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The contribution of circulating factors to epigenetic inheritance in mice

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1 Abstract

An organism is sensitive to the environment, particularly in early life. Traumatic experiences occurring in postnatal until adolescent periods can result in the development of altered cognitive and physiological function in adulthood. Similar functions may also be affected in the offspring across consecutive generations, even when they were never exposed to similar traumatic exposures. Experimental evidence indicates that this type of inheritance involves epigenetic marks in the germline. The mechanism(s) by which the environment communicates with the germline to establish these marks is unknown. It is likely that in response to traumatic experiences, factors are released into the bloodstream that can relay messages throughout the body. While this may be intended to increase biological fitness at the time of exposure, long-term consequences may manifest. In particular, germ cells may receive signals from the released factors, and undergo heritable epigenetic remodeling. The factors involved in the induction of such remodeling are not well understood. To study this, we used an established mouse model of postnatal unpredictable maternal separation combined with unpredictable maternal stress (MSUS). We performed an assessment of this model and confirmed its consistency and reproducibility in transmitting behavioral and metabolic disturbances until the fourth generation through the patriline. Then, we collected blood from MSUS and control males and performed a metabolomics analysis to identify perturbations to extracellular circulating factors that may interact with germ cells. We found that pathways primarily involving polyunsaturated fatty acids, but also bile acid biosynthesis and steroidogenesis, were altered in MSUS mice. These pathways are major ligand contributors for nuclear receptors, in particular the peroxisome proliferator-activated receptors (PPARs). In naïve mice, pharmacologically mimicking the changes in paternal polyunsaturated fatty acid pathways with a PPAR agonist could reproduce metabolic phenotypes in the second and third generations. Injecting serum collected from MSUS males into naïve mice could also induce transmission of similar metabolic phenotypes in the offspring. Then, using blood and saliva collected from a cohort of children who

suffered paternal loss and subsequent maternal separation in the form of admittance to an orphanage (PLMS), we found that polyunsaturated fatty acid, bile acid biosynthesis and steroidogenesis pathways were also altered, similar to MSUS mice. Consistent with the effects of trauma exposure, we also found high-density lipoproteins (HDLs) were decreased and corresponding HDL-associated miRNAs were differentially expressed (miR-16, miR-29a, miR-375) in both MSUS and PLMS serum, which correlate with altered miRNAs in MSUS sperm. Exposing germ cell-like cells to MSUS serum could mimic the altered expression of miR-375, which could be reversed after *in vitro* knockdown of the HDL receptor. Together, our data demonstrate causal evidence for serum factors to induce transmission of metabolic disturbances to offspring. Changes in polyunsaturated fatty acid metabolites, and other pathways involving nuclear receptor ligands, provide a mechanism for the environment to relay messages to the germline, since PPAR activation could transmit metabolic phenotypes transgenerationally. HDLs may also carry miRNAs to the germline, and induce alterations to the germline transcriptome. Lastly, early-life exposure to traumatic experiences has, in part, conserved effects on the circulating metabolome and transcriptome in mice and humans.

2 Zusammenfassung

Ein Organismus ist besonders im frühen Leben empfindlich für Umwelteinflüsse. Traumatische Erfahrungen, die in postnatalen bis adoleszenten Phasen auftreten, können im Erwachsenenalter zu veränderten kognitiven und physiologischen Funktionen führen. Deren Nachkommen in den darauffolgenden Generationen können ebenfalls von ähnlichen Veränderungen der Funktionen betroffen sein, obwohl sie nie der ursprünglichen, traumatischen Erfahrungen ausgesetzt waren. Experimentelle Hinweise deuten darauf hin, dass epigenetische Markierungen in der Keimbahn massgeblich zu dieser Art der Vererbung beitragen. Die Mechanismen, welche die Umwelteinflüsse in eine epigenetische Markierung in der Keimbahn übersetzen, sind unbekannt. Es ist anzunehmen, dass als Reaktion auf traumatische Erfahrungen Botenstoffe in den Blutkreislauf freigesetzt werden und auf diese Weise ein Signal systemisch weitergeleitet werden kann. So ein Mechanismus könnte zum Zeitpunkt der Exposition zur Verbesserung der biologischen Fitness beitragen, allerdings könnte es auch langfristige Konsequenzen nach sich tragen. Insbesondere können Signale von solchen freigesetzten Botenstoffen die Keimzellen erreichen und vererbbar, epigenetische Markierungen beeinflussen. Es ist bisher nicht bekannt, welche Botenstoffe solche Änderungen in den Keimzellen auslösen können. Um dies zu untersuchen, verwendeten wir ein etabliertes Mausmodell namens MSUS (engl. unpredictable maternal separation combined with unpredictable maternal stress), welches ein frühkindliches Trauma nachahmt. Dabei werden die Jungtiere unvorhersehbar von der Mutter getrennt und die Mutter wird zusätzlich unvorhersehbar gestresst. Wir führten eine Bewertung dieses Modells durch und bestätigten seine Beständigkeit und Reproduzierbarkeit bei der Übertragung von Verhaltens- und Stoffwechseländerungen bis zur vierten Generation durch die väterliche Linie. Das Metabolom des Blutes von Männchen aus der MSUS und Kontrollgruppe wurde analysiert, um Änderungen der extrazellulären, zirkulierenden Botenstoffe zu identifizieren, welche Keimzellen beeinflussen könnten. Bei MSUS-Männchen wurden

Änderungen bei den Signalwegen für mehrfach ungesättigte Fettsäuren, Gallensäurebiosynthese und Steroidgenese festgestellt. Diese Signalwege tragen massgeblich zur Bildung von Liganden der nuklearen Rezeptoren bei, insbesondere der Gruppe der Peroxisom-Proliferator-aktivierte Rezeptoren (PPAR). Der metabolische Phänotyp, welcher in der zweiten und dritten Generation von MSUS Mäusen beobachtet wird, konnte bei naiven Mäusen mittels eines PPAR-Agonisten pharmakologisch nachgeahmt werden. Der metabolische Phänotyp konnte ebenfalls in den Nachkommen von naiven Männchen, welche einer Transfusion mit Serum von MSUS-Männchen unterzogen wurden, induziert werden. Weiter konnte in Blut- und Speichelproben von Kindern, welche wegen des Verlustes von Mutter und Vater in ein Waisenheim eingewiesen wurden (PLMS, engl. Paternal loss and maternal separation), ähnliche Änderungen wie im MSUS-Modell an den Signalwegen für mehrfach ungesättigte Fettsäuren, Gallensäurebiosynthese und Steroidgenese festgestellt werden. Ferner waren mehrere miRNAs, welche mit Lipoproteinen hoher Dichte (engl. HDL) assoziiert sind, darunter miR-16, miR-29a und miR375, in den MSUS- und PLMS-Gruppen unterschiedlich exprimiert und korrelierten mit veränderten miRNAs im Sperma von MSUS-Männchen. Die veränderte Expression von miR-375 konnte in einer keimzellenähnlichen Zelllinie nachgeahmt werden, in dem die Zellen mit dem Serum von MSUS-Mäusen ausgesetzt wurden. Diese veränderte Expression konnte in vitro mittels Gen-Knockdown der HDL-Rezeptoren rückgängig gemacht werden. Zusammengenommen zeigen unsere Resultate einen kausalen Zusammenhang zwischen Botenstoffen im Serum und der Vererbung von Stoffwechselstörungen an die Nachkommen. Da die Aktivierung von PPAR die metabolischen Phänotypen transgenerational übertragen konnte, stellen die Veränderungen in Fettsäuremetaboliten und nuklearen Rezeptorliganden einen möglichen Mechanismus dar, um Umweltsignale an die Keimbahn weiterzuleiten. Ferner könnten miRNAs, welche mit Lipoproteinen hoher Dichte assoziiert sind, in die Keimbahn transportiert werden und so Veränderungen im Transkriptom der Keimbahn führen. Schlussendlich scheint ein frühkindliches Trauma teilweise ähnliche Auswirkungen auf zirkulierende Metaboliten und das Transkriptom in Mäusen wie auch Menschen zu haben.

3 Table of Contents

1 Abstract	2
2 Zusammenfassung	4
3 Table of Contents	6
4 List of abbreviations.....	10
5 Introduction.....	14
5.1 Epigenetics	14
5.2 Transgenerational epigenetic inheritance in mammals.....	14
5.3 Effects of early-life adversity across generations.....	15
5.4 Epigenetic marks in germ cells	17
5.5 Environmental communication with germ cells	20
5.6 Hypothesis	21
5.7 Aims	21
6 Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: Evidence in the 4th generation	22
6.1 Abstract	23
6.2 Introduction.....	24
6.3 Methods	25
6.4 Results.....	30
6.5 Discussion	37
6.6 Acknowledgments	40
6.7 Authors Contributions	40
6.8 Conflict of Interest Statement	40
6.9 Supplementary data	41
7 Nuclear receptor signaling in the communication between blood and the germline	45
7.1 Abstract	46
7.2 Main Text	47
7.3 Methods	55

7.4 Acknowledgments	65
7.5 Authors Contributions	65
7.6 Conflict of Interest Statement	66
7.7 Supplementary Data.....	66
8 Similar alterations in behavior, lipid metabolism, and circulating microRNAs in mice and humans after early-life trauma.....	76
8.1 Abstract	77
8.2 Main Text	78
8.3 Methods	84
8.4 Acknowledgments	89
8.5 Authors Contributions	89
8.6 Conflict of Interest Statement	90
8.7 Supplementary Data.....	90
9 Discussion.....	97
9.1 Depth of inheritance in mammals	97
9.2 The conserved effects of childhood adversity on blood factors in mouse and human	98
9.3 Nuclear receptors	100
9.4 Potential origins of germ cell RNA	104
9.5 Serum factors and germ cell modifications	105
9.6 Sources of altered metabolites in blood	106
9.7 Potential basis for altered HDL cholesterols	109
9.8 PPAR mechanisms of action	110
10 Significance	115
11 References	116
12 Annex 1: Brain metabolic alterations in mice subjected to postnatal traumatic stress and in their offspring.....	142
12.1 Abstract	143
12.2 Introduction.....	144
12.3 Methods	145
12.4 Results.....	148
12.5 Discussion	155

12.6 Acknowledgments	160
12.7 Authors Contributions	161
12.8 Conflict of Interest Statement.....	161
13 Annex 2: Alterations in sperm long RNA contribute to the epigenetic inheritance of the effects of postnatal trauma.....	162
13.1 Abstract	163
13.2 Introduction.....	163
13.3 Methods	164
13.4 Results.....	171
13.5 Discussion	183
13.6 Acknowledgments	185
13.7 Author contributions	185
13.8 Conflict of interests	185
13.9 Supplementary information	186
14 Dedication	200
15 Acknowledgements	201

“And those who were seen dancing were thought to be insane by those who could not hear the music.” -Nietzsche

4 List of abbreviations

° C	Degrees Celsius
AA	Arachidonic acid
ACE	Adverse childhood experience
Acetyl CoA	Acetyl Coenzyme A
ADHD	Attention deficit hyperactivity disorder
AFAST	Aberdeen folic acid supplementation trial
Ala	Alanine
ALA	Alpha-linolenic acid
AP1	Activator protein 1
Asc	Ascorbate
Asp	Aspartate
ATAC(seq)	Assay for transposase-accessible chromatin (sequencing)
BH	Bonferroni-Hochberg
BMI	Body mass index
BPA	Bisphenol A
BPD	Borderline personality disorder
BSL2	Biosafety level 2
BTB	Blood-testis barrier
CES-DC	Center for Epidemiological Studies Scale for Depression in Children
cm	centimeters
COX1/COX2	Cyclooxygenase-1/ -2
Cr	Creatine
DNA	Deoxyribonucleic acid
DNAme	DNA methylation
ELISA	Enzyme-linked immunosorbent assay
EMBL-EBI	European molecular biology laboratory-European bioinformatics institute
EPM	Elevated plus maze

ETH	Eidgenoische Technologische Hochschule (Swiss Federal Institute of Technology)
eWAT	Epididymal white adipose tissue
F0	Filial generation 0
F1	Filial generation 1
F2	Filial generation 2
F3	Filial generation 3
F4	Filial generation 4
F5	Filial generation 5
FDR	False discovery rate
FST	Forced swim test
<i>g</i>	G-force
GABA	Gamma-aminobutyrate
Glc	Glucose
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GPC	Glycerophosphorylcholine
GSH	Glutathione
HDL	High-density lipoproteins
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
i.p.	Intraperitoneal
i.v.	Intravenous
inj	Injection
Ins	Myo-inositol
kd	Knockdown
LA	Linoleic acid
Lac	Lactate
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
LDB	Light dark box
LDL	Low-density lipoproteins
LXR	Liver X receptor

m/z	Mass to charge ratio
min	Minutes
miRNA/miR	micro RNA
MRS	Magnetic resonance spectroscopy
MSUS	Unpredictable maternal separation combined with unpredictable maternal stress
NAA	N-acetylaspartate
NAAG	N-acetylaspartyglutamte
ncRNA	Non-coding RNA
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
NR	Nuclear receptor
NW	Normal weight
OW	Overweight
PCho	Phosphorylcholine
PCr	Phosphocreatine
PE	Phosphorylethanolamine
PGC	Primordial germ cells
PLMS	Paternal loss and subsequent maternal separation
PND	Postnatal day
PPAR	Peroxisome proliferator-activated receptor
PTM	Posttranslational modification
PTSD	Posttraumatic stress disorder
PUFA	Polyunsaturated fatty acids
PXR	Pregnane X receptor
RNA	Ribonucleic acid
ROUT	Robust regression and outlier removal
RXR	Retinoid X receptor
SCARED	Scale of anxiety-related disorders in children
Scyllo	Scyllo-inositol
sec	Seconds

siRNA	Small interfering RNA
SSC	Spermatogonial stem cell
STAT	Signal transducer and activator of transcription
Tau	Taurine
TBT	Tributyltin
TCA	Tricarboxylic acid
Tesa	Tesagliptazar
TF	Transcription factor
TNF α	Tumor necrosis factor alpha
TOFMS	Time of flight mass spectrometry
tRF	tRNA fragments
tRNA	transfer RNA
TSS	Transcription start site
TZD	Thiazolidindione
UW	Underweight
UZH	University Zürich
VDR	Vitamin D receptor
VOI	Volumes of interest
wt/vol	Weight to volume ratio

5 Introduction

5.1 Epigenetics

The DNA sequence is considered stable across the lifetime. Events that result in sequence remodeling, for example transposition¹, repeat expansions² or in response to mutagens³, are often deleterious for the organism. Epigenetics refers to the chromosomal changes that alter gene expression, without altering the DNA sequence itself⁴, thus allowing DNA to function with plasticity through mechanisms that alter gene expression in response to inputs from the environment. Classically, these mechanisms involve the molecular modifications occurring directly on the chromatin, but the definition has more recently expanded to include all processes that inhibit or promote gene expression patterns independent of sequence remodeling⁵. This includes DNA methylation (DNAm) and hydroxymethylation, histone composition, retention or post-translational modifications (PTMs), chromatin conformation, and several classes of non-coding RNAs and their modifications (termed epitranscriptome⁶). In somatic cells, epigenetic mechanisms regulate vital cellular functions, such as differentiation⁷, or adjusting cell function in response to environmental inputs⁸. However, in germ cells, epigenetic regulation can result in altered phenotypes across several consecutive generations, termed transgenerational epigenetic inheritance⁹, and may contribute to altered health and disease susceptibility.

5.2 Transgenerational epigenetic inheritance in mammals

Disease etiology is highly complex in mammals. In particular, non-communicable diseases with high heritability rates cannot always be explained by genetic factors alone¹⁰. Evidence indicates that several neuropsychiatric, metabolic and immunologic disorders can originate from environmental exposures, and co-occur in progeny that never experienced comparable environmental exposures^{11–13}. While the Lamarckian premise that evolutionary adaptations can occur within generations can be viewed as hyperbole¹⁴, there is evidence that epigenetic inheritance may provide an

adaptive advantage in the offspring¹⁵. Altered phenotypes are thought to increase fitness in progeny exposed to similar environmental exposures as their parents. However, in the absence of these exposures, offspring may instead become mal-adapted to the environment¹⁶. Evidence suggests that over several generations some phenotypes may also revert^{17,18}. Alternatively, in the directly exposed, counteracting exposures such as environmental enrichment may reverse detrimental effects¹⁹, whereas additive effects of exposures across consecutive generations has also been reported^{20,21}. The depth and severity of penetrance across generations seems to be related to the number of consecutive generations exposed, the length and type of exposure, the sex and the developmental period of the exposed.

5.2.1 Types of exposures

In humans, studies have identified dietary changes²², stress²³, medications²⁴ and environmental toxins²⁵ to altered phenotypes in offspring. However, many of the human studies reveal effects from *in utero* exposures, or are intergenerational (parent to child). Given limitations such as data availability for consecutive generations, rodent models have become essential to corroborate findings in humans, investigate the penetrance of effects across generations and to provide mechanistic insight. Indeed, several studies in rodents have confirmed phenotypic disturbances in offspring following parental high fat¹² or low protein diets²⁶, pre- and postnatal stress^{11,27}, exposure to environmental toxicants^{28–30}, and prescription medications³¹. Moreover, rodent studies extend this list to other types of environmental exposures, such as, immune activation³² and deficient maternal care³³. Unfortunately, only a few exposure models have been investigated beyond the 3rd generation, and include prenatal stress²¹, environmental toxicants³⁴ and obesogens³⁵, drugs^{31,36} and the *Mtrr* hypomorphic genetic mutation³⁷. To fully understand the impact of diverse environmental exposures, it is therefore necessary to extend current research models beyond the 3rd generation in mammals and, in parallel, begin to collect comprehensive data sets for rigorous transgenerational studies in humans.

5.3 Effects of early-life adversity across generations

Early-life adversity is particularly well known to have consequences on development and the acquisition of cognitive and physiological disturbances in adulthood³⁸. In mammals, these disturbances are known to transmit phenotypes across generations, and often involve either prenatal or postnatal exposures.

Prenatal exposures, beginning both pre- and post-conception, can be quite severe since the developing primordial germ cells (PGCs) are exposed and may be permanently reprogrammed during exposure. Therefore, the depth of penetrance and severity of the phenotypes across generations can be quite robust. In a rat model of prenatal maternal stress, metabolite alterations in urine were present up to the fourth generation in males²¹, while immune activation using the compound poly I:C in pregnant females resulted in behavioral deficits reminiscent of schizophrenia in the 3rd generation of males³². Dietary interventions have also been shown to modulate phenotypes across generations, including maternal undernutrition³⁹, and folic acid supplementation^{37,40}. Environmental toxins such as the endocrine disruptor vinclozolin²⁹, or the obesogen tributyltin (TBT)³⁵ demonstrate altered phenotypes up to the 4th generation, while the medication valproic acid, used to treat epilepsy, could induce autism-like behaviors transgenerationally³¹ and nicotine exposure in grand mothers could produce symptoms of attention deficit hyperactivity disorder (ADHD) in mice⁴¹. Interestingly, many of these findings are corroborated by human studies. Maternal undernutrition during the Dutch Hunger Winter (1944-45) resulted in higher prevalence of grand offspring with increased ponderal index (adiposity)⁴². More recently, the Aberdeen folic acid supplementation trial (AFAST) demonstrated that maternal folate supplementation could alter DNA methylation in offspring at genes relevant for embryonic development, immune function and cellular proliferation⁴³. Additionally, smoking or nicotine exposure during gestation can also result in transmission of asthma^{44,45} or autism-associated²⁵ symptoms to grand children.

Like prenatal exposures, postnatal exposures can also result in transmission of phenotypes across generations, but are unique in that they avoid

interference with gestational developmental processes and epigenetic reprogramming occurring during embryogenesis⁴⁶. However, postnatal exposures are vulnerable to pre pubertal developmental reprogramming, which may result in more robust consequences compared with adult exposures. Postnatal exposure to maternal separation with^{11,47,48} or without maternal stress^{49,50} results in transmission of behavioral, metabolic and/or cognitive phenotypes across generations. Along the lines of maternal separation, maternal care involving licking and grooming in rats can also affect stress response across generations³³. Because rodents are breastfed until weaning, postnatal nutritional interventions are more difficult to evaluate. Perhaps in future studies the effects of breast milk composition will reveal disturbances to offspring health that lead to transmission of phenotypes across generations⁵¹. In humans, extensive analysis has been performed on food-access records from Överkalix, Sweden. It was found that food shortages during childhood (the slow growth period) in both boys and girls could produce cardiovascular and diabetes-related health risks in their grandchildren^{22,52}. In regards to stress exposure, the offspring of men and women who were evacuated from a Finnish town during childhood in World War 2 had an increased risk of psychiatric hospitalizations⁵³. Additionally, epigenetic modifications resulting from childhood trauma were reported at loci (FKBP5)⁵⁴ later found to be associated with intergenerational inheritance of the effects of trauma in holocaust survivors²³. Notably, such retrospective studies in humans provide associations, but need controlled mammalian models alongside robust longitudinal studies across generations in humans in order to get closer to finding causal evidence for true epigenetic transmission of phenotypes through the germline.

5.4 Epigenetic marks in germ cells

Owing to confounds inherent to the female germline, such as hormonal fluctuations, intrauterine factors and availability of oocytes, research into the epigenetic mechanisms occurring in germ cells has preferentially been in sperm⁴⁶. So far, several epigenetic marks have been identified in sperm that are associated with altered phenotypes in offspring or grand offspring.

DNAme was one of the first identified to be correlated with altered phenotypes in offspring⁵⁵, and has since been implicated in several animal models, and in humans⁵⁶. Additional epigenetic marks such as histone PTMs⁵⁷, differential histone retention^{58,59} and chromatin 3-dimensional structure⁶⁰ (i.e. looping) have more recently been implicated, but causal evidence has not been demonstrated. For this, advanced techniques need to be developed in order to isolate and induce specific changes to the epigenome and monitor the effects. With the advancement of CRISPR technologies, causal evidence for the role of DNAme in particular may not be far away. So far, the only causal evidence for epigenetic inheritance rests on RNA⁶¹. Sperm total or small RNAs injected into naïve embryos can result in offspring with altered phenotypes, similar to the offspring of mice from which the RNA was taken⁴⁸. Several labs using different models have been able to confirm such causal evidence^{62,63}, however only total RNA or small non-coding RNA, such as miRNAs and transfer RNA (tRNA) fragments (tRFs)^{12,26}, have been demonstrated to replicate this phenomenon. The influence of other RNA species, such as long (non-coding) RNAs, will also be necessary to delineate. Lastly, while epigenetic processes ultimately function to regulate transcription, transcription factors themselves are not traditionally considered an epigenetic ‘unit’. However, their DNA-binding abilities and role in transcriptional regulation are compelling endorsements for their epigenetic status^{64,65}, therefore they are included in some definitions. Consistent with this premise, it was demonstrated that the poised transcriptional state of the chromatin in mature sperm could influence post-fertilization gene expression⁶⁰, demonstrating that transcription factors may also have molecular tendencies relevant to transgenerational inheritance⁶⁶, regardless of their classification.

5.4.1 Establishment of germ cell epigenetic marks: potential mechanisms of induction

5.4.1.1 Metabolites

While it is invariably important to understand which epigenetic marks can result in the manifestation of altered phenotypes in offspring, the mechanism of how they are established is currently not investigated with the same

intensity. However, the type of epigenetic marks thought to contribute to transmission of phenotypes helps to identify which pathways are involved in their establishment. For example, DNA or histone methylation requires a steady cycling of methyl groups, which are supplied by one-carbon metabolism involving nutrients such as B-vitamins and amino acids (i.e. methionine, folic acid and homocysteine)⁶⁷. Thus, dietary disturbances to these nutrients may result in a molecular cascade eventually affecting DNA or histone methylation. Other epigenetic marks, such as histone acetylation and DNA hydroxymethylation can be modified by acetyl-CoA⁶⁸, supplied by β -oxidation, or α -ketoglutarate⁶⁹, supplied by the TCA cycle, respectively. Thus, disturbances to glucose and fatty acid metabolism resulting from diet, exercise, or diseases such as diabetes and obesity, may ultimately contribute to these epigenetic changes in the germline⁷⁰. Several types of metabolic signaling pathways maintain metabolites vital for epigenetic mechanisms⁷¹ and may explain why environmental exposures that perturb metabolic homeostasis often demonstrate epigenetic inheritance of altered phenotypes.

5.4.1.2 Hormones

Further to metabolites, several types of hormones have also been implicated in epigenetic inheritance. Gestational testosterone levels were associated with increased risk for autism-like behaviors in children⁷². Similarly, both mineralocorticoid and glucocorticoid receptors have been associated with inheritance of the effects of stress exposure in both rodents^{73,74} and humans⁷⁵. Since hormones are sensitive to the environment and can initiate robust signaling cascades through binding with their receptors, nuclear receptors also make strong candidates. Nuclear receptors are particularly apt to receive signals from the environment, and upon ligand binding can translocate to the nucleus to interact directly with chromatin. These chromatin interactions are not restricted to classical roles involving transcription activation or transrepression, but are now known to also involve structural modifications and the recruitment of enzymes that can independently modify the epigenome⁶⁵. Similarly, hormone derivatives and other lipophilic metabolites can also interact with nuclear receptors. Such molecules including fatty acids, oxysterols (cholesterols), bile acids, retinols (vitamin A) and

calcitriol (vitamin D) can also bind various nuclear receptors⁷⁶. Importantly, nuclear receptors control vast physiological processes, and their ligands are strongly linked with environmental inputs. A full characterization of such metabolites has the potential to reveal several pathways dysregulated by the environment that may then interact with the sperm epigenome through nuclear receptor activity.

5.4.1.3 RNA

While RNA is the only epigenetic mark causally linked to phenotype inheritance, the exact origins of sperm RNAs remain unknown. Of course some RNAs are most certainly endogenously expressed within germ cells, but since small RNAs have also been demonstrated to regulate gene expression outside their tissue of origin⁷⁷, it stands to reason that RNAs contained within germ cells might not have originated there. In particular, tRFs found in mature sperm were shown to originate in the epididymis and be taken up by the developing sperm during epididymal transit²⁶. Notably, these tRFs were essential for embryogenesis⁷⁸, demonstrating the importance of germline uptake of RNA. The uptake of RNA by the germline from other sources cannot be excluded; in fact they may also be of vital importance. Because RNAs are easily degraded, they likely travel with protein chaperones, or even more likely, enclosed in extracellular vesicles⁷⁹. So far, aside from epididymosomes, RNAs have been identified in exosomes and high-density lipoproteins⁸⁰ (HDLs) within the circulation, as well as from within vesicles secreted by prostate cells⁸¹ (prostosomes) and Sertoli cells⁸², which come in direct contact with developing sperm. In the context of transgenerational inheritance, these particular interactions have not been explored.

5.5 Environmental communication with germ cells

Transgenerational inheritance has been demonstrated using several paradigms that target diverse tissues and organ systems, yet signals still manage to reach the germline. Therefore, the circulation likely contains the factors that are released in response to various environments, which can communicate with germ cells. In blood, the ability for circulating factors to induce epigenetic changes to the germline may originate from several types of

signaling molecules, as outlined above. Together, these molecules are mostly extracellular, and comprise the metabolome (fatty acids, hormones, sterols, etc.), transcriptome (small and long RNA) and proteome (HDLs, exosomal proteins, cytokines, chemokines, etc.). It is likely that identifying alterations to these classes of molecules will reveal patterns about the types of signals that are interacting with germ cells to perturb the epigenome. Further, assessment of these signaling factors at different time points may provide evidence for differential manifestation of epigenetic changes across lifespan.

5.6 Hypothesis

Circulating factors released in response to the environment can impact the germline to induce transgenerational phenotypic consequences.

5.7 Aims

Using an established mouse model of early-life adversity in the form of paternal postnatal exposure to a chronic traumatic experience (MSUS), we will assess the depth and severity of phenotypes passed through consecutive generations (Chapter 6). Then, with metabolomic and transcriptomic approaches we will identify the circulating factors altered by MSUS treatment at various time points, and assess their contribution to altered phenotypes across generations (Chapter 7; Chapter 8). Finally, we will determine whether similar changes to circulating factors following early-life adversity are conserved across mouse and human (Chapter 7; Chapter 8).

As a follow-up to previous work in our lab, we will also investigate additional outstanding questions in the field. First, using the MSUS model, we will evaluate the contribution of altered brain metabolites to behavioral outcomes across generations (Annex 1). Second, and more relevant to the mechanisms of transgenerational inheritance, we will assess the contribution of sperm long RNA to transmitting phenotypic effects of early-life adversity to offspring (Annex 2).

6 Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: Evidence in the 4th generation

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6.1 Abstract

In the past decades, evidence supporting the transmission of acquired traits across generations has reshaped the field of genetics and the understanding of disease susceptibility. In humans, pioneer studies showed that exposure to famine, endocrine disruptors or trauma can affect descendants, and has led to a paradigm shift in thinking about heredity. Studies in humans have however been limited by the low number of successive generations, the different conditions that can be examined, and the lack of mechanistic insight they can provide. Animal models have been instrumental to circumvent these limitations and allowed studies on the mechanisms of inheritance of environmentally-induced traits across generations in controlled and reproducible settings. However, most models available today are only intergenerational and do not demonstrate transmission beyond the direct offspring of exposed individuals. Here, we report transgenerational transmission of behavioral and metabolic phenotypes up to the 4th generation in a mouse model of paternal postnatal trauma (MSUS). Based on large animal numbers (up to 124 per group) from several independent breedings conducted 10 years apart by different experimenters, we show that depressive-like behaviors are transmitted to the offspring until the third generation, and risk-taking and glucose dysregulation until the fourth generation via males. The symptoms are consistent and reproducible, and persist with similar severity across generations. These results provide strong evidence that adverse conditions in early postnatal life can have transgenerational effects, and highlight the validity of MSUS as a solid model of transgenerational epigenetic inheritance.

6.2 Introduction

The concept of transgenerational epigenetic inheritance implies that epigenetic signatures induced by exposure can be maintained across generations and may be responsible for the manifestation of phenotypes in parents and their offspring. This stands in contrast to the Mendelian model of inheritance by which genetic factors are the sole hereditary vectors of trait variation. Transgenerational epigenetic inheritance has major implications for disease etiology in humans and helps explain complex conditions like neuropsychiatric, metabolic and immunological disorders whose heritability cannot be explained only by genetic factors¹⁰. Although the concept initially met reservation due to conceptual limitations such as the fact that the epigenome is reprogrammed in developing germ cells and the early embryo, evidence has accumulated to indicate that environmentally-induced epigenetic alterations and phenotypes can indeed be transmitted across generations in mammals (for recent reviews see ^{46,83–85}).

Epidemiological studies in humans have implicated grandparental and parental environmental conditions such as nutrient availability, exposure to endocrine disruptors or traumatic experiences in disease susceptibility in descendants^{86,87}. In mammals, rodent models of coat color linked to the agouti^{vy} locus and DNA methylation⁵⁵ or exposure to the endocrine disruptor vinclozolin³⁴, have shown altered phenotypes that are stably transmitted to non-exposed offspring. Other exposure models including various dietary regimes^{12,88–95}, environmental toxins^{30,35,96–101}, postnatal trauma¹¹, prenatal glucocorticoids⁷³, prenatal immune activation³², psychotropic medication³¹ or olfactory stimulation¹⁰² have linked exposure to altered traits in non-exposed offspring. However today, most models are intergenerational and exhibit traits that are transmitted only to the direct offspring of exposed individuals but not to further generations. Moreover, most models use exposure until breeding, and thus have effects in the progeny that may be due to the acute exposure of sperm at the time of breeding that may not persist beyond exposure, which is a limitation.

To gain understanding of the mechanisms of transgenerational epigenetic inheritance, models with transmission up to the 3rd generation or possibly to further generations, are needed. Our lab has developed a mouse model of early postnatal trauma based on unpredictable maternal separation combined with unpredictable maternal stress (MSUS) in which multiple effects were documented in the offspring up to three generations. Mice exposed to MSUS and their offspring have increased risk-taking behaviors^{11,19,48,49,74,103,104}, depressive-like symptoms^{11,19,48,49,74,103,104}, altered social recognition¹⁰⁵, memory deficits¹⁰³ and insulin/glucose dysregulation⁴⁸. They also show stress resilience¹⁰⁵ and improved behavioral flexibility⁷⁴ in some conditions. The symptoms are transmitted through both males and females^{11,49}.

Because this model is robust and epigenetic changes have been detected in the male germline and in tissues in the offspring^{11,48} (female germline not tested), we examined whether symptoms are also present in the 4th generation produced from the patriline. The results show that both behavioral and metabolic symptoms induced by MSUS are present in mice from the 4th generation, indicating that MSUS is a solid and reproducible transgenerational model of early-life adversity.

6.3 Methods

6.3.1 Mice

C57Bl/6J mice were obtained from Janvier (France) and housed in a temperature and humidity-controlled facility on a 12h reversed light-dark cycle (white light from 20h00 until 8h00, dark from 8h00 until 20h00). Mice were housed in individually ventilated cages (SealSafe PLUS, Tecniplast, Germany) with bedding made from wood chips (LIGNOCEL SELECT, J. Rettenmaier & Söhne GmbH Co.KG, Germany) and nesting material consisting of paper tissue and cardboard houses. Animals had access to food (M/R Haltung Extrudat, Provimi Kliba SA, Switzerland, Cat. #3436) and water *ad libitum*, and cages were changed weekly. Experimental procedures were performed during the animals' active cycle (dark cycle) in accordance with guidelines and regulations of the cantonal veterinary office in Zürich and

the Swiss Animal Welfare Act (Tierschutzgesetz). All animal experiments were approved under license number 116/2005, 210/2008, 55/2012, 57/2015.

6.3.2 Unpredictable maternal separation combined with unpredictable maternal stress (MSUS) paradigm

C57Bl/6J naïve dams (3-4 month old) were mated with age-matched naïve males to produce MSUS mice. After one week, males were separated from females to avoid any interference with gestation. Two days prior to predicted delivery, nesting material was removed from each cage until PND21. At delivery, dams and pups were randomly assigned to MSUS or control groups, taking into account the number of male pups born within each day to balance male group sizes. Mouse pups (F1) were separated from their mother for 3 hours per day from postnatal day 1 (PND1) to PND14. Separation occurred at unpredictable times during the dark (active) cycle. In addition, mothers were subjected to either a modified forced swim test in cold water (18 °C for 5 min, more details in section “forced swim test”) or restraint in a plastic tube (20 min, 3.18 cm diameter with sliding nose restraint, Midsci) (randomly) at an unpredictable time during the 3 hours of separation. Mothers were subjected to only one stressor per day. Control animals were left undisturbed except for weekly cage changes and weight measurements. Pups were weaned at PND21 and assigned to sex- and treatment-matched cages (4 to 5 mice) between PND22 and PND28. Siblings were assigned to different cages to avoid litter effects. Breeding of F1, F2 or F3 males was usually conducted after phenotyping following several weeks of rest. It was however also conducted occasionally before phenotyping to confirm that testing has no influence on the offspring (in other animals than reported here).

6.3.3 Breeding size and litter numbers

For all F0 breedings conducted to generate F1 offspring, 40 naïve males were paired with 40 naïve females. The number of litters produced for each F1 breeding is as follows: Breeding 22 produced 17 control and 15 MSUS litters, Breeding 23 produced 12 control and 14 MSUS litters and Breeding 28 produced 14 control and 13 MSUS litters. Litter size ranged between 1-14 animals (females and males). For generating F2 and F3 mice, the number of

males chosen for breeding depended on the number of offspring needed for planned experiments, the availability of space in animal housing facilities and the number of males available for breeding. For Breeding 20, 18 control and 16 MSUS F1 male mice were each paired with one naïve female and produced 13 control and 14 MSUS F2 litters. In Breeding 23, 25 control and 17 MSUS F1 males were each paired with one naïve female and produced 17 control litters and 11 MSUS F2 litters. In Breeding 25, 14 control and 14 MSUS F1 males were each paired with one naïve female and produced 13 control and 12 MSUS F2 litters. For generating F3 males, breeding and litter sizes are as follows: Breeding 7 consisted of 15 control and 15 MSUS F2 males that were each paired with one naïve female and produced 11 control and 7 MSUS litters, Breeding 21 consisted of 10 control and 12 MSUS F2 males that were each paired with one naïve female and produced 5 control and 9 MSUS litters and Breeding 23 consisted of 20 control and 20 MSUS F2 males that were each paired with one naïve female and produced 14 control and 14 MSUS litters. For generating F4 males and females, Breeding 7 consisted of 16 control and 14 MSUS F3 males that were each paired with one naïve female and produced 11 control and 7 MSUS litters and Breeding 23 consisted of 16 control and 16 MSUS F3 males that were each paired with one naïve female and produced 14 control and 13 MSUS litters. The reported number of litters include only litters containing males used for final cage assignment (or females in the case of Breeding 7), and do not include litters that were not used. Litters with less than 5 or more than 10 pups were excluded, or if the mother was ill or died before weaning.

6.3.4 Behavioral Testing

At all times, the experimenter was blind to the identity and treatment of animals. Experiments were conducted during the animals' active cycle (dark phase). Control and MSUS animals were alternately tested to avoid circadian effects or group bias.

6.3.4.1 *Elevated plus maze*

The elevated plus maze is a platform raised 60 cm above the ground with two opposing open arms and two opposing closed arms. Closed arms have 15 cm

walls and are closed at their extremity, such that a mouse can enter the arm from the center of the maze, but cannot see or reach over the sides. Each arm is 30x5 cm. Open arms were exposed to 18 ± 1 lux and closed arms to 9 ± 1 lux. The start of each test was remotely activated when each mouse was individually placed in the center of the platform, facing an open arm. Mice were left on the maze for 5 min and their behavior was monitored with a video tracking system (ViewPoint Behavior Technology, France) and viewed by the experimenter through a screen in a separate room. The latency to first enter an open arm was recorded manually from the screen while time spent in center and on each arm, and total distance covered on the maze were recorded automatically by the video tracking system.

6.3.4.2 Forced swim test

Mice were placed in a 5 liter cylindrical plastic tank of water for 6 min. Tank height measures 25 cm high and 19 cm in diameter, and was filled up to 3.4 liters with water at a temperature of 18 ± 1 °C. For maternal stress during the MSUS paradigm, slightly different conditions were applied. Mothers were placed for 5 minutes in a 3 liter cylindrical plastic tank, measuring 18 cm high and 13 cm in diameter, which was filled to 2.2 liters with water at a temperature of 18 ± 1 °C. In both cases the water level was sufficient to ensure that no mouse could touch the bottom of the tank, or reach the top and jump away to escape. For behavioral phenotyping, but not for maternal stress, video recording was initiated remotely when the mouse was placed in water, floating duration was scored manually. For statistical analysis, minute 3 to 6 was scored. The tank was cleaned and water was refreshed regularly.

6.3.5 Metabolic Assays

At all times, the experimenter was blind to the identity and treatment of animals. If more than one experimenter was required for a given test, it was designed so that each experimenter had balanced numbers of control and MSUS mice to avoid experimenter-specific effects. Experiments were conducted during the animals' active cycle (dark phase). Control and MSUS animals were alternately tested or tested side-by-side to avoid circadian effects or group bias.

6.3.5.1 Glucose in response to restraint challenge

Mice were temporarily single-housed at least 4 h prior to testing, but not more than 18 h. Each individual mouse was placed for 30 min in a cylindrical plastic tube (3.18 cm diameter with sliding nose restraint; Midsci) for physical restraint, and blood was drawn by a tail prick, within 1 cm of tail end using a 28G needle, 0, 15, 30 and 90 min after initiation of restraint. After 30 min, mice were released from the tube and placed in a temporary cage, until all samplings were completed (until 90 min time point). At 90 min, mice were confined under an inverted 1 liter glass beaker (dimensions 14.5 cm high and 12 cm diameter) with their tail exiting from under the spout to allow access by the experimenter for blood sampling. Mice were then immediately (within 10 sec) placed back into their cage. Glucose was measured from fresh blood droplets with an Accu-Chek Aviva glucometer (Roche, Switzerland). Blood collection was conducted under red lighting (637 nm), which is invisible to mice, to maintain the dark cycle. Testing started at 14:00 for mice from F3 breedings, and at 09:00 for mice from F4 breeding.

6.3.5.2 Food intake and weight measurement

Food pellets were measured in each cage (maximum 5 animals/cage) daily for 72 h, and averaged per animal. Pellets were replaced with fresh pellets every 24 h to limit crumb spillage. Animals were weighed prior to the onset of the experiment and at the time of the last measurement under red lighting.

6.3.6 Statistics

Statistical analyses were performed using GraphPad Prism software (Version 7). For all analyses, except for glucose in response to restraint, independent sample *t* test was used. For analyzing glucose values in response to restraint, 2-way ANOVA with repeated measures was performed. For outlier removal, predetermined criteria were selected as follows: For behavioral data, outliers were removed according to the robust regression and outlier removal (ROUT) test, with Q set at 5%. For metabolic data, outliers were defined as values that lie outside of 2 standard deviations from the group mean. P<0.1 was considered a trend, indicated by a hashtag, and P<0.05 was considered

significant, indicated by asterisks. The use of blue (control) and red (MSUS) colors in Figures 2 to 5 and Supplementary Figures 1 to 3 was matched to the breeding and experimental scheme shown in Figure 1.

6.4 Results

6.4.1 MSUS paradigm

The MSUS paradigm was designed to mimic in mice, exposure to traumatic experiences during childhood in humans. MSUS is based on the combination of adverse conditions subjected to young mouse pups during early postnatal life and to their mother during the same period (Fig. 1). The paradigm consists of separating mouse pups (F1) from their mother (F0) unpredictably each day for 3 hours from postnatal day 1 (PND1) to PND14 (Fig. 1A). In this paradigm, unpredictability is critical because it avoids that mothers predict separation and compensate for their absence by providing more maternal care before separation. Instead, it leads to an overall decreased and disorganized maternal care, especially between PND1 and PND7¹¹. Further to separation, dams are also exposed to an additional stressor unpredictably (anytime during the 3 hours of separation) (Fig. 1B). Here again, unpredictability is important as it increases the severity of the stressor, and the combination of unpredictable maternal separation with such unpredictable maternal stress was shown to induce stronger behavioral phenotypes in the offspring than separation alone⁴⁹. Breeding was conducted until the 4th generation by mating adult males at each generation (F1, F2 and F3) with naïve primiparous control females (Fig. 1C).

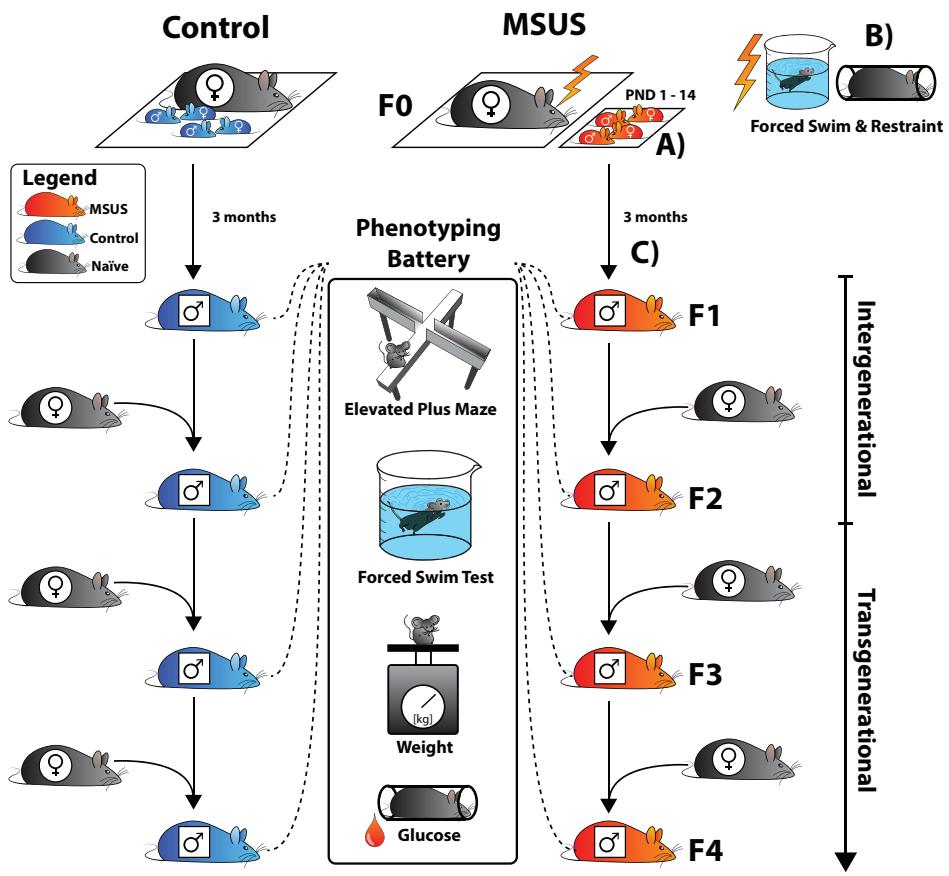


Figure 1: MSUS paradigm. MSUS consists of (A) separating mouse pups (F1) from their mother (F0, naïve primiparous control females mated with naïve males) daily for 3 hours per day at an unpredictable time during the 12h active cycle, starting 1 day after birth (postnatal day 1, PND1) until PND14. (B) During separation, dams are exposed to an additional unpredictable stressor by being subjected to either, a forced swim in 18°C water for 5 minutes or a 20 minute physical restraint in a tube, anytime (unpredictably) during the 3h. From PND15, mice are left undisturbed with their mother until PND21 (no further MSUS), are then weaned at PND21 and raised normally until adulthood (C). Control litters are raised normally (left). Males used to generate the pups are removed from the breeding cage shortly after mating thus, fathers never encounter their offspring and do not contribute to their rearing. When adult (3-8 months of age), F1 males are paired with naïve primiparous control females to sire the F2 generation, then F2 and F3 males are bred with naïve primiparous control females to generate an F3 and F4 offspring, respectively. Males from each generation are tested on the elevated plus maze, forced swim test, weight measurements and glucose response after physical restraint. MSUS is applied only to F1 mice, mice from F2, F3 and F4 generations are not exposed to any manipulation. Phenotypes transmitted from father to offspring are intergenerational,

phenotypes that persist from father to offspring then grand-offspring or great grand-offspring are transgenerational.

6.4.2 Transgenerational effects of MSUS are robust and reproducible, and observed until the 4th generation

The MSUS paradigm has been repeatedly demonstrated to cause behavioral and metabolic alterations in adulthood, not only in directly exposed mice (F1) but also in their (unexposed) offspring (F2)^{11,19,48,49,103,105} and grand-offspring (F3)^{11,105}. We consolidated these findings by obtaining different cohorts of mice from several MSUS breedings across several years. The mice were tested on different tasks by different experimenters and the data were pooled to reach large n (Supplementary Table 1). When tested on an elevated plus maze, F1 and F2 MSUS males spent more time on the open arms of the maze (Fig. 2A), confirming transmission of reduced aversion to open space from father to offspring. The grand-offspring (F3) did not show this phenotype. However, mice from F1, F2 and F3 generations had decreased latency to first enter an open arm (Fig. 2B), suggesting increased risk-taking behavior that is transgenerationally transmitted to F3. Further to the elevated plus maze, F3 mice were also tested on a forced swim test to assess passive coping, a trait characteristic of depressive-like behaviors. Consistent with that observed previously¹¹, F3 MSUS males spent more time floating than controls (Fig. 3A), confirming that depressive-like symptoms are reproducible in the 3rd generation.

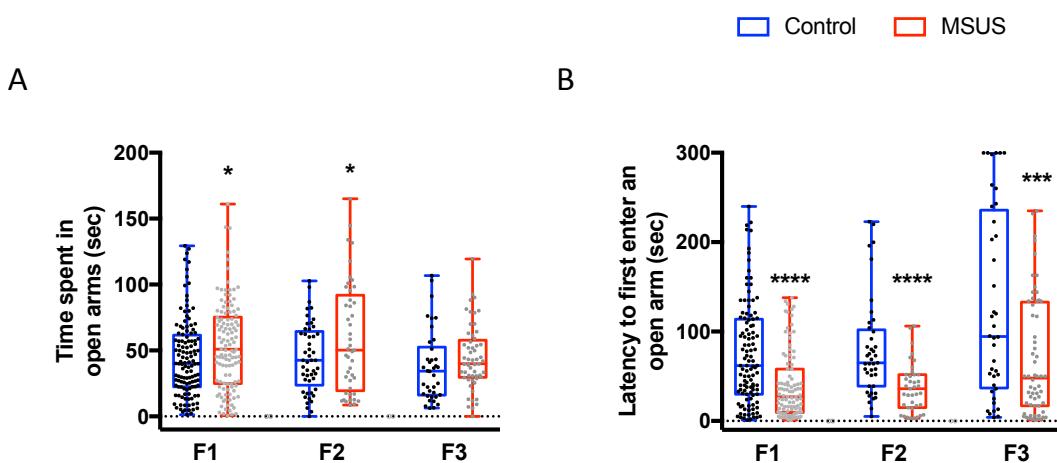


Figure 2: Persistent behavioral effects of MSUS across 3 generations on the

elevated plus maze. MSUS treatment (A) increases the amount of time spent on the open arms of an elevated plus maze in F1 and F2 mice but not F3 mice (F1 control n=124, MSUS n=118, $t_{240}=2.26$ P=0.025; F2 control n=49, MSUS n =45, $t_{92}=2.096$ P=0.039; F3 control n=38, MSUS n=57, $t_{93}=1.244$ P=0.217) and (B) decreases the latency to first enter an open arm in F1, F2 and F3 mice (F1 control n=111, MSUS n=101, $t_{210}=5.298$ P<0.0001; F2 control n=41, MSUS n=39, $t_{78}=4.353$ P<0.0001; F3 control n=40, MSUS n=59, $t_{97}=3.51$ P=0.0007). Data represent mean \pm whiskers. Reported n represent data after outlier removal using the ROUT test at Q=5%. *P<0.05, ***P<0.001, ****P<0.0001

While transgenerational transmission across 3 generations has been reported twice with different behavioral tests in MSUS mice^{11,105}, transmission to the 4th generation has not yet been examined. We produced a 4th generation of MSUS and control mice by breeding F3 males to control females and generated 2 batches of mice, 10 years apart (2007 and 2017; Supplementary Table 2) then tested the male offspring when adult (different experimenters). When tested on the elevated plus maze, F4 males from both batches spent significantly more time in the open arms of the maze and had significantly shorter latency to first enter an open arm, similar to F1, F2 and F3 males (Fig. 3B). Total distance covered was not changed, as expected (Supplementary Fig. S1A). Then, when tested on the forced swim test, F4 MSUS males (Batch 2 only, Batch 1 not tested in 2008) spent a similar amount of time floating to controls (Fig. 3C), suggesting no depressive-like symptoms thus no apparent transmission of this trait beyond F3.

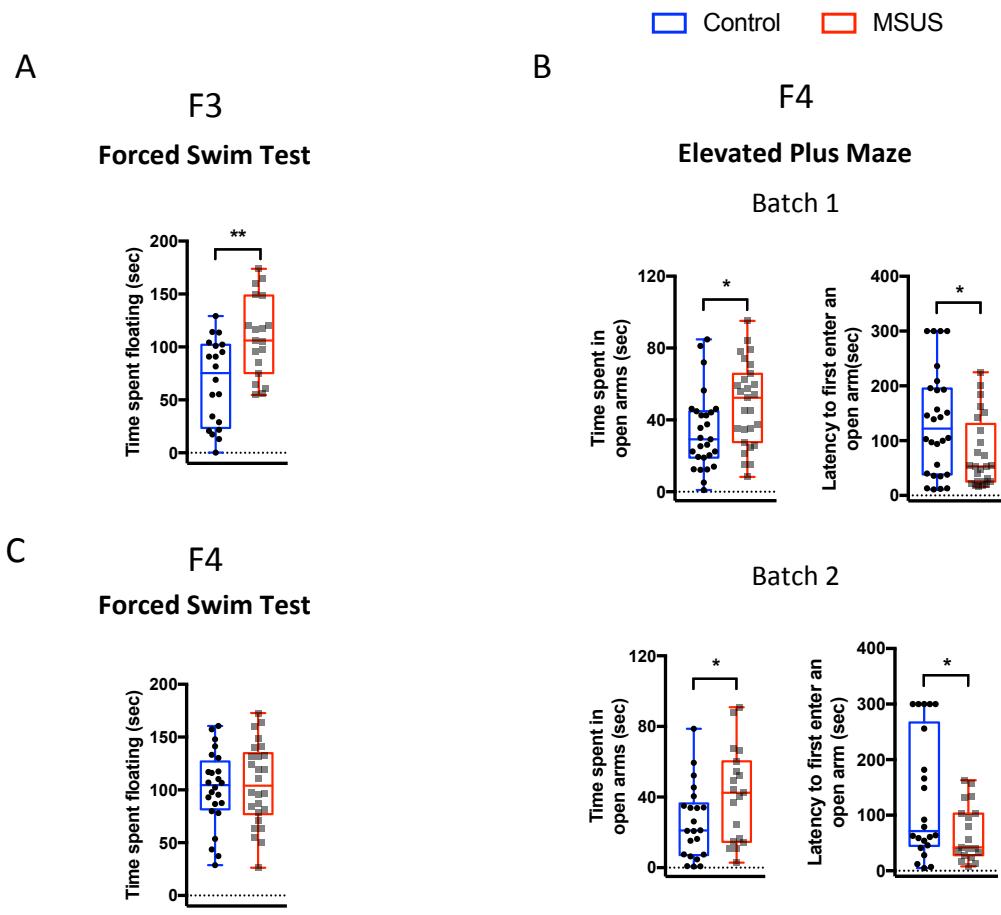


Figure 3: Reproducible behavioral alterations by MSUS in F3 and F4 generations. Depressive-like symptoms shown by increased time spent floating on a forced swim test in MSUS males from (A) F3 generation but not from (C) F4 generation (F3: control n=20, MSUS n=19 $t_{37}=3.37$ P=0.0018; F4: Batch 2 control n=24, MSUS n=26 $t_{48}=0.424$ P=0.6732). In (B), separate batches of F4 males (Batch 1 and 2) were tested on the elevated plus maze. Time spent on the open arms and latency to first enter an open arm are similarly altered in both batches. (Batch 1 for time spent on open arms: control n=22, MSUS n=19, $t_{52}=2.49$ P=0.0161; Batch 1 for latency to enter an open arm: control n=22, MSUS n=19, $t_{51}=2.432$ P=0.019; Batch 2 for spent time on open arms: control n=27, MSUS n=27, $t_{39}=2.159$ P=0.037; Batch 2 latency to first enter an open arm: control n=28, MSUS n=25, $t_{39}=2.209$ P=0.033). Data represent mean \pm whiskers. Reported n represent data after outlier removal using the ROUT test at Q=5%. *P<0.05, **P<0.01

To examine if these effects affect both sexes, we also tested F4 MSUS and control females. Similar to males, F4 MSUS females spent more time in the open arms and had shorter latency to first enter an open arm on the elevated

plus maze compared to F4 controls (Fig. 4B), but had normal locomotor activity (Supplementary Fig. S1B). F4 females spent a similar amount of time floating as control females in a forced swim test (Fig. 4A), suggesting no depressive symptoms, similar to F4 MSUS males. These findings establish that the effects of paternal MSUS persist across generations, some until the F3 and F4 generations. Further, they highlight risk-taking behavior as a highly consistent trait that similarly affects males and females up to the 4th generation.

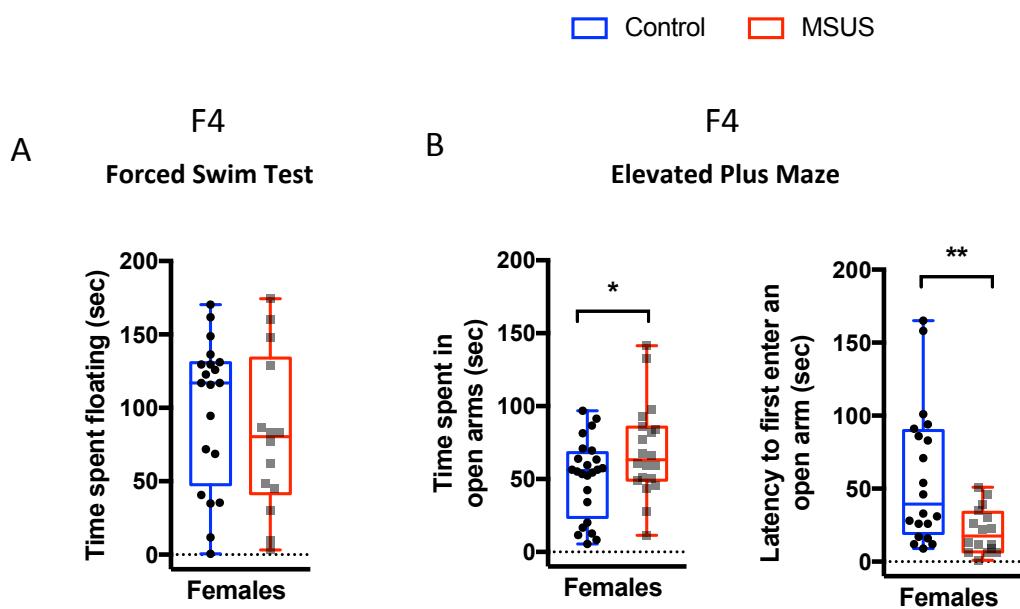


Figure 4: Behavioral phenotypes in F4 female progeny. (A) F4 MSUS females do not significantly differ from control females in time spent floating during the forced swim test (control n=20, MSUS n=14, $t_{32}=0.918$ P=0.366). (B) Time spent in the open arms of the elevated plus maze was increased (control n=24, MSUS n=20, $t_{42}=2.09$ P=0.043), while latency to first enter an open arm was decreased in F4 MSUS females (control n=20, MSUS n=16, $t_{34}=3.01$ P=0.005). Data represent mean \pm whiskers. Reported n represents data after outlier removal using the ROUT test at Q=5%. *P<0.05, **P<0.01

6.4.3 Metabolic effects of MSUS are transmitted transgenerationally

MSUS has been shown to dysregulate glucose and insulin levels in F1 mice and their offspring⁴⁸. We examined if these symptoms are also present in mice from the F3 and F4 generations. In both F3 and F4 MSUS males, there

was a trend for increased glucose at baseline compared to control males (Fig. 5A), unlike F2 MSUS males which had decreased glucose levels⁴⁸. However, during a physical restraint challenge, the mounting of glucose response was modestly but significantly attenuated in F4 MSUS males compared to controls, similar to that observed in F2 MSUS males⁴⁸ (Fig. 5B). This effect was not observed in F3 MSUS males (Supplementary Fig. S2). Interestingly in F4 MSUS mice, body weight was lower than controls at PND21 but slightly increased in adulthood (Fig. 5C), suggesting a rebound response. This response may be due to increased food consumption as indicated by higher food intake in MSUS mice (Fig. 5D), but does not result from an inherently larger body size since tail length was normal in MSUS mice (Fig. 5E). Body weight and food intake in F4 females was unaffected by MSUS (Supplementary Fig. S3).

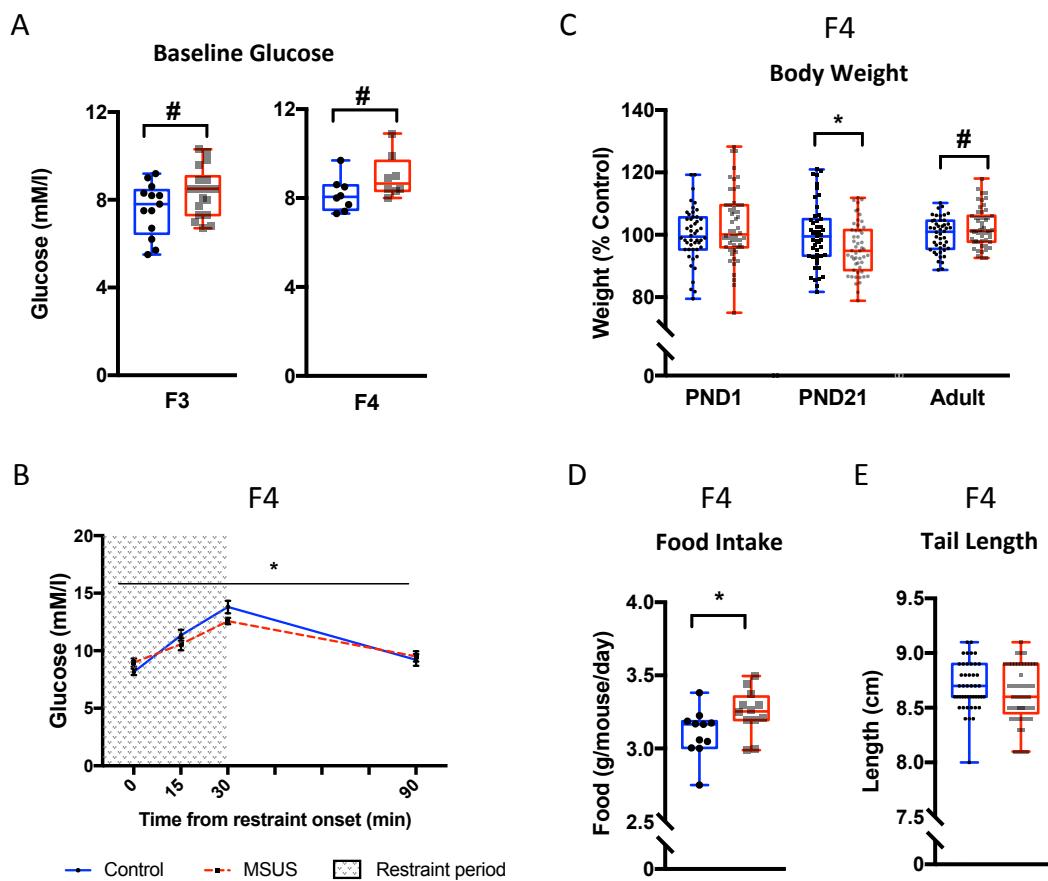


Figure 5: Transgenerational effects of MSUS treatment on glucose level. (A) Baseline glucose was measured in whole blood following tail prick in F3 (left) and F4 males (right) (F3 control n=13, MSUS n=18, $t_{29}=1.891$ P=0.069; F4 control n=8, MSUS n=8, $t_{14}=1.84$ P=0.087). Continuing from (A), glucose concentrations in F4

blood (B) was measured at 15-minute intervals during a 30-minute physical restraint challenge, and 60 minutes after release from the restraint tube (control n=8, MSUS n=8, for interaction $F_{3,42}=2.99$ P=0.042). (C) Body weight of F4 males was measured at PND1, PND21 and in adulthood (PND1: control n=48, MSUS n=52, $t_{99}=1.29$ P=0.199; PND21: control n=46, MSUS n=50, $t_{94}=2.27$ P=0.025; adult: control n=46, MSUS n=51, $t_{95}=1.86$ P=0.065). (D) Food intake (control n=11, MSUS n=12, $t_{21}=2.185$ P=0.04; n represents number of cages) and (E) tail length were measured in adult mice (control n=46, MSUS n=51, $t_{92}=1.38$ P=0.172). Data represent mean ± whiskers (a, c-e) or s.e.m (b). Reported n represents data after outlier removal. #P<0.1, *P<0.05

6.5 Discussion

This study provides evidence that exposure to traumatic stress in early postnatal life in mice induces several behavioral alterations that are transmitted across several successive generations. While increased risk-taking and glucose dysregulation affect mice up to the 4th generation, depressive-like behaviors affect F3 but not F4 MSUS males. This indicates that risk-taking is a robust trait that perpetuates in descendants, suggesting that it may be more penetrant than other traits. This may be because, although disadvantageous in some conditions, it may be beneficial in challenging situations and provide a form of active coping advantage. In contrast, passive coping associated with behavioral despair as observed on the forced swim test was not expressed by F4 MSUS mice. This however does not mean that this trait has disappeared, since we observed in the past that some F2 MSUS males did not show any depressive-like symptoms (were asymptomatic) but were still able to transmit this trait to their offspring¹¹. The manifestation of both risk-taking and depressive-like behaviors in MSUS mice is interesting because, together with antisocial behaviors observed in F3 males¹⁰⁵, these traits are typical of a common psychiatric disorder, borderline personality disorder (BPD), which has a lifetime prevalence of 6.4% in the population¹⁰⁶. BPD is a severe condition characterized by impulsive behaviors leading to risky and potentially life-threatening conduct¹⁰⁷, emotional lability including depressed mood¹⁰⁷, and impaired social functioning^{108,109}. MSUS

mice also have memory deficits¹⁰³ and stress-induced analgesia (unpublished data), which are other prominent BPD symptoms^{110, 111}. Notably, BPD has a strong heritability component that cannot be explained by genetic factors alone¹¹², and instead, the risk to develop the disorder has been associated with adverse childhood experiences^{113,114}. Traumatic experiences in humans are known to result in maladaptive coping strategies¹¹⁵ and in increased risk-taking behavior when occurring in childhood¹¹⁶. The environmental etiology of BPD and its known heritability suggest that it likely involves epigenetic factors, possibly in the germline. While in humans, germline-dependent inheritance is difficult to prove and cannot easily be distinguished from social and rearing factors e.g. being raised by a parent with a psychiatric illness can predispose a child to psychiatric illness¹¹⁷, germline-dependent inheritance implicating sperm RNA has been causally demonstrated in our MSUS model⁴⁸.

Further to behavioral deficits, MSUS also causes metabolic alterations across generations. Metabolic symptoms have been reported in humans exposed to trauma, and metabolic syndrome can develop in response to prolonged stress¹¹⁸ and in people suffering from BPD¹¹⁹ and post-traumatic stress disorder¹²⁰. Notably, the effects of MSUS on metabolism are expressed differently across generations. While in F2 MSUS males, baseline glucose is downregulated⁴⁸, it is slightly upregulated in F3 and F4 MSUS mice. This is in contrast to behavioral traits which are similarly expressed across generations but is a phenomenon already observed in other transgenerational models^{34,94}. Differential expression of phenotypes across generations has been reported in other models of transgenerational epigenetic inheritance¹²¹.

In addition to male phenotypes, we also extend the previously reported transgenerational effects to MSUS females¹¹ until the fourth generation. Behavioral differences in F4 MSUS females are directly comparable to F4 MSUS males, suggesting similar trait penetrance through the patriline in females. However, this is not the case for metabolic phenotypes, suggesting different mechanisms of transmission depending on the phenotype. Regarding potential mechanisms of transmission, our past work demonstrated a causal role for sperm RNA in the transfer of phenotypes⁴⁸, and correlated

changes in DNA methylation in sperm with transgenerational phenotypes¹¹, suggesting that several epigenetic factors are likely implicated. Others factors or mechanisms may also be involved^{58,60}.

A distinct and unique feature of the MSUS paradigm is that it is postnatal, which provides a significant advantage over prenatal models because it avoids interference with gestational developmental processes and epigenetic reprogramming occurring during embryogenesis⁴⁶. MSUS exposure is also brief and limited to a specific time window between PND1 and PND14, allowing for easier identification of the cells affected during that time, in particular in developing gonads which have only a limited number of different cell types at this stage. Most other models have exposure extending from before conception throughout embryogenesis and postnatal development until adulthood^{88,91–93,102}, making interpretation more difficult as each developmental stage is likely to be affected, producing cumulated effects. Further, in models with adult exposure extending until breeding, the effects can result from acute factor(s) in gonads, supporting cells or seminal fluid at the time of conception, and may not just implicate germ cells. With MSUS, breeding occurs many months after exposure, eliminating any acute changes and selecting for effects that persist until adulthood. This persistence suggests that spermatogonial cells may be affected. Another unique advantage of MSUS is that it does not involve any drug, chemical, nutritional insult or invasive manipulation, and relies on “natural” aspects of childhood mistreatment such as neglect, attachment disruption and abuse. Other physiological parameters like altered maternal milk composition or lower body temperature due to separation may also be implicated.

The use of a battery of behavioral tasks including the elevated plus maze, forced swim test and in previous studies, the open field test, light-dark box, emergence test, fear conditioning, social interaction task, operant conditioning, object recognition and social defeat, and of several parameters on some of the tests e.g. latency to enter and time spent in open arms on the elevated plus maze, generated a comprehensive and thorough behavioral profiling of MSUS mice across generations. After conducting MSUS treatment

in 30 independent experiments since 2001 with up to 40 breeding pairs each time, this study identifies the elevated plus maze and forced swim test as reliable tests to validate the effects of MSUS across generations. The effects observed on these tasks are robust, consistent and highly reproducible. MSUS is currently one of the few available mouse models of transgenerational epigenetic inheritance with transmission up to the 4th generation, a depth of inheritance previously demonstrated in rodents with prenatal stress²¹, toxicants^{30,34}, drugs^{31,36} or genetic mutation (*Mtrr* hypomorphic)³⁷. Studies of the mechanisms of transgenerational inheritance are expected to have important implications for public health in the future.

6.6 Acknowledgments

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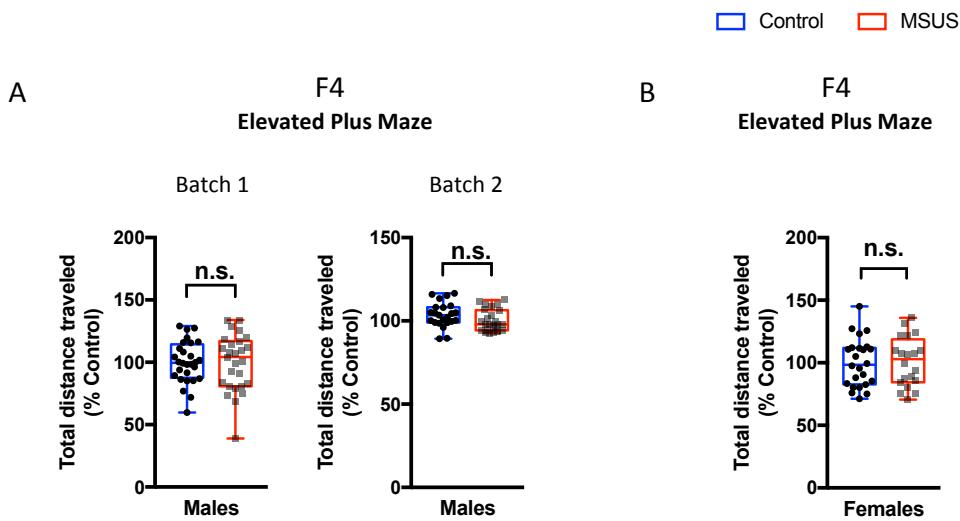
6.7 Authors Contributions

GvS, TF and IMM conceived and designed the study. GvS and IMM wrote the manuscript. GvS and MR compiled and analyzed raw data, and prepared figures. GvS, FM and TF performed behavioral experiments. FM organized and managed breedings. GvS, MR and FM performed metabolic experiments. IMM raised funds to support this project.

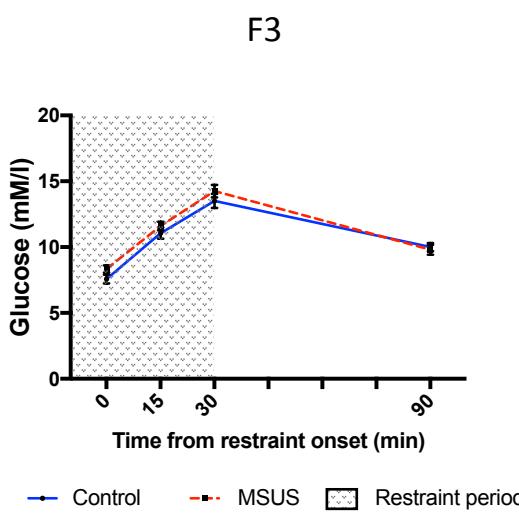
6.8 Conflict of Interest Statement

The authors declare no conflict of interest

6.9 Supplementary data

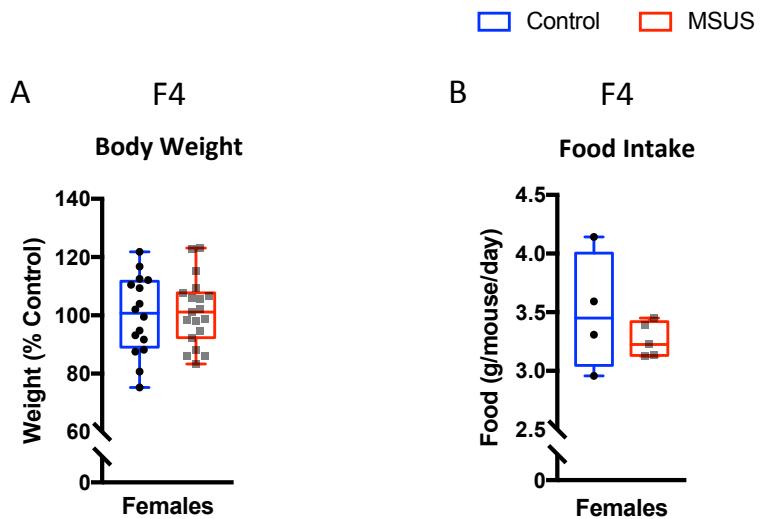


Supplemental Figure 1: Locomotor activity is not altered by MSUS in F4 males or females. (A) Normal locomotor activity in F4 males from Batch 1 or 2 on the elevated plus maze (Batch 1: control n=28, MSUS n=27, $t_{53}=0.06$ P=0.9521; Batch 2: control n=24, MSUS n=24, $t_{45}=1.61$ P=0.115). (B) Females also do not demonstrate altered locomotor activity on elevated plus maze (control n=24, MSUS n=20, $t_{42}=0.133$ P=0.89). Data represent mean \pm s.e.m. Reported n represent data after outlier removal using the ROUT test at Q=5%. n.s., not significant.



Supplemental Figure 2: F3 MSUS males have normal blood glucose level during and after a restraint challenge. (A) Glucose concentration in blood measured at 15-minute intervals during a 30-minute restraint stress, and 60 minutes after release from the restraint tube (control n=13, MSUS n=17, for

interaction $F_{3, 90} = 0.89$ $P=0.45$). Data represent mean \pm s.e.m. Reported n represents data after outlier removal.



Supplemental Figure 3: F4 females have unaltered body weight and normal food intake. (A) Body weight (control n=16, MSUS n=19, $t_{33}=0.319$ $P=0.751$) and (B) food intake (control n=4, MSUS n=5, $t_7=1.01$ $P=0.347$; n represents number of cages) were not significantly different in F4 MSUS females compared to controls. Data represent mean \pm s.e.m. Reported n represent data after outlier removal using the ROUT test at Q=5%.

Supplementary Table 1: Animal numbers and technical parameters of breedings used for elevated plus maze analyses in Figure 2. Table represents data and breedings/generations for experiments using the elevated plus maze. The total number of animals in each group is shown, and the numbers used for statistical analysis after outlier removal by ROUT test set at Q=5%. For each breeding, we report the name of experimenters (defined by their initials) who prepared the animals using the MSUS paradigm, and the name of experimenters who conducted the phenotyping when the mice were adult. The column “Date” reports month and year of birth of animals for each generation. Total numbers are calculated separately at the bottom. Data shown does not contain previously published data.

Breedings	Animals tested		Time spent in open arms		Latency to first enter an open arm		Experimenter(s)		Date
	Control	MSUS	Control- after ROUT	MSUS- after ROUT	Control- after ROUT	MSUS- after ROUT	MSUS treatment	Phenotyping	
F1									
Breeding 22	31	47	31	47	28	42	GvS, FM, MR	FM	07.2015
Breeding 23	40	33	40	33	38	29	FM, GvS, IL	FM	10.2015
Breeding 28	53	38	53	38	45	30	MR, IL	GvS	08.2017
F2									
Breeding 20	13	13	13	13	10	12	FM, KG, JB, GvS	FM	07.2014
Breeding 23	16	13	16	13	13	9	FM, GvS, IL	FM	05.2016
Breeding 25	20	19	20	19	18	18	FM, MR, IL, JB, GvS	GvS	05.2017
F3									
Breeding 7	20	19	20	19	20	16	TF	TF	06.2007
Breeding 21	9	28	8	24	9	27	GvS, FM	FM	09.2015
Breeding 23	11	16	10	14	11	16	FM, GvS, IL	FM	11.2016
Total									
F1	124	118	124	118	111	101			
F2	49	45	49	45	41	39			
F3	40	63	38	57	40	59			

Supplementary Table 2: Technical parameters for F3 and F4 batches of males used for forced swim task and elevated plus maze in Figure 3. Table showing technical differences between independent batches of mice used for the forced swim test in F3 mice and elevated plus maze in F4 mice. Each batch was separated by 10 years, and different experimenters (defined by their initials) performed the experiments. Dates indicate when animals were phenotyped.

Test	F3 Forced swim test		F4 Elevated plus maze	
Batch	Batch 1	Batch 2	Batch 1	Batch 2
Experimenter	TF	FM	TF	GvS
MSUS Breeding	7	23	7	23
Date (month.year)	11.2007	03.2017	07.2008	01.2018

Supplementary Table 3: Technical parameters for F4 females used for forced swim task and elevated plus maze in Figure 4. Table indicates the number of female mice used for behavioral phenotyping. Using the ROUT test at Q=5%, outliers were only removed for the parameter measuring the latency to first enter an open arm of the elevated plus maze. Only F4 females from Breeding 7 were tested.

F4 females	Forced swim test	Elevated plus maze	
MSUS Breeding	7	7	
Parameter measured	Time spent floating	Time spent in open arms	Latency to first enter an open arm
Number of mice tested	Control=20, MSUS=14	Control=24, MSUS=20	Control=24, MSUS=20
Number of mice after outlier removal	Control=20, MSUS=14	Control=24, MSUS=20	Control=20, MSUS=16

7 Nuclear receptor signaling in the communication between blood and the germline

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7.1 Abstract

In many species, environmental stimuli can leave molecular traces in the germline and contribute to phenotypic changes in the offspring, in some cases across generations^{11,34,98,122,123}. So far, little is known about which signals from the periphery are involved in the communication with the germline. We postulate that these signals are induced by circulating factors released in response to environmental exposure. Here, we show that when serum from adult mice exposed to postnatal trauma is injected in naïve mice it can induce aberrant metabolic phenotypes in the offspring, similar to those expressed by the offspring of directly exposed mice. Metabolomics analysis identified polyunsaturated fatty acid metabolism and nuclear receptor activation involving peroxisome proliferator-activated receptors (PPARs) as potential mediators for such transmission. Mimicking PPAR activation with a dual PPAR α/γ agonist affects the sperm transcriptome in fathers, similar to sperm of traumatized fathers, and reproduces the metabolic phenotypes in the offspring and grand-offspring. Similar metabolomic pathway alterations could be validated in humans, in both serum and saliva from children exposed to early childhood adversity. These results suggest conserved effects of early life adversity on fatty acid metabolism, and a causal involvement of paternal blood factors and PPAR nuclear receptors in transgenerational transmission of phenotypes.

Key words: transgenerational inheritance, nuclear receptors, PPAR, serum, fatty acids, MSUS

7.2 Main Text

We used an established transgenerational mouse model of traumatic stress in postnatal life to examine whether blood can be a carrier of signals induced by environmental exposure, which interact with germ cells. This model is based on daily exposure to unpredictable maternal separation combined with unpredictable maternal stress (MSUS) from 1 day after birth until postnatal day 14 (PND14) (Fig. 1, left). It induces phenotypic alterations in exposed animals and their offspring when adult up to the fourth generation^{11,74,104,124}. To assess if circulating factors are linked to transmission of phenotypes, we collected blood from 4-month old MSUS and control males, prepared serum and injected 90 µl intravenously (i.v.) in naïve adult males twice per week for 4 weeks (Fig. 1, middle). Following this regime, males were bred with naïve females to generate an offspring that were phenotyped when adult. The offspring of males injected with MSUS serum trended towards reduced weight ($P = 0.059$; Fig. 2a) and had significantly lower glucose during a restraint challenge (Fig. 2c), similar to MSUS offspring⁴⁸ (Fig. 2a-b). These results suggest that factors in blood are sufficient to induce the transmission of trauma effects to the offspring.

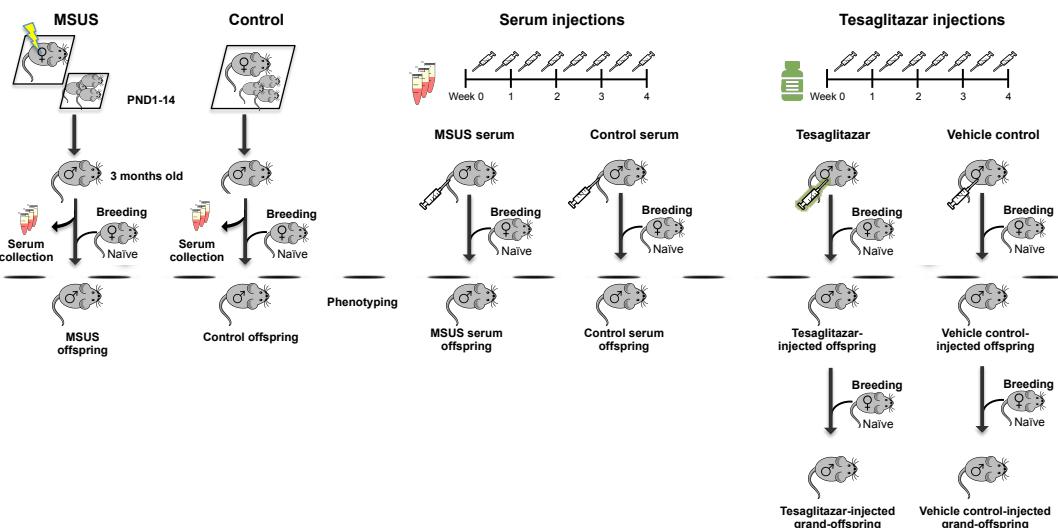


Figure 1. Overview of experimental design.

At 3 months of age, serum was prepared from blood taken from MSUS and control animals (left) and injected twice per week for 4 weeks into naïve age-matched males (middle). After injections, males were paired with naïve control females and their offspring were phenotyped when 3-month old and compared to the offspring of

MSUS males. Following a similar schedule as serum injections (2 per week for 4 weeks), naïve males were either injected with tesaglitazar (green syringe) or vehicle control (gray syringe; right). Males were paired with naïve females and the offspring and grand-offspring were phenotyped and compared to the offspring of MSUS males.

To identify factors in circulation that could be responsible, we conducted unbiased metabolomic analyses of blood. In adult blood, metabolites include several classes of potent signaling molecules such as hormones, lipids, organic acids and antioxidants, which are sensitive to the environment and in many cases, involved in epigenetic mechanisms of genome regulation^{70,125,126}. We conducted methanol extraction of metabolites from plasma from control and MSUS males followed by high-throughput time-of-flight mass spectrometry (TOF-MS). The results showed that metabolomic pathways involving polyunsaturated fatty acids (PUFAs), α -linolenic and linoleic acid (ALA/LA), and arachidonic acid (AA) were significantly upregulated in adult MSUS males compared to controls. In contrast, bile acid biosynthesis and to a lesser extent steroidogenesis, were downregulated (Fig. 3, full table in Extended Data Fig. 1). Lower bile acid biosynthesis is consistent with the reported ability of PUFAs to reduce the expression of genes involved in such biosynthesis¹²⁷ while altered steroidogenesis is consistent with our previous data showing reduced expression of the mineralocorticoid receptor, a receptor for the steroidogenic metabolite aldosterone. Aldosterone was itself downregulated in plasma by MSUS (Extended Data Fig. 2) and pharmacological blockade of its receptor was previously shown to induce effects similar to MSUS⁷⁴. Remarkably, except for AA metabolism, these pathways were also altered in plasma from the adult offspring of MSUS males (Fig. 3). Given the persistence of these alterations across generations, we next examined if they were directly affected in postnatal life or emerged as a secondary effect later in adulthood. We conducted metabolomic analysis similar to adults in plasma from pups after 7 days of MSUS or control conditions (PND8). Consistent with that observed in adults, ALA/LA and AA pathways were significantly altered in MSUS pups, however in an opposite direction. Bile acid and steroidogenesis pathways

were unchanged (Fig. 3). These results suggest that PUFAs metabolism is perturbed by MSUS in early postnatal life and is remodeled during development, possibly through compensatory mechanisms, and may lead to an alteration of downstream cascades in adulthood¹²⁸.

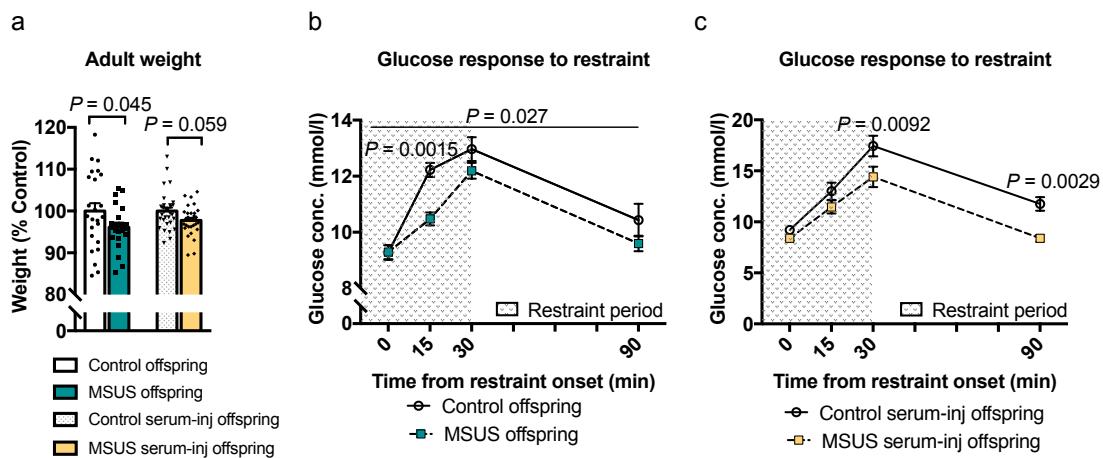


Figure 2. Serum injections transmit phenotypes to offspring.

a. Adult male weight is decreased in both, MSUS offspring and the offspring of males injected with MSUS serum compared to respective control groups. For MSUS offspring n = 22, for Control offspring n = 24, one-tailed Student's t-test (data reproduced) $P = 0.045$, $t = 1.734$, df = 44. For MSUS serum-injected offspring n = 30 and for Control serum-injected offspring n = 31, two-tailed Mann-Whitney U = 334.5, $P = 0.059$. b. There is an attenuated rise in blood glucose level in MSUS offspring following a 30-min restraint challenge. At 15 min, blood glucose is significantly lower than in Control offspring, and there is an interaction across all time points. For MSUS offspring n = 13, for Control offspring n = 12, repeated measures ANOVA, for interaction $P = 0.027$, $F(3,69) = 3.25$, at 15-min adjusted $P = 0.0015$, $t = 3.693$, df = 92. c. There is also an attenuated rise in blood glucose level in the offspring of MSUS serum-injected males following a 30-min restraint challenge. At 30 and 90 min, blood glucose is significantly lower than in the offspring of Control serum-injected males. MSUS serum-injected offspring n = 17, and Control serum-injected offspring n = 14, repeated measures ANOVA, for interaction $P = 0.2$ (not significant), $F(3,116) = 1.57$, at 30-min adjusted $P = 0.0092$, $t = 3.117$, df = 116, at 90-min adjusted $P = 0.0029$, $t = 3.48$, df = 116. Data reported as mean ± s.e.m.

Fatty acids, especially PUFAs, and their metabolites are important for membrane structure and are involved in many processes including cognition¹²⁹, spermatogenesis¹³⁰, and epigenetic regulation¹³¹. They are also potent ligands for peroxisome proliferator-activated receptors (PPARs), a class of nuclear receptor that forms transcription factor complexes with retinoid X receptor (RXR) to regulate gene expression, chromatin structure, and interacts with epigenetic modifying enzymes^{65,132}. Further, bile acids and steroid metabolites are ligands for farnesoid X receptors (FXRs) and liver x receptors (LXRs), belonging to the same family of nuclear receptors as PPAR and RXR, with strong biological interactions¹²⁸. To test aberrant ligand activation of PPAR receptors in MSUS we performed a transcription factor binding assay. In white adipose tissue, we found increased binding of PPAR γ to its consensus sequence in MSUS compared with controls (Extended Data Fig. 3), confirming increased ligand activation.

Group		Alpha linolenic and linoleic acid metabolism	Arachidonic acid metabolism	Bile acid biosynthesis	Steroidogenesis
MSUS	PND8	+	/	/	/
		-	****	****	/
	Adult	+	****	****	/
		-	/	/	***
	Offspring	+	*	/	/
		-	**	/	***
PLMS	Serum	+	/	****	/
		-	/	/	#
	Saliva	+	***	****	/
		-	/	/	/

Figure 3. Metabolomics analysis of serum and plasma.

Pathway enrichment of differentially identified metabolites in MSUS plasma from PND8 pups, adults and offspring compared to controls (n = 5 for each group), and from serum and saliva in PLMS children compared to controls (for serum, PLMS n = 20, Control n = 14; for saliva, PLMS n = 25, Control n = 14). Asterisk and hashtag represent FDR after multiple testing corrections using the Benjamini-Hochberg (BH) test. Columns indicate significance for positive (+) and negative (-) enrichment. #FDR < 0.1, *FDR < 0.05, **FDR < 0.01, ****FDR < 0.0001. (/) symbolizes non-significance.

To test causality between nuclear receptor activation and phenotypes in offspring, we used an *in vivo* approach and injected the dual PPAR α/γ agonist tesaglitazar (10 μ g/kg) intraperitoneally (i.p.) into naïve adult males (Fig. 1, right). Notably, pharmacological activation of PPAR was favored over other nuclear receptors to mimic the increase in fatty acid metabolites present in MSUS at the time of breeding. Following 4 weeks of injections (2 per week) we allowed a full spermatogenesis cycle to complete prior to breeding males, in order to isolate only the persistent effects to the germline. Males were bred to naïve control females to generate offspring, and when adult, the offspring were bred to naïve control females to generate grand-offspring. Strikingly, both the offspring and grand-offspring of tesaglitazar-injected males had decreased body weight when adult (Fig. 4a), similar to MSUS offspring and to the offspring of males injected with MSUS serum. Further, like MSUS offspring⁴⁸, glucose levels during a glucose tolerance test (GTT) were significantly lower in the offspring and grand-offspring of tesaglitazar-injected males compared to their respective controls (Fig. 4b, c). Glucose levels during a restraint challenge were not altered (Extended Data Fig. 4). These results suggest that both, serum injection and PPAR activation can mimic phenotypic effects of MSUS across generations. Weight at PND7 in female and male offspring of tesaglitazar-injected males was also increased compared to control groups (Extended Data Fig. 5), confirming that the phenotypic consequences of paternal exposure to PPAR ligands can appear early in offspring development¹³³.

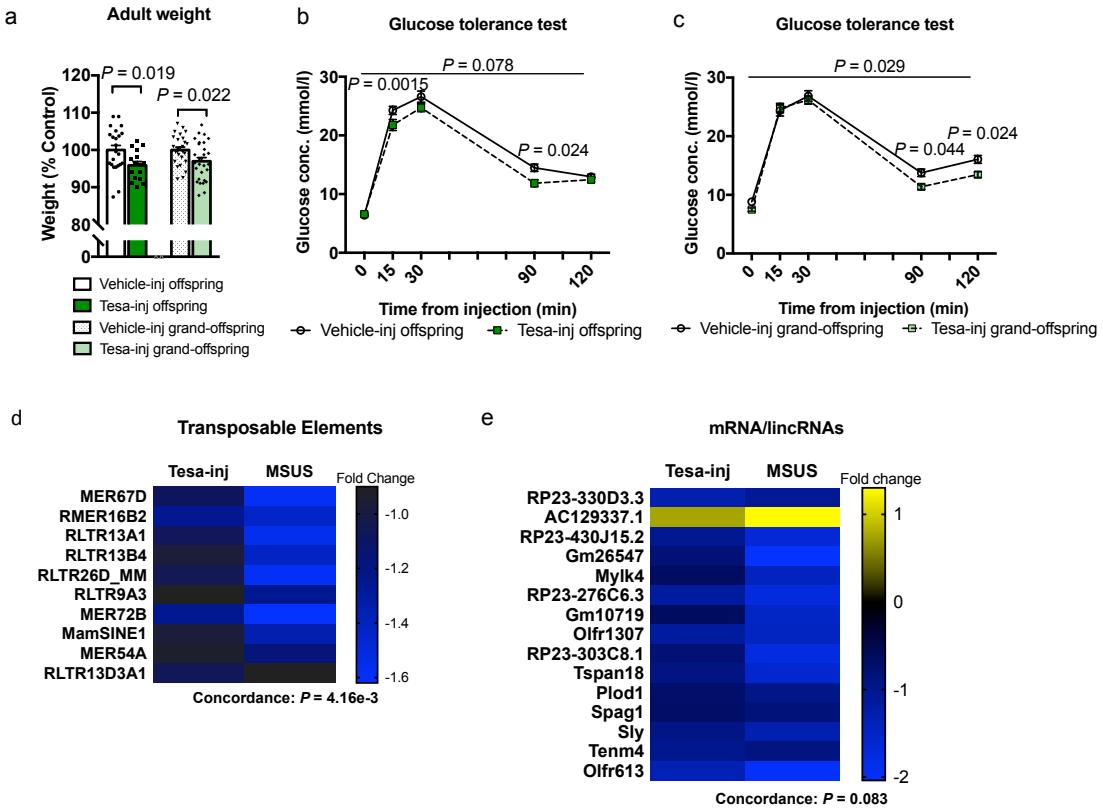


Figure 4. Tesaglitazar reproduces MSUS phenotypes in offspring alongside altered sperm RNA expression in fathers.

a. Adult weight in offspring and grand-offspring of tesaglitazar-injected (Tesa-inj) males is reduced compared to the offspring and grand-offspring of vehicle-injected (Vehicle-inj) males. Tesa-inj offspring $n = 16$, Vehicle-inj offspring $n = 23$, two-tailed Student's *t*-test, $P = 0.019$, $t = 2.44$, $df = 37$; Tesa-inj grand-offspring $n = 28$, Vehicle-inj grand-offspring $n = 26$, two-tailed Student's *t*-test, $P = 0.022$, $t = 2.37$, $df = 52$. b-c. Glucose level in offspring (b) and grand-offspring (c) from tesaglitazar- (Tesa-inj) and vehicle-injected, (Vehicle-inj) males during a glucose tolerance test lasting 120 min. For (d), Tesa-inj offspring $n = 14$, Vehicle-inj offspring $n = 21$, repeated measures ANOVA, interaction $P = 0.077$, $F(4, 132) = 2.16$, at 15-min adjusted $P = 0.036$, $t = 2.71$, $df = 165$, at 90-min adjusted $P = 0.024$, $t = 2.85$, $df = 165$; for (e), Tesa-inj grand-offspring $n = 16$, Vehicle-inj grand-offspring $n = 13$, repeated measures ANOVA, for interaction $P = 0.029$, $F(4, 108) = 2.82$, at 90-min adjusted $P = 0.044$, $t = 2.65$, $df = 135$, at 120-min adjusted $P = 0.024$, $t = 2.86$, $df = 135$. Conc.; concentration. Data reported as mean \pm s.e.m. d. Overlap of differentially expressed transposable elements (TEs) in sperm from tesaglitazar-injected (Tesa-inj) and MSUS males. Data represents TEs with $P < 0.05$ and similar fold change. Fold change represented in heat map represents $\log_2(\text{fold change})$. Total overlap is presented in Extended Data Fig. 6a. For Tesa-inj $n=5$ and Vehicle-inj sperm $n = 7$,

Control sperm n = 3, MSUS sperm n = 4. e. Overlap of differentially expressed mRNA/lincRNAs in sperm from tesaglitazar-injected (Tesa-inj) and MSUS males. Data represents mRNA/lincRNAs with $P < 0.05$ and similar direction of fold change. Fold change represented in heat map represents log₂(fold change). Total overlap is presented in Extended Data Fig. 6b. For sperm from Tesa-inj n=5 and Vehicle-inj n = 7, Control sperm n = 3, MSUS sperm n = 4.

Since sperm RNA is causally involved in the transmission of the effects of MSUS^{48,134}, we sequenced sperm RNA from tesaglitazar- and vehicle-injected males (Extended Data Fig. 7-8). Differential expression analysis revealed global downregulation of transposable elements (TEs), consistent with recent data involving *in vivo* oral administration of tesaglitazar in mice¹³⁵. Interestingly, a similar pattern of decreased TEs was found in MSUS sperm¹³⁴. To directly compare TEs across data sets, we split RNAs by type: TEs and mRNA/lincRNA. We found significant concordance of TE expression across tesaglitazar-injected and MSUS sperm (Fig. 4b; Extended Data Fig. 6a), including several LTRs. We also found modest concordance ($P = 0.083$) of mRNAs/lincRNAs (Fig. 4c; Extended Data Fig. 6b), with several long non-coding RNAs being the most enriched across data sets (Fig. 4c). Additionally, in sperm from tesaglitazar-injected males several genes were altered (FDR < 0.05), in particular involving the mitochondrial respiratory chain complex (Extended Data Fig. 8), suggesting an effect of PPAR on mitochondrial metabolism¹³⁶. Together, this data demonstrates that PPAR ligands in the periphery can have lasting effects on gene expression in the germline, and in partial concordance with gene expression changes in sperm following early life trauma exposure.

To relate these findings to trauma conditions in human, we assessed blood metabolites in a cohort of children (6-12 years old girls and boys) from an SOS Children's Village in Lahore, Pakistan. These children have been exposed to early life trauma in the form of paternal loss and maternal separation (PLMS). Control children were schoolmates of PLMS children living with both parents, and groups were matched for body mass, age and gender (Extended Data Fig. 9b-d). This Pakistani population is advantageous because of high rates of consanguinity in

Pakistan¹³⁷ ensuring a higher degree of genetic homogeneity. PLMS children had increased depressive symptoms compared to controls (Extended Data Fig. 9a), consistent with depressive-like behaviors observed in MSUS mice¹¹. Similar to MSUS mice, serum metabolites in PLMS children showed significant positive enrichment for AA metabolism and modest negative enrichment for bile acid biosynthesis (Fig. 3). Because not all children could tolerate the procedures necessary for blood sampling, we also collected saliva and conducted parallel metabolomic analyses. In saliva, both ALA/LA and AA metabolism and steroidogenesis, were altered in PLMS children compared to controls (Fig. 3; Extended Data Fig. 10). These results showing co-occurrence of metabolomic alterations in mouse and in serum and saliva in human suggest conserved mechanisms across species and consistent pathways across body fluids.

Germ cells are the carrier of heredity and pass information from parent to progeny via their genome and epigenome¹³⁸. Because they are sensitive to environmental factors especially in early life¹³⁹, they can be altered by exposure and then transfer traces of exposure across generations. Nuclear receptors are critical for such traces because they can relay signals from the environment to the chromatin and contribute to establish molecular features on chromatin⁶⁵. Here, we identify PPAR as causally involved in the induction of differential RNA expression in the germline and the propagation of altered phenotypes across generations, a new finding that extends its previous association with transgenerational transmission of metabolic phenotypes^{35,98,123}. Recently transcription factors were demonstrated to have an effect on the poised transcriptional state in gametes, and influence the developmental trajectory of zygotes⁶⁰. Thus, activation of PPAR in MSUS sperm may be partially responsible for the observed differential gene expression in their zygotic offspring¹³⁴. Nuclear receptor signaling in support cells such as Sertoli, Leydig and epididymal cells that cross-talk with spermatogenic cells may also contribute to epigenetic remodeling in gametes¹⁴⁰. The evidence that this can be mediated by blood-borne factors in a heritable manner further underscores the importance of blood components for physiology and cognition^{98,141–143}. The induction of phenotype transmission via blood components also challenges the Weismann barrier theory¹⁴⁴ by showing that relevant information can be transferred from soma to the

germline. Finally, high-throughput metabolomic methods used in this study to identify and monitor metabolites altered by trauma provide novel insight for epigenetic inheritance. When applied to other environmental exposures and body fluids like sperm, blood, seminal fluid, etc¹⁴⁵, it may help to identify potential mechanisms of transmission of the effects of exposures. Understanding these mechanisms will ultimately lead to prevention and treatment strategies for related diseases.

7.3 Methods

7.3.1 Mice

C57Bl/6J mice were kept under a 12-hour reverse light/dark cycle in a temperature and humidity-controlled facility. Animals had access to food and water *ad libitum*. Experimental procedures were performed during the animals' active cycle (reverse light cycle in the facility, light on at 9 am and off at 9 pm) in accordance with guidelines and regulations of the cantonal veterinary office, Zürich, except for those involving serum-injected mice and their offspring that were performed during the animals' inactive cycle as approved by the Home office, United Kingdom.

7.3.2 MSUS

To produce MSUS mice, 3-month old C57Bl/6J primiparous females were paired with age-matched control males for one week in a total of 40 breeding pairs. Following birth of pups, dams were randomly assigned to MSUS or control groups, with assignment done in a way to balance litter size and number of animals across groups. Dams assigned to MSUS treatment group were separated from their pups for 3 hours per day unpredictably from postnatal day (PND) 1 to 14. Separation onset was at an unpredictable time within the 3 hours, and during separation, each mother was randomly exposed to an acute swim in cold water (18 °C for 5 min) or restraint in a tube (20 min). Control animals were left undisturbed apart from cage changes once per week (similar to MSUS). At PND21, all pups were weaned from their mother and assigned to cages in groups of 4-5 mice/cage housed by gender

and treatment. Siblings were distributed in different cages and mixed with pups from different mothers to avoid litter effects.

7.3.3 Serum sampling and intravenous injection

4-month old MSUS and control males were sacrificed by decapitation and trunk blood was collected into non-coated Eppendorf tubes. Clotting was allowed overnight at 4 °C. After centrifugation for 10 min at 2,000 g at 4 °C, serum was collected and stored at -80 °C. When two months old, naïve mice received 8 tail vein injections of 90 ul of serum from adult MSUS or control mice over the course of 4 weeks. For each group, serum from 43 mice was pooled before injections. In total, 16 males were injected with the pooled serum from MSUS and 16 with the pooled serum from controls. For injections, mice were restrained in a tube and placed in a heating chamber at 38 °C. Injections were alternated between opposing lateral veins of the tail. Proper insertion of the injection needle was successful on the first attempt in most cases, and never more than 3 attempts were necessary. Males were paired one-to-one with primiparous naïve females 12 days after receiving the last injection and removed after one week.

7.3.4 Metabolic testing

Before testing, cages were labeled such that the experimenter was blind to treatment group. When more than one experimenter was required to conduct a given test, each experimenter used a similar number of control and MSUS animals to exclude experimenter-specific effects. Control and MSUS treatment groups were tested alternately or side-by-side to avoid circadian effects. All glucose measurements were performed using fresh blood droplets with an Accu-Chek Aviva glucometer (Roche, Switzerland).

7.3.4.1 Glucose in response to restraint challenge

Mice were single-housed for minimum 4 hours but no more than 18 hours prior to testing with access to food and water. For physical restraint, each mouse was confined individually in a cylindrical plastic tube (3.18 cm diameter with sliding nose restraint; Midsci) for 30 min. Blood was drawn at 0, 15, 30 and 90 min after initiation of restraint using a 28G needle to prick the tail from within 1 cm of the tip. After blood was measured at the 30-min time point, the

mouse was released and placed in an individual temporary cage. At 90 min, the mouse was briefly (10 sec) placed under an inverted 1-liter glass beaker (dimensions 14.5 cm high and 12 cm diameter) with its tail positioned to protrude from the beaker spout for easy access by the experimenter. The mouse was then placed back into its temporary cage for 1 hour, before returning to its original group cage. Data were analyzed with repeated-measurements ANOVA and corrected for multiple comparisons using the Šidák post-hoc test.

7.3.4.2 Glucose tolerance test

Mice were single housed without food starting between 5-6pm and onset of test experiment began at 9am the next morning. Glucose was measured in blood samples at 0, 15, 30, 90 and 120-min following intraperitoneal (i.p.) injection of sterile glucose solution containing 2 mg per g of body weight in 0.45% (wt/vol) saline. Each mouse was kept under an inverted 1-liter beaker with its tail in the spout as described above. After taking the 30-min measurement, the mouse was placed in an individual cage and then taken out briefly (10 sec) to take measurements at 90- and 120-min time point. The mouse was placed back into its temporary cage for 1 hour before returning to its original group cage, to reduce fighting caused by experimental stress. Data were analyzed with repeated-measurements ANOVA and corrected for multiple comparisons using the Šidák post-hoc test.

7.3.4.3 Food intake and weight measurement

All animals were weighed using the same scale at the same time of day. Following weight measurements, food intake was also measured. Food pellets were weighed at the beginning and end of three consecutive days and replaced every 24 hours to limit crumb spillage. Food consumption was calculated per cage (maximum 5 animals), and averaged per animal. No difference in food intake was observed (data not shown). Data were analyzed with two-tailed Students *t*-test, except for MSUS offspring, which was analyzed with one-tailed Student's *t*-test to confirm previous reports⁴⁸.

7.3.5 Blood and tissue collection

Mouse pup siblings at postnatal day 8 (after 7 days of MSUS treatment) were sacrificed from each cage after removing the mother, and all pups (up to 10) were sacrificed within 2 min. For blood and tissue collection in adults, males were single-housed overnight with food and water to avoid activating their stress response by the sequential removal of littermates. Details of serum/plasma processing are described in different sections of the methods.

7.3.5.1 *Blood collection for metabolomic analyses*

Trunk blood was collected in EDTA coated tubes (Microvette, Sarstedt) from PND8 pups and 4-6 months old mice after decapitation. Samples were stored at 4 °C for 1-3 hours and centrifuged at 2,000 g for 10 min. Plasma was collected and stored at -80 °C until processed for metabolomic analysis. For analyses, samples from pups from different litters (and not from siblings) or from adult mice from different cages (and not cage mates) were chosen.

7.3.5.2 *Sperm collection*

Sperm was collected using the swim-up method as described previously. Briefly, cauda epididymis was dissected and perforated with dissection scissors and placed in M2 medium (7002230, Sigma M7167) for 1 hour to allow mature sperm to swim out of the tissue. Medium was collected and spun down at 2,000 x g for 6 minutes at 4 °C. Sperm pellets were then resuspended in 15ml somatic cell lysis buffer (contains 1% SDS (10%) and 0.5% Triton X-100 in Milli-Q water) and left to incubate on ice for 10 minutes. Sperm was re-pelleted by centrifugation at 2,000 x g for 6 minutes at 4 °C and washed twice with ice cold PBS (spun after each wash at 2,000 x g for 6 minutes at 4 °C). After the final wash, pellets were stored at -80 °C until further use.

7.3.5.3 *Tissue collection*

Following decapitation and blood collection, mice were pinned by their feet to a sterilized dissection board. A midline incision was made from the subcostal region to the pubic zone where two transverse incisions were made bilaterally to the femur, exposing internal organs. Biopsies from white adipose tissue were collected proximal to the epididymis on each side.

7.3.6 Metabolomic measurements

Metabolites were extracted from plasma 3 times with 70% ethanol at a temperature >70 °C. Extracts were analyzed using flow injection – time of flight mass spectrometry (Agilent 6550 QTOF) operated in negative mode, described previously¹⁴⁶. Distinct mass-to-charge (*m/z*) ratio could be identified in each batch of samples (typically with 5,000-12,000 ions). Ions were annotated by aligning their measured mass to compounds defined by the KEGG database, allowing a tolerance of 0.001 Da. Only deprotonated ions (without adducts) were considered in the analysis. When multiple matches were identified, such as in the case of structural isomers, all candidates were retained. For enrichment analysis, metabolites with *P* < 0.05 and log₂(fold change) > 0.25 or < -0.25 were used. Enrichments were considered significant when FDR < 0.05 after multiple testing corrections. Enrichment analysis was performed with in-house software (Zamboni Lab, Institute of Molecular Systems Biology, ETH Zürich).

7.3.7 PPAR γ transcription factor binding assay

PPAR γ transcription factor binding activity was assessed using nuclear extracts from epididymal white adipose tissue (eWAT). Nuclear proteins were collected from 10 mg eWAT using a Nuclear Extraction kit (Abcam ab113474). Protein concentration in nuclear extracts was measured using Qubit protein assay kit (ThermoFischer Scientific), and volumes were adjusted to have similar protein concentration across samples. For measurement of PPAR γ binding activity, we used the PPAR γ transcription factor assay kit (Abcam ab133101), according to the manufacturer's protocol. Briefly, 10 ul of nuclear extracts were added with binding buffer to individual wells of a plate conjugated with DNA sequences containing known PPAR γ binding motifs, and incubated at 4 °C overnight. Wells were washed 5x with wash buffer and primary antibody against PPAR γ was added for 1 hour at room temperature without agitation. Wells were washed again 5x with wash buffer and an HRP-containing secondary antibody was added to wells and incubated at room temperature for 1 hour. Following another 5x wash, developing solution was

added and left to incubate under gentle agitation for 45 min. The reaction was stopped by adding stop buffer and absorbance was measured immediately with NOVOstar plate reader (BMG Labtech). Samples were measured in duplicate, alongside a positive control supplied by the manufacturer to confirm assay success. Reagents described above were provided in the kit. Data were analyzed with two-tailed Students *t*-test.

7.3.8 Aldosterone enzyme-linked immunosorbent assay (ELISA)

Aldosterone concentration in serum was measured using a competitive ELISA (Abnova, KA1883) according to manufacturer's protocol. 50 ul of standards, control and serum samples from adult MSUS and control males were added to wells containing aldosterone antibody, and an HRP-conjugated aldosterone antigen was added. After 1-hour incubation at room temperature for competitive binding, cells were washed and tetramethylbenzadine (TMB) substrate was added for color development, followed by addition of stop solution. Absorbance was measured at 450 nm with a NOVOstar plate reader (BMG Labtech), and sample absorbance (inversely related to concentration) was determined by plotting a standard curve with manufacturer-supplied standards and controls. Samples were measured in duplicate and averaged. Concentrations were analyzed with two-tailed Students *t*-test.

7.3.9 Tesaglitazar injections

3-month old mice were injected i.p. with either tesaglitazar solution at 10 ug per kg body weight or vehicle control twice per week for 4 weeks. Tesaglitazar (Sigma Aldrich, now Merck) was solubilized in DMSO at 10ug per ul and resuspended in sterile 0.9% saline (DMSO concentration at 0.01%). Vehicle consisted of 0.01% DMSO in 0.9% sterile saline. Mice were weighed at baseline, before each injection and 6 weeks after the last injection. 11 tesaglitazar-injected and 12 vehicle-injected males were paired with 3-month old primiparous control females 46 days after the last injection. Pairing lasted 1 week, and males were grouped back with their original littermates. The offspring were subjected to standard rearing conditions, with cage changes occurring once per week, and were weaned at PND21 in social cages with

pups from other litters. When 3 months old, mice were subjected to phenotyping followed by a minimum 1-week rest before being bred to naïve primiparous control females. 9 tesaglitazar-injected male offspring and 8 vehicle-injected offspring were paired for one week with 2 females each to produce a sufficient number of grand-offspring (Extended Data Fig. 11). After pairing, females were separated into individual cages.

7.3.10 RNA sequencing

RNA was extracted from sperm using the Trizol/chloroform method, as per the manufacturers recommendations, and analyzed using Bioanalyzer (Agilent 2100). Sequencing was performed using Illumina Genome analyzer. RNA libraries were prepared with the TruSeq Stranded Total RNA kit according to the manufacturers instructions with the following minor modifications: 220 ng total RNA was depleted from rRNA using RiboZero Gold. Indices were diluted to 1:3. High-throughput sequencing was performed on Genome Analyzer HiSeq 2500 in Rapid run mode for 37 cycle + 7 cycles to read indices. Samples were sequenced in 2 x 50 bp runs.

7.3.11 RNA-Seq data processing and expression analysis

Single-end reads were assessed for quality using FastQC (version 0.11.5)¹⁴⁷. Quality control was performed with Trim Galore (version 1.16)¹⁴⁸, and bases with quality score less than 30 (-q 30), reads shorter than 30 bp were also removed (--length 30) and adapters were removed. Filtered reads were pseudo-aligned with Salmon (version 0.11.2)¹⁴⁹ with library type parameter (-l SR), on a transcriptome index prepared with 1) the GENCODE¹⁵⁰ annotation (version M18), 2) piRNA precursors¹⁵¹, and 3) transposable elements (TEs) from repeat masker (concatenated by family).

For differential expression analysis, normalization factors were calculated using the TMM method¹⁵² on all quantified RNAs aggregated at the gene (or repeat element family) level, and repeat elements or mRNA and lincRNAs were selected for testing. Only features with more than 20 reads in at least a number of samples equivalent to 80% of the size of the smallest experimental

group were considered. In the case of MSUS sperm, where two libraries per sample were available, we used the voom/dupCor method of the limma R package v.3.34.9¹⁵³ as previously¹³⁴ to account for non-independence of the samples; in all other cases edgeR v.3.24.0 was used with the exact test.

For data concordance, a Pearson correlation coefficient was generated using genes with $P < 0.05$ in both data sets.

7.3.12 Human cohort

To assemble a cohort of children exposed to trauma, we contacted the administration of an orphanage (SOS Children's Village) in Lahore, Pakistan and selected children fulfilling the following criteria at the time of assessment: 1) Age between 6-12 years, 2) Paternal death, 3) Maternal separation in the form of adoption by the SOS village, 4) Entry of the child to the SOS village within 12 months preceding the assessment. Maternal separation was forced because mothers could no longer provide sufficient support to their child and had to transfer their care to the SOS village. They had no or minimal contact with their child at the time of assessment. Maternal suffering during the first 13 years of age of a child is known to affect mental health in adulthood¹⁵⁴. Paternal loss was used as an inclusion criteria, as spousal death is a critical life stressor in human¹⁵⁵, and serves to mimic unpredictable maternal stress of the MSUS model. Exclusion criteria included 1) History of abuse, 2) History of traumatic brain injury, intellectual disability or cerebral palsy. Based on these criteria, a total of 26 children with paternal loss and maternal separation (PLMS) were selected. A control group (n=16) was recruited among schoolmates of PLMS children, and comprised 6-12 year old children living with both parents and with no history of trauma, traumatic brain injury, intellectual disability or cerebral palsy. Complete confidentiality of participants was maintained at all stages of data collection and analyses. The administration of the SOS village, Lahore, Pakistan was informed and approved all study procedures.

7.3.12.1 Demographics

Detailed demographic information for PLMS and control groups was provided by the administration of the SOS village for the PLMS children and from

parents for control children. This included age, gender, parental consanguinity (defined by 1st or 2nd cousin parental union) and physical health records. Weight and height were measured in all children by two research interns blinded to the study design. Children were classified as underweight, healthy weight, or overweight based on their correspondence to the ‘less than 5th percentile’, ‘between 5th and 85th percentile’ and ‘85th to 95th percentile’ reference ranges defined for Pakistani children of the same age and sex¹⁵⁶.

7.3.12.2 Assessment of depressive behaviors

Depressive symptoms were evaluated using the Center for Epidemiological Studies Scale for Depression in Children (CES-DC). CES-DC is a validated tool to screen for depressive symptoms in children aged 6-13 years based on 20 self-report items scored on a likert-like scale: 0 corresponding to ‘not at all’ and 3 corresponding to ‘a lot’. A score of 15 or higher on this scale indicates a high risk of depression and warrants clinical evaluation and intervention¹⁵⁷. CES-DC assessment of children was conducted through interviews by an unblinded investigator. A confirmation of the childrens’ responses was obtained from their foster mother. In case of disagreement between responses (<1% of cases), the responses of the foster mother were considered valid.

7.3.12.3 Serum and saliva samples collection

Blood was collected by a trained phlebotomist blinded to the study design. Blood withdrawal took place during the morning school hours for both groups approximately 1 hour after their last oral intake. All children received a brief explanation of the blood withdrawal procedure and were promised a gift basket for their cooperation. Children showing reluctance or despair were excluded (n=6 PLMS, n=2 Controls). After sterilization with a swab, axillary vein venipuncture was performed through a butterfly syringe and 6 ml blood was collected per child in serum separating tube (BD vacutainer, ThermoFisher Scientific). After 1-hour incubation at room temperature, the tubes were centrifuged at 1,300 g for 10 min at 4 °C for serum separation. Extracted serum was aliquoted into 1.5 ml tubes and stored at -80 °C. Saliva was collected by two research interns blinded to the study design. Children who had active upper respiratory tract infections (identified through the

symptoms of fever, rhinorrhea, or cough) at the time of sample collection were excluded (n=1 PLMS, n=2 Controls). All children received a brief explanation of the saliva collection procedure and were promised a gift basket for their cooperation. After a 1-hour period of no oral intake, the children were asked to rinse their mouth with clear water twice. Saliva was collected 5 min after rinsing through passive drooling in salivette tubes (Sarstedt) over a period of 5 min. Collected saliva was aliquoted into 1.5 ml tubes and stored at -80 °C. Serum and saliva aliquots were shipped to Zürich in packages containing 20 kg of dry ice for analysis.

7.3.13 Statistical analysis

Samples size was estimated based on our previous work with the MSUS model^{11,19,48,74,103–105}. Bodyweight, PPAR γ transcription factor binding assay and aldosterone ELISA measurements were assessed using two-tailed student *t*-tests. For some experiments, the data presented were reproduced from previous findings, in which case one-tailed tests were used. In all such cases, this is clearly stated in text. Glucose tolerance tests and glucose in response to restraint challenge were analyzed using repeated-measurements ANOVA and corrected for multiple comparisons using the Šidák post-hoc test. Most data matched the requirements for parametric statistical tests (normal distribution and homogeneity of variance). For data not normally distributed, Mann-Whitney U test was used. This was the case for serum-injected offspring weight measurements, PLMS depression scale and PLMS age. Outliers were determined using the pre-defined criteria of adding and subtracting twice the standard deviation from the mean. Reported *n* represents number of animals after outlier removal. For metabolomics enrichments, FDR was calculated using Benjamini-Hochberg post-hoc test. Statistics were mainly computed with GraphPad Prism unless stated otherwise. Reported *n* represent biological replicates. Error bars represent s.e.m. in all figures. For all data, significance was set at a minimum $P < 0.05$. For a trend $\#P < 0.1$. Asterisks represent significance as follows, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Type of analysis and descriptive data for each statistical test is presented in figure legends.

7.4 Acknowledgments

We thank the University Zürich, the ETH Zürich, the Swiss Science National Foundation (grant 31003A-135715), ETH grants (ETH-10 15-2 and ETH-17 13-2), Novartis Foundation (#16B097), Roche Postdoctoral Fellowship Program (ID233). We thank Anastasiia Efimova for experimental support and Deepak Tanwar for bioinformatics support, Martin Roszkowski and Irina Lazar-Contes for assisting the maintenance of MSUS breedings, Silvia Schelbert for lab organization in Zürich and maintaining the animal license, and Yvonne Zipfel for animal care in Zürich. We also thank Paul Green for help with serum intravenous injections, and Paweł Zieleński for help with general animal care in Cambridge. We thank Chris Lelliot for conceptual support and recommendations for tesaglitazar injections, Darren Logan, Wayo Matsushima and Tomas diDomenico for advice on early bioinformatics analysis. We would also like to extend our gratitude to the administration of the SOS village, Pakistan (Mrs. Saba Faisal, Mrs. Rubina Asghar Ali, and Mrs. Almas Butt) and The Educators school, Lahore, Pakistan (Mrs. Sajida Makhdoom) for allowing us the assessments of PLMS and the control children respectively; Anooshay Abid and Mehr Shafique (Lahore University of Management Sciences, Lahore, Pakistan) for technical help; Chughtai Laboratories (Lahore, Pakistan) for their assistance with blood collection and human lipid profiling; and Prof. Aziz Mithani and Prof. Shaper Mirza (Lahore University of Management Sciences, Lahore, Pakistan) for organizational support.

7.5 Authors Contributions

GvS, KG and IMM conceived and designed the study. GvS and IMM wrote the manuscript. GvS conducted MSUS treatments together with FM, collected plasma for metabolomic analyses, performed molecular analyses of MSUS liver and adipose, performed tesaglitazar injections, and organized breedings for tesaglitazar-injected mice and their offspring and phenotyped mice from MSUS and tesaglitazar breedings together with FM. KG collected and prepared serum, organized serum injections, bred serum-injected mice, and phenotyped the offspring. AJ collected serum and saliva samples from

children at the SOS village in Lahore, Pakistan, and performed all related data measurements including analysis of CES-DC results, and assisted with writing sections of the manuscript. PLG performed bioinformatic analysis, helped to prepare figures and assisted with statistical analyses, and provided key insight into manuscript development. FM organized animal housing and breeding logistics in Zürich, tracked animal welfare and performed phenotyping with GvS. NZ measured metabolites in plasma, serum and saliva through LC-MS and analyzed the data. NG helped GvS with molecular analyses. IMM and EM provided essential conceptual support throughout the project and raised funds to finance the project.

7.6 Conflict of Interest Statement

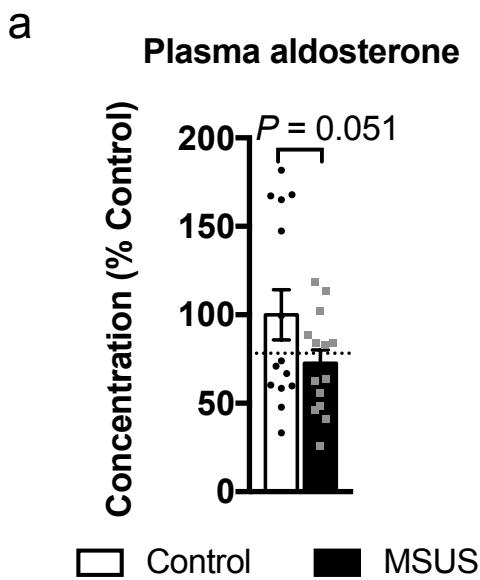
The authors declare no conflict of interest

7.7 Supplementary Data

Enrichment	PND8		F1 Adults		F2 Adults	
	-	+	-	+	-	+
Alpha linolenic acid and linoleic acid metabolism	2.2E-19	/	/	5.2E-05	5.0E-03	2.1E-02
Arachidonic acid metabolism	7.4E-16	/	/	3.5E-05	/	/
Arginine and proline metabolism	/	/	2.9E-02	/	/	/
Bile acid biosynthesis	/	/	1.9E-09	/	1.4E-14	/
Galactose metabolism	/	/	/	/	/	5.1E-05
Pentose phosphate pathway	/	/	2.1E-02	/	/	/
Retinol metabolism	/	/	/	/	/	/
Steroidogenesis	/	/	8.8E-02	/	1.2E-02	/

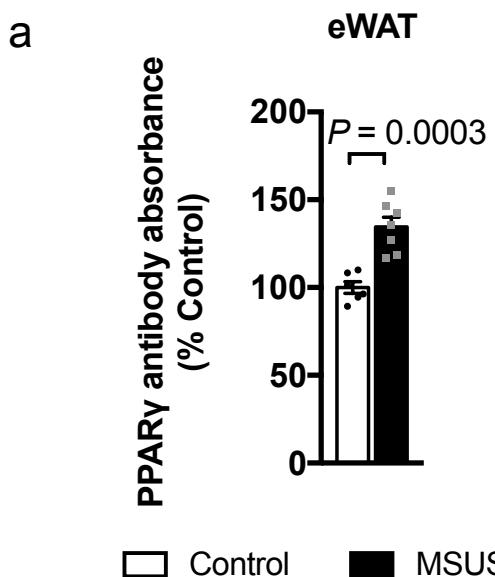
Extended Data Figure 1

Table expanded from Figure 2 to show all metabolomic enrichment pathways with false discovery rate (FDR) <0.1 after multiple testing corrections using the Benjamini-Hochberg (BH) test. (+) denotes a positive enrichment, (-) denotes a negative enrichment. FDR reported with scientific numbering system. (/) symbolizes non-significance.



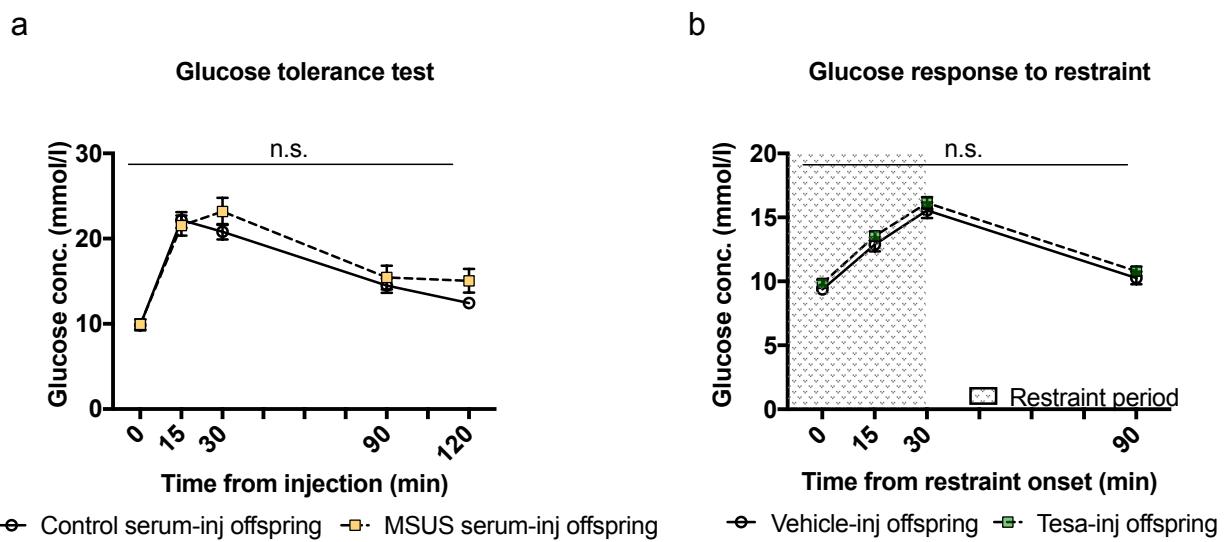
Extended Data Figure 2

a. Concentration of aldosterone in plasma from MSUS and Control males measured by ELISA. The dotted line at 78.2% indicates fold change observed by previous TOF-MS measurement (FDR = 0.02). One-tailed Student's *t*-test, $n = 14$ per group, $t = 1.70$, $df = 26$. FDR; false discovery rate. Data reported as mean \pm s.e.m.



Extended Data Figure 3

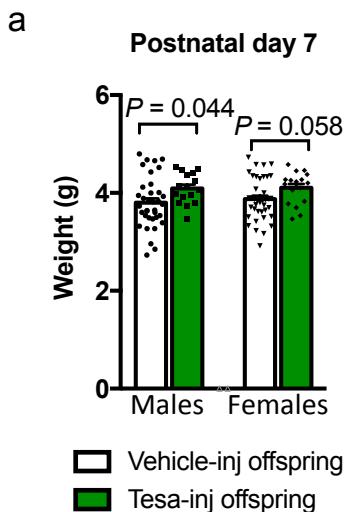
a. Nuclear extracts from epididymal white adipose tissue collected from MSUS males have higher PPAR γ transcription factor binding on consensus sequences than controls. MSUS n = 7, Control n = 6, two-tailed Student's *t*-test, P = 0.0003, t = 5.23, df = 11, eWAT; epididymal white adipose tissue. Data reported as mean \pm s.e.m.



Extended Data Figure 4

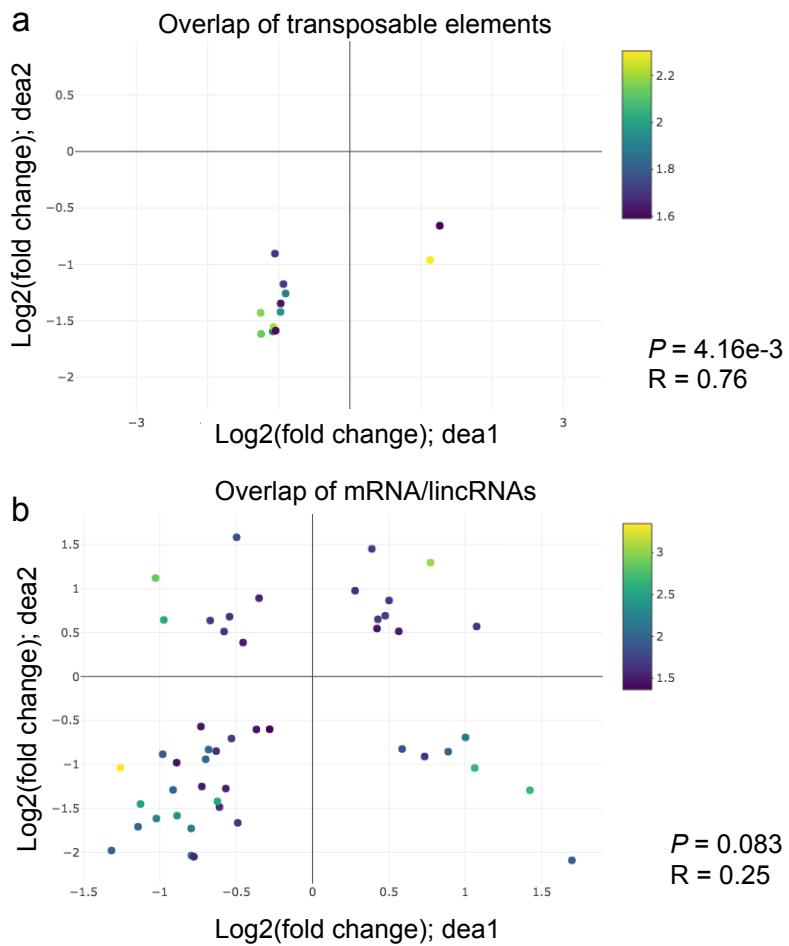
a. Glucose level during a glucose tolerance test was comparable in the offspring of MSUS serum-injected males compared to controls, n = 16 per group, repeated measures ANOVA, for interaction P = 0.46, F (4, 150) = 0.914.

b. Glucose level during and after a 30-min restraint challenge was comparable in male offspring from tesaglitazar-injected (Tesa-inj) fathers compared to vehicle-injected (Vehicle-inj) fathers, Tesa-inj offspring n = 17, Vehicle-inj offspring n = 16, repeated measures ANOVA, for interaction P = 0.99, F (3, 90) = 0.024. n.s.; not significant. Data reported as mean \pm s.e.m.



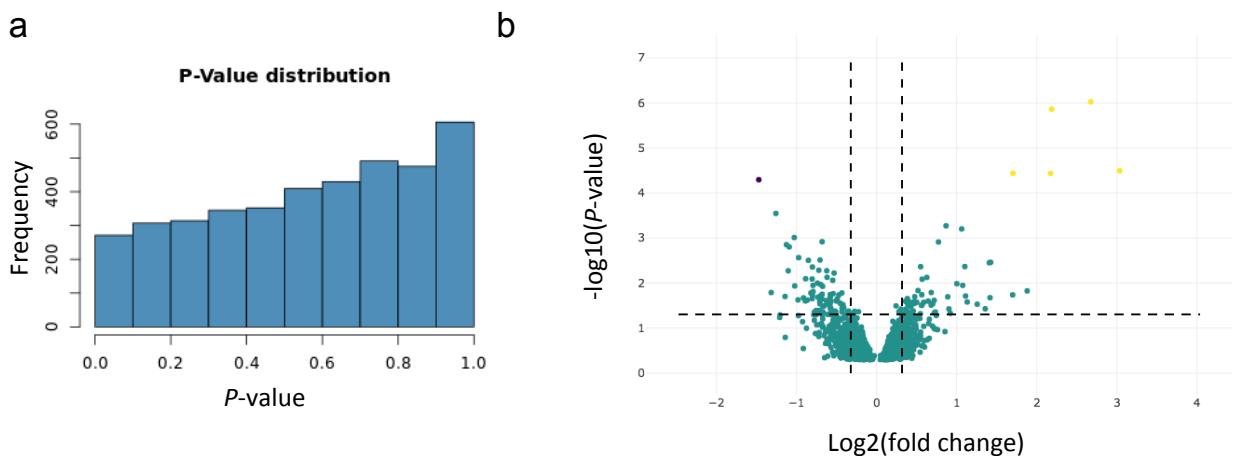
Extended Data Figure 5

- a. Male and female weight of PND7 offspring of tesaglitazar- (Tesa-inj) and vehicle-injected (Vehicle-inj) males. For males, Tesa-inj offspring $n = 16$, Vehicle-inj offspring $n = 36$, two-tailed Student's t -test, $P = 0.044$, $t=2.06$, $df=50$; for females Tesa-inj offspring $n = 17$, Vehicle-inj offspring $n = 39$, $P = 0.058$, $t = 1.94$, $df = 54$. Data reported as mean \pm s.e.m.



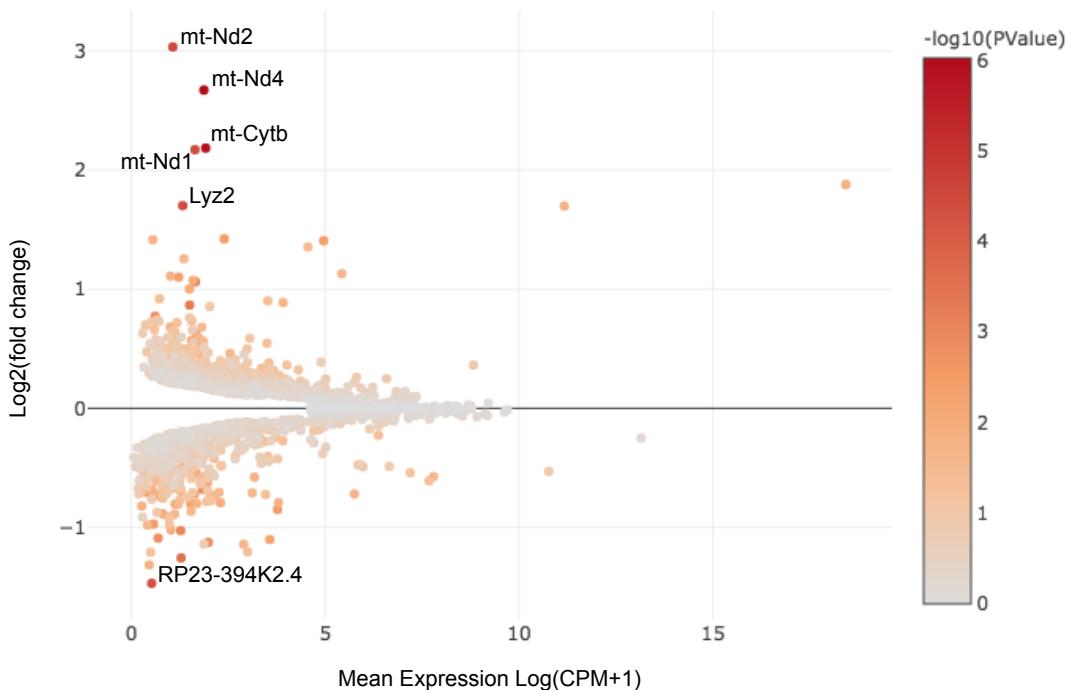
Extended Data Figure 6

a-b. Overlap of differentially expressed (a) transposable elements and (b) mRNA/lincRNAs in sperm from tesaglitazar-injected and MSUS males. A P -value cutoff of $P < 0.05$ for both data sets was used for the analysis. The P -value of the Pearson correlation between data sets are written on the figure next to the graph. The x-axis represents the fold change in tesaglitazar-injected males (dea1), while the y-axis represents the fold change in MSUS males (dea2). Color legend represents $-\log_{10}(p\text{-value})$ for each individual gene.



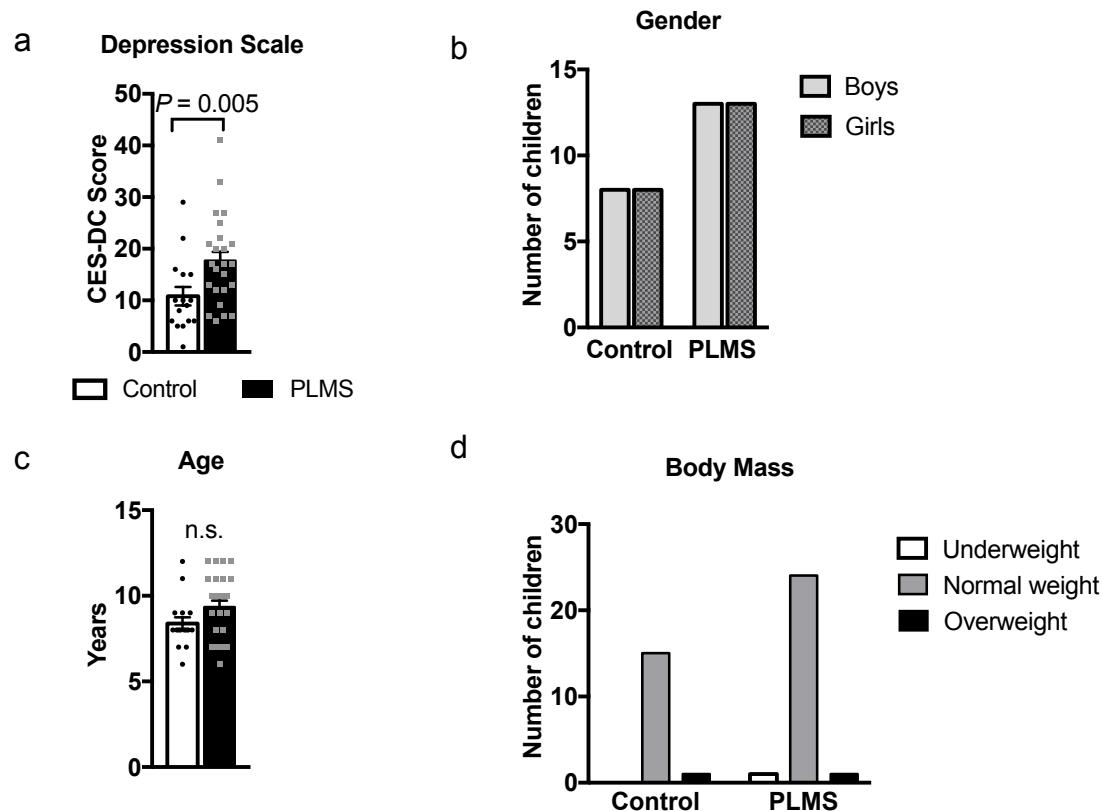
Extended Data Figure 7

Descriptive data of sperm RNA sequencing from tesaglitazar- and vehicle-injected males. a. P -value distribution and b. volcano plot of annotated genes. Genes in yellow (upregulated) and purple (downregulated) represent significance level of FDR < 0.05.



Extended Data Figure 8

MA plot of differentially expressed mRNA/lncRNAs in sperm from tesaglitazar-injected males. Genes shown represent those with a significance level of FDR < 0.05. n = 7 per group.



Extended Data Figure 9

- Depressive symptoms were screened using the Center for Epidemiological Studies Scale for Depression in Children (CES-DC) tool, and were significantly higher in PLMS children compared to controls. PLMS n = 25, Control n = 16, two-tailed Mann-Whitney U = 97, $P = 0.005$.
- Mean age was comparable between groups. PLMS n = 26, Control n = 16, two-tailed Mann-Whitney U = 148, $P = 0.12$. n.s.; not significant.
- Each group is represented by an equal number of girls and boys to balance gender-specific effects. PLMS male n = 13, female n = 13; Control male n = 8, female n = 8.
- Body mass in children was classified as underweight, normal weight, or overweight based on their correspondence to the reference ranges defined for Pakistani children of same age and sex. In both groups, one child was

overweighted, while in the PLMS group, one child was underweighted. All other children were within the range of normal body mass. PLMS n = 26, Control n = 16. Data reported as mean \pm s.e.m., n.s., not significant

Enrichments in PLMS children	Serum		Saliva	
	-	+	-	+
Alanine Metabolism	/	8.7E-04	/	/
Alpha Linolenic Acid and Linoleic Acid Metabolism	/	/	/	6.1E-04
Amino Sugar Metabolism	8.0E-02	1.7E-04	/	/
Ammonia Recycling	/	2.5E-05	/	/
Androgen and Estrogen Metabolism	/	2.6E-02	/	/
Arachidonic Acid Metabolism	/	6.1E-28	/	6.3E-16
Arginine and Proline Metabolism	/	4.6E-07	4.7E-04	/
Aspartate Metabolism	/	6.9E-04	/	/
Beta Oxidation of Very Long Chain Fatty Acids	/	4.3E-02	/	/
Beta-Alanine Metabolism	/	6.3E-03	/	/
Betaine Metabolism	/	2.9E-02	/	/
Butyrate Metabolism	/	/	2.6E-02	/
Carnitine Synthesis	/	1.0E-03	/	/
Catecholamine Biosynthesis	/	/	2.1E-02	/
Citric Acid Cycle	/	3.6E-03	6.9E-06	/
Cysteine Metabolism	/	8.7E-04	/	/
Fatty Acid Biosynthesis	/	9.4E-02	2.0E-02	/
Folate Metabolism	/	8.0E-03	/	/
Fructose and Mannose Degradation	2.4E-05	/	6.2E-03	/
Galactose Metabolism	2.8E-11	/	9.0E-13	/
Gluconeogenesis	5.7E-05	6.3E-03	1.8E-05	/
Glucose-Alanine Cycle	3.2E-02	4.8E-03	/	/
Glutamate Metabolism	/	3.3E-03	2.6E-02	/
Glutathione Metabolism	/	3.8E-04	/	/
Glycerol Phosphate Shuttle	/	2.7E-02	/	/
Glycerolipid Metabolism	/	4.5E-03	2.8E-02	/
Glycine and Serine Metabolism	6.8E-02	1.1E-06	3.4E-02	/
Glycolysis	4.4E-05	8.5E-02	4.7E-04	/
Histidine Metabolism	/	1.7E-02	/	/
Homocysteine Degradation	/	/	4.4E-02	/
Inositol Metabolism	4.0E-02	4.6E-02	4.0E-02	/
Inositol Phosphate Metabolism	4.5E-02	6.9E-02	/	/
Lactose Degradation	5.7E-05	/	6.7E-04	/
Lactose Synthesis	3.4E-02	/	1.2E-03	/
Malate-Aspartate Shuttle	/	4.3E-04	/	/
Methionine Metabolism	/	3.6E-03	/	/
Mitochondrial Electron Transport Chain	/	4.8E-03	1.2E-02	/
Nucleotide Sugars Metabolism	7.1E-04	/	4.7E-04	/
Phenylalanine and Tyrosine Metabolism	/	7.0E-02	5.8E-03	/
Phosphatidylinositol Phosphate Metabolism	3.2E-02	/	/	/
Phospholipid Biosynthesis	/	8.0E-03	/	/
Plasmalogen Synthesis	/	4.5E-03	/	/
Porphyrin Metabolism	/	8.0E-03	/	/
Propanoate Metabolism	/	6.3E-03	4.7E-04	/
Pyrimidine Metabolism	/	1.3E-05	/	/
Pyruvaldehyde Degradation	/	1.0E-02	8.0E-03	/
Pyruvate Metabolism	/	6.0E-05	4.0E-04	/
Retinol Metabolism	4.0E-02	/	/	/
Sphingolipid Metabolism	4.9E-02	/	/	/
Starch and Sucrose Metabolism	1.9E-03	8.2E-02	4.7E-04	/
Steroidogenesis	/	/	/	2.5E-03
Taurine and Hypotaurine Metabolism	/	4.9E-04	/	/
Transcription/Translation	/	2.0E-05	/	/
Transfer of Acetyl Groups into Mitochondria	3.2E-02	2.8E-02	2.8E-03	/
Trehalose Degradation	3.4E-02	/	2.0E-02	/
Tyrosine Metabolism	/	9.1E-02	1.6E-05	/
Urea Cycle	5.2E-02	1.5E-03	/	/
Valine, Leucine and Isoleucine Degradation	/	2.0E-02	4.3E-04	/

Extended Data Figure 10

Table expanded from Figure 4 to show all metabolomic enrichment pathways with FDR < 0.1 after multiple testing corrections using the Benjamini-Hochberg (BH) test. (+) denotes a positive enrichment, (-) denotes a negative enrichment. FDR reported with scientific numbering system. (/) symbolizes non-significance.

Group	Injected	Offspring	Grand-offspring
Tesaglitazar	11	20	64
Vehicle	12	39	45

Extended Data Figure 11

Table of animal numbers used in tesaglitazar breeding, as described in Methods.

8 Similar alterations in behavior, lipid metabolism, and circulating microRNAs in mice and humans after early-life trauma

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8.1 Abstract

Early-life traumatic experiences have a long-lasting impact on physiological and psychological functioning. In mice, postnatal trauma in the form of unpredictable maternal separation combined with unpredictable maternal stress (MSUS) leads to behavioral and metabolic perturbations in adulthood, and in non-traumatized offspring across several generations, likely involving epigenetic mechanisms. Here, we demonstrate that several physiological perturbations in MSUS mice can be validated in a human cohort of 6-12 year old children (n=26) who were exposed to early-life trauma in the form of paternal loss and maternal separation (PLMS) within the preceding year. Compared to matched controls (n=16), these children show behavioral impairments and reduced levels of serum high-density lipoproteins (HDL), similar to the MSUS mice. Altered expression of HDL-associated miRNAs in MSUS serum (miR-16, miR-29a, miR-375) is also apparent in both serum and saliva of PLMS children. In particular, miR-375 is upregulated in serum, corresponding to previous reports of its upregulation in MSUS sperm. Treatment of developing germ cell-like cells *in vitro* with serum from MSUS mice increases miR-375, which is reversed after siRNA-mediated knockdown of HDL receptor, SCARB1. These results suggest that similar behavioral and metabolic effects and vectors of non-genomic inheritance of early trauma may exist in mice and humans.

Key words: Early-life trauma, maternal separation, depression, high-density lipoproteins, microRNAs, serum, saliva

8.2 Main Text

Exposure to traumatic experiences during early-life has long-lasting effects on physical and mental health in animals¹⁵⁸. Our previous work on a mouse model of early-life trauma, induced through maternal separation and unpredictable maternal stress (MSUS) from postnatal day 1 (PND1) to PND14 demonstrates depressive-like symptoms, increased risk-taking, antisocial behaviors, impaired object memory, and metabolic perturbations in adulthood. Importantly, many of these symptoms are transmitted to their progeny up to three generations in a germline dependent manner^{11,48,103,105}. Changes in germline epigenome, in particular, germ cell non-coding RNAs (ncRNAs) have recently been identified as vectors of such transgenerational epigenetic inheritance (TEI) after different environmental exposures^{11,159}. Injecting RNA extracted from the sperm of male mice exposed to MSUS when pups, into fertilized control oocytes recapitulates trauma-induced behavioral and metabolic symptoms in the resulting progeny¹¹. The importance of germline ncRNAs in TEI in humans is not yet fully established but initial results suggest their potential implication. Alterations in sperm microRNAs (miRNAs), similar to those observed in mice exposed to stress, were recently demonstrated in men with adverse childhood experiences (ACE)¹⁶⁰. However, this study did not address how such experiences induce changes in germline ncRNAs. Circulating miRNAs appear as attractive candidates for carrying the centrally acting stimuli to the germline after early-life trauma but this has not been studied to the best of our knowledge¹⁵⁹.

To investigate a conservation of the effects of early-life trauma between mice and humans, we studied a cohort of 6-12 year old children (n=26) residing in the SOS village (an orphanage) in Lahore, Pakistan. This cohort has many similarities to the MSUS mouse model. The cohort closely resembles the inbred C57bl/6J MSUS mice because of high levels of parental consanguinity (Supplementary Table 1). The children have experienced paternal loss and maternal separation (PLMS) within the last one year. Paternal loss was chosen as an exposure because spousal death is a significant stressor in humans¹⁵⁵ and hence mimics the maternal stress in the MSUS model.

Similarly, the adoption of the children by the SOS village recapitulates the maternal separation component of MSUS. The age group of 6-12 years was selected because the influences of sex hormones on behavior and physiology are minimal in this age. A control group (n=16) who were living with both their parents was recruited from the schoolmates from the PLMS children. The PLMS children and the controls were matched on age, gender, body mass index, and parental consanguinity (Supplementary Table 2).

Both the groups underwent detailed behavioral assessments through center for epidemiological studies depression scale for children (CES-DC)¹⁵⁷ and scale for anxiety related disorders in children (SCARED)¹⁶¹. The risk for depression and anxiety disorders (Fig. 1a, Supplementary Table 3) are more prevalent in the PLMS children compared to controls. These symptoms overlap with those observed in adult MSUS mice that show increased depressive-like symptoms, as well as, altered sociability^{11,48}.

We then investigated molecular similarities between MSUS and PLMS, focusing in particular on circulating markers. We previously demonstrated changes in glucose metabolism in the adult MSUS mice and their progeny⁴⁸. We extended this characterization by including assessment of serum lipids in adult MSUS mice and performed similar assessment for serum lipids in the PLMS children. Compared to controls, a significantly higher proportion of PLMS children show high-density lipoproteins (HDL) below the normal range (Fig. 1b). This finding is similar to MSUS mice also showing a decrease in HDL (Fig. 1c). Changes in low-density lipoproteins (LDL) are not observed in either MSUS mice (Supplementary Fig. 1) or PLMS children (Supplementary Fig. 2). Furthermore, among the PLMS children, the mean score on CES-DC was significantly higher in those with HDL levels<35 (Fig. 1d).

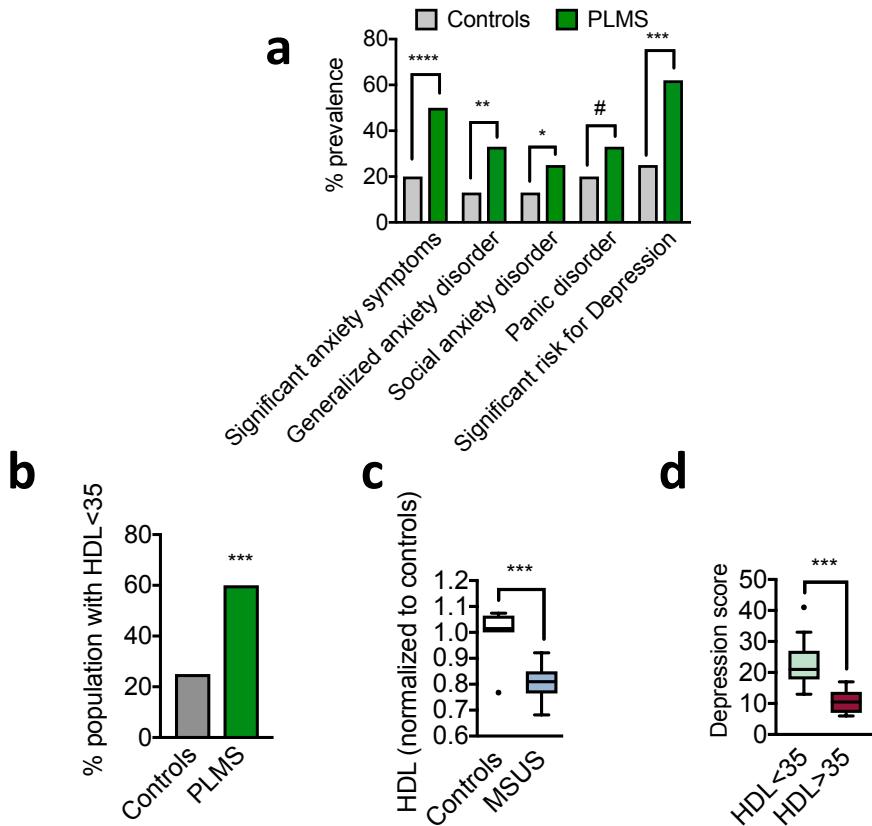


Figure 1: Similar psychological and lipid impairments in humans and mice with early-life trauma.

(a) Prevalence (%) of different psychological conditions as assessed by the Scale of Anxiety Related Disorders in Children (SCARED) and Center for Epidemiological Studies- Depression scale (CES-DS) in PLMS children compared to controls (Fisher's exact test: Significant anxiety symptoms, **** $p<0.0001$, Generalized anxiety disorder, ** $p<0.01$, # $p<0.1$, * $p<0.05$, *** $p<0.001$. n=16 Controls, n=26 PLMS. (b) Prevalence (%) of individuals with HDL<35 in PLMS cohort compared to matched controls. Fisher's exact test *** $p<0.001$. n=14, Controls. N=20 PLMS. (c) Serum HDL in MSUS mice normalized to controls (Unpaired t test *** $p<0.001$). n=8 Controls, n=8 MSUS. Box indicates IQR, whiskers drawn by Tukey's method. (d) Comparison of Depression scores in PLMS children with HDL levels < and > 35 (Unpaired t test *** $p<0.001$). n=12 HDL<35, n=8 HDL>35. Box indicates IQR, whiskers drawn by Tukey's method.

Along with extra-cellular vesicles, HDLs are considered a major carrier of circulating ncRNAs in mammals⁸⁰. Similarly, anatomical locations relevant to lipid metabolism, such as white and brown adipose tissue are a major source

of circulating microRNAs (miRNAs)⁷⁷. Therefore, we next investigated if changes in HDL are related to an altered circulating miRNA profile in PLMS children and MSUS mice. For this, we developed a list of 9 candidate miRNAs (Supplementary Table 4), which have been previously associated with lipid metabolism and were detectable in a serum miRNA sequencing screen in a cohort of adults with traumatic exposure (unpublished data). While four of our candidates (let-7, miR-138, miR-200c, miR-370) show low baseline expression (Ct values>35), four other miRNAs (miR-15a, miR-16, miR-29a, and miR-375) are significantly altered in the serum of PLMS children (Fig. 2a). Furthermore, miRNA assessments of the serum of MSUS mice show comparable alterations in three of these miRNAs (Fig. 2b). These changes likely lead to functional consequences as the mRNA targets of these miRNAs show corresponding altered expression in the target tissue (Supplementary Fig. 3). Importantly, the miRNAs altered in both MSUS mice and PLMS children; miR-16, miR-375, and miR-29a are either known to be carried by HDL or regulate HDL levels and are implicated in behavior and metabolism (Supplementary Table 4).

HDLs and miRNAs can be detected in different body fluids, such as saliva, semen, and milk; in both mice and humans^{162–164}. To evaluate miRNA changes in additional body fluids we checked their expression in saliva from PLMS cohort. Three miRNAs (miR-15a, miR-16, miR-375) altered in the serum are also altered in the saliva, in the opposite direction (Fig. 2c). However, opposite changes in serum and saliva are not surprising, as similar findings have been previously reported¹⁶⁵. While an assessment of HDL-carried miRNAs in the milk or seminal fluid could be of relevance, it was not possible to test these in the PLMS cohort. In particular, similar to serum, HDL-carried miRNAs present in seminal fluid may be directly taken up through the HDL receptor, SCARB1, present in both the developing and mature sperm cells¹⁶⁶.

Finally, to assess the relevance of serum HDL in carrying circulating miRNAs to the sperm, we treated developing sperm-cell like (GC-1) cells with serum from MSUS mice. Serum treatment induced changes in the HDL-carried

miRNA miR-375 but not in miR-16 or miR-29a in the GC-1 cells (Fig. 2d). The change in miR-375 was reversed in the GC-1 cells treated with MSUS serum when HDL- receptor SCARB1 was knocked down (Fig. 2e, Supplementary Fig. 4).

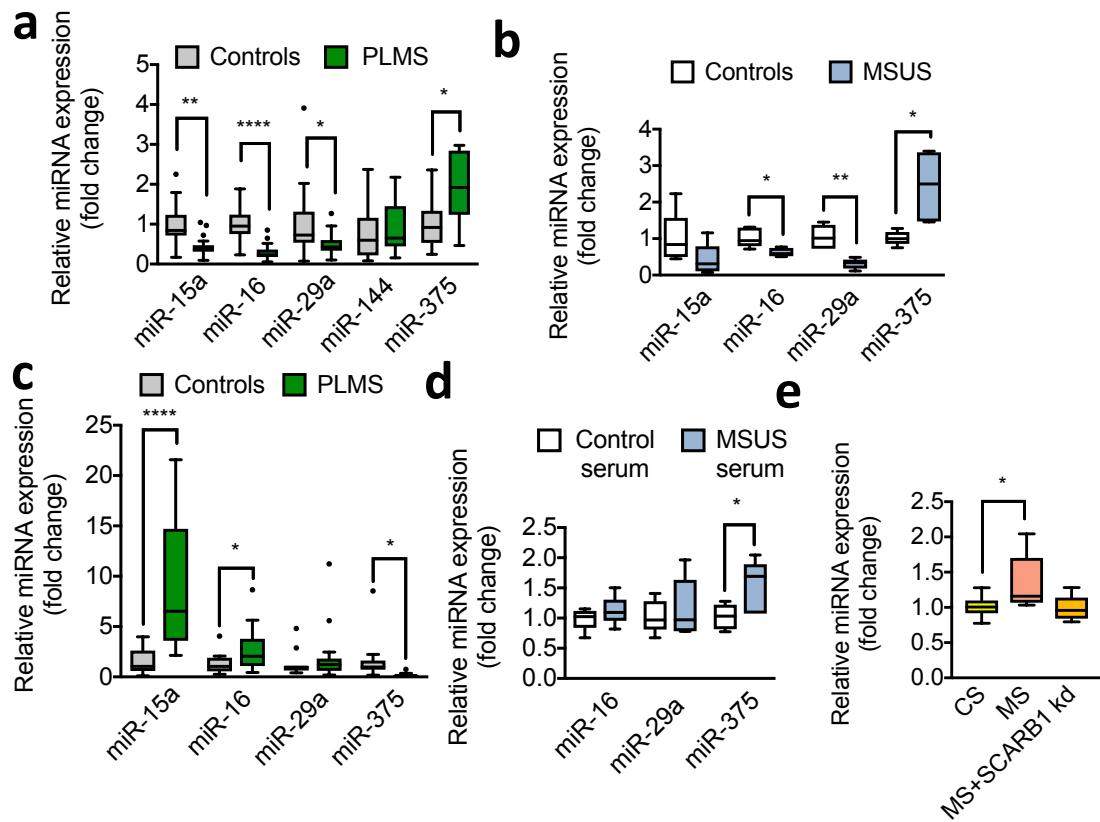


Figure 2: Alterations in HDL-related miRNAs in humans and mice with early-life trauma and their uptake by developing germ cell-like cells *in vitro*

(a) Expression of miRNAs in serum from PLMS children compared to controls (Unpaired t test: miR-15a ** $p<0.01$, miR-16 **** $p<0.0001$, miR-29a * $p<0.05$, miR-144 $p=0.73$, miR-375 * $p<0.05$). n=14 Controls, n=20 PLMS. Box indicates IQR, whiskers drawn by Tukey's method. (b) Expression of miRNAs in serum from MSUS mice compared to controls (Unpaired t test: miR-15a $p=0.17$, miR-16 * $p<0.05$, miR-29a ** $p<0.01$, miR-375 * $p<0.05$). n=6 Controls, n=6 MSUS. Box indicates IQR, whiskers drawn by Tukey's method. (c) Expression of miRNAs in saliva from PLMS children compared to controls (Unpaired t test: miR-15 **** $p<0.0001$, miR-16 * $p<0.05$, miR-29a $p=0.47$, miR-375 * $p<0.05$). n=14 Controls, n=25 PLMS. Box indicates IQR, whiskers drawn by Tukey's method. (d) Expression of miR-16, miR-29a, and miR-375 in GC-1 cells treated with serum from MSUS mice compared to those treated with serum from controls (Unpaired t test: miR-16 $p=0.35$, miR-29a

p=0.62, miR-375 *p<0.05). n=5 Controls serum (in duplicates), n=5 MSUS serum (in duplicates). Box indicates IQR, whiskers drawn by Tukey's method. (e) Expression of miR-375 in GC-1 cells treated with serum from MSUS mice (MS) with and without siRNA-mediated knockdown of SCARB1 compared to GC-1 cells treated with serum from control mice (CS) (One way ANOVA *p<0.05, F=4.47, post-hoc: CS vs. MS *p<0.05, CS vs. MS+SCARB1 kd p=0.98). n=5 CS, n=5 MS, n=5 MS+SCARB1 kd.

Our results indicate conserved differential expression of HDL and certain circulating miRNAs after early-life trauma in mice and humans. The changes in HDL is one of the likely mechanisms underlying the changes in circulating miRNAs as the differentially regulated miRNAs are either carried by HDL or are known regulators of HDLs. Furthermore, our *in vitro* study in spermatogonial stem cell-like cells suggests that HDLs have the potential to induce miRNA changes in the developing germline. A possibility that HDLs have a mediating role in induction of germline changes for TEI is highly conceivable considering that different exposures associated with TEI in mammals, such as high-fat diet, exercise, acute or chronic stress are associated with changes in serum lipoprotein, including HDLs^{167,168}. An *in vivo* confirmation of this finding could yield specific targets for intervention (ranging from whole body metabolism to specific HDL-ncRNA interactions) for preventing transmission of harmful effects to the progeny of trauma-exposed populations.

A limited sample size and single time-point assessments in the human are major limitations of our study. However, we intend to follow up the current study with longitudinal assessments of an expanded cohort of PLMS children to track their behavioral, metabolic, and circulating miRNAs trajectories with an eventual goal to analyze their semen specimens post-puberty. Nevertheless, this study is the first to identify novel serum and saliva miRNA changes after early-life traumatic exposure in humans, which interestingly matches the findings from an established mouse model of TEI. The wide-ranging long-term consequences of early-life traumatic experiences have lead to increased appreciation that their screening should be incorporated into

clinical practice. However, there could be reservations about divulging such experiences among some individuals. Furthermore, a recall bias is an important consideration in such screens, which may be even more important in the context of possible traumatic amnesia¹⁶⁰. Hence, non-invasive biomarkers are required to allow reliable screening of early-life adversity. Changes in salivary miRNAs in PLMS children raises the possibility of using saliva as a body fluid for such screenings. Overall, our findings indicate a conservation of pathways related to lipid metabolism and circulating miRNAs across species and raises the possibility that similar vectors of early trauma exist in mice and humans.

8.3 Methods

8.3.1 Human Study

The administration of an orphanage (SOS childrens' village) in Lahore, Pakistan was approached for assessment of children with early-life parental separation. The children were included in the assessment if they fulfilled the following criteria at the time of assessment: 1) Age between 6-12 years, 2) Paternal death, 3) Maternal separation in the form of adoption of the child by the SOS village, 4) Entry of the child to the SOS village within 12 months preceding the assessment. Exclusion criteria included; 1) History of abuse, 2) History of traumatic brain injury, intellectual disability or cerebral palsy. Based on these criteria, a total of 26 children with paternal loss and maternal separation (PLMS) were selected for the assessments. A control group (n=16) was recruited from the schoolmates of the PLMS children, which comprised 6-12 year old children who were living with both their parents and did not have a history of traumatic brain injury, intellectual disability or cerebral palsy.

8.3.2 Demographic Assessment

Detailed demographic information was acquired for both the groups from the administration of the SOS village for the PLMS group and parents for the controls. This included age, gender, parental consanguinity (defined by 1st or 2nd cousin parental union), and physical health records of the children. All the children underwent weight and height assessment by two research interns

blinded to the study design. Children were classified as under-weight (UW), healthy- weight (HW), or over-weight (OW) based on their correspondence to the ‘less than 5th percentile’, ‘between 5th and 85th percentile’ and ‘85th to 95th percentile’ reference ranges defined for Pakistani children of same age and sex¹⁵⁶.

8.3.3 Behavioral Assessment

The children were evaluated for presence of depressive symptoms through Center for Epidemiological Studies Scale for Depression in Children (CES-DC). CES-DC is a validated tool to screen for depressive symptoms in children aged 6-13 years based on 20-self report items, which are scored on a likert-like scale: 0 corresponding to ‘not at all’ to 3 corresponding to ‘a lot’. A score of 15 or higher indicates a high risk of depression and warrants clinical evaluation and intervention¹⁵⁷. The anxiety symptoms were evaluated in children through Scale for Anxiety Related Disorders (SCARED)¹⁶¹. SCARED is a validated 41-item inventory rated on a likert-like scale: 0 corresponding to ‘never/rarely’ to 3 corresponding to ‘very often’. Two questions related to the parents were not evaluated. CES-DC and SCARED assessments were performed in the form of interviews by an unblinded investigator. Furthermore, a confirmation of childrens’ responses was obtained from their foster mothers. In case of a disagreement between the responses on rare occasions, foster mothers’ responses were considered valid.

8.3.4 Saliva Collection

Saliva collection was performed by two research interns blinded to the study design. Children who had active upper respiratory tract infections (identified through the symptoms of fever, rhinorrhea, or cough) at the time of sample collection were excluded (n=1 PLMS, n=2 controls). All the children received a brief explanation of the saliva collection procedure and were promised a gift basket comprising different eateries for their co-operation. After a one-hour period of no oral intake, the children were asked to rinse their mouths with clear water twice. Saliva was collected 5 minutes after rinsing through passive drooling in salivette tubes (Sarstedt) over a period of 5 minutes. Collected saliva was aliquoted into 1.5 ml tubes and stored at -80 C.

8.3.5 Human Serum Collection

Blood collection was performed by a trained phlebotomist blinded to the study design. Blood withdrawal took place during the morning school hours for both the groups approximately 1 hour after their last oral intake. All the children received a brief explanation of the blood withdrawal procedure and were promised a gift basket comprising different eateries for their co-operation. Children who still showed high levels of reluctance or despair were excluded (n= 6 PLMS, n=2 controls). After sterilization with a swab, axillary vein venipuncture was performed through a butterfly syringe and 6 ml blood was collected per child in serum separating tube (BD vacutainer, Thermo Fisher scientific). After one-hour incubation at room temperature, the tubes were centrifuged for 10 minutes at 1,300 rcf for serum separation. Extracted serum was aliquoted into 1.5 ml tubes and stored at -80 C. Serum and saliva aliquots were shipped in packages containing 20 kilograms of dry ice for further analysis to Zurich.

8.3.6 Confidentiality Statement

Complete confidentiality of the participants was maintained through all the stages of data collection and analysis. The administration of the SOS village, Lahore, Pakistan was appraised of and approved all the listed study procedures.

8.3.7 Animals

C57Bl/6J mice were maintained under a reverse light-dark cycle in a temperature and humidity-controlled facility with food and water *ad libitum*. All experimental manipulations were performed during the animals' active cycle in accordance with guidelines and regulations of the cantonal veterinary office, Zurich. All animal experiments were approved under license number 55/2012, 57/2015.

8.3.8 Maternal Separation and Unpredictable Maternal Stress

For unpredictable maternal separation combined with maternal stress (MSUS), C57Bl/6J dams (2-3 months-old) and litters were selected at random

and subjected to daily 3hr proximal separation from postnatal day 1 to 14. Control animals were left undisturbed apart from a cage change once a week until weaning (postnatal day 21). Once weaned, pups were reared in social groups (4-5 mice/cage) composed of animals subjected to the same treatment but from different dams to avoid litter effects.

8.3.9 Animal Behavioral Assessment

Behavioral assessment (elevated plus maze) was performed in adult male mice by an experimenter blinded to the treatment. Behaviors were monitored by direct observation and videotracking (Viewpoint, France).

The elevated plus maze consisted of a platform with two open (without walls) and two closed (with walls) arms (dark gray PVC, 30cm × 5cm) elevated 60 cm above the floor. All experiments were performed in red light (15W). Each mouse was placed on the central platform, facing a closed arm, and observed for a 5-min period. The latency to enter an open arm, the time spent in each arm and the total distance moved were automatically recorded by a video-tracking system.

8.3.10 Animal Serum Collection

Adult MSUS and control males (3-4 months old) were sacrificed by decapitation and trunk blood was collected into non-coated Eppendorf tubes. Blood was allowed to clot for 30 mins, and serum was separated from hematocrit by centrifugation for 10 min at 2,000 g at 4° C. Serum was stored at -80° C until further processing.

8.3.11 Cell culture and siRNA mediated knockdown

GC-1 cells were obtained from ATCC (CRL-2053), and cultured according to accompanying datasheet at 37°C with 5% CO₂ in a biosafety level 2 (BSL2) cell culturing laboratory. Cell medium contained high-glucose DMEM with 10% FBS (HyClone) and 40 ug/ml gentamycin (Sigma Aldrich).

For serum exposure, medium was supplemented with 10% MSUS or control serum and sterile filtered with 0.22um Durapore PVDF membrane filter units (MillexGV, Merck) immediately before use. Cells were plated onto 12 or 24-

well plates. At 40% confluence regular medium was removed and replaced with 1 ml serum-enriched medium per well. After 24 hours serum-enriched medium was removed and cells were harvested with Trizol for downstream RNA extraction. Cells were exposed to serum from independent animals and performed in duplicate.

For transfections, approximately 8,000 cells were plated in 24-well plates. Transfection of a pool of siRNAs targeting SR-B1 (Flexitube siRNA, Qiagen) or negative control siRNA (All Star negative control, Qiagen) was carried out with HiPerfect® transfection reagent (Qiagen). Then the cells were returned to the incubator after transfection and grown for 48 hours prior to harvest or further treatment. The cells were harvested by removing the medium, washing with ice-cold PBS three times, and lysing with Trizol reagent for RNA extraction.

8.3.12 RNA extraction and RT-qPCR

For RNA extraction from cells, the medium was removed; the cells were washed three times with ice cold-PBS, lysed and homogenized by adding 1 mL Trizol reagent (Invitrogen) directly to the plates. For RNA extraction from human and mouse serum, 1 mL QIAzol reagent (Qiagen) was added to 200 uL of serum. After 5 minutes incubation, 3.5 uL of miRNAeasy Serum/Plasma spike-in control (Qiagen, 1.6×10^8 copies of cel-mir-39 per uL) was added. This was followed by Chloroform addition, aqueous phase separation, and Isopropanol-based precipitation of RNA. 500 ug total RNA was reverse transcribed using miScript II RT kit (Qiagen). Miscript primer assays (Qiagen) for mature miRNAs were used to amplify the respective transcripts from a cDNA pool. For mRNA quantification, Quantitect (Qiagen) gene specific primers were used. Real time PCR was performed on LightCycler 480® (Roche). Small nuclear RNA (RNU6) or spike-in control were used as endogenous controls for miRNA analysis, whereas GAPDH or rRNA18 were used as endogenous controls for mRNA analysis and quantification was performed as previously described¹⁶⁹.

8.3.13 Statistical analyses

For behavioral/molecular data comparison between two groups, unpaired Student t test was used, with additional Welsch correction in cases where the variances were unequal between the two groups. For data involving comparison between more than two groups, one-way ANOVA was used. Significant ANOVA analyses were followed by post-hoc pair-wise comparisons. Tukey's or Sidak's post-hoc was used for pair-wise comparisons. Outliers were defined by the ROUT test and were removed from the analyses. Significance was set at $p < 0.05$ for all tests and two-sided tests were performed. Statistical analysis was performed using GraphPad prism version 7 and verified by SPSS version 25. All graphs were drawn with GraphPad prism version 7.

8.4 Acknowledgments

This work was supported by the University of Zurich, the Swiss Federal Institute of Technology, the Swiss National Science Foundation. We would like to thank the administration of the SOS village, Pakistan (Mrs. Saba Faisal, Mrs. Rubina Asghar Ali, and Mrs. Almas Butt) and The Educators school, Lahore, Pakistan (Mrs. Sajida Makhdoom) for very kindly allowing us the assessments of the PLMS and the control children respectively; Mehr Shafique (Lahore University of Management Sciences, Lahore, Pakistan), Francesca Manuella, Niharika Gaur, and Irina Lazar-Contes (University of Zurich) for technical help; Chughtai Laboratories (Lahore, Pakistan) for their assistance with blood collection; and Prof. Aziz Mithani and Prof. Shaper Mirza (Lahore University of Management Sciences, Lahore, Pakistan) for organizational support.

8.5 Authors Contributions

AJ and IMM conceived and designed the study, analyzed the results and wrote the manuscript. AJ and AA collected behavioral data and blood/saliva samples from humans. AJ and SR performed molecular analyses of human samples. AJ, LH, and GvS performed molecular analyses of mice samples. GvS performed the MSUS paradigms, collected samples from the mice and

helped in the interpretation of mice data. KT assisted AJ in performing and analyzing *in vitro* experiments. IMM provided funding for the project.

8.6 Conflict of Interest Statement

The authors declare no conflict of interest

8.7 Supplementary Data

Supplementary Table 1:

	Gender	Age (years)	BMI	Parental Consanguinity	Duration spent in the SOS village (months)	Medical history
1	F	7	13.98	1 st cousin	2	Food allergy/ intolerance
2	M	10	16.28	1 st cousin	6	Asthma
3	F	12	15.38	2 nd cousin	2	No remarks
4	M	7	14.46	Unrelated	7	No remarks
5	M	12	15.69	1 st cousin	3	No remarks
6	M	10	15.18	2 nd cousin	3	No remarks
7	M	11	14.53	Unrelated	3	Nocturnal enuresis
8	F	7	13.64	1 st cousin	8	No remarks
9	F	10	16.85	1 st cousin	12	Food allergy/ intolerance
10	F	6	14.95	2 nd cousin	2	Food allergy/ intolerance
11	M	7	14.26	1 st cousin	2	Nocturnal enuresis
12	F	8	15.07	1 st cousin	5	No remarks
13	F	10	16.07	Unrelated	6	Food allergy/ intolerance
14	F	11	20.44	1 st cousin	2	No remarks
15	F	11	18.06	2 nd cousin	5	No remarks
16	F	11	14.06	2 nd cousin	7	No remarks
17	F	9	15.39	Unrelated	7	Food allergy/ intolerance
18	M	10	13.98	Unrelated	5	Food allergy/ intolerance Nocturnal enuresis
19	M	12	14.97	Unrelated	2	No remarks
20	F	9	14.65	1 st cousin	6	No remarks

21	M	9	13.78	Unrelated	5	No remarks
22	F	12	15.23	1 st cousin	3	No remarks
23	M	11	15.71	1 st cousin	3	No remarks
24	M	9	13.88	Unrelated	1	No remarks
25	M	7	14.38	Unrelated	2	No remarks
26	M	7	14.21	Unrelated	1	No remarks

Supplementary Table 2:

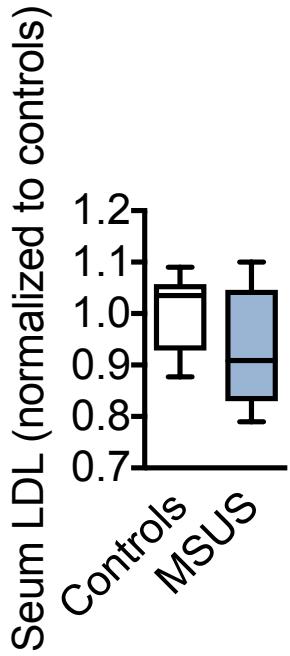
	Controls (n=16)	PLMS (n=26)	p value
Gender (n)			
Males	8	13	ns
Females	8	13	
Age (mean±SD)	8.38 ± 0.36	9.35 ± 0.37	ns
BMI (n)			
Under-weight	1	1	ns
Healthy-weight	14	24	
Over-weight	1	1	
Parental consanguinity (n)			
1 st or 2 nd cousin parents	10	16	ns
Unrelated parents	6	10	

Supplementary Table 3:

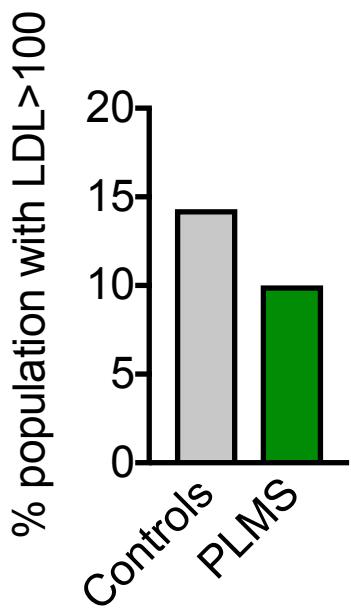
	Relative risk	95% Confidence Interval
Generalized anxiety symptoms	1.86	1.43-2.43
Generalized anxiety disorder	1.65	1.25-2.11
Social anxiety disorder	1.42	1.04-2.84
Panic disorder	1.37	1.02-1.78
Significant risk for Depression	2.12	1.60-2.86

Supplementary Table 4:

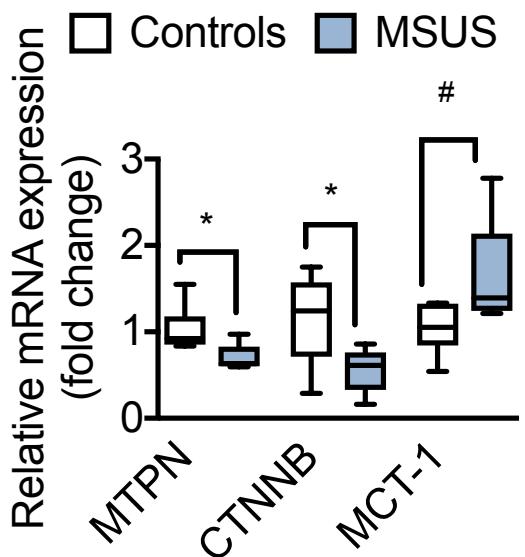
Candidate miRNAs	Association with traumatic stress	Association with lipid metabolism	References
let-7a	Down-regulated in serum of PTSD patients	Known HDL-miRNA	Vickers et al. 2011 Zhou et al. 2014
miR-15a	Important for coping with chronic stress in mice	Associated with cardiovascular conditions	Volk et al. 2016
miR-16	Serum fluctuations of miR-16 associate with resilience against chronic mild stress in mice	Known HDL-miRNA	Vickers et al. 2011 Zurawek et al. 2016
miR-29a	Elevated in the serum after academic stress in humans	Regulates lipogenic programs in the liver	Honda et al. 2013, Kurtz et al. 2015
miR-138	Increased in hippocampus after chronic stress in mice		Konopka et al. 2010
miR-144	Regulates fear-extinction memory in mice	Regulator of ABCA1 and HDL biosynthesis	Ramirez et al. 2013
miR-200c	Increased in serum of patients with major depressive disorder	Upregulated in pediatric patients with hypercholesterolemia	Belzeaux et al. 2012
miR-370	Increased in serum of patients with PTSD	Regulates cholesterol and fatty acid metabolism	Zhou et al. 2014
miR-375	Increased in serum and sperm after traumatic stress in the MSUS model	Known HDL-miRNA	Vickers et al. 2011 Gapp et al. 2014



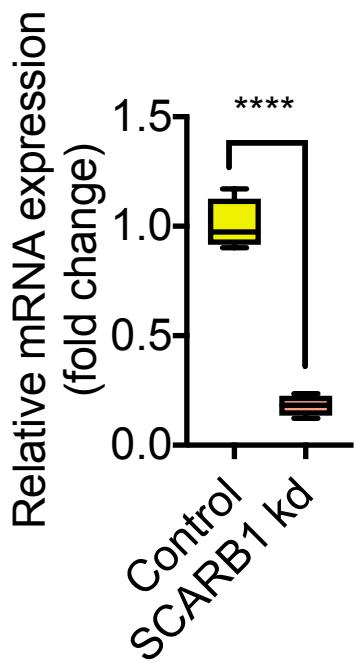
Supp. Fig. 1. No change in serum LDL in MSUS mice. Serum HDL in MSUS mice normalized to controls (unpaired t test: $p=0.168$). n= 8 Controls, n=8 MSUS



Supp. Fig. 2. No change in serum LDL in PLMS children. Prevalence (%) of individuals with LDL>100 in PLMS cohort compared to matched controls (Fisher's exact test $p=0.37$). n=14 Controls, n=20 PLMS.



Supp. Fig. 3. Expression of mRNAs in the pancreas from MSUS mice compared to controls (Unpaired t test: MTPN: miR-15 * $p<0.05$, CTNNB: * $p<0.05$, MCT-1 # $p<0.1$). n=6 Controls, n=6 MSUS. Box indicates IQR, whiskers drawn by Tukey's method.



Supp. Fig. 4. Expression of SR-B1 in GC-1 cells after siRNA- mediated knock down SCARB1 (SCARB1 kd) compared to those treated with control siRNA (Unpaired t test: **** $p>0.0001$). n=4 Control, n=4 SCARB1 kd. Box indicates IQR, whiskers drawn by Tukey's method.

9 Discussion

9.1 Depth of inheritance in mammals

To date, only few studies have addressed the question regarding the depth of inheritance in mammals. Mostly, there is a greater research focus on the mechanisms of transmission, than on evaluating the number of consecutive generations that may be affected. Owing to biological limitations such as the longer gestational periods and lifespan of mammals, and for technical limitations such as the high turnover of researchers in labs and the bottlenecking of mammalian lineages across generations¹⁷⁰, the majority of studies remain intergenerational (parent to offspring). Invertebrates have demonstrated transmission of exposures up to 80 generations¹⁷¹, but such penetrance has yet to be defined in mammals. Because the epigenome is regarded as plastic¹⁷², it is important to understand how different exposures can leave varying degrees of permanence. Indeed, testing the depth of penetrance across different exposures may provide key insight into mechanisms, which would never be discovered by performing intergenerational studies alone. Certain phenotypes may be present intergenerationally but disappear transgenerationally. In MSUS, the persistence of risk-taking behaviors, but not fear-memory¹⁰³, alongside glucose dysregulation may help to narrow down the molecular origins of inheritance to mechanisms preferentially involving those phenotypes. Similarly, truly transgenerational studies have made compelling contributions towards understanding specific exposures that contribute to specific disease susceptibilities. Prenatal immune activation led to schizophrenia-like behaviors in grand offspring³², while environmental toxins such as BPA, tributyltin, methoxychlor and vinclozolin can lead to metabolic disease and infertility in the 3rd and 4th generations^{35,173}. Prenatal stress could also induce changes in metabolites correlated with anxiety in the fourth generation of offspring resulting from single generational and multigenerational stress exposure- thus also contributing a cumulative effect of stress across generations²¹. However, these studies represent prenatal exposures. Thus we expand the current data to include a postnatal exposure with penetrance of

phenotypes to the fourth generation. Interestingly, only after assessing the fourth generation were we able to ascertain that the total observed symptomology of MSUS closely resembles BPD. Understanding the types of exposure that predispose consecutive generations to specific diseases may provide preferential therapeutic advantages, and reduce the burden of disease on the medical community. Perhaps treating MSUS for BPD in the first generation could target against the spread of these phenotypes in the fourth generation more effectively than treating MSUS for symptoms that would anyways disappear in the second or third generation. Of course this would assume that the inheritance of one phenotype is independent from the presence of another in the previous generation, but these questions have not been fully investigated. It would be interesting to determine whether preventing or directly treating exposed individuals with current therapies represents a better strategy than trying to develop new drugs and therapies for reversing inherited diseases, especially when their molecular etiology is not yet clear. While such an approach has been partially explored in MSUS, for example using the antidepressant Desipramine¹¹, or environmental enrichment¹⁹, only certain phenotypes were reversed. Assessment of the full battery of phenotypes would be necessary to fully explore this potential.

9.2 The conserved effects of childhood adversity on blood factors in mouse and human

Both PUFA and bile acid biosynthesis pathways were altered in blood (and saliva in the case of the human cohort) from mouse and human cohorts of early-life adversity, suggesting a conserved effect. In humans, studies having identified PUFA supplementation as a therapeutic treatment for post-traumatic stress disorder¹⁷⁴, confirming a connection between adverse experiences and fatty acid regulation in blood¹⁷⁵. In the periphery, PUFAs regulate several metabolic and immunologic functions¹⁷⁶, while in the brain, PUFAs are important for cognition and their dysregulation has been linked with depression and anxiety¹⁷⁷, which may also help to explain some of the aberrant behaviors in MSUS¹¹. In addition to fatty acids, bile acid dysregulation is similarly altered in both MSUS and PLMS cohorts.

Interestingly, bile acids are derived from steroid metabolites, consistent with the co-occurrence of altered steroidogenesis in MSUS blood and PLMS saliva. Further, cholesterol is an intermediate in the bile acid biogenesis pathway¹⁷⁸, and the major component of HDLs and LDLs. We also found that HDLs are altered and that HDL-associated miRNAs have similar expression profiles in both MSUS and PLMS cohorts. The fact that several prominent perturbations across mouse and human are associated with a common signaling pathway (miRNAs-HDLs-cholesterols-bile acids-steroids), suggests that the induction of this cascade may arise from a similar event. In MSUS, fatty acid perturbations were the first to be perturbed, during the middle of the MSUS treatment. While fatty acids have different molecular properties to steroid-derived metabolites, their contribution to metabolic function may explain how their initial perturbation could result in activation of the steroid-derived metabolites; the most compelling explanation for this involves nuclear receptor activity. PUFAs, bile acids, and steroid derivatives are all endogenous ligands for PPARs, farnesoid X receptors (FXR) and liver X receptors (LXR), respectively. Upon ligand binding, these nuclear receptors bind with retinoid X receptors (RXRs), and translocate to the nucleus to activate transcription. These receptors have similar DNA-binding motifs resulting in a strong overlap of genes that can be transcribed by each of them, including regulating the expression of each other. In the case of PPARs, LXR and FXR expression can be modulated by PPAR activity¹⁷⁹, and because they share similar DNA binding motifs, access to specific loci may be disrupted due to competitive binding¹⁸⁰. Thus, altered PPAR activity may alter the expression, and consequently provoke the activity of related receptors resulting in perturbations to downstream processes- involving bile acids and steroid derivatives. Such compensatory mechanisms may function to regain homeostasis, however long exposure may reprogram the system. Indeed, in MSUS pups PUFA metabolites were downregulated, but rebounded to an upregulation in adulthood. During this time perturbations to bile acid biosynthesis and steroidogenesis emerged. Thus, early adversity may have set in motion a physiological response that involves multiple nuclear receptors, resulting in the modulation of bile acids, cholesterol, and hormones, with relevance for phenotype transmission.

It is worth noting that while PUFA metabolites were downregulated in MSUS pups, they were upregulated in adult MSUS and in PLMS children. Here, we compare the dysregulation in PLMS children with adult MSUS, rather than with MSUS pups. The downregulation of PUFAs may be a result of current or immediate trauma exposure¹⁸¹, while the long-term effects are better represented by the adults. In the case of the PLMS children, their recovery in the SOS village began in the year before blood measurements, and the paternal loss occurred prior to admittance, suggesting that the physiological effects of adversity may have already entered into the long-term effects. For this reason we believe that adult MSUS is more comparable with PLMS.

9.3 Nuclear receptors

Several nuclear receptors have been associated with transgenerational inheritance in two distinct ways. The first involves an epigenetic mark occurring at or near the promoter of the gene while the second involves ligand activation of the protein. Regarding epigenetic marks at the promoter, altered histone variants and H3K27me3 at PPAR γ were found in sperm from rats with liver fibrosis which altered wound-healing properties in offspring⁹⁸. In another study, gestational protein restriction modified PPAR α promoter methylation in offspring liver¹⁸². In further support of this effect but involving pre-conception exposures, paternal protein restriction also resulted in aberrant promoter methylation of PPAR α , which was associated with adverse lipid and cholesterol homeostasis¹²³. Further to nuclear receptors, another model of gestational under-nutrition altered LXR methylation in sperm from first generation males, which was also found in liver in the second generation³⁹. These studies demonstrate alteration of epigenetic marks at genomic loci relevant for nuclear receptor function, which result in consequences for their downstream activity and an association with inheritance. However several keystone papers implicate the direct activation of the receptors themselves via ligand activation may be the key initiator, rather than the result, of an epigenetic effect. Perhaps the best examples come from endocrine disrupting chemicals such as BPA. BPA primarily activates estrogen receptors alpha and

beta (ER α , ER β), but can also bind RXR and PXR⁶⁶. Similarly, vinclozolin, known for its effects on spermatogenesis up to the fourth generation, is an androgen receptor ligand, but may also have effects on estrogen and progesterone receptors¹⁸³. Because such chemicals have variable actions on several receptors, it is difficult to say whether direct ligand activation of a particular nuclear receptor is causal in phenotype transmission. Further, we can't exclude off target effects that may confound interpretations, such as mutagenesis¹⁸⁴, which are common to environmental toxicants since their pharmacological properties are not screened with the same intensity as medical drugs.

The first example of the specific activation of a nuclear receptor came from injecting mice with the synthetic glucocorticoid Dexamethasone. In this study, they found differential DNAme and RNA expression of nuclear receptor genes in the offspring brain and kidney, and non-CpG changes in DNAme in sperm from the injected fathers⁷⁵. Similarly, a recent study could show that Dexamethasone injections altered phenotypes in rats intergenerationally¹⁸⁵, but they found no evidence of altered DNAme (using MeDIP-seq), or histone PTMs at specific loci, and small RNAs were unchanged in the sperm of the injected. They did however observe that non-promoter methylation levels were more variable in the Dexamethasone-injected animals, thus presenting a similar finding to the original study in mice. Intriguingly, DNAme was shown to be intimately linked to chromatin organization¹⁸⁶, suggesting that there may have been a functional influence of this finding. Unfortunately, no follow-up analysis was ever performed to implicate functional relevance. However, another study using the environmental toxin, TBT, was shown to affect metabolic phenotypes up to four generations³⁵. Notably, TBT is an obesogen and acts on PPAR γ and RXR, specifically, providing more evidence that direct activation of nuclear receptors can affect transgenerational phenotypes. Uniquely, they found transmissible effects on chromatin organization in third and fourth generation sperm using ATAC-sequencing, a consequence arguably more congruent with the epigenetic impact of nuclear receptor activity since nuclear receptors bind DNA directly. Similarly, tesaglitazar is a

dual PPAR α/γ agonist with verified binding properties showing higher binding affinity for PPAR α compared with PPAR γ . Repeated injection with tesaglitazar could confirm transgenerational transmission resulting from ligand activation of PPAR. However, in the tesaglitazar-injected mice, we have not ruled out other types of epigenetic regulation, such as chromatin organization, or whether PPAR PTMs are involved¹⁸⁷. Instead, transcription factor activation resulting in concordance of differentially expressed genes in sperm of tesaglitazar-injected mice provides compelling evidence. In particular, PPAR activation by tesaglitazar demonstrates a rare case of a specific environmental manipulation on an adult mammal producing transgenerational effects, highlighting the robust consequences of environmental manipulations resulting in the specific modulation of nuclear receptors. Moreover, we show that even transient changes in circulating factors are sufficient to induce persistent, and perhaps permanent, changes to the germline. Because mature sperm exposed to the direct effects of tesaglitazar would not have been present at the time of breeding, it is likely changes in sperm were established in a permanent manner, potentially through chromatin remodeling. In contrast, one study found that miRNAs were altered in the sperm of mice treated with Corticosterone¹⁸⁸. It would be interesting to see whether these miRNAs are directly altered by Corticosterone, or if this is a downstream effect. In this study, additional epigenetic perturbations were not measured.

It seems that ligand activation of nuclear receptors may have potent epigenetic effects that involve, but also may extend beyond, traditional readouts (DNAm, RNAseq, histone PTMs). Newer techniques and creative strategies may be necessary to obtain a better readout of their epigenetic activity. For this we performed long RNA sequencing, rather than small, which allowed us to better correlate transcription factor binding activity and chromatin accessibility with follow-up experiments like ATAC-seq and ChIP-seq. This is a long way from demonstrating causal evidence. Alternatively, since sperm are considered to have reduced transcriptional activity, we could simply quantify PPAR proteins bound to chromatin. This technique assumes that ligand activation of a nuclear receptor always results in its' translocation

to the nucleus and subsequent binding to the chromatin. Such a method requires the specific isolation of only the chromatin-bound proteins, and is currently being developed in the lab. If successful, such an approach could result in the isolation of all chromatin-bound proteins, which could be measured with a mass-spectrometry approach to unbiasedly detect all chromatin-bound proteins. This could then be combined with ATAC-sequencing data to correlate chromatin-bound proteins with changes to accessible chromatin. Finally, if nuclear receptors were found to be differentially bound to chromatin in sperm, evidence would have to demonstrate that this has a functional impact on offspring. Monitoring of embryogenesis would therefore be necessary. However, the common argument against epigenetic inheritance is that several epigenetic marks repeatedly undergo erasure and therefore one particular mark is unlikely to persist throughout development. While we know there are exceptions to this, nuclear receptors nonetheless provide an interesting loophole to this mentality. Since nuclear receptors carried by sperm would be present post-fertilization, and since they can interact with DNA structure and even recruit epigenetic modifying enzymes as well as regulate transcription⁶⁴, epigenetic marks don't have to persist, but rather just be re-established by similar mechanisms pre- and post-fertilization. This type of mechanism could also explain RNA-mediated inheritance, but would need extensive evaluation for its merit towards transgenerational epigenetic inheritance. Because RNA-mediated inheritance of phenotypes has been repeatedly demonstrated^{12,26,48,61,62,189}, transcription factor activity by nuclear receptors may be crucial in the initial establishment of aberrant RNA transcripts in sperm, which then lead to transmission of phenotypes. Moreover, we found that injecting both the small and long RNA fraction of F1 MSUS sperm into fertilized oocytes reproduced MSUS phenotypes in the offspring (Annex 2), emphasizing that probing the mechanisms globally regulating the transcriptome is essential for revealing the source of aberrant RNA profiles. Further, if post-fertilization transcriptional activity by nuclear receptors resulted in a similar change in RNA profiles as in pre-fertilization sperm, then it could mimic the same effect as injecting sperm RNA into zygotes.

9.4 Potential origins of germ cell RNA

The question of the origin of altered RNA in sperm is still under debate. While some believe sperm is transcriptionally silent because of the tight compaction of chromatin¹⁹⁰, RNA sequencing nonetheless identifies small⁴⁸ and long (Annex 2) RNA transcripts in sperm. So the question remains, are they endogenously expressed or not? We could show that the majority of long RNA transcripts identified in sperm could also be found in spermatozoa along different stages of spermatogenesis (Annex 2), suggesting that the transcripts could be endogenously expressed by germ cells, and remain until maturity. This is a favorable explanation given the implicated role of nuclear receptor transcriptional activity in germ cells, but cannot account for all possible origins of sperm RNA⁷⁸.

HDLs are another type of vesicle that carries RNAs, and their contribution of RNAs to the sperm transcriptome is not known. HDLs and known HDL-associated miRNAs were altered in serum in both MSUS and PLMS cohorts, and similarly altered in sperm from MSUS mice⁴⁸. In cell culture, serum from MSUS mice induced an upregulation of miR-375 in germ cell-like cells, which was reversed after inhibition of the HDL receptor through siRNA-mediated knockdown, confirming that HDL can deliver transcripts to cells⁸⁰. These findings, however, were done *in vitro* and are only correlative to the findings found *in vivo*. Several additional experiments are necessary to corroborate the findings. First, HDLs from MSUS and PLMS serum samples need to be isolated in order to confirm certain RNA transcripts are enriched or depleted inside. Second, HDL particles containing differential RNA content would need to be injected into the circulation of naïve males, and the altered RNAs measured in sperm to confirm HDL-to-germ cell uptake. Lastly, the transcripts delivered by HDL to the germ cells would have to demonstrate a contribution to inheritance of altered phenotypes by zygotic injection, and phenotyping of the resulting offspring. Nevertheless, our findings provide a strong proof of principle for the potential of HDL-associated RNAs in serum to deliver transcripts to germ cells.

9.5 Serum factors and germ cell modifications

Building on several keystone studies, we are the first to demonstrate that serum factors can induce the transmission of phenotypes in progeny. In 2011, a breakthrough was presented in how blood factors from aged mice could induce cognitive signs of early aging in young mice¹⁴¹. Follow-up studies have since demonstrated that parabiosis, serum transfer or plasma proteins could also affect cognition^{143,191,192}. Then in 2014, Zeybel et al could show that injecting serum collected from rats with induced liver damage, could produce histone variant enrichment and modifications in sperm of the injected rats similar to sperm from rats with the liver damage⁹⁸. This was the first study to implicate serum factors could alter the sperm epigenome, however, offspring were not generated. Thus, there was no confirmation that epigenetic alterations induced by serum factors have functional relevance to phenotype inheritance.

While we posit that the initiation of transmission is caused by metabolites, in the studies outline above, several implicate the serum-induced phenotype to be caused by a protein (i.e. CCL11, TIMP2). In Zeybel et al, they concluded that the effect was caused by an unknown factor secreted by hepatic stellate cell-derived myofibroblast. Interestingly, we present novel evidence for several lipid-based molecules such as fatty acids, sterols, hormones and bile acids. PUFAs in particular have been shown to remodel late-stage spermatozoa protected by the blood-testis barrier¹⁴⁰. This feature of lipid-based compounds may be why acute preconception stress exposures can still result in phenotypic alterations in offspring¹⁹³, so that last minute changes to the environment can be accounted for in the offspring. Lipophilic molecules in germ cells or in support cells such as Sertoli, Leydig and epididymal cells, that cross-talk with developing spermatogenic cells, or other extracellular-to-nucleus signaling components, may also contribute¹⁴⁰. However, whether circulating proteins in MSUS can communicate with germ cells, similar to the aforementioned studies, is yet to be determined. In fact, very little is known in general about the interaction of the circulating proteome with germ cells and presents an interesting opportunity for future studies.

9.5.1 Developmental stage and the serum-to-germ cell interaction

The ability for an environmental exposure to affect the male germline relies on several components. First, the environmental exposure must initiate the release of a signal into the bloodstream. This means that regardless of its origin, the effects of an environment must be severe enough such that the target tissue releases a signal into the circulation in order to access the germline. Second, the signal must be able to reach the germline. Prior to PND21, when the development of the blood-testis-barrier (BTB) is incomplete¹⁹⁴, circulating factors have access to all spermatogonial cells. At this time point however, the first full spermatogenesis cycle has not yet completed, leaving only cells up until the pachytene stage to be present, and not mature sperm⁴⁶. After full development of the BTB (after PND20⁴⁶), only the spermatogonial stem cells (SSCs) are directly accessible, while differentiated spermatozoa are enclosed in an immune privileged state. Thus, unless a signal interacts directly with the SSCs in adulthood, then it must pass through several supporting cells (i.e. Sertoli cells) or tissue structures (i.e. epididymis) in order to reach differentiated spermatozoa, including mature sperm. The ability for a signal to reach the germline in early-life therefore differs greatly from postnatal periods to adulthood, as long as the BTB remains intact¹⁹⁵. Lastly the germline must be able to receive and interpret the signal. Regardless of the time or location of the signal-to-cell interaction, the cell must be equipped to receive the message. In the case of ligand signals, the cell must express their specific receptors. Similarly, immune signals, such as cytokines and chemokines acting through their receptors¹⁹⁶, and extracellular vesicles from blood (exosomes and high-density lipoproteins), may also reach developing spermatozoa to impact the sperm epigenome. Importantly, in germ cells, nuclear receptors are expressed^{196,197}.

9.6 Sources of altered metabolites in blood

Several considerations need to be made in order to identify the source(s) of altered metabolites in the blood. These include dietary factors across lifespan, especially considering that childhood nutritional status can program metabolic

function with lingering consequences into adulthood¹⁹⁸. Another prominent consideration is the microbiome, which similar to diet can dictate which nutrients reach your organs for proper function. Diet and microbiome will be discussed in more detail below, however other mechanisms are possible. External stimuli may also alter metabolic function, either acutely or persistently, thus favoring certain metabolic pathways over others. The best example of this is the Warburg effect, in which cancer cells prefer aerobic glycolysis over oxidative phosphorylation in order to reduce oxidative stress and favor proliferation¹⁹⁹. Lastly, environmentally induced epigenetic alterations to genes vital for metabolite processing may become perturbed such that downstream signaling is affected.

9.6.1 Diet

In the context of our research, using inbred mice allows us to exclude genetic variables, so the first and most obvious source of altered metabolites might be diet. Indeed diets common to certain cultures can alter metabolites that are strongly linked with the associated diseases – or disease protection – within that culture²⁰⁰. However, both MSUS and control mice receive the same chow, and food intake has not revealed any differences between groups⁴⁸, nor was pup weight at weaning altered¹¹, suggesting that breast milk consumption was similar between groups. But, this is not the case during the MSUS paradigm itself. While the pups still receive their nutrition from their mother's milk, the mothers are being stressed on a daily basis. In fact, the stress hormone cortisol in breast milk was found to be inversely associated with BMI in early childhood²⁰¹. Notably, at PND8, MSUS pups showed a decrease in PUFA metabolites, which rebounded into adulthood suggesting acquisition of compensatory mechanisms. Specifically, the PUFAs ALA/LA are essential fatty acids, and must come from dietary sources. This suggests that the deficit originated from the mothers milk, as that is the only source of nutrients for the pups at this time. Although evidence for the direct effect of stress on fatty acid content in milk has not been sufficiently revealed, milk fatty acids have been linked with infant serum phospholipids²⁰² suggesting a possible link between milk composition and metabolic signaling in early development. In regards to MSUS, the only dietary link to altered metabolites

would have to have originated in postnatal life, resulting from milk composition. For this it would be important to conduct a metabolomic, transcriptomic and proteomic screen comparing MSUS and control breast milk before confidently ruling out an interaction with offspring developmental programming.

9.6.2 Microbiome

Another consideration regarding the source of altered metabolites in blood stems from the microbiome. Microbiota could be remodeled through diet (i.e. breast milk), or through sharing a cage with a mother whose microbiome remodels over the course of chronic stress exposure²⁰³. During the MSUS paradigm pups are susceptible to both of these conditions, thus presenting an additional possibility of compounding effects. Recently the microbiome has been associated with cognitive and immunological diseases²⁰⁴, in particular regarding the gut-brain axis. Therefore, the effects of the microbiome on cognitive effects of MSUS may be particularly interesting for future studies. The gut-brain axis has also been linked to metabolic syndrome through acetate production²⁰⁵. Interestingly, acetate is the major constituent of acetyl CoA, the founder molecule of fatty acid and cholesterol biosynthesis. Additionally, transplantation of the fecal microbiome from lean donors can reverse insulin resistance in obese recipients, mainly involving the short-chain fatty acid butyrate²⁰⁶. Several additional studies have also revealed a strong interaction between the microbiome and changes in the production of metabolites relevant to health and disease²⁰⁷, the majority however seem to involve short- and long-chain fatty acids^{208–211}. Such prominent interactions between the microbiome and metabolites form the basis for considerations for treatment of disease through microbiome-mediated regulation of metabolites²¹².

In regards to nuclear receptor signaling, the bile acid receptor FXR has been extensively associated with microbiome homeostasis. Importantly, the microbiome was found to regulate bile acid metabolism through metabolite interactions with FXR²¹³. Since bile acids are downstream of cholesterol synthesis, these results are consistent with findings from another group

showing that the microbiome can affect cholesterol absorption, however the authors suspect this effects was a result of altered gene expression of LXR target genes²¹⁴, suggesting an interaction between common nuclear receptors¹²⁸. Interestingly, it was found that certain bacteria produce metabolites, which act on PPAR γ to prevent overexpansion of pathological bacterial species²¹⁵. These studies indicate a clear connection between the microbiome, metabolite homeostasis and nuclear receptor signaling. Such microbiome mediated effects, remarkably, provides a unified explanation for the major effects observed in MSUS and constitutes an important mechanism worth exploring in follow-up studies.

9.7 Potential basis for altered HDL cholesterols

Cholesterol is essential for all cells in the body, in particular for membrane function. However, it is also a precursor for steroids, bile acids and vitamin D. This widespread importance likely underlies the observation that all cells express the enzymes for cholesterol synthesis. A marked amount of cholesterol comes from dietary sources, however the majority comes from biosynthesis in the liver and intestines. Biosynthesis occurs from acetyl CoA, the molecule produced from both beta-oxidation and glycolysis, and is shuttled through the mevalonate pathway requiring several enzyme reactions that utilize NADPH cofactors. When considering the basis for aberrant cholesterol concentrations in the body, typically the first consideration is diet. However, diet is an unlikely explanation, as reviewed in previous sections. This would then suggest that there is an inherent biological mechanism for the change in cholesterol, particularly HDL bound cholesterols, identified in MSUS mice. HMG CoA reductase (HMGR) is the primary rate-limiting enzyme in cholesterol biosynthesis, and produces HMG CoA from acetyl CoA. Thus, any disruption in HMGR function or availability of acetyl CoA would therefore affect downstream cholesterol synthesis.

In the case that diet is not the contributor to altered cholesterol, it stands to reason that either cholesterol absorption or biosynthesis pathways are perturbed. Intestinal function represents a large contribution to total body

cholesterol, since it is the site for dietary cholesterol absorption and a major site of biosynthesis. Since the microbiome can regulate cholesterol absorption²¹⁴, microbiome composition and proteins involved in cholesterol absorption are important to quantify.

9.8 PPAR mechanisms of action

PPARs can activate and inhibit the expression of genes involving diverse functions across metabolism and immune signaling. Several mechanisms of transcription regulation have been identified, but are still researched with strong intensity. Uniquely, ligand activation of PPARs is actually a form of de-repression, while several mechanisms of transrepression (not involving direct PPAR-DNA interactions) have additionally been identified. In the absence of a ligand, PPAR is bound by a co-repressor complex, resulting in the inhibition of target gene expression. This may explain why, paradoxically, PPAR γ knockouts can sometimes reveal an upregulation of target gene expression²¹⁶. Upon ligand binding, PPARs undergo a conformational change resulting in the release of the co-repressor complex in exchange for a co-activator complex, recruitment of RNA polymerase and the subsequent initiation of target gene expression²¹⁷. Transrepression, on the other hand, is not so straightforward. PPARs have been shown to interact with the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) to inhibit its binding on the DNA²¹⁸, and in another study ligand-bound PPAR γ could prevent the degradation of an NF- κ B co-repressor complex, thus preventing target gene expression²¹⁹. Notably, PPAR target genes seem to preferentially involve metabolic signaling, while its involvement in immune function more often involves transrepression. Apart from NF- κ B, PPARs have been identified to repress activator protein 1 (AP1), nuclear factor of activated T-cells (NFAT) and signal transducer and activators of transcription (STATs)²¹⁶. These interactions were often associated with a specific signal, implying that different ligands may exert subtle differences in downstream action of PPARs following their activation and may help to explain the diversity of effects following PPAR activation using synthetic, natural and endogenous ligands.

9.8.1 Synthetic agonists

Since PPARs are important in controlling metabolic processes involving lipid homeostasis, adipogenesis, insulin resistance and glucose control, they make for compelling pharmaceutical targets to treat diabetes. Indeed, PPARs have also been noted for their role in inflammation, as they are expressed in the vasculature and in circulating immune cells, such as mast cells. Since diabetics often experience vascular (usually involving advanced glycation end products related to glucose control) and inflammatory complications, PPARs emerge as an optimal target. Two classes of drugs, the thiazolidinediones (TZDs) and fibrates, have been developed as agonists for PPAR γ and PPAR α , respectively. PPAR γ agonists are more specifically known to act on adipocytes (where tissue specific expression is highest), and initiate adipogenesis. Their therapeutic effect lies in the ability to increase insulin sensitivity and cellular glucose uptake. In the early 1990's TZDs went through several clinical trials for the treatment of diabetes and vascular complications, and troglitazone was the first to be approved in 1997. Due to previously undetected evidence of hepatotoxicity following approval of the drug, it was consequently removed from market. Another TZD, Pioglitazone (currently in use for diabetes treatment) has common side effects involving weight gain (adipogenesis) and fluid retention²²⁰. However, these specific side effects can increase the psychological burden on diabetics, who often experience these issues independently of drug treatment. PPAR α agonists known as fibrates were first discovered serendipitously when it was discovered that farm workers exposed to a fibrate-containing pesticide developed improved fatty acid and cholesterol profiles²²¹. Shortly thereafter the first drug, clofibrate, was synthesized. Interestingly however, the mechanism of action was thought to involve androgen receptors, and wasn't until the 1990s that it was tied to PPARs²²². Several different fibrates have emerged since, with fenofibrate being the predominant agonist used clinically. Interestingly however, PPAR α is expressed most widely in the liver, and have a greater beneficial effect on fatty acid and cholesterol profiles than on insulin and glucose sensitivities, compared with PPAR γ , but are often surpassed by statins for treatment of cholesterol and cardiovascular disease. Since PPAR α treatment also showed

some initial promise in treating obesity it was hypothesized to create dual PPAR α/γ agonists whereby the effects of the PPAR α would counteract the negative side effects of a PPAR γ -only treatment. Indeed, these drugs termed the glitazars were, and still are, being developed for treatment of diabetes and dyslipidemia, and often show absence of weight gain common to PPAR γ treatment alone. Only saroglitazar has been approved for clinical use. As common to synthetic drug development, not all drugs of the same class have similar effects. In the case for the fibrates, some drugs actually demonstrate a paradoxical decrease in circulating HDLs²²³. Since the ligand-binding socket is considered larger and more promiscuous in order to accommodate inherent size and diversity of fatty acids, several different binding interactions could affect the overall outcome. The broad availability of diverse endogenous ligands likely has even more complex interactions. This may explain why metabolic readouts in MSUS offspring differ slightly from the offspring of males injected with tesaglitazar. Indeed, PPAR ligand binding affects the size and shape of the DNA binding domain and may affect its preference for certain genomic loci. Therefore tesaglitazar treatment may not be sufficient to mimic the diversity of ligands present in MSUS. Moreover, this may also help to explain the paradoxical evidence for downregulation of HDL after treatment with some PPAR α agonists. Our finding that HDL was lower in both MSUS and PLMS serum raised some conflicting concerns regarding a potential increase in PPAR fatty acid-based ligands. Here, we see that this paradoxical effect seems to have scientific precedence.

9.8.2 Natural ligands

Aside from the synthetic agonists, studies have also identified a variety of natural ligands. The idea into researching these compounds was based on the question of whether the side effects from synthetic ligands resulted from ‘intense’ binding of one molecule to PPAR proteins, when the large ligand-binding domain would suggest that PPAR function relies preferentially on weaker interactions but from several classes of molecules. Thus, research into naturally occurring PPAR ligands was borne. As expected, hundreds of dietary sources of PPAR ligands have been found, and several classes of molecules. These include compounds from green tea, soybeans, ginger, wine,

and several herbs such as oregano, thyme and rosemary²²⁴. Interestingly, several of these compounds, such as resveratrol from red wine, have been previously linked to improved metabolic health²²⁵. However, unlike synthetic chemicals which are screen to have specific binding partners, many of the natural compounds have binding interactions with a number of other receptors, for example genistein (from soybeans) is also an estrogen receptor ligand²²⁶. These interactions may be beneficial or contraindicated if diet were to be considered as a treatment strategy. Overall, these data confirm that diet can have a significant impact on PPAR signaling and may further provide sufficient diversity of ligands so as not to acquire adverse side effects predicted to result from one specific ligand-protein interaction.

9.8.3 Endogenous ligands

Endogenous ligands can be distinguished from natural ligands in that they originate within the organism, although they can rely on external precursors, and are drivers of crucial metabolic processes. Endogenous ligands of PPAR include the fatty acids derived eicosanoids, as well as oxidized low-density lipoproteins, the essential fatty acids, ALA/LA, AA, EPA and (poly)unsaturated fatty acids. Notably, the supply of endogenous ligands can be altered by perturbations in cell signaling, independent of dietary sources. In the case of PPAR ligands, metabolic shifts favoring beta-oxidation, aerobic versus anaerobic respiration, inflammation and stress can shift the supply of ligands. For example, PUFAs can be processed into eicosanoids, prostaglandins and leukotrienes in response to inflammation²²⁷, thus diverting fatty acids into immune sentinels. However, many of these downstream derivatives are also endogenous ligands for PPAR, thus unifying PPAR function across metabolic and immune pathways²²⁸. However, regarding the effects of PPAR on immune signaling, some PPAR ligands also have PPAR-independent effects on immune function and have thus made it difficult to uncouple the PPAR-ligand specific effects. Nonetheless, there is substantial evidence to indicate that PPARs regulate key elements of immune function, specifically regarding vascular disease²²⁹. The variability of the effects of PPARs seems to correspond with the cell type. For example, cells that express more cyclooxygenases 1 and 2 (COX1 & COX2, respectively), such as immune

cells, will have higher conversion of eicosanoids from fatty acids. The result would be that eicosanoids, rather than fatty acids, preferentially elicit immunological effects of PPAR, whereas in adipose tissue, where lower conversion of fatty acids to eicosanoids occurs, cells respond with more metabolic-related outcomes of PPAR signaling. Indeed, PPAR activation in adipose tissue elicits gene expression changes related to adipogenesis and insulin sensitization²³⁰. In contrast, PPARs in macrophages and T-cells have mechanistic relevance to vascular and immune function²³¹. While we demonstrate the MSUS have clear alterations in metabolism, we have not completed a full characterization of immune function. Preliminary data (not shown) suggests a beneficial immune profile in MSUS indicated by a significant decrease in C-reactive protein and downregulation of tumor necrosis factor alpha (TNF α), suggesting PPAR activation is co-occurring in metabolic and immune related tissue. It would be interesting to characterize the metabolites across different tissues and/or cells types in order to associate certain endogenous ligands with different downstream effects of PPAR activation.

10 Significance

The data presented in this thesis demonstrate that behavioral and metabolic consequences of early-life adversity are consistent and reproducible up to four generations through the patriline. Our data also contribute essential insight into the molecular mechanisms of such inheritance by demonstrating that serum factors can communicate with the germline to causally induce transmission of phenotypes to offspring. This likely involves nuclear receptor signaling since we could show that ligand activation of the nuclear receptor PPAR can impact metabolic phenotypes transgenerationally. Lastly, several classes of metabolites known to interact with nuclear receptors, such as fatty acids, bile acids and steroids, and small RNA species, are similarly altered in mouse and human cohorts of early-life adversity, highlighting the potential for these mechanisms to be conserved across species.

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12 Annex 1: Brain metabolic alterations in mice subjected to postnatal traumatic stress and in their offspring

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12.1 Abstract

Adverse environmental and social conditions early in life have a strong impact on health. They are major risk factors for mental diseases in adulthood and, in some cases, their effects can be transmitted across generations. The consequences of detrimental stress conditions on brain metabolism across generations are not well known. Using high-field (14.1 T) magnetic resonance spectroscopy, we investigated the neurochemical profile of adult male mice exposed to traumatic stress in early postnatal life and of their offspring, and of undisturbed control mice. We found that, relative to controls, early-life stress-exposed mice have metabolic alterations consistent with neuronal dysfunction, including reduced concentration of N-acetylaspartate, glutamate and g-aminobutyrate, in the prefrontal cortex in basal conditions. Their offspring have normal neurochemical profiles in basal conditions. Remarkably, when challenged by an acute cold swim stress, the offspring has attenuated metabolic responses in the prefrontal cortex, hippocampus and striatum. In particular, the expected stress-induced reduction in the concentration of N-acetylaspartate, a putative marker of neuronal health, was prevented in the cortex and hippocampus. These findings suggest that paternal trauma can confer beneficial brain metabolism adaptations to acute stress in the offspring.

Keywords: Traumatic stress, transgenerational, neurodevelopmental, metabolism, neurochemical profile, magnetic resonance spectroscopy

12.2 Introduction

The brain is the key organ in appraising the environment and in triggering adequate physiological and behavioural responses to potential adverse and dangerous factors. Although stress responses allow adaptation to environmental threats, they can be maladaptive when excessively intense and/or persistent, becoming a risk factor for the development of psychopathology. Indeed, traumatic stress experienced during a sensitive period of brain development increases the susceptibility to develop psychosis, mood and anxiety disorders in adulthood^{232–235}. Further, the progeny of individuals exposed to stress early in life is often predisposed to psychopathology^{16,236–238}. Notwithstanding, there is also evidence for resilience in offspring of individuals exposed to trauma²³⁹. In mice, early-life stress can also have beneficial effects on the offspring, such as improved goal-directed behaviours, flexibility and adaptation to novel rules⁷⁴.

Regional concentrations of brain metabolites, the so-called neurochemical profiles, vary with the developmental stage, oscillate with local functional state, and are affected by acute injury or prolonged disease²⁴⁰. Neurochemical profiles measured non-invasively by ¹H magnetic resonance spectroscopy (MRS) have been widely used to probe specific metabolic alterations in neurological disorders²⁴¹. Here, we used state-of-the-art high resolution MRS to determine whether brain metabolism is altered by postnatal traumatic stress in adult mice and their offspring. We applied an established model of postnatal trauma based on unpredictable maternal separation combined with unpredictable maternal stress (MSUS). This paradigm causes depressive symptoms, antisocial behaviours, risk-taking and cognitive deficits, which are transmitted across several generations by males or females^{11,49,103}. Since trauma is known to impact limbic and reward circuits²⁴², we examined the neurochemical profiles of mice exposed to MSUS in the dorsal hippocampus, prefrontal cortex and striatum.

12.3 Methods

12.3.1 Animals

All experiments were conducted in C57BL/6JRj mice according to the Swiss animal welfare legislation under approval of the local ethics committee (Kanton Zürich Gesundheitsdirektion Veterinäramt) and are reported according to the ARRIVE guidelines. Mice were housed in the Zürich facility with controlled temperature (20–22° C) and humidity (50–56%) and free access to food and water, under a 12-h reverse light–dark cycle (light off at 08:00). One week before MRS experiments, mice were transferred to Lausanne and housed under similar conditions but with light off at 19:00.

The study involved control and MSUS-exposed mice (F1) and their offspring (F2). Sample size estimation was based on previous experiments^{243,244}. MRS experiments were performed on 3-month-old male mice (F1: 13 MSUS and 13 controls; F2: 14 MSUS and 15 controls). A second set of 7-month old F2 male mice also underwent brain MRS experiments (12 MSUS and 10 controls) in combination with an acute stress. One week after baseline MRS, mice from this second group were exposed to cold swim stress, and MRS experiments were immediately repeated after warming up for 10 min under a heat lamp.

12.3.2 MSUS paradigm

Male and female mice (F0; from Janvier SAS, Berthevin, France) were bred on one-to-one pairing, and gestating females were single-housed and either maintained in regular conditions and allowed to raise their pups undisturbed (controls), or subjected to MSUS from postnatal day 1 to 14. Female mice were randomly assigned to either experimental group. For MSUS, dams and litters were subjected to 3-h daily separation unpredictably, during which dams were randomly and unpredictably exposed to restraint stress for 20 min or forced swim stress for 5 min¹¹. Control mice were left undisturbed except for weekly cage changing. Once weaned on postnatal day 21, pups (F1) were housed in groups of 4–5 of the same gender and treatment, but from different mothers to avoid litter effects. To obtain second-generation mice (F2), adult F1 control or MSUS males were mated to naïve adult females then removed

after mating, so F1 male mice were not in contact with the gestating female or with their progeny.

12.3.3 Acute swim stress

Mice were single-housed one day before handling. Exposure to cold swim stress prior to MRS experiments was performed as previously described²⁴⁵. Briefly, mice were placed in a plastic cylinder (20 cm high, 16 cm diameter) filled with $18 \pm 1^\circ\text{C}$ water up to 12 cm height for 6 min.

12.3.4 MRS

Investigators performing MRS experiments were blind to the group identity of each animal until all spectra were quantified. All experiments were carried out in a 14.1 T magnet with a horizontal bore of 26 cm (Magnex Scientific, Abingdon, UK), equipped with a 12-cm internal diameter gradient coil insert (400 mT/m, 200 μs), and interfaced to a DirectDrive console (Agilent Technologies, Palo Alto, CA, USA). Radio frequency transmission and reception were achieved with a home-built quadrature surface coil composed of two geometrically decoupled single-turn loops of 12 mm inner diameter resonating at 600 MHz. Spontaneously breathing mice were anaesthetised with 1–1.5% isoflurane (Animalcare Ltd., York, UK) in a 1:1 O₂:air mixture, and fixed in a home-built mouse holder with a bite bar and two ear inserts. Body temperature was maintained at 37°C by warm water circulation. Respiration and temperature were continuously monitored using a MR-compatible system (Small Animal Instruments, Inc., Stony Brook, NY, USA). Volumes of interest (VOI) were precisely placed in the dorsal hippocampus ($1.2 \times 2 \times 2.1 \text{ mm}^3$), frontal cortex ($0.8 \times 4 \times 1.6 \text{ mm}^3$) or striatum ($1.8 \times 1.5 \times 2 \text{ mm}^3$) according to anatomical landmarks in T2-weighted fast-spin-echo images. Field homogeneity in the VOI was achieved with FAST(EST)MAP²⁴⁶. Spectra were acquired using SPECIAL with echo time of 2.8 ms and repetition time of 4 s²⁴⁷. We accumulated 210 scans for the cortex and striatum, and 320 scans for the hippocampus. The order of scanning the regions was random, except for MR experiments after stress, which followed the order: cortex, hippocampus, striatum. In this case, the average scanning time after stress did not differ between experimental groups for each brain region (Table 1).

Table 1. Timing (in min) of MRS acquisitions after cold swim stress

	Control		MSUS	
	Scan start	Scan end	Scan start	Scan end
Cortex	32 ± 1	46 ± 1	32 ± 1	46 ± 1
Hippocampus	55 ± 4	75 ± 3	58 ± 11	77 ± 11
Striatum	82 ± 4	96 ± 4	85 ± 12	99 ± 12

Metabolite concentrations were determined with LCModel (Stephen Provencher Inc., Oakville, Ontario, Canada), including a macromolecule (Mac) spectrum in the database and using the unsuppressed water signal measured from the same VOI as internal reference.¹⁷ The following metabolites were included in the analysis: alanine (Ala), ascorbate (Asc), aspartate (Asp), creatine (Cr), γ-aminobutyrate (GABA), glutamine (Gln), glutamate (Glu), glutathione (GSH), glycine (Gly), glycerophosphorylcholine (GPC), glucose (Glc), lactate (Lac), myo-inositol (Ins), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphorylethanolamine (PE), phosphorylcholine (PCho), phosphocreatine (PCr), scyllo-inositol (Scyllo), taurine (Tau). The Cramér-Rao lower bound (CRLB) was provided by LCModel as a measure of the reliability of the quantification for each metabolite. In most measured spectra, scyllo-inositol was below the detection limit, and was excluded from subsequent data analyses. Remaining metabolites had CRLB below 30%.

Nine mice (out of 77) were excluded from the study because of abnormally high glutamine levels in all brain regions investigated, which suggests the occurrence of congenital portosystemic shunting²⁴⁴: 1 F1 control, 2 F1 MSUS, 4 F2 control and 2 F2 MSUS mice.

12.3.5 Statistical analysis

Data are presented as mean ± SEM unless otherwise stated and were analysed using the analysis of variance (ANOVA) for all metabolites together (repeated measures), followed by independent Student's t tests for post hoc

comparisons. Significance was considered for $P < 0.05$. Effect of MSUS-exposure on baseline neurochemical profiles (for either F1 or F2 mice) was tested with independent ANOVAs for the hippocampus, cortex and striatum because their neurochemical profiles are distinct²⁴³. The effect of acute stress was tested in a single multivariate ANOVA together with the effects of brain region and MSUS-exposure, as well as their interactions. Statistical analyses were performed with IBM SPSS 21 (IBM Corp., Armonk, New York, USA).

12.4 Results

Localised MRS at 14.1 T in the hippocampus, anterior cortex and striatum *in vivo* resulted in spectra with signal-to-noise ratios of 21 ± 3 , 21 ± 3 and 18 ± 2 , and spectral line widths of 11 ± 3 , 12 ± 3 and 11 ± 2 Hz, respectively (averaged for all acquired spectra, errors in SD of 101 scans/region; Figure 1). This excellent spectral quality allowed us to quantify a neurochemical profile composed of 19 metabolites in the three brain structures.

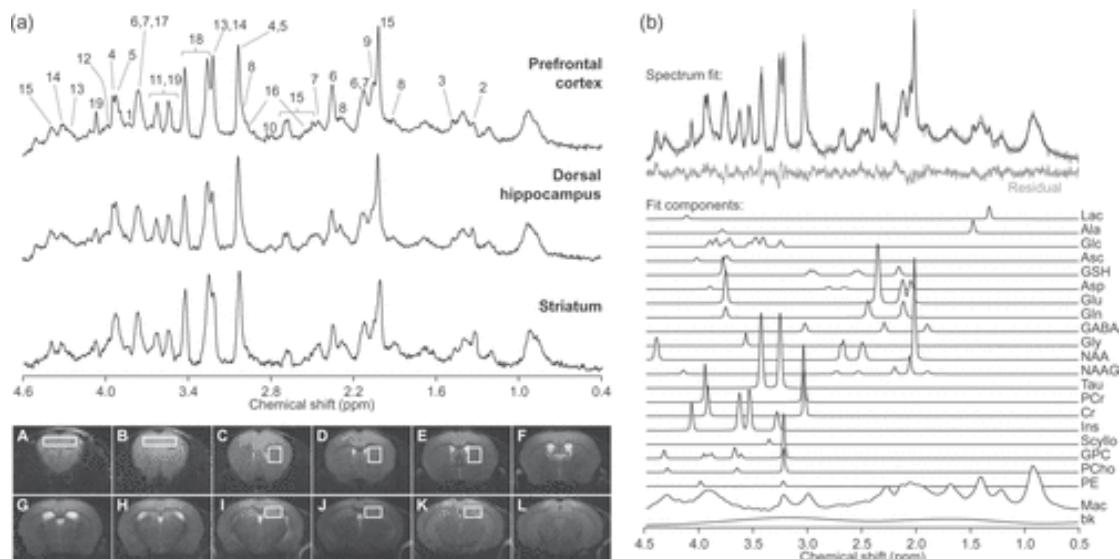


Figure 1. Representative ^1H spectra acquired *in vivo* at 14.1 T from the mouse prefrontal cortex, dorsal hippocampus and striatum (a). For display, spectra were processed with a shifted Gaussian function ($gf = 0.12$, $gfs = 0.02$) prior to Fourier transformation. Peak assignment is as follows: 1, glucose; 2, lactate; 3, alanine; 4, phosphocreatine; 5, creatine; 6, glutamate; 7, glutamine; 8, GABA; 9, N-acetylaspartylglutamate; 10, aspartate; 11, glycine; 12, phosphorylethanolamine; 13, phosphorylcholine; 14, glycerylphosphorylcholine; 15, N-acetylaspartate; 16, glutathione; 17, ascorbate; 18, taurine; 19, myo-inositol. Consecutive mouse brain slices from fast-spin-echo images (6-mm thickness) are displayed below. Typical

placement of VOIs in the cortex, striatum and hippocampus is represented in slices A–B, C–E and I–K, respectively. LCModel fit of the top spectrum (prefrontal cortex) is represented in (b). The fit is overlaid on the spectrum and the resulting residual is shown below. Mac: macromolecules; bk: background baseline; see text for metabolites.

MSUS induced neurochemical alterations in the prefrontal cortex ($F(1,22) = 9.574$, $P = 0.002$) but not in the dorsal hippocampus ($F(1,22) = 0.553$, $P = 0.457$) or striatum ($F(1,22) = 0.004$, $P = 0.951$) of adult mice (F1; Figure 2). In particular, MSUS-exposed mice (F1) had reduced cortical levels of the putative neuronal marker NAA (-9.2% , $P = 0.023$), the neurotransmitters glutamate (-10.8% , $P < 0.022$) and GABA (-12.3% , $P < 0.027$) and the phospholipid precursor phosphorylcholine (-33.0% , $P < 0.046$) when compared with controls.

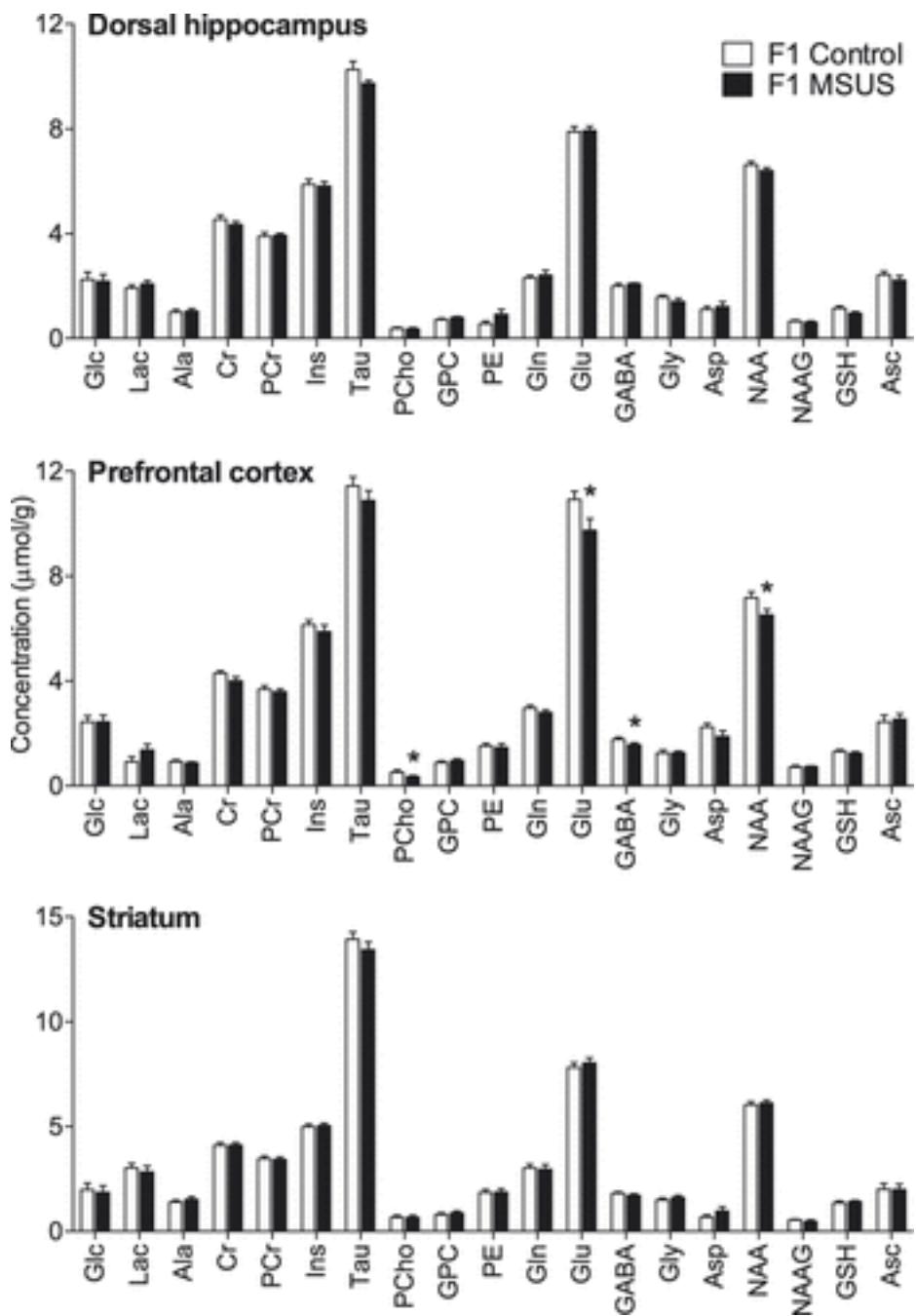


Figure 2. Neurochemical profiles in the hippocampus, prefrontal cortex and striatum of MSUS-exposed mice (F1, n = 11) and non-stressed controls (n = 12) mice. Data are mean \pm SEM. *P < 0.05 for MSUS-exposed mice vs. controls. Ala: alanine; Asc: ascorbate; Asp: aspartate; Cr: creatine; GABA: γ -aminobutyrate; Glc: glucose; Gln: glutamine; Glu: glutamate; Gly: glycine; GPC: glycerophosphorylcholine; GSH: glutathione; Ins: myo-inositol; Lac: lactate; NAA: N-acetylaspartate; NAAG: N-acetylaspartylglutamate; PCho: phosphorylcholine; PCr: phosphocreatine; PE: phosphorylethanolamine; Tau: taurine.

To investigate whether the observed effects are transmitted to the following generation, we bred MSUS-exposed males to naïve control females (non-stressed) to generate F2 progeny. MRS in the offspring of MSUS-exposed mice (F2) revealed no baseline alteration of metabolite concentrations in any of the three brain regions analysed when compared with controls: $F(1,44) = 0.642$, $P = 0.423$ for cortex; $F(1,44) = 1.277$, $P = 0.259$ for hippocampus; $F(1,44) = 0.070$, $P = 0.781$ for striatum (Figure 3).

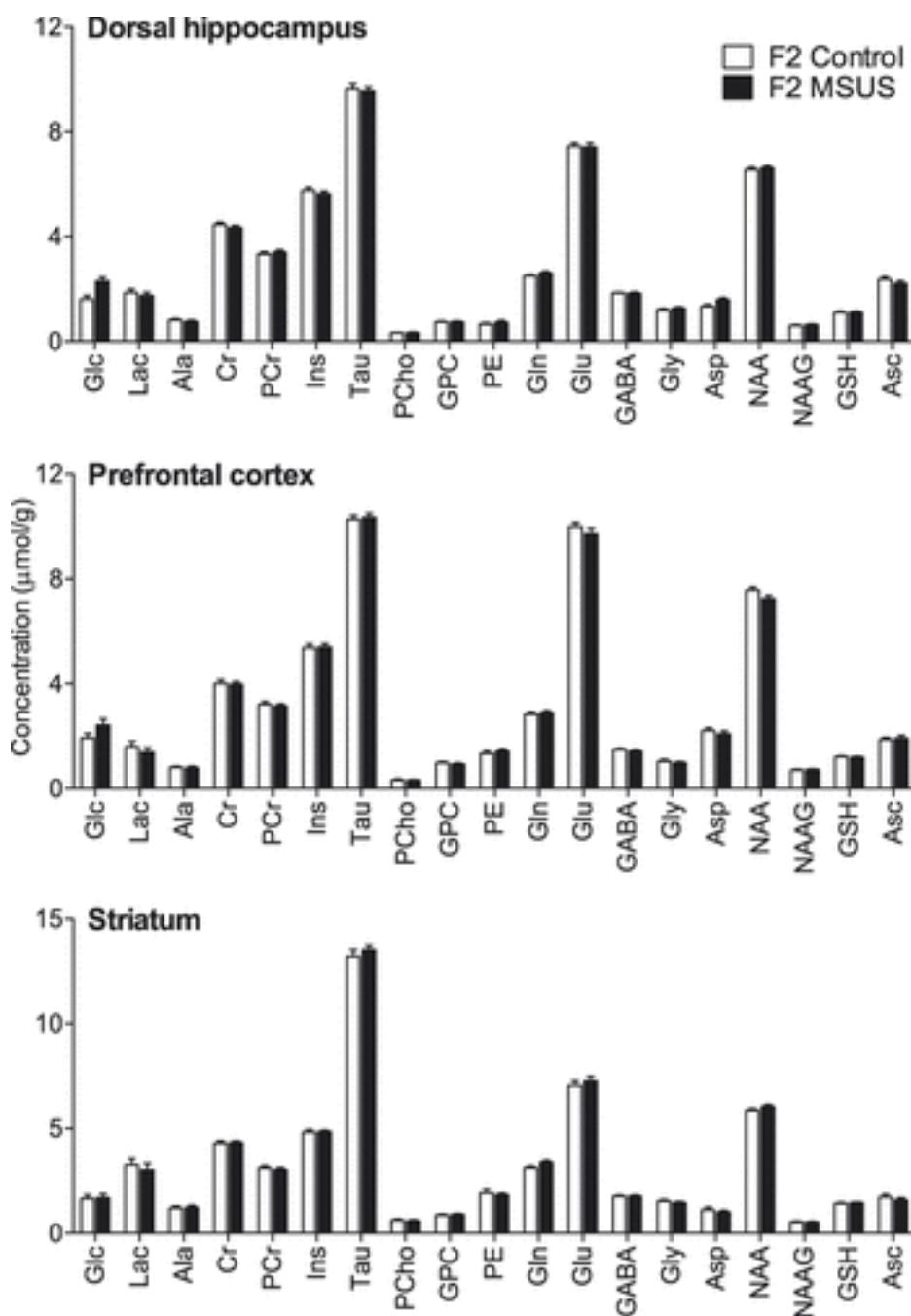


Figure 3. Neurochemical profiles in the hippocampus, prefrontal cortex and striatum of the offspring (F2) of MSUS-exposed ($n = 24$) and control mice ($n = 21$). Data are mean \pm SEM. Abbreviations are as in Figure 2.

Since some systems may reveal their dysfunctions only when activated, we tested whether exposure to an acute stress affects brain metabolism in F2 MSUS offspring. Upon acute swim stress, we observed that the concentrations of several brain metabolites were modified in F2 MSUS mice compared with controls, and metabolic modifications were region-specific (Figure 4). Repeated-measures ANOVA with acute swim stress, brain region and paternal MSUS as fixed factors, as well as their interactions revealed the expected regional specificity of the neurochemical profile ($F(36,182) = 101.0$, $P < 0.001$), a strong effect of acute swim stress exposure ($F(18,90) = 9.524$, $P < 0.001$) that was region-specific (region-stress interaction: $F(36,182) = 2.552$, $P < 0.001$), and an effect of paternal MSUS ($F(18,90) = 2.725$, $P = 0.001$) that significantly interacted with the effect of acute stress exposure (stress-MSUS interaction: $F(18,90) = 1.803$, $P = 0.032$). Interactions of paternal MSUS exposure with brain region were not significant (interaction MSUS-region: $F(36,182) = 0.816$, $P = 0.773$; interaction MSUS-region-stress: $F(36,182) = 1.166$, $P = 0.248$).

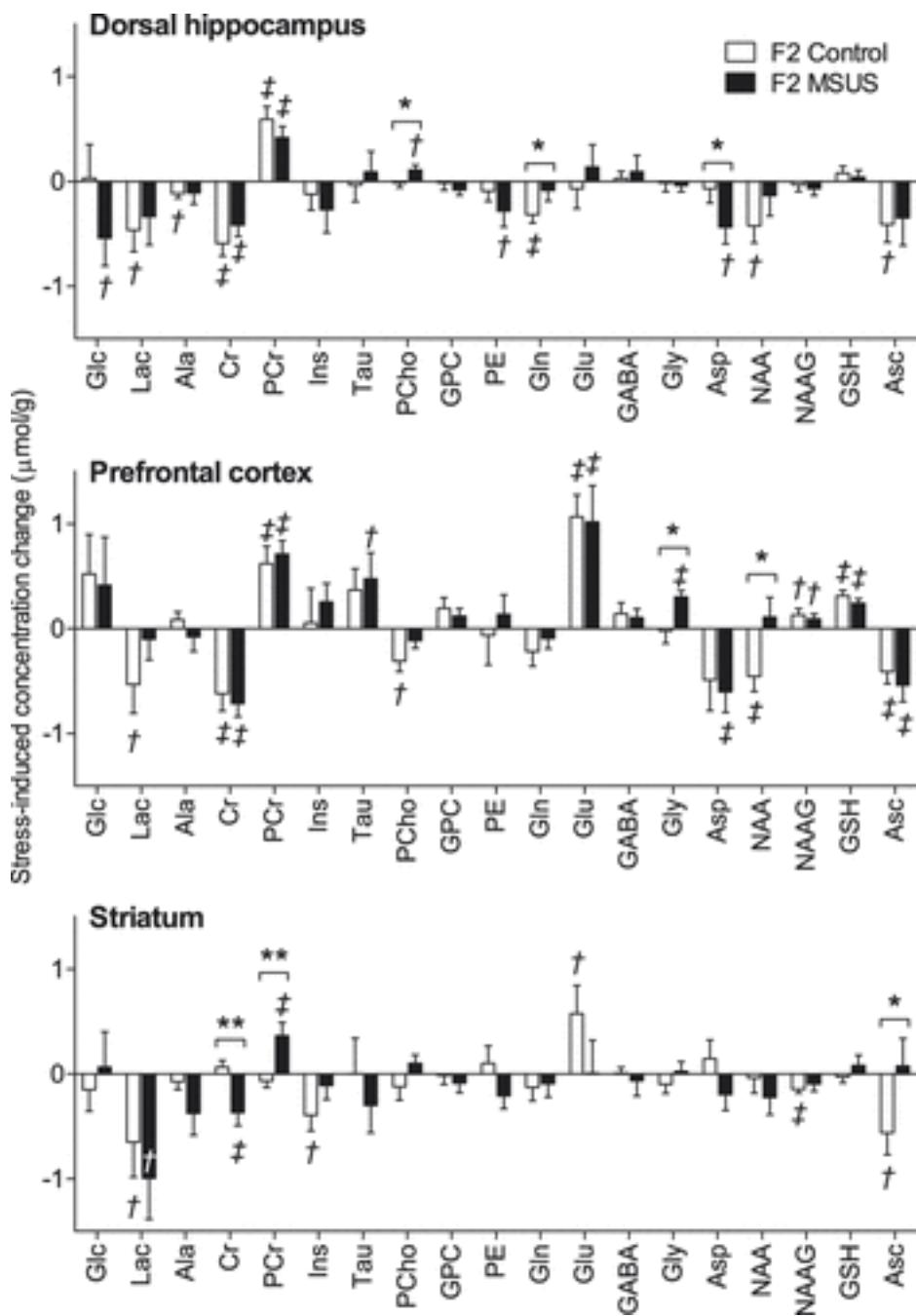


Figure 4. Acute swim stress-induced variation of metabolite concentrations in the hippocampus, prefrontal cortex and striatum of the offspring (F2) of MSUS-exposed ($n = 11$) and control ($n = 9$) mice. Data are mean \pm SEM. Multivariate ANOVA results: region $F(36,182) = 101.0$, $P < 0.001$; stress $F(18,90) = 9.524$, $P < 0.001$; MSUS $F(18,90) = 2.725$; $P = 0.001$; region*MSUS $F(36,182) = 0.816$, $P = 0.773$; stress*MSUS $F(18,90) = 1.803$; $P = 0.032$; region*stress $F(36,182) = 2.552$; $P < 0.001$; region*stress*MSUS $F(36,182) = 1.166$, $P = 0.248$. Post-hoc testing significant stress-induced modifications are depicted by $\dagger P < 0.05$ and $\ddagger P < 0.01$ (baseline vs. post-stress); MSUS-exposure effect was tested on concentration differences between post-stress and baseline (* $P < 0.05$, ** $P < 0.01$). Abbreviations are as in Figure 2.

Modifications of metabolite concentrations caused by acute swim stress in F2 control animals were specifically in the dorsal hippocampus, reduced lactate (-30% , $P = 0.028$), alanine (-18% , $P = 0.017$), creatine (-14% , $P < 0.001$), glutamine (-13% , $P = 0.003$), NAA (-6% , $P = 0.018$) and ascorbate (-18% , $P = 0.025$), and increased phosphocreatine ($+19\%$, $P < 0.001$); in the prefrontal cortex, reduced lactate (-37% , $P = 0.047$), creatine (-16% , $P = 0.003$), phosphorylcholine (-70% , $P = 0.014$), NAA (-6% , $P = 0.008$) and ascorbate (-23% , $P = 0.005$), and increased phosphocreatine ($+18\%$, $P = 0.003$), glutamate ($+11\%$, $P < 0.001$), NAAG ($+18\%$, $P = 0.030$) and glutathione ($+28\%$, $P < 0.001$); in the striatum, reduced lactate (-27% , $P = 0.047$), myo-inositol (-8% , $P = 0.017$), NAAG (-24% , $P = 0.003$) and ascorbate (-30% , $P = 0.016$), and increased glutamate ($+9\%$, $P = 0.035$).

These neurochemical responses to acute swim stress were modified by paternal MSUS exposure. Namely, in the offspring of MSUS mice but not controls, acute swim stress exposure caused a 26% reduction in aspartate ($P = 0.011$ for pre- vs. post-stress; $P = 0.047$ for MSUS vs. control) and a 40% increase in phosphorylcholine ($P = 0.027$ for pre- vs. post-stress; $P = 0.045$ for MSUS vs. control) in the hippocampus. In contrast, it caused a 13% reduction of glutamine levels in the hippocampus of controls ($P = 0.003$ for pre- vs. post-stress), but not in the offspring of MSUS mice ($P = 0.217$ for pre- vs. post-stress; $P = 0.046$ for MSUS vs. control).

Interestingly, in prefrontal cortex, there was a 6% reduction in NAA levels ($P = 0.008$ for pre- vs. post-stress in controls) by acute swim stress in control offspring but not in the offspring of MSUS mice ($P = 0.284$ for pre- vs. post-stress; $P = 0.014$ for MSUS vs. control). In addition, a 31% increase in glycine concentration was induced in F2 MSUS mice ($P = 0.011$ for pre- vs. post-stress) but not in F2 controls ($P = 0.433$ for pre- vs. post-stress; $P = 0.014$ for MSUS vs. control).

In the hippocampus and prefrontal cortex of both control and MSUS mice, there was a reduction of creatine concentration caused by acute swim stress that was accompanied by an increase in phosphocreatine. In the particular

case of the striatum, this effect occurred only in MSUS progeny, which had an 8% reduction in creatine ($P = 0.008$ for pre- vs. post-stress; $P = 0.003$ for MSUS vs. control) and 13% increase in phosphocreatine ($P = 0.009$ for pre- vs. post-stress; $P = 0.004$ for MSUS vs. control) concentrations. While ascorbate concentration in striatum was reduced by acute swim stress in the control offspring (-30% , $P = 0.013$ for pre- vs. post-stress), it was not modified in MSUS offspring ($P = 0.389$ for pre- vs. post-stress; $P = 0.037$ for MSUS vs. control).

12.5 Discussion

This study shows that traumatic stress in early postnatal life (MSUS) induces neurochemical alterations in the prefrontal cortex of adult mice and strikingly modifies the metabolic response to acute stress in their paternal line offspring.

In F1 adult mice, the alterations included reduced concentration of metabolites that are mainly synthesized in neurons, specifically the excitatory neurotransmitter glutamate, the inhibitory neurotransmitter GABA, and the putative neuronal marker NAA, suggesting neuronal dysfunction or neurodegeneration in the prefrontal cortex.¹¹ These results extend a previous report showing a reduction in NAA, glutamate and glutamine in the prefrontal cortex of rats exposed to maternal separation in the first 2 weeks of life²⁴⁸. Importantly, our observations in the prefrontal cortex parallel findings from MRS studies in the anterior cingulate cortex and other prefrontal areas of adult humans suffering from neurodevelopmental or psychiatric disorders^{249–251}. Particularly, meta-analysis of MRS studies of neurodevelopmental disorders including autism spectrum disorders, attention deficit hyperactivity disorder and obsessive compulsive disorder, found a general reduction of glutamate and GABA in areas of patients' prefrontal cortex, relative to healthy individuals²⁵¹. A reduction in NAA levels was generally observed in psychiatric disorders, most consistently in the anterior cingulate cortex, and often the decreased NAA was reversed with adequate therapy^{250,252}. Although MRS measurements of glutamate levels in schizophrenia patients are contradictory, there is a general consensus for an association between chronic

schizophrenia and reduced glutamate in areas of the frontal cortex²⁵³. Likewise, the majority of MRS studies reported lower GABA concentrations in schizophrenia patients relative to healthy individuals, regardless of the brain area analysed and illness stage²⁵³. Also patients suffering from mood disorders display a functional impairment in the prefrontal cortex^{254,255}, that is accompanied by reduced glutamate and/or glutamine MRS signals²⁵⁶.

In addition to GABA, glutamate and NAA, also phosphorylcholine was reduced in the prefrontal cortex of early-life stress-exposed mice relative to controls. Phosphorylcholine is a water-soluble choline-containing compound involved in membrane lipid metabolism²⁴⁰. Namely, it is precursor of phosphatidylcholine and, in turn, of sphingomyelin, which is necessary for adequate myelination of axons²⁵⁷. In addition of integrating membranous myelin sheaths surrounding axons, sphingomyelin is implicated in immune responses²⁵⁸. Notably, the prefrontal cortex of mice exposed to postnatal maternal separation was shown to display a neuroinflammatory response with microgliosis²⁵⁹. However, baseline low-grade cortical inflammation in MSUS-exposed mice remains to be demonstrated. Overall, these data in adult mice exposed to MSUS integrate well with previous observations of prefrontal cortex integrity loss and a possible inflammatory state after early chronic life stress.

Neurochemical modifications elicited by MSUS exposure were observed only in prefrontal cortex in F1 mice. These results are also consistent with the observation that early-life stress reduces the number of parvalbumin-containing interneurons in the prefrontal cortex but not the hippocampus of the stress-exposed mice in adulthood^{260,261}. The absence of metabolic alterations in other brain regions contrasts with the fact that other phenotypes are observed across the brain, i.e. impaired synaptic plasticity in hippocampus¹⁰³ and altered serotoninergic signaling in different brain areas¹⁰⁵ in MSUS mice. This may suggest that the observed neurochemical modifications are not contributing to these phenotypes. Likewise baseline neurochemical profiles were not affected in any tested brain area in the F2 offspring. But because the F2 offspring does display many behavioural

alterations including depressive-like symptoms, antisocial behaviours, impaired memory and altered risk assessment in adulthood^{11,74,103,105} we explored the comprehensive neurochemical profile following an acute swim stress challenge. This indeed revealed broad modifications in the neurochemical profile in MSUS offspring.

Acute stress is known to prompt an increase in norepinephrine and glucocorticoids thereby rapidly modulating glutamatergic neurotransmission. It stimulates glutamate release in the hippocampus and prefrontal cortex, which results in learning facilitation²⁶². In line with this, we observed a prominent increase of glutamate concentration in the prefrontal cortex of F2 MSUS mice after acute swim stress. In a study at low magnetic field (4.7 T), Kim et al.²⁶³ reported similar variation in glutamate levels within the rat cortex and hippocampus immediately after 1 h immobilisation stress. Stimulation of glutamatergic neurotransmission is energetically demanding and requires increased glucose metabolism in neurons and astrocytes, namely to fuel glutamate synthesis, restore membrane potentials, clear synaptic glutamate and convert it into glutamine²⁶⁴. To match this glucose demand, stress simultaneously increases blood glucose levels and stimulates brain glucose utilisation²⁶², which likely results in lactate production and release from the brain parenchyma. Indeed, stress is known to increase lactate release in the medial prefrontal cortex, hippocampus and striatum^{265,266}. After the stress event, the lactate in the tissue can be avidly consumed by neurons for energy production and production of neurotransmitter amino acids²⁶⁷. As expected in F2 animals, lactate levels after stress were generally lower than in the baseline scan.

Stress caused a general reduction of ascorbate (MRS-detected signal includes both oxidised and reduced forms) and a concomitant increase of glutathione levels in the prefrontal cortex. In line with this, administration of ascorbate to cultured neurons results in its rapid intracellular oxidation to dehydroascorbate and decreased glutathione levels²⁶⁸. Although the fate of ascorbate in the post-stress period is not evident, its reduction may result in stimulation of neuronal glycolysis. In fact, Cisternas et al. further showed that

exposing cultured neurons to ascorbate inhibits glycolysis, stimulates glucose oxidation through the pentose phosphate pathway (probably to maintain redox homeostasis), and favours lactate utilisation²⁶⁸. In sum, the reduction of ascorbate levels is consistent with a stress-induced stimulation of brain metabolism, leading to a reduction of energy substrates such as lactate and alanine, and accumulation of phosphocreatine after stress.

The post-stress readjustment of brain energy metabolism to basal rates resulted in an excess of available energy reflected by higher phosphocreatine-to-creatine ratios in the prefrontal cortex and hippocampus. Delayed effects (in the range of hours) of the glucocorticoids released upon stress include regulation of gene expression, namely reduction of neuronal expression of genes involved in ATP synthesis, and of lactate dehydrogenase B that converts lactate into pyruvate for further mitochondrial oxidation²⁶². Reducing ATP synthesis and lactate oxidation in the brain would later contribute to a normalisation of phosphocreatine, creatine and lactate concentrations. MRS in the striatum of acute stress-exposed F2 mice was performed 90 min after the acute swim stress challenge. Interestingly, levels of creatine and phosphocreatine in the striatum were modified after acute swim stress in F2 MSUS mice but not in controls. Possibly, the late effects of glucocorticoids on genes that regulate energy metabolism already took place at this time in controls but not yet in F2 MSUS mice. In agreement with this interpretation, we have identified MSUS-induced cerebral epigenetic changes involving histone post-translational modifications at the mineralocorticoid receptor gene, as well as decreased mineralocorticoid receptor expression in the offspring of MSUS-exposed mice⁷⁴. In opposition, levels of ascorbate were reduced by acute swim stress in the striatum of controls but not of F2 MSUS mice, suggesting that the proposed stress-induced redox response that regulates neuronal metabolism (see above) is blunted in the striatum of F2 MSUS mice.

NAA was reduced after the acute swim stress episode in the dorsal hippocampus and the prefrontal cortex of control mice but not in the offspring of MSUS-exposed mice. Aside from an acute metabolic response to stress, NAA reductions may also result from impaired mitochondrial integrity in

neurons and degeneration of neuronal processes²⁴⁰. A substantial retraction of dendritic branches was found in the medial prefrontal cortex after a single episode of forced swim²⁶⁹, and loss of excitatory synapses (PSD95-positive) may also occur in the hippocampus after acute stress²⁷⁰. NAA was also found to be specifically reduced in the anterior cingulate cortex and in the hippocampus of mice one day after exposure to fear conditioning²⁷¹, and in the hippocampus of adult rats after repeated forced swim for 28 consecutive days²⁷². Interestingly, the absence of a stress-induced decline in NAA levels in the prefrontal cortex and hippocampus in MSUS offspring alludes to an improved response to stress. This improvement suggests a beneficial adaptation in MSUS-exposed offspring, which may confer resistance to stress-induced neurodegeneration.

Glutamate levels in the prefrontal cortex of both MSUS-exposed offspring and controls were increased, consistent with the effects of acute swim stress. Curiously, this was accompanied by an increase in glycine in the offspring of MSUS-exposed mice, but not in controls. Glycine is a co-agonist of N-methyl-D-aspartate (NMDA) receptors, and tonic activation of the glycine modulatory site has an important role in the response to stress²⁷³. Although stress can increase the potency of glycine at the NMDA receptor²⁷⁴, its involvement in inhibitory neurotransmission through glycine receptors within the prefrontal cortex is notably robust^{275,276}. Increased levels of glycine in the prefrontal cortex upon stress can thus counterbalance excessive excitatory glutamatergic transmission, positing a further mechanism of adaptive protection from deleterious effects of acute stress in MSUS-exposed offspring.

Hippocampal and cortical aspartate levels were reduced in F2 MSUS mice after acute swim stress, relative to basal concentrations (pre-stress scan). Conversely, such stress-induced modifications were not observed in non-stressed controls. Aspartate is synthesised from oxaloacetate, is essential to transport reducing equivalents into the mitochondrial matrix (through the malate-aspartate shuttle) and is also a precursor for NAA synthesis in neurons²⁴⁰. While NAA can be used by oligodendrocytes to produce myelin, aspartate released extracellularly damages myelinated axons through

activation of NMDA receptors in oligodendrocytes²⁷⁷. Again, the reduction of aspartate levels upon stress in the prefrontal cortex and hippocampus of F2 MSUS mice appears to be linked to protective effects contributed by higher NAA. Moreover, post-stress levels of the sphingomyelin precursor phosphorylcholine²⁵⁸ tended to be higher in F2 MSUS mice than controls, which may further result in protection of myelinated axons.

Acute swim stress induced a reduction of glutamine levels in the hippocampus, possibly reflecting impaired glutamatergic transmission and reduced glutamate clearance by astrocytes that are responsible for glutamine synthesis. In line with the beneficial effect of paternal MSUS exposure⁷⁴, the offspring of MSUS-exposed mice was devoid of glutamine alterations after stress in the hippocampus.

In conclusion, we show that acute swim stress unmasks the heritable effects of early-life stress on brain metabolism. Since acute stress blunted the reduction of a neuronal health proxy, NAA concentration, in the hippocampus and prefrontal cortex in MSUS offspring only, our study demonstrates a beneficial adaptation of the stress response following paternal early-life stress.

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12.7 Authors Contributions

KG, IMM and JMND designed the study. KG and GvS performed MSUS experiments. AC and JMND performed MRS experiments. JMND analysed MRS data. All authors interpreted results and contributed to writing the final manuscript.

12.8 Conflict of Interest Statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

13 Annex 2: Alterations in sperm long RNA contribute to the epigenetic inheritance of the effects of postnatal trauma

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13.1 Abstract

Psychiatric diseases have a strong heritable component known to not be restricted to DNA sequence-based genetic inheritance alone but to also involve epigenetic factors in germ cells^{48,189}. Initial evidence suggested that sperm RNA is causally linked^{48,189} to the transmission of symptoms induced by traumatic experiences. Here we show that alterations in long RNA in sperm contribute to the inheritance of specific trauma symptoms. Injection of long RNA fraction from sperm of males exposed to postnatal trauma recapitulates the effects on food intake, glucose response to insulin and risk-taking in adulthood whereas the small RNA fraction alters body weight and behavioral despair. Alterations in long RNA are maintained after fertilization, suggesting a direct link between sperm and embryo RNA.

13.2 Introduction

Adverse experiences can have long-lasting transgenerational effects on mental and physical health, and often increase disease risk^{278,279}. Traumatic stress in early-life in particular, can induce pathologies like psychosis, depression and metabolic dysfunctions in adulthood across generations²⁸⁰. To examine the biological factors involved, we recapitulated heritable behavioural and metabolic effects of postnatal trauma across several generations using a previously established model of unpredictable maternal separation combined with unpredictable maternal stress (MSUS) in the mouse, that shows symptoms through up to three generations (Fig.1)^{11,19,48,49,74,103–105}. We have shown that such postnatal trauma alters small RNA in sperm and that injection of total sperm RNA from exposed male mice into naïve fertilized oocytes elicits symptoms reminiscent of those observed in natural offspring of exposed fathers⁴⁸. Other studies have demonstrated that adult stress^{63,188} and environmental insults like altered diet or vinclozolin exposure, or positive factors such as exercise or environmental enrichment can affect small RNA in sperm^{89,92,188,281–283} and somatic tissues²⁸⁴ in the offspring. Recently, tRNA fragments and their modifications were also found to be affected by nutritional insult, and unmodified or modified sperm small RNA injected into fertilized oocytes could mimic metabolic changes resulting

from altered parental diet in the progeny^{12,78,92}. These studies therefore suggest that small RNA in sperm can be carrier of heritable information. Here we sought to determine whether long RNA in sperm also contributes to the transmission of the effects of previous exposure.

13.3 Methods

13.3.1 Mice

C57BL/6J mice were housed in a temperature and humidity-controlled facility under a reverse light-dark cycle, and food and water were provided *ad libitum*. Experimental procedures were performed during the animals' active cycle. All experiments were approved by the cantonal veterinary office, Zurich (license 55/12 then 57/15).

13.3.2 MSUS paradigm

C57BL/6J primiparous females and males were mated at 2.5-3 months of age. Randomly selected dams and litters were subjected to daily 3h proximal unpredictable separation combined with unpredictable maternal stress (MSUS) from postnatal day 1 to 14 as described previously¹¹ or left undisturbed (Controls). Cage changes took place once a week until weaning (postnatal day 21) for both MSUS and control animals. At weaning, pups were assigned to social groups (4-5 mice per cage) of the same treatment and sex, but from different dams to prevent litter effects.

13.3.3 Sperm collection

Males used for sperm collection did not undergo any behavioural or metabolic testing and were approximately 3 months old at the time of sampling. For sperm RNA sequencing, mature sperm cells were collected from cauda epididymis and purified by counterflow centrifugal elutriation using a Beckman JE-5.0 elutriation rotor in a Sanderson chamber and a Beckman Avanti J-26 XPI elutriation centrifuge as described previously⁴⁸. Purity of samples was confirmed by inspecting eluted sperm cells under a light microscope. For RNA injection experiments, sperm cells were separated from potential somatic contamination by swim-up²⁸⁵.

13.3.4 Zygotes collection

C57Bl/6J primiparous females underwent superovulation consisting of intraperitoneal injection of 5 IU pregnant mare serum gonadotropin followed by 5 IU human chorionic gonadotrophin 48 h later. Females were then left to mate with either an age-matched MSUS or control male. After plug detection, zygotes were scraped out of uterine horn and collected directly into Trizol LS solution.

13.3.5 RNA extraction

Total RNA was prepared from adult mouse sperm using a standard Trizol protocol. Total RNA was prepared from zygotes and 4-cell embryos using the Trizol LS protocol. The quantity and quality of RNA were determined by Agilent 2100 Bioanalyser (Agilent Technologies) and Qubit fluorometer (Life Technologies).

13.3.6 RNA size separation

500 ng sperm RNA pooled from 4 control or 4 MSUS males each were subjected to Agencourt AMPure XP (Beckman Coulter) following the manufacturer's instructions. Total RNA contained in 50 ul water was mixed with 125ul AMPure XP bead solution, incubated for 5 minutes at room temperature then placed on a magnetic stand to allow magnetic beads to bind long RNA. When the solution appeared clear, the supernatant containing small RNA was aspirated and saved for later processing. Beads with bound long RNA were washed twice with 200ul of 70% EtOH. Long RNA was eluted by addition of 100 ul elution buffer. Samples were placed again on the magnetic stand to capture magnetic beads while aspirating the supernatant containing long RNA and transferring it to a new tube for subsequent processing. Small and long RNA fractions were subjected to EtOH precipitation for purification. Efficiency of size separation was determined using Agilent 2100 Bioanalyzer (Supplementary Figure 2a).

13.3.7 RNA fragmentation

130 ul of long RNA in Tris-HCL, pH 8.0 buffer was transferred into Crimp cap Micro tubes and subsequently fragmented using a Covaris Ultrasonicator

E210 containing an AFA intensifier using the following settings: time: 450sec, 175 Watt, 200 cycles/burst, intensity 5, duty cycle 10%, temperature 4-8°C, Power mode: frequency sweeping, degassing mode: continuous, water lever: 6. Successful fragmentation was determined using Agilent 2100 Bioanalyzer (Supplementary Figure 2b).

13.3.8 RNA injection in fertilized oocytes and embryo culture

Control fertilized oocytes were collected from C57BL/6J females (Janvier, France) after superovulation (intraperitoneal injection of 5 IU pregnant mare serum gonadotropin followed by 5 IU human chorionic gonadotrophin 48h later), and mating with C57BL/6J males. 1-2 pl of 0.5 ng/µl solution of small or long RNA isolated and pooled from sperm from of 4 adult MSUS or control males dissolved in 0.5 mM Tris-HCl, pH 8.0, 5 µM EDTA were microinjected into the male pronucleus of fertilized oocytes using a standard microscope and DNA microinjection method ²⁸⁶. Injection of fragmented long RNA from sperm used the same protocol and the same samples but fragmentation. This amount of injected RNA corresponds to the amount of sperm RNA estimated to be delivered to the oocyte by a sperm cell ²⁸⁷. The experimenter was blind to treatment (double blinding method), and injections were balanced across groups and were all performed between 2 and 4 pm. For molecular analysis at the 4-cell stage, embryos were placed in KSOM medium (EmbryoMax Powdered Media Kit, Millipore Cat# MR-020P-5F) supplemented with essential amino acids (Millipore) and cultured for 48h in 5% CO₂ at 37C°.

13.3.9 Behavioural testing

The experimenter was double-blinded to treatment, meaning the group assignment was coded twice prior to experiments, once by the experimenter preparing RNA samples and once by the experimenter performing RNA injections. Behaviours were videotaped and scored both manually and automatically by tracking software (Viewpoint). All behavioural tests were conducted in adult age-matched male animals.

13.3.9.1 *Elevated plus maze*

The elevated plus maze consisted of a platform with two open arms (no walls) and two closed arms (with walls) (dark gray PVC, 30x5 cm) elevated 60 cm above the floor. Mice were placed in the center of the platform facing a closed arm, and tracked for 5 minutes. The latency to enter an open arm was manually scored and total distance moved was automatically recorded by a videotracking system.

13.3.9.2 *Light-dark box*

Each mouse was placed in the lit compartment (white walls, 130 lux) of a box (40x42x26 cm) split into two unequal compartments (2/3 lit, 1/3 dark compartment with black walls and covered by a black lid and by a divider with a central opening (5x5 cm). Mice could freely move from the lit to the dark compartment during a 10-min period. Time spent in each compartment and latency to enter the dark compartment were measured manually.

13.3.9.3 *Forced swim test*

Mice were placed in a beaker of cold water (18+/-1°C) for 6 min. Floating duration was scored manually.

13.3.10 Metabolic testing

Experimenters were double-blinded to treatment, meaning both the experimenter conducting RNA injections and the experimenter conducting phenotyping on resulting offspring was not aware of groups assignment. All metabolic tests were conducted in adult age-matched males.

13.3.10.1 *Caloric intake measurement*

The amount of consumed food was measured for each cage in 4 months old animals every 24h. Caloric intake was calculated as the mean amount of food intake over 72h in relation to mean body weight (caloric intake = mean food intake/mean body weight).

13.3.10.2 *Glucose (GTT) and insulin (ITT) tolerance test*

Mice were fasted for 5h. For GTT, glucose was measured in blood at baseline and 0, 15, 30 and 90 min after intraperitoneal injection of 2 mg per g body

weight of glucose in sterile 0.45% (wt/vol) saline (injection started at 2 pm). For ITT, glucose was measured in blood at baseline, and 0, 15, 30, 90 and 120 min after intraperitoneal injection of 1 mU per g body weight of insulin (NovoRapid Novo Nordisk A/S) in sterile 0.9% saline (injection started at 2 pm). If blood glucose decreased below 1.7 mM/ml, it was rescued by intraperitoneal injection of 2 mg/g of glucose. For both GTT and ITT, glucose level was determined in fresh tail blood using an Accu-Chek Aviva device (Roche).

13.3.11 RNA sequencing (RNAseq)

Sequencing was done using an Illumina Genome Analyzer HiSeq 2500 (Illumina) in Rapid run mode for 51 cycles plus 7 cycles to read the indices in two separate runs (run 1 consisted of libraries representing biological replicates of control sperm RNA each pooled from 5 males and libraries representing biological replicates of sperm RNA from males exposed to MSUS, each pooled from 5 males. Run 2 consisted of technical replicates of run 1 plus one additional sample representing sperm RNA from 5 males exposed to MSUS. RNA libraries for first sequencing run were prepared with the NEBNext Ultra Directional RNA Library Prep Kit (NEB#E7420S, New England BioLabs Inc.) following the manufacturer's recommendations. Libraries for the second run were prepared using the TruSeq Stranded Total RNA kit according to the manufacturer's instructions with indices diluted at 1:3. 220 ng of total sperm RNA was subjected to removal of rRNA using Ribozero (first run) and Ribozero gold kit (second run). Efficiency of Ribozero treatment was validated by Agilent 2100 Bioanalyzer. An average of 6.4 ng of zygotic RNA and 3.7 ng of 4-cell embryo RNA per sample were not subjected to ribosomal depletion but immediately used for first strand synthesis. After demultiplexing and adaptor removal, an average of 60,948,122 pass filter reads was obtained in each sperm library and 43,346,362,5 in the zygotes libraries.

13.3.12 Bioinformatic analysis

RNAseq reads were trimmed of adapter sequences on the 3' end using cutadapt v1.14 with a 5% error rare and using adapter sequences of the

Truseq universal adapter, the Truseq indexed adapters (with sample-specific barcodes) and their reverse complement. Reads with a length of less than 15bp were discarded. Samples were then directly quantified using Salmon v0.9.1¹⁴⁹ on an index created from the GRCm38 genome using, as transcripts: 1) GENCODE vM16 features, 2) transposable elements from repeat masker (concatenating stranded features associated to the same family of elements), and 3) piRNA precursors¹⁵¹. Given the weak strand bias (roughly 65/35) of the libraries, they were treated as unstranded. RNA composition estimates (Supplementary Figure 4) were obtained by summing the reads of transcripts belonging to the same RNA biotype and dividing by the total number of assigned reads in the sample. For all downstream analyses, transcript counts were then summed by gene symbol. Expression profiles during spermatogenesis and from Sertoli cells were obtained from the Gene Expression Omnibus (respectively GSE100964 and GSM1069639)^{288,289} and quantified as indicated above. Library type was specified as respectively SF (strand-specific reads coming from the forward strand) and ISR (inward strand-specific reads coming from the reverse strand). For differential expression analysis, only lincRNAs and protein-coding genes that had at least 20 reads in at least 3 samples were tested. Differential expression analysis was performed with edgeR, using the exact test for zygotes and 4-cell embryos, while for sperm, generalized linear models were used to account for the technical differences between batches (~batch+condition). Given the presence of technical replicates in the sperm dataset, we performed two additional control analyses showing that largely the same results could be obtained by either, i) using only the most recent batch (Supplementary Figure 7a), or ii) accounting for the incomplete independence of the samples as described in²⁹⁰ (Supplementary Figure 7b). Gene ontology enrichment analysis was performed using the goseq R package²⁹¹ (Fisher's exact test) to account for the length bias in RNAseq experiments, using GO terms with 10 to 1000 annotated genes. Only genes with existing GO annotations were used, and for enrichments analysis performed on differentially expressed genes, only tested genes (i.e. after the aforementioned count filtering) were used as background. When indicated, most specific enrichments were obtained by removing from the results, terms

that had significantly enriched related sub-terms. For heatmaps and PCA, log(1+normalized counts) were used. Small RNAseq libraries were pre-processed with cutadapt to remove adapters. Subsequently, only reads ending in CCA-3' were selected for enrichment analysis of tRNA-derived fragments. CCA-3' was trimmed off, and reads were quantified using Salmon. For this, an index of tRNA sequences was constructed using the mouse tRNA sequences from GtRNAdb²⁹² with parameters “--perfectHash --kmerLen 15”. Differential expression analysis was performed using DESeq2 and tximport^{293,294}. tRNA gene loci were grouped into genes by trimming off the trailing number from the GtRNAdb annotation names, and tRNA with pseudo-counts equal to 0 in all replicates were removed from the analysis prior to running DESeq2 with default parameters.

13.3.13 Statistical analyses

Samples size was estimated based on previous work on the MSUS model^{11,19,48,49,74,105}. Two-tailed Student *t* tests were used to assess statistical significance for behavioural, body weight, caloric intake and GTT measurements. Chi-square test for 2 binomial populations with Yates continuity correction was used to analyze the proportion of animals rescued on the ITT before 90 minutes post-injection. When data did not match the requirements for parametric statistical tests (normal distribution), Mann Whitney U test was applied. If variance was not homogenous between groups, adjusted *P* value, *t* value and degree of freedom (Welch correction) were determined in parametric tests. Data were screened for outliers using prism’s ROUT test (for total distance moved on the elevated plus maze, time spent in bright on light-dark box, time spent floating on forced swim test, weight, food intake and glucose response) and identified animals were excluded from analysis. All statistics of behavioural and metabolic tests were computed with Prism and SPSS. All reported replicates were biological replicates, or pooled samples from biological replicates in the case of sequencing samples and RNA injection samples. Significance was set at *p* < 0.05 for all tests. Boxplot whiskers represents the Tukey method.

13.3.14 Data availability

Small RNAseq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5834 (sperm), E-MTAB-6589 (zygotes) and E-MTAB-6587 (4-cell embryos). All relevant data are available from the authors. Previously published datasets used for comparisons in this study are available under Gene Expression Omnibus (respectively GSM1069639, GSE100964 and GSE50132)^{48,288,289}.

13.3.15 Code availability

Code used to generate results is available from the authors upon request.

13.4 Results

13.4.1 The impact of MSUS on behaviour.

First, using the MSUS paradigm, we produced a cohort of mice exposed to traumatic stress in early postnatal life and confirmed their behavioural phenotype in adulthood described previously^{11,48} (Supplementary Figure 1). As expected, F1 MSUS adult males had increased risk-taking behaviours reflected by shorter latency to first enter an open arm on an elevated plus maze, and more time spent in the bright field of a light dark box (Figs.S1a,c) with increased total locomotor activity (Supplementary Figure 1b) but no change in latency to first enter the dark compartment of the light dark box (Supplementary Figure 1d). They also had a tendency for increased behavioural despair shown by more time spent floating on a forced swim test (Supplementary Figure 1e).

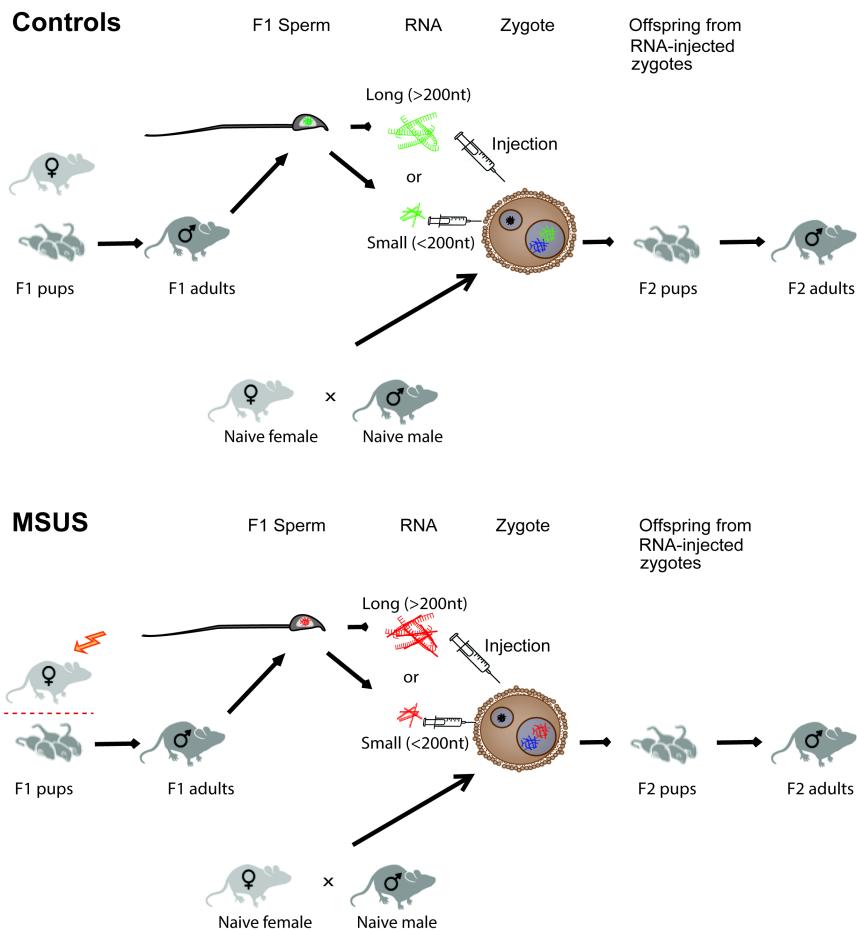


Figure 1: Generation of control and MSUS F1 males and of animals resulting from sperm RNA injections into zygotes. Between postnatal day (PND) 1 to 14, mouse pups are exposed to unpredictable maternal separation combined with unpredictable maternal stress (MSUS) while control mice are left undisturbed. When adult, sperm of F1 MSUS and control males is harvested, RNA is extracted, size selected then used for injection into naïve fertilized oocytes. Long RNA (>200 nt) includes mRNA, lncRNA, TE RNA among others. Small RNA (<200 nt) includes miRNAs, tRNA fragments, and piRNAs among others. Injected zygotes are implanted into foster mothers to produce offspring. (nt = nucleotides).

13.4.2 The effects of small versus long RNA from sperm on behaviour and metabolism

We then harvested sperm from adult control and MSUS males, extracted total RNA and fractionated the RNA into small (<200 nucleotides (nt)) and long (>200 nt) RNA using Ampure beads. Effective separation was confirmed by Bioanalyzer analysis (Supplementary Figure 2a). We then used these fractions for pronuclear injection into fertilized oocytes. Injection of the small

or long RNA fraction from MSUS sperm replicated different hallmarks of the MSUS phenotype in the resulting offspring ¹¹ (Figs.2,3, S3a,b). Injection of small or long RNA alone was insufficient to reproduce changes in risk-taking behaviors on the elevated plus maze observed in natural MSUS animals (Fig.2a) with small RNA inducing a decrease in locomotor activity inconsistent with natural MSUS offspring ⁴⁸. However, injection of long but not small RNA induced a tendency for increased time spent in the bright field of the light dark box (Fig.2b), similar to natural MSUS offspring, whereas the injection of small RNA resulted in a tendency for a decreased time spent in the bright field. The small RNA fraction mimicked increased behavioural despair on the forced swim test (Fig.2c) ⁴⁸. Thus, analysis of the behavioural phenotype in the offspring resulting from sperm RNA injections into zygotes indicate the necessity of both RNA fractions to mimic behavioural changes known to be induced by MSUS in natural offspring.

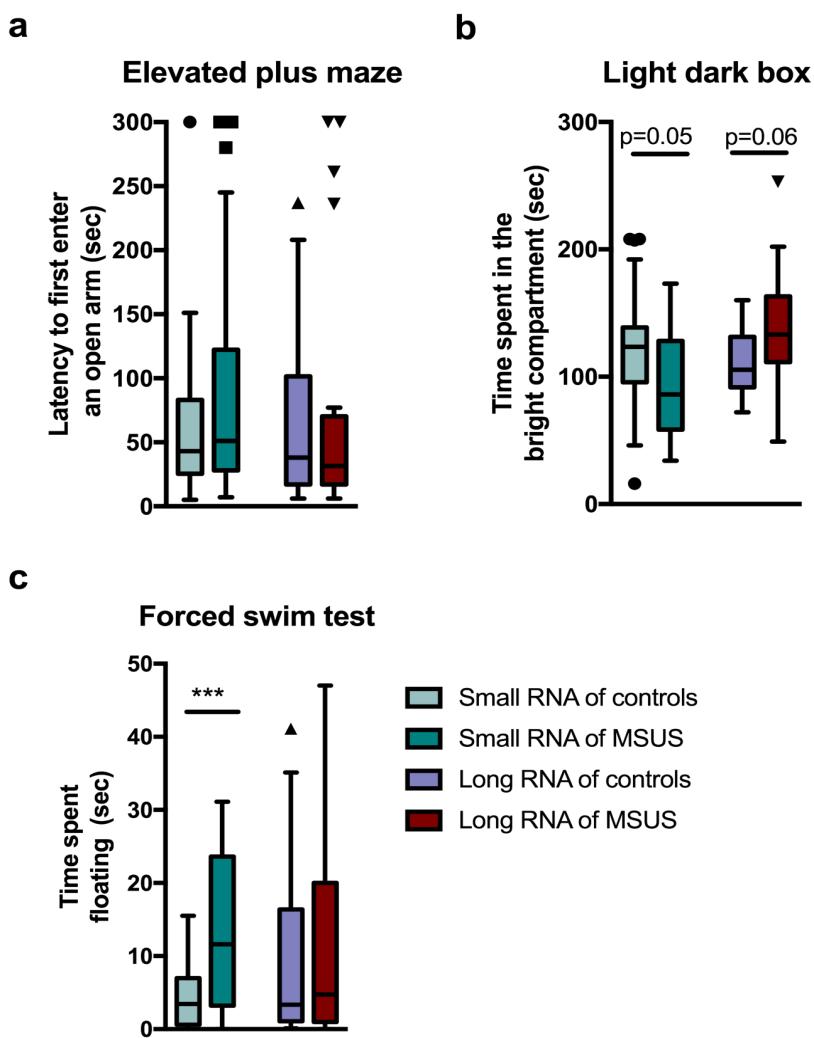


Figure 2: The long RNA fraction from sperm of males exposed to MSUS is sufficient to mimic some behavioural alterations observed in natural offspring of MSUS fathers. Injection of small or long sperm RNA of F1 MSUS males into naïve zygotes has no effect on (a) the latency to first enter an open arm on an elevated plus maze (Small RNA of controls n=25, Small RNA of MSUS n=20, Mann Whitney U=214.5, p>0.05); Long RNA of controls n=17, Long RNA of MSUS n=22, Mann Whitney U=171, p>0.05). Injection of small sperm RNA but not long sperm RNA increases (b) time spent in the bright compartment in a light dark box (Small RNA of controls n=24, Small RNA of MSUS n=17, t(39)=2.02 p=0.05; Long RNA of controls n=18, Long RNA of MSUS n=21, t(32,19)=-1.95, p>0.05) and (c) time spent floating minutes 3 to 6 on a forced swim test (Small RNA of controls n=20, Small RNA of MSUS n=19, t(22.59)=-3.84, p=0.001; Long RNA of controls n=16, Long RNA of MSUS n=22, Mann Whitney U=158, p>0.05) in the resulting male offspring in adulthood. Data are median ± whiskers. Dots, boxes and triangles: values that lie outside the sum of the 75th percentile and 1.5 x the interquartile range or the 25th

percentile minus $1.5 \times$ the interquartile range (all values included in statistical analysis). * $p<0.05$, *** $p<0.001$.

Further, injection of small RNA increased body weight (Fig.3a) contrary to that in natural MSUS offspring, which has lower body weight. Long RNA increased food consumption (Fig.3b) and the sensitivity to insulin challenge (Fig.3c), similar to natural MSUS offspring ⁴⁸. Injection of small or long RNA fraction alone was insufficient to elicit altered glucose clearance in response to a glucose challenge (Fig.3d), indicating again, that both small and long RNA together are required to mimic all aspects of the metabolic changes. Given the fact that total RNA injection does mimic alterations observed in natural MSUS offspring ⁴⁸, these results strongly suggest that alterations in both small RNA and long RNA together mediate the effects of postnatal trauma from father to offspring, while alone, they do not or can even cause different effects.

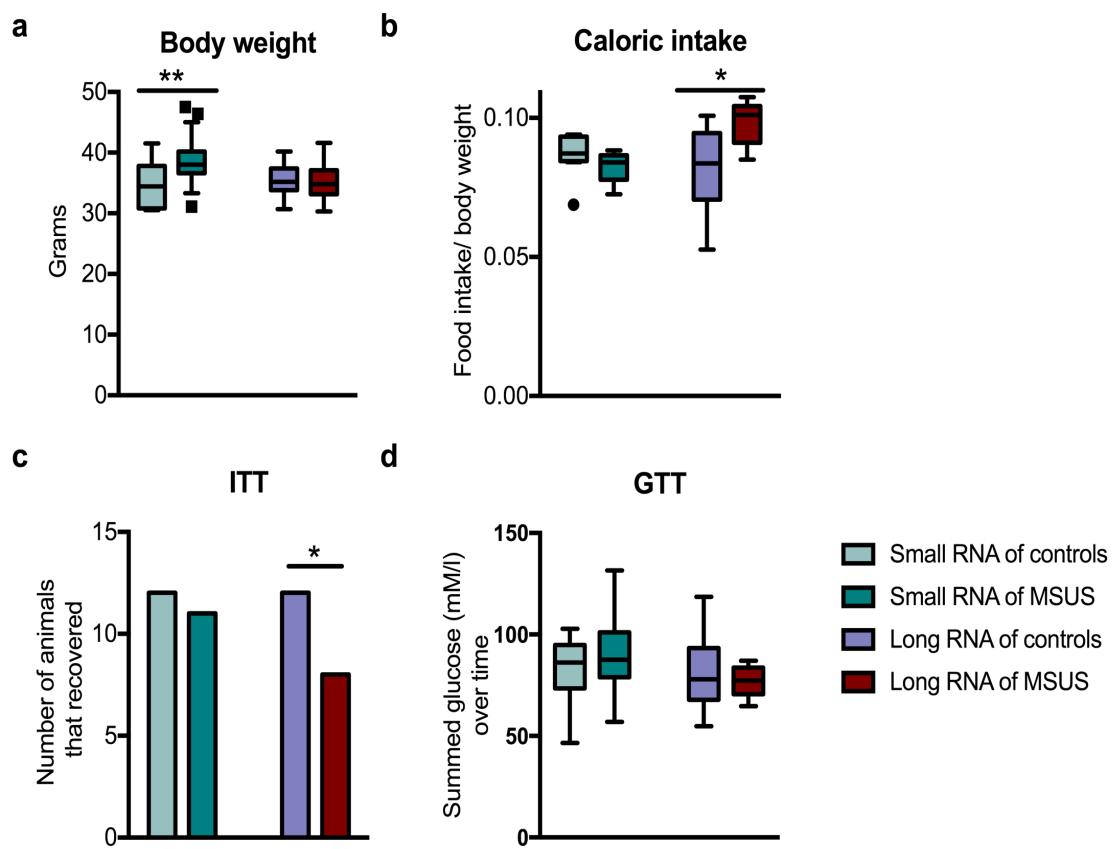


Figure 3: The long RNA fraction from sperm of males exposed to MSUS is sufficient to mimic some metabolic alterations observed in natural offspring of MSUS fathers. Injection of sperm small RNA of F1 MSUS males into naïve zygotes

(a) decreases body weight (Small RNA of controls n=16, Small RNA of MSUS n=19, $t(33)=3$, ** $p<0.01$; Long RNA of controls n=15, Long RNA of MSUS n=21, $t(34)=0.37$; $p>.05$). Injection of long RNA (b) increases average caloric intake measured over 72 hours (Small RNA of controls n=8, Small RNA of MSUS n=6, $t(12)=1.08$, $p>0.05$; Long RNA of controls n=8, Long RNA of MSUS n=6, $t(12)=2.29$; $p<0.05$) and (c) increases sensitivity to insulin after an insulin challenge (Small RNA of controls n=12, Small RNA of MSUS n=12, $\chi^2 (1, n=24)=0.6$, $p>0.05$; Long RNA of controls n=12, Long RNA of MSUS n=12, $\chi^2 (1, n=24)=2.7$, $p=0.05$). (d) Neither RNA fraction affects the glucose response after a glucose challenge (Small RNA of controls n=12, Small RNA of MSUS n=12, $t(22)=1.17$; Long RNA of controls n=12, Long RNA of MSUS n=12, $t(22)=0.77$; $p>0.05$) in the resulting male offspring when adult. Data are median \pm whiskers. Triangles: values that lie outside the 25th percentile minus 1.5 \times the interquartile range (all values included in statistical analysis). * $p \leq 0.05$, ** $p<0.01$.

13.4.3 Consequences of MSUS on sperm transcriptome

Assessment of the sperm transcriptome by next-generation sequencing revealed that long RNA accumulated in mature sperm is dramatically altered in adult males exposed to MSUS compared to control conditions. Besides ribosomal RNA, mitochondrial ribosomal RNA and repeat elements reads mapping to coding and non-coding regions could be detected, consistent with previous reports ²⁹⁵ (Figs.S4). The transcripts giving rise to the detected reads were intact and not fragmented as indicated by their distribution spanning the entire transcripts size range (Supplementary Figure 5) and the expected RNA size profile observed by bioanalyzer (Supplementary Figure 2a). Gene ontology (GO) term enrichment analysis of reads mapping to genes in control sperm revealed enrichment for RNA processing, cellular macromolecular complex assembly and chromatin organization among others (Table S1), suggesting gene and transcript regulatory functions. Principal component analysis (PCA) revealed that sperm samples from control and MSUS males segregate (Supplementary Figure 6).

Further quantitative comparison of long RNA from control and MSUS sperm showed significant differential load of several mRNAs and long intergenic non-coding RNAs (lincRNAs) (Fig.4a,b). GO term analysis revealed an enrichment for cell adhesion and extracellular matrix organization, among others (Fig.4c,

Table S2, Supplementary Figure 7). Interestingly, reads mapping to transposable elements (TEs) were dysregulated in the sperm of MSUS males (Supplementary Figure 8a). When analyzing TEs separately, we found that the relative abundance of several relatively young retro-TEs²⁹⁶ is higher in sperm of males exposed to MSUS (Supplementary Figure 8b). Whether this indeed reflects an activation of these TEs in response to MSUS remains to be determined. Consistent with the induction of some aspects of the MSUS phenotype by sperm small RNA (Fig.3c), we previously demonstrated sperm transcriptomic alterations in miRNA and piRNA⁴⁸. Surprisingly, tRNA 3' fragments were not significantly altered in MSUS sperm (Supplementary Figure 9).

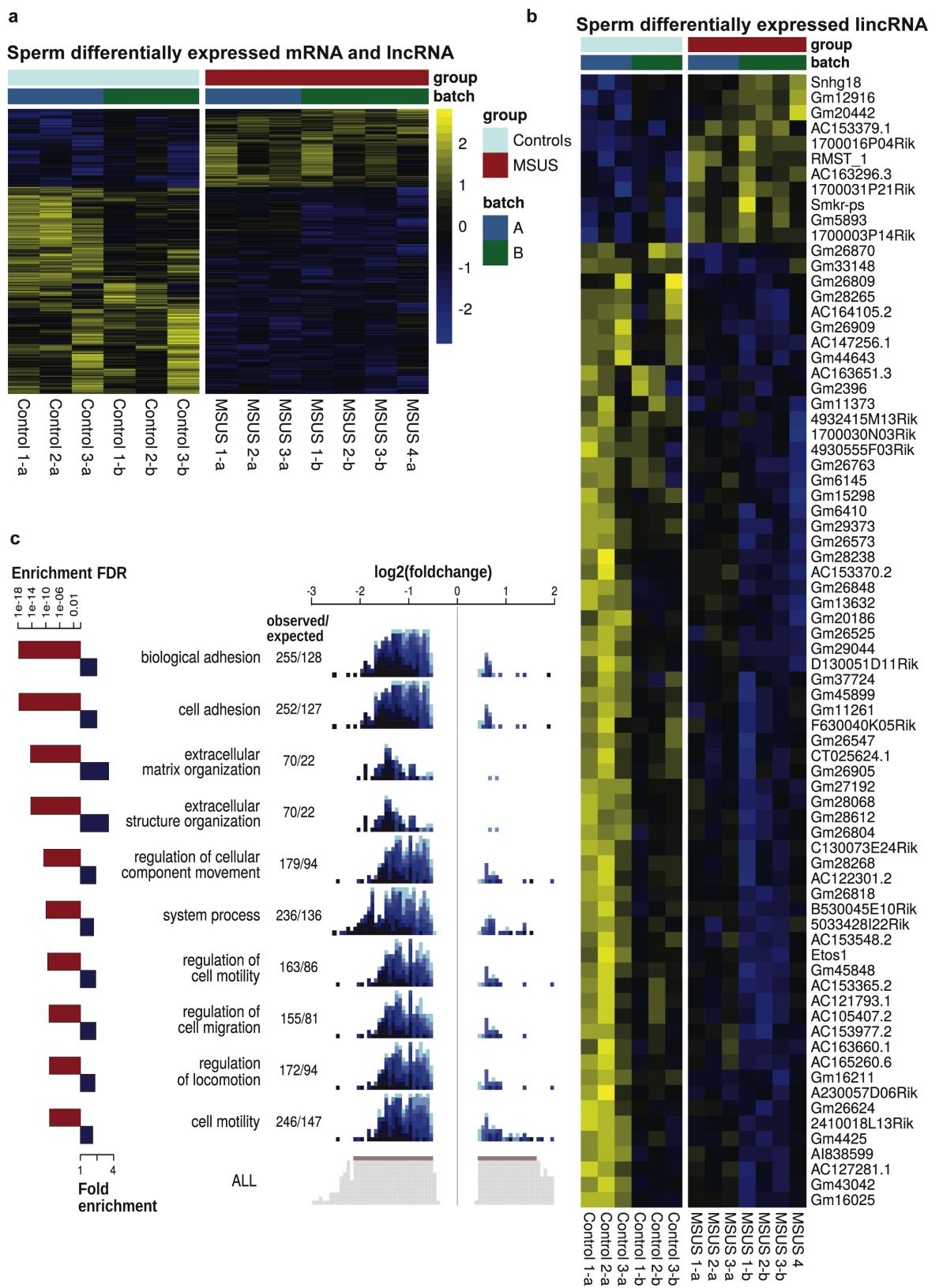


Figure 4: MSUS affects sperm long RNA composition. (a) Heatmap showing that MSUS induces significant changes in a range of protein coding and long non-coding RNA (lncRNA) in sperm. Plotted are the row z-scores of log normalized counts. (b) Heatmap showing single long intergenic non-coding (linc), RNA transcripts with

differential accumulation in sperm of MSUS and control males (controls n=4 with 2 biological replicates, MSUS n=3 with 3 biological replicates, each replicate consists of sperm RNA pooled from 5 mice; multiple testing corrected p<0.01). (c) GO term analysis reveals enrichment of differentially expressed protein coding genes in several categories in sperm RNA from MSUS males. Red bars represent p-values of each GO term enrichment after multiple comparison correction. Blue bars represent fold enrichment of each GO term. Each blue square depicts a differentially expressed sperm transcript. Shades of blue represents the significance of its differential expression. ob= observed, exp= expected. Sequencing was done once with 2 batches of different libraries (a or b).

13.4.4 The potential origin of mRNA and long non-coding RNA (lncRNA) differentially accumulated in sperm by MSUS

Based on the assumption that sperm is transcriptionally silent²⁹⁷, we investigated the potential cellular origin of the RNA altered by MSUS in adult sperm. Meta-analyses of previously published datasets^{288,289} on the transcriptome of spermatogonia, pachytene spermatocytes and round spermatids, three different spermatogenic populations that differentiate successively during spermatogenesis to give rise to sperm revealed the presence of almost all differentially expressed gene transcripts in spermatogonia, pachytene spermatocytes (all but 3 out of 1201) and in round spermatids (all but 9 out of 1201), suggesting that transcripts differentially expressed in mature sperm originate from preceding spermatogenic cells at an earlier stage of spermatogenesis (Fig.5). Consistent with a spermatogenic origin of some transcripts found to be altered by MSUS, analyses of Sertoli cells, somatic cells in testes, showed no expression of 112 genes, that were differentially expressed in MSUS sperm. Sertoli cells did not contain any of the 9 transcripts altered in MSUS sperm but not detectable in round spermatids, indicating potential uptake during epididymal transit (3 upregulated) or a decreased supply (6 downregulated) of immature sperm. For the 3 up-regulated transcripts, we cannot exclude uptake by for instance “plasma bridges”²⁹⁸ after induction of expression upon exposure to MSUS in Sertoli cells.

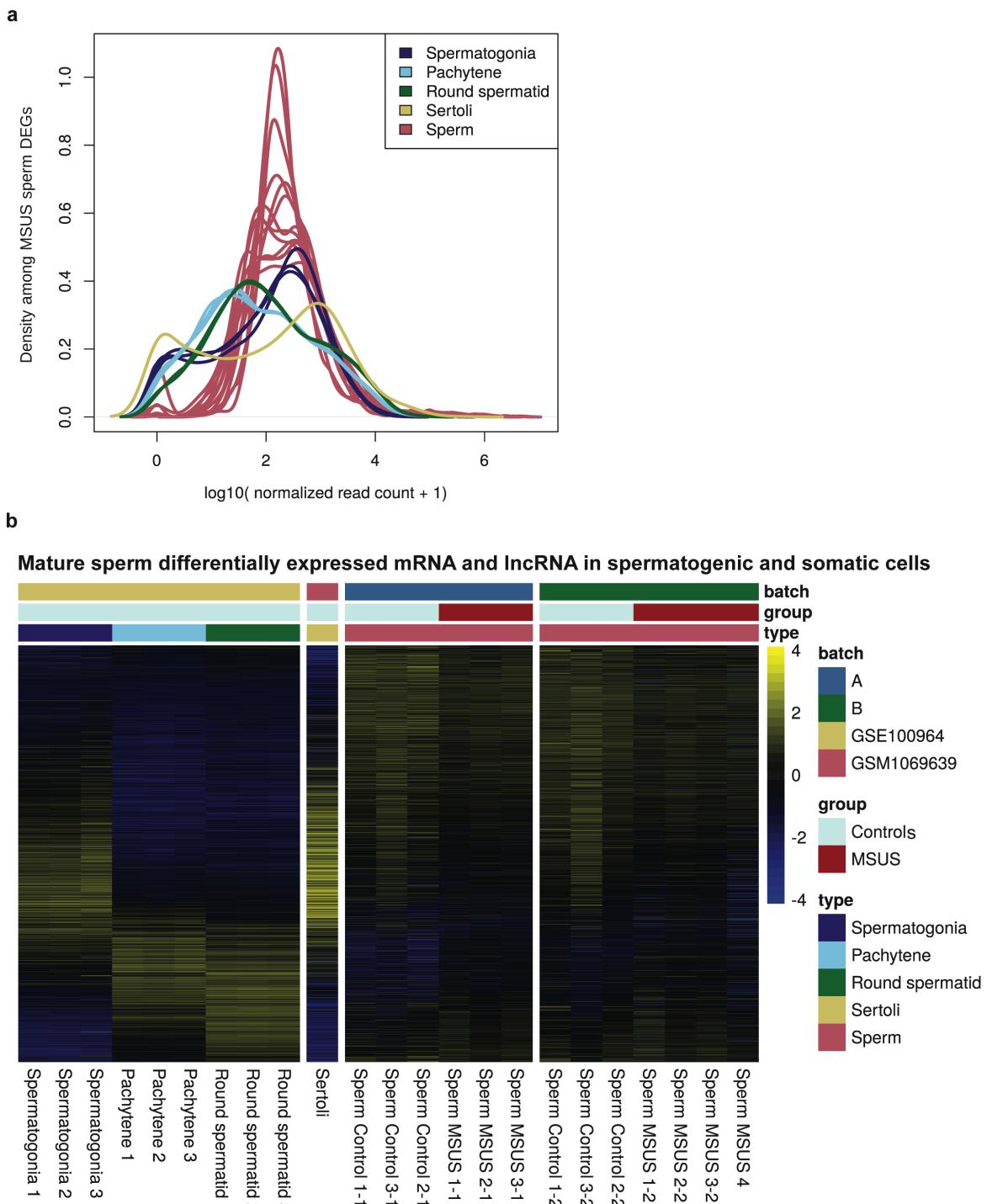


Figure 5: Expression of long RNA transcripts affected by MSUS in sperm at different developmental spermatogenic stages and in Sertoli cells. (a) Density plot shows the presence of mRNA and long non-coding RNA (lncRNA) found to be differentially accumulated in MSUS sperm in spermatogonia, pachytene spermatocytes (pachytene), round spermatids and Sertoli cells. (b) Heatmap shows single differentially accumulated mRNA and long non-coding RNA in F1 control and

MSUS sperm in comparison to spermatogonia (3 samples), pachytene spermatocytes (3 samples), round spermatids (3 samples) and Sertoli cells (1 sample) in control conditions (published data^{288,289}). Sequencing was done once with 2 batches of different libraries (a or b). Plotted are the row z-scores of log normalized counts.

13.4.5 The fate and function of mRNA and lncRNA differentially accumulated in sperm

Besides providing insight into the potential origin of altered sperm RNA, we examined the fate of these RNA after fertilization. We assessed whether RNA differentially expressed in sperm can be detected in 1-cell zygotes after mating of control females with MSUS males. Next-generation sequencing of RNA pooled from 7-10 zygotes revealed many reads mapping to protein-coding genes and long non-coding genomic regions (Supplementary Figure 4a). A majority of differentially expressed genes in MSUS sperm and zygotes had correlated fold changes in expression in zygotes (Fig.6a), suggesting a delivery of those long sperm RNA to the zygote at fertilization. Additional differentially-expressed transcripts in MSUS zygotes, suggest early downstream effects of sperm RNA (Fig.6b). GO term analysis of differentially expressed genes in zygotes showed an enrichment for genes involved in reproduction and import into cell among others (Supplementary Figure 10, Table S3).

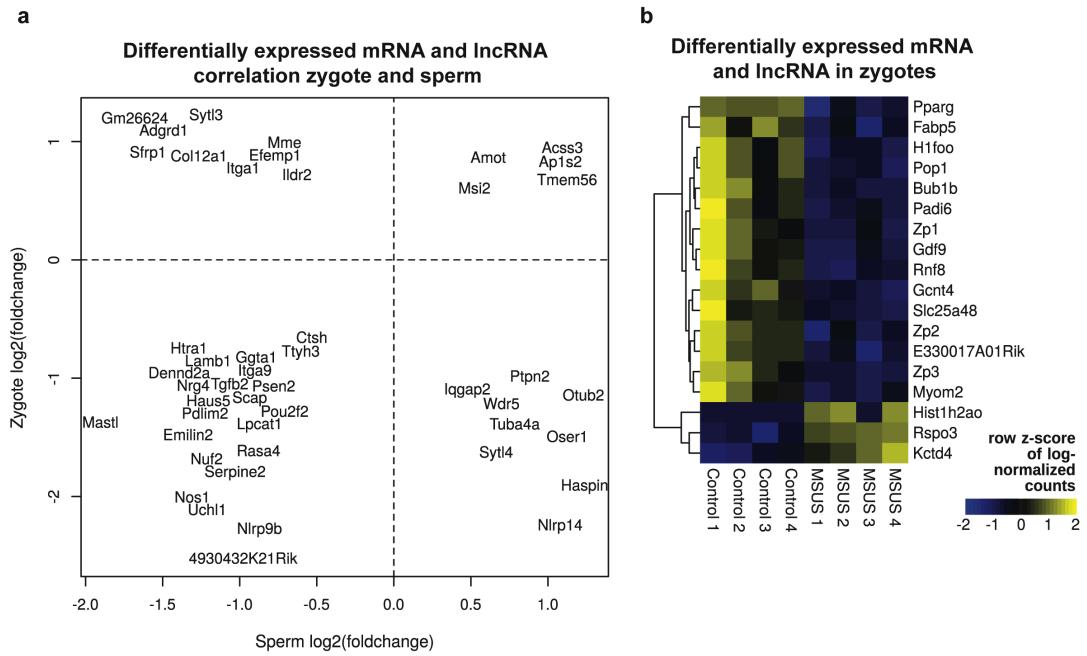


Figure 6: Detection of long RNA affected by MSUS in zygotes. (a) Scatter plot reveals correlation of 47 mRNA and long non-coding RNA (lncRNA) with a p-value less than 0.01 (multiple testing uncorrected) in zygotes resulting from mating of MSUS males with naïve females and a q-value less than 0.05 (multiple testing corrected) in MSUS sperm (overlap p~0.55). (b) Heatmap representing expression of 18 differentially expressed genes in F2 control and MSUS zygotes (multiple testing corrected, p<0.05). Each library reflects a pool of 7-10 zygotes (Control n=4 and MSUS n=4).

13.4.6 The importance of sperm long RNA integrity to induce effects in the offspring

Because long RNA may be subjected to processing or cleavage, and generate small RNA fragments that could mediate some of the effects observed, we assessed the importance of the integrity of RNA. We fragmented long RNA from control and MSUS sperm to a size of <200nt, injected these fragmented RNA into control fertilized oocytes and assessed gene expression in resulting 4-cell embryos after injection (Supplementary Figure 4). 4-cell stage was chosen over 1-cell zygotic stage to allow enough time for injected RNA to produce their effects if any. No statistically significant alteration could be identified in embryos injected with fragmented long RNA from control or MSUS sperm (Table S4). Comparison of transcripts with putative differential expression (p>0.05 but p<0.1, not corrected for multiple

comparison) in 4-cell embryos after injection of fragmented long RNA from MSUS sperm showed no changes compared to controls (Supplementary Figure 11a). Consistently, genes differentially expressed in MSUS zygotes are not dysregulated in 4-cell embryos resulting from fertilized oocytes injected with fragmented sperm long RNA (Supplementary Figure 11b). These results strongly suggest that sperm long RNA needs to be full length to induce transcriptional changes in the embryo.

13.5 Discussion

Here we show that sperm long RNA is impacted by postnatal trauma in adulthood and provide evidence that this RNA contributes to the transmission of some of the effects of trauma in the offspring. The data also show that reproducing an excess of long RNA alone or small RNA alone by injection into fertilized oocytes is not sufficient to recapitulate all symptoms in adulthood, indicating that the combination of small and long RNA (by injecting total RNA) is necessary⁴⁸ and suggesting a synergistic action of long and small RNA. However, beyond a required combined action, it is also possible that a decrease (and not just an increase as reproduced by injection) in specific small and/or long RNA is also necessary to produce some of the effects, which is not recapitulated by RNA injection. In the future, manipulations mimicking down-regulation of specific small or long RNA using for instance, the CRISPR-dCas9 technology would help address this point²⁹⁹. The results also show that MSUS effects are transmitted independently of alteration of tRNA 3' fragments in sperm contrary to that reported in dietary models (high fat or low protein diet)^{12,78,92}. These differences in the contribution of RNA might be due to the different nature and time window of interventions, which span preconception to adulthood in the case of dietary models but only a short postnatal period (P1 to P14), 3-4 months before mating and sperm collection in our model. This highlights the complexity of the mechanisms of transmission from the directly exposed animals to the first generation of offspring. Transmission in the MSUS model, is transgenerational (up to the third generation^{11,48,49,74}). More complete phenotypic and epigenetic profiling of the dietary models would help better understand the underlying

mechanisms. We speculate that a complex interplay between different sperm RNA fractions and other factors like DNA methylation, known to be altered by MSUS in adult sperm and brain across generations^{19,74,103} may contribute to transmission, with long RNA playing an important role for some symptoms e.g. metabolic. Our data also provide evidence that a subset of altered sperm RNA is important for the early embryo since it remains altered in the zygote.

A previous study in a model of paternal low protein diet suggested regulation of MERVL elements based on data showing downregulation of MERVL targets in the offspring at embryonic stage⁷⁸. Our data suggest a regulation of TEs in sperm of males exposed to MSUS, potentially indicating a common denominator across different models of epigenetic inheritance. TE expression in the brain, a highly steroidogenic tissue, is responsive to acute and chronic stress³⁰⁰. This process was suggested to involve glucocorticoid-mediated epigenetic remodeling³⁰¹. TE regulation in gametes, highly steroidogenic cells as well, might contribute to the observed changes in sperm in response to MSUS. Stress-induced regulation of TE expression can lead to transposition and mutagenesis in bacteria, fish and mice^{302–304}.

Long RNA affected by MSUS in sperm is expressed in round spermatids, suggesting that their alteration can occur earlier during spermatogenesis and persist until mature sperm. Further, some small RNAs are known to be transferred to maturing sperm from exosome-like vesicles called epididymosomes during epididymal transit³⁰⁵. Such transfer may also occur in our model, and further, RNA may be provided through yet unexplored mechanisms involving “plasma-bridges” for instance²⁹⁸. Our data further indicate that loss of RNA integrity prevents an induction of transcriptional changes in embryos associated with MSUS. The information carrier in our model is therefore different from that reported in another model of transgenerational inheritance involving c-Kit, in which truncated versions of the involved transcript could allow transmission of the phenotype⁶¹. Our study hence underscores the complex repertoire of potential signals not based on DNA sequence for intergenerational transmission. Our findings might have major implications for disease susceptibility induced by early-life experiences

and its transmission. They may also help explore the development of RNA-based approaches to prevent the molecular transfer of the effects of early trauma onto disease risk.

13.6 Acknowledgments

We thank Heiko Hörster for help with animal husbandry, Alexandra Sapetschnig for advice on sequencing library preparation and Tomás Di Domenico for advice on sequencing data analysis. This work was supported by the Austrian Academy of Sciences (fFORTE), the University of Zürich, the Swiss Federal Institute of Technology (grant: ETH-10 15-2), the Swiss National Science Foundation, Roche, Novartis Foundation, the Slack-Gyr Foundation, Cancer Research UK (C13474/A18583, C6946/A14492) and Wellcome (104640/Z/14/Z, 092096/Z/10/Z).

13.7 Author contributions

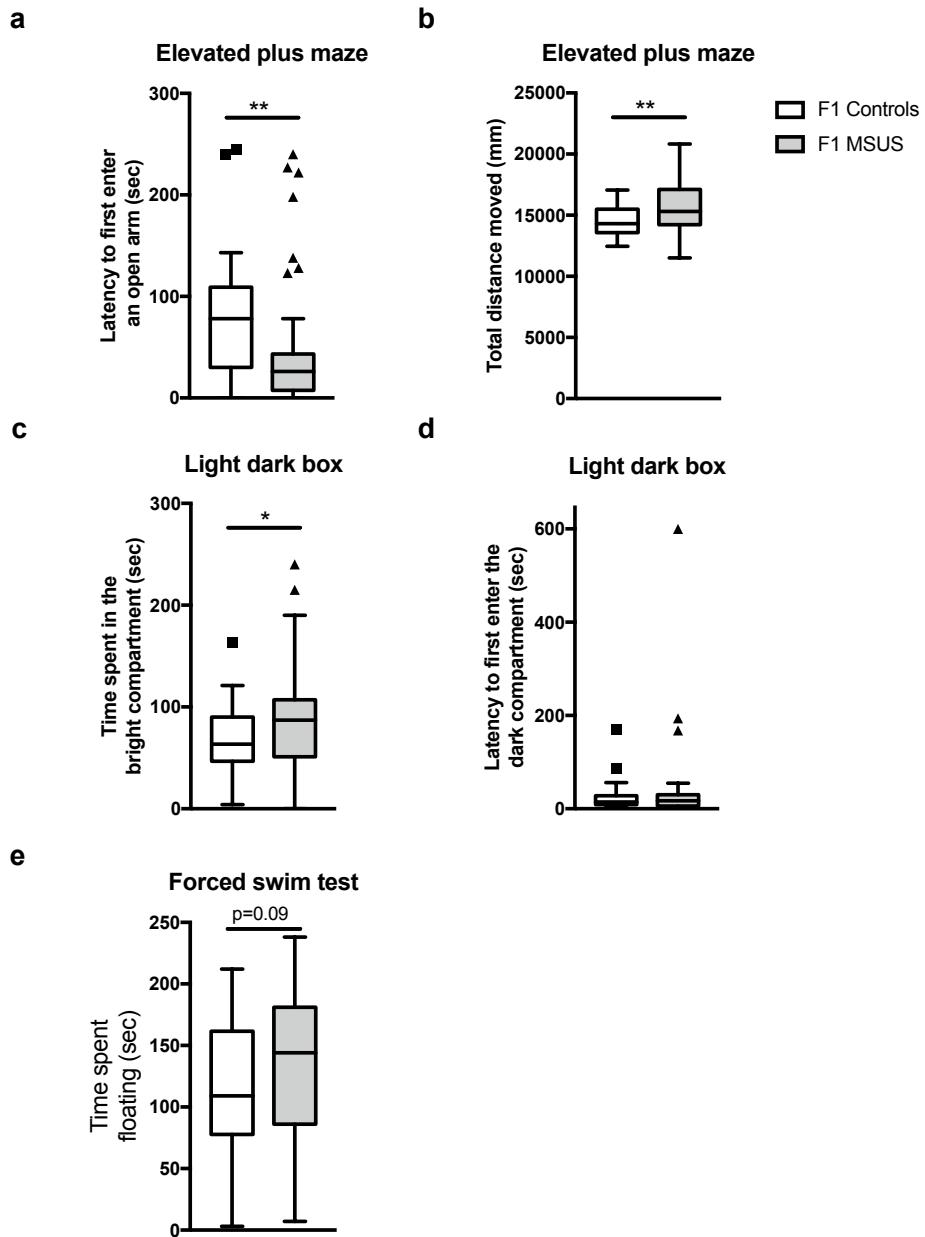
K.G. carried out MSUS paradigm, metabolic measurements, sperm RNA preparation for sequencing libraries and for RNA injection into fertilized oocytes, zygote collection and sequencing library preparation. G. vS. assisted with metabolic tests and carried out MSUS paradigm with F.M.. F.M. did behavioural testing. P.L.G, K.R., W.M. and T.G. performed RNA sequencing analyses. P.P. carried out the RNA microinjection experiments. G.V. helped with sequencing library preparation. M.R. helped with sequencing results quality control. K.G., I.M.M. and E.A.M. designed the study, interpreted the results and wrote the manuscript. I.M.M. and E.A.M. raised funds to support the project.

13.8 Conflict of interests

The authors declare no conflicts of interests.

13.9 Supplementary information

Supplementary figure 1

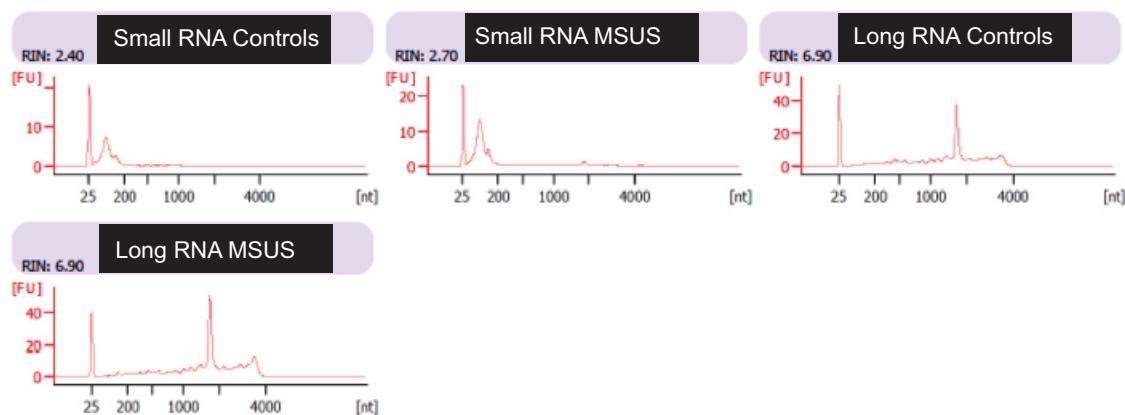


Effect of MSUS on behavior. F1 males exposed to MSUS (a) have significantly reduced latency to first enter an open arm on an elevated plus maze (Mann–Whitney U=437.5, F1 controls n=31, F1 MSUS n=46, p<0.01) and (b) covered a longer distance on the maze (Mann–Whitney U = 446, F1 controls n=31, F1 MSUS n=46, p<0.01. (c) They spent significantly more time in the bright compartment of a light dark box (F1 controls n=34, F1 MSUS n=43, t(74,14)=−2.01, p<0.05) independent of (d) their latency to first enter the dark compartment (Mann–Whitney U=681, F1 controls n=34, F1 MSUS n=43,

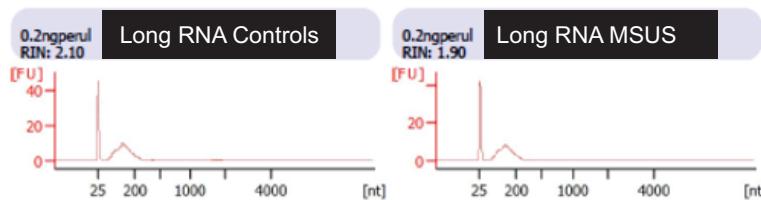
$p>0.05$). They also (e) showed a tendency to spent significantly more time floating on the forced swim test (F1 controls $n=36$, F1 MSUS $n=47$, $t(1.69)=81$ $p=0.09$). Data are median \pm whiskers. * $p<0.05$, ** $p<0.01$, t-test or Mann-Whitney test, as appropriate. Boxes and triangles: values that lie outside the sum of the 75th percentile and 1.5 \times the interquartile range or the 25th percentile minus 1.5 \times the interquartile range (all values included in statistical analysis).

Supplementary figure 2

a



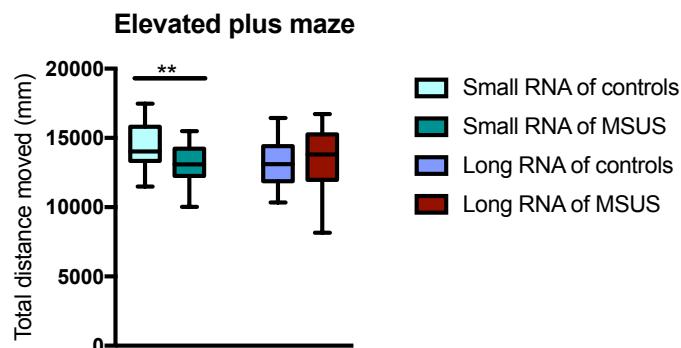
b



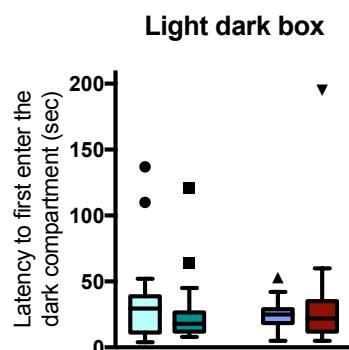
Size distribution of sperm RNA used for injections into fertilized oocytes. (a) Bioanalyzer profiles of size-selected small (<200 nt) and long (>200 nt) RNA purified from sperm samples, each being a pool from 4 males/group, showing well-segregated size populations for both control (co) and MSUS (MS) sperm RNA. (b) Bioanalyzer profiles of size-selected long RNA after fragmentation showing a clear shift of size populations to values below 200 nt for both control and MSUS sperm RNA.

Supplementary figure 3

a

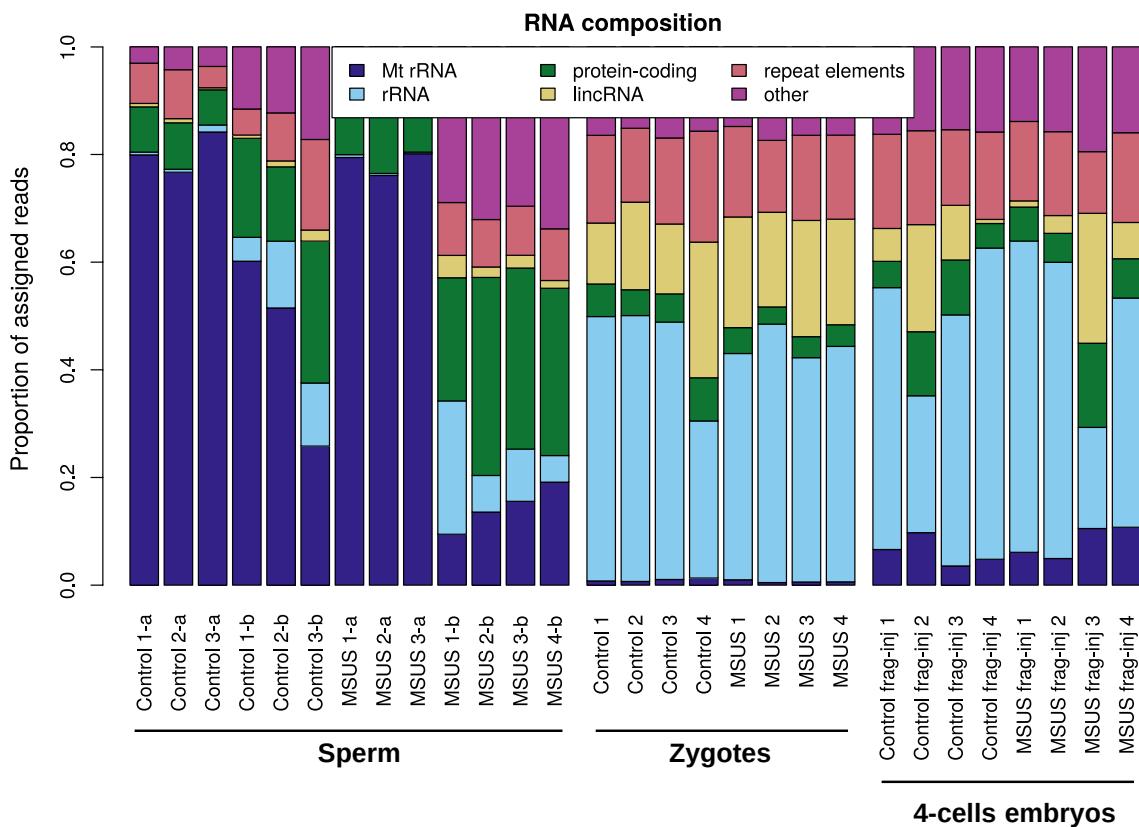


b



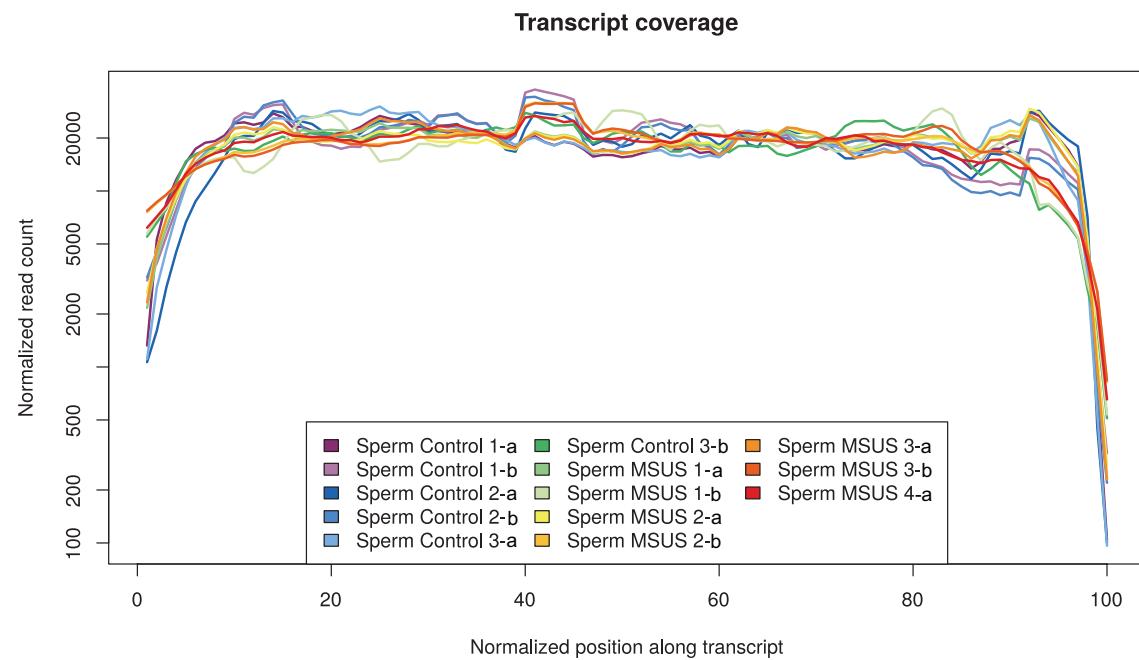
Effects of the injection of sperm long and small RNA into zygotes on behavior of the resulting animals when adult. Injection of small RNA (<200 nt) of F1 MSUS males into naïve zygotes affects (a) the total distance moved on an elevated plus maze (Small RNA of controls n=25, small RNA of MSUS n=20, $t(43)=2.96$, $**p<0.01$; Long RNA of controls n=17, Long RNA of MSUS n=22, $t(37)=0.48$, $p>0.05$) but (b) not the latency to first enter the dark compartment of a light dark box (Small RNA of controls n=24, small RNA of MSUS n=17, Mann Whitney U=182; $p>0.05$; Long RNA of controls n=18, Long RNA of MSUS n=21, Mann Whitney U=171.5 $p>0.05$) in the resulting male offspring when adult. Data are median \pm whiskers. Dots, boxes, triangles and circles: values that lie outside the sum of the 75th percentile and 1.5x the interquartile range (all values included in statistical analysis).

Supplementary figure 4



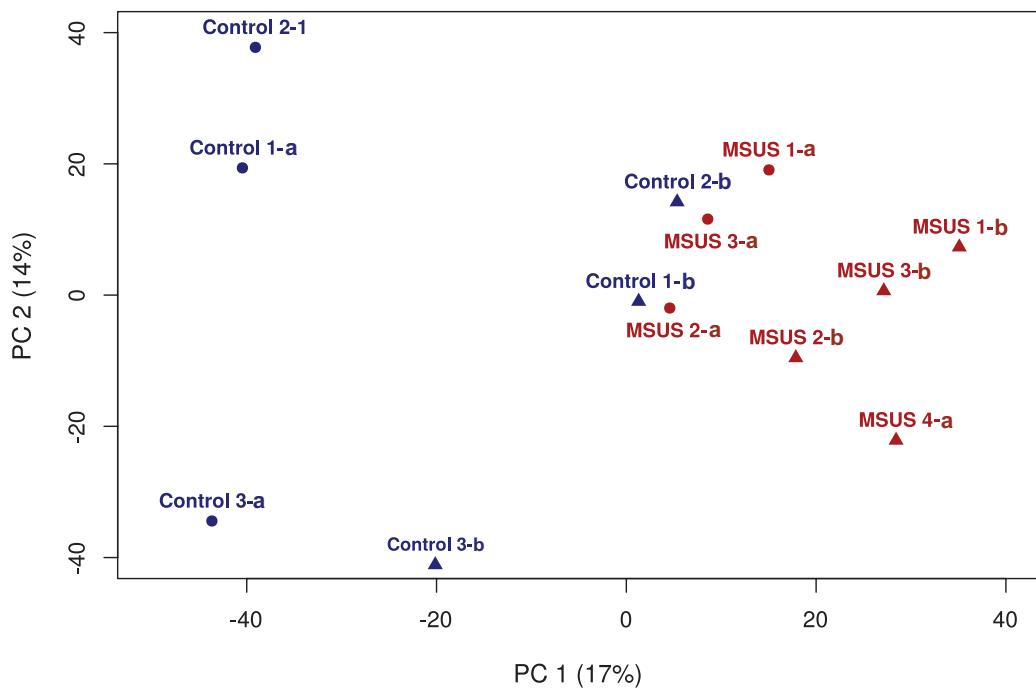
RNA composition revealed by next generation sequencing in sperm, zygotes and 4-cell embryos. Transcriptome of sperm and zygotes of control and MSUS males, zygotes resulting from mating of control or MSUS males with naïve control females, and 4-cell embryos resulting from the injection of fragmented long RNA from sperm of control or MSUS males into naïve control zygotes reveals variable levels of transcripts mapping to mitochondrial ribosomal RNA (Mt rRNA), ribosomal RNA (rRNA), protein coding RNA (protein-coding), long intergenic non-coding RNA (lincRNA), repeat elements (repeat elements) and others. Sperm libraries first batch: a; second batch: b are technical replicates.

Supplementary figure 5



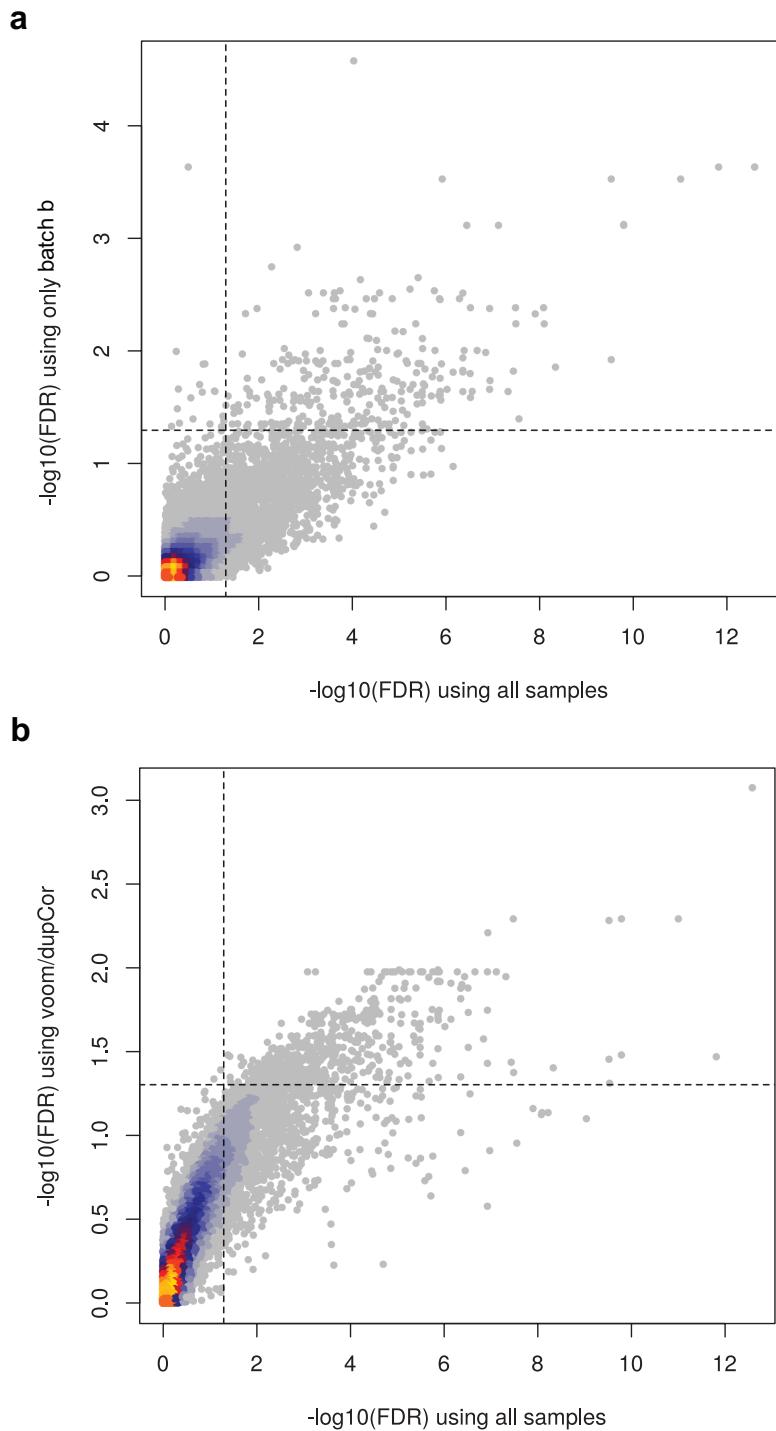
Integrity of sperm long RNA transcripts. Overall mapping of sequencing reads across normalized length of long sperm transcripts shows consistent coverage across all exons.

Supplementary figure 6



Principal component (PC) analysis of next-generation sequencing experiment. Reads mapping to any class of long RNA shows clustering of RNA populations according to treatment (Control or MSUS) (principal component 1). Sperm libraries first batch: a; second batch: b are technical replicates.

Supplementary figure 7

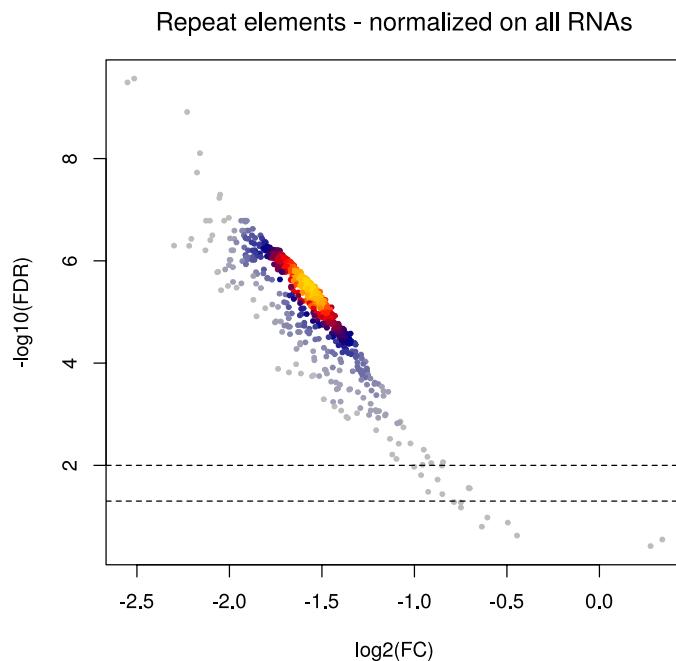


Assessment of the impact on MSUS sperm differential expression of different ways to analyze the technical replicates. While the different libraries created from each sample do not represent full biological replicates, it helps to estimate part of the technical variability, and we therefore compared

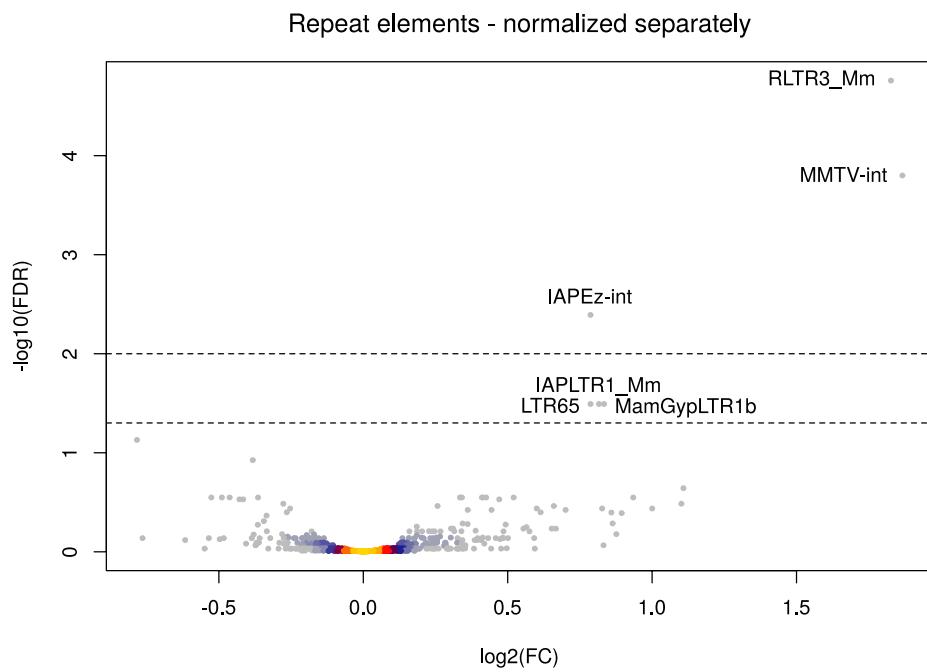
the robustness of the main results when trying different approaches to account for this. Each dot represents a gene tested for differential expression, colored according to the density of points. The x-axis indicates the FDR when using all samples in the standard fashion (as done for Figure 4 and described in the methods), while the y-axes indicate the corresponding FDRs when using only the second set of libraries (panel a), or when using a random effect variable to account for the nested replication model (panel b; the exact model used was $\sim\text{batch}+(1|\text{sample})+\text{group}$). Both approaches correlate well with the standard analysis used in Figure 4.

Supplementary figure 8

a



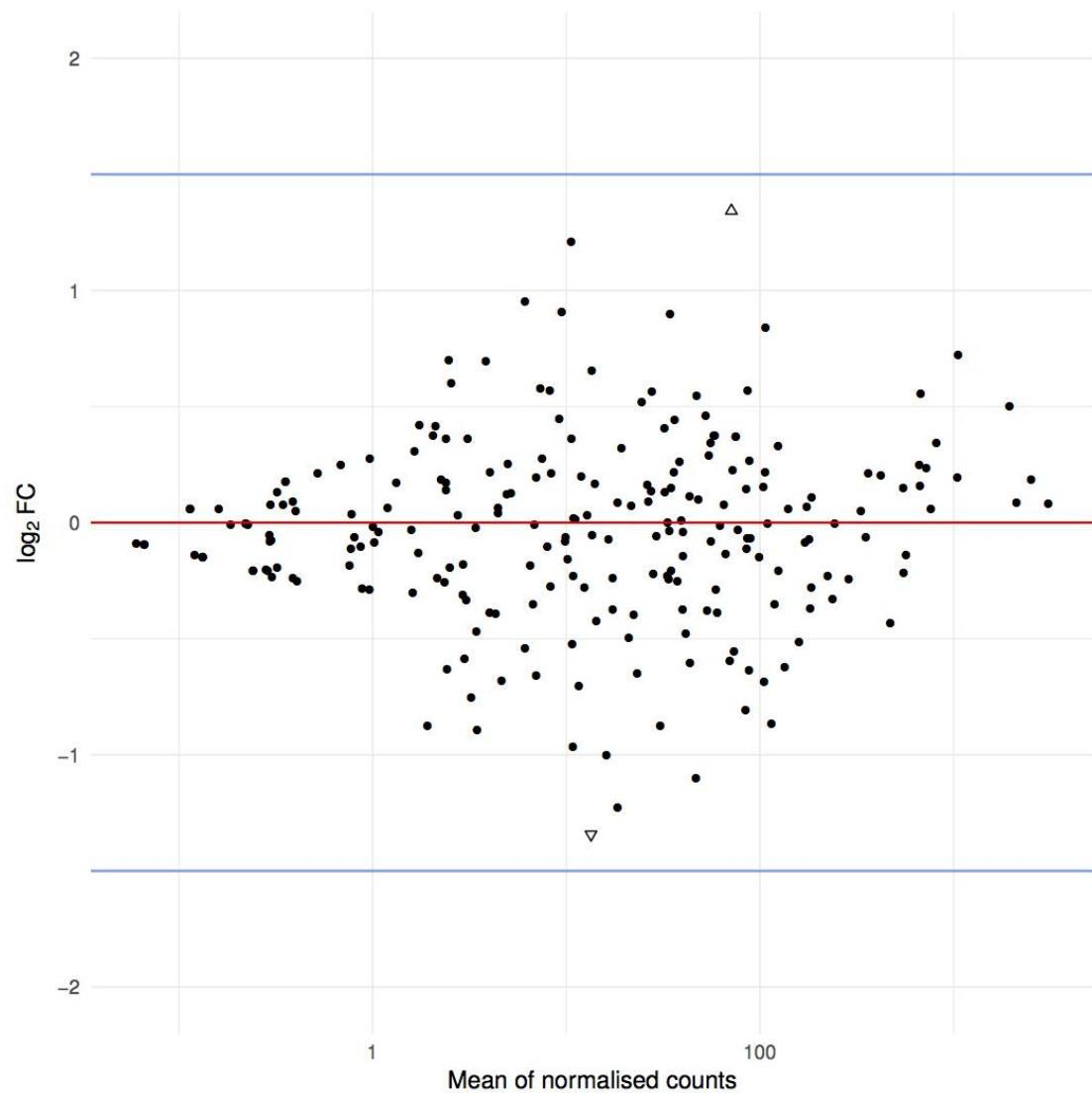
b



Differential expression of transposable elements (TEs) in sperm of control and MSUS animals. (a) When normalized to all transcripts, the vast majority of TEs appear downregulated in MSUS (only TEs are plotted). (b)

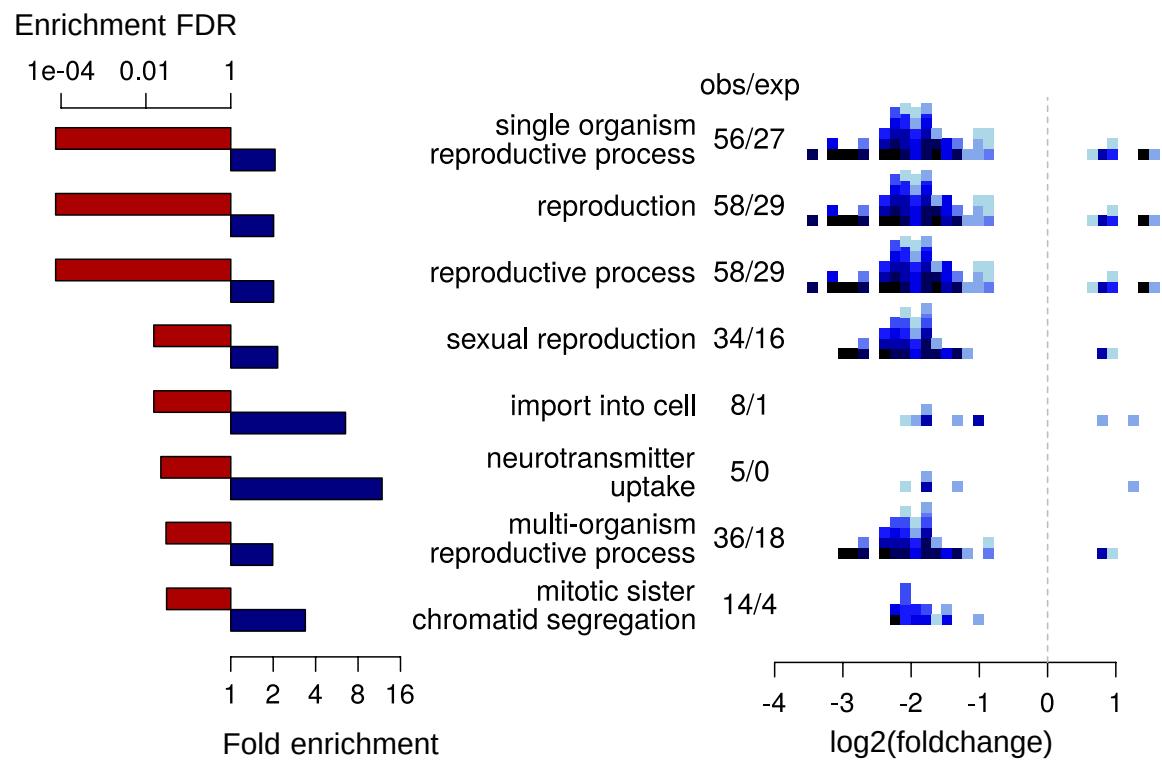
When TEs are normalized separately from other RNA types (assuming that the global difference in TEs is technical, and that most TEs are in similar amount in the two groups), some TEs instead are upregulated.

Supplementary figure 9



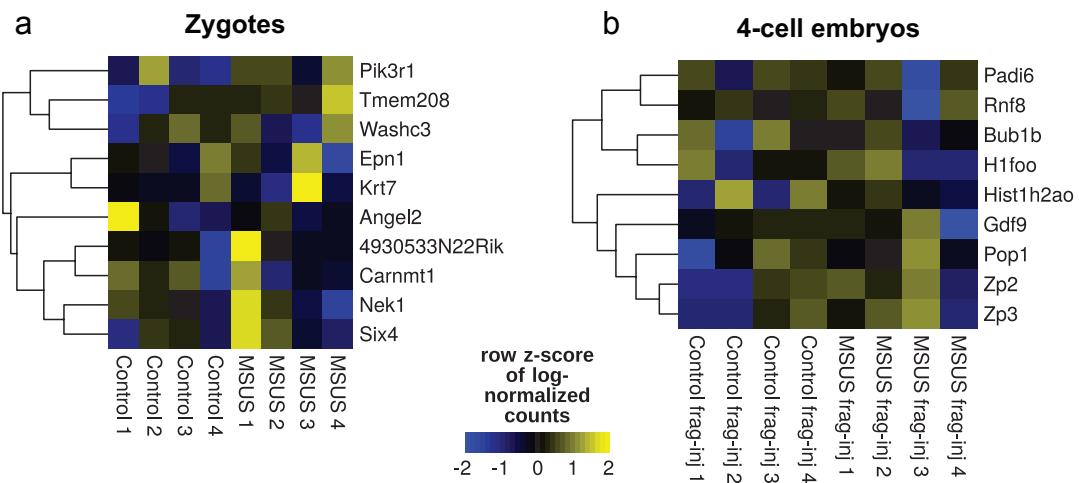
Effect of MSUS on sperm tRNA fragments. Analysis of next generation sequencing data reveals no change in the expression of tRNA fragments in animals exposed to MSUS (Control n=3, MSUS n=3, each sample represents pooled RNA from the sperm of 5 males, 3 biological replicates/group). Data used for this analysis have been published previously⁴⁸.

Supplementary figure 10



Effect of MSUS on gene expression in the offspring at zygotic stage. GO term analyses of differentially expressed genes in F2 control and MSUS zygotes shows that 18 genes are affected by MSUS (multiple testing corrected, $p<0.05$). Each library reflects a pool of 7-10 zygotes (Control n=4 and MSUS n=4). Top most specific GO terms enriched among differentially expressed genes are related to reproduction, but also include import into cell and neurotransmitter uptake. Red bars represent p-values of each GO term enrichment after multiple comparison correction. Blue bars represent fold enrichment of each GO term. Each blue square depicts a differentially expressed sperm transcript. Shades of blue represents the significance of its differential expression. ob= observed, exp= expected.

Supplementary figure 11



Gene expression in zygotes and 4-cell embryos revealed by next generation sequencing. (a) Genes with a trend ($p<0.01$ before correction for multiple testing) in 4-cell embryos injected with fragmented MSUS sperm RNA vs fragmented control sperm RNA do not show any similar trend in zygotes from natural MSUS and controls. (b) Expression of protein coding and long non-coding transcripts identified as differentially expressed in MSUS or control zygotes show no difference between 4-cell embryos resulting from zygotes injected with fragmented long RNA from MSUS vs control sperm ($n=4$ for each group, each library reflecting a pool of 7-10 embryos).

Supplementary table 1

(separate file) GO term enrichment analysis of control sperm RNAs.

Supplementary table 2

(separate file) GO term enrichment of differentially expressed genes between controls and MSUS (sheet a) including enrichments specific to up-regulated and down-regulated genes (sheets b and c), as well as similar enrichments for analyses including only the second library batch (sheet d) or using a random effect variable to account for the relatedness of technical replicates (sheet e).

Supplementary table 3

(separate file) GO term enrichment analysis of differentially expressed genes in MSUS zygotes.

Supplementary table 4

(separate file) Differential expression in 4-cell embryos resulting from naïve zygotes injected with sperm fragmented long RNA from control or MSUS males.

14 Dedication

For the wild ones.
For the ones with soul.
For the poets.
For the rebels.
For the courageous.
For the abused.
For the intellectuals.
For the artists.
For the mad.
For the loyal.
For the melancholy.
For the blissful.
For the imperfect.
For the fighters.
For the ones we lost.
And for the ones we have.

15 Acknowledgements

Mom (madre, mama, MAHMI!), Popi, Lala, Bro, Mike and Allegra, I can't believe I am blessed to have a family of such unique, supportive and completely ridiculous humans. I am so happy I get to go through life with you. Amber, Caleb, Micah and Adeline, you remind me every day what life is really about and I am so honored to be your aunt! Matthias (and Rose) you take care of me, guide me, support me, challenge me and love me. You are my home. Sarah, your unconditional friendship has brought so much light into my life, it was a true (Christmas) miracle the day you came into my life! Harri you have proven friendship has no bounds, and you are medicine for my soul. Tahlia, despite being half a world away, you have always been here with me right by my side every step of the way. Irina, there is absolutely zero chance I could have done this without you. Please don't ever leave me! Franci and Martin, I deeply cherish our friendship, and our many many hours spent in E52 will hold a special place in my heart. Isabelle, you have been a mentor and a role model, and also a friend. I am so grateful for having had the opportunity to learn from you and develop in your lab. Thank you Urs and Nicola, for the mentorship and advice that was essential for this work. Kathi, you have also taught me much about life in science, thank you for putting time and effort into me. To the rest of the lab, thank you for all the good times, you have made this period of my life truly memorable!

Josef, Johanna, Martin and Ale, you took me in as family and gave me love, support and endless supplies of Italian food, for which I am eternally grateful. You are my Swiss sanctuary. Thank you to my expat crew. You are the most insane group of wonderful humans I wouldn't trade in for anything! Jess and Tobi, so many adventures, and so many more to come. Thank you for your love and support. Gene Rostermundt, rest in peace. Your encouragement and friendship, even if only for a short time, will always have a special place in my heart. To my friends in California, I am so sorry I missed out on so many life events the last few years. But you nonetheless supported and encouraged me. Thank you to Jesse, Christy, Jackie L, Jackie A, Keri, Vanessa, Heather, Kirstin, Kristen, Stacie, Amanda and Kristi. I miss you!