MicroRNA expression in the developing human brain and its role in neuropsychiatric disorders

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À miúda que em pequena comeu acendalhas, vês como consegues!

Summary

Foetal brain development is a critical period for future brain function where highly dynamic gene expression patterns give rise to the cellular diversity and complexity of the human brain. As a consequence, this is also likely to be an important period of vulnerability for neurodevelopmental and neuropsychiatric disorders. microRNAs (miRNAs) are a class of small noncoding RNA molecules with a prominent role in shaping and fine-tuning gene expression. In this thesis, I have used small-RNA sequencing to evaluate how variation in miRNA expression in 2nd trimester foetal brain might contribute to risk for neuropsychiatric disorders. I detected 1449 miRNAs in 2nd trimester foetal brain (corresponding to 55% of all known miRNAs) and assessed the effects of sex and gestational age on miRNA expression. Combining these data with genome-wide genotyping, I performed an eQTL analysis and identified 30 miRNAs where expression is associated with common genetic variation (miR-eQTLs) at FDR < 0.05. Finally, I related the identified miR-eQTLs to neuropsychiatric disorders and other brain traits using summary data-based Mendelian randomization. I identified 3 miRNAs for which eQTL are pleiotropically, and potentially causally associated with psychiatric traits. The A-allele of rs112622797 and the A-allele of rs12880925 were associated with higher miR-6840-5p and miR-4707-3p expression respectively, and both alleles were associated with decreased adult brain volume. The C-allele of rs174561 was associated with increased miR-1908-5p expression and increased risk for bipolar disorder, increased irritability, and increased sleep duration. Predicted gene targets of miR-1908-5p were also found to be enriched for genetic association with bipolar disorder. Further dissecting this association may translate to more effective treatments and a better quality of life for affected individuals.

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Chapter 1 – General Introduction

1.1 microRNAs

MicroRNAs (miRNAs) are a class of conserved small noncoding RNA molecules, 19 - 25 nucleotide in length, with a crucial role in the post-transcriptional regulation of gene expression by targeting specific mRNAs for degradation or translation repression (Bartel, 2004). Moreover, some miRNAs also have essential roles in transcriptional gene silencing (Kim et al., 2008) and mRNA-specific upregulation (Valinezhad et al., 2014). Through their actions, miRNAs shape and fine-tune the transcriptome with downstream effects on the proteome and, consequently, on developmental and cellular processes (Baek et al., 2008).

1.1.1 miRNA nomenclature

All published miRNA sequences from all available species are currently annotated and publicly available in the miRbase database. The most recent miRbase release (V22.1) contains hairpin precursor miRNAs and mature miRNAs from 271 species, including 1917 hairpin precursor miRNAs and 2654 mature miRNA sequences for humans (Kozomara et al., 2019).

miRNA nomenclature (Figure 1.1) follows a convention set out by Griffith – Jones and colleagues (Ambros et al., 2003), where the term "miR" refers to a mature miRNA species which is named numerically in order of discovery with the notable exemption of let-7 (lethal target 7) and lin (abnormal cell lineage) miRNAs. These miRNA families were named after the phenotype under study before this convention was implemented (Reinhart et al., 2000; Lee et al., 1993). miRNA names usually have a three-letter prefix which denotes its organism of origin. For instance, hsa-miR-9 stands for human miRNA 9, where hsa stands for *Homo Sapiens*. In contrast, mmu-miR-9 stands for its

homologue in mouse, where mmu stands for *Mus Musculus*. Some miRNA species derive from the same common ancestor and constitute a family of miRNAs, where they usually have similar physiological functions and similar sequences. miRNAs from the same family have a letter suffix added to their name. For example, miR-30a and miR-30b are members of the same miRNA family (miR-30). In addition, miRNAs encoded in several genomic locations, such as miR-30c-1 and miR-30c-2, have a numerical suffix added after the family suffix. Finally, a 3p or 5p suffix is added to denote if the mature miRNA is derived from the 3' arm or the 5' arm of the same miRNA precursor, as both can be biologically active – for instance, miR-30-c-1-3p and miR-30c-1-5p.

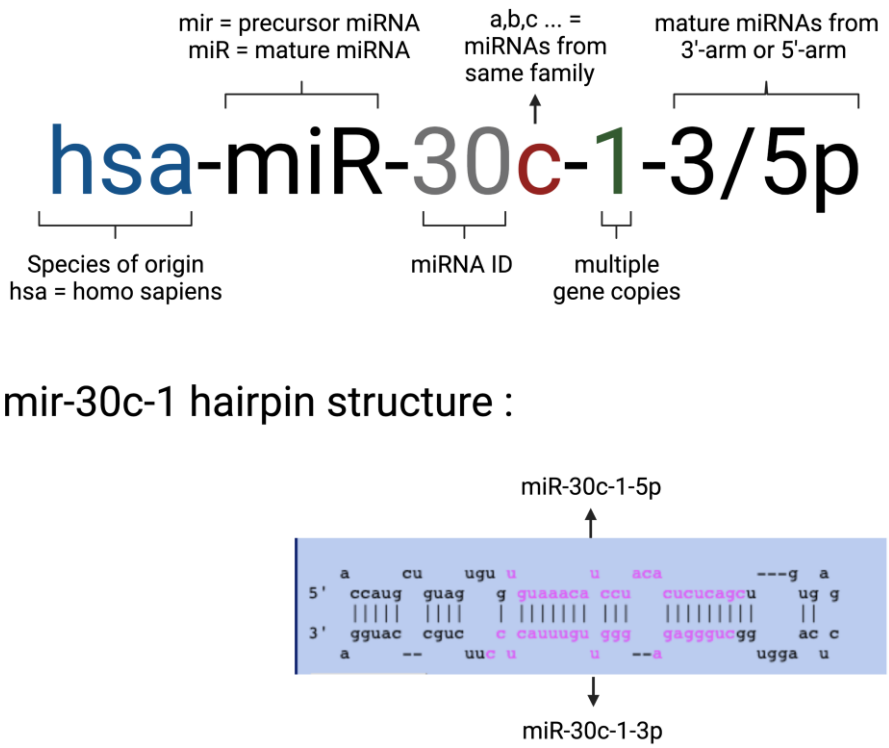


Figure 1.1 - Overview of miRNA nomenclature.

Schematic of miRNA nomenclature rules and example of a miRNA hairpin structure – mir-30c-1 obtained from miRbase (https://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0000736) that, once processed, will give rise to 2 mature miRNAs – miR-30c-1-5p originating from the 5' arm and miR-30c-1-3p originating from the 3p arm. Created with Biorender.com

1.1.2 miRNA biogenesis

1.1.2.1 miRNA gene location and miRNA gene transcription

Most human miRNAs are located in clusters in the genome, which can localize to intergenic, intronic or exonic genomic regions. In most cases, miRNAs are transcribed into long primary miRNAs (pri-miRNAs) by RNA polymerase II and, in rare cases, by RNA polymerase III (Lee et al., 2004; Borchert et al., 2006; Ramalingam et al., 2014).

miRNAs can be transcribed either as individual miRNAs or as part of a cluster in which a much larger polycistronic transcript containing multiple miRNAs is transcribed (Tanzer & Stadler, 2004; Hertel et al., 2006) and, as a consequence, can be co-regulated and co-expressed (Altuvia et al., 2005; Kim et al., 2009).

Nearly half of all known human miRNA genes are intergenic and are transcribed from their own miRNA promoters by RNA polymerase II (Lee et al., 2004; Schanen & Li, 2011; Ha & Kim, 2014; de Rie et al., 2017). A notable exception is a cluster of intergenic miRNAs located interspersed among repetitive Alu elements on chromosome 19, which is transcribed by RNA polymerase III (Borchert et al., 2006). The only other miRNAs transcribed by RNA polymerase III originate from a handful of viruses, such as the murid herpesvirus 4 (MuHV4) and the bovine leukaemia virus (BLV) (Bogerd et al., 2010; Kincaid et al., 2012). The other half of human miRNA genes are intragenic, located within host genes, and transcribed by RNA polymerase II. Most intragenic miRNAs are located within introns, and only a small number are located in exons of protein-coding genes. The pri-miRNA of intronic miRNAs is its host gene's heterogeneous nuclear RNA (hnRNA) (Kim & Kim, 2007). Intronic miRNAs can either be processed from the introns of their host transcription units and have the same expression pattern as their respective host gene as well as sharing common regulatory mechanisms,

including promoters (Lee et al., 2004; Rodriguez et al., 2004; Baskerville & Bartel, 2005), or be transcribed and regulated as independent transcription units that do not show concordance in expression patterns with their host gene (Wang et al., 2009a; Radfar et al., 2011; Ramalingam et al., 2014) and have been shown to have their own promoters independent of their host gene.

Moreover, alternative splicing has been shown to play a role in uncoupling the expression amongst clustered miRNAs and between miRNAs and their host genes (Ramalingam et al., 2014). The biogenesis of miRNAs can be classified into canonical and non-canonical pathways. An overview of both these pathways are depicted in Figure 1.2 (canonical pathway) and Figure 1.4 (non-canonical pathway).

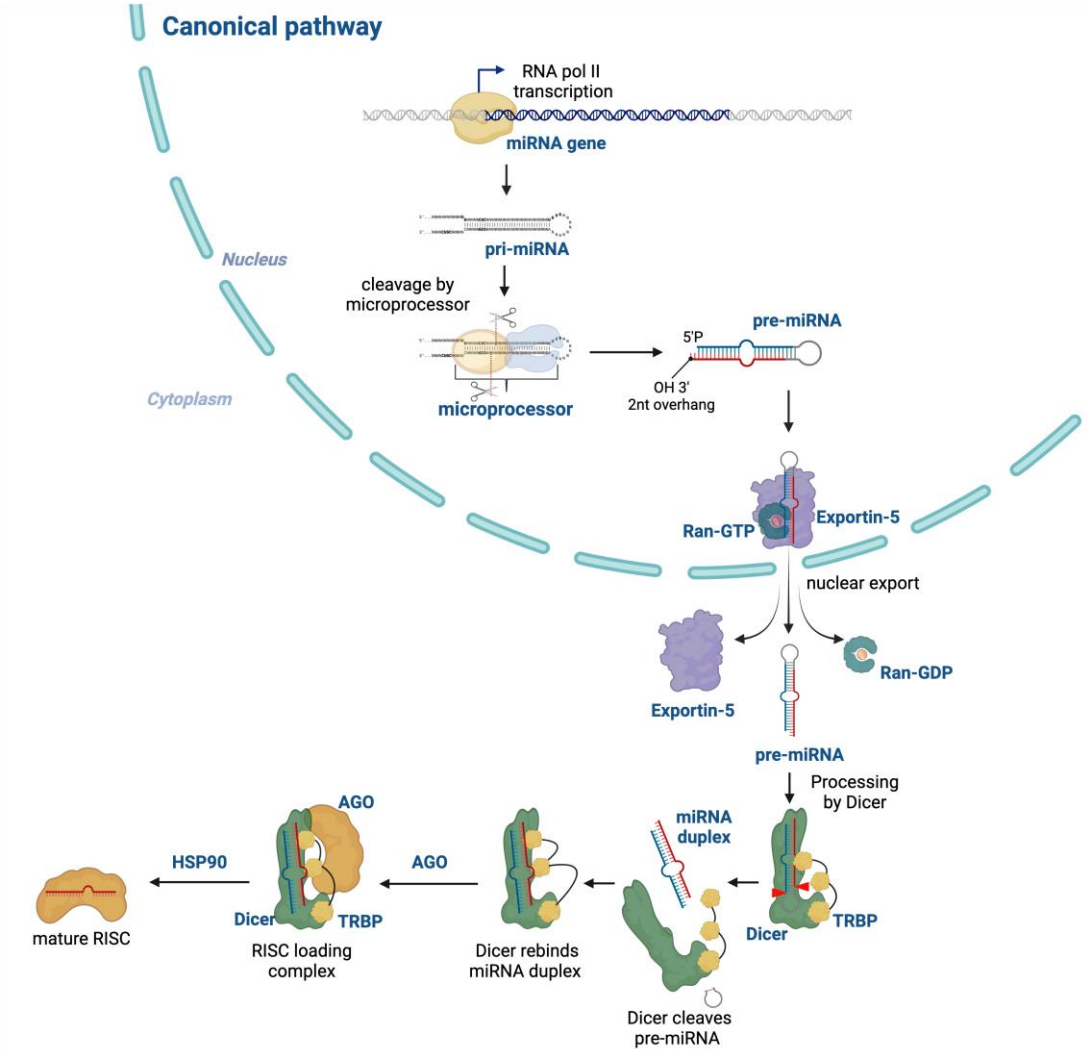


Figure 1.2 - Overview of the canonical pathway of miRNA biogenesis.

miRNAs are transcribed by RNA polymerase II and are either post- or co-transcriptionally processed by the nuclear microprocessor complex, consisting of Drosha and a DiGeorge critical region 8 (DGCR8) dimer. The microprocessor cleaves pri-miRNAs in the nucleus leading to the release of the pre-miRNA that has a characteristic 3'-OH 2nt overhang. This motif is recognized by Exportin-5 which binds to pre-miRNAs and, coupled with Ran-GTP, mediates the export of pre-miRNAs to the cytoplasm. In the cytoplasm, the terminal loop of pre-miRNAs is cleaved by Dicer and its co-factor trans-activation responsive RNA binding protein (TRBP), originating a miRNA duplex intermediary. Dicer, TRBP and an Argonaute (AGO) protein form the RISC loading complex (RLC), which mediates the loading of one strand of the miRNA duplex to the AGO protein, forming the RNA-induced silencing complex (RISC). Subsequently, RISC is stabilized by HSP90 and co-chaperones leading to a mature RISC which scans mRNAs for complementarity with the miRNA seed region. Adapted from: Treiber et al., 2018. Created with Biorender.com

1.1.2.2 Canonical Pathway of miRNA Biogenesis

1.1.2.2.1 Pri–miRNA processing by the microprocessor complex

The majority of human miRNAs are processed through the canonical pathway. In this pathway, pri-miRNAs are processed into precursor miRNAs (pre-miRNAs) post- or co-transcriptionally by the nuclear microprocessor complex (Conrad et al., 2014; Suzuki et al., 2017; Louloupi et al., 2017). This complex is composed of the RNA binding protein DGCR8 (DiGeorge critical region 8, or Pasha – partner of Drosha) and the ribonuclease III enzyme Drosha (Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Ha & Kim, 2014; Nguyen et al., 2015; Kwon et al., 2016). Cryo-EM studies determined that the core of the microprocessor complex is composed of a closely packed DGCR8 dimer that interacts with the basal region of Drosha and the terminal pri-miRNA loop (Partin et al., 2020; Jin et al., 2020). Other proteins like helicases, certain splicing factors, and heterogeneous nuclear ribonucleoproteins (hnRNPs) can interact with the microprocessor complex, modulating miRNA biogenesis (Denli et al., 2004; Gregory et al., 2004; Guil & Caceres, 2007; Trabucchi et al., 2009; Creugny et al., 2018; Kwon et al., 2020).

DGCR8 is a double-stranded RNA binding protein that acts as an anchor for Drosha via its' C-terminus (Han et al., 2006) and is essential for Drosha-dependent miRNA biogenesis (Wang et al., 2007). Moreover, DGCR8 is able to recognize several regulatory motifs within the pri-miRNA sequence, improving both the efficiency and the accuracy of pri-miRNA processing (Figure 1.3). For example, DGCR8 recognizes and binds to the apical UGU motif of pri-miRNAs through an RNA-binding heme domain (Rhed) (Partin et al., 2017; Dang et al., 2020), which is thought to guide Drosha towards the basal junction and prevent abortive pri-miRNA cleavages (Nguyen et al., 2015; Herbert et al., 2016).

Recent studies have demonstrated that the microprocessor complex exists as a heterotrimer even before pri-miRNA recognition and binding (Nguyen et al., 2015; Herbert et al., 2016), and that both the apical and basal junctions of the pri-miRNA cooperatively coordinate Drosha's cleavage site (Ma et al., 2012; Burke et al., 2014).

Within the microprocessor complex, Drosha surrounds the basal segment of the pri-miRNA and binds to pri-miRNAs at the ssRNA-dsRNA junction (Nguyen et al., 2015; Kwon et al., 2016). This binding positions the Drosha cut site 11bp from the basal junction of the pri-miRNA hairpin stem and acts as a "molecular ruler" (Nguyen et al., 2015). Subsequently, Drosha cleaves the pri-miRNA via intramolecular dimerization of its RNAse III domains (RIIIDa cleaves the 3' strand and RIIIDb domain cleaves the 5' strand), leading to the release of a ~ 65 - 70 nucleotide pre-miRNA (Landthaler et al., 2004; Ha & Kim, 2014; Nguyen et al., 2015) that contains a characteristic 2nt 3' overhang (Lee al., 2003; Han et al., 2004; Zhang et al., 2004).

Whilst a small number of non-miRNA transcripts containing hairpins can be processed by the canonical pathway (Karginov et al., 2010; Macias et al., 2012; Kim et al., 2017), the vast majority are not processed by this pathway. The microprocessor complex is able to specifically recognize and bind to pri-miRNAs, as opposed to other secondary structures present in transcripts, and several studies, described below, have elucidated the mechanisms behind this process.

1.1.2.2.2 Pri-miRNA structure and regulatory motifs that allow and enhance pri-miRNA processing

Pri-miRNAs have characteristic secondary structures consisting of a hairpin or stem-loop (also known as the apex) flanked by a long ssRNA sequence on both sides (also known as the basal junction) (Auyeung et al., 2013). This structure, along with the presence of several regulatory motifs,

allows pri-miRNAs to be recognized and distinguished from other hairpin-containing transcripts by the microprocessor complex and be subsequently processed. Several studies have reported that efficient processing of pri-miRNAs by the microprocessor requires a specific stem-loop length (Han et al., 2006; Nguyen et al., 2015; Fang & Bartel, 2015; Roden et al., 2017) with an optimal stem length of 36 ± 3 nt, equivalent to 3 helical turns (Roden et al., 2017). In addition, a pri-miRNA apical loop ≥ 10 nt is thought to allow efficient processing (Ma et al., 2013) up to a maximum of 15 nt (Zeng et al., 2005; Zhang & Zeng, 2010). A single-stranded flanking sequence longer than 9 nt on either side of the pri-miRNA hairpin has also been shown to be required for its processing (Zeng & Cullen, 2005), as well as bulge-enriched and bulge-depleted regions within the hairpin (Sperber et al.,2014; Roden et al., 2017).

Pri-miRNAs also contain several primary sequence regulatory motifs that guide and enhance their processing (Figure 1.3). In humans, nearly 79% of pri-miRNAs contain either a basal UG motif, a UGU/UGUG motif in their terminal loop or a 3' CNNC motif (Auyeung et al., 2013). The basal UG and terminal UGU/UGUG motifs have been shown to increase pri-miRNA processing (Auyeung et al., 2013; Nguyen et al., 2015), which has been proposed to enhance the processing of hairpins with non-optimal lengths (Fang & Bartel, 2005). The CNNC motif is associated with SRp20/SRSF3 and DDX17 binding (Auyeung et al., 2013; Mori et al., 2014) and is enriched in and selectively enhances the processing of optimal length pri-miRNAs (Roden et al., 2017).

More recently, Alarcon and colleagues (2015a, b) demonstrated that methylation of a subset of pri-miRNAs by methyltransferase-like 3 (METTL3), originating an N6-methyl-adenosine mark in GGAC motifs, is a critical post-transcriptional modification for pri-miRNA recognition. N6-methyl-adenosine are recognized by hnRNPA2B1, which recruits the microprocessor complex via interaction with DGCR8 and promotes pri-miRNA processing (Alarcon et al. 2015a, b).

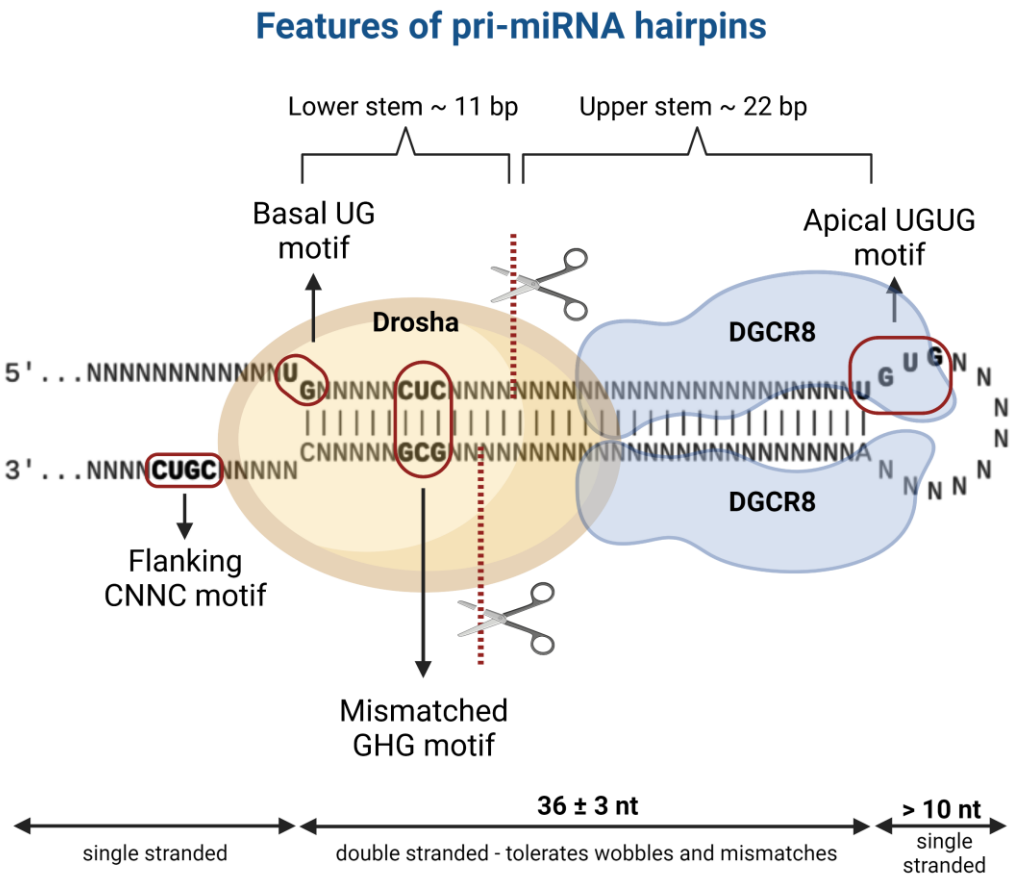


Figure 1.3 – Overview of the structure of pri-miRNA and regulatory motifs that enhance miRNA processing by the microprocessor complex.

Pri-miRNAs are capped and polyadenylated (Lee et al., 2004; Cai et al., 2004). Several regulatory sequence motifs involved in pri-miRNA recognition have been described in humans. The basal UG motif located at positions -14 and -13 from the 5' Drosha cleavage site has been found present in 24% of miRNAs tested. Drosha interacts with the UG motif strengthening the binding of Drosha to the ssRNA-dsRNA junction, leading to enhanced pri-miRNA processing and ensuring accuracy (Nguyen et al., 2015; Auyeung et al., 2013). The apical UGUG motif was found to be present in 20% of miRNAs. The DGCR8 dimer binds to this motif with the help of hemin via the Rhed domain, leading to the correct positioning of Drosha at the basal junction (Nguyen et al., 2015; Nguyen et al., 2018; Auyeung et al., 2013; Partin et al., 2017). The 3' CNNC motif was found present in 30% of miRNAs. The distance between the CNNC motif and the basal junction is vital for pri-miRNA processing (Roden et al., 2017). This motif is found ~17–18 nt downstream from 3p mature miRNA and is associated with SRp20/SRSF3 binding, which recruits Drosha to the basal junction enhancing Microprocessor activity (Auyeung et al., 2013; Mori et al., 2014; Fernandez et al., 2017; Kim et al., 2018). The mismatched GHG motif (where H can be any nucleotide other than G) is located 7-9 nucleotides from the basal junction on the 3' arm. Drosha

interacts with this motif via its dsRNA-binding domain to precisely find pri-miRNA cleavage sites (Fang & Bartel, 2015; Kwon et al., 2019). Given that ~20% of human microRNAs lack any of these sequence motifs, it is plausible that there are other regulatory motifs yet to be discovered. In addition, many pri-miRNAs possess alternative Drosha cleavage sites and the mechanisms governing the choice of cleavage sites by Drosha, and possible motifs that influence that choice are poorly understood (Li, S. et al., 2020). Adapted from: Fang & Bartel, 2015. Created with Biorender.com

1.1.2.2.3 Pre-miRNA export from the nucleus

Pre-miRNAs are exported to the cytoplasm through the nuclear envelope by Exportin-5, a Ran-GTP-dependent RNA binding protein (Yi et al., 2003; Bohnsack et al., 2004). The 2nt 3' overhang is required for Exportin-5 to be able to recognize the pre-miRNA (Zheng & Cullen, 2004), which is transported as an exportin 5 – Ran-GTP – pre-miRNA complex (Yi et al., 2003; Bohnsack et al., 2004). Once in the cytoplasm, the Ran-GTP is hydrolyzed leading to the release of both Ran and the pre-miRNA, which will be subsequently processed into mature miRNA (Bohnsack et al., 2004).

1.1.2.2.4 Pre-miRNA processing by Dicer

Once in the cytoplasm, pre-miRNAs are further processed by Dicer. Dicer is an L-shaped RNase III endonuclease with the ability to recognize the 3' overhang of pre-miRNAs, but it can also recognize the 5' end in some instances (Zhang et al., 2004; Lau et al., 2009; Park et al., 2011; Taylor et al., 2013; Liu et al., 2018). The 3' 2nt overhang of the pre-miRNA is anchored in Dicer's PAZ domain (Tian et al., 2014), and the double-stranded hairpin structure aids the binding between Dicer and the pre-miRNA (Tsutsumi et al., 2011; Feng et al., 2012). Dicer-associated proteins such as TRBP (transactivation-response (TAR) RNA binding protein) are bound to another

Dicer domain (DexD/H-box helicase domain) and act as a co-factor for Dicer, regulating its dicing activity as well as substrate selection and pre-miRNA cleavage site (Chendrimada et al., 2005; Lee et al., 2006b; Du et al., 2008; Daniels et al., 2009; Lee & Doudna, 2012; Wilson et al., 2015). This region has also been shown to interact with pre-miRNA terminal loops (Liu et al., 2018), which may have implications in terms of allosteric effects by different terminal loop lengths on Dicer's activity. Dicer's cleavage site also depends on pre-miRNA structure (Gu et al., 2012). Dicer cleaves pre-miRNAs between 21-25 nt from the base, removing the terminal loop (Bernstein et al., 2001; Feng et al., 2012; Denli et al., 2004; Okada et al., 2009) and creating a mature miRNA duplex (Zhang et al., 2004).

1.1.2.2.5 RISC assembly

The miRNA duplex is loaded to an Argonaute (AGO) protein (AGO1 - 4 in humans) to form the RNA-induced silencing complex (RISC), which represses target gene expression (Bernstein et al., 2001; Schwarz et al., 2003; for review, see: Nakanishi, 2016; Iwakawa & Tomari, 2022). The RISC-loading complex (RLC) is the essential structure required for loading miRNA duplexes into RISC and consists of Dicer, AGO2 and TRBP. Following pre-miRNA cleavage by Dicer, the miRNA duplex is released. Subsequently, the duplex is rebound by Dicer in another position (Noland et al., 2011). TRBP is a dsRNA binding protein that acts as a strand asymmetry sensor (Chendrimada et al., 2005) and orientates the miRNA duplex within Dicer so it can be loaded onto an AGO protein in an ATP-dependent manner (Tomari et al., 2004; Nakanishi, 2016). TRBP has also been shown to recruit AGO2 to load the RNA duplex generated by Dicer (Gregory et al., 2005). The Hsc70/HSP90 chaperone machinery is required to keep AGO proteins in an open conformation until the miRNA duplex is loaded (Johnston et al., 2010; Iwasaki et al., 2010), which leads to the unwinding of the duplex.

miRNA duplexes have directionality - a 3p or 5p suffix designates miRNAs derived from the 3′ end and 5' end, respectively. Both strands can be loaded into AGO and form a functional RISC complex, and strand selection is a tightly regulated process. The fraction of 3p or 5p strand RISC complexes varies significantly for each miRNA depending on cell type, developmental stage, and cellular context (including several diseases) and ranges from equal amounts to predominantly one form (for review, see: Meijer et al., 2014). Generally, the strand with lower thermodynamic stability at its 5' - end (weakest binding) between the 5p and the 3p strand, or the strand with an uracil 5'-end and an excess of purines is preferentially loaded into the AGO and becomes the guide strand (Khvorova et al., 2003). The choice of strand can also be affected by the type of AGO present, as the PAZ and MID domains of AGO3 have been shown to specifically enhance the passenger strand expression and activity of let-7a in comparison with let-7a processed by AGO1, AGO2 or AGO4, independent of the 5′-end thermodynamic stability (Winter & Diederichs, 2013). The unloaded strand, also known as the passenger strand, is usually degraded by several mechanisms, depending on the degree of complementarity between the duplex (Schwarz et al., 2003; Khvorova et al., 2003; Ha & Kim, 2014). It is also possible for both the passenger and the guide strand to co-accumulate as a miRNA pair and target different sets of mRNAs separately (Ro et al., 2007). Moreover, a study in *C. elegans* demonstrated that the presence or absence of specific target mRNAs protected their cognate miRNA from degradation, culminating in specific miRNA passenger strand accumulation, which suggests mRNAs and miRNAs can mutually regulate each other (Chatterjee et al., 2011).

1.1.2.3 Non-canonical miRNA Biogenesis Pathways

Several non-canonical miRNA biogenesis pathways have been described. These can be grouped into Drosha/DGCR8-independent and Dicer-independent pathways (Figure 1.4).

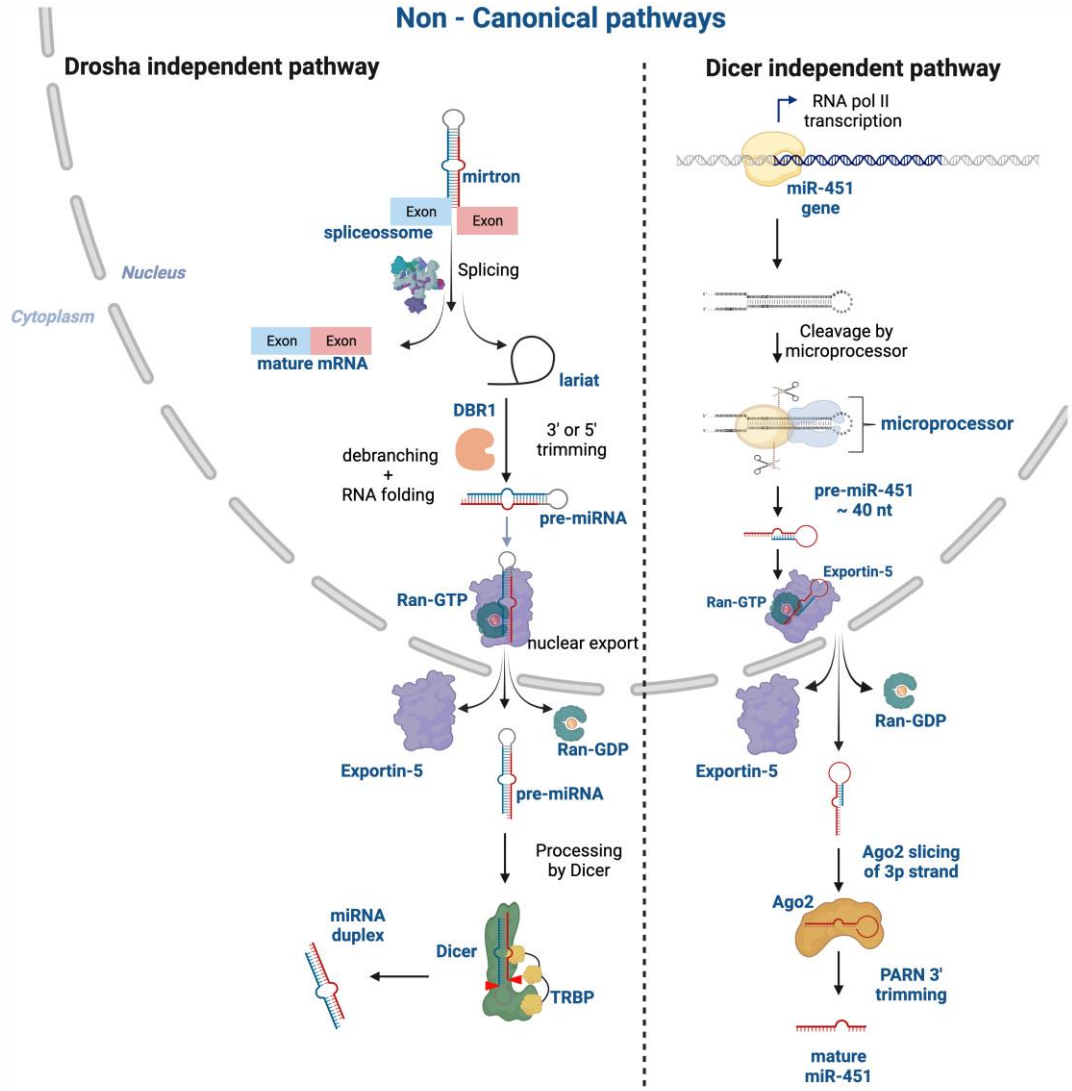


Figure 1.4 – Non-canonical miRNA biogenesis pathways - Drosha independent pathway and Dicer independent pathway.

Drosha independent pathway - MiRtrons are processed by the Drosha independent pathway and are spliced out by the spliceosome, which originates an intron lariat. The intron lariat is subsequently debranched by the lariat debranching enzyme (DBR1). If a mirtron is 3' or 5' tailed, it also undergoes trimming. Once exported via exportin-5, miRtron-derived pre-miRNAs are cleaved by Dicer and its co-factor TRBP, leading to the formation of a miRNA duplex. Dicer independent pathway – miR-451 is processed through the Dicer-independent pathway. After being processed by the microprocessor complex, the pre-miRNA is too small to be recognized by Dicer (~ 40 nt). In the cytoplasm, the entire miR-451 pre-miRNA is loaded into AGO2, which

cleaves half of the passenger strand (3’ arm) via AGO2-mediated slicing (Yang et al., 2010). This cleavage is followed by 3’-5’ trimming of the remainder of the passenger strand by polyA-specific ribonuclease (PARN) (Yoda et al., 2013). Adapted from: Yang & Lai, 2011; Miyoshi et al., 2010. Created with Biorender.com

1.1.2.3.1 Drosha/DGCR8-independent pathway

Pre-miRNAs generated by the Drosha/DGCR8-independent pathway are similar to other Dicer substrates, for example, miRtrons (short introns) which are produced by excision of small mRNA introns (~56nt) by the spliceosome (Ruby et al., 2007; Okamura et al., 2007; Berezikov et al., 2007). Splicing leads to the formation of an intron lariat, creating a stable hairpin with a shorter stem than canonical pri-miRNAs (Westholm & Lai, 2011). Due to being shorter hairpins, mirtron-derived pri-miRNAs cannot be processed by Drosha/DGCR8 and are instead debranched by the lariat debranching enzyme (DBR1) (Okamura et al., 2007; Ruby et al., 2007) and subsequently exported to the nucleus for further processing.

Moreover, 7-methylguanosine (m7G)-capped pre-miRNAs are also processed by the Drosha/DGCR8- independent pathway. These pre-miRNAs are directly transcribed by RNA pol II, bypassing cleavage by Drosha and are exported to the cytoplasm through exportin-1. After Dicer cleavage, the presence of the m7G cap prevents the 5p miRNA from being loaded into AGO, which creates a strong 3p strand bias (Xie et al., 2013).

1.1.2.3.2 Dicer-independent pathway

Drosha processes pri-miRNAs generated by the Dicer-independent pathway from endogenous short hairpin RNA (shRNA) transcripts (Yang et al., 2010). The originating pre-miRNAs are too small to be Dicer substrates,

and the entire pre-miRNA is loaded into AGO2 (Yang et al., 2010). AGO2 has an intrinsic endonuclease ability, and these pre-miRNAs undergo AGO2-dependent slicing of the 3p strand, leading to the formation of a 30nt intermediary, followed by 3′-5′ trimming of the 3p strand (Cheloufi et al., 2010; Yoda et al., 2013). This process is promoted by the activity of the eukaryotic translation initiation factor (EIF1A), another component of RISC (Yi et al., 2015). The only miRNA known to be processed by this pathway is miR-451, a miRNA essential for erythropoiesis (Dore et al., 2008), which is processed by the Dicer-independent pathway due to its pre-miRNA structure being so short that it cannot be recognized by Dicer (Cheloufi et al., 2010).

1.1.3 miRNAs target recognition

miRNAs were originally thought to predominantly bind to the 3′-UTR of their mRNA targets (Bartel, 2009). Plant miRNAs require extensive complementarity with their targets (Rhoades et al., 2002). In contrast, in animals, complementarity between the 5' proximal "seed region" (nucleotides 2 to 7) of the miRNA and its target is sufficient for RISC mRNA recognition and binding through Watson-Crick base pairing (Lewis et al., 2003; Bartel, 2009). Currently, there are 2654 mature miRNAs in humans annotated in miRbase v22.1, and over 45,000 miRNA response elements (MRE) have been found in the 3'-UTR of more than 60% of all coding genes, suggesting that miRNAs regulate the majority of genes (Friedman et al., 2009). Interestingly, isomiRs can contain untemplated post-transcriptional modifications within their seed region, which may increase the number of coding genes regulated by miRNAs (Guo & Chen, 2014).

A microarray study has demonstrated that some miRNAs that are preferentially expressed in the brain (miR-124) and muscle (miR-1) can moderately downregulate hundreds of mRNA targets in cell culture (Lim et

al., 2005). In most cases, miRNAs fine-tune gene expression and have mild to moderate effects (Lewis et al., 2003), often repressing the expression of target mRNAs by < 20% (Baek et al., 2008; Selbach et al., 2008).

mRNAs can have multiple target sites and be regulated cooperatively (Krek et al., 2005; Friedman & Burgue, 2014) by multiple miRNAs in a log-additive manner (Grimson et al., 2007). This phenomenon is prominent in the brain, as neuronal mRNAs possess longer 3'-UTRs and an increased density of miRNA response elements (Cacchiarelli et al., 2008; Barbash et al., 2014). Some mRNAs have multiple target sites for the same miRNA species and can be regulated by multiple copies of the same miRNA (Lee et al., 1993; Reinhart et al., 2000; Mayr et al., 2007). A small number of mRNAs (7% of genes with MREs) have more than one conserved site for the same miRNA family, whereas the majority of mRNAs (72% of genes with MREs) have target sites for multiple independent miRNA families (Friedman et al., 2009). In addition, miRNAs located in the same polycistronic cluster are usually coexpressed and have either overlapping predicted targets or target members of the same pathway (Tsang et al., 2010). A recent study by Cherone and colleagues (2019) has demonstrated that specific genetically independent brain-enriched miRNAs can act together to cooperatively repress mRNA targets robustly by 5-10 fold (Cherone et al., 2019). These phenomena make validating miRNA targets difficult (Alvarez-Saavedra & Horvitz, 2010).

1.1.4 miRNA target prediction

An absence of high-throughput methods for miRNA target identification and the complexity of validating miRNA targets lead to the development of several algorithms that predict miRNA target sites (Lewis et al., 2003; Kiriakidou et al., 2004; John et al., 2004; Krek et al., 2005).

Because target sites were thought to be predominantly located in the 3'-UTR (Bartel et al., 2009), these algorithms initially only mined miRNA target sequences in the 3'-UTR of mRNAs by identifying evolutionarily conserved sites with complementarity to miRNA seed regions (a 7nt match) (Lewis et al., 2003; Lewis et al.,2005; Rajewsky, 2006). This view predominated, despite several studies providing evidence of widespread miRNA binding to mRNA protein coding sequence (CDS) (Easow et al., 2007; Hausser et al., 2013; Liu et al., 2015), miRNA binding to individual 5'-UTR and CDS (Easow et al., 2007; Forman et al., 2008; Lytle et al., 2007; Orom et al., 2008; Tay et al., 2008; Qin et al., 2010) and even miRNA binding to promoters (Place et al., 2008). Several more recent studies have indicated that both this view and miRNA target predictions are, at best, an incomplete picture. Chi and colleagues (2009) generated interaction maps for the 20 most abundant mouse brain miRNAs, including miR-124, by HITS-CLIP (high-throughput sequencing of RNA isolated by crosslinking and immunoprecipitation). This method allows for the sequencing of mRNA segments bound to AGO by using UV to crosslink proteins with RNA, followed by immunoprecipitation of AGO proteins and cDNA sequencing. The authors reported that 27% of mRNAs bound to AGO had no predicted seed match among the top 20 most abundantly expressed miRNAs (Chi et al., 2009). Subsequently, Hafner and colleagues (2010) modified this method (PAR-CLIP) in HEK293 cells to perform a transcriptome-wide survey of the binding sites of several RNA binding proteins, including AGO1-4. The authors reported that most miRNA binding sites were present within exonic regions (84%). Of these, only 46% of binding sites were located in the 3'-UTR of mRNA targets, with 50% of binding sites in the CDS and 4% in the 5'- UTR (Hafner et al., 2010). Inhibition of the top 25 most abundantly expressed miRNAs in these cells had a negligible effect on the expression of mRNAs with miRNA binding sites present exclusively in their CDS (Hafner et al., 2010), which suggests CDS miRNA binding sites help fine-tune mRNA expression. These findings were subsequently validated by crosslinking ligation and Sequencing of Hybrids (CLASH), where similar findings were reported (Helwak et al., 2013). Helwak and colleagues (2013) also reported that 18% of miRNA-mRNA pairs did not bind via the seed region located in

the 5’ end and displayed Watson-Crick base pairing at the 3’ end instead. This finding suggests that some miRNAs do not pair through the seed region and that the initial algorithms developed to predict miRNA targets were incomplete.

Several studies proposed that miRNA binding to these non-canonical binding sites led to mRNA repression (Chi et al., 2012; Loeb et al., 2012; Helwak et al., 2013; Grosswendt et al., 2014). However, a subsequent meta-analysis demonstrated that this is not the case, suggesting that most miRNA-binding functional sites are canonical (Agarwal et al., 2015).

Direct assays of miRNA-target interactions based on CLIP methods have led to the development of improved miRNA target prediction algorithms (Liu et al., 2013; Erhard et al., 2013) and bioinformatic tools that include CDS and 5’-UTR binding regions have been developed (Reczko et al., 2012; Dweep & Gretz, 2015; Riolo et al., 2021). Moreover, analysis of miRNA-mRNA interactions derived from the CLASH method has been employed to develop and improve TargetScan (Agarwal et al., 2015; McGeary et al., 2019). TargetScan is the current state-of-the-art sequence-based miRNA target prediction tool (Riffo-Campos et al., 2016), outperforming other miRNA target-predicting tools (Kern et al., 2021).

TargetScan predicts miRNA targets by searching for an exact match between the seed region of a miRNA and 8mer, 7mer, and 6mer sites in the 3’-UTR of its potential targets (Agarwal et al., 2015; McGeary et al., 2019; Riolo et al., 2021) (Figure 1.5). Additional predictive features associated with target repression, such as seed-pairing stability, target site abundance and conservation and presence of 3’ compensatory pairing, are taken into consideration, and potential miRNA targets are ranked based on the predicted efficacy of targeting, which is computed into context ++ scores (Agarwal et al., 2015; McGeary et al., 2019).

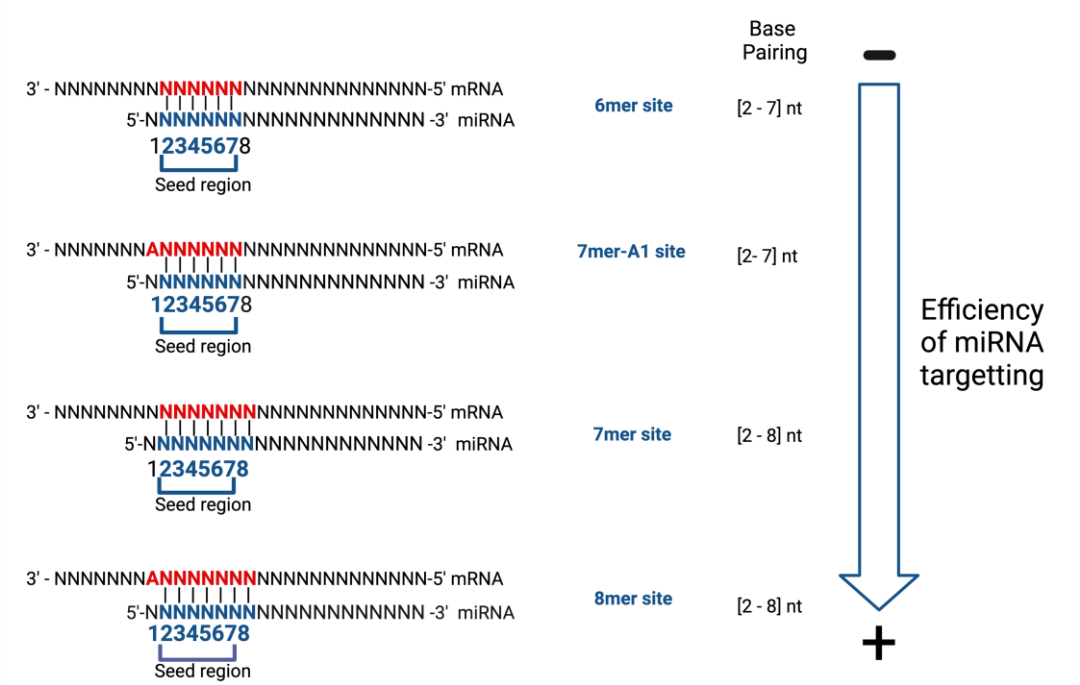


Figure 1.5 – Types of miRNA response element sites in mRNA 3'-UTR employed by TargetScan to predict miRNA targets in order of repressive efficacy from less repressive (6mer site) to more repressive (8mer site).

6mer site: Perfect match position 2–7 (the seed), the least stringent; 7mer-A1 site: Perfect match from nucleotide 2 to nucleotide 7 (the seed) with an A opposite position 1; 7mer-m8 site: Perfect match from nucleotide 2 to nucleotide 8 (the seed + position 8) without the adenosine "A" opposite position 1; 8mer site - perfect match from 2-8 nt of the mature miRNA (seed + position 8), with an adenosine "A" in mRNA opposite position 1, the most stringent (Friedman et al., 2009). The presence of adenosine opposite position 1 facilitates target recognition within AGO2 (Schirle et al., 2014) and enhances miRNA repression (Nielsen et al., 2007). Red mRNA sequence - mRNA positions that determine the type of site (8mer, 7mer, 6mer). Blue miRNA sequence (N) – miRNA positions with perfect Watson-Crick complementarity to target. Blue numbers – nucleotide positions that determine the type of site. Adapted from: Riolo et al., 2021. Created with Biorender.com

1.1.5 Primate specific miRNAs

Novel miRNA genes can arise by multiple mechanisms (for review, see: Lu et al., 2008; Berezikov, 2011), such as duplication of an existing miRNA gene which subsequently gains a new function (Hertel et al., 2006; Ruby et al., 2007; Lu et al., 2008), mutations in the seed region (Lu et al., 2008) or antisense transcription of an existing miRNA gene (Tyler et al., 2008). Additionally, introns can give rise to unstructured transcripts that may gradually evolve and form hairpin structures identifiable by the microprocessor complex, originating a new miRNA gene (Berezikov et al., 2011). This event commonly occurs in species-specific miRNAs (Campo-Paysaa et al., 2011). Moreover, transposable elements, as well as snoRNAs and tRNAs, can originate transcripts that may also give rise to miRNA-like hairpin structures identifiable by the microprocessor complex, originating new miRNA genes (Berezikov et al., 2011; Smalheiser & Torvik, 2005; Hertel et al., 2006; Yuan et al., 2011).

Phylogenetic analysis of 1433 miRNAs expressed in humans indicates that a large proportion of these miRNAs (53%) originated in primates, with 28% of these first arising in the hominid lineage (Iwama et al., 2013). The emergence of primate- and human-specific miRNAs is likely to have had an essential role in shaping gene networks involved in brain size expansion and the increased complexity and sophistication observed in primate and human brains (Arcila et al., 2014; Prodromidou & Matsas, 2019). In the developing brain, over 100 primate-specific miRNAs and 14 human-specific miRNAs have been identified (Berezikov, 2011; Hu et al., 2012), the majority of which are involved in neuronal progenitor proliferation, generation of neurons, brain function and cognition (Nowakowski et al., 2013; Arcila et al., 2014; Nowakowski et al., 2018; Prodromidou & Matsas, 2019).

1.1.6 miRNAs function: post-transcriptional gene regulation

Post-transcriptional gene silencing (PTGS) by RISC can occur through repression of translation, sequential degradation or direct target mRNA cleavage by a catalytically active AGO2 protein (Lee et al., 1993; Rhoades et al., 2002; Llave et al., 2002; Bartel, 2004; Bushati & Cohen, 2007). Moreover, the biological outcome of miRNA-mediated PTGS can be altered by factors contributing to the strength of the miRNA:mRNA binding and the repressive effect of the target site (Carroll et al., 2014).

AGO2-mediated RNA cleavage occurs when there is perfect complementarity between the miRNA and the mRNA target. This mechanism commonly occurs in plants (Llave et al., 2002), but there is evidence of at least one case of AGO2 - mediated cleavage occurring in humans between miR-196 and its target HOXB8 (Yekta et al., 2004). In mammals, studies have reported that sequential degradation of mRNA targets accounts for the majority (66 – 90%) of miRNA-mediated PTGS (Guo et al., 2010; Eichhorn et al., 2014), with translational repression contributing only a small proportion (10 – 25 %) of the overall repression (Hendrickson et al., 2009; Guo et al., 2010).

1.1.6.1 miRNA mediated mRNA destabilization and mRNA degradation

miRNA-mediated mRNA destabilization and mRNA degradation are the primary means of miRNA PTGS in mammals (Guo et al., 2010; Eichhorn et al., 2014). Sequential degradation of mRNA involves mRNA destabilization through a two-step deadenylation process, 5’ - decapping and 5’ - 3’ target mRNA degradation (Eulalio et al., 2009). For mRNA degradation to occur,

RISC binds to the 3'-UTR of target mRNAs which leads to AGO recruiting scaffold protein glycine-tryptophan protein of 182 KDa (GW182) (Rehwinkel et al., 2005). GW182 then recruits the PAN2-PAN3 (PolyA-specific Nuclease subunit) complex and guides it to the poly-A tail of the target mRNA, where the PAN2 subunit performs an initial deadenylation of the target mRNA (Braun et al., 2011). In the second step of this process, a complex formed between a 3'-5' exonuclease (CCR4a) and a scaffolding protein (NOT1) removes the remainder of the Poly(A) tail. Following this, GW182 sequesters the target mRNA in processing-bodies (P-bodies) and the mRNA 5' cap is removed by the decapping protein 2 (DCP2) followed by destabilized mRNA degradation by exoribonuclease 1 (XRN1) (Eystathioy et al., 2003; Andrei et al., 2005; Jakymiw et al., 2005; Liu et al., 2005; Sen & Blau, 2005). Target mRNA sequestration is a dynamic occurrence, and sequestered mRNAs are released from P-bodies and translated when cells are under stress (Bhattacharyya et al., 2006).

1.1.6.2 miRNA mediated translational repression

Most studies on translational repression of endogenous mRNAs support the notion that miRNAs repress translation primarily by blocking the initiation of translation (Pillai et al., 2005; Humphreys et al., 2005; Hendrickson et al., 2009; Eichhorn et al., 2014).

RISC can block the initiation of translation via interaction with translation initiation factor eIF4e (Humpfreys et al., 2005; Richter & Sonenberg, 2005; Mathonnet et al., 2007; Wakiyama et al., 2007; Chen & Gao, 2017), which is part of the eukaryotic initiation complex eIF4F. This blocking will prevent the eIF4F complex from recruiting the ribosomal 40S subunit and forming a 43S pre-initiation complex (Richter & Sonenberg, 2005; Ricci et al., 2013; Fukaya et al., 2014). Moreover, GW182 proteins are crucial for translation repression and interact with PABP (poly(A)-binding protein) to recruit several auxiliary proteins such as PAN2-PAN3 and CCR4:NOT deadenylases along with

Dcp1:Dcp2 decapping complexes to inhibit the initiation of translation (Behm-Ansmant et al., 2006; Braun et al., 2011; Fabian et al., 2009; Kuzuoglu-Ozturk et al., 2012; Huntzinger et al., 2013; Mathys et al., 2014).

The mechanisms behind translational repression have not been fully elucidated. miRNAs and their targets have been observed to associate with polysomes in sucrose sedimentation gradients. This observation led to post-initiation translational repression mechanisms being proposed (Maroney et al., 2006; Petersen et al., 2006), such as inhibition of elongation, co-translational degradation and premature termination of translation (for review, see: Filipowicz et al., 2008). These proposed mechanisms have caveats. For instance, the notion that miRNAs inhibit elongation is mainly derived from studies using reporter constructs and artificial systems, which usually do not behave like endogenous mRNAs (Eichhorn et al., 2014).

In contrast, several studies support the notion that repression of translation is functionally linked to mRNA destabilization and decay (Hendrickson et al., 2009; Bazzini et al., 2012; Tat et al., 2016). It has been suggested that miRNA-mediated PTGS occurs in a “two-hit model”, with translational repression being the first hit leading to an immediate stop in translation, followed by mRNA destabilization and degradation, which would complete mRNA silencing (Zdanowicz et al., 2009; Bazzini et al., 2012; Djuranovic et al., 2012; Bethune et al., 2012). This model is consistent with observations that translational repression occurs quite rapidly but has a weak effect on mRNA silencing (Eichhorn et al., 2014).

1.1.6.3 Non-canonical miRNA functions

1.1.6.3.1 Nuclear miRNA functions

Several components of the miRNA RISC machinery have been detected in the nucleus, including AGO, Dicer and GW182 (Hwang et al., 2007; Ohrt et

al., 2008; Tan et al., 2009; Sinkkonen et al., 2010; Till et al., 2007; Ahlenstiel et al., 2012; Nishi et al., 2013). Moreover, several studies have reported the presence of specific AGO-loaded miRNAs in the nuclei of animal cells (Politz et al., 2009; Hwang et al., 2007; Kim et al., 2008; Foldes-Papp et al., 2009), which have been found by some (but not all) studies to form complexes with RNAi factors (Ohrt et al., 2008; Khudayberdiev et al., 2013; Gagnon et al., 2014). In neural stem cells, a substantial number of miRNAs have been found in the nucleus, with some miRNAs being enriched in the nucleus (Jeffries et al., 2011). Specific miRNAs are enriched in the nuclei (or nucleoli) of several cancer lines (Park et al., 2010; Li et al., 2013), myoblasts (Politz et al., 2009) and post-mitotic neurons (Khudayberdiev et al., 2013). These observations suggested novel non-canonical miRNA functions.

The mechanisms of mature miRNA transport into the nucleus have not been fully elucidated. However, it is thought that mature miRNAs are loaded into AGO in the cytoplasm and can shuttle in and out of the nucleus via importin-8 and exportin-1, where they accumulate depending on the presence of a target (Castanotto et al., 2009; Weinmann et al., 2009). A 3′ hexanucleotide regulatory motif (AGUGUU) has been shown to direct miR-29b to the nucleus in HeLa cells (Hwang et al., 2007). However, this motif is not conserved between other nuclear miRNAs (Hwang et al., 2007; Jeffries et al., 2011). In the nucleus, miRNAs have been found to regulate gene expression through multiple mechanisms. These include AGO2-mediated cleavage of circular antisense transcripts, leading to a decrease in mRNA levels (Hansen et al., 2011), cleavage of long non-coding RNAs (Leucci et al., 2013) or binding to other pri-miRNAs and preventing their processing (Tang et al., 2012). Moreover, several miRNAs can bind to complementary promoters of target genes to regulate gene expression at the transcriptional level: either inducing gene expression by competing with promoter-binding repressors (Place et al., 2008) or repressing gene expression by implementing silent–state histone modifications such as an increase in promoter H3K27me3, linked to heterochromatin formation (Kim et al., 2008; Tan et al., 2009; Benhamed et al., 2012).

1.1.6.3.2 miRNA mediated translation activation

In some cases, such as in quiescent cells, miRNAs can promote the translation of specific mRNAs via the formation of a micro-ribonucleoprotein (microRNP) complex with Fragile X mental retardation related protein 1 (FXR1) (Vasudevan et al., 2007; Vasudevan & Steitz, 2007; Lin et al., 2011; Truesdell et al., 2012; Valinezhad et al., 2014). GW182 is downregulated in the G0 state, which results in the absence of interaction between GW182 and AGO2. As a consequence, AGO2 is then free to interact with FXR1, which eventually results in miRNA upregulation of translation of target mRNAs (Yang et al., 2004; Vasudevan et al., 2007).

1.1.7 Regulation of miRNA biogenesis

miRNA biogenesis is tightly regulated both spatially and temporally (Lee et al., 2016) by cofactor proteins, RNA precursors, and post-translational modifications (PTMs) (Lee et al., 2006; Heale et al., 2009; Lee & Doudna, 2012; Heo et al., 2012). miRNAs are also extensively regulated by RNA binding proteins (RBPs) such as lin-28 homologue A (LIN28A) and hnRNPA1 (for review, see: Ha & Kim, 2014; Treiber et al., 2019) and by long non-coding RNAs (Krol et al., 2015; Jiang et al., 2017).

PTMs of miRNA biogenesis machinery, such as phosphorylation, ubiquitylation and SUMOylation of DGCR8, Drosha, TRBP and AGO, can remodel miRNA activity following an external stimulus (for review, see: Treiber et al., 2019). For instance, DGCR8 is heavily phosphorylated by mitogenic MAPKs, which increases the activity of the microprocessor and results in increased biogenesis of miRNAs with a pro-growth profile (Herbert et al., 2013). TRBP is also phosphorylated by ERK and S6K, which increases the stability of the TRBP-Dicer complex and stimulates mature miRNA

production (Paroo et al., 2009; Warner et al., 2016). In contrast, phosphorylation of Dicer by ERK has been shown to switch-off Dicer activity and is a requirement for embryonic gene expression transition from oocytes in *C.elegans* (Drake et al., 2014).

1.1.8 miRNA turnover

The expression level of miRNAs is regulated not only by their biogenesis but also by their degradation. Association with AGO proteins is critical to miRNA function and increases the stability of mature miRNAs, thereby regulating their abundance (Grishock et al., 2001; Diederichs & Haber, 2007; Winter & Diederichs, 2011). While miRNAs are remarkably stable and possess a decay period ten times longer than mRNAs (Gantier et al., 2011), they also decay in response to different cellular cues. miRNA turnover is significant during development, where the expression of miRNAs changes quickly as cell types convert (Ruegger & Grosshans, 2012). Target mRNAs have been shown to promote post-transcriptional miRNA modifications that control the rate of miRNA decay (Baccarini et al., 2011; Marcinowski et al., 2012). In neurons, miRNA decay periods are more variable than in other cells (Krol et al., 2010) and can be modulated by several physiological stimuli, including blocking glutamate receptors (Krol et al., 2010; Kocerha et al., 2009) and synaptic stimulation (Wibrand et al., 2010), which hints at the role miRNAs have in neuronal function.

1.2 microRNAs in brain development and function

1.2.1 Foetal brain development

Human brain development starts during the third post-conceptional week (PCW) with the folding and fusion of ectoderm to form the neural tube (Ladher & Schoenwold, 2005) and continues into early adulthood through a series of accurately organized processes, which include the differentiation, migration, and maturation of diverse cell types, and the formation and refinement of functional neuronal circuits which underlie the mechanisms behind dramatic structural changes (Hofman, 2012; Workman et al., 2013; Taverna et al., 2014; Prodromidou & Matsas, 2019).

The formation of the forebrain, the midbrain, and the hindbrain vesicles from the neural tube and subsequent formation of the telencephalon (cerebral cortex) vesicle and the diencephalon (thalamus, hypothalamus, and other structures) vesicle from the forebrain vesicle (Rash & Grove, 2006; Rhinn et al., 2006) is accompanied by a series of highly complex and dynamic sequences of temporally overlapping cellular events (Tau & Peterson, 2010).

1.2.1.1 Neuron formation

The human cortex consists of billions of neurons and glia that emerge from a monolayer of uniform proliferating neuroepithelial cells (NECs) populating the neural tube (Bayer & Altman, 1991; Bayer & Altman, 2005; Namba & Hunter, 2017). NECs become apical radial glia cells (aRGCs), which are the primary type of neural progenitor cells (NPCs) of the

telencephalon and are located in the ventricular zone (VZ), marking the beginning of cortical neurogenesis (Silbereis et al., 2016). Radial glia are embryonic neural stem cells and will give rise to the lineages of all neuronal and glial cells, depending on their location within a matrix of different molecular gradients in the ventricular zone layer (Noctor et al., 2001; Anthony et al., 2004; Kowalczyk et al., 2009). Radial glia start by increasing their pool size through symmetric divisions in the ventricular zone that lines the cerebral ventricles (Hatten, 1993; Kornack & Rakic, 1995; Rakic, 1995; Rakic, 2000; Kriegstein & Götz , 2003; Rash & Grove, 2006; Ghashghaei et al., 2007; Bystron et al., 2008). As their proliferative potential decreases, they can divide asymmetrically to generate one radial glia which will remain anchored and either a neuron or a basal progenitor cell (Kowalczyk et al., 2009; Chen & Walsh, 2002; Kingsbury et al., 2003; Miyata et al., 2001; Noctor et al., 2001; Liu & Rao, 2004; Wonders & Anderson, 2006). Basal progenitor cells delaminate and start accumulating on the basal side of the VZ, giving rise to the subventricular zone (SVZ) (Noctor et al., 2001; Haubensak et al., 2004), which rapidly expands; leading to the exponential production of a large number of neurons (Garcia-Moreno et al., 2012; Betizeau et al., 2013). At the end of neurogenesis, apical radial glia switch to gliogenesis, giving rise to oligodendrocytes, astrocytes and ependymal cells (Kriegstein & Alvarez-Buylla, 2009).

1.2.1.2 Neuron migration

Neuronal migration occurs predominantly between PCW 12-20, is mostly complete by PCW 26-29, and is crucial to the formation of functional neuronal circuits with stereotyped connectivity patterns leading to proper brain function (Gupta et al., 2005; de Graaf-Peters & Hadders-Algra, 2006; Tau & Peterson, 2010). Newly formed immature neurons migrate radially out of the VZ (or SVZ) along radial glial fibres, which are protrusions of apical radial glia that guide neurons to the cortical plate in an “inside-out” fashion,

leading to the formation of 6 cortical layers where the first generated neurons are located in deeper cortical layers; and more recently formed neurons are located in superficial cortical layers (Sidman & Rakic, 1973; Hatten, 1993; Kornack & Rakic, 1995; Rakic, 1995; Takahashi et al., 1999; Rakic, 2000). All GABAergic neurons of the cerebral cortex are formed in another, foetal-specific region called the ganglionic eminences and migrate parallel to the outer cortical surface to their final destination in the developing cortex (Van Eden et al., 1989; Monk et al., 2001; McManus et al., 2004).

1.2.1.3 Synapse and neuronal circuit formation

Once a neuron stops migrating, it begins terminal differentiation encompassing dendritic branching, axonal extension and branching to synaptic partners and the formation of dendritic spines and synaptic bouttons (Tau & Peterson, 2010; Fernandez et al., 2016; Prieto-Colomina et al., 2021). Synaptic connections occur by PCW 5 between neurons located in a temporary cortical layer structure called the preplate (Wood et al., 1992). These synaptic connections are initially transient and further refined and modified as neuronal circuits mature. The development of neuronal circuits requires the coordination of highly complex neurodevelopmental events, which are continuously refined and modified (Tau & Peterson, 2010).

1.2.2 Gene expression profiles in brain development

The complexity and cellular diversity of the human brain arise from the orchestration of gene expression patterns during foetal brain development (Silbereis et al., 2016). Several studies have shown that gene expression is more dynamic during foetal brain development than at any other life stage

(Johnson et al., 2009; Colantuoni et al., 2011; Kang et al., 2011). In addition, significant regional differences in gene expression patterns in the developing neocortex have been identified (Johnson et al., 2009; Miller et al., 2014; Pleitikos et al., 2014). Foetal brain gene expression patterns are driven by genetic factors during the first six months of foetal development (Kang et al., 2011; Pletikos et al., 2014; Bakken et al., 2016), with environmental factors having a bigger influence in the last phases of foetal brain development, as well as, early postnatal brain development (Pletikos et al., 2014). Proper brain development and function rely on the tight balance between self-amplification, self-renewal and differentiation of cortical progenitor cells (Taverna et al., 2014), with even minor departures from this balance and/or the timing of this balance producing significant differences in the cortical phenotype, including decreased or increased cortical thickness, surface area and cortical folding (Fernandez et al., 2016; Prieto-Colomina et al., 2021). This balance is achieved by tight spatial and temporal regulation of gene expression patterns mediated through several regulatory mechanisms, including the direct action of transcription factors which drive gene expression (Davuluri et al., 2008), epigenetic mechanisms such as DNA methylation and histone modifications (Henikoff & Matzke, 1997; Jaenisch & Bird, 2003; Hirabayashi & Gotoh, 2010; Maze et al., 2014; Spiers et al., 2015) and regulatory non-coding RNAs including miRNAs (Nowakowski et al., 2018; Prodromidou & Matsas, 2019).

1.2.3 Expression of miRNAs in the brain

Nearly 50% of all known miRNAs are expressed in the mammalian brain (O'Carroll and Schaefer, 2013; 70% in Adlakha and Saini, 2014), and many miRNAs are brain-enriched (Sempere et al., 2004; Landgraf et al., 2007; Bartel, 2018). Moreover, miRNAs are dynamically regulated during brain development, and many have brain region-specific expression profiles

(Sempere et al., 2004; Landgraf et al., 2007; Bak et al., 2008; He et al., 2012; Ziats & Rennert, 2014). miRNAs are also differentially expressed in different subtypes of neurons, such as in glutamatergic versus GABAergic neurons or between subtypes of GABAergic neurons (He et al., 2012). In addition, several studies have demonstrated that miRNAs are enriched in dendritic spines, axons and synapses and possess differential expression dependent on neuronal compartments (Lugli et al., 2008; Natera-Naranjo et al., 2010; O'Carrol & Schaefer, 2013; Sasaki et al., 2013). Neuronal activity has been shown to regulate the expression of synaptic miRNAs (Eacker et al., 2011; Siegel et al., 2011; Pichardo-Casas et al., 2012). These studies strongly implicate miRNAs in brain development and function (Barca-Mayo & De Pietri-Tonelli, 2014; Rajman & Schratt, 2017; Gebert & MacRae, 2019; Prodromidou & Matsas, 2019; Cho et al., 2019).

1.2.4 miRNAs and brain development

There is a substantial body of evidence indicating that miRNAs are highly expressed in the brain, where they are vital in regulating processes pertaining to brain development and neuronal function, including neurogenesis, neuronal cell–type determination and migration, axonal pathfinding, synapse formation and neuronal circuit development, among others (Nowaskowski et al., 2018; for review see: Rajman & Schratt, 2017; Prieto – Colomina et al., 2021), by targeting regulatory molecules encompassing TFs, chromatin modifiers and components of signalling pathways (Nowaskowski et al., 2018). In the developing brain, miRNAs can either be master regulators of gene expression or fine-tuners, depending on the specific miRNA and the cellular context. miRNAs act as master regulators when they promote developmental transitions by repressing transcripts associated with the previous stage. In contrast, miRNAs act as fine-tuners when they act to reduce variability in the gene expression levels

of their targets, leading to a decreased level of noise and increased robustness in signalling (Schratt, 2009a; Hornstein & Shamron, 2006; Ebert & Sharp, 2012; Rajman & Schratt, 2017).

1.2.4.1 Ablation models of miRNA biogenesis pathway in brain development

Initially, the role of miRNAs in brain development was studied by their overall ablation by targeting critical elements of the miRNA biogenesis pathway. The complete knockout of Dicer in mice is embryonically lethal, with mice dying at embryonic day (E) 7.5 before neurulation occurs, indicating the importance of miRNAs in development (Bernstein et al., 2003; Murchison et al., 2005). Moreover, in mice, the conditional knockout of Dicer at late embryonic stages (> E10.5) leads to reduced neurogenesis and cell proliferation which translates into a smaller cortex (De Pietri Tonelli et al., 2008), increased apoptosis, impaired neuronal migration and differentiation, resulting in disorganized cortical circuits (Kawase-Koga et al., 2009; Nowakowski et al., 2011; McKoughlin et al., 2012; Saurat et al., 2013). In contrast, loss of Dicer at early embryonic stages (E7.5) in the telencephalon leads to a severe histological disruption of its rostral-ventral organization due to the inhibition of miRNA let-7 (Fernandez et al., 2020). DGCR8 is exclusive to the miRNA biogenesis pathway, and homozygous DGCR8 knockout is also embryonically lethal in mice, which die at E6.5 (Wang et al., 2007; Stark et al., 2008), whilst heterozygous DGCR8 knockouts display abnormal miRNA biogenesis, accompanied by several neuronal and behavioural deficits (Stark et al., 2008). Moreover, the ablation of Drosha in forebrain neural progenitors resulted in early differentiation and loss of progenitor stemness (Knuckles et al., 2012), and AGO2 knockout mice display aberrant neural tube closure and die early during development (Liu et al., 2004).

1.2.4.2 Roles of miRNAs in foetal brain development

It is important to note that because of miRNA co-targeting and cooperative mRNA repression discussed in section 1.1.3, individual miRNAs are often not essential for viability or development (Miska et al., 2007; Kutsche et al., 2018). This effect is pronounced during brain development, where unrelated miRNAs act together to regulate key developmental genes, thereby increasing the robustness of foetal gene expression programs. For instance, neuronal differentiation induces the expression of miR-138 and miR-137, both brain-enriched miRNAs that act as a co-targetting pair of crucial neuronal differentiation-associated genes as well as one another and drive neuronal differentiation together (Cherone et al., 2019). Nonetheless, overexpression and inhibition studies have identified several miRNAs with crucial roles in brain development, some of which will be briefly discussed below. An overview of some of the key miRNAs involved in foetal brain development is provided in Table 1.1, along with their mRNA targets and the functional consequences of miRNA regulation where “+” denotes activation/ promotion of a process (in green) and “-“ denotes inhibition. Moreover, Figure 1.6 highlights some of the miRNAs crucial to different processes of foetal brain development, including NPC proliferation and differentiation, neurogenesis, neuronal migration, neuronal maturation and gliogenesis.

Table 1.1, pt. I – Key miRNAs in foetal brain development.

NSC- neural stem cell; aRGC – apical radial glia cell; IPC – intermediate progenitor cell. Adapted from: Prieto-Colomina et al., 2021

Biological process	Effect of miRNAs	mRNA targets	miRNAs	References
NSC proliferation	+	Nanog, Jarid1b, p57	miR-134, miR-137, miR-25	Meza-Soza et al., 2014; Tomasello et al., 2022
		Pten	miR-17/92	Fang et al., 2017
	-	Tlx , CyclinD1 (Wnt pathway)	miR-9, miR-137, miR-20a/20b, miR-23, and miR15-b	Zhao et al., 2009; Sun et al., 2011; Ghosh et al., 2014;
	-	Lin28, Irs-2, Hmga2, Tlx, and CyclinD1	let-7	Zhao et al., 2010;2013; Rybak et al., 2008; Fernandez et al., 2020
	-	Jag1, SCP1, PTBP1, coREST (Notch pathway)	miR-124, miR-34a, miR-23b/24/27b, and miR-9	Cheng et al., 2009; Makeyev et al., 2007; Visvanathan et al., 2007; Packer et al., 2008
Cell survival	+	Tp53	miR-29, miR-134	Park et al., 2009; Bonev et al., 2011
	-	Tp53	miR-9, miR-34a, and miR-29	
aRGC identity	-	Pax6	miR-7 and miR-9	Shibata et al., 2011; Needhamsen et al., 2014
IPC production	-	Pten , Tbr2	miR-17/92	Bian et al., 2013

Table 1.1, pt. II – Key miRNAs in foetal brain development.

Biological process	Effect of miRNAs	mRNA targets	miRNAs	References
	+	N-cadherin	miR-379-410	Rago et al., 2014
Neuron Migration	-	Dcx, REST/CoREST	miR-22, miR-124, miR-34c, miR-204, miR-134	Gaughwin et al., 2011; Volvert et al., 2014; Veno et al., 2017
		Foxp2	miR-9, miR-132	Rajman & Schratt, 2017
Neuron differentiation	+	Cyclin D1, Tlx, Sox9, and Scp1	let-7, miR-124, miR-9	Meza-Sosa et al., 2014; Volvert et al., 2014
		Sirt1, Syntaxin1A, Synaptotagmin1	miR-34a	Volvert et al., 2012
		Hmga2, Upf1, FoxG1, Meis2, Tlx	miR-128, miR-9, let-7	Volvert et al., 2012; Shu et al., 2019
		Lsd1	miR-137	Sun et al., 2011; Volvert et al., 2012; Tomasello et al., 2022
Oligodendrocyte differentiation	+	PDGFRα, Sox6, Hes5, FoxJ3, ZFP238, Lingo1, Etv5	miR-219, miR-23a, miR-338, miR-297	Dugas et al., 2010; Rajman & Schratt, 2017, Wang et al., 2017
Astrocyte differentiation	+	NfiA/B, Igf2bp2, Plagl2, Hmga2	let-7, miR-125, miR-92, miR-124	Shimazaki et al., 2016
	-	Mapk14	miR-153, miR-17/106	

1.2.4.3 Roles of miRNAs in neuronal differentiation

miR-124 and miR-9 are highly conserved miRNAs that constitute the most highly expressed miRNAs in the brain (Krichevsky et al., 2006; Gao, 2010; Han et al., 2020; Radhakrishnan & Anand, 2016) with crucial roles in neuronal development (Visvanathan et al., 2007; Radhakrishnan & Anand, 2016). miR-124 is minimally expressed in NPCs, and its expression increases during foetal brain development and upon neuronal differentiation, reaching maximum levels in mature neurons (Lagos-Quintana et al., 2002; Deo et al., 2006; Visvanathan et al., 2007; Cheng et al.,2009). In contrast, miR-9 is primarily expressed in neuronal precursors where it controls NPC numbers (Delaloy et al., 2010; Akerblom et al., 2013; Coolen et al., 2013; Radhakrishnan & Anand, 2016).

NPC maintenance depends on the Notch pathway, which promotes NPC self-renewal and inhibits neuronal differentiation. This pathway is initiated by the Notch ligand Jagged1 (Jag1) binding to the Notch transmembrane receptor (Louvi & Artavanis-Tsakonas, 2006; Imayoshi & Kageyama, 2011). miR-124 turns off the Notch pathway by suppressing Jag1, allowing neuronal differentiation to occur (Cheng et al., 2009; Liu et al., 2011; Jiao et al., 2017). Overexpression of miR-124 in HeLa cells leads to a global shift in the transcriptome towards brain-specific mRNA expression (Lim et al., 2005). This phenomenon was observed in a variety of other systems, such as neuronal progenitors, embryonic stem cells and fibroblasts (Krichevsky et al., 2006; Silber et al., 2008; Xia et al., 2012; Yoo et al., 2011), demonstrating that miR-124 can act as a master regulator of neurogenesis. miR-124 drives neurogenesis by inhibiting key transcriptional repressors of neuronal-specific genes; specifically, miR-124 targets small C-terminal domain phosphatase 1 (SCP1), preventing the phosphorylation and stabilization of the RE1 silencing transcription factor (REST) (Conaco et al., 2006; Visvanathan et al., 2007; Packer et al., 2008; Nesti et al., 2014). REST is an anti-neural transcriptional repressor, which forms a complex with its cofactors mSin3A (Grimes et al., 2000) and CoREST (Andres et al., 1999) and binds to repressor element 1

(RE1) sites upstream of neuronal genes. Through this mechanism, REST strongly represses neuronal gene transcription via histone deacetylases (HDACs) and thus must be inhibited for neuronal differentiation to occur (Ballas et al., 2005). miR-124 also inhibits the expression of the polypyrimidine-tract-binding protein (PTBP1), a splicing factor that represses neuron-specific splicing (Makeyev et al., 2007). In undifferentiated cells, high levels of REST repress the expression of several miRNAs, including miR-124 leading to a double-negative feedback loop that operates as a molecular switch to drive neuronal differentiation (Visvanathan et al., 2007; Cheng et al., 2009). PTBP1 has also been shown to block pri-miR-124-1 cleavage by the microprocessor leading to the inhibition of miR-124 biogenesis (Yeom et al., 2018), which suggests that additional miRNAs control these negative feedback loops by targeting REST and/or PTBP1 leading to increased miR-124 expression and neuronal fate specification. One such miRNA is miR-9, which has been shown to target CoREST in neurons (Packer et al., 2008). miR-124, in conjunction with miR-9, regulate the switch to neuron-specific chromatin remodelling complexes (nBAF) by downregulating the BAF53a subunit expressed in non-neuronal cells and NPCs, which is replaced by the neuron-specific subunit BAF53b (Yoo et al., 2009) and is required for post-mitotic events such as dendritogenesis (Wu et al., 2007).

Several miRNAs promote neurogenesis via regulation of the Wnt pathway. For instance, miR-9 and let-7 downregulate the nuclear receptor TLX (Zhao et al., 2009; 2010; Roese-Koerner et al., 2013), an upstream activator of the Wnt pathway expressed in neurogenic niches in both foetal and adult brain. TLX prevents premature neuron differentiation and promotes NPC self-renewal (for review, see: Islam & Zhang, 2015) and its downregulation inhibits proliferation and promotes neuronal differentiation. Through a negative feedback loop, TLX directly represses miR-9 expression (Zhao et al., 2009) and represses miR-137 expression via recruitment of the histone demethylase LSD1. In turn, when expressed, miR-137 promotes neuronal differentiation by targeting LSD1 (Sun et al., 2011) and preventing LSD1 from erasing activating H3K4me3 epigenetic marks (Yokoyama et al., 2008). Moreover, several miRNAs, such as let-7, miR-20a/20b, miR-23 and

miR-15b, downregulate cyclin D1, a downstream effector of the Wnt pathway, either directly (Ghosh et al., 2014) or via regulating the methylation status of the cyclin D1 promoter (Lv et al., 2014) thereby inhibiting cell proliferation.

1.2.4.4 Roles of miRNAs in neuronal migration and maturation

Neuronal migration is regulated by the coordinated action of several miRNAs that can promote, inhibit or protect neuronal migration along radial glial cells (Rajman & Schratt, 2017). Several members of the miR-379-410 cluster, including miR-369-3p, miR-496 and miR-543, promote neuronal migration by downregulating N-cadherin, a neuronal adhesion molecule (Rago et al., 2014). In contrast, miR-134, miR-22 and miR-124 work cooperatively to inhibit neuronal migration by targeting doublecortin (Dcx) either directly (Gaughwin et al., 2011) or indirectly via members of the REST/CoREST transcriptional repressor complex (Volvert et al., 2014). As neurons migrate, they begin to polarize and convert from a multipolar morphology into a bipolar morphology (Noctor et al., 2004) via miR-22 and miR-124 regulation (Volvert et al., 2014). Once neurons reach their final destination, axons and dendrites start forming for neurons to establish functional connections. miR-9 regulates neurite outgrowth by targeting forkhead transcription factors 1 and 2 (Foxp1 and Foxp2) (Otaegi et al., 2011; Clovis et al., 2012). In addition, miR-9 increases axon branching by locally regulating the expression of microtubule-associated protein 1b (Map1b) in axons (Dajas-Bailador et al., 2012). Several miRNAs modulate axonal branching and dendrite morphogenesis by targeting components of the actin-remodelling complex. miR-124 promotes axonal and dendritic branching by inhibiting the expression of RhoG GTPase (Franke et al., 2012). miR-134 decreases dendritic spine size by targeting Limk1, which inhibits actin polymerization (Schratt et al., 2006). Moreover, miR-134 promotes dendritic branching by targeting Pumilio2 (Pum2), a translational

repressor involved in dendritogenesis (Fiore et al., 2009). miR-132 promotes BDNF – dependent axonal branching and increases dendritic spines by inhibiting the expression of p250GAP, a repressor of RAC1, resulting in increased actin remodelling (Wayman et al., 2008; Marler et al., 2014).

1.2.4.5 Roles of miRNAs in gliogenesis

Gliogenesis is the process by which radial glia give rise to astrocyte precursor cells, astrocytes and oligodendrocyte precursor cells during foetal brain development (Molnár et al., 2019). This process is thought to occur sequentially and to partially overlap with neurogenesis throughout the second half of human foetal development (Kadhim et al., 1988; deAzevedo et al., 2003). Recent studies have demonstrated that truncated radial glial cells (tRGs) originate glial progenitor cells at 18 PCW (Yang et al., 2022), which undergo mitosis and sharply increase in numbers after 20 PCW, suggesting that the neurogenesis-to-gliogenesis switch occurs at 20 PCW in human foetal development (Fu et al., 2021). Several studies have highlighted miRNAs as essential regulators of oligodendrocyte and astrocyte differentiation. For instance, oligodendrocyte-specific miRNAs miR-219 and miR-338 promote oligodendrocyte differentiation and myelination (Shin et al., 2009; Dugas & Notterpek, 2010; Zhao et al., 2010) by repressing oligodendrocyte differentiation inhibitors such as platelet-derived growth factor alpha (PDGFRα), Hes5, Sox6, FoxJ3 and pro-neuronal genes such as Zfp238 (Stolt et al., 2006; Dugas & Notterpek, 2010; Wang et al., 2017). Moreover, several miRNAs involved in neuronal fate determination can also regulate glial differentiation, depending on cellular competence. For instance, miR-92a regulates neuronal fate determination (Bian et al., 2013) and is also required for astrocyte differentiation (Selvi et al., 2015). In addition, miR-23a, which promotes neurogenesis, also promotes oligodendrocyte differentiation and myelination (Lin et al., 2013).

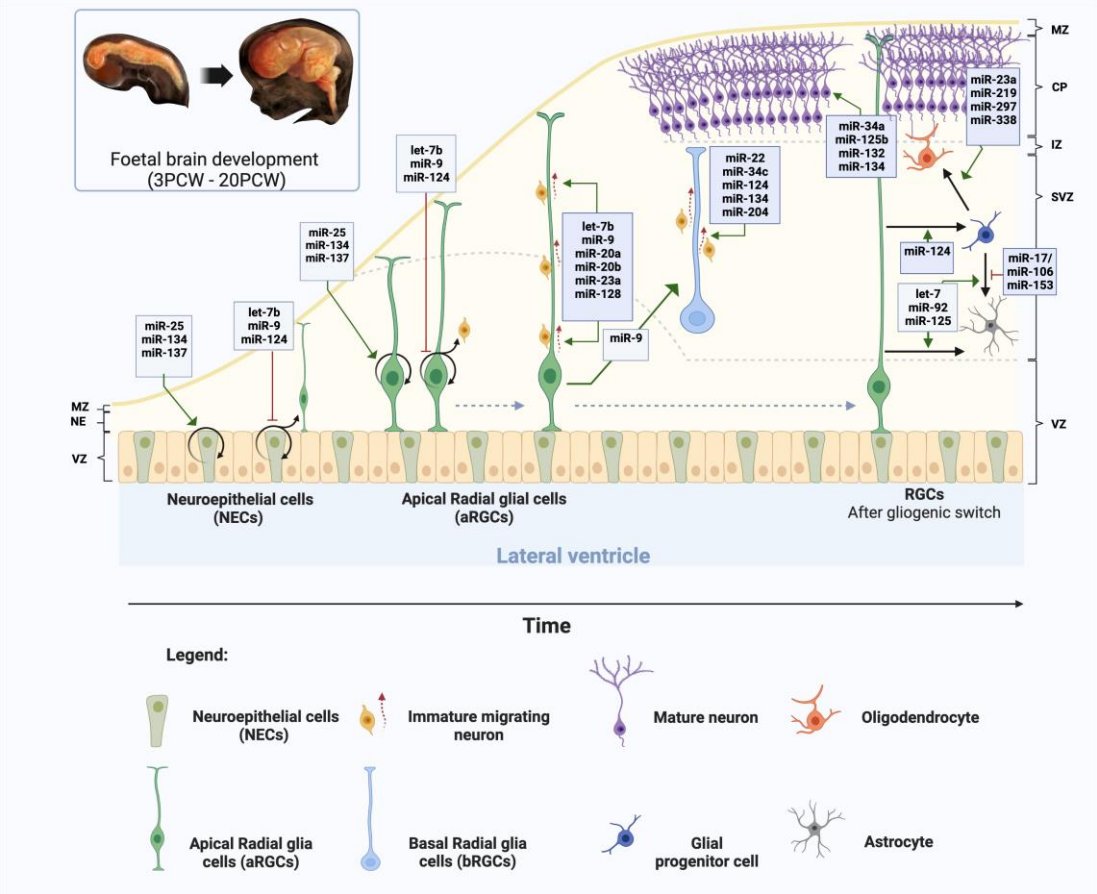


Figure 1.6 – miRNAs involved in foetal brain development.

Foetal brain development starts with the formation of the neural tube at 3 PCW (Ladher & Schoenwolf, 2005). Subsequently, at 5-6 PCWs, neuronal progenitors rapidly proliferate in the ventricular zone (VZ) that lines the cerebral ventricles (Rakic, 1978; Rakic, 1988; Rakic, 1995; Bystron et al., 2008). miR-25, miR-134, and miR-137 induce the proliferation of neural stem cells by regulating the expression of several proteins, including pluripotency factors (Nanog and Sox2) and cell-cycle inhibitors (p57) (Meza Sosa et al., 2014). By PCW 8, neuronal progenitors begin to differentiate, and radial glia can give rise to neurons (Malatesta et al., 2000; Noctor et al., 2001; Miller, 2002; Liu & Rao, 2004). Several miRNAs, including miR-124, miR-9 and let-7, promote differentiation of both radial glia and neurons by several mechanisms, including inhibiting TLX, which would otherwise stimulate neuronal progenitor self-renewal and cyclin D, which controls cell proliferation (Zhao et al., 2009). The inhibition of SCP1, BAF53a, and PTBP1, among others, by miR-124 leads to a shift from non-neuronal into neuronal gene expression profiles (Lang & Shi, 2012). Moreover, the negative feedback regulatory loops between miRNAs and their targets during neurogenesis allow for the spatiotemporal control of gene expression and give rise to a border between progenitor cell pools and differentiated neurons. After differentiation, post-mitotic neurons migrate along radial glia cells (Rakic, 2000) in an “inside-out” manner, where

deeper cortical layers are formed first (Hatten, 1993; Rakic, 1978; Rakic, 1988; Rakic, 1995). This process peaks at 12-20 PCW and continues up to 26-29 PCW (Gupta et al., 2005) and requires a complex interaction between neurons and scaffolding glia (Hatten, 1999; Chao et al., 2009). Several miRNAs work cooperatively to regulate neuronal migration. For instance, miR-134 can inhibit neuronal migration by targeting doublecortin (Dcx) directly (Gaughwin et al., 2011), whilst miR-22 and miR-124 target Dcx by downregulating members of the CoREST/REST transcriptional repressor complex (Volvert et al., 2014). In contrast, several other members of the miR-379-410 cluster, such as miR369-3p, miR-496 and miR-543, promote neuronal migration by downregulating N-cadherin, a neuronal adhesion molecule (Rago et al., 2014). As neurons reach the end of their migration, they mature and extend dendrites and axons to other neurons to form synaptic connections, a process dependent on several molecular gradients. Refinement of synapses occurs later, starting at 20 PCW. Several miRNAs regulate these processes by promoting axonal branching (e.g. miR-29, miR-124 and miR-132), dendritogenesis (e.g. miR-132, miR-185 and miR-134), regulating spine maturation (e.g. inhibition - miR-125, miR-134, miR-138; promotion – miR-132) and regulating synapse development and function (e.g. miR-137) (Rajman & Schratt, 2017). Adapted from: Prieto-Colomina et al., 2021. Created with Biorender.com

1.3 Role of miRNAs in neuropsychiatric disorders

1.3.1 Neuropsychiatric disorders

Neuropsychiatric disorders are a group of complex disorders that, despite lacking obvious neuropathology, can result in significantly altered affect, perceptions, cognition, personality and behaviour. Common examples include attention-deficit hyperactivity disorder (ADHD), schizophrenia (SZ), autism spectrum disorder (ASD), obsessive-compulsive disorder (OCD),

Tourette syndrome (TS), intellectual disability (ID), major depression disorder (MDD), bipolar disorder (BD), anxiety disorders, eating disorders, post-traumatic stress disorder (PTSD), Alzheimer's disease (AD) and substance use disorders. These disorders are highly debilitating, leading to significant functional impairments, substantial morbidity and mortality, and a tremendous public health burden. Most neuropsychiatric disorders are common in the population and possess a complex, largely unknown aetiology. Moreover, many affected individuals frequently respond poorly to medication and relapse, pressing the need for new and more effective therapies.

Some neuropsychiatric disorders such as ASD, ADHD, OCD, TS and ID are classed as neurodevelopmental disorders, and symptoms typically present during childhood. These disorders are associated with altered neurodevelopmental trajectories and a significant disruption of prenatal brain development is assumed. Neuropsychiatric disorders, such as SZ, BD and even MDD, are also thought to have a neurodevelopmental component. In these disorders, alterations during critical periods of brain development are hypothesized to predispose to the development of the condition in adulthood (Weinberger, 1987; Murray & Lewis, 1987; Basset et al., 2001; Walsh et al., 2008; Scholtz & Phillips, 2009; Cristino et al., 2013; Jamuar et al., 2014; Silbereis et al., 2016; Wray et al., 2018; O'Brien et al., 2018; Parenti et al., 2020; Kloiber et al., 2020; Hall et al., 2020).

1.3.2 The genetics of neuropsychiatric disorders

1.3.2.1 Heritability of neuropsychiatric disorders – Twin and adoption studies

Neuropsychiatric disorders tend to aggregate in families due to genetic and environmental factors. Adoption and twin studies disentangle the relative contribution of genetic and environmental effects on the trait by comparing monozygotic (identical) and dizygotic (non-identical) twins. Monozygotic twins are genetically identical, share the same prenatal environment, and, if raised together, also share a familial environment. In contrast, dizygotic twins share 50% of their segregating genes like other siblings, share the same prenatal environment and also share a familial environment. The extent to which monozygotic twins are more concordant for a neuropsychiatric disorder than dizygotic twins can be used to compute heritability, which is the proportion of disease liability in the population due to genetic factors (Merikangas & Merikangas, 2016). Once genetic factors have been considered, any remaining similarities between twins are due to shared familial environments, whilst discordance between monozygotic twins can be attributed to nonshared environmental influences.

Epidemiological data from twin and adoption studies strongly argues for an important genetic architecture underlying neuropsychiatric disorders. These studies demonstrated substantial heritability for SZ (60 to 80%) (Sullivan et al., 2003; Hilker et al., 2018), BD (79 to 93%) (McGuffin et al., 2003; Kieseppa et al., 2004), ASD (83% to 90%) (Sandin et al., 2017; Tick et al., 2016) and ADHD (74%) (Rietveld et al., 2003; Faraone & Larsson, 2019). The heritability of MDD is estimated to be lower, at around 37 % (Sullivan et al., 2000), but is increased for recurrent, early-onset, and postpartum depression (Kendler et al., 2007; Sullivan et al., 2000). Taken together,

quantitative genetic studies suggest that the aetiology of neuropsychiatric disorders can be elucidated using genetic approaches.

1.3.2.2 Genetic architecture of neuropsychiatric disorders

Neuropsychiatric disorders possess a complex genetic architecture and are highly polygenic, involving hundreds to thousands of risk variants spread across the genome. The frequency of a risk variant in the population is inversely proportional to its effect size, with high-risk variants being rarer in the population due negative selection (Keller & Miller, 2006; Park et al., 2011b; Rees et al., 2011). Moreover, neuropsychiatric disorders are multifactorial, and thousands of genetic risk factors aggregate and interact with each other and with environmental risk factors such as early-life adversity to mediate risk (Stilo & Murray, 2019; Klei et al., 2021) .

Recent advances in genomics have allowed for the identification of both rare variants by CNV analysis and exome sequencing and common risk variants by GWAS in neuropsychiatric disorders. These will briefly be described in turn.

1.3.2.3 Rare risk variants in neuropsychiatric disorders

1.3.2.3.1 CNV analysis

CNVs are structural rearrangements of chromosomes and can be defined as a segment of DNA of 1 kilobase (kb) or larger that are present in a genome at a variable copy number compared to a reference genome (Redon et al., 2006). CNVs encompass insertions, deletions and duplications that can span up to several dozen genes and can be analyzed using standard

SNP genotyping arrays. Several studies have identified rare CNVs as risk factors for neuropsychiatric disorders with large effect sizes (OR 2-57 for SZ) (Rees et al., 2014; for review, see: Rees & Kirov, 2021), particularly in SZ (n=12) (Marshall et al., 2017), ASD (n=15) (Sanders et al., 2015; Clements et al., 2017; Iakoucheva et al., 2019), ADHD (n=8) (Schneider et al., 2014; Gudmundsoon et al., 2019; Martin et al., 2020) and ID (n=70) (Coe et al., 2014), and to a lesser extent in BD (Green et al., 2016) (n=1) and MDD (n=3) (Kendall et al., 2019). Smaller studies have also implicated CNVs in the risk for OCD (n=1) and TS (n=2) (McGrath et al., 2014; Gazzellone et al., 2016; Huang et al., 2017). Despite their large effect size, most CNVs lack specificity and confer risk for several psychiatric disorders and are, therefore, pleiotropic. CNV pleiotropy suggests a shared genetic architecture and aetiology between several neuropsychiatric disorders; for example, CNVs have highlighted a significant overlap between ASD and SZ (Kushima et al., 2018). The majority of identified CNVs conferring risk for neuropsychiatric disorders encompass several genes. For example, 22q11.2 deletions lead to a congenital condition called velocardiofacial (VCF) or DiGeorge syndrome (Cancrini et al., 2014). Individuals with DiGeorge syndrome are at higher risk of developing SZ as adults (Murphy, Jones, and Owen 1999), as well as ASD, ADHD, anxiety, depression and ID (Phillip & Basset, 2011; Fabbro et al., 2012; Schneider et al., 2014; Fung et al., 2015; McDonald-McGinn et al., 2015; Cascella & Muzio, 2015; Clements et al., 2017). Currently, the only CNV associated with neuropsychiatric disorders that disrupts a single gene are deletions on chromosome 2p16. These deletions disrupt NRXN1, a gene involved in synapse formation and synaptic plasticity, and are associated with TS (Huang et al., 2017), ID, SZ and ASD (Castronovo et al., 2020), which also suggests that synaptic dysregulation is a common feature in these disorders.

1.3.2.3.2 Exome sequencing

Exome sequencing has allowed for rare coding variants to be studied in the context of neuropsychiatric disorders, and both CNV analysis and exome sequencing have highlighted the importance of *de novo* mutations in the aetiology of these conditions (Sanders et al., 2012; Sanders et al., 2015; Fromer et al., 2014). Several studies have identified rare *de novo* loss of function (LOF) mutations associated with SZ (Takata et al., 2014; Singh et al., 2022; Howrigan et al., 2020; Rees et al., 2020), ASD (Iossifov et al., 2014; Sanders et al., 2015; Takata et al., 2018; Satterstrom et al., 2020), BD (Nishioka et al., 2021) and ID (Singh et al., 2016; Kummeling et al., 2020). Moreover, rare truncating mutations have also been associated with ASD, BD, SZ, ID and ADHD (Krumm et al., 2015; Ganna et al., 2018).

Rare de novo heterozygous loss-of-function (LoF) mutations in the SETD1A gene, a histone methylase with developmental roles, are associated with a high risk for the development of SZ (Takata et al., 2014; Singh et al., 2016), learning difficulties and developmental disorders (Singh et al., 2016). Mutations in SETD1A are also associated with early-onset epilepsy (Yu et al., 2019), disruption of speech development (Eising et al., 2019) and global developmental delay (Kummeling et al., 2020). Recently, an exome sequencing study encompassing 24,248 SZ patients and 97,332 controls by the Schizophrenia Exome Sequencing Meta-Analysis (SCHEMA) Consortium reported 10 genes with ultra-rare LOF mutations that surpassed the genome-wide significance threshold of association in SZ (Singh et al., 2022). Of note, despite the level of significance of the association, the proportion of patients carrying these variants will be extremely small.

1.3.2.4 Common risk variation in neuropsychiatric disorders

1.3.2.4.1 Genome-wide association studies

Genome-wide association studies (GWAS) are a type of case versus control association study using large cohorts of thousands of individuals that enables a systematic, hypothesis-free and unbiased population-based evaluation of individual DNA variants in relation to a given trait. GWAS are widely used to study complex diseases because they incorporate the merits of association (power to detect small effects) with that of linkage (no requirement for specific knowledge of pathogenesis). In GWAS, common genetic variants, mostly single nucleotide polymorphisms (SNPs, defined as point mutations with a population frequency of at least 1%), located throughout the entire genome, are tested for association with a trait or disease (Newton-Cheh & Hirschhorn, 2005). If a variant is more frequent in cases than controls, the variant is said to be associated with the disease and marks a region of the genome that influences risk. Associated SNPs reported in these studies typically have individual odds ratios (OR) < 1.2 for neuropsychiatric disorders (for review, see: Cichon et al., 2009).

In order to prevent false positives, GWAS imposes a stringent statistical significance threshold of $p < 5 \times 10^{-8}$ through Bonferroni correction for 1 million independent tests (Dudbridge & Gusnanto, 2008). This threshold considers both the number of SNPs tested and the linkage disequilibrium (LD) between SNPs. The stringent threshold of genome-wide significance, and the small effect sizes of the risk SNPs, mean that the statistical power to detect association requires very large sample sizes. The need for large sample sizes precipitated the creation of collaborative consortia, such as the Psychiatric Genomics Consortium (PGC), with the aim of uniting genetic researchers in order to combine genotypes from many thousands of patients and controls.

1.3.2.4.2 Current common risk loci identified by GWAS in neuropsychiatric disorders

With sufficiently large sample sizes, GWAS have yielded a considerable number of significant results for neuropsychiatric disorders. Currently, 287 loci have been associated with SZ at genome-wide significance, with SNPs estimated to account for 0.24 of SZ heritability (Trubetskoy et al., 2022). A recent GWAS meta-analysis preprint has identified 27 genome-wide significant loci in ADHD (Demontis et al., 2022). Moreover, 64 genomic loci have been associated with BD (Mullins et al., 2021); 75 risk loci with AD (Bellenguez et al., 2022), 102 loci with MDD (Howard et al., 2019), 8 risk loci for anorexia (Watson et al., 2019), and 5 risk loci with ASD (Grove et al., 2019). Despite smaller samples sizes, 1 risk locus has been identified in TS (Yu et al., 2019), 3 risk loci have been identified in PTSD – 2 in the European population, and 1 in the African population (Nievergelt et al., 2019) - and 2 risk loci have been identified in an anxiety GWAS metanalysis (Otowa et al., 2016). In contrast, GWAS of panic disorder (Forstner et al., 2021) and OCD (IOCDF-GC and OCGAS, 2017) have so far failed to find genome-wide significant loci associated with these conditions. With regards to substance abuse, 29 independent loci have been reported to be associated with problematic drinking (Zhou et al., 2020), 18 loci with alcohol consumption level and alcohol use disorder (AUD) (Kranzler et al., 2019), 5 risk loci with nicotine dependence (Quach et al., 2020), 2 risk loci with cannabis – use disorder (CanUD) (Johnson et al., 2020) and 18 independent risk loci were found to be associated with opioid use disorder (OUD) in a recent multi-trait analysis GWAS (Deak et al., 2022).

1.3.2.4.3 Limitations of GWAS and the need for functional genomic studies

GWAS studies have allowed for the systematic identification of genetic loci associated with neuropsychiatric disorders and other traits. However, because GWAS rely on linkage disequilibrium between SNPs, a shortcoming of this method is that the most significant risk SNP at each locus (index SNP) is not necessarily the causal variant. Moreover, GWAS is unable to distinguish the top variant signal from many other variants that are in high LD with it, as any of these could be the causal variant (Hormozdiari et al., 2014)

Most (> 90%) of the top associated variants identified through GWAS are located in non–coding regions of the genome and are enriched in regulatory regions such as enhancers, promoters, silencers and insulators, which can be cell-type specific (Maurano et al., 2012; Kumar et al., 2012). This implies that most GWAS risk variants do not affect protein structure but rather affect gene regulation. The effect of causal risk variants on gene regulation is likely to be highly context-specific with regard to the tissues, cells, and developmental stages, depending on the disease (Hindorff et al., 2009; Encode Project Consortium, 2012; Eicher et al., 2015). All these limitations make GWAS results challenging to interpret in terms of disease biology.

It has been demonstrated that SNPs associated with complex traits by GWAS, such as neuropsychiatric disorders, have a three-fold likelihood of being an expression quantitative locus (eQTL) (Nica et al., 2010; Nicolae et al., 2010; Fehrmann et al., 2011; Hernandez et al., 2012; Porcu et al., 2019) which is a variant associated with changes in gene expression.

Whilst GWAS typically report the closest gene as a likely candidate, this is usually not the gene that is differentially regulated in association with the risk genotype (Musunuru et al., 2010; Zhu et al., 2016). Functional genomic studies are therefore required to elucidate the affected genes mediating the GWAS association, identify causal variants and interpret their biological

impact on diseases and traits (ENCODE Project Consortium, 2012; Schaid et al., 2018; Broekema et al.,2019).

1.3.2.4.4 Gene prioritization of GWAS risk loci

Gene prioritization aims to link disease-associated genetic variants to putative target genes and typically involves an eQTL study in disease relevant tissues and cells (Westra et al., 2013; Westra & Franke, 2014; Zhernakova et al., 2017; Võsa et al., 2018). In an eQTL study, genome-wide genotyping is combined with transcriptomic data from a given tissue to identify variants associated with altered gene expression (Schadt et al., 2003; Fehrmann et al., 2011). eQTL variants can act on phenotypes by affecting expression of local (cis - eQTLs) or distant (trans - eQTLs) gene targets. Identified eQTLs can be subsequently analyzed by different statistical approaches to ascertain whether the eQTL affects both gene expression and the trait of interest in order to identify potential causal effects. Of note, the top eQTL variant or SNPs in high LD with the top eQTL may not be the same as the index SNP in the GWAS being analyzed, usually due to multiple causal variants at the locus in high LD affecting the gene expression and the trait of interest independently (Yang et al., 2012; Hormozdiari et al., 2014; Porcu et al., 2019). Common approaches employed to identify eQTLs influencing complex traits and diseases are colocalization, transcriptome-wide association studies (TWAS), and mendelian randomization (MR) (for review, see: Cano-Gomez & Trynka, 2020; Li & Ritchie, 2021; Walker et al., 2022).

1.3.2.5 Foetal brain development and neuropsychiatric disorders

As mentioned in section 1.3.1, many neuropsychiatric disorders are either classed as neurodevelopmental disorders or are thought to have a neurodevelopmental component. The majority of neurons present in the adult human cortex are generated between the end of 1st trimester through the 2nd trimester of foetal development (Byston et al., 2008), making this developmental window a potential critical period of vulnerability.

Human foetal brain development is complex and highly dynamic regarding spatial and temporal expression trajectories of individual transcripts (Gulsuner et al.,2013), meaning that early developmental insults that affect these processes would alter the brain development trajectory predisposing towards neural circuits dysfunction and psychiatric illness. There is a growing body of evidence suggesting that both rare and common risk genes for neurodevelopmental disorders such as SZ, ASD and ID are highly expressed and dynamically regulated in foetal brain development (Hill & Bray, 2012; Miller et al., 2014; Birnbaum et al., 2015; Li et al., 2018; Liu et al., 2018). These studies support the notion that foetal expression profiles are essential for future brain function.

1.3.2.6 eQTL studies in foetal brain and risk for neuropsychiatric disorders

The majority of common variation associated with risk for neuropsychiatric disorders is non-coding and likely to affect gene expression, and gene expression is highly specific to tissue, cell type and developmental period. These factors make eQTL analysis in foetal brain important in understanding how changes in gene expression patterns during prenatal brain development may contribute to the aetiology of these disorders. O'Brien and colleagues

(2018) published the first eQTL study in foetal brain, in which expression of 28,875 genes was assayed in 120 2nd trimester samples. They identified a total of 1329 genes that were subject to foetal eQTLs (at FDR < 0.05) and found these eQTLs to be enriched among risk variants for neuropsychiatric disorders, including SZ, ADHD and BD (O'Brien et al., 2018). These results were subsequently corroborated by Walker and colleagues (2019) in a larger study involving 201 mid-gestation foetal brains, that identified 6546 genes that were subject to foetal eQTLs and 4635 genes that were subject to spliceQTLs (sQTLs). In addition to mRNA species, eQTL analysis can be extended to other RNA species, such as miRNAs.

1.3.2.7 miRNAs in neuropsychiatric disorders

Altered miRNA expression has been reported in the post-mortem brains of individuals with various psychiatric disorders, including substance abuse (Im & Kenny, 2012), MDD (Smalheiser et al., 2012), ASD (Ander et al., 2015; Wu et al., 2016), SZ and BD (Moreau et al., 2011). Several miRNAs have been reported to be differentially expressed in the dorsolateral prefrontal cortex (DLPFC) of people with SZ compared to controls (Santarelli et al., 2011; Moreau et al., 2011). The targets of these miRNAs showed enrichment for genes involved in axon guidance, long-term potentiation, and other processes associated with neuropsychiatric disorders. In addition, 59 miRNAs were reported to be differentially expressed in the DLPFC and superior temporal gyrus in SZ, which targeted and down-regulated the expression of mRNAs coding for proteins involved in neurodevelopmental pathways and cell-cell signalling (Beveridge et al., 2008, 2010). Moreover, genes encoding miRNAs and molecules involved in miRNA post-transcriptional gene regulation and processing are located at several high-confidence genomic risk loci for psychiatric disorders, consistent with an aetiological role of miRNAs in these conditions. These include FXR1, MIR548AJ2 and MIR137 at genome-wide significant risk loci for SZ (Ripke et

al., 2014; Duan et al., 2014; Strazisar et al., 2015). To date, published studies implicating miRNA in neuropsychiatric disorders have been carried out exclusively using adult brain tissue. Therefore, little is known about how the expression of miRNAs in foetal brain might contribute to risk for neuropsychiatric disorders.

1.4 Aims of thesis

The aims of this thesis are as follows:

1) Identify miRNA expressed in human foetal brain and assess the effects of sex and gestational age on miRNA expression (Chapter 2).
2) Map common genetic variants associated with miRNA expression in human foetal brain (Chapter 3).
3) Test association between identified miR-eQTL and neuropsychiatric disorders and other brain-related traits (Chapter 4).

Chapter 2 - Expression of microRNAs in human 2nd trimester foetal brain and investigation of effects of sex and gestational age

2.1 Introduction

The methods most commonly employed to profile miRNA expression are reverse transcription quantitative PCR (RT- qPCR), microarrays and RNA sequencing (RNA-Seq). RT-qPCR is a low throughput method, mainly employed for targeted miRNA analysis of a small number of miRNAs (Shi & Chiang, 2005; Mestdagh et al., 2014; Salone & Rederstorff, 2015). Due to its high sensitivity and specificity, this method is usually employed to verify results obtained by microarrays and next-generation sequencing, including RNA-Seq (Mestdagh et al., 2014).

Microarrays are a high-throughput method for miRNA quantification, where hundreds of miRNA-specific probes are attached to fixed locations on a glass or polymer slide. After mature miRNA purification and reverse transcription, the resulting cDNA is labelled (with either biotin, fluorophores, or less commonly radioactive compounds). The labelled cDNA and miRNA probes are hybridized and scanned for signals, allowing for the relative quantification and normalization of miRNA expression from each probe on the slide in relation to a control experiment (Yin et al., 2008). Whilst microarrays can profile hundreds of miRNAs simultaneously, this technique does not allow absolute quantification, nor is it able to identify novel miRNAs as it is necessary to have prior knowledge of the miRNAs to be interrogated for probe design. Moreover, the sensitivity of microarrays is considerably lower, which necessitates large quantities of starting material, and its specificity can be compromised due to the high similarity between miRNA species.

RNA sequencing (RNA-Seq) is a high-throughput alternative to microarrays that does not require *a priori* knowledge and allows for the unbiased quantification of the transcriptome (Wang et al., 2009; Ching et al., 2014). RNA-seq is commonly employed to perform differential gene expression (DGE) analysis by comparing the expression of genes between conditions and identifying altered genes and pathways. RNA-Seq also allows to identify alternative splicing events (Wang et al., 2008; Pan et al., 2008;

Tremblay et al., 2016), perform allele-specific expression analysis (Degner et al., 2009), discover and annotate new transcripts (Guttman et al., 2010) and study the regulation of gene expression by non-coding RNAs (Djebali et al., 2012; Li et al., 2016). Mature miRNAs are small and lack a poly(A) tail. As such, they are either not captured or poorly represented by standard RNA-Seq library preparations (Dard-Dascot et al., 2018; Stark et al., 2019) and require their own specific method.

Small-RNA sequencing (Small-RNA seq) is a modification of the RNA-seq method that allows the sequencing of small-RNAs by use of sequential RNA ligation in cDNA library preparation (Hafner et al., 2008). This method quantifies miRNAs and detects novel miRNAs, isomiRs, and other small RNA species unbiasedly compared to microarrays and RT-qPCR (Dard-Dascot et al., 2018; Benesova et al., 2021). Small RNA sequencing has been successfully employed to study miRNA expression in the adult human brain and identify novel miRNAs (Wake et al., 2016), perform differential analysis between brain regions in AD (Dobricic et al., 2022), identify miRNA editing in Huntington's disease (Guo et al., 2022), integrate miRNA expression with mRNA expression and protein levels in Parkinson's Disease (Caldi Gomes et al., 2022), among others. To the best of my knowledge, the present investigation was the first study employing small RNA-sequencing to study miRNA expression in 2nd trimester foetal human brain. In this chapter, I describe the small RNA sequencing protocol I employed to profile miRNA expression in 2nd trimester foetal brain and the methods used to analyze small RNA-sequencing data and assess variables responsible for driving miRNA expression, including analyzing the effects of sex and gestational age.

Sex differences have been reported in the brains of adolescents and adults at the morphological (Ruigrok et al., 2014; Ritchie et al., 2018), functional (Ingalhalikar et al., 2014), biochemical (Laakso et al., 2002), and behavioural levels (Zell et al., 2015; Gur & Gur, 2017). More recently, sex differences in foetal brain functional connectivity have been reported (Wheelock et al., 2019). These differences are likely to arise due to sexual

differentiation of the human brain caused by different gene expression patterns between males and females that begin during foetal development. In animal studies, foetal development is a crucial time for the sexual differentiation of the brain, which will subsequently lead to behavioural sex differences (Arnold, 2009).

Recently, the Genotype-Tissue Expression (GTEx) Project reported that sex influences both gene expression patterns and cellular composition of tissues throughout the body, with 37% of all genes having sex-biased gene expression in at least one tissue (Oliva et al., 2020). The human genome differs between sexes with males possessing a Y-chromosome and an X-chromosome, whereas females possess 2 copies of the X-chromosome. According to the most recent Ensembl release 108, the human X-chromosome has approximately 1575 genes, whilst the Y-chromosome has approximately 203 genes (Cunningham et al., 2022). Of these, 29 genes are located in pseudoautosomal regions (PAR1 and PAR2) and are homologous between females and males (Ross et al., 2005; Monteiro et al., 2021). Because females have 2 copies of the X-chromosome, during early embryonic development, one copy of the X-chromosome in female cells is randomly inactivated (XCI) to balance the dosage of X-chromosome linked genes between sexes. However, several studies have demonstrated that at least 23 - 30 % of X-chromosome linked genes escape XCI to some extent (Balaton et al., 2015; Tukiainen et al., 2017) and therefore contribute to sex-biased gene expression of X-chromosome linked genes (Balaton et al., 2018).

Sex differences in gene expression can occur by several mechanisms, such as gene expression of Y-chromosome linked genes, differences in dosage of X-chromosome genes and epigenetic mechanisms such as non-random X-chromosome silencing and imprinting of X-chromosome linked genes (Arnold, 2017). Sex hormones can regulate gene expression and contribute to the sex-biased expression of autosomal genes and to X-chromosome dependent epigenetic regulation of gene expression. Whilst most transcription factors are not differentially expressed between males and

females, a recent study demonstrated significant sex differences in chromatin accessibility and that many transcription factors have sex-biased regulatory targeting patterns (Kukurba et al., 2016).

Sex differences have also been observed in miRNA expression in gonadal (Mishima et al., 2008) and somatic tissues, including the brain (Morgan & Bale, 2012; Pak et al., 2013; Ziatts & Rennert, 2014). In neonatal mouse brain, 149 miRNAs have been found to be differentially expressed between females and males, of which 32% were regulated by a sex-chromosome epigenetic mechanism, and 48% were regulated by sex hormones (Morgan & Bale, 2012; Morgan & Bale, 2011). Ziatts and Rennert (2014) performed an RNA-Seq study to investigate miRNA expression patterns in human brain from infancy to adolescence. This study reported a total of 40 miRNAs differentially expressed between females and males in the prefrontal cortex over development, with the period of adolescence having the most sex-biased miRNA expression (n=26 miRNAs, 1 – upregulated in males and 25 upregulated in females).

The observed prevalence and presentation of several psychiatric disorders differ substantially by sex (Rutter et al., 2003; Zagni et al., 2016), including neurodevelopmental disorders such as ASD and ID (Werling & Geschwind, 2013; Polyak et al., 2015). However, little is known regarding sex differences in human foetal brain and throughout human foetal brain development. Understanding how the foetal expression of miRNAs differs by sex in normal brain development may provide insight into the mechanisms underlying this disparity.

Human foetal brain development is complex and highly dynamic concerning spatial and temporal expression trajectories of individual transcripts (Gulsuner et al.,2013). In fact, prenatal transcriptional changes in the brain occur more rapidly than at any other stage of life (Johnson et al., 2009; Colantuoni et al., 2011; Kang et al., 2011; Jaffe et al., 2018). Recent studies support the notion that foetal expression profiles are important for future brain function and that the degree of dysfunction will be inversely proportional to disease onset due to the failure of compensatory mechanisms

(Birnbaum & Weinberger, 2017). Under this model, early developmental insults that affect foetal brain expression profiles are proposed to alter the brain development trajectory predisposing towards neural circuit dysfunction and neuropsychiatric disorders. There is a growing body of evidence suggesting that both rare and common risk genes for neurodevelopmental disorders such as SZ, ASD and ID are highly expressed and dynamically regulated in foetal brain development (Hill & Bray, 2012; Miller et al., 2014; Birnbaum et al., 2015; Li et al., 2016).

Given the crucial role of miRNA in regulating processes pertaining to brain development and neuronal function (Rajman & Schratt, 2017; Prieto-Colomina et al., 2021), I sought to determine which miRNAs are expressed in 2nd trimester foetal brain via small-RNA sequencing and the potential effects of sex and gestational age.

2.2 Materials and Methods

2.2.1 Samples

Initially, a total of 148 2nd trimester human foetal brain samples were used (12-20 post-conception weeks [PCW]) obtained from the MRC / Wellcome Trust Human Developmental Biology Resource (http://www.hdbr.org/). Samples were derived from elective abortions due to non-medical reasons and possessed a normal karyotype. PennCNV (Wang et al., 2007) was employed to call CNVs from samples for which signal intensity data (log R ratio and B allele frequency) was available (n = 68), in order to assess if any carried known risk CNVs for neurodevelopmental disorders listed in Kendall et al., (2019). None of the samples where CNVs were investigated possessed neurodevelopmental CNVs. However, a limitation of the present analysis is that not all samples were able to be screened for neurodevelopmental CNVs for lack of signal intensity data.

Ethical approval for use of the HDBR samples was granted by the Royal Free Hospital research ethics committee under reference 08/H0712/34 and Human Tissue Authority (HTA) material storage licence 12220. In addition, ethical approval for investigating genetic effects on gene expression in human brain was obtained from the Psychiatry, Nursing and Midwifery Research Ethics Subcommittee (PNM RESC/12/13 -102). Samples had been received as undissected frozen brain tissue. Total RNA was extracted from a homogenate of half a section of brain tissue from each foetus using Trizol (Ambion). The other half was used for genomic DNA extraction using standard phenol-chloroform extraction. All tissue samples were stored at – 80C.

2.2.2 Total RNA extraction, processing and quantification

Total RNA was extracted following the standard Trizol (Ambion) method. The purity and concentration (ng/µl) of the extracted total RNA samples was determined by spectrophotometry; to ensure RNA quality free from protein and solvent contamination, a 260/ 280 ratio ≥ 1.8 and 260/ 230 ≥ 1 was required. Subsequently, total RNA was DNAse treated using the TURBO DNA – free^{TM} kit (Ambion) to remove genomic DNA from the samples. RNA quality was accessed through an Agilent RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer System in the form of an RNA integrity number (RIN). RNA concentration was accurately quantified by fluorometry using Qubit (Thermo Fisher Scientific).

2.2.3 TruSeq Small RNA Library Preparation

Small RNA libraries were constructed in accordance with Illumina guidelines, using 1 ug of DNAse treated total RNA. Figure 2.1 illustrates the TruSeq Small RNA Library preparation workflow.

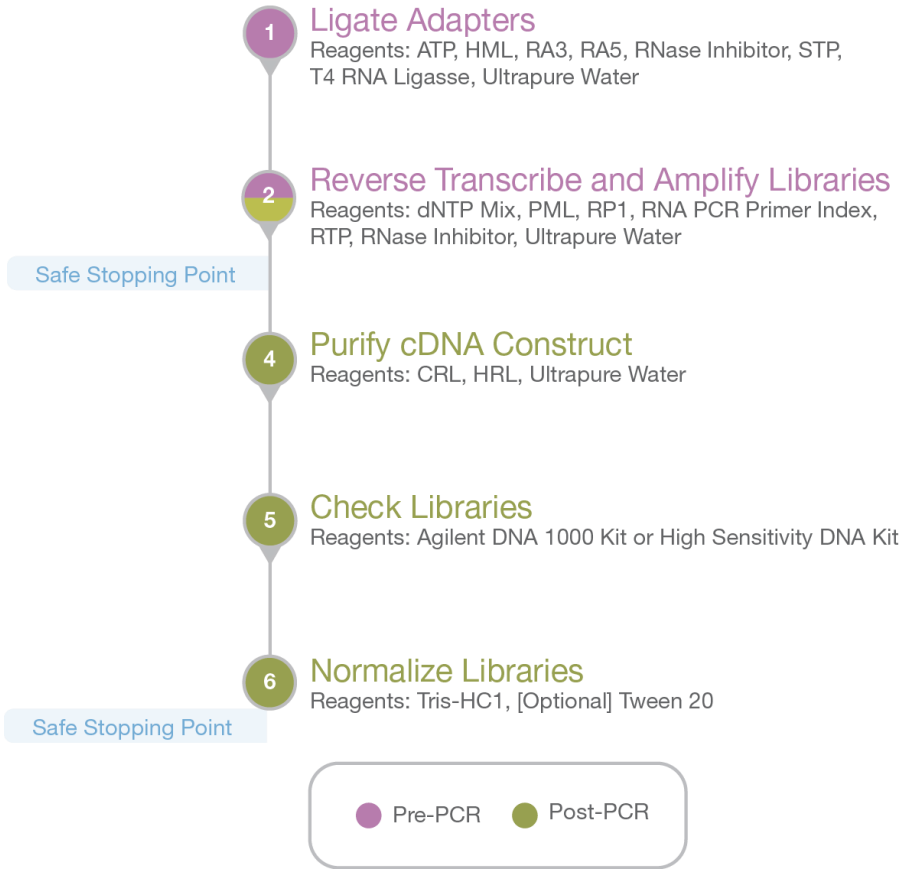


Figure 2.1 - TruSeq Small RNA Prep workflow.

Briefly, 1ug of DNAse treated total RNA was initially ligated with 3' and 5' adapters, followed by reverse transcription. Subsequently, the resulting cDNA was amplified by PCR using different PCR indexes for each sample in order to discern between them. This was followed by the purification of the cDNA construct by size selection using agarose gel extraction. Library quality was assessed using the Agilent High Sensitivity DNA kit. At this point, samples were normalized and stored at - 20 C (Adapted from: Illumina TruSeq Small RNA Library guidelines).

2.2.4 Purification of cDNA construct and library quality control

Small RNA libraries were purified by agarose gel extraction. This method allowed specific isolation of libraries with sizes between 145 -160 nucleotides

which contain mature miRNA generated from ~22 nt and piwi-interacting RNAs (piRNAs) generated from ~30 nt, as well as, small RNA fragments, and other regulatory small RNAs (in addition to the ligated adapters). Subsequently, the agarose containing the libraries of interest was dissolved, and the small RNA libraries were collected using the MinElute Gel Extraction Kit (Qiagen). Quality control of purified libraries was performed using Bioanalyzer and the Agilent High Sensitivity DNA kit. Small - RNA Seq libraries underwent a 2:1 concentration step using a vacuum concentrator prior to purification.

2.2.5 Normalization and pooling of miRNA libraries

Libraries were quantified through qPCR using NEBNext Library quantification kit for Illumina (New England Biolabs) and pooled together in equimolar amounts in batches of 20 samples. Pooled libraries were concentrated using a vacuum concentrator and quantified by fluorometry using Qubit. Subsequently, pooled libraries were normalized to a concentration of 2 nM using Tris-HCl 10 mM, pH 8.5. 0.1% Tween 20 was added to each pooled library to prevent cDNA absorption by the Eppendorf tube and stored at -20 C.

2.2.6 Small RNA Sequencing and data pre-processing

Pooled libraries were sequenced on an Illumina Hi-Seq 4000 to achieve approximately 15 million (M) reads per library. The resulting 50 bp single-end sequencing FASTQ files were processed according to a recently published standardized protocol for miRNA - sequencing studies (Potla et al., 2021)

which was adapted to my Small-RNA sequencing. Figure 2.2 explains the main steps of this pipeline.

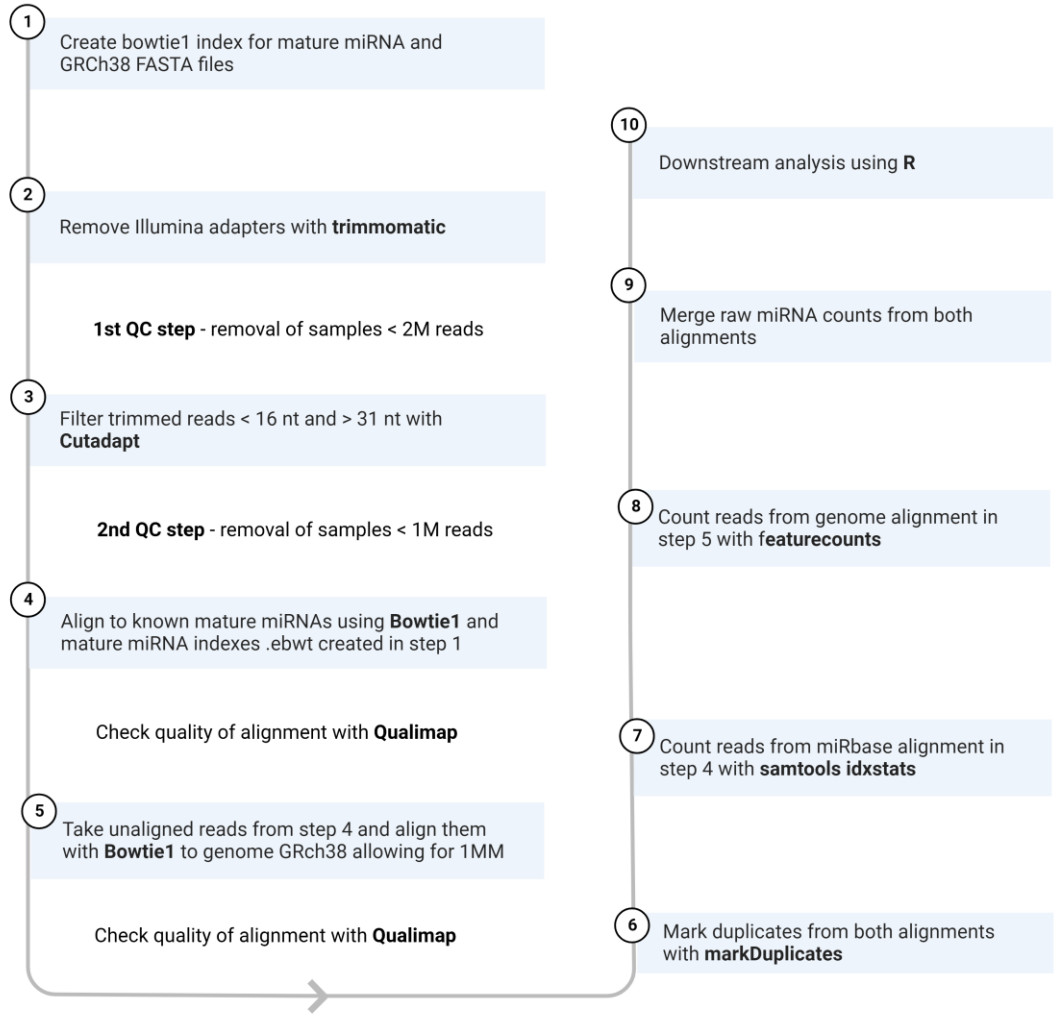


Figure 2.2 – Outline of the main steps of the small RNA-sequencing data pre-processing pipeline employed in this study adapted from Potla et al.(2021).

M - million, nt – nucleotide, MM – mismatch. Created with Biorender.com

Briefly, Illumina adapters were trimmed using trimmomatic version 0.38 (Bolger et al., 2014), followed by quality control (QC) of sequenced reads. FastQC version 0.11.8 was employed to create and visualize plots based on individual base sequence quality scores, sequence length distribution, individual sequence GC content, and duplicate sequences (Andrews, 2010),

which were subsequently aggregated by multiQC version 1.12 (Ewels et al., 2016). Samples with less than 2M trimmed reads were eliminated (See section 2.2.7). Post-trimming samples that survived initial QC had an average of 10.36M reads, and a median of 9.2M reads.

In order to only retain miRNAs (19–25 nt) and piwiRNAS (26–31 nt) reads, after trimming Illumina adapters, the trimmed reads were filtered to remove reads that were too short (< 16 nt) and too long (> 31 nt) using Cutadapt v3.2. Filtered reads were mapped using Bowtie aligner version 1.3.1 in a two-step approach, as documented by Potla and colleagues (2021). Firstly, mature miRNA sequences were downloaded from miRbase v22 (Kozomara et al., 2019) as FASTA files and used to create six “ebwt” Bowtie indexes. Filtered reads were aligned to mature miRNA sequences using Bowtie with stringent criteria by not allowing mismatches, using the following bowtie parameters: -n 0 -l 30 --norc --best --strata -m 1. These options tell Bowtie to allow no mismatches in a seed of read length of 30 bp, do not align reverse-complement reference strand and report the best hits found in a stratum of reads.

Subsequently, the unaligned reads from the first alignment were aligned to the Bowtie indexes created from the GRCh38 reference genome allowing for 1 mismatch between the miRNA reads and the reference genome, using the Bowtie parameters: -n 1 -l 30 --norc --best --strata -m 1 in order to account for isomiRs. Mapping quality from both alignment steps was ascertained by employing Qualimap 2.2.1 (Okonechnikov et al., 2016).

SAM files originating from both alignment steps were converted into BAM files via Samtools version 1.9 (Li et al., 2009), and duplicates were identified via MarkDuplicates Picard 2.20.2 (Picard Tools). Markduplicates does not distinguish between PCR duplicates or biological duplicates. The removal of duplicates is associated with decreased power to detect differentially expressed genes and, as such, duplicates were not removed from the analysis (Parekh et al., 2016). BAM files were indexed using bamtools 2.3.0 (Barnet et al., 2011) to allow for faster processing using the .bai index created.

Aligned reads were quantified into raw counts using samtools 1.9 idxstats for miRbase aligned counts (Li et al., 2009) and featurecounts for genome aligned counts (Liao et al., 2014) with parameters: -F GTF -t miRNA -g Name -O -M and using a GTF file which contains the genomic coordinates of mature microRNAs attained from miRbase v22. Raw miRNA counts derived from aligning miRNA reads to miRbase and to the GRCh38 reference genome were merged, and miRNAs with 0 counts in more than 90% of the samples were eliminated. A total of 1449 miRNAs were detected.

2.2.7 Sample QC and exclusion of samples

Sample libraries underwent a series of QC analyses based on measures from FASTQC version 0.11.8 (Andrews, 2010) and MultiQC version 1.12 (Ewels et al., 2016). Initially, I sequenced 148 samples to a depth of 15M reads per sample. However, there were considerable differences in the number of sequenced reads between samples, despite samples being pooled in equimolar amounts. After trimming, sample reads ranged from 0M reads to 66.9M reads. As a first QC pass, all samples < 2M trimmed reads were eliminated. A total of 14 samples were eliminated at this step, 11 of which had 0M trimmed reads where the sequencing had failed and possessed an average read length between 41 bp to 50 bp after trimming. The filtering step performed after trimming ensured that all the remaining reads were either miRNAs or piwi-RNAs. After this step, a second QC was performed where all samples with < 1M filtered reads were eliminated. A total of 15 samples were eliminated. Of the remaining 119 samples, one sample was eliminated due to being 26 PCW and outside the 2nd trimester age range of 12-20 PCW employed in this study.

An additional 6 samples were either missing associated genotypes or failed genotyping QC (had anomalous heterozygosity, were missing > 0.05 of

genotyped SNPs (--geno) or had sex assignment discrepancies) and, as such, were eliminated from the analysis. A total of 112 samples passed QC and were included in this study. These samples had on average, 6.54M filtered reads, with a median of 5.6M filtered reads. However, 6 of these samples had lower sequencing depth with < 2M filtered reads (2 samples with 1.2M reads, 1.3M reads, 1.8M reads, and 2 samples with 1.9M reads).

Due to power constraints, I did not perform additional sample exclusion based on FASTQC results. A Principal Component Analysis (PCA) of TMM normalized miRNA counts was performed to identify heterogeneity in miRNA expression between samples (Figure 2.3) using FactoMineR (Husson et al., 2017). Subsequently, I attempted to identify outlier samples in PCA by employing R packages bigstatsr and bigutilsr (Privé et al.,2018; 2020), which have been successfully employed for outlier detection in the UK Biobank genotype data (Privé et al.,2020). No outlier samples were identified.

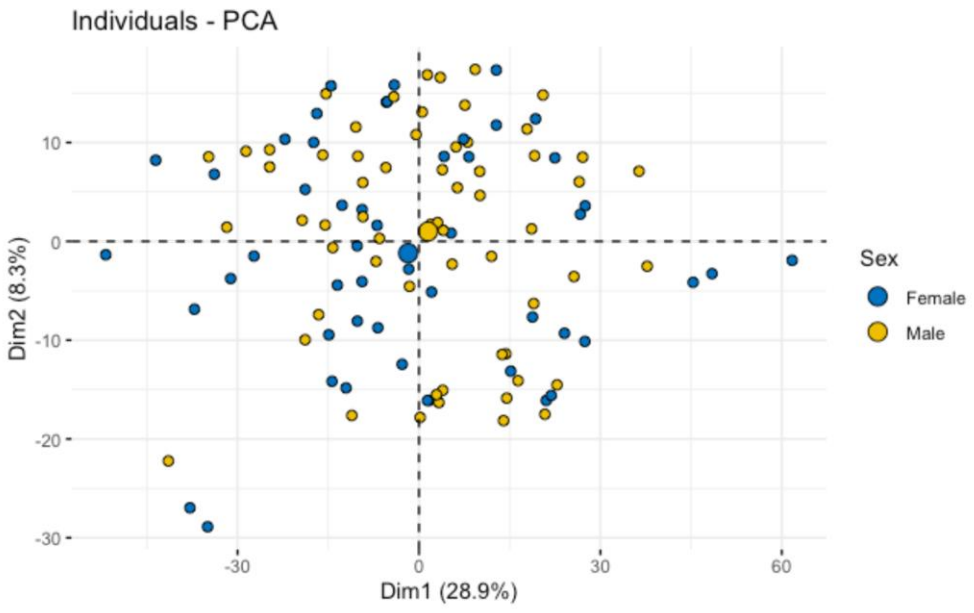


Figure 2.3 – PCA of TMM normalized miRNA counts (log_2 CPM + 1) scaled and centered.

PCA was performed using FactoMineR (Husson et al., 2017). A cut-off of more than 6 SD from the mean was employed for outlier detection, due to this being considered the standard cut-off for outlier detection in previous studies (Patterson et al, 2006, Privé et al., 2020). No outlier samples were detected using this cut-off. Of note, assuming principal components (PCs) have a normal distribution, approximately 99.999% of samples would lie within 6 SD from the mean.

2.2.8 Sample demographics

The sex of the majority of the samples used in this study had been previously determined by karyotyping, expression of genes on the Y-chromosome in males and heterozygosity for genetic X-chromosome markers in females (O'Brien et al., 2018). Of the 112 samples employed in this study, 51 were female, and 61 were male. Foetal age in PCW had previously been determined by the HDBR through foot length and knee-to-heel length measurements. In this study, sample age ranged from 12-20 PCW, with an average of 14.63 PCW in females and 14.90 PCW in males. The vast majority of samples employed in this study had been genotyped previously (Hannon et al., 2016; O'Brien et al., 2018). An additional 19 samples were specifically genotyped for this study (see Chapter 3, section 3.2.2). After genotyping, sex was empirically determined in these samples based on X -chromosome heterozygosity rates using the "--check-sex" flag in PLINK 1.9 (Purcell et al., 2007). Genotyped SNPs were employed to determine ancestry by Principal Component (PC) analysis using Peddy (Pedersen & Quilan, 2017).

2.2.9 Choice of normalization method

The R package DANA (DAta-driven Normalization Assessment) was employed to identify the method best suited to normalize miRNA raw counts in my dataset between eight commonly used normalization methods – Total Count (TC), Upper Quantile (UQ), Median, Trimmed Mean of M-values (TMM), DESeq, PoissonSeq, Quantile Normalization (QN) and Remove Unwanted Variation (RUVg, RUVr and RUVs) (Duren et al., 2022). The choice of the best normalization method depends on the $cc+$ and the $mscr-$ metrics, where $cc+$ is a ratio between 0 and 1 that quantifies the preservation of true biological variation; and $mscr-$ is a ratio between 0 and 1 that quantifies the reduction of handling effects. The goal was to identify which normalization method was the best compromise between maximally removing depth variation from experimental handling (high $mscr-$) whilst preserving true biological variation ($cc+$ close to 1). After DANA analysis (Table 2.1), I decided to choose TMM normalization for my dataset, given that it is the method that preserves biological variation the most whilst still effectively correcting for handling effects, such as library size and composition.

Table 2.1 – DANA summary metrics mcrs- and cc+ for the assessment of the suitability of different normalization methods in my miRNA dataset.

Normalization method	**cc+** preservation of true biological variation	**msrc-** relative reduction of handling effects
RUVs	0.9086	0.5728
QN	0.9154	0.7497
RUVr	0.9246	0.8337
DeSeq	0.9595	0.7589
UQ	0.9671	0.6036
Median	0.9682	0.5807
TC	0.9702	0.7426
PoissonSeq	0.9703	0.7410
RUVg	0.9707	0.7885
TMM	0.9721	0.7634

2.2.10 Normalization of raw miRNA counts

Reads were normalized using the trimmed mean of M-values (TMM) method, which normalizes libraries by their relative size (Robinson & Oshlack, 2010), and transformed into log2 (CPM + 1) using edgeR (Robinson et al., 2010) where CPM stands for counts per million and 1 is a prior count to avoid taking the log of 0. Subsequently, the distribution of samples before and after normalization was analyzed (Figure 2.4). As can be seen in Figure 2.4, comparing between samples, none appears to have an abnormal distribution. Initially, I had detected 1449 miRNAs, and all of these were employed in chapter 3 to detect cis-eQTL even in lowly expressed miRNAs. However, for the differential expression (DE) analysis described in this chapter, miRNAs with less than 1 CPM in at least 10% of samples were removed from the analysis. The reasoning behind this was that low-expressed miRNAs have a higher degree of associated noise and can decrease the sensitivity for detecting differentially expressed genes (DEGs). Moreover, I employed limma voom to detect differential expression (Law et al., 2014), which assumes low counts have been removed from the dataset prior to the analysis. After removing low-expressed miRNAs, out of the initial 1449 miRNAs detected, 838 miRNAs were kept for DE analysis.

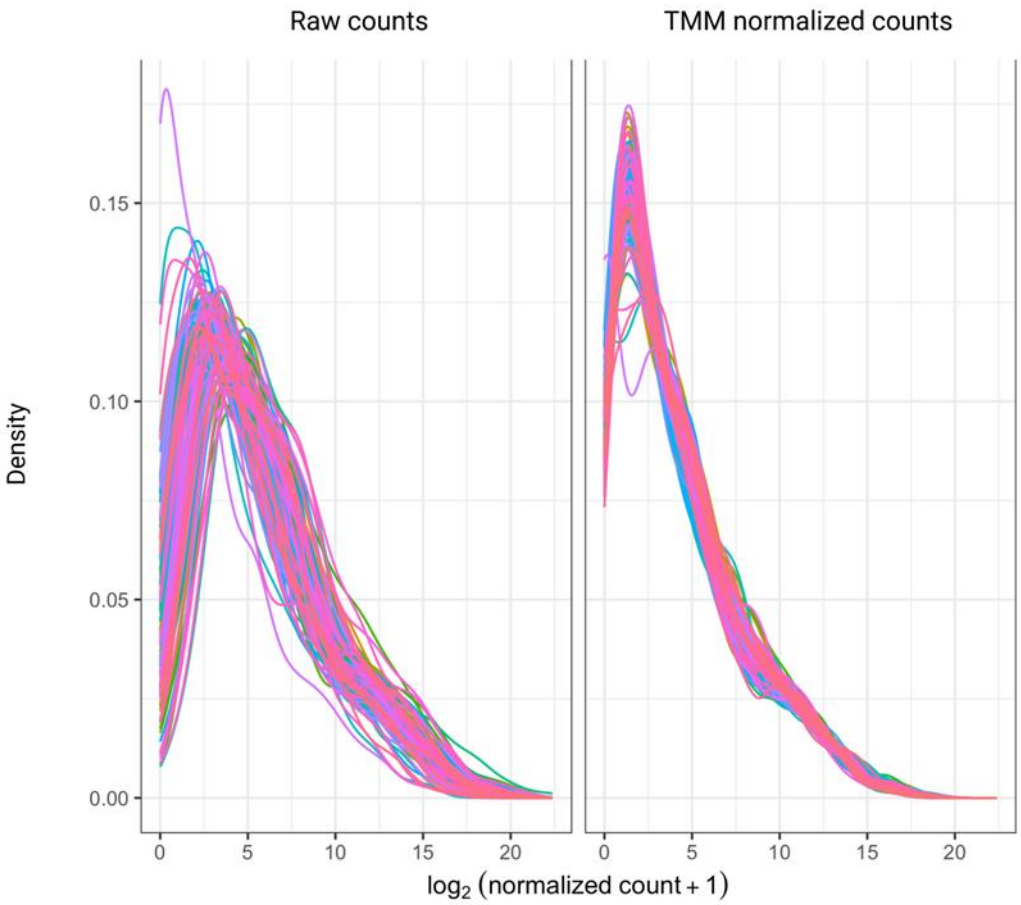


Figure 2.4 – Distribution of miRNA expression across samples before and after normalization.

2.2.11 Sample variables

Sample demographics such as sex, age (PCW) and the first 3 PCs of ethnicity (PC1, PC2 and PC3) were included as covariates. Moreover, sequencing batch, several library statistics such as average read quality, % Duplicates after filtering, % GC after filtering, average read length after filtering, total reads after filtering, % mapped reads, and 10 hidden confounders estimated by PEER analysis were also employed as covariates.

2.2.12 PEER analysis

Hidden confounders affecting the expression of mature miRNAs were estimated through the use of probabilistic estimation of expression residuals (PEER; Stegle et al., 2012) after TMM normalization. Sample covariates were added to the PEER model, and a total of 10 PEER factors were estimated.

2.2.13 Quantification of technical and biological variation by VariancePartition.

The proportion of variance in miRNA expression explained by each confounder was quantified using the VariancePartition R package (Hoffman & Schadt, 2016) after TMM normalization, removal of low-expressed miRNAs and PEER analysis. Variation within sex, PCW, RIN, sequencing batch, ethnicity (PC1, PC2 and PC3), and library statistics ascertained by FASTQC and Qualimap (such as average read quality, % duplicates after filtering, % GC after filtering, average read length after filtering, total reads after filtering and % mapped reads), as well as the 10 PEER hidden confounders were examined via a linear mixed model where continuous variables were modelled as fixed effects and categorical variables such as sex as random effects. VariancePartition ascertains the effect of each variable on gene expression whilst accounting for all the others. This analysis was performed after removing low-expressed miRNAs to account for only the miRNAs employed in the differential expression analysis.

2.2.14 Assessment of correlation between variables by VariancePartition

VariancePartition was further employed to evaluate the correlation between the covariates employed in this study. If two variables are highly correlated, VariancePartition cannot correctly estimate each variable's contribution and will instead divide the contribution over multiple variables. This was done to interpret the results from VariancePartition correctly and to ascertain if any of the PEER hidden confounders were correlated with the 2 variables I subsequently explored – sex and gestational age. Recently, it has been demonstrated that PEER factors encode for transcriptome diversity within samples (García-Nieto et al., 2022) which likely arises from varying proportions of different cell-types between samples (Zhabotynsky et al., 2022). If this transcriptome diversity is secondary to the effects of sex and age, using PEER factors as covariates would remove part of the gene expression changes I wish to study. Figure 2.5 shows a correlation matrix between all pairs of variables employed in this study. Of note, PCW is highly correlated with PEER 6 ($r = 0.49$) and PEER 9 ($r = 0.51$), which suggests these PEER factors correct for transcriptome diversity that arises from foetal development progression.

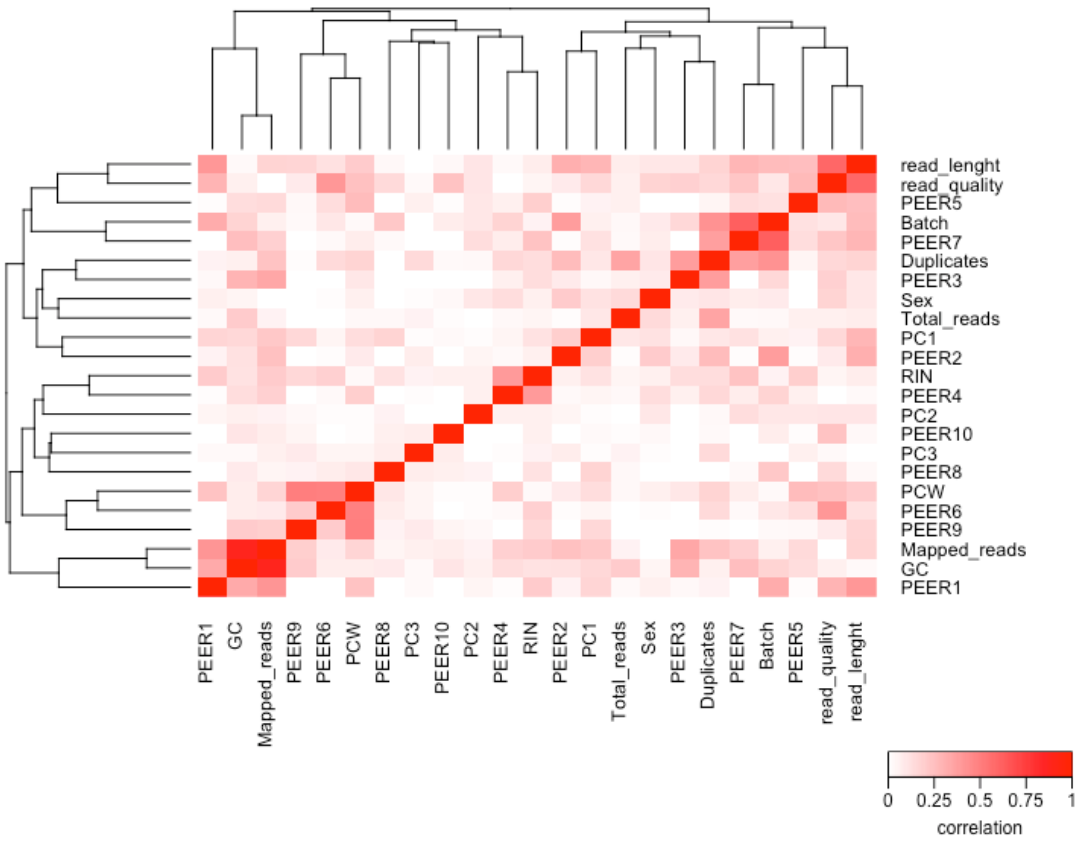


Figure 2.5 - Results from Canonical Correlation Analysis of variables employed in this study using Variance Partition.

The % of GC after filtering is highly correlated with Total mapped reads (r = 0.87). PCW is highly correlated with both PEER 6 (r=0.49) and PEER 9 (r=0.51), whilst sex does not seem to be highly correlated to any covariates

VariancePartition analysis was repeated after the removal of correlated variables in order to ascertain if the proportion of variance attributed to age was unmasked. In addition, I introduced an interaction term between SexxAge to investigate if there is an interaction between sex and age in my dataset.

2.2.15 Choice of covariates for differential expression (DE) analysis

All the covariates described in section 2.2.11 were employed in the eQTL study described in Chapter 3 because the FASTQTL method employed uses the resulting PEER residuals. However, it is not feasible to use an extensive number of covariates in a model for differential expression analysis due to the expenditure of available degrees of freedom. Moreover, collinearity should be avoided, and covariates should be independent. By analyzing the results from the VariancePartition analysis, I decided to employ the following criteria to choose which covariates to use in my DE analysis:

1) Drop one of the covariates highly correlated with each other (> 0.8) – Dropped % GC
2) Remove covariates with a correlation > 0.25 with sex and/or age – Dropped PEER5, PEER6, PEER9
3) Apart from sex and age (PCW), which are the covariates whose effects I wished to study, remove covariates that on average explain < 5% of the total variance in miRNA expression – Dropped PC1, PC2 and PC3, average read quality, % duplicates after filtering, average read length after filtering, total reads after filtering and % mapped reads, as well as PEER4 and PEER10.

As such, the covariates employed to construct a simplified model for DE analysis were sex and age (PCW), the effects of which I wanted to investigate, and hidden confounders PEER1, PEER2 and PEER3.

2.2.16 Differential gene expression analysis

Differential gene expression analysis was conducted in 112 samples that survived QC using limma – voom. Limma-voom was chosen for DE analysis because it allows for flexible model specification and adequately controls for FDR, as opposed to DESeq2 and edgeR, which originate a disproportionate amount of false positives (Li et al., 2022). Effects of sex and age on miRNA gene expression were assessed via a linear model using weighted least squares for each gene with the function lmFit, followed by empirical Bayes smoothing of standard errors (Smyth, 2004; Law et al., 2014).

838 miRNAs with > 1CPM in at least 10% of samples were included in the differential expression analysis. Within each differential expression analysis, multiple comparisons were corrected by the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995) and miRNAs with a BH-adjusted p-value (FDR) < 0.05 were deemed significant.

2.2.16.1 Sex-biased expression - design matrix

A design matrix was created where sex was used as the group variable. Age (PCW), PEER1, PEER2 and PEER3 were added as covariates to the design matrix, as demonstrated, to control for these effects. The model was constructed with an intercept term and employed females as reference (mean-reference model). Consequently, this model will calculate the mean miRNA expression of the male group relative to the female group (Law et al., 2020).

$$\text{design\_matrix} <\text{- model.matrix}\ (\sim \text{Sex} + \text{PCW} + \text{PEER1} + \text{PEER2} + \text{PEER3})$$

2.2.16.2 Effects of age on miRNA expression – Design matrix

Initially, I wanted to test which miRNAs were correlated with age in PCW by using a regression model. For this purpose, I kept age as a continuous variable and created a design matrix as follows:

$$\text{design\_matrix} <\text{- model.matrix}(\sim \text{PCW} + \text{Sex} + \text{PEER1} + \text{PEER2} + \text{PEER3})$$

This design matrix allowed me to estimate the rate of change in each miRNA expression per unit increase in age (PCW). After identifying miRNAs that were differentially expressed with the progression of foetal brain development, I calculated Pearson's correlation coefficients (r) to identify linear relationships between miRNA expression in differentially expressed miRNAs and age (PCW). Pearson's correlation coefficients (r) were calculated using the cor() function in R (v. 4.1.2).

2.2.17 miRNA Target prediction and GO analysis

miRNA targets were retrieved from TargetScan 8.0 (McGeary et al., 2019) for miRNAs that were found to be differentially expressed between females and males. TargetScan 8.0 only reports genes with a match to the miRNA seed region in their 3'- UTRs and their orthologs. All predicted miRNA targets for each sex-biased miRNA were employed for GO analysis using ShinyGO v0.76.2, a graphical tool for gene-set enrichment analysis (Ge et al., 2020). GO analysis was restricted to GO Biological processes (BP) and GO terms with a minimum of 2 and a maximum of 2000 genes. In addition, GO term

redundancies were removed from the analysis. Enrichment p-values FDR corrected and FDR < 0.01 was considered significant.

2.2.18 Graphical outputs

Plots were created using ggplot2 (Wickham et al., 2016) unless stated otherwise. The PCA plot (Figure 2.3) was created with the R package FactoMineR (Husson et al., 2007) and ggplot2. Functions plotVarPart and plotPercentBars from the VariancePartition package (Hoffman & Schadt, 2016) were employed to create VariancePartition plots (Figure 2.6, Figure 2.7, Figure 2.8); whilst function plotCorrMatrix was employed to create the correlation matrix of variables (Figure 2.5). The volcano plot (Figure 2.9) was created using the R package Enhanced Volcano v1.8.0 (Blighe et al., 2018). GO analysis enrichment plot (Figure 2.10) was created with ShinyGO (Ge et al., 2020). Age correlation plots and plots of TMM normalized mature miRNA counts between female and male samples (Figure 2.11B, Figure 2.12) were created with ggstatsplot (Patil, 2021).

2.3 Results

2.3.1 miRNAs detected in 2nd trimester foetal brain

Small-RNA sequencing data was used to quantify mature miRNA expression in 112 2nd trimester foetal brain samples between 12-20 PCW. The abundance of individual mature miRNAs was quantified using miRNA human (hsa) annotation from miRbase v.22, which contains 2644 mature microRNAs (including miR-3p and miR-5p). After filtering miRNAs with 0 counts in at least 90% of samples, a total of 1449 miRNAs were detected. This corresponds to 55% of all known miRNAs, highlighting the importance that miRNAs have in brain development, even if a large number of these are expressed at low levels. After filtering for low-expressed miRNAs (< 1 CPM in 90% of samples), a total of 838 miRNAs were kept for downstream analysis. This corresponds to 32% of all known miRNAs present in miRbase v22. Table 2.2 contains the top 10 most highly expressed miRNAs in my small RNA sequencing data, their respective genomic locations (chr), their average expression (log2 CPM + 1) across samples and their described function in the literature with regards to brain development. Together these 10 miRNAs account for ~ 62% of the detected miRNA expression. All of these miRNAs, except for miR-92a-3p, were also among the top 10 expressed miRNAs in a recent miRNA-Seq study in prenatal mouse brain (Rahmanian et al., 2019).

Table 2.2 – Top 10 most highly expressed miRNAs in my small-RNA sequencing study involving 112 2nd trimester foetal brains (12-20 PCW).

Chr - chromosome where miRNA gene is located.

miRNA	Chr	Average expression (log_2 CPM + 1)	Function in brain development	Reference
miR-9-5p	Chr1 Chr5 Chr15	17.25	Regulates balance between neural progenitor proliferation and neuron differentiation. Regulates neuronal migration.	Radhakrishnan & Anand, 2016; Rajman & Schratt, 2017
miR-181a-5p	Chr1 Chr9	17.18	Retinal axon specification and growth, axonal pathfinding, modulates synaptic plasticity Regulates apoptosis and mitochondrial function in neurons and astrocytes.	Carrella et al., 2015; Corradi & Baudet, 2020; Sambandan et al., 2017; Zhang et al., 2017; Khanna et al., 2011; Ouyang et al., 2012; Liu et al., 2013
miR-26a-5p	Chr3 Chr12	15.65	Regulates neuronal development, morphogenesis, and dendritic arborization.	Li and Sun, 2013; Luarte et al., 2020
miR-99b-5p	Chr19	15.22	Regulates mTOR.	Ye et al., 2015; Cao et al., 2017
miR-125b-5p	Chr11 Chr21	15.16	Promotes neuronal differentiation and neuronal migration, regulates synapse structure and function.	Cui et al., 2012; Edbauer et al., 2010
miR-92a-3p	Chr13 ChrX	15.10	Controls proliferation and differentiation of neural stem cells. Enhances axonal outgrowth.	Zhang et al., 2013; Xia et al., 2022; Jin et al., 2016
hsa-let-7a-5p	Chr9 Chr11 Chr22	14.92	Controls cerebral cortex development. Controls cell-cycle length and cell-cycle dynamics. Regulates neuronal differentiation.	Fairchild et al., 2019; Schwamborn et al., 2009
miR-92b-3p	Chr1	14.37	Controls proliferation and differentiation of neural stem cells. Enhances axonal outgrowth.	Zhang et al., 2013; Chen et al., 2019; Xia et al., 2022; Jin et al., 2016
hsa-let-7f-5p	Chr9 ChrX	14.01	Controls cerebral cortex development. Controls cell-cycle length and cell-cycle dynamics. Regulates neuronal differentiation.	Fairchild et al., 2019; Deng et al., 2020; Caygill & Johnson, 2008
hsa-let-7c-5p	Chr21	13.84	Regulates synaptic and neuronal function.	McGowan et al., 2018

2.3.2 VariancePartition of expression of miRNAs detected in 2nd trimester foetal brain

VariancePartition allowed me to quantify the proportion of variance in miRNA expression explained by each covariate (Hoffman & Schadt, 2016). This analysis was performed after removing low-expressed miRNAs to account for only the miRNAs employed in the differential expression analysis.

As can be seen in Figure 2.6, the effect of sex on miRNA expression as a whole is negligible after correcting for variation due to other variables, whilst the effect of gestational age (PCW) is small.

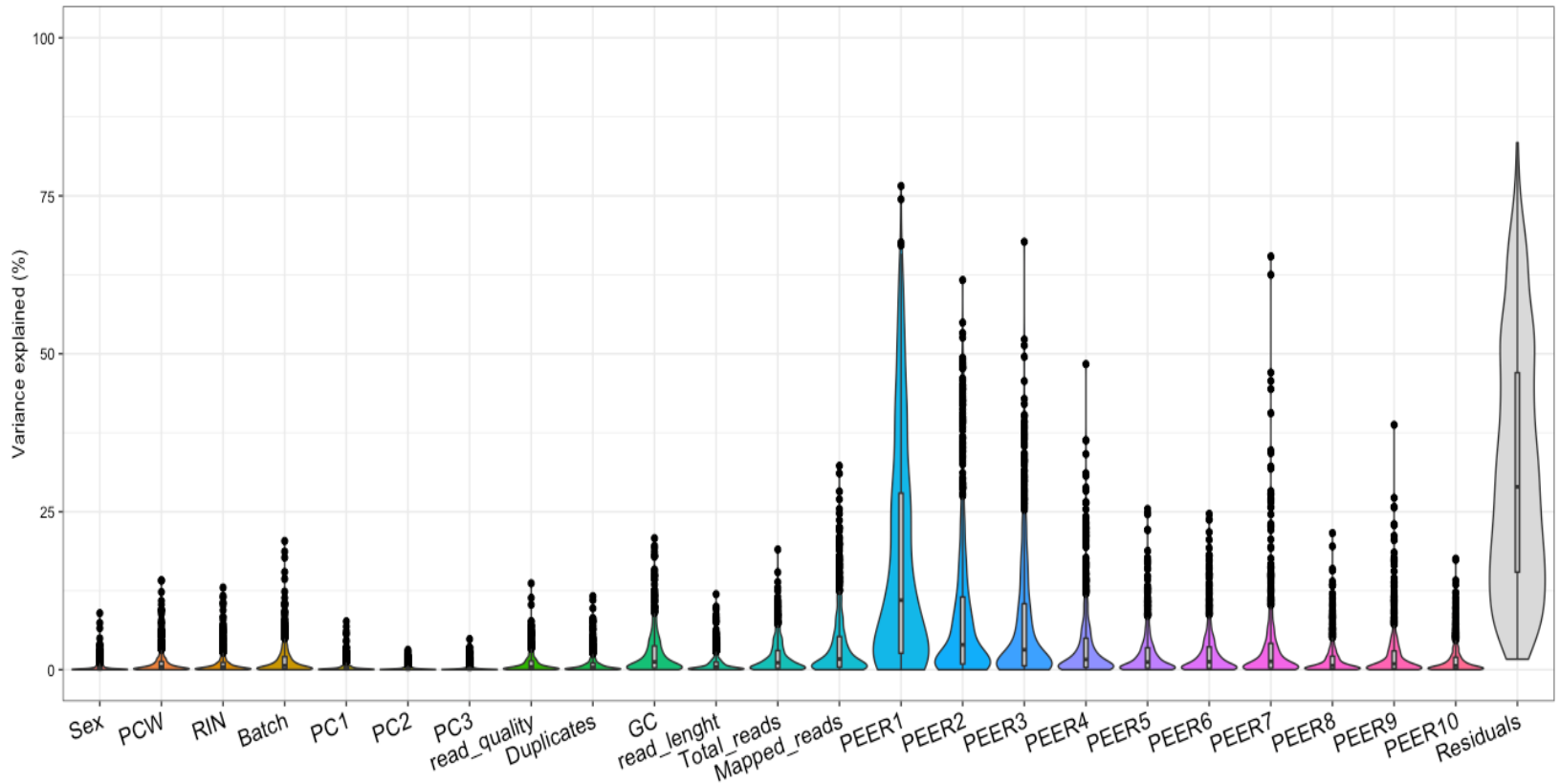


Figure 2.6 – Results from Variance Partition analysis showing major drivers of miRNA expression after removal of poorly expressed miRNAs (< 1 CPM in 10% of samples) in this study.

All terms were modelled as fixed effects with the exception of sex, which was modelled as a random effect. PEER1-PEER10 are the 10 hidden confounders estimated through PEER analysis. When correcting for all the other variables, on average, sex accounts for 0.37% of total miRNA expression variance, whilst PCW accounts for 1.08%, and the 3 PC components of ethnicity (PC1, PC2 and PC3) as a whole account for < 1% of total miRNA variance.

When correcting for all the other variables, on average, sex accounts for less than 1% of the variance of total miRNA expression, and gestational age accounts for 1.08% of the variance. The 3 PC components of ethnicity (PC1, PC2 and PC3) account for < 1% of the total miRNA variance. My sample population is an admixed population, and these results suggest that ethnicity does not contribute to differences in miRNA expression and as such, using an admixed population in my study is not a limitation for the eQTL analysis I will perform in chapter 3. This finding is consistent with a study by Quach and colleagues (2009), where mature miRNAs showed a lack of diversity in different human populations.

2.3.3 Identification of miRNAs where sex, age and the interaction between sex and age contributed more to variance of miRNA expression

Although my VariancePartition analysis indicated that sex did not significantly impact miRNA expression in foetal brain in general, it remained possible that individual miRNA show sex differences in expression. By performing VariancePartition analysis on individual miRNAs, I found a small subset of miRNAs where sex explained a larger proportion of variance in gene expression (Figure 2.7). For instance, miR-373-3p was identified as the miRNA with the highest variance attributable to sex, where sex explains ~ 9.75% of the variability in miR-373-3p expression.

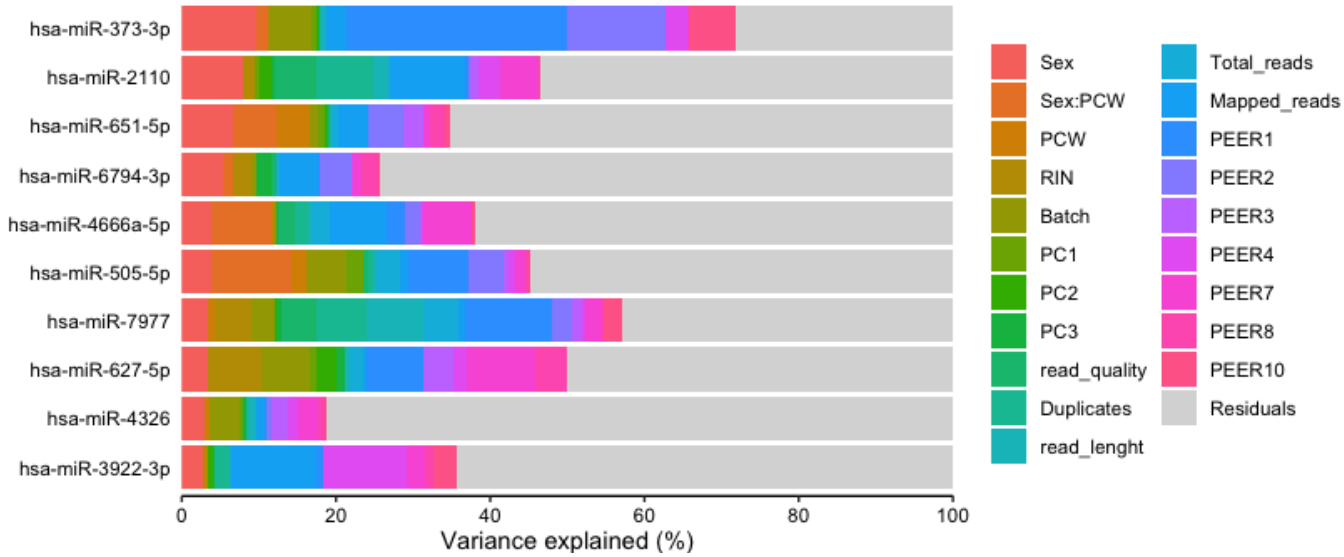


Figure 2.7 – Top 10 miRNAs whose expression is influenced by sex according to Variance Partition analysis of individual miRNAs

On a global level, the contribution of age for miRNA expression in 2nd trimester foetal brain was small. However, by performing VariancePartition on individual miRNAs, I identified a subset of miRNAs where age considerably impacts their expression. As can be seen in Figure 2.8, age is responsible for over 25% of the variance in the expression of both miR-195-3p and miR-195-5p.

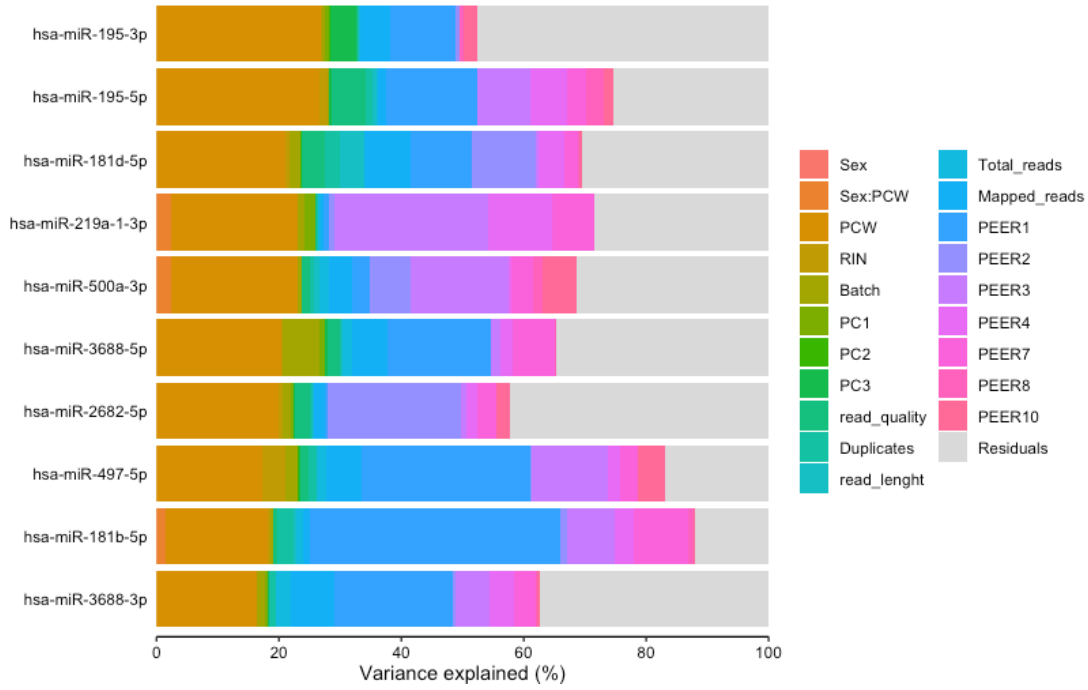


Figure 2.8 – Top 10 miRNAs whose expression is influenced by age (PCW) according to VariancePartition analysis.

VariancePartition analysis on individual miRNAs identified miRNAs whose expression was influenced by sex and age at a higher degree. Subsequently, I performed differential expression analysis to investigate if these miRNAs were differentially expressed in terms of sex and age in 2nd trimester foetal brain.

2.3.4 Differential expression analysis between females and males

To test if there were differentially expressed miRNAs due to sex, differential expression analysis was performed on 838 mature miRNAs between females (n=51) and males (n=61), using limma-voom. In this study, sample age ranged from 12-20 PCW, with an average of 14.63 PCW in females and 14.90 PCW in males.

At FDR < 0.05, only miR-373-3p was found to be differentially expressed between female and male 2nd trimester foetal brains, with lower expression in males (Table 2.3, Figure 2.9). This finding is consistent with my VariancePartition analysis (Figure 2.7).

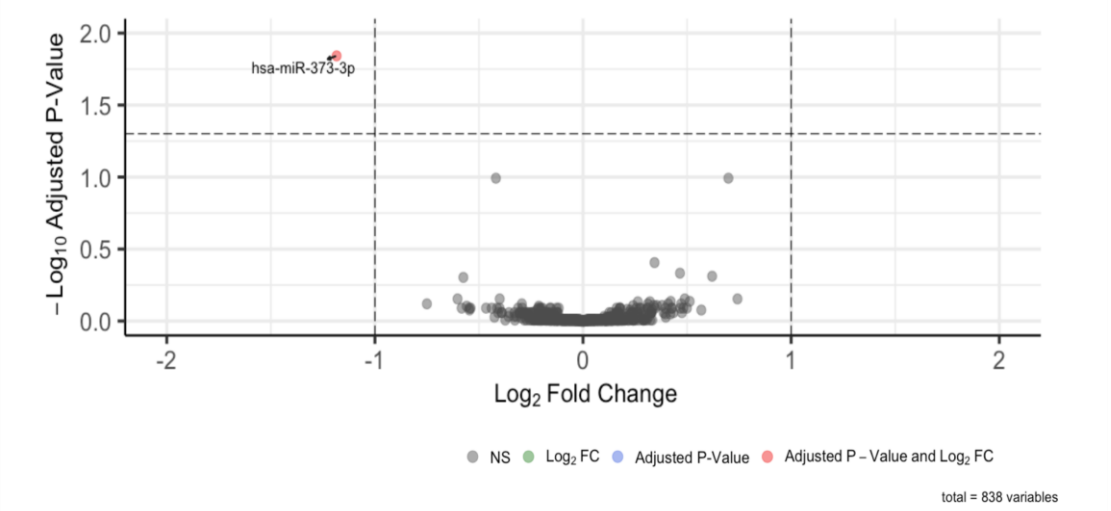


Figure 2.9 – Volcano plot of sex-biased gene expression in 2nd trimester foetal brains.

Adjusted P-value cut-off =0.05, log2FC cut-off = ±1. The miRNA miR-373-3p was the only miRNA found to be DE between female and male samples, with miR-373-3p being downregulated in males.

Table 2.3 – Results of differential expression analysis between female and male samples by limma-voom in 2nd trimester foetal brain, using females as reference.

miRNA - miRNA ID, Chr - chromosome location, Log_2FC – log2 fold change of miRNA expression between female and male samples, P-value – uncorrected p-value, Padj – corrected p-value by BH method, B - B-statistic which corresponds to the log-odds of miRNA being differentially expressed.

miRNA	chr	Log_2FC	P - value	Padj	B
hsa-miR-373-3p	19	-1.18	1.72E-05	0.014	2.35

miR-373-3p is located within the miR-371-3 gene cluster on chromosome 19q13.4 and is transcribed into a polycistronic pri-miRNA which is subsequently processed into 3 distinct pre-miRNAs (pre-miR-371, pre-miR-372 and pre-miR-373) (Suh et al., 2004). This miRNA is highly expressed in embryonic stem cells and has been shown to regulate cell proliferation (Tanaka et al., 2011), apoptosis (Eichelser et al., 2014; Lv et al.,2020) and mesendoderm differentiation (Rosa et al., 2014).

TargetScan v8.0 (McGeary et al., 2019) predicted that miR-373-3p (and its miRNA family members miR-302-3p/372-3p/520-3p) can target the 3'- UTR of 1019 transcripts. ShinyGO (Ge et al., 2020) was employed to perform a GO analysis of BP terms using all of miR-373-3p predicted targets and restricting GO analysis to pathways with $\leq$ 2000 genes. At FDR < 0.01, a total of 111 GO terms were found enriched for miR-373-3p targets. Figure 2.10 displays the top 20 most significant pathways. In addition to terms relating to transcription, "neuron differentiation" ($F_{enrichment}$=1.9, padj=1.7 x 10^{-11}), "generation of neurons" ($F_{enrichment}$=1.9, padj=1.3 x 10^{-11}) and "neurogenesis" ($F_{enrichment}$=1.9, padj=7.9 x 10^{-12}) were BP GO terms highly enriched in miR-373-5p targets.

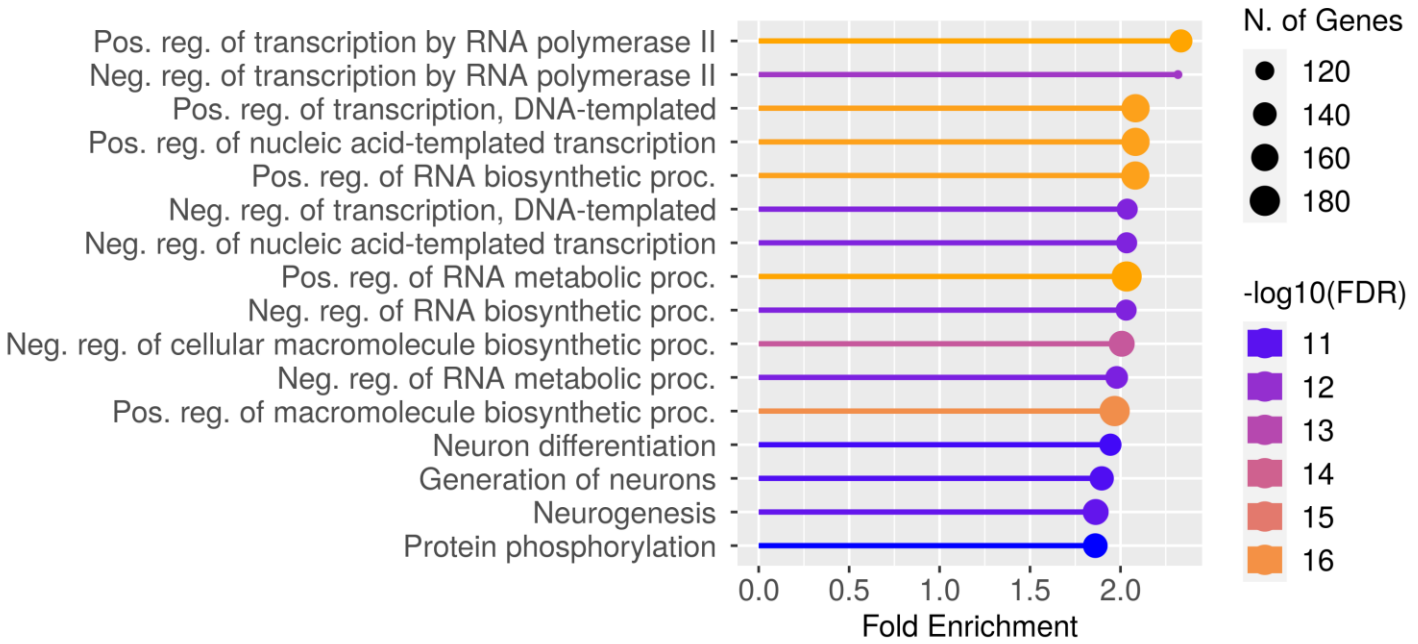


Figure 2.10 - Top 20 most enriched GO BP pathways of miR-373-3p predicted targets stratified by fold enrichment.

miRNA targets obtained with TargetScanv8.0 (McGeary et al., 2019); GO analysis was performed with ShinyGO (Ge et al., 2020).

Taken together, the results from VariancePartition and differential expression analysis between female and male samples suggest that mature miRNAs do not contribute significantly to observed sex differences in 2nd trimester foetal brain. This observation is in contrast with what was found for mRNAs in an RNA-Seq study by our lab employing largely overlapping samples, where prominent sex differences in the expression of 2558 autosomal genes, 155 genes on the X-chromosome and 43 genes on the Y-chromosome were found at a false discovery rate (FDR) < 0.1 (O'Brien et al., 2019).

2.3.5 Lack of consistency between sex differences in pri-miRNA expression assessed by RNA-Seq and mature miRNA expression assessed by Small-RNA Seq.

A previous RNA – Seq study from our lab (O'Brien et al, 2019) found that the primary transcripts (pri-miRNAs) of 14 miRNAs were found to be differentially expressed between female and male samples at FDR < 0.05 in 2nd trimester foetal brain (Table 2.4). Of note, the pri-miRNA for miR-373-3p was not amongst them.

Table 2.4 – Results of differential expression analysis by DESeq2 between female and male samples in 2nd trimester foetal brain, using females as reference (O'Brien et al, 2019).

miRNA - miRNA ID, Chr - chromosome location, log2FC – log2 fold change of miRNA expression between female and male samples, P-value – uncorrected p-value, Padj – corrected p-value by FDR.

Pri-miRNA	Chr	Female Counts	Male Counts	log_2FC	p-value	p_{adj}
MIR3682	chr2	67	45	-0.257	3.6E-05	0.00325
MIR873	chr9	32	20	-0.276	4.9E-05	0.00401
MIR651	chrX	3	1	-0.223	0.00018	0.00918
MIR383	chr8	7	4	-0.255	0.00018	0.0094
MIR570	chr3	38	25	-0.257	0.00019	0.00973
MIR2681	chr13	18	12	-0.247	0.00028	0.0124
MIR646HG	chr20	178	121	-0.243	0.00043	0.0165
MIR539	chr14	6	3	-0.231	0.00055	0.0193
MIR4697HG	chr11	1688	1302	-0.187	0.00077	0.0239
MIR154	chr14	9	5	-0.221	0.00087	0.0254
MIR181A1HG	chr1	1600	1861	0.147	0.00129	0.0327
MIR548AY	chr3	4	2	-0.207	0.00136	0.0337
MIR186	chr1	52	40	-0.215	0.00167	0.0385
MIR670HG	chr11	23	29	0.214	0.00191	0.042

MIR3682, the most significantly sex-biased pri-miRNA in the RNA-Seq study, had very low expression counts in this study. Its expression was below the cut-off of low expression of > 1 CPM in at least 10% of samples and consequently had been eliminated from the DE analysis. As such, depicted in Figure 2.11 are the side-by-side comparison of normalized counts between females and males of MIR873, the 2nd most significant sex-biased pri-miRNA, which showed sex-biased expression in the RNA-Seq study (obtained from http://fgen.psycm.cf.ac.uk/FBSeq1/) and their corresponding mature miRNA normalized counts between females and males in this study.

These findings suggest that changes in pri-miRNA expression do not necessarily lead to changes in expression in the corresponding mature miRNAs, which seem to be maintained at a reduced level of variability, possibly via regulation of mature miRNA biogenesis by modulation of Drosha and Dicer/TRBP.

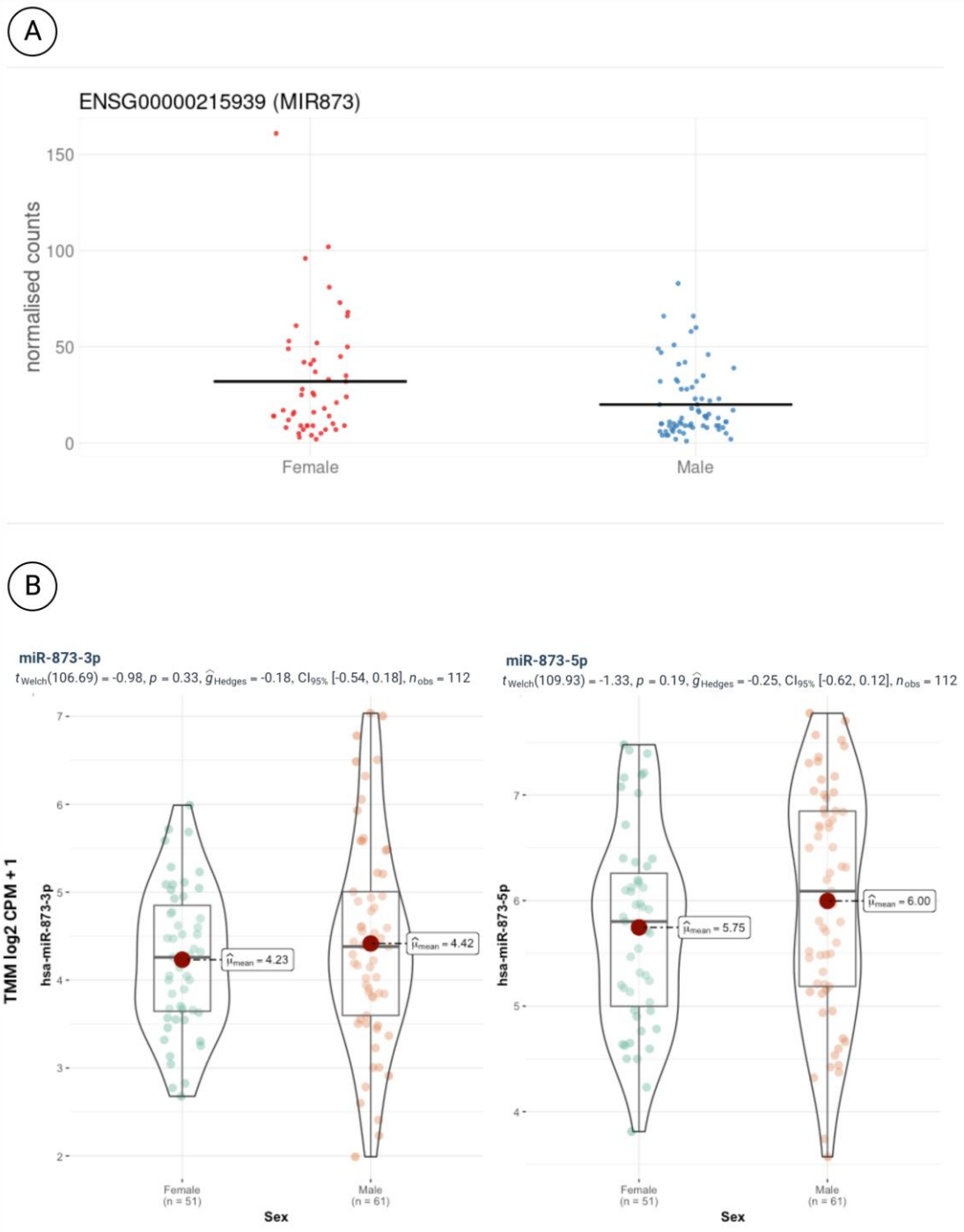


Figure 2.11 – Comparison of pri-miRNA sex-biased effects with lack of sex-biased effects in respective mature miRNAs.

A - Normalized counts between females and males of MIR873 pri-miRNA showing sex biased expression in the RNA-Seq study at FDR < 0.05. **B -** Their respective mature miRNAs miR-873-3p and miR-873-5p normalized counts.

2.3.6 Effect of gestational age on mature miRNA expression in 2nd trimester foetal brain

The effect of gestational age on gene expression was ascertained by building a linear model accounting for sex and PEER1, PEER2 and PEER3 factors via limma-voom. Age was used as a continuous variable; by doing this, I wanted to ascertain if there were miRNAs that had differences in gene expression as age increased. At FDR < 0.05, a total of 171 miRNAs were found to be differentially expressed with age increase. Pearson correlations were subsequently calculated for these miRNAs using the expression residuals obtained from the linear model. Of these 171 miRNAs, 83 were negatively correlated with age meaning their expression decreases as time progresses, and 88 were positively correlated with age, meaning their expression increases as time progresses. Table 2.5 shows the top 20 miRNAs that were differentially expressed with age increase. I focused on the top 2 differentially expressed miRNAs with age and plotted the correlations between miR-195-5p expression and age (positively correlated) and miR-219a-1-3p expression and age (negatively correlated) (Figure 2.12). Of note, circulating levels of miR-195-5p have been shown to be altered in ASD (Vasu et al., 2014; Hu et al., 2017), SZ (Wei et al., 2015) and ADHD (Zadehbagheri et al., 2019). Moreover, this miRNA is disrupted by an ASD-associated CNV (Vaishnavi et al., 2013) and has been found to be upregulated in the entorhinal cortex of AD patients (Dobricic et al., 2022).

Table 2.5 – Top 20 miRNAs that are differentially expressed with age in 2nd trimester foetal brain, where age was employed as a continuous variable.

miRNA - miRNA ID, Chr - chromosome location, log_2FC – log_2 fold change of miRNA expression per timepoint (PCW), P-value – uncorrected p-value, Padj – corrected p-value by BH method, B - B-statistic which corresponds to the log-odds of miRNA being differentially expressed, R – Pearson's correlation.

miRNA	log_2FC	P - value	Padj	B	R
hsa-miR-195-5p	0.34	4.16E-16	3.48E-13	26.12	0.63
hsa-miR-219a-1-3p	-0.22	5.91E-12	2.47E-09	16.85	-0.48
hsa-miR-497-5p	0.32	1.06E-11	2.96E-09	16.29	0.48
hsa-miR-22-5p	0.18	2.26E-11	4.74E-09	15.55	0.33
hsa-let-7b-5p	0.18	9.59E-11	1.61E-08	14.07	0.56
hsa-miR-301b-3p	-0.19	1.83E-10	2.56E-08	13.47	-0.28
hsa-miR-128-3p	0.17	1.37E-09	1.64E-07	11.46	0.54
hsa-miR-181a-5p	0.12	2.20E-09	2.31E-07	10.96	0.48
hsa-let-7c-5p	0.12	2.59E-09	2.41E-07	10.84	0.51
hsa-miR-195-3p	0.30	3.47E-09	2.91E-07	10.66	0.51
hsa-miR-500a-3p	-0.17	8.04E-09	6.13E-07	9.81	-0.33
hsa-miR-181a-3p	0.14	1.41E-08	9.82E-07	9.22	0.49
hsa-miR-2682-5p	0.21	1.99E-08	1.28E-06	9.04	0.42
hsa-miR-660-5p	-0.14	2.79E-08	1.67E-06	8.62	-0.49
hsa-miR-301a-3p	-0.14	3.07E-08	1.72E-06	8.47	-0.27
hsa-miR-1251-5p	-0.25	2.40E-07	1.24E-05	6.65	-0.46
hsa-miR-135a-2-3p	-0.21	2.52E-07	1.24E-05	6.65	-0.41
hsa-miR-148a-3p	0.13	2.96E-07	1.38E-05	6.29	0.35
hsa-miR-887-3p	-0.16	3.35E-07	1.48E-05	6.25	-0.25
hsa-miR-3688-3p	0.23	4.22E-07	1.69E-05	6.18	0.47

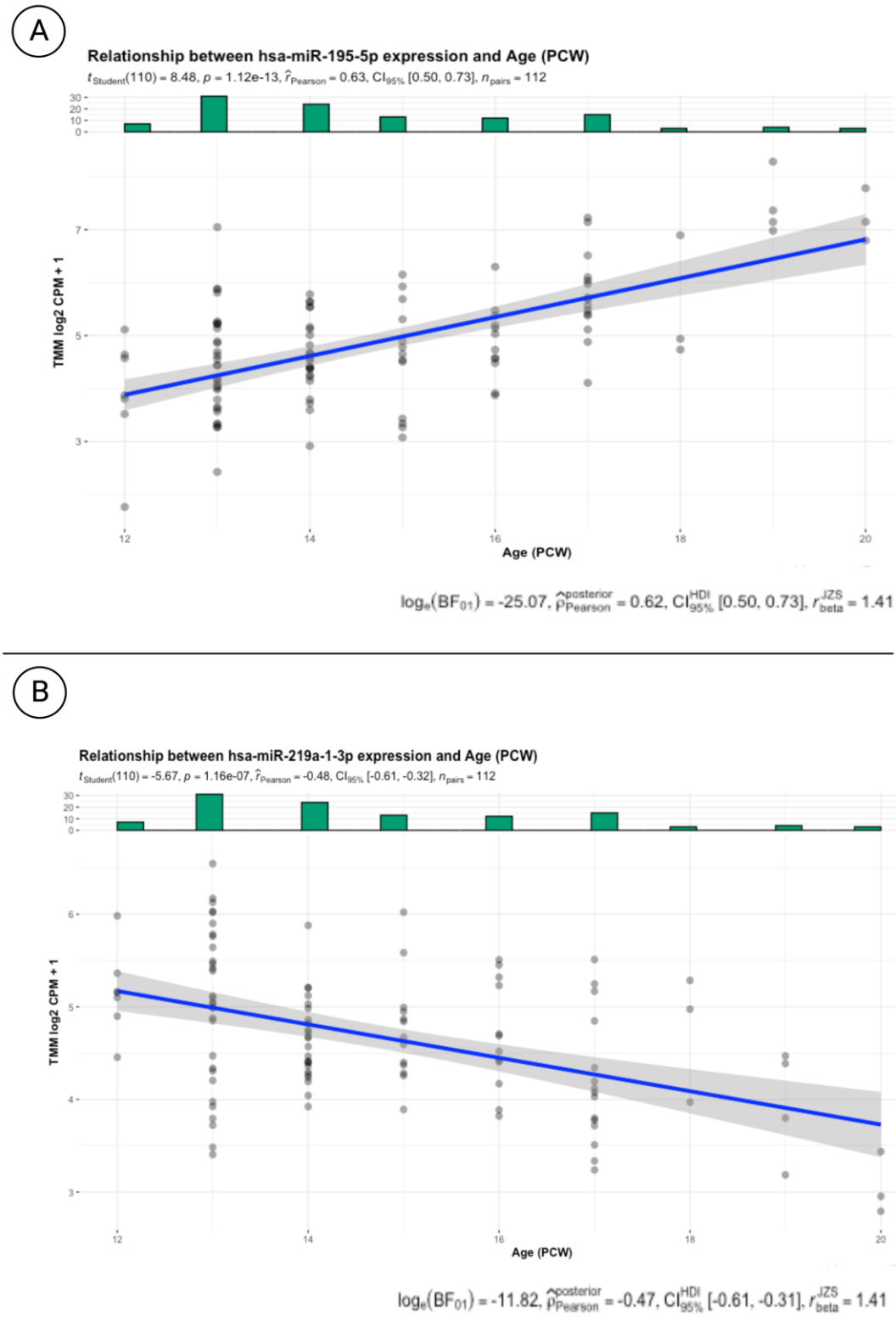


Figure 2.12 – Correlation of individual miRNA expression with age.

Correlation of miR-195-5p the miRNA with the highest positive correlation with age **(A)** and miR-219-a-1-3p with the highest negative correlation with age **(B)**.

2.4 Discussion

2.4.1 miRNAs detected in 2nd trimester foetal brain

This chapter explored the expression of miRNAs in human 2nd trimester foetal brain (12 – 20 PCW) and the influence of sex and gestational age. Small RNA Sequencing was performed on bulk tissue in a collection of 112 foetal brain samples, and 1449 miRNAs were detected after removing miRNAs with 0 counts in > 90% of samples. Subsequently, low-expressed miRNAs (< 1 CPM in at least 10% of samples) were eliminated from the downstream differential expression analysis, leaving 838 miRNAs. As previously shown in adult mouse tissues and both human and mouse prenatal tissues (Lagos-Quintana et al., 2002; Landgraf et al., 2007; Rahmanian et al., 2019), a handful of highly expressed miRNAs account for the majority of the detected miRNA expression, with ~62% of miRNA expression in my study originating from the top 10 most highly expressed miRNAs.

Some of the top 10 highly expressed miRNAs identified in this study have a high tissue specificity index (TSI) in human adult tissues (Ludwig et al., 2016), adult mouse tissue (Lagos-Quintana et al., 2002; Gao et al., 2011) and prenatal mouse tissues (Rahmanian et al.,2019). For instance, miR-9-5p, miR-125b-5p, and miR-92b-3p are highly brain-specific, whereas others such as miR-26a-5p are ubiquitously expressed at high levels across tissues in prenatal mouse (Rahmanian et al.,2019).

The FANTOM5 project created a miRNA expression atlas which allowed the calculation of a cell-type specificity index for each miRNA by deep-sequencing 47 mouse and 396 human samples, including 121 distinct human cell types (De Rie et al., 2017). By using this metric, I ascertained that the

majority of the miRNAs highly expressed in 2nd trimester human brain consist of miRNAs that are moderate to highly cell-type specific (Table 2.6). A notable exception are members of the let-7 family, such as miR-let7f-5p and miR-let7a-5p, which are ubiquitously expressed at high levels across tissues and cell types (McCall et al., 2017).

Table 2.6 – Cell-type specificity index obtained from De Rie et al., (2017) for the top 10 most highly expressed miRNAs identified in this study.

miRNA	Cell type specificity index	First most enriched in cell ontology cluster	P-value
hsa-miR-9-5p	0.99	neural cell	2.65E-42
hsa-miR-181a-5p	0.863	leukocyte	1.64E-16
hsa-miR-26a-5p	0.767	leukocyte	1.17E-50
hsa-miR-99b-5p	0.61	endothelial cell	8.75E-05
hsa-miR-125b-5p	0.825	muscle cell	1.02E-17
hsa-miR-92a-3p	0.739	pluripotent stem cell	1.13E-11
hsa-let-7a-5p	0.544	mesodermal cell	1.76E-07
hsa-miR-92b-3p	0.911	pluripotent stem cell	6.04E-21
hsa-let-7f-5p	0.555	mesodermal cell	7.71E-12
hsa-let-7c-5p	0.96	neural cell	4.31E-14

Lafferty and colleagues (2022) recently released a preprint in *bioRxiv* where they performed small-RNA Sequencing in 212 2nd trimester foetal brain samples. Using a cut-off of 10 counts across at least 10 samples, the authors reported detecting 621 miRNAs present in miRbase v.22 (Lafferty et al., 2022). In my dataset, after removing low-expressed miRNAs, I detected all but 5% of the miRNAs detected by Lafferty and colleagues (2022) (Figure 2.13). My detection cut-off seems more lenient, which would explain why I detected a higher number of miRNAs.

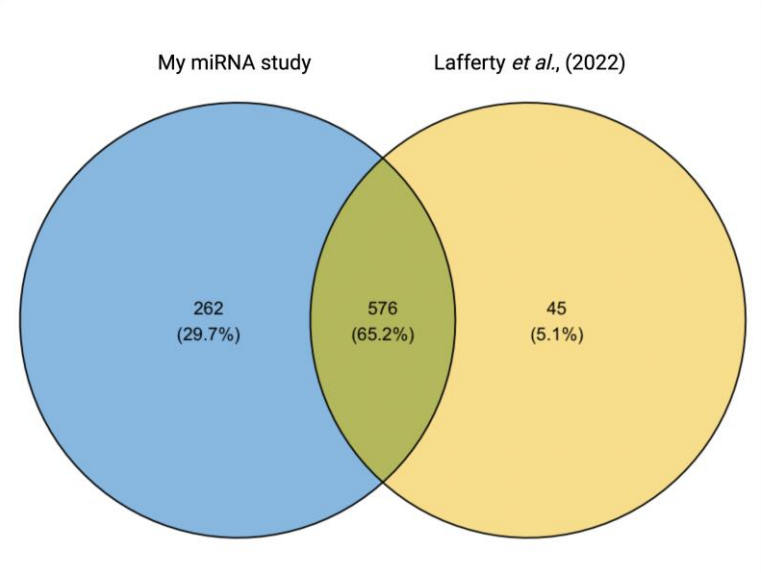


Figure 2.13 – Venn Diagram of the miRNAs detected in my study after removal of low miRNA counts and the miRNAs detected in a similar study in 2nd trimester foetal brain (Lafferty et al., 2022, preprint).

By comparing which miRNAs were more highly expressed between my small RNA-sequencing study and the one performed by Lafferty and colleagues (2022) (Table 2.7), I found that 9 out of 10 miRNAs were concordant between both studies.

Table 2.7 – Top 10 most highly expressed miRNAs in the Lafferty and colleagues (2022) small RNA sequencing study involving 212 2nd trimester foetal brains.

Mean VST – Mean variance stabilizing transformation (VST) normalized counts (log2 CPM + 1)

miRNA	Mean VST
hsa-miR-181a-5p	17.68
hsa-miR-9-5p	17.16
hsa-miR-92a-3p	17.09
hsa-miR-26a-5p	16.79
hsa-let-7a-5p	16.27
hsa-let-7c-5p	16.27
hsa-miR-92b-3p	15.27
hsa-miR-125b-5p	15.09
hsa-let-7f-5p	14.99
hsa-miR-128-3p	14.91

2.4.2 Sex differences in global miRNA expression in 2nd trimester foetal brain

Both the analysis of variance in miRNA expression via variancePartition and the differential expression analysis by sex combining all samples aged 12-20 PCW indicate that sex has a negligible effect on global miRNA expression in 2nd trimester foetal brain. At FDR < 0.05, only miR-373-3p was found to be differentially expressed between females and males, with a lower expression in males. Interestingly, this miRNA has been suggested as a candidate regulator of differentially expressed genes in AD, a disease that displays higher incidence in females (Abyadeh et al., 2022). These results suggest that miRNAs do not contribute significantly to sex differences in foetal brain development. However, there is still the possibility of sex differences in miRNA expression in specific cell-types that I could not detect, given that my analysis was performed on bulk brain tissue. Recently, a miRNA-Seq study reported 87 miRNAs with sex-biased expression in microglia derived from adult mouse brains (Kodama et al., 2020). It's possible that the expression of miRNAs in human foetal microglia also differs substantially by sex, and that this effect was missed, due to microglia being a minor cell population in the developing brain (Hatori, 2022) and microglial density substantially decreasing due to apoptotic refinement during the 2nd trimester (from 12-16 PCW) of foetal brain development (Menassa et al., 2022). Moreover, it is possible that sex differences in miRNA expression occur at later foetal development stages, such as during the 3rd trimester.

The paucity of sex-biased miRNA expression in 2nd trimester foetal brain is surprising, given that the X-chromosome is highly enriched in miRNAs with approximately 6% of human miRNAs originating from the X-chromosome (118 miRNAs), whereas 4 miRNAs originate from the Y-chromosome (Di Palo et al., 2020). Moreover, at least in reproductive tissues, both oestrogen and progesterone can regulate the expression of key components of miRNA biogenesis, such as exportin-5, Dicer and AGO2, and consequently regulate

mature miRNA levels (Bhat-Nakshatri et al., 2009; Nothnick et al., 2010; Cheng et al., 2009).

None of the 4 Y-linked miRNAs were expressed in my 2nd trimester foetal brain samples, which contributed to the lack of observed sex differences in miRNA expression. However, several of the X-linked miRNAs expressed in my dataset are intronic to genes that escape X-inactivation (Matarrese et al., 2019; Di Palo et al., 2020) and, therefore, likely to also escape gene silencing and be differentially expressed between sexes at the pri-miRNA level.

I found no evidence of differences in expression of X-linked miRNAs between sexes at the mature miRNA level. A possible explanation for this observation and for the sparsity in sex-biased miRNA expression in 2nd trimester foetal brain is that miRNAs can regulate hundreds of target genes, and their expression needs to be tightly regulated across development. If miRNAs varied widely between females and males throughout all of foetal development, they would cause widespread downstream transcriptional effects, creating a cascade of events that would be difficult to regulate. This hypothesis is consistent with the principle of “canalization” (Waddington, 1959), where development tends to reach the same endpoint despite minor environmental and genetic variation. miRNAs have been suggested to act as agents of canalization when they dampen and fine-tune gene expression (Posadas & Carthew, 2014) and, as such, are a lot less amenable to sex-biased expression. An RNA-Seq study by Ziatts and Rennert (2014) reported 40 miRNAs with sex-biased expression over brain development between infancy to adolescence. However, the largest proportion of these miRNAs (n=26) was found in the prefrontal cortex of adolescent brains. In infancy (4 months – 1 year), only two miRNAs were differentially expressed between females and males, with one miRNA upregulated in each sex. This observation further supports the notion that miRNAs do not contribute significantly to sex differences during early brain development (foetal and perinatal/neonatal period).

2.4.3 Sex differences in pri-miRNA expression do not correlate with changes in mature miRNA expression

A previous RNA-seq study from our lab, employing largely the same samples, reported that 14 miRNA primary transcripts (pri-miRNAs) were found to be differentially expressed between males and females in 2nd trimester foetal brain at FDR < 0.05 (O'Brien et al., 2019). Notably, miR-373-3p was not among them. This suggests that the sex-biased expression of miR-373-3p I observed is not a consequence of sex differences in transcriptional regulation. Therefore, it is likely that sex differences in mature miR-373-3p levels arise from differential regulation of the miRNA biogenesis machinery (e.g., Drosha, Dicer/TRBP) or differential regulation of miRNA turnover.

Moreover, none of the pri-miRNAs found to be differentially expressed in the RNA-Seq study (O'Brien et al., 2019) were differentially expressed at the mature miRNA level in largely the same samples. A possible explanation for the discrepancy with the present findings could be that miRNA expression is tightly regulated during the biogenesis of mature miRNAs and resists change within a specific range of differences in pri-miRNA expression. This would mean that miRNA expression would usually be "buffered" within certain limits to maintain stable, mature miRNA levels. Given that miRNA up- and down-regulation is associated with multiple disorders, most notably cancer, a self-regulatory loop of this sort is likely in place to prevent dysregulated expression of miRNAs. Considering both these findings, it seems that changes in pri-miRNA expression do not necessarily lead to changes in mature miRNA expression and subsequent downstream effects on miRNA target downregulation. This is again consistent with the role of miRNAs in canalization (Posadas & Carthew, 2014) and would explain the lack of sex differences in mature miRNA expression of miRNAs that escape X-inactivation in 2nd trimester foetal brain. In this light, sex-biased pri-miRNA

expression and mature miRNA expression in 2nd trimester foetal brain can be seen as uncoupled from one another.

In breast cancer cell lines, oestrogen can regulate the expression of several miRNAs via ERa, ERb and c-Myc at the transcriptional level (Castellano et al., 2009; Bhat-Nakshatri et al., 2009; Maillot et al., 2009). Most studies report that oestrogen treatment is associated with a global decrease in miRNA expression, particularly in miRNAs with guanine-rich apical loop precursors (Maillot et al., 2009; Cohen et al., 2017). In contrast, only a small subset of miRNAs was found to be upregulated (and some downregulated) in response to oestrogen in aging rat brain tissue (Rao et al., 2013; Rao et al., 2015), without a corresponding change in either the pri-miRNA or the pre-miRNA levels (Rao et al., 2015). A subsequent study by the same group confirmed that, at least in the aging rat brain, oestrogen seems to selectively regulate miRNA levels by stabilization of mature miRNAs instead of affecting pri-miRNA transcription or Drosha processing (Kim et al., 2021). It is plausible that the same occurs in foetal brain, which would explain why miR-373-3p is differentially expressed between sexes without a corresponding change in its pri-miRNA levels and why the sex differences in pri-miRNA expression observed by O'Brien and colleagues (2019) do not correlate with changes in mature miRNA levels.

2.4.4 Downstream effects of Sex differences in miRNA expression in 2nd trimester foetal brain development

miR-373-3p, the only miRNA with sex-biased expression identified in this study, possesses a small fold change between males and females with a 1.18 $\log_2$FC difference between sexes. However, this seems to be common for autosomal genes (Mayne et al., 2016; Gershoni & Pietrokoski, 2017;

O'Brien et al., 2019) and small changes in miRNA levels will likely drive further differences in mRNA expression between females and males.

This miRNA is predicted to target the 3'-UTR of 1019 transcripts, according to Targetscan v8.0, that are enriched for GO BP terms, including neuron differentiation (padj=1.7 x 10^{-11}), generation of neurons (padj=1.3 x 10^{-11}) and neurogenesis (padj=7.9 x 10^{-12}) as well as terms relating to transcription. miR-373-3p is downregulated in male 2nd trimester foetal brain samples in my dataset. This could lead to increased expression of miR-373-3p targets in males, potentially affecting neuronal development.

2.4.5 Effects of age on miRNA expression

VariancePartition analysis indicated that the effect of age on global miRNA expression in my 2nd trimester foetal brain samples is small.

However, I found 171 individual miRNA that did show a correlation in expression with post-conceptional week, with approximately half upregulated and half downregulated with increasing gestational age. Examples include miR-22-5p, which was upregulated with age and is known to regulate neuronal migration and morphology (Volvert et al., 2014). In contrast, miR-92b was downregulated with age, has known roles in maintaining NSC proliferation, and is expressed at higher levels in neural progenitors than neurons (Nowakowski et al., 2003). Therefore, it is likely that the miRNAs found to be downregulated with increasing gestational age are likely to be more critical for neural stem cell proliferation. In contrast, the miRNAs upregulated with increasing gestational age are likely to be more critical for processes like neuronal differentiation.

Moreover, only a subset of miRNAs likely contributes to temporal gene expression dynamics and the coordinated cascade of events that govern

foetal brain development. This hypothesis is supported by a recent study in mice foetal development where the expression gradients of 3 miRNAs – miR-128/miR-9 and let-7 were reported to be sufficient to specify neuronal laminar fates. A decreasing gradient of miR-128 and miR-9 is required to generate neurons in layer VI and layer V. In contrast, an increasing gradient of let-7 is necessary to generate neurons in layers IV-II (Shu et al., 2019).

Given that the developmental window in this study was only 9 weeks, it is possible that gestational age effects on the expression of other miRNAs were missed. Also, given that this study was performed in bulk tissue, I could only ascertain the effects of gestational age on global miRNA expression in the 2nd trimester foetal brain. As a consequence, cell-specific miRNA dynamics were missed. Moreover, it is possible that region-specific miRNA dynamics in important foetal brain regions such as the VZ, SVZ and CP were also missed.

In chapter 3, I will perform a miR-eQTL study in 2nd trimester foetal brain using the small-RNA sequencing data described in this chapter with genome-wide genotyping of these samples, controlling for the variables identified in this chapter.

Chapter 3: microRNA cis - eQTL in 2nd trimester human foetal brain

3.1 Introduction

Genome-wide association studies (GWAS) have identified thousands of genetic variants associated with common traits and diseases (MacArthur et al., 2017). The majority of the most significantly associated variants reside in non-coding regions (intronic or intergenic) of the genome and are therefore assumed to alter gene regulation (gene expression or splicing) (Hindorff et al., 2009; Encode Project Consortium, 2012; Eicher et al., 2015).

Gene expression levels have been shown to contribute to disease outcomes (Giraud et al., 2007) and are quantitative traits that can be seen as intermediate phenotypes (Cheung & Spielman, 2002). The variability in gene expression itself is a heritable trait in humans (Yan et al., 2002) and, therefore, amenable to association analysis (Brem et al., 2002; Schadt et al., 2003). Variants associated with altered gene expression can be mapped on a genomic scale by combining genome-wide genotyping with transcriptomic data from a given tissue. These are commonly referred to as expression quantitative trait loci (eQTLs) (Schadt et al., 2003; Fehrmann et al., 2011). eQTL studies can help identify to which extent a variant can affect gene expression. These variants can act on phenotypes by affecting the expression of local (cis) or distant (trans) gene targets.

The first human eQTL studies employed lymphoblastoid cell lines (LCL) transformed with Epstein–Barr virus (EBV) from individuals that had been extensively genotyped by the HapMap Consortium (Cheung et al., 2003; Spielman et al., 2007; Stranger et al., 2007). In this study, 888 genes were found to have cis-eQTLs at FDR < 0.2 (Stranger et al., 2007).

Subsequent studies on non–transformed primary blood cells (Goring et al., 2007; Emilsson et al., 2008) and primary tissues such as adipose tissue, liver and cortical brain have not only largely corroborated the previous eQTL studies in LCLs; but also revealed that 29–80% of the reported eQTLs are

tissue– and cell-specific (Myers et al., 2007; Emilsson et al., 2008; Schadt et al., 2008; Dimas et al., 2009; Nica et al., 2011).

Several large–scale studies have been undertaken to catalogue human brain eQTLs. The Genotype-Tissue Expression (GTEx) consortium examined RNA sequencing from 838 donors across 49 different tissues, including 11 distinct brain regions (GTEx Consortium, 2020). This study identified cis–eQTLs, tissue-specific trans–eQTLs, eQTL interactions across cell types, and sex-biased eQTLs. GTEx detected eQTLs for 1,260 –18,795 genes per tissue, leading to eQTL discovery for 95% of protein-coding RNAs and > 60% of long non-coding RNAs. Nearly a third of the reported eQTLs were estimated to be active in all or almost all assayed tissues; whilst a fifth were estimated to be active in five or fewer tissues (GTEx Consortium, 2015; GTEx Consortium, 2017; GTEx Consortium, 2020). Using cell–type deconvolution, Kim–Hellmuth and colleagues (2020) reported that a substantial proportion (~ 3000) of GTEx eQTLs are actually cell–type-specific eQTLs.

Recent efforts to understand altered gene expression in neuropsychiatric and neurogenerative diseases have employed eQTL studies to link disease-associated genetic variants to putative target genes. Fromer and colleagues performed an eQTL analysis on RNA-Seq data from the dorsolateral prefrontal cortex (DLPFC – Brodmann areas 9 and 46) of 279 controls and 258 individuals with SZ (Fromer et al., 2016). The authors reported that 80% of the genes assayed (13,137) had cis-eQTLs at FDR < 0.05. eQTL SNPs (eSNPs) were depleted in intergenic regions and enriched in gene elements (introns, exons, UTRs) and non-coding RNAs, mainly within 100 kb of the transcription start and end sites. Moreover, this study reported that in 20 out of 108 schizophrenia GWAS risk loci (Ripke et al., 2014), altered expression of one or more genes in adult DLPFC could at least partially explain the association with schizophrenia (Fromer et al., 2016).

Ng and colleagues (2017) used the ROSMAP cohort to generate a multiomic resource combining genotyping, RNA sequencing, DNA

methylation and histone acetylation data from the DLPFC of 411 older adults. They identified 3,388 genes with eQTLs, 56,973 CG dinucleotides with meQTLs and 1,681 H3K9Ac peaks with haQTLs at FDR < 0.05 in DLPFC and reported that epigenomic features mediate the effects of SNPs on gene expression in 9% of cases. Moreover, the authors employed a weighted GWAS (wGWAS) analysis approach to published genome-wide association studies to discover new susceptibility loci and identified 18 new schizophrenia and 2 new bipolar susceptibility variants (Ng et al., 2017).

The PsychENCODE Consortium generated a comprehensive resource of genomic, epigenomic and transcriptomic data from an extensive collection of brains (1,866 donors), including controls and individuals diagnosed with schizophrenia, bipolar disorder and autism. PsychEncode combined data from adult cortex datasets, including GTEx, the CommonMind Consortium (CMC), ENCODE and Roadmap, to map eQTL in the adult cerebral cortex (Wang et al., 2018). This allowed for the identification cis-eQTLs involving ~33,000 eGenes (expressed genes) in the PFC at FDR < 0.05, which encompasses almost the total number of genes expressed in adult brain.

A small number of eQTLs have been shown to be sex and age-dependent (Yao et al., 2014; Viñuela et al., 2018), and age-dependent changes in the variance of gene expression have been reported (Viñuela et al., 2018). eQTLs have also been shown to be developmental–stage specific in both Drosophila (Cannavo et al., 2016) and humans (O'Brien et al., 2018; Walker et al., 2019).

In the first eQTL study in foetal brain, O'Brien and colleagues (2018) identified 1,329 eQTLs in a dataset consisting of 120 2nd trimester foetal brains. 79% of the observed eQTLs in foetal brains were also found in adult brains, with 172 genes regulated by putative foetal–specific eQTLs (O'Brien et al., 2018). These results were subsequently corroborated by Walker and colleagues (2019) in a larger dataset of 201 foetal brains, where significant eQTLs were highly concordant between studies (effect size correlation, r = 0.67). However, Walker and colleagues identified a significantly higher number of eQTLs (n = 6,546) due to their larger sample size.

eQTL analysis can be extended to other phenotypes related to gene expression and regulation, such as miRNAs.

In the context of miRNAs and QTLs, there are two distinct types of QTLs, miRNA –binding QTLs and miRNA eQTLs. miRNA – binding QTLs are located within the 3'–UTR of their target transcript and affect miRNA binding, constituting 25% of all eQTLs in 3'– UTR in LCLs and blood cells (Liu et al., 2012; Gamazon et al., 2012). These are enriched in several psychiatric disorders, namely schizophrenia, depression, bipolar disorder, and anorexia nervosa (Geaghan et al., 2022). In contrast, miRNA eQTLs (sequence variants associated with miRNA expression levels) have been less studied due to the limited availability of miRNA sequencing data. Most of the earlier miR-eQTL studies were modestly powered and reported only a small number of miR-eQTLs.

The first published study to map miR-eQTLs was performed in primary fibroblasts from 180 European newborns of the GenCord project, where it was shown that out of 121 miRNAs interrogated, ~10% showed evidence for cis-regulatory variation (permutated *P*-value < 0.05; estimated FDR = 0.5) (Borel et al., 2011). The study also identified 18 significant trans–eQTLs for 13 miRNAs (estimated FDR = 0.3), including a SNP (rs1522653) that was an eQTL for 5 different miRNAs (Borel et al., 2011). Subsequently, Parts and colleagues (2012) performed a miR-eQTL study using the adipose tissue from 131 individuals from the Multiple Tissue Human Expression Resource (MuTHER) cohort consisting of LCLs, skin and fat tissue of approximately 60 twin pairs. This study reported a total of 14 eQTLs associated with the altered expression of 8 miRNAs at FDR < 0.05, which showed enrichment for variants associated with body mass index in GWAS. Half of these miR-eQTLs were also associated with mRNA transcript levels in the same region (Parts et al., 2012). An additional study on adipose tissue was performed by Civelek and colleagues (2013) on a population of 200 male subjects that were part of the Metabolic Syndrome in Men (METSIM) study. This study identified 9 miRNAs with significant *cis-eQTLs* at FDR < 0.05 but found no evidence of trans–eQTLs on miRNA expression (Civelek et al., 2013).

Increasingly larger sample sizes lead to more miR-eQTLs being discovered, as Lappalainen and colleagues (2013) reported a large number of eQTLs in 3,773 genes and a large number of cis-eQTLs involving 60 miRNAs at FDR < 0.05 in B–lymphoblastoid cell lines of 462 individuals from the 1000 Genomes Project.

In an effort to understand how the genetic control of miRNA expression changes in response to external stimuli, Siddle and colleagues (2014) mapped miR-eQTLs in monocyte-derived dendritic cells from 65 healthy individuals of European descent before and after infection with *Mycobacterium tuberculosis*. In this study, ~40% of miRNAs were shown to be differentially expressed upon infection, with 3% of miRNAs being affected by local genetic factors. Moreover, the authors report two infection-specific response eQTLs for miRNAs, illustrating the impact of genotype-environment interactions on miRNA expression (Siddle et al., 2014). Subsequently, the same group surveyed 977 genome-wide miRNA sequencing profiles from primary human monocytes derived from 100 individuals of African descent and 100 individuals of European descent at the basal state and upon immune activation. The authors identified eQTLs associated with 122 miRNAs at FDR < 0.05, with only a small proportion of these found in highly expressed miRNAs and/or miRNAs with conserved promoters. Moreover, most miR-eQTLs were unaffected by immune stimulation (85 % of miR-eQTLs). In contrast, 53% of the mRNA eQTLs in the same study displayed context-dependent effects. These results suggest that miR-eQTLs are largely context-independent in monocytes (Rotival et al., 2020).

Huan and colleagues (2015) compiled the first comprehensive genome-wide map of miRNA eQTLs in whole blood, where they performed miR-eQTL mapping of 5,239 individuals from the Framingham Heart Study (FHS) and identified cis-eQTLs for 76 mature miRNAs at an FDR < 0.1. Of these, 49 miRNAs (64%) were intragenic and located in exons, introns or untranslated regions of the host genes, and 27 (36%) were intergenic. Using family data from the study cohort, the authors estimated that genetic factors, on average, explain 11% of the variation in miRNA level under an additive model. The

majority of cis-eQTLs identified (58% in intragenic miRNAs and 83% in intergenic miRNAs) were located 5'-upstream of their associated mature miRNA/ primary miRNA sequence, with 49% of cis-eQTLs being located distally 300 – 500 kb upstream of their associated intergenic miRNA. Functional analysis of the cis-eQTLs identified in this study showed that a large proportion of these were located in regulatory elements such as CpG islands (2%), promoters (9%), enhancers (35%), and transcription factor (TF) binding regions (15%). Interestingly, this study and several others reported that 20% – 33% of miR-eQTLs were also associated with variation in the expression of their mRNA targets in immune cells (Gamazon et al., 2012; Lappalainen et al., 2013; Huan et al., 2015).

Budach, Heinig and Marsico (2016) combined miRNA eQTL data from the Lappalainen study (2013) with epigenetic and regulatory annotations for the B–lymphoblastoid cell line under study from the ENCODE project (2012) and determined the relationship between regulatory functional elements and miRNA regulation. The authors showed miR–eQTL enrichment for regulatory regions such as promoters, enhancers, transcription factor binding sites, and precursor miRNAs. miRNA–only eQTLs were enriched for intronic promoters, confirming the existence of alternative promoters for intragenic miRNAs.

Moreover, despite a significant overlap between miR-eQTLs and mRNA eQTLs of host genes, 74% of these eQTLs affected miRNA and host gene expression independently (Budach et al., 2016).

Nikpay and colleagues (2019) performed an eQTL analysis of circulating plasma miRNAs in a sample of 710 unrelated subjects of European ancestry and found miR-eQTLs for 143 miRNAs, including cis-eQTLs for 60 miRNAs ($P < 5 \times 10^{-8}$). Most of the cis-eQTLs identified (62%) were located distally, more than 50 kb away from their associated miRNA, corroborating previous studies (Huan et al., 2015). Moreover, the authors denoted that cis-eQTLs were associated with both miRNA primary transcripts and their corresponding mature miRNAs ($P < 0.0001$). In contrast, trans–eQTLs lacked such an association ($P = 0.7$) (Nikpay et al., 2019). These results suggest

that, at least in plasma, cis-miR-eQTLs mainly regulate the expression of pri-miRNAs, whilst trans-miR-eQTLs affect mature miRNA stability.

White and colleagues (2021) performed an eQTL study between maternal genotypes and plasmatic miRNA levels measured during the first trimester of pregnancy of 369 women from the prospective pre-birth Genetics of Glucose Regulation in Gestation and Growth (Gen3G) cohort. The authors reported cis-eQTLs associated with the expression of 147 miRNAs at FDR < 0.05. Interestingly, despite this study mapping cis-eQTLs within 2 Mbs of mature miRNAs (1Mb upstream and 1 Mb downstream), roughly 33% of the miR-eQTLs identified in this study were within 50 kb of their corresponding mature miRNA (White et al., 2021, preprint).

In recent years, efforts have been made to curate associations between miRNAs, SNPs and human diseases into databases, namely the MiRNA SNP Disease Database (MSDD), the ncRNA-related eQTL database (ncRNA-eQTL) and the JAMIR-eQTL database.

The MiRNA SNP Disease Database (MSDD) is a manually curated comprehensive database of SNPs in functional regions of miRNA regulation, such as mature miRNAs, promoter regions, pri–miRNAs, pre–miRNAs and target gene 3′–UTRs. The database has reviewed over 2000 peer-reviewed publications and has 525 associations among 182 miRNAs, 197 SNPs, 153 genes and 164 human diseases (Yue et al., 2018).

A ncRNA–related eQTL database (ncRNA–eQTL) has been developed for 33 different cancer types (Li et al., 2020). Li and colleagues performed an eQTL analysis of > 8700 samples from the Cancer Genome Atlas (TCGA) consortium, encompassing genotyping, transcriptomics and survival data from patients from > 10,000 primary tumours across 33 cancer types. Most of the non-coding RNAs in this dataset are lncRNAs (> 40,000); however, the TCGA also has miRNA expression profiles. In this study, the authors identified 952 miRNAs with trans-eQTLs and 3,586 miRNAs with cis-eQTLs at FDR < 0.05 across all tissues with some cancers such as lymphoid neoplasm diffuse large B-cell lymphoma (DLBC) displaying only one cis-

regulated miRNA. In contrast, thyroid cancer (THCA) had a total of 301 cis-regulated miRNAs. Moreover, the authors reported 116 miRNA–eQTLs associated with patient overall survival times across 33 cancer types (FDR < 0.05).

The JAMiR-eQTL database is a publicly available repository of cis- and trans-miR-eQTLs from a study of a large (3,448 subjects) cohort of Japanese individuals representing different types of dementia, including Alzheimer's disease, Lewy body dementia, vascular dementia and MCI. The authors analysed cis- and trans-eQTLs in 6 types of dementia and across all subjects. Cis-eQTLs were associated with 125 miRNAs in AD (n= 1,314), 1 miRNA in Lewy body dementia (n=134), 19 miRNAs in vascular dementia (n=69) and 319 miRNAs in MCI (n=504) at FDR < 0.05). Moreover, 120 miRNAs were associated with cis-eQTLs in all subjects, independent of dementia type (Akiyama et al., 2021).

Sonehara and colleagues (2021) performed small RNA-sequencing of peripheral blood mononuclear cells (PBMCs) and whole–genome sequencing (WGS) of 141 Japanese individuals. This study identified cis-miR-eQTLs associated with the expression of 40 miRNAs at FDR < 0.2. Of the identified emiRNAs, 63% had not been reported previously, including 5 miRNAs whose lead cis-miR-eQTL is monomorphic in the European populations. These results highlight the importance of performing miR-eQTL analysis in diverse populations (Sonehara et al., 2021).

To the best of my knowledge, only three published miRNA eQTL studies have been performed in the human brain. Williamson et al. (2015) mapped miR-eQTLs using 78 adult post–mortem brains from the Stanley Medical Research Institute, of which 27 were from individuals with schizophrenia, 29 were from individuals with bipolar disorder, and 22 were from control individuals. miR-eQTLs were detected for 32 miRNAs at FDR < 0.1, which were enriched for GWAS SNPs associated with SZ (empirical p=0.018) (Williamson et al., 2015). In a study led by Mamdani et al. (2015), the same group mapped miRNA eQTLs in 36 adult nucleus accumbens (NAc) of 18 subjects with alcohol dependence and 18 controls. Despite a small sample

size, the authors discovered cis-eQTLs for 68 miRNAs at FDR < 0.1. More recently, the same group performed a gene network analysis of mRNAs and miRNAs from PFC and NAc of matched cases with alcohol dependence (n=18) and controls (n=17). As part of this study, the impact of genetic variants on gene expression in a disease-dependent manner was investigated. In the alcohol dependence mediated eQTL analysis, 4 miRNAs were found to be associated with cis-eQTLs in the NAc, and 7 miRNAs were identified in the PFC at FDR < 0.1. The authors suggest that miRNA expression differences between alcohol dependence cases and controls might be under brain region-specific genetic control, which potentially mediates alcohol's effect in a brain region-specific manner (Vornholt et al., 2020).

The only study of miR-eQTLs in foetal development was performed using 40 full-term pregnancy placentas. This study identified miR-eQTLs associated with the expression of 16 miRNAs, including 4 miRNAs that are differentially expressed in pre-eclampsia (Inno et al., 2021).

Of the 2654 mature miRNAs identified in humans (Kozomara et al., 2019), an estimated 50-70% are expressed in the brain (O'Carroll & Schaefer, 2013; Fineberg et al., 2009; Adlakha & Saini, 2014). Given the role of miRNAs as both master regulators and fine–tuners of gene expression in brain development (for review, see: Rajman & Schratt, 2017; Prieto-Colomina et al., 2021), the present study aimed to identify common associated variants with miRNA expression in human foetal brain development. To my knowledge, this is the first miR-eQTL study to be performed in human foetal brain.

3.2 Methods

3.2.1 Samples

This study employed 112 2nd trimester human foetal brain samples from elective terminations (12–20 PCW) obtained from the MRC / Wellcome Trust Human Developmental Biology Resource (http://www.hdbr.org). Ethical approval for the HDBR was granted by the Royal Free Hospital research ethics committee under reference 08/H0712/34 and Human Tissue Authority (HTA) material storage licence 12220. In addition, ethical approval for assessing genetic effects on gene expression in human brain was obtained from the Psychiatry, Nursing and Midwifery Research Ethics Subcommittee (PNM RESC/12/13 -102) at King's College London. Samples were received as undissected frozen brain tissue. Total RNA was extracted from a homogenate of half a section of brain tissue from each foetus using Trizol (Ambion). The other half was used for genomic DNA extraction using standard phenol-chloroform extraction. Foetal age was determined by the HDBR through foot length and knee-to-heel length measurements. Foetal sex had been previously determined for most samples by karyotyping, expression of genes on the Y-chromosome in males and heterozygosity for genetic X-chromosome markers in females (O'Brien et al., 2018). I empirically determined sex in the remaining samples based on X -chromosome heterozygosity rates using the "--check-sex" flag in PLINK 1.9 (Purcell et al., 2007). Figure 3.1 depicts a schematic overview of the main methods employed in this chapter.

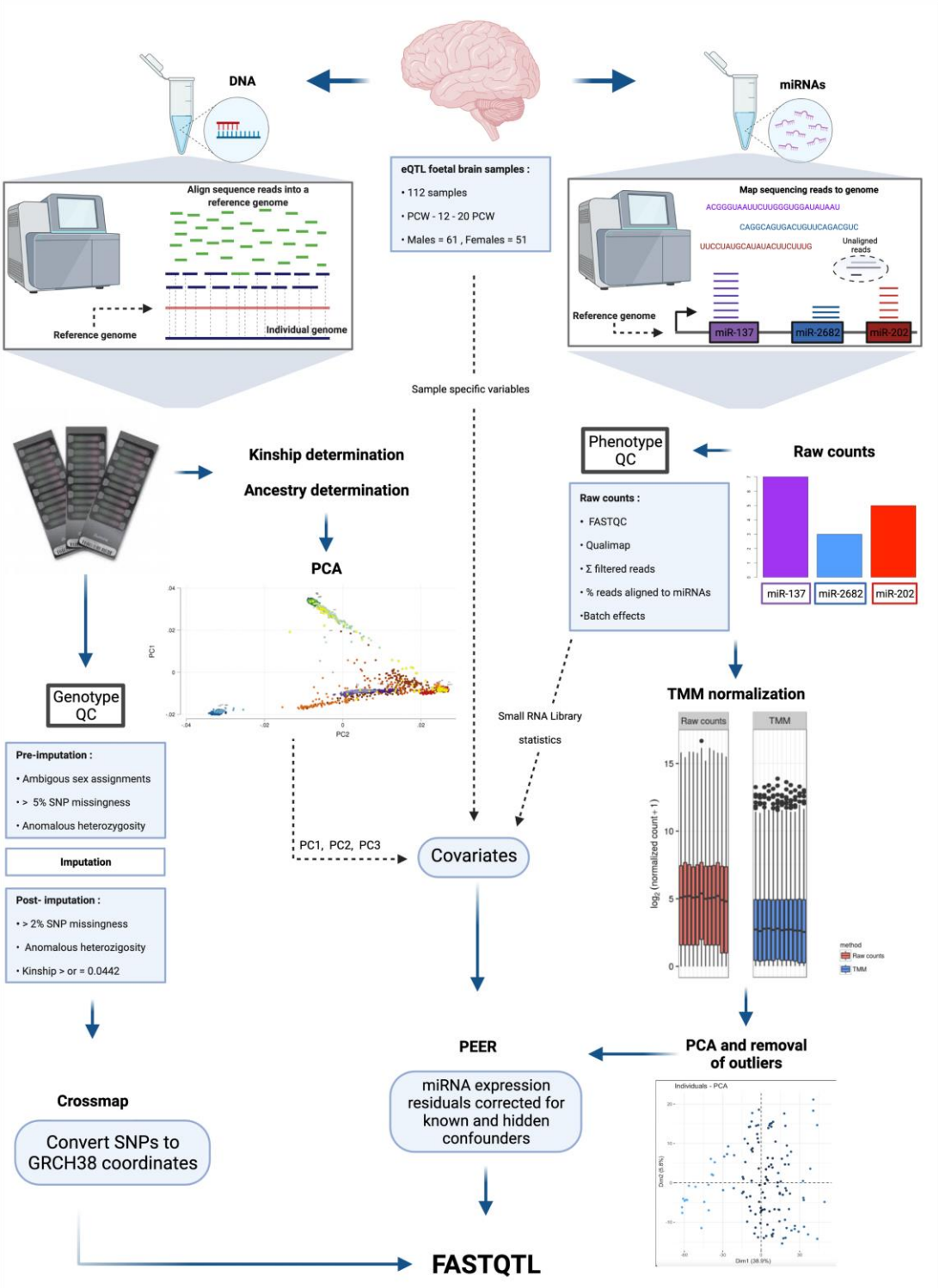


Figure 3.1 - Schematic overview of the main methods employed in this chapter.

A total of 112 samples from 2nd trimester foetal brain were employed in this study. Undissected frozen brain tissue from these samples was used to extract both DNA and total RNA according to

established protocols. Extracted DNA was used for genome-wide genotyping of ~710,000 SNPs. Subsequently, genotypes underwent extensive QC rounds and were imputed for additional SNPs using minimac3 and Eagle v2.3 phasing through the Michigan server, which was followed by another series of QC of the imputed SNPs and samples. A total of 6,571,705 SNPs passed QC. Genotyped SNPs were also employed to determine kinship and ancestry of the samples employed in this study. Kinship was used as a QC filter post-imputation, whilst PC1, PC2 and PC3 derived from ancestry determination were employed as covariates for eQTL analysis. Total RNA was employed in this study for the generation of Small RNA - Sequencing libraries according to Illumina guidelines. After sequencing libraries on an Illumina Hi–Seq 4000, reads were first mapped to miRbase, allowing no mismatch. Unaligned reads were subsequently aligned to reference genome GRCH38 allowing for 1 mismatch. Raw reads from both alignments for each miRNA were added together. Small RNA Sequencing libraries underwent a series of QC filters based on sequencing depth, library size and mapping quality. Raw counts were normalized by the TMM method implemented in edgeR and converted into log2 cpm+1. PCA analysis of normalized reads was employed to identify and eliminate outliers. Normalized reads, genotyping PC1, PC2 and PC3, library demographics and sample-specific variables, including sex and age (PCW), were employed to build a linear model of miRNA expression and infer 10 hidden confounders in my data through PEER. Residuals of PEER analysis consisting of miRNA expression corrected by known and hidden confounders for the 112 samples and their respective genotypes converted into GRCH38 coordinates were employed in permutation analysis to find cis-miR-eQTLs in 2nd trimester foetal brain through FASTQTL. Created with BioRender.com

3.2.2 Genotyping

The vast majority of samples employed in this study had already been genotyped in previous studies by our lab (Hannon *et al*., 2016; O'Brien *et al*., 2018). An additional 19 samples were genotyped specifically for this study using the Infinium OmniExpress-24 BeadChip array (Illumina), which genotypes approximately 710,000 SNPs.

3.2.2.1 Quality control and genotype imputation

Initially, there were genotypes for 151 samples available. All these genotypes were subjected to strict quality control and genotype imputation. Pre - imputation quality control involved using PLINK v1.9 to check for consistency of sex assignments, as well as to inspect the subject-level missingness rates. The Haplotype Reference Consortium v1.1 (HRC) panel was used for imputation. As part of quality control, a checking tool developed by Will Rayner (check–bim) was employed to check for consistency of strand, alleles, positions, Ref/Alt assignments and frequencies between the genotyped SNPs and the HRC panel. A total of 8 samples with ambiguous sex assignments and/ or with > 5% missing markers or anomalous heterozygosity were eliminated. SNPs that were missing in > 5% of samples or had minor allele frequency less than 0.01 were removed. In addition, A/T and G/C SNPs with minor allele frequencies > 0.4 were also removed.

Subsequently, the SNP strand and ref/alt assignment were updated to match the Haplotype Reference Consortium (HRC) version 1.1, and SNPs where the minor allele frequency differed by > 0.2 from the HRC version were removed. 143 samples were imputed for additional SNPs from the HRC panel using minimac3 and Eagle v2.3 phasing through the Michigan Imputation Server (https://imputationserver.sph.umich.edu/index.html).

Following imputation, genotypes underwent additional QC by applying filters based on minor allele frequency (MAF), Hardy-Weinberg equilibrium (HWE), heterozygosity, SNP missingness rate and kinship. After imputation, 5 samples were eliminated, including 3 samples that were 3rd-degree relatives. SNPs were initially annotated with rsID numbers from dbSNP v149 and converted to GRCh38 coordinates using CrossMap (Zhao et al., 2014), using chain files from the UCSC Genome Browser (https://genome.ucsc.edu/). Subsequently, checkVCF (https://github.com/zhanxw/checkVCF) was employed to identify and remove

non-SNP positions, duplicate sites and loci with an imputation r2 less than 0.8, minor allele frequency (MAF) less than 0.05, or Hardy-Weinberg Equilibrium (HWE) violation p-values < 1×10^{-4}. In total,138 samples and 6,571,705 SNPs passed genotyping QC filters.

3.2.2.2 Ancestry determination

Genotyped SNPs were employed not only to infer the relatedness of samples but also to determine ancestry. The outcome of the ancestry determination procedure is a set of principal components (PCs) describing the sample structure in terms of genetically defined populations; these PCs are then included as covariates when fitting the genome-wide regression models in an eQTL study. The brain samples employed in this study are an admixed population, as shown in Figure 3.2 and are representative of the London population.

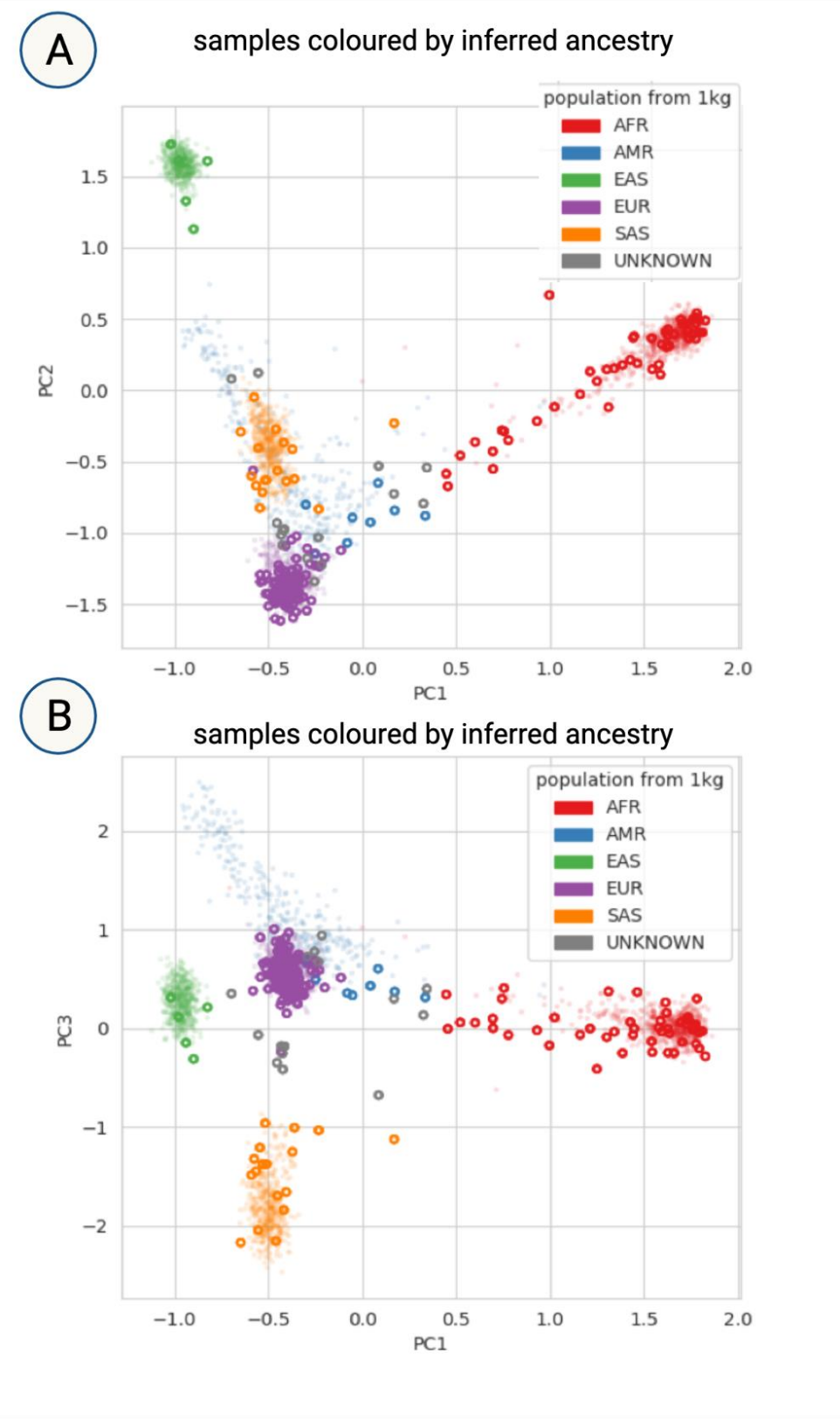


Figure 3.2 – Ancestry determination of the foetal brain samples employed in this study.

Plot of Principal components (PCs) calculated using Peddy (Pedersen & Quinlan, 2017) based on the 1000 genomes project (1000 Genomes Project Consortium, 2015). A – PC1 and PC2 of ancestry determination; B – PC1 and PC3 of ancestry determination. N=151 samples. AFR - African; AMR - Ad mixed American; EUR - European; SAS - South Asian; UNK - Unknown.

In order to maximize the number of samples employed in this study, foetal brain samples were not excluded based on ancestry. Admixed populations have complex population substructures that may confound eQTL analysis and need to be adequately controlled to prevent spurious associations (Hellwege et al., 2017; Gay et al., 2020). Population stratification was controlled by including the first 3 genotype principal components (PC1, PC2 and PC3) as fixed effects in the linear model implemented by PEER (Steagle et al., 2012) as previously described (O'Brien et al., 2018). The resulting miRNA expression residuals (corrected for population stratification and additional known and hidden confounders) were subsequently employed in my miR - eQTL analysis (see: 3.2.5 – miR - eQTL discovery).

3.2.3 Small–RNA sequencing

As described in depth in chapter 2, Total RNA was extracted following the standard Trizol (Ambion) method. Subsequently, small - RNA libraries were generated in accordance with Illumina guidelines, using 1 ug of DNAse treated total RNA. Libraries were purified by agarose gel extraction, allowing for specific isolation of libraries with sizes between 145 - 160 nucleotides which contain mature miRNA generated from ~22 nt and piwi-interacting RNAs (piRNAs) generated from ~30 nt, as well as, small RNA fragments, and other regulatory small RNAs (in addition to the ligated adapters). Following quantification through qPCR, libraries were pooled together in equimolar amounts in batches of 20 samples and sequenced on an Illumina Hi–Seq 4000.

3.2.3.1 Small-RNA data processing

50 bp single-end sequencing reads, in the form of FASTQ files, were pre-processed according to a recently published standardized protocol for miRNA - sequencing studies (Potla et al., 2021). Briefly, adapters were trimmed using trimmomatic (Bolger, Lohse and Usadel, 2014), followed by quality control (QC) of sequenced reads. FastQC and multiQC (Ewels et al., 2016) were employed to create and visualize plots based on individual base sequence quality scores, sequence length distribution, individual sequence GC content, and duplicate sequences. Samples with low-quality sequencing data were eliminated.

In order to only retain miRNAs (21–25 nt) and piwiRNAS (21–31 nt) reads, trimmed reads were filtered to remove reads that are too short (< 16 nt) and too long (> 31 nt).

Filtered reads were mapped using the Bowtie aligner in a two-step approach, as documented by Potla and colleagues (2021). Firstly, reads were aligned to mature miRNA reads in miRbase v. 22 (Kozomara et al., 2019) with stringent criteria by not allowing mismatches. Subsequently, the unaligned reads from the first alignment were aligned to the GRCh38 reference genome allowing for 1 mismatch between the miRNA reads and the reference genome. Mapped reads were visualized using IGV (Thorvaldsdottir et al., 2013), and mapping quality was ascertained using Qualimap (Okonechnikov et al., 2016). Aligned reads were quantified into raw counts using samtools idxstats for miRbase aligned counts and featurecounts for genome-mapped reads (Liao et al., 2014).

3.2.4 Normalisation of raw miRNA counts

Raw miRNA counts derived from aligning each miRNA to miRbase and to the GRCh38 reference genome were merged, and miRNAs with 0 counts in more than 90% of the samples were eliminated. A total of 1449 miRNAs were detected. Sample libraries underwent a series of QC analyses based on measures from FASTQC, MultiQC, and Qualimap. Of the samples that survived small-RNA seq QC, 112 had associated genotypes.

Reads were normalized using the trimmed mean of M-values (TMM) method and transformed into log2 CPM +1 (Robinson & Oshlack, 2010) using edgeR. A PCA of TMM normalized counts was performed to identify and remove outliers. No outliers were detected (see: 2.2.7 - Sample QC and exclusion of samples).

3.2.5 miR – eQTL discovery

eQTL analyses were carried out using FastQTL (Ongen et al., 2016) in 112 samples with both genotype and small - RNA sequencing measures that passed QC. TMM normalized miRNA expression measures were corrected for age, sex, RIN, sequencing batch, the first three principal components of genotype (PC1, PC2, PC3), average read quality, % duplicates after filtering, % GC content after filtering, average read length after filtering (bp), total amount of reads after filtering, % of mapped reads to miRNAs and 10 hidden confounders estimated through PEER (Stegle et al., 2012). The resulting PEER miRNA expression residuals were employed to test the association between single nucleotide polymorphisms (SNPs) located within a ±500 kb window extending in both directions from the first nucleotide of the mature miRNA sequence for each of the miRNAs expressed in 2nd trimester

foetal brain (n= 1449). FDR for each eQTL was calculated by first correcting p-values for the number of SNPs tested per miRNA (within 500kb on either side of the start of mature miRNA) through estimation of a beta distribution using a minimum of 1000 permutations (maximum 10,000 permutations), and then correcting these P - values for the number of miRNAs tested using Storey's q-value method (Storey & Tibshirani, 2003).

3.2.6 Power of eQTL study

The power of an eQTL study is dependent on multiple factors, some of which are difficult to quantify, including effect sizes. I used the R package PowereQTL (Dong et al., 2021) to estimate the power of my eQTL study. To this end, I employed the same effect size and noise level (log standard deviation of 0.13) as the GTEx Consortium Power analysis (GTEx Consortium, 2013). I assumed a linear association between miRNA expression and genotype. Moreover, I incorporated a Bonferroni correction for the 2,608,405 cis-SNP-miRNA pairs tested in my eQTL analysis. Through this analysis, I could ascertain that my study is sufficiently powered to detect cis-miR-eQTLs with MAF > 19.37% but lacks the power to detect all of the miR-eQTLs with lower frequencies (Figure 3.3). For this study to be adequately powered to detect cis-miR-eQTLs with MAF > 5%, I would require a total of 410 foetal brain samples.

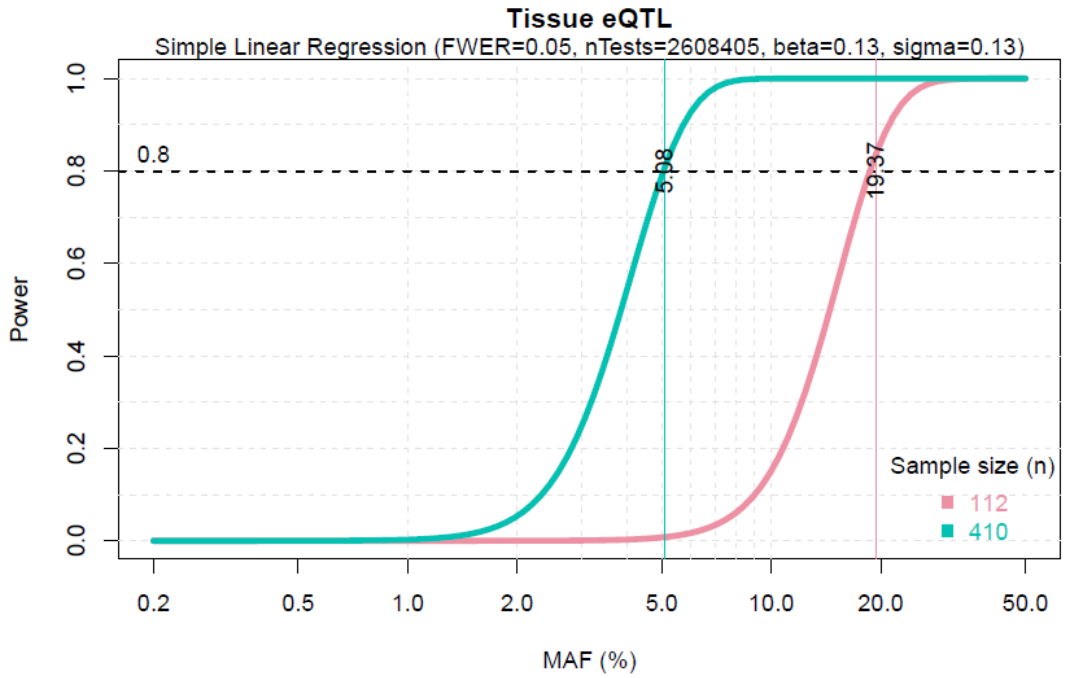


Figure 3.3 – Power of the current bulk tissue eQTL study employing 112 samples versus power of this study if it employed 410 samples, assuming the same effect size and associated noise as the GTEx Consortium Power analysis (GTEx Consortium, 2013) and a linear association between miRNA expression and genotypes.

MAF- minor allele frequency, beta – effect size, sigma – level of noise

3.3 Results

Using FASTQTL (Ongen et al., 2016), I investigated the associations between ~ 5.31M SNPs and the expression levels of 1449 miRNAs in 112 2nd trimester brain samples. Cis-eQTL analysis was targeted to a ±500 kb window extending in both directions from the start of mature miRNA loci, annotated based on miRbase v22 (Kozomara et al., 2019). The genomic regions flanking the 1449 miRNAs under analysis included 1,115,053 unique SNPs. In total, I tested 2,608,405 cis-SNP-miRNA pairs. After permutation analysis, I tested if the beta-approximated permutation p-values were well calibrated (Figure 3.4). As can be seen in Figure 3.4, beta-approximated p-values are highly correlated with empirical P-values ($R=1$, $p < 2.2 \times 10^{-16}$).

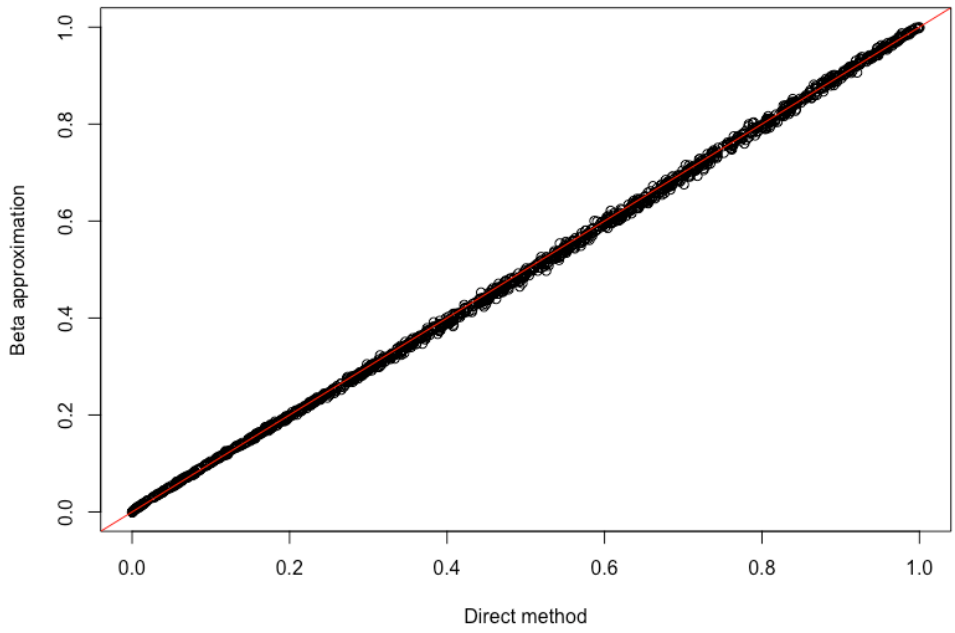


Figure 3.4 – Correlation between beta approximated p-values and empirical p-values of eQTL analysis performed in a ±500 kb window of mature miRNAs (Pearson's correlation R=1, p < 2.2 x 10^{-16}).

At FDR < 0.05, I identified miRNA cis-eQTLs associated with the expression of 30 miRNAs, all of which mapped to unique genomic locations. Table 3.1 lists the 30 miRNAs identified in this study, the chromosome (chr) where they are located, the most significant miR-eQTL identified by permutation analysis that is associated with miRNA expression (Top miR-eQTL), the distance of the miR-eQTL to the start of mature miRNA, the q value of each association and the miRNA type (I – Intronic; N – Non-intronic).

Table 3.1 – miRNAs associated with miR-eQTLs in second trimester foetal brain.

miRNA	Chr	Top miR-eQTL	Distance to mature miRNA	q-value	miRNA type
hsa-miR-5683	chr6	rs7769202	79	8.9156E-13	I
hsa-miR-4707-3p	chr14	rs2273626	15	3.2119E-12	N
hsa-miR-544b	chr3	rs3821536	32618	1.8093E-08	I
hsa-miR-6868-3p	chr17	rs2243486	-10072	1.8093E-08	I
hsa-miR-618	chr12	rs10862209	-2621	2.8352E-08	N
hsa-miR-1908-5p	chr11	rs174544	-14929	9.2764E-08	N
hsa-miR-4326	chr20	rs7263455	15722	5.8552E-07	I
hsa-miR-3615	chr17	rs745666	-5	6.4315E-07	N
hsa-miR-548ba	chr2	rs2140551	-464362	7.4047E-07	I
hsa-miR-1269a	chr4	rs72641631	-6625	2.3255E-06	N
hsa-miR-1287-5p	chr10	rs942803	13602	1.6373E-05	N
hsa-miR-3117-3p	chr1	rs1925342	21061	5.9812E-05	I
hsa-miR-4467	chr7	rs6971245	-3908	6.9581E-05	I
hsa-miR-4803	chr5	rs1561401	-450	0.00021958	I
hsa-miR-323b-3p	chr14	rs56103835	-51	0.00021958	N
hsa-miR-7854-3p	chr16	rs2927318	-303	0.00024305	I
hsa-miR-4662a-5p	chr8	rs7006762	-82859	0.00085809	N
hsa-miR-548at-5p	chr17	rs11653901	-10624	0.00105387	I
hsa-miR-3161	chr11	rs74236456	-66010	0.00123203	I
hsa-miR-3125	chr2	rs6717278	6563	0.00207331	I
hsa-miR-3176	chr16	rs116698525	-15147	0.00266069	I
hsa-miR-1270	chr19	rs28576121	-18408	0.00277493	N
hsa-miR-4761-3p	chr22	rs4680	-67	0.00325894	N
hsa-miR-641	chr19	rs41275750	-50416	0.01652916	I
hsa-miR-4423-5p	chr1	rs709777	-13198	0.01653772	N
hsa-miR-6886-5p	chr19	rs1003723	25	0.03145733	I
hsa-miR-202-5p	chr10	rs11101657	-3583	0.03955397	N
hsa-miR-6840-5p	chr7	rs112622797	-36864	0.03955397	N
hsa-miR-6826-3p	chr3	rs6788178	54097	0.03955397	I
hsa-miR-3938	chr3	rs55852613	-23443	0.04153267	I

In order to visualize the genome-wide distributions of miR-eQTLs, I performed a Manhattan plot of all significant and non-significant miR-eQTLs identified in this study (Figure 3.5). As can be seen in Figure 3.5, the miR-eQTLs are evenly distributed throughout the genome. Interestingly, this study identified a miR-eQTL for hsa-miR-323b-3p, a miRNA located in the paternally imprinted cluster C14MC.

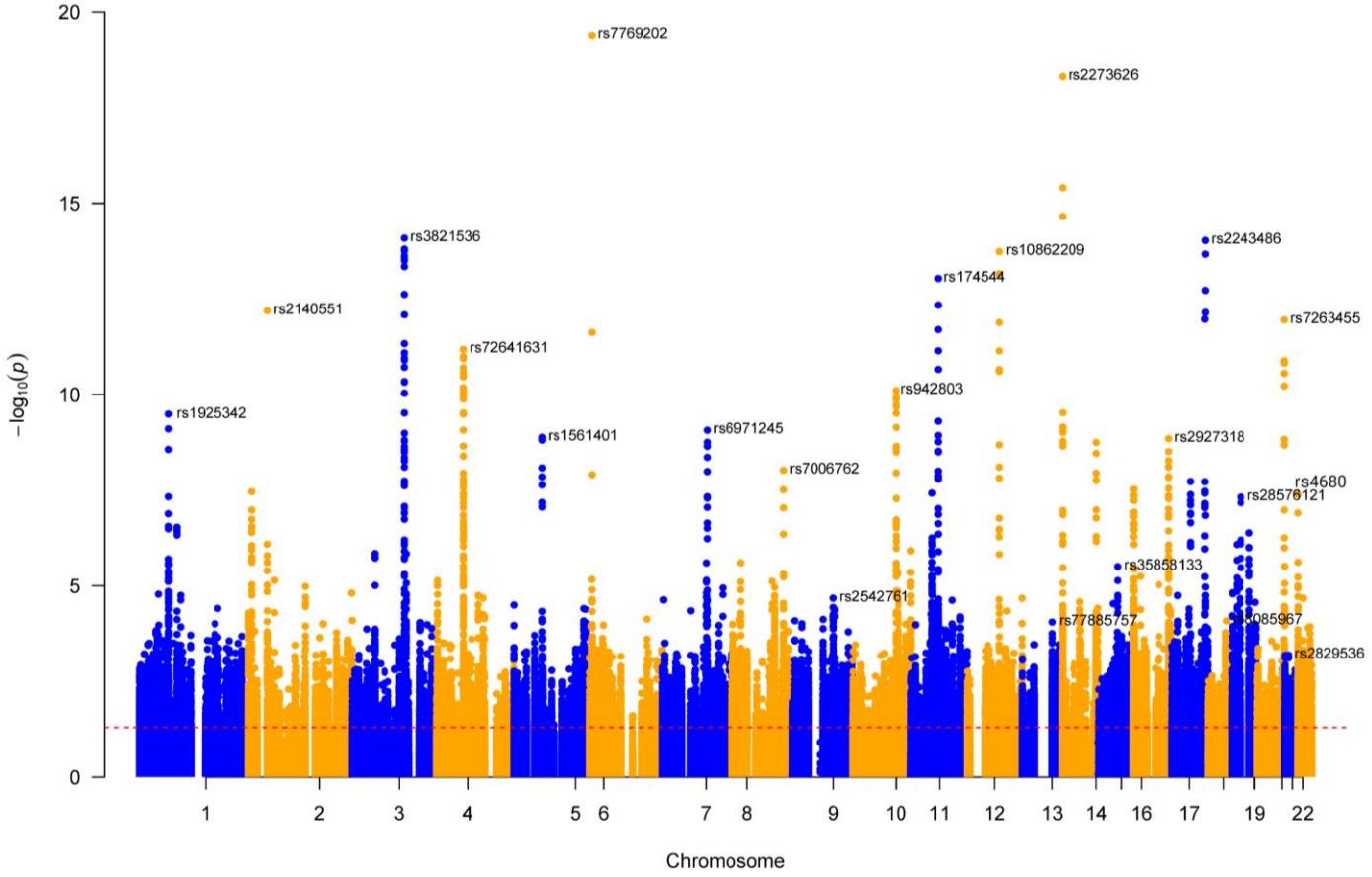


Figure 3.5 – Manhattan plot of miR-eQTL distribution throughout the genome.

Dashed red line represents FDR < 0.05 cut off. All SNPs above the dashed line are statistically significant miR-eQTLs.

Using PhastCon (Siepel et al.,2005), I determined sequence conservation of the miRNAs with associated miR-eQTLs using the UCSC genome browser Table browser tool and the 100 Vertebrate Conservation group of organisms for comparison. PhastCon scores were dichotomized into low- and high - conservation using a cut-off point of 0.5. Interestingly, 90% of the identified miRNAs with an associated miR-eQTL are poorly conserved across vertebrate species (PhastCon Score < 0.5). In contrast, 3 miRNAs – miR-1287-5p, miR-323b-3p and miR-202-5p have PhastCon scores > 0.95 and are highly conserved throughout vertebrates.

70% of the miR-eQTLs identified (n = 21) were located upstream of their associated mature miRNA, and 83% (25 out of 30) miR-eQTLs were within 50 kb of their corresponding mature miRNA (Figure 3.6). Only one miR-eQTL was located more than 100kb away from its associated mature miRNA: rs2140551, which was 464 kb upstream from its associated mature miRNA (hsa-miR-548ba) in a region that Haploreg v4 predicts to be an enhancer (Ward & Manolis, 2015).

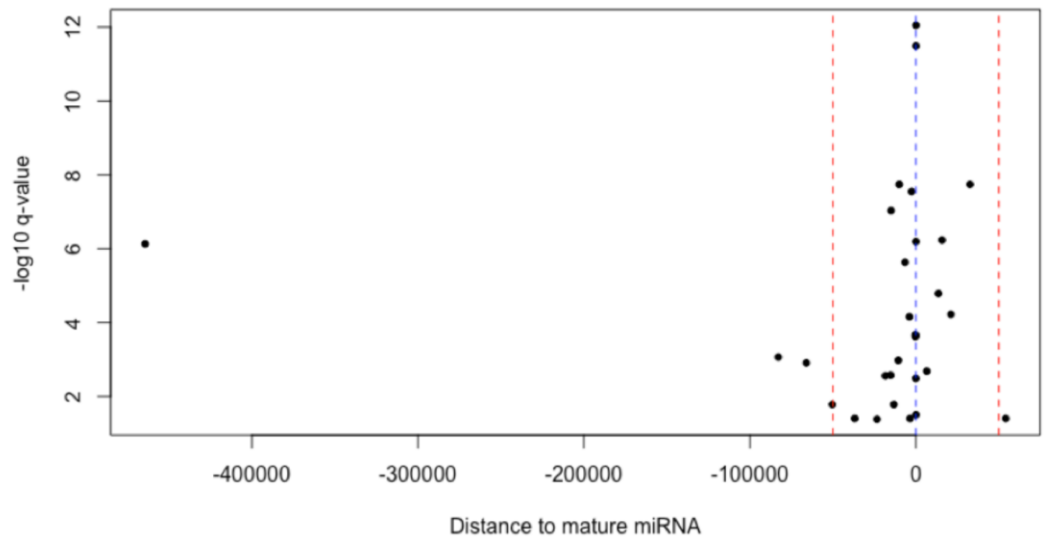


Figure 3.6 – Distance of miR-eQTLs to start of their associated mature miRNA.

Dashed blue line – start of mature miRNA. Dashed red lines – 50 kb and -50kb from mature miRNA.

Of the identified miR-eQTLs, 6 were within 100 bp of their respective mature miRNA, and 4 were located either within the mature miRNA itself (miR-4707-3p) or within the pri-miRNA hairpin sequence (miR-323b-3p, miR-3615 and miR-6686-5p). In order to ascertain if the association between genotype at rs2273626 and expression of miR-4707-3p was due to sequence alignment bias, I performed the same eQTL analysis in mapped reads that were allowed 1 mismatch during the alignment phase to both miRbase and genome (1MM method). As can be seen in Table 3.2, the q–values obtained from both alignment methods are highly similar, indicating that the observed miR-eQTL association is not explainable by sequence alignment bias.

Table 3.2 – Comparison of q-values obtained from eQTL analysis of both alignment methods in eQTLs located within microRNAs identified in this study.

miRNA final	miR-eQTL	Distance to mature miRNA	Location of top eQTL	q-value original method	q-value 1MM method
hsa-miR-4707-3p	rs2273626	15	Seed region of mature miRNA	3.2119E-12	4.27E-12

To explore the effect of the 4 miR-eQTLs located within pri-miRNA sequences, I analyzed where these SNPs are located in relation to the structure of pri-miRNA hairpins by searching these against the miRNASNP-v3 database (Chun–Jie et al., 2020), a database for miRNA-related SNP and SNP effects. The location of these miR-eQTLs in the context of the 3D structure of pri-miRNA hairpins is shown in Figure 3.7.

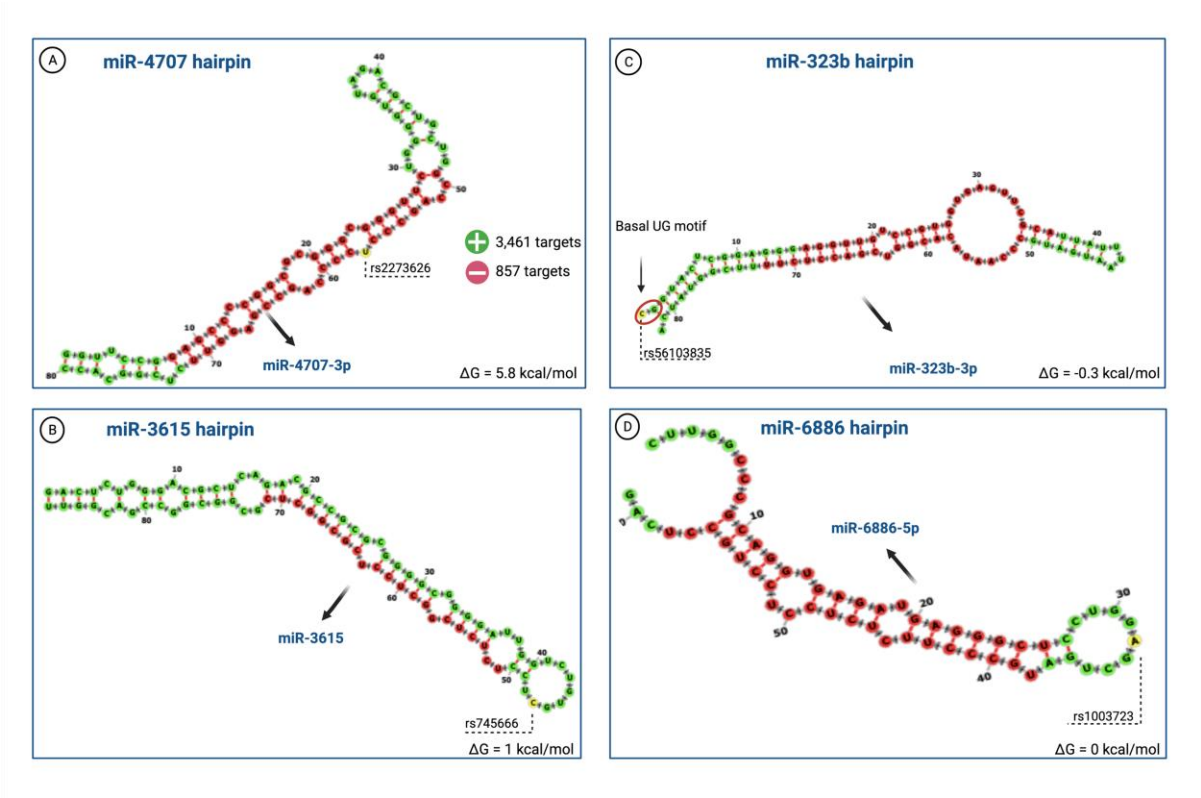


Figure 3.7 – Location of miR-eQTLs in the context of the 3D structure of miRNA hairpins identified in this study.

Red – mature miRNA bases; Green – pri-miRNA bases not in mature miRNAs; Yellow – miR-eQTL position. **A -** miR-4707 hairpin structure – Can give rise to 2 mature miRNAs: miR-4707-5p and miR-4707-3p identified in this study. SNP rs2273626 is located in the seed region of miR-4707-3p and is associated with an increase in minimum free energy ΔG =5.6 Kcal/mol. This SNP also alters the seed region of miR-4707-3p and leads to changes in target post-transcriptional regulation. TargetScan analysis predicts this SNP leads to the gain of 3461 targets and the loss of 857 targets. **B –** miR-3615 hairpin structure – Gives rise to miR-3615. SNP rs745666 is located in the apical loop of the miR-3615 hairpin within a regulatory structure called the pre-element. This SNP is associated with an increase in minimum free energy ΔG = 1 kcal/mol. **C –** miR-323b hairpin structure – The paternally imprinted hairpin can give rise to 2 mature miRNAs: miR-323b-5p and the identified miR-323b-3p. SNP rs56103835 modifies the regulatory basal UG motif (red circle) of the miR-323b hairpin and is associated with a small decrease in minimum free energy ΔG = -0.3 kcal/mol. **D –** miR-6886 hairpin structure - Can give rise to 2 mature miRNAs: the miR-6886-5p identified and miR-6886-3p. SNP rs1003727 is located in the apical loop of the miR-6886 hairpin within a regulatory structure called the pre-element and is not associated with changes in minimum free energy ΔG = 0 kcal/mol.

The expression of miR-4707-3p is associated with SNP rs2273626 located in its seed region (see Figure 3.7A). This SNP is associated with a significant increase in minimum free energy predicted by *RNAfold* (Gruber et al., 2008), which leads to the miR-4707 hairpin being less thermodynamically stable and is predicted to decrease miRNA expression. Moreover, given that this SNP is located in the seed region, it will also lead to altered post-transcriptional regulation of miR-4707-3p targets. TargetScan 8.0 (McGeary et al., 2019) analysis predicts that this SNP is associated with a gain in 3,461 mRNA targets and a loss in 857 mRNA targets. The remaining 3 miR-eQTLs are located in regulatory regions of their associated miRNA hairpins. The miR-eQTLs associated with miR-3615 (Figure 3.7B) and miR-6886 (Figure 3.7D) are located in the apical stem-loop of their respective hairpins. The apical stem-loop is part of a regulatory region called the pre-element. This area is critical for defining miRNAs and regulating their production from primary transcripts by Drosha and Dicer (Zhang & Zeng, 2010). *RNAfold* analysis predicts that one of these SNPs - rs745666, associated with miR-3615 expression, is also associated with a slight minimum free energy increase predicted to lead to a small change in miR-3615 expression (Figure 3.7B). Finally, the miR-eQTL associated with miR-323b-3p alters a conserved basal regulatory UG motif at the base of the miR-323b hairpin. This region marks the boundary of the basal junction between ssRNA and dsRNA and enhances pri-miRNA processing (Auyeung et al., 2013). *RNAfold* analysis predicts this SNP is associated with a slight minimum free energy decrease (ΔG= -0.30 kcal/mol), leading to a small change in miRNA expression.

As can be seen in Table 3.1, 56% of the miRNAs with eQTLs are intronic (n=17). The remainder are either intergenic (n=8) or exonic miRNAs (n=5). There are no significant differences in q-values based on miRNA location (intronic versus non–intronic) (p=0.9332) (Figure 3.8).

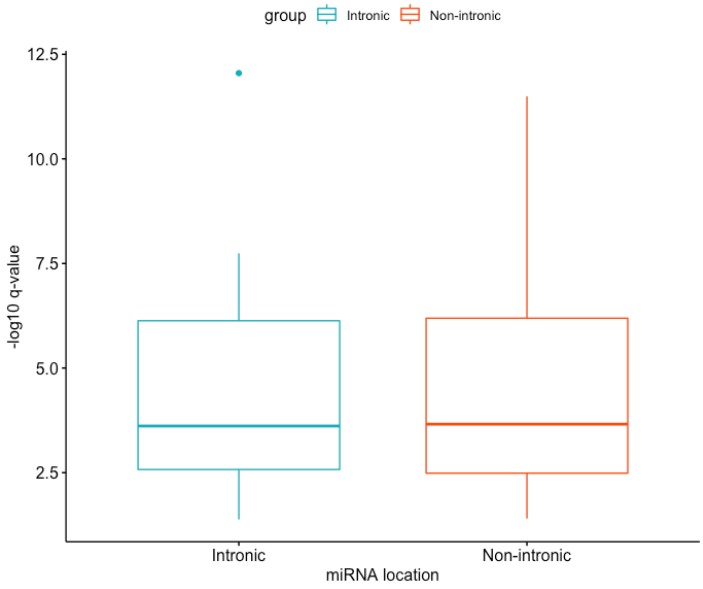


Figure 3.8 – Comparison of miR-eQTL strength between miR-eQTLs associated with intronic miRNAs compared to those associated with non-intronic miRNAs.

There are no significant differences between the two groups (chi square = 0.0070, df = 1, p = 0.9332)

Both intronic and exonic miRNAs are located within host genes. In this study, 73 % of the miRNAs with associated miR-eQTLs were found within host mRNAs (22 of 30 miRNAs). Table 3.3, displays miRNAs within host genes and their associated miR-eQTL, the strength of the association (q - value), name of host gene, location of miRNA within the host gene, distance of the miR-eQTL to the start of mature miRNA, and the start of the host gene TSS.

Table 3.3 – miRNAs associated with miR-eQTLs that are located within host genes.

miRNA	miR-eQTL	q-value	Host Gene	Location of miRNA within host gene	Distance to mature miRNA	Distance to host gene TSS
miR-5683	rs7769202	8.9156E-13	F13A1	Intron 12	79	149195
miR-4707-3p	rs2273626	3.2119E-12	HAUS4	Exon 0	15	-99
miR-544b	rs3821536	1.8093E-08	UMPS	Intron 1	32618	34677
miR-6868-3p	rs2243486	1.8093E-08	EXOC7	Intron 4	-10072	-15743
miR-1908-5p	rs174544	9.2764E-08	FADS1	Exon 0	-14929	3419
miR-4326	rs7263455	5.8552E-07	ARFGAP1	Intron 13	15722	-29752
miR-3615	rs745666	6.4315E-07	SLC9A3R1	Exon 1	-5	-39
miR-548ba	rs2140551	7.4047E-07	FSHR	Intron 2	-464362	559247
miR-1287-5p	rs942803	1.6373E-05	PYROXD2	Exon 7	13602	6269
miR-3117-3p	rs1925342	5.9812E-05	SGIP1	Intron 2	21061	-115905
miR-4467	rs6971245	6.9581E-05	LRWD1	Intron 11	-3908	-2642
miR-4803	rs1561401	0.00021958	MAP1B	Intron 2	-450	26108
miR-7854-3p	rs2927318	0.00024305	CMIP	Intron 1	-303	-88841
miR-548at-5p	rs11653901	0.00105387	ATP6V0A1	intron 11	-10624	-25295
miR-3161	rs74236456	0.00123203	PTPRJ	Intron 1	-66010	-50258
miR-3125	rs6717278	0.00207331	TRIB2	Intron 2	6563	-27424
miR-3176	rs116698525	0.00266069	CAPN15	intron 2	-15147	-493
miR-4761-3p	rs4680	0.00325894	COMT	Exon 2	-67	-21987
miR-641	rs41275750	0.01652916	AKT2	Intron 1	-50416	11121
miR-6886-5p	rs1003723	0.03145733	LDLR	Intron 9	25	-24092
miR-6826-3p	rs6788178	0.03955397	COPG1	Intron 19	54097	29000000
miR-3938	rs55852613	0.04153267	ERC2	Intron 14	-23443	344622

In order to infer if miR-eQTLs could mediate their effects on miRNA expression by altering host gene expression, I compared my results with an eQTL analysis performed by my group in a largely overlapping sample (O'Brien et al., 2018). Despite the vast majority of miRNAs with eQTLs identified in this study being located within a host-gene; the genetic signal associated with miRNA expression was only colocalized with host-gene expression in one case (Table 3.4).

Table 3.4 – Effects of miR-eQTLs on host-gene expression.

Host Gene	miRNA	miR-eQTL	p-value (nominal) of eQTL and host - gene expression
HAUS4	hsa-miR-4707-3p	rs2273626	0.000220459
PYROXD2	hsa-miR-1287-5p	rs942803	0.0619005
FADS1	hsa-miR-1908-5p	rs174544	0.0721365
PTPRJ	hsa-miR-3161	rs74236456	0.112123
SGIP1	hsa-miR-3117-3p	rs1925342	0.124956
ATP6V0A1	hsa-miR-548at-5p	rs11653901	0.159921
ARFGAP1	hsa-miR-4326	rs7263455	0.226399
COMT	hsa-miR-4761-3p	rs4680	0.230298
UMPS	hsa-miR-544b	rs3821536	0.258039
ERC2	hsa-miR-3938	rs55852613	0.276531
EXOC7	hsa-miR-6868-3p	rs2243486	0.283885
F13A1	hsa-miR-5683	rs7769202	0.29282
CMIP	hsa-miR-7854-3p	rs2927318	0.40442
LDLR	hsa-miR-6886-5p	rs1003723	0.453057
AKT2	hsa-miR-641	rs41275750	0.453431
CAPN15	hsa-miR-3176	rs116698525	0.598684
MAP1B	hsa-miR-4803	rs1561401	0.642137
LRWD1	hsa-miR-4467	rs6971245	0.751098
SLC9A3R1	hsa-miR-3615	rs745666	0.815106
COPG1	hsa-miR-6826-3p	rs6788178	0.833882
TRIB2	hsa-miR-3125	rs6717278	0.966066

As can be seen, in Table 3.4, only rs2273626 - the eQTL SNP for miR-4707-3p - is an eQTL for its host gene (HAUS4) at $P < 0.05$ (not FDR adjusted). Therefore, most of the identified miR-eQTLs exert their effects independent of host gene expression. It will be interesting to check if the shared eQTL rs2273626 affects both the expression of miR-4707-3p and HAUS4 independently.

I used Haploreg v4 (Ward & Manolis, 2015) to characterize the miR-eQTLs in this study functionally. Interestingly, 5 of the miR-eQTLs identified in this study are not in high LD with other SNPs ($R^2 > 0.8$) (Table 3.5). I decided to focus my analysis on these miR-eQTLs, as they are likely to be causal SNPs. Table 3.5 displays their functional characterization, including conservation score of SNP (SiPhy cons), promoter and enhancer histone

marks, DNase marks, association with regulatory protein binding and regulatory motif alterations. The identification as eQTLs in previous studies, as well as, in which genes these SNPs are located and their functional annotation according to dBSNP is also shown.

Table 3.5 – Functional characterization of miR-eQTLs without high LD identified in this study.

SiPhy cons – SNP conservation score.

miR-eQTL	miRNA	SiPhy cons	Promoter histone marks	Enhancer histone marks	DNAse	Proteins bound	Motifs changed	Selected eQTL hits	Gencode genes	dbSNP func annot
rs7769202	miR-5683	No	-	4 tissues	4 tissues	TCF12	Nkx2	-	F13A1	intronic
rs745666	miR-3615	No	24 tissues	-	53 tissues	20 bound proteins	Evi-1, RREB-1, ZNF263	1 hit	MIR3615	5'-UTR
rs2140551	miR-548ba	Yes	-	GI smooth muscle	-	-	4 altered motifs	107 hits	STON1	synonymous
rs7006762	miR-4662a-5p	No	-	liver	-	-	Pax-1, STAT	1 hit	11kb 5' of MTSS1	-
rs74236456	miR-3161	No	Blood	14 tissues	Skin, lung	-	4 altered motifs	1 hit	PTPRJ	intronic

As can be seen in Table 3.5, all these miR-eQTLs affect regulatory motifs and are present in areas of the genome that have tissue-specific histone marks that infer some regulatory function. Moreover, 3 of these SNPs are located in DNAse I hypersensitive sites, further supporting their association with transcriptional activity.

Of note, SNP rs7769202 (T > C) associated with miR-5683 expression is also associated with enhancer histone marks in 4 tissues, including H3K4me1 and H3K27ac in adult brain. There was no evidence for foetal brain. This SNP also alters a motif in the transcription factor Nkx2 and is predicted to increase its binding affinity (PWM score increases from 2.4 to 13.6), which theoretically would lead to an increased expression of miR-5683.

In addition, SNP rs745666 (G > C) associated with miR-3615 expression is also associated with promoter histone marks in 24 tissues, including H3K4me3 in TSS active promoters in foetal brain (and TSS bivalent promoters exclusively in female foetal brain). This SNP has also been reported to be an eQTL for RAB37 in testis by GTEx and is predicted to decrease the affinity of transcription factors Evi-1 and RREB-1; whilst increasing the affinity of ZNF263. Interestingly, miR-3615 is located downstream of RAB37 and has been shown to switch host genes (switches co-expression partners) in different cancer types, suggesting alternative promoter usage (Liu et al., 2021). Of note, this SNP is located in the regulatory apical loop of miR-3615 itself, and it leads to a slight destabilization of the hairpin (Figure 3.7B). Therefore, the effect in miR-3615 is likely mediated by affecting regulatory motifs in the miR-3615 secondary structure instead of affecting transcription.

In figure 3.9, I show the genotypic association between these 5 miR-eQTLs and the expression of their associated miRNA. As can be seen in Figure 3.9A, the C-allele of SNP rs7769202 is associated with a significant increase in the expression miR-5683 ($p=9.43 \times 10^{-11}$). This observation is consistent with the increase in Nkx2 binding affinity predicted by Haploreg V4 (Ward & Manolis, 2015). This SNP explains 78 % of miR-5683 gene

expression variability ($\omega^2p = 0.78$). Another miR-eQTL that explains much of the variability in the expression of its associated miRNA is rs2140551 ($\omega^2p = 0.55$). This SNP is associated with a significant increase in miR-548ba expression ($p=3.64 \times 10^{-9}$) (Figure 3.9B). The remaining miR-eQTLs with no LD partners identified in this study seem to explain a smaller fraction of the expression of their associated miRNA (Figure 3.9C and 3.9D). A similar phenomenon was reported by Nikpay and colleagues (2019) in plasma, where, on average, each miR-eQTL explained < 1% of the variation in miRNA levels, with a small number of miR-eQTLs explaining a larger proportion (4-20%) of the variation in mature miRNA levels in plasma. However, this effect appears to be much more pronounced in foetal brain. Of note, the expression of miR-3161 was either absent or extremely low in homozygotes for the G– allele at SNP rs74236456 (Figure 3.9E).

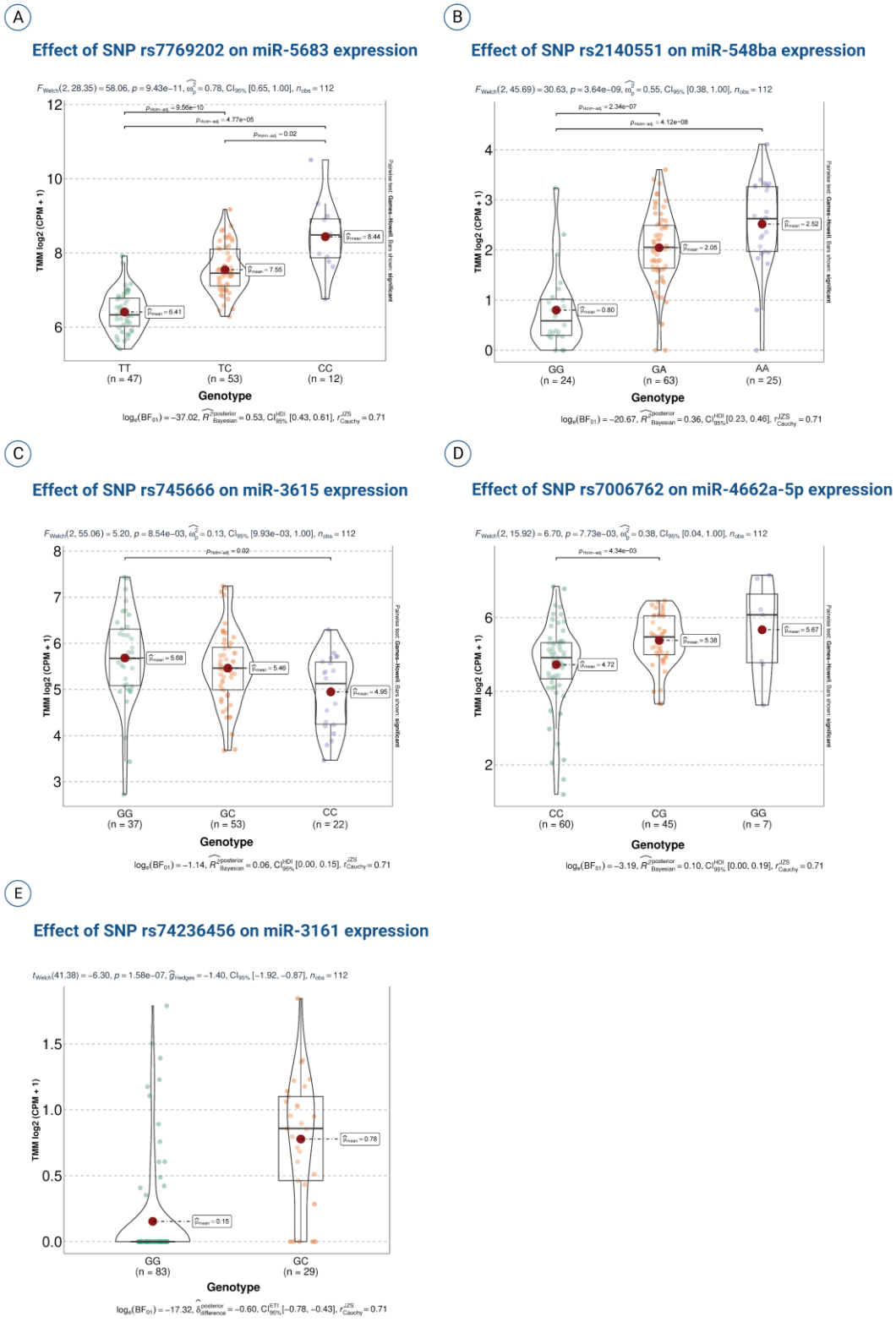


Figure 3.9 – Genotypic associations with miRNA expression of miR-eQTL that do not possess high LD.

The x-axis of each plot corresponds to the 3 observed SNP genotypes. The reference allele is on the left-hand side and alternative allele is on the right-hand side. The y-axis represents log_2-cpm + 1 TMM normalized miRNA expression values. The sample size (n) for each genotype is given below the respective alleles. All p-values are adjusted by the Holm method. Only statistically significant associations are shown. **A-** Effect of SNP rs7769202 on miR-5683 expression. This SNP is associated with a significant increase in miR-5683 expression ($p=9.43 \times 10^{-11}$) with a 1.14 log_2FC in miR-5683 expression between homozygotes for the reference allele (TT) and heterozygotes (TC) and a 2.03 log_2FC in miR-5683 expression between homozygotes for the reference allele (TT) and homozygotes for the alternative allele (CC). rs7769202 genotype explains 78 % of the variability in miR-5683 expression. **B -** Effect of SNP rs2140551 on miR-548ba expression. This SNP is associated with a significant increase in miR-548ba expression ($p=3.64 \times 10^{-9}$) with a 1.25 log_2FC in miR-548ba expression between homozygotes for the reference allele (GG) where expression is barely detectable and heterozygotes (GA) and a 1.72 log_2FC in miR-5683 expression between homozygotes for the reference allele (GG) and homozygotes for the alternative allele (AA). rs2140551 genotype explains 55 % of the variability in miR-548ba expression. **C -** Effect of SNP rs745666 on miR-3615 expression. This SNP is associated with a small but significant decrease in miR-3615 expression ($p=8.54 \times 10^{-3}$) with a -0.22 log_2FC in miR-3615 expression between homozygotes for the reference allele (GG) and heterozygotes (GC) and a -0.73 log_2FC in miR-3615 expression between homozygotes for the reference allele (GG) and homozygotes for the alternative allele (CC). Of note several homozygotes for the reference allele have a negatively skewed expression of miR-3615. rs745666 genotype explains 13 % of the variability in miR-3615 expression. **D -** Effect of SNP rs7006762 on miR-4662a-5p expression. This SNP is associated with a significant increase in miR-4662a-5p expression ($p=7.73 \times 10^{-3}$) with a 0.66 log_2FC in miR-4662a-5p expression between homozygotes for the reference allele (CC) and heterozygotes (CG) and a 0.95 log_2FC in miR-4662a-5p expression between homozygotes for the reference allele (CC) and homozygotes for the alternative allele (GG). Of note several homozygotes for the reference allele have a negatively skewed expression of miR-4662a-5p. rs7006762 genotype explains 38 % of the variability in miR-4662a-5p expression. **E-** Effect of SNP rs74236456 on miR-3161 expression. This SNP seems to contribute to the activation of expression of miR-3161 at low levels, as most homozygotes for the reference allele (GG) have no detectable expression of miR-3161 whereas heterozygotes (GC) have a 0.63 log_2FC change in expression in comparison with the GG genotype. This SNP has a MAF=0.161542 according to the 1000 genomes project, which explains why there are no homozygotes for the alternative allele in our sample.

I also show genotypic associations with miRNA expression for two miR-eQTLs located within the mature miRNA (rs2273626) and pri–miRNA (rs56103835) sequences of their associated miRNAs, given that my RNAfold analysis predicted SNP rs2273626 would decrease the expression of miR-4707-3p; and SNP rs56103835 modifies the regulatory basal UG motif in the pri-miRNA hairpin of its associated miRNA miR-323b-3p (Figure 3.10).

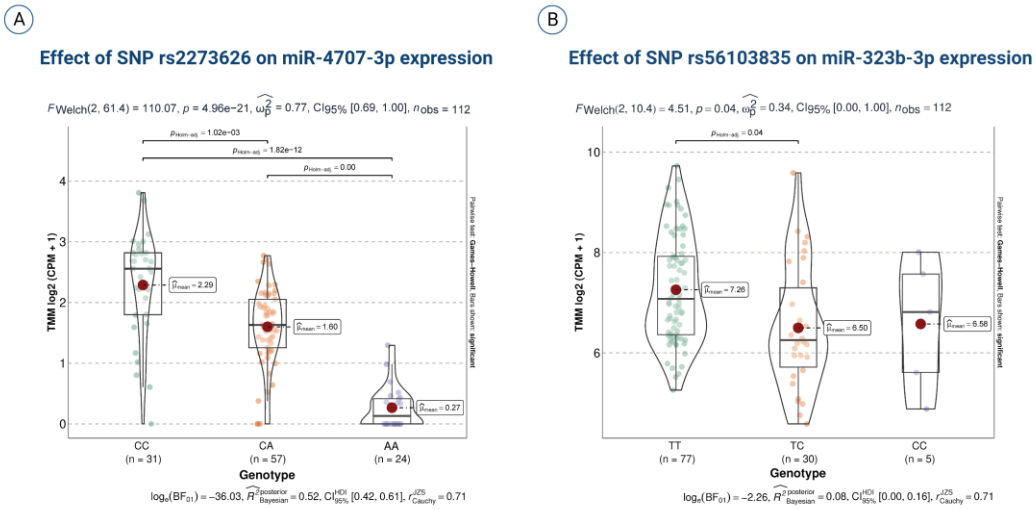


Figure 3.10 - Genotypic associations with miRNA expression of miR-eQTL located within miRNA hairpins.

The x-axis of each plot corresponds to the 3 observed SNP genotypes. The reference allele is on the left-hand side and alternative allele is on the right-hand side. The y-axis represents log_2-cpm +1 TMM normalized miRNA expression values. The sample size (n) for each genotype is given below the respective alleles. All p-values are adjusted by the Holm method. Only statistically significant associations are shown. **A -** Effect of SNP rs2273626 on miR-4707-3p expression. This SNP is associated with a significant decrease in miR-4707-3p expression ($p=4.96 \times 10^{-21}$) which is consistent with my RNAfold analysis. rs2273626 is associated with a -0.69 log_2FC in miR-4707-3p expression between homozygotes for the reference allele (CC) and heterozygotes (CA) and a -2.02 log_2FC between homozygotes for the reference allele (CC) and homozygotes for the alternative allele (AA). Homozygotes for the risk allele have very low or no expression on miR-4707-3p. rs2273626 genotype explains 77 % of the variability in miR-4707-3p expression. **B -**

Effect of SNP rs56103835 on miR-323b-3p expression. This SNP is associated with a small but significant decrease in miR-323b-3p expression (p=0.04) which is consistent with it altering a regulatory motif in its associated miRNA hairpin. rs56103835 is associated with -0.68 log_2FC between homozygotes for the reference allele (TT) and heterozygotes (TC). rs56103835 genotype explains 34 % of the variability in miR-323b-3p expression. There is no difference between heterozygotes (TC) and homozygotes for the alternative allele (CC) because this miRNA is paternally imprinted.

As can be seen in Figure 3.10, the A-allele of SNP rs2273626 significantly decreases the expression of miR-4707-3p ($p=4.96 \times 10^{-21}$) with homozygotes for the A-allele having null or very low levels of miRNA expression. Despite this SNP being in LD with 31 other SNPs with R2 > 0.8, it is likely that rs2273626 is the causal variant as rs2273626 leads to a significant destabilization of the miR-4707-3p hairpin, providing mechanistic reasoning for this miR-eQTL. This SNP explains 77% of the variability in miR-4707-3p expression (ω^2p =0.77). The C–allele of SNP rs56103835 slightly decreases miR-323b-3p expression (p=0.04), which is consistent with it altering a regulatory motif within miR-323b-3p that enhances processing. Together, these results indicate that there are significant miR-eQTLs in 2nd trimester foetal brain, most of which are located within 50 kb of their associated mature miRNA and independent of host gene expression. Some of the identified miR-eQTLs have large effect sizes and explain the majority of the variability in miRNA expression that is attributable to genetic effects. Moreover, I provide evidence that some of the miR-eQTLs identified in this study affect the stability and processing of mature miRNAs instead of affecting the transcription of primary miRNA transcripts.

3.4 Discussion

This Chapter identifies common variants associated with miRNA expression in human foetal brain. Foetal brain development is marked by extremely dynamic transcription patterns where transcriptional changes occur more rapidly than at any other stage of life in the brain (Johnson et al., 2009; Colantuoni et al., 2011; Kang et al., 2011; Jaffe et al., 2018) and these patterns are crucial for proper future brain function (Gulsuner et al.,2013). miRNAs are highly expressed in the brain and regulate several processes pertaining to brain development and neuronal function, including neurogenesis, neuronal cell–type determination and migration, axonal pathfinding, synapse formation and neuronal circuit development (for review, see: Rajman & Schratt, 2017; Prieto-Colomina et al., 2021). In this context, identifying how common variants affect miRNA expression in foetal brain and how this might predispose towards a neurodevelopmental disorder by altering a brain development trajectory is important. Moreover, the assessment of miR-eQTL allows us to shed light on the role of miRNA regulation in the brain.

Using FASTQTL, I identified 30 miRNAs whose expression is associated with common genetic variation (miR-eQTLs) at FDR < 0.05. This constitutes ~ 2% of the miRNAs under study (n= 1449). This percentage of miRNA with cis-regulatory genetic influences approximates that found in adipose tissue (Rantalainen et al., 2011; Parts et al., 2012; Civelek et al., 2013) and monocyte-derived dendritic cells (Siddle et al., 2014), but is lower than the percentage of circulating miRNAs in plasma with miR-eQTLs (7% of the examined miRNAs), reported by Nikpay and colleagues (2019). This discrepancy might be due to the large sample size of the Nikpay study (n=710) in comparison with the Rantalainen (n=70), Parts (n=131), Civelek (n=200), Siddle (n=65) and current (n=112) studies.

My group performed an mRNA eQTL study in 2nd trimester foetal brain with a similar sample size (n=120) on primarily the same samples as this

study and identified eQTLs in 4.6% of genes investigated (O'Brien et al.,2018). This indicates that foetal brain miRNAs have fewer cis-eQTLs than mRNAs and are under less genetic control. This finding is consistent with several other studies (Su et al., 2011; Civelek et al., 2013; Lappalainen et al., 2013) and might originate from miRNAs being under higher selective pressure than protein-coding genes, given their role in regulating gene expression. As evidence of this, human miRNA sequences and mature miRNA sequences, in particular, have shown a lack of diversity in different populations (Quach et al., 2009), which points to high selective pressure in miRNAs. Changes in miRNA expression will have significant downstream effects on genes regulated by that miRNA, given that ~60% of protein-coding genes have at least one conserved miRNA binding site (Friedman et al., 2009).

None of the miRNAs associated with miR-eQTLs in this study were reported in any of the other miR-eQTL studies performed in adult brain tissue (Williamson et al., 2015; Mamdani et al., 2015; Vornholt et al., 2020). Interestingly, 2 were reported in the context of pregnancy – miR-323b-3p in the plasma of 1st trimester pregnant women (White et al., 2021) and miR-1269a in full–term placenta (Inno et al., 2021). Moreover, miR-1908-5p expression has been associated with rs174561 in plasma (Nikpay et al., 2019), a SNP in perfect LD with the one reported in my miR- eQTL study (rs174544), with both SNPs having the same nominal p-value reported by FASTQTL in this study. Of note, this SNP (rs174561) has also been found to be associated with FADS1 (the host gene of miR-1908) and FADS3 expression in adult brain (GTEx consortium, 2020), but not in foetal brain (O'Brien et al., 2018).

Most of the miRNAs with eQTLs identified in this study are expressed either at low or moderate levels in 2nd trimester foetal brain. This is consistent with previous studies (Rotival et al., 2020) and may suggest that these miRNAs are more amenable to expression variation and under less selective pressure because they are expressed at lower levels and therefore are probably fine-tuners of gene expression, may be redundant miRNAs or,

involved in the regulation of pathways that will not have significant deleterious implications for the cell if their expression changes. In fact, several studies indicate that lowly expressed miRNAs tend to be species-specific (Berezikov et al., 2006; Fahlgren et al., 2007; Lu et al., 2008) and that in humans, these miRNAs are under a less strong purifying selection than highly expressed miRNAs (Liang & Lee, 2009).

In this study, 29 of the 30 miR-eQTLs identified were located less than 100 kb away from the mature miRNA, with 83% (25 out of 30) of miR-eQTLs being within 50 kb of their corresponding mature miRNA. Only 1 miR-eQTL was located more than 100kb away from its mature miRNA. These results indicate that the majority of miR-eQTLs in foetal brain concentrate near miRNA genes and their transcription sites. In contrast, several miR-eQTL studies in other tissues have reported that the majority of identified cis-miR-eQTLs are located further away. Huan et al. (2015) reported that 49% of their identified miR-eQTLs in whole blood were located 300–500kb away from their target miRNA (Huan et al., 2015). Nikpay et al. (2019) reported that 62% of their identified miR-eQTLs in plasma were located > 50kb away, and White et al. (2021) reported that 77% of their identified miR-eQTLs in plasma were located > 50kb. One possible explanation for this is the possibility of miRNAs in foetal brain using alternative intronic promoters that are tissue and/or developmental stage specific. miRNAs are transcribed from intronic regions within a host gene or from intragenic regions (Steiman-Shimony et al., 2018). Intragenic miRNAs have been reported to have independent (mainly intronic) alternative promoters. These promoters are usually tissue-specific, in contrast to host gene promoters which are usually ubiquitously expressed (Marsico et al., 2013; Budach et al., 2016). This suggests that miR-eQTLs may affect promoter activity in a tissue-specific fashion and that alternative promoters might be a source of genetic variation.

The miR-eQTLs identified in this study seem to be evenly distributed throughout the genome, and I did not find evidence of a hotspot for miR-cis-eQTLs. This is surprising because I identified miR-eQTLs associated with miRNAs that are co-expressed as clusters, such as miR-323b-3p.

miR-323b-3p is located in a paternally imprinted cluster on chromosome 14 (C14MC) consisting of 52 miRNAs (Morales-Prieto et al., 2013). miRNA clusters are usually transcribed as a single transcriptional unit (Baskerville & Bartel, 2005). Moreover, this miRNA, along with several miRNAs from this cluster, have been found to have miR-eQTLs in the plasma of women during the first trimester of pregnancy (White et al., 2021). Despite a large number of the members of this cluster being expressed in my samples, I only found a miR-eQTL associated with miR-323b-3p expression, which suggests this miR-eQTL affects miR-323b-3p exclusively, most likely by affecting processing by Drosha instead of affecting primary miRNA transcription. This hypothesis is further supported by the fact that rs56103835 associated with miR-323b-3p expression is actually one of the 4 miR-eQTLs identified in this study that is located within the pri-miRNA hairpin structure of its associated miRNA .

Mature miRNAs are derived from long primary transcripts (pri–miRNAs) that always contain one or several RNA stem-loop hairpin secondary structures in which the mature miRNA resides. For the majority of miRNAs, pri–miRNAs are processed in the nucleus by the Microprocessor complex formed by Drosha and DGCR8 (Lee et al., 2003; Ha & Kim, 2014; Kwon et al., 2016), giving rise to precursor miRNAs (pre-miRNAs). Pre-miRNAs are further processed by Dicer (and TRBP) into mature miRNAs (Bernstein et al., 2001; Ha & Kim, 2014). Several studies have identified regulatory motifs within the pri-miRNA hairpins that dictate processing efficiency and impact mature miRNA biogenesis. These include an optimal hairpin stem length of ~36 nucleotides ± 3nt and apical loop sizes of ~10-15 nucleotides. Moreover, miRNA processing is enhanced by regulatory motifs (Figure 3.11), such as the UG motif at the base of the stem and the UGUG motif in the apical loop, which are recognized by the Microprocessor complex and the CNNC motif (below the basal junction) where accessory protein SRp20/SRSF3 binds (Auyeung et al., 2013; Fang & Bartel., 2015; Roden et al., 2017).

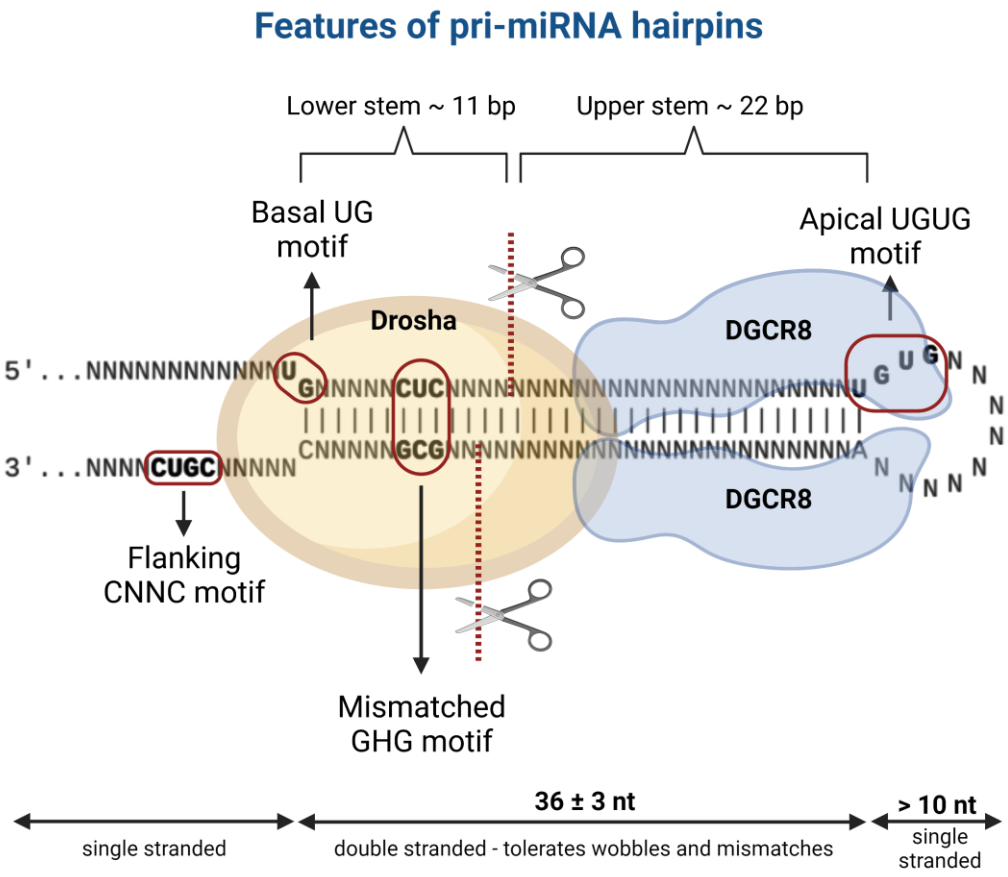


Figure 3.11 – Defining features of pri-miRNAs hairpins that dictate miRNA processing.

Optimal miRNA processing requires a hairpin stem length of ~36 nucleotides ± 3nt and apical loop sizes of ~10-15 nucleotides (apical loop sizes can range from 3-23 nucleotides). Moreover, several regulatory motifs such as the CNNC motif and the UG motif enhance processing of mature miRNAs. Adapted from: Fang & Bartel, 2015. Created with Biorender.com

SNPs that lead to changes in RNA stability might alter RNA secondary structure and as a consequence pri-miRNA structural motifs such as bulge enriched and bulge depleted areas of the hairpin, affecting pri-miRNA processing and leading to altered mature miRNA expression. In this study, I used *RNAfold* and predicted that miR-eQTL rs2273626 located in the seed region of miR-4707-3p leads to a considerable increase in minimum free energy ΔG =5.6 Kcal/mol within the hairpin, which will destabilize the

structure and be associated with a decrease in processing efficiency of the miR-4707-3p hairpin, leading to a decrease in mature miR-4707-3p expression. In the case of miR-323b-3p, SNP rs56103835 alters the regulatory basal UG motif of its pri-miRNA. This motif acts as a marker of the boundary of the basal junction that divides ssRNA and dsRNA and interacts with Drosha to ensure correct positioning of the microprocessor complex and leads to enhanced generation of pre-miRNAs (Auyeung et al., 2013; Nguyen et al., 2015; Jin et al., 2020). Studies have shown that mutations in the basal UG motif reduce the accumulation of mature miRNA (Auyeung et al., 2013). Therefore, this SNP will lead to a decrease in Drosha's processing efficiency and, consequently, a decrease in mature miR-323b-3p levels.

73% of the miRNAs with associated miR-eQTLs in this study were found within host mRNAs (22 of 30 miRNAs) in intronic and exonic regions Intragenic miRNAs and their host genes can share the same promoter, in which case the miRNA is likely to be co-expressed with the host gene (Lutter et al., 2010). Despite the vast majority of miRNAs with eQTLs identified in this study being located within a host–gene, the genetic signal associated with miRNA expression was only colocalized with host-gene expression in the case of hsa-miR-4707-3p and HAUS4. This means that most intragenic miRNAs do not use the host gene promoter and must use intronic promoters, consistent with the vast majority of miR-eQTLs in this study being < 50kb from the mature miRNA. This also indicates that SNPs in intronic miRNA promoters that affect expression of pri-miRNA hairpins will affect miRNA biogenesis independently from the host gene expression. It will be interesting to investigate whether this miR-eQTL will affect miRNA (miR-4707-3p) and host gene (HAUS4) expression independently (expression not correlated within genotypes).

The majority of miRNAs with associated miR-eQTLs identified in this study are poorly conserved miRNAs in vertebrates (n=27), and most are exclusive to primates. Over 100 primate-specific miRNAs and 14 human-specific miRNAs have been identified in foetal brain (for review, see: Berezikov, 2011), the majority regulating progenitor proliferation and neuronal

differentiation (Nowakowski et al., 2013, 2018; Arcila et al., 2014). miRNAs are crucial to shaping gene expression patterns that are suggested to underpin the accelerated evolution of the human brain (Berezikov E., 2011; Chakraborty et al., 2018; Prodromidou & Matsas, 2019) and even determine anatomical regions during brain development (Arcila et al., 2014; Nowakowski et al., 2018) and brain maturation (Ziats & Rennert, 2014). miRNAs mediate these effects by controlling and fine-tuning spatiotemporal gene expression patterns during brain development (Schratt, 2009a,b) despite being expressed at low levels in adult prefrontal cortex and cerebellum (Hu et al., 2012) and in this study in foetal brain. Therefore, it is possible that miR-eQTLs that affect the expression of mature human and primate-specific miRNAs are associated with neocortical expansion, cognition and neurodevelopmental and neuropsychiatric disorders. In the next Chapter, I will perform a PheWAS and SMR study on the identified miR-eQTLs to ascertain if they are associated with these traits.

Chapter 4 - Association between microRNA expression in 2nd trimester human foetal brain and neuropsychiatric disorders and brain phenotypes

4.1 Introduction

4.1.1 Overview

In the previous chapter, I identified 30 miRNAs that are subject to variable genetic influences on their expression, in the form of cis-miR-eQTLs, in 2nd trimester foetal brain. Given the regulatory role of miRNAs on processes crucial for both brain development and function, as well as their widespread downstream effects on post-transcriptional gene regulation, these cis-miR-eQTLs may be relevant to brain traits, including neuropsychiatric disorders.

Alterations in miRNA abundance have been reported in brain tissue and blood from individuals with neuropsychiatric disorders (Issler & Chen, 2015; Kocerha et al., 2015). Moreover, genetic risk variants have been shown to alter miRNA expression and function (Strazisar et al., 2015; Duan et al., 2014) and risk exposomes such as sleep deprivation alter miRNA expression (Davis et al., 2007; Maccani et al., 2010; Maccani & Marsit, 2011; Maccani & Knopik, 2012; Wang & Cui, 2012).

Trait-associated SNPs are three times more likely to be an eQTL (Hernandez et al., 2012; Nica et al., 2010; Nicolae et al., 2010). For this study, I investigated whether my identified cis-miR-eQTLs were associated with neuropsychiatric disorders and neurological/cognitive phenotypes by screening summary data from GWAS carried out by the Psychiatric Genomics Consortium and the Complex Trait Genetics lab at VU University Amsterdam Lab.

The Psychiatric Genomics Consortium (PGC) is a large collaborative international consortium that studies the genetic architecture of major neuropsychiatric/neurodevelopmental disorders, including attention-deficit hyperactivity disorder (ADHD), schizophrenia (SZ), autism spectrum disorder

(ASD), obsessive-compulsive disorder (OCD), Tourette syndrome (TS), major depressive disorder (MDD), bipolar disorder (BD), anxiety disorders, eating disorders, post-traumatic stress disorder (PTSD), Alzheimer's disease (AD) and substance use disorders.

The Complex Trait Genetics (CTG) Lab at VU University Amsterdam aims to explore the genetic architecture and gene x environment interactions of several brain phenotypes, including brain volume, intelligence, sensitivity to environmental stress and adversity (SESA), neuroticism, neuroticism subclusters and depression, Alzheimer's dementia, antisocial behaviour and insomnia. The GWAS performed by this group often employ UK Biobank samples. The UK Biobank is a large prospective cohort database of genome-wide genotyping, biological and health data of ~ 500,000 healthy individuals across the UK.

After screening both the PGC3 GWAS and the CNCR - CTG Lab GWAS summary data for suggestive evidence of association with miR-eQTLs, I will ascertain whether these associations are consistent with causality/pleiotropy or linkage. A common approach to identify potential causal effects of eQTLs is based on mendelian randomization (MR) (for review, see: Cano-Gomez & Trynka, 2020; Li & Ritchie, 2021; Walker et al., 2022).

4.1.2 Summary data-based Mendelian randomization (SMR) and heterogeneity in dependent instruments (HEIDI) tests

The Mendelian randomization (MR) method derives from epidemiological studies and uses genetic variation to test a causal inference between a modifiable exposure and disease risk (for review, see: Sanderson et al., 2022; Walker et al., 2022). A modification of this method commonly used in

functional genomics uses eQTLs as a proxy for a modifiable variable (exposure, which in this case is gene expression) to estimate the possible causal relationship between gene expression and disease risk (Zhu et al, 2016). Summary data-based Mendelian randomization (SMR; Zhu et al, 2016) uses summary statistics from GWAS of a trait of interest and tests for joint associations with variants associated with gene expression (or another potential mediating phenotype) in a relevant tissue or cell due to a shared and potentially causal variant at a locus. A significant association between an eQTL and trait detected by the SMR test can arise either due to causal effects of the eQTL on the trait (the genetic variant influences gene expression which then influences the trait), pleiotropic effects (the genetic variant influences gene expression and the trait independently) or linkage (the genetic variant is in linkage disequilibrium with the actual causal variant, which might operate through different gene) (Figure 4.1).

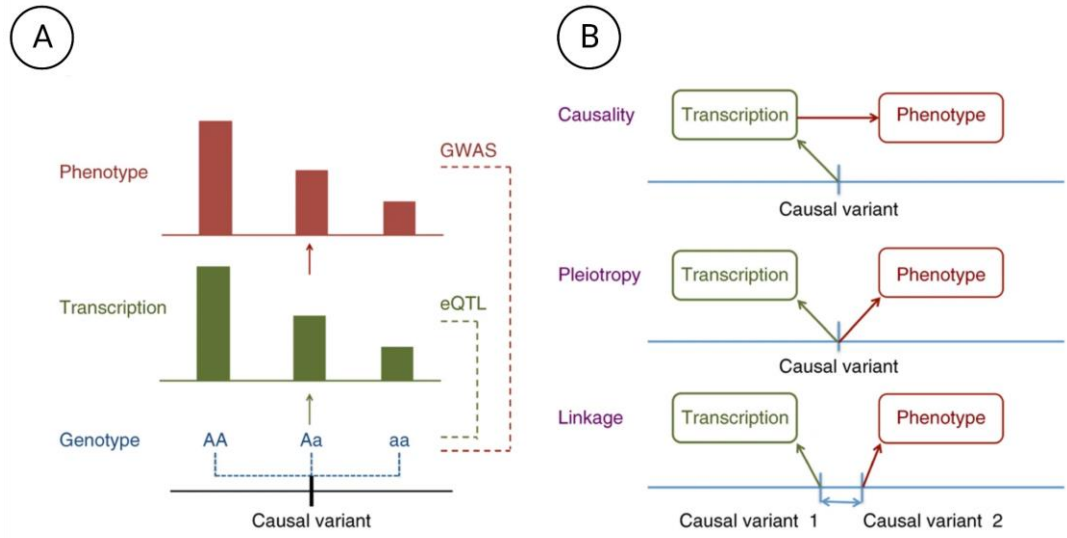


Figure 4.1 – Models of association between genomic variants influencing gene expression (eQTLs) and phenotypes (traits)

A – Causality between a genetic variant and phenotype (trait) occurs when the genetic variant influences a trait via altering gene expression. B – Possible mechanisms underlying an observed association between a phenotype (trait) and gene expression. Adapted from: Zhu et al., 2016, with permission (Appendix 1).

SMR, in itself, is unable to disentangle a causative association from pleiotropy, although it has successfully identified causative associations between gene expression and complex traits (Zhu et al., 2016; Porcu et al., 2019) that had been previously experimentally confirmed (e.g. SORT1 expression and cholesterol levels [Zhu et al., 2016; Porcu et al., 2019; Musunuru et al., 2010]).

An additional test, dubbed heterogeneity in dependent instruments test (HEIDI test), uses the top ~ 20 associated SNPs within a cis-eQTL region to distinguish causal/pleiotropic effects from linkage. This test assumes only one causal variant (affecting both gene expression and the trait of interest) in the cis-eQTL region. HEIDI test results with small heterogeneity test p-values (e.g. $p < 0.05$) are disregarded, as these are likely to arise due to linkage (a homogenous pattern indicates a single shared causal variant) (Zhu et al., 2016).

4.1.3 Multi-marker Analysis of GenoMic Annotation (MAGMA)

As an independent test of whether an associated miRNA's expression plausibly influences a trait, I will perform a Multi-marker Analysis of GenoMic Annotation (MAGMA) analysis of predicted miRNA targets on GWAS of the associated neuropsychiatric disorders and neurological/cognitive traits. MAGMA is a gene and gene-set analysis method that uses a multiple linear principal components regression approach to include the LD between markers in the analysis. The gene-set analysis is divided into two parts. Firstly, a gene analysis is performed to quantify how strongly each gene is associated with the trait of interest. Gene correlations, which reflect the LD between genes, are also estimated. These correlations are needed to compensate for the dependencies between genes during the gene-set

analysis. Secondly, the gene p-values and the gene correlation matrix are used to perform the gene-set analysis (de Leeuw et al., 2015).

4.1.4 Aims of Chapter

The aims of this Chapter are:

1) To ascertain if any of the cis-miR-eQTLs identified in 2nd trimester foetal brain are additionally associated with psychiatric disorders and neurological / cognitive phenotypes.

2) To test whether these associations are consistent with causality / pleiotropy or linkage.

3) To test for enrichment of trait-associated genetic variation in genes that are predicted targets of any miRNA that is causally / pleiotropically associated with that trait.

4.2 Methods

4.2.1 Initial screening of PGC GWAS and CNCR - CTG Lab GWAS

I performed an initial screening of GWAS summary statistics to see if any of the miR-eQTLs I identified in the previous chapter were associated with psychiatric disorders and neurological/cognitive phenotypes, using a $p < 5 \times 10^{-5}$ as indicative of suggestive association.

4.2.1.1 Initial screening of PGC GWAS

The PGC GWAS summary statistics employed in this study were obtained from the PGC website repository at https://www.med.unc.edu/pgc/download-results/, and the GWAS used in this study are indicated in Table 4.1.

The summary statistics of the PGC AD study (Jansen et al., 2019) were missing the majority of the SNPs under study, so I also screened the summary statistics of the newest AD GWAS (Bellenguez et al., 2022) obtained from the GWAS catalogue https://www.ebi.ac.uk/gwas/studies/GCST90027158, to ascertain if any of the miR-eQTLs were associated with AD.

Table 4.1 – Details of summary statistics and Neuropsychiatric disorders analysed in this study contained in the PGC repository.

Neuropsychiatric Disorder	GWAS reference
Attention Deficit Hyperactivity Disorder (ADHD)	Demontis et al., 2018
Schizophrenia (SZ)	Trubetskoy et al., 2022
Autism Spectrum Disorder (ASD)	Grove et al, 2019
Major Depressive Disorder (MDD)	Howard et al., 2019
Anorexia	Watson et al., 2019
Bipolar Disorder (BD)	Mullins et al., 2021
Obsessive - Compulsive Disorder (OCD)	International OCD Foundation Genetics Collaborative (IOCDF-GC) and OCD Collaborative Genetics Association Studies (OCGAS), 2018
Tourette Syndrome (TS)	Yu et al., 2019
Cross - Disorder	Cross Disorder Group, 2019
Post -Traumatic Stress Disorder (PTSD)	Nivergelt et al., 2019
Anxiety	Otowa et al., 2016
Panic Disorder	Forstner et al., 2021
Alzheimer's Disease	Jansen et al., 2019
Alcohol Dependence	Walters et al., 2018
Cannabis use disorder	Jonhson et al., 2020
Opioid Dependence	Polimanti et al., 2020

4.2.1.2 Initial screening of CNCR-CTG Lab GWAS

The CNCR - CTG Lab GWAS summary statistics employed in this study were obtained from the CTG Lab website repository at https://ctg.cncr.nl/software/summary_statistics, and the GWAS employed in this study are indicated in Table 4.2. SNP rs7263455 (eQTL for miR-4326) and SNP rs745666 (eQTL for miR-3615) were absent in all of the CNCR-CTG GWAS.

Table 4.2 – Details of summary statistics and Neurological / Cognitive traits analyzed in this study contained in the CTG Lab repository.

Neurological / Cognitive trait	GWAS reference
Brain Volume (metanalysis)	Jansen et al., 2020
Intelligence	Savage et al., 2018
Sensitivity to Environmental Stress and Adversity (SESA)	Nagel et al., 2020
Neuroticism, neuroticism subclusters and depression	Nagel et al., 2018b
Insomnia	Jansen et al., 2019
Neuroticism - item-level analysis	Nagel et al., 2018a

4.2.1.3 PheWas scan of miR-eQTLs associated with traits in PGC GWAS and CNCR-CTG Lab GWAS

Subsequently, I tested for the presence of pleiotropic effects of the miR-eQTLs associated with either neuropsychiatric disorders and brain / cognitive endophenotypes by performing a Phenome-Wide Association study (PheWas) nominal scan on an Atlas of GWAS summary statistics from the CNCR-CTG lab at https://atlas.ctglab.nl/PheWAS which contains 600 GWAS performed on UK Biobank samples (Watanabe et al., 2019). A p-value cut-off of 5×10^{-5} was taken as indicative of suggestive association. Only Neurological, Cognitive, Psychiatric and Environment – Education traits were considered.

4.2.2 Effect of miR-eQTL genotype on associated miRNA expression

The structure of pri-miRNA hairpins and analysis of the effect of SNPs on pri-miRNA hairpin thermodynamic stability were performed by the software ViennaRNA *RNAfold* (Lorenz et al., 2011) using the default parameters in the miRNASNP-v3 database (http://bioinfo.life.hust.edu.cn/miRNASNP/) (Liu et al., 2020). The plot of the genotypic association of miR-eQTL with miRNA expression in 2nd trimester foetal brain was generated using the R package ggstatsplot (Patil, I., 2021).

4.2.3 SMR and HEIDI analysis of miR-eQTLs associated with traits in PGC and CNCR-CTG Lab GWAS

SMR and HEIDI analysis was performed with the SMR software (Zhu et al., 2016) using my 2nd trimester foetal brain miR-cis-eQTL data obtained by FASTQTL analysis that was described in the previous Chapter and summary statistics from the GWAS for bipolar disorder (Mullins et al., 2021), neuroticism-IRR (Nagel et al., 2018a), sleep duration (Jansen et al., 2019), intelligence (Savage et al., 2018) and brain volume metanalysis (Jansen et al., 2020) to test for pleiotropic association between miRNA expression and these traits due to a shared and potentially causal variant at the miR-eQTL loci. Only miR-eQTL found to be associated with a trait at $p < 5 \times 10^{-5}$ were assessed, and therefore a total of 5 separate SMR tests were performed. miR-eQTLs were originally annotated in build hg38, whilst SNPs from GWAS were annotated in build hg19. The R package rtracklayer (Lawrence, Gentleman, and Carey, 2009) was employed to perform a liftOver of miR-eQTL coordinates into hg19 build using chain hg38TOHg19.over.chain.

1000 Genomes EUR samples were used as a reference sample for pairwise SNP LD estimation. I employed most of the default settings of SMR, such as only allowing SNPs with a minor allele frequency (MAF) > 0.01, removing SNPs in very strong linkage disequilibrium (LD, $R^2 > 0.9$) with the top associated miR-eQTL, and removing SNPs in low LD or not in LD $R^2 < 0.05$) with the top associated miR-eQTL. Given that I only had 30 miR-eQTLs to test at $p < 0.05$ and the exploratory nature of this study, I decided to relax the threshold of eQTL p-value ($P_{eQTL} < 0.05$) from the default of $P_{eQTL} < 5.0 \times 10^{-8}$ to select the top associated cis-eQTL for SMR analysis. SNPs with allele frequency difference > 0.2 between any pairwise data sets - LD reference, eQTL summary data and the GWAS summary data were eliminated. Heterogeneity in dependent instruments (HEIDI) test was performed to evaluate whether linkage with other variants can explain the observed association between the trait and the miR-eQTL. Rejection of the null hypothesis ($P_{HEIDI} < 0.05$) indicates that the observed association could be due to two distinct genetic variants in linkage disequilibrium with each other. The HEIDI test was performed after removing SNPs in very strong LD ($R^2 > 0.9$) or absent LD ($R^2 < 0.05$) with the top associated miR-eQTL. Moreover, a threshold of $P_{eQTL} = 0.2$ for the HEIDI test was employed to get an adequate number of SNPs along with number of cis-SNPs ≥ 3 and maximum eQTLs in a HEIDI test = 20. P_{SMR} values were corrected for multiple testing by Bonferroni correction of the 30 miR-eQTLs analyzed and the 23 GWAS studies in the initial screening, totaling 690 tests. The P_{HEIDI} was not corrected for multiple testing to be conservative, as only non-significant ($p > 0.05$) observations are retained.

The SMR locus and effect plots were performed using R, and the thresholds p-smr=1 and p-heidi=0 were employed, as suggested by the authors (Zhu et al., 2016). Moreover, all available SNPs were included in the SMR effect graphs (Figure 4.8), allowing for a visualization of the HEIDI test.

4.2.4 MAGMA analysis of miR-1908-5p targets with causal / pleiotropic associations by SMR with traits in PGC GWAS and CNCR-CTG Lab GWAS

Predicted targets of miR-1908-5p were retrieved from TargetScan 8.0 (McGeary et al., 2019), a miRNA target prediction tool that searches for the presence of conserved miRNA response elements, namely 8mer, 7mer and 6mer sites, in the 3'-UTR of mRNAs that match the seed region of the specified miRNA (Lewis et al., 2005).

Target gene names were converted into Entrez IDs using the Biomart package (Durinck et al., 2009). All miR-1908-5p targets predicted by Targetscan 8.0 were treated as a gene set. A competitive MAGMA-based gene-set analysis was utilized to test the association of that gene set using the summary statistics from the PGC GWAS and CNCR-CTG Lab GWAS, where a pleiotropic association with miRNA expression by SMR was discovered. $p < 0.05$ was considered significant.

SNPs were annotated to genes based on NCBI (37.3) gene definitions (NCBI Resource Coordinators, 2017), and variants were mapped to a gene if they were located within a window of 35kb upstream and 10kb downstream of each gene, as previously described (Network Pathway Analysis Subgroup of the Psychiatric Genomics Consortium, 2015; Pardiñas et al., 2018; Sey et al., 2020). The European ancestry samples from the 1000 Genomes Phase 3 data (http://www.1000genomes.org) were employed as a reference dataset for pairwise SNP linkage disequilibrium (LD) estimation. MAGMA accounts for the potentially confounding effects of gene size, number of SNPs in a gene and LD between markers in a generalized regression model.

When raw genotype data are unavailable, MAGMA employs a SNP-wise model, combining SNP p-values into a gene test-statistic. The SNP model chosen for the MAGMA analysis was the multi-model. This model runs both a SNP-wise mean analysis which uses the sum of squared SNP Z-statistics as

test statistic and a SNP-wise top SNP analysis, which uses sum of -log (SNP p-value) for top SNPs as statistic for each gene and combines the resulting gene p-values into an aggregate p-value. This method gives rise to a more even distribution of statistical power and possesses sensitivity for a multitude of different genetic architectures.

4.2.5 Gene Ontology (GO) analysis of miR-1908-5p targets

All miR-1908-5p targets predicted by Targetscan were employed for Gene Ontology (GO) analysis using ShinyGO v0.76.2, a graphical tool for gene-set enrichment analysis (Ge et al., 2020). GO analysis was restricted to GO Biological processes (BP) and GO terms with a minimum of 2 and a maximum of 2000 genes. In addition, GO term redundancies were removed from the analysis. Enrichment p-values were FDR corrected, and FDR < 0.01 was considered significant.

4.2.6 MAGMA analysis of miR-1908-5p targets by GO terms in bipolar disorder

Enriched GO terms of miR-1908-5p targets at FDR < 0.01 were used to create individual GO term gene sets. Target gene names were converted into Entrez IDs using the Biomart package (Durinck et al., 2009). A total of 238 gene sets were created. A competitive MAGMA-based gene-set analysis of miR-1908-5p targets by GO term was utilized to test the association of each GO term using the summary statistics from the BD PGC GWAS (Mullins et

al., 2021). Multiple comparisons were corrected by FDR, and FDR < 0.05 was considered significant.

4.3 Results

4.3.1 Nominal screening of association of 2nd trimester foetal brain miR-eQTLs and neuropsychiatric disorders / brain endophenotypes:

Initially, I performed a screen of association using GWAS summary statistics to see if any of the identified miR-eQTLs were associated with any psychiatric disorders and brain endophenotypes, using a p-value cut-off of 5 x 10^{-5} as indicative of suggestive association. Tables 4.3 and 4.4 show the association p-values for the miR-eQTLs investigated from the available PGC GWAS that encompass 11 major psychiatric disorders (attention-deficit hyperactivity disorder (ADHD), schizophrenia (SZ), autism spectrum disorder (ASD), obsessive-compulsive disorder (OCD), Tourette Syndrome (TS), major depressive disorder (MDD), bipolar disorder (BD), anxiety disorders, eating disorders, post-traumatic stress disorder (PTSD), Alzheimer's disease (AD) and substance use disorders). The summary statistics of the PGC AD study were missing most of the SNPs under investigation, so I also screened the newest AD (2022) GWAS (Bellenguez et al., 2022) to ascertain if any of the miR-eQTLs were associated with this condition. As can be seen in Table 4.3, I identified a single miR-eQTL associated with psychiatric disorders at the $p < 5 X 10^{-5}$ threshold. The SNP rs174561 associated with expression of miR-1908-5p in 2nd trimester foetal brain (q-value=9.28 x 10^{-8}), is also significantly associated with BD ($p = 1.83 x 10^{-11}$).

Table 4.3, pt. I – P-values of association between the ADHD (2019), SCZ (2022), ASD (2019), MDD (2019), Anorexia (2019), BD (2021), OCD (2018) and TS (2019) GWAS from the PGC used in this study and the SNPs (SNP ID) identified as miR-eQTLs in 2nd trimester foetal brain and their associated miRNAs.

In bold – associations with $p < 5 \times 10^{-5}$. P-values rounded to 2 decimal places.

SNP ID	miRNA	ADHD (2019)	SCZ (2022)	ASD (2019)	MDD (2019)	Anorexia (2019)	BD (2021)	OCD (2018)	TS (2019)
rs7769202	miR-5683	NA	0.85	0.04	0.02	0.38	0.78	0.55	0.28
rs2273626	miR-4707-3p	0.10	0.04	1.00	0.12	3.66E-03	0.07	0.26	0.1
rs3821536	miR-544b	0.90	0.12	0.83	0.78	0.31	0.19	0.06	0.97
rs2243486	miR-6868-3p	0.08	0.98	0.19	0.26	0.62	0.46	0.2	0.92
rs10862209	miR-618	0.51	0.39	0.51	0.42	0.42	0.43	0.09	0.07
rs174561	miR-1908-5p	0.85	0.03	0.80	1.35E-03	0.89	**1.83E-11**	0.69	0.32
rs7263455	miR-4326	NA	0.21	0.09	NA	NA	0.24	0.42	0.89
rs745666	miR-3615	NA	0.77	0.47	NA	NA	0.90	0.12	0.22
rs2140551	miR-548ba	0.77	0.02	0.10	0.43	0.83	0.65	0.43	0.74
rs72641631	miR-1269a	0.79	0.80	0.67	0.88	0.51	0.44	0.81	0.08
rs942803	miR-1287-5p	0.89	0.56	0.33	0.33	0.31	0.03	0.84	0.74
rs1925342	miR-3117-3p	0.86	0.97	0.19	0.03	0.25	0.22	0.88	0.99
rs6971245	miR-4467	0.65	0.59	0.04	0.78	0.86	0.14	0.25	0.71
rs1561401	miR-4803	0.95	0.98	0.87	0.18	0.49	0.99	0.27	0.41
rs56103835	miR-323b-3p	0.77	0.11	0.99	0.01	0.90	0.41	0.16	0.58
rs2927318	miR-7854-3p	0.44	0.66	0.54	0.99	0.86	0.72	0.64	0.53
rs7006762	miR-4662a-5p	0.97	0.59	0.89	0.53	0.28	0.07	0.16	0.31
rs11653901	miR-548at-5p	0.28	6.72E-04	0.28	0.78	0.23	0.27	0.18	0.97
rs74236456	miR-3161	0.38	0.98	0.01	0.14	0.10	0.06	0.37	0.11
rs6717278	miR-3125	0.23	0.03	0.57	0.97	0.59	0.60	0.89	0.53

Table 4.3, pt. II – P-values of association between the ADHD (2019), SCZ (2022), ASD (2019), MDD (2019), Anorexia (2019), BD (2021), OCD (2018) and TS (2019) GWAS from the PGC used in this study and the SNPs (SNP ID) identified as miR-eQTLs in 2nd trimester foetal brain and their associated miRNAs.

SNP ID	miRNA	ADHD (2019)	SCZ (2022)	ASD (2019)	MDD (2019)	Anorexia (2019)	BD (2021)	OCD (2018)	TS (2019)
rs116698525	miR-3176	NA	0.04	0.58	6.23E-03	0.92	8.45E-03	0.29	0.18
rs28576121	miR-1270	0.21	0.55	0.15	0.43	NA	0.57	0.08	0.09
rs4680	miR-4761-3p	0.98	0.02	0.59	0.67	0.94	0.48	0.92	0.09
rs41275750	miR-641	0.05	0.90	0.16	0.31	0.36	0.38	0.25	0.65
rs709777	miR-4423-5p	0.04	0.82	0.27	0.51	0.86	0.59	0.09	0.89
rs1003723	miR-6886-5p	0.27	0.20	0.09	0.09	0.85	0.87	0.56	0.18
rs11101657	miR-202-5p	NA	0.14	NA	NA	NA	0.16	0.9	9.60E-03
rs112622797	miR-6840-5p	0.34	0.15	0.32	0.38	0.09	0.60	0.9	0.52
rs6788178	miR-6826-3p	0.09	2.51E-04	0.51	0.73	0.24	0.23	0.03	0.86
rs55852613	miR-3938	0.24	0.25	0.29	0.13	0.26	0.70	0.46	0.08

Table 4.4, pt. I – P-values of association of the SNPs found to be miR-eQTLs in 2nd trimester foetal brain in the Cross-disorder (2019), PTSD (2019), Anxiety (2019), Alcohol dependence (2018), Cannabis use disorder (2020), Opioid dependence (2020) GWAS from the PGC used in this study and the AD (2022) GWAS.

The AD (2019) GWAS from the PGC is not depicted as all but 3 SNPs were missing from the summary statistics. P-values rounded to 2 decimal places.

SNP ID	miRNA	Cross-disorder (2019)	PTSD (2019)	Anxiety (2016)	Panic Disorder (2019)	AD (2022)	Alcohol dependence (2018)	Cannabis use disorder (2020)	Opioid dependence (2020)
rs7769202	miR-5683	NA	0.33	0.12	0.05	0.14	0.85	0.90	NA
rs2273626	miR-4707-3p	0.05	0.52	0.17	0.30	0.03	0.03	0.59	NA
rs3821536	miR-544b	0.97	0.17	0.22	0.49	0.32	0.61	0.53	0.71
rs2243486	miR-6868-3p	0.60	0.29	0.42	0.04	0.74	0.64	0.78	NA
rs10862209	miR-618	0.23	0.23	0.07	0.09	0.25	0.70	0.69	NA
rs174561	miR-1908-5p	3.15E-04	0.78	0.48	0.06	0.67	0.17	0.45	0.78
rs7263455	miR-4326	NA	0.50	0.29	0.20	0.89	0.29	0.87	NA
rs745666	miR-3615	NA	0.15	0.13	0.32	0.85	0.78	0.97	NA
rs2140551	miR-548ba	3.35E-02	0.50	0.40	7.08E-03	0.22	0.70	0.08	0.09
rs72641631	miR-1269a	0.87	0.35	0.70	0.64	0.62	0.86	0.23	0.78
rs942803	miR-1287-5p	0.07	0.54	0.23	1.00	0.86	0.55	0.33	0.64
rs1925342	miR-3117-3p	0.43	0.47	0.84	0.78	0.27	0.39	0.77	0.77
rs6971245	miR-4467	0.18	0.71	0.75	0.52	0.86	0.20	0.65	NA
rs1561401	miR-4803	1.00	0.10	0.50	0.94	0.71	0.10	0.79	NA
rs56103835	miR-323b-3p	0.8	0.36	0.26	0.09	0.48	0.78	0.01	0.59
rs2927318	miR-7854-3p	0.45	0.22	0.88	0.39	0.60	0.63	0.27	NA
rs7006762	miR-4662a-5p	0.13	0.66	0.84	0.14	0.73	0.98	0.54	0.64
rs11653901	miR-548at-5p	0.05	0.09	0.62	0.20	0.66	0.88	0.95	0.42

Table 4.4, pt. II – P-values of association of the SNPs found to be miR-eQTLs in 2$_{nd}$ trimester foetal brain in the Cross-disorder (2019), PTSD (2019), Anxiety (2019), Alcohol dependence (2018), Cannabis use disorder (2020), Opioid dependence (2020) GWAS from the PGC used in this study and the AD (2022) GWAS.

SNP ID	miRNA	Cross-disorder (2019)	PTSD (2019)	Anxiety (2016)	Panic Disorder (2019)	AD (2022)	Alcohol dependence (2018)	Cannabis use disorder (2020)	Opioid dependence (2020)
rs74236456	miR-3161	0.07	0.54	0.90	0.78	0.56	0.65	0.61	NA
rs6717278	miR-3125	0.79	0.82	0.16	0.74	1.64E-03	0.56	0.88	0.47
rs116698525	miR-3176	NA	0.89	0.37	0.26	0.44	0.90	0.79	NA
rs28576121	miR-1270	0.26	0.20	0.76	0.40	0.79	0.65	0.72	NA
rs4680	miR-4761-3p	1.00	0.11	0.94	7.07E-04	0.95	0.61	0.14	0.93
rs41275750	miR-641	0.50	0.28	0.73	0.14	0.65	0.45	0.84	NA
rs709777	miR-4423-5p	0.27	0.21	0.93	0.12	0.84	0.36	0.46	0.57
rs1003723	miR-6886-5p	0.33	0.11	0.04	0.63	1.92E-02	0.97	0.43	0.86
rs11101657	miR-202-5p	NA	0.14	NA	0.14	0.85	0.15	NA	NA
rs112622797	miR-6840-5p	1.00	0.89	0.64	0.82	0.55	0.57	0.92	NA
rs6788178	miR-6826-3p	5.79E-04	0.11	0.26	0.92	8.30E-03	0.71	0.78	0.62
rs55852613	miR-3938	0.30	0.87	0.18	0.26	0.4112	0.84	0.94	0.86

As can be seen in Table 4.5, the association between miR-eQTL (for miR-1908-5p) rs174561 and bipolar disorder seems to be mostly driven by an association with bipolar disorder type I ($p = 1.28 \times 10^{-8}$), where the C – allele of rs174561 is the risk allele for BD. There is suggestive evidence that rs174561 is also associated with BD type II (p =0.01), but this analysis lacks power due to the limited sample size of BD type II cases (n = 6154).

Table 4.5 - P-values of association of SNP rs174561 and BD subtypes in the BD GWAS of the PGC3.

Chr - chromosome, A1 – effect allele, A2 – non-effect allele, BETA – Direction of effect, SE- Standard error, NCAS - number of cases, NCON - number of controls.
In bold – associations with $p < 5 \times 10^{-5}$. P-values rounded to 2 decimal places.

SNP ID	Chr	A1	A2	BD subtypes	BETA	SE	NCAS	NCON	P-value
rs174561	11	T	C	BD (I + II)	-0.07	0.01	41917	371549	**1.83E-11**
				BD type I	-0.07	0.01	23842	163521	**1.28E-08**
				BD type II	-0.05	0.02	6154	81091	0.01

To check if any of the miR-eQTLs I identified were associated with brain and cognitive endophenotypes, I screened GWAS summary statistics from the CNCR - CTG Lab performed on UK Biobank samples, which encompass brain volume, intelligence, sensitivity to environmental stress and adversity (SESA), neuroticism, neuroticism subclusters and depression, and insomnia.

As can be seen in Table 4.6, I found 2 miR-eQTLs, which have suggestive associations with brain volume - rs2273626 on chromosome 14 ($p = 1.06 \times 10^{-5}$), which is also associated with miR-4707-3p expression, and rs112622797 on chromosome 7 ($p = 4.59 \times 10^{-5}$), which is also associated with miR-6840-5p expression. Interestingly, SNP rs174561, which is

associated with miR-1908-5p expression and shown to be associated with bipolar disorder (Table 4.4), is also associated with intelligence ($p = 3.49 \times 10^{-5}$) (Table 4.6), sleep duration ($p = 8.69 \times 10^{-7}$) (Table 4.7) and the irritability (IRR) component of neuroticism ($p = 1.29 \times 10^{-8}$) (Table 4.8).

Table 4.6, pt. I - P-values of association of the SNPs that were found to be miR-eQTLs in 2nd trimester foetal brain in the Brain Volume GWAS (2020), Intelligence (2018) GWAS, SESA (2020) GWAS and Neuroticism, Neuroticism subclusters (worry and depressed affect) and depression (2018b) GWAS of the CNCR – CTG group.

In bold – associations with $p < 5 \times 10^{-5}$. P-values rounded to 2 decimal places.

SNP ID	miRNA	Brain volume (2020)	Intelligence (2018)	SESA (2020)	Neuroticism subclusters and depression Nagel (2018b)			
					Depression	Depressed affect	Neuroticism	Worry
rs7769202	miR-5683	0.44	2.75E-03	0.34	0.13	0.46	0.27	0.38
rs2273626	miR-4707-3p	**1.06E-05**	0.53	0.38	0.81	0.61	0.42	0.04
rs3821536	miR-544b	0.29	0.02	0.02	0.91	0.96	0.23	0.78
rs2243486	miR-6868-3p	0.85	0.28	0.60	0.20	0.16	0.26	0.87
rs10862209	miR-618	0.76	0.12	0.35	0.64	0.74	0.50	0.22
rs174561	miR-1908-5p	0.05	**3.49E-05**	0.32	0.29	0.06	0.28	0.97
rs2140551	miR-548ba	0.48	0.63	0.84	0.74	0.60	0.23	0.09
rs72641631	miR-1269a	0.79	0.12	5.73E-03	0.71	0.92	0.61	0.59
rs942803	miR-1287-5p	0.88	0.36	0.37	0.44	0.33	0.37	0.30
rs1925342	miR-3117-3p	0.82	0.48	0.13	0.50	0.82	0.19	0.08
rs6971245	miR-4467	0.05	0.13	0.40	0.45	0.86	0.38	0.34
rs1561401	miR-4803	0.06	NA	0.98	0.33	0.91	0.65	0.18
rs56103835	miR-323b-3p	0.80	0.82	0.16	8.67E-03	0.02	0.07	0.18
rs2927318	miR-7854-3p	0.39	0.66	0.03	0.02	0.03	0.02	0.47
rs7006762	miR-4662a-5p	0.34	0.58	0.89	0.66	0.64	0.98	0.32
rs11653901	miR-548at-5p	0.87	0.23	0.59	0.99	0.02	0.43	0.07
rs74236456	miR-3161	0.62	0.02	0.82	0.60	0.12	0.64	0.73
rs6717278	miR-3125	0.33	0.10	0.78	0.86	0.14	0.29	0.19

Table 4.6, pt. II- P-values of association of the SNPs that were found to be miR-eQTLs in 2nd trimester foetal brain in the Brain Volume GWAS (2020), Intelligence (2018) GWAS, SESA (2020) GWAS and Neuroticism, Neuroticism subclusters (worry and depressed affect) and depression (2018b) GWAS of the CNCR – CTG group.

SNP ID	miRNA	Brain volume (2020)	Intelligence (2018)	SESA (2020)	Neuroticism subclusters and depression Nagel (2018b)			
					Depression	Depressed affect	Neuroticism	Worry
rs116698525	miR-3176	0.62	0.37	0.09	0.62	0.81	0.46	0.88
rs28576121	miR-1270	0.40	0.13	0.40	0.89	0.52	0.17	0.34
rs4680	miR-4761-3p	0.02	0.03	0.11	0.22	0.47	0.07	1.15E-03
rs41275750	miR-641	0.95	0.80	0.30	0.24	0.17	0.10	0.24
rs709777	miR-4423-5p	0.56	0.53	0.95	0.99	0.27	0.16	0.19
rs1003723	miR-6886-5p	0.58	0.52	0.67	0.67	0.72	0.71	0.01
rs11101657	miR-202-5p	0.95	0.04	0.30	0.70	0.35	0.12	0.06
rs112622797	miR-6840-5p	**4.59E-05**	0.03	0.60	0.18	0.68	0.68	0.93
rs6788178	miR-6826-3p	0.34	0.08	0.11	0.47	0.06	0.16	0.45
rs55852613	miR-3938	0.77	0.76	0.59	0.97	0.70	0.66	0.42

Table 4.7, pt. I - P-values of association of the SNPs that were found to be miR-eQTLs in 2nd trimester foetal brain with different sleep-related traits of the Insomnia GWAS (Jansen et al., 2019) from the CNCR – CTG lab.

In bold – associations with $p < 5 \times 10^{-5}$. P-values rounded to 2 decimal places.

SNP ID	miRNA	Insomnia (Jansen et al., 2019)						
		Insomnia	Napping	Getting - up	Dozing	Morningness	Snoring	Sleep duration
rs7769202	miR-5683	0.01	0.80	0.10	0.78	0.63	0.61	0.55
rs2273626	miR-4707-3p	0.99	0.80	0.16	0.18	0.04	0.41	0.52
rs3821536	miR-544b	0.66	0.47	0.13	0.25	0.47	0.09	0.18
rs2243486	miR-6868-3p	0.22	0.19	0.41	0.76	0.77	0.89	0.01
rs10862209	miR-618	0.47	0.56	0.45	0.12	0.52	0.79	0.38
rs174561	miR-1908-5p	0.11	0.02	1.76E-04	0.78	9.57E-03	0.31	**8.69E-07**
rs2140551	miR-548ba	0.10	3.72E-03	8.82E-05	0.97	2.39E-04	0.03	0.12
rs72641631	miR-1269a	0.76	0.25	0.17	0.01	0.02	0.94	0.06
rs942803	miR-1287-5p	0.77	0.10	0.75	0.56	0.38	0.15	0.21
rs1925342	miR-3117-3p	0.30	1.00	0.47	0.87	0.96	0.28	1.19E-03
rs6971245	miR-4467	0.34	0.62	0.25	0.25	3.31E-03	0.40	0.27
rs1561401	miR-4803	0.79	0.79	0.87	0.83	0.26	0.12	0.54
rs56103835	miR-323b-3p	0.35	0.40	0.21	0.42	0.47	0.76	0.39
rs2927318	miR-7854-3p	0.78	0.78	1.83E-03	0.56	0.91	0.78	0.78
rs7006762	miR-4662a-5p	0.58	0.09	0.07	0.62	0.13	0.62	0.01
rs11653901	miR-548at-5p	0.10	0.32	0.23	0.87	0.47	5.52E-03	0.66
rs74236456	miR-3161	0.11	0.10	6.53E-03	0.55	0.11	0.86	0.75
rs6717278	miR-3125	0.02	0.82	0.47	0.14	0.74	0.45	0.68

Table 4.7, pt. II - P-values of association of the SNPs that were found to be miR-eQTLs in 2nd trimester foetal brain with different sleep-related traits of the Insomnia GWAS (Jansen et al., 2019) from the CNCR – CTG lab.

SNP ID	miRNA	Insomnia (Jansen, 2019)						
		Insomnia	Napping	Getting - up	Dozing	Morningness	Snoring	Sleep duration
rs116698525	miR-3176	0.72	0.89	0.76	0.37	0.47	0.53	0.90
rs28576121	miR-1270	0.22	0.70	0.62	0.53	0.41	0.14	0.13
rs4680	miR-4761-3p	0.68	0.04	0.01	0.27	1.00	0.20	9.51E-03
rs41275750	miR-641	0.30	0.70	0.49	1.00	0.60	0.24	0.50
rs709777	miR-4423-5p	0.19	0.97	0.41	0.34	0.10	0.47	0.46
rs1003723	miR-6886-5p	0.83	0.96	0.66	0.31	0.90	0.47	0.27
rs11101657	miR-202-5p	0.68	0.23	0.40	0.72	0.77	0.87	0.77
rs112622797	miR-6840-5p	0.57	0.50	0.40	0.73	0.15	6.28E-03	0.07
rs6788178	miR-6826-3p	0.89	0.64	0.34	0.32	0.37	0.64	0.33
rs55852613	miR-3938	0.20	0.80	0.47	0.42	0.63	0.94	0.01

Table 4.8, pt. I - P-values of association of the SNPs that were found to be miR-eQTLs in 2nd trimester foetal brain with components of item level analysis of Neuroticism GWAS (Nagel et al., 2018a) from the CNCR – CTG lab.

In bold – associations with $p < 5 \times 10^{-5}$. SUM – Neuroticism sum score, MOOD – Mood swings, MIS – feeling miserable, IRR - Irritability, HURT – sensitivity /hurt feelings, FED-UP - fed-up feelings, NERV-FEEL – Nervous feelings, WORRY – Anxious, TENSE – Tense/ highly strung, WORR-EMB –Worried too long after embarrassment, SUF-NERV – suffering from nerves, LONE – loneliness, GUILT – guilty feelings. P-values rounded to 2 decimal places.

SNP ID	Item-levels of Neuroticism (Nagel et al., 2018a)												
	SUM	MOOD	MIS	IRR	HURT	FED-UP	NERV-FEEL	WORRY	TENSE	WORR-EMB	SUF-NERV	LONE	GUILT
rs7769202	0.49	0.66	0.78	0.15	0.50	0.47	0.36	0.98	0.38	0.19	0.87	0.47	0.02
rs2273626	0.39	0.36	0.84	0.19	0.30	0.83	0.02	0.58	0.44	0.65	0.01	0.84	0.67
rs3821536	0.51	0.81	0.99	0.94	0.16	0.67	0.54	0.43	0.52	0.01	0.29	0.60	0.39
rs2243486	0.24	0.19	0.04	0.03	0.76	0.65	0.92	0.74	0.51	0.42	0.46	0.32	0.85
rs10862209	0.65	0.81	0.78	0.13	0.74	0.50	0.21	0.92	0.86	0.42	0.03	0.75	0.42
rs174561	7	0.22	3.08E-04	**6.20E-08**	0.39	0.20	0.98	0.67	0.35	0.06	0.37	0.19	0.72
rs2140551	0.08	0.28	0.99	0.04	0.28	0.09	0.08	4.38E-03	0.21	0.42	0.30	0.61	0.51
rs72641631	0.69	0.91	0.99	0.26	0.01	0.90	0.47	0.99	0.08	2.47E-03	0.67	0.13	0.62
rs942803	0.25	0.18	0.30	0.83	0.62	0.74	0.41	0.45	0.32	0.15	0.80	0.84	0.71
rs1925342	0.10	0.80	0.95	0.38	0.51	0.97	0.18	0.04	0.27	0.01	0.19	0.60	0.32
rs6971245	0.48	0.86	0.16	0.64	0.77	0.41	0.45	0.10	0.82	0.73	0.13	0.98	0.13
rs1561401	0.62	0.23	0.80	0.85	0.25	0.96	0.43	0.61	0.13	0.41	0.49	0.94	0.37
rs56103835	0.07	0.68	2.45E-03	0.50	0.17	0.21	0.67	0.24	0.31	0.36	0.22	0.01	0.53
rs2927318	0.01	0.01	0.24	0.21	0.28	0.02	0.16	0.64	0.93	0.02	0.32	0.10	0.02
rs7006762	0.86	0.58	1.00	0.15	0.70	0.41	0.73	0.10	0.19	0.61	0.96	0.12	0.86

Table 4.8, pt. II - P-values of association of the SNPs that were found to be miR-eQTLs in 2nd trimester foetal brain with components of item level analysis of Neuroticism GWAS (Nagel et al., 2018a) from the CNCR – CTG lab.

SNP ID	SUM	MOOD	MIS	IRR	HURT	FED-UP	NERV-FEEL	WORRY	TENSE	WORR-EMB	SUF-NERV	LONE	GUILT
rs11653901	0.40	0.01	0.41	0.09	0.24	0.04	0.02	0.79	0.07	0.81	0.20	0.04	0.56
rs74236456	0.75	0.34	0.26	0.82	0.27	0.02	0.75	0.17	0.08	0.87	0.21	0.49	0.38
rs6717278	0.49	0.02	0.08	0.77	0.54	0.66	0.34	0.47	0.16	0.35	0.36	0.22	0.76
rs116698525	0.50	0.19	0.56	0.09	0.02	0.82	0.39	0.90	0.49	0.05	0.38	0.96	0.15
rs28576121	0.16	0.11	0.87	0.05	0.72	0.92	0.19	0.96	0.07	0.45	0.13	0.63	0.94
rs4680	0.05	0.60	0.08	0.27	0.09	0.39	0.00	0.01	0.11	0.20	0.28	0.41	0.38
rs41275750	0.10	0.06	0.94	0.12	0.87	0.06	0.24	0.18	0.27	0.64	0.95	0.85	0.07
rs709777	0.32	0.65	0.02	0.76	0.86	0.60	0.77	0.39	0.21	0.84	0.31	0.86	0.82
rs1003723	0.59	0.91	0.91	0.06	0.80	0.38	0.01	0.12	0.46	0.72	0.01	0.68	0.96
rs11101657	0.10	0.11	0.39	0.78	0.80	0.72	0.19	0.03	0.02	0.69	0.22	0.82	0.02
rs112622797	0.80	0.96	0.06	0.85	0.05	0.07	0.75	0.16	0.81	0.30	0.38	0.32	0.02
rs6788178	0.20	0.60	0.07	0.12	0.34	0.01	0.41	0.79	0.17	0.10	0.32	0.27	0.34
rs55852613	0.50	0.90	0.48	0.41	0.73	0.69	0.35	0.39	0.58	0.16	0.38	0.18	0.47

In order to test for the presence of pleiotropic effects of the miR-eQTLs associated with neuropsychiatric disorders and brain / cognitive endophenotypes, I further performed a Phenome-Wide Association study (PheWas) nominal scan of SNPs rs174561, rs112622797 and rs2273626 on an Atlas of GWAS summary statistics from the CNCR-CTG lab involving 600 GWAS performed on UK Biobank samples (Watanabe et al., 2019).

As can be seen in Table 4.9 and Figure 4.1, rs174561, which is associated with both miR-1908-5p expression and bipolar disorder, is also associated with cognitive endophenotypes, such as cognitive performance ($p = 7.84 \times 10^{-6}$) and verbal numerical reasoning ($p = 2.08 \times 10^{-5}$). This SNP has also been associated with sleep duration in multiple studies and is also associated with daytime napping ($p = 2.55 \times 10^{-11}$). Moreover, rs174561 has been associated with irritability in another GWAS ($p = 2.26 \times 10^{-7}$) and is also associated with depressive symptoms in two studies. SNP rs112622797, which is associated with miR-6840-5p expression and brain volume, is also associated with educational attainment ($p = 1.27 \times 10^{-5}$) and alcohol intake frequency ($p = 2.19 \times 10^{-5}$), whereas SNP rs2273626, which is associated with brain volume and miR-4707-3p expression is significantly associated with educational attainment ($p = 2.66 \times 10^{-10}$).

Table 4.9 - P-values of cognitive, psychiatric and brain related PheWas of the miR-eQTLs rs174561- associated with Bipolar Disorder, Intelligence, Sleep duration and Neuroticsm (IRR), and miR-eQTLs rs112622797 and rs2273626 associated with Brain Volume.

A1 – effect allele, A2 – non-effect allele, PMID - Pubmed ID number, N - number of samples analysed. In bold – GWAS summary stats employed in this study.

SNP ID	Domain	Trait	P-value	A1	A2	PMID	Year	N
rs174561	Cognitive	Cognitive performance	7.84E-06	C	T	30038396	2018	257828
rs174561	**Cognitive**	**Intelligence**	**3.49E-05**	**C**	**T**	**29942086**	**2018**	**269867**
rs174561	Cognitive	Verbal-numerical reasoning	2.08E-05	NA	NA	29844566	2018	168033
rs174561	Psychiatric	Depressive symptoms (MA GWAMA)	1.49E-06	T	C	30643256	2019	1067913
rs174561	Psychiatric	Depressive symptoms (univariate)	6.16E-08	C	T	30643256	2019	1067913
rs174561	Psychiatric	Irritability	2.26E-07	C	T	31427789	2019	369232
rs174561	**Psychiatric**	**Irritability (IRR)**	**6.20E-08**	**C**	**T**	**29500382**	**2018**	**260369**
rs174561	Psychiatric	Nap during day	2.55E-11	C	T	31427789	2019	386124
rs174561	Psychiatric	Sleep duration	4.25E-07	C	T	31427789	2019	384225
rs174561	Psychiatric	Sleep duration	4.10E-08	C	T	30846698	2019	446118
rs174561	**Psychiatric**	**Sleep duration**	**8.69E-07**	**C**	**T**	**30804565**	**2019**	**384317**
rs112622797	Environment	Educational attainment	1.27E-05	G	A	27225129	2016	328917
rs112622797	Neurological	Total brain volume	1.21E-05	G	A	31676860	2019	19629
rs112622797	Psychiatric	Alcohol intake frequency	2.188E-05	G	A	31427789	2019	386082
rs2273626	Environment	Educational attainment	2.66E-10	A	C	30038396	2018	328917

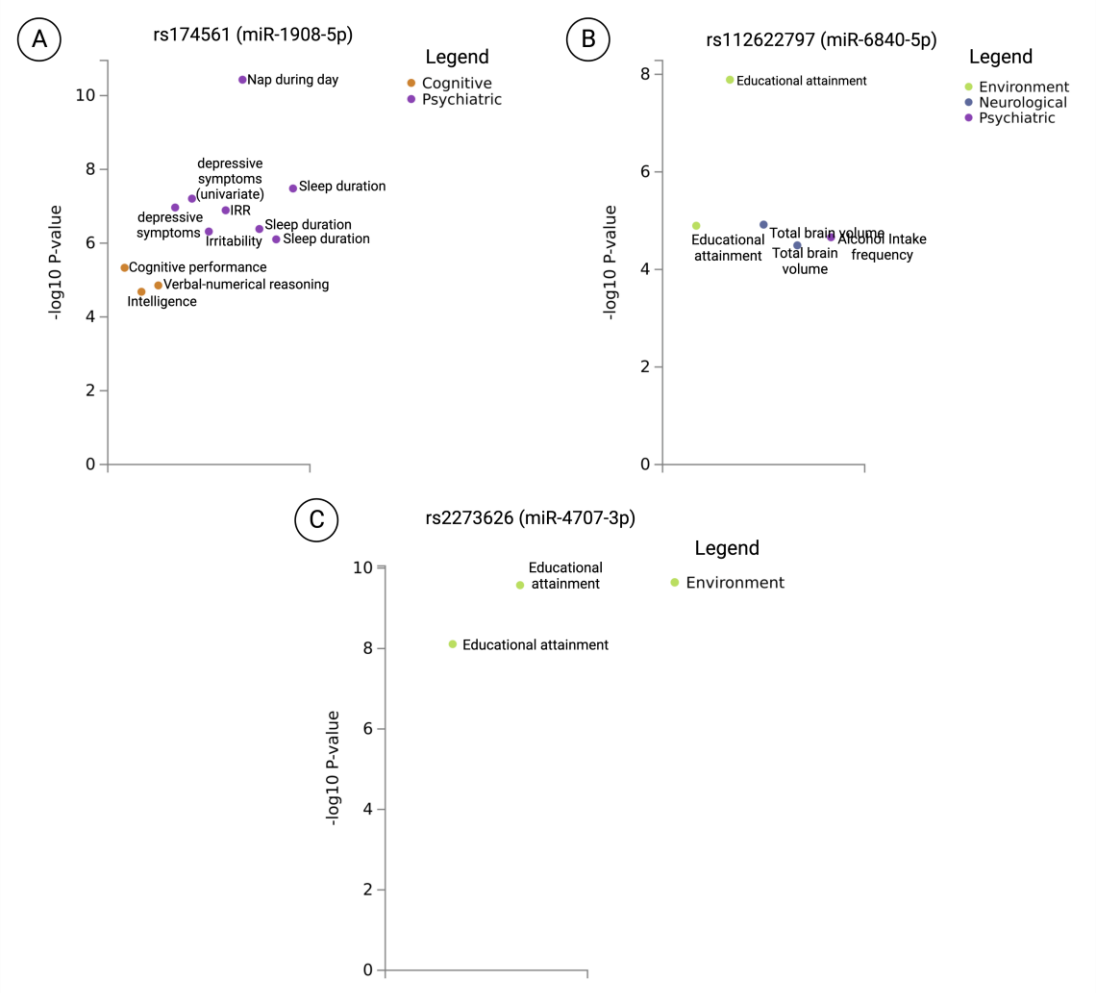


Figure 4.2 – Cognitive, psychiatric and educational attainment PheWas plot of miR-eQTLs under analysis.

A – miR-eQTL rs174561 associated with miR-1908-5p expression. **B -** mir-eQTL rs112622797 associated with miR-6840-5p expression. **C –** miR-eQTL rs2273626 associated with miR-4707-3p. Phewas plots created with GWAS Atlas database https://atlas.ctglab.nl/PheWAS (Watanabe et al., 2019).

These results suggest that these miR-eQTLs are associated with multiple psychiatric, cognitive and brain phenotypes. Of note, SNP rs174561 is associated with bipolar disorder and several other traits, such as intelligence and cognitive abilities, neuroticism and sleep variables. As can be seen in

Table 4.10, the C allele is associated with a higher risk for BD, higher intelligence, higher irritability and longer sleep duration.

Table 4.10 – Summary of association of SNP rs174561 with multiple BD and BD - associated trait GWAS.

A1 – effect allele, A2 – non-effect allele, BETA – direction of effect, SE – standard error (SE), p-value – p-value of association and N – number of samples analysed.

GWAS	A1	A2	BETA	SE	p-value	N
Bipolar disorder	T	C	-0.0674	0.01	1.83E-11	413466
Neuroticism - IRR	T	C	-0.0138	0.00255	6.20E-08	365644
Intelligence	T	C	-0.0124	0.00299	3.49E-05	266038
Sleep duration	C	T	0.01303	0.00265	8.69E-07	383641

This SNP is located within the pri-miRNA hairpin sequence of miR-1908 and the C-allele is associated with a significant decrease in minimum free energy (ΔG= - 3.20 kcal/mol) predicted by *RNAfold* (Figure 4.2); this suggests that the C-allele of rs174561 makes the miR-1908 pri-miRNA more stable, which is associated with an increase in mature miR-1908-5p expression.

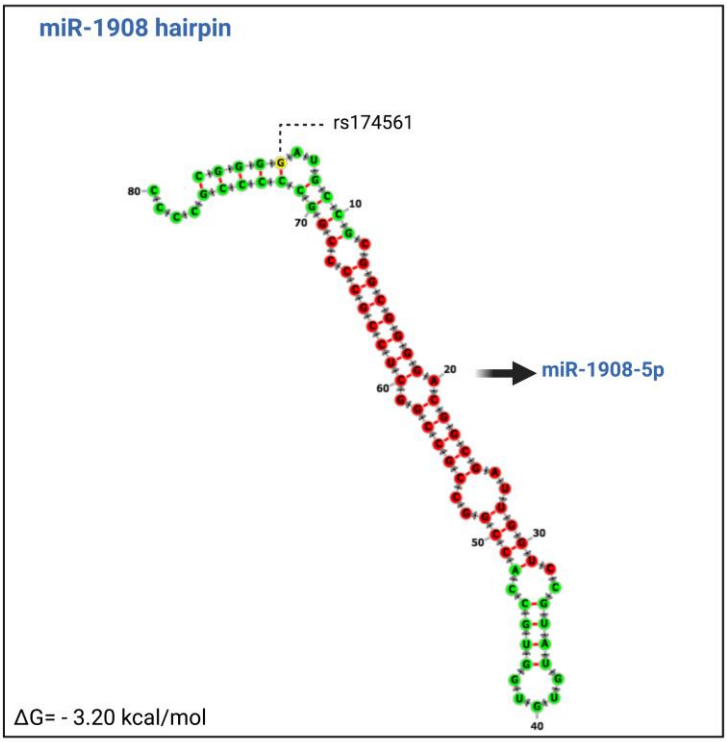


Figure 4.3 - Location of miR-eQTL rs174561 in the context of the 3D structure of miRNA hairpin miR-1908 identified in this study.

Red – mature miRNA bases; Green – pri-miRNA bases not in mature miRNAs; Yellow – miR-eQTL position. The miR-1908 hairpin can give rise to 2 mature miRNAs: miR-1908-5p identified in this study and miR-1908-3p. SNP rs174561 (T > C) is located in position 5 of the miR-1908 hairpin and the C-allele is associated with a decrease in minimum free energy ΔG = -3.20 Kcal/mol.

As can be seen in Figure 4.3, the effects of rs174561 genotype on mature miR-1908-5p expression in 2nd trimester foetal brain from my eQTL analysis support this prediction, as the C allele of rs174561 is associated with higher miR-1908-5p expression.

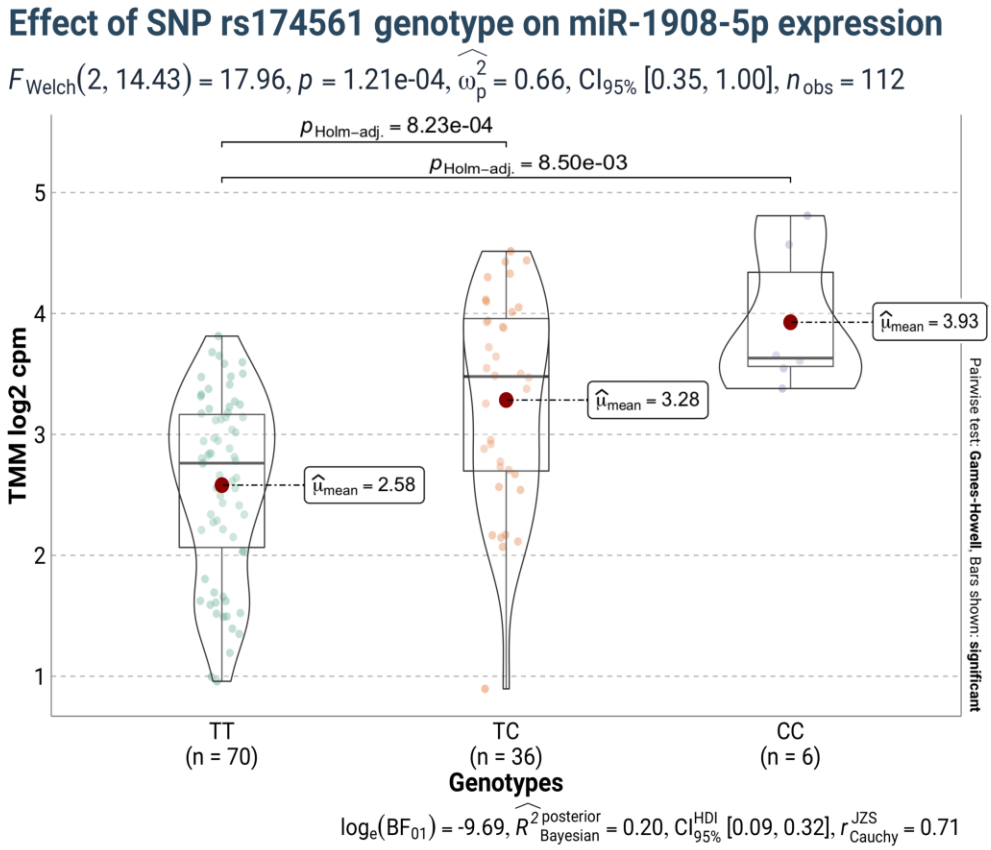


Figure 4.4 - Genotypic associations of rs174561 with miR-1908-5p expression.

The x-axis corresponds to the 3 observed SNP genotypes. The reference allele (T) is on the left-hand side and alternative allele (C) is on the right-hand side. The y-axis represents log_2-CPM TMM normalized miRNA expression values. The sample size (n) for each genotype is given below the respective alleles. All p-values are adjusted by the Holm method. Only statistically significant associations are shown.

A Welch's ANOVA test revealed that the C- allele of rs174561 is significantly associated with increased miR-1908-5p expression in 2nd trimester foetal brain ($p = 1.21 \times 10^{-4}$) with a 0.7 log_2FC in miR-1908-5p expression between homozygotes for the reference allele (TT) and heterozygotes (TC) and a 1.35 log_2FC in miR-1908-5p expression between homozygotes for the reference allele (TT) and homozygotes for the alternative allele (CC). rs173561 genotype explains 66 % of the variability in

mature miR-1905-5p expression within the 112 foetal brain samples analyzed ($\omega^2p = 0.66$), which is considered a large effect size (Cohen J., 1988; Field, AP., 2013).

A SNP can be associated with multiple traits either because it influences those traits through causality / pleiotropy or because of linkage disequilibrium with a causative variant. I performed summary data-based Mendelian randomization (SMR) and HEIDI analysis to ascertain if changes in miRNA expression associated with SNPs rs174561, rs112622797 and rs2273626 in 2nd trimester foetal brain could mediate genetic risk for these conditions, by testing if the association between miR-eQTL rs174561 and BD, intelligence, sleep duration and irritability and miR-eQTLs - rs112622797 and rs2273626 and brain volume arises due to pleiotropic effects due to a shared and potentially causal variant at these loci or linkage.

4.3.2 SMR and HEIDI analysis of 2nd trimester foetal brain miR-eQTLs and neuropsychiatric disorders/brain endophenotypes:

I only performed SMR analysis for traits with which miR-eQTLs were found to be associated (at $p < 5 \times 10^{-5}$) in the PGC / CNCR-CTG GWAS. As can be seen in Table 4.11, increased expression of miR-1908-5p associated with the C allele of rs174561 is pleiotropically associated with increased risk for bipolar disorder (P-SMR = 5.78×10^{-7}) (Figure 4.5), increased irritability (P – SMR = 1.19×10^{-5}), and increased sleep duration (P-SMR = 4.02×10^{-5}) (Figure 4.6), which survive Bonferroni correction for 690 tests (30 miR-eQTLs and 23 GWAS screened), as well as increased intelligence (P – SMR = 2.97×10^{-4}), which does not survive Bonferroni correction for 690 tests (Figure 4.8). With regards to brain volume, I found 2 miRNAs that were pleiotropically and potentially causally associated with Brain volume. The A-allele of

rs112622797 was associated with higher miR-6840-5p expression and decreased brain volume (P-SMR= 1.84 x 10^{-3}). The G-allele of rs12880925 was associated with lower expression of miR-4707-3p and an increase in brain volume (P-SMR = 2.54 x 10^{-4}). Neither of these survived Bonferroni correction for 690 tests.

Table 4.11 – SMR and HEIDI analysis of miR-eQTL rs174561 affecting miR-1908-5p expression in Bipolar (type I + type II), Irritability (Neuroticism), Sleep duration and Intelligence GWAS and of miR-eQTLs rs112622797 affecting miR-6840-5p expression and rs12880925 affecting miR-4707-3p expression in the Brain volume GWAS meta-analysis.

GWAS – trait analysed, top SNP – miR-eQTL SNP ID, Chr – SNP chromosome, A1 – the effect (coded) allele, A2 – the other allele, Freq – frequency of A1 allele (estimated from the 1000G reference samples), $BETA_{GWAS}$ – effect size from GWAS, SE_{GWAS} – standard error from GWAS, P_{GWAS} – p-value from GWAS, $BETA_{eQTL}$ – effect size from eQTL study, SE_{eQTL} – standard error from eQTL study, P_{eQTL} – p-value from eQTL study, $BETA_{SMR}$ – effect size from SMR analysis , SE_{SMR} – standard error from SMR analysis , P_{SMR} – p-value from SMR analysis, $P_{SMR\ Bonferroni}$ – Bonferroni corrected $P_{SMR,}$ P_{HEIDI} – p-value from HEIDI test, $N_{SNPS\ HEIDI}$ – number of SNPs used in the HEIDI test.

GWAS	top SNP	Chr	A1	A2	Freq	BETA GWAS	SE GWAS	P_{GWAS}	BETA eQTL	SE eQTL	P_{eQTL}	BETA SMR	SE SMR	P_{SMR}	P_{SMR} Bonferroni	P_{HEIDI}	N_{SNPs} HEIDI
Bipolar (I + II)	rs174561	11	C	T	0.31	0.067	0.01	1.83E-11	1.056	0.14	9.26E-14	0.064	0.013	5.78E-07	3.99E-04	8.45E-02	20
Irritability	rs174561	11	C	T	0.31	0.014	2.55E-03	6.20E-08	1.056	0.14	9.26E-14	0.013	0.003	1.19E-05	0.00821	1.89E-01	20
Sleep duration	rs174561	11	C	T	0.31	0.013	2.65E-03	8.69E-07	1.056	0.14	9.26E-14	0.012	0.003	4.02E-05	0.0277	5.58E-01	20
Intelligence	rs174561	11	C	T	0.31	0.012	2.99E-03	3.49E-05	1.056	0.14	9.26E-14	0.012	0.003	2.97E-04	0.205	3.60E-02	20
Brain Volume	rs112622797	7	A	G	0.18	-0.032	7.66E-03	3.23E-05	0.856	0.18	2.52E-06	-0.037	0.012	1.84E-03	1.00	5.82E-01	20
Brain Volume	rs12880925	14	G	A	0.53	0.031	7.68E-03	6.05E-05	-1.023	0.11	4.92E-19	-0.030	0.008	2.54E-04	0.175	1.84E-01	20

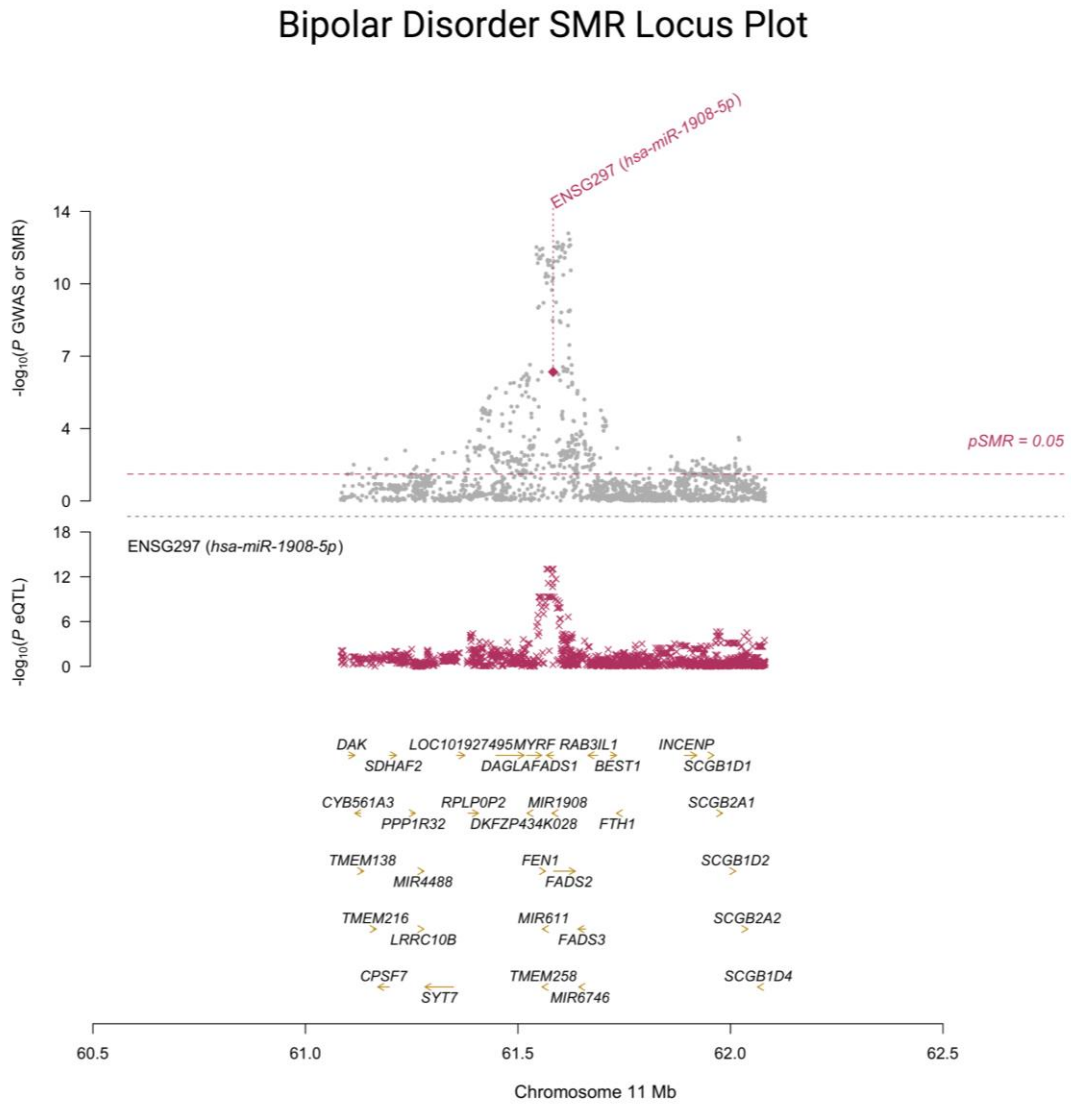


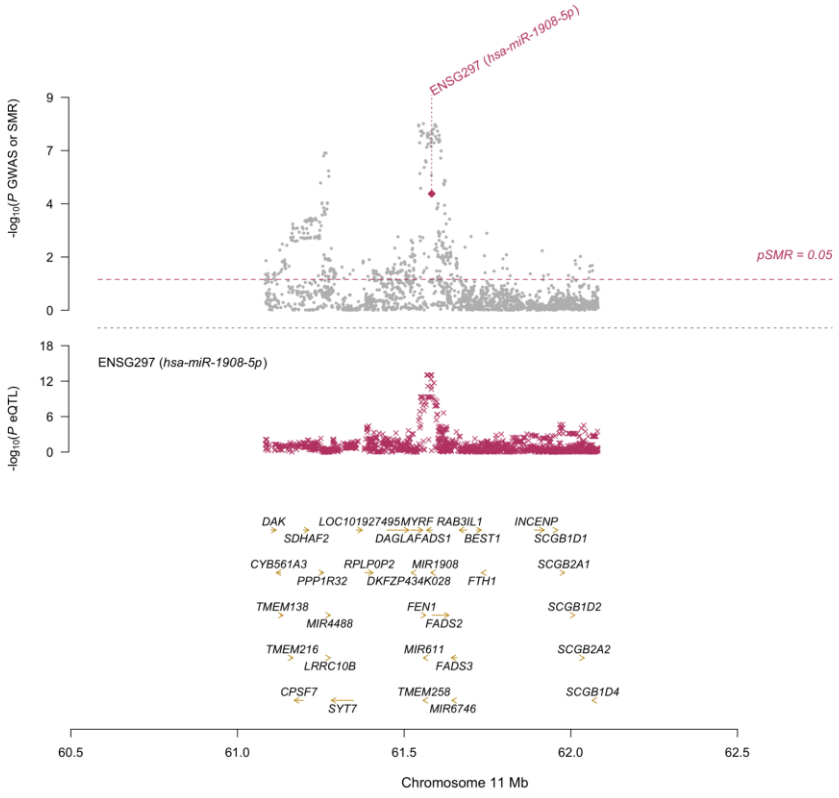
Figure 4.5 – SMR Locus plot for rs174561 in bipolar disorder (combined) in a ± 500kb window of miR-1908-5p. The top plot constitutes the GWAS layer, where the grey dots represent P-values for all the SNPs associated with Bipolar Disorder (all samples) reported in Mullins et al., (2021) GWAS.

The red colour represents the p-value for the probe that passed the SMR threshold of significance (hsa-miR-1908-5p); the solid diamond indicates that this probe passed the HEIDI threshold (P > 0.05). The middle plot constitutes the eQTL layer where the red crosses represent p-values of the associations of SNPs with miR-1908-5p expression in a ± 500kb window in 2nd trimester foetal brain. The bottom plot shows the location of genes in the locus. Gene positions are in hg19.

As shown in Figure 4.5, miR-1908-5p passed the HEIDI test for BD, which signifies I cannot reject the null hypothesis that there is a single causal variant affecting both miR-1908-5p expression and BD risk. As such, miR-1908-5p should be prioritized in future functional studies.

A

Neuroticism IRR SMR Locus Plot



B

Sleep duration SMR Locus Plot

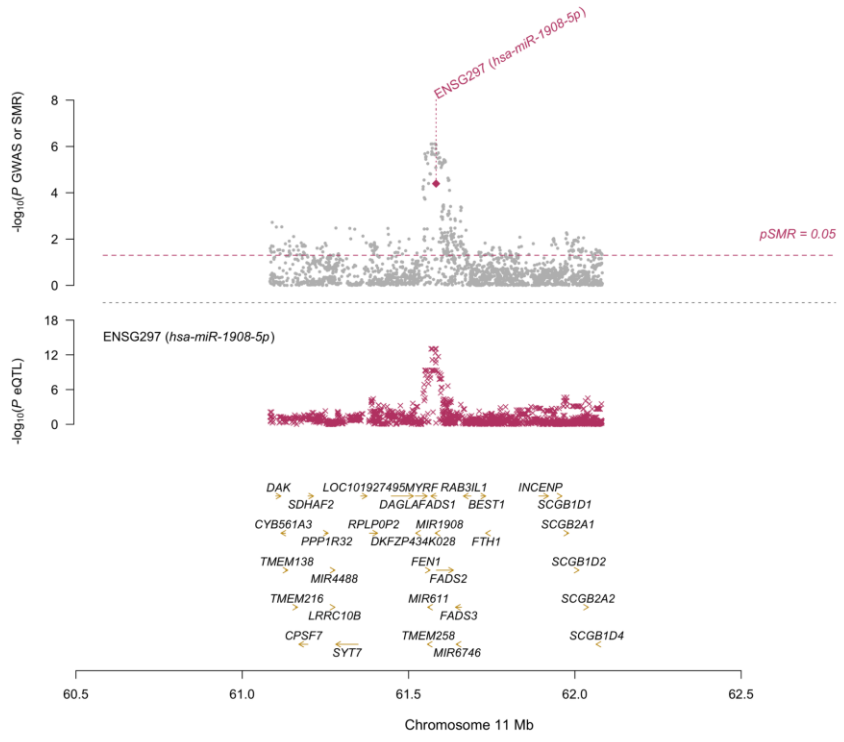


Figure 4.6 – SMR Locus plot for rs174561 in a ± 500kb window of miR-1908-5p in irritability and sleep duration.

A – In neuroticism (IRR). The top plot constitutes the GWAS layer, where the grey dots represent P-values for all the SNPs associated with the Irritability component (IRR) of the item level analysis of neuroticism GWAS reported by Nagel et al., (2018a). The red colour represents the p-value for the probe that passed the SMR threshold of significance (hsa - miR-1908-5p); the solid diamond indicates that this probe passed the HEIDI threshold (P > 0.05). The middle plot constitutes the eQTL layer where the red crosses represent p-values of the associations of SNPs with miR-1908-5p expression in a $\pm$ 500kb window in 2nd trimester foetal brain. The bottom plot shows the location of genes in the locus. Gene positions are in hg19. **B –** In sleep duration. The top plot constitutes the GWAS layer, where the grey dots represent P-values for all the SNPs associated with Sleep duration in the Insomnia GWAS reported by Jansen et al., (2019); The red colour represents the p-value for the probe that passed the SMR threshold of significance (hsa-miR-1908-5p); The solid diamond indicates that this probe passed the HEIDI threshold (P > 0.05). The middle plot constitutes the eQTL layer where the red crosses represent p-values of the associations of SNPs with miR-1908-5p expression in a $\pm$ 500kb window in 2nd trimester foetal brain. The bottom plot shows the location of genes in the locus. Gene positions are in hg19.

As seen in Figure 4.6, miR-1908-5p also passed the HEIDI test in irritability and sleep duration, suggesting pleiotropic effects affecting not only BD risk but also these BD-related traits likely via a shared molecular mechanism. In contrast with other traits associated with miR-1908-5p expression, the association between expression of miR-1908-5p and increased intelligence seems to be driven by linkage between the eQTL and independent risk variants, given the HEIDI (heterogeneity in dependent instruments) test is significant ($p < 0.05$) (Figure 4.7). These results suggest that miR-1908-5p is pleiotropically associated with bipolar disorder risk and bipolar-associated traits (irritability and sleep duration). Moreover, this suggests miR-1908-5p is a relevant gene underlying the GWAS hit and that the genetic factors mediating this association and influencing bipolar disorder risk could exert their effects during 2nd trimester foetal brain development.

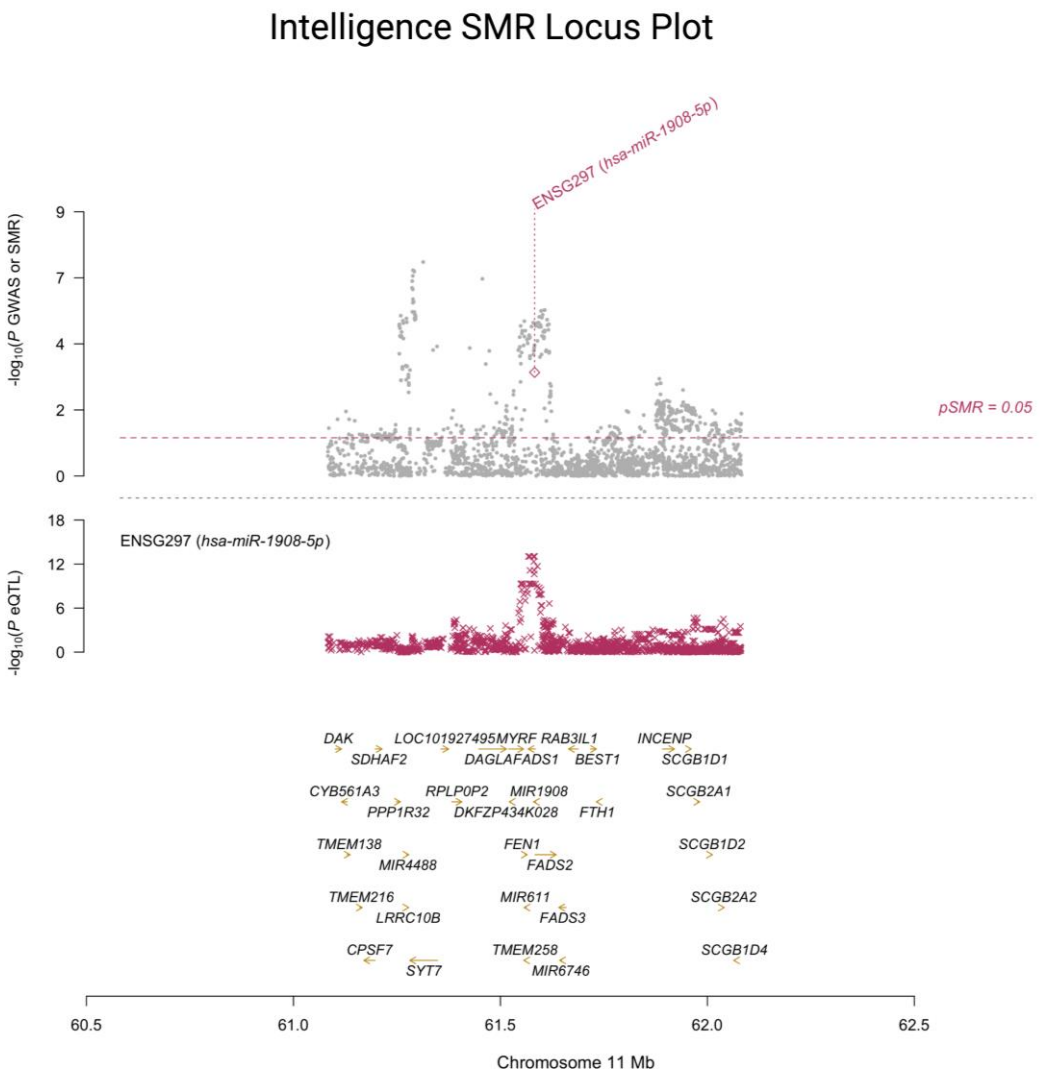


Figure 4.7 – SMR Locus plot for rs174561 in a ±500kb window of miR-1908-5p in intelligence.

The top plot constitutes the GWAS layer, where the grey dots represent P-values for all the SNPs associated with the intelligence metanalysis GWAS reported by Savage *et al*., (2018). The red colour represents the p-value for the probe that passed the SMR threshold of significance; the hollow diamond indicates that this probe did not pass the HEIDI threshold (P > 0.05). The middle plot constitutes the eQTL layer where the red crosses represent p-values of the associations of SNPs with miR-1908-5p expression in a ± 500kb window in 2nd trimester foetal brain. The bottom plot shows the location of genes in the locus. Gene positions are in hg19.

Figure 4.8 allows for a visual interpretation of the HEIDI test and to confirm the directionality of the effects found in my SMR analysis between miR-1908-5p expression and risk of BD (Figure 4.8A), irritability (Figure 4.8B), sleep duration (Figure 4.8C) and intelligence (Figure 4.8D). As can be seen in figure 4.8 there are clear pleiotropic effects between miR-1908-5p expression and both BD and irritability. There seem to be other SNPs acting on sleep duration, that don't influence miR-1908-5p expression, albeit there is also evidence of pleiotropic effects between miR-1908-5p expression and sleep duration. There is a positive correlation between higher expression of miR-1908-5p and increased risk for BD, increased irritability, increased sleep duration, and increased intelligence. The association between increased miR-1908-5p expression and intelligence is at least partly driven by linkage. Having identified miR-1908-5p as showing significantly pleiotropic association with bipolar disorder and other traits, I performed a Multi-marker Analysis of GenoMic Annotation (MAGMA) analysis on miR-1908-5p targets on the basis that if miR-1908-5p expression is causally related to these traits, the genes that miR-1908-5p regulates should also be enriched for genetic association with them.

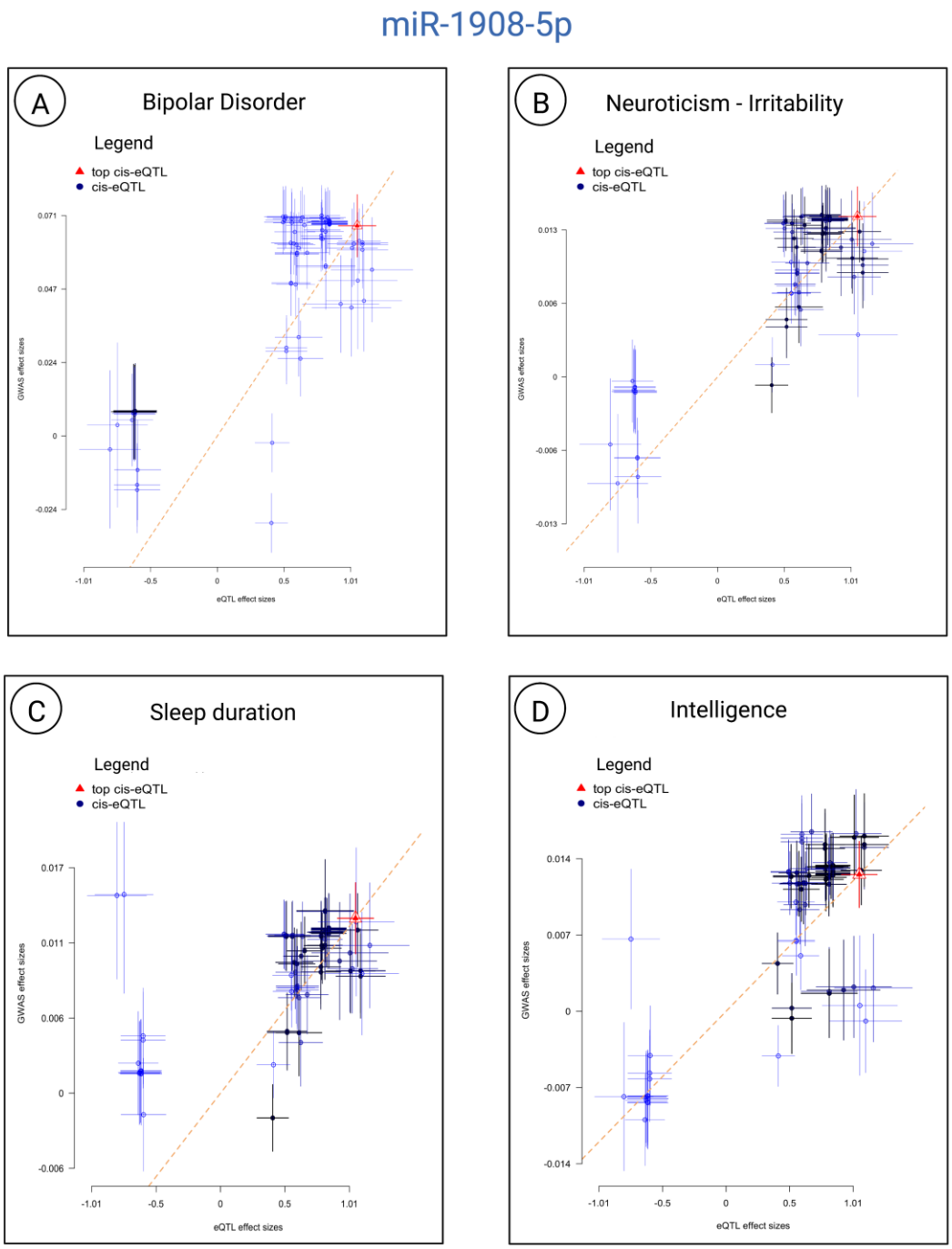


Figure 4.8 – SMR Effect sizes for miR-1908-5p of SNPs used for the HEIDI test from BD GWAS(A) and Irritability (B), Sleep duration (C) and Intelligence (D) GWAS plotted against those for SNPs from the miR-eQTL in 2nd trimester foetal brain analysis.

The x-axis represents miR-cis-eQTL effect sizes while the y-axis represents GWAS effect sizes. These plots display the correlation between the miR-eQTLs effect sizes and GWAS effect sizes.

The orange dashed lines represent the estimate of b_{xy} at the top *cis*-eQTL. Blue circles represent cis-miR-eQTLs, red triangle represents to top cis-miR-eQTL (rs174561). Error bars are the standard errors of each SNP effect.

4.3.3 MAGMA analysis of enrichment of miR-1908-5p targets in bipolar disorder, irritability and sleep duration.

Initially, I retrieved predicted targets of miR-1908-5p from TargetScan 8.0 (McGeary et al., 2019), a miRNA target prediction tool that searches for the presence of conserved miRNA response elements (namely 8mer, 7mer and 6mer sites) in the 3'-UTR of mRNAs that match the seed region of the specified miRNA (Lewis et al., 2005). A total of 2444 predicted targets with a corresponding Entrez ID formed the gene set. Subsequently, a competitive gene-set association analysis was conducted by MAGMA using a gene analysis multi-SNP model in the bipolar disorder, irritability and sleep duration GWAS.

Gene targets of miR-1908-5p were found to be significantly enriched for genetic association with bipolar disorder ($p = 0.003$) and sleep duration ($p = 0.015$), but not irritability ($p = 0.186$).

Subsequently, I wanted to identify if there were specific biological pathways that drove the association of predicted miR-1908-5p targets with bipolar disorder. To this end, I performed a GO analysis using ShinyGO (Ge et al., 2020) on the 2444 miR-1908-5p predicted targets to identify GO BP molecular terms that are enriched. At FDR < 0.01, 238 enriched BP GO terms were identified. Figure 4.9 displays the top 20 most significantly enriched GO terms, the majority of which are related to synaptic signalling ($p = 1.27 \times 10^{-10}$, Fold enrichment = 1.82), "neurogenesis" ($p = 2.61 \times 10^{-14}$,

Fold enrichment = 1.63) and both negative ($p = 4.4 \times 10^{-10}$, Fold enrichment =1.72) and positive regulation of transcription by RNA pol. II ($p = 9.56 \times 10^{-12}$ Fold enrichment =1.69).

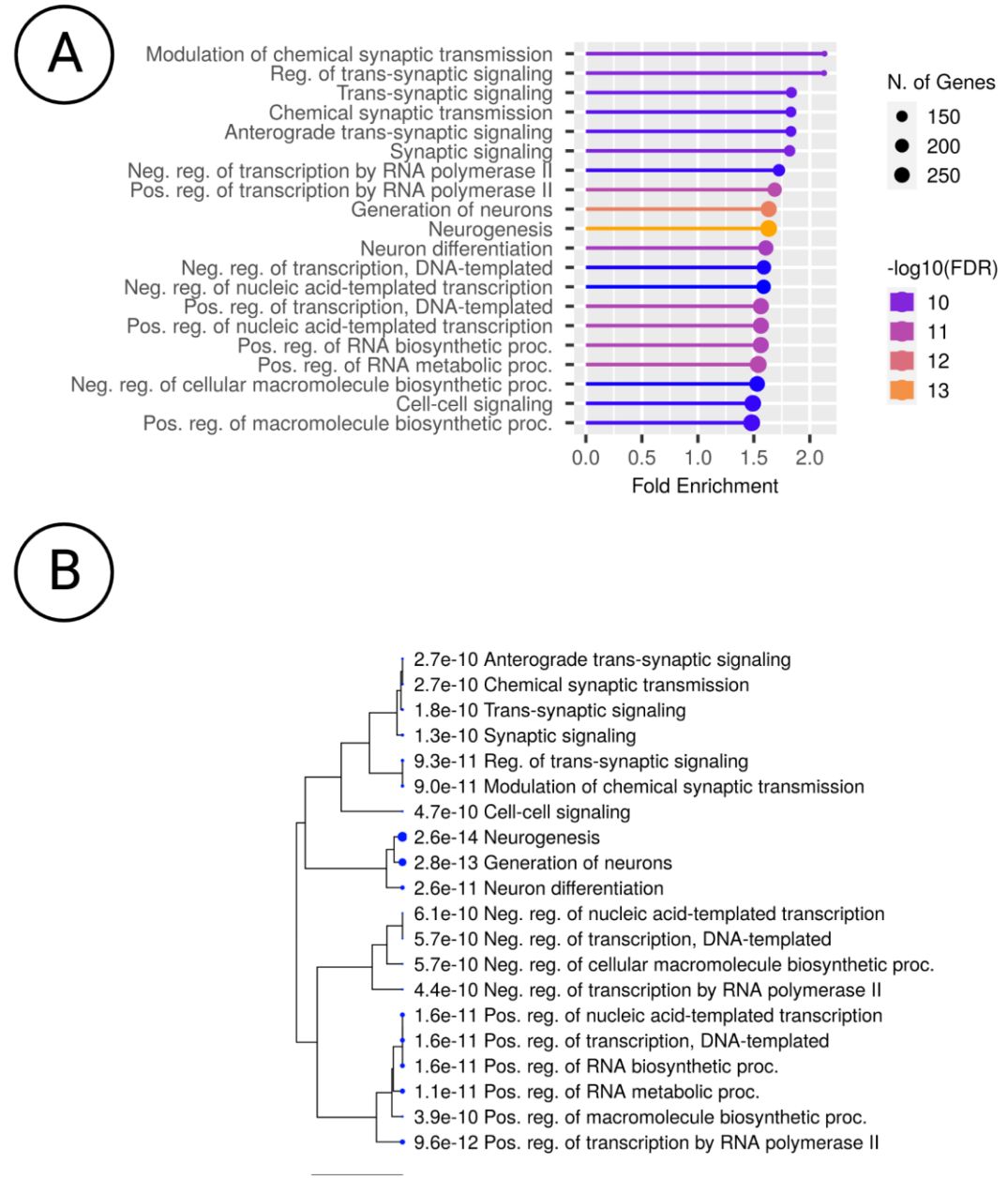


Figure 4.9 – BP GO analysis of miR-1908-5p predicted targets using ShinyGo.

A – Top 20 most significantly enriched GO BP pathways. Fold Enrichment is defined as percentage of miR-1908-5p targets belonging to specific pathway, divided by the corresponding

percentage in the background (all protein coding genes) and indicates overrepresentation. The most overrepresented BP pathways of miR-1908-5p targets are synaptic transmission, regulation of transcription, neurogenesis and neuron differentiation. **B –** Correlation via hierarchical clustering of the top 20 most significant pathways enriched for miR-1908-5p targets. Pathways with large numbers of shared genes are clustered together. Size of blue solid circle represents enrichment FDR.

To identify biological processes targeted by miR-1908-5p that are most relevant to bipolar disorder, I used the enriched GO terms to create 238 gene sets of miR-1908-5p targets segmented by GO term and performed a MAGMA analysis of these in BD. The GO BP terms of miR-1908-5p targets most significantly enriched for genetic association with bipolar disorder are displayed in Table 4.12. At FDR < 0.05, only GO terms related to the regulation of transport and ion, and cation transport (FDR p-value = 4.53×10^{-2}) were significantly enriched for genetic association with bipolar disorder within miR-1908-5p targets. I also found evidence of nominal enrichment in several pathways related to the regulation of trans-synaptic signalling (FDR p-value = 5.75×10^{-2}), which suggests miR-1908-5p influences risk for bipolar disorder by altering these pathways and that both ionic transport and synaptic signalling are essential pathways in BD.

Table 4.12 – MAGMA analysis of miR-1908-5p targets by GO term in Bipolar disorder.

N – number of genes in gene-set, BETA – regression coefficient of gene-set, BETA STD – standardized regression coefficient (dividing BETA by standard deviation of gene-set), SE – standard error of BETA, p-value – uncorrected p-value of association and FDR – FDR corrected p-value.

GO BP term	N	BETA	BETA STD	SE	P	FDR
Regulation of transport	274	0.21	0.026	0.062	3.11E-04	4.53E-02
Ion transport	218	0.22	0.023	0.067	5.56E-04	4.53E-02
Cation transport	176	0.23	0.022	0.074	9.10E-04	4.53E-02
Endosomal transport	47	0.47	0.023	0.150	9.49E-04	4.53E-02
Cation transmembrane transport	138	0.25	0.021	0.082	9.53E-04	4.53E-02
Intracellular transport	254	0.18	0.020	0.060	1.99E-03	5.75E-02
Metal ion transport	138	0.23	0.020	0.083	2.44E-03	5.75E-02
Modulation of chemical synaptic transmission	103	0.27	0.020	0.097	2.57E-03	5.75E-02
Regulation of trans-synaptic signalling	103	0.27	0.020	0.097	2.57E-03	5.75E-02
Regulation of ion transmembrane transport	82	0.30	0.020	0.108	2.98E-03	5.75E-02
Chemical synaptic transmission	140	0.23	0.020	0.084	3.01E-03	5.75E-02
Anterograde trans-synaptic signalling	140	0.23	0.020	0.084	3.01E-03	5.75E-02
Trans-synaptic signalling	142	0.23	0.020	0.084	3.14E-03	5.75E-02
Regulation of ion transport	115	0.26	0.020	0.095	3.47E-03	5.89E-02
Synaptic signalling	148	0.20	0.018	0.082	6.13E-03	9.72E-02
Homeostatic process	248	0.16	0.018	0.063	7.09E-03	1.05E-01
Protein transport	262	0.15	0.017	0.060	7.59E-03	1.06E-01
Neuron differentiation	241	0.16	0.018	0.065	8.56E-03	1.11E-01
Behaviour	102	0.25	0.018	0.104	8.85E-03	1.11E-01
Neurogenesis	292	0.14	0.017	0.060	1.03E-02	1.22E-01

4.4 Discussion

In this Chapter, I identified 3 miRNAs whose expression levels regulated by cis-miR-eQTLS are pleiotropically and potentially causally associated with psychiatric / neurocognitive traits.

I found evidence that both miR-eQTL rs112622797, associated with miR-6840-5p expression and miR-eQTL rs2273626, associated with miR-4707-3p expression, are associated with brain volume. I found rs174561 to be pleiotropically associated with miR-1908-5p expression and bipolar disorder, irritability and sleep duration.

SNP rs174561 accounts for 66% of the variability in the expression of miR-1908-5p in the 2nd trimester foetal brain samples used in this study, with the C-allele associated with increased expression. Increased expression of miR-1908-5p due to the C-allele has also been described in both HuH-7 cells (Beehler et al., 2021) and circulating plasma (Nikpay et al., 2019).

The large effect size of rs174561 on miR-1908-5p expression in 2nd trimester foetal brain is likely caused by this SNP being located within the pri-miRNA hairpin structure of miR-1908 and the C - allele significantly increasing the stability of the pri-miRNA hairpin (ΔG = -3.20 Kcal/mol) as predicted by *RNAfold*. This suggests that rs174561 affects pri-miRNA processing, a finding previously reported by Ghanbari and colleagues (2015) in the context of cardiometabolic phenotypes. Recently, the pri-miRNA of miR-1908 has been shown to undergo significant levels of a type of abortive processing, called inverse processing, where Drosha cleaves the apical loop of the pri-miRNA leading to no mature miRNA being produced (Kim et al., 2021). One of the proposed reasons for this finding was a lack of a stable lower stem in the pri-miRNA (Kim et al., 2021). Therefore, it's likely that rs174561 increases the levels of mature miR-1908-5p by impeding inverse

processing of the hairpin and making it easier for the microprocessor to distinguish the polarity of the miR-1908 pri-miRNA.

Bipolar disorder (BD) is a common and debilitating neuropsychiatric disorder with a large and complex genetic component characterized by extreme mood swings which cycle between profound depression and mania. Moreover, BD displays significant heterogeneity in clinical manifestations, disease trajectories and pharmacological response.

The association between rs174561 and BD is mainly driven by Bipolar Disorder type I (BD I), where episodes of mania predominate. Interestingly, in several studies, BD type I has been shown to be more heritable than Bipolar type II (BD II) (Parker et al., 2018; Song et al., 2018; Coleman et al., 2020). However, it is important to note that in the most recent BD GWAS, the number of BD I cases analyzed (n=23,842) was significantly higher than the number of BD II analyzed (n=6154), which means BD II was underrepresented and underpowered. Of note, I did not find any evidence of an association between rs174561 and SZ, even though BD and SZ have a substantial overlap in common genetic variation, with a genetic correlation of 0.68 (Brainstorm Consortium, 2018) and relatives of both BD and SZ patients having a higher relative risk for both disorders (Lichtenstein et al., 2009). This means that rs174561 is located in a genomic region associated exclusively with BD and will contribute to biological differences between both disorders.

rs174561 was also associated with the irritability (IRR) component of neuroticism and sleep duration. Neuroticism and sleep duration can be seen as BD - associated traits since a recent meta-analysis of 18 separate studies reported that individuals with BD exhibit significantly higher scores of neuroticism (z-value= 8.59) in comparison with control individuals ($p < 0.0001$) (Hanke et al., 2022). Moreover, sleep disturbances are quite common in BD, with reduced sleep duration being a hallmark symptom and a trigger of manic episodes (Lewis et al., 2017), and both insomnia and hypersomnia commonly occur during depressive episodes, which continue in the period between episodes (Harve et al., 2009).

Using the GWAS ATLAS database (Watanabe et al., 2019), I performed a genetic correlation between a previous BD GWAS (Ruderfer et al., 2018) which corresponds to the most recent BD GWAS in the GWAS ATLAS database and both the Irritability GWAS (Nagel et al., 2018a) and Sleep duration GWAS (Jansen et al., 2019) employed in this study. I found a positive genetic correlation between bipolar disorder and irritability and sleep duration (Figure 4.9), which further supports that neuroticism and sleep duration are BD-associated traits.

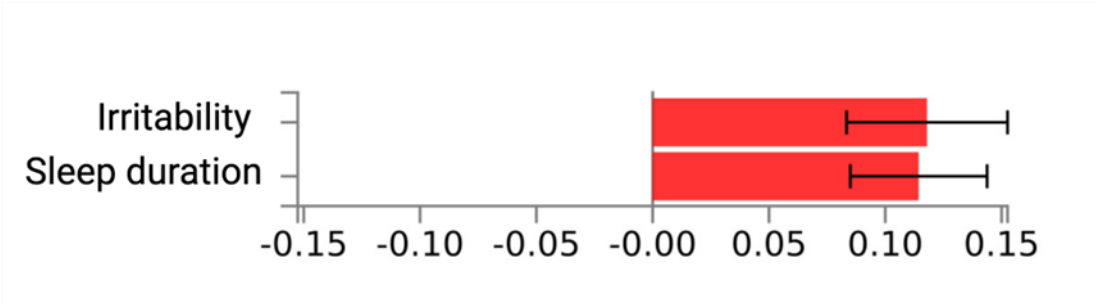


Figure 4.10 – Genetic correlation of the 2018 BD GWAS (Ruderfer et al., 2018) with the Irritability GWAS (Nagel et al., 2018a) and Sleep duration GWAS (Jansen et al., 2019) employed in this study.

Genetic correlations were performed using the GWAS ATLAS database (https://atlas.ctglab.nl/traitDB) (Watanabe et al., 2019). The genetic correlation (rg) between Bipolar disorder and Irritability is 0.118 (SE = 0.025, Z = 3.406, p-value = 6.60×10^{-4}). The genetic correlation (rg) between Bipolar disorder and Sleep duration is 0.114 (SE = 0.029, Z = 3.884, p-value = 1.03×10^{-4}).

In addition, both sleep duration and insomnia GWAS studies have reported a positive genetic correlation between BD and sleep duration (Dasthi et al., 2019; Jansen et al., 2019). More recently, it has been demonstrated that the genetic liability in the form of polygenic risk scores (PRS) to both insomnia and longer sleep duration depends on the BD subtype, where PRS for sleep duration was shown to be associated with BD I, and with an increased relative risk for BD I compared with controls, but not for BD II (Lewis et al., 2020). In contrast, the PRS for insomnia was

associated with an increased relative risk for BD II compared to controls, but not for BD I (Lewis et al., 2020).

I subsequently explored whether any of these 3 miR-eQTLs had pleiotropic effects on other neuropsychiatric neurological / cognitive phenotypes. To this end, I performed a Phenome-Wide Association study (PheWas) nominal scan of SNPs rs174561, rs112622797 and rs2273626 on an Atlas of GWAS summary statistics from the CNCR-CTG lab involving 600 GWAS performed on UK Biobank samples. Interestingly, rs174561 was shown to be associated with cognitive performance, verbal-numerical reasoning and nap during the day, the latter contributing to sleep duration. This SNP was also associated with depressive symptoms in a multivariate genome-wide association meta-analysis of the well-being spectrum (Baselmans et al., 2019).

Regarding the SNPs previously associated with brain volume, SNP rs2273626, associated with miR-4707-3p expression, is also associated with educational attainment, whereas SNP rs112622797, associated with miR-6840-5p expression is associated with educational attainment and alcohol intake frequency.

miR-1908-5p is located within intron 1 of fatty acid desaturase 1 (FADS1), and rs174561 is an eQTL for FADS1 and several other genes in adult brain (GTeX V8 data – GTeX consortium, 2020), suggesting that the association with bipolar disorder and other BD - related traits could be caused by variation in FADS1 expression, rather than on miR-1908-5p. This scenario is unlikely because, firstly, the expression of the miR-1908 pri-miRNA is not closely related to its host gene, FADS1 (Kuang et al., 2015). Secondly, the plausible mechanism of action by which rs174561 leads to an increase of mature miR-1908-5p is by making the miR-1908 hairpin more thermodynamically stable, which leads to more mature miR-1908-5p being produced, a mechanism independent of its host gene and any shared regulatory elements. Thirdly, MAGMA analysis indicated an enrichment of bipolar disorder genetic associations in miR-1908-5p target genes. Some of these miR-1908-5p targets have been validated, including genes such as

GRIN1, STX1A, GRM4, CLSTN1 and DLGAP4 that function in glutamatergic synapses (Kim et al., 2016).

Gene targets of miR-1908-5p were found to be significantly enriched in bipolar disorder ($p = 0.003$), sleep duration ($p = 0.015$) but not irritability ($p = 0.186$), again providing independent evidence for a role of miR-1908-5p in brain traits. The lack of enrichment of miR-1908-5p in irritability might be caused by only a subset of targets involved in particular pathways being involved in irritability instead of all targets of miR-1908-5p. MAGMA gene-set analysis stratified by GO biological processes highlighted ion and cation transport and trans-synaptic signalling as three of the most enriched pathways of gene targets of miR-1908-5p targets in bipolar disorder, suggesting these pathways drive this association. Of note, both trans-synaptic signalling and regulation of ion transport GO pathways have been reported to be associated with cortical structural abnormalities in paediatric bipolar disorder (Lei et al., 2022). Loss of cationic homeostasis is a well-known feature of bipolar disorder pathophysiology and is a proposed mechanism for mood swings due to altered neuron resting potentials causing relatively depolarized neuronal membrane potentials (El-Mallakh & Wyatt, 1995; El-Mallakh, Yff, and Gao, 2016; El-Mallakh, Gao and You, 2021). The mechanism of action of mood-stabilizing drugs might correct this ionic balance, mainly by reducing intracellular Na^+ levels (El-Mallakh & Paskitti, 2001). Therefore, it is likely that increased miR-1908-5p expression increases the risk for BD by decreasing the expression of genes involved in ionic homeostasis. In addition, trans-synaptic signalling is crucial for synapse formation, refinement and diversification, as well as regulating synaptic plasticity (Fossati et al., 2019). An excitatory and inhibitory synaptic imbalance has been proposed as a mechanism involved in mania (Lee et al., 2018), and synaptic signalling genes have been found to be downregulated in comparison to controls in a sizeable RNA-Sequencing study in the amygdala and anterior cortex of bipolar patients (Zandi et al., 2022). This suggests that miR-1908-5p further contributes to bipolar risk via the downregulation of genes involved in trans-synaptic signalling.

Interestingly, miR-1908-5p is a primate-specific miRNA, and the risk variants associated with an increased miR-1908-5p expression associated with BD risk are active in 2nd trimester foetal brain. To the best of my knowledge, this is the first time this finding has been reported. It is likely that these variants are also active in the adult brain, where they could continue to influence BD risk along with irritability and sleep duration.

miR-1908 has been shown to be abnormally expressed in several diseases, including cancer (for review, see: Shen et al., 2022). In the peripheral blood of AD patients, high levels of miR-1908-5p have been detected, which leads to impairment of ApoE-dependent AB clearance, which is associated with risk for AD (Wang et al., 2018). Interestingly, miR-1908-5p has been shown to be downregulated in a very small study (15 BP subjects) in peripheral blood in depressive episodes of female BD patients compared to remission ($p = 0.004$) (Banach et al., 2017). Moreover, the expression of miR-1908-5p was decreased in response to valproate, a drug commonly used to treat BD, in neuroprogenitor cells (NPCs) derived from dermal fibroblasts from a BD patient. In contrast, the opposite effect was observed in NPCs derived from dermal fibroblasts from a control subject (Kim et al., 2016).

My SMR analysis indicated that the A allele of rs112622797 is associated with an increase in miR-6840-5p expression and is also associated with decreased brain volume. In contrast, the G allele of rs12880925 is associated with decreased expression of miR-4707-3p and is associated with increased brain volume. Both these associations have $P_{HEIDI} > 0.05$, suggesting the presence of causal/pleiotropic effects on brain volume by both miR-6840-5p and miR-4707-3p. It is important to note that the top SNP reported in my FASTQTL analysis at the miR-4707-3p locus was rs2273626, whereas the SNP reported by my SMR analysis was rs12880925. These SNPs are in high linkage disequilibrium (LD; $r2 > 0.99$, $D'=1$) according to Haploreg v4.1 (Ward et al., 2016) and have the same nominal p-value and beta in my eQTL analysis due to having the identical genotypes in my samples.

miR-4707-3p is located within the 5'-UTR of the HAUS4 gene. The miR-eQTL associated with miR-4707-3p expression (rs2273626 C > A) co-localizes with an eQTL for its host gene HAUS4 in 2nd trimester foetal brain in a largely overlapping sample where the eQTL p-value (nominal) is p=0.0002 (not FDR adjusted) (O'Brien et al., 2018). This SNP is located within the seed region of miR-4707-3p and is associated with a significant increase in minimum free energy (ΔG =5.6 Kcal/mol) predicted by *RNAfold* (Gruber et al., 2008), which leads to the miR-4707 hairpin being less thermodynamically stable and is predicted to decrease miRNA expression. In the previous Chapter (Figure 3.10A), I showed that the rs2273626 genotype explains 77% of the variability in miR-4707-3p expression in 2nd trimester foetal brain and that the A-allele is associated with a significant decrease in miR-4707-3p expression, with the majority of homozygotes for the A-allele having low to no miR-4707-3p expression. Moreover, given that this SNP is located in the seed region of miR-4707-3p (which binds to the target mRNA sequence), the A–allele could alter post-transcriptional regulation in the samples where it is expressed even at considerably low levels. A preprint performing a miR-eQTL study in 212 mid-gestation neocortical brain samples recently released on bioRxiV by Lafferty and colleagues (2022) provides independent validation for my miR-4707-3p findings. The authors also found a miR-eQTL (rs4981455) for miR-4707-3p that colocalized with an eQTL for the host gene HAUS4, brain volume, and educational attainment. In that study, the alleles associated with increased expression of miR-4707-3p (and HAUS4) were both associated with decreased brain volume and decreased educational attainment (Lafferty et al., 2022). Given that the miR-eQTL of miR-4707-3p co-localizes with the eQTL of the host gene HAUS4, I cannot ascertain if the association to brain volume I found is associated solely with miR-4707-3p expression, particularly considering that HAUS4 has been shown to regulate mitotic spindle assembly and cell proliferation (Lauo et al., 2009; Uehara et al., 2009). The study of Lafferty et al. (2022) also found a miR-eQTL for miR-1908-5p (rs2015950 G > A), but the authors did not follow up on this finding.

In conclusion, I have identified 3 miR-eQTLs that are pleiotropically and potentially causally associated with neuropsychiatric / brain-related traits. Further investigation of these miRNAs and their targets may shed light on the biological underpinnings of these traits. The work described in this thesis suggests that miR-1908-5p is involved in the pathogenesis of BD and that the risk variants for BD are active in 2nd trimester foetal brain. Exploring the associations among bipolar disorder, BD-related traits and miRNA expression influenced by genetic risk variants can provide useful etiological insights, help prioritize potential causal relationships, and advance the understanding of its biological underpinnings. BD is associated with premature death due to significant comorbidities and a high risk of suicide (Hayes et al., 2015). Moreover, clinical heterogeneity and treatment response translates into this disorder being frequently misdiagnosed and sub-optimally treated. It is possible that disentangling the association between 2nd trimester foetal brain miR-1908-5p expression and bipolar disorder risk could translate into more effective treatments and a better quality of life for the affected individuals.

Chapter 5 – General Discussion

5.1 Summary of main findings

5.1.1 Detection of mature miRNA expression in 2nd trimester human brain

The aim of this thesis was to evaluate how variation in miRNA expression in 2nd trimester foetal brain might contribute to risk for neuropsychiatric disorders. To this end, I first performed small-RNA sequencing to quantify mature miRNA expression in 112 2nd trimester foetal brain samples with gestational ages between 12-20 PCW. I detected a total of 1449 miRNAs in 2nd trimester foetal brain, which corresponds to 55% of all known miRNAs, highlighting the importance of miRNAs in foetal brain development. A study employing high-throughput RNA sequencing bound to AGO2 via crosslinking immunoprecipitation (HITS-CLIP) in 9 foetal brain samples at 15 - 16.5 PCW and 19-20.5 PCW detected a total of 921 human miRNAs expressed in 2nd trimester foetal brain (Nowakowski et al., 2018). Another recent study performing small RNA sequencing in cortical samples from 212 samples found 907 miRNAs (621 miRNAs were present miRbase v22; the remainder were newly discovered miRNA species) (Lafferty et al., 2022, preprint). The larger number of miRNAs detected in my study, as opposed to Nowakowski et al., 2018 and Lafferty et al., 2022 is likely due to me using whole brain homogenates instead of cortical tissue (Lafferty et al., 2022) and having a less stringent cut-off for miRNA detection.

5.1.2 Factors influencing miRNA expression in 2nd trimester human brain

Subsequently, I identified the main drivers of miRNA expression variation at the global level in 2nd trimester foetal brain. This analysis indicated that, in general, age and sex did not have a significant impact on miRNA expression in 2nd trimester foetal brain. However, when analyzed individually, I found evidence of sex-biased miRNA expression in a single miRNA (miR-373-3p) downregulated in males. This finding suggests that miRNAs do not contribute significantly to sex differences in foetal brain development and is consistent with previous studies on sex-biased miRNA expression in the neonatal brain, where only two miRNAs were found to be differentially expressed between females and males (Ziatts & Rennert, 2014). I also found 171 individual miRNAs that showed a correlation in expression with post-conception week, with approximately half upregulated and half down-regulated with increasing gestational age, suggesting important roles in prenatal brain development.

5.1.3 cis-miR-eQTL analysis in 2nd trimester foetal brain

The expression of the detected 1449 miRNAs, controlled for influences driving miRNA expression, was combined with genome-wide genotyping in order to map eQTLs operating on miRNAs in 2nd trimester foetal brain. I identified 30 miRNAs whose expression is associated with common genetic variation (miR-eQTLs) at FDR < 0.05, all of which mapped to unique genomic locations. This constitutes ~ 2% of the miRNAs under study (n = 1449) and is a smaller proportion than mRNA eQTLs (4.6%) identified by our group on

primarily the same samples (O'Brien et al.,2018), suggesting that miRNAs are under tighter regulatory control and negative selective pressure.

5.1.4 miR-eQTL associated with neuropsychiatric disorders and other brain traits

Finally, I related the identified miR-eQTLs to common genetic variants associated with neuropsychiatric and other common brain traits (Chapter 4). SMR and HEIDI tests were employed to ascertain if altered miRNA expression arising from genetic variants is pleiotropically and/or potentially causally associated with these traits and not the result of genetic linkage. I identified 3 miRNAs whose expression levels regulated by cis-miR-eQTLS are associated with 5 psychiatric/neurocognitive traits, namely miR-1908-5p with bipolar disorder, irritability, sleep duration and intelligence, and miR-6840-5p and miR-4707-3p with brain volume.

My SMR analysis indicated that the C-allele of rs174561 is associated with increased expression of miR-1908-5p, increased risk for bipolar disorder, increased irritability scores and increased sleep duration even after stringent Bonferroni correction for 690 tests. The HEIDI test for these associations indicated that they were unlikely to have arisen due to linkage with other variants that could be operating through different genes. In contrast, the association of miR-1908-5p expression in 2nd trimester foetal brain with increased intelligence is likely to arise, at least partly due to linkage between rs174561 and another variant.

As an independent test of whether miR-1908-5p expression could influence bipolar disorder and associated traits, I performed a MAGMA analysis on miR-1908-5p targets, assuming that if miR-1908-5p is associated with bipolar disorder, then the genes miR-1908-5p regulates will also be

associated with the condition. Predicted gene targets of miR-1908-5p were found to be significantly enriched for association with bipolar disorder, sleep duration and intelligence, but not irritability. This provides independent evidence of a causal role of miR-1908-5p expression in bipolar disorder and some associated traits because it tests the regulatory function of miR-1908-5p. To the best of my knowledge, this is the first time this finding has been reported. MAGMA gene-set analysis stratified by GO biological processes highlighted ion and cation transport and trans-synaptic signaling as three of the most enriched pathways of gene targets of miR-1908-5p targets in bipolar disorder, suggesting these pathways drive this association.

5.2 Independent validation of miR-eQTLs in 2nd trimester foetal brain findings

When writing this thesis, a preprint performing a miR-eQTL study in 212 mid-gestation neocortical brain samples was released on bioRxiV by Lafferty and colleagues (2022). I decided to use this study as independent validation for my miR-eQTL analysis. The authors found 70 miRNAs whose expression was influenced by cis-miR-eQTLs, 59 of which are present in miRbase. The larger number of miR-eQTLs found in this study stems from it being better powered to detect eQTLs, given its larger sample size (n=212). 8 of the miRNAs whose expression is influenced by miR-eQTLs in 2nd trimester foetal brain identified in this study were also reported by Lafferty et al., (2022).

In contrast, the remaining 22 miRNAs were exclusive to my study. This discrepancy might stem from my study using whole brain homogenates instead of cortical tissue, and some of the miRNAs I identified may be poorly

expressed in the cortex. Moreover, it is possible that some of the miRNAs I identified as being influenced by miR-eQTLs were below the detection cut-off level employed by Lafferty and colleagues (2022). Of note, this study validates my miR-4707-3p findings. The authors also found a miR-eQTL (rs4981455) for miR-4707-3p that colocalized with an eQTL for the host gene HAUS4, brain volume, and educational attainment. Where the alleles associated with increased expression of miR-4707-3p (and HAUS4) were both associated with decreased brain volume and decreased educational attainment (Lafferty et al., 2022). This study also found a miR-eQTL (rs2015950) for miR-1908-5p, but the authors did not follow up on this finding.

5.3 Limitations of this study

5.3.1 Small RNA-Sequencing method employed

The method employed in this project for small RNA sequencing library preparation (two-adaptor ligation method) is the most commonly used method in small RNA sequencing studies (Benesova et al., 2021). However, this method is associated with ligation bias (Raabe et al., 2014) due to different affinities of adaptors and miRNAs caused by secondary structures of both species (Fuchs et al., 2015). This may cause some miRNAs to not be accurately detected and may confound results. There are now improved two-adaptor ligation methods utilizing randomized adaptors that minimize this bias and have better performance (Giraldez et al., 2018; Wright et al., 2019; for review, see: Benesova et al., 2021). Moreover, the use of unique

molecular identifiers (UMIs) further refines Small RNA Sequencing studies by mitigating PCR bias, the significance of which is only now being acknowledged (Fu et al., 2018; Wright et al., 2019). Recent studies comparing the performance of all 3 methods (the original two-adaptor ligation method employed in this study, the use of randomized adaptors and the use of UMI) have ranked the original two-adaptor ligation method as having the worst performance; whereas using randomized adaptors performed best (Herbert et al., 2020; Heinicke et al., 2020). Ideally, as future work, I would have repeated this study using randomized adaptors and assessed if there were miRNAs that I could not detect because of ligation bias. However, I am confident that by performing PEER corrections (Stegle et al., 2012) on my Small RNA Sequencing data, I was able to minimize the bias associated with differences in ligation affinity in the miRNAs I identified in this study.

5.3.2 Statistical power of the eQTL Study

In this thesis, I found 30 mature miRNAs whose expression was influenced by cis-miR-eQTLs in 2nd trimester foetal brain. All but one of the miR-eQTLs identified have global minor allele frequencies (MAF) > 5% (dbSNP alfa). Given that the number of samples employed is quite limited (n = 112), this study is underpowered to detect eQTLs with lower frequencies. Power analysis of my eQTL study indicates that my study is sufficiently powered to detect cis-miR-eQTLs with MAF > 20 %, assuming the same effect size (beta = 0.13) that was used by the GTeX Consortium (2013).

Increasing the sample size will lead to more miR-eQTLs being detected in foetal brain, as previous studies reported a strong correlation between sample size and the number of eQTLs discovered (GTEx Consortium, 2017). This can be exemplified by the Lafferty et al., (2022) preprint, where they reported 70 miRNAs whose expression was influenced by miR-eQTLs in 2nd

trimester foetal brain employing 212 cortical samples. However, it is possible that identifying more miR-eQTLs operating in foetal brain will not lead to identifying more pleiotropic effects of miR-eQTLs on risk for neuropsychiatric traits, given that Lafferty et al. (2022) were unable to identify any such association.

5.3.3 Use of Bulk brain tissue homogenates for DE and eQTL analysis

The analyses described in this thesis were based on the sequencing of bulk tissue from 2nd trimester foetal brains, where miRNA expression levels were averaged across all cell types. Foetal brains are precious resources, and using available bulk frozen tissue allowed me to have enough samples to map miR-eQTLs operating in 2nd trimester foetal brain with high confidence. Consequently, cell-specific and region-specific effects at the eQTL and DEG levels will be diluted and possibly masked by the bulk RNA sequencing expression measures. As such, a caveat of this method is that I cannot relate the miR-eQTLs or differentially expressed genes identified to specific cell types or brain regions nor determine which cell types contribute to risk of bipolar disorder in 2nd trimester foetal brain. Further refinement of the brain regions and cell types in which the miR-eQTLs identified in this study are active is required to improve the understanding of the cellular context of miRNA expression in both brain development and neuropsychiatric disorders. A future study could employ single-cell small RNA sequencing, which would allow me to investigate the cellular context in which miR-eQTLs influence miRNA expression, study miR-eQTLs in rare cell types, and identify cell-type-specific DEG and cell-type-specific eQTLs not detected using bulk RNA – Sequencing (Kang et al., 2018; Soneson & Robinson, 2018; Potter, 2018; van der Wijst et al., 2018, 2020). Moreover, integrating single-cell

small RNA sequencing with spatial transcriptomics would enable me to map miR- eQTLs to specific brain areas and subsets of cells.

5.3.4 Predicted miRNA targets using TargetScan

The extent to which miR-eQTLs influence downstream miRNA target expression is difficult to measure and impossible to access using only small RNA-Sequencing. The miRNA targets employed for GO analysis and MAGMA analysis in this thesis were predicted by Targetscan 8.0, which predicts biological targets by searching for the presence of conserved 8mer, 7mer, and 6mer sites in the 3'-UTR of genes that match the seed region of each miRNA. This method has several limitations (Peterson et al., 2014; Riffo-Campos et al., 2016). Possibly, some of the predicted targets employed in this study are false positives. It is also possible that some of the predicted miRNA targets are not expressed in 2nd trimester foetal brain. miRNAs can also target genes by binding to the coding sequence (CDS) of a gene (Tay et al., 2008; Anneke et al., 2014), and therefore any genes targeted by miRNAs due to complementarity of the seed region with the CDS will have been missed by this analysis. Indeed, Nowakowski and colleagues (2018) identified miRNA–mRNA interactions in the developing brain and reported that 27% of AGO2 binding sites were located in the CDS on target mRNAs. The use of databases of validated miRNA targets has similar limitations and is biased in favour of miRNA:mRNA interactions assessed in assayed tissues. A follow-up study using miRNA transfection for miRNA upregulation and miRNA sponges for miRNA downregulation in appropriate cell lines (NSC, early neurons, late neurons, glia) could elucidate the mechanisms by which the miR-eQTLs identified in this study have pleiotropic effects on risk for neuropsychiatric disorders and other brain traits. Alternatively, I could identify miRNA–mRNA interactions via AGO1- 4 crosslinked immunoprecipitation followed by NGS in different cell types and foetal brain

regions, similar to the assay performed by Nowakowski and colleagues (2018).

5.4 Future perspectives

Apart from the future work outlined above to overcome the limitations of this study, there are additional interesting potential future investigations. Extending miR-eQTL analysis to sex chromosomes may identify sex-specific miR-eQTLs (Shen, Wang and Yang, 2019). Of the miRNAs with miR-eQTLs reported by Lafferty and colleagues (2022), 5 were located in the X-chromosome. It is plausible that sex-specific miR-eQTLs may underlie sex-specific genetic liability to neuropsychiatric disorders. However, a recent study reported that sex-specific eQTLs in whole blood do not translate to detectable sex-specific trait associations (Porcu et al., 2022). By performing an eQTL analysis at the mature miRNA level, I assessed the total effects of variants on the entire miRNA biogenesis process. Performing an eQTL analysis at the pri-miRNA level via RNA Sequencing in the same samples would allow me to ascertain which miR-eQTLs affect miRNA expression by altering the transcription of pri-miRNAs and which would affect the biogenesis of mature miRNAs, further refining the mechanism of action of the identified miR-eQTLs.

Moreover, given the role of miRNAs in gene expression regulation, the identified cis-miR-eQTLs are likely trans-eQTLs for downstream miRNA targets. Performing a trans-eQTL analysis using the identified miR-eQTLs could shed light on how miR-eQTLs influence the gene expression of miRNA targets. However, this analysis would have a sizeable multiple-testing burden unless restricted to predicted mRNA targets. Small RNA sequencing also profiles other small RNA species, such as piwi-RNAs. Extending my analysis

to piwi-RNAs in 2nd trimester foetal brain may shed light on their role in brain development and neuropsychiatric disorders (Chavda et al., 2022)., Performing similar analyses in earlier and later stages of gestation would allow for a more comprehensive assessment of miRNA expression in brain development and the potential role of miR-eQTLs in brain function and neuropsychiatric disorders.

5.5 Conclusion

In conclusion, the work described in this thesis provide novel insights regarding the role of miRNAs in 2nd trimester foetal brain development and how variation in miRNA expression during this developmental phase might contribute to risk for neuropsychiatric disorders. This is the first report implicating miR-1908-5p expression in 2nd trimester foetal brain with risk for bipolar disorder and highlights the need for further investigation into the subject.

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