Universität Zürich[UZH]

Master's Thesis

Study of the neurobiological basis of trauma in a Dutch military cohort and a mouse model: a unique cross-species perspective

by

Marta J. Koper

Research Master in Cognitive and Clinical Neuroscience

Specialization: Fundamental Neuroscience

Supervised by

Prof. Dr. Isabelle Mansuy and Prof. Dr. Bart Rutten

Laboratory of Neuroepigenetics, UZH/ETH, Zürich

Maastricht University

2016/2017

ABSTRACT

While the physiological fight-or-flight response to acute stress contributes to the survival advantage in evolutionary terms, a long-lasting exposure to traumatic stress may induce enduring dysfunctions of the body systems. Both early-life and adult trauma lead to the neuroendocrine and epigenetic changes, which are the basis for the development of mental and physiological disorders. A recent Dutch military cohort study has provided evidence on molecular substrates underpinning clinical conditions after combat trauma. Based on these data, a cross-species analysis was carried out to compare whether biological alterations found in traumatized soldiers overlap with those induced by early-life trauma exposure. For this, a mouse model combining early-life unpredictable maternal separation with unpredictable maternal stress (MSUS) was used, and candidate genes, such as ZFP57 and RNF39, were investigated in terms of mRNA expression and DNA methylation. The human trauma-induced DNA methylation changes were not found in MSUS for these two candidate genes. Serum microRNA profiling revealed aberrant expression of miR-34b, miR-146a, miR-146b and miR-370 in MSUS mice relative to controls. A subsequent literature analysis of microRNA targets suggested its potential implication in the neuroendocrine, immune and metabolic downstream signalling pathways. Bridging the bench with the bedside, further research in both rodents and humans would benefit from the current findings by continuing the investigation of trauma-induced phenotypic changes.

Key words: microRNAs, DNA methylation, gene expression, traumatic stress

TABLE OF CONTENTS

1. INTRODUCTION

A growing body of evidence suggests an adverse effect of traumatic stress on physical and mental health. Physiological and emotional adaptations in acute stress serve an adaptive function; however, in the long term they can lead to chronic alternations of the body systems, including metabolic, endocrine, immune and nervous system (Chrousos, 2009). The neurobiological sequelae of early-life trauma have been shown to play a relevant role in the emergence of psychiatric disorders, such as schizophrenia, bipolar disorder, and depression during lifetime (Aas et al., 2014; Álvarez et al., 2011; Pace et al., 2006). In addition to contributing to the pathogenesis, childhood trauma may render an increased susceptibility for developing physical and psychiatric disorders (Anda et al., 2006; Heim, Owens, Plotsky, & Nemeroff, 1997). Notably, it has been demonstrated that individuals with a history of childhood trauma, who were once more exposed to traumatic stress in adulthood, showed higher vulnerability for post-traumatic stress disorder (PTSD) (Bremner, Southwick, Johnson, Yehuda, & Charney, 1993; Mehta et al., 2013). Whereas the association is suggestive, it does not prove causality, since not all trauma-exposed individuals develop PTSD (deRoon-Cassini, Mancini, Rusch, & Bonanno, 2010; L. A. King, King, Fairbank, Keane, & Adams, 1998).

Based on epidemiological data, the prevalence of early childhood trauma, i.e. exposure to any traumatic event before the age of 13, was estimated at 38,5% (Koenen, Roberts, Stone, & Dunn, 2010). High lifetime exposure rates to potentially traumatic events were observed in the general population; 74% of the women and 81% of the men reported to experience at least one traumatic event that met the American Psychological Association (APA) diagnostic criteria of a traumatic stressor (Stein, Walker, Hazen, & Forde, 1997). On the other hand, lifetime (and 12-month) prevalence for trauma-related disorders, such as PTSD, range from 1 to 9,2% (0.4 - 3.8%) in high-income populations, and are higher compared to rates of around 2% (0.2-2%) in low- and middle-income countries (Atwoli, Stein, Koenen, & McLaughlin, 2015; Dorrington et al., 2014; Karam et al., 2014). These data together may suggest individual-specific differences towards the vulnerability to trauma-induced disorders. Importantly, the overall cost of PTSD treatment in Europe was estimated at € 8,4 billion (Olesen et al., 2012). The socio-economic burden of trauma-related disorders includes not only patient's functional impairment and disability or lower work performance, but also an increased use of health services. Given that, trauma-related disorders have become a pronounced public health problem, which compels the development of more effective evidence-based interventions.

An increasing number of researchers have focused on the biological bases of trauma implicated in developing and maintaining the pathological state. Instead of direct causal mecha-

nisms between trauma and disease, the emphasis is on identifying the intermediary neurobiological targets by which clinical states of trauma manifest themselves. For instance, the findings from pre-clinical and clinical studies suggest that the genetic variations related to the neurobiological stress response mediate risk for developing adult psychiatric disorders in relation to trauma exposure (Binder et al., 2008; J. A. King, Abend, & Edwards, 2001). Additionally, functional analyses of DNA methylation (DNAme) have revealed epigenetic marks at stress-response genes (McGowan et al., 2009; Palma-Gudiel, Córdova-Palomera, Eixarch, Deuschle, & Fañanás, 2015), which can be transgenerationally inherited (Franklin et al., 2010). Moreover, an aberrant microRNA (miRNA) expression has been reported in both early-life stress and PTSD (Bam et al., 2016; Gapp et al., 2014). Despite the short sequence length (~20 nucleotide-long RNA molecules), literature emphasises an important role of miRNAs in post-transcriptional regulation of target genes, thereby having a broad effect on downstream signalling cascades (He & Hannon, 2004).

The integration of information from these multiple lines of evidence is necessary to bridge the bench with the bedside. Over the last decades, a large number of rodent models have been developed to investigate the neuroendocrine, immune, and (epi-)genetic pathways underpinning trauma-related disorders (Schöner, Heinz, Endres, Gertz, & Kronenberg, 2017); however, there has been limited success in translating animal models to the clinic. Therefore, the current project approached research questions working back from the human findings to a mouse model of trauma. The clinical findings were derived from the PRISMO study (n=1.032), a prospective cohort study of Dutch soldiers involved with the International Security Assistance Force (ISAF) and deployed to Afghanistan between 2008 and 2010. Changes in DNAme profiles examined in whole-blood samples from 93 male individuals before and after military deployment were shown to mediate between trauma exposure and the subsequent development of psychopathology. The genome-wide analyses identified decreased methylation levels in 17 CpG sites and 12 regions in the human genome, showing a negative association with PTSD symptoms over time. The data replication in an independent cohort of combat trauma-exposed US Marines revealed the same whole-blood DNAme patterns for two genomic regions close to the transcription start site of ZFP57 and RNF39 (Nievergelt et al., 2015). Additionally, a post-mortem examination of the dorsolateral PFC tissue from schizophrenic patients showed significantly lower DNAme in the abovementioned genomic regions. Finally, cross-species validation revealed an association between transcript levels of RNF39 and ZFP57 and behavioural phenotypes of differential susceptibility to traumatic stress (Daskalakis, Bagot, Parker, Vinkers, & de Kloet, 2013).

Based on the above-mentioned PRISMO findings, the neurobiological targets were selected to investigate in a mouse model of early life trauma combining unpredictable maternal separation with unpredictable maternal stress (MSUS). Previous evidence has shown that MSUS mice in addition to behavioural alterations, displayed aberrant epigenetic profiles of genes in the male germline as well as in the brain, which could be transmitted across generations (Franklin et al., 2010). Furthermore, changes in the miRNA expression were found in different tissues, and some of these alterations were transmitted to the progeny (Gapp et al., 2014). The current master's thesis project consisted of a cross-species exploratory analysis. In this analysis research questions (vs directional hypotheses) were posted whether there are similarities between neurobiological bases of human adulthood trauma and animal early-life trauma, in terms of gene expression, DNAme, and miRNA expression. The dysregulation of circulating miRNA expression was determined in MSUS. Based on literature review, function of each miRNA was explored in relation to their potential involvement in the immune, neuroendocrine and metabolic system.

2. METHODS

Animals. C57BL/6 mice (Janvier, France) were used in this project. Cages were placed in a temperature and humidity-controlled facility under reversed light-dark cycle. Mice were given ad libitum access to food and water.

Animal treatment. MSUS was performed as previously described (Franklin et al., 2010). Briefly, C57Bl/6J dams (F0, 2-3 months old) and their offspring (F1) were randomly selected and subjected to unpredictable maternal separation combined with unpredictable maternal stress for 3 hours daily, from postnatal day 1 to 14 (PND 1-14). During separation, dams were exposed either to restraint stress or forced swim test. The control group was left undisturbed except for a cage change once a week until weaning (PND21). After weaning, mice subjected to the same treatment but from different mothers were randomly assigned to be housed 4-5 per cage. The second generation (F2) offspring were obtained by breeding adult control or MSUS males (F1) with wild-type C57Bl/6J females.

Tissue collection. Mice, aged 6 months, were anesthetized with isoflurane and sacrificed by decapitation. Trunk blood was collected in EDTA-coated tubes (BD Vacutainer Systems, Plymouth, UK) for whole blood or in DNA-low bind Eppendorf tubes for serum. Hippocampal tissue was isolated and immediately frozen in liquid nitrogen, followed by storage at -80°C until further processing.

2.1. Gene expression analysis

Whole blood RNA isolation. A standard phenol-chloroform extraction was used to isolate total RNA from the whole blood. Different blood input volumes (30-140 µl) were adjusted to 250 µl using nuclease-free water (AppliChem, Darmstadt, Germany) and homogenized in 750 µl of TRIzol LS (Invitrogen, CA, USA) using a 26G needles and 1 ml syringes. Double chloroform extraction was carried out to maximise the amount of RNA extracted, followed by precipitation with isopropanol to recover total RNA. The pellet was washed twice with 70% ethanol, dried in a heat block at 50°C for approximately 5 min, then dissolved in 20 µl of nuclease-free water and incubated at 55°C for 15 min. The purity and concentration of RNA were determined by a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Subsequently, the RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Reverse Transcription. Total RNA was adjusted to 50 ng/µl and subjected to reverse transcription with oligo(dT) primer (10 µM) and M-MLV reverse transcriptase (200 U/µl; Promega, Madison, WI, USA) in a reaction volume of 25 µl. Briefly, 650 ng of total RNA was incubated with 2 µl oligo(dT)s at 70°C for 5 min and cooled down on ice. The reverse transcription reaction consisted of 5 µl M-MLV RT 5X Buffer, 0.5 µl RNasin, 3.5 µl dNTPs (2.5 mM) and 1 µl M-MLV reverse transcriptase. Reverse transcription was carried out at 37°C for 90 min, followed by incubation at 65°C for 10 min to inactivate the enzyme.

RT-qPCR. Real-time qPCR based on SYBR Green I technology (Roche, Mannheim, Germany) was performed to amplify target sequences in triplicate, on a Roche LightCycler® 480 qPCR system (Roche). The expression of the candidate genes (Table 1) was normalized using Rplp0 as a housekeeping gene (ribosomal protein, large, P0; QuantiTect primer assay, Qiagen, Hilden, Germany). Each RT-qPCR reaction consisted of 7.5 µl containing 3 µl of 1:10-

Table 1. Primer sequences and PCR conditions for gene expression analysis.

FUNCTION	GENE	FORWARD PRIMER*	REVERSE PRIMER*
ENDOGENOUS CONTROL	RPLP0**	QuantiTect primer assay (Qiagen)	
	TUBD1	TCTCTTGCTAACTTGGTGGTCCTC	GCTGGGTCTTTAAATCCCTCTACG
TARGET GENES	ZFP57	GGCATTACCAAACAATGGCAG	TTCCCATTCTTCCTGGGTGAAA
	RNF39	GCTCATTGGACTTGCTCTTCG	GCTTCTCCAGGCAAGATACG
	PPP1R11	GGTGTCTCATCCCGCTTTCT	TCTGATTCTCTGGCTCGGTTG
	PEG3	TACCATCACGAAGACGACACC	GTTTCTCTCCCACTTCGGCT
	NNAT	AGCCGGGAACAAAGACTCAG	GAGTACCTGAACACCGCGT
	H19	CCTCAAGATGAAAGAAATGGTGCTA	TCAGAACGAGACGGACTTAAAGAA

* *The primer pairs were designed to span exon-exon junctions to ensure amplification of only mRNA*

diluted cDNA, 0.6 µl forward-reverse primer mix (10 µM each primer), 0.15 µl nuclease-free water, and 3.75 µl SYBR Green I Master. For each RT-qPCR run, a reverse transcription negative control and a non-template negative control were used to confirm the absence of genomic DNA, and to check for primer-dimer, respectively. The cycling conditions were as follows: an initial pre-incubation step at 95°C for 10 min, 50 cycles with denaturation (10s at 95°C), annealing (10s at 60°C) and elongation (10s at 72°C), followed by
a final cooling phase at 4°C.

Hippocampal RNA isolation. RNA extraction from the hippocampus followed a similar protocol as described above, with slight modifications. Tissue lysis was performed using stainless steel beads (5mm diameter, Qiagen) in a TissueLyser II (Qiagen). An additional RNA clean-up step was carried out using the butanol/ether extraction method. Briefly, RNA was mixed with 500 µl of water-saturated 1-butanol, which allowed for phase separation of potential organic contaminates. The organic upper phase was removed and discarded. After three extractions with water-saturated 1-butanol, 500 µl of water-saturated diethylether was used to clear out residual 1-butanol. This step was repeated once and then the diethylether was allowed to evaporate leaving the open tubes under a fume hood for 15 min until the characteristic odour of the diethylether was gone. RNA concentration and quality assessment was performed again using the NanoDrop, and 260/280 and 260/230 ratios of over 1.8 were obtained for all samples.

RT and RT-qPCR. Reverse transcription reactions were performed using the GoScript Reverse Transcription System (Promega). RNA (25 µg/µl) was mixed with 1 µl random primers and nuclease-free water for a total volume of 5 µl. The samples were heated at 70°C for 5 minutes and then chilled on ice for 5 minutes. Reverse transcription reaction mix consisted of 4 µl GoScript^{TM} 5X Reaction Buffer, 3 µl MgCl2, 1 µl PCR nucleotide mix (10mM), 0.5 µl Recombinant RNasin® Ribonuclease Inhibitor, 1 µl GoScriptTM Reverse Transcriptase, and 5.5 µl nuclease-free water. Tthe samples were incubated at 25°C for 5 min (annealing), 42°C for 60 min (extention), and 70°C for 15 min (reverse transcriptase inactivation). The samples were stored at −20°C. Real-time quantification was performed following the same procedure as described above, except Tubd1 was used as an internal reference gene.

Statistical analysis. Differences in gene expression were calculated from triplicates Ct values following the $2^{-\Delta\Delta CT}$ method. Statistical comparisons between conditions were performed using a two-tailed Student's t-test, with significance levels at $p<0.05$. All results were reported as mean ± standard error of the mean (SEM).

2.2. DNA methylation analysis

DNA isolation. Whole blood DNA was isolated from whole blood by using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. The amount of whole blood used as source of genomic DNA varied between 80 and 200 µl, and the samples with lower volume were adjusted to 200 µl with PBS. DNA quality and concentrations were assessed using spectrophotometry (NanoDrop Technologies, Inc.).

Bisulfite treatment and PCR. DNA denaturation and bisulfite treatment were performed into one-step using the EZ DNA Methylation-Gold™ Kit according to the manufacturer's instructions (Zymo Research, Hornby, Canada). The PCR and sequencing primers were designed using the PyroMark Assay Design Software (Version 2.0, Qiagen). Then, 150 ng of DNA was amplified with fragment-specific 5'-3' primers for the ZFP57 and RNF39 genes (Table 2). Each PCR mix contained 0.3 µl of each primer (100 µM), 0.6 µl dNTPs (10 mM), 2.4 µl $MgCl_2$, 6 µl of 5x Flexi Buffer, 0.3 µl HotStar Taq polymerase, and 1 µl bisulfited treated DNA in a total volume of 30 µl. PCR conditions were as follows: an initial pre-incubation at 95°C for 10 min and 45 cycles of denaturation (95°C for 30 s), annealing (58°C for 30 s) and elongation (72°C for 60 s).

Pyrosequencing. The percentage of DNA methylation (DNAme) at each CG site was quantified by pyrosequencing using PyroMark Q24 Reagents (Qiagen) according to the manufacturer's instructions. Briefly, to purify and render the single-stranded PCR product, a biotinylated reverse primer was immobilized on Sepharose beads. The Sepharose beads containing the PCR product were purified, washed, and denatured. Then, the sequencing primer was hybridized to the complementary single-stranded template and the pyrosequencing reaction was carried out by sequential addition of nucleotides in a specific order on a PyroMark Q24 machine (Qiagen). The software provided the quality control and the percentage of methylation at each

Table 2. Primer sequences and PCR conditions for bisulfite pyrosequencing analysis.

GEN	REGION	PRIMER SET	FORWARD PRIMER	REVERSE PRIMER *	SIZE	SEQUENCING PRIMER
ZFP57	Promoter Region	ZFP57-1	AAGTGTTAGGATGA-TAGGTATGTATT	ACACTCCAC-CTTTATCTAACTCAA	248	TTTTTTATATTTTTTAAATTAAGTT
	Exon 2	ZFP57-2	AGTAGAAAGGAAGTAGAGGGA-TAAAG	AACCACAAC-CACACAAAAAACTAA	129	AGGGTTTTTTTTTGGGTTTTA
		ZFP57-3	TGGTAAGGGTTTTTTTTT-GGGTTTTA	ACTTCTCCCACCCAAATAT	235	GTAGGATTGTAGAGTTTTAAGTAG
RNF39	CTCF4	RNF39-1	AGAGTTTATTTT-GTTAGGTGGTGTTA	ACTAAAAAC-CTAAAACTCAAACTTCT	230	TTTTTTTGTTTTGTATTTGTGAT
		RNF39-2	AGAGTTTATTTT-GTTAGGTGGTGTTA	ACTAAAAAC-CTAAAACTCAAACTTCT	230	GGATTTGGATTGGGAG
		RNF39-3	AGAGTTTATTTT-GTTAGGTGGTGTTA	ACCCCCCTCCCAATCCAAAT	73	GGTGGTGTTAGGTTT

** Reverse primers were 5'-biotinylated for subsequent pyrosequencing analysis*

CpG site, and the methylation of target CpGs was evaluated by comparing the MSUS averaged methylation levels to the controls at each CpG. To detect regions of statistically significant differential methylation, the multiple t-test analysis was applied, with the level of significance at $p<0.05$.

2.3. miRNA expression analysis

miRNA targets. Two-step selection was performed to identify serum miRNAs for further analysis. Based on the sequencing data from the human cohort PRISMO, miRNA targets were selected according to differential expression above a fold-change threshold (the cut-off values for differential expression were $-1 \geq \log2$ fold change ≥ 1). Then, the selected miRNAs were screen for their expression in mouse serum using the miRDB and miRBase databases. The expression of the selected miRNA targets, i.e. miR-34b, miR-138, miR-146a, miR-370, was profiled for each RT sample.

Blood serum isolation. To prepare serum, blood was allowed to clot overnight at 4°C, centrifuged for 10 min at 2,000 x *g* at 4°C, and then serum was collected and stored at -80°C until further processing.

miRNA spike-in control. Using an endogenous synthetic miRNA mimic, that is only expressed in *Caenorhabditis elegans* and not in mammals, is currently the method of choice for a reliable relative quantification of serum miRNAs in both human and rodent studies (Tomasetti et al., 2012) The expression levels of target miRNAs were normalized to a synthetic cel-miR-39 spike-in control (miRNeasy Serum/Plasma Spike-In Control, Qiagen). It was reconstituted in 300 µl nuclease-free water, and then diluted to prepare the working solutions for RNA purification and generation of standard curve.

Serum RNA extraction. For total RNA extraction, 150-200 µl of serum was diluted with nuclease-free water to adjust the reaction volume to 250 µl, and 1 ml of TRI-Reagent (Sigma-Aldrich, Poole, United Kingdom) was added. The mixture was homogenized using a sterile 1 ml syringe and a 26G needle and incubated at room temperature for 5 min. Then, 3.5 µl (1.6×10^8 copies/µl,) of synthetic cel-miR-39 mimic was spiked into the mixture prior to addition of 200 µl chloroform. To note, fresh cel-miR-39 working solutions were prepared for each set of isolation experiments as recommended by the manufacturer. Samples were mixed and incubated at room temperature for 3 min. Phase separation was performed by centrifugation at 12,000 × *g* at 4°C for 15 min in an Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany). After an additional chloroform extraction, RNA precipitation was performed by overnight incubation at -20°C in a solution containing 500 µl isopropanol and 15 µl glycogen. To recover the precipitated RNA, the samples were centrifuged, the supernatant discarded,

and the pellet washed with 70% ethanol twice by centrifugation at 7,600 × *g* at 4°C for 5 min. The RNA pellet was dried for 10 min at room temperature and eluted in 13 µl of nuclease-free water. Due to the low amount of RNA obtained from serum samples, the quality and quantity of extracted RNA were assessed using two different technologies: spectrophotometry (NanoDrop Technologies, Inc.) and fluorometry, more sensitive for low input RNA samples (Qubit® RNA HS Assay Kit with a Qubit® 2.0 Fluorometer, Life Technologies). In general, the RNA yield was 3-13 ng per 100 µl serum.

Reverse transcription. To detect and quantify mature miRNAs a single-step cDNA synthesis was performed using the miScript II RT Kit (Qiagen) according to the manufacturer's instructions. In brief, 12 µl of total RNA (2.5 ng/µl) was combined with 4 µl 5x HiSpec Buffer, 2 µl 10x Nucleic Mix and 2 µl miScript RT Mix in a total reaction volume of 20 µl. The mixture was incubated at 37°C for 60 min, followed by enzyme deactivation at 95°C for 5 min, and then held at 4°C. RT products were diluted in 200 µl nuclease-free water and stored at -20°C until further RT-qPCR analysis.

microRNA RT-qPCR. RT-qPCR was performed in triplicates for each sample, using the miScript SYBR Green PCR kit (Qiagen) and the miScript Primer Assays (Qiagen) according to the manufacturer's recommendations. Spiked-in cel-miR-39 was detected with the Ce_miR-39_1 miScript Primer Assay (Qiagen). In more detail, each reaction consisted of a 10 uL mix containing 2 µl of diluted RT product, 5 µl 2x QuantiTect SYBR Green PCR Master Mix, 1 µl 10x miScript Universal Primer, and 1 µl of the corresponding 10x miScript Primer Assay. RT qPCRs were run in 384-well plates on the Roche LightCycler® II 480 (Roche). The thermal cycling parameters included pre-incubation at 95°C for 15 min; 52 cycles of amplification at 94°C for 15 s (denaturation), 55°C for 30 s (annealing) and 70°C for 30 s (elongation).

Absolute quantification. To generate a standard curve, 2 µl ($4x10^9$ copies/µl) of cel-miR-39 spike-in control were mixed with 78 µl RNase-free water containing 10 ng/µl MS2 (Roche). Reverse transcription was performed using the Qiagen miRNeasy Serum/Plasma protocol. The synthesized cDNA was diluted in 200 µl nuclease-free water resulting in concentration of $1x10^6$ copies/µl, and subsequently was subjected to four serial dilutions according to the manufacturer's instruction. Then, 2 µl of each dilution ($1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$ copies) was amplified in triplicate. After extracting Ct values and estimating the mean Ct for each dilution, the standard curve was generated by plotting the concentration of cel-miR-39 used in each PCR reaction against the mean Ct value. Then, to assess cel-miR-39 recovery after miRNA purification, a fixed amount of input RNA from biological samples were first re-

verse-transcribed and then amplified by PCR. The levels of cel-miR-39 were determined from the Ct values obtained using biological samples.

3. RESULTS

To perform a comparative analysis of trauma-induced biological changes in humans and mice, molecular effects of MSUS were examined in adult mice and compared to the data previously obtained in the PRISMO human cohort, in terms of mRNA expression, DNAme, and miRNA expression.

3.1. Expression of candidate genes in mouse blood

To investigate the involvement of candidate genes in MSUS mice response to trauma, gene expression analysis was carried out by RT-qPCR in F1 MSUS male whole blood samples. Six target genes were pre-selected from the PRISMO study, namely ZFP57, RNF39, PPP1R11, NNAT, PEG3, H19. Interestingly, the mRNA expression of ZFP57, NNAT, PEG3, and H19 was not quantifiable by means of RT-qPCR. Additionally, a low mRNA expression was found for RNF39, however not suitable for any statistical analysis. To note, the only possible intergroup analysis was performed for the PPP1R11 gene. However, no significant change ($p=0.67$) was detected in the MSUS group compared to controls (Fig.1).

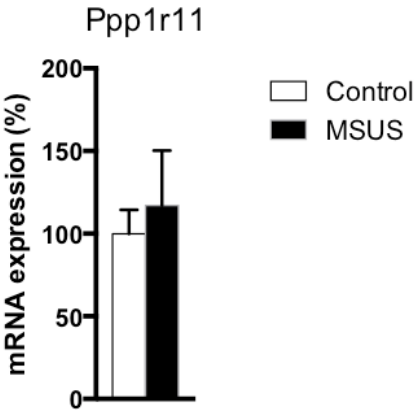


Figure 1. PPP1R11 expression in mouse whole blood. Male MSUS (n=8) compared to controls (n=8). Data represent mean ± SEM. Data is expressed as relative to the control group; Rplp0 was used as an internal reference gene.

3.2. ZFP57 and RNF39 methylation levels in mouse blood

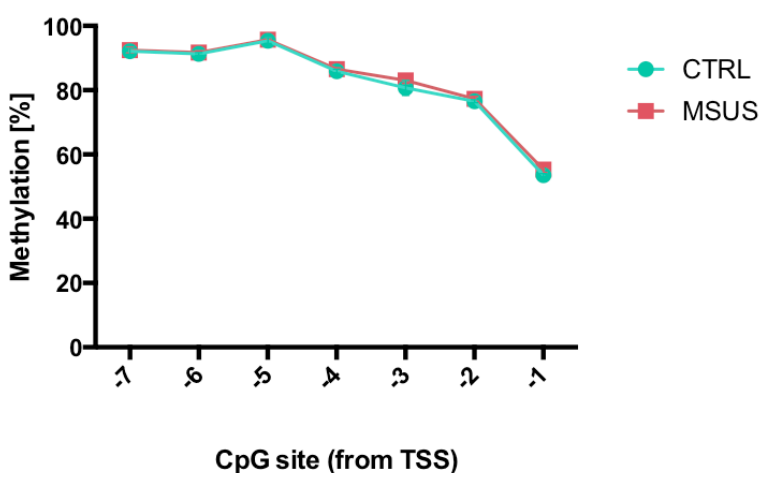
To discern whether similar alterations arise in DNAmet profiles between human and mice after trauma exposure, a targeted quantitative CpG methylation analysis for ZFP57 and RNF39 was performed. Several regions of potential regulatory function were chosen for both genes using the Ensembl genome browser. Since promoter DNAmet is known to repress gene transcription, CpGs within the ZFP57 promoter region were selected for further analysis. The

second exon of the ZFP57 gene was also included, as it was found to be highly homologous to the human region, showing an aberrant methylation pattern in the PRISMO cohort. The CCCTC-binding factor (CTCF) is known to affect gene regulatory mechanisms by blocking the communication between an enhancer and gene promoter, thereby inhibiting transcriptional activation. Additionally, the CTCF-binding sequence contains CpGs and therefore can be epigenetically controlled via DNAme. While CTCF preferably binds to unmethylated regions, loss of CTCF binding and thus its insulator function has been detected in methylated DNA sequences (Holwerda & de Laat, 2013). Given that, out of five CTCF-binding sites in the RNF39 gene, the CTCF-binding site 4 was selected for further examination due to its proximal localization to the PPP1R11 gene. It was hypothesised that under an aberrant methylation within the CTCF-binding site 4, the abrogation of RNF39 and/or PPP1R11 gene activity would be observed. Importantly, the CTCF-binding site also partially overlaps with the second RNF39 CpG island, which makes it an interesting target.

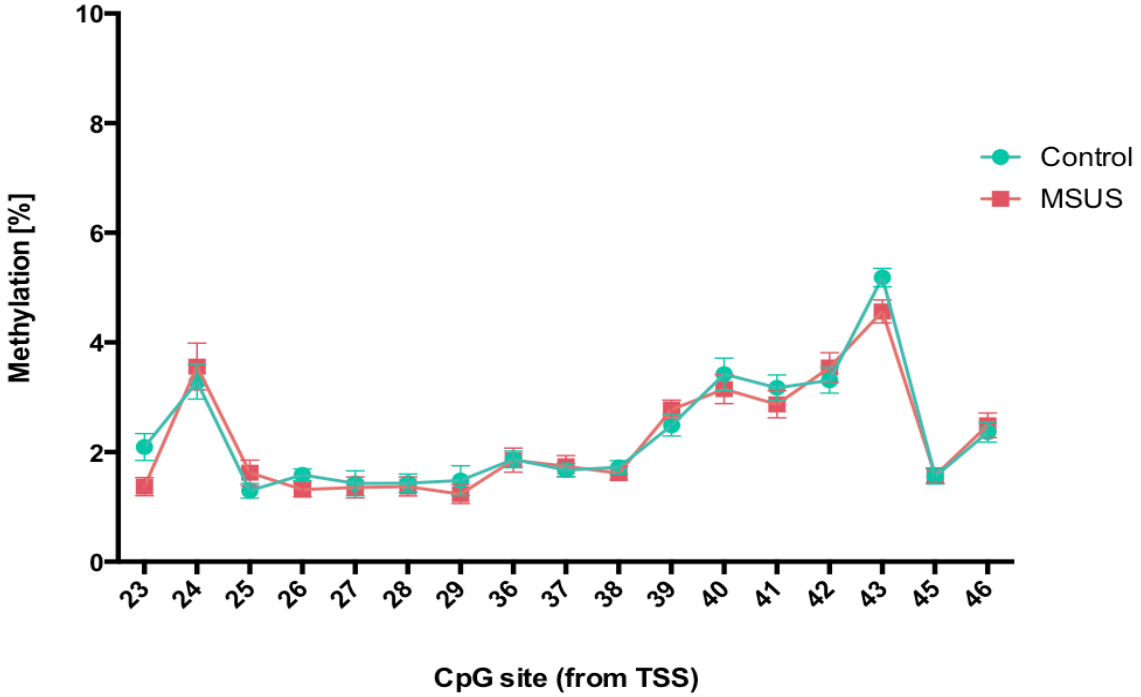
Both groups MSUS and controls showed high levels (>50%) of DNAme in the ZFP57 promoter region, nonetheless, the difference between the groups remained non-significant (Fig.2A). As represented in Fig.2B, non-significant group effect was found in the CpGs within the second exon of the ZFP57 gene in MSUS. Also, analysing the RNF39 gene, no significant change in the methylation levels in the CTCF-binding site 4 was detected (Fig.2C).

Figure 2. Methylation levels in the ZFP57 and RNF39 genes. (A) ZFP57 promoter region. (B) ZFP57 Exon 2. (C) RNF39 CTCF-binding site 4. MSUS males (n=10) compared to controls (n=10); whole blood.

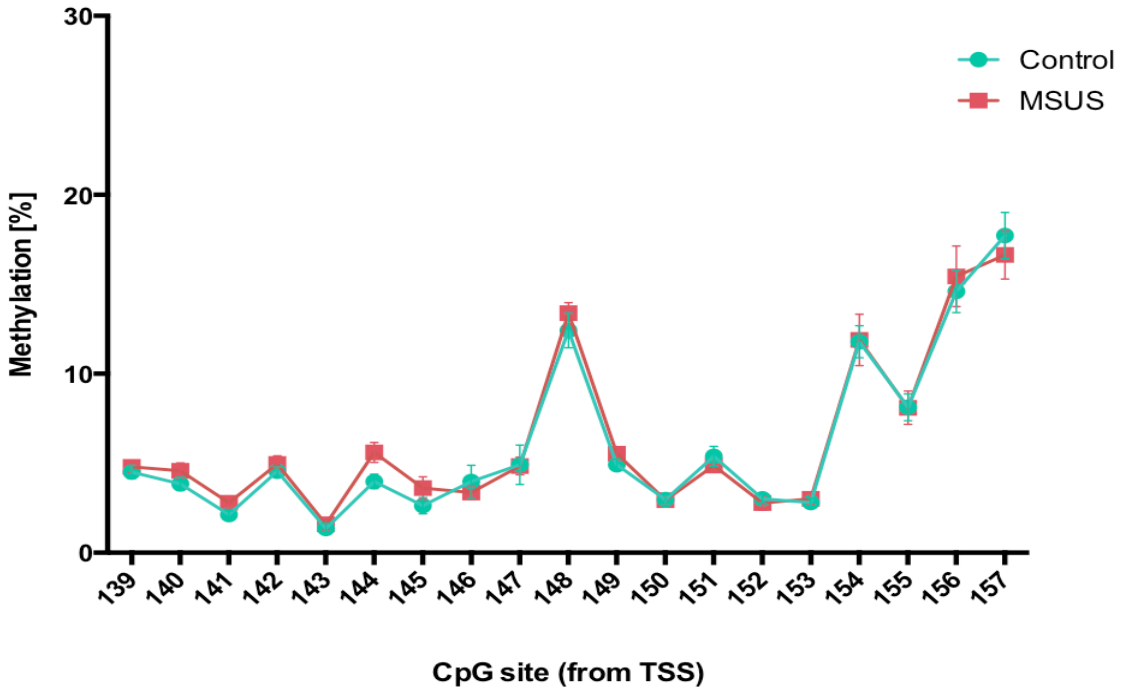
A.



B.



C.



3.3. PRISMO derived serum miRNAs are down-regulated in F1 MSUS males

To investigate whether miRNAs, found to have an altered expression in the PRISMO sequencing data, are also aberrantly expressed in the MSUS model, an analysis of miRNA expression profiles from serum samples was performed. RT-qPCR analysis showed that miR-34b, (p=0.04), miR-146a (p=0.02), miR-146b (p=0.03), miR-370 (p=0.01) were significantly decreased in the serum of MSUS males relative to controls (Fig. 3). No significant changes in serum miR-138, miR-195 and miR-144 (p>0.05) were observed between the MSUS group and controls.

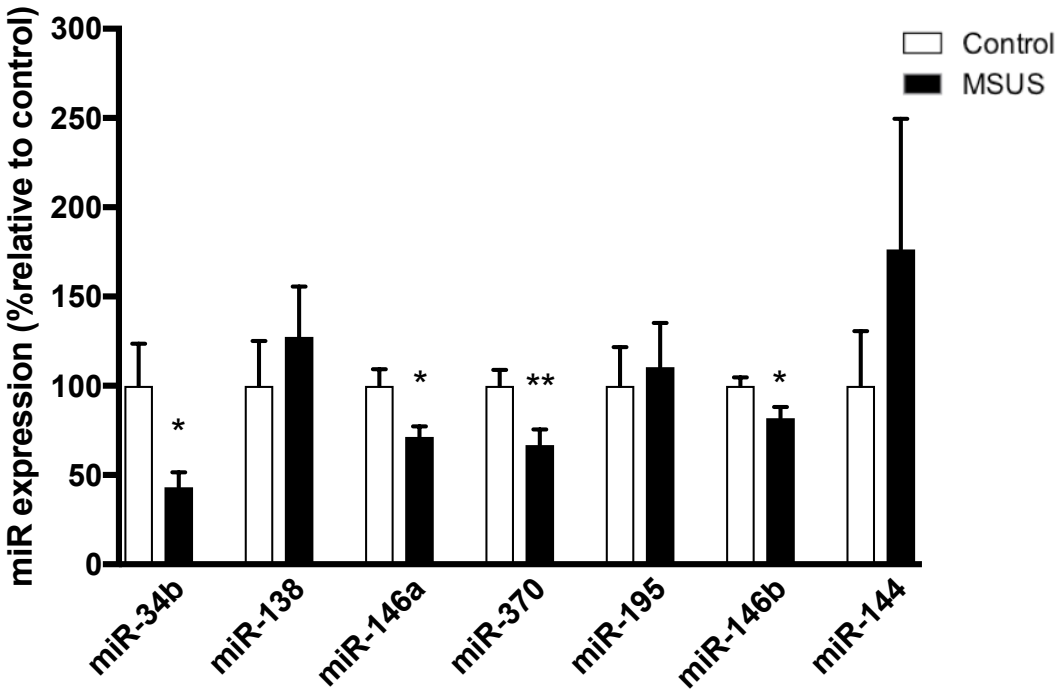


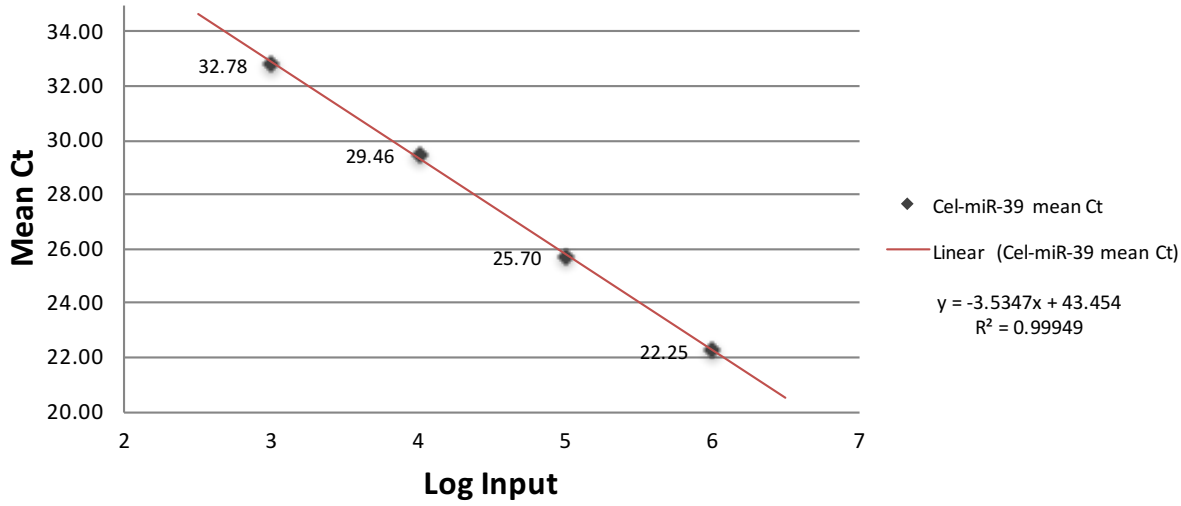
Figure 3. miRNA expression in F1 control and MSUS adult males. RT-qPCR in serum: miR-34b, miR-138, miR-146a, miR-146b, miR-370, miR-195, miR-144. MSUS males (n=12) compared to controls (n=13). Data represent mean ± SEM. *p ≤ 0.05, **p ≤ 0.01.

3.4. Spike-in control recovery after RNA purification

To control for RNA recovery and RT efficiency a standard curve was generated. Data presented in the Fig. 4A suggest a good linear relationship ($R^2 > 0.999$). Also, high amplification efficiency (91.82%, slope = -3.535) and consistency across replicate reactions proved the well-optimized qPCR assay. The amount of synthetic cel-miR-39 added to the sample during RNA isolation was compared to the concentration of cel-miR-39 recovered from the sample

after miRNA extraction. The extraction efficiency expressed as a recovery percentage was determined. Two groups of samples are represented in the Fig.4. Unlike MSUS1, MSUS2 sera were subjected to multiple freezing and thawing cycles, which reduced recovery efficiency. Without repeated sample handling, MSUS1 showed cel-miR-39 levels of 60±11% compared to 45±9% in MSUS2, suggesting that multiple freeze-thaw cycles affect serum RNA quality.

A.



B.

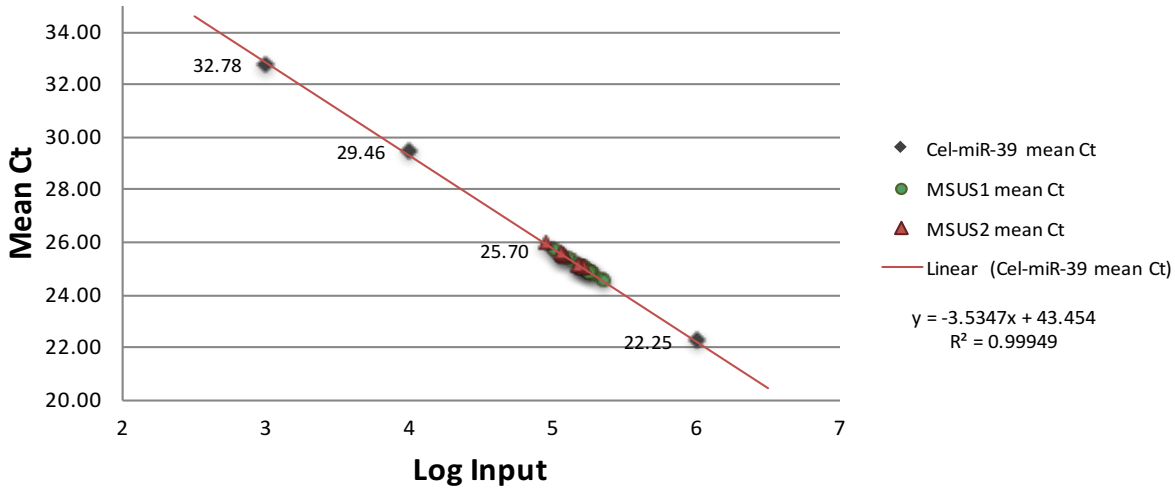


Figure 4. Cel-miR-39 recovery after miRNA purification. (A) Generation of the standard curve. (R^2 > 0.999; amplification efficiency = 91.82%; slope = -3.535). (B) Quantification of cel-miR-39 levels in the biological samples.

3.5 Insults in adult life affect gene expression in F2 MSUS male hippocampus

To evaluate the extent to which an additional exposure to stressful stimuli in adult life could affect the mRNA expression of the same candidate genes in MSUS male mice, an additional RT-qPCR analysis on hippocampus samples from F2 MSUS exposed to forced swim in adulthood (6 months-old at the time of testing) was performed. As shown in Fig.4A, when non-exposed MSUS adult males were compared to controls, only RNF39 gene expression was significantly increased ($p=0.04$). By contrast, MSUS mice after additional challenges in adult life revealed not only elevated RNF39 transcript levels ($p=0.02$), but also significantly decreased expression of ZFP59 ($p=0.01$) and its imprinted gene NNAT ($p=0.000$), as depicted in Fig.5B.

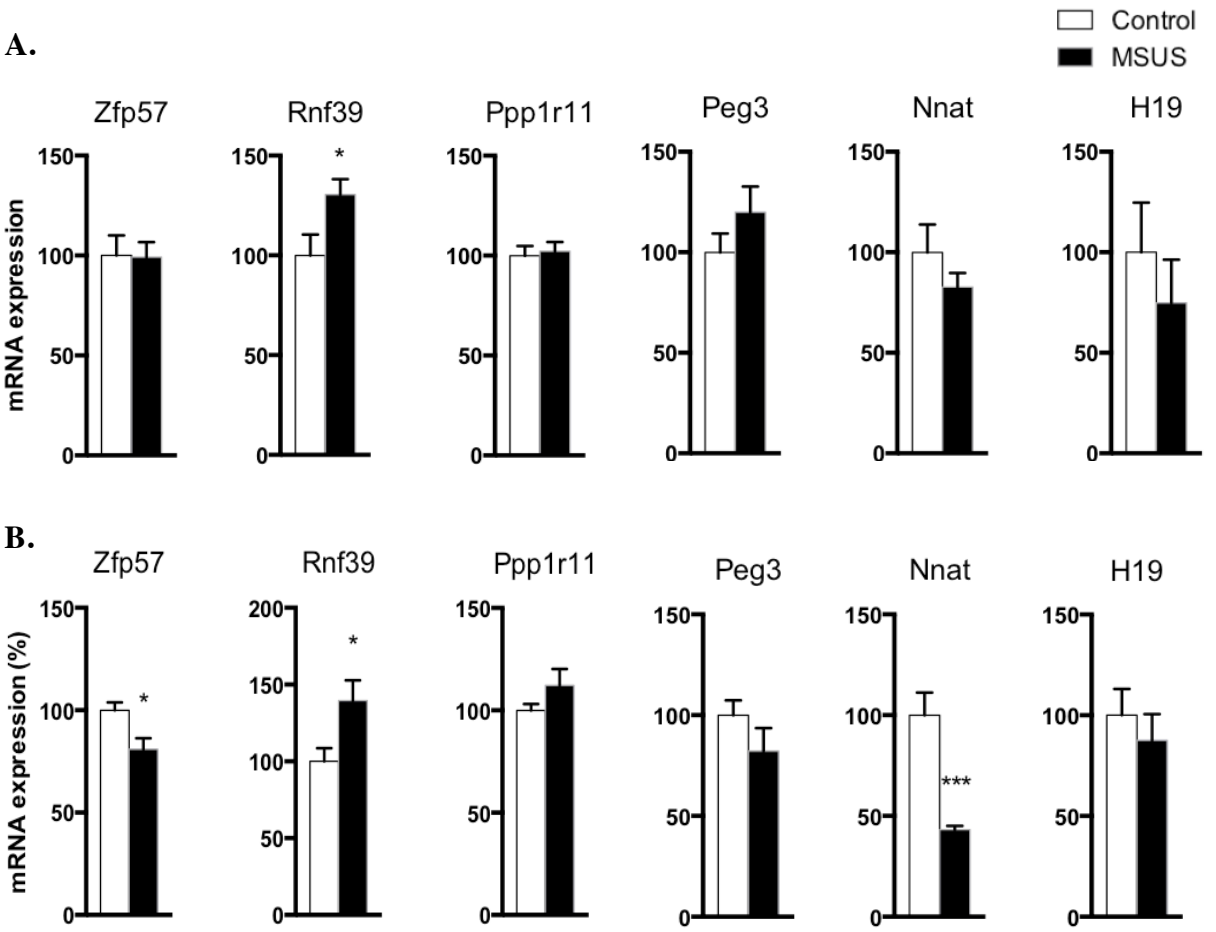


Figure 5. Altered gene expression in F2 MSUS males. RT-qPCR in hippocampal tissue: ZFP57, RNF39, PPP1R11, NNAT, PEG3, H19. (A) Non-exposed MSUS adults (n=8) compared to controls (n=8). (B) Exposed MSUS adults (n=8) compared to controls (n=10). Data is expressed as relative to the control group; Tubd1 was used as an internal reference gene. Data represent mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$.

4. DISCUSSION

There is strong evidence that early-life trauma can be a trigger factor for the development of physiological and/or psychiatric disorders (Aas et al., 2014; Álvarez et al., 2011; Pace et al., 2006). In turn, adult trauma has also drawn research attention since its neurobiological and psychological sequelae can lead to health impairments (Wagner, Wolfe, Rotnitsky, Proctor, & Erickson, 2000). Importantly, both early-life trauma and adult trauma have been shown to trigger neuroendocrine, immune and metabolic dysfunction (Chrousos, 2009), which suggests the underlying mechanisms would overlap or early-life trauma would be merely a risk factor for PTSD. In this cross-species comparison of trauma-induced molecular changes, specific biological substrates of early-life trauma were investigated in MSUS model and compared to those reported in adult soldiers with PTSD.

4.1. Interspecies differences in DNA methylation following trauma exposure

ZFP57, a gene encoding a zinc finger protein 57, has been identified as a key factor in genomic imprinting by maintaining germline-derived methylation during not only early development, but also in later life (Li et al., 2008; Plant et al., 2014; Quenneville et al., 2012). Mechanism by which ZFP57 controls the somatic maintenance of DNAme involves KRAB-associated protein 1 (KAP1), and KAP1-deficient mice exhibited an anxiety-like phenotype (Jakobsson et al., 2008). RNF39 encodes ring finger protein 39 and is regarded as a plasticity-associated gene, since its implication has been documented in studies involving the long-term potentiation (LTP) (Matsuo, Asada, Fujitani, & Inokuchi, 2001). Notably, RNF39 implication has been investigated in the context of fear memory formation and synaptic plasticity, and its increased expression has been induced in the dentate gyrus and lateral amygdala, following LTP and classical conditioning, respectively (Ploski, Park, Ping, Monsey, & Schafe, 2010). Recently, another study has reported RNF39 up-regulation, relative to non-stressed controls, following glucocorticoid challenge in animals previously exposed to chronic restraint stress (Datson et al., 2013).

Significant and consistent changes in DNAme of ZFP57 and RNF39 reported in the PRISMO study encouraged the further investigation of these genes in MSUS. However, no significant DNAme alterations of ZFP57 and RNF39 were found in adult MSUS male mice relative to controls. This apparent discrepancy in the results could be explained by few factors. Firstly, DNAme levels were measured exclusively in selected regions of the abovementioned genes in MSUS model, compared to the genome-wide DNAme profiling in the PRISMO study. Secondly, there exists the difference between the human and rodent physiology which would contribute to divergent trauma-induced neurobiological effects. Finally, these

inconsistent findings could suggest the biological dissimilarity between the biological substrates underlining early-life and adult trauma.

4.2. Hippocampal gene expression alterations in MSUS offspring exposed to an additional stress during adulthood

DNAme was analysed together with gene expression of ZFP57, RNF39 and other genes. Surprisingly, mRNA analysis in both whole blood and white blood cells (WBC) revealed no expression of ZFP57 and its imprinted genes (NNAT, PEG3, H19), and very low expression of RNF39. The fact that ZFP57 was not expressed in blood remains consistent with the previous observations in humans (Dr Rutten's laboratory) and rats (Dr Yehuda's laboratory). Also, literature supports a crucial role of ZFP57 in embryogenesis and its expression primarily limited to ovaries and testes in adult tissues (Li et al., 2008). An additional analysis of transcription levels of the same genes but in the hippocampus provided interesting findings. The second generation MSUS mice were exposed to a further stress in adult life, which negatively affected the transcription of ZFP57 and NNAT genes, already found significant in F2 but not in F1 hippocampal tissue of unchallenged adult mice. Interestingly, an increased RNF39 transcriptional response has been previously detected following glucocorticoid challenge only in adult male rats with a history of chronic restraint stress (Datson et al., 2013). A growing evidence supports the RNF39 role in fear memory consolidation and the reported here findings show an aberrant RNF39 expression exclusively in F2, but not in F1 directly MSUS exposed mice. This raises the question of whether the hippocampal changes suggest a specific trauma-induced memory formation across generations, which would contribute to disease vulnerability following traumatic stress in adulthood.

4.3. From mice to humans: microRNA profiling and role analysis

One of the main findings in this project is the demonstration of aberrant miRNA levels in adult mice in association with early-life trauma exposure. A significant decrease in the expression of four miRNAs, i.e. miR-34b, miR-146a, miR-146b, and miR-370, was found. The miR-146 family is implicated in the control of inflammation signalling and innate immune response, and consists of two members, miR-146a and miR-146b, which as mature miRNAs differ by only two nucleotides at 3'UTR and thus their target mRNAs may overlap (Hurst et al., 2009; Saba, Sorensen, & Booth, 2014; Taganov, Boldin, Chang, & Baltimore, 2006). MiR-146a was shown to be a negative regulator of the TLR4-NFκB pathway in macrophages by targeting the IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) genes, thereby indirectly repressing the production of pro-inflammatory cytokines (Taganov et al., 2006). Furthermore, miR-146a expression serves as a negative regulato-

ry mechanism in the type I interferon (IFN) production by down-regulating IRAK1, IRAK2 and TRAF6 genes (Hou et al., 2009).

Here, the study's results show significantly reduced levels of both miR-146a and miR-146b, consistent with previous findings indicating an association between decreased miR-146 expression and overstimulated inflammatory state leading to pathophysiological conditions (Baldeón R. et al., 2014; Sato et al., 2010). To date, no evidence exists linking directly the miR-146 expression with either early-life or adult trauma; however it has been shown that inflammation plays a role in the pathophysiology of psychiatric diseases, including trauma-related disorders (Debnath et al., 2011). For instance, up-regulated pro-inflammatory cytokines responsible for chronic inflammation in PTSD patients have been related to cortisol dysfunction and an increased susceptibility to PTSD (Gill, Saligan, Woods, & Page, 2009). Moreover, individuals exposed to early-life trauma have revealed augmented inflammatory responses in adulthood (Baumeister, Akhtar, Ciufolini, Pariante, & Mondelli, 2016). Considering this, a further analysis could examine the mRNA expression of the miR-146 target genes, i.e. IRAK1, IRAK2 and TRAF6, and measure levels of pro-inflammatory molecules.

The mir-34 miRNA precursor family gives rise to three mature miRNAs, i.e. miR-34a transcribed from chromosome 1, and miR-34b and -34c encoded by the same primary transcript on chromosome 11, which have been shown to be involved in developmental pathways regulating cell-cycle restraint and apoptosis after stress, metabolic homeostasis in health and disease, but above all their role in tumour suppression has been demonstrated (Achari, Winslow, Ceder, & Larsson, 2014; Chakraborty, George Priya Doss, & Bandyopadhyay, 2013; Corney et al., 2010; Rokavec, Li, Jiang, & Hermeking, 2014; Xu et al., 2015). Notably, several studies have elucidated a potential function of the miR-34 family in the stress response pathway, however, the evidence does not converge on a single conclusion. The current results revealed a significant down-regulation of miR-34b in relation to early-life trauma. A very recent study has demonstrated an anxiolytic-like effects of overexpressed miR-34b on behavioural and neurochemical phenotypes in rats by targeting corticotropin-releasing factor receptor 1 (CRF_1 receptor). That is, an increased expression of miR-34b negatively regulated CRF_1 receptor mRNA levels, thereby decreasing hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis and anxiety-like behaviour (Zhu et al., 2017).

The HPA axis activation under stress triggers the secretion of hypothalamic corticotropin-releasing factor (CRF), which stimulates the release of pituitary adrenocorticotropin hormone (ACTH), followed by the secretion of glucocorticoids from the adrenal glands. The glucocorticoid negative control exerted upon ubiquitously distributed membrane receptors in the hypo-

thalamus, hippocampus, amygdala and prefrontal cortex (PFC) terminates the stress response and facilitates physiological homeostasis (Chrousos, 1998; Herman, McKlveen, Solomon, Carvalho-Netto, & Myers, 2012). However, attenuation of the feedback and HPA hyperactivity have been proposed as the brain-based hallmarks of trauma-related conditions, since chronically-stressed individuals' increased GC levels result in down-regulation of GC receptors and disinhibition of CRF release (Mizoguchi, Ishige, Aburada, & Tabira, 2003). Additionally, CRF hypersecretion can be enhanced by the CRF-CRF_1 receptor system itself as a positive feedback mechanism exists between them stimulating CRF production during stress (Ono et al., 1985). Unlike beneficial activity of the CRF-CRF_1 receptor signalling in acute threats, in the chronic stress responses it has been identified as a potential pathogenic factor in trauma-related disorders (Contoreggi, 2015), and interestingly, CRF_1 receptor antagonists efficiently minimised the neuroendocrine and behavioural responses to stress, without disturbing the HPA axis functioning (Habib et al., 2000). Furthermore, evidence suggests a critical role of forebrain CRF_1 receptor in mediating early-life trauma-induced neurobiological alterations leading to emotional and cognitive impairments in adulthood (X.-D. Wang et al., 2011; Xiao-Dong Wang et al., 2012). Intriguingly, the lack of forebrain CRF_1 receptor has been demonstrated to have a stress-protective effect on both cortical damage and cognitive function, and also to inhibit the hippocampal GR down-regulation after chronic stress exposure (Xiao-Dong Wang et al., 2011).

Taken together, the above discussion suggests that a further MSUS research could benefit from investigating trauma-induced phenotypic changes by exploring biological correlations involving miRNAs and HPA axis, especially with focus on miR-34b and CRF_1 receptor mRNA. It is important to note that also other members of the miR-34 family have been examined within the context of trauma-related research, and accordingly to the abovementioned findings, an advantageous effect of elevated miRNA-34c levels have been found in mouse models of both chronic and acute stress. The study has revealed that miRNA-34c silencing promoted anxiety-like behaviour, which was reverted by ectopic expression of miR-34c in vitro (Haramati et al., 2011). Contrariwise, it has been recently shown that deficiency of all three members of miR-34 family has a protective effect upon neurochemical signalling and behaviour, and is able to induce resilience to stress-induced anxiety-like phenotype (Andolina et al., 2016). Therefore, additional evidence is needed to address conclusively the issue whether the all miR-34 family plays role in adaptation to stressful encounters. In more detail, the following questions could be addressed, whether 1) all miR-34s are involved in trauma-induced neurobiological alterations in MSUS model, 2) there are differences in the mir-34

serum and brain expression levels, and 3) the miR-34 function is protective in healthy conditions and, thus the down-regulation would be proposed as a potential pathogenic factor after postnatal traumatic stress.

MiR-370 has been shown to be involved in several biologic and pathological processes, such as angiogenesis and tumorigenesis (Mollainezhad, Eskandari, Pourazar, Salehi, & Andalib, 2016; H. Zhang, Sun, & Hao, 2016). However, increasing evidence indicates its role in lipid metabolism, with emphasis on fatty acid oxidation and biosynthesis. Whereas miR-370 indirectly stimulates lipogenesis through up-regulation of miR-122 and its target genes, the fatty acid β-oxidation is directly regulated by miR-370-induced down-regulation of carnitine palmitoyl transferase 1α (Cpt1α), a key enzyme in fatty acid catabolism (Iliopoulos, Drosatos, Hiyama, Goldberg, & Zannis, 2010). Interestingly, it has been proposed that miR-370 up-regulation, which reduces the fatty acid β-oxidation and promotes the biosynthesis of lipids, could contribute to the accumulation of hepatic triglycerides, and thereby inducing disease (Iliopoulos et al., 2010). Additionally, there is research evidence that supports the miR-370 role in type 2 diabetes mellitus and coronary artery disease that is a lipid-driven, chronic inflammatory disease of the vessels with fatty deposits. The data suggest a positive association between miR-370 levels and metabolic markers, including body mass index, fasting plasma glucose, total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, LDL/HDL ratio, (Liu et al., 2016; Motawae et al., 2015), and a negative relationship with high-density lipoprotein (HDL), that is considered anti-atherogenic good cholesterol (Vergeer, Holleboom, Kastelein, & Kuivenhoven, 2010). Taken together, these findings may indicate a deleterious impact of miR-370 overexpression on the metabolic pathways. Consistent with these findings, an increased amounts and activity of miR-370 have been related to aberrant lipid metabolism in type 2 diabetic mice (X. Zhang et al., 2016). Nonetheless, an opposite pattern of miR-370 expression was found in the present research, i.e., relative to controls, it was significantly decreased. Since there have been observed metabolic abnormalities in relation to early-life traumatic stress (Vargas et al., 2016), it would be a potential research line to investigate whether miR-370 down-regulation in MSUS mice affects the downstream metabolic gene targets, leading to their overexpression and thereby increasing fatty acid β-oxidation and reducing lipogenesis. As previously shown, imbalance between fatty acid oxidation and synthesis involves mainly hepatic and adipose tissue processes leading to metabolic disturbances. Therefore, serum mir-370 expression should be compared with its hepatic and adipose tissue expression. Notably, miR-122 has been found highly abundant in the liver (Lagos-Quintana et al., 2002), hence it would be interesting to examine the miR-122 expres-

sion and identify its relationship to miR-370 in the regulation of metabolic pathways in MSUS model. Finally, studies have also identified a link between the presence of lipid alterations and a diagnosis of PTSD (Maia et al., 2008), which encourages the validation by real-time expression profiling of miR-370 in the PRISMO cohort.

To conclude, these findings provide evidence on the dysregulation of circulating miRNAs in relation to early traumatic stress. Further research would be necessary to explore their potential implication in downstream effectors of the neuroendocrine, immune and metabolic pathways. Furthermore, their validation in serum from PTSD patients would be crucial providing more insight into the underlying mechanisms of PTSD.

5. CONCLUSIONS

The purpose of the current master's thesis was to investigate whether there are similarities between neurobiological bases of human adulthood trauma and rodent early-life trauma, in terms of gene expression, DNAme, and miRNA expression. The DNAme changes in the ZFP57 and RNF39 genes resulting from combat trauma were not found in MSUS male blood. Serum miRNA profiling showed aberrant expression of miR-34b, miR-146a, miR-146b and miR-370 in MSUS male mice. A subsequent literature review of these miRNAs suggested its potential implication in the neuroendocrine, immune and metabolic downstream signalling pathways. Future research would benefit from the current findings by continuing the investigation of trauma-induced phenotypic changes.

6. REFERENCES

Aas, M., Haukvik, U. K., Djurovic, S., Tesli, M., Athanasiu, L., Bjella, T., … Melle, I. (2014). Interplay between childhood trauma and BDNF val66met variants on blood BDNF mRNA levels and on hippocampus subfields volumes in schizophrenia spectrum and bipolar disorders. *Journal of Psychiatric Research*, *59*, 14–21. https://doi.org/10.1016/j.jpsychires.2014.08.011

Achari, C., Winslow, S., Ceder, Y., & Larsson, C. (2014). Expression of miR-34c induces G2/M cell cycle arrest in breast cancer cells. *BMC Cancer*, *14*(1), 538. https://doi.org/10.1186/1471-2407-14-538

Álvarez, M.-J., Roura, P., Osés, A., Foguet, Q., Solà, J., & Arrufat, F.-X. (2011). Prevalence and Clinical Impact of Childhood Trauma in Patients With Severe Mental Disorders. *The Journal of Nervous and Mental Disease*, *199*(3), 156–161. https://doi.org/10.1097/NMD.0b013e31820c751c

Anda, R. F., Felitti, V. J., Bremner, J. D., Walker, J. D., Whitfield, C., Perry, B. D., … Giles, W. H. (2006). The enduring effects of abuse and related adverse experiences in childhood. *European Archives of Psychiatry and Clinical Neuroscience*, *256*(3), 174–186. https://doi.org/10.1007/s00406-005-0624-4

Andolina, D., Di Segni, M., Bisicchia, E., D'Alessandro, F., Cestari, V., Ventura, A., … Ventura, R. (2016). Effects of lack of microRNA-34 on the neural circuitry underlying the stress response and anxiety. *Neuropharmacology*, *107*, 305–316. https://doi.org/10.1016/j.neuropharm.2016.03.044

Atwoli, L., Stein, D. J., Koenen, K. C., & McLaughlin, K. A. (2015). Epidemiology of posttraumatic stress disorder. *Current Opinion in Psychiatry*, *28*(4), 307–311. https://doi.org/10.1097/YCO.0000000000000167

Baldeón R., L., Weigelt, K., de Wit, H., Ozcan, B., van Oudenaren, A., Sempértegui, F., … Leenen, P. J. M. (2014). Decreased Serum Level of miR-146a as Sign of Chronic Inflammation in Type 2 Diabetic Patients. *PLoS ONE*, *9*(12), e115209. https://doi.org/10.1371/journal.pone.0115209

Bam, M., Yang, X., Zumbrun, E. E., Zhong, Y., Zhou, J., Ginsberg, J. P., … Nagarkatti, M. (2016). Dysregulated immune system networks in war veterans with PTSD is an outcome of altered miRNA expression and DNA methylation. *Scientific Reports*, *6*(1), 31209. https://doi.org/10.1038/srep31209

Baumeister, D., Akhtar, R., Ciufolini, S., Pariante, C. M., & Mondelli, V. (2016). Childhood

trauma and adulthood inflammation: a meta-analysis of peripheral C-reactive protein, interleukin-6 and tumour necrosis factor-α. *Molecular Psychiatry*, *21*(5), 642–649. https://doi.org/10.1038/mp.2015.67

Binder, E. B., Bradley, R. G., Liu, W., Epstein, M. P., Deveau, T. C., Mercer, K. B., … Ressler, K. J. (2008). Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *JAMA*, *299*(11), 1291–305. https://doi.org/10.1001/jama.299.11.1291

Bremner, J. D., Southwick, S. M., Johnson, D. R., Yehuda, R., & Charney, D. S. (1993). Childhood physical abuse and combat-related posttraumatic stress disorder in Vietnam veterans. *American Journal of Psychiatry*, *150*(2), 235–239. https://doi.org/10.1176/ajp.150.2.235

Chakraborty, C., George Priya Doss, C., & Bandyopadhyay, S. (2013). miRNAs in insulin resistance and diabetes-associated pancreatic cancer: the 'minute and miracle' molecule moving as a monitor in the 'genomic galaxy'. *Current Drug Targets*, *14*(10), 1110–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23834149

Chrousos, G. P. (1998). Stressors, Stress, and Neuroendocrine Integration of the Adaptive Response: The 1997 Hans Selye Memorial Lecture. *Annals of the New York Academy of Sciences*, *851*(1 STRESS OF LIF), 311–335. https://doi.org/10.1111/j.1749-6632.1998.tb09006.x

Chrousos, G. P. (2009). Stress and disorders of the stress system. *Nature Reviews Endocrinology*, *5*(7), 374–381. https://doi.org/10.1038/nrendo.2009.106

Contoreggi, C. (2015). Corticotropin releasing hormone and imaging, rethinking the stress axis. *Nuclear Medicine and Biology*, *42*(4), 323–39. https://doi.org/10.1016/j.nucmedbio.2014.11.008

Corney, D. C., Hwang, C.-I., Matoso, A., Vogt, M., Flesken-Nikitin, A., Godwin, A. K., … Nikitin, A. Y. (2010). Frequent downregulation of miR-34 family in human ovarian cancers. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *16*(4), 1119–28. https://doi.org/10.1158/1078-0432.CCR-09-2642

Daskalakis, N. P., Bagot, R. C., Parker, K. J., Vinkers, C. H., & de Kloet, E. R. (2013). The three-hit concept of vulnerability and resilience: toward understanding adaptation to early-life adversity outcome. *Psychoneuroendocrinology*, *38*(9), 1858–73. https://doi.org/10.1016/j.psyneuen.2013.06.008

Datson, N. A., van den Oever, J. M. E., Korobko, O. B., Magarinos, A. M., de Kloet, E. R., & McEwen, B. S. (2013). Previous history of chronic stress changes the transcriptional

response to glucocorticoid challenge in the dentate gyrus region of the male rat hippocampus. *Endocrinology*, *154*(9), 3261–72. https://doi.org/10.1210/en.2012-2233

Debnath, M., Doyle, K., Langan, C., McDonald, C., Leonard, B., & Cannon, D. (2011). Recent advances in psychoneuroimmunology: Inflammation in psychiatric disorders. *Translational Neuroscience*, *2*(2), 121–137. https://doi.org/10.2478/s13380-011-0019-0

deRoon-Cassini, T. A., Mancini, A. D., Rusch, M. D., & Bonanno, G. A. (2010). Psychopathology and resilience following traumatic injury: a latent growth mixture model analysis. *Rehabilitation Psychology*, *55*(1), 1–11. https://doi.org/10.1037/a0018601

Dorrington, S., Zavos, H., Ball, H., McGuffin, P., Rijsdijk, F., Siribaddana, S., … Hotopf, M. (2014). Trauma, post-traumatic stress disorder and psychiatric disorders in a middle-income setting: prevalence and comorbidity. *The British Journal of Psychiatry : The Journal of Mental Science*, *205*(5), 383–9. https://doi.org/10.1192/bjp.bp.113.141796

Franklin, T. B., Russig, H., Weiss, I. C., Gräff, J., Linder, N., Michalon, A., … Mansuy, I. M. (2010). Epigenetic Transmission of the Impact of Early Stress Across Generations. *Biological Psychiatry*, *68*(5), 408–415. https://doi.org/10.1016/j.biopsych.2010.05.036

Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., … Mansuy, I. M. (2014). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nature Publishing Group*, *17*(5). https://doi.org/10.1038/nn.3695

Gill, J. M., Saligan, L., Woods, S., & Page, G. (2009). PTSD is Associated With an Excess of Inflammatory Immune Activities. *Perspectives in Psychiatric Care*, *45*(4), 262–277. https://doi.org/10.1111/j.1744-6163.2009.00229.x

Habib, K. E., Weld, K. P., Rice, K. C., Pushkas, J., Champoux, M., Listwak, S., … Gold, P. W. (2000). Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(11), 6079–84. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10823952

Haramati, S., Navon, I., Issler, O., Ezra-Nevo, G., Gil, S., Zwang, R., … Chen, A. (2011). microRNA as Repressors of Stress-Induced Anxiety: The Case of Amygdalar miR-34. *Journal of Neuroscience*, *31*(40), 14191–14203. https://doi.org/10.1523/JNEUROSCI.1673-11.2011

He, L., & Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nature Reviews Genetics*, *5*(7), 522–531. https://doi.org/10.1038/nrg1379

Heim, C., Owens, M. J., Plotsky, P. M., & Nemeroff, C. B. (1997). Persistent changes in corticotropin-releasing factor systems due to early life stress: relationship to the pathophysiology of major depression and post-traumatic stress disorder. *Psychopharmacology Bulletin*, *33*(2), 185–92. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9230630

Herman, J. P., McKlveen, J. M., Solomon, M. B., Carvalho-Netto, E., & Myers, B. (2012). Neural regulation of the stress response: glucocorticoid feedback mechanisms. *Brazilian Journal of Medical and Biological Research = Revista Brasileira de Pesquisas Medicas E Biologicas*, *45*(4), 292–8. https://doi.org/10.1590/s0100-879x2012007500041

Holwerda, S. J. B., & de Laat, W. (2013). CTCF: the protein, the binding partners, the binding sites and their chromatin loops. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *368*(1620), 20120369. https://doi.org/10.1098/rstb.2012.0369

Hou, J., Wang, P., Lin, L., Liu, X., Ma, F., An, H., … Cao, X. (2009). MicroRNA-146a Feedback Inhibits RIG-I-Dependent Type I IFN Production in Macrophages by Targeting TRAF6, IRAK1, and IRAK2. *The Journal of Immunology*, *183*(3). https://doi.org/https://doi.org/10.4049/jimmunol.0900707

Hurst, D. R., Edmonds, M. D., Scott, G. K., Benz, C. C., Vaidya, K. S., & Welch, D. R. (2009). Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis. *Cancer Research*, *69*(4), 1279–83. https://doi.org/10.1158/0008-5472.CAN-08-3559

Iliopoulos, D., Drosatos, K., Hiyama, Y., Goldberg, I. J., & Zannis, V. I. (2010). MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism. *Journal of Lipid Research*, *51*(6), 1513–23. https://doi.org/10.1194/jlr.M004812

Jakobsson, J., Cordero, M. I., Bisaz, R., Groner, A. C., Busskamp, V., Bensadoun, J.-C., … Trono, D. (2008). KAP1-mediated epigenetic repression in the forebrain modulates behavioral vulnerability to stress. *Neuron*, *60*(5), 818–31. https://doi.org/10.1016/j.neuron.2008.09.036

Karam, E. G., Friedman, M. J., Hill, E. D., Kessler, R. C., McLaughlin, K. A., Petukhova, M., … Koenen, K. C. (2014). Cumulative traumas and risk thresholds: 12-month PTSD in the World Mental Health (WMH) surveys. *Depression and Anxiety*, *31*(2), 130–142. https://doi.org/10.1002/da.22169

King, J. A., Abend, S., & Edwards, E. (2001). Genetic predisposition and the development of

posttraumatic stress disorder in an animal model. *Biological Psychiatry*, *50*(4), 231–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11522256

King, L. A., King, D. W., Fairbank, J. A., Keane, T. M., & Adams, G. A. (1998). Resilience-recovery factors in post-traumatic stress disorder among female and male Vietnam veterans: Hardiness, postwar social support, and additional stressful life events. *Journal of Personality and Social Psychology*, *74*(2), 420–434. https://doi.org/10.1037/0022-3514.74.2.420

Koenen, K. C., Roberts, A. L., Stone, D. M., & Dunn, E. C. (2010). The epidemiology of early childhood trauma. In R. A. Lanius, E. Vermetten, & C. Pain (Eds.), *The Impact of Early Life Trauma on Health and Disease: The Hidden Epidemic.* (pp. 157–165). Cambridge University Press. https://doi.org/10.1017/CBO9780511777042.019

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., & Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. *Current Biology : CB*, *12*(9), 735–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12007417

Li, X., Ito, M., Zhou, F., Youngson, N., Zuo, X., Leder, P., & Ferguson-Smith, A. C. (2008). A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. *Developmental Cell*, *15*(4), 547–57. https://doi.org/10.1016/j.devcel.2008.08.014

Liu, H., Yang, N., Fei, Z., Qiu, J., Ma, D., Liu, X., … Li, S. (2016). Analysis of plasma miR-208a and miR-370 expression levels for early diagnosis of coronary artery disease. *Biomedical Reports*, *5*(3), 332–336. https://doi.org/10.3892/br.2016.726

Maia, D. B., Marmar, C. R., Mendlowicz, M. V, Metzler, T., Nóbrega, A., Peres, M. C., … Figueira, I. (2008). Abnormal serum lipid profile in Brazilian police officers with post-traumatic stress disorder. *Journal of Affective Disorders*, *107*(1–3), 259–63. https://doi.org/10.1016/j.jad.2007.08.013

Matsuo, R., Asada, A., Fujitani, K., & Inokuchi, K. (2001). LIRF, a Gene Induced during Hippocampal Long-Term Potentiation as an Immediate-Early Gene, Encodes a Novel RING Finger Protein. *Biochemical and Biophysical Research Communications*, *289*(2), 479–484. https://doi.org/10.1006/bbrc.2001.5975

McGowan, P. O., Sasaki, A., D'Alessio, A. C., Dymov, S., Labonté, B., Szyf, M., … Meaney, M. J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nature Neuroscience*, *12*(3), 342–8. https://doi.org/10.1038/nn.2270

Mehta, D., Klengel, T., Conneely, K. N., Smith, A. K., Altmann, A., Pace, T. W., … Binder, E. B. (2013). Childhood maltreatment is associated with distinct genomic and epigenetic

profiles in posttraumatic stress disorder. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(20), 8302–7. https://doi.org/10.1073/pnas.1217750110

Mizoguchi, K., Ishige, A., Aburada, M., & Tabira, T. (2003). Chronic stress attenuates glucocorticoid negative feedback: involvement of the prefrontal cortex and hippocampus. *Neuroscience*, *119*(3), 887–97. https://doi.org/10.1016/S0306-4522(03)00105-2

Mollainezhad, H., Eskandari, N., Pourazar, A., Salehi, M., & Andalib, A. (2016). Expression of microRNA-370 in human breast cancer compare with normal samples. *Advanced Biomedical Research*, *5*, 129. https://doi.org/10.4103/2277-9175.186987

Motawae, T. M., Ismail, M. F., Shabayek, M. I., Seleem, M. M., Kuiper, J., & Aukrust, P. (2015). MicroRNAs 9 and 370 Association with Biochemical Markers in T2D and CAD Complication of T2D. *PLOS ONE*, *10*(5), e0126957. https://doi.org/10.1371/journal.pone.0126957

Nievergelt, C. M., Maihofer, A. X., Mustapic, M., Yurgil, K. A., Schork, N. J., Miller, M. W., … Baker, D. G. (2015). Genomic predictors of combat stress vulnerability and resilience in U.S. Marines: A genome-wide association study across multiple ancestries implicates PRTFDC1 as a potential PTSD gene. *Psychoneuroendocrinology*, *51*, 459–471. https://doi.org/10.1016/j.psyneuen.2014.10.017

Olesen, J., Gustavsson, A., Svensson, M., Wittchen, H.-U., Jönsson, B., CDBE2010 study group, & European Brain Council. (2012). The economic cost of brain disorders in Europe. *European Journal of Neurology*, *19*(1), 155–162. https://doi.org/10.1111/j.1468-1331.2011.03590.x

Ono, N., Samson, W. K., McDonald, J. K., Lumpkin, M. D., Bedran de Castro, J. C., & McCann, S. M. (1985). Effects of intravenous and intraventricular injection of antisera directed against corticotropin-releasing factor on the secretion of anterior pituitary hormones. *Proceedings of the National Academy of Sciences of the United States of America*, *82*(22), 7787–90. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2999777

Pace, T. W. W., Mletzko, T. C., Alagbe, O., Musselman, D. L., Nemeroff, C. B., Miller, A. H., & Heim, C. M. (2006). Increased Stress-Induced Inflammatory Responses in Male Patients With Major Depression and Increased Early Life Stress. *American Journal of Psychiatry*, *163*(9), 1630–1633. https://doi.org/10.1176/ajp.2006.163.9.1630

Palma-Gudiel, H., Córdova-Palomera, A., Eixarch, E., Deuschle, M., & Fañanás, L. (2015).

Maternal psychosocial stress during pregnancy alters the epigenetic signature of the glucocorticoid receptor gene promoter in their offspring: a meta-analysis. *Epigenetics*, *10*(10), 893–902. https://doi.org/10.1080/15592294.2015.1088630

Plant, K., Fairfax, B. P., Makino, S., Vandiedonck, C., Radhakrishnan, J., & Knight, J. C. (2014). Fine mapping genetic determinants of the highly variably expressed MHC gene ZFP57. *European Journal of Human Genetics*, *22*(4), 568–571. https://doi.org/10.1038/ejhg.2013.244

Ploski, J. E., Park, K. W., Ping, J., Monsey, M. S., & Schafe, G. E. (2010). Identification of plasticity-associated genes regulated by Pavlovian fear conditioning in the lateral amygdala. *Journal of Neurochemistry*, *112*(3), 636–650. https://doi.org/10.1111/j.1471-4159.2009.06491.x

Quenneville, S., Turelli, P., Bojkowska, K., Raclot, C., Offner, S., Kapopoulou, A., & Trono, D. (2012). The KRAB-ZFP/KAP1 system contributes to the early embryonic establishment of site-specific DNA methylation patterns maintained during development. *Cell Reports*, *2*(4), 766–73. https://doi.org/10.1016/j.celrep.2012.08.043

Rokavec, M., Li, H., Jiang, L., & Hermeking, H. (2014). The p53/miR-34 axis in development and disease. *Journal of Molecular Cell Biology*, *6*(3), 214–230. https://doi.org/10.1093/jmcb/mju003

Saba, R., Sorensen, D. L., & Booth, S. A. (2014). MicroRNA-146a: A Dominant, Negative Regulator of the Innate Immune Response. *Frontiers in Immunology*, *5*, 578. https://doi.org/10.3389/fimmu.2014.00578

Sato, T., Liu, X., Nelson, A., Nakanishi, M., Kanaji, N., Wang, X., … Rennard, S. I. (2010). Reduced miR-146a Increases Prostaglandin E2 in Chronic Obstructive Pulmonary Disease Fibroblasts. *American Journal of Respiratory and Critical Care Medicine*, *182*(8), 1020–1029. https://doi.org/10.1164/rccm.201001-0055OC

Schöner, J., Heinz, A., Endres, M., Gertz, K., & Kronenberg, G. (2017). Post-traumatic stress disorder and beyond: an overview of rodent stress models. *Journal of Cellular and Molecular Medicine*. https://doi.org/10.1111/jcmm.13161

Stein, M. B., Walker, J. R., Hazen, A. L., & Forde, D. R. (1997). Full and partial posttraumatic stress disorder: findings from a community survey. *American Journal of Psychiatry*, *154*(8), 1114–1119. https://doi.org/10.1176/ajp.154.8.1114

Taganov, K. D., Boldin, M. P., Chang, K.-J., & Baltimore, D. (2006). NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences of the United*

States of America, *103*(33), 12481–6. https://doi.org/10.1073/pnas.0605298103

Tomasetti, M., Staffolani, S., Nocchi, L., Neuzil, J., Strafella, E., Manzella, N., … Santarelli, L. (2012). Clinical significance of circulating miR-126 quantification in malignant mesothelioma patients. *Clinical Biochemistry*, *45*(7), 575–581. https://doi.org/10.1016/j.clinbiochem.2012.02.009

Vargas, J., Junco, M., Gomez, C., Lajud, N., Tailleux, A., Mullins, J., & López, M. (2016). Early Life Stress Increases Metabolic Risk, HPA Axis Reactivity, and Depressive-Like Behavior When Combined with Postweaning Social Isolation in Rats. *PLOS ONE*, *11*(9), e0162665. https://doi.org/10.1371/journal.pone.0162665

Vergeer, M., Holleboom, A. G., Kastelein, J. J. P., & Kuivenhoven, J. A. (2010). The HDL hypothesis: does high-density lipoprotein protect from atherosclerosis? *Journal of Lipid Research*, *51*(8), 2058–73. https://doi.org/10.1194/jlr.R001610

Wagner, A. W., Wolfe, J., Rotnitsky, A., Proctor, S. P., & Erickson, D. J. (2000). An investigation of the impact of posttraumatic stress disorder on physical health. *Journal of Traumatic Stress*, *13*(1), 41–55. https://doi.org/10.1023/A:1007716813407

Wang, X.-D., Chen, Y., Wolf, M., Wagner, K. V., Liebl, C., Scharf, S. H., … Schmidt, M. V. (2011). Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling. *Neurobiology of Disease*, *42*(3), 300–310. https://doi.org/10.1016/j.nbd.2011.01.020

Wang, X.-D., Labermaier, C., Holsboer, F., Wurst, W., Deussing, J. M., Müller, M. B., & Schmidt, M. V. (2012). Early-life stress-induced anxiety-related behavior in adult mice partially requires forebrain corticotropin-releasing hormone receptor 1. *European Journal of Neuroscience*, *36*(3), 2360–2367. https://doi.org/10.1111/j.1460-9568.2012.08148.x

Wang, X.-D., Rammes, G., Kraev, I., Wolf, M., Liebl, C., Scharf, S. H., … Schmidt, M. V. (2011). Forebrain CRF1 Modulates Early-Life Stress-Programmed Cognitive Deficits. *Journal of Neuroscience*, *31*(38), 13625–13634. https://doi.org/10.1523/JNEUROSCI.2259-11.2011

Xu, Y., Zalzala, M., Xu, J., Li, Y., Yin, L., & Zhang, Y. (2015). A metabolic stress-inducible miR-34a-HNF4α pathway regulates lipid and lipoprotein metabolism. *Nature Communications*, *6*, 7466. https://doi.org/10.1038/ncomms8466

Zhang, H., Sun, X., & Hao, D. (2016). Upregulation of microRNA-370 facilitates the repair of amputated fingers through targeting forkhead box protein O1. *Experimental Biology and Medicine (Maywood, N.J.)*, *241*(3), 282–9.

https://doi.org/10.1177/1535370215600549

Zhang, X., Fu, X., Zhu, D., Zhang, C., Hou, S., Li, M., & Yang, X. (2016). Salidroside-regulated lipid metabolism with down-regulation of miR-370 in type 2 diabetic mice. *European Journal of Pharmacology*, *779*, 46–52. https://doi.org/10.1016/j.ejphar.2016.03.011

Zhu, J., Chen, Z., Tian, J., Meng, Z., Ju, M., Wu, G., & Tian, Z. (2017). miR-34b attenuates trauma-induced anxiety-like behavior by targeting CRHR1. *International Journal of Molecular Medicine*. https://doi.org/10.3892/ijmm.2017.2981