UNIVERSIDADE FEDERAL DE PELOTAS
Faculdade de Veterinária
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Tese

O papel dos microRNAs exossomais no metabolismo e envelhecimento ovariano usando camundongos como modelo de menopausa

Bianka Machado Zanini

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Tese apresentada ao Programa de Pós-Graduação em Veterinária da Faculdade de Veterinária da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área de concentração: Sanidade Animal)

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Com toda minha paz, aos meus avós Luciano Pedro Zanini e Clea Ferreira

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Resumo

O declínio da reserva ovariana leva à menopausa e à redução dos estrogênios séricos. MicroRNAs são pequenos RNAs não codificantes, que podem regular a expressão gênica, sendo secretados pelas células e transportados no soro via exossomos (EXO). Os EXO são demonstrados como potencial terapêutico, tendo grande potencial como plataformas de entrega de medicamentos para tratamento de doenças. No entanto, nenhum estudo avaliando os efeitos do estradiol (E2), miRNAs e injeção de EXO na fertilidade de camundongos havia sido relatado até o momento. Portanto, nesta tese, objetivamos identificar perfis de miRNA em EXO séricos de camundongos induzidos à estropausa e tratados com E2 e caracterizar o papel dos EXO na modulação do metabolismo pós-menopausa. No Artigo 1 utilizamos camundongos fêmeas divididos em três grupos Controle (CTL), injetados com diepóxido de 4-vinilciclohexeno (VCD), para indução da estropausa e VCD+E2, que foram induzidos a estropausa e receberam suplementação com E2. No Artigo 2 os camundongos foram divididos em CTL e VCD também, porém um dos grupos VCD recebeu suplementação com EXO. Estes EXO foram extraídos de camundongos cíclicos de mesma idade e injetados por 21 dias. Em ambos os artigos a estropausa resultou em ganho de massa corporal em comparação com os CTL. A partir do sequenciamento dos miRNAs realizado dos exossomos extraídos do soro, no Artigo 1 402 miRNAs foram detectados no Artigo 2 355 miRNAs. Nossos resultados sugerem que a estropausa induzida por VCD e a reposição de E2 têm impacto no perfil de miRNAs exossômicos séricos. Além disso, miR-200a-3p e miR-200b-3p que foram regulados no soro dos animais também foram regulados no tecido ovariano. Ainda realizamos caracterização dos exossomos e sequenciamento do tecido hepático no Artigo 2. No grupo VCD+EXO, ao total 1285 genes foram regulados no fígado em comparação ao grupo VCD. Isto destaca uma regulação significativa dos EXO no transcriptoma hepático. Algumas vias foram reguladas exclusivamente no EXO em comparação com VCD, sugerindo um efeito direto do tratamento com EXO. Entre essas vias de inflamação e reparação tecidual. Nossos achados representam um ponto de partida para o uso de exo-miRs como reguladores da saúde metabólica em mulheres acíclicas. As análises mostraram que a atividade ovariana pode modular modificações epigenéticas mediadas por miRNAs e a reposição de estradiol têm um impacto no perfil de miRNAs exossômicos séricos. Ou seja, E2 e EXO podem vir a ser usados como potencial candidatos à desenvolvimento de nova terapias.

Palavras-chave: menopausa; ovário; envelhecimento; vcd; estradiol; micro

Abstract

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The decline in ovarian reserve leads to menopause and a reduction in serum estrogen levels. MicroRNAs are small non-coding RNAs that can regulate geneexpression, being secreted by cells and transported in the serum via exosomes(EXO). Exosomes are demonstrated as having potential therapeutic effects, with significant potential as drug delivery platforms for disease treatment.However, no study evaluating the effects of estradiol (E2), miRNAs, and EXO injection on mouse fertility had been reported until now. Therefore, in this thesis,our objective was to identify miRNA profiles in serum exosomes of mice inducedinto estrous and treated with E2, as well as to characterize the role of EXO in modulating post-menopausal metabolism.In Article 1, female mice were divided into three groups: Control (CTL), injected with 4-vinylcyclohexene diepoxide (VCD) to induce estrous, and VCD+E2, induced into estrous and supplementedwith E2. In Article 2, mice were divided into CTL and VCD groups, but one VCDgroup received EXO supplementation. These EXOs were extracted from cyclicmice of the same age and injected for 21 days. In both articles, estrous resultedin weight gain compared to CTL.From the miRNA sequencing of exosomes extracted from serum, 402 miRNAs were detected in Article 1 and 355 miRNAsin Article 2. Our results suggest that VCD-induced estrous and E2 replacementimpact the profile of serum exosomal miRNAs. Additionally, miR-200a-3p and miR-200b-3p, regulated in the animals' serum, were also regulated in ovarian tissue. We also conducted exosome characterization and liver tissuesequencing in Article 2. In the VCD+EXO group, a total of 1285 genes were regulated in the liver compared to the VCD group, highlighting significant regulation of EXOs in the hepatic transcriptome. Some pathways were exclusively regulated in EXOs compared to VCD, suggesting a direct effect of EXO treatment, including pathways related to inflammation and tissue repair.Our findings serve as a starting point for using exo-miRs as regulators ofmetabolic health in acyclic women. Analyses showed that ovarian activity can modulate miRNA-mediated epigenetic modifications, and estradiol replacementhas an impact on serum exosomal miRNA profiles. In other words, E2 and EXOmay emerge as potential candidates for the development of new therapies.

Keywords: menopause; ovary; aging; vcd; estradiol; microvesicle

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Artigo 1

Artigo 2

Lista de Abreviaturas e Siglas

17β-E2	17 beta-Estradiol
AGO2	Argonauta
Akt	*Serine/Threonine Kinase 1*
Amh	*Anti-Mullerian Hormone Gene*
B2m	*Beta-2-Microglobulin Gene*
Cdkn2a	*Cyclin Dependent Kinase inhibitor 2a*
DCNT	Doenças crônicas não transmissíveis
DCV	Doenças cardiovasculares
df/df	Ames Dwarf
DICER	Ribonuclease Dicer 1
DROSHA	Ribonuclease de Drosha
EXO	Exossomos
EXO-miR	microRNA exossomico
FC	Fold Change
FDA	Food and Drug Administrations
FSH	Hormônio Folículo Estimulante
Foxo3	*Forkhead Box O3 Gene*
HDL	Lipoproteína de Alta Densidade
Igf1	Insulin-Like Growth Factor 1 Gene
Il-1β	Interleucina 1 beta
Il-6	*Interleucina-6*
IU	Internation Unity
KIT	*Proto-OncoGene Receptor Tyrosine Kinase Gene*
LIF	Fator Inibidor de Leucemia
mRNA	RNA mensageiro
miRNA	microRNA

Mtor	*Mechanistic Target Rapamycin Kinase GenemTOR*
Map14	*Mitogen-activated protein kinase 14*
OVX	Ovariectomia
PBS	Phosphate-buffered saline
Pi3k	*Phosphatidylinositol-4,5-Bisphosphate 3-Kinase*
P53	*Tumor protein 53*
RISC	Complexo de Silenciamento Induzido por RNA
RNA	Ácido Ribonucleico
Stat3	*Signal Transducer and Activator O Transcription 3*
Tgf-β	*Transforming growth factor beta*
Tnf-α	*Transforming growth factor alfa*
TRH	Terapia de Reposição Hormonal
VCD	4-Diepóxido de vinilciclohexeno
ZEB1	*Zinc Finger E-Box Binding Homeobox*
ZEB2	*Zinc Finger E-Box Binding Homeobox 2*

Sumário

1 Introdução

Diversas teorias já foram propostas para explicar o processo de envelhecimento, pode-se destacar duas: a Teoria Programada, segundo o envelhecimento segue um cronograma biológico dependendo da expressão gênica associada aos processos responsáveis pelo reparo e defesa. E a Teoria do Acúmulo de Danos, defende que os fatores ambientais induzem ao acúmulo de danos, levando potencialmente ao envelhecimento. Estes danos celulares são derivados de diversos agravos sofridos pelo organismo ao longo da vida, incluindo instabilidade genômica, diminuição dos telômeros, alterações epigenéticas e perda da proteostase (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013. Como resposta a este dano, o organismo inicia uma série de adaptações antagonistas como a desregulação na sensibilidade a nutrientes, disfunção mitocondrial e senescência celular (Lopez-Otin et al., 2013), que predispõem as doenças crônicas não transmissíveis (DCNT) com o avançar da idade.

Nas fêmeas além do envelhecimento natural do organismo, o envelhecimento dos órgãos reprodutivos também é uma questão biológica importante, de especial interesse na pesquisa sobre os mecanismos gerais do envelhecimento. Em geral as fêmeas tem uma longevidade maior que machos (Austad & Fischer, 2016). Assim, fica claro que existem alguns efeitos sexualmente dimórficos que não estão claramente compreendidos. Diferenças nos esteroides sexuais podem refletir na ativação do eixo hipotálamo-hipófise-adrenal e respostas diferentes ao estresse oxidativo quando comparamos camundongos machos com fêmeas (Symonds, 2007). Assim, entender a biologia das fêmeas e o papel dos ovários pode fornecer informações moleculares do envelhecimento em vários órgãos e tecidos. Associado ao envelhecimento, ocorre um declínio da atividade ovariana que leva à infertilidade das fêmeas com o avançar da idade. A queda na atividade ovariana relaciona-se com o declínio progressivo na quantidade e na qualidade dos folículos, definidos pela reserva ovariana, o qual em humanos é formado durante o período fetal e em roedores logo após o nascimento (Finch, 2014; J. Zhang et al., 2016).

Os estrogênios, hormônios esteroides sexuais femininos, têm amplos efeitos sistêmicos em diferentes tipos de células por meio de seus receptores. Os níveis de

estrogênios sistêmicos, especialmente 17β-estradiol (E$_2$), mudam drasticamente no momento da menopausa em mulheres (O'Connor et al., 2009). Embora a terapia com estrogênio esteja clinicamente disponível há mais de seis décadas, os riscos e benefícios da terapia de reposição hormonal (TRH) com estrogênio ainda são motivo de estudos. O E$_2$ um hormônio utilizado como contraceptivo oral e hormônio de reposição. Milhões de mulheres são tratadas com TRH para alívio dos sintomas da menopausa, para os quais o estrogênio é único e altamente eficaz (Rozenberg, Vandromme, & Antoine, 2013). As terapias hormonais contendo compostos de estrogênio são o único tratamento aprovado pela Food and Drug Administrations (FDA) que alivia o amplo espectro de sintomas associados à menopausa (Baber, Panay, & Fenton, 2016; Neves-e-Castro et al., 2015; Obstetricians & Gynecologists, 2014; Society, 2017; Stuenkel et al., 2015). Estudos observacionais dos efeitos metabólicos e vasculares dos estrogênios sugeriram um benefício potencial por aumentar a expectativa de vida, diminuir a adiposidade e a inflamação crônica de baixo grau, melhorar a homeostase da glicose, diminuir a resistência à insulina e as taxas de produção de glicose hepática (Lobo, 2013; Rozenberg et al., 2013; Sprague, Trentham-Dietz, & Cronin, 2012). Porém, estudos randomizados não demonstraram evidência de benefício em mulheres (Hulley et al., 1999; Rossouw et al., 2002; Salpeter, Cheng, Thabane, Buckley, & Salpeter, 2009). A relação risco benefício precisa ser melhor compreendida caso a caso antes da recomendação da terapia.

A grande maioria das mulheres passa por uma transição natural para menopausa, retendo o tecido ovariano inativo. No entanto, os modelos para indução desta condição em roedores são limitados. Uma possibilidade é a remoção cirúrgica dos ovários e a outra é o uso do composto 4-Diepóxido de vinilciclohexeno (VCD), que leva à falha gradual da função ovariana (Kappeler and Hoyer 2012). Isto resulta em animais depletados de folículos, mas que retém o tecido ovariano residual, assim como ocorre em mulheres na menopausa. Recentemente, a sensibilidade da regulação dos microRNAs (miRNAs) ao estrogênio ,em diferentes tipos celulares, foi demonstrada em alguns estudos (Gómez-Gómez, Organista-Nava et al. 2016, Jiang, Li et al. 2016, Micheli, Palermo et al. 2016). Isto indica que os miRNAs são diretamente influenciados pela menopausa. miRNAs são uma classe de reguladores epigenéticos, pequenos RNAs não codificantes, que se ligam aos seus RNA mensageiros (mRNAs) alvo levando ao silenciamento do RNA e supressão da tradução (McGinnis, Luense et al. 2015). Os miRNAs podem ser secretados em fluidos extracelulares e transportados

para c células-alvo por meio de vesículas, como exossomos (EXO) (Makarova, Shkurnikov et al. 2016). Os miRNAs extracelulares funcionam como mensageiros químicos para mediar a comunicação célula-célula similarmente a hormônios endócrinos e abrem muitas possibilidades em termos de terapia com miRNAs (Fu, Ye et al. 2013). Um estudo demonstrou que miRNAs específicos diferiram com a idade em mulheres, incluindo miR-21 e miR-146a (Kangas, Pöllänen et al. 2014). Os dados deste estudo sugerem que o estradiol pode ser um mediador da mudança no conteúdo de miRNA nos exossomos (exo-miR) que, por sua vez, está associado à mudança no estado metabólico de saúde.

Exossomos são vesículas extracelulares nanométricas (<100nm de diâmetro) que circulam na corrente sanguínea para transferir sinais moleculares de tecido para o tecido (Clayton et al., 2004; Luga et al., 2012; C. Yang e Robbins, 2011). Essas estruturas esféricas são cercadas por uma bicamada lipídica e carregam uma carga biológica ativa, que inclui proteínas, lipídios, e RNA (Théry, 2002; Valadi, 2007). O conteúdo dos exossomos é afetado por sua origem e condição fisiológica da célula secretora (Guduric-Fuchs, 2012; Nolte-'t Hoen, 2012). Os RNAs exossomicos secretados podem gerar alterações na atividade das células receptoras, sugerindo a funcionalidade do conteúdo dos exossomos nas células-alvo (D'Anca et al., 2019). Estudos demonstram que o isolamento de exossomos do soro de animais jovens e posterior injeção em animais velhos tem ação de reduzir e retardar os efeitos do envelhecimento (Lee, 2018; Q. Yang et al., 2020). Dessa forma sugere-se que os exossomos tenham um papel ativo na regulação do envelhecimento. Assim os exo-miRs tem sidos propostos como biomarcadores não invasivos para envelhecimento e doenças relacionadas (Basu & Ludlow, 2016; Rani, Ryan, Griffin, Ritter, 2015) e podem fornecer evidências para elucidar e reverter certos mecanismos de envelhecimento. Com base nisso, nossos objetivos foram, Artigo 1: identificar perfis de miRNA em exossomos séricos de camundongos induzidos à estropausa e tratados com 17β-estradiol e caracterizar o papel dos exossomos na modulação do metabolismo pós-menopausa em camundongos.

2 Objetivos

Experimento 1
Caracterizar os miRNAs secretados pelo ovário e seu papel no envelhecimento gonadal e somático, especificamente:

- Identificar o perfil de miRNAs circulantes no soro em camundongosinduzidos a menopausa, recebendo ou não estradiol;

Experimento 2
Caracterizar o papel dos exossomos no metabolismo pós-menopausa em camundongos, especificamente:

- Descrever o papel dos exossomos na regulação das alteraçõesmetabólicas pós menopausa em camundongos;

3 Revisão de Literatura

Envelhecimento e menopausa

As doenças crônicas não transmissíveis (DCNT) representam a principal causa de morte no mundo e, no Brasil, correspondem a 70% das mortes (Malta et al., 2014). Inclusive, as doenças cardiovasculares (DCV's) são responsáveis por 28% dos óbitos em adultos (Malta et al., 2014). Assim o risco de ocorrência de doenças crônicas como diabetes tipo II, DCV, câncer, entre outros, aumenta dramaticamente com o envelhecimento da população e tem se tornado um problema econômico grave em diversos países. O significado evolutivo e os mecanismos moleculares básicos envolvidos na determinação da longevidade continuam sendo um problema não resolvido. Neste sentido, diversas teorias já foram propostas para explicar o processo de envelhecimento, e podem ser agrupadas naquelas que defendem o envelhecimento não programado e aquelas que propõem a existência do envelhecimento programado (Pamplona, Jové et al. 2023). Os danos celulares derivados de diversos agravos sofridos pelo organismo ao longo da vida, incluindo instabilidade genômica, diminuição dos telômeros, alterações epigenéticas e perda da proteostase fazem parte destas teorias (LopezOtin, Blasco, Partridge, Serrano, & Kroemer, 2013). Como resposta a estes danos, o organismo inicia uma série de adaptações antagonistas como a desregulação na sensibilidade a nutrientes, disfunção mitocondrial e senescência celular (Lopez-Otin et al., 2013), que predispõem as DCNT com o avançar da idade.

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a biologia das fêmeas e o papel dos ovários pode fornecerinformações moleculares do envelhecimento em vários órgãos e tecidos Associado ao envelhecimento, ocorre um declínio da atividade ovariana que levaà infertilidade das fêmeas com o avançar da idade. A queda na atividade ovariana relaciona-se com o declínio progressivo na quantidade e na qualidade dos folículos, definidos pela reserva ovariana, o qual em humanos é formado duranteo período fetal e em roedores logo após o nascimento (Finch, 2014; J. Zhang et al., 2016). A reserva ovariana é constituída de folículos primordiais, que são continuamente recrutados para ativação seguindo o processo de foliculogênese até a ovulação ou atresia em qualquer etapa do desenvolvimento, não podendo retornar à reserva (Chen et al., 2014; Weenen et al., 2004). A reserva ovariana é finita, e sua depleção leva ao fim da vida reprodutiva, que em mulheres é chamadade menopausa (Downs & Wise, 2009). Como resultado da inatividade ovariana, osníveis de estrogênio são severamente reduzidos seguido pela dominância androgênica na pós-menopausa (Trémollieres, Pouilles, & Ribot, 1996). Essa deficiência hormonal leva a alterações no perfil lipídico sérico, bem como na composição corporal e distribuição de gordura (Trémollieres et al., 1996). A quantidade excessiva de tecido adiposo leva a um perfil de citocinas desequilibrado, com aumento de interleucina-6 (IL-6), fator de necrose tumoral alfa (TNF-α), interleucina 1 beta (IL-1β), resistina e leptina entre outras moléculas inflamatórias (Ghigliotti et al., 2014). Essas citocinas pró-inflamatórias circulantes formam uma base para a inflamação sistêmica de baixo grau também conhecida por estar associada ao envelhecimento e a resistência à insulina (De Luca & Olefsky, 2008). É interessante destacar que a idade e a menopausa têm uma relação direta com a longevidade. Mulheres com menopausa precoce tem menor expectativa de vida, considerando mortalidade por diversas causas (Jacobsen, Knutsen, & Fraser, 1999). Especificamente mulheres com menopausa precoce temmaior risco de morte por DCV (Jacobsen et al., 1999). Isso sugere que o ovário pode influenciar ativamente a saúde geral da mulher.

Em camundongos também há declínio da reserva ovariana e da fertilidade, na linhagem C57BL/6 a reserva é reduzida pela metade até os 10 meses de idade e, aos 18 meses é reduzida em aproximadamente 10 vezes em comparação com camundongos adultos jovens (Kevenaar, 2006). Entre 11 e 16 meses a ciclicidade também é interrompida, semelhante à linha do tempo observada em mulheres (Felicio, 1984). Por volta dos 18 meses de idade, as fêmeas deixarão de produzir filhotes (Felicio, 1984; Selesniemi, 2008). Isso indica que, embora a menopausa não seja um fenômeno observado em camundongos, há uma redução severa na reserva ovariana, diminuição na ovulação e fertilidade, semelhante ao que ocorre em mulheres, comprovando que camundongos são um modelo valioso para estudar o envelhecimento reprodutivo (Broekmans, 2009). Foi observado que o transplante de ovários jovens para fêmeas mais velhas, aumentou a expectativa de vida das fêmeas mais velhas (J. B. Mason, Cargill, Anderson, & Carey, 2009). Além disso, estudos mais recentes demonstraram que o transplante de ovários jovens com depleção de células germinativas aumentou ainda mais a expectativa de vida das fêmeas receptoras (Habermehl et al., 2019). Isto levanta a questão de que outros fatores, além do estradiol, podem ser responsáveis por este efeito protetor do ovário. Curiosamente, independentemente da presença de células germinativas, as alterações de citocinas inflamatórias associadas ao envelhecimento foram revertidas após quatro meses de exposição ao ovário jovem (Habermehl et al., 2019). Então estas evidências nos sugerem que o ovário jovem produz fatores intrínsecos que são capazes de prevenir o envelhecimento e, portanto, podem ter um efeito protetivo contra diversas doenças crônicas do envelhecimento e menopausa.

Os estrogênios, hormônios esteroides sexuais femininos, têm amplos efeitos sistêmicos em diferentes tipos de células por meio de seus receptores. Como se sabe, os níveis de estrogênios sistêmicos, especialmente 17β-estradiol (E2), mudam drasticamente no momento da menopausa em mulheres (O'Connor et al., 2009). Embora a terapia com estrogênio esteja clinicamente disponível há mais de seis décadas, os riscos e benefícios da terapia de reposição hormonal (TRH) com estrogênio ainda são motivo de estudos. O estradiol é um hormônio utilizado como contraceptivo oral e hormônio de reposição. Milhões de mulheres são tratadas com terapia hormonal para alívio dos sintomas da menopausa, para os quais o estrogênio é único e altamente eficaz (Rozenberg, Vandromme, & Antoine, 2013). As terapias hormonais contendo compostos de estrogênio são o único tratamento aprovado pela

Food and Drug Administrations (FDA) que alivia o amplo espectro de sintomas associados à menopausa (Baber, Panay, & Fenton, 2016; Neves-e- Castro et al., 2015; Obstetricians & Gynecologists, 2014; Society, 2017; Stuenkel et al., 2015). Estudos observacionais dos efeitos metabólicos e vasculares dos estrogênios sugeriram um benefício potencial por aumentar a expectativa de vida, diminuir a adiposidade e a inflamação crônica de baixo grau, melhorar a homeostase da glicose, diminuir a resistência à insulina e as taxas de produção de glicose hepática (Lobo, 2013; Rozenberg et al., 2013; Sprague, Trentham-Dietz, &Cronin, 2012). Porém, estudos randomizados não demonstraram evidência de benefício em mulheres (Hulley et al., 1999; Rossouw et al., 2002; Salpeter, Cheng,Thabane, Buckley, & Salpeter, 2009). A relação risco benefício precisa ser melhor compreendida caso a caso antes da recomendação da terapia.

Neste sentido, pesquisas em modelos animais indicam que a perda de hormônios ovarianos, particularmente estrogênios após a ovariectomia (OVX), é prejudicial para vários órgãos e tecidos (Daniel, 2013; Finch, 2014; Frick, 2015; Koebele & Bimonte-Nelson, 2015; Korol & Pisani, 2015; Luine, 2014). Em roedores a OVX resulta em aumento dos triglicerídeos hepáticos, que foi normalizado após o tratamento de reposição de estradiol (Galmés-Pascual et al., 2017). Além disso,animais OVX têm função mitocondrial hepática prejudicada, além de expressão e atividade reduzida da superóxido dismutase (Ballestri et al., 2017; Galmés-Pascualet al., 2017). Assim, a OVX resulta em um fenótipo de disfunção metabólica, marcado por esteatose, aumento de peso corporal e ganho de massa gorda, maioreficiência alimentar, com menor acoplamento mitocondrial e excreção fecal de ácidos biliares. A reposição de estradiol foi eficaz em neutralizar esses fenótipos induzidos por OVX (Zhu et al., 2013). Contudo, as mulheres naturalmente na menopausa possuem uma vantagem na saúde sobre as mulheres cirurgicamentena menopausa. A OVX parece causar dislipidemia e grave perda de densidade mineral óssea em apenas 1 ano em mulheres, aumentando o risco de desenvolvimento de algumas doenças como, depressão, osteoporose, síndrome metabólica. Assim, manter o tecido ovariano no organismo, mesmo com os ovários envelhecido pode fornecer um benefício metabólico, independentemente das células germinativas ativas (Yoshida, Takahashi, Yamatani, Takata, & Kurachi, 2011). Dado que a grande maioria das mulheres passa por uma transição natural para a menopausa, retendo o tecido ovariano e não sofre uma OVX, o composto 4-Diepóxido de vinilciclohexeno (VCD) pode ser usado para induzir um

modelo demenopausa mais fiel em roedores, melhorando o entendimento de como as terapias interagem com um sistema reprodutivo intacto, mas com depleção de folículos.

O VCD é um resíduo químico industrial produzido a partir da síntese de inseticidas, antioxidantes, pneus de borracha, entre outros, tem sido utilizado comercialmente para reduzir a fertilidade e proliferação de roedores. O VCD tem efeitos ovotóxicos que foram percebidos pela primeira vez em operárias que trabalhavam em fabricas e apresentavam menopausa precoce (Bhardwaj & Saraf, 2014; Hoyer, Devine, Hu, Thompson, & Sipes, 2001; Mayer et al., 2002; Program, 1989). A exposição de animais ao composto leva à falha gradual da função ovariana, resultando em animais depletados de folículos, mas que retém o tecido ovariano residual, assim como ocorre em mulheres na menopausa (Kappeler & Hoyer, 2012; Liu, Wang, Xing, & Fan, 2015). O VCD no ovário de roedores leva a apoptose dos folículos primordiais e primários causando assim falência ovariana (Reis et al., 2014; Kappeler & Hoyer, 2012). O ciclo estral de camundongos tratados com VCD cessa 2-3 meses após a conclusão das injeções com VCD, o que por sua vez resulta em uma queda gradual do estrogênio, mimetizando o período de transição da peri a pós-menopausa em mulheres (Mayer et al., 2002). Assim, um animal com depleção de folículos e ovários intactos se aproxima muitoda progressão humana natural através da transição pré, peri e pós-menopausa (Brooks, Pollow, & Hoyer, 2016). Além disso, fornece uma alternativa não cirúrgicaao modelo OVX, reduzindo complicações pós-cirúrgicas, como contaminações. Com doses diárias de VCD por 20 dias, níveis aumentados do hormônio folículo-estimulante (FSH) são observados após 60-90 dias do início da exposição (Reis etal., 2014) caracterizando a transição para menopausa. O que torna os folículos ovarianos primordiais e primários alvos seletivos do VCD é a presença de fatores de crescimento essenciais para o desenvolvimento e sobrevivência das células foliculares, como fatores de associação de células da granulosa, o ligante do receptor KIT (KITL) e fator inibidor de leucemia (LIF) (Fernandez et al., 2008; Mark-Kappeler et al., 2011). É importante ressaltar que o modelo VCD mimetiza tanto operfil hormonal (Carroll, 2010; Pestana-Oliveira et al., 2018; Reis et al., 2014) quanto comportamental de ansiedade (Reis et al., 2014), memória prejudicada (Koebele & Bimonte-Nelson, 2016), depressão (Kalil, Pestana-Oliveira, Santos, Carolino, & Anselmo-Franci, 2020) manifestadas tipicamente por mulheres na perimenopausa. Portanto, o modelo de depleção folicular induzida por VCD, seguido de falência ovariana, tem sido

amplamente utilizado em pesquisas experimentais sobre perimenopausa e menopausa.

<u>microRNAs e exosomos</u>

A regulação da expressão gênica desempenha papel fundamental no desenvolvimento folicular e no envelhecimento dentro do ovário. Alguns processos celulares importantes, incluindo a transição e estabilidade do RNA mensageiro (mRNA), são regulados por pequenos RNAs não codificantes. A descoberta de miRNAs e seus mecanismos de ação ampliou e esclareceu à compreensão da regulação gênica. Eles são uma classe de reguladores epigenéticos, pequenos RNAs não codificantes, que se ligam aos seus mRNAs alvo levando ao silenciamento do RNA e supressão da tradução (Catalanotto, Cogoni et al. 2016). A maioria são transcritos de sequências de DNA em miRNAs primários e processados em miRNAs precursores e, finalmente, miRNAs maduros (Luense et al., 2009). A interação de miRNAs com seus genes alvo é dinâmica e dependente de muitos fatores, como a localização subcelular de miRNAs, a abundância de miRNAs e mRNAs alvo e a afinidade das interações miRNA-mRNA (Vasudevan, 2012). Alguns estudos recentes sugerem um papel importante para os miRNAs na regulação do processo de envelhecimento (Jin Jung & Suh, 2012; Olivieri, Rippo, Procopio, & Fazioli, 2013; Y. Zhang et al., 2017). Os miRNAs podem ser codificados por genes separados ou incorporados em regiões intrônicas ou exônicas de genes codificadores de proteínas e são transcritos como genes únicos ou cluster policistrônico para formar estruturas secundárias de alça (Tam et al., 2008; Watanabe et al., 2008). A ribonuclease de Drosha tipo III (DROSHA) e o gene da região crítica da síndrome de DiGeorge 8 (DGCR8) clivam a alça em pré-miRNA, que é exportado para o citoplasma para ser posteriormente cortada pela ribonuclease Dicer 1 tipo III (DICER1), resultando em um RNA duplex. Uma fita do RNA duplex (miRNA maduro) se liga ao complexo de silenciamento induzido por RNA (RISC) e regula a expressão do gene alvo. A segunda fita é degradada (Ha & Kim, 2014), embora também possa ser selecionada para se tornar um miRNA maduro, mas o mecanismo pelo qual uma fita é selecionada não é completamente compreendido. Alternativamente o miRNA também pode ser gerado

por vias independentes de DROSHA/DGCR8 ou independentes de DICER1 (Luense et al., 2009). O miRNA maduro se liga por complementaridade de pares de bases na região não traduzida 3' ou 5' do mRNA para inibir sua tradução, seja por indução de degradação do mRNA ou bloqueando sua tradução (Tang et al., 2007). No entanto, existem algumas evidências de que o miRNA também pode atuar aumentando a expressão de alguns genes (Broughton, Lovci, Huang, Yeo, & Pasquinelli, 2016). A função do miRNA é muitas vezes redundante, um único miRNA pode regular vários genes e um mRNA pode ser regulado por vários miRNAs (Vasudevan, 2012).

Os miRNAs têm um papel predominantemente intracelular. No entanto, podem ser secretados em fluidos extracelulares e transportados para células-alvo por meio de vesículas, como exossomos, ou por ligação a proteínas (Makarova et al., 2016). Os miRNAs extracelulares funcionam como mensageiros químicos para mediar a comunicação célula-célula similarmente a hormônios endócrinos e abrem muitas possibilidades em termos de terapia com miRNAs. Os miRNAs extracelulares também têm sido amplamente relatados como potenciais biomarcadores para uma variedade de doenças (Fu et al., 2013). Muitos estudos detectaram miRNAs extracelulares/circulantes em fluidos biológicos, como plasma e soro, líquido cefalorraquidiano, saliva, leite materno, urina, lágrimas, colostro, líquido peritoneal, lavagem brônquica, fluido seminal e fluido folicular ovariano (Arroyo et al., 2011; X. Chen, Liang, Zhang, Zen, & Zhang, 2012). Ao contrário de outras espécies de RNA, os miRNAs são altamente estáveis, resistindo à degradação em temperatura ambiente por até 4 dias, descongelamento e pH alto ou baixo (Makarova et al., 2016).

Duas populações de miRNAs extracelulares existem em fluidos biológicos. Um pode ser encontrado em vesículas, como exossomos, microvesículas e corpos apoptóticos,enquanto o outro está associado a proteínas, como a argonauta 2 (AGO2) (Arroyo et al., 2011). Enquanto vários estudos sugerem que a maioria dos miRNAs extracelularesnão estão associados a exossomos/microvesículas, mas sim ligados a AGO2, outro estudo relatou que miRNAs extracelulares estão presentes predominantemente em exossomos no soro humano (Arroyo et al., 2011; Turchinovich, Weiz, Langheinz, & Burwinkel, 2011). Uma vez que esses estudos mediram apenas um grupo selecionadode miRNAs em algumas amostras de plasma, é possível que a existência de miRNApredominantemente exossômicos (exo-miR) ou livres de vesículas seja dependente do próprio miRNA, do tipo de célula de que se originam e/ou outros fatores que afetama secreção de miRNAs em indivíduos. Os

miRNAs extracelulares podem também serencontrados lipoproteína de alta densidade (HDL) e nucleofosmina 1 (NPM1) (Wang et al., 2010). A presença de miRNAs em vesículas ou com proteínas acompanhantesé geralmente considerada para proteger miRNAs extracelulares e aumentar sua estabilidade no meio extracelular.

Nesse sentido, exossomos são vesículas extracelulares nanométricas (<100nm de diâmetro), conhecidas por circularem pela corrente sanguínea para transferir sinais moleculares de tecido para tecido (Clayton et al., 2004; Luga et al., 2012; C. Yang & Robbins, 2011). Essas estruturas esféricas são cercadas por uma bicamada lipídica e carregam uma carga biológica ativa, incluindo proteínas, lipídios, carboidratos e material genético. Os exosomos se originam dos endossomos tardios no citoplasma formando corpos multivesiculares e permanecem nas células em que são formados ou são fundidos com a membrana plasmática e exportados para matriz extracelular e posteriormente para a circulação (Heijnen, Schiel, Fijnheer, Geuze, & Sixma, 1999). O conteúdo dos exossomos é afetado por sua origem e condição fisiológica da célula secretora. Estudos recentes demonstraram que as mensagens veiculadas pelos RNAs exossômicos secretados podem gerar alterações na expressão gênica e nas concentrações de proteínas das células receptoras, sugerindo a funcionalidade do conteúdo do exossomos nas células-alvo (D'Anca et al., 2019). Especialmente o papel imunoestimulante dos exossomos foi enfatizado, por exemplo, mostrando que as células dendríticas se comunicam por meio de miRNAs presentes nos exossomos em camundongos (Montecalvo et al., 2012). Ainda, estudos demonstram que o isolamento de exossomos do soro de animais jovens e posterior injeção em animais velhos tem ação de reduzir e retardar os efeitos do envelhecimento (Lee et al., 2018; 2018; Yang et al., 2020), sugerindo um papel central no envelhecimento do organismo.

Os exo-miR têm sido investigados para identificar como podem modular vias de sinalização associadas ao envelhecimento ligando-se a mRNAs alvo. Para tanto, miRNAs diferencialmente expressos, originários de uma fração específica do sangue, como células, soro ou microvesículas, foram identificados em animais velhos em comparação a jovens (Lee et al., 2018). Estes poderiam fornecer moléculas-chave para elucidar e reverter certos mecanismos do envelhecimento. Os exo-miR também

foram propostos como biomarcadores não invasivos para envelhecimento (Basu & Ludlow, 2016; Rani, Ryan, Griffin, & Ritter, 2015). Em um estudo seis miRNAs foram transfectados para o fígado, miR-16-5p, miR-17-5p, miR-21-a-5p, miR-30c-5p, miR-103-3p e miR-130a-3p, e levaram a diminuição da expressão de p16 e mTOR, indicando uma reversão de moléculas relacionadas ao envelhecimento (R. Kangas et al., 2017). A alteração nos níveis de moléculas relacionadas ao envelhecimento durou apenas 1 dia após as injeções, portanto, o efeito observado foi agudo e não crônico. Estes dados sugerem que a transfecção de miRNA via sistema circulatório apresenta potencial para induzir alterações em moléculas relacionadas ao envelhecimento em tecidos alvos.

Os miRNAs também são diretamente influenciados pela menopausa. Um estudo demonstrou que a associação da terapia de reposição hormonal à base de estrogênio com miRNAs selecionados e marcadores de inflamação do soro comparados entre mulheres na peri versus pós menopausa, dois miRNAs diferiram com a idade (Kangas et al., 2014). Os dados deste estudo sugerem que a menopausa pode ser um mediador da mudança no conteúdo de miRNA nos exossomos associado à mudança no estado metabólico de saúde. O miR-10b-5p foi associado ao início de doenças motoras como Parkinson e Huntington (Myers et al., 2016), indicando sua função no desenvolvimento de distúrbios neurodegenerativos associados à idade. Além disso, o papel do miR-10b-5p circulante na diferenciação osteogênica após fratura no período pós-menopausa foi reconhecido (Weilner et al., 2015). Alterações dos níveis de miR-28-3p durante a senescência precoce foram observadas em células endoteliais, indicando assim o possível papel regulador de miRNAs no envelhecimento. Em 2017, (Kangas et al., 2017) conseguiram demonstrar à influencia da TRH ao conteúdo de miRNAs, ou seja, miRNAs mudaram com a menopausa em mulheres e destes alguns foram revertidos pela TRH. Os resultados sugerem que os miRNAs dos exossomos séricos são sensíveis às mudanças hormonais em mulheres perimenopausa e carregam mensagens regulatórias importantes entre as células. Esses miRNAs demonstraram funcionar como reguladores da homeostase celular.

Embora pouco se saiba sobre como os miRNAs são regulados durante o processo de envelhecimento ovariano, alguns miRNAs desempenham papel essencial no desenvolvimento do folículo (Ahn et al. 2010). Sendo alguns miRNAs conhecidos por estarem envolvidos na insuficiência ovariana prematura em ratos (Kim et al. 2017).

Estudos do nosso grupo indicam importante papel do hormônio de crescimento (GH) neste processo, já que camundongos Ames Dwarf (df/df) apresentam reserva ovariana de folículos primordiais aumentada (Schneider, Matkovich, Saccon, et al., 2017). Interessantemente, ovários de camundongos df/df tem um perfil único de expressão de miRNAs nos associados a este atraso no envelhecimento ovariano (Schneider, Matkovich, Victoria, et al., 2017). Portanto, é essencial entender o papel do ovário no contexto geral da longevidade.

4 Artigos

4.1 Artigo 1

Dynamics of serum exosome microRNA profile altered by chemically induced estropause and rescued by estrogen therapy in female mice

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Dynamics of Serum Exosome microRNA Profile Altered by Chemically Induced Estropause and Rescued by Estrogen Therapy in Female Mice

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Abstract

The decline in the ovarian reserve leads to menopause and reduced serum estrogens. MicroRNAs are small non-coding RNAs, which can regulate gene expression, and be secreted by cells and trafficked in serum via exosomes. Serum miRNAs regulate tissue function and disease development. Therefore, the aim of this study was to identify miRNA profiles in serum exosomes of mice induced to estropause and treated with 17β-estradiol (E2). Female mice were divided into three groups including control (CTL), injected with 4-Vinylcyclohexene diepoxide (VCD) and injected with VCD plus E2 (VCD+E_2). Estropause was confirmed by acyclicity and a significant reduction in the number of ovarianfollicles ($p<0.05$). Body mass gain during estropause was higher in VCD and VCD+E2 compared to CTL females ($p=0.02$). Sequencing of miRNAs was performed from exosomes extracted from serum and 402 miRNAs were detected. Eight miRNAs were differentially regulated between CTL and VCD groups, seven miRNAs regulated between CTL and VCD+E2 groups and ten miRNAs regulated between VCD and VCD+E2 groups. Only miR-200a-3p and miR-200b-3p were up-regulated in both serum exosomes and ovarian tissue in both VCD groups, suggesting that these exosomal miRNAs couldbe associated to ovarian activity. In the hepatic tissue, only miR-370-3p ($p=0.02$) was upregulated in theVCD+E2 group, as observed in serum. Our results suggest that VCD induced estropause and E2 replacement have an impact in the profile of serum exosomal miRNAs. The miR-200 family was increased in serum exosomes and ovarian tissue and may be a candidate biomarker of ovarian function.

Introduction

Understanding the role of the aging ovaries in female biology can provide insights into aging across various organs and tissues. In females, aging is associated with a decline in ovarian activity leading to infertility and menopause in women (Wang, Wang et al. 2023) and estropause in mice (Finch 2014). As a result of ovarian inactivity, estrogen levels are severely reduced, followed by androgen dominance in postmenopausal women (Trémollieres, Pouilles et al. 1996). This hormonal imbalance leads to changes in serum lipid profiles and body composition (Trémollieres, Pouilles et al. 1996). Excessive postmenopausal adipose tissue results in increased levels of pro-inflammatory cytokines (Ghigliotti, Barisione et al. 2014) and insulin resistance (Mauvais-Jarvis, Clegg et al. 2013). Menopause is a risk factor for the development of insulin resistance independent of age, due to the reduction in circulating estrogens (Pu, Tan et al. 2017). As systemic estrogen levels undergo drastic changes during menopause (Gordon, Peltier et al. 2019), hormonal replacement therapy (HRT) is often applied in menopausal women (Rozenberg, Vandromme et al. 2013). HRT has been shown to influence life expectancy, reduce adiposity and low-grade chronic inflammation, and improving glucose homeostasis. (Sprague, Trentham-Dietz et al. 2012, Mauvais-Jarvis, Clegg et al. 2013, Rozenberg, Vandromme et al. 2013).

Ovariectomy (OVX) is the most widely used method to mimic menopause in mice. OVX abruptly reduces estrogens levels (Koebele and Bimonte-Nelson 2017), resulting in increased body mass gain, insulin resistance (Zidon, Padilla et al. 2020), dyslipidemia and metabolic syndrome (Fuller, McCoin et al. 2021). Given that most women experience a gradual natural transition to menopause, estrogen levels drop over a period of time and the ovarian tissue is retained. As an E2-reducing alternative to OVX, exposure to 4-Vinylcyclohexene diepoxide (VCD) can be used to mimic a more natural menopause transition in mice. Exposure of mice to VCD leads to gradual ovarian failure, resulting in follicle depletion with retention of residual ovarian tissue (Kappeler and Hoyer 2012, Li, Fang et al. 2015). Importantly, mice receiving VCD have a similar hormonal profile to perimenopause women (Reis, Pestana-Oliveira et al. 2014, Pestana-Oliveira, Kalil et al. 2018), as well as body mass gain (Ávila, Zanini et al. 2023) and metabolic changes (Romero-Aleshire, Diamond-Stanic et al. 2009). However, mice induced to estropause using VCD increased fat mass and developed insulin resistance only when exposed to a high-fat diet (Keck, Romero-Aleshire et al. 2007). Under regular chow diet most metabolic parameters remain unchanged between cyclic and acyclic estropausal mice (Keck, Romero-Aleshire et al. 2007, Ávila, Zanini et al. 2023). This is interesting as we can study cessation of ovarian activity in young mice with minimal resulting metabolic changes.

MicroRNAs (miRNAs) are small (19-25 bp) RNAs that regulate messenger RNA (mRNA) stability or translation and consequently gene expression (Catalanotto, Cogoni et al. 2016). Despite their main intracellular role, miRNAs can be secreted to the extracellular space and transported in the bloodstream (Makarova, Shkurnikov et al. 2016), functioning as messengers to mediate tissue communication, like endocrine hormones. miRNAs can be secreted from the cell packaged inside exosomes. Exosomes are small extracellular vesicles (<100nm in diameter) that transfer molecular signals from tissue to tissue (Clayton, Turkes et al. 2004, Yang and Robbins 2011, Luga, Zhang et al. 2012). The content of exosomes is influenced by its tissue of origin and the physiological condition of the secreting cells (Kalluri and LeBleu 2020). Therefore, miRNAs carried by exosomes can induce changes in gene expression and cell function in distant recipient tissues (D'Anca, Fenoglio et al. 2019).

Ovaries can be a source of exosomal miRNAs, while also influencing exosomal miRNA serum profile through changes in estrogen production. In women, menopause is accompanied by a change in the serum profile of miRNAs (Kangas, Pöllänen et al. 2014). Ten miRNAs were differentially regulated between

and pre- and post-menopausal women, of which only four were normalized by HRT (Kangas, Pöllänen et al. 2014). Our group also identified 22 miRNAs that change with age in mouse ovaries (Schneider, Matkovich et al. 2017), indicating that the ovarian miRNA profile changes in response to declining ovarian activity and age. However, we could not find any studies evaluating serum miRNA profile in OVX or VCD estropausal mice. In addition, estradiol secreted by active ovaries and used in HRT was shown to affect the expression of miR-125b in mice neurons (Micheli, Palermo et al. 2016) and miR-21, miR-143 and miR-124 in vitro (Gómez-Gómez, Organista-Nava et al. 2016, Jiang, Li et al. 2016). Therefore, it is essential to understand changes observed in serum miRNA profile after depletion of the ovarian reserve or estrogen replacement. This can be an important step in identifying serum miRNA markers of ovarian function, as well as possible candidates for clinical replacement therapies for postmenopausal women.

Methodology

Animals and Experimental Design
The study was approved by the Animal Experimentation Ethics Committee from Universidade Federal de Pelotas (number 0902860). For this, two-month-old female C57BL/6 mice (n=31) were used. Mice were maintained under controlled conditions of temperature and light (22±2ºC, 12-hour light/12-hour dark cycles) and provided with a standard diet and ad libitum access to water.
For estropause induction, female mice (n=21) were treated daily with i.p. injections of VCD (160 mg/kg, Sigma, St. Louis, MO) for 20 consecutive days, while control (CTL) mice received a placebo solution. VCD-treated mice were further divided into a VCD control group (VCD; n=10) and a VCD β-estradiol group (VCD-E2; n=11). Two-months after the end of VCD treatment mice received s.c. injections of placebo or E2 (5 µg/kg in sesame oil, Sigma, St. Louis, MO) three times a week for three months. CTL and VCD mice received placebo s.c. injections. Vaginal cytology was performed before the beginning of E2 treatment to confirm mice were in estropause. Body weight was evaluated every two weeks. The amount of body fat was determined by weighing the fat tissue immediately after euthanasia, relative to body weight.
At the end of the experiment, following a 4-hour fasting period, mice were anesthetized using isoflurane. Exsanguination was performed by cardiac puncture followed by cervical dislocation. Mice were dissected, and blood, ovaries, visceral fat and liver were collected and stored in 10% buffered formalin and at -80ºC.

Vaginal Cytology
Vaginal smears were obtained 60 days after the end of VCD injections. Daily smears were performed at the same time for five consecutive days. The collected material was stained using rapid panoptic. Females in diestrus for all five days were considered to be in estropause.

Glucose and Insulin Tolerance Test
For the glucose tolerance test (GTT), mice received an i.p. injection of glucose (2 g/kg body weight) after a 4-hour fast. Blood was collected 0, 15, 30, 60, and 120 minutes after injection, and glucose levels were measured using a glucometer (AccuChek Active, Roche Diagnostics®, USA). For the insulin tolerance test (ITT), mice received i.p. injection of insulin (0.5 IU/kg body weight) after a 2-hour fast. Blood was collected 0, 5, 20, and 35 minutes after injection, and glucose levels were measured using a glucometer. The percentage of glucose decay between 5 and 20 minutes after insulin injection was calculated.

Follicle counting

The ovaries were removed from 10% buffered formalin, dehydrated in alcohol, cleared in xylene, embedded in paraffin, and then cut into 5 μm sections using a semiautomatic microtome (RM2245 38, Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK). One out of every six sections were selected and placed on standard histological slides. After drying in a 56 °C oven for 24 h, the slides were stained with hematoxylin and eosin and mounted with coverslips and synthetic resin (Sigma Chemical Company®, St. Louis, MO, USA). Images of the ovarian sections were captured using microscope (Nikon Eclipse E200, Nikon Corporation, Japan) with 4×, 10×, and 40× objectives. Follicles with clearly visible oocyte nuclei were quantified. The number of follicles counted was divided by the total number of sections for each ovary. The follicle classification protocol was based on Myers et al. (2004).

Exosome Extraction and Characterization

Exosomes were isolated from serum using a commercial kit (Total Exosome Isolation Reagent from serum, Invitrogen, Vilnius, Lithuania) following the manufacturer's protocol. Briefly, a buffer was added to the serum sample and incubated at 5°C for 30 minutes. Precipitated exosomes were recovered by centrifugation at 10,000 xg for 10 minutes. The pellet was resuspended in phosphate-buffered saline (PBS).

Particle concentration and particle size distribution of extracted exosomes were determined by NTA using a NanoSight NS300 with 488 nm laser set at 25°C and camera level 11 (Malvern instruments). The NanoSight sample pump was used in conjunction with 1 mL syringes at flow speed of 30. Samples were run immediately after dilution in 0.1um filtered HPLC grade water. Each analysis consists of five 60-second video captures per sample (University of Florida – ICBR, Cytometry Core Facility; RRID:SCR_019119).

miRNA Library Preparation, Sequencing and Processing

For exosome RNA extraction, a commercial kit for total RNA and protein isolation from exosomes (Total Exosome RNA & Protein Isolation Kit, Invitrogen, Vilnius, Lithuania) was used. miRNA libraries from total RNA were prepared using the NEXTFLEX® Small RNA-Seq Kit V4 (Perkin Elmer, Seer Green, Beaconsfield, United Kingdom) and submitted to sequencing on a HiSeq 2500 instrument. Alignment and quantification of miRNA libraries was performed using cutadapt and bowtie2 (Martin 2011) as described before (Saccon, Schneider et al. 2021).

miRNAs target prediction and enriched pathways and GO Terms

The mirPath tool (version 3.0) was used to predict target genes of the differentially regulated miRNAs using the microT-CDS v. 5.0 database (Vlachos, Zagganas et al. 2015) and for retrieving KEGG molecular pathways (Kanehisa and Goto 2000, Kanehisa, Sato et al. 2016), considering p values < 0.05 as significant. For pathway analysis, the miRNAs were analyzed separately as down- or up-regulated.

Gene expression analysis

The ovarian and liver RNA extraction was performed using the Trizol protocol. The final RNA was dissolved in 20 μL RNAse free water and its concentration and quality were estimated by spectrophotometry (EpochTM Microplate Spectrophotometer, BioTek, Winooski, VT, USA). Complementary DNA (cDNA) was synthesized from 1 ug of total RNA using random hexamer nucleotide (RH) primers (iScript cDNA Synthesis Kit Biorad, Hercules, CA, USA). After that, samples were used for real-time PCR analysis to detect the expression of target genes. Real-time PCR was performed using Sybr Green qPCR Master Mix (ThermoFisher, Vilnius, Lithuania) in a 20 μL reaction

using the Quant studio 7 system (Applied Biosystems). Each reaction was performed in duplicate, using 2 μL of cDNA (20 ng), 10 μL of Sybr Green, 0.4 μL of each primer (10 μM) and 7.2 μL of ultrapure water. For each assay, 45 cycles (95ºC for 15 sec and 60ºC for 1 min) were performed and a dissociation curve was included at the end of reaction to detect the amplification specificity of a single PCR product. To measure miRNA expression, 10 ng of total RNA was reverse transcribed using TaqMan® Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. For the RT-qPCR reaction a 1:10 dilution of cDNA template was prepared. RT-qPCR was performed in duplicates using the QuantStudio™ 7 Flex System. We used TaqMan® Advanced miRNA Assays (Applied Biosystems, Foster City, CA, USA) with primers specificfor selected miRNA: miR-200a-3p, miR-370-3p, miR-7072-5p, miR-23a-5p, miR-5107-5p, miR-6950-3p, miR-24-1-5p, miR-200b-3p.

The $2^{-\Delta\Delta Ct}$ method was used to calculate and normalize expression of each target mRNA (Table 1) using the internal control *B2m* as a reference, and for miRNA using miR-16-5p as a reference.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 10. One-way ANOVA was used for follicle count, glucose metabolism, and gene expression analyses. Repeated measures ANOVA was used for body mass analysis. P values lower than 0.05 were considered statistically significant.

Differentially expressed miRNAs were analyzed using the EdgeR package (Robinson, McCarthy et al. 2010) in the R software. P values lower than 0.05 and FC <0.75 or >1.5 were considered significantly down or up-regulated, respectively.

Results

Body composition and glucose metabolism

Estropause mice had higher body mass compared to cyclic mice (**Fig. 1-A;** p<0.0001), as well as increased body mass gain after estropause confirmation (**Fig. 1-B,** p=0.0155). Estropause mice also had higher abdominal fat mass (**Fig. 1-C;** p=0.01) compared to CTL mice. Liver weight was not different between groups (**Fig.1-D**; p=0.06).

Lower basal glucose levels were observed for VCD-E2 group compared to the CTL and VCD groups (p=0.0002; **Fig. 2-A**)**.** However, during the ITT, the VCD+E2 group had a higher glucose level at 20 and 35 min (**Fig. 2-B)** and a lower glucose decay rate (p=0.02) compared to CTL and VCD groups. No difference between groups for glucose levels and the area under the curve in GTT was observed (**Fig. 2-C**).

Estropause confirmation

All females injected with VCD were acyclic prior to initiation of E2 treatment, as confirmed by vaginal cytology (5 consecutive days in diestrus). The number of follicles at all stages of development was reduced in estropause compared to cyclic females, and no effect of E2 was observed (**Fig.3**)

miRNA Serum Sequencing

Absolute quantification of exosomes particle had average 166.4 ± 2.3 nm in diameter and concentration was $1.5^{12} \pm 4.5^{10}$ particles/mL (**Figure 4**). A total of 402 miRNAs were detected in the serum exosomes in this study. Heatmap and principal component analysis (**Figure 5)** show a clear distinction in the profile of the three groups. The differentially regulated miRNAs between CTL, VCD and VCD+E2 groups are shown in **Table 2**. There were eight miRNAs differentially regulated between CTL and VCD groups, seven miRNAs regulated between CTL and VCD+E2 groups and ten miRNAs regulated between VCD and VCD+E2 groups. The Venn diagram (**Fig. 6**) indicate little overlap in regulated miRNAs is observed

among groups. Only one miRNA (miR-200a-3p) was commonly regulated between CTL/VCD and CTL/VCD+E2, suggesting an effect of VCD not reversed by E2. Two miRNAs (miR-7072-5p and miR-370-3p) were commonly regulated between CTL/VCD-E2 and VCD/VCD+E2, suggesting a role of E2 treatment. miR-874-3p was down-regulated in VCD compared to CTL mice, however it was up-regulated in VCD-E2 compared to VCD mice.

Next, we examined the regulated pathways from the predicted target genes of regulated miRNAs. We observed that the Hippo and TGF-β signaling pathway were targets of down-regulated miRNAs in both VCD groups compared to the CTL group. The FoxO and insulin signaling pathways were targets of up-regulated miRNAs in VCD-E2 mice (**Table 3**). Additionally, inflammation related pathways were targets of up-regulated miRNAs in both VCD groups compared to CTL (**Table 3**).

miRNA expression in ovarian and hepatic tissue

Based on the sequencing results, we select the strongest regulated miRNAs in serum exosomes for validation in ovarian and liver tissue. miR-370-3p (p=0.04), miR-200a-3p (p=0.002), miR-24-1-5p (p=0.04), miR-1249-3p (p=0.05) and miR-451 (p=0.03) were differentially regulated between CTL and VCD-E2 groups in ovarian tissue (**Fig. 7**). miR-200b-3p (p=0.001) was up-regulated in the VCD and VCD+E2 groups compared to the CTL group **(Fig. 7)** and we did not detect miR-7072-5p expression in ovarian tissue.

In the hepatic tissue, only miR-370-3p (p=0.02) was upregulated in the VCD+E2 compared to CTL and miR-7072-5p (p=0.001) in the VCD group compared to CTL and VCD-E2 groups (**Fig. 8**).

Gene expression in ovarian and hepatic tissue

Based on the miRNAs differentially regulated in serum we selected a group of target gene according to pathway analysis. In the ovarian tissue, expression of *Amh* (p=0.03), *TGF*-β (p=0.05), *P53* (p=0.03) and *Mtor* (p=0.03) was down-regulated in the VCD and VCD+E2 groups, while *Foxo3a* (p=0.05) *and Cdkn2a* (p=0.03) were up-regulated in the VCD+E2 group. *Stat3 (p=0.003)* was up-regulated only in the VCD group (**Fig.9**).

In the hepatic tissue *Pi3K* (p=0.02), *mTOR* (p=0.03), *Foxo3a* (p=0.05), *Stat3* (p=0.007), *Cdkn2a* (p=0.04), *P53* (p=0.01*), TGF*β (p=0.05) were up-regulated in the VCD and VCD+E2 groups, while *Zeb1-v3* (p=0.003) was down-regulated in the VCD+E2 group **(Fig. 10).**

Discussion

Serum exosomal miRNAs have been suggested to play a role in intercellular communication (Kosaka, Iguchi et al. 2010) but can also serve as a biomarker for diseases that affect postmenopausal women, such as osteoporosis (Sugatani and Hruska 2013), breast cancer (Kumar, Keerthana et al. 2013), and metabolic diseases (Vinciguerra, Sgroi et al. 2009). The serum miRNA profile can be affected by age independently of disease in humans (Tchernof, Desmeules et al. 2004) and mice (Tara, Souza et al. 2005). Therefore, the use of VCD in young mice to induce estropause enabled us to isolate the effect of ovarian activity in the serum exosomal miRNA profile from the effects of aging. Additionally, as VCD mice retain the ovarian tissue, we can also determine its contributions to the serum miRNA profile, which would not be possible with the use of ovariectomized mice.

We detected a total of 402 miRNAs in serum exosomes. There were eight miRNAs regulated betwee

CTL and VCD mice, six miRNAs regulated between CTL and VCD-E2 mice and nine miRNAs regulated between VCD and VCD-E2 mice. Only miR-200a-3p and miR-200b-3p were up-regulated in both serum exosomes and ovarian tissue, suggesting that these exosomal miRNAs could be associated to ovarian activity. miR-370-3p and miR-24-1-5p were regulated in opposite directions between serum and ovarian tissue. No common miRNAs were observed when we compared miRNAs regulated in our study with those regulated in pre and postmenopausal women (Kangas, Törmäkangas et al. 2017). This can indicate a divergence by species or even confounding effects of age at natural menopause in women when comparing to chemically induced ovarian inactivity in very young mice. Our model is interesting as it dismisses the age factor as a variable. In the liver, only miR-370-3p was up-regulated as in serum exosomes from E2 treated mice. Among the pathways commonly regulated by serum exosomalmiRNAs, Hippo and TGF-β signaling pathway were targets of down-regulated miRNAs in both VCD groups compared to CTL. FoxO and insulin signaling pathways were targets of up-regulated miRNAs in E2 treated VCD mice. Previous findings (Yan, Yang et al. 2019) indicated that E2 improves insulin sensitivity through activation of the Foxo1 transcription factor in ovariectomized mice. However, we observed that VCD-E2 mice had a slight insulin resistance in the ITT test. In addition, inflammation related pathways were targets of up-regulated miRNAs in both VCD groups compared to CTL. Circulating pro-inflammatory cytokines and low-grade systemic inflammation are known to be associated with aging (Franceschi, Capri et al. 2007). Inflammation is increased in VCD treated rats (Muhammad, Goode et al. 2009), as well as ovariectomized mice (Rogers, Perfield et al. 2009), further indicating the role of exosomal miRNAs in mediating this process.

miR-200a-3p and 200b-3p were increased in serum exosomes of VCD and VCD-E2 compared to CTL mice. These same miRNAs were the up-regulated in ovarian, but not liver tissue. This suggest these miRNAs can be secreted from the ovary to circulation, and serve as potential biomarkers of ovarian activity, although further studies are needed to validate this. Interestingly, the miR-200 family is also involved in folliculogenesis and steroidogenesis (Alvi, Zayed et al. 2021), further suggesting its association to follicle exhaustion. Upregulation of miR-200a-3p and miR-200b-3p has been observed in the ovaries of old compared to young mice before (Kim and You 2022). In our study all mice had the same age, despite VCD mice had follicle depletion, suggesting that the ovarian tissue of our mice was aged. Nevertheless, our data suggest that miR-200 is a marker of ovarian aging, both locally and systemically, not affect by E2 treatment. The miR-200 family is also involved in the diagnosis and prognosis of ovarian and breast cancer (Li, Fang et al. 2015). In ovarian tissue, miR-200 is upregulated in ovarian tumors compared to normal ovarian tissue (Choi and Ng 2017). This is interesting, as the risk of ovarian cancer increases dramatically in postmenopausal women (Rodriguez, Calle et al. 2002), suggesting increased miR-200 expression can be involved in its pathogenesis. We identified *Zeb1* and *Zeb2* as direct targets of miR-200 family and its expression was not affected in ovarian tissue. In the liver, however, *Zeb1* expression was lower in the VCD+E2 compared to CTL mice. A previous study predicted ZEB1 and ZEB2 as genes regulated by serum exosomal miRNAs in postmenopausal women (Kangas, Törmäkangas et al. 2017), although miR-200a-3p and miR-200b-3p were not among the regulated miRNAs (Kangas, Törmäkangas et al. 2017). These genes are members of the zinc finger family and act as transcription factors (Choi and Ng 2017) and are associated with the development and progression of cancer, including epithelial ovarian cancer (Choi and Ng 2017). The identification of these miRNAs and genes could be a crucial step in identifying serum markers for ovarian function and disease biomarkers for postmenopausal women.

miR-370-3p was increased in serum exosomes of E2 treated mice in comparison to both CTL and VCD mice. Expression of miR-370-3p was reduced in the ovary and increased in the liver of VCD-E2 compared to CTL mice. This pattern of regulation suggests that miR-370-3p expression is regulated by

E2 treatment and may not be of ovarian source, as no difference between CTL and VCD mice was observed. miR-370-3p regulates steroidogenic factor 1 (SF1) and was reduced in serum of women diagnosed with endometriosis (Hu, Mamillapalli et al. 2019). SF1 is a transcription factor that regulates E2 synthesis (Attar, Tokunaga et al. 2009), which further suggest a role for miR-370-3p in response to E2. miR-7072-5p was also up-regulated in serum exosomes of E2 treated mice in comparison to both CTL and VCD mice. We did not detect miR-7072-5p expression in ovarian tissue and in the liver it was strongly up-regulated only in VCD compared to both CTL and VCD-E2 mice. We found no evidence in the literature for expression of miR-7072-5p in liver or ovarian tissue, nor its association to menopause and aging, highlighting the need for further studies. Predicted targets genes of miR-7072-5p, include *Akt, Foxo3a, Igf1, Tgf-*β*, Stat3*. We observed that *Tgf-*β and *Stat3* were down-regulated in liver tissue of E2 treated mice and *Foxo3a* was increased in ovarian tissue of E2 treated mice.

We also evaluated the target genes of miRNAs regulated in serum. Changes in the expression of *Igf1, Akt, Foxo3a*, *Tgf-*β*, p53, Mtor*, are known to be associated with age in several tissues (Kim, Bang et al. 2021). However, the pattern of gene expression that we observed in the ovary is not the same we observed in the liver. *Foxo3a*, for example, increased its expression in liver of VCD mice, while in the ovary it was higher in the VCD+E2 compared to CTL females. *Mtor* was down-regulated in the ovary and up-regulated in the liver of both VCD groups. Changes in *Mtor* expression are associated to aging, as mice with reduced *Mtor* expression have increased survival (Wu, Liu et al. 2013), suggesting a negative effect in the liver tissue of our mice. This divergent tissue response can be a consequence of ovarian failure. We previously observed that expression of Foxo3a, mTOR, Pi3k and Akt were reduced in the ovaries of old compared to young mice (Schneider, Zhi et al. 2014). Our current study confirms that some of these changes are associated to follicle depletion rather than age itself, as we observed reduce expression in mice of the same age with depleted ovarian reserve. However, changes in liver tissue gene expression can indicate the negative effects of estropause. For example, *Cdkn2a*, *P53* and *Tgf*β were up-regulated in the liver of VCD mice and are senescence and aging markers (Pruitt, Freeland et al. 2013, Rapisarda, Borghesan et al. 2017, Mylonas, O'Sullivan et al. 2021), suggesting increased aging of the liver tissue of estropause mice.

We observed that VCD induced the cessation of cyclicity in females two months after the end of VCD injections, consistent with findings by others (Mayer, Pearsall et al. 2002, Chen, Perez et al. 2014). In addition to cytology, the reduction in the number of follicles at all stages of development and decreased ovarian *Amh* expression, confirmed that all VCD females were acyclic before the start of E2. VCD females had increased body and fat mass compared to control females, which could be due to low estradiol levels, as previous observed for VCD treated mice (Ávila, Zanini et al. 2023) and menopausal women (Lovejoy, Champagne et al. 2008) . However, others observed that E2 treatment can decrease weight and fat gain using the OVX model (Roesch 2006). We observed lower basal glucose levels in the E2-supplemented group, as reported before in mice (Qi, Guo et al. 2018) and humans (Depypere, Dierickx et al. 2020). VCD+E2 females also had higher uterine mass compared to CTL and VCD females as expected, confirming the effectiveness of exogenous E2 administration (Koebele, Nishimura et al. 2020). It is important to highlight that changes observed in serum exosome miRNAs can be also associated to other metabolic changes observed. For example, VCD females had increased body mass gain, and others suggested serum miRNA are regulated by obesity in mice (Ji and Guo 2019). Therefore, functional studies must be performed in order to establish a causal association between these miRNAs and ovarian activity

Conclusion

Our integrative analysis showed that ovarian activity can modulate miRNA-mediated epigenetic modifications independently of age. This could be a crucial step in identifying serum markers of ovarian function and potential candidates for therapeutic replacement therapies for postmenopausal women. Our results suggest that VCD induced estropause and E2 replacement have an impact in the profile of serum exosomal miRNAs. The miR-200 family was increased in serum exosomes and ovarian tissue of estropause mice and may be a potential candidate biomarker of ovarian function.

Data availability
The data is available from the corresponding author upon request.

Competing interests
The authors declare no competing interests related to this work.

Tables and figures
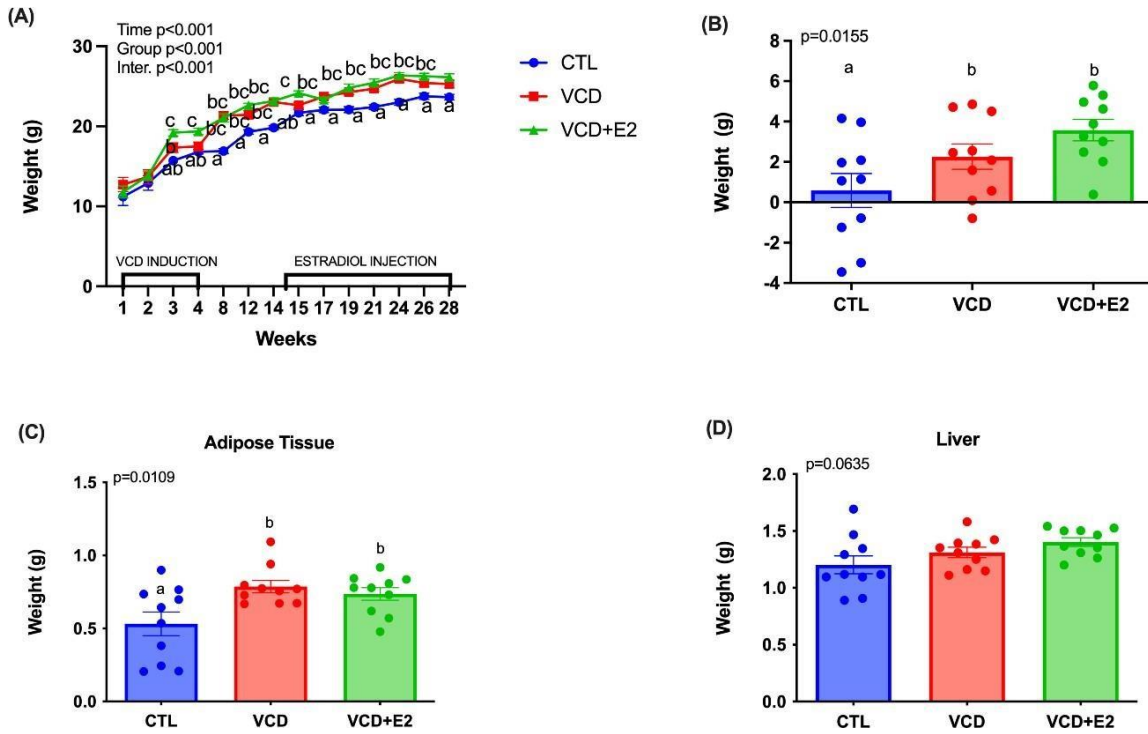
Figure1. (A) Body weight during the experiment period for control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice. VCD was injected from week one to four to induce estropause. At week 11, estrogen therapy was started. (B) Body weight gain from confirmation of estropause to the end of the experiment. Weight of (C) adipose (D) and liver tissue. Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at p<0.05.
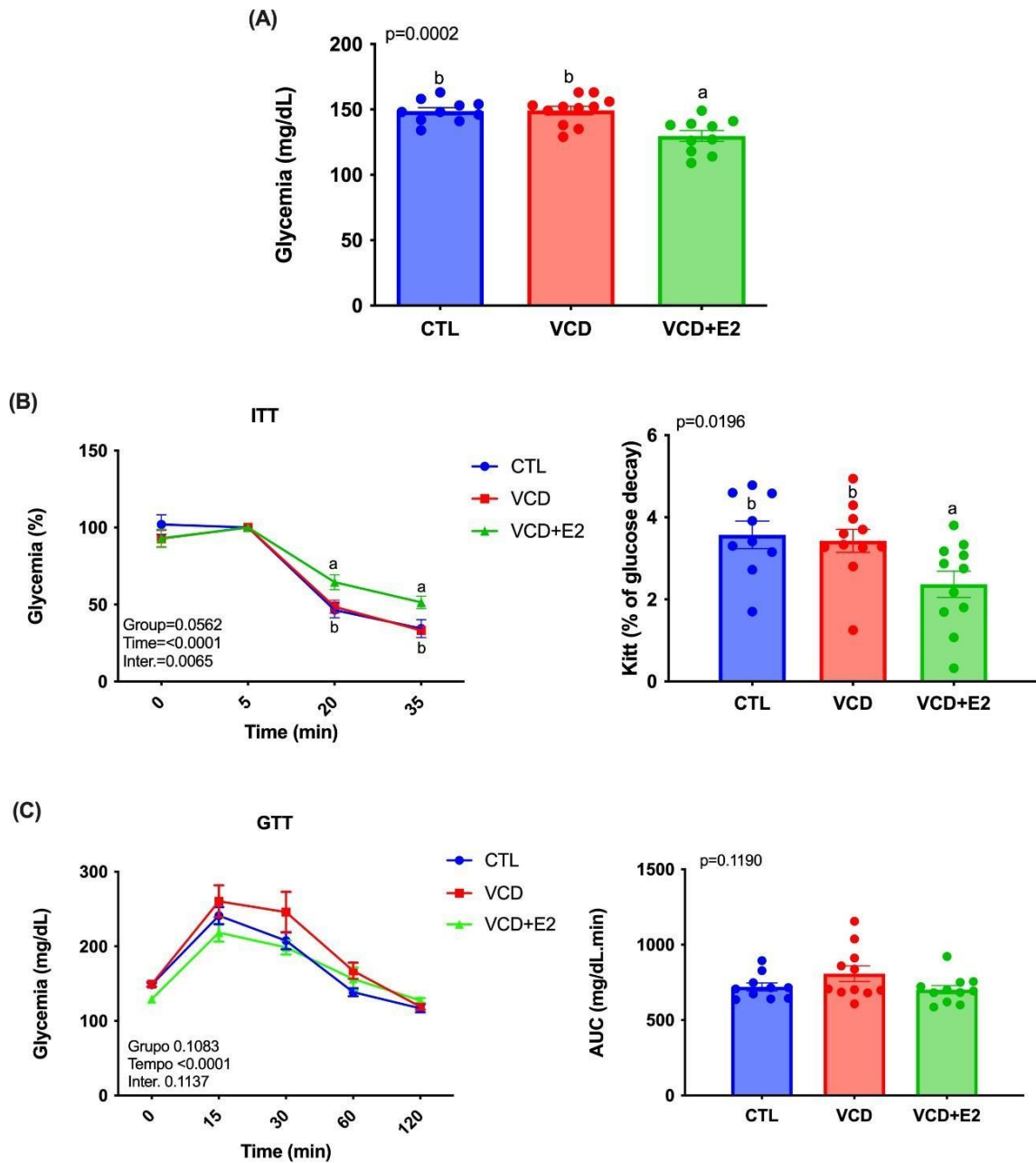
Figure 2. (A) Fasting glucose levels for control (CTL), estropause (VCD) and estropause receiving estradiol (VCD-E2) mice. (B) Insulin tolerance test (ITT) and glucose decay constant (KITT). (C) Glucose tolerance test (GTT) and glucose area under the curve. Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at p<0.05.
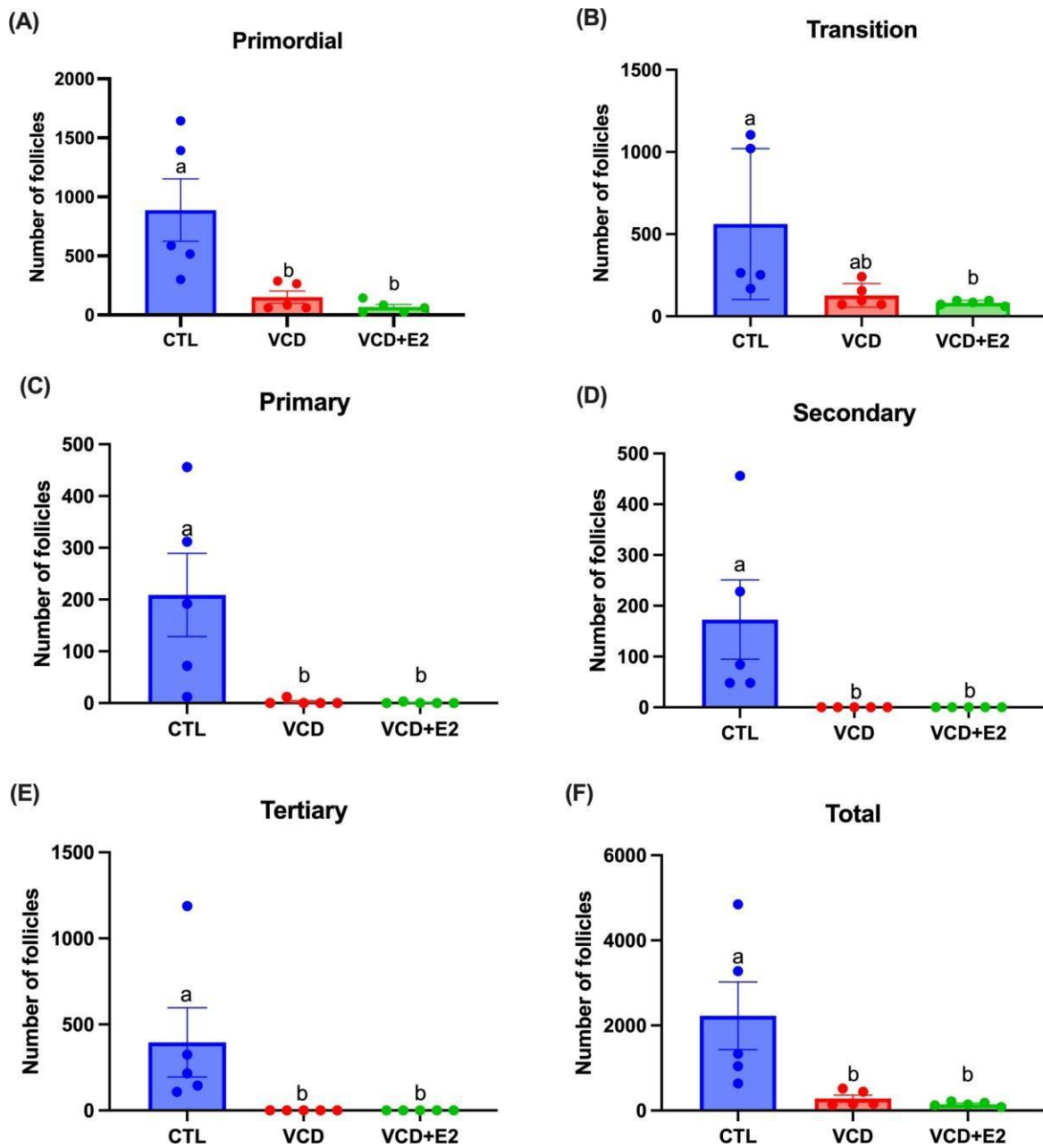
Figure 3. Number of (A) primordial, (B) transition, (C) primary, (D) secondary(E) tertiary, and (F) total follicles per ovarian section for control (CTL), estroupause (VCD) and estropause receiving estradiol (VCD-E2) mice. Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at p<0.05.
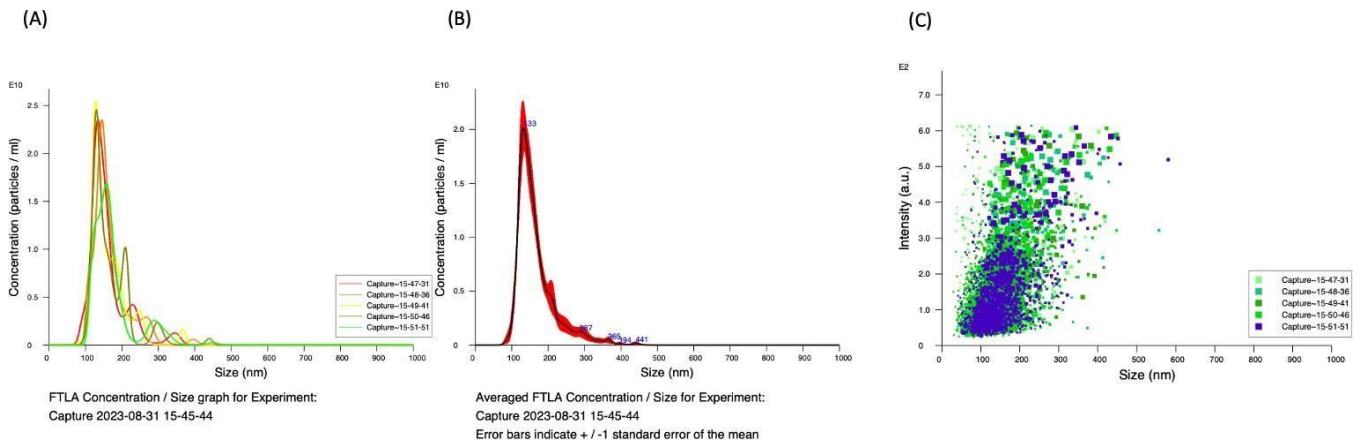
Figure 4. Exosome nanoparticle tracking analysis using NanoSight NS300 system. Five videos (60 s duration each) of Brownian motion of nanoparticles were recorded and analyzed. The samples were measured with manual shutter and gain adjustments. The sample are from exosomes extracted from mouse serum. (A) and (B) show size and concentration and (C) size and intensity.
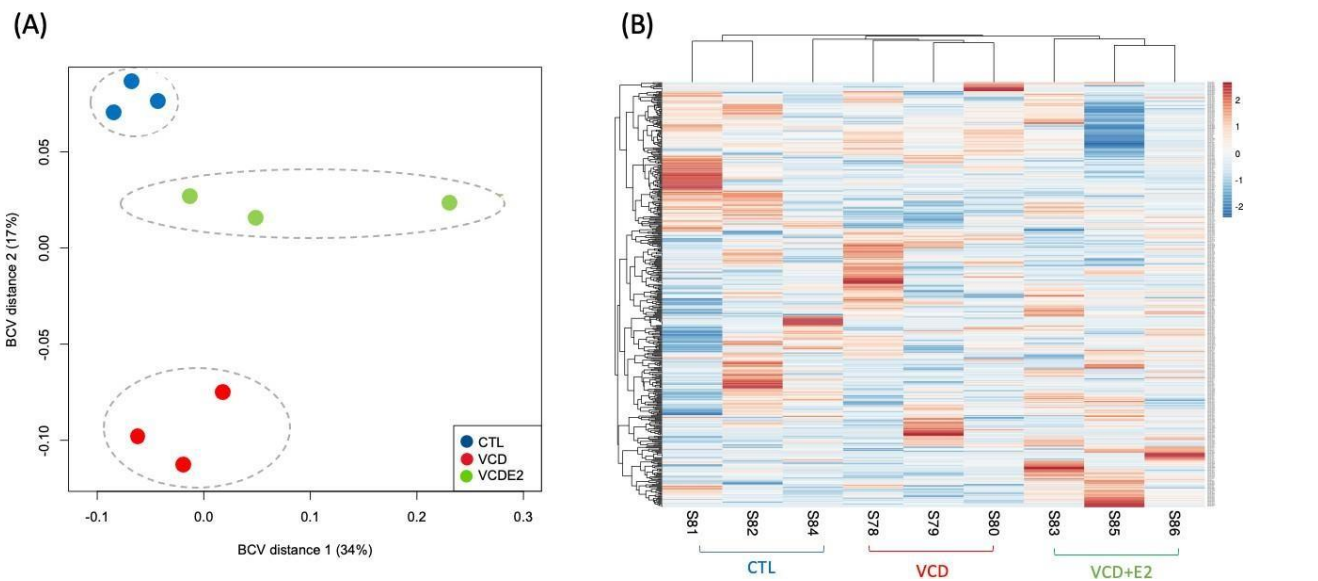
Figure 5. (A) Principal component analysis of serum exosomal miRNAs for control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice (B) Unsupervised hierarchical clustering ordered by the adjusted level of miRNA expression for all expressed miRNAs in serum.
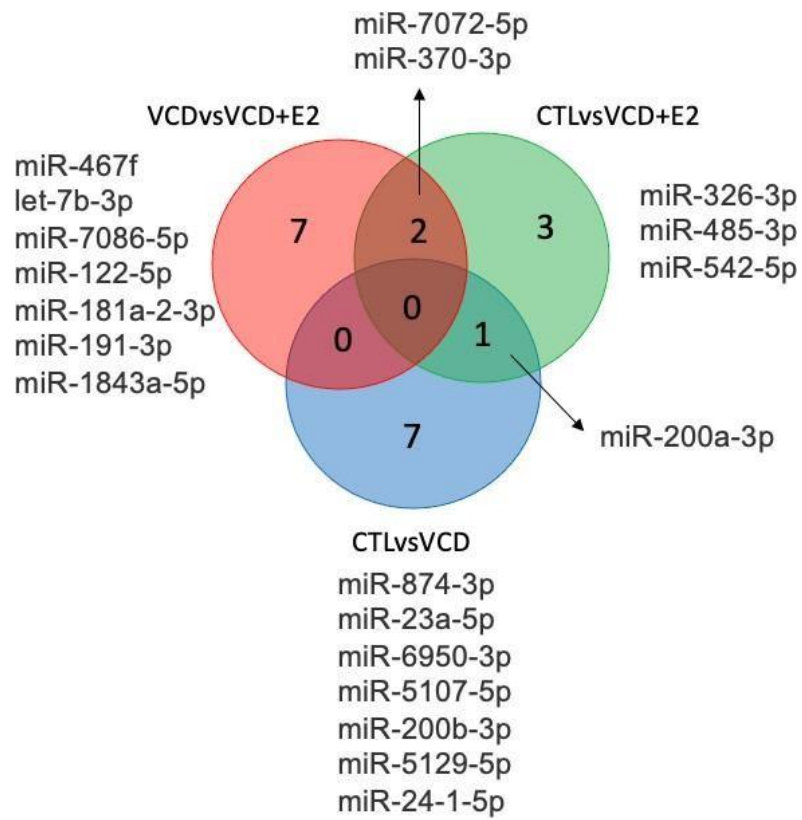
Figure 6. Venn diagram indicating miRNAs significantly regulated miRNAs commonly regulated between control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice.
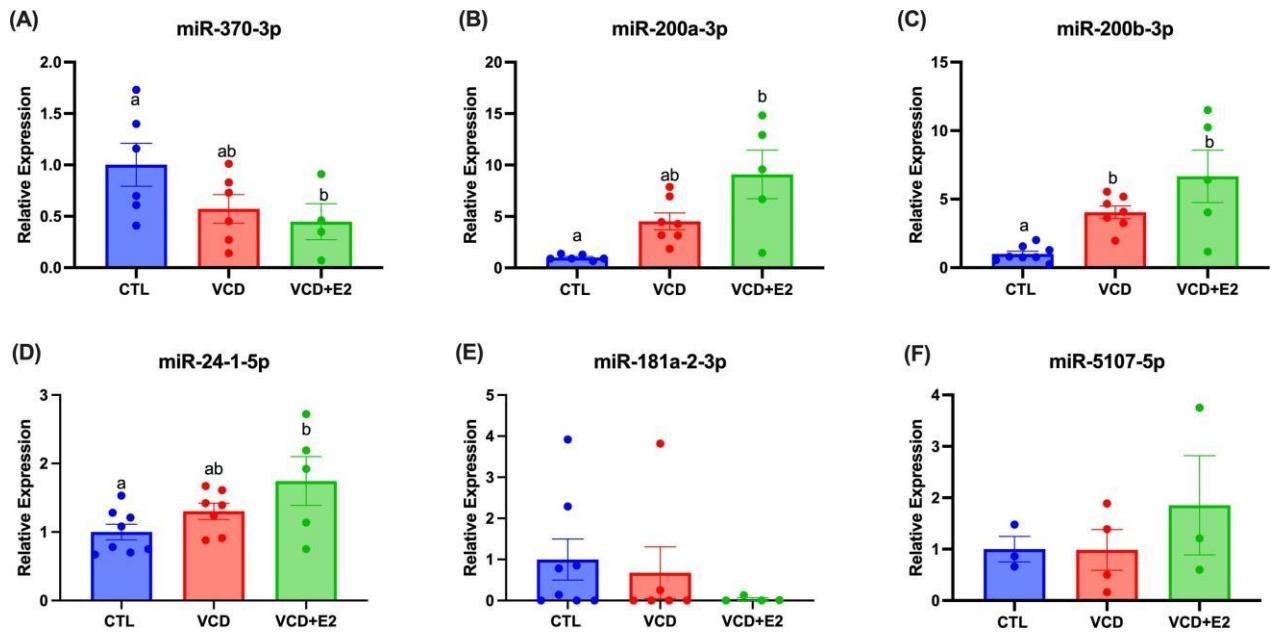
Figure 7. Expression of miRNAs in the ovarian tissue for control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice. Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at p<0.05.
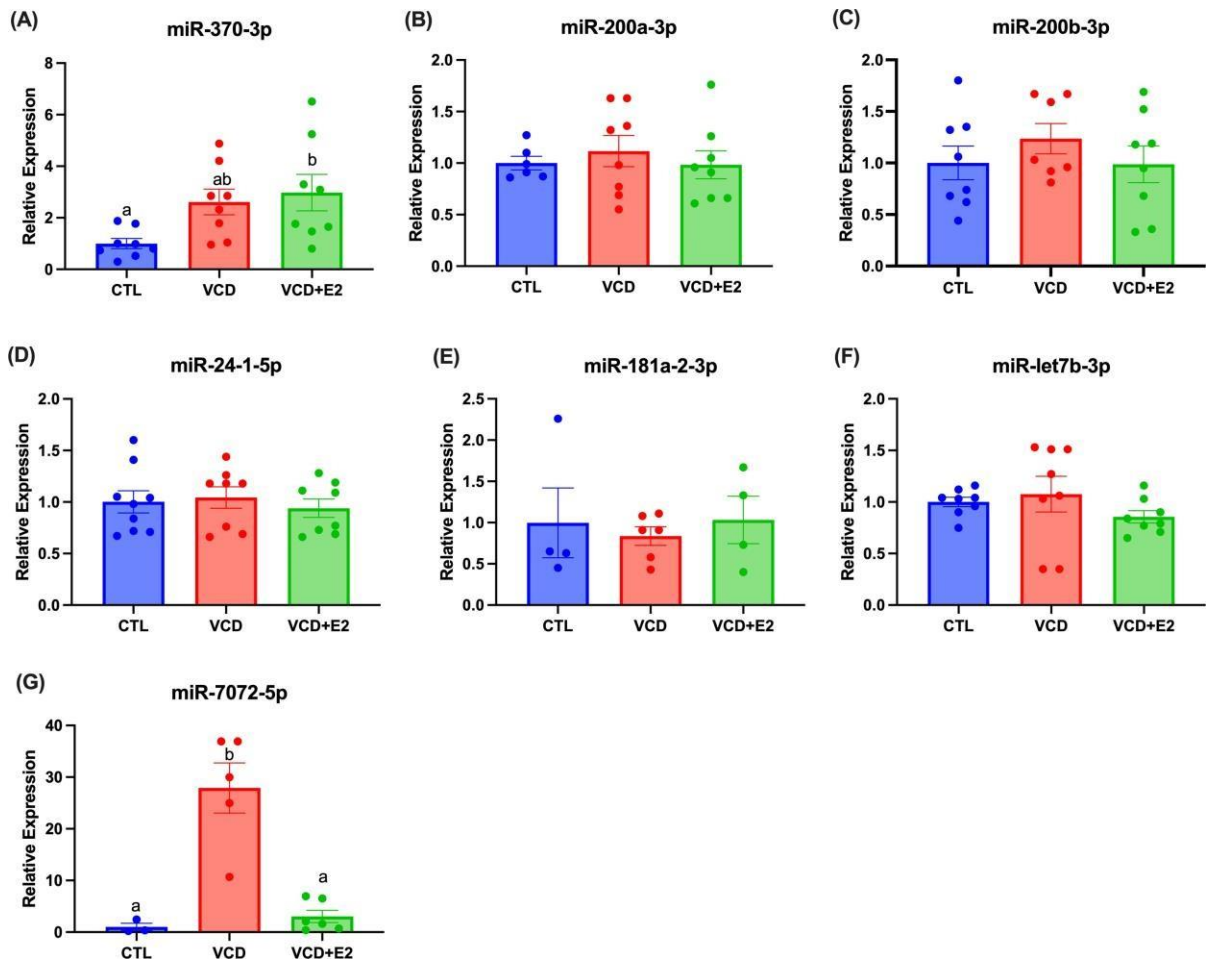
Figure 8. Expression of miRNAs in the liver for control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice. Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at $p < 0.05$.
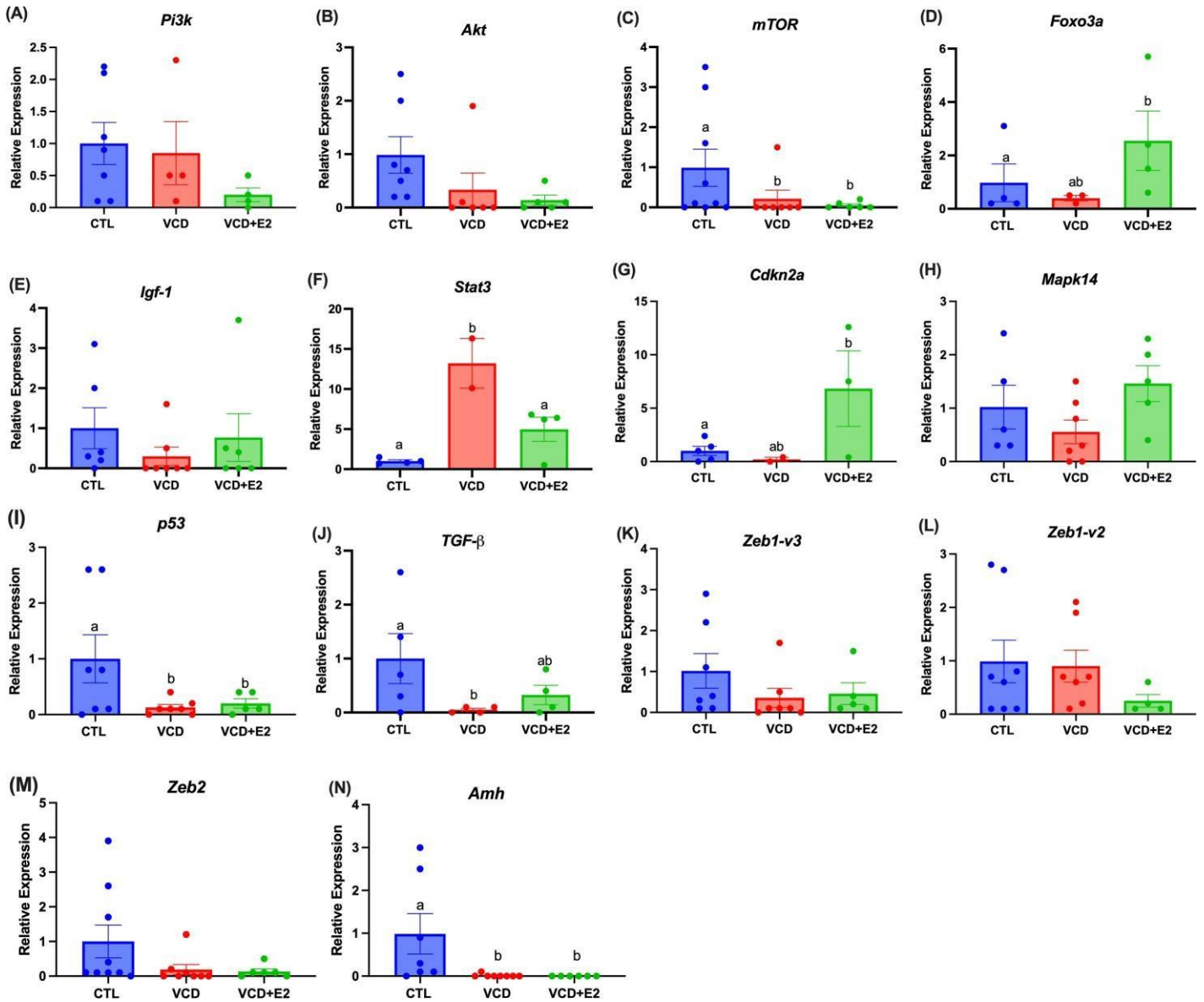
Figure 9. Gene expression in the ovarian tissue for control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice. Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at p<0.05.
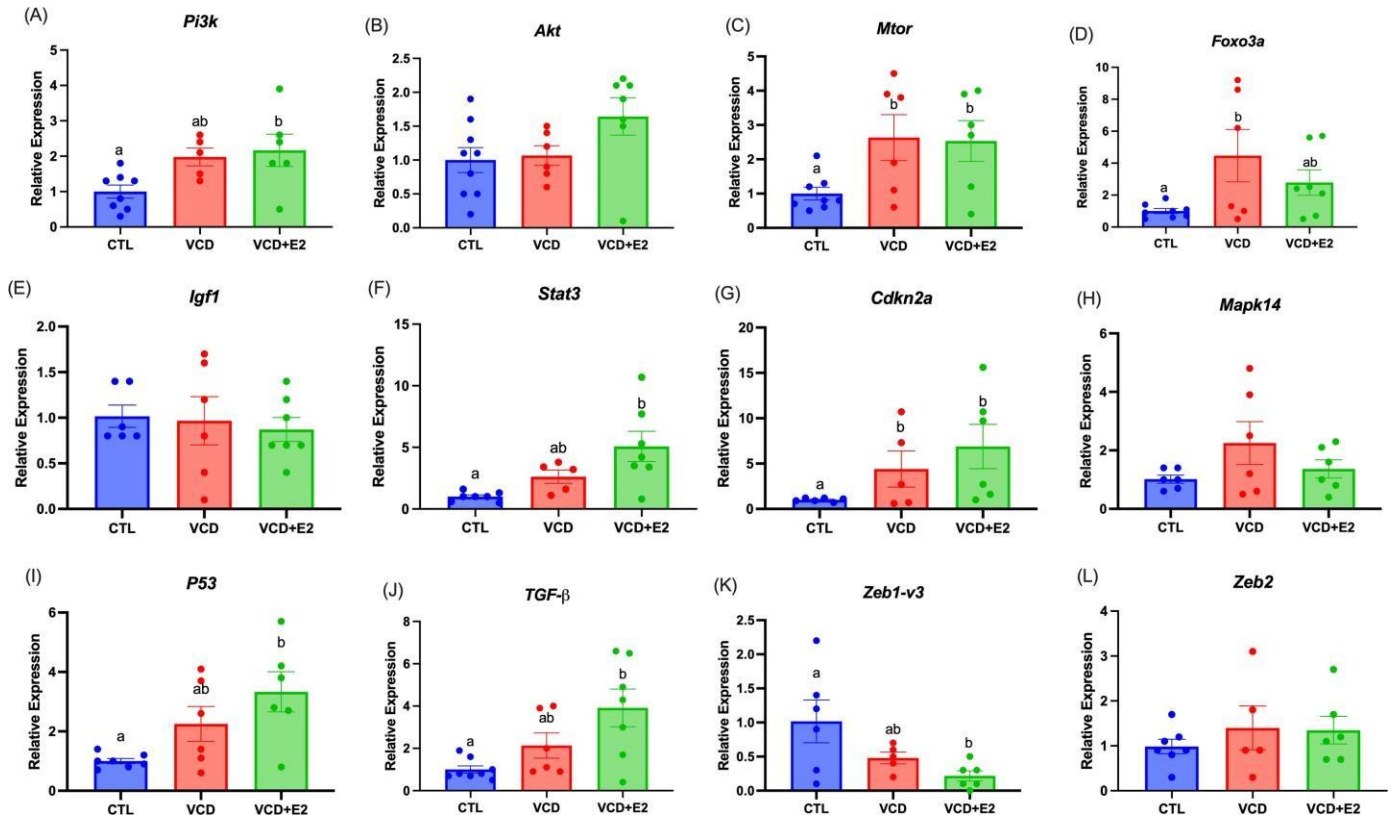
Figure 10. Gene expression in the liver tissue for control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice. Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at p<0.05.

Table 1. Sequence of primers used in RRq-PCR

Primer	Foward	Reverse	NCBI
AKT-1	CCGCCTGATCAAGATGACAGCA	TGATCCATGCGGGGCTTCTG	NM_001409449
AMH	TCCTACATCTGGCTGAAGTGATATG	CAGGTGGAGGCTCTTGGAACT	NM_007445.3
β2m	AAGTATACTCACGCCACCCA	AAGACCAGTCCTTGCTGAAG	NM_009735.3
FOXO3A	TCCCAGATCTACGAGTGGATGG	CCTTCATTCTGAACGCGCAT	NM_001376967.1
IGF-1	CTGAGCTGGTGGATGCTCT	CACTCATCCACAATGCCTGT	NM_001111275.2
IRS-2	AGCCAGGAGACAAGAACTCC	AGTGATGGGACAGGAAGTCG	NM_001081212.2
Mtor	CGGCAACTTGACCATCCTCT	TGCTGGAAGGCGTCAATCTT	NM_020009.2
p16	CCCAACGCCCCGAACT	GCAGAAGAGCTGCTACGTGAA	NM_009877.2
p38	TGGAAGAGCCTGACCTATGA	GACACACACACACACACAAAC	NM_001357724.1
p53	CCATGGCCCCTGTCATCTTT	TGAGGGGAGGAGAGTACGTG	NM_001127233.1
Pi3k	TAGCTGCATTGGAGCTCCTT	TACGAACTGTGGGAGCAGAT	NM_011083.2
STAT3	GATGGGGCTGAGAGCAGAAG	TGAAAGTGCAGAGCCAGGAG	NM_011486.5
TGFβ	GGGGCTGATCCCGTTGATTT	ACTGGAGTTGTACGGCAGTG	NM_011577.2
TNF	ATGAGAAGTTCCCAAATGGC	CTCCACTTGGTGGTTTGCTA	NM_001278601.1
ZEB1-v1	GCGGCGCAATAACGTTACAAA	AAAGCGGTTCTTGAAGGGGT	NM_011546.3
ZEB1-v2	GGAGAGGTGACTGGTTGTGG	GCCACATCAGCAATAGCAGC	NM_001360981.1
ZEB1-v3	GGGTGTGTGCGTGGACTC	GGTCTGCTGGCAGTTCATCA	NM_001360982.1
ZEB2	CCCTTTATGAACGGTGGGCT	TGTCTTCCGTCTTGCAGTCC	NM_001355289.2

AMH (Anti-Mullerian hormone), AKT-1 (Serine/Threonine Kinase 1),β2m (Beta-2-Microglobin), FOXO3a (Forkhead Transcription Factor O Subfamily Member 3a), IGF-1 (Insulin-like growth factor 1), IRS-2 (Insulin receptor substrate-2), Mtor(mammalian target of rapamycin), p16 (Cyclin Dependent Kinase Inhibitor 2A), p38 (Mitogen-activated protein kinase 14), p53 (Tumor protein 53), Pi3k (Phosphatidylinositol-4,5- Bisphosphate 3-Kinase), STAT3 (Signal Transducer and Activator O Transcription 3), Tnfa (Tumor Necrosiss Factor alpha), Tgfb1 (Transforming growth factor beta 1), ZEB1-v1 (Zinc Finger E-Box Binding Homeobox 1 - variant 1), ZEB1-v2 (Zinc Finger E-Box Binding Homeobox 1 - variant 2), ZEB1-v3 (Zinc Finger E-Box Binding Homeobox 1-variant 3), ZEB2 (Zinc Finger E-Box Binding Homeobox 2).

Table 2.miRNAs differentially expressed (p<0.05) for control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice.

miRNA name	CTL vs VCD		CTL vs VCD+E2		VCD vs VCD+E2	
	logFC	pValue	logFC	pValue	logFC	pValue
mmu-miR-24-1-5p	↓ -1.611	**0.001**	-0.620	0.153	0.990	0.099
mmu-miR-874-3p	↓ -0.702	**0.004**	-0.173	0.465	0.530	**0.031**
mmu-miR-6950-3p	-2.966	**0.018**	-2.098	0.054	0.861	1.000
mmu-miR-23a-5p	-1.135	**0.019**	-0.503	0.251	0.632	0.208
mmu-miR-5129-5p	↓ -0.494	**0.038**	-0.256	0.297	0.238	0.405
mmu-miR-5107-5p	↓ -2.184	**0.045**	-1.220	0.196	0.961	0.693
mmu-miR-7086-5p	-0.981	0.057	0.065	0.945	1.046	**0.049**
mmu-miR-467f	-3.871	0.066	0.485	0.782	▲ 4.343	**0.017**
mmu-miR-191-3p	-0.753	0.072	0.143	0.722	▲ 0.895	**0.032**
mmu-miR-181a-2-3p	-0.780	0.221	0.426	0.456	▲ 1.206	**0.030**
mmu-miR-122-5p	0.134	0.281	↓ -0.332	**0.013**	▲ -0.465	**0.000**
mmu-let-7b-3p	- 0.539	0.294	0.574	0.198	↓ 1.113	**0.010**
mmu-miR-542-5p	-0.322	0.443	↓ -0.95	**0.044**	▲ -0.633	0.287
mmu-miR-200a-3p	1.339	**0.008**	1.200	**0.035**	-0.138	0.888
mmu-miR-200b-3p	1.098	**0.026**	-1.133	0.107	-0.684	0.138
mmu-miR-1843a-5p	▲ 0.457	0.233	-0.381	0.365	↓ -0.837	**0.028**
mmu-miR-326-3p	0.387	0.242	0.680	**0.031**	0.293	0.347
mmu-miR-485-3p	0.744	0.256	▲ 1.466	**0.015**	0.724	0.152
mmu-miR-7072-5p	0.109	0.812	▲ 0.873	**0.022**	0.764	**0.041**
mmu-miR-370-3p	0.062	0.853	▲ 0.673	**0.014**	▲ 0.611	**0.023**

Table 3. Enriched KEGG pathways for target genes of differentially expressed miRNAs for control (CTL), estroupause (VCD) and estropause receving estradiol (VCD-E2) mice.

Kegg Patway	p-value	Genes	miRNAs
Hippo signaling pathway	2.9E+02	10	1
Regulation of actin cytoskeleton	1.2E-02	9	1
Axon guidance	3.2E-02	8	
Thyroid hormone signaling pathway	9.8E-03	7	1
TGF-beta signaling pathway	1.2E-02	2	1
	Up regulated miRNAs		
FoxO signaling pathway	1.0E+05	1	1
Intestinal immune network for IgA production	3.3E-03	3	2
Inflammatory bowel disease (IBD)	2.8E-02	1	1
Lysine degradation	4.0E-02	1	1
Hippo signaling pathway	4.8E-02	1	1
CTL vs VCD	**Down-regulated miRNAs**		
Hippo signaling pathway	3.6E-01	7	1
ECM-receptor interaction	3.6E-04	6	2
Viral carcinogenesis	6.7E-01	6	1
TGF-beta signaling pathway	5.2E-01	4	1
microRNAs in cancer	5.8E-01	2	1
	Up regulated miRNAs		
Glycosaminoglycan biosynthesis-heparan sulfate/heparin	5.1E+03	1	1
Intestinal immune network for IgA production	7.8E-03	1	1
Inflammatory bowel disease (IBD)	1.0E-02	1	1
Pathways in cancer	3.1E-02	3	1
Hepatitis B	5.0E-02	3	1
CTL vs VCDE2	**Down-regulated miRNAs**		
Pathways in cancer	1.7E-02	33	1
Hippo signaling pathway	5.9E-06	21	1
Thyroid hormone signaling pathway	1.6E-03	19	1
Cell cycle	2.0E-02	16	1
TGF-beta signaling pathway	2.8E-04	10	1
	Up regulated miRNAs		
Pathways in cancer	4.5E-02	38	2
FoxO signaling pathway	4.7E-01	30	2
Ras signaling pathway	7.3E-03	24	3
Jak-STAT signaling pathway	8.6E-03	20	1
Insulin signaling pathway	8.6E-03	19	3
Estrogen signaling pathway	1.4E-02	12	2

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4.2 Artigo 2

THE ROLE OF EXOSSOMES FROM CYCLIC MICE IN MODULATION OF LIVER METABOLISM IN ESTROUPAUSE MICE

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Abstrat

Exosomes are extracellular vesicles that circulate through the bloodstream to transfer molecular signals from tissue to tissue. The messages conveyed by exosomal RNAs can induce changes in gene expression and protein concentrations in recipient cells. The aim of this study was to characterize the role of exosomes from cyclic mice in modulating liver metabolism in estropause mice. Female C57BL/6 mice were induced to estropause using 4-vinylcyclohexene diepoxide (VCD). VCD-treated mice were divided in control group and treatment group, with the latter receiving 10 injections of exosomes at 3-day intervals. Exosomes were extracted from the serum of untreated cyclic mice of the same age. Mice in estropause had higher body mass compared to cyclic mice (p=0.04). We observed ten miRNAs differentially regulated between CTL and VCD mice, fifteen between CTL and EXO, and none between VCD and EXO. Commonly regulated between VCD and EXO compared to the CTL group, were observed miR-103-3p and miR-320-3p down-regulated, and miR-150-5p and miR-20a-3p up-regulated. In the liver tissue we observed more genes regulated between EXO and VCD groups. This highlights a significant regulation of exosomes on hepatic transcriptome. Some pathways were exclusively regulated in EXO compared to VCD mice, suggesting a direct effect of EXO treatment. Among those PPAR signaling pathway, Antigen processing and presentation, B cell receptor signaling pathway, T cell receptor signaling pathway, apoptosis, cytosolic DNA-sensing pathway, NOD-like receptor signaling pathway, and *VEGF* signaling pathway. Our findings represent a starting point for the use exo-miRs as regulators ofmetabolic health in acyclic females.

Introduction

Exosomes are nanometric extracellular vesicles (<100nm in diameter) that circulate in the bloodstream to transfer molecular signals from tissue to tissue. Exosomes are nanometric extracellular vesicles (<100nm in diameter) that circulate in the bloodstream to transfer molecular signals from tissue to tissue. (Clayton, Turkes et al. 2004, Yang and Robbins 2011, Luga, Zhang et al. 2012). These spherical structures are surrounded by a lipid bilayer and carry an active biological content, including proteins, lipids, and RNA (Théry, Zitvogel et al. 2002, Valadi, Ekström et al. 2007). The content of exosomes is influenced by their origin and the physiological condition of the secreting cell (Guduric-Fuchs, O'Connor et al. 2012, Nolte-'t Hoen, Buermans et al. 2012). RNAs inside secreted exosomes can induce changes in the activity of recipient cells, suggesting the functionality of its cargo in target cells (D'Anca, Fenoglio et al. 2019). Studies demonstrate that isolating exosomes from the serum of young mice and injection them into old mice can affect expression of age-related genes (Lee, Kim et al. 2018, Yang, Cong et al. 2020). Therefore, it is suggested that exosomes play an active role in the regulation of aging.

MicroRNAs (miRNAs) are small RNAs (19-25 nucleotides) that regulate the stability or translation of messenger RNA (mRNA) and, consequently, gene expression (Luense, Carletti et al. 2009). Specifically, the content of microRNAs in exosomes (exo-miRs) to modulate signaling pathways in distant target cells has been investigated. miRNAs originating from a specific blood fraction, such as cells (Raposo and Stoorvogel 2013), serum (Arroyo, Chevillet et al. 2011) or microvesicles (Turchinovich, Weiz et al. 2012), chages in old compared to young mice. Therefore, exo-miRs have been proposed as non-invasive biomarkers for aging and age-related diseases (Rani, Ryan et al. 2015, Basu and Ludlow 2016) and can provide evidence of pathways to reverse age-related conditions.

Associated with aging, there is a decline in ovarian activity that leads to female infertility with advancing age. The ovarian reserve is finite its depletion results in the end of reproductive life, referred to as menopause in women (Te Velde, Scheffer et al. 1998). Early onset of menopause is associated with accelerated epigenetic age (Wellons, Ouyang et al. 2012). Women with early menopause have a shorter life expectancy, considering mortality from various causes (Jacobsen, Heuch et al. 2003). Specifically, women with early menopause have a higher risk of death from cardiovascular diseases (Jacobsen, Knutsen et al. 1999). This suggests that the ovary can actively influence overall female health. In mice, ovariectomy reduces longevity (Benedusi, Martini et al. 2015), while the transplantation of young ovaries into old females increased longevity (Mason, Cargill et al. 2009). Additionally, the transplantation of young ovaries with depleted germ cells further increased the life expectancy of the recipient females (Habermehl, Parkinson et al. 2019). This raises the question of whether factors other than estradiol produced by active ovaries may be responsible for this protective effect. Recently, our group conducted a study (unpublished data - Zanini, B et al., 2024), demonstrating that chemically induced estropause and estradiol replacement had an impact on the profile of serum exosomal

miRNAs. This indicates that changes in the exo-miR profile may regulate metabolism in females experiencing estropause.

These evidence suggest that the young ovary produces intrinsic factors that can prevent aging, which becomes especially important when ovarian activity is reduced. Given that the vast majority of women undergo a natural transition to menopause while retaining ovarian tissue, the compound 4-vinylcyclohexene diepoxide (VCD) can be used to induce a better menopausal model compared to ovariectomy in mice, as it maintains an intact reproductive system but with follicular depletion (Kappeler and Hoyer 2012, Liu, Wang et al. 2015). VCD is an industrial chemical residue and has been used to reduce the fertility of rodents (Mayer, Pearsall et al. 2002, Bhardwaj and Saraf 2014). The follicular loss induced by VCD in rodents occurs through the acceleration of the normal atresia process, which occurs via apoptosis (Reis, Pestana-Oliveira et al. 2014).

Since their discovery, exosomes have sparked interest, and their investigation has grown. Exosomes can have therapeutic potential due to their low immunogenicity and toxicity, and serve as a drug delivery platform (Alvarez-Erviti, Seow et al. 2011). Advancements in nanotechnology enable the encapsulation of therapeutic agents, such as chemotherapeutic drugs, small molecules, miRNAs, and siRNAs, in exosomes. (Alvarez-Erviti, Seow et al. 2011, Cooper, Wiklander et al. 2014). In ovarian cancer, exosomes derived from adipose mesenchymal stem cells inhibited the cell proliferation of human ovarian cancer cells, blocking the cell cycle and activating mitochondria-mediated apoptosis (Reza, Choi et al. 2016). Although there are still many challenges for their clinical use, such as large-scale manufacturing, cell sources, and target specificity, exosome engineering remains a promising therapeutic strategy for treatment of several diseases. The administration of exo-miRs also has therapeutic potential in neurodegenerative diseases (Frühbeis, Fröhlich et al. 2013), inflammatory (Lin, Li et al. 2015) and cardiovascular (Waldenström and Ronquist 2014). These are diseases commonly observed in postmenopausal women. Therefore, our aim was to characterize the role of exosomes derived from cyclic females in modulating post-estropause liver metabolism in estropause mice.

Methodology

Animals and experimental design

This study was approved by the Animal Experimentation Ethics Committee from Universidade Federal de Pelotas (number 1379560). For this, C57BL/6 female mice (1-month-old, n=181) were kept under controlled conditions of temperature and light (22± 2ºC, cycles 12 hours light/12 hours dark) and provided with *ad libitum* access to a standard chow and water. Mice were randomly allocated to a control and experimental group to receive VCD and exosomes (**Figure 1**). Females (n=21) were treated daily with intraperitoneal injection of VCD (160 mg/kg) for 20 days to induce estropause, while control females (n=10) received placebo (sesame oil) at the same frequency.

Exosome extraction and injection

At 6 months of age, 70 untreated control females were euthanized to collect serum for exosomes extraction. Exosomes were isolated from serum using a commercial kit (Total Exosome Isolation Reagent from serum, Invitrogen, Vilnius, Lithuania) following the manufacturer's protocol. Briefly, a buffer was added to the serum samples and incubated at 5°C for 30 minutes. Precipitated exosomes were recovered by centrifugation at 10,000 xg for 10 minutes. The pellet was resuspended in phosphate-buffered saline (PBS).

Exosomes were extracted from serum of individual mice and combined in a tube for dilution and measurement of protein and RNA concentration. Each mouse in the VCD exosome treated group received one injection every three days, in a total of 10 injections/mice in a 30-day interval (100 µL/mouse). Mice were divided in control group (CTL; n=10), estropause control group (VCD; n=11) and estropause treated with exosomes group (EXO; n=10**).** Body weight was evaluated every two weeks from the beginning of VCD treatment to the end of the experiment.

The amount of body fat was determined by weighing the intraabdominal fat tissue immediately after euthanasia, relative to body weight. After 24hr of the last exosome injection mice were anesthetized using isoflurane and euthanasia was performed following a 4-hour fasting period. Exsanguination was performed by cardiac puncture followed by cervical dislocation. Mice were dissected, and ovaries, visceral fat and liver were collected and stored in 10% buffered formalin and at -80ºC. Blood samples were centrifuged at 10000xg for 10 min for serum separation.

Exosome Characterization

Particle concentration and size distribution of extracted exosomes from cyclic mice were determined by NTA using a NanoSight NS300 with 488 nm laser set at 25°C and camera level 11 (Malvern instruments). The NanoSight sample pump was used in conjunction with 1 mL syringes at flow speed of 30. Samples were run immediately after dilution in 0.1um filtered HPLC grade water. Each analysis consists of five 60-

second video captures per sample (University of Florida – ICBR, Cytometry Core Facility; RRID:SCR_019119).

Part of the isolated exosomes was stained with SYTO™ RNASelect™ Green Fluorescent Cell Stain (Invitrogen - S32703), following the manufacturer's instructions. Exosomes were stained one day before administration as overnight incubation was necessary. Mice (2 EXO and 1 CTL) were euthanized 24 hours post-injection, and blood and adipose tissue were collected. Both were freshly fixed on slides and subsequently visualized using a confocal fluorescence microscope (FluoView 1000; Olympus).

Vaginal Cytology

Vaginal smears were obtained 60 days after the end of VCD injections in CTL, VCD and VCD-EXO groups (n=31). Daily smears were performed at the same time for five consecutive days. The collected material was stained using rapid panoptic. Females in diestrus all five days were considered to be in estropause.

Insulin Tolerance Test

Insulin tolerance test was performed one day before euthanasia. For the insulin tolerance test (ITT), mice received i.p. injection of insulin (0.5 IU/kg body weight) after a 2-hour fast. Blood was collected 0, 5, 20, and 35 minutes after injection, and glucose levels were measured using a glucometer. The percentage of glucose decay between 5 and 20 minutes after insulin injection was calculated.

Follicle counting

The ovaries were removed from 10% buffered formalin, dehydrated in alcohol, cleared in xylene, embedded in paraffin, and then cut into 5 µm sections using a semiautomatic microtome (RM2245 38, Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK). One out of every six sections were selected and placed on standard histological slides. After drying in a 56 °C oven for 24 h, the slides were stained with hematoxylin and eosin and mounted with coverslips and synthetic resin (Sigma Chemical Company®, St. Louis, MO, USA). Images of the ovarian sections were captured using microscope (Nikon Eclipse E200, Nikon Corporation, Japan) with 4×, 10×, and 40× objectives. Follicles with clearly visible oocyte nuclei were quantified. The number of follicles counted was divided by the total number of sections for each ovary. The follicle classification protocol was based on Myers et. al. (2004).

miRNA Library Preparation, Sequencing and Processing

For exosome RNA extraction, a commercial kit for total RNA and protein isolation from exosomes (Total Exosome RNA & Protein Isolation Kit, Invitrogen, Vilnius, Lithuania) was used. The protein was extracted from exosomes and measured using a commercial kit (Pierce BCA Protein Assay) according to the manufacturer's instructions. In the same samples, total RNA was measured using Qubit (ThermoFisher).

miRNA libraries from total RNA from serum exosomes of CTL, VCD and EXO mice were prepared using the NEXTFLEX® Small RNA-Seq Kit V4 (Perkin Elmer, Seer Green, Beaconsfield, United Kingdom) and submitted to sequencing in a Illumina NovaSeq X Plus instrument. Alignment and quantification of miRNA libraries was performed using cutadapt and bowtie2 (Martin 2011) as described before (Saccon, Schneider et al. 2021). Statistical analyses for differentially expressed miRNAs was performed using the software R (4.3.2) and the Bioconductor package EdgeR. Read counts were normalized for library depth, and pairwise comparisons, measuring fold change, uncorrected P-values from the negative binomial distribution, and adjusted P values (false discovery rate; FDR) were obtained. Principal components analysis (PCA) was also performed using R to observe sample distribution in a two-dimensional plot. miRNAs with a FDR<0.1 and fold change (FC) >2.0 were considered upregulated; and with FDR<0.1 and FC<0.5 were considered down-regulated.

The mirPath tool (version 3.0) was used to predict target genes of the differentially regulated miRNAs using the microT-CDS v. 5.0 database (Vlachos, Zagganas et al. 2015) and for retrieving KEGG molecular pathways (Kanehisa and Goto 2000, Kanehisa, Sato et al. 2016), considering p values < 0.05 as significant. For pathway analysis, miRNAs were analyzed separately as down- or up-regulated.

<u>mRNA Library Preparation, Sequencing and Processing</u>
Hepatic tissue was collected from CTL, VCD and EXO mice 24 hours after the last injection, and total RNA was extracted using the TRIzol protocol. RNA was dissolved in 20 µL RNAse free water and its concentration and quality were estimated by spectrophotometry (EpochTM Microplate Spectrophotometer, BioTek, Winooski, VT, USA). Libraries were prepared using poly-A selected mRNA library using the xGen RNA library preparation kit (Integrated DNA Technologies, IA, USA) with NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, USA). Library sequecing was performed in a NovaSeq X Plus equipment.

The mapping of sequencing reads to the mouse transcriptome was performed using HiSat2 (Pertea, Kim et al. 2016). The number of reads aligned to its corresponding gene was calculated by HTSeq 0.6.1 (Anders, Pyl et al. 2015). Statistical analyses for differentially expressed mRNAs was performed using the software R (4.3.2) and the Bioconductor package EdgeR using the HTSeq output count. Read counts were normalized for library depth, and pairwise comparisons, measuring fold change, uncorrected P-values from the negative binomial distribution, and FDR were obtained. PCA was also performed using R to observe sample distribution in a two-dimensional plot. Genes with a FDR<0.1 and FC>2.0 were considered upregulated; and with FDR<0.1 and FC<0.5 were considered down-regulated. mRNAs were further processed for pathway analysis using the Generally Applicable Gene-set Enrichment (GAGE), which uses log-based fold changes as per gene statistics, and Pathview packages in R (Luo and Brouwer 2013). P values lower than 0.05 were considered significant for pathways and GO Terms analysis.

Statistical Analyses
Statistical analyses were performed using GraphPad Prism 10. One-way ANOVA was used for follicle count, glucose metabolism, and gene expression analyses. Repeated measures ANOVA was used for body mass analysis. P values lower than 0.05 were considered statistically significant.

Results

Body composition and insulin metabolism
Mice in estropause had higher body mass compared to cyclic mice (**Fig. 2A**; p=0.04), however, exosomes did not affect body weight (**Fig. 2B**). The VCD and EXO groups had lower uterine weight (**Fig. 2-C**; p<0.0001), as well as lower peri-ovarian fat (**Fig. 2D**; p=0.01). Perigonadal adipose tissue (**Fig. 2E**; p=0.07), peri-renal (**Fig. 2F**; p=0.08), kidney (**Fig. 2G**; p=0.65), and liver (**Fig. 2H;** p=0.18) were not different between groups.

Higher basal glucose levels were observed in the EXO (**Fig., 3A**; p=0.02) compared to the CTL group. However, during the ITT, the rate of glycemic decay was not different between groups (**Fig. 3B and 3C**; p=0.38).

Estropause confirmation
All females injected with VCD were acyclic before the start of exosome treatment, confirmed by vaginal cytology (5 consecutive days in diestrus). The number of follicles in all stages of development was reduced in estropause mice, especially with no secondary and tertiary follicles observed **(Fig. 4)**. No effect of exosomes on follicle numbers was observed.

Exosome characterization
We observed isolated and stained exosomes were transferred to blood and adipose tissue after intraperitoneal injection by fluorescent emission **(Fig. 5)**. The absolute quantification of exosome particles had an average diameter of 166.4 ± 2.3 nm, and the concentration was 1.68E+10 ± 9.31E+08 particles/mL **(Fig. 6)**. The protein content was 19,472.66 µg/mL and 78 ng/ml of RNA after the extraction of exosome content.

miRNA Serum Sequencing
A total of 355 miRNAs were detected in serum. The heatmap and PCA **(Fig. 7A-B)** illustrate the small difference in the profile of the three groups. Number of differentially regulated miRNAs among CTL, VCD, and EXO groups is shown in the Venn diagram **(Fig. 8).** There were ten differentially regulated miRNAs between CTL and VCD, fifteen between CTL and EXO, and none between VCD and EXO. Commonly regulated miRNAs between VCD and EXO compared to the CTL group are down-regulated miR-103-3p and miR-320-3p, and up-regulated miR-150-5p and miR-20a-3p.

Next, we examined the pathways regulated by the predicted target genes of regulated miRNAs. We observed that the EXO group had 45 pathways for down-regulated miRNAs compared to the CTL group, while the VCD group regulated only 11 pathways compared to the CTL group. The top 10 pathways regulated by these miRNAs are described in **Table 1.**

Gene expression in hepatic tissue

We performed mRNA sequencing to compare expression patterns in liver of CTL, VCD and EXO mice. We identified 13,832 genes expressed in liver **(Fig. 9).** Nineteen genes were regulated between the CTL and VCD groups, with 8 up-regulated and 11 down-regulated. In the EXO group, 270 genes were regulated compared to CTL group, with 158 up-regulated and 112 down-regulated. In the VCD and EXO group, a total of 1285 genes were regulated, with 767 up-regulated and 518 down-regulated **(Fig. 10-A).** This highlights a significant regulation of liver transcriptome by exosomes. The top 10 regulated genes among groups is shown in **Table 2**. KEGG pathway analysis revealed that up-regulated genes in VCD compared to CTL mice were involved in endocytosis, protein coding and signaling processes, insulin signaling, and amino acid biosynthesis. Meanwhile, up-regulated pathways in EXO compared to CTL mice were involved in cholesterol and steroid synthesis, apoptosis, insulin signaling, and protein coding. Most pathways regulated in EXO compared to VCD mice were also regulated in EXO compared to CTL mice, indicating a greater effect of exosome treatment on these pathways. Some pathways were exclusively regulated in EXO compared to VCD mice, suggesting a direct effect of exosome treatment. Among those PPAR signaling pathway, Antigen processing and presentation, B cell receptor signaling pathway, intestinal immune network for IgA production, T cell receptor signaling pathway, Hematopoietic cell lineage, apoptosis, glycosaminoglycan degradation, cytosolic DNA-sensing pathway, NOD-like receptor signaling pathway, vasopressin-regulated water reabsorption, and VEGF signaling pathway (**Fig. 10-B**).

Comparative liver mRNAs and serum miRNAs analysis

An additional enrichment analysis using the Mienturnet and TargetScan tools identified possible direct interactions between differentially expressed mRNAs and miRNAs regulated in our study. In EXO compared to CTL group, for up-regulated miRNAs we have 87 validated target genes, 2 predicted genes, and none in common. Regarding down-regulated miRNAs in the same group, we have 621 validated genes, 15 predicted genes, and two in common (*Msi2 and Meis2*). In VCD compared to CTL group, for up-regulated miRNAs, we had 58 validated target genes, 17 predicted genes, and none in common. For down-regulated miRNAs, we found 80 validated genes, 16 predicted genes, and only one in common (*Msi2*). And for the VCD and EXO group, there was no regulator.

The genes *Cyp3a11* and *Cyp4a12a* were up-regulated in liver of VCD and EXO compared to CTL group. Both genes are predicted to interact directly with miR-103-3p,

which was down-regulated in serum exosomes from both groups. In the EXO group, in addition to miR-103-3p, miR-224-5p, miR-20a-5p, mmu-let-7a-5p, mmu-let-7f-5p, and mmu-let-7d-5p were down-regulated. These 6 miRNAs are predicted to interact with *Cyp3a11* also. The gene *Cyp4a12a* is also a predicted target of miR-224-5p and miR-20a-5p, also regulated in serum exosomes in our study.

Similarly, in the EXO compared to CTL group, 6 validated, 43 predicted, and 3 common pathways were down-regulated (fatty acid degradation, valine, leucine, and isoleucine degradation, and fatty acid metabolism). Up-regulated pathways included one validated, 40 predicted, and none in common. For the VCD compared to CTL group, 7 validated, 6 predicted, and 5 common down-regulated pathways were observed (caffeine metabolism, retinol metabolism, metabolic pathways, fatty acid biosynthesis, fatty acid metabolism). For up-regulated pathways we observed 9 validated, 49 predicted, and one in common (glycosaminoglycan biosynthesis heparan sulfate/heparin).

Interestingly, when observing mIRNA regulated in serum exosomes from the VCD compared to the CTL group, six up-regulated miRNAs are not up-regulated in the EXO compared to CTL group. These are miR-345-5p, miR-297a-3p, miR-324-3p, miR-30b-5p, miR-466f-3p, and miR-181a-1-3p. The predicted target genes of these miRNAs were also observed to be regulated in liver tissue in our study. We observed that 154 predicted target genes were actually regulated in the tissue of EXO compared to VCD mice. This highlights an effective role of exosomes in the regulation of liver gene expression.

Discussion

Our target organ for analyzing the effects of exo-miRs was the liver, as it primarily responds to changes in the circulating exosomal profile. The liver was chosen based on previous studies (Lee, Kim et al. 2018) that also used exosome injections and demonstrated higher uptake by the liver and lungs than other tissues. Sequencing identified 13,832 genes expressed in live, of which 1285 were regulated in the EXO group compared to VCD (767 upregulated and 518 downregulated). Recent literature suggests that exosomes play a crucial role in liver physiology and pathophysiology. Multiple effects of exosomes on cell-to-cell communication in the liver are described (Kogure, Lin et al. 2011, Ibrahim, Hirsova et al. 2016, Kakazu, Mauer et al. 2016, Nojima, Freeman et al. 2016) and can lead to a variety of biological responses, including inflammation, angiogenesis, proliferation, and tissue remodeling. The liver is one of the largest organ in the body, but its large size and high blood flow in mice are not sufficient justifications to explain the high distribution of exosomes (Gjedde and Gjeode 1980, Edmond Hui, Fisher et al. 1994). There is a specific up-regulation of certain miRNAs in liver of mice, which may be important for cellular aging (Maes, An et al. 2008). Genes involved in drug metabolism and clearance, such as cytochromes P450, exhibit sex-specific expression in rodents (Waxman, Lapenson et al. 1991, Buckley and Klaassen 2007, Cheng, Yu et al. 2011). In our study, we observed that these genes were also regulated by the estropause state and treatment with

exosomes, further indicating its role in metabolism for acyclic females.

The intense change in genes expression in the liver of exosome treated mice in our study may represent a response to process the content of the injections. In the liver, biochemical processes that regulate divergent patterns of gene expression by sex usually result from endogenous hormones, particularly growth hormone (GH). GH represses live-specific expression of CYP genes in females (Delesque-Touchard, Park et al. 2000, Wiwi and Waxman 2005, Waxman and O'Connor 2006). CYP3a11 and CYP4a12a were differentially expressed in estropause females in our study. Therefore, considering that miRNAs and mRNAs control multiple signaling pathways, even subtle changes in their expression can have a significant impact on biological processes. The pathways exclusively regulated in EXO compared to VCD mice (not regulated in EXO or VCD compared to CTL) include B cell receptor signaling pathway, intestinal immune network for IgA production, T cell receptor signaling pathway, hematopoietic cell lineage, apoptosis, glycosaminoglycan degradation, cytosolic DNA-sensing pathway, NOD-like receptor signaling pathway, vasopressin-regulated water reabsorption, and VEGF signaling pathway. These pathways are related to the inflammatory response and tissue remodeling, suggesting a stimulatory effect of exosomes on these processes. This pathways may be suppressed by the estropause state and rescued by exosome treatment.

The identification of miRNAs in serum is still an emerging field. Few data on miRNAs, and circulating exo-miRs, is available in animal menopause models. Thus, we presented the profiles of serum exosomal miRNA expression in females in estropause and receiving exosomes from cyclic animals of the same age. In this way, we attempted to associate how changes in liver gene expression profiles may be linked to circulating exosomal miRNAs. A total of 355 miRNAs were detected in serum, with ten miRNAs differentially regulated in VCD compared to CTL mice, fifteen in EXO compared to CTL mice, and none detected in EXO compared to VCD mice. We observed miR-103-3p and miR-320-3p commonly down-regulated VCD and EXO compared to CTL mice, while miR-150-5p and miR-20a-3p were up-regulated. The discovery of miRNAs in circulation has led to important research to determine whether these small non-coding RNAs can be used as diagnostic and prognostic tools for various diseases. Although circulating miRNAs have been examined in diseases such as cancer and cardiovascular conditions, the role of miRNAs in aging, particularly in menopause, has only recently begun to be explored. Here, we provide evidence that circulating exo-miRs in serum are differentially expressed between mice in estropause compared to cyclic mice. Interestingly, we observed that serum miRNAs that were increased in the VCD compared to the CTL group were not increased in the EXO compared to CTL group. These include miR-345-5p, miR-297a-3p, miR-324-3p, miR-30b-5p, miR-466f-3p, and miR-181a-1-3p. Among the targets of these exclusive regulated miRNAs are the FoXO, TGF-beta, mTOR, type II diabetes, TNF-alpha

signaling pathways. This indicates a possible effect of these miRNAs on liver gene regulation of these pathways, as observed in this study.

According to the literature, miR-345-5p is involved in adipocyte differentiation by suppressing the expression of VEGF-B. VEGF-B was observed in our study as one of the pathways exclusively regulated by exosomes in liver. VEGF-B is highly expressed in cells with high metabolic activity, such as brown adipocytes, skeletal myocytes, myocardium, and pancreatic beta cells (Aase, von Euler et al. 2001, Bry, Kivelä et al. 2014, Liu, He et al. 2020). *VEGF-B* induces the expression of fatty acid transport protein 3 (*FATP3*) and *FATP4*, which are specific vascular fatty acid transport proteins. (Rafii and Carmeliet 2016). Both FATP3 and FATP4 were observed to be regulated in liver in EXO compared to VCD mice. It is noteworthy that the mice in estropause had higher body mass and fat content compared to CTL group. Some authors suggest that miRNA and its target genes may potentially regulate the pathological progression of obesity-related diseases (Liu, He et al. 2020). Therefore, further studies are needed to charactrize the eeffects of miRNAs in the development of estropause increased body and fat mass. As a direct consequence of ovarian failure, hormonal and metabolic changes occur due to the decrease in estrogens, resulting in increased visceral fat and body weight. As females age they produce lower levels of estradiol (Gosden and Faddy 1994). However, we observed few metabolic changes beyond slightly increased body weight and fat mass in VCD mice overall. This allowed us to isolate the effect of ovarian failure and reaffirm its effects on the exosome profile and the liver transcriptome. Our data is in alignment with a previous study from our groups, where VCD mice had minimal metabolic changes beyond increased body mass (Ávila, Zanini et al. 2023).

miR-324-3p was found to be upregulated in postmeopausal women and associated with bone mineral density (Shi, Jiang et al. 2022). Therefore, it could serve as a biomarker for this condition. In our study miRNA-324-3p was also upregulated in VCD mice. However, after in mice receiving exosome injections it was not up-regulated, indicating that exosomes may have an effect on the expression of this marker. miRNA-30b-5p was also evaluated in postmenopausal women, rats, and rhesus macaques and down-regulated in osteopenia and/or osteoporosis (Chen, Li et al. 2016). Rats subjected to ovariectomy (OVX) also had downregulation of miRNA-30b-5p. (Chen, Li et al. 2016) Mir-30 is sensitive to estrogen levels in different tissues (Bhat-Nakshatri, Wang et al. 2009, Kuokkanen, Chen et al. 2010, Mellios, Galdzicka et al. 2012). We observed that miR-30b-5p was upregulated in the VCD group but notin the EXO group. Interestingly, as described, all miRNAs upregulated in the VCD group that were not regulated in the EXO group were found to be dependent on E2. MiR-181a is also na age-related miRNA associated with E2 (Benedetti, Papulino et al.2021). Our findings demonstrate that E2 and ciclity is a critical regulator of exosomal miRNA expression levels.

We observed exosomes injected ex vivo in adipose tissue and blood, indicatingthat they reached target tissues. Moreover, the injection time was relatively short, allowing

for the observation of strong changes in liver gene expression levels although no changes in body composition. Our findings represent a starting point for the use exo-miRs as regulators of metabolic health in acyclic females. Further investigations into the pathways altered by exosome injection are still needed to develop exosome based therapie.

Data availability
The data is available from the corresponding author upon request.
Competing interests
The authors declare no competing interests related to this work.

Figures
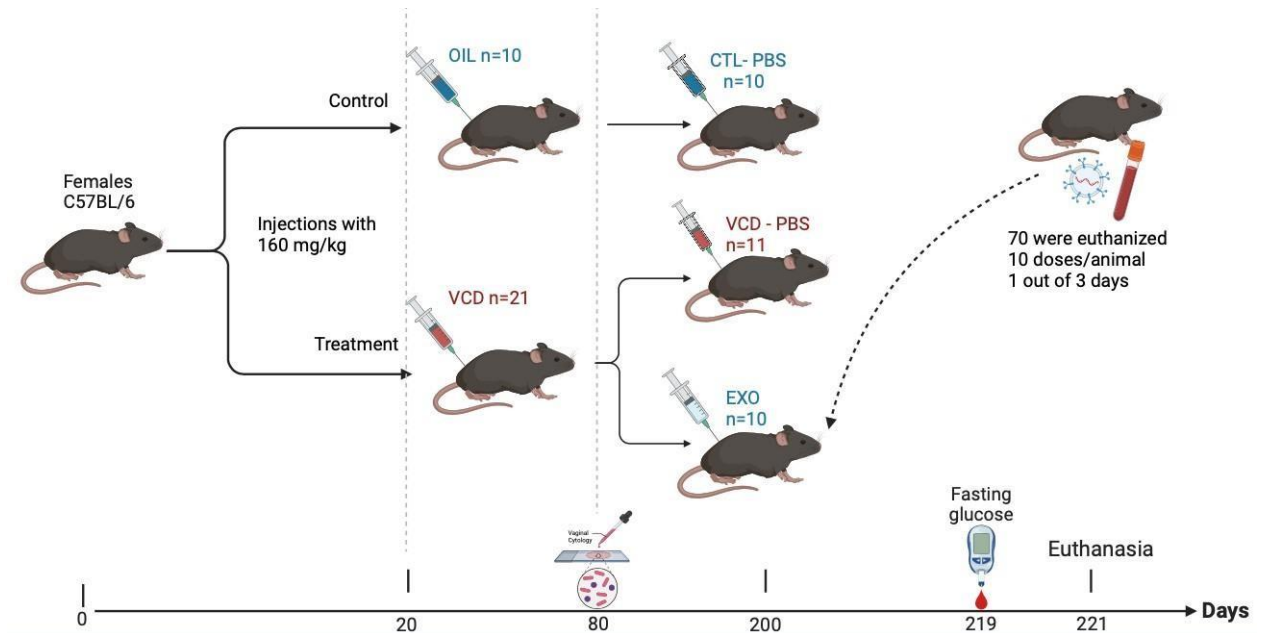
Figure 1 Experimental Design: Female mice at 60 days of age received injections of sesame oil or 4-vinylcyclohexene diepoxide (VCD) for 20 consecutive days. Sixty days after the end of the VCD/oil injection, estrous cycle assessment was conducted to confirm estropause. After confirmation, mice were divided into VCD and EXO groups. After six months, the EXO group started exosome therapy every three days via intraperitoneal injection for 30 days. One day before the end of therapy, all groups underwent an insulin tolerance test, and the next day, wereeuthanized.
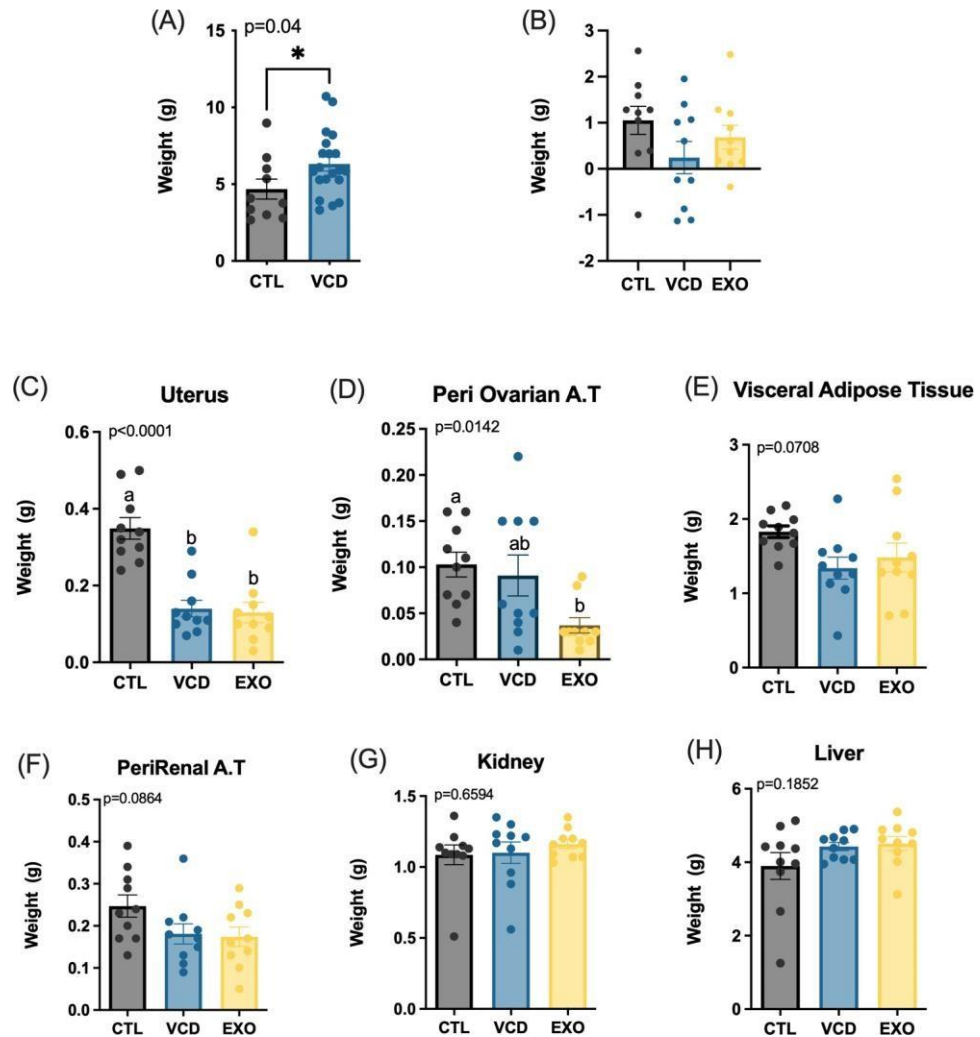
Figure 2 (A) Body weight from the end of VCD treatment until the beginning of exosome treatment (B) Body weight gain from the beginning to the end of injections with exossomes. Weight of (C) uterus (D) peri ovarian adipose rissue(E) visceral (F) peri renal adipose tissue (G) kidney and (H) liver for control (CTL),estropause (VCD) and estropause receiving exosome (EXO) mice. Values are represented as mean ± standar error of the mean (SEM). Different letters indicatestatistical difference at p<0.05).
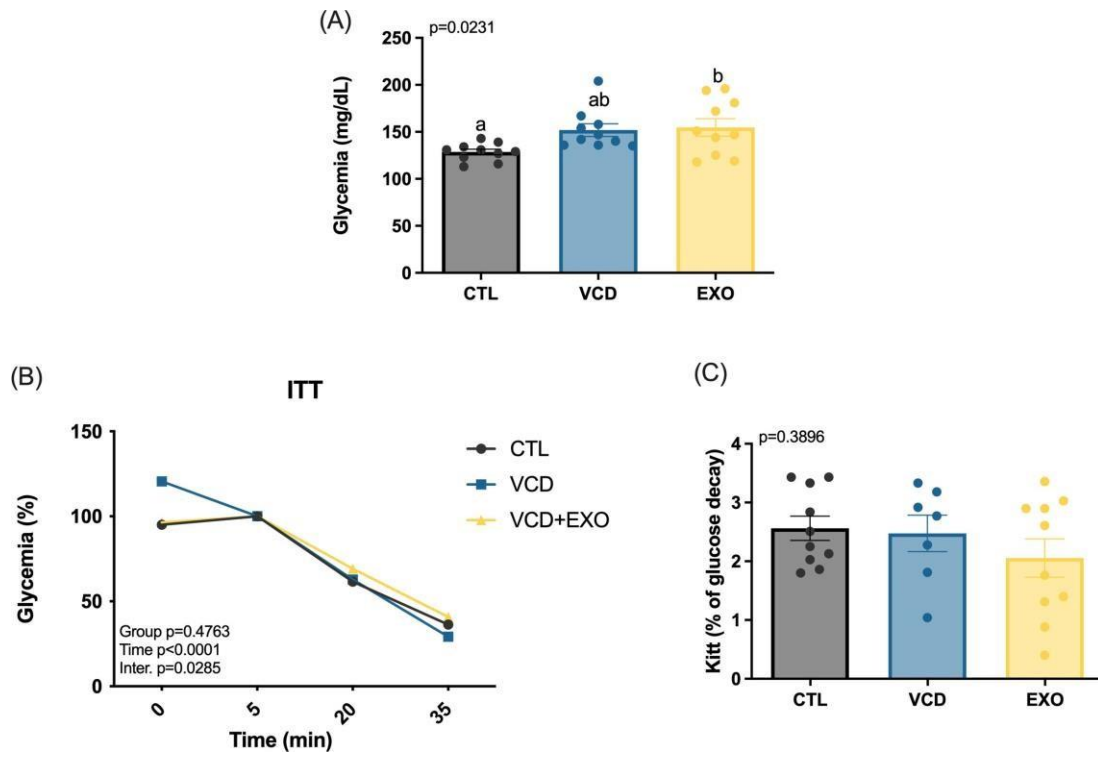
Figure 3 (A) Fasting glucose levels for control (CTL), estropause (VCD) and estropause receiving exosome (EXO) mice. (B) Insulin tolerance test (ITT) and(C) glucose decay constant (KITT). Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at p<0.05).
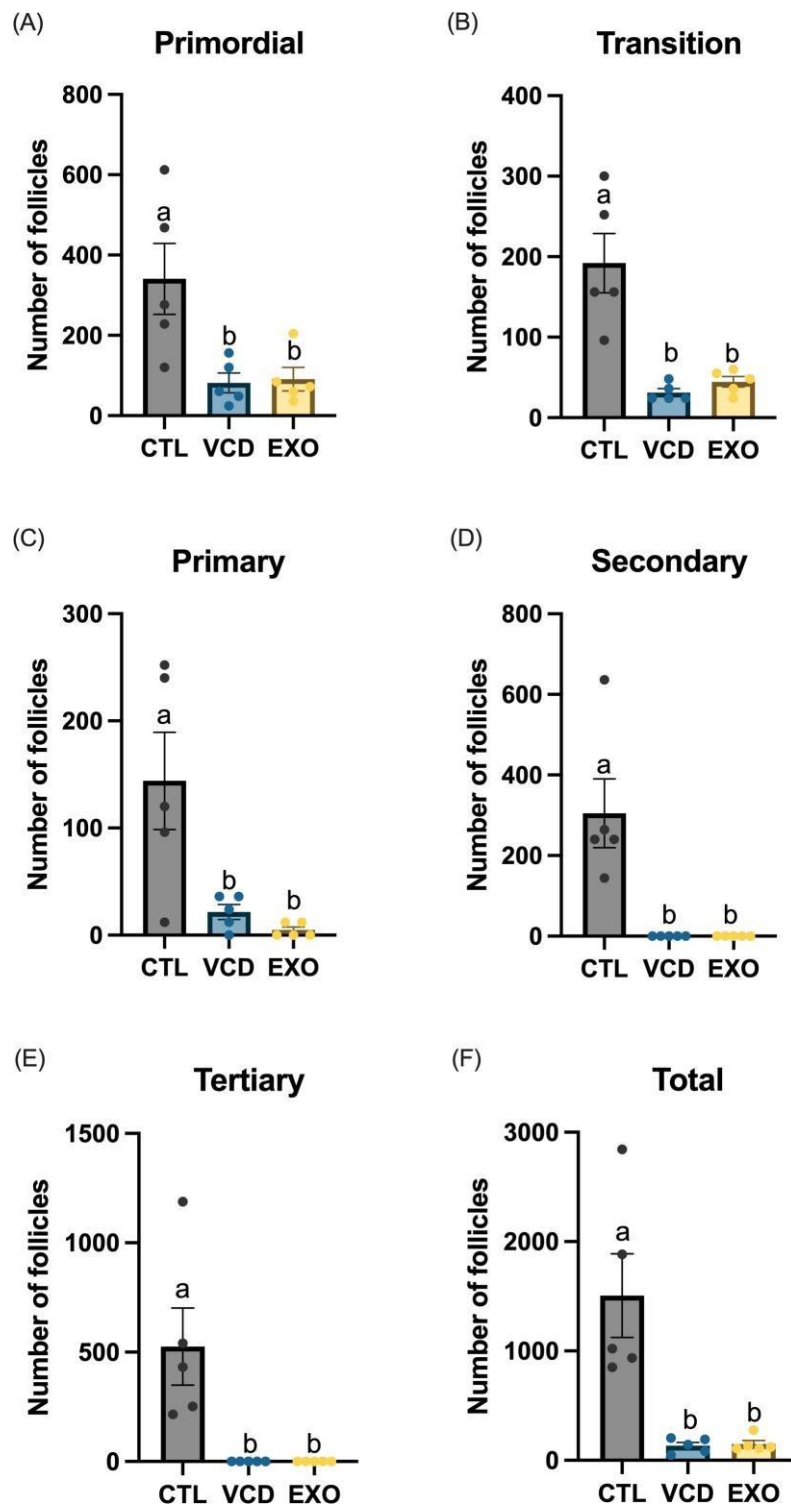
Figure 4 Number of (A) primordial, (B) transition, (C) primary, (D) secondary, (E) tertiary and (F) total folicles per ovarian section for control (CTL), estropause (VCD) and estropause receiving exosome (EXO) mice. Values are represented as mean ± standard error of the mean (SEM). Different letters indicate statistical difference at p<0.05.
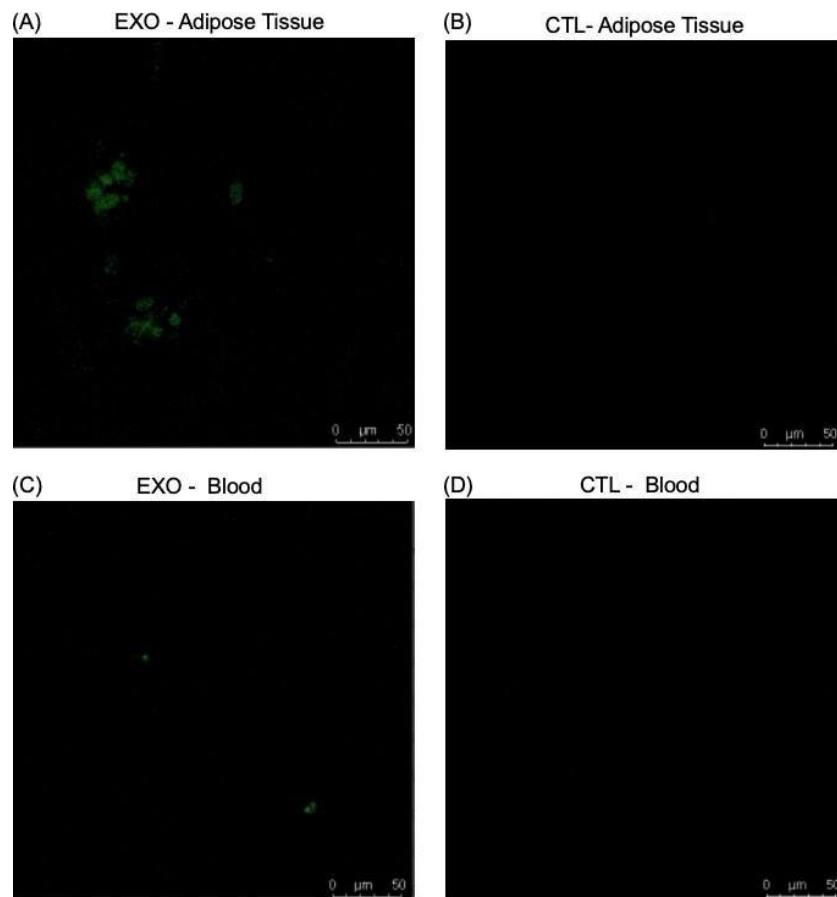
Figure 5 Ex vivo images of the distribution of exogenous exosomes in adiposetissue and blood of mice after staining with SYTO Select. (A) Image of stainedexosomes in fresh adipose tissue 24 hours post-injection; (B) image of control(CTL) animal without exosome staining in fresh adipose tissue; (C) blood smear image of mice after exogenous exosome administration; and (D) control(CTL) blood smear image without markings.

(A)

FTLA Concentration / Size graph for Experiment:
Capture 2023-08-31 15-45-44

(B)

Averaged FTLA Concentration / Size for Experiment:
Capture 2023-08-31 15-45-44
Error bars indicate + / -1 standard error of the mean

(C)
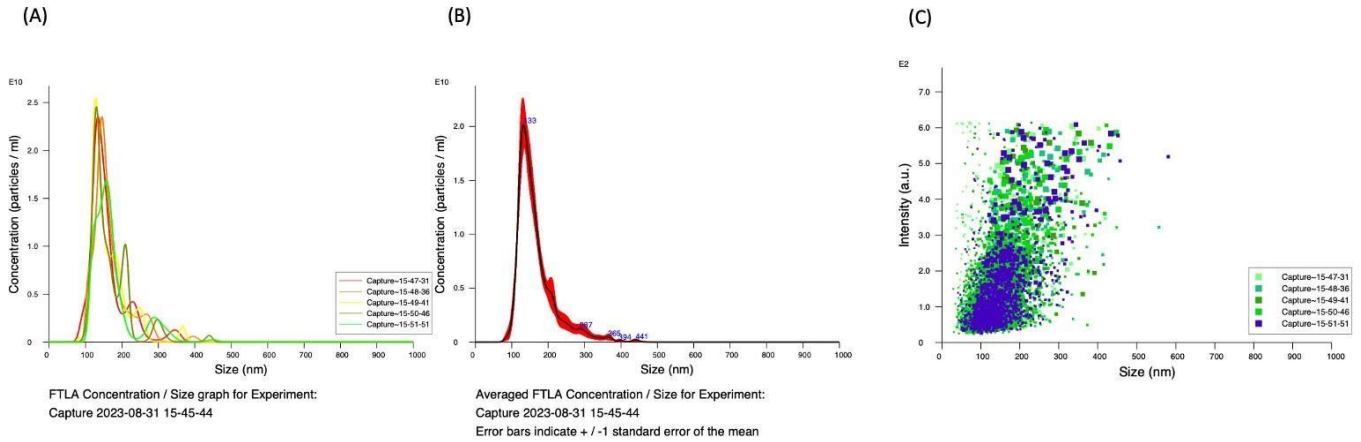
Figure 6 Exosome nanoparticle tracking analysis using NanoSight NS300 system. Five videos (60sduration each) of Brownian motion of nanoparticles were recorded and analyzed. The samples were measured with manual shutter and gain adjustments. The sample are from mice serum. (A) and (B) show size and concentration and (C) size and intensity.
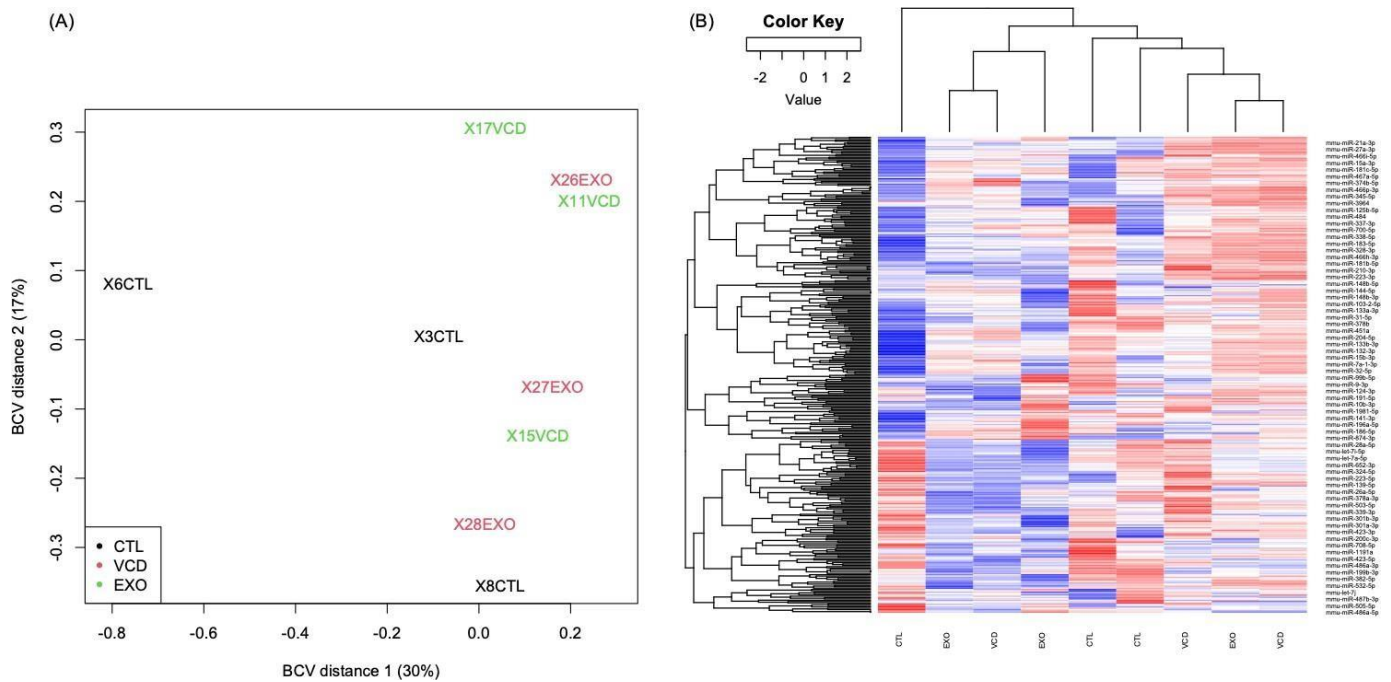
Figure 7 (A) Principal component analysis of serum exosomal miRNAs for control (CTL), estropause (VCD) and estropause receiving exosomes (EXO) mice. (B) Unsupervised hierarchical clustering ordered by the adjusted level of miRNA expression for all expressed miRNA in serum
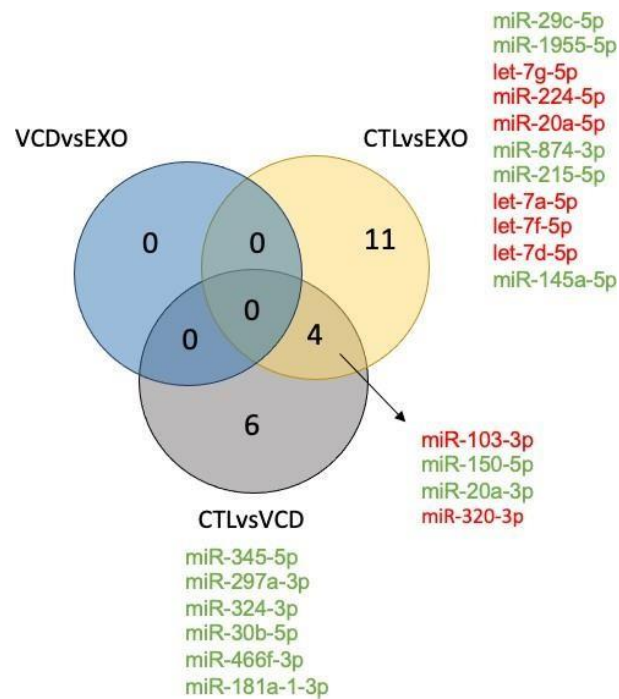
Figure 8 Venn diagram showing significantly regulated miRNAs commonly regulated between control (CTL), estrogen depletion (VCD), and estrogen-depleted mice receiving exosomes (EXO). For each group, upregulated miRNAs (indicated in red) and downregulated miRNAs (indicated in green) are represented in the diagram.
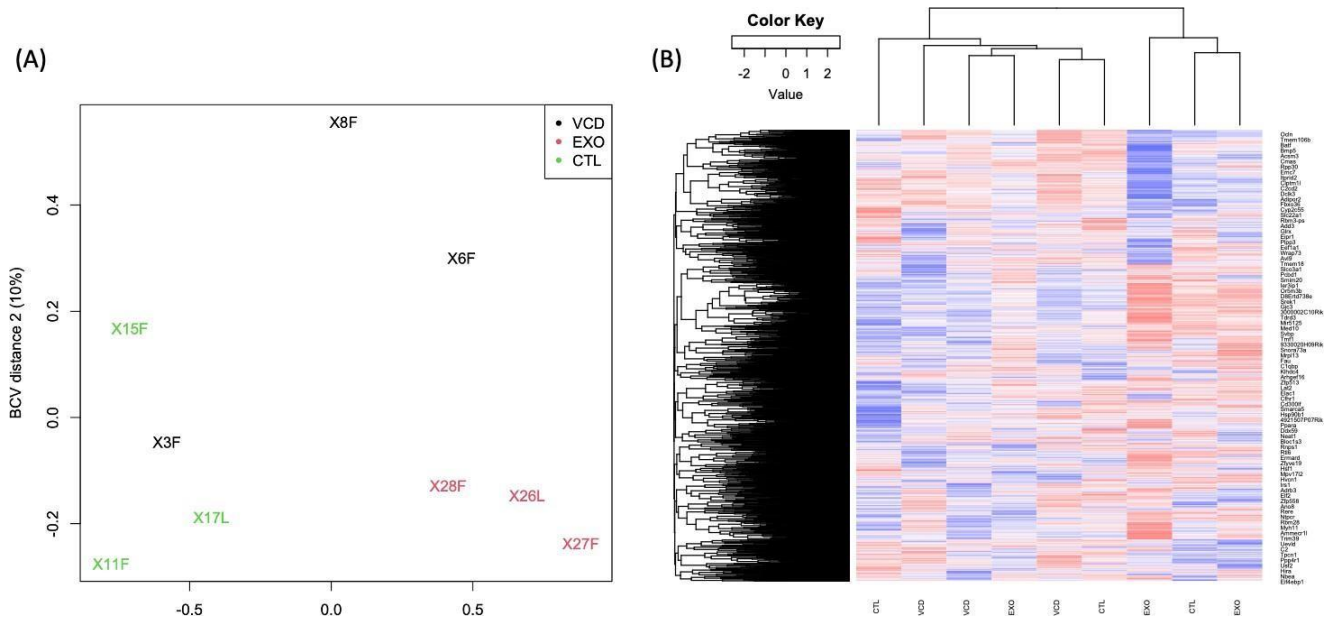
Figura 9 (A) Principal componente analysis of liver mRNA for control (CTL), estropause (VCD)and estropause receiving exosomes (EXO) mice (B) Unsupervised hierarchical clustering ordered by the adjusted level of mRNA expression for all expressed mRNA from liver tissue.
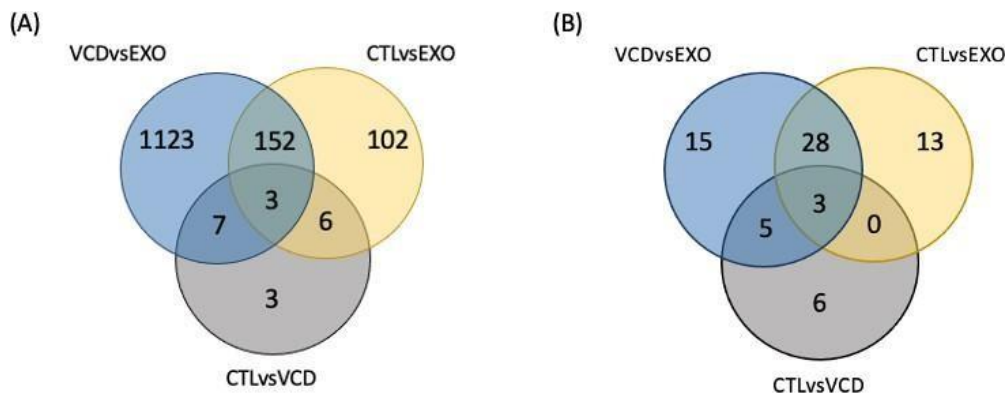
Figure 10 Venn diagram (A) indicating mRNAs significantly regulated from hepatic tissuebetween control (CTL), estropause (VCD) and estropause reciving exossomes (EXO) mice (B) indicating pathways from mRNAs significantly regulated from hepatic tissue between control (CTL), estropause (VCD) and estropause reciving exossomes (EXO) mice.

Tables

Table 1. Enriched KEGG pathways for target genes differentially expressed miRNAs for control (CTL), estropause (VCD) and estropause receiving exosomes (EXO) mice.

CTL vs VCD	Down-regulated		
KEGG pathway	p-value	#genes	#miRNAs
Metabolic pathways	3.19E-06	59	2
Fatty acid metabolism	6.43E-24	7	2
Lysine degradation	0.0001	7	2
Valine Leucine and Isoleucine degradation	0.004	7	2
Fatty acid degradation	1.11E-04	6	1
Retinol metabolism	0.03	5	2
Propanoate metabolism	0.02	3	2
Fatty acid biosynthesis	2.58E-20	1	2
Lysine biosynthesis	0.004	1	1
Caffeine metabolism	0.008	1	1
	Up regulated		
HTLV-infection	0.005	59	6
MAPK signaling pathway	0.013	53	6
Protein processing in endoplasmic reticulum	0.003	43	6
FoxO signaling pathway	0.0002	40	6
Insulin signaling pathway	0.02	33	5
Hippo signaling pathway	0.006	30	5
Oocyte meiosis	0.005	28	5
TNF signaling pathway	0.006	27	5
GnRH signaling pathway	0.04	21	6
mTOR signaling pathway	0.004	20	5

CTL vs EXO	Down-regulated		
KEGG pathway	p-value	#genes	#miRNAs
Pathways in cancer	0.0006	116	7
PI3K-Akt signaling pathway	0.03	100	8
MAPK signaling pathway	0.005	77	7
HTLV- infection	0.03	77	7
FoxO signaling pathway	0.0006	53	7
Insulin signaling pathway	0.01	49	7
Cell cycle	0.01	44	7
mTOR signaling pathway	0.01	24	7
Fatty acid degradation	0.001	17	6
Valine Leucine and isoleucine degradation	0.03	17	7

		Up regulated	
Pathway in cancer	0.03	46	3
Viral carcionogenesis	0.003	30	3
FoxO signaling pathway	0.0002	27	3
Protein processing in endiplasmic reticulum	0.006	26	3
Insulin signaling pathway	0.02	21	2
Hippo signaling pathway	0.0006	18	3
Oocyte meiosis	0.04	16	3
mTOR signaling pathway	0.004	14	3
Estrogen signaling pathway	0.04	12	3
Endometrial cancer	0.02	10	2

Table 2. Top 10 mRNAs differentially expressed in hepatic tissue from control (CTL), estropause (VCD) and estropause receiving exosomes (EXO) mice.

CTL vs VCD	Down-regulated	
mRNA	**p-value**	**FDR**
Tnp	1.93E-07	0.00266957
Prm2	3.21E-06	0.012434102
Pcp4l1	4.06E-06	0.012434102
Spata4	9.74E-06	0.021840492
Fam216a	3.05E-05	0.052711426
Tnp1	4.13E-05	0.057195081
Meig1	4.75E-05	0.059717293
Gli1	6.41E-05	0.072010156
Lilrb4b	8.27E-05	0.072010156
Clec2m	8.69E-05	0.072010156
	Up regulated	
Mup18	3.04E-06	0.012434102
Mup17	4.49E-06	0.012434102
Cyp4a12a	1.11E-05	0.021840492
Mup16	3.89E-05	0.057195081
Mup11	7.86E-05	0.072010156
Cyp3a11	9.31E-05	0.072010156
Fam131c	9.37E-05	0.072010156
Lcn2	0.000106934	0.077847706
Mup1	0.000166034	0.104470573
Mup13	0.00017883	0.104470573
CTL vs EXO	Down-regulated	
mRNA	**p-value**	**FDR**
Cyp3a41a	1.96E-12	4.12E-16
Sult2aa1	2.34E-09	4.43E-0.6
Cyp17a1	2.88E-09	4.43E-0.6
Sult2a5	3.08E-09	4.43E-0.6
Cyp3a44	5.62E-08	4.90E-02
Pcp4l1	9.42E-08	6.85E-05
Hamp2	1.23E-07	8.18E-05
Cfp	1.24E-07	8.18E-05
Cd5l	1.88E-07	0.000118373
Cyp3a16	2.96E-07	0.000177871
Cyp3a41b	1.08E-06	0.000514383
	Up regulated	
Cyp4a12a	2.42E-16	1.96E-12
Mup14	4.24E-16	1.96E-12
Mup20	5.86E-11	5.86E-11
Mup1	1.65E-10	1.65E-10

	p-value	FDR
Socs2	7.12E-09	7.12E-09
Mup12	4.43E-06	4.43E-06
Mup13	1.48E-05	1.48E-05
Serpina4-ps1	1.90E-05	1.90E-05
Mup17	1.90E-05	1.90E-05
Snhg11	3.86E-05	3.86E-05

VCD vs EXO — **Down-regulated**

mRNA	p-value	FDR
Cyp3a41a	1.79E-10	3.69E-07
Crebl2	5.76E-09	4.69E-06
Cyp17a1	6.20E-08	2.77E-5
Sntb1	2.93E-07	0.000103787
Cps1	4.97E-07	0.000150934
Cyp3a41b	5.68E-07	0.000163642
Gfra1	8.21E-07	0.000199466
Mia2	8.22E-07	0.0001994466
Ugt1a5	1.26E-06	0.000273243
Acss3	1.35E-06	0.00028337

Up regulated

mRNA	p-value	FDR
Prm2	1.31E-13	1.81E-09
Fabp9	9.12E-13	6.31E-09
Spz1	3.87E-11	1.61E-07
Tnp2	4.66E-11	1.61E-07
Ybx2	7.57E-11	2.09E-07
Clec2m	1.90E-10	3.69E-07
Spata4	2.14E-10	3.69E-07
Gk2	7.29E-10	1.12E-06
Snhg11	9.41E-10	1.30E-06
Ccdc136	1.31E-09	1.64E-06

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5 Considerações Finais

Em síntese, nossas análises mostraram que a atividade ovariana pode modular modificações epigenéticas mediadas por miRNAs independentemente da idade. A reposição de estradiol têm um impacto no perfil de miRNAs exossômicos séricos, destacando a família miR-200 no artigo 1 que podem vir a ser usados como um potencial candidato a biomarcador da função ovariana. As injeções de exossomos, quando fornecidas de forma crônica são eficazes para alcançar a corrente sanguínea e o tecido adiposo, indicando que apodem atingir outros tecidos alvos. Esta intervenção mesmo sendo em um período relativamente curto, permitiu observar fortes alterações nos níveis de expressão gênica hepática. Nossos achados representam um ponto de partida para o uso de exo-miRs como reguladores da saúde metabólica em mulheres pós-menopausa. No entanto,estudos adicionais ainda são necessários para melhor entender sobre as vias alteradas pela injeção de exossomos, para que assim possa haver desenvolvimentos de novas terapias.

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Anexos

Anexo I – Documento da Comissão de Ética e Experimentação Animal (Artigo 1)

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº   **121/2019/CEEA/REITORIA**
PROCESSO Nº   23110.040879/2019-07

Certificado

Certificamos que a proposta intitulada **"Adição de 17α e 17β estradiol ao cultivo *in vitro* de ovários de camundongos : Efeito na ativação de folículos primordiais "**, registrada com o nº **23110.040879/2019-07,** sob a responsabilidade de **Augusto Schneider** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) **–** encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de **07 de outubro de 2019.**

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	01/11/2019 a 31/12/2020
Espécie/linhagem/raça	*Mus musculus*/C57BL/6
Nº de animais	36
Idade	21 dias

Anexo II - Documento da Comissão de Ética e Experimentação Animal (Artigo 2)

PARECER N°   **99/2021/CEUA/REITORIA**
PROCESSO N°   23110.023313/2021-27

Certificado

Certificamos que a proposta intitulada **"Exossomos: moduladores do metabolismo pós-menopausa em camundongos"**, registrada com o n° **23110.023313/2021-27**, sob a responsabilidade de **Augusto Schneider** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei n° 11.794, de 8 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética no Uso de Animais, em reunião de 15 de setembro de 2021.

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	Início = **01/10/2021** Término = **31/12/2023**
Espécie/linhagem/raça	*Mus musculus*/ **C57BL/6**
N° de animais	**195**
Idade	**2 meses**
Sexo	Fêmeas