BNX compared to methadone-treated participants. BNX treatment was associated with a longer length of stay, which could indicate greater treatment adherence. The potential influence of treatment and non-treatment related parameters were also examined. Treatment related factors (e.g., time since last dose,

life-time length of treatment) had a significant relationship with cognitive performance in BNX-treated participants, but not methadone treated participants. Neurocognitive performance was also significantly influenced by non-treatment related demographics factors, such as age and BMI. Together, these findings demonstrate cognitive impairment in people undergoing residential rehabilitation for opioid addiction and highlight treatment and demographics parameters that could potentially influence cognitive outcomes and should be considered in future studies.

In Chapter 3, a systematic literature review was conducted to investigate the effect of opioids on the gut microbiota. Results demonstrated that opioid use resulted in dysbiosis of the gut microbiota, and identified specific microbes that were repeatedly dysbiotic across clinical and preclinical studies for the first time. Opioid use also resulted in alterations to key signalling pathways of the microbiota-gut-brain axis, suggesting the potential for opioid induced dysbiosis of the gut microbiota to influence cognition. These results may have significant implications for future research aiming to better understand the pathology of opioid dependence, and may inform the development of future novel treatments that improve the lives of people with opioid dependence.

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CHAPTER 2

List of Names or Abbreviations

AA – Amino Acid

Ar. AA – Aromatic Amino Acid

ACE – Abundance-based Coverage Estimators

BA, SBA – Bile acids, Secondary bile acids

BACS-SC – Brief Assessment of Cognition in Schizophrenia Symbol Coding

BBB – Blood Brain Barrier

BCAA – Branched Chain Amino Acid

B.I.D. - bis in die i.e. twice a day

BMI – Body Mass Index

BMT – Buprenorphine Maintenance Therapy

BVMT-R – Brief Visuospatial Memory Test Revised

CBT – Cognitive Behavioural Therapy

CD – Compulsory Detention

CF Animal Naming – Category Fluency Animal Naming

CPP – Conditioned Place Preference

DNA – Deoxyribonucleic Acid

DSM – Diagnostic and Statistical Manual

DU – Drug Users

FMT – Faecal Microbiota Transplant

GF – Germ Free

GM – Gut Microbiota

HC – Healthy Control

HCAD – Histone Deacetylase

HCV – Hepatitis C Virus

HDACi – Histone Deacetylase Inhibitor

HE – Hepatic Encephalopathy

HIV – Human Immunodeficiency Virus

HVLT-R – Hopkins Verbal Learning Test Revised

ICD – International Classification of Diseases

I.M. – Intramuscular

I.P. – Intraperitoneal

KEGG – Kyoto Encyclopaedia of Genes and Genomes

LNS – Letter Number Span, or Letter Number Sequencing

LPS – Lipopolysaccharide

MCCB – MATRICS Consensus Cognitive Battery

MCoA – Montreal Cognitive Assessment

MGB – Microbiota Gut Brain

MMT – Methadone Maintenance Therapy

NAB-Mazes – Neuropsychological Assessment Battery Mazes

NASH – Non-alcoholic Steatohepatitis

OR – Opioid receptor

OUT – Operational Taxonomic Unit

OUD – Opioid use disorder

PCA – Principal Component Analysis

PCoA – Principal Coordinates Analysis (also known as Multidimensional Scaling; MDS)

PICRUSt - Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

PGN – Peptidoglycan

PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PUFA – Polyunsaturated Fatty Acids

RNA – Ribonucleic Acid

S.C. – Subcutaneous

SCFA – Short Chain Fatty Acid

SIV – Simian Immunodeficiency Virus

SPF – Specific Pathogen Free

SUD – Substance use disorder

T2DM – Type 2 Diabetes Mellitus

T.I.D. - ter in die, i.e. three times a day

TMT-A – Trail Making Test A

WGS – Whole Genome Sequencing

WMS III-SS – Wechsler Memory Scale III Spatial Span

WT – Wild Type

Glossary

16S rRNA – (also referred to as rDNA) a highly conserved, and universally present, subunit of the ribosomal RNA gene, present in bacteria, utilised for taxonomic classification

Abundance – the number of individuals of a distinct species within an ecosystem

ACE – (Abundance-based Coverage Estimators) measure of alpha diversity, measures community richness

Alpha Diversity – the richness of a community within a single, distinct site

Beta Diversity – the difference of richness between two or more distinct sites

Bray-Curtis Dissimilarity – a measure of beta diversity

Chao1 – a measure of alpha diversity, measures community richness

Diversity – the richness and evenness of a particular site or, the number of distinct species present in a given community and the variations in the populations of these distinct species e.g. if there are three species at site A, and two at site B, site A has a higher diversity

Evenness – the uniformity of the population (or uniformity of abundance) of an individual species compared to other species in a given community e.g. the number of *Bacteroides* compared to the number of *Prevotella* in a given site

Faith's Phylogenetic Diversity – a measure of alpha diversity,

Jaccard Similarity Index – a measure of beta diversity

Observed OTU's – (Observed Operational Taxonomic Units) a measure of alpha diversity, measures community richness

Opiates – Drugs derived from the poppy plant e.g. morphine

Opioids – Opiate analogue drugs of synthetic (e.g. methadone), or partially synthetic (e.g. buprenorphine) origin that act as ligands to opioid receptors and exert similar effects

PICRUSt – (Phylogenetic Investigation of Communities by Reconstruction of Unobserved

States) a method of predicting the metagenomics content of microbes through analysis of 16S rRNA data

Relative Abundance – the amount an individual species contributes to the whole community of its ecosystem

Richness – the number of distinct organisms present in a community e.g. if a given site has two different species, there is a richness of two. If another site has three species, it has a greater richness

Shannon Index – a measure of alpha diversity, measures community evenness

Simpsons Index – a measure of alpha diversity, measures community evenness

Conference Presentation

The following presentation arose directly from work contained within this thesis.

Nair M, Yu Y, Weston-Green K (2020). A systematic review investigating the effect of opioid use on the gut microbiome. Biological Psychiatry Australia (online presentation), 19 – 21 Oct 2020.

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Chapter 1

1.1 Introduction

1.1.1 *Overview of Opioid Use*

Chronic opioid use, including Opioid Use Disorder and Opioid Misuse, is a potentially fatal practice that is occurring at epidemic levels, and as such is an issue requiring greater attention and understanding. Opioid use disorder (OUD) is a Diagnostic and Statistical Manual (DSM)-5 substance use disorder (SUD) characterized by chronic, relapsing use of drugs such as morphine, hydrocodone and fentanyl, in spite of the severe negative cognitive, physical, social and economic consequences they may incur. OUD is a spectrum SUD (Strang et al., 2020), the severity of which depends on the number of criteria (Table 1) that an individual presents with, with diagnoses ranging from mild (2-3), moderate (4-5), to severe (6-7). Apart from the DSM-5, the DSM-IV and ICD-10 can used to diagnose opioid addiction. Unlike the DSM-5, the DSM-IV considers opioid abuse and dependence to be two distinct disorders (Peer et al., 2013). Similarly, the ICD-10 also considers abuse and dependence to be two separate disorders (Strang et al., 2020), though it uses many of the same diagnostic criteria (Table 1.1). Opioid misuse is the administration of prescribed opioid medications (such as codeine) not in line with prescriber direction, such as using higher doses than is recommended or more frequently than directed (Tetrault and Butner, 2015). Chronic opioid use commonly involves opiates and opioids. Opiates refer to substances of natural origin derived from the poppy plant, such as morphine. Opioids refer to substances that act on opioid receptors in the body more broadly, and can include semi-synthetic compounds such as hydrocodone, and synthetic compounds such as fentanyl and heroin (Strang et al., 2020). While technically incorrect, the two terms will be used interchangeably for the remainder of this thesis.

Table 1.1 **Diagnostic criteria for chronic opioid use, including DSM-IV and DSM-5 criteria for Opioid Use Disorder and ICD-10 criteria for Opioid Dependence**

DSM-IV	DSM-5	ICD-10
Persistent use despite negative health consequences	Persistent use despite negative health consequences	Strong desire to use opioids
Failure to meet work, school or social obligations due to opioid use	Failure to meet work, school or social obligations due to opioid use	Difficulty controlling use of opioids
Persistent use of opioids despite recurring social issues resulting from or exacerbated by effects of opioids	Persistent use of opioids despite recurring social issues resulting from or exacerbated by effects of opioids	Development of tolerance (i.e., higher doses are needed to achieve desired effects
	Craving for opioids	Persistent use despite negative consequences (i.e. impaired cognition
Taken in larger amounts or for longer than intended	Taken in larger amounts or for longer than intended	Neglecting other interests to spend greater time pursuing opioids or recover from their impact
Persistent, but unsuccessful desire to reduce or control use	Persistent, but unsuccessful desire to reduce or control use	Withdrawal upon cessation of use, evidenced by either, a) characteristic withdrawal syndrome, or b) use of opioids for purpose of relieving or avoiding withdrawal
Spending large amounts of time trying to obtain, use, or recover from use	Spending large amounts of time trying to obtain, use, or recover from use	
Reduced or failure to uphold social, work, or recreational activities due to use	Reduced or failure to uphold social, work, or recreational activities due to use	
Continued use despite awareness of physical or psychological issues onset from or exacerbated by drug	Continued use despite awareness of physical or psychological issues onset from or exacerbated by drug	
Tolerance defined as either, a) diminished effects with continued use of same dose or, b) need for higher doses to achieve intoxication or desired effect	Tolerance defined as either, a) diminished effects with continued use of same dose or, b) need for higher doses to achieve intoxication or desired effect	
Withdrawal upon cessation of use, evidenced by either, a) characteristic withdrawal syndrome, or b) use of opioids for purpose of relieving or avoiding withdrawal	Withdrawal upon cessation of use, evidenced by either, a) characteristic withdrawal syndrome, or b) use of opioids for purpose of relieving or avoiding withdrawal	

Notes**:** Diagnostic and Statistical Manual (DSM) IV allows for diagnosis of abuse or dependence, whereas DSM-5 only diagnoses OUD. DSM-IV criteria for abuse include the first three criteria as well as substance-related legal issues (not listed). Dependence involves meeting three or more italicised symptoms in a concurrent 12-month period. DSM-5 added the criteria of craving and severity of OUD, not included in DSM-IV.

The prevalence of harmful opioid use is immense and is on the rise. OUD is occurring at an epidemic level with an estimated 40.5 million individuals presenting with the disorder globally in 2017 (Degenhardt et al., 2019, James et al., 2018), and an estimated 2 million adults

qualifying for a DSM-IV diagnosis of OUD in the United States alone (Han et al., 2017). In addition to this, an increasing rate of mortality has been linked to these drugs, rising 292% from 2001 to 2016 in the United States (Gomes et al., 2018). Globally, opioids contributed to approximately one-third of drug related deaths in 2015 (Nolan, Socias and Wood, 2018), with approximately 40 000 deaths related to opioids and 24 000 deaths related to heroin or synthetic opioids (Ruhm, 2018). Prescription opioids serve a unique problem as, whilst they are a vital option for pain management, they are often misused (used in higher doses or for longer than directed), and are a risk factor for the development of a full OUD (Brummett et al., 2017, Cicero and Ellis, 2017, Deyo et al., 2017). While cases are more concentrated in the US, there is a need for further investigation into Australian cohorts.

Misuse of opioids is exceedingly high, with an estimated 11.5 million U.S. adults reporting misuse of prescription opioids in 2015 (Han et al., 2017). Use of hydrocodone and oxycodone, commonly prescribed pain management drugs, has increased two- and five-fold respectively over the last fifteen years (Kolodny et al., 2015). Several other comorbidities often result from harmful opioid use including: increased risk of infection with blood borne diseases such as Human Immunodeficiency Virus (HIV) and Hepatitis C (HCV); development of other comorbid diseases (Strang et al., 2020); progression to use of stronger opioids such as heroin and riskier drug use practises (Cicero and Ellis, 2017, Strang et al., 2020); greater risk of involvement in the criminal justice system (Pryor, Boman and Hemez, 2021), and an increased risk of mortality. Additionally, the societal costs of OUD and misuse are immense, with approximately a trillion dollars lost through the health care system, criminal justice system and lost work hours in the United States alone (Florence, Luo and Rice, 2021). Hence, misuse of opioids is occurring at an epidemic rate, in part due to increasing use of synthetic and prescription opioids, leading to severe health, social and economic consequences.

1.1.2 *Opioid Use and Cognition*

While definitions vary, cognition most commonly refers to the processes involved in the acquisition, processing, storage and retrieval of information (Lyon et al., 2021). More recently, Lyon (2020) put forward a new definition, suggesting cognition can be referred to as "…the sensory and other information-processing-mechanisms an organism has for becoming familiar with, valuing, and interacting productively with features of its environment […] in order to meet existential needs…". Cognitive processes are classified by domains (Harvey, 2019) and are usually defined by the processes involved. These domains are hierarchical in nature; more general domains, such as attention, contain subdomains, such as selective attention (Table 1.2). Domains are not independent as processes in some domains may rely on the functioning of others. Performance in these domains is assessed through the use of neurocognitive tests. Poorer performance in these tests reflects impairments in cognition, and is frequently reported in patients engaging in chronic use of substances such as opioids (discussed in detail below). For example, Darke et al. (2012) reported poorer performance in tests measuring the domains of executive function, processing speed, verbal learning and non-verbal learning in patients engaging in either methadone and buprenorphine use compared to healthy controls not using opioids. Mechanisms by which these impairments may occur are also discussed below. Addiction is also conceptualised as a form of maladaptive learning, suggesting even a role for intact processes in the development and maintenance of addiction (Belin et al., 2013, Gould, 2010, Milton and Everitt, 2012). The effect of medications for opioid treatment on cognitive performance remains is the subject of much contemporary research; however, it is unclear whether treatment improves or further impairs cognition.

Table 1.2 **A brief list of general cognitive domains and their subdomains, as well as some neurocognitive tests used to assess performance in those domains**

General Cognitive Domain	Subdomain	Further Subdomains	Example Neurocognitive Tests used for Assessment
Executive Function	Reasoning and problem solving		WCTS, NAB-Mazes
Processing Speed	Coding and tracking		TMT-A and B, BACS-Symbol Coding
Motor Skills	Drawing Copying		MCoA-Clock Drawing BVMT-R
Attention	Selective attention Vigilance		Dual processing CPT
Memory	Working memory Episodic/Declarative	Verbal Non-verbal Verbal Non-verbal	LNS WMS III-SS HVLT-R
Language and Verbal Skills	Naming and fluency		CF-Animal Naming

Notes: This is not an exhaustive list, and there is much debate over the categorisation and classification of the various cognitive domains. For example, reasoning and problem solving are considered two distinct subdomains by (Harvey, 2019), but are assessed as one domain by the MATRICS Consensus Cognitive Battery. This table was adapted from HARVEY, P. D. 2019. Domains of cognition and their assessment. *Dialogues in Clinical Neuroscience*, 21, 227-237.

1.1.3 *Existing Treatments for OUD and their Limitations*

While the existing treatment options of OUD have some efficacy, medication options have several clinically significant limitations (Table 1.3). Pharmacological treatments for opioid addiction include methadone, buprenorphine, buprenorphine-naloxone (BNX, a mixture of buprenorphine and the μ-opioid receptor antagonist naloxone) and naltrexone (Volkow and Blanco, 2020). Although there are three types of opioid receptors (μ, δ, and κ), literature suggests that μ-opioid receptors have the greatest involvement in OUD and addiction (Fields and Margolis, 2015), and as such, are the primary target for medication assisted therapies.

Methadone is a full μ-opioid-receptor (μ-OR) agonist with an extended pharmacodynamic profile. Treatment with methadone can control withdrawal symptoms and its long-lasting effects are able to reduce craving and diminish the rewarding effects of illicit opioids (Lobmaier et al., 2010). While methadone has the highest efficacy out of all available treatment options, treatment programmes using methadone have high rates of drop out and

Table 1.3 **Summary of existing treatment options for opioid addiction, their receptors and their limitations**

	Methadone	Buprenorphine	BNX	Naloxone	Naltrexone
Overview	μ-OR agonist	Partial μ-OR agonist, full κ-OR antagonist	Sublingual mixture of buprenorphine and naloxone	μ-OR antagonist IV injection, IM injection	μ-OR antagonist
Limitations	Abuse potential, may cause neonatal abstinence syndrome	Can precipitate withdrawal, efficacy is dose dependent	Potentially harmful during pregnancy, lower treatment adherence compared to other treatments	Can precipitate withdrawal	High dropout especially in early treatment periods, can precipitate withdrawal

relapse, especially within the first twelve months of treatment (Salsitz and Wiegand, 2016, Nosyk et al., 2010, Cao et al., 2014). Risk factors for early methadone treatment dropout include inadequate dosing and prior treatment dropout (Durand et al., 2021). Buprenorphine is a partial μ-OR agonist and κ-opioid receptor (κ-OR) antagonist. While higher doses are needed to match the efficacy of methadone, it is safer to administer during overdose due to its reduced potency (Lobmaier et al., 2010). A more recent formulation using buprenorphine is buprenorphine-naloxone (BNX). BNX, generally delivered sublingually, has less abuse potential than buprenorphine and has lower risk of diversion (channelling of prescription medications to unintended users) due to an extended pharmacological profile and lower bioavailability, and precipitation of withdrawal if injected (Doran, 2005). In spite of its varied benefits, lower treatment adherence has been observed in patients administered these options compared to patients undergoing treatment with methadone (Gryczynski et al., 2013, Mattick et al., 2014). Naltrexone, unlike methadone and buprenorphine, is a μ-opioid receptor antagonist. Treatment with naltrexone can result in precipitated withdrawal if a patient has not undergone detoxification to ensure a prior lack of drugs in their system. To reduce the risk of precipitated withdrawal, treatment with naltrexone often requires stepped escalation in dosage over the course of treatment, is used later in the course of treatment for opioid addiction and is

often reserved to maintain abstinence in patients following treatment exit (Blanco and Volkow, 2019). Finally, behavioural therapies may confer some benefits when combined with pharmacological treatments, but results are mixed. For example, a study by Pan et al. (2015), reported a greater number of opioid-negative urine tests in patients treated with cognitive behavioural therapy (CBT) and methadone maintenance therapy (MMT) compared to patients treated with MMT alone, but did not report any difference in retention rates. Conversely, in their review of the literature, Amato et al. (2011) did not find a significant improvement in therapy retention, abstinence or therapy attendance for patients undergoing combined psychosocial and pharmacological treatment compared to those undergoing pharmacological treatment alone. Hence, results regarding the benefits of psychobehavioural treatments are mixed. In short, while there is some efficacy with the existing treatments for opioid addiction, these treatments have several limitations, and new treatment options that address these gaps are needed to further improve patient outcomes.

1.1.4 *Summary*

Chronic opioid use is occurring at epidemic rates, and are continuing to rise, contributing to an alarming rate of mortality as well as other health, social and economic consequences. The literature suggests that existing pharmacological treatments for OUD have greater efficacy than psychobehavioural treatments, but fails to demonstrate a significant improvement of combined (pharmacological and psychobehavioural) therapy programmes on treatment outcomes. In addition to this, pharmacological treatments such as methadone, buprenorphine and naltrexone have several clinically significant limitations. These include high rates of dropout in treatment programmes, especially early in the treatment course, high rates of relapse, significant abuse potential, and poorer efficacy with incorrect dosing. As such, there is a need to address the shortcomings of existing treatments, and determine the factors that may contribute to poorer clinical outcomes in order to improve these treatments.

Considering chronic opioid use through a neuropsychological framework is helpful in highlighting the factors that may contribute to poorer treatment outcomes in patients undergoing chronic opioid use. A growing body of literature has described cognitive impairments, underpinned by drug-induced alterations in brain structures and neurocircuitry, as major contributing factors to the pathology of opioid addiction.

1.2 The Three-Stage Model of Addiction, Neurobiology and Cognition in Chronic Opioid Use

OUD can be considered a neuropsychological disorder involving maladaptive learning, impaired cognitive functioning and behaviour, underpinned by drug-induced adaptations in brain regions and signalling pathways related to motivation and reward processing (Strang et al., 2020). The current three-stage model of addiction is useful in explaining how these psychological and neurological adaptations may develop and further contribute to the maintenance of OUD (Koob and Volkow, 2010). The three-stage cycle consists of a binge/intoxication stage, a withdrawal/negative affect stage, and a preoccupation/anticipation stage (Figure 1.1), each of which is mediated by different brain structures and signalling between structures (Table 1.4), leading to impaired cognition. Cognition (the input, processing, storage and retrieval of information) involves executive functions, such as attentional control, working memory, inhibitory control and attention shifting (Bickel et al., 2012). Compared to healthy individuals, patients with addiction disorders have impaired functioning in these processes (i.e., poorer performance in tests sensitive to these processes) which may further contribute to the course of the disorder. The theoretical model of addiction, the allostatic (compensatory) neurobiological changes that occur as a result of addiction and OUD, and the cognitive impairments of OUD will now be reviewed below.

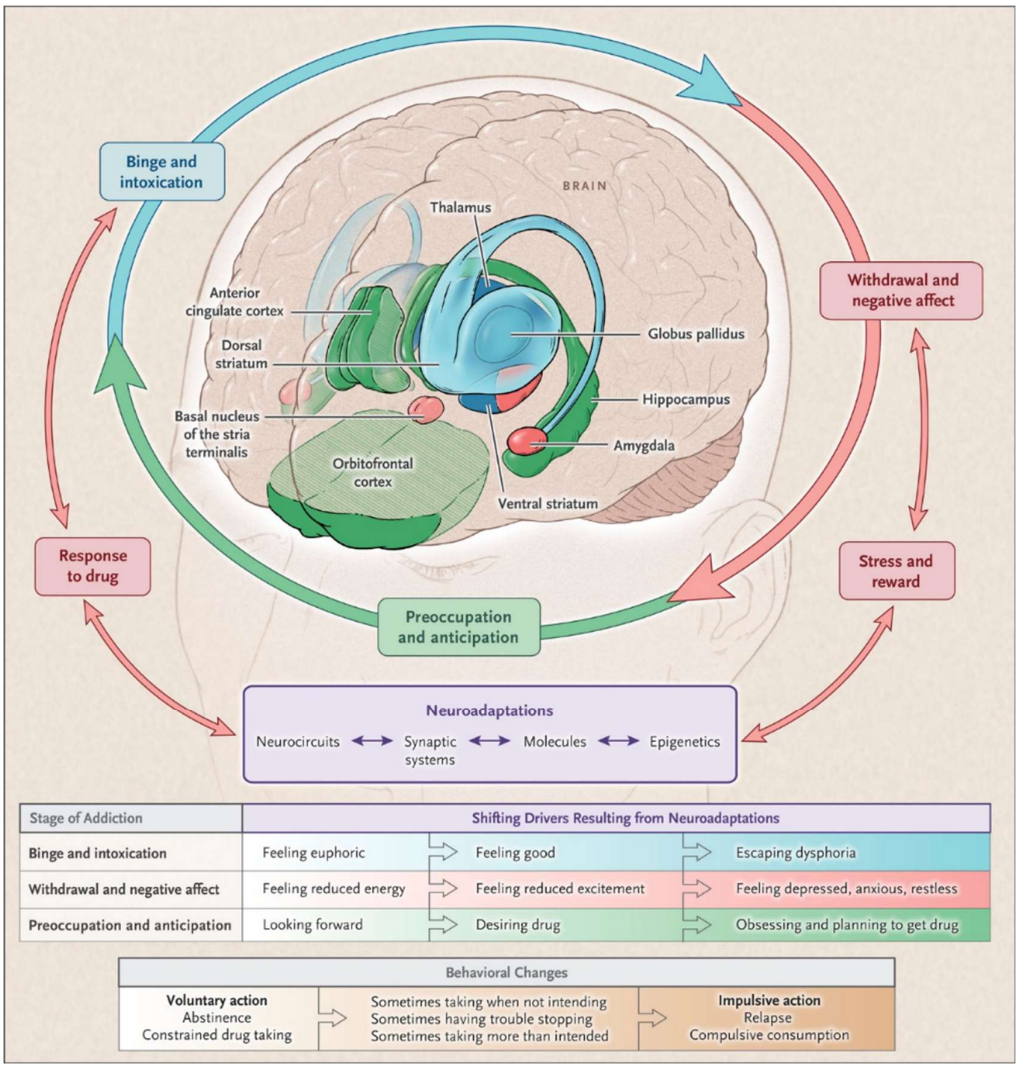

Figure 1.1 **The three-stage model of addiction.** While this model is proposed as being ubiquitous in addiction, the model outlines the brain structures and processes involved in chronic opioid use. Not included in the figure are the psychological processes that underlie the three stages. The Binge/Intoxication stage is mediated in part by the psychological attribute of Incentive Salience, where a cue is attributed motivation properties, for example. Incentive Salience orients an individual to cues that reliably predict opioids (e.g., a needle). The Withdrawal/Negative Affect stage involves hyperkatifeia and hyperalgesia, factors that contribute to relapse and further drug seeking. Finally, the Preoccupation/Anticipation stage is mediated by failures in cognition and executive processes. This stage characterises addiction as a cyclical process by contributing to relapse. Adapted from VOLKOW, N. D., KOOB, G. F. & MCLELLAN, A. T. 2016. Neurobiologic Advances from the Brain Disease Model of Addiction. *N Engl J Med*, 374, 363-71.

Table 1.4 **Overview of the brain structures and mechanisms involved in the three stages of opioid addiction and their role in the stage**

	Mechanisms Involved	Role in Addiction
Binge/Intoxication **VTA, NAcc, Amyg, Hipp, VP, Hypo, DSt, PFC**	Dopamine, D_1 receptors GABAergic interneurons	Opioids inhibit GABAergic neurons causing disinhibition of dopaminergic neurons. Dopaminergic signalling facilitates rewarding effects of drugs and reinforces drug seeking behaviour
Withdrawal/Negative Affect **VTA, NAcc, PaG, DTh, eAmyg, Sh. of NAcc, VSt, DSt**	Dopamine, D_2 receptors, GABA, Glutamate, CRT, Dynorphin	Continued opioid use results in diminishing returns as tolerance and withdrawal occurs. At the cellular and molecular level this is mediated through the development of allostatic adaptations. Allostatic adaptations include receptor internalisation and desensitization. Hyperkatifeia (negative affect) occurs as a result of brain stress system activation which contributes to withdrawal as does activation of the pain pathway
Preoccupation/Anticipation **PFC, VTA, NAcc, Sh. of NAcc, eAmyg, Insula, Hipp**	CRF, glutamate, GABA	Failures in executive processes (seated in the PFC) contribute to relapse. Mechanisms at the cellular and molecular level that may facilitate this includes CRF signalling and disruption of glutamatergic homeostasis. Drug associated-cues and stress may contribute to relapse through these mechanisms.

Notes: *This table serves as a brief overview outlining the key structures and mechanisms involved in the three stages of opioid addiction (it is not a comprehensive list).* **Abbreviations:** *Amyg=Amygdala, eAmyg=Extended Amygdala, CRF=Corticotropin-Releasing Factor, DSt=Dorsal Striatum, VSt=Ventral Striatum, GABA=γ-aminobutyric acid, Hipp=Hippocampus, Hypo=Hypothalamus, PaG=Periaqueductal Grey, PFC=Prefrontal Cortex, NAcc=Nucleus Accumbens, Sh. Of NAcc=Shell of the NAcc, VP=Ventral Pallidum, VTA=Ventral Tegmental Area*

1.2.1 *Binge/Intoxication Stage*

In the binge/intoxication phase, the use of opioids activates reward related neurocircuitry that positively reinforces drug-seeking and administration by inducing feelings of euphoria or sedation (Koob and Volkow, 2010, Moningka et al., 2019). Concurrently, maladaptive learning occurs wherein an individual becomes sensitized to drug-associated cues that further motivates drug-seeking and administration – the motivational property of these cues is known as incentive salience. At the neurobiological level, drug use induces an influx of the reward related neurotransmitter dopamine into the mesocorticolimbic system, including the ventral tegmental area (VTA), ventral striatum, nucleus accumbens (NAc), and the prefrontal cortex (PFC). Neurotransmitters other than dopamine suggested to be involved in the

binge/intoxication phase include GABA, serotonin and opioid peptides (Koob and Volkow, 2016). Additionally, glutamate is reported to mediate dopaminergic signalling, suggesting a role of this neurotransmitter in reward learning (Wise and Robble, 2020). With prolonged use of opioids, allostatic adaptations develop at the neurobiological level leading to tolerance of the drug (Williams, Christie and Manzoni, 2001). At the cellular level, these allostatic adaptations include rapid recycling of μ-OR, which may drive increasing dosage as patients receive diminishing returns from current doses.

Other mechanisms at the cellular level that may potentially contribute to opioid tolerance include pro-inflammatory signalling leading to neuronal sensitization (Latremoliere and Woolf, 2009, Eidson and Murphy, 2019). In their study, Ikeda, Kiritoshi and Murase (2012) report that astrocytes contribute to central sensitization through hyperexcitability in rats subjects to inflammatory pain. Elsewhere, studies report that microglial release of cytokines such as TNFα, IL-1β and Il-6 may contribute to neuronal sensitization (Basbaum et al., 2009), which may contribute to tolerance (Hutchinson et al., 2011). In their preclinical study, Kawasaki et al. (2008) evidenced a potential link between these pro-inflammatory cytokines and altered synaptic transmission; IL-1β and TNFα enhanced excitatory signalling, whereas IL-1β and IL-6 reduced inhibitory synaptic signalling in spinal neurons. Further, microglia express TLR4 which can be activated by morphine, fentanyl and oxycodone (as well as bacterial components, as discussed throughout this thesis) – hence these substances have the potential to directly induce the release of proinflammatory cytokines (Eidson and Murphy, 2019). Finally, recent evidence suggests such central sensitization may be an integral component of opioid dependence (Cahill and Taylor, 2017, Hall et al., 2022), potentially through inflammatory mechanisms.

These neurobiological changes may drive the cognitive processes that cause a shift from voluntary drug use to addiction (Koob and Volkow, 2010). The cognitive processes that

facilitate this shift include maladaptive learning and the attribution of incentive salience to drug-associated cues. A cue is a stimulus that frequently occurs with, and reliably predicts the drug. For example, a needle may be a cue the reliably predicts the presence of heroin. With prolonged drug use, the cue develops the motivational attribute of incentive salience, and may illicit craving and expectation of the drug, stimulating further drug seeking as a result. In fact, cues themselves can activate reward related circuitry. Evidence for this can be found in a non-drug primate study that demonstrated a shift in dopaminergic neuron signalling from presentation of a reward to a cue (Schultz, Dayan and Montague, 1997). Initially, dopaminergic neuron signalling was observed when animals were presented with a reward (i.e., food). Over extended presentations, when the reward was paired with a cue, and the cue could reliably predict the reward, reward-related dopaminergic signalling occurred in response to the cue itself and not the reward (Schultz et al., 1997). Similarly, in the context of drug addiction, a cue (for example, a needle) that is associated with, and reliably predicts, a reward (for example, heroin) may induce drug-seeking as the reward is expected in the context of the cue.

1.2.2 *Withdrawal/Negative Affect Stage*

By the withdrawal/negative affect stage, allostatic adaptations have developed as a result of chronic drug administration. These adaptations increase the reward threshold for opioid drugs (meaning higher doses of drugs are needed to achieve the desired effects) leading to tolerance. Opponent processes result in a decreased pain threshold (hyperalgesia) and the development of negative affect (hyperkatifeia) wherein a patient may feel dysphoria, malaise and irritability (Koob, 2020). Normally, the presence of opioids in the system keeps these opponent processes in balance. Cessation of opioid use, or even an inadequate dosage, results in an emphasised effect of these negative processes, resulting in symptoms of withdrawal and

craving. These withdrawal symptoms negatively reinforce opioid use (Moningka et al., 2019). At the neurobiological level, areas associated with the withdrawal/negative affect stage include the ventral striatum extended amygdala, the nucleus accumbens, and ventral tegmental area (Koob, 2020, Koob and Volkow, 2010).

1.2.3 *Preoccupation/Anticipation Stage*

The preoccupation/anticipation stage characterises the chronic, relapsing nature of substance addiction, facilitated by failures in executive functions. At the neurobiological level, this stage of the addiction cycle involves the prefrontal cortex and neurotransmitters such as dopamine and serotonin which together underlie the executive functions that are impaired in addiction (Logue and Gould, 2014). Deficits in executive functions are associated with negative treatment outcomes, such as poor treatment retention and relapse back into drug use, making them critical to the course of addiction (Mahoney, 2019, Ramey and Regier, 2019, Sampedro-Piquero et al., 2019). Cognitive impairment may also predict treatment adherence. For example, a study by Aharonovich, Nunes and Hasin (2003) investigating a cohort of patients with cocaine use disorder found poorer attention and reasoning performance at baseline in patients who dropped out of a treatment programme compared to those who completed treatment. The finding that cognitive impairment predicts poorer treatment adherence has also been observed in patients engaging in abuse of alcohol (Copersino et al., 2012, Teichner et al., 2002, Manning, Verdejo-Garcia and Lubman, 2017), and in heroin users (Katz et al., 2005). There is a lack of research in other substances, but cognitive impairment in nicotine, methamphetamine, cannabis and opiates may also predict worse treatment outcomes (Stevens et al., 2014). In short, cognitive impairment is a central component of several forms

of substance use disorders due to its relevance to treatment outcomes, and as such, these impairments need to be addresses and treated.

Research has highlighted several domains that are impaired in OUD (Wollman et al., 2019). For example, a meta-analysis by Baldacchino et al. (2012) reported that patients engaging in chronic opioid use performed worse on tasks measuring verbal working memory, verbal fluency and impulsivity/inhibition compared to opioid free controls. Additionally, chronic opioid users had poorer performance in tasks measuring attention and long-term memory compared to controls, although these deficits were not statistically significant. A separate meta-analysis reported impaired performance in tasks measuring impulsivity, cognitive flexibility, short-term memory and long-term memory in chronic methadone users (as part of Methadone Maintenance Treatment; MMT) compared to healthy controls (Baldacchino et al., 2017). Abstinence from opioid use appears to recover cognitive functioning as short-, and long-term memory performance was greater in abstinent patients compared to chronic methadone users (Baldacchino et al., 2017). While the research demonstrates cognitive impairment in chronic opioid use, studies often use different neurocognitive batteries to assess performance, which themselves have vastly different methods of classifying cognitive domains. This is a limitation that makes it difficult to standardise results and determine patterns across the literature. Regardless, a brief summary by of the findings by Wollman et al. (2019) are included in this thesis as a general overview of the impairments observed in the literature (Table 1.5).

While impairments in cognitive functioning are a component of OUD, existing treatment options are unproven in their ability to recover them. In fact, medication assisted therapy for opioid use disorder (MOUD) may result in further impairments in cognitive functioning (Pujol et al., 2018). For example, patients undergoing methadone maintenance treatment (MMT) perform worse on tasks measuring impulsivity, processing speed, social

Table 1.5 **Neurocognitive domains identified as being impaired in patients with OUD**

Cognitive Domain	Tests Used for Assessment
Simple Attention	Digit Span Forward
Decision Making	Iowa Gambling Test
Immediate Visual Memory	Benton Visual, Retention Test,
Immediate Verbal Memory	Rey Auditory Verbal, Learning, Logical Memory
Delayed Verbal Memory	Rey Auditory Verbal, Learning, Logical Memory
Working Memory	WAIS-Digit Span-Backwards, WAIS-Letter Number Sequencing, 2 Back test
Visuospatial Ability	Block Design
Complex Psychomotor Processing	Digit Symbol, Substitution Test, Symbol Digit, Modality Test
Planning	Tower of London, Porteus Maze
Verbal Fluency	Phonemic Fluency, Semantic Fluency
Inhibition	Stroop Task, Go/No Go Test
Cognitive Flexibility	Trail-Making Test-B, WCST Perseverative Error

Notes: This is not a comprehensive list. The ability to collate impaired domains is limited due to studies utilising different neurocognitive batteries and a lack of consistency in defining cognitive domains. Adapted from WOLLMAN, S. C., HAUSON, A. O., HALL, M. G., CONNORS, E. J., ALLEN, K. E., STERN, M. J., . . . FLORA-TOSTADO, C. 2019. Neuropsychological functioning in opioid use disorder: A research synthesis and meta-analysis. *American Journal of Drug and Alcohol Abuse*, 45, 11-25.

cognition, cognitive flexibility and working memory, compared to healthy, opioid free controls. Other recent studies conversely suggest a beneficial effect of methadone treatment on cognitive performance. The first study by Wong et al. (2021) reported an improvement in cognitive functioning as a result of methadone treatment after a four-week period, measured by the Montreal Cognitive Assessment, though this study did not compared patients to an opioid free control group. The second study by Li et al. (2021) also reported improvements in some measures of impulsivity but not others (delayed discounting task) in patients undergoing MMT, compared to controls. Finally, a study by Nikraftar et al. (2021a) reported some beneficial effect of MMT and buprenorphine treatment on cognition compared to current users, though patients in treatment still performed worse than healthy controls on some tasks. Compared to controls, buprenorphine treated patients performed worse on tasks measuring memory, but were not significantly different in tasks measuring set shifting and attention. Compared to controls, methadone patients performed worse on tasks measuring set shifting and attention, but were not significantly impaired on tasks measuring memory (Nikraftar et al. (2021a). Therefore, while there is some evidence for a therapeutic effect of MOUD on cognitive impairment in chronic opioid users, studies appear conflicting. The limited efficacy

of existing treatments in addressing these cognitive impairments needs to be addressed due the clinical significance of these deficits.

1.2.4 *Summary*

Neurobiological alterations and psychological impairments occur at the various stages of chronic opioid use. Initial hijacking of the dopaminergic reward pathway by opioids leads to the development of addiction. This process occurs in concert with maladaptive cognitive processes that sensitize an individual to drug associated cues, further motivating drug seeking. Adaptations at the neurobiological level result in diminishing returns from opioids, contributing to tolerance. In addition, symptoms of withdrawal such as hyperkatifeia and hyperalgesia negatively reinforce drug seeking. Finally, the chronicity of addiction is facilitated by failures in cognitive functioning, especially executive functions such as attention and impulse control. Performance in these executive functions is clinically significant as they can predict treatment retention and dropout. Existing treatments for OUD do not adequately address these cognitive impairments, and may even exacerbate them, potentially driving the cycles of addiction forward. As such, addressing the limited ability of existing therapies to target these cognitive impairments should be a central concern for future treatments. First, a greater understanding of the pathology of OUD is required, especially with regards to the development and maintenance of these cognitive impairments. Recent research has suggested dysbiosis of the gut microbiota in patients with chronic opioid use, but the contribution of this dysbiosis to neurobiology and cognitive aspects of opioid addiction are not fully understood.

1.3 Overview of the Gut Microbiota

Accumulating evidence implicates the gut microbiota, i.e., the complex and dynamic community of bacteria that inhabits the gastrointestinal tract in host health, and in brain development and cognition (Diaz Heijtz et al., 2011). This community of bacteria outnumbers both the cell and gene count of humans and, locally, are vital in nutrition extraction, vitamin synthesis and overall gastrointestinal health (Wang and Wang, 2016). A growing body of research implicates the gut microbiota in brain functioning, as the gut microbiota and brain can engage in bidirectional communication through several, often overlapping pathways, collectively known as the microbiota-gut-brain (MGB) axis (Figure 1.2). These pathways include the immune system (Fung, Olson and Hsiao, 2017), nervous system (Carabotti et al., 2015), neuroendocrine system (Cussotto et al., 2018), metabolic products including short chain fatty acids (Dalile et al., 2019, Silva, Bernardi and Frozza, 2020) and bile acids (Monteiro-Cardoso, Corliano and Singaraja, 2021), and production and modulation of neurotransmitters (Strandwitz, 2018, Liu and Huang, 2019).

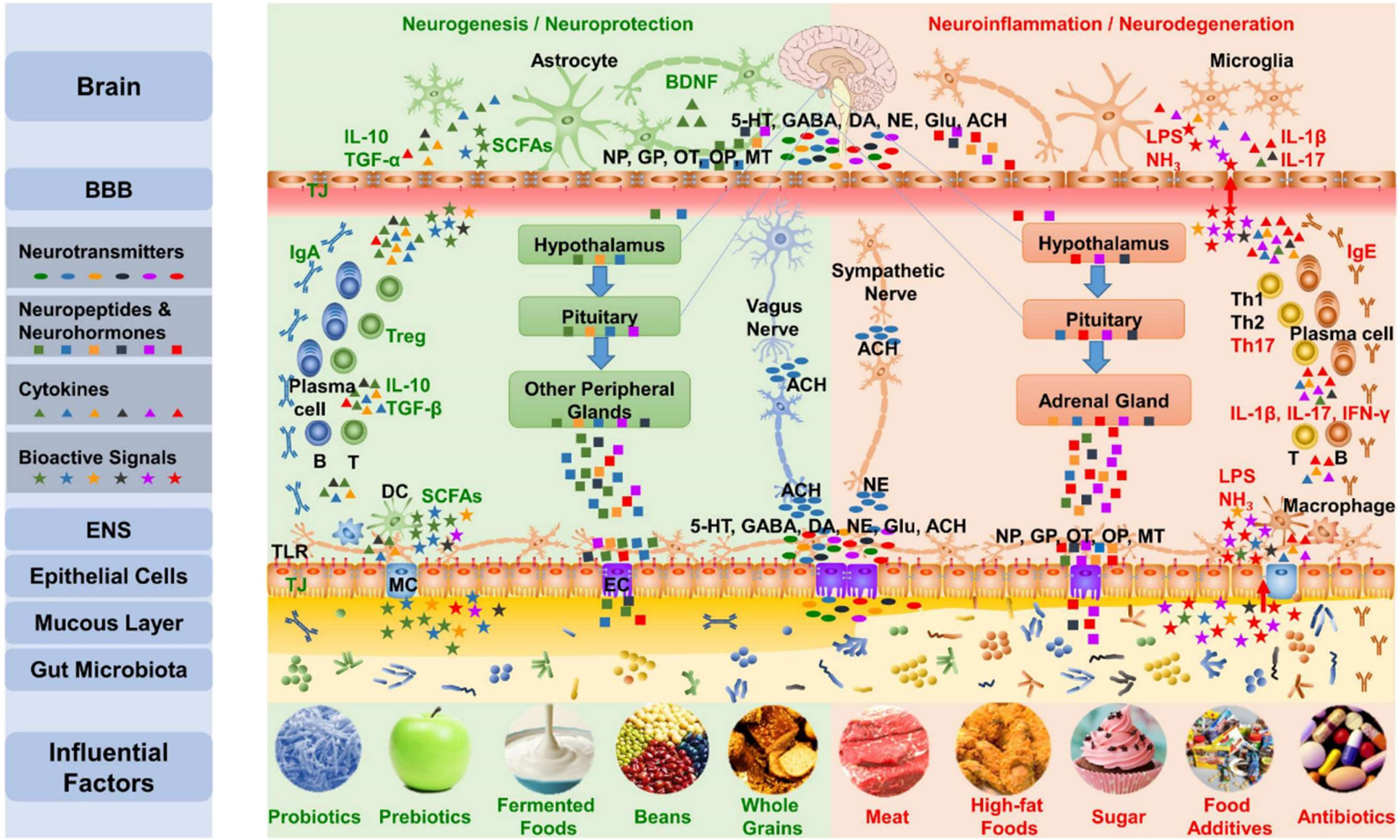

Figure 1.2 **An overview of the microbiota-gut-brain (MGB) axis.** Adapted from LIANG, S., WU, X. & JIN, F. 2018. Gut-Brain Psychology: Rethinking Psychology From the Microbiota-Gut-Brain Axis. *Front Integr Neurosci*, 12, 33.

1.3.1 *Gut Microbiota and the Immune System*

Within the immune system the gut microbiota can influence microglia, which are CNS localised immune cells that are crucial in synaptic pruning and, neuronal development and homeostasis (Lannes et al., 2017). Evidence has shown that microglia development and function is impaired in animals that do not possess a gut microbiota (known as germ free animals), compared to animals with an intact microbiota (Erny et al., 2015). The same study also observed recovery of microglia morphology and activity upon colonisation of the gastrointestinal tract in germ-free animals, further evidencing the role of the microbiota in CNS immune system functioning. Elsewhere, the potential significance of microglia in cognition has been demonstrated in a rodent model of Parkinson's disease, where microglial activation was associated with cognitive impairment in this model (Zhang et al., 2021a). Interestingly, an altered gut microbiota has been observed in patients with this neurodegenerative disease (Scheperjans et al., 2015). The microbiota is critically important in immune system development and functioning, and may have less direct influence on the brain through other mechanisms, such as influencing the differentiation of immune cells and regulating inflammatory responses (Brown, Kenny and Xavier, 2019).

1.3.2 *Gut Microbiota and the Nervous System*

Nervous system and gut microbiota interactions primarily involve the vagus nerve and the enteric nervous system. The vagus nerve serves as an interface between the enteric nervous system (ENS) and the central nervous system (Breit et al., 2018), primarily receiving input from the ENS; however, neither are in direct contact with the microbiota (Hyland and Cryan, 2016). Instead, communication between the microbiota, gut and brain involves intermediary mechanisms such as Toll-Like Receptors (TLR), which are expressed in the ENS (Barajon et

al., 2009), and vagus nerve activation by microbially-derived products (such as endotoxins and short-chain fatty acids (SCFAs). The vagus nerve serves as a primary mediator of interoceptive cues, from sites such as the gut (Paciorek and Skora, 2020, Weng et al., 2021), thereby serving as a mechanism by which the microbiota can influence the brain.

1.3.3 *Gut Microbiota and the Endocrine/Neuroendocrine System*

Another pathway through which the gut microbiota can influence the brain involves the neuroendocrine system. The neuroendocrine pathway includes the hypothalamic-pituitary-adrenal (HPA) axis (a system involved in the regulation of a number of processes, including mood (Bao and Swaab, 2019) and memory (Wolf, 2003)), which is regulated by corticotrophin releasing factor (CRF) signalling, but also includes components of the immune and nervous system pathways (Cussotto et al., 2018). In the gut, the neuroendocrine system includes cells such as enteroendocrine and enterochromaffin cells (EEC; ECC) secrete hormones (such as glucagon-like peptide 1 and 2, GLP-1, GLP2; and peptide YY, PYY) and neuropeptides (Toni, 2004, Holzer and Farzi, 2014, Farzi, Frohlich and Holzer, 2018), allowing them to influence appetite and gut barrier homeostasis. More importantly, the activity of these cells can be influenced by the gut microbiota and their metabolic products. For example, a study by Cani et al. (2009) reported an increase in the production of the EEC-derived hormone GLP-2 following prebiotic supplementation in a model of obesity, compared to mice without a prebiotic supplemented diet. The hormone GLP-2 improves gut barrier integrity, possibly through upregulating tight junction proteins (Cani, Everard and Duparc, 2013, Kuwahara et al., 2020). As such, the literature suggests a role for the microbiota in influencing various actions of EECs, such as hormone secretion, which may directly or indirectly influence host behaviour. The activity of EECs can reach the brain by way of the vagus nerve (Dockray, 2013), thereby

allowing for a path of communication between the gut microbiota and the brain. In addition to influencing the activity of EECs, the microbiota exerts a measure of control over colonic ECCs, which synthesize the majority of serotonin in the body. For example, a study by Yano et al. (2015) reported deficient serum and plasma serotonin concentrations in germ-free (GF) mice compared to specific pathogen free (SPF) control mice, a finding attributed to ECCs. Hence, it appears that the gut microbiota influences neuroendocrine cells such as EECs and ECCs, which serve as intermediaries in the MGB axis.

1.3.4 *Gut Microbiota and Neurotransmitters*

In addition to modulating synthesis of neurotransmitters by the host, the gut microbiota possesses the ability to produce and metabolise neurotransmitters (Strandwitz, 2018, Strandwitz et al., 2019). Neurotransmitters linked to the gut microbiota include dopamine, serotonin, tryptamine, glutamate and GABA, all of which are implicated in cognition and addiction. Evidence for the link between neurotransmitters and the gut microbiota comes primarily from preclinical studies. For example, a study by Asano et al. (2012) reported lower levels of catecholamines (including dopamine) in GF animals compared to SPF animals, albeit the majority of these catecholamines existed in a biologically inactive form. Exposing GF mice to *Clostridia* genus bacteria possessing GUS (β-glucuronidase) resulted in an increase in lumen levels of dopamine. Elsewhere, the gut microbiota has been linked to dopamine receptor expression in the brain, and to addiction related behaviours. For example, in their preclinical study, Jadhav et al. (2018) examined the relationship between vulnerability to alcohol addiction, dopamine receptor availability in the dorsal striatum and the gut microbiota. Animals with an increased vulnerability to addiction reportedly had increased alpha diversity (i.e., within site diversity) compared to more resilient animals, increased D_1, and decreased D_2

dopamine receptor expression in the dorsal striatum (Jadhav et al. (2018). Together these papers suggest a role of the gut microbiota in dopamine production, and a link between the gut microbiota and dopamine receptor expression in the context of addiction. In addition to dopamine, the gut microbiota has been linked to production and regulation of serotonin. As mentioned above, the gut microbiota is able to control the production of serotonin through interactions with ECC cells (Yano et al., 2015), potentially through their upregulation of tryptophan hydroxylase by SCFAs (Reigstad et al., 2015) and tryptamine production (Williams et al., 2014), but various strains have been identified serotonin-producing (O'Mahony et al., 2015). In addition to these neurotransmitters, certain bacteria are able to produce glutamate, though strains capable of this are yet to be identified in the gut (Baj et al., 2019) and gut bacteria possess the ability to produce GABA (Barrett et al., 2012, Pokusaeva et al., 2017). Therefore, gut bacteria may play a role in modulating major inhibitory and excitatory neurotransmitter signalling pathways. Finally, addictive behaviour may also modulate neurotransmitter production and metabolism by microbiota. Therefore, further research to understand the role of the gut microbiota in addiction is required.

1.3.5 *Gut Microbiota and Metabolic Products*

Finally, gut microbiota derived products (such as SCFAs and secondary bile acids), as well as their constituents (such as lipopolysaccharides (LPS) and peptidoglycans (PGN)), have the potential to directly and indirectly influence the brain. SCFAs, including acetate, propionate and butyrate, are produced by the gut microbiota through fermentation of dietary fibres (Macfarlane and Macfarlane, 2003) and serve several important roles. For example, SCFAs serve as a source of energy, protect from inflammation, maintain blood brain barrier (BBB) and gastrointestinal barrier integrity, promote mucous production in the gastrointestinal tract

and protect from inflammation (Dalile et al., 2019). In addition to regulating BBB integrity, SCFAs can cross the BBB through via monocarboxylate transporters where they exhibit histone deacetylase inhibitor (HDACi) properties on various genes relevant to cognition, such as brain derived neurotrophic factor (BDNF). Other products of the gut microbiota that may influence the brain include secondary bile acids, which are involved in gut barrier homeostasis and inflammation (Lajczak-McGinley et al., 2020, Liu et al., 2018). Studies also report links between altered bile acids profiles and cognitive impairment in Alzheimer's disease (MahmoudianDehkordi et al., 2019b, Nho et al., 2019). In short, these studies outline a link between microbially-derived products and the brain, with potential influence on cognition. Bacterial cell components (such as LPSs and PGNs) may also influence brain function. LPS are toxic components of the outer membrane of microbes (Raetz and Whitfield, 2002) that can activate TLR-4 (part of the innate immune system) and initiate immune responses, leading to inflammation (Hoshino et al., 2016, Park and Lee, 2013). A study by Zhao et al. (2019) reported cognitive impairment in measures of memory in mice as a result of LPS induced neuro-inflammation. PGN also are a component of bacterial cell walls (Tosoni, Conti and Heijtz, 2019) that are able to cross the blood brain barrier. Within the CNS, PGN can activate part of the innate immune system, and furthermore, research suggests a role of PGN in the development of the brain (Arentsen et al., 2018). In short, microbial products and components serve as another pathways enabling communication between the gut and the brain.

1.4 Studying the Gut Microbiota

1.4.1 *Profiling the Gut Microbiota*

Profiling the gut microbiota provides insight into how the composition and functional potential of a bacterial community is altered in normal and pathological states. Following

collection of faecal samples from subjects, gene sequences are extracted and analysed. The majority of studies utilise 16S rRNA gene sequences, though an increasing number of studies are beginning to utilise whole genome sequencing (WGS). Sequences extracted are amplified by polymerase chain reaction (PCR) and grouped into operational taxonomic units (OTUs). OTUs are cross referenced to databases to then identify which microbes they represent. This is accomplished by determining which genes present in an OTU also occur in identified microbes. Following identification of the bacteria present, the alpha (α) and beta (β) diversity of the gut microbiota can be determined, outlining the structure of a community (Figure 1.3).

1.4.1.1 α-Diversity

Alpha diversity is a measure of within sample diversity, and can be broken down further into richness and evenness (Kim et al., 2017). Richness considers the number of distinct species present in a community, but is not concerned with what the species actually are. Evenness considers the relative abundance of the individual species – that is, it considers the differences in the number of the individual species present, and how much of a community they occupy. To explain these concepts, consider three farms. One farm may have three species present – cows, goats, and pigs. This site has a richness of three. The second farm may have four species – cows, sheep, chickens and ducks. The second farm has a greater richness, due to the greater number of species occupying the site. Within this second farm there may be two cows, four sheep, seven pigs and eleven ducks. The third farm may have the same number of

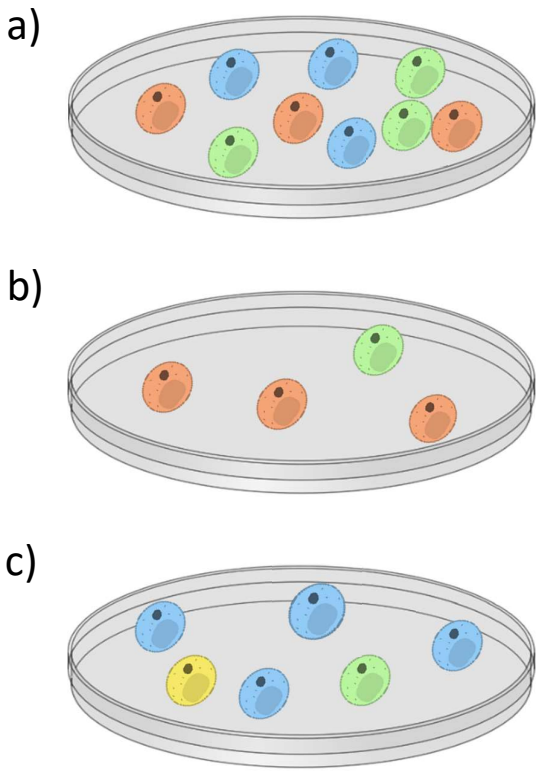

Figure 1.3 **Alpha and Beta Diversity.** Alpha Diversity refers to the within-sample structure of a community, whereas beta diversity refers to the differences in community structure between samples. Alpha diversity can be further broken down in richness and evenness. Richness is the number of different species present in a sample. In the figure, sample a) and sample c) have the same richness (both have 3 species present, but sample a) and b) have a different richness. Evenness (or, relative abundance) is the distribution of the species present. While sample a) and c) have the same richness, the evenness is different as sample a) has an equal distribution of species, whereas sample c) does not. Finally, beta diversity is different between all three sample as they do not all possess the same species.

species but may have an equal number of each. In this case, the richness is the same, but there is a disparity in evenness. Numerous indices exist that can be used to measure alpha diversity, and include Chao1, Simpson's Index, Shannon's Index and Observed OTUs.

1.4.1.2 β-Diversity

Beta diversity considers the similarity and dissimilarity (or overlap) of two or more different sites by considering the number of shared species between sites (Lozupone et al., 2011). In the example of the farms, the first two farms share only one species, cows, and therefore have little overlap i.e., there is a difference in beta diversity. Conversely, the second and third farm have complete overlap as they share all species i.e., there is no difference in beta diversity. Indices for measuring beta diversity include Bray-Curtis and UniFrac (Wong, Wu and Gloor, 2016), the latter of which can be Weighted (considering repeat counts of bacterial strains) or Unweighted (not considering repeat counts of bacterial strains).

1.5 Converging Evidence of Gut Microbiota in Cognition and SUD

Dysbiosis of the gut microbiota has been reported in several neuropsychiatric disorders such as autism (Strati et al., 2017a), bipolar disorder (Sublette et al., 2021), schizophrenia (Li et al., 2020b, Yuan et al., 2019), depression (Cheung et al., 2019) and anxiety (Simpson et al., 2021), all of which present with impairments in cognitive ability (Alabdali, Al-Ayadhi and El-Ansary, 2014, Bora and Ozerdem, 2017, Castaneda et al., 2008, Perini et al., 2019). Additionally, disruption of the commensal gut microbiota has been reported in patients misusing cocaine, opioids, alcohol and methamphetamine, cohorts which also frequently present with cognitive impairment (discussed earlier). For example, a clinical study by Volpe et al. (2014) reported increased abundance of *Bacteroidetes* in cocaine users compared to non-users. While the results regarding alpha diversity are inconclusive, preclinical studies reliably show alterations in beta diversity in pre-clinical models chronically exposed to cocaine

(Chivero et al., 2019, Scorza et al., 2019). Interestingly, studies are suggesting a potential link between microbiota and addiction-related behaviours in preclinical models. For example, a study by Suess et al. (2021) found certain bacteria (such as the genera *Allobaculum*, *Ruminococcus* and *Turicibacter*) to have increased abundance at baseline in rats with higher sensitivity to cocaine compared to rats with lower sensitivity. In another study, Kiraly et al. (2016) reported that mice with their microbiota depleted through exposure to antibiotics develop conditioned place preference at lower doses than mice not exposed to antibiotics. The development of conditioned place preference is an indication of the rewarding effects of the drug (Huston et al., 2013), and as such, this study suggests that the microbiota may be involved in cognitive processing of the rewarding effects of a drug. Dysbiosis of the gut microbiota has also been reported in cohorts engaging in chronic opioid use, though the pattern of dysbiosis is inconclusive (discussed in Chapter 3). For example, clinical studies have found dysbiosis in the gut microbiota and enrichment of bacteria such as *Bifidobacterium* (Acharya et al., 2017, Barengolts et al., 2018); however, the effect of opioids on specific microbes is inconclusive. Similarly, preclinical studies report inconsistent findings when examining the effect of opioids on diversity and specific microbes (Sharma et al., 2020a, Simpson et al., 2020).

As dysbiosis of the gut microbiota has been reported in addiction, which is associated with cognitive impairment, and given that research demonstrates communication between microbiota and the brain, it is possible that microbiota may influence addiction-related behaviours. Therefore, it is important to examine the effect of opioids on the gut microbiota and seek to understand how the microbiota may influence cognition. Elucidating the nature of this relationship may provide new insights aiding in the development of novel treatments for opioid addiction and associated brain dysfunction.

1.6 Summary

The harmful use of opioids is occurring at an epidemic rate, resulting is severe health, social and economic outcomes. Impairment in cognitive functioning is a core component of OUD, and these impairments can exacerbate and perpetuate the state of addiction. While there is some efficacy of current medications available for chronic opioid use, these treatments have several clinically significant limitations such as their inability to treat the cognitive impairments resulting from opioid addiction. As such there is need to address the limitations of these existing treatments.

Research shows that the gut microbiota is disrupted in several neuropsychiatric diseases that present with impairments in cognitive functioning, suggesting that the two may be linked. Such findings have been reported in patients with autism (Strati et al., 2017a), schizophrenia (Li et al., 2020b, Yuan et al., 2019), bipolar disorder (Sublette et al., 2021) and depression (Cheung et al., 2019). In addition, the gut microbiota is disrupted in patients addicted to various substances of abuse – including alcohol, cocaine, methamphetamine and opioids – and further evidence shows a link to drug-related learning in preclinical models, further supporting this link. A growing body of literature demonstrates that the gut microbiota is able to communicate with the brain along a number of distinct but often overlapping pathways. Therefore, disruption of the microbiota with opioid use may impact the brain and contribute to the cognitive impairment. While this link is plausible, the effect of opioid use on the gut microbiota is inconclusive as studies provide confounding evidence. Furthermore, the link between the gut microbiota and cognition in opioid use disorder has not yet been examined in the literature. Addressing these gaps may provide novel insight into the pathology of addiction in opioid users, and may aid in treating the cognitive sequalae that contributes to the chronic nature of this disorder; however, further research is needed.

1.7 Thesis Aims

1.7.1 General Aims

The general aim of this thesis was to examine cognition in chronic opioid users, investigate changes to the gut microbiota with opioid use, and identify whether changes to the microbiota by opioids may be related to cognition. Given the role of gut microbiota in brain function, including cognition and addiction, understanding changes in microbiota may provide clues that could underpin the development of novel therapeutics to improve the treatment of opioid dependence and associated cognitive decline. This thesis was significantly impacted by COVID and required alteration to the project design (as detailed in COVID Impact Statements* under the Specific Aims, below).

1.7.2 Specific Aims

The specific aims of this thesis were to:

1. Investigate the effect of opioid use on cognition through clinical testing of participants undergoing treatment for opioid dependence in a residential care setting;

   *COVID Impact Statement Aim 1: due to COVID, participant testing for the control group could not be completed and data sets were incomplete resulting in a smaller sample size.

2. Investigate the influence of opioids on specific strains of the gut microbiota;

   *COVID Impact Statement Aim 2: microbiota analyses was to be conducted in opioid vs control participants via Whole Genome Sequencing (WGS); however, analyses could not be conducted due to incomplete data sets. Instead, this aim was addressed through extraction of existing data during a systematic literature review.

3. Outline the potential functional outcomes of altered gut microbiota and metabolites in relation to brain function and cognition.

    *COVID Impact Statement Aim 3: Cognitive data sets collected from opioid vs control participants were to be correlated with WGS data (which yields functional information) from participant gut microbiota samples; however, this could no longer occur. Instead, functional outcomes of microbiota alterations during opioid use were inferred from data extracted during the systematic literature review, where available, and further literature investigations were conducted to identify their potential role in signalling pathways of the microbiota-brain-gut axis

1.7.3 Hypotheses

1. Cognition will be impaired in patients undergoing chronic opioid use compared to the control cohort
2. Opioid use will result in a dysbiosis of the microbiota and will result in alterations in specific strains
3. Opioid use will result in changes in the functional potential of the gut microbiota in pathways relevant to the microbiota-gut-brain axis that may in turn influence cognition

1.7.4 Significance

Chronic opioid use is occurring at an alarming rate, and whilst existing treatments have proven efficacy, there are several inherent limitations. Addiction to opioids may be considered a neuropsychological disorder, presenting with a varied range of cognitive deficits. Existing treatments do not address these deficits and may even result in further impairments, thus contributing to poorer treatment outcomes.

In order to address the limitations of the existing treatment options, a greater

understanding of the nature of chronic opioid use is needed. The gut microbiota is increasingly being implicated in host health and brain function. Further, research has reported alterations in the gut microbiota in cohorts engaging in chronic use of other substances and in neuropsychological disorders. Investigating the gut microbiota in individuals with a history of chronic opioid use may provide novel insights into our understanding of the nature of the disorder and inform the development of novel therapies that may better address the limitations of current treatments.

1.8 General Methods

To address aim 1 listed in Section 1.7.2 of this thesis, a cohort of patients undergoing mandated treatment for opioid addiction in an Australian residential setting were tested. Participants underwent testing with a neurocognitive battery to determine the effect of chronic opioid use on cognitive performance and to examine how various treatment related factors (i.e., dosage, length of stay, length of treatment, time since last opioid treatment dose) might influence test performance, as well as non-treatment-related demographic factors (age, BMI and years of education) (Chapter 2). To address aims 2 and 3 listed in Section 1.7.2 of this thesis, a systematic literature review in line with PRISMA guidelines was conducted. The systematic literature identified studies from three major scientific databases to determine how chronic opioid use effects specific strains of the gut microbiota and how the functional potential of the gut microbiota may be affected (Chapter 3).

Chapter 2

The Effect of Chronic Opioid Use on Cognition in a Residential Care Setting

2.1 Abstract

Impaired cognitive functioning is a core component of addiction; however, it is unclear whether cognitive dysfunction occurs with opioid use due to confounding results in the literature. Pharmacological options for opioid addiction can negatively affect cognitive performance; however, the mechanisms are unknown. The present study aimed to investigate the effect of opioid-based pharmacological treatments for opioid dependence (methadone and BNX), treatment-factors (i.e., dosage, lifetime duration of treatment, length of stay, time since last treatment), and non-treatment related factors (age, years of education and BMI) on multiple cognitive domains. Participants who recently entered residential rehabilitation, receiving daily methadone (n=15) or BNX (n=7), underwent testing to assess multiple cognitive domains. Raw test scores were converted to *T*-scores and percentiles as an indicator of performance compared to the general population. Below-average to average test performance was found in both groups, with no difference between groups (all $p > .05$). The BNX (not methadone) group showed a significant positive correlation between time since last treatment and verbal learning performance ($r = .770$, $p = .043$); and between lifetime history of treatment and non-verbal working memory ($r = .920$, $p = .027$). Non-treatment (demographic)-related factors including age positively correlated to processing speed ($r = .631$, $p = .002$) across both groups, and BMI negatively correlated to problem solving ($r = -.857$, $p = .014$) in the BNX group. BNX resulted in a longer length of stay, suggesting greater treatment adherence compared to methadone. This

study demonstrated cognitive impairment in individuals undergoing treatment for chronic opioid addiction in an Australian rehabilitation setting, and identified several treatment and demographic-related parameters influenced cognition. While there were no differences in cognitive performance between methadone and BNX-treated participants overall, BNX appeared to confer several treatment benefits. Further research using adequate sample sizes and an opioid-naïve control group is required.

2.2 Introduction

As discussed in Chapter 1, chronic opioid use is associated with impaired neurocognitive functioning which can have a significant influence on daily functioning and may also impact the course of the addiction cycle, as well as treatment outcomes (Aharonovich et al., 2006, Copersino et al., 2012, Katz et al., 2005). While there have been consistent reports of impairment in patients exhibiting chronic opioid use, the existing literature appears to be confounding and the specific domains impaired differs between studies. In a meta-analysis by Baldacchino et al. (2012), verbal working memory, cognitive impulsivity and verbal fluency were consistently impaired in chronic opioid users compared to opioid naïve controls, whereas visual working memory, long-term memory, attention and cognitive flexibility did not differ significantly. A subsequent meta-analysis by the same group (Baldacchino et al., 2017) reported impaired cognitive flexibility, attention, short- and long-term memory, and impulsivity in chronic methadone users compared to healthy controls. Wollman et al. (2019) reported impairments in complex psychomotor functioning, attention, memory and working memory, visuospatial memory, verbal fluency and executive functioning, but not motor and processing speed in patients with OUD compared to controls. Hence, it appears that certain domains might be more readily affected by chronic opioid use compared to others. Inconsistencies may be explained by methodological differences and heterogeneity of participants (particularly age and body mass index that are negatively correlated with cognitive function (Drag and Bieliauskas, 2010, Gunstad et al., 2010, Prickett, Brennan and Stolwyk, 2015, Smith et al., 2011).

Methadone and buprenorphine are two-well studied opioid medications utilised for the pharmacological treatment of opioid dependence. These drugs have distinct receptor binding profiles; the former is a μ-OR (μ-OR) agonist, whereas the latter is a high-affinity, partial μ-

OR binding agonist, but a κ-opioid receptor (κ-OR) antagonist. Furthermore, contemporary therapeutic regimes utilise naloxone, a μ-OR antagonist in conjunction with buprenorphine in a formulation known as buprenorphine-naloxone (BNX), further changing the binding profile. These differences in pharmacology may contribute to variability in cognitive performance; however, little is known about the cognitive effects of BNX. Indeed, the literature often reports poorer cognitive functioning in patients treated with methadone compared to those administered buprenorphine, though these results are only limited to several domains such as attention (Loeber et al., 2008, Nikraftar et al., 2021b, Rapeli et al., 2011). Data reveals that methadone users also had poorer short-term memory compared to short-term abstinent individuals (Baldacchino et al., 2017). Regardless, individuals undergoing pharmacological treatment for opioid use seem to outperform active chronic opioid users (Baldacchino, Balfour and Matthews, 2015), suggesting some benefit of treatment; however, unfortunately these levels appear to remain lower compared to healthy controls (Rapeli et al., 2011, Soyka et al., 2008).

Elsewhere, the literature suggests that other clinical factors relating to opioid addiction medications, such as dose, duration of treatment and time since administration (i.e., considering peaks and troughs in circulating drug levels), may influence cognition in patients; however, findings appear to be conflicting. For example, Rass et al. (2014) reported a negative effect of increasing methadone dosage on neurocognitive performance, a finding not replicated by other studies investigating patients undergoing methadone or buprenorphine treatment (Soyka et al., 2008, Haight et al., 2019). In addition, the cross-sectional study by Rass et al. (2014) reported a positive correlation between length of treatment and a select number of cognitive domains, such as working memory in a cohort of patients undergoing methadone maintenance therapy (MMT). Conversely, other cross-sectional studies such as those by Li et al. (2021) and Motazedian et al. (2021) failed to replicate these findings in cohorts undergoing MMT. Time

since treatment administration could influence cognitive function as peak levels of methadone and buprenorphine occur from approximately one- to one and a half hours after administration (Baewert et al., 2007, Bullingham et al., 1981). For example, patients maintained on buprenorphine exhibited poorer neurocognitive functioning in the domains of verbal fluency and set shifting (a subdomain of cognitive flexibility) when tested during peak levels of buprenorphine compared to trough levels (Singh et al., 2021). In individuals undergoing methadone treatment, peak level testing sessions were also associated with poorer performance in psychomotor speed, working memory and divided attention compared to trough level testing sessions (Rass et al., 2014). However, to date, no study has compared cognitive performance in patients administered methadone compared to BNX-treated patients. Overall, the gaps and apparent inconsistencies in the literature demonstrate a need for further research examining cognition in individuals undergoing treatment for opioid misuse, and the potential influence of treatment related factors (i.e., dosage, length of treatment, time since dosage). Furthermore, there is limited investigation in this field in the Australian population, particularly in people who have recently entered rehabilitation and commencing pharmacological treatment with opioid-based therapies, especially BNX.

The overall aim of the present study was to examine cognition in individuals undergoing treatment for opioid dependence. Specifically, this study aimed to examine the effect of; (1) opioid-based pharmacological treatment for opioid dependence (methadone vs BNX), (2) clinical parameters pertaining to treatment (dosage, time since last dose, length of treatment and length of stay), (3) non-treatment related demographic parameters (such as age, BMI and years of education) on multiple domains of cognitive function. It was hypothesised that: a) the cognitive performance of patients undergoing treatment with either intervention (methadone or BNX) would not be equal, b) neurocognitive functioning would be influenced by clinical parameters pertaining to treatment (i.e. patients with longer durations of treatment and length

of stay would have more intact cognitive functioning; and neurocognitive functioning would be different based on dosage and time since administration), c) non-treatment related parameters would influence cognition with a negative relationship between increasing age and/or BMI, and reduced cognitive functioning.

COVID IMPACT STATEMENT: This study originally aimed to examine alterations in the gut microbiota and cognitive function of 2 groups: 1) individuals undergoing treatment for chronic opioid use disorder, 2) matched (age, sex, BMI and education) drug-naïve controls without a history of substance use. Differences between groups in relation to gut microbiota profiles and functioning in specific cognitive domains were to be investigated, as well as correlational analyses between these factors. As a result of COVID, this clinical research could not be continued and complete data sets could not be collected within the timeframe of this 1 year Master's degree. At the point of lock-down, the cognitive data had been collected for a smaller sample of a patient cohort (individuals undergoing treatment for opioid misuse), without controls, and, due to missing data, microbiota analyses also could not proceed at this time. In order to fulfil the research component of this Master's thesis, the aim was altered to investigate the cognitive data for the cohort containing individuals undergoing treatment for opioid use disorder, converted to percentiles to infer cognitive performance of this participant group compared to the general population (and specific aims above).

2.3 Methods

2.3.1 *Participants*

A single site cohort study was conducted to examine cognitive function in individuals undergoing treatment for opioid dependence. The population consisted of participants (n=22) receiving treatment for chronic opioid misuse at a residential rehabilitation clinic in NSW,

Australia. A standard requirement for admission to the facility was a history of chronic opioid misuse. The opioid treatment program included pharmacological intervention (methadone or BNX treatment (see Table 2.1 for dose range), administered daily at 10am) in addition to therapeutic community-based treatment. The goal of treatment in the facility was to attain stabilised methadone or BNX treatment, which patients are encouraged to achieve within 90 days (this population was recruited to the present study). After this, participants progress to a different part of the facility where the goal of treatment is to reduce methadone or BNX use (but did not form part of the present study). While participants in the present study were new to the rehabilitation program, they may have been undergoing methadone or BNX use prior to admission, therefore length of treatment (days) representing the self-reported lifetime length of treatment with either BNX or methadone was collected. To be eligible for inclusion, participants were required to be: (1) able to give Informed Consent; (2) proficient in English reading, writing and speaking, (3) be able to participate in neurocognitive tests and surveys, and (4) 18 years or older. Participants with a history of poly substance use, and past history of mental illness were included in this study.

2.3.2 *Procedure*

Participants were recruited through flyers placed in the rehabilitation clinic by staff. Participants provided two sets of informed consent: 1) consent for the researcher to visit and discuss the project, 2) informed consent to participate in the project following a briefing and question/answer session with the researchers. Demographics data were collected from participants including age, gender, BMI, handedness, years in education, parent's years of education, life-time length of treatment (methadone or BNX). Participants then underwent testing with a neurocognitive battery. The total testing period took approximately two hours,

with breaks as needed, and were conducted on the clinic premises. Participants were provided with compensation in the form of a $20 gift voucher at the end of the sessions. This project was approved by the Joint University of Wollongong and Illawarra Shoalhaven Local Health District Health and Medical Human Research Ethics Committee (2019/ETH03695).

2.3.3 *Apparatus*

The neuropsychological tests conducted were:

- *Trail-Making Test A (TMT-A)*: TMT-A measures speed of processing. Participants are presented with a sheet of paper containing randomly positioned numbered circles (1 to 25) and are requested to rapidly draw a line connecting the circles in consecutive order. Time taken to connect the lines was recorded in seconds (Bowie and Harvey, 2006).
- *Brief Assessment of Cognition-Symbol Coding (BACS-SC)*: BACS-SC measures speed of processing. In this test, participants are presented with a range of symbols, and numbers that correspond to each symbol (i.e., a code). Participants are then provided with a set of symbols and are requested to match each symbol with its corresponding number within 90 seconds. The number of correct matches were recorded (Keefe et al., 2008).
- *Hopkins Verbal Learning Test-Revised (HVLT-R)*: HVLT-R measures verbal learning. In this test, participants are orally presented a list of 12 nouns derived from three semantic categories that they are asked to recite. Participants are presented the list three times and asked to recite as many words as possible. The number of correct recalls were recorded after each of the three trials and a total score was calculated (Benedict et al., 1998).
- *Wechsler Memory Scale III (WMS-III) Spatial Span (WMS-III SS)*: WMS-III SS measures non-verbal working memory. The participant is requested to reproduce a sequence pattern that was demonstrated by the test administrator on an array of 10 blocks using their pointer

finger. The first trial begins with a sequence pattern length of two blocks, and one additional block is added with each successful trial. The trials are discontinued when the participant cannot successfully reproduce the pattern sequence. The participant is then requested to perform trials involving reproducing the sequence pattern in reverse order to the administrator. The number of correct trials was recorded (Wiechmann, Hall and O'Bryant, 2011).

- *Letter-Number Span (LNS)*: LNS measures verbal working memory. Participants are orally presented with a mixed sequence of letters and numbers and are requested to verbally repeat a reordered sequence with letters presented first in alphabetical order, followed by numbers in sequential order. The sequence length begins at two symbols, with an additional symbol added with each trial. Every trial consists of four tests. The test ends upon completion of all trials or until participants are unable to complete any of the four tests of a given trial. The number of correct tests was recorded (Mielicki et al., 2018).
- *Neuropsychological Assessment Battery (NAB-Mazes)*: NAB-Mazes measures reasoning and problem-solving. Participants are presented a series of seven mazes of escalating complexity and are requested to complete them in the shortest period of time possible. Participants cannot cross lines and must correct mistakes to adequately complete a maze. Testing is concluded if participants complete the mazes within a 3-minute period, or if completion was unable to be reached. Scores were awarded for each based on time to completion (i.e., shorter time to completion is awarded a larger score) and the total score across the completed mazes was calculated (Pietrzak, Sprague and Snyder, 2008).
- *Brief Visuospatial Memory Test-Revised (BVMT-R)*: BVMT-R measures visual learning. In this test, participants are presented with an array of six figures on a page for 10 seconds, and are required reproduce them exactly as they appear on the page. Three attempts are

given. A score for each correct figure and its positioning was awarded and a total of the three attempted was calculated (Tam and Schmitter-Edgecombe, 2013).

- *Category Fluency-Animals (CF-Animals)*: CF-Animals measures speed of processing. Participants are required to name as many animals as possible with a 60 second period. The number of unique animals named was calculated (Gladsjo et al., 1999).

2.3.4 *Statistical Analyses*

Statistical analyses were performed using IBM SPSS Statistics 27. For cognitive data, *T*-scores and percentiles (%ile) were calculated from the participant's raw scores for each test. This was done by determining which *T*-score and %ile the raw scores corresponded to according to published normative data, corrected for age and gender. In this way, participant's performance could be compared to the general population in the absence of a control cohort (as per COVID impact statement above). One-Way ANOVAs were used to examine treatment (methadone or BNX) effects on neurocognitive test performance and demographic parameters. Pearson's Correlation tests were used to investigate the relationship between test scores and treatment-related parameters (dosage, time since last treatment, life-time length of pharmacological treatment, length of stay) and non-treatment demographic parameters (age, BMI, years of education). In the event that data did not meet the assumption of normality, Spearman's rho tests were used for correlation analyses. Correlation analyses were conducted on the cohort as a whole, and for the methadone and BNX groups individually. Significant differences were accepted when $p < .05$; however, non-significant trends in the data ($p = .05$ to .07) were also noted.

2.4 Results

2.4.1 *Demographics*

Demographics are outlined in Table 2.1. The cohort included 22 subjects, with 15 undergoing methadone treatment (11 male), and 7 undergoing BNX treatment (2 male). The mean age was 37.32 ± 1.40 years for the whole cohort, 37.60 ± 1.59 years for the methadone group and 36.71 ± 2.80 years for the BNX group with no significant difference between the groups ($F(1, 20) = .087$, $p = .771$) (Table 2.1). The mean BMI for the whole cohort was 27.67 ± 1.49, including 28.49 ± 1.40 for the methadone group and 25.92 ± 3.70 for the BNX group that were not significantly different between groups ($F(1, 20) = .637$, $p = .434$) (Table 2.1).

Mean years of education was 11.36 ± 0.45 years for the whole cohort, and when analysed based on treatment group, the methadone group had 10.67 ± 0.42 years of education while the BNX group had 12.86 ± 0.91. A one-way ANOVA revealed a statistically significant difference in years in education ($F(1, 20) = 6.344$, $p = .020$) between these groups (Table 2.1). There was no significant difference in total years of education for the father ($F(1, 20) = .408$, $p = .536$), or mother ($F(1, 20) = .232$, $p = .638$) in the methadone compared to the BNX treatment groups (Table 2.1).

Mean length of treatment (life-time treatment with methadone or BNX) for the whole cohort was 1097 ± 403.20 days, and there were no significant differences between the length of treatment in the BNX- compared to the methadone-treated group ($F(1, 15) = 2.695$, $p = .121$) (Table 2.1). When considering length of stay within the treatment facility, the average number of days was 60.68 ± 10.94 for the cohort as a whole, 44.07 ± 9.67 days for the methadone-treated group whereas the BNX-treatment group exhibited a significantly longer stary

Table 2.1 Cohort demographics

	Whole Cohort	(Methadone Group)	(BNX Group)	*F*	*p*
N	22	15	7		
Sex					
Male	13	11	2		
Female	9	4	5		
Age ($\bar{x}$ ± SEM)	37.32 ± 1.40	37.60 ± 1.59	36.71 ± 2.80	.087	.771
Handedness					
Right	22	15	7		
Left	0	0	0		
BMI ($\bar{x}$ ± SEM)	27.67 ± 1.49	28.49 ± 1.40	25.92 ± 3.70	.637	.434
Education (Years, $\bar{x}$ ± SEM)	11.36 ± 0.45	10.67 ± 0.42	12.86 ± 0.91	6.344	.020
Parent's Education (Years, $\bar{x}$ ± SEM)	10.81 ± 1.22	11.44 ± 1.00	9.80 ± 2.89	.408	.536
Father	11.10 ± .99	11.50 ± 0.62	10.50 ± 2.40	.232	.638
Mother					
Length of Treatment (Days, $\bar{x}$ ± SEM)	1097.12 ± 403.20	1503.42 ± 532.81	122.00 ± 23.96	2.695	.121
Length of Stay (Days, $\bar{x}$ ± SEM)	60.68 ± 10.94	44.08 ± 9.67	96.67 ± 22.35	6.529	.020
Time since last Treatment (Minutes, $\bar{x}$ ± SEM)	246.73 ± 29.64	242.40 ± 37.96	256.00 ± 49.39	.044	.837
Dosage (Range, mg)		30 - 135	6 - 32		
(mg, $\bar{x}$ ± SEM)	-	95.36 ± 7.89	19.71 ± 3.10		
Self-Reported Psychiatric Illness					
Depression	16	10	6		
Anxiety	17	12	5		
Bipolar	3	3	0		
Schizophrenia	2	1	1		
Panic Disorder	1	2	0		
PTSD	7	5	3		
Psychosis	2	2	0		
Personality/Behavioural	3	2	1		
Other	2	1	1		
Self-Reported Substance Use in Past 6 Months	3	3	-		
Alcohol	16	10	6		
Cigarettes	11	6	5		
Cannabis	11	7	4		
Ice (Crystal Meth)	14	9	5		
Heroin	5	4	1		
Other Opioids	6	5	1		
Other (Cocaine, Methamphetamine, MDMA, GHB, LSD, etc.)	7	3	4		

Abbreviations: BNX=buprenorphine and naloxone, MDMA=3, 4-Methylenedioxymethamphetamine or, Ecstasy, GHB=Gamma-hydroxybutyrate

of 96.67 ± 22.35 days ($F(1, 20) = 6.529$, $p = .020$) (Table 2.1). The mean time since last treatment (i.e. most recent dose of methadone or BNX) for the whole cohort was 246.73 ± 29.64 minutes and there was no statistically significant difference in the time since last treatment ($F(1, 20) = .044$, $p = .837$) between the treatment groups (Table 2.1). Self-reported psychiatric illnesses and substance use over the past 6 months are shown in Table in 2.1, with each participant reporting use of at least one substance; however, given that participants enter the facility due to opioid dependence, opioid misuse was underreported (Table 2.1).

2.4.2 *Neurocognitive Test Scores of Participants: Whole Cohort and Pharmacological (Methadone or BNX) Treatment Groups*

The mean neurocognitive test *T*-scores and percentiles are shown in Table 2.2. In the absence of a control group, the ability to examine the scores of the participants undergoing treatment compared to non-treated subjects was not possible; however, examination of the percentiles identified average-low cognitive performance across the cohort as a whole. Scores in the highest percentile were apparent in the CF-Animal Naming test (52.85 ± 5.25 percentile), and lowest performance was observed in the HVLT-R test (19.85 ± 3.65 percentile) (Table 2.2). A one-way ANOVA was conducted to investigate the effect of treatment on neurocognitive test performance; however, there were no significant differences between scores in the methadone or BNX treatment groups for any of the tests conducted (TMT-A, $F(1, 20) = .046$, $p = .832$; BACS-SC, $F(1, 20) = 1.283$, $p = .271$; HVLT-R, $F(1, 20) = .158$, $p = .696$; WMS III-SS $F(1, 20) = 1.205$, $p = .285$; LNS, $F(1, 20) = .001$, $p = .971$; NAB Mazes, $F(1, 20) = .529$, $p = .476$; BVMT-R, $F(1, 20) = .174$, p = .681; and CF Animal Naming, $F(1, 20) = 1.291$, $p = .269$) (Table 2.2).

Table 2.2 **Mean neurocognitive test *T*-scores and percentiles by treatment groups**

	Whole Cohort	(Methadone Group)	(BNX Group)
Test Scores ($\bar{x}$ ± SEM)			
TMT-A			
T-score	45.23 ± 1.88	44.87 ± 2.36	46.00 ± 3.27
%ile	35.95 ± 5.76	35.08 ± 7.21	37.80 ± 10.19
BACS-SC			
T-score	40.64 ± 2.45	38.13 ± 3.18	46.00 ± 2.90
%ile	27.46 ± 6.22	22.68 ± 7.87	37.71 ± 9.55
HVLT-R			
T-score	38.96 ± 1.52	38.53 ± 2.09	39.86 ± 1.88
%ile	19.85 ± 3.65	20.86 ± 5.23	17.69 ± 2.96
WMS III-SS			
T-score	40.91 ± 2.06	39.73 ± 2.34	43.43 ± 4.22
%ile	26.53 ± 5.06	22.75 ± 5.66	34.61 ± 10.25
LNS			
T-score	40.18 ± 2.34	40.20 ± 2.71	40.14 ± 4.86
%ile	25.59 ± 5.22	25.45 ± 5.95	25.87 ± 11.11
NAB Mazes			
T-score	46.32 ± 1.83	45.40 ± 2.09	48.29 ± 3.73
%ile	38.00 ± 5.96	35.00 ± 6.64	44.43 ± 12.64
BVMT-R			
T-score	45.09 ± 2.57	45.33 ± 3.17	44.57 ± 4.74
%ile	42.93 ± 7.29	45.05 ± 9.05	38.39 ± 13.08
CF-Animal Naming			
T-score	51.45 ± 1.89	49.73 ± 1.90	55.14 ± 4.23
%ile	52.85 ± 5.25	48.80 ± 6.01	61.53 ± 10.22

No significant difference between the treatment groups (all $p>0.05$).

2.4.3 *Correlation between Treatment Factors (Dosage, Time Since Last Dose, Length of Treatment and Length of Stay) and Test Score*

Correlation analyses were conducted to investigate the relationship between dosage and neurocognitive test scores for each treatment group. Pearson's correlation tests reveal no significant relationship between dosage and neuropsychological test performance in the methadone or BNX groups (all $p>0.05$) (Table 2.3).

Table 2.3 **Correlation between Dosage (mg) and neurocognitive test performance (%ile)**

Test	Methadone Group	BNX Group
TMT-A	r = -.446, p = .110	r = -.195, p = .676
BACS-SC	r = .055, p = .853	r = -.110, p = .815
HVLT-R	r = -.148, p = .615	r = .066, p = .888
WMS III-SS	r = -.098, p = .740	r = -.300, p = .514
LNS	r = -.090, p = .758	r = .025, p = .957
NAB Mazes	r = .171, p = .559	r = .666, p = .103
BVMT-R	r = .136, p = .643	r = -.388, p = .390
CF-Animal Naming	r = .387, p = .172	r = -.403, p = .370

To investigate the relationship between time since last treatment dosage and neurocognitive test performance Pearson's correlation tests were conducted. No significant correlations were identified between the variables when analysing the cohort as a whole (all $p > 0.05$); however, a trend towards a positive correlation between time since last treatment and NAB Mazes scores was identified when observing the data across the whole cohort ($r = .374$, $p = .068$) (Table 2.4). When splitting the data based on treatment, there was a significant and strong positive correlation between time since last treatment and HVLT-R test performance ($r = .770$, $p = .043$) within the BNX treatment group (Figure 2.1), with no further significant correlations noted in either treatment group (all $p > .05$) (Table 2.4).

Table 2.4 **Correlation between Time since last Treatment (minutes) and neurocognitive test performance (%ile)**

Test	Whole Cohort	(Methadone Group)	(BNX Group)
TMT-A	$r = .301$, $p = .174$	$r = .204$, $p = .466$	$r = .558$, $p = .193$
BACS-SC	$r = .197$, $p = .378$	$r = .075$, $p = .792$	$r = .565$, $p = .187$
WMS III-SS	$r = -.159$, $p = .478$	$r = -.215$, $p = .442$	$r = -.100$, $p = .831$
LNS	$r = .251$, $p = .260$	$r = .108$, $p = .703$	$r = .557$, $p = .194$
NAB Mazes	$r = .374$, $p = .068$^	$r = .272$, $p = .326$	$r = .592$, $p = .162$
BVMT-R	$r = .033$, $p = .884$	$r = -.060$, $p = .831$	$r = .298$, $p = .517$
CF-Animal Naming	$r = .149$, $p = .509$	$r = -.024$, $p = .933$	$r = .519$, $p = .232$

Notes: ^ results trended towards significance ($p = .05$ - $.07$)

Spearman's rho tests were utilised when examining the relationship between length of treatment (i.e. self-reported life-time length of methadone or BNX treatment) and test performance as the assumption of normality was not met; however, no significant correlations were found across the cohort as a whole (all $p > .05$) (Table 2.5, Figure 2.2). When considering data for each treatment group, a significant and strong positive correlation was found between WMS III-SS performance and length of treatment for the BNX group ($r = .920$, $p = 027$), but this was not significant for the methadone group (Figure 2.2) and no other correlations were identified for length of treatment (all $p > .05$).

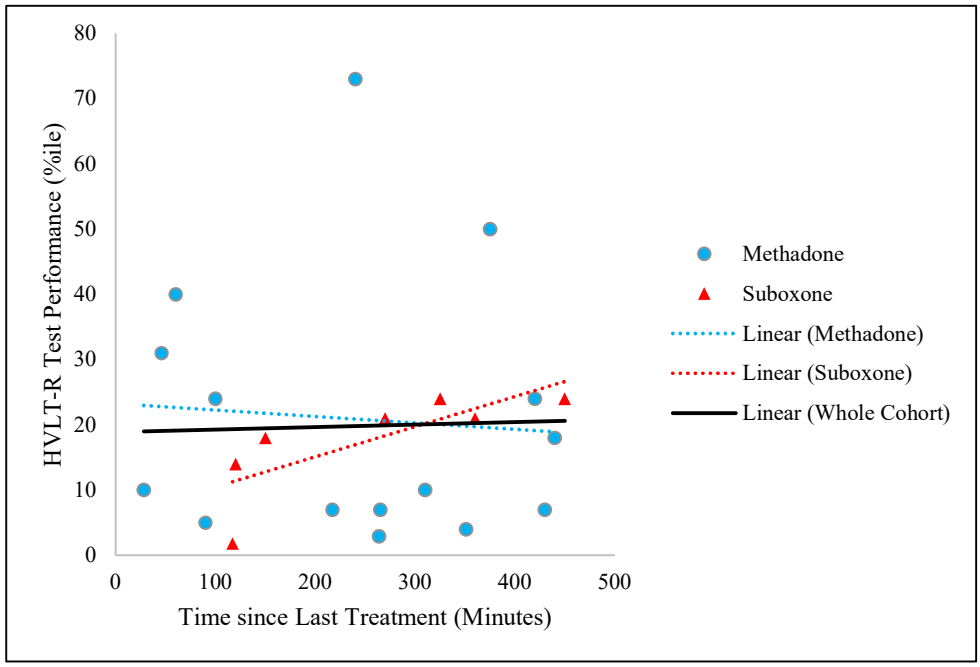

Figure 2.1 **Correlation between time since last treatment (minutes) and HVLT-R test performance (%ile) for the whole cohort and by treatment.** A significant and strong positive correlation was found between time since last treatment and HVLT-R performance for the BNX treatment (r = .770, p = .043). No significant correlation was found for the whole cohort (r = .031, p = .891 or the methadone group (r = -.071, p = .801).

Table 2.5 **Correlation between length of treatment (days) and neurocognitive test performance (%ile)**

Test	Whole Cohort	(Methadone Group)	(BNX Group)
TMT-A	r = -.022, p = .933	r = .060, p = .853	r = -.214, p = .730
BACS-SC	r = .310, p = .226	r = .341, p = .278	r = .781, p = .119
HVLT-R	r = .221, p = .395	r = .338, p = .283	r = -.445, p = .453
LNS	r = .031, p = .907	r = .062, p = .849	r = .373, p = .536
NAB Mazes	r = .278, p = .279	r = .468, p = .125	r = -.194, p = .754
BVMT-R	r = .400, p = .111	r = .312 p = .323	r = .214, p = .730
CF-Animal Naming	r = .076, p = .773	r = .412, p = .183	r = -.463, p = .433

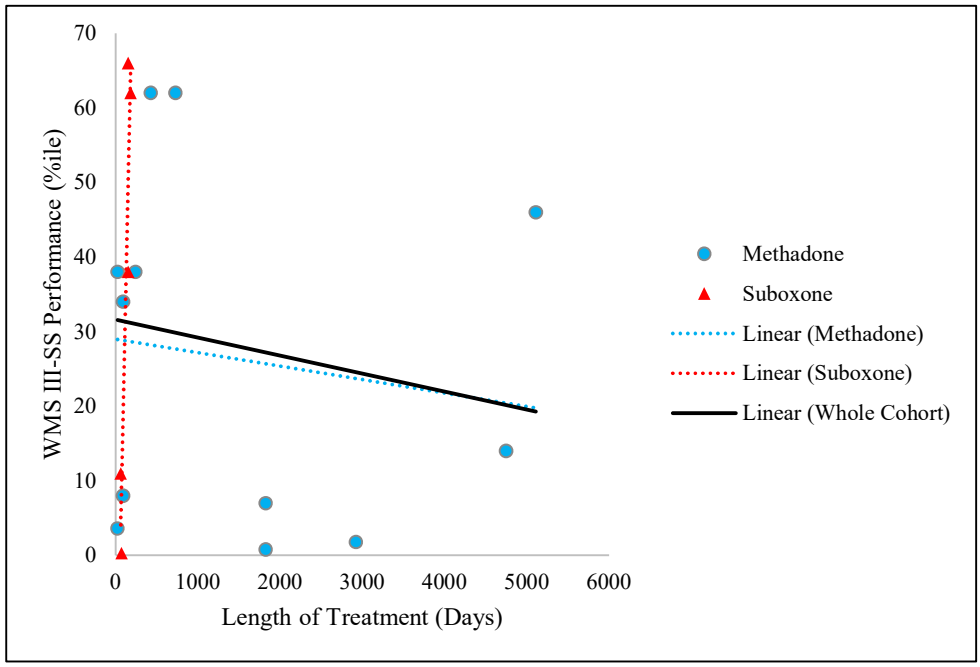

Figure 2.2 **Correlation between life-time length of treatment (days) and WMS III-SS test performance (%ile) for the whole cohort and by treatment.** A significant and strong positive correlation was found between life-time length of pharmacological treatment and WMS III-SS performance for the BNX group, (r = .920, p = .027). No significant correlation was found for the whole cohort (r = .111, p = .671) or the methadone group, (r = -.032, p = .922).

To assess the statistical relationship between length of stay and neurocognitive test performance for the whole cohort, bivariate correlation analyses were conducted. The assumption of normality was not met so Spearman's rho were used for the analyses. No significant correlation was found between length of stay and performance in any cognitive test (all p > .05) (Table 2.6); however, there was a non-significant trend towards a positive relationship between length of stay and BACS-SC (r = .435, p = .062) and a negative correlation with HVLT-R scores (r = -.455, p = .050) (Table 2.6). Further analyses were conducted to examine the relationship between length of stay and test performance within each treatment group. No significant correlation was found between length of stay and performance in any test within the methadone treatment group (all p>0.05) (Table 2.6). Furthermore, there were no significant correlations in the BNX group (all p > 0.05); however, a trend towards a

positive correlation was observed in the WMS II-SS scores in the BNX group (r = .787, p = .063) (Table 2.6).

Table 2.6 **Correlation between Length of Stay (days) and neurocognitive test performance (%ile)**

Test	Whole Cohort	(Methadone Group)	(BNX Group)
TMT-A	r = .412, p = .080	r = .434, p = .138	r = -.388, p = .447
BACS-SC	r = .435, p = .062^	r = .270, p = .372	r = .234, p = .644
HVLT-R	r = -.455, p = .050^	r = -.333, p = .266	r = -.745, p = .089
WMS III-SS	r = .349, p = .143	r = .290, p = .336	r = .787, p = .063^S
LNS	r = -.005, p = .983	r = -.057, p = .854	r = -.257, p = .623
NAB Mazes	r = -.043, p = .862	r = .149, p = .627	r = -.695, p = .133
BVMT-R	r = .098, p = .689	r = .393, p = .184	r = .006, p = .991
CF-Animal Naming	r = -.153, p = .531	r = -.247, p = .416	r = -.598, p = .210

Notes: ^ results trended towards significance (p = .05 - .07)

2.4.4 *Correlation between Non-Treatment Related Demographic Factors (Age, BMI and Years of Education) and Test Score*

To assess the statistical relationship between demographic factors (i.e. participant age, BMI and years of education) and neurocognitive test performance, bivariate correlation analyses were conducted. A Pearson's correlation test was conducted to examine age and neurocognitive test scores across the whole cohort as the assumption of normality was met. Analyses revealed a significant and strong positive correlation between age and TMT-A performance (r = .631, p = .002) (Figure 2.3); however, no further significant correlations with age were reported when considering the cohort as a whole (Table 2.7). Further analyses were then conducted to investigate correlations between age and neurocognitive test performance for each treatment group. A significant and positive correlation was found between age and TMT-A performance for both the methadone (r = .530, p = .042) and the BNX group (r = .859, p = .013) (Figure

2.3), with no significant correlations reported between age and any other neurocognitive tests across the treatment groups (Table 2.7).

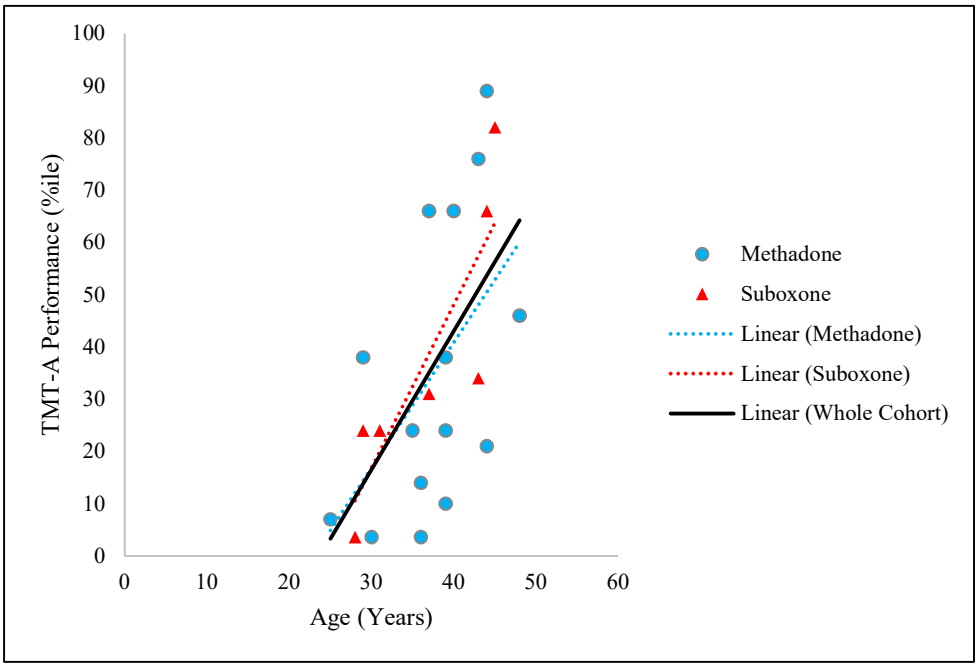

Figure 2.3 **Correlation between age (years) and TMT-A test performance (%ile) for the whole cohort and by treatment.** A significant positive correlation was found between age and TMT-A performance for the whole cohort (r = .631, p = .002), with a moderate correlation in the methadone group, (r = .530, p = .042), and a strong correlation in the BNX group, (r = .859, p = .013).

Table 2.7 **Correlation between age (years) and neurocognitive test performance (%ile)**

Test	Whole Cohort	(Methadone Group)	(BNX Group)
BACS-SC	r = .317, p = .150	r = .325, p = .237	r = .408, p = .363
HVLT-R	r = -.225, p = .314	r = -.352, p = .198	r = .240, p = .605
WMS III-SS	r = -.108, p = .633	r = -.205, p = .463	r = .078, p = .869
LNS	r = -.328, p = .136	r = .093, p = .742	r = .691, p = .086
NAB Mazes	r = .165, p = .464	r = .161, p = .566	r = .204, p = .661
BVMT-R	r = .164, p = .467	r = .115, p = .683	r = .246, p = .596
CF-Animal Naming	r = .020, p = .928	r = -.307, p = .266	r = .518, p = .233

Spearman's rho tests were utilised when examining the relationship between BMI and test performance as the assumption of normality was not met, with no significant correlations

observed across the cohort as a whole (all $p > .05$) (Table 2.8, Figure 2.4). However, further analyses examining these variables for each treatment group revealed a significant and strong negative correlation between BMI and NAB-Mazes performance for the BNX group ($r = -.817$, $p = .014$) (Figure 2.4), but no other correlations between test scores and BMI were noted in the treatment groups (Table 2.8).

Table 2.8 **Correlation between body mass index and neurocognitive test performance (%ile)**

Test	Whole Cohort	(Methadone Group)	(BNX Group)
TMT-A	$r = -.167$, $p = .458$	$r = -.362$, $p = .185$	$r = .324$, $p = .478$
BACS-SC	$r = -.245$, $p = .272$	$r = -.098$, $p = .727$	$r = -.036$, $p = .939$
HVLT-R	$r = -.264$, $p = .234$	$r = -.080$, $p = .776$	$r = -.546$, $p = .205$
WMS III-SS	$r = .020$, $p = .928$	$r = -.278$, $p = .315$	$r = .468$, $p = .289$
LNS	$r = -.145$, $p = .518$	$r = -.350$, $p = .200$	$r = .378$, $p = .403$
BVMT-R	$r = .054$, $p = .812$	$r = .137$, $p = .626$	$r = -.107$, $p = .819$
CF-Animal Naming	$r = -.049$, $p = .830$	$r = .305$, $p = .269$	$r = .036$, $p = .939$

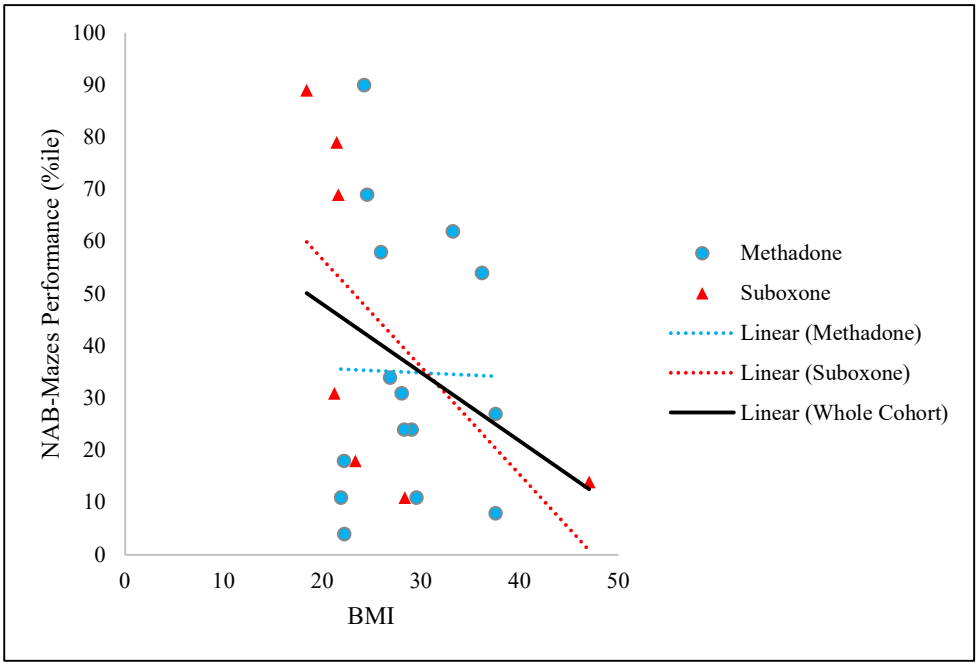

Figure 2.4 **Correlation between body mass index and NAB-Mazes test performance (%ile) for the whole cohort and by treatment.** A significant and strong negative correlation was found between body mass index and NAB-Mazes performance for the BNX group, ($r = -.857$, $p = .014$). No significant correlation was found across the cohort as a whole ($r = -.351$, $p = .109$) or for the methadone group, ($r = -.019$, $p = .948$).

Finally, there were no significant correlations between years of education and neurocognitive test scores across the cohort as a whole (all $p > .05$) (Table 2.9); however, correlations between CF-Animal Naming score and years of education trended towards significance for the whole cohort ($r = .408$, $p = .059$) and the BNX group ($r = .741$, $p = .057$), as did BVMT-R and years of education for the BNX group ($r = .741$, $p = .057$), with no other correlations observed (Table. 2.9).

Table 2.9 **Correlation between education (years) and neurocognitive test performance (%ile)**

Test	Whole Cohort	(Methadone Group)	(BNX Group)
TMT-A	$r = .143$, $p = .527$	$r = .141$, $p = .615$	$r = .278$, $p = .546$
BACS-SC	$r = .217$, $p = .332$	$r = -.030$, $p = .915$	$r = .330$, $p = .469$
HVLT-R	$r = .388$, $p = .075$	$r = .429$, $p = .111$	$r = .215$, $p = .643$
WMS III-SS	$r = .221$, $p = .323$	$r = .036$, $p = .898$	$r = .241$, $p = .603$
LNS	$r = -.085$, $p = .707$	$r = -.123$, $p = .662$	$r = .259$, $p = .574$
NAB Mazes	$r = .185$, $p = .410$	$r = .464$, $p = .081$	$r = -.385$, $p = .393$
BVMT-R	$r = .329$, $p = .134$	$r = .405$, $p = .134$	$r = .753$, $p = .051$
CF-Animal Naming	$r = .408$, $p = .059$^	$r = .145$, $p = .607$	$r = .741$, $p = .057$^

Notes: ^ results trended towards significance ($p = .05$ - $.07$)

2.5 Discussion

The present study aimed to investigate neurocognitive functioning in a cohort of individuals following chronic opioid exposure (i.e., undergoing pharmacological treatment using the opioid medications methadone and BNX, for chronic opioid dependence). Clinical parameters relating to treatment, such as drug treatment administered, dosage, time since last treatment, life-time length of pharmacological treatment and current length of stay in the residential facility, as well as non-treatment related parameters including age, years of education and BMI were examined using correlational analyses to infer the major factors influencing cognitive function in specific domains. In the present population-based study, there were no significant differences in cognitive test scores between methadone and BNX-treated individuals; however, overall test scores were below-average to average across all tests utilised when assessing percentiles as an indicator of performance compared to the general population. Individuals undergoing BNX treatment had a higher self-reported number of years in education; however, correlational analyses did not identify a significant relationship between years of education and cognitive performance in either of the treatment groups or the cohort as a whole. Participants undergoing BNX treatment also had a longer length of stay compared to the methadone group, indicating greater compliance to the rehabilitation therapy program; however, length of stay within the facility did not appear to significantly impact cognitive performance in either group. This differed to the self-reported lifetime history of treatment with either methadone or BNX, as this study revealed a strong positive relationship between the length of BNX treatment and non-verbal working memory (WMS III-SS). Treatment dosage did not correlate to cognitive performance but timing between the last dose and the start of cognitive testing was a factor following BNX treatment, as there was a strong positive correlation with verbal learning (i.e., HVLT-R performance) in the BNX group that was not apparent in the methadone group. Non-treatment related demographics factors, such as age and

BMI, impacted cognition with an unexpected significant improvement in processing speed (TMT-A scores) with increasing age in the cohort as a whole and across both treatment groups, and a negative correlation between BMI and reasoning / problem solving (NAB-Mazes) in the BNX group that was not apparent in the methadone group.

The finding that the two treatment groups did not significantly differ in cognitive performance is in agreement with a finding by Rapeli et al. (2011), who reported no significant difference in verbal memory performance (Logical Memory subtest from the WMS III) in participants treated with either methadone, buprenorphine or BNX (also known as suboxone). However, several other studies that reported superior performance in the domains of verbal memory, as well as impulsivity, cognitive flexibility, executive function and attention in patients undergoing buprenorphine treatment compared to methadone-treated patients (Hill, Garner and Baldacchino, 2018, Nikraftar et al., 2021b, Rapeli et al., 2007). For example, a study by Rapeli et al., (2011) examined patients treated with buprenorphine or buprenorphine/naloxone and revealed improvements in the LNS task from 6 – 9 months post-treatment to 12 – 17 months post-treatment, a finding that was not replicated in methadone-treated patients or controls. Although there was no control group included in the present study, results revealed below-average to average cognitive performance in participants treated with either methadone or BNX compared to the general population when utilising percentiles as an indicator of test performance. Other studies report mixed results when comparing cognitive performance to drug naïve patients. For example, our findings are in line with prior reports of impairments in speed of processing, verbal fluency, working memory as well as attention and impulsivity in cohorts undergoing treatment with methadone compared to non-treated controls (Li et al., 2021, Mintzer and Stitzer, 2002, Sant, Camilleri and Dimech, 2020). Motazedian et al. (2021) also reported impairments in executive functioning, short-term memory and attention in a cohort of MMT patients compared to abstinent opioid users, suggesting that cognitive

function could recover following cessation of opioid intake. However, other studies have also reported no significant impairment in certain domains, such as non-verbal memory and visuospatial skills, as well as attention and executive function in participants undergoing methadone treatment compared to healthy controls (Elkana et al., 2019, Sant et al., 2020). Interestingly, the results of the present study showed that CF-Animal Naming performance was slightly higher than average in the present cohort compared to the general population (particularly in the BNX-treated group), which also seems converse to the established research. For example, impaired performance in similar paradigms have been reported in patients undergoing methadone (Elkana et al., 2019, Mazhari et al., 2015) and buprenorphine treatment (Messinis et al., 2009, Soyka et al., 2008). However, the present study appears to be the first to reveal cognitive improvements in processing speed linked to increased length of treatment with BNX. Overall, the contrasts between findings across studies may be due to the use of different neurocognitive tests. For example, the present study tested verbal memory performance with the Letter Number Span paradigm, whereas the Logical Memory subtest of the WMS III was utilised in other studies (Rapeli et al., 2007, Rapeli et al., 2011). Inconsistencies may also be due to differences in drug treatments (most studies have focussed on methadone or buprenorphine alone), dosage, the populations studied, and also due to the small sample size used in the present study. These inconsistencies highlight the need to utilise a standard testing battery to investigate the effects of opioid on cognitive performance in research going forward. The inconsistences also demonstrate the need to further investigate how cognitive performance varies in BNX-treated cohorts compared to other opioids and controls (using adequately powered study designs), as this is under investigated in the literature.

The present study did not find any significant correlations between dosage and test performance for either treatment. This finding was unexpected, as we hypothesised that dosage would impact cognition. Indeed, higher methadone dosage has been reported to impair

performance in episodic memory (Curran et al., 2001), attention and working memory (Rass et al., 2014), but improve performance in executive functioning (Rass et al., 2014). The dosage range of methadone administered in our cohort was 30-135mg, lower than the range of 40 – 200mg administered in the study conducted by Rass et al. (2014). However, much like the present study, no significant correlation was found between test performance and buprenorphine dosage by Saroj et al. (2020); or between test performance and either buprenorphine or methadone dosage by Soyka et al. (2008). Therefore, further studies are required to clarify the effects of dosage on cognitive performance during treatment. On the other hand, the present study identified a significant and strong positive correlation between self-reported lifetime history of BNX treatment and working memory (WMS III-SS), and between time since last treatment and verbal learning in the BNX treatment group (not in the methadone treatment group). These results suggest that increased BNX adherence could improve aspects of cognitive function and that timing between dosing and testing should be a consideration in future studies. However, it is also important to note the limitations of correlational analyses and that controlled longitudinal studies would be helpful in understanding whether cognitive benefits exist during BNX treatment over time. Nevertheless, a positive correlation between verbal working memory (Sternberg task) and length of methadone treatment was also reported by Rass et al. (2014), who also demonstrated a positive correlation between length of treatment, and episodic memory and meta-memory. However, while the present study found that increased time since last treatment correlated to improved verbal working memory performance, Rass et al. (2014) found that increasing time since last treatment resulted in poorer reaction times during a working memory task (*n*-back task). Other research has demonstrated a negative correlation between time since last treatment and performance in psychomotor function, attention and episodic memory (Rass et al., 2014) and cognitive flexibility (Barahmand et al., 2016), while Elkana et al. (2019) did not find a

significant correlation between duration of methadone use and cognitive performance, or time since last treatment and cognitive performance. Taken together, the results of the present study and others could reflect methodological differences between studies (i.e., the present study included participants with self-reported mental illness, whereas others excluded patients with psychiatric illnesses (Rass et al. (2014), Curran et al. (2001)), and drug treatment examined (i.e., other studies examine methadone while the present study found significance in the BNX treatment group which was not administered to participants in these prior studies). Alternatively, it is possible that cognitive performance follows an inverted U-shaped curve in the time following treatment, where scores are lower immediately following treatment (i.e. a possible sedative/euphoric effect), improving within the hours after the dose, then lowering over time prior to the need for the next dose; however, longitudinal studies are required to confirm this suggestion.

In the present study, although the BNX group had a significantly longer length of stay compared to the methadone-treated group, no significant correlation was found between performance in any test and this factor. As with our study, Elkana et al. (2019) did not find a correlation between length of methadone maintenance treatment on verbal and non-verbal memory, attention and executive function or psychomotor functioning in methadone-treated patients. Similarly, our findings are supported by Saroj et al. (2020) who did not find a correlation between length of treatment and verbal learning, speed of processing, and verbal- or non-verbal working memory, in patients undergoing buprenorphine treatment compared to healthy controls. Conversely, Li et al. (2021) reported impairment in some tasks measuring impulsivity (such as delayed discounting) but not others (such as the beads task) in patients undergoing MMT compared to healthy controls. Also in contrast to our findings, Rass et al. (2014) report a positive correlation between treatment duration and working memory, the free recall component subtest measuring episodic memory and meta-memory, as well as increased

false alarm rates during tasks measuring recognition memory. In the present study, while an increased length of stay could reflect greater adherence to the rehabilitation program, we were unable to determine whether the lower mean length of stay in the methadone group reflected higher drop-out rates, or increased rate of progression through the rehabilitation program. The literature does not suggest a significant difference in compliance in patients treated with methadone compared to those treated with buprenorphine (Kinsky et al., 2019, Soyka et al., 2008, Strain et al., 1994); however, this is the first report in BNX patients. It should also be noted that inconsistencies between our findings and the literature may be explained by the large standard error for length of days for the methadone group, but further research is needed to validate these conclusions.

Non-treatment related demographic factors (age and BMI) were found to have a significant influence on cognitive performance in some tests. The present study found that age was positively correlated to speed of processing (TMT-A) in both treatments and when the cohort was analysed as a whole. We hypothesised that increased age would negatively impact cognitive function, similar to the findings of Waters and Caplan (2005), who reported a negative correlation between age and speed of processing utilising a digit symbol substitution task (a paradigm similar to the BACS-SC test used in the present study), and deficits in working memory and executive functioning (Murman, 2015). However, the average age of the population investigated in the present study was approximately 37 years; therefore, while increasing age could be a factor contributing to cognitive impairment, it may not have been a negative influence in the present study due to the younger age of the participants. Indeed, Kennedy (1981) reported poorer TMT-A performance in participants aged 50 – 69 compared to participants aged 20 – 49 from the general population. In addition to age, BMI was negatively correlated to reasoning / problem-solving performance (NAB-Mazes) in the BNX group but not the methadone treatment group. The established literature supports our finding that

increased BMI negatively impacts cognitive performance (Smith et al., 2011). For example, BMI was negatively correlated to reasoning / problem-solving and executive functioning in children (Gray, Schvey and Tanofsky-Kraff (2020) and adults (Gunstad et al., 2007). Studies by Gunstad et al. (2010) and Nilsson and Nilsson (2009) also reported a negative effect of higher BMI on performance in prospective memory, verbal fluency (letter and category fluency) and semantic memory in adults; however, Gunstad et al. (2010) also reported superior visuospatial attention and processing speed in participants with a higher BMI compared to lower BMI participants. While studies do report higher BMI in chronic opioid users (Diasso et al., 2019), few studies have investigated how BMI and chronic opioid use may influence cognition together. The average BMI in our cohort was 27.67, which is considered overweight and having excess adiposity (Flegal et al., 2005, Okorodudu et al., 2010). A possible mechanism by which BMI may lead to impaired cognition is through the inflammatory effect of adipose tissue. For example, Cannavale et al. (2021) found that C-reactive protein (an inflammatory marker) mediated the relationship between visceral adipose tissue and impairments in performance in attentional inhibitory control as assessed by a modified flanker task. Opioids similarly have a pro-inflammatory effect (Hofford, Russo and Kiraly, 2019), inducing upregulation of cytokines such as TNF-α and IL-6 (Johnston et al., 2004) and altering neuro-inflammation related pathways in the brains of chronic opioid users (Seney et al., 2021). Hence, a high BMI in the context of chronic opioid use may further exacerbate cognitive impairment through an inflammatory process; however, further research is required to confirm. Interestingly, while age and BMI did affect cognitive performance, other demographic factors such as years of education (which were significantly different between the treatment groups) did not influence test performance. Given the results of the present study, future research should consider the potential influence of demographic co-variates on cognitive performance.

There are several shortcomings of the present study. Firstly, the present study utilised a small population-based sample that may have impacted the results and larger, multi-site studies are required. Secondly, the present study was not able include a drug-naïve control group (see COVID Impact Statement), which is a limitation that should be addressed in future research in order to determine whether cognitive function is reduced in people undergoing opioid treatment compared to people without a history of opioid use, and to examine differences between drug treatment groups. Importantly, correlational analyses can only infer statistical relationships and may not represent cause or effect. In addition, the present study included participants with a self-reported history of comorbid psychiatric illness, comorbid substance use or history of psychiatric medication use, which could influence cognitive outcomes. Furthermore, while each participant in this study was admitted to the residential rehabilitation site for opioid misuse, few participants recalled a history of opioid use during the collection of demographic data. Accurate demographic data should be incorporated as co-variates in future studies. In addition, a consensus on the gold standard of tests to use when assessing cognitive performance in individuals with chronic opioid misuse should be made in order to assist comparison of results across studies.

In conclusion, the present study revealed cognitive deficits in individuals undergoing methadone or BNX treatment for chronic opioid misuse in an Australian residential rehabilitation setting, with no significant differences between treatment groups. Clinical parameters pertaining to treatment, such as increased time since last dosage and length of treatment with BNX was associated with improved cognition in key domains, while dosage and length of stay did not alter test scores. None of the parameters examined influenced cognition during methadone treatment. BNX treatment may increase adherence to the residential rehabilitation program, evidenced through an increased length of stay compared to the methadone treatment group. Non-treatment related demographic parameters, such as age

and BMI influenced cognition, while years of education did not. This study is among the first to investigate the effects of BNX treatment on cognitive functioning and examine other factors that may affect neurocognitive performance. The results of this small-sample population study demonstrate reason for further studies to investigate cognition and influencing factors in opioid misuse, ideally employing longitudinal study designs. Understanding the factors that influence cognitive performance in cohorts engaging in chronic opioid use may help to guide the development of future novel treatments and improve the lives of people with opioid addiction.

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Chapter 3

Dysbiosis of the Commensal Gut Microbiota in Chronic Opioid Use

3.1 Abstract

Chronic opioid use is occurring at an epidemic rate, resulting in several severe consequences such as impaired cognition. Existing medications for treating opioid addiction have inherent limitations such as high abuse potential, relapse and dropout, the potential to induce withdrawal symptoms and to further impair cognition. A growing body of literature has demonstrated that the gut microbiota engages in bidirectional communication with the brain, and is also altered (dysbiotic) in patients engaging in chronic alcohol and cocaine use compared to healthy controls. Clinical and preclinical studies similarly report dysbiosis in cohorts engaging in chronic opioid use, though the pattern of dysbiosis is inconclusive. Therefore, the present study aimed to determine: a) what the effect of opioid use is on the gut microbiota, and b) outline the changes in functional potential to the gut microbiota by opioid use and how they may relate to the signalling pathways of the microbiota-gut-brain axis. A systematic literature search of three databases (SCOPUS, PubMed, and Web of Science) was conducted for studies investigating the effect of chronic opioid use on the gut microbiota. 20 articles were included in the present review (4 clinical, 16 preclinical). Chronic opioid use consistently resulted in alterations in beta, but not alpha, diversity. Seven genera were repeatedly dysbiotic in clinical studies, as well as 36 microbes at various levels of taxonomy in preclinical studies. Four bacteria (Dialister, Lachnospiraceae, Peptostreptococcaceae and Ruminococcaceae) had a consistent pattern of dysbiosis across both clinical and preclinical studies. Four phyla (Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes), four families (Bacteroidaceae,

Lachnospiraceae, Peptostreptococcaceae, Ruminococcaceae), and 13 genera (Alistipes, Alloprevotella, Anaerostipes, Bacteroides, Bifidobacterium, Dialister, Haemophilus, Lactobacillus, Parabacteroides, Parasutterella, Prevotella, Roseburia, Ruminococcus) were repeatedly dysbiotic as a result of opioid use, in addition to several metabolites (including short chain fatty acids and bile acids) and metabolic pathways, all of which may contribute to brain function. Future research would benefit from determining the relationship between these microbes, their functional potential and cognition in the context of opioid addiction and cognition.

3.2 Introduction

Harmful use of opioids, opioid misuse and opioid use disorder (OUD) are growing public health concerns that require treatment options facilitated by a deeper understanding of the pathophysiology of the disorders. An alarming increase in prevalence of OUD and a high rate of misuse of opioids have been reported, with an estimated 40.5 million individuals misusing opioids globally in 2017 (Degenhardt et al., 2019, James et al., 2018). Major factors contributing to this increase are the misuse of prescription opioids (such as codeine) (Han et al., 2017, Han et al., 2015, Han, Sherman and Palamar, 2019), and synthetic opioids (such as fentanyl) (Jones, Einstein and Compton, 2018, O'Donnell, Gladden and Seth, 2017), over-prescription for minor pain, as well as a shift to stronger and longer acting opioids (such as oxycodone and methadone) (Karanges et al., 2016, Gisev et al., 2018, Larance et al., 2018, Han et al., 2017, Han et al., 2015, Han et al., 2019). Opioid misuse is defined as the use of opioids outside of prescriber direction (Elliott and Jones, 2019), whereas OUD is a DSM-5 substance use disorder characterised by the continued and harmful use of opioid drugs despite the presence of detrimental outcomes (Blanco and Volkow, 2019, Strang et al., 2020). These include greater likelihood for, and rates of, polysubstance misuse and dependence (Compton, Valentino and DuPont, 2021, D'Amico et al., 2021), neonatal abstinence syndrome (Martins et al., 2019), potential involvement with the criminal justice system (Pryor et al., 2021, Subramaniam and Stitzer, 2009, Winkelman, Chang and Binswanger, 2018), negative economic consequences (Oderda et al., 2015, Edwards et al., 2020, Florence et al., 2021) and an increased risk of mortality (Gaither, Shabanova and Leventhal, 2018, Olfson et al., 2019, Mattson et al., 2021). Existing treatments for opioid use disorders include pharmacological interventions (methadone maintenance treatment, buprenorphine/naloxone treatment) and psychobehavioural interventions (such as cognitive behavioural therapy, CBT), however these treatments have limitations (Dugosh et al., 2016). Methadone is a μ-opioid receptor agonist

useful in opioid detoxification and in opioid maintenance therapy programmes (Ayanga, Shorter and Kosten, 2016). Whilst methadone has shown efficacy in treating opioid dependent patients (Salsitz and Wiegand, 2016), treatment efficacy is dependent on early treatment adherence (i.e., during first 12 months), which is when treatment dropout is highest (Salsitz and Wiegand, 2016, Nosyk et al., 2010, Cao et al., 2014), and incorrect dosage can result in relapse or poor treatment adherence (D'Aunno, Park and Pollack, 2019). Buprenorphine is a partial μ-opioid receptor agonist also used in maintenance therapy, often in conjunction with the opioid receptor antagonist naloxone (i.e., BNX) (Ayanga et al., 2016). Although buprenorphine has many benefits over methadone, such as a decreased likelihood of being misused (Ayanga et al., 2016), evidence indicates poorer retention (Burns et al., 2015, Gryczynski et al., 2013, Hser et al., 2014), especially at lower or unfixed doses (Mattick et al., 2014). In addition, high levels (up to 59%) of treatment drop-out have been reported during early phases of buprenorphine treatment (Ponizovsky et al., 2010, Hakansson and Hallen, 2014). Psychobehavioural therapies (such as Cognitive Behavioural Therapy; CBT) alone (Mayet et al., 2005, Veilleux et al., 2010), or in combination with pharmacotherapies (Amato et al., 2011, Strang et al., 2020) also have limited efficacy. Therefore, novel treatments for opioid dependence are required.

Poorer cognitive performance in several domains has been reported in patients engaging in harmful opioid use (see Chapter 2). For example, performance in attention, executive function, psychomotor speed, and working memory are reportedly below the levels of healthy patients (Baldacchino et al., 2012, Wollman et al., 2019, Sanborn et al., 2020, Kroll et al., 2018). This is an issue that requires more attention due to the potential importance of cognition in the course of substance use disorders and their treatment. Indeed, research on other commonly abused substances, such as alcohol and cocaine, demonstrate an inverse relationship between cognitive performance and positive treatment outcomes including treatment adherence

(Teichner et al., 2002, Aharonovich et al., 2006, Streeter et al., 2008, Copersino et al., 2012, Manning et al., 2017, Mahoney, 2019, Caballeria et al., 2020). It is unclear whether existing treatments for OUD improve the cognitive dysfunctions associated with the disorder, and some evidence suggests that pharmacological treatments for opioid addiction may further worsen cognitive outcomes (Pujol et al., 2018). Patients undergoing methadone maintenance treatment (MMT) can present with poorer cognitive performance compared with both healthy controls and abstinent patients (Baldacchino et al., 2017, Motazedian et al., 2021, Sanborn et al., 2020). Buprenorphine-treated patients show poorer performance in tasks measuring cognitive flexibility, set shifting, working memory and executive function (Soyka et al., 2008, Saroj et al., 2020). A study by Rapeli et al. (2007) investigated the cognitive performance of patients undergoing either methadone, or combined buprenorphine / naloxone treatment, compared to untreated, non-addicted healthy controls. Results indicated poorer performance in working memory in both the treatment groups compared to the controls, with lower attention and verbal memory also reported in participants undergoing methadone treatment (Rapeli et al., 2007). In a subsequent longitudinal study, neither methadone nor buprenorphine were able to recover cognitive performance over the long term, as neither treatment group showed improvement in working memory performance (Rapeli et al., 2009). Overall, there is a need to address the shortcomings of these existing treatments in terms of efficacy for addiction and cognitive impairments associated with OUD, and novel treatment options that may potentially target these pathologies are required.

There has been a growing body of evidence in recent years to show that the commensal microflora community inhabiting the gastrointestinal tract (known as the gut microbiota) plays an integral role in host health, including brain function and cognition (Bienenstock, Kunze and Forsythe, 2015, Liang, Wu and Jin, 2018, Cryan et al., 2019). A central part of microbiota research involves characterising the structure of the gut microbiota, and generally utilises 16S

rRNA gene sequencing (Bjorkhaug et al., 2019, Wang et al., 2018b) or shotgun (whole genome) sequencing (Dubinkina et al., 2017), and considers many factors including alpha and beta diversity. Alpha diversity (measured by indices such as Chao1, the Shannon index, the Simpson index and the ACE index), considers the richness (number of species present) and evenness (uniformity of the abundance of the species present) of the microbiota within a site. On the other hand, beta diversity considers the differences and overlap in community structure between distinct sites or individuals.

The microbiota engages in bidirectional communication with the body and brain along the microbiota-gut-brain axis (MGB) through several pathways including the immune, neuroendocrine and nervous systems (Carabotti et al., 2015), the ability of microbes to produces neurotransmitters (Strandwitz, 2018), and their role in gastrointestinal tract health (Aziz et al., 2013) and can thus influence brain function. For example, germ-free mice have impaired microglia function (e.g., abnormal cell growth, and impaired activation) compared to control animals, and reconstitution of the microbiota in germ-free animals is able to return microglia to normal after several weeks (Cryan et al., 2019). Microglia, found exclusively in the CNS, are important for healthy synaptic development and neuronal health (Morris et al., 2013), while impaired microglia are associated with both neuropsychiatric diseases (Blank and Prinz, 2013, Kato et al., 2013) and impaired hippocampal-dependent learning and memory (Reshef et al., 2014). In addition, the gut microbiota can influence host health through metabolites such as short chain fatty acids (Dalile et al., 2019, Silva et al., 2020), which are able to influence the permeability of the blood brain barrier by regulating tight junction proteins (Braniste et al., 2014), and can cross the blood brain barrier (Kim et al., 2013, Stilling et al., 2016), where they may act as histone deacetylase (HDAC) inhibitors (Davie, 2003, Kim, Leeds and Chuang, 2009). While the gut microbiota is associated with normal brain function and behaviour (Diaz Heijtz et al., 2011, Stilling, Dinan and Cryan, 2014, Borre et al., 2014, Ceppa,

Mancini and Tuohy, 2019) including neurodevelopment (Sampson and Mazmanian, 2015) and cognitive function (Bajaj et al., 2012, Magnusson et al., 2015), an altered microbiota profile (known as dysbiosis) is associated with negative health consequences, including neuropsychiatric disorders such as autism (Strati et al., 2017b), schizophrenia (Nguyen et al., 2021), bipolar disorder (Nguyen et al., 2018), anxiety (Crumeyrolle-Arias et al., 2014, Jiang et al., 2018) depression (Li et al., 2019)**,** and the severity symptoms (Madan et al., 2020, Li et al., 2020b). A dysbiotic gut microbiota has also been associated with addiction as substance abuse alters the gut microbiota. For example, alcohol (Dubinkina et al., 2017, Wang et al., 2018b, Leclercq et al., 2019, Litwinowicz, Choroszy and Waszczuk, 2020)**,** cocaine (Volpe et al., 2014, Scorza et al., 2019) and methamphetamine (Forouzan, Hoffman and Kosten, 2020) misuse result in dysbiosis of the microbiota. In addition to this, a study in alcohol dependence found a link between severity of behavioural symptoms in alcohol dependent patients and an increase in gut intestinal tract permeability, a finding which was associated with a dysbiotic gut microbiota (Yang et al., 2019). These papers outline a relationship between the gut microbiota and substance dependence, and the potential of the microbiota to contribute to the cognitive impairments observed in these disorders.

Research suggests that opioid misuse also causes dysbiosis in the gut. For example, a clinical study by Acharya et al. (2017) found beta diversity to be different between patients engaging in opioid use compared to patients not engaging in opioid use in a cohort with hepatic encephalitis, but did not report results regarding alpha diversity. Preclinical studies have also found alterations in alpha and beta diversity as a result of morphine treatment as well as enrichment in several strains (Wang et al., 2018a), however other studies do not observe any patterns of dysbiosis in alpha diversity as a result of morphine treatment (Lee et al., 2018). Therefore, results on the effect of opioid use on the gut microbiota remain unclear. Addressing this gap may identify microbes that could serve as candidates for targets in succeeding research

aiming to understand the pathology of opioid addiction and developing novel therapeutics for opioid addiction and associated cognitive dysfunction. While thematic reviews have explored opioid use and the gut microbiota (Wang and Roy, 2017, Ren and Lotfipour, 2020), to the author's knowledge, a systematic review investigating the gut microbiota and opioid use has not yet been conducted. As such, the aim of the present systematic literature review was to: a) determine the effect of opioids on specific strains of the gut microbiota; and, b) outline the potential functional outcomes of altered gut microbiota and metabolites by investigating their role in key signalling pathways of the microbiota-gut brain axis and how they may potentially affect cognition.

3.3 Methods

A systematic review following PRISMA guidelines (Moher et al., 2009, Moher et al., 2015) was conducted to examine research investigating the relationship between opioid use and the commensal gut microbiota. Opioids were selected as the sole focus of this chapter as these substances were consistently utilised by the cohort of patients investigated in Chapter 2. An outline detailing the screening phases of this systematic review is provided in Figure 3.1.

3.3.1 *Search Strategy*

Three electronic databases (PubMed, SCOPUS and Web of Science; WOS) were searched for relevant literature. Databases were searched for original articles, written in English up to September, 2020. Search terms used to identify literature investigating opioid use and gut microbiota included "*heroin, opiate**, *opioid**" paired with either "*gut microbiota*, *microbiome*, or *microbiota*". As an example, WOS database searches for studies examining heroin and microbiota were: "*heroin* AND *gut microbiota*",

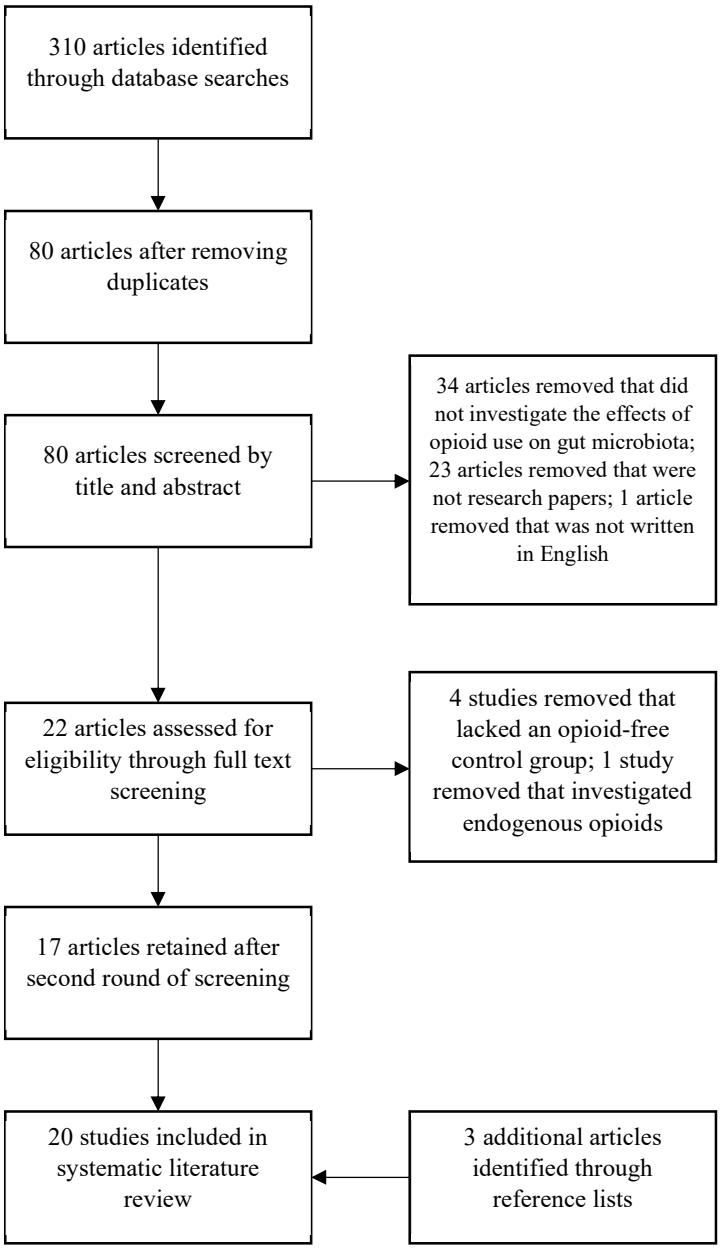

Fig. 3.1 **PRISMA Flow Chart outlining process for identification of eligible studies for inclusion into systematic literature review.**

"*heroin* AND *microbiome*" and "*heroin* AND *microbiota*." After removing duplicates, a first round of screening was conducted based on title and abstract. The second round of screening was a full text article screening against the eligibility criteria. Review articles were excluded but reference lists were screened for further studies.

3.3.2 *Eligibility Criteria*

To be eligible for inclusion in this systematic review, studies must have investigated the relationship between opioid use and the gut microbiota. Studies were excluded if: 1) they did not report the microbiota profiles of the cohorts being investigated, or 2) did not compare opioid use compared to controls.

3.3.3 *Data Extraction and Analysis*

After screening, eligible studies were further reviewed for data extraction. The following data were extracted from the articles: substance examined, cohort demographic data, species and strain (for preclinical studies), study design including dosage of substances administered, route of administration, treatment duration and timelines, cognitive and behavioural tests used, methods of microbiota sampling and analyses, microbiota structure and composition (i.e., alpha and beta diversity), dysbiotic strains and mechanistic data (where available), as well as standard publication data (authors, year of publication, journal of publication).

3.4 Results

3.4.1 *Search Results*

The literature search yielded 310 returns (SCOPUS: 193, WOS: 111, PubMed: 6). Duplicates were removed, and 80 articles were retained for first round of screening by title and abstract. After first round of screening, 58 articles were excluded; 34 articles were removed as they did not investigate opioid use or did not report on the gut microbiota, 23 articles were removed as they were not research papers (reviews, conference proceedings etc.), and one was removed as it was not written in English. Twenty-two articles were retained for a second round

of screening by full text screening. After a second round of screening, 5 articles were excluded; four articles were removed because they lacked an opioid free control group (Iglesias-Santamaria, 2020, Jackson et al., 2018, Pettigrew et al., 2019, Zaborin et al., 2014), and one article was removed as it investigated endogenous opioids (Lee et al., 2017). Seventeen articles were retained after full text screening. Three additional papers were sourced from references (Li et al., 2020a, O'Sullivan et al., 2019, Xu et al., 2017). A total of 20 articles passed the screening process and were included in this systematic literature review (outlined in Table 3.1 and Table 3.2). Four articles were clinical studies (Acharya et al., 2017, Barengolts et al., 2018, Li et al., 2020a, Xu et al., 2017), and are outlined in Table 3.1. The remaining 16 were preclinical studies, including one primate study (Sindberg et al., 2019), 12 mice studies (Banerjee et al., 2016, Hakimian et al., 2019, Kang et al., 2017, Lee et al., 2018, Meng et al., 2020, Meng, Sindberg and Roy, 2015, Sharma et al., 2020b, Touw et al., 2017, Wang et al., 2018a, Wang et al., 2020a, Zhang et al., 2019a, Zhang et al., 2021b), and three rat studies (O'Sullivan et al., 2019, Simpson et al., 2020, Zhang et al., 2020a), and are outlined in Table 3.2.

3.4.2 *Alterations to the Microbiota by Opioid/Opiate Use*

3.4.2.1 Alterations to the Microbiota by Opioid/Opiate Administration in Clinical Studies

Acharya et al. (2017) reported dysbiosis of the gut microbiota as a result of opioid use in patients with cirrhosis (n=72, including patients with non-alcohol steatohepatitis (NASH) and hepatic encephalopathy (HE)) who have engaged in chronic opioid use (daily use for 3 months) compared to cirrhotic controls not using opioids (Table 3.1). Faecal samples were analysed by 16S rRNA sequencing and results showed that opioid use resulted in a shift in beta diversity, regardless of HE-status. Patients using opioids exhibited reduced abundance of several bacterial families (*Clostridiales* XIV, *Lachnospiraceae*, *Bacteroidaceae* and

Ruminococcaceae) compared to non-opioid using patients, reflecting the disruptive effects that opioid use can have on microbiota, especially native microbes. Opioid users who had a diagnosis of HE exhibited increased *Bifidobacterium* abundance compared to patients using opioids without comorbid HE, whereas opioid users without HE exhibited increased *Peptostreptococcaceae* and reduced *Parasutterella* compared to non-HE patients not using opioids. In patients with a comorbid diagnosis of NASH, opioid use was not associated with disruption of the composition of the microbiota, whereas dysbiosis was observed in patients not using opioids. The functional potential of the altered microbiota was also investigated using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; (Acharya et al., 2017) (Table 3.1). Opioid users showed significantly increased potential for metabolism of branched and aromatic amino acids compared to patients not using opioids, whereas microbiota of non-opioid using patients had increased potential for functions related to bioenergetic processes and branched chain amino acid production. When examining the HE-positive subgroup, opioid using patients had increased potential of pathways related to aromatic amino acid metabolism and branched amino acid degradation, endotoxin synthesis, branched chain amino acid motility, and nitrogen metabolism compared to non-opioid patients. Finally, when comparing patients without diagnosis of comorbid HE, there was predicted upregulation of pathways involved in aromatic acid metabolism in opioid using patients compared to non-opioid using patients. These results suggest an effect of opioid use on amino acid metabolism, as opioid-using patients repeatedly presented with increased functional potential of pathways related to metabolism of these molecules compared to non-opioid using patients, even in the presence of comorbidity. In short, this paper reports shifts in the structure and functional potential of the microbiota as a result of opioid use in a cohort of cirrhotic patients, with composition also affected by NASH and HE (Acharya et al., 2017).

Barengolts et al. (2018) investigated a cohort of African American men with a DSM-

IV diagnosis of opioid use disorder and co-morbid type 2 diabetes mellitus (T2DM) compared to controls (Table 3.1). Patient microbiota profiles were analysed through 16S rRNA gene sequencing of faecal samples. Alpha diversity, determined using the Shannon Index, was not altered as a result of opioid use but trended towards a higher diversity in the non-opioid T2DM group treated with metformin compared to all other groups (Table 3.1). Similarly, beta diversity did not change between groups. When examining specific bacterial populations, *Bifidobacterium* abundance was decreased in the T2DM group compared to the non-T2DM controls, but there was no difference between opioid users and non-opioid users overall. However, opioid using patients with T2DM had a significant increase in *Bifidobacterium* compared to patients not using opioids with T2DM (Table 3.1). The authors also investigated interactions between metformin (diabetes medication) and opioids in the T2DM subgroup. Metformin treatment of T2DM in opioid users significantly decreased *Bifidobacterium* compared to levels observed in opioid users with T2DM without metformin, and compared to levels observed in healthy (non-T2DM or opioid) controls (Table 3.1). There was a significant effect of opioid use on *Prevotella*, which was decreased in opioid users compared to non-using controls (Table 3.1); however, *Bifidobacterium* and *Prevotella* were not influenced by T2DM comorbidity in opioid users (i.e., no change in the opioid user group compared to the opioid and T2DM comorbidity group (Table 3.1). These results suggest that opioids did not shift alpha or beta diversity in this population, but did influence the genera *Bifidobacterium* and *Prevotella* uniquely in the presence of comorbid T2DM and metformin treatment.

Dysbiosis as a result of methadone was reported in a study by Li et al. (2020a). The study investigated patients undergoing one or two years of compulsory detention (CD; a government enforced rehabilitation program) for drug use (n=28, male); patients undergoing methadone maintenance treatment (MMT; n=16, 12 of which were male); current users of heroin or methamphetamine (drug using, DU; n=27, all male); and healthy non-drug using

controls (HC; n=28, 21 of which were male) were examined. Patient faecal samples were analysed by 16rRNA sequencing. Alpha diversity was not significantly different between groups (Table 3.1). Beta diversity was significantly different between groups. Community structure differed significantly between the CD group and controls. MMT and DU groups showed similar, overlapping community structure. The phyla *Actinobacteria* and *Cyanobacteria*, and the genera *Bifidobacterium*, *Fusicatenibacter*, *Intestinibacter*, *Lactobacillus*, *Streptobacillus* and *Veillonella* increased abundance in MMT patients. *Aestuariispira* was highly abundant in the HC group, while *Collinsella*, *Roseburia*, *Ruminococcus* and *Succinivibrio* was increased in the DU group. Finally, *Alloprevotella*, *Erysipelotrichaceae* incertae sedis and *Flavonifractor* were increased in abundance in CD. MMT patients had an increased abundance of *Actinobacteria* compared to the CD group and genera that were present in significantly different abundance depending on treatment group included: *Anaerostipes* (depleted in CD compared to MMT and CD groups), *Bifidobacterium* (greatest in MMT), *Fusicatenibacter* (greatest in MMT), *Haemophilus* (greatest in DU), *Intestinibacter* (greater in MMT compared to CD and control groups), *Klebsiella* (greatest in MMT), *Lactobacillus* (greatest in MMT), *Megasphaera, Roseburia* (greatest in DU, depleted in MMT), *Ruminococcus* (greatest in DU, depleted in CD), *Sporobacter* and *Streptococcus* (greatest in MMT; Table 3.1). In short, these results reflect unique enrichment and depletion of several bacterial genera as a result of rehabilitation regime, drug use, or absence of use. These results do not reflect a difference in evenness and richness between individuals undergoing various rehabilitation programmes, compared to patients undergoing active drug use and healthy controls, and instead reflects differences in the abundance of specific microbes.

Finally, Xu et al. (2017) investigated the effects of heroin (n=26), methamphetamine (n=15), ephedrine (n=4) or other drug use (n=5) on the gut microbiota in males patients in rehabilitation, compared to healthy male controls (n=48). Patient faecal samples were collected

and analysed by 16S rRNA sequencing. Alpha diversity was altered in SUD compared to controls. Chao1 increased in SUD, compared to controls; however, no changes were observed between SUD groups (across substance types). There was also an increase in observed species diversity index in SUD compared to controls. The report then conducted analyses on a subgroup of age matched participants (29 SUD patients and 28 controls aged from 19 to 37) to remove age a potential cofounder. In this age matched subgroup there was an increase in Chao1 in the SUD group compared to controls, with no significant difference between drug use groups (i.e., regardless of drug type consumed). There was also a significant difference in beta diversity between the substance use disorder group and controls (Table 3.1). At the genus level *Alistipes, Bacteroides, Barnesiella, Blautia, Clostridium* XI, *Dialister, Escherichia*/*Shigella*, *Faecalibacterium, Gemmiger, Haemophilus, Megasphaera, Parabacteroides* and *Paraprevotella*, were decreased; and, *Alloprevotella, Clostridium* XIVa*, Megamonas, Phasolarctobacterium, Prevotella, Roseburia* and *Ruminococcus* were increased in the substance use cohort. Further analysis revealed an effect of age on *Barnesiella, Blautia, Clostridium* XI and *Megasphaera*. In addition, length of history of substance use had an effect on the abundance of a number of genera, including *Prevotella, Phascolarctobacterium* and *Ruminococcus,* which were increased while *Bacteroidetes* and *Haemophilus* were decreased in longer term substance use compared shorter term substance use disorder. Xu et al. (2017) also outlined some potential functional consequences through PICRUSt. Metabolic pathways associated with cell growth and death, DNA replication and repair, and translation were upregulated with substance use, and those associated with cellular signalling and processing, and metabolism were diminished (Table 3.1). This study demonstrates a dysbiotic effect of substance use, but no differences based on substance type used, compared to controls. Length of history of substance use had a significant effect on community structure.

Table 3.1 **Clinical studies investigating the effect of chronic opioid use on the commensal gut microbiota**

Study Reference	Opioids Investigated	Study Participants	Comorbidity	Method of Analysis	α and β Diversity
Acharya et al. (2017)	Oxycodone (n=42) Morphine (n=11) Hydromorphone (n=8) Tramadol (n=7) Methadone (n=4)	Opioid positive cirrhotic patients, opioid negative cirrhotic patients (n=72 per group)	Cirrhosis Opioid Positive HE (n=40), Diabetes (n=20), NASH (n=7), Alcohol Use (n=20); Opioid Negative HE (n=38), Diabetes (n=21), NASH (n=11), Alcohol Use (n=13)	16S rRNA sequencing β: UniFrac Functional Potential: PiCRUST	β: Δ between opioid and non-opioid groups
Barengolts et al. (2018)	Opioids (Unspecified)	African American Men OP-/T2DM- (n=24), OP-/T2DM+/Met- (n=11), OP-/T2DM+/Met (n=19), OP+/T2DM- (n=28), OP+/T2DM+/Met- (n=5), OP+/T2DM+/Met+ (n=6)		16S rRNA gene sequencing of stool samples α: Shannon β: Bray-Curtis	α: Trended ↑ in T2D+/OP-/Met+ vs all other groups β: ~ between groups
Li et al. (2020a)	Heroin, Methadone, Methamphetamine	Compulsory Detention (n=28; all Male), Drug Users (n=27; all Male), MMT (n=16; 12 Male), Healthy Controls (n=28; 21 Male)		16S rDNA sequencing α: ACE, Chao1, Observed Species, Shannon, Simpson β: Jaccard	α: ~ between groups β: Δ between groups. CD Δ from HC; ~ between MMT, DU CD
Xu et al. (2017)	Heroin, Methamphetamine, Ephedrine,	(n=101, all male) Heroin (n=26) Methamphetamine (n=15) Ephedrine (n=4), Others (n=5; Heroin and Ephedrine, Methamphetamine, Heroin and Methamphetamine) Drug Free Controls (n=48)	Drug Addiction Rehabilitation	16S rRNA gene sequencing α: Chao 1 and Observed Species Diversity β: Unweighted UNIFRAC	α: ↑ (non-sig) in SUD (Chao1); ↑ but not when age matched (Observed species index) β: Δ between SUD and Controls; ~ between substances of abuse

Study Reference	Dysbiotic Strains	Functional Potential
Acharya et al. (2017)	↓ Bacteroideaceae, Clostridiales XIV, Lachnospiraceae and Ruminococcaceae in opioid positive patients compared to opioid negative group ↑ Bifidobacterium in Op+/HE+ vs /OP+/HE- ↑ Peptostreptococcaceae; ↓ Parasutterella in OP+/HE- vs OP-/HE-	↑ AA Acid metabolism, BCAA degradation; ↓ BCAA production in OP+ vs OP- ↑ AA Acid metabolism, BCAA degradation, endotoxin synthesis, BCAA motility, nitrogen metabolism in HE+/OP+ vs HE+/OP- ↑ AA Acid metabolism in HE-/OP+ vs HE-/OP+
Barengolts et al. (2018)	↓ Bifidobacterium in T2DM+ vs T2DM- ↑ Bifidobacterium in OP+/T2DM+ vs OP-/T2DM+ ↑ Bifidobacterium in OP+/T2DM+/Met+ vs OP+/T2DM+/Met-, and OP- and T2DM+ groups ↓ Prevotella in OP+/T2DM+/Met- vs OP-/T2DM+/Met- Trend between Bacteroides caccae abundance and Op+, Met+	N/A
Li et al. (2020a)	↑ Cyanobacteria and Actinobacteria, and Bifidobacterium, Fusicatenibacter, Intestinibacter, Lactobacillus, Klebsiella, Streptobacillus, Sporobacter, Streptococcus and Veillonella; ↓ Megasphaera, Roseburia abundance in MMT ↑ Collinsella, Haemophilus, Megasphaera, Roseburia, Ruminococcus and Succinivibrio in DU ↑ Alloprevotella, Erysipelotrichaceae incertae sedis and Flavonifractor; ↓ Anaerostipes, Ruminococcus in CD ↓ Aestuariispira in all groups compared to HC Firmicutes ↑ DU, compared to CD	N/A
Xu et al. (2017)	↑ Alloprevotella, Clostridium XIVa, Megamonas, Phasolarctobacterium, Prevotella, Roseburia and Ruminococcus; ↓ Alistipes, Bacteroides, Barnesiella, Blautia, Clostridium XI, Dialister, Escherichia/Shigella, Faecalibacterium, Gemmiger, Haemophilus, Megasphaera, Parabacteroides and Paraprevotella in SUD cohort Barnesiella, Blautia, Clostridium XI and Megasphaera abundance was influenced by patient age ↑ Prevotella, Phascolarctobacterium and Ruminococcus; ↓ Bacteroidetes and Haemophilus in Longer term SUD	N/A

Abbreviations: α=Alpha, β=Beta, Δ =Different, ~=Not Different, N/A=Not Available; OP=Opioids, OP+=Opioid Negative, OP-=Opioid Negative; HE=Hepatic Encephalitis; NASH= Non-Alcoholic Steatohepatitis; T2DM=Type 2 Diabetes Mellitus, T2DM+=T2DM Positive, T2DM-=T2DM Negative; Met=Metformin, Met+=Met Positive, Met-=Met Negative; MMT=Methadone Maintenance Treatment, DU=Drug Users, CD=Compulsory Detention, HC=Healthy Controls; SUD=Substance Use Disorder; AA Acid=Aromatic Amino Acids, BCAA=Branched-Chained Amino Acids

Table 3.2 **Preclinical studies investigating the effect of chronic opioid use on the commensal gut microbiota**

Study Reference	Opioid Investigated	Treatment Groups	Species/Strain	Comorbidity and Diet	Experimental Paradigm	Method of Analysis
Banerjee et al. (2016)	Morphine	Morphine, Morphine and Naltrexone, Placebo (n=6 per group) FMT Morphine or Placebo donor (n=10 per group); Morphine or Placebo recipient (n=32)	Male C57BL/6, NSG, TLR2KO and MORKO, 8-10 week old		Morphine, Naltrexone (25mg slow-release pellet implant) or Placebo, microbiota tested after 5-6 days FMT Morphine or Placebo pelleted faecal matter administered to morphine or placebo pelleted recipient mice, once daily, three times, by oral gavage, microbiota tested 24h after final FMT.	16S rDNA sequencing α: PD
Hakimian et al. (2019)	Remifentanil, Oxycodone	n-3 PUFA Diet and Saline (n=5) n-3 PUFA Diet and Opioid (n=10) Control Diet and Saline (n=14) Control and Opioid (n=14)	Male C57B16/J, 6-8 weeks old	Anxiety, Standard lab chow or n-3 PUFA supplemented	Acquisition with remifentanil (0.05mg/kg/infusion) self-administed (i.v.) for 2h sessions or until 50 infusions were administered, paired with audio/visual reinforcing cues (3 days) Maintenance with oxycodone (0.25mg/kg/infusion, 10 days), First extinction (cues with no drug), 5 days Reinstatement (oxycodone, 2 days) Reinstatement (oxycodone, 2 days) Second extinction (cues with no drugs, 5 days)	16S rRNA sequencing α: Faith's Phylogenetic Diversity, Chao1, and Shannon Index β: Bray-Curtis
Kang et al. (2017)	Morphine	Morphine, Morphine and ABX, Placebo, Placebo plus ABX (n=7 per group)	Male Swiss Webster	Antinociception	Antibiotic (ABX) treatment for 10 days with Vancomycin (5mg/mg), Neomycin (10mg/kg), Metronidazole (10mg/kg) and Streptomycin (10mg/kg) by oral gavage every 12 hours, and Ampicillin (1g/L) in drinking water. Morphine (75mg pellet, s.c.) or placebo implanted at day 5. Mice sacrificed at day 10.	16s rRNA sequencing

Study Reference	α and β Diversity	Dysbiotic Microbes	Functional Potential
Banerjee et al. (2016)	α: Non-sig Δ between groups β: Δ between morphine and placebo mice, ~ between naltrexone and placebo, Placebo-Placebo, Placebo-Morphine, Morphine-Morphine, Morphine-Placebo	↑ *Bacillaceae*, *Enterococcaceae*, *Erysipelotrichaceae*, *Staphylococcaceae* and *Streptococcaceae*; ↓ *Bacteroidetes* in morphine group vs both other groups	↑ Coprostanol, Cholesterol; ↓ Cholate, CDCA, DCA, UDCA and Unconjugated UDCA, bile salt hydrolase and free taurine in WT morphine vs WT placebo and WT morphine and WT naltrexone
Hakimian et al. (2019)	α:. n-3 PUFA ↑ species richness (Chao1, Faiths PD and Shannon Index) vs control diet β: Δ between diet groups when adjusting for study phase; Δ between OXY maintenance and both extinction phases; ~ between D1 and D10. ~ between extinction phases	<u>During Oxy Maintenance</u> ↑ *Allobaculum*, *Alistipes*, *Bifidobacterium*, *Coprobacillus*, *Coriobacteriaceae*, *Dorea*, *Erysipelotrichaceae*, *Lactobacillus*, *Oscillospira*, and *Streptococcus*; ↓ *Akkermansia*, *Clostridium*, *Coprococcus*, *Enterobacteriaceae* and *Parabacteroides* in n3P vs control diet <u>During Oxy Extinction</u> ↑*Bifidobacterium* and *Desulfovibrio*; ↓ *Parabacteroides*, and *Clostridiaceae*, in n3P diet vs control diet <u>Within n-3P diet treatment</u> ↑ *Enterococcus*; ↓ *Akkermansia, Allobaculum, Bifidobacterium*, and *Coriobacteriaceae* during extinction phase, compared to maintenance phase <u>Within control diet treatment</u> ↓ *Akkermansia, Coprococcus*, family *Enterobacteriaceae, Parabacteroides and Bifidobacterium* during extinction phase, compared to maintenance phase <u>Opioid extinction</u> ↓*Akkermansia* and *Bifidobacterium*, regardless of diet, but ↓ *Parabacteroides*, within the control diet group.	N/A
Kang et al. (2017)	↓ total bacterial abundance in non-ABX morphine-treated mice vs non-ABX placebo mice	↓ *Bacteroidales*, *Clostridiales* and *Lactobacillales*; ↑ *Enterobacteriales* in non-ABX morphine vs non-ABX placebo mice	N/A

Study Reference	Opioid Investigated	Treatment Groups	Species/Strain	Comorbidity and Diet	Experimental Paradigm	Method of Analysis
Lee et al. (2018)	Morphine	Morphine Injection (n=4) Morphine Pellet (n=4) Saline Injection (n=8) Vehicle Pellet (n=4)	Male C57BL/6J, 6-8 weeks old		Intermittent morphine sulphate i.p. escalating dosage (10, 20, 30, 40mg/kg) b.i.d., for 4 days, saline i.p., b.i.d. Continuous morphine sulfate (25mg, s.c.) or placebo pellet.	16S rDNA sequencing α: Observed OTUs β
Meng et al. (2020)	Morphine	Morphine, HIV only, HIV and Morphine and Controls (n=6 per group)	Humanized Bone Marrow-Liver-Thymus mice generated by implanting xenogenic Thymus and Liver tissue into NOD-SCID IL2R Gamma Null, from 4 weeks old	HIV	HIV infection for 4 weeks, Combined treatment, morphine administered 21 days into HIV infection Morphine (75mg slow-release implant) or placebo. Mice sacrificed and microbiota tested after 7 days.	16S rRNA sequencing of caecal contents α: Oberved OTUs, Shannon Index β: Bray-Curtis
Meng et al. (2015)	Morphine	Morphine, Morphine and Naltrexone, Placebo (n=6)	Male C57BL/6 and TLR2KO, 8-10 weeks old	Sepsis	Caecal Ligation and Puncture 24 prior to pellet implant Morphine (25mg slow-release pellet), morphine and naltrexone (30mg pellet) or placebo	16S rDNA sequencing
O'Sullivan et al. (2019)	Morphine	Morphine, Morphine and Naltrexone, Naltrexone, and Placebo (n=4 per group)	Sprague-Dawley		Morphine (75mg slow release, s.c.) pellet or placebo for 6 days, then sacrificed and microbiota analysed, or Naltrexone (100mg/kg, i.p.) for Naltrexone and Withdrawal groups at day 6, then sacrificed and microbiota analysed.	qPCR of caecal DNA

Study Reference	α and β Diversity	Dysbiotic Microbes	Functional Potential
Lee et al. (2018)	α: ~ between morphine (sustained and intermittent) and controls β: Δ between morphine (sustained and intermittent) and controls	Intermittent treatment ↑ Ruminococcus spp.; ↓ Lactobacillus spp. compared to controls. Sustained treatment ↑ Clostridium spp. and Rikenellaceae vs controls	N/A
Meng et al. (2020)	α: ↓ Combined morphine and HIV compared to all other groups β: Δ morphine and placebo	HIV/Morphine group vs other groups ↑ Firmicutes, Proteobacteria and Enterococcus, Staphylococcus ↓Actinobacteria, Bacteroidetes and Tenericutes, Muribaculaceae, Lachnospiraceae, Lactobacillus and Ruminococcaceae. HIV infected animals ↑ Proteobacteria, Staphylococcus; ↓ Bacteroidetes Morphine facilitated ↑ Enterobacteriaceae in H-BLT mice	N/A
Meng et al. (2015)	N/A	↑ Firmicutes, Staphylococcus sciuri, S. cohnii, and S. aureus and Enterococcus durans, E. casseliflavus, E. faecium, and E. faecalis in morphine-treated mice vs both other groups	N/A
O'Sullivan et al. (2019)	N/A	↑ Bacteroides thetaiotaomicron, Bacteroides fragilis, Enterococcus faecalis, Enterococcus gallinarum, and Bacteroides vulgatus; ↓ Bifidobacterium, Clostridium coccoides, Clostridium leptum, Butyricicoccus genus and Butyricicoccus pullicaecorum, Faecalibacterium prausnitzii in withdrawal group vs other groups ↑ Anaerotruncus colihominis in morphine vs withdrawal mice ↓ Prevotella in morphine vs placebo mice	N/A

Study Reference	Opioid Investigated	Treatment Groups	Species/Strain	Comorbidity and Diet	Experimental Paradigm	Method of Analysis
Sharma et al. (2020b)	Hydromorphone	Hydromorphone (H), Dextran Sodium Sulphate (DSS), or H and DSS, and Saline (n=4 per group)	Male PF-WT C57BL/6 and IL-10KO C57BL/6, 10-16 weeks old	Colitis and IBD	Hydromorphone (7.5 mg/kg, b.i.d., i.p.) for 7 days. DSS (to induce colitis) in water from day 3 for 5 days.	16S rDNA sequencing α: Chao1 β: Unweighted UniFrac Functional Potential: PiCRUST, KEGG
Simpson et al. (2020)	Oxycodone	Oxy, Sal, No ABX (n=9, 5 female) Oxy, Sal, ABX (n=10, 5 female) Oxy, Nal, No ABX (n=10, 6 female) Oxy, Nal, ABX (n=9, 5 female) Sal and Nal (n=9, 5 female)	Sprague-Dawley		Initial ABX (including Vancomycin, 2mg/mL; Bacitracin, 0.5mg/mL; Neomycin, 2mg/mL; Natamycin 1.2µg/mL) in water, or normal water for 2 weeks. Then, ABX or water with oxycodone (2mg/kg, s.c. injection, b.i.d., for 5 days) or saline. Final treatment with naloxone (1mg/kg, s.c. injection) 2h following final injection, or saline Faecal samples analysed prior to ABX or water, and at study conclusion.	16S rRNA sequencing α: Shannon, Chao1 β: Bray-Curtis
Sindberg et al. (2019)	Morphine	Morphine (n=4) SIV (n=4) SIV and Morphine (n=6)	Male Indian-Origin Rhesus Macaques, 3-4 weeks old	Simian Immunodeficiency Syndrome (SIV)	Morphine (50mg/mL, escalating dose, 2mg/kg to 3mg/kg first 2 weeks, 4mg/kg 2nd week onwards, i.m., t.i.d.) SIV infection at day 0 for SIV alone group, or day 70 into morphine treatment for morphine + SIV group Faecal samples collected pre-treatment and, day 21, 64 and 84 (from initial treatment in morphine alone group), 3, 8, 15 and 22 (post-SIV infection in SIV alone group). For combined SIV and morphine group, collected day 21 and 64 from initial morphine treatment, then day 3, 8, 14 and 22 post-SIV infection.	16S rDNA sequencing α: Shannon Index, Observed OTUs β: Weighted UniFrac

Study Reference	α and β Diversity	Dysbiotic Microbes	Functional Potential
Sharma et al. (2020b)	α: ↓ in H and DSS compared to H, DSS and Controls. β: Δ between DSS and H and DSS	↑ Proteobacteria, Verrucomicrobia, Bacteroidaceae, Enterobacteriaceae, Enterococcaceae, Peptostreptococcaceae, Porphyromonadaceae, Verrucomicrobiaceae, Akkermansia, Bacteroides, Bilophila, Enterococcus, Parabacteroides, Ruminococcus, Sutterella and Turicibacter, Bacteroides acidfaciens, Ruminococcus gnavus and Akkermansia municiphila ↓ Firmicutes, Lachnospiraceae, Lactobacillaceae, Odoribacteraceae, Rikenellaceae, Ruminococcaceae, S24-7, Adlercreutzia, Anaerostipes, Odoribacter, AF12, Lacobacillus, Lactobacillus reuteri and Mucispirillum schaedleri, in H and DSS vs controls	N/A
Simpson et al. (2020)	α: ~ between Oxy+/ABX- and saline post-treatment. ↓Oxy+/ABX+ vs OXY+/ABX-, and Saline β: ~ between Saline and Oxycodone groups, Δ between ABX and non-ABX groups	↓ Bacteroidetes in Oxy, and Oxy and ABX (non-sig) post-treatment vs vehicle ↓ Bacteroidetes in Oxy and ABX compared to Oxy ↓ Firmicutes in Oxy, and Oxy and ABX (non-sig) post-treatment vs vehicle ↓ Firmicutes in Oxy and ABX compared to Oxy ↑ Cyanobacteria, Proteobacteria, and Verrucomicrobia in Oxy and ABX post-treatment vs Oxy and vehicle	N/A
Sindberg et al. (2019)	α: ~ between groups. β: Δ between treatments. Δ between pre-treatment and morphine post-treatment samples. ~ between SIV and morphine, and morphine groups	↑ Methanobacteriaceae; ↓ Streptococcaceae and Ruminococcaceae post-morphine treatment ↑ Ruminococcaceae post-SIV infection. ↑ Methanobacteriaceae; ↓ Streptococcaceae in morphine and SIV vs pre-treatment samples. ↓ Leuconostocaceae family post-treatment for all groups ↑ Veillonellaaceae, Fibrobacteraceae fibrobacter, Veillonellaceae Megasphaera, Ersipelotrichaceae RFN20, and BS11; ↓ Order TM7-3, Paraprevotellaceae YRC22 in Terminal SIV samples (Day 22) ↓ Streptococcaceae streptococcus and Pasteurellaceae Aggregatibacter in Terminal Morphine samples (Day 84) ↓ Veillonellaceae dialister, Actinobacillus, Pasteurellacea haemophilus and Methanobacteriaceae Methanosphaera in Terminal SIV and Morphine samples (Day 92)	↓ Primary bile acids, such as cholate and glycocholate; ↑ ketolithocholate, dehydrocholate, taurocholenate sulfate and 3 b-hydroxy-5-cholenoic acid secondary bile acids in morphine post-treatment vs pre-treatment ↑ sphingolipid metabolites, such as sphinganine and sphingosine in morphine ↑ Serotonin and N-acetylserotonin in SIV group SIV and Morphine treatment altered serotonin, N-acetylserotonin, N-acetylkynurenine, tricarballylate, and secondary bile acid deoxycholate

Study Reference	Opioid Investigated	Treatment Groups	Species/Strain	Comorbidity and Diet	Experimental Paradigm	Method of Analysis
Touw et al. (2017)	Loperamide	Loperamide (n=24) or control (n=27) SPC FMT recipients Loperamide FMT recipients (n=17) or control FMT recipients (n=19)	Male and Female SPF C57Bl/6, 8-10 weeks old (donors), GF (recipients), 12-14 weeks old	Constipation	Loperamide (0.1%) in drinking water for 7 days. Mice were sacrificed and faecal samples analysed after 7 days. FMT (1.5mL, gavaged) caecal homogenate from donor to recipient mice for 3-4 weeks. Mice were sacrificed and faecal samples analysed after 7 days.	16S rRNA gene sequencing α: Shannon β: BrayWeighted and Unweighted UniFrac Functional Potential: Biolog Gen III
Wang et al. (2018a)	Morphine	Morphine, Naltrexone, Morphine and Naltrexone, and Placebo (n=4 per group)	Female C57BL/6J, 8-10 weeks old		Morphine (25mg pellet, s.c.), naltrexone (30mg pellet, s.c.), morphine and naltrexone, or placebo for 6 days	16S rRNA sequencing of faecal samples α: Chao1 β: Unweighted UniFrac
Wang et al. (2020a)	Morphine	Morphine, Placebo, Infection and Placebo, Infection and Morphine (n=4 per group)	Female Pathogen-Free C57BL/6J, 8-10 weeks old	Hospital Infections	Morphine (25mg pellet, s.c.) or placebo. C. rodentium infection (200µL oral gavage) 24h after pellet implantation. Faecal samples collected daily for 6 days.	16S rRNA sequencing of faecal samples α: Chao1 β: Unweighted UniFrac
Zhang et al. (2019a)	Morphine	TLR2KO morphine (n=23) TLR2KO saline (n=19) TLR4KO morphine (n=11) TLR4KO saline (n=8) WT morphine (n=7) WT saline (n=6)	TLR2KO, TLR4KO, and C57Bl/6 (WT) mice	Analgesic Tolerance	Initial pan-ABX for 7-10 days in drinking water. Morphine sulphate (b.i.d.) escalating (5, 10, 15, 20, 25, 30, 35, 40mg/kg) or constant dose (15mg/kg), or saline for 8 days with pan-ABX	16S rRNA sequencing of faecal samples β: Bray-Curtis

Study Reference	α and β Diversity	Dysbiotic Microbes	Functional Potential
Touw et al. (2017)	α: ~ between groups. ~ between GF recipient mice β: Δ between groups. Δ (weighted UniFrac) between GF recipient mice	↑ Bacteroidetes, Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, Bacteroidales S-24-7, Bacteroidales ovatus and Parabacteroidales distasonis; ↓ Firmicutes, Clostridiales, Lachnospirace and Ruminococcaceae in loperamide mice vs controls	↓ Butyrate, Acetate and Propionate in opioid animals compared to controls. Loperamide community showed ↑ potential for metabolizing amino acids, carboxylic acids, hexose acids, and various sugars.
Wang et al. (2018a)	α: ↓ in morphine-treated group compared to placebo at Day 3 β: ~ at Day 0. Δ between morphine and placebo at Day 3. Naltrexone reduced morphine-induced dysbiosis, but naltrexone treatment group Δ from placebo at Day 3	↑ Clostridium, Enterococcus, Flavobacterium, Fusobacterium, and Sutterella in morphine group vs other groups ↑ Enterococcus faecalis in morphine group at Day 3	↓ bile acids; ↑ phosphatidylethanolamines and saturated fatty acids in morphine vs placebo The morphine induced decrease of secondary bile acid, deoxycholic acid, and phosphatidylethanolamines were reversed by naltrexone Enterococcus and Erysipelotrichaceae were negatively associated with cholic and octadecanedioic acid, both of which were conversely positively correlated with Bacteroidales. Phosphatidylethanolamines and steric acid were conversely positively associated with Erysipelotrichaceae and Enterococcus, and negatively with the order Bacteroidales.
Wang et al. (2020a)	α: ↓ C. rodentium vs placebo, but not further affected by morphine β: Δ between groups	N/A	N/A
Zhang et al. (2019a)	β: Δ between WT morphine and saline. ~ between morphine and saline mice in TLR2KO and TLR4KO	↑Allobaculum, Peptostreptococcaceae and Prevotellaceae; ↓ Actinobacteria, Firmicutes, Bifidobacteriaceae, Lactobacillaceae, Bifidobacterium and Lactobacillus in WT Morphine vs WT saline	N/A

Study Reference	Opioid Investigated	Treatment Groups	Species/Strain	Comorbidity and Diet	Experimental Paradigm	Method of Analysis	α and β Diversity
Zhang et al. (2021b)	Morphine	Morphine (n=10) Vehicle (n=10)	Male C57BL/6J mice, 8 weeks old		3 stage (acquisition, extinction and reinstatement) CPP paradigm. Morphine (1mL of 10mg/kg, i.p.) or equal saline, placed inside A for 45 min, days 3, 5, 7, 9, 11, 13. Saline i.p., placed inside B, days 4, 6, 8, 10, 12 and 14 for both groups. Place preference days 15, 22, 29, 36, 43 until PP extinct. Reinstatement by final morphine challenge (same conc., i.p., and day 44) and final PP test.	16S rRNA gene sequencing of faecal samples α: ACE, Chao1, Observed OTUs, Shannon and Simpsons β: Weighted UniFrac Functional Outcome: PiCRUST	α: ↑ Observed OTUs, Chao1, ACE at morphine acquisition vs vehicle group; ↓ Shannon at extinction stage vs acquisition; ~ between reinstatement and extinction β: Δ between groups, Δ between CPP stages
Zhang et al. (2020a)	Morphine	Morphine (n=24) Vehicle (n=7)	Male Sprague-Dawley rats		Morphine (1mL of 10mg/mL, i.p.) on days 6, 8 10 and 12, or saline on days 7, 9, 11, and 13 CPP paradigm. Morphine or saline i.p. then placing into non-preferred side (pairing side to morphine). Saline i.p. for both groups then placing into preferred side on alternate days. Free access to both compartments on day 14 to test side preference.	16S rRNA sequencing of faecal samples α: ACE, Chao1, Shannon and Simpsons	α: ~ between groups

Study Reference	Dysbiotic Microbes	Functional Potential
Zhang et al. (2021b)	↑ Verrucomicrobia; ↓ Bacteroides in morphine acquisition compared to vehicle group	↑ signal transduction mechanisms and replication; recombination and repair proteins; ↓ nicotinate and micotinamide metabolism; nitrogen metabolism and cyanoamino acid metabolism pathways at morphine acquisition vs controls
	↑ Bacteroidetes, Bacteroides and Coprobacter; ↓ Verrucomicrobia, Candidatus Saccharibacteria, Akkermansia, Saccharibacteria genera incertae sedis, Eisenbergiella and Ruminococcus at extinction compared to acquisition	
	↑ Bacteroides and Coprobacter; ↓ Candidatus Saccharibacteria, Eisenbergiella, Saccharibacteria genera incertae sedis and Clostridium XlVa at reinstatement compared to acquisition	↑ Alanine, Aspartate, Glutamate and Histidine Metabolism; Amino Acid related enzymes; Protein Export; and Ribosome Biogenesis; ↓Two Component System at morphine extinction vs acquisition
	Acquisition was characterised by abundance of Aestuariispira, Alistipes, Akkermansia, Anaerovorax, Clostridium IV and Ruminococcus	↑ Other ion-coupled transporters; glycolysis/gluconeogenesis; ↓ Phenylalanine, Tryptophan and Tyrosine Biosynthesis; Arginine and Proline Metabolism; Bacterial Secretion System; and Oxidative Phorphorylation at morphine reinstatement vs extinction
	The control group was characterised by abundance of the genera Anaerotruncus, Bacteroides, Bilophila, Clostridium_XIVb, Eisenbergiella, Parabacteroides and Rhizobium	
	Extinction was characterised by abundance of Anaerovorax, Corpobacter, Escherichia_Shigella, Lactobacillus and Parvibacter.	↑ Ribosome Biogenesis; Purine Metabolism; Cysteine and Methionine Metabolism; DNA Repair and Recombination Proteins; Amino Acid Related Enzymes; and, Alanine, Aspartate and Glutamate Metabolism Pathways; ↓ Two Component Systemat reinstatement vs acquisition
	Reinstatement was characterised by abundance of Anaerovorax, Escherichia_Shigella and Lactobacillus	
Zhang et al. (2020a)	↑ Coriobacteriaceae, Peptococcaceae_1, Allobaculum and Parasutterella; ↓ Alloprevotella, Desulfovibrio and Rikenella in Morphine post-treatment compared to morphine pre-treatment	N/A
	↑Clostridium XIVa, Coriobacteriaceae, Corynebacterium and Parasutterella, Peptococcaceae_1 and Streptococcaceae; ↓ Desulfovibrio in vehicle post-treatment compared to vehicle pre-treatment	
	↓ Corynebacterium, Clostridium_XlVa, Enterococcaceae, Staphylococcaceae and Streptococcaceae in morphine post-treatment compared to vehicle post-treatment	
	↑ Alloprevotella, Peptostreptococcaceae and Romboutsia; ↓Anaerofilum, Catabacter, Catabacteriaceae, Christensenella, Christensenellaceae, Clostridium_IV, Dorea, Elusimicrobium, Elusimicrobiaceae, Roseburia, Schwartzia, Spirochaetaceae and Veillonellaceae in Hi-CPP compared to lo-CPP	
	Alloprevotella and Romboutsia postively; Elusimicrobiaceae, Elusimicrobium, Lachnospiraceae, Roseburia and Ruminococcaceae negatively, correlated with CPP score	
	↑ Helicobacteraceae, Helicobacter; ↓ Olsenella, Puniceicoccaceae and Rothia	
	Rothia abundance negatively correlated to CPP score	

Abbreviations: α=Alpha, β=Beta, Δ =Different, ~=Not Different, N/A=Not Available; Spp.=several species; FMT=Faecal Matter Transplant; CPP=Conditioned Place Preference; GF=Germ-Free; PD=Phylogenetic Diversity; SPF=Specific Pathogen free; n3P=n-3 PUFA=n-3 Polyunsaturated Fatty Acids; ABX=antibiotics; OXY=Oxycodone; WT= Wild Type, TLRKO=Toll-Like Receptor Knockout; BLT=Bone-Marrow, Liver Thymus; i.p.=intraperitoneal, i.m.=intramuscular, s.c.=subcutaneous; b.i.d.=twice daily, t.i.d.=thrice daily

3.4.2.2 Alterations of the Microbiota by Opioid/Opiate Administration in Preclinical Studies

A) Effect of opioids on gut microbiota

- *Morphine*

Dysbiosis of several strains were reported as a result of morphine administration in a study by Zhang et al. (2020a). Male Sprague-Dawley rats were grouped into either morphine (n=24) or vehicle (n=7) treatments. Rats were administered morphine (1mL/kg of a 10mg/mL solution) on days 6, 8, 10, and 12, or saline on days 7, 9, 11 and 13 via intraperitoneal injection. Faecal samples were collected and analysed via 16S rRNA sequencing. Alpha diversity, reported as abundance-based coverage estimator (ACE), Chao1, Shannon and Simpson's Index, was not significantly different between morphine and saline-treated rats (Table 3.2). Morphine treatment resulted in dysbiosis of *Allobaculum* and *Parasutterella*, which were increased in abundance, and *Alloprevotella, Desulfovibrio* and *Rikenella*, which were depleted post-treatment. At the family level, *Coriobacteriaceae* and *Peptococcaceae*_1 increased in abundance in post-treatment samples compared to pre-treatment samples. *Clostridium*_XIVa, *Corynebacterium* and *Parasutterella* increased in post-treatment samples in saline-treated rats, whereas *Desulfovibrio* decreased post-treatment. Additionally, at the family level *Coriobacteriaceae, Peptococcaceae*_1 and *Streptococcaceae* increased in abundance post-treatment in the saline group. The study considered the relationship between gut microbiota and drug associated learning through a model of condition place preference (CPP). In this paradigm animals are placed in chambers paired or not paired a drug for several sessions, during a conditioning period. Following this, animals were given free access to either chamber. Animals that preferred drug paired chambers at this stage were considered to have associated the chamber with the rewarding effects of the drug. Rats with a high sensitivity to CPP had increased levels *of Alloprevotella, Peptostreptococcaceae and Romboutsia* and decreased levels of *Anaerofilum, Catabacter, Catabacteriaceae, Christensenella, Christensenellaceae,*

Clostridium_IV, Dorea, Elusimicrobium, Elusimicrobiaceae, Roseburia, Schwartzia, Spirochaetaceae and *Veillonellaceae* compared rats with low sensitivity to CPP. *Alloprevotella* and *Romboutsia* positively correlated with CPP scores, whereas *Elusimicrobiaceae*, *Elusimicrobium*, *Lachnospiraceae*, *Roseburia* and *Ruminococcaceae* correlated negatively with CPP score, suggesting a role of these microbes in facilitating reward learning (Table 3.2). Finally, the authors investigated which bacteria may contribute to morphine sensitivity by investigating baseline composition of high and low CPP score rats (i.e., pre-treatment samples) and correlating strains to CPP score. At baseline, high CPP rats had increased abundance of *Helicobacteraceae* and *Helicobacter* but decreased *Olsenella, Puniceicoccaceae and Rothia*. The abundance of *Rothia* at baseline was negatively correlated to CPP score. These results suggest a potential protective effect of *Rothia* as CPP sensitivity decreased with as the abundance of this genera increased (Table 3.2). Overall, Zhang et al. (2020a) do not report changes in alpha diversity, but do report dysbiosis as a result of morphine administration, and also outline unique patterns of dysbiosis based on sensitivity to CPP, associated with learning i.e. cognitive function. Finally, sensitivity to CPP was associated with specific strains.

Dysbiosis of the microbiota by morphine hydrochloride administration was reported by Zhang et al. (2021b). Male C57BL/6 mice were grouped into either morphine hydrochloride (n=10) or saline (n=10) treatment groups. Morphine hydrochloride (1mL of a 10mg/kg solution) was administered via i.p. injection. During the acquisition/conditioning phase mice were administered morphine or saline (controls) on days 3, 5, 7, 9, 11 and 13 and placed into side A of the apparatus. Both groups received saline on days 4, 6, 8, 10, 12 and 14 and were placed into side B of the apparatus. Following the final injection, a place preference test was conducted weekly, until CPP extinction. After achieving extinction, mice received a final morphine injection and a final place preference test (the reinstatement test). Faecal samples were collected and analysed by 16S rRNA sequencing. Alpha diversity, reported as ACE,

Chao1, Observed Species, Coverage Indices, Shannon and Simpsons Indices, was different between study stages (Table 3.2). During the acquisition phase, morphine treatment increased the richness, but not diversity, of the microbiota compared to the controls. The richness and diversity of the microbiota was decreased during the extinction phase compared to the acquisition phase, in morphine treated mice. Beta diversity was different in morphine-treated mice during the acquisition stage compared to morphine-treated mice during the extinction stage and control group mice. Conversely, beta diversity was similar between morphine-treated mice during extinction compared to morphine-treated mice during reinstatement and controls. These results suggests that subsequent morphine challenges following a period of abstinence do not produce the same effects on the gut microbiota as initial morphine use. *Bacteroides* was decreased in the morphine group at acquisition compared to the control group, whereas *Verrucomicrobia* was increased (Table 3.2). During the extinction phase, *Bacteroides, Bacteroidetes* and *Coprobacter* were more abundant, whereas *Akkermansia*, *Eisenbergiella, Ruminococcus, Candidatus Saccharibacteria, Saccharibacteria_incertae_sedis* and *Verrucomicrobia* were depleted, compared to the acquisition phase. During the reinstatement phase, *Bacteroides* and *Coprobacter* were more abundant, whereas *Clostridium*_XIVa, *Eisenbergiella, Candidatus Saccharibacteria* and *Saccharibacteria_incertae_sedis* were decreased in prevalence, compared to the acquisition phase. The control group was characterised by abundance of the genera *Anaerotruncus, Bacteroides, Bilophila, Clostridium*_XIVb, *Eisenbergiella, Parabacteroides* and *Rhizobium*. The acquisition phase of the morphine group was characterised by *Aestuariispira, Akkermansia, Alistipes, Anaerovorax, Clostridium*_IV and *Ruminococcus*. The extinction phase of the morphine group was characterised by *Anaerovorax, Corpobacter, Escherichia_Shigella, Lactobacillus* and *Parvibacter*. Finally, the instatement phase was characterised by an abundance of *Anaerovorax*, *Escherichia*_Shigella and *Lactobacillus*. Next, the authors investigated the

functional potential of the gut microbiota. During the acquisition phase, the morphine treatment group had enrichment of pathways involving recombination and repair proteins, signal transduction mechanisms and replication with a downregulation of pathways involving metabolism of cyanoamino acid, nitrogen, nicotinate and nicotiamide compared to the control group. During the extinction phase, pathways involved in metabolism of alanine, aspartate, glutamate and histidine, protein export, enzymes related to amino acids, and ribosome production were upregulated, whereas the two-component signal transduction system (a system bacteria use to respond to external stimuli) was downregulated, compared to the acquisition phase. During the reinstatement phase pathways involved in glycolysis/gluconeogenesis and ion-couple transporters were increased, whereas pathways involved in bacterial secretion, arginine and proline metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, and oxidative phosphorylation were downregulated, compared to the extinction phase. Finally, during reinstatement, pathways related to ribosome biogenesis, DNA repair and recombination proteins, alanine, aspartate and glutamate metabolism, purine metabolism, cysteine and methionine metabolism and amino acid related enzymes were upregulated, whereas pathways related to the two-component system was downregulated, compared to the acquisition phase (Table 3.2). Overall, these results reflect a dysbiotic effect of morphine hydrochloride treatment. However, cessation from drug administration allowed a shift in community structure back towards control levels. Finally, a unique community structure arose with each study phase in addition to unique functional outcomes.

In another study, morphine treatment resulted in dysbiosis of the microbiota when administered via intraperitoneal injection and pellet implantation (Lee et al., 2018). A group of male C57BL/6J mice were administered morphine (n=4) or saline (n=8), twice daily, via intraperitoneal injection at escalating doses (10, 20, 30 and 40mg/kg) with intermittent withdrawal periods for four days. Another group of mice underwent continuous morphine

administration (25mg/kg pellet implant, n=4) or saline implant (n=4). Faecal samples were analysed by 16S rRNA sequencing to determine microbiota composition. Alpha diversity did not change as a result of drug administration regardless of route of administration (Table 3.2). Beta diversity was different between morphine-treated mice and controls. Interestingly, the authors noted different patterns of dysbiosis in the microbiota based on route of administration. While both saline groups showed overlap in composition, microbiota composition between the morphine-treated groups were distinct, and did not overlap with each other or the saline groups. Furthermore, abundance of *Lactobacillus* was decreased in intermittent morphine-treated mice, whereas *Ruminococcus* was increased compared to saline i.p. injected controls. Abundance of *Clostridium* and *Rikenellaceae* was increased in morphine-pelleted mice compared to saline implanted controls (Table 3.2). Next, the authors investigated the effect of FMT from saline and morphine-treated mice to drug-naïve recipient animals, reporting no difference in alpha diversity between saline and morphine recipient groups. In addition to this, intermittent treatment by injection resulted in increased permeability of the intestinal tract. Compared to morphine-treated mice, opioid naïve mice displayed preference for cocaine-paired chambers, a finding replicated in mice receiving FMT from saline-treated mice but not morphine-treated mice. Furthermore, intermittent, but not sustained, morphine-treatment impaired cocaine-induced CPP. In short, this study identified unique effects of different routes of morphine administration on the composition of the gut microbiota, and drug-related reward learning.

Treatment with morphine disturbed the commensal gut microbiota in a primate model with comorbid simian immunodeficiency virus (modelling HIV; Sindberg et al. 2019). Male Indian-Origin Rhesus Macaques were administered morphine (n=4), SIV (n= 4) or comorbid morphine and SIV infection (n=6), and faecal samples collected were analysed by mass spectrophotometry of 16S rDNA. Administration of morphine (50mg/mL) occurred via intramuscular injection three times daily, at escalating doses; from 2mg/kg to 3mg/kg over first

two weeks, then 4mg/kg until study endpoint at 12 weeks. Alpha diversity, as determined by Observed Operational Taxonomic Units (OTUs) and Shannon Index, was not significantly different between groups (Table 3.2) and no individual treatment group showed alterations in alpha diversity when comparing post-treatment samples to pre-treatment samples. However, beta diversity was significantly different between treatments. *Ruminococcaceae* and *Streptococcaceae* were increased in pre-morphine treated samples, whereas *Methanobacteriaceae* was increased in samples from post-morphine treated animals. While SIV treatment alone did not significantly alter microbiota composition, combined SIV and morphine treatment did alter composition. Furthermore, combined treatment post-treatment microbiota structure was similar to structure of primates treated with morphine alone. Combined treatment also resulted in increased *Methanobacteriaceae* but decreased *Streptococcaceae* in post-treatment samples compared to pre-treatment samples. Next, the authors compared final samples to initial samples from each treatment (Day 64 for morphine treatment alone, Day 22 for SIV treatment alone, and Day 92 for combined treatment). *Leuconostocaceae* was depleted in all post-treatment samples compared to pre-treatment samples. Morphine alone resulted in decreased *Aggregatibacter* and *Streptococcus*. SIV alone resulted in increased *BS11, Ersipelotrichaceae RFN20, Fibrobacteraceae fibrobacter, Veillonellaceae, Veillonellaceae Megasphaera,* and decreased *Paraprevotellaceae YRC22* and *TM7-3* abundance. Combined treatment resulted in decreased *Actinobacillus, Dialister, Haemophilus* and *Methanosphaera* (Sindberg et al., 2019). The authors also report altered levels of several metabolites in faeces (Table 3.2). For example, morphine treatment resulted in increased levels of several secondary bile acids including 3 b-hydroxy-5-cholenoic acid, 7-ketolithocholate, 12-dehydrocholate, taurocholate sulphate and sphingolipid metabolites sphinganine and sphingosine; but decreased levels of primary bile acids cholate and glycocholate compared to pre-treated samples. SIV treatment resulted in increased levels of N-

acetyl serotonin and serotonin, compared to pre-treatment samples. Compared to samples from pre-treatment, combined treatment resulted in even greater increases in N-acetyl serotonin and serotonin than in SIV alone in addition to N-acetyl kynurenine, tricarballylate, and secondary bile acids such as deoxycholate. In short, unique patterns of dysbiosis on microbial strains are reported here as a result of morphine administration, SIV infection, or both, though no effect on alpha diversity is apparent.

Similarly, Meng et al. (2020) reported dysbiosis following morphine pellet implantation in humanised bone marrow-liver-thymus (BLT) mice with HIV. Treatments included HIV infection, HIV and morphine (75mg pellet), morphine alone, or controls (n=6). Animals were sacrificed 7 days after implantation and faecal samples were analysed by 16S rRNA sequencing. Alpha diversity, analysed as Observed OTUs and the Shannon Index, was decreased in the HIV and morphine (combined) treatment group compared to controls (Table 3.2). Further, beta diversity was different between morphine and combined treatment mice compared to placebo-treated mice. The combined treatment group had a significantly greater relative abundance of *Firmicutes* and *Proteobacteria* compared to placebo-treated mice. Conversely, abundance of *Bacteroidetes*, *Actinobacteria, Tenericutes, Lachnospiraceae, Muribaculaceae* and *Ruminococcaceae* was depleted in combined treatment mice; the latter three of which are associated with SCFA production (Meng et al., 2020). *Enterobacteriaceae* was enriched in both groups of morphine-treated mice, compared to non-morphine treated mice. At the genus level, *Enterococcus* and *Staphylococcus* were increased in abundance in HIV-treated mice, whereas relative abundance of *Enterococcus* was increased and *Lactobacillus* abundance was decreased in HIV and morphine-treated mice (Table 3.2).

Dysbiosis of specific bacterial strains due to morphine administration were reported by Kang et al. (2017) in their study investigating the relationship between chronic morphine administration, the microbiota and pain tolerance (antinociception). Male Swiss Webster mice

were assigned to either morphine (75mg pellet), morphine pellet with antibiotics (ABX, an antibiotic cocktail), placebo (pellet without drug), or a placebo pellet and ABX groups (n=7 per group). Faecal samples were analysed by 16S rRNA qRT-PCR to determine bacterial abundance. Overall bacterial abundance was depleted in morphine-treated mice (Table 3.2). *Enterobacteriales* increased in relative abundance in non-ABX morphine treated-mice compared to non-ABX controls, whereas *Bacteroidales*, *Clostridiales* and *Lactobacillales* were depleted. In short, this study reports an overall deleterious effect of morphine administration on the abundance of the commensal gut microbiota.

In another study by Wang et al. (2020), female C57BL/6J mice were administered morphine (25mg pellet implant), placebo, morphine with infection (by *C. rodentium*, modelling hospital infections), or placebo with infection (n=4 per group) over 6 days. Alpha diversity, reported as Chao1, was decreased with infection, but further disruption by morphine treatment was not found. Beta diversity was different between all groups (Table 3.2). Overall, this study outlines a dysbiotic effect of morphine treatment on the community structure of the microbiota.

Morphine disrupted the commensal microbial community according to a study conducted by Banerjee et al. (2016). C57B16/J mice were treated with either 25mg morphine, morphine with naltrexone, or placebo pellet (n=6 per group). Faecal samples were collected 72 hours post-treatment and analysed through 16S rRNA sequencing. There was a non-significant depletion in alpha diversity, analysed as Phylogenetic Diversity index, in morphine-treated mice compared to both other groups (Table 3.2). Beta diversity, was significantly different between morphine-treated mice and both other groups, but not between naltrexone-treated mice and controls, suggesting a potential remediating effect of naltrexone on the commensal microbial community structure. Morphine treatment resulted in a significant increase in phyla *Firmicutes*, with a concomitant reduction in *Bacteroidetes*, thus demonstrating a decreased *Firmicutes*/*Bacteroidetes* ratio. Several families belonging to *Firmicutes* increased in

abundance after morphine treatment compared to all other groups, including: *Bacillaceae*, *Enterococcaceae*, *Erysipelotrichaceae*, *Staphylococcaceae* and *Streptococcaceae*.

In their study, Meng et al. (2015) investigated the effect of morphine (25mg slow-release pellet), morphine and naltrexone (30mg pellet), or placebo (n=6 per group) on gut microbiota in WT or TLR2KO mice modelling sepsis (caecal ligation and puncture). Faecal samples were analysed by 16S rRNA gene sequencing. Morphine treatment resulted in increases in several gram-positive species belonging to *Firmicutes*, including: *Enterococcus casseliflavus, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Staphylococcus cohnii* and *Staphylococcus sciuri* (Meng et al., 2015; Table 3.2), which was reversed by naltrexone treatment.

Several changes in bacterial abundance at the species level as a result of morphine administration were also reported in rats by O'Sullivan et al. (2019). Sprague-Dawley rats were divided into four treatment groups (n=4 per group), including morphine pellet (75mg slow-release implant), placebo (75mg drug free implant), morphine and naltrexone (modelling withdrawal; morphine pellet and 100mg/kg naltrexone via i.p. injection), or naltrexone (injection only). Rats implanted with a pellet were sacrificed after 6 days, while the naltrexone and morphine and naltrexone (withdrawal) groups received injections at day 6 and were sacrificed 24 hours later. Microbiota was analysed by qPCR of caecal contents. *Anaerotruncus colihominis* abundance significantly increased in morphine-treated mice compared to withdrawal mice, but morphine alone resulted in depletion of *Prevotella* compared to placebo. Finally, the authors noted that the abundance of genus *Bifidobacterium*, and species *Faecalibacterium prausnitzii*, both of which possess anti-inflammatory properties (Riedel et al., 2006, Khokhlova et al., 2012, Quevrain et al., 2016), were decreased in the withdrawal treatment (Table 3.2). Therefore, in addition to reporting dysbiosis as a result of morphine

administration, this study also reported dysbiosis of various beneficial bacteria as a result of naltrexone, after initiation of addiction.

Morphine administration resulted in dysbiosis of the commensal microbiota in a study by Wang et al. (2018). Female C57Bl/6J mice were randomised into either a morphine (25mg pellet implant), placebo pellet (25mg implant), naltrexone (30mg pellet implant), or a morphine and naltrexone group (n=4 per group), for 6 days. Faecal matter was analysed through 16S rRNA sequencing. Alpha diversity, reported as Chao1, was decreased in morphine-treated animals compared placebo at Day 3 (Table 3.2). Beta diversity was also different between morphine-treated and placebo mice at Day 3. Morphine-treated mice had enrichment of potentially pathogenic bacteria compared to controls from Day 3, including *Clostridium, Enterococcus, Flavobacterium, Fusobacterium* and *Sutterella*. Compared to controls, there was a significant increase in the species *Enterococcus faecalis* in morphine-treated mice, but not naltrexone-treated mice. Metabolites were also disrupted depending on treatment (Table 3.2). Phosphatidylethanolamines and saturated fatty acids were upregulated in the faecal samples of morphine-treated mice, whereas bile acids were decreased. The secondary bile acid deoxycholic acid, was decreased with morphine treatment. The authors outlined correlations between certain microbes and metabolites. The genus *Enterococcus* and family *Erysipelotrichaceae* were negatively associated with cholic and octadecanedioic acid, which was conversely positively correlated with *Bacteroidales*. Phosphatidylethanolamines and steric acid were positively associated with *Erysipelotrichaceae* and *Enterococcus*, but negatively associated with the order *Bacteroidales* (Table 3.2). In short, this study reports a dysbiotic effect of morphine treatment, which may include enrichment of harmful bacteria, which was not observed in naltrexone-treated mice.

- *Other Opioids*

Dysbiosis of the commensal microbiota as a result of oxycodone administration was reported by Simpsons et al. (2020), but a protective effect of the receptor antagonist naloxone was not found. Adult Sprague-Dawley rats were randomly assigned to either; an oxycodone and saline (n=9, 5 female), oxycodone and saline with antibiotics to deplete the microbiota (n=10, 5 female), oxycodone and naloxone without antibiotics (modelling withdrawal; n=10, 6 female), oxycodone and naloxone with antibiotics (modelling withdrawal with antibiotics; n=9, 5 female), or a saline and naloxone (control) group (n=9, 5 female) treatment. The microbiota was either depleted by antibiotics in drinking water, or kept intact by supplying normal drinking water (placebo) for two weeks, after which oxycodone (2mg/kg) was administered every 12 hours, for five days by subcutaneous injection. Depending on treatment group, withdrawal or intoxication was induced by administration of naloxone (1mg/kg) or saline, respectively. Faecal samples were obtained and analysed by 16S rRNA sequencing following each injection. There was no difference in alpha or beta diversity between saline and naloxone, and oxycodone-treated groups not administered antibiotics suggesting no significant dysbiotic effect of the opioid administration. Alpha diversity was decreased in the oxycodone and antibiotic treatment group compared to both the oxycodone without antibiotics and saline and naloxone treatment groups. Beta diversity was different between antibiotic-treated rats and rats not treated with antibiotics. *Bacteroidetes* and *Firmicutes* were depleted in the oxycodone compared to the oxycodone plus antibiotics groups and (control) group, but were not significantly different between the oxycodone without antibiotics group and the saline group. *Cyanobacteria*, *Proteobacteria*, and *Verrucomicrobia* were increased in oxycodone and antibiotic-treated rats post-treatment compared to oxycodone and saline and naloxone-treated rats (Table 3.2).

In their rodent study, Hakimian et al. (2019) report shifts in the commensal microbiota

as a result remifentanil and oxycodone administration. The authors investigated the effect of opioid administration on microbiota and behaviour, and the potential remediating effects of n-3 polyunsaturated fatty acids (PUFA). Male C57B16/J mice were grouped into four treatments, including: control diet with saline (n=14), control diet and opioids (n= 14), n-3 PUFA with saline (n=5), and n-3 PUFA with opioids (n=10). Mice self-administered remifentanil (0.05mg/kg/infusion) for 3 days during the acquisition phase, then oxycodone (0.25mg/kg/infusion) for 10 days during the maintenance phase through intravenous catheters via a lever press. Following this, mice underwent an extinction period of 5 days, then reinstatement for 2 days with oxycodone, and finally an additional 5 days of extinction. Faecal samples were analysed by 16S rRNA sequencing at several intervals (baseline, days one and ten of oxycodone treatment, day five of first extinction period, day one of second extinction period). Alpha diversity, analysed as Chao1, Faith's Phylogenetic Diversity, and the Shannon Index, was enriched as a result of n-3 PUFA supplementation, regardless of opioid status but was not different between study periods (Table 3.2). The composition of the microbiota was significantly different between the n-3 PUFA and control diet mice overall when controlling for drug intervention period, suggesting a significant effect of diet on microbial composition independent of the influence of opioids. When controlling for diet, microbiota composition was significantly different between oxycodone maintenance and both extinction periods. The microbiota composition was not significantly different between days 1 and 10 of the maintenance phase nor was it significantly different between the two extinction periods. During oxycodone maintenance, n-3 PUFA supplementation resulted in increases in *Allobaculum, Alistipes, Bifidobacterium, Coprobacillus, Coriobacteriaceae* (family)*, Dorea, Erysipelotrichaceae* (family)*, Lactobacillus, Oscillospira,* and *Streptococcus*, and decreases in *Akkermansia, Clostridium, Coprococcus, Enterobacteriaceae* (family) and *Parabacteroides* compared to control diet mice (Hakimian et al., 2019). During the extinction phase, n-3 PUFA

diet resulted in increases in *Bifidobacterium* and *Desulfovibrio*, and depletion of *Parabacteroides*, and *Clostridiaceae* (family) compared to control diet animals. Within the n-3 PUFA diet subgroup depletion of *Akkermansia, Allobaculum, Bifidobacterium* and *Coriobacteriaceae*, and enrichment of *Enterococcus* was reported during the extinction phase compared to the maintenance phase. Similarly, in the opioid-treated control diet group, depletion of *Akkermansia, Coprococcus,* family *Enterobacteriaceae, Parabacteroides* and *Bifidobacterium* was reported during the extinction phase compared to the maintenance phase (Table 3.2). Finally, diet supplementation reduced oxycodone seeking (as determined by reduced number of lever presses) and anxiety compared to control diet mice. According to these results, diet and opioids influence the shape of the microbiota community, but diet may also exert a protective effect on opioid seeking. Structure of the microbiota appears to remain consistent over the course of addiction, as it did not differ significantly from day 1 and 10. As abstinence (extinction phase) resulted in a shift in community structure compared to the maintenance phase, the results suggest that abstinence alone may also have a remediating effect. Taken together, a targeted diet administered during abstinence may improve the behavioural symptoms of addiction. Further research should elucidate the biological changes that underlie these improvements to enhance these targeted treatment options.

The commensal microbiota was disrupted by hydromorphone in a study investigating opioid use in the context of colitis and inflammatory bowel disease (Sharma et al., 2019). Male pathogen-free, or interleukin-10 knockout, C57BL/6 mice were assigned to either a hydromorphone, hydromorphone and dextran sodium sulphate (DSS; which induces colitis), DSS alone, or placebo group (n=4 per group). Hydromorphone was administered twice daily at 7.5mg/kg, via i.p. injection for 7 days. Alpha diversity, analysed as Chao1, was significantly reduced in hydromorphone and DSS treated mice compared to the three other groups (Table 3.2). Beta diversity was different between the four groups. In the combined hydromorphone

and DSS treatment group there was a decreased abundance of *Firmicutes*, and an increased abundance of *Proteobacteria* and *Verrucomicrobia* compared to control mice. At the family level, these included increases in *Bacteroidaceae, Enterobacteriaceae, Enterococcaceae, Peptostreptococcaceae, Porphyromonadaceae* and *Verrucomicrobiaceae*, and decreases in *Lachnospiraceae, Lactobacillaceae, Odoribacteraceae, Rikenellaceae, Ruminococcaceae* and *S24-7* (Table 3.2). At the genus level *Akkermansia, Bacteroides, Bilophila, Enterococcus, Parabacteroides, Ruminococcus, Sutterella* and *Turicibacter* were increased in abundance, whilst *Adlercreutzia, AF12, Anaerostipes, Lactobacillus and Odoribacter* were depleted in combined treatment compared to controls. The genus *Akkermansia* accounted for the family level increase of *Verrucomicrobiaceae*, *Bacteroides*, at the genus level, accounted for the increase of *Bacteroidaceae* at the family level; and the genus *Sutterella* for accounted for increased abundance of the family *Enterobacteriaceae*. Finally, compared to controls, the species *Akkermansia muciniphila, Bacteroides acidfaciens* and *Ruminococcus gnavus* were increased in hydromorphone and DSS mice compared to controls, whereas *Lactobacillus reuteri* and *Mucispirillum schaedleri* were decreased (Table 3.2). Prevalence of opioid use is high among individuals with colitis (Niccum et al., 2021) and is a risk factor for developing OUD (Cohen-Mekelburg et al., 2018). As evidenced here, colitis and comorbid opioid use had a confounding effect on dysbiosis. Controlling the gut microbiota in colitis through FMT shows promise according to the literature (Mankowska-Wierzbicka et al., 2020, Narula et al., 2017) and utilising a similar approach may control the additional dysbiosis induced by opioids reported here. The effect of opioids and colitis on cognition and the course of addiction has yet to be investigated, however. Other research in similar models of colitis report alterations in hippocampal microglia (Gampierakis et al., 2021), the morphology of which are influenced by the gut microbiota. Hence, severe dysbiosis of the gut microbiota by opioids and colitis may influence cognition through these mechanisms and contribute to the cycle of addiction.

Administration of the opioid loperamide (used here to induce constipation) resulted in dysbiosis of the microbiota in a study by Touw et al. (2017). C57BL/6 mice were administered loperamide (0.1%) in water (n=24), or normal drinking water (controls; n=27) for 7 days. Faecal samples were analysed by 16S rRNA sequencing. Alpha diversity, reported as Shannon's Index, was not different between groups. Beta diversity, was significantly different between loperamide-treated mice and controls (Table 3.2). In loperamide-treated animals, *Bacteroidetes, Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, Bacteroidales S-24-7, Bacteroidales ovatus and Parabacteroidales distasonis* were increased post-treatment, whereas *Firmicutes, Clostridiales, Lachnospiraceae,* and *Ruminococcaceae* were depleted compared to controls. Further, a depletion of the SCFAs acetate, butyrate and propionate, was observed in opioid-treated mice. Analysis of bacterial function showed upregulation of pathways involved in metabolism of amino acids, carboxylic acids, hexose acids, and various sugars in loperamide treated mice (Table 3.2). A FMT was conducted next where recipient GF mice received loperamide (n=17) or control (n=19) faecal samples from donor mice. Beta diversity was different between GF-loperamide and GF-control recipient mice as determined by weighted, but not unweighted, UniFrac. In short, loperamide has a dysbiotic effect on microbiota structure, and influences availability of microbe-produced metabolites and several key metabolic pathways.

B) Effect of Medications for Opioid Addiction

The literature suggests that medications for opioid addiction may confer some beneficial effects to the gut microbiota. According to Banerjee et al. (2016), beta diversity was not significantly different between naltrexone-treated mice and controls, suggesting a protective effect against dysbiosis. Further, Meng et al. (2015) report the dysbiosis caused by morphine was reversed by naltrexone administration. In their study, Wang et al. (2018) report

that beta diversity in both naltrexone-treated groups overlapped but did not overlap with morphine and placebo-treated mice by Day 3. Further, the enrichment of pathogenic bacteria observed in morphine-treated mice was not observed in naltrexone-pelleted mice (Table 3.2). The depletion of deoxycholic acid resulting from morphine treatment was attenuated by naltrexone, as was the increase in phosphatidylethanolamine by morphine. O'Sullivan et al. (2019) reported dysbiosis in mice implanted with morphine and naltrexone pellets. The authors report enrichment in *Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides vulgatus, Enterococcus faecalis* and *Enterococcus gallinarum* and depletion of *Butyricicoccus pullicaecorum, Clostridium leptum* and *Clostridium coccoides* in mice treated with morphine and naltrexone compared to mice treated with morphine alone, naltrexone alone, or placebo. Finally, in their study, Simpsons et al. (2020) do not report any beneficial effect of naloxone as there was no difference in alpha or beta diversity in mice treated with naloxone and saline compared to those treated with oxycodone.

C) Effect of Receptor Knockout

Banerjee et al. (2016) also investigated the role of opioid receptors in morphine-induced microbial dysbiosis using toll-like receptor 2 knockout (TLR2KO) mice and μ-opioid receptor knockout (MORKO) mice treated with morphine or placebo. Microbiota composition of TLR2KO mice treated with either placebo or morphine overlapped with wild-type placebo-treated mice, and morphine and naltrexone-treated mice, suggesting TL2R knockout prevented dysbiosis. Wild-type morphine-treated mice showed distinct clustering compared to placebo and both TLR2KO treatment groups. MORKO mice administered morphine or placebo had a distinct community structure compared to wild-type mice administered morphine or placebo (controls), indicating unique influence of MORKO on the commensal microbiota (Table 3.2). In the same study, the potential influence of peripheral immune cells was examined, using

immunocompromised mice (non-obese, severe combined immune-deficient (NOD-SCID) with interleukin-2 (IL-2) receptor gamma knockout (NOD SCID Gamma [NSG]) mice). NSG mice community structure overlapped with MORKO. Furthermore, NSG mice clustered distinctly from morphine and placebo-treated wild-type mice; however, placebo and morphine-treated NSG groups overlapped in composition, indicating a unique effect of the mouse model in community structure, but no additional dysbiotic effect of morphine administration. Following this, FMT was conducted three times from morphine or placebo pelleted wild-type donor mice to morphine or placebo pelleted wild-type recipient mice. Transplantation occurred from placebo-to-placebo, placebo-to-morphine, morphine-to-placebo, and morphine-to-morphine mice (n=8 per recipient group). Following transplantation, the community structure of recipient animals shifted towards that observed in donor animals (Table 3.2). Finally, the authors investigated the faecal levels of metabolites post-morphine treatment in wild-type and TLR2KO mice implanted with morphine or placebo pellets (Table 3.2). Coprostanol, derived from cholesterol, was significantly increased in morphine pelleted mice compared to placebo, and morphine and naltrexone-pelleted mice (Banerjee et al., 2016). Conversely, primary and secondary bile acids were depleted in the faeces of morphine-treated mice compared to morphine with naltrexone, and placebo-treated mice, including chenodeoxycholate, deoxycholate, cholate and tauroursodeoxycholate. These results were not replicated in TLR2KO morphine-treated mice, suggesting an integral role of the TLR2 receptor mediating morphine-induced alterations of bile acid and lipid metabolites. Results observed may have been due to reduced activity of bile salt hydrolase in the gut bacteria (Banerjee et al. 2016). Overall, these results suggest a dysbiotic effect of morphine administration on beta diversity only, which may be protected by naltrexone. Additionally, the gut microbiota is able to induce the phenotype of opioid addiction in FMT recipient mice. Finally, these results suggest a role of TLR2 and MOR in causing dysbiosis of the microbiota, as knockout prevented dysbiosis.

While this study reported a protective effect of TLR2KO on the microbiota, this finding may be in contrast to Meng et al. (2015) who do report dysbiosis of several bacteria in TLR2KO mice treated with morphine compared to TLR2KO control mice (Table 3.2).

Zhang et al. (2019) investigated the microbiota of TLR2KO mice administered morphine (n=23), TLR2KO mice administered saline (n=19), TLR4KO mice administered morphine (n=11), TLR4KO mice administered saline (n=8), and C57BL/6 (wild-type; WT) administered morphine (n=7), and WT mice administered saline (n=6), following treatment with antibiotics. Morphine was administered at a constant (15mg/kg) or escalating dose (5, 10, 15, 20, 25, 30, 35, 40mg/kg), twice daily for 8 days alongside antibiotics. Faecal samples were derived from the small intestine and analysed by 16S rRNA gene sequencing. Beta diversity was different between morphine tolerant and saline-treated wild-type mice. However, no significant difference was found between morphine-treated and saline-treated mice within the TLR2KO and TLR4KO strains, suggesting that these receptors mediated dysbiosis. Wild-type morphine-treated mice had a reduction in *Actinobacteria*, *Firmicutes*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Bifidobacterium* and *Lactobacillus*, but expansion of *Allobaculum, Peptostreptococcaceae* and *Prevotellaceae* was also observed in this group, compared to wild type saline treated animals (Table 3.2). These findings were not found in TLR2KO or TLR4KO mice. In short, this study reports dysbiosis as a result of morphine administration, but implicates TLR2 and TLR4 in facilitating these changes.

Table 3.3 **Overview of dysbiosis of the gut microbiota as a result of opioid use in clinical and preclinical studies**

OUTCOME	CLINICAL	PRECLINICAL
α – Diversity	~ in all studies	↓ in 5; ↑ in 2; ~ in 4; NR in 4
β – Diversity	Δ in 3; ~ in 1	Δ in 9; ~ in 1; NS ~ in 1; NR in 6
Dysbiotic Microbes	(Genera) Alloprevotella, Bifidobacterium, Haemophilus, Megasphaera, Prevotella, Roseburia and Ruminococcus	Phyla: 7 Class: 1 Families: 14 Genera: 13 Species: 1
Alterations in Metabolites		↓ Primary bile acids ↑ Secondary bile acids; ↓ in ↓ SCFA, SCFA producing microbes
Changes in Functional Potential	↑ Ar. A Acid metabolism ↑ BCAA degradation ↑ Endotoxin production ↑ Functions carried out by the digestive system Cell health and signalling	↑ Signal transduction ↑ Protein repair ↓ Nicotinamide (Vitamin B3 variant) metabolism ↑, ↓ Amino acids metabolism

Abbreviations: ↑=Increase, ↓=Decrease, Δ =Different/Difference, ~=Not Different, NR=Not Reported, NS=Non-Significant; SCFA=Short Chain Fatty Acids, Ar. A Acid=Aromatic Amino Acids, BCAA=Branched Chain Amino Acids

Table 3.4 **Bacteria disrupted in clinical and preclinical studies as a result of chronic opioid use**

Dysbiotic Microbes	Taxonomic Ranking	Clinical	Preclinical
Actinobacteria	Phylum	↑	↓
Bacteroidetes	Phylum	↓	↑, ↓
Cyanobacteria	Phylum	↑	↓
Firmicutes	Phylum	↑	↑, ↓
Bacteroidaceae	Family	↓	↑
Lachnospiraceae	Family	↓	↓
Peptostreptococcaceae	Family	↑	↑
Ruminococcaceae	Family	↓	↓
***Clostridium* XIVa**	Sub-Family Lachnospiraceae	↑	↓
Alistipes	Genus	↓	↑
Alloprevotella	Genus	↑	↓
Anaerostipes	Genus	↑, ↓	↓
Bacteroides	Genus	↓	↑, ↓
Bifidobacterium	Genus	↑, ↓	↑, ↓
Dialister	Genus	↓	↓
Haemophilus	Genus	↑, ↓	↓
Lactobacillus	Genus	↑	↑, ↓
Parabacteroides	Genus	↓	↑, ↓
Parasutterella	Genus	↓	↑
Prevotella	Genus	↑, ↓	↓
Roseburia	Genus	↑, ↓	↓
Ruminococcus	Genus	↑, ↓	↑

3.5 Discussion

The present systematic review aimed to examine the influence of opioids on the gut microbiota, determine which strains were effected and outline the predicted functional outcomes of altered microbiota on metabolites and MGB axis signalling pathways. The effect of opioid use on alpha diversity and beta diversity were investigated, specific microbes that were enriched or depleted were identified, and the functional potential of the altered microbiota was examined. Opioid use did not significantly disrupt alpha diversity in clinical studies, while preclinical results regarding alpha diversity are inconclusive due to seemingly confounding reports. Depletion in alpha diversity following opioid administration was reported in five studies, whereas enrichment was reported in two studies; no significant difference was reported in four studies and the remaining four did not report on this outcome. Depletion in alpha diversity was generally reported in cohorts with comorbid diseases (Meng et al., 2020; Sharma et al., 2019; Simpson et al., 2020), as only two studies reported depletion as a result of opioid treatment alone (Wang et al., 2018; Zhang et al., 2020b). Enrichment of alpha diversity was found in response to n-3 PUFA diet regardless of opioid status in Hakimian et al. (2019), and as a result of combined HIV and morphine treatment in Meng et al. (2020). Taken together, these studies may suggest a minimal influence of opioids on alpha diversity, though more research is needed to validate this conclusion. In the present review, results were analysed to determine how the two individual components of alpha diversity, richness and evenness, were effected by opioids. Richness was increased in four studies, depleted in two and not significantly different in six; evenness was increased in one and not significantly different in six. Based on these results, opioid use may facilitate an increase in the number of species present, reflected by an increased richness; however, confounding result necessitate further research. In addition, the mechanisms through opioids alter species richness warrants further investigation, but may involve impaired gut barrier integrity and inflammatory responses,

which often result from chronic opioid use (Banerjee et al., 2016, Meng et al., 2013). These results are in line with studies in other substances of abuse. For example, there appears to be no significant effect of harmful alcohol use on the alpha diversity of the gut microbiota (Mutlu et al., 2012; Dubinka et al., 2017; Ciocan et al., 2018a; Bjørkhaug et al., 2019). Furthermore, studies on cocaine report depletion (Scorza et al., 2019) or no change (Volpe et al., 2014) in alpha diversity. On the other hand, opioid use did impair beta diversity in three clinical studies, with no difference reported by Barengolts et al. (2018), suggesting that opioid use causes a shift in the community structure. However, comorbidity may contribute to these results more than use of opioids. Dysbiosis has been reported in studies examining T2D (Umirah et al., 2021) and the particular finding of dysbiosis in *Bifidobacterium* reported by Barengolts et al. (2018) is reported in numerous other studies in patients with T2D not using opioids (Gurung et al., 2020). Elsewhere, cirrhosis and hepatic encephalopathy are associated with a dysbiotic gut microbiota (Dhiman, 2013, Rai, Saraswat and Dhiman, 2015). This comorbidity may explain the dysbiosis reported in Acharya et al. (2017), who do not report changes in beta diversity in patients without cirrhosis, suggesting that opioid use worsens dysbiosis with comorbidity, opioids alone may have a negligible influence on this outcome. Clinical trials investigating patients with OUD without comorbidity will be necessary to determine how opioids affect beta diversity, and a lack of such a study is a limitation to enabling clear conclusions in the present review and literature. Lastly, Li et al. (2020) report differences in beta diversity between patients undergoing compulsory detention and healthy controls, but overlap in patients actively engaging in drug use and patients undergoing methadone maintenance, suggesting a comparable effect of methadone administration and harmful drug use on beta diversity. Further investigation of such programmes is warranted to determine the factors that result in the different compositions observed, as they may be clinically significant. Stronger evidence for an effect of opioid use on beta diversity can be found in preclinical

research. Beta diversity was significantly different between opioid-treated animals and non-opioid treated animals in nine out ten studies that reported on this outcome. A non-significant trend towards differences in beta diversity was reported by Hakimian et al. (2019), and the remaining six studies did not explicitly report on this outcome. In short, chronic opioid use appears to induce a shift in the composition of the microbiota away from that observed in drug naïve group though a myriad of other factors may contribute to these results, such as treatment conditions and comorbidity. Conversely, chronic opioid use does not appear to influence alpha diversity overall, and may only facilitate an increase in richness. The implications of these alterations and how they may relate to cognition should be a focus of future research in addiction, as it a current gap in knowledge.

Specific microbes were altered as a result of opioid administration. In the clinical studies (Table 3.3**)**, the genera *Alloprevotella, Bifidobacterium, Haemophilus, Megasphaera, Prevotella, Roseburia and Ruminococcus* were dysbiotic in more than one study, though the patterns of dysbiosis were not consistent across these papers. *Alloprevotella, Bifidobacterium* and *Ruminococcus* were generally increased in cohorts administering opioids. In preclinical studies (Table 3.3), 36 strains were enriched or depleted as a result of opioid use overall. These included seven phyla (*Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria, Tenericutes* and *Verrucomicrobia*), one class (*Clostridiales*), 14 families (*Bacteroidaceae, Coriobacteriaceae, Enterobacteriaceae, Enterococcaceae, Erysipelotrichaceae, Lachnospiraceae, Lactobacillaceae, Methanobacteriaceae, Peptostreptococcaceae, Porphyromonadaceae, Rikenellaceae, Ruminococcaceae, Streptococcaceae, Veillonellaceae*), 13 genera (*Akkermansia, Allobaculum, Bacteroides, Bifidobacterium, Clostridium, Desulfovibrio, Dorea, Enterococcus, Lactobacillus, Parabacteroides, Ruminococcus, Streptococcus, Sutterella*), and one species (*E. faecalis*). Interestingly, there was a high degree of overlap in dysbiotic strains between clinical and

preclinical studies (Table 3.4), though the pattern of dysbiosis was rarely consistent. This review identified *Lachnospiraceae*, *Peptostreptococcaceae* and *Ruminococcaceae* (at the family level), and *Dialister* (at the genus level) that had a common pattern of dysbiosis between clinical and preclinical studies. Several of these bacteria are directly or indirectly linked to cognition according to the literature, and therefore may contribute to cognitive impairments in OUD. For example, *Bacteroidetes* was associated with impaired memory in a rodent model of obesity (Zhang et al., 2019b), a disorder underpinned by neurocircuitry commonly involved in drug addiction such as dopaminergic reward signalling (Kenny, 2011, Volkow, Wise and Baler, 2017). In addition to this, depletion of *Firmicutes* was reportedly associated with impaired visual memory performance in a cohort of older (aged 50 to 85) adults (Manderino et al., 2017), and evidence from a cohort of depressed patients has linked depletion of *Firmicutes* to decreased levels of SCFA (Huang et al., 2018). As discussed previously, SCFA can act on the CNS, and other preclinical evidence has linked SCFA to performance in memory (Lee et al., 2020a), suggesting a link between these microbes and cognition. There is a scarcity of literature reporting on such a link in opioid addiction, but the literature discussed here supports a role of these specific microbes in the cognitive impairments observed in patients with OUD, warranting further investigation.

The dysbiosis of the gut microbiota resulting from opioid use was linked to alterations in various metabolic pathways and metabolic products (Table 3.1, 3.2 and 3.3). In clinical studies, opioid use was linked to increased potential for aromatic amino acid metabolism, and branched chain amino acids degradation; endotoxin synthesis, nitrogen metabolism; cell growth and death; DNA replication and repair, and translation. Downregulation of pathways involved in cellular signalling and processing, and metabolism were also reported. In preclinical studies opioid use was linked to potential upregulation of pathways involved in signal transduction; recombination and repair proteins, and potential downregulation of

pathways involved in nicotinate and nicotinamide metabolism; nitrogen and cyanoaminoacid metabolism. Several metabolites important to host health and function were also disrupted. In opioid-treated animals altered levels of primary and secondary bile acid production, sphingolipid metabolism, neurotransmitter (serotonin and *N*-acetylserotonin) levels, cholesterol levels, and SCFAs levels were reported. According to the literature, bile acids act as a regulator of the commensal gut microbiota (Ridlon et al., 2015). For example, the secondary bile acid deoxycholic acid, which was reportedly depleted as a result of morphine administration by Wang et al. (2018), is a strong antimicrobial compound and is associated with impaired gut barrier integrity (Stenman et al., 2013). Secondary bile acids can be produced by several bacterial strains, including *Bacteroides, Bifidobacterium, Clostridium, Enterococcus* and *Lactobacillus* (Zeng et al., 2019), all of which were found to be dysbiotic in the present review. As such, these microbes may mediate opioid-induced dysbiosis, impair gut barrier integrity and facilitate translocation of microbes, metabolites and toxins through production of bile acids. The role of bile acids in cognition is less explored. Mahmoudiandehkordi et al. (2019a) reported lower serum levels of primary bile acids, but increased serum levels of secondary bile acids, including deoxycholic acid, in patients with Alzheimer's disease compared to patients with normal cognitive performance. As increased levels in secondary bile acids, especially deoxycholic acid, are reported in opioid studies, and are associated with impaired cognition in other disorders, future studies should investigate the relationship between these key metabolites and cognition in opioid use. Sphingolipids, key signalling and structural molecules, were increased as a result of morphine treatment by Sindberg et al. (2019). The literature has outlined a contributing role for sphingolipids in the development of morphine tolerance, hyperalgesia and antinociception (Kalinichenko et al., 2018). *Bacteroides* and *Prevotella,* noted as being able to produce these molecules (Heaver, Johnson and Ley, 2018), were dysbiotic as a result of morphine treatment. Altogether, the

relationship between sphingolipids, gut microbiota, cognition and opioid dependence is under examined. SCFAs and SCFA-producing microbes, which serve roles in host health, were disrupted as a result of opioid use. A depletion of SCFAs (butyrate, acetate and propionate) was reported by Touw et al. (2017), whereas a depletion in SCFA producing strains (*Muribaculaceae*, *Lachnospiraceae*, and *Ruminococcaceae*) was reported by Meng et al. (2020). Research has demonstrated the ability of the SCFA butyrate to act as a histone deacetylase inhibitor (HDACi), in the brain (Bourassa et al., 2016). Specifically, butyrate has been reported to facilitate the expression of neurotropic factors such as BDNF in several studies (Lee et al., 2019, Wu et al., 2008). BDNF may facilitate the rewarding aspect of several drugs, including morphine (Ghitza et al., 2010); BDNF levels increase as a result of drug use, and; administration of BDNF enhances drug seeking behaviours (Vargas-Perez et al., 2009, Bolanos and Nestler, 2004). Further, supplementation with beneficial microbes was found to rescue cognitive performance in a rodent study of vascular dementia, a result associated with increased butyrate and BDNF (Liu et al., 2015). Therefore, disruption of microbiota metabolites may be integral to cognitive performance in opioid use through their interaction with BDNF. However, the role of SCFA in facilitating cognitive impairment in opioid addiction directly (by acting on the CNS and via epigenetics), or indirectly (by controlling BBB and gut barrier integrity) requires further investigation as they may serve as an ideal candidate for future novel treatments.

Innate immune system and opioid receptors appear to be integral in facilitating opioid-induced dysbiosis. Zhang et al. (2019) and Banerjee et al. (2016) reported overlap in microbiota community structure between morphine-treated TLR2KO, TLR4KO mice and placebo mice, regardless of route of administration. However, while alpha and beta diversity do not appear to be affected in receptor knockout mice, Meng et al. (2015) suggest that several species of bacteria (specifically Gram-Positive species) are susceptible to dysbiosis even with TLR2KO.

TLR are pattern recognition receptors, part of the innate immune system that are activated by bacterial components. Specifically, TLR4 is activated by lipopolysaccharides (LPS) and TLR2 by peptidoglycans (PGN), although PGN activation of TLR2 is disputed (Dziarski and Gupta, 2005, Schwandner et al., 1999, Travassos et al., 2004). Regardless, gram-positive bacteria possess a thicker PGN layer and no outer lipid membrane, compared to gram negative bacteria which possess a thinner PGN layer but do have lipid outer membrane. The finding that gram positive bacteria were increased in TLR2KO mice by Meng et al. (2015) suggests that TLR2 may regulate levels of these microbes, potentially through PGN-induced production of IL-17A, in a TLR2 manner. This interaction is evidenced by the finding that morphine administration reportedly increased production of IL-17A production, a pro-inflammatory cytokine, by way of TLR2 activation by translocated microbes, peripherally. Other studies link gram-positive bacteria such as *Firmicutes*, which were reportedly enriched by opioids, to the immune system by way of their metabolites. For example, *Firmicutes*, able to produce beneficial products such as SCFA (Dalile et al., 2019), are to facilitate Treg cell generation (Arpaia et al., 2013), which supress immune responses. Numerous immune system cells, including Treg cells expressed TLR2 (Sutmuller et al., 2006). Finally, TLR have been linked to synaptic plasticity during alcohol addiction (Crews et al., 2017), sensitivity to cocaine-induced CPP (Zhu et al., 2018) and development of morphine tolerance (Eidson et al., 2017). In short, this evidence suggests a role of TLRs and opioid receptors in mediating gut microbiota dysbiosis and opioid addiction, most likely through the immune system and inflammation. While there is some evidence linking these receptors to addiction-related behaviours and brain function, further research is needed to investigate these relationships.

These results outline a disruption of several metabolic pathways and production of metabolites that are integral to microbe and host cross-communication and health. There are several directions for further research, such as determining a) the functional potential of the

microbes disrupted by opioid use, b) the significance of alterations in metabolites and pathways in contributing to the cycle of addiction and, c) the mechanisms by which opioids facilitate an enrichment or depletion of these strains. Addressing this gap may inform a better understanding of opioid addiction pathology and the development of novel treatments.

There are several limitations to the current literature on opioid use and gut microbiota. Firstly, the majority of studies utilised 16S rRNA sequencing to profile the commensal microbiota. A limitation of 16S rRNA sequencing is that it cannot provide accurate species level identification, nor can it provide accurate prediction of the functional potential of the microbe's genome (Ranjan et al., 2016, Johnson et al., 2019). As such, future research should endeavour to utilise whole genome sequencing when profiling the microbiota. The research also did not investigate the influence of sex on microbiota composition. Sex influences risk of opioid misuse (Jamison et al., 2010, Serdarevic, Striley and Cottler, 2017), and also treatment efficacy (Huhn, Berry and Dunn, 2019), which warrants investigation into how the gut microbiota may also be effected by opioid use as consequence of gender. The unique effect of different opioids should also be investigated in future studies. Morphine was the most commonly investigated substance in the literature, but there has been an alarming rise in the use of other opioids, including fentanyl, heroin, codeine, oxycodone, and other prescription opioids. Studies should also examine the effect of opioid addiction medications, particularly buprenorphine-naloxone (BNX) that is under investigated in the literature, and whether successful treatment approaches result in restored microbiota balance. Future studies investigating the effect of opioid use on the microbiota in models without comorbidity are needed. Several of the studies included in this review included cohorts with comorbid diseases, many of which are linked to dysbiosis of the commensal microbiota, making it difficult to assign any results specifically to opioids. Finally, larger sample sizes are needed to give more power and confidence to the results found.

In conclusion, the present review has contributed to our understanding of how chronic opioid use impacts the commensal gut microbiota, and has identified a number of mechanisms through which this may influence brain function and cognition. The review has found that beta, but not alpha, diversity is frequently impaired by opioid use. Finally, this review has identified a panel of microbes that are frequently dysbiotic in the presence of opioids that could serve as key candidates for future research into novel therapeutics.

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Chapter 4

Overall Discussion and Conclusions

4.1 Overview

An important feature of opioid addiction is cognition due to the role of cognitive function in the various stages of the addiction cycle and treatment outcomes. The literature demonstrates cognitive impairment in opioid use; however, results are inconclusive, and little research has examined particularly sensitive populations, such as individuals commencing rehabilitation program and pharmacological treatment, particularly in the Australian population. While existing pharmacological treatments for opioid addiction (such as methadone and buprenorphine-naloxone) have demonstrated efficacy, there are limitations including an inability to improve cognitive impairments, or even further enhance cognitive detriments in some individuals. Hence, there is a need for greater understanding of the effect of opioid-based pharmacological treatments for opioid addiction on cognition, and to determine whether other parameters interplay in cognitive outcomes, such as dose, duration of treatment, length of stay in rehabilitation programs, etc. Furthermore, given the shortfalls of existing pharmacological interventions, there is a need for novel treatment options. Research is increasingly demonstrating a role for gut microbiota in host health, including brain development and functioning; indeed, the gut microbiota engages in bidirectional communication with the brain through various pathways, termed the microbiota-gut-brain axis. However, individuals presenting with disorders with cognitive dysfunction, such as schizophrenia, depression and anxiety also present with an altered gut microbiota. Furthermore, the existing literature demonstrates alterations in microbiota in studies of addiction (e.g. cocaine and alcohol), which also frequently present with impaired cognition. There is a body

of literature that has investigated the effect of chronic opioid use on microbiota; however, results appear inconclusive and further examination is required. Taken together, the literature suggests a potential contribution of the microbiota to the cognitive impairment associated with opioid addiction; however, further research is required. The present thesis aimed to 1) examine cognition in an Australian population of people undergoing early stages of a rehabilitation program (incorporating opioid-based pharmacological treatment) for opioid misuse; 2) investigate the effects of opioid use on the gut microbiota, and; 3) examine the potential functional outcomes of altered gut microbiota and metabolites with regards to opioid addiction and cognition by investigating their role in key signalling pathways of the microbiota-gut-brain axis.

4.1.1 *Summary of Findings*

In Chapter 2, the results demonstrated cognitive performance that ranged from a low score in the bottom 19.85 ± 3.65th percentile of the general population for verbal learning (HVLT-R) to a high score in the 52.85 ± 5.25th percentile of the general population for processing speed (CF-Animals), demonstrating a level of cognitive dysfunction in this population compared to the normative data. There was no significant difference in cognitive performance between patients undergoing treatment with methadone compared to patients undergoing treatment with buprenorphine-naloxone (BNX). Correlational analyses were conducted to determine whether cognitive performance was correlated to various treatment related factors (i.e., dosage (of either BNX or methadone), time since last treatment, life-time length of pharmacological treatment, length of stay). Dose of methadone or BNX did not influence cognitive performance. However, there was a strong and positive correlation between time since last treatment and verbal learning performance (i.e., HVLT-R) in the BNX group,

demonstrating the importance of considering the immediate effects of opioid treatment on cognition as a potential confounder across studies in this field. In addition, the BNX group had a positive correlation between lifetime history of treatment and non-verbal working memory performance (i.e., WMS III-SS), suggesting a beneficial effect of BNX in this cognitive domain (albeit, not significantly different to methadone). Neither of these relationships were replicated in the methadone group. Non-treatment related demographics factors, such as age were positively correlated to speed of processing (i.e., TMT-A) for each treatment and the whole cohort, whereas BMI was negatively correlated to reasoning / problem-solving for the BNX group only (refer to Chapter 2). Interestingly, there was a significant difference in years of education and length of stay between the two groups, but neither of these factors significantly affected neurocognitive performance in either group (with no significant correlations observed, all $p > .05$). Furthermore, there was a longer length of stay in the BNX treatment group compared to the methadone group; although length of stay did not correlate to improved cognitive outcomes; this finding suggests greater adherence to the rehabilitation program (i.e., lower drop-out rates) with BNX treatment compared to methadone. Overall, these findings demonstrate cognitive impairment in individuals undergoing rehabilitation for opioid addiction; however, an important limitation was the lack of an opioid-free control group and further, adequately powered, studies are needed. Nevertheless, the results of Chapter 2 suggest several benefits of BNX over methadone, as well as a role for treatment-related parameters and demographic factors in the cognitive functioning of patients. Given the importance of cognition in the addiction cycle and treatment outcomes, further studies investigating the role of factors that may influence a patient's course of addiction, treatment response and recovery are warranted.

The second major aim of the present thesis was to investigate the influence of opioids on the gut microbiota. This was initially to be addressed using whole genome sequencing

(WGS) of faecal samples obtained from participants in the cognitive study of Chapter 2; however, due to COVID lockdown, data sets remained incomplete and WGS was no longer feasible. Therefore, Chapter 3 addressed this gap in knowledge through a systematic literature review of the existing literature. The review identified that beta diversity, but not alpha diversity, was commonly altered as a result of chronic opioid use in both clinical and preclinical studies; 22 microbes were repeatedly dysbiotic across clinical and preclinical research; several microbiota metabolites and functional pathways (including those related to immune and neurotransmitter signalling) were affected by opioids. Hence, the hypothesis that opioid administration is associated with a dysbiosis of the microbiota and altered metabolites that could have functional implications was supported. Further research is required to determine the role (if any) of these dysbiotic strains in addiction, treatment and recovery; however, we highlight for the first time, key candidate strains of interest for further research. Some of the other key findings of the review included reports that Toll-Like receptors (TLR) were found to have a central role in mediating dysbiosis, as knockout of the receptors prevented dysbiosis from occurring (Banerjee et al., 2016), addiction medications could either recover or induce dysbiosis (Banerjee et al., 2016, O'Sullivan et al., 2019), and reward learning was influenced by chronic opioid use (a finding that was associated with microbiota dysbiosis (Zhang et al., 2021b)). Overall, these findings indicate that dysbiosis of the gut microbiota occurs as a result of chronic opioid use and highlight several pathways through which the gut microbiota could be involved in the cycle of addiction. Dysbiosis of the gut microbiota may act as a negatively reinforcing, interoceptive cue in chronic opioid use (Ren and Lotfipour, 2020).

The third aim was to outline the potential functional outcomes of an altered gut microbiota and metabolites by investigating their role in key signalling pathways of the microbiota-brain-gut (MGB) axis, which was also addressed in Chapter 3 through functional data extracted from the existing literature. The final hypothesis was that opioid use would result

in changes in the functional potential of the gut microbiota, and that these changes may involve the MGB axis to potentially influence cognition. The systematic literature supported this hypothesis, as dysbiosis of the microbiota by chronic opioid use led to disruption of various metabolites involved in MGB axis signalling. For example, short chain fatty acids (SCFAs) were reportedly disrupted by chronic opioid use, which is an important finding given the ability of microbiota-derived SCFAs to influence neuronal and microglial function, learning and memory (reviewed in Silva, Bernardi and Frozza (2020)). The results reported lends support to the notion that dysbiosis of the gut microbiota may influence cognition and the cycle of addiction, which could be relevant to the clinical observations reported in Chapter 2. The following sections will further discuss the integration of the gut microbiota in the addiction cycle and cognitive dysfunction during opioid use and potential mechanisms.

4.2 Gut Microbiota and the Immune System in OUD

The systematic literature review (Chapter 3) highlighted several mechanisms by which the gut microbiota may influence cognition during opioid use via the immune system, summarised in Figure 4.1. These include depletion of metabolic products (such as SCFA) that help maintain gut homeostasis and epithelial integrity, and enrichment of metabolic products (such as secondary bile acids and endotoxins) that disrupt this homeostasis and trigger the immune system through activation of local receptors such as TLR (Table 4.1).

Depletion of SCFA and SCFA-producing bacteria as a result of chronic opioid administration may contribute to increased permeability of the gastrointestinal tract. Research has demonstrated that SCFAs improve gastrointestinal barrier function and can protect against

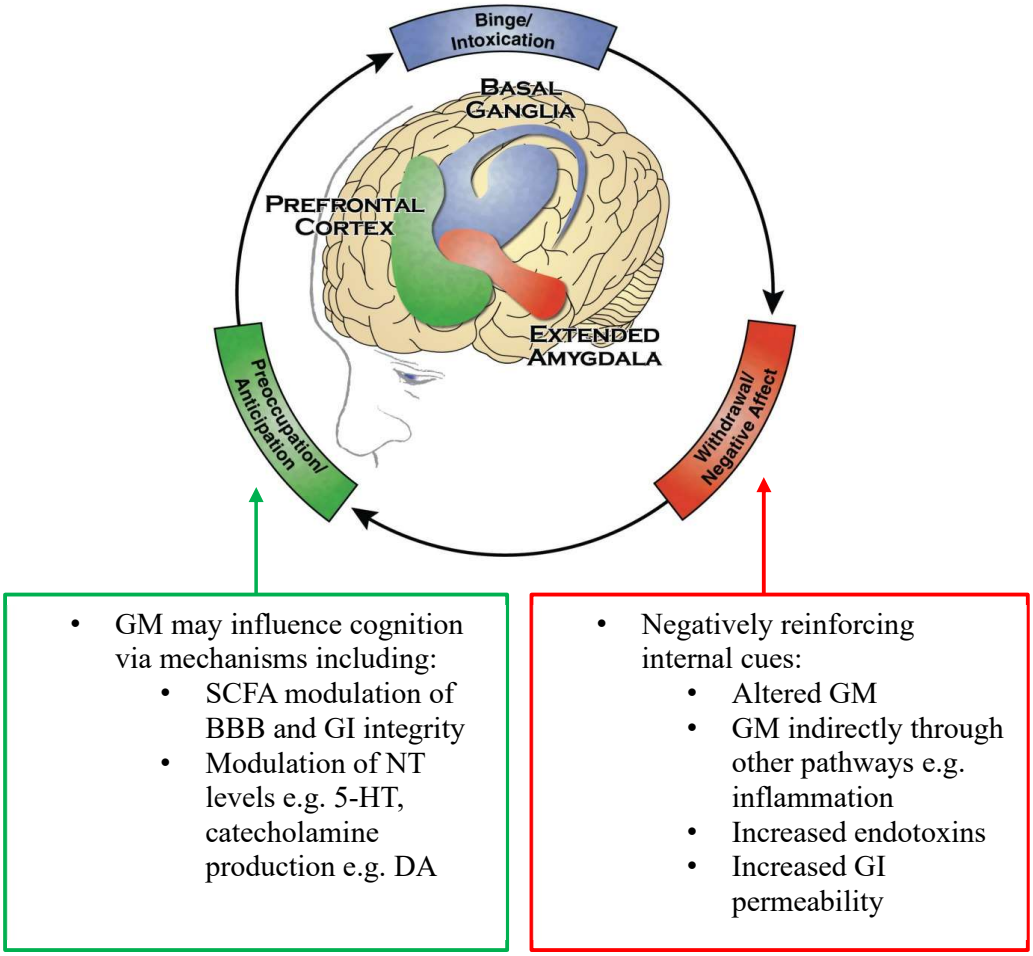

Figure 4.1 **Proposed mechanisms through which dysbiosis of microbiota by chronic opioid use may influence cognition and contribute to the cycle of addiction.** Chronic opioid use results in dysbiosis of the gut microbiota. This dysbiosis may contribute to several negative outcomes, such as: a) altering gut homeostasis, b) impairing the gastrointestinal tract allowing for translocation of microbes and products, c) altering the composition of the microbiota and output of metabolic products. Such outcomes may trigger the immune system, one of the many pathways of the MGB axis. Dysbiosis in the gut, an altered microbiota, translocation of products may influence the brain by acting as interoceptive cues processed by structures such as the insula. In the context of addiction, these cues may drive addiction-related behaviours as a patients seeks to alleviate these issues. Alternatively, cognition may be influenced by some of the many other products of the microbiota involved in other pathways of the MGB axis that were reportedly altered in Chapter 3. For example, SCFAs, which were altered in chronic opioid users, can influence BBB and GI barrier integrity. The GM also produces modulates or produces precursors to many neurotransmitters such as serotonin and dopamine, which are integral to brain function. Hence, the microbiota may also influence cognition through other pathways such the neuroendocrine pathway. Linking to the role of cognition in the model of addiction introduced in Chapter 1, these opioid induced alterations may altogether perpetuate the cycle of opioid dependence. ***Abbreviations*****:** GM=Gut Microbiota; BBB=Blood Brain Barrier; SCFA=Short Chain Fatty Acids; DA=Dopamine; GI=Gastrointestinal.

Table 4.1 Overview of the potential pathways through which the gut microbiota may contribute to chronic opioid use and influence cognition and the potential molecules involved, based on the findings of Chapter 3.

MGB Axis Pathways	Molecules Involved	Potential Structures Involved
Immune Pathway	• Short Chain Fatty Acids (Butyrate) • Secondary Bile Acids • Endotoxins, Peptidoglycans and Lipopolysaccharides • Microbes	• Toll-Like Receptors • Microglia • Vagus Nerve • Insula
Neuroendocrine	• Short Chain Fatty Acids (Butyrate) • Neurotransmitters • Amino Acids (Aromatic and Branched Chain)	• Vagus Nerve • Gastrointestinal Tract

ethanol-induced disruption to the gut (Eamin et al., 2013). SCFAs increase barrier integrity through upregulation of Claudin-1, a tight junction protein, in-vitro (Wang et al., 2012), thus providing evidence of a mechanistic linking between SCFAs and gut barrier integrity. In the present context, the opioid-induced depletion of SCFA and SCFA producing bacteria may lead to disruption in gut barrier permeability; a symptom present in many disorders with impaired cognition, including Parkinson's disease (Clairembault et al., 2015), autism spectrum disorders (de Magistris et al., 2010), schizophrenia (Julio-Pieper et al., 2014, Maes et al., 2019, Yuan et al., 2019). Reports demonstrate that these disorders also have reported depletion of SCFA or SCFA-producing bacteria (Aho et al., 2021, Huang et al., 2021, Wang et al., 2020, Zhang et al., 2020). Together, these studies evidence a link between SCFAs, gut barrier permeability and potentially, cognition. Similarly, clinical and preclinical studies have reported impaired gut barrier integrity as a result of chronic alcohol consumption (Leclercq et al., 2014, Lee et al., 2020, Yang et al., 2019). In the systematic review conducted in Chapter 3, one preclinical study reported the depletion of SCFAs as a result of opioids (Touw et al., 2017), whereas the majority of studies reported dysbiosis of SCFA-producing bacteria, such as *Firmicutes*. SCFAs may contribute to cognitive impairments in OUD through their ability to act on the CNS, and to maintain not only GI tract permeability, but also BBB permeability (discussed later); thus

impacting brain function and perpetuating the cycle of addiction. Future studies should focus on SCFAs when investigating the relationship between the gut microbiota and cognition in OUD as a potential mechanism by which microbiota dysbiosis can cause a functional effect on behavioural outcomes.

In addition to SCFAs, the studies investigated in Chapter 3 reported alterations in bile acids (BA), though secondary BAs (SBA) were observed to both increase (Sindberg et al., 2019) and decrease (Banerjee et al., 2016) as a result of chronic opioid use. Study design is likely to explain these inconsistencies as Sindberg et al. (2019) utilised morphine injections in a primate model, whereas Banerjee et al. (2016) utilised morphine pellet implants in a mice model. Studies in alcohol, however, consistently report increases in SBA in addicted patients (Bajaj, 2019, Kakiyama et al., 2014). Much like SCFAs, BAs influence gut barrier integrity but do so through farnesoid X receptors (FXR). These receptors are located in various tissues (e.g., intestinal tissues) and cells (e.g., CD4, CD8 immune cells), and are activated by primary (PBA) and secondary BA (SBA), such as deoxycholic acid and ursodeoxycholic acid (Shaik, Prasad and Narala, 2015, Tripathi et al., 2018). Upon activation by BAs, the receptors can induce the production of antimicrobial agents, exert anti-inflammatory action and improve gut barrier integrity (Gadaleta et al., 2011, Inagaki et al., 2006, Shaik et al., 2015). Therefore, disruption of bacterial products (such as SCFAs and BAs) may contribute to the increased permeability of the intestinal barrier observed in opioid addiction (Gicquelais et al., 2020, Kang et al., 2017, Rueda-Ruzafa et al., 2020, Salavrakos et al., 2021).

Mechanisms by which disruption of these metabolites and increased permeability of the gut epithelium may contribute to the cycle of addiction appear to converge on the immune system, one of the major pathways of the microbiota-gut-brain (MGB) axis (Figure 4.2). As noted previously, SCFAs and BAs regulate intestinal permeability and a depletion of these metabolites may impair permeability and allow for translocation of microbes and endotoxins

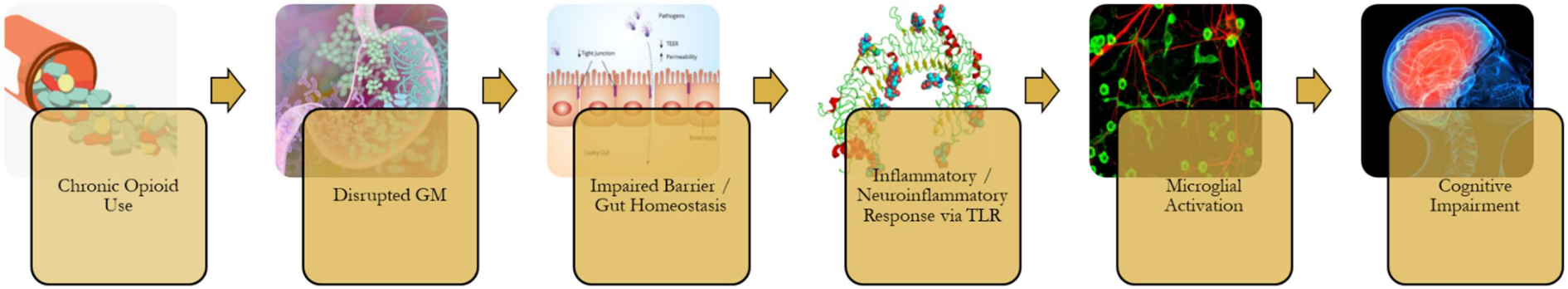

Figure 4.2 **Proposed mechanisms through which opioid induced perturbations in the gut may influence the course of opioid addiction and cognition through neuroinflammation.** Chronic opioid use induces disruption of the gastrointestinal tract causing dysbiosis, and impaired barrier integrity, facilitating translocation of microbes and endotoxins outside of the gut lumen. This translocation induces a neuroinflammatory response via CNS components such as TLRs on microglia, which are involved in various cognitive processes. Thus, chronic neuroinflammation resulting from persistent opioid use, impaired gut health and microbiota dysbiosis may potentially lead to impaired cognition, perpetuating the cycle of addiction.

from the gut lumen to induce a systemic immune response. TLR are activated by bacterial constituents (such as peptidoglycans (PGN) and lipopolysaccharides (LPS)), playing a vital role in immune responses by activating transcription factors such as nuclear factor kappa B (NF-κB) (Kawasaki and Kawai, 2014), and are evidently central to opioid addiction (Hutchinson et al., 2007). For example, a preclinical study by Hutchinson et al. (2012) found that either TLR4 knockout or antagonism by naloxone diminished oxycodone-induced place preference (place preference is a model of drug-associated learning that also reflects the development of addiction). In another study, Zhang et al. (2011) found TLR2 knockout diminished the development of tolerance to morphine, and morphine-induced microglia activation. Furthermore, research demonstrates a relationship between SCFAs (Huuskonen et al., 2004), BAs (Jena et al., 2018), and microglia, outlining a more direct relationship by which microbially derived metabolites may influence the CNS. Microglial activation is a component of neuro-inflammation, which itself is common in opioid addiction (Bachtell et al., 2017, Eidson et al., 2017). Neuro-inflammation in the context of opioid addiction contributes to the development of tolerance via TLR4 activation (Wang et al., 2021). In studies of alcohol abuse, neuro-inflammation is linked to poorer cognitive performance (Coppens et al., 2019), and in preclinical opioid research, perinatal exposure to methadone increased TLR4 and microglial activation in Sprague-Dawley pups and resulted in poorer cognitive function during adulthood (Jantzie et al., 2020). These findings seem to not only place TLRs and neuroinflammation as a potential component in the development of opioid addiction, but also to cognitive function. The findings of Chapter 3 also suggested a role of TLRs in opioid-induced microbiota dysbiosis. Knockout of these receptors prevented dysbiosis according to Banerjee et al. (2016) and Zhang et al. (2019), and in another study by Meng et al. (2013), TLR2KO and TLR4KO prevented morphine-induced bacterial translocation due to impaired barrier integrity. This converging line of evidence suggests a role for TLRs in mediating dysbiosis of the gut microbiota, and in

contributing to opioid addiction through microglial activation (Figure 4.2). Interestingly, the results of Chapter 2 demonstrated an overweight phenotype in the cohort of participants tested, based on the average BMI. Existing literature demonstrates that visceral adipose tissue is associated with an upregulation of inflammatory markers (e.g., C-reactive protein, interleukin-6), and is linked to impaired cognition function through this state of systemic inflammation (Cannavale et al., 2021). This coincides with the finding of the present study that BMI negatively correlated to cognitive performance. Importantly, taken together with the literature, our findings of cognitive impairment in Chapter 2 could be related to microbiota dysbiosis and systemic inflammation in this overweight population; however, further research is required to confirm. This thesis highlights a necessity to investigate cause and effect relationships between diet, adiposity, BMI, gut microbiota, inflammation and brain function and opioid use.

4.2.1 *The Three Stage Model of Addiction, Neuro-inflammation and Interoception*

As suggested above, the gut microbiota may contribute to the cycle of opioid addiction through its role in neuro-inflammation, which serves as a negatively reinforcing interoceptive cue (Figure 4.1). Interoception is the awareness of the internal state of the body (Verdejo-Garcia, Clark and Dunn, 2012), and in the context of opioid use this may include sensitivity to pain and other symptoms associated with tolerance and withdrawal. Inflammation and increased intestinal permeability (Ganci et al., 2019), the enrichment of certain microbes that upregulate intestinal permeability, enrichment of pathogenic bacteria and translocation of microbes by chronic use of opioids may contribute to these internal sensations, as suggested by Ren and Lotfipour (2020), thereby outlining a role of the microbiota in the three stage model of addiction. Research suggests a central role for the insula, which processes internal cues, in addiction. For example, one imaging study reported stronger connections between the insula

and amygdala, and increased impulsivity in abstinent heroin users (Xie et al., 2011). Clinical studies have reported that interoception is impaired in patients with OUD (Stewart et al., 2020), and in abstinent males (Subay and Sonmez, 2021) compared to healthy controls. Furthermore, damage to the insula interrupts addiction related behaviours, as outlined in a review by Droutman, Read and Bechara (2015). More recent research even posits the gut microbiota as directly exerting interoceptive cues (Critchley and Garfinkel, 2017, Mayer et al., 2014). The current thesis proposes that disruption of the gut microbiota by chronic opioid use contributes to the cycle of addiction, in-part by acting as, or contributing to negatively reinforcing interoceptive cues. The systematic literature review of Chapter 3 suggested that neuro-inflammation, induced by changes in the gut microbiota, may be one such cue that leads to impaired cognition, thereby further potentiating harmful opioid use. Hence, through the immune system, the gut microbiota may be involved in the withdrawal/negative affect stage and preoccupation/anticipation stage of addiction in the context of chronic opioid use.

4.3 Gut Microbiota and Neurotransmitters in OUD

Disruption of the gut microbiota by chronic opioid use may also affect the brain through the neuroendocrine pathway (Table 4.1). The microbiota and products such as SFCAs influence the production of neurotransmitters and neuropeptides by enteroendocrine (EEC) and enterochromaffin cells (ECC; refer to Chapter 1). This is evidenced through a study by Yano et al. (2015), who reported decreased levels of serotonin in germ-free compared to control mice, and another by Reigstad et al. (2015), who reported increased ECC production of serotonin in mice colonised with a human microbiota, in comparison to GF mice. Furthermore, compared to germ-free mice, humanised gut microbiota mice had increased levels of colonic *Tph1* protein and mRNA, which codes for tryptophan hydroxylase 1, the rate limiting enzyme of serotonin

production in the gut (Reigstad et al., 2015). The same study also reported promotion of *TPH1* transcription in a model of human ECCs following treatment with microbiota metabolites, acetate (10-50mM) and lower concentrations (0.5mM and 1mM) of butyrate (Reigstad et al., 2015). In the context of opioid use, there is evidence to suggest that opioids can increase serotonin by inhibiting serotonin reuptake from the synaptic cleft (reviewed in Baldo and Rose (2020)). In addition, compared to controls, chronic opioid users exhibit increased metabolism of aromatic amino acids (Acharya et al., 2017), such as tyrosine and tryptophan that are precursors to dopamine and serotonin, respectively. Furthermore, recent studies have discovered the presence of tyrosine hydroxylase coding genes in microbes such as *Lactobacillus* and *Enterococcus* (van Kessel et al., 2019), which were identified in Chapter 3 as dysbiotic in OUD through numerous studies (Hakimian et al., 2019, Meng et al., 2020, Sharma et al., 2020). Therefore, microbiota metabolites could modulate precursors to neurotransmitters that are implicated in brain function, including cognition (Jenkins et al., 2016, Jongkees et al., 2015). As such, there is cause to further investigate the contribution of the gut microbiota to cognitive impairment via neurotransmitter dysfunction in OUD.

4.4 Limitations and Future Directions

The present thesis identified cognitive dysfunction in individuals undergoing treatment for chronic opioid addiction in an Australian rehabilitation setting, demonstrated imbalances in the gut microbiota associated with opioid use and a potential role for the gut microbiota in the mechanisms of cognitive dysfunction associated with opioid use. However, considering the complexity of interactions between the microbiota-gut-brain axis, further research is needed to understand the relevance of microbiota alterations in addiction and cognition in people with an opioid use disorder. Future studies may examine the functional outcomes (i.e., effects on

cognition, the addiction cycle, treatment response and recovery) of treatment with bacterial supplements containing key species identified as dysbiotic in Chapter 3.

The findings of cognitive impairment in the population examined in Chapter 2 are important; however, further studies could consider an increased sample size, collecting data from multiple sites, examining participants longitudinally (i.e., at the start of treatment, throughout the rehabilitation cycle and post-recovery), and utilise a demographically-matched (e.g. age, sex, education) opioid-naïve control group. Chapter 2 was limited by the use of correlational analyses and future research should include a more robust statistical approach. Data sets containing self-reported parameters could be matched to medical records in order to identify and manage some aspects of under/over-reporting. Future studies could also examine the role of diet on cognitive parameters and the gut microbiota.

Despite uncovering important findings in the systematic literature review of Chapter 3, research into the relationship between the gut microbiota and OUD have several limitations. Firstly, studies frequently investigate cohorts presenting with comorbidity such as diabetes (Barengolts et al., 2018), HIV (Sindberg et al., 2019) or bowel disorders (Sharma et al., 2020), which themselves can result in microbiota dysbiosis. As such, it is hard to parse out the individual contribution of chronic opioid use. The research frequently utilised 16 rRNA sequencing and more robust whole genome sequencing (WGS) methods exist. 16S rRNA sequencing gives has lower taxonomic resolution and is limited in providing species level identification (Ranjan et al., 2016, Rizal et al., 2020). For example, a study by Ranjan et al. (2016) reported that 16S rRNA sequencing detected 1800 species whereas WGS detected over 3000 when analysing the same sample. Due to the superior capacity for WGS to also describe the genomic content of the microbiota, it may also provide functional data to give insight into how the microbiota is causally related to disease. As such, future research into OUD should

transition to WGS methods, enabling clearer understanding of potential impacts on brain function and identify potential microbial targets for intervention.

There are several further research questions that succeeding studies could address, including whether different opioids, such as morphine and codeine, or buprenorphine-naloxone and methadone, uniquely affect the composition of the gut microbiota. As limited studies in the present in the review of Chapter 3 suggest that medications for opioid use protect and reverse dysbiosis, it would be important to examine the mechanisms by which this occurs to enhance understanding of disease pathogenesis. The role of the treatment- and non-treatment-related parameters examined in Chapter 2 (such as length of treatment, dosage, BMI, age etc.) on dysbiosis and cognitive performance during opioid use is another potential question for future research.

4.5 Conclusions

In conclusion, the results of this thesis have demonstrated: a) cognitive impairment in an Australian cohort undergoing early stages of treatment compared to a general population, b) that opioid use impairs balance of the gut microbiota, and c) alterations to the microbiota by opioid use involves key signalling pathways of the microbiota-gut-brain axis and thus may influence brain functioning in individuals engaging in opioid use. Importantly, while there was no significant difference between performance between participants treated with methadone and BNX, results suggested BNX treatment may confer some benefits to cognitive functioning over methadone treatment. The study also highlighted the importance of key treatment (time since last dosage and lifetime history of treatment) and non-treatment (e.g., age and BMI) related factors in cognitive performance of individuals undergoing opioid-assisted therapy in early stages of rehabilitation treatment. Although further research is needed, these are key

findings that may inform the future of opioid-based pharmacological treatment. The result of this thesis also demonstrates for the first time that opioid use caused dysbiosis of specific microbes; and also disrupted key signalling pathways of the microbiota-gut-brain axis that may be involved in cognition. These are significant results that support the potential to address not just opioid misuse, but addiction more broadly, through targeting the microbiota. Overall, the findings of this thesis will potentially inform future studies and the development of novel therapies that could improve the lives of individuals with opioid addiction.

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