UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

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VZOREC IZRAŽANJA GENOV V ENDOMETRIJU V STANJU RECEPTIVNOSTI PRI PREISKOVANKAH Z ADENOMIOZO

DOKTORSKA DISERTACIJA

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UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA
INTERDISCIPLINARNI DOKTORSKI ŠTUDIJ PROGRAMA BIOMEDICINA
ZNANSTVENO PODROČJE GENETIKA

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GENE EXPRESSION PROFILE OF ENDOMETRIUM IN RECEPTIVE STATE IN WOMEN WITH ADENOMYOSIS

DOCTORAL DISSERTATION

Ljubljana, 2022

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AI Nižja stopnja zanositve pri ženskah z adenomiozo maternice se povezuje z ovirano endometrijsko receptivnostjo za vgnezdenje zarodka. Pripadajoči molekularni vzroki so slabo raziskani zaradi preteklih tehnoloških omejitev neinvazivnih diagnostičnih metod za odkrivanje adenomioze. S pristopi sistemske biologije smo zato identificirali kandidatne biološke poti/gene spremenjene endometrijske receptivnosti pri adenomiozi. Izvedli smo analizo RNA sekvenciranja (RNA-seq) endometrija v pričakovanem stanju receptivnosti ženskam z (n = 10) in brez (n = 10) ultrazvočnih znakov adenomioze. Identificirane spremenjeno izražene gene smo dalje integrirali s podatki iz pregleda literature, da bi razumeli njihov pomen v povezavi z molekularno biologijo endometrija. Zbrali smo poročane transkripte in proteine, povezane z endometrijsko receptivnostjo pri adenomiozi, pri sorodni, a bolje raziskani endometriozi in pri zdravi maternici, ter poenotili njihovo poimenovanje po genski nomeklaturi zbirke HGNC. S primerjavo podatkov RNA-seq samo potrjeno receptivnih vzorcev (8 adenomioznih in 5 kontrolnih) smo zaznali 382 spremenjeno izraženih genov ($p < 0,05$), ki so bili v največji meri obogateni v poteh, povezanih z odzivi na signalizacijo interferonov, in v poti celične adhezije. Z integracijo zbranih 382 (RNA-seq), 42 (adenomioza), 173 (endometrioza) in 151 (zdrava maternica) genov smo identificirali obogatene poti *organizacija zunajceličnega matriksa*, *regulacija reproduktivnih procesov*, *odziv na VEGF* in *signalizacija z interlevkini*, ki jih predlagamo kot dodatne kandidate za preučevanje endometrijske receptivnosti pri adenomiozi. Rezultate RNA-seq omejuje neznačilno spremenjeno izražanje genov po popravku vrednosti p (FDR > 0,05), kar bi lahko bila posledica nizkega števila uporabljenih vzorcev. Na podlagi analize podatkov iz dosedanje literature in lastnih rezultatov RNA-seq sklepamo, da je endometrijska receptivnost pri ženskah z adenomiozo spremenjena na ravni signalizacije s citokini imunskega sistema.

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AB Lower pregnancy rate in women with uterine adenomyosis is associated with impaired endometrial receptivity for embryo implantation. The underlying molecular causes are poorly understood due to past technological limitations of non-invasive methods to diagnose adenomyosis. We therefore applied systems biology approaches to identify candidate biological pathways/genes of altered endometrial receptivity in adenomyosis. We performed RNA sequencing (RNA-seq) analysis of the endometrium in the expected receptive state in women with (n = 10) and without (n = 10) ultrasound signs of adenomyosis. The identified differentially expressed genes were further integrated with data from the literature mining to understand their relevance in the context of endometrial molecular biology. We gathered reported transcripts and proteins associated with endometrial receptivity in adenomyosis, in related but better-studied endometriosis and in healthy uterus, and adopted their gene nomenclature according to the HGNC database. The comparison of RNA-seq data of only confirmed receptive samples (8 adenomyosis and 5 controls) identified 382 differentially expressed genes ($p < 0.05$) further mostly enriched in pathways associated with responses to interferon signalling and in pathway related to cell adhesion. By integration of gathered 382 (RNA-seq), 42 (adenomyosis), 173 (endometriosis) and 151 (healthy uterus) genes we identified enriched pathways *extracellular matrix organisation*, *regulation of reproductive processes*, *VEGF response* and *signalling by interleukins*, which we propose as additional candidate pathway for studying endometrial receptivity in adenomyosis. Our RNA-seq results are limited by insignificant gene expression difference after p-value correction (FDR > 0.05), which may be due to the small sample size. Based on the analysis of existing literature and own RNA-seq results, we conclude that endometrial receptivity in adenomyosis is altered at the level of immune cytokine signalling.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

Str.

KAZALO PREGLEDNIC

KAZALO SLIK

PRILOGE

Priloga A: Seti genov, uporabljeni v naši raziskavi

Priloga B: Pregled molekularnih raziskav, povezanih z adenomiozo

OKRAJŠAVE IN SIMBOLI

CPM	štetje na milijon (angl. *count per million*)
DAVID	podatkovna zbirka za anotacijo, vizualizacijo in integrirano odkrivanje (angl. *The Database for Annotation, Visualization and Integrated Discovery*)
EEC	endometrijske epitelijske celice (angl. *endometrial epithelial cells*)
ESC	endometrijske stromalne celice (angl. *endometrial stromal cells*)
E2	estradiol
EMT	prehod iz epitelija v mezenhim (angl. *epithelial-to-mesenchymal transition*)
FDR	delež napačnih uvrstitev (angl. *false discovery rate*)
GO	ontologija funkcij genov (angl. *Gene Ontology*)
HGNC	odbor za nomenklaturo genov HUGO (angl. *HUGO Gene Nomenclature Committee*)
HOXA10	protein homebox Hox-A10 (angl. *homebox protein Hox-A10*)
qPCR	kvantitativna verižna reakcija s polimerazo (angl. *quantitative polymerase chain reaction*)
IFN	iterferon
IL	interlevkin
KEGG	kjotska enciklopedija genov in genomov (angl. *Kyoto Encyclopedia of Genes and Genomes*)
LH	luteinizirajoči hormon (angl. *luteinizing hormone*)
lncRNA	dolga nekodirajoča RNA (angl. *long noncoding RNA*)
M	menstruacijska faza menstruacijskega ciklusa
MMP	matriks metaloproteinaza (angl. *matrix metalloproteinase*)
MR	magnetna resonanca
mRNA	informacijska RNA (angl. *messenger RNA*)
miRNA	mikro RNA (angl. *micro RNA*)
ncRNA	nekodirajoča RNA (angl. *noncoding RNA*)
NGS	sekvenciranje naslednje generacije (angl. *next generation sequencing*)
OBMP	oploditev z biomedicinsko pomočjo
P4	progesteron
P	proliferacijska faza menstruacijskega ciklusa
PP	pozna proliferacijska faza menstruacijskega ciklusa
PS	pozna sekrecijska faza menstruacijskega ciklusa
RNA-seq	RNA sekvenciranje (angl. *RNA sequencing*)
S	sekrecijska faza menstruacijskega ciklusa
STRING	iskalno orodje za pridobivanje interakcijskih genov/protein (angl. *Search Tool for the Retrieval of Interacting Genes/Proteins*)
scRNA-seq	RNA-seq posameznih celic (angl. *single-cell RNA sequencing*)
SP	srednja proliferacijska faza menstruacijskega ciklusa
SS	srednja sekrecijska faza menstruacijskega ciklusa
TVUZ	transvaginalni ultrazvok
TIAR	poškodba in celjenje tkiva (angl. *tissue injury and repair*)
NK/uNK	naravna ubijalka / maternična naravna ubijalka (angl. (*uterine*) *natural killer*)
ZP	zgodnja proliferacijska faza menstruacijskega ciklusa
ZS	zgodnja sekrecijska faza menstruacijskega ciklusa

SLOVARČEK

Adenomioza	Patološko stanje maternice, kjer se endometriju podobno tkivo nahaja v mišični steni maternice.
EvEA	Evtopični endometrij pri adenomiozi. To je sluznica endometrija, ki se nahaja na pravilnem mestu, obdaja maternično votlino, in predstavlja ciljno tkivo vgnezdenja zarodka.
EkEA	Ektopični endometrij pri adenomiozi. Endometriju podobno tkivo, ki se nahaja na nepravilnem mestu, in sicer v mišični steni maternice, predstavlja patološke lezije, ki označujejo to bolezen.
Endometrioza	Adenomiozi sorodna bolezen, kjer se endometriju podobno tkivo nahaja na ektopičnih mestih izven maternice (v trebušni votlini).
Endometrijska receptivnost	Čas srednje sekrecijske faze menstruacijskega ciklusa, ko je endometrij dovzeten za vgnezdenje zarodka.
Datiranje	Beleženje faze menstruacijskega ciklusa v kateri je bil pridobljen vzorec endometrija.
RNA-seq	Določanje globalnega transkriptoma biološkega vzorca z metodo sekvenciranja.

1 UVOD S PREDSTAVITVIJO PROBLEMATIKE, CILJEV IN HIPOTEZ

Adenomioza je patologija maternice, za katero je značilna prisotnost endometriju podobnega tkiva v mišični steni maternice. Po trenutno najbolj raziskani teoriji se nastanek te bolezni povezuje s pridobljenimi invazivnimi lastnostmi celic endometrija, ki migrirajo in obstanejo v spodaj ležečem miometriju (Vannuccini in sod., 2017). Absolutna diagnoza adenomioze je mogoča s histološko analizo vzorcev histerektomije (operativna odstranitev maternice), ki se opravi pri starejših simptomatskih ženskah (Ferenczy, 1998). Z izboljšanjem občutljivosti ultrazvočne tehnologije pa se znaki adenomioze lahko opazijo že pri mlajših ženskah (Bazot in Daraï, 2018). Pri zdravljenju neplodnosti se adenomioza povezuje z zmanjšano možnostjo zanositve (Salim in sod., 2012; Puente in sod., 2016; Sharma in sod., 2019; Nirgianakis in sod., 2020), domnevo zaradi vpliva te bolezni na zmanjšano endometrijsko receptivnost za vgnezdenje zarodka (Campo in sod., 2012). Endometrij, ki obdaja maternično votlino, namreč predstavlja ciljno tkivo vgnezditve zarodka (Altmäe in sod., 2012). Molekularni mehanizmi, ki bi pojasnili domnevno ovirano endometrijsko receptivnost pri adenomiozi, so slabo poznani zaradi malo opravljenih raziskav kot posledica tehnoloških omejitev neinvazivnih diagnostičnih metod adenomioze v preteklosti (Puente in sod., 2016). Zato smo v tej doktorski disertaciji uporabili pristope sistemske biologije, da smo identificirali kandidatne biološke poti in gene spremenjene endometrijske receptivnosti pri adenomiozi maternice. Iz znanstvene literature smo pridobili podatke, ki so se navezovali na genetske vzroke spremenjenega endometrijskega izražanja pri adenomiozi in sorodni, a bolje raziskani endometriozi ter izvedli analizo RNA sekvenciranja (RNA-seq) biopsij endometrija, da smo lahko integrirali podatke in predstavili molekularno ozadje endometrijske receptivnosti pri adenomiozi. Ker se v literaturi uporabljajo različni sistemi poimenovanje proteinov in transkriptov RNA, smo opisane proteine poimenovali po zbirki Uniprot (The UniProt ..., 2019), gene pa po zbirki HGNC (HUGO, 2019). Navedli smo tudi pogosta uporabljena alternativna imena oz. sinonime.

Izziv molekularnih raziskav endometrija predstavlja zagotavljanje homogenosti preiskovanih vzorcev, v smislu zagotavljanja enake stopnje fiziološke zrelosti endometrija v trenutku vzorčenja posamezne biopsije. Le primerjava vzorcev, datiranih v isto fazo menstruacijskega ciklusa, omogoča identifikacijo in primerjavo lokusov, povezanih s prisotnostjo endometrijske patologije (Devesa-Peiro in sod., 2021). V genetsko molekularnem delu te doktorske disertacije je bilo preiskovankam vzorčenje endometrija izvedeno med 7. in 9. dnem po izmerjenem vrhu luteinizirajočega hormona (dnevi LH+7–LH+9), ko se pričakuje pojav endometrijske receptivnosti. S primerjavo RNA-seq podatkov smo ugotovili, da je takšno datiranje vzorcev premalo natančno za identifikacijo spremenjeno izraženih genov, povezanih z adenomiozo. Zato smo vzorce endometrija dodatno datirali z uporabo novega molekularnega testa (Saare in sod., 2019), ki deluje na podlagi analize izražanja izbranih genov endometrijske receptivnosti. S ponovno analizo RNA-seq podatkov samo potrjeno receptivnih vzorcev smo identificirali spremenjeno izražene gene in obogatene poti, ki

predstavljajo močne kandidate za nadaljnje raziskave endometrijske receptivnosti pri adenomiozi.

1.1 PREDSTAVITEV PROBLEMATIKE

1.1.1 Maternica

Maternica je ženski reproduktivni organ, ki omogoča vgnezdenje zarodka v stadiju blastociste in njegov nadaljnji razvoj. Leži v mali medenici med danko in sečnim mehurjem. Spodnji ožji del se imenuje vrat, zgornji širši del telo, zaobljen zgornji del telesa pa fundus. Znotraj maternice se nahaja maternična votlina, ki je prekrita s sluznico, imenovano endometrij, ki predstavlja ciljno tkivo vgnezdenja zarodka. Endometrij je sestavljen iz dveh plasti; bazalna (angl. *bazalis*) in funkcionalna (angl. *functionalis*). Bazalna oz. osnovna plast je stalno prisotna in je sestavljena iz žlez, strjene strome in krvnih žil. Nad bazalnim endometrijem se nahaja funkcionalna oz. povrhnja plast, ki jo sestavljajo površinski epitelij, žlezni epitelij in ožiljena stroma. Pod bazalnim endometrijem se nahaja gladko mišičevje maternice ali miometrij, ki podpira stromalno in vaskulatorno tkivo ter omogoča krčenje maternice. Miometrij je razdeljen na notranji in zunanji del. Notranji miometrij imenovan tudi junkcijska cona (angl. *junctional zone*) se nahaja tik pod bazalno plastjo endometrija in predstavlja sluznično-mišično mejo med endometrijem in zunanjim miometrijem (angl. *endometrial-myometrial interface*). Notranji miometrij sestavljajo krožna mišična vlakna, zunanji miometrij pa vzdolžna mišična vlakna. Zunanji miometrij obdaja seroza, ki predstavlja najbolj zunanjo plast maternice (Naftalin in Jurkovic, 2009).

1.1.2 Menstruacijski ciklus endometrija

Menstruacijski ciklus endometrija označuje rodno dobo ženske. Predstavlja proces, ko je funkcionalna plast endometrija podvržena periodičnim ciklom luščenja v obliki menstrualne krvi, ki mu sledi njegova regeneracija (debeljenje in diferenciacija tkiva) iz bazalne plasti, s čimer je zagotovljeno optimalno okolje za vgnezdenje zarodka v vsakem ciklusu. Dolžina menstruacijskega ciklusa se med ženskami razlikuje in lahko traja od 21 do 35 dni, v povprečju okoli 28 dni. Dinamično spreminjanje endometrija po posameznih fazah menstruacijskega ciklusa sovpada s hormonskim procesom dozorevanja jajčne celice v jajčniku, ki ga narekujejo spreminjajoče se ravni izražanja gonadotropin sproščujočega hormona (angl. *gonadotropin releasing hormone*, GnRH), folikel stimulirajočega hormona (angl. *follicle stimulating hormone*, FSH), luteinizirajočega hormona (LH), estradiola (E2) in progesterona (P4) (Sherman in Korenman, 1975; Hawkins in Matzuk, 2008; Makieva in sod., 2018).

Pri dolžini 28 dni se menstruacijski ciklus prične z menstruacijsko (M) fazo (dnevi 1-4), kjer se tkivo endometrija lušči iz maternice in kot menstrualna kri zapusti telo. Sledi

proliferacijska (P) faza (dnevi 4-14), kjer povišano izločanje E2 iz rastoče granuloze v jajčnem mešičku spodbuja mitotično delitev endometrijskih celic strome in epitelija. Posledično se tkivo endometrija debeli, stroma pa se ožili s spiralnimi arterijami. Nagel porast LH (dan LH+0) okoli 36 ur pred ovulacijo (okoli 14. dne ciklusa) omogoči jajčnemu mešičku, da poči in jajčna celica se lahko sprosti iz jajčnika. Granuloza v jajčnem mešičku se pretvori v rumeno telesce, kjer se sinteza E2 preusmeri v sintezo P4. Hormon P4 ustavi debeljenje endometrija in spodbuja izločanje glikogena in mukusa iz endometrijskih žlez, kar označuje zgodnjo sekrecijsko (ZS) fazo (dnevi 15-20). V srednje sekrecijski (SS) fazi (dnevi 21-24) se pojavi t.i. okno vgnezdenja (angl. *window of implantation*), ki predstavlja kratek časovni okvir endometrijske receptivnosti za vgnezditev zarodka. Časovni okvir okna vgnezdenja je med ženskami različen in naj bi trajal med dnevi LH+6 in LH+11 (Tan in sod., 2018). Istočasno se endometrijske stromalne celice (ESC) v procesu decidualizacije diferencirajo v novo tkivo, imenovano decidua, kamor se lahko vgnezdi zarodek. Decidua zagotavlja vir rastnih dejavnikov in citokinov, ki nadzirajo vgnezdenje in nadaljnji razvoj zarodka, uravnavajo imunski odziv in podpirajo angiogenezo (Okada in sod., 2018). V pozni sekrecijski (PS) fazi (dnevi 25-28) decidua dokončno dozori. Če nosečnost ne nastopi, rumeno telesce propade in posledično upadeta nivoja E2 ter P4, decidua pa je podvržena procesu apoptoze in degradaciji. Menstruacijski ciklus se ponovi (Hawkins in Matzuk, 2008; Makieva in sod., 2018).

1.1.3 Molekularni dejavniki vgnezdenja zarodka

Za uspešno vgnezdenje zarodka je potrebna apozicija, adhezija in invazija blastociste v receptivni endometrij. Receptivnost endometrija označuje izgubo komponent mucinov, ki zavirajo pritrditev zarodka, in pa pridobitev adhezijskih molekul kaderinov in integrinov, ki omogočajo njegovo pritrditev (Simón in sod., 2000). V nadaljevanju je predstavljenih nekaj molekularnih dejavnikov, ki sodelujejo pri vgezdenju zarodka.

Hormon P4 in receptor za progesteron (angl. *progesterone receptor*, *PGR*) imata pomembno vlogo pri vzpostavitvi mikrookolja receptivnega endometrija. Le-to mora biti bogato s citokini in kemokini, ki spodbujajo apozicijo in adhezijo blastociste (Makieva in sod., 2018). Pomembni citokini so zaviralni dejavnik levkemije (angl. *leukemia inhibitory factor*, *LIF*), beta interlevkina 1 (angl. *interleukin-1 beta*, *IL1B*), spodbujevalni dejavnik rasti kolonij (angl. *colony stimulating factor*, *CSF*) in heparin-vezavni epidermalni rastni dejavnik (angl. *heparin-binding epidermal growth factor*, HB-EGF) (Cullinan in sod., 1996; Florio in sod., 2007; Halasz in Szekeres-Bartho, 2013). Pomembni kemokini, ki sodelujejo pri navzkrižni komunikaciji med endometrijem in zarodkom, pa so IL-8 (angl. *interleukin-8; CXCL8*), kemokin 2 s C-C motivom (angl. *C-C motif chemokine 2*, *CCL2*; pogosto uporabljeno alternativno ime MCP-1), z rastjo uravnavan alfa protein (angl. *growth-regulated alpha protein*; *CXCL1*; pogosto uporabljeno alternativno ime C-X-C motif chemokine 1) in receptor

tipa 4 za C-X-C kemokin (angl. *C-X-C chemokine receptor type 4*, *CXCR4*) (Hess in sod., 2007).

Signalizacija P4 preko izražanja zaviralnega dejavnika, induciranega s progesteronom (angl. *progesterone-induced blocking factor*, *PIBF*), zagotavlja imunotolerantno okolje endometrija za uspešno vgnezdenje blastociste. Izražanje PIBF zavira delovanje celic naravnih ubijalk (NK) in spodbuja citokinski odziv celic T pomagalk tipa 2 (angl. *T helper 2*, Th2), ki so infiltrirane v stromi endometrija in vzdržujejo protivnetno okolje (Halasz in Szekeres-Bartho, 2013).

Hormon P4 nadzira delovanje prepisovalnega dejavnika (angl. *transcription factor*) proteina homebox Hox-A10 (*HOXA10*), ki je pomemben za procese vgnezdenja. Povišano izražanje HOXA10 v epiteliju endometrija tekom okna vgnezdenja vodi v izražanje integrinov alfa 5/beta 3 (angl. *integrin alpha-V/beta-3*, ITGAV:ITGB3; pogosto uporabljeno alternativno ime $\alpha_v\beta_3$) in ITGA4:ITGB1 ter tvorbo pinopodov na apikalni strani epitelija, kamor se lahko blastocista vsidra (Nikas in Makrigiannakis, 2003; Halasz in Szekeres-Bartho, 2013). Nekatere raziskave so pokazale, da zarodek in endometrij matere v času vgnezdenja aktivno komunicirata preko zunajceličnih veziklov (angl. *extracellular vesicle*) (Ng in sod., 2013). Endometrij naj bi v zunajcelične vezikle izločal adhezivne molekule, kot so integrin-vezavni fibronektin in člane poti kinaz fokalne adhezije (angl. *focal adhesion kinase*, *FAK*), ki jih sprejmejo celice trofoblasta, da si izboljšajo adhezivne lastnosti (Greening in sod., 2016).

Po apoziciji in adheziji sledi invazija blastociste v deciduo, kar sproži migracijo stromalnih celic na mesto vgnezdenja. Stromalne celice obkrožijo zarodek in tako aktivno pripomorejo k njegovemu nadaljnjemu vgnezdenju (Schwenke in sod., 2013). Premikanje in polarnost endometrijskih celic nadzoruje družina proteinov Rho GTPaz, ki s tvorbo stresnih vlaken polimeriziranega aktina in tvorbo lameliopodija omogočijo premikanje celic skozi zunajcelični matriks (angl. *extracellular matrix*) (Makieva in sod., 2018). Član družine Rho GTPaz, RAC1 (angl. *Ras-related C3 botulinum toxin substrate 1*, *RAC1*, s pogostim alternativnim imenom Rac-1) v interakciji s p21-aktivirajočo kinazo oz. PAK (spada med serin/treonin kinaze), omogoči napredovanje lamelipodijev preko integrinske adhezije v smeri migriranja celic. Medtem pa na drugem koncu celice protein RHOA (angl. *transforming protein RhoA*, *RHOA*, s pogostim alternativnim poimenovanjem RhoA) omogoči prekinitev adhezijskih stikov. Protein ROCK1 (angl. *Rho-associated protein kinase 1*, *ROCK1*), ki jo aktivira RHOA, tvori krčne sile preko interakcij aktina in miozina. Krčenje in odmik zadnjih robov omogoča premikanje telesa celice. Hitro preurejanje citoskeleta in olajšano migracijo stromalnih celic preko signalne kaskade RHOA/ROCK in RAC1/PAK omogoča negenomsko delovanje P4 (Grewal in sod., 2010; Makieva in sod., 2018). Hormon P4 v decidui spodbuja tudi tvorbo vezavnega proteina 1 za inzulinu podoben rastni dejavnik (angl. *insulin-like growth factor-binding protein 1*, *IGFBP1*), ki se veže na specifične integrine ITGA5:ITGB1

trofoblasta in izboljša migracijske in invazivne lastnosti zarodka (Halasz in Szekeres-Bartho, 2013).

Za uspešno migracijo stromalnih celic in globoko invazijo trofoblasta je pomembno preurejanje zunajceličnega matriksa (laminini in kolageni). Aktivno preurejanje zunajceličnega matriksa decidue je doseženo s pomočjo proteolitskih encimov matriks metaloproteinaz (MMP). Celice trofoblasta izločajo MMP-2 in -9 za razgradnjo kolagena tipa 4, ki je glavna komponenta bazalnih membran. Hormon P4 lahko preko različnih mehanizmov nadzira aktivnost encimov MMP. S tem deluje kot negativni regulator invazije zarodka, s čimer prepreči njegovo prekomerno agresivnost. Signalizacija PGR normalno deluje preko vezave na specifične P4 odzivne elemente, ki se nahajajo v promotorski regiji ciljnih genov. Hormon P4 pa lahko regulira tudi promotorje brez teh elementov. Tako lahko P4 v stromalnih celicah spodbuja izražanje proproteina beta 1 transformirajočega rastnega dejavnika (angl. *transforming growth factor beta-1 proprotein*, *TGFB1*; pogosto uporabljen sinonim TGF-β), ki pa v epitelijskih celicah zavira izražanje *MMP7* in aktivira izražanje inhibitorja 3 metaloproteinaz (angl. *metalloproteinase inhibitor 3*, *TIMP3*). Po drugem mehanizmu lahko P4 ovira vezavo prepisovalnega dejavnika p65 (angl. *transcription factor p65*, *RELA*, pogosto uporabljen sinonim jedrni dejavnik kapa B (angl. *nuclear factor NF-kappa-B*, NF-κB) na promotorske regije *MMP1*, *MMP3* in *MMP9* ter prepreči njihovo transkripcijo. Pri nadziranju izražanja *MMP2* hormon P4 spodbuja degradacijo prepisovalnega dejavnika Sp4 (angl. *transcription factor Sp4*, *SP4*), s čimer ne pride do tvorbe kompleksa SP4/PGR. Ker ni vezave kompleksa na promotorsko regijo *MMP2*, je nadaljnja transkripcija tega gena zavrta. Posredno, preko regulacije nivoja leptina (*LEP*), pa lahko P4 vpliva na signalno pot pretvornika signala in aktivatorja transkripcije 3 (angl. *signal transducer and activator of transcription 3*, *STAT3*), ki uravnava transkripcijo *MMP2* in *MMP9* (Simón in sod., 2000; Halasz in Szekeres-Bartho, 2013).

1.1.4 Adenomioza

1.1.4.1 Patogeneza

Adenomioza je patologija maternice, kjer se endometriju podobno tkivo (celice žleznega epitelija in fibroblasti strome) nahajajo na ektopičnih mestih v mišični steni maternice (miometriju) (Ferenczy, 1998). Adenomiozno tkivo je lahko znotraj miometrija razpršeno (difuzna adenomioza) ali pa se nahaja na enem ali več posameznih mestih (fokalna adenomioza) (Van den Bosch in sod., 2015). Pogosti simptomi adenomioze so močne menstrualne krvavitve, bolečine v spodnjem delu trebuha in neplodnost (Vannuccini in sod., 2017). Absolutna diagnoza adenomioze je mogoča le s histološkim pregledom vzorcev histerektomije. S tehnološkim razvojem tehnik slikanja, kot sta transvaginalni ultrazvok (TVUZ) (Andres in sod., 2018) in magnetna resonanca (MR) (Kissler in sod., 2008), pa je v

zadnjem času omogočeno zgodnejše neinvazivno odkrivanje te bolezni (Van den Bosch in sod., 2015).

Kishi in sod. (2012) so s pomočjo MR opazovali mesta pojavljanja adenomioznega tkiva v miometriju in predlagali štiri podtipe klasifikacije adenomioze. Le-ti so podrobneje opisani v nadaljevanju s pripadajočimi molekularnimi dejavniki patofiziologije.

1.1.4.1.1 Podtip I ali notranja adenomioza (angl. *intrinsic adenomyosis*)

Ta podtip označuje prisotnost adenomiozega tkiva v notranjem miometriju (Kishi in sod., 2012). Je najbolj raziskan podtip adenomioze, čigar nastanek se povezuje s poškodbo notranjega miometrija, kar omogoča celicam bazalne plasti endometrija invazijo spodaj ležečega miometrija (Kishi in sod., 2012). Invazija bazalnega endometrija naj bi bila mogoča, ker za razliko od drugih tkiv sluznice (npr. črevesja) interfaza med endometrijem in miometrijem nima vmesne bazalne membrane (Fusi in sod., 2006; Zhai in sod., 2020b). Do poškodb notranjega miometrija bi lahko prišlo zaradi invazivnih kirurških posegov ali carskih rezov. Poškodbe miometrija pa bi lahko bile tudi fiziološkega izvora zaradi ciklične peristaltične aktivnosti maternice tekom reproduktivne dobe ženske (Kunz in sod., 2007).

Poškodovan notranji miometrij naj bi preko lokalno povišanega *IL1B* izzval aktivacijo sintaze 2 prostaglandina G/H (angl. *prostaglandin G/H synthase 2*, *PTGS2*; s pogostim alternativnim imenom cyclooxygenase-2 oz. COX-2), ki sproži tvorbo prostaglandina E2 (PGE2). Le-ta v poškodovanem tkivu dalje aktivira steroidogeni akutni regulatorni protein (angl. *steroidogenic acute regulatory protein*, *STAR*) in aromatazo (angl. *aromatase*, *CYP19A1*; pogosto uporabljeno alternativno ime P450 aromatase). Proteina STAR in CYP19A1 sprožita tvorbo in aromatizacijo testosterona v E2, kar vodi v hiperestrogeno okolje endometrija. Hormon E2 preko vezave na izoobliko beta receptorja za estrogen (angl. *estrogen receptor beta*, *ESR2*) izzove delitve celic in s tem celjenje poškodbe. Vendar pa povišana raven E2 hkrati deluje tudi na izoobliko alfa receptorja za estrogen (angl. *estrogen receptor*, *ESR1*, s pogostim alternativnim imenom ER-alfa), s čimer se poviša sinteza oksitocina. Oksitocin zviša peristaltično aktivnost maternice (hiperperistaltika), s čimer se zvišajo mehanski pritiski v maternici. To pa poškoduje celice miometrija in proces celjenja tkiva se ustavi. Ustvari se mehanizem pozitivne povratne zanke, kjer kronična hiperperistaltika notranjega miometrija vodi v ponavljajoče se cikle samopoškodbe in celjenja tkiva (TIAR). Mehanizem TIAR poškoduje mišična vlakna miometrija, s čimer je olajšan vdor endometrijskih celic (García-Solares in sod., 2018).

Raziskave na molekularnem nivoju kažejo, da imajo ESC žensk z adenomiozo v primerjavi z ženskami brez adenomioze višjo zmožnost migriranja (Mehasseb in sod., 2010b). Na podlagi analize izražanja gena regulatorja apoptoze Bcl2 (angl. *apoptosis regulator Bcl-2*, *BCL2*) in pripadajočega proteina je bila dokazana tudi njihova boljša odpornost na apoptozo in hitrejše delitve celic (proliferacija) (Jones in sod., 1998; Li J. in sod., 2019). Tudi v raziskavah

globalne analize transkriptoma endometrija med ženskami z in brez adenomioze so bili identificirani spremenjeno izraženi geni, ki so bili dalje obogateni v bioloških poteh, povezanih z regulacijo apoptoze. Identificirali pa so tudi obogateno signalno pot tarče rapamicina pri sesalcih (angl. *mammalian target of rapamycin*, mTOR), ki uravnava delitve celic in njihovo preživetje (Herndon in sod., 2016; Xiang in sod., 2019).

Pred kratkim so Liu in sod. (2021) izvedli analizo RNA-seq posameznih celic (scRNA-seq) na vzorcih parnega evtopičnega in ektopičnega endometrija ženske z adenomiozo (EvEA in EkEA) ter na kontrolnem vzorcu endometrija ženske z laparaskopsko odstranitvijo mioma (kontrola). S primerjavo transkriptomskih podatkov endometrijskih epitelijskih celic (EEC) tako med EvEA in EkEA kot tudi med EvEA in kontrolnim endometrijem so zaznali spremenjeno izražene gene, ki so bili dalje obogateni v biološke poti, povezane s celičnim gibanjem, celično proliferacijo, angiogenezo in vnetnim procesom. Avtorji so zaključili, da so patološke spremembe prisotne že v EvEA, kar potrjuje teorijo o nastanku adenomioze z invazijo in migracijo endometrijskih celic v miometrij. Dodatno so bili spremenjeno izraženi geni med EvEA in EkEA, obogateni v poteh, povezanih z rakavimi značilnostmi (angiogeneza, celična migracija, vnetje, proliferacija in signalni poti NF-κB ter fosfoinozitida 3 kinaze (angl. *phosphoinositide-3-kinase/protein kinase B (Akt)*, PI3k/AKT). Avtorji so te poti predlagali kot kandidatne mehanizme, ki omogočajo obstoj adenomioznih lezij v miometriju (Liu in sod., 2021). Migracijske in invazivne lastnosti EEC pri adenomiozi naj bi omogočil mehanizem epitelijsko mezenhimskega prehoda (EMT), ki ga sprožijo povišane vrednosti E2 (Chen in sod., 2010). Za proces EMT je značilno zmanjšano izražanje molekularnega označevalca epitelijskih celic kaderina 1 (angl. *cadherin-1*, *CDH1*; pogosto uporabljeno alternativno ime E-cadherin), kar povzroči izgubo celične polarnosti in adhezije. Hkrati pa je povišano izražanje mezenhimskih označevalcev, kot so beta katenin 1 (angl. *catenin beta-1*, *CTNNB1*) in drugih članov signalne poti Wnt (Oh in sod., 2013), vimentina (Chen in sod., 2010) ter kinaze vezane na integrin (angl. *integrin-linked kinase*, *ILK*) (Zhou in sod., 2018), ki pa celicam omogočajo gibanje (mobilnost). Pri ženskah z adenomiozo je bilo s spremenjenimi vrednostmi izražanja genov lizil oksidaze (angl. *lysyl oxidase*, *LOX*), *MMP7* (Herndon in sod., 2016), MMP2 in MMP9 (Li in sod., 2006) pokazano tudi moteno delovanje zunajceličnega matriksa EvEA in EkEA, kar bi lahko dodatno pripomoglo k olajšanemu vdoru in preživetju endometrijskih celic v miometrij.

1.1.4.1.2 Podtip II ali zunanja adenomioza (angl. *extrinsic adenomyosis*)

Podtip označuje prisotnost adenomioznega tkiva v zunanjem miometriju, ki se širi proti notranjosti organa (Kishi in sod., 2012). Do infiltracije endometrijska tkiva iz zunanje strani maternice (preko seroze) naj bi prišlo zaradi retrogradne menstruacijske krvavitve preko jajcevodov v trebušno votlino. Od tam pa endometrijske celice (mezenhimske matične celice ali odrasle celice) prodrejo v zunanji miometrij in razvijejo endometrijske zasevke (Zhai in

sod., 2020b). Teorija etiologije tega podtipa je podprta z visokim deležem žensk s fokalno adenomiozo v zunanjem miometriju na dorzalni (hrbtni) strani maternice in pridruženo globoko infiltrativno endometriozo (angl. *deep infiltrating endometriosis*) (Chapron in sod., 2017; Khan in sod., 2019).

1.1.4.1.3 Podtip III ali intramuralna adenomioza (angl. *intramural adenomyosis*)

Podtip označuje prisotnost adenomioznega tkiva v centralnem miometriju, ki ga obdaja nepoškodovano mišično tkivo; poškodovan ni notranji miometrij niti seroza (Kishi in sod., 2012). Do nastanka tega podtipa adenomioze naj bi prišlo zaradi *de novo* transformacije pluripotentnih embrionalnih ostankov Müllerjevih vodov, ki so napačno locirani v miometriju (Ferenczy, 1998). Druga teorija nastanka tega podtipa adenomioze se povezuje z diferenciacijo odraslih endometrijskih matičnih celic, ki naj bi migrirale v miometrij. Pri tej teoriji naj bi endometrijske progenitorne epitelijske celice in mezenhimske matične celice prečkale bazalno plast endometrija in se nato v miometriju dalje delile in diferencirale v epitelijske oz. stromalne celice (Zhai in sod., 2020b).

1.1.4.1.4 Podtip IV ali nedoločena adenomioza (angl. *indeterminate adenomyosis*)

Podtip označuje hudo obliko adenomioze in sestoji iz kombinacij podtipov I–III (Kishi in sod., 2012).

1.1.4.2 Vpliv na plodnost

S prelaganjem nosečnosti na kasnejše reproduktivno obdobje je adenomioza pogosto zaznana v diagnostičnih postopkih zdravljenja neplodnosti (Maheshwari in sod., 2012). Puente in sod. (2016) so z uporabo TVUZ diagnosticirali adenomiozo pri 24,4 % žensk (n = 248/1015), ki so se med leti 2009 in 2013 zdravile za neplodnostjo. Od tega jih je imelo 167 (67,3 %) blago, 56 (22,6 %) zmerno in 25 (10,1 %) hudo obliko adenomioze. Adenomioza je bila v višji meri značilno zastopana med ženskami starejšimi od 40 let (29,7 %; n = 94/316) kot pa med mlajšimi od 40 let (22,0 %; n = 154/699). Adenomioza je bila pogostejša pri ženskah s pridruženo endometriozo (35,1 %; n = 34/97) in/ali miomi (18,0 %; n = 48/266). Hashim in sod. (2020) pa so poročali o 7,5 % (n = 24/320) deležu na novo diagnosticirane adenomioze med mladimi neplodnimi ženskami (Hashim in sod., 2020). Razlike o poročanih deležih pojavnosti adenomioze v populacijah bi lahko bile posledica še neoblikovanih popolnih kriterijev za njeno ultrazvočno diagnozo (Bazot in Daraï, 2018).

Puente in sod. (2016) so poročali, da je adenomioza pogostejša pri ženskah, ki so imele ponavljajoče neuspešne poizkuse vgnezdenja zarodkov (angl. *recurrent implantation failure*) (34,7 %; n = 107/308), ponavljajoče splave (38,2 %; n = 26/68) in neuspešne predhodne

postopke oploditve z biomedicinsko pomočjo (OBMP), (34,7 %, n = 107/305). Avtorji so zaključili, da adenomioza negativno vpliva na plodnost bolnice (Puente in sod., 2016). Podobno so poročali tudi drugi raziskovalci (Salim in sod., 2012; Thalluri in Tremellen, 2012; Puente in sod., 2016; Sharma in sod., 2019). Po drugi strani pa nekatere raziskave teh povezav niso zaznale (Mijatovic in sod., 2010; Costello in sod., 2011; Benaglia in sod., 2014). Vercellini in sod. (2014) so na podlagi meta analize osmih kliničnih raziskav napovedali 28 % manjšo verjetnost za uspešno nosečnost v postopkih OBMP pri ženskah z adenomiozo kot pa brez adenomioze. Tudi meta analiza, ki so jo opravili Horton in sod. (2019), povezuje adenomiozo maternice z zmanjšanimi stopnjami vgnezdenja zarodkov, nosečnosti in rojstvi otrok ter z zvišano stopnjo splavov.

1.1.4.3 Dejavniki vpliva na endometrijsko receptivnost

Molekularnih raziskav o vplivu adenomioze na plodnost je malo. Večina jih je opravljena na posameznem ali manjšem številu kandidatnih genov in/ali pripadajočih proteinih, kar smo povzeli v naši objavljeni raziskavi (Prašnikar in sod., 2020b). V nadaljevanju je naštetih več strukturnih in molekularnih nepravilnosti, s katerimi naj bi adenomiozne lezije negativno vplivale na plodnost bolnice.

- Poškodovan notranji miometrij. Prisotnost adenomioznih lezij bi lahko vplivala na organizacijo mišičnih vlaken notranjega miometrija, s čimer naj bi se spremenil vzorec krčenja maternice. Posledično bi to lahko oviralo tako potovanje semenčic proti jajcevodoma kot tudi invazijo zarodka v endometrij (Mehasseb in sod., 2010a; Zhang in sod., 2015).

- Prekomerne vrednosti prostih radikalov v maternici. Prisotnost adenomioznih lezij naj bi vodilo v zvišane vrednosti prostih radikalov. Le-to naj bi sprožilo vnetni odziv in nadaljnjo aktivacijo makrofagov in celic T, ki škodujejo spolnim celicam, ovirajo razvoj zarodka in sprožijo splav (Ota in sod., 1998a). V primerjavi s kontrolnimi skupinami so v endometriju žensk z adenomiozo zaznali povišane vrednosti različnih encimov, ki tvorijo ali odstranjujejo proste radikale: superoksid dismutazo (Ota in sod., 1999), glutationin peroksidazo (Ota in sod., 2000), ksantin oksidazo (Ota in sod., 2001a), katalazo (Ota in sod., 2002) in sintazo dušikovega oksida (Ota in sod., 1998b). V endometriju žensk z adenomiozo so poročali o povišanih ravneh izražanja vnetnih citokinov (Sotnikova in sod., 2002; Ulukus E.C. in sod., 2005) kot tudi zvišani citotoksičnosti maternič nih celic NK (uNK) (Yang in sod., 2004).

- Lokalno povišana koncentracija E2 v maternici. Nenormalno delovanje CYP19A1 v EvEA in EkEA vodi v izražanje od E2 odvisnih genov in s tem ovira pravilno molekularno delovanje endometrija tekom menstruacijskega ciklusa (Maia in sod., 2006; Mehasseb in sod., 2011).

- Prekomerno ožiljenje endometrija. Prekomerno izražanje regulatornih dejavnikov (VEGF, E2, eNOS in prostaglandini), vključenih v proliferacijo žilnih celic, vodi v prekomerno ožiljenje endometrija v P in S fazi. To naj bi bil tudi vzrok hudih menstruacijskih krvavitev oz. hipermenoreje pri ženskah z adenomiozo (Ota in Tanaka, 2003; Vannuccini in sod., 2017).

Pri ženskah z adenomiozo so v primerjavi z ženskami brez adenomioze poročali o znižanem izražanju nekaterih genov in proteinov (navedeni spodaj), ki se povezujejo z vlogo pri endometrijski receptivnosti in decidualizaciji strome.

- ITGB3 in SPPP1 (angl. *secreted phosphoprotein 1*; pogosto uporabljen sinonim osteopontin). Znižano izražanje na ravni gena in proteina tega integrina in njegovega liganda je bilo povezano z zmanjšano adhezijsko kapaciteto decidue za vezavo zarodka (Xiao in sod., 2013).

- LIF in LIFR. Znižano izražanje tega citokina in pripadajočega receptorja je bilo povezano z ovirano fosforilacijo in nadaljnjo aktivacijo signalne poti STAT3. Ovirana pot STAT3 pa naj bi zavrla prekinitev medceličnih stikov v lumnu endometrijskega epitelija, kar ovira proces vgenzdenja zarodka (Yen in sod., 2016).

- IL-10. Tudi zmanjšano izražanje protivnetnega citokina IL-10 je bilo povezano z ovirano aktivacijo signalne poti STAT3. Posledično naj bi se zmanjšala stopnja izražanja prepisovalnega dejavnika HOXA10, kar zniža izražanje njegovih pripadajočih genov, povezanih s tvorbo pinopodov (pomembni dejavniki adhezije zarodka) in genov, povezanih s procesom decidualizacije strome (Fischer in sod., 2011; Wang in sod., 2018).

- Prepisovalni dejavniki z vlogo pri decidualizaciji. Peng in sod. (2021) so z analizo svežih vzorcev biopsij endometrija med ženskami z in brez adenomioze zaznali značilno zmanjšano izražanje genov za prepisovalne dejavnike HOXA10, FOX01 (angl. *forkhead box protein O1*), KLF5 (angl. *Krueppel-like factor 5*), CEBPB (angl. *CCAAT/enhancer-binding protein beta*) in HAND2 (angl. *heart- and neural crest derivatives-expressed protein 2*). Na primarni kulturi decidualiziranih ESC (dESC), ki so jih pridobili iz žensk z adenomiozo, pa so zaznali spremenjene vrednosti izločanih citokinov CXCL-1, -2, -3, IL-6, -8, SSP1, CCL-2, -12, -20 in VEGFA ter spremenjeno razmerje med izražanjem encimov preoblikovanja zunajceličnega matriksa *MMP9* in *TIMP1* (Peng in sod., 2021).

- miR-21. Zmanjšano izražanje tega transkripta je bilo povezano s posledičnim prekomernim izražanjem gena *KLF12* in znižanim izražanjem gena za jedrni receptor NR4A1 (angl. *nuclear receptor subfamily 4 group A member 1*). To pa naj bi dalje vodilo v znižano

izražanje pripadajočih *PRL* in *IGFBP1* (molekularna dejavnika decidualizacije) (Yan in sod., 2019).

1.1.5 Omske raziskave endometrija pri različnih ginekoloških stanjih

Iz Priloge B, v kateri so zbrane objavljene molekularne raziskave adenomioze, se kaže kompleksnost bioloških in celičnih procesov patofiziologije endometrija pri tej bolezni. Kompleksen pa je tudi fiziološki razvoj endometrija tekom menstruacijskega ciklusa (Makieva in sod., 2018). Zato je na podlagi analize posameznih kandidatnih lokusov težko opredeliti, ali je spremenjeno endometrijsko izražanje vzrok ali posledica prisotnosti adenomioze. Molekularno kompleksnost endometrija je tako smiselno raziskovati z omskimi pristopi, ki omogočajo globalno (angl. *genome-wide*) molekularno analizo tkiva (Altmäe in sod., 2014). Izraz omika se nanaša na uporabo visoko zmogljivih (angl. *high-throughput*) tehnik, ki lahko v biološkem vzorcu preiskujejo razlike v genomu (določevanje raznolikosti v DNA zaporedju; genomika), epigenomu (določanje epigenetskih sprememb DNA; epigenomika), transkriptomu (določevanje sestave in obsežnosti izražanja genov iz genoma pod posebnimi pogoji ali v določenem časovnem obdobju; transkriptomika), proteomu (določevanje sestave in razsežnosti proteinov pod posebnimi pogoji ali v določenem časovnem obdobju) in metabolomu (določevanje raznolikosti v sestavi in razsežnosti metabolitov; metabolomika) (Pirih in Kunej, 2017).

Tehnologija analize transkriptoma je do danes močno napredovala, saj je iz uporabe mikromrež, ki temelji na tehnologiji hibridizacije, prešla na tehnologijo sekvenciranja naslednje generacije (NGS) (Zhao in sod., 2014). Tehnologija RNA-seq omogoča tvorbo več milijonov kratkih RNA odčitkov, ki se nato s pomočjo bioinformatskih programov poravnajo na referenčni genom, referenčne transkripte ali na novo sestavljene referenčne genome. Na tak način je mogoče preveriti izražanje vseh transkriptov in bolje oceniti njihovo stopnjo izražanja v preiskovanem vzorcu (Wang Z. in sod., 2009). Z nadaljnjimi obogatitvenimi analizami spremenjeno izraženih genov med preiskovanima skupinama pa je mogoče ugotoviti njihovo vlogo v bioloških sistemih. Obogatitvene analize genov je mogoče izvesti z različnimi bioinformacijskimi orodji, kot je npr. DAVID (Huang in sod., 2009), ClueGO (Bindea in sod., 2009) in CluePedia (Bindea in sod., 2013), ki delujejo na podlagi algoritmov, statističnih testov in biološkega znanja različnih podatkovnih zbirk. Najbolj je razširjena uporaba zbirke Gene Ontology (GO), s katero je mogoče za preiskovane gene pridobiti podatke o treh ontologijah: biološki proces (angl. *biological process*, GO-BP), celična komponenta (angl. *cellular component*, GO-CC) in molekularna vloga (angl. *molecular function*, GO-MF) (Ashburner in sod., 2000). Z dodatnimi zbirkami, kot sta npr. KEGG (Kanehisa in sod., 2017) in Reactome (Fabregat in sod., 2016) pa je mogoče analizirati preiskovane gene za dodatne obogatene biološke poti in reakcije.

1.1.5.1 Endometrijska receptivnost (zdrava maternica)

Namen omskih raziskav fiziološkega razvoja endometrija v SS fazi menstruacijskega ciklusa je prepoznati genske družine in pripadajoče signalne poti, ki sodelujejo v procesih endometrijske receptivnosti (Borthwick in sod., 2003; Kao in sod., 2003b; Díaz-Gimeno in sod., 2011; Altmäe in sod., 2012; Sigurgeirsson in sod., 2017; Yu in sod., 2021). Altmäe in sod. (2017) so iz devetih raziskav, ki so primerjale transkriptom vzorcev endometrija med receptivnim (LH+7) in nereceptivnim (LH+2) stanjem, zbrali poročane spremenjeno izražene gene ter med njimi identificirali 57 močnejših kandidatov endometrijske receptivnosti (Altmäe in sod., 2017). Te gene so vključili v komercialni diagnostični test za določevanje pojava receptivne faze (SS faza) v menstruacijskem ciklusu endometrija (Saare in sod., 2019). Poznavanje personaliziranega časa optimalne endometrijske receptivnosti posamezne ženske omogoča načrtovanje optimalnega časa prenosa zarodka v maternico s postopki OBMP. Na tak način naj bi se zagotovila sinhrona komunikacija med zarodkom in endometrijem matere, kar bi zvišalo možnost zanositve. S pomočjo transkriptomskih raziskav in algoritmov strojnega učenja se na tržišču pojavlja vse več takšnih testov (Díaz-Gimeno in sod., 2011; Enciso in sod., 2018; Haouzi in sod., 2021; Zhang in sod., 2021), saj nizka stopnja vgnezditve kljub visoko kvalitetnim zarodkom ostaja eden izmed izzivov zdravljenja neplodnosti (Edwards, 1995; Ruiz-Alonso in sod., 2013; Wyns in sod., 2020).

1.1.5.2 Adenomioza

S pregledom literature smo zasledili šest transkriptomskih raziskav endometrija med ženskami z in brez adenomioze (Martinez-Conejero in sod., 2011; Herndon in sod., 2016; Jiang J.F. in sod., 2016; Dior in sod., 2018; Xiang in sod., 2019; Liu in sod., 2021). Večina jih je bila opravljenih na vzorcih endometrija P faze menstruacijskega ciklusa (Herndon in sod., 2016; Jiang J.F. in sod., 2016; Xiang in sod., 2019) z namenom raziskovanja patofiziologije adenomioze. Herndon in sod. (2016) so identificirane spremenjeno izražene gene povezali s sodelovanjem v bioloških poteh apoptoze, odzivom na steroidne hormone in preoblikovanjem zunajceličnega matriksa (Herndon in sod., 2016). Xiang in sod. (2019) so spremenjeno izražene gene povezali s celično rastjo in proliferacijo, celičnim gibanjem in spremenjenim uravnavanjem signalnih poti IL-6 in ERK/MAPK (Xiang in sod., 2019). Jiang J.F. in sod. (2016) pa so poleg mRNA analizirali tudi profil izražanja dolgih nekodirajočih RNA (lncRNA) in izpostavili nekaj kandidatnih lncRNA z vplivom na stopnjo izražanja pripadajočih genov (Xiang in sod., 2019). Pri raziskavi, ki so jo opravili Liu in sod. (2021) in je podrobneje predstavljena v poglavju 1.1.4.1.1, pa datiranje endometrija ni bilo zabeleženo (Liu in sod., 2021). Omenjene raziskave so bile opravljene pri naprednih oblikah adenomioze, pri bolnicah srednjih 40 let ali starejših.

Vpliv adenomioze na molekularno ozadje endometrijske receptivnosti pri ženskah v rodni dobi pa sta določevali le dve transkriptomski raziskavi, ena na ženskah z ultrazvočno potrjenimi znaki adenomioze (Martinez-Conejero in sod., 2011) in druga pri ženskah s

pridruženo endometriozo (Dior in sod., 2018). Martinez-Conejero in sod. (2011) so z metodo mikromrež zaznali 34 genov spremenjenega endometrijskega izražanja med adenomiozno in kontrolno skupino, katerih vloge pa takrat niso povezovali z vplivom na endometrijsko receptivnost.

1.1.5.3 Sorodna bolezen endometrioza

V primerjavi z adenomiozo je bilo več omskih raziskav endometrija opravljenih pri endometriozi. Te raziskave smo zbrali in njihove podatke uporabili v naši raziskavi (Prašnikar in sod., 2020a). Endometrioza je sorodna patologija adenomiozi (Benagiano in sod., 2014), kjer pa se endometriju podobno tkivo nahaja na ektopičnih mestih izven maternice, tj. v trebušni votlini in v mali medenici (Del Frate in sod., 2006). Po mednarodni statistični razvrstitvi bolezni in z njimi povezanimi zdravstvenimi težavami (ICD-10, 2019) je adenomioza razvrščena kot »endometrioza maternice«. Adenomioza in endometrioza sta pri bolnicah pogosto sopojavni (Kunz in sod., 2005). Meuleman in sod. (2009) so ocenili, da za endometriozo trpi okoli 47 % (n = 104/221) žensk, ki se zdravijo za neplodnostjo s parterji z normalnim izvidom spermiograma. Nakazuje se negativen vpliv endometrioze na plodnost bolnice (Strathy in sod., 1982; Macer in Taylor, 2012).

V omskih raziskavah endometrija v pričakovanem času receptivnosti med ženskami z in brez endometrioze so bili identificirani številni spremenjeno izraženi geni (Kao in sod., 2003a; Burney in sod., 2007; Tamaresis in sod., 2014; Houshdaran in sod., 2016), miRNA (Zhou in sod., 2016) in proteini (Stephens in sod., 2010). Kao in sod. (2003a) so med 206 spremenjeno izraženimi geni identificirali tudi gene zmanjšane stopnje izražanja, ki sodelujejo pri celični adheziji in pri izločanju proteinov endometrijskega epitelija. Avtorji so zaključili, da lahko endometrij pri endometriozi predstavlja toksično okolje, ki zaradi porušenega delovanja imunskega sistema in zvišanega vnetnega ter apoptoznega odziva ovira vezavo zarodka (Kao in sod., 2003a). Burney in sod. (2007) so pri ženskah z endometriozo opazili spremenjeno uravnavanje prehoda endometrija iz P v S fazo menstruacijskega ciklusa. V vzorcih endometrija SS faze so namreč zaznali povišano izražanje genov, vključenih v procesa sinteze DNA in celične mitoze, kar je značilnost P faze. Avtorji so predlagali, da je endometrij žensk z endometriozo odporen na delovanje P4, kar posledično vpliva na izražanje od P4 odvisnih genov. To pa bi lahko dalje oviralo decidualizacijo strome in stanje receptivnosti (Burney in sod., 2007). Tamaresis in sod. (2014) so spremenjeno izražene gene povezali z aktivacijo imunskih celic, aktivnostjo citokinov, spremenjeno signalno potjo steroidnih hormonov in s spremenjenimi signalnimi potmi rastnega dejavnika, Wnt, MAPK in NFKB (Tamaresis in sod., 2014). Zhou in sod. (2016) so poleg endometrijskih transkriptov mRNA izvedli tudi profiliranje transkriptov miRNA in identificirali zvišano izražanje miR-196a pri endometriozni skupini. Na kulturi dESC, ki so jih pridobili iz žensk z endometriozo, so pokazali, da lahko povišane vrednosti miR-196a zavirajo izražanje gena izooblike beta PGR (pogosto uporabljen simbol v literaturi *PGR-B*) preko vezave na njegovo 3'-neprevedno regijo

(angl. *untranslated region*, *UTR*). Avtorji so zaključili, da bi lahko bilo spremenjeno izražanje miRNA eden od mehanizmov progesteronske odpornosti endometrija pri endometriozi (Zhou in sod., 2016). Houshdaran in sod. (2016) so vzorcem endometrija hkrati določevali DNA metilom in transkriptom. Pokazali so, da lahko endometrioza vpliva na mehanizme preoblikovanja kromatina in spremeni vzorce metilacije CpG otočkov. Spremembe v metilaciji DNA so dalje povezali s spremenjeno izraženimi geni, ki sodelujejo v celični delitvi, angiogenezi, vnetnemu in imunskemu odzivu ter v odzivu na steroidne hormone (Houshdaran in sod., 2016). Stephens in sod. (2010) pa so s proteomsko analizo pokazali, da v endometriju žensk z endometriozo potekajo potranslacijske modifikacije, ki vodijo v specifične izooblike proteinov.

1.2 NAMEN RAZISKAVE IN DELOVNE HIPOTEZE

Namen naloge je bil s pristopi sistemske biologije razširiti znanje o molekularnem ozadju endometrijske receptivnosti za vgnezdenje zarodka pri slabo raziskani adenomiozi.

Delovni hipotezi sta bili:

Hipoteza 1: Vzorca izražanja genov endometrija v stanju receptivnosti se razlikujeta med skupinama preiskovank z adenomiozo in preiskovank brez adenomioze.

Hipoteza 2: Izražanje genov z domnevno vlogo sodelovanja pri vzpostavitvi receptivnega stanja in genov povezanih s patofiziologijo adenomioze je spremenjeno pri preiskovankah z adenomiozo.

1.3 CILJI

Cilji doktorske disertacije so bili:

1. Z metodo RNA-seq sekvencirati transkriptom endometrija v pričakovanem stanju receptivnosti ženskam z ultrazvočno diagnozo adenomioze in kontrolni skupini žensk brez patologij maternice.
2. Iz znanstvenih člankov pridobiti gene, nekodirajoče RNA (ncRNA) in proteine, ki se povezujejo s spremenjenim izražanjem v endometriju žensk z adenomiozo v primerjavi z ženskami brez adenomioze.
3. Iz znanstvenih člankov pridobiti gene, ncRNA in proteine, ki se povezujejo s spremenjenim izražanjem v endometriju žensk z endometriozo v primerjavi z ženskami brez endometrioze.
4. Integrirati rezultate izvedene analize RNA-seq s trenutnim znanjem molekularnega ozadja endometrijske receptivnosti pri normalnih in patoloških stanjih.

2 ZNANSTVENA DELA

Doktorska disertacija je sestavljena iz treh originalnih znanstvenih člankov in ostalega povezovalnega dela (preverjanje hipoteze 2 in izdelava koekspresijske mreže lncRNA-mRNA). Slika 1 prikazuje grafičen povzetek poteka raziskave doktorske disertacije. Uporabili smo pristope sistemske biologije (sinteza objavljenih podatkov s pregleda literature, izvedba analize RNA-seq in integracija rezultatov RNA-seq s pridobljenimi podatki iz literature), da smo identificirali kandidatne biološke poti in gene, povezane s spremenjeno endometrijsko receptivnostjo pri adenomiozi.

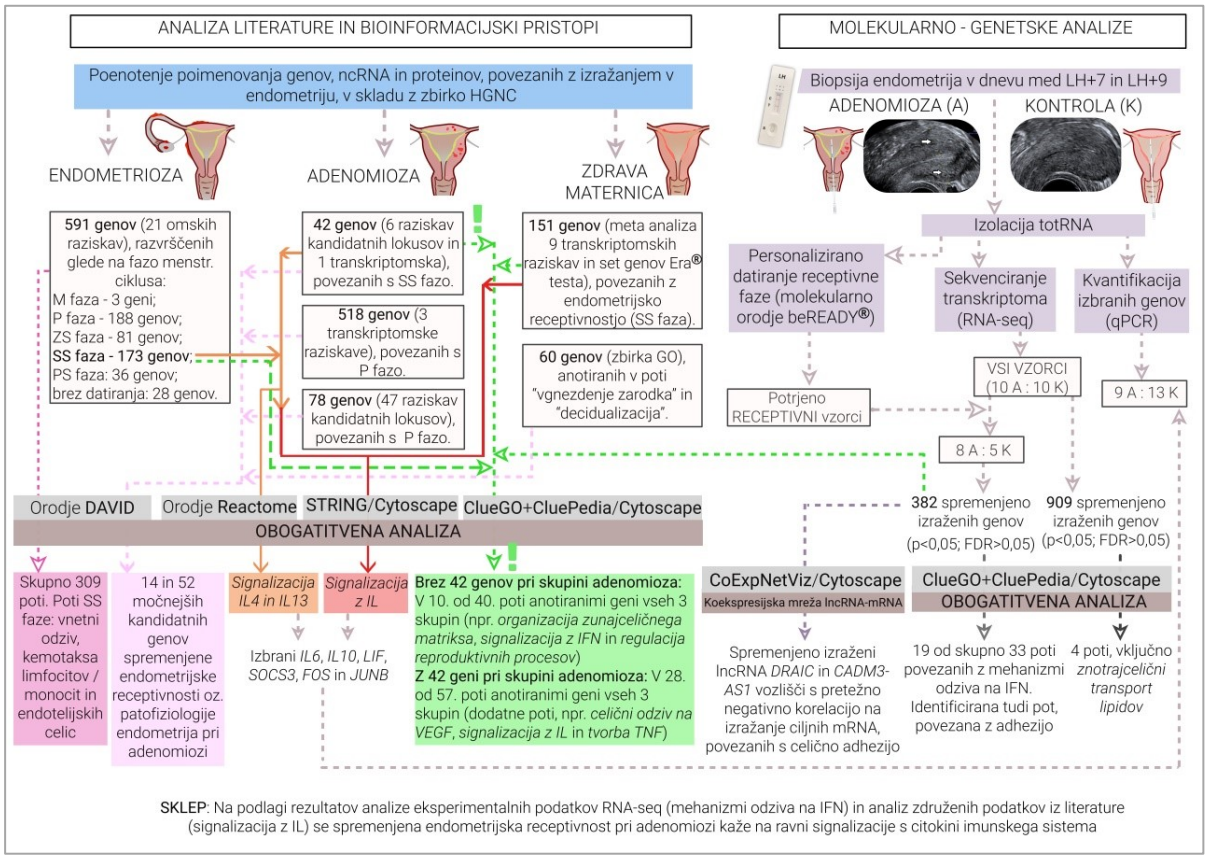
Iz znanstvene literature smo pridobili podatke o transkriptih in proteinih, ki se povezujejo z endometrijskim izražanjem v stanju receptivnosti pri različnih ginekoloških stanjih (adenomioza, endometrioza in zdrava maternica) ter s spremenjenim izražanjem v proliferacijskem endometriju pri adenomiozi. Heterogeno poimenovanje lokusov smo poenotili po genski nomenklaturi podatkovne zbirke HGNC (HUGO, 2019). Oblikovali smo sete genov (Priloga A), povezane z endometrijsko receptivnostjo pri adenomiozi (42 genov), endometriozi (173 genov) in zdravi maternici (seta 151 in 60 genov) ter seta, povezana s patofiziologijo endomertija pri adenomiozi (seta 78 in 518 genov). Genske sete smo obogatili, da smo identificirali kandidatne poti ali gene, povezane s patofiziologijo oz. spremenjeno endometrijsko receptivnostjo pri adenomiozi.

Izvedli smo analize izražanja na vzorcih endometrija, ki so bili pridobljeni v pričakovanem stanju receptivnosti (med dnevi LH+7 in LH+9) žensk z in brez ultrazvočno potrjene adenomioze. Kvantifikacijo šestih izbranih kandidatnih genov (*IL10*, *IL6*, *LIF*, *SOCS3*, *JUNB* in *FOS*) smo izvedli z metodo kvantitativnega PCR (qPCR) in sekvenciranjem transkriptoma (mRNA in lncRNA) z metodo RNA-seq. Kandidatne gene za qPCR smo izbrali na podlagi anotiranja v bioloških poteh *signalizacija z IL* in *signalizacija z IL4 in IL13*, ki smo ju identificirali z obogatitvenimi analizami oblikovanih setov z 42 (adenomioza), 173 (endometrioza) in 151 (zdrava maternica) geni. Zaznali smo znižano izražanje izbranih genov pri adenomiozni v primerjavi s kontrolno skupino, a je bila razlika statistično neznačilna. Analizo RNA-seq endometrija v pričakovanem stanju receptivnosti med adenomiozno in kontrolno skupino žensk smo izvedli z 10 vzorci vsake skupine. Identificirali smo 909 spremenjeno izraženih genov ($p < 0,05$; popravek vrednosti p (FDR > 0,05) in štiri obogatene biološke poti (Bonferroni vrednost $p < 0,05$). Te poti so bile nespecifične, zato jim nismo mogli pripisat podporne literature v povezavi z biologijo endometrija. Uporabljenim vzorcem za RNA-seq endometrija smo dodatno datirali fazo menstruacijskega ciklusa ob času vzorčenja biopsije. Natančno datiranje je bilo izvedeno z novim komercialnim molekularnim orodjem beREADY®, ki na podlagi vzorca izražanja izbranih genov omogoča personalizirano datiranje stanja receptivnosti. Od skupno 20 vzorcev je bilo 13 vzorcev datiranih v receptivno fazo, dva v zgodnjo in pet v pozno receptivno fazo. S ponovno primerjavo transkriptomskih podatkov samo 13 potrjeno receptivnih vzorcev endometrija med adenomiozno (n = 8) in kontrolno (n = 5) skupino smo zaznali 382 spremenjeno izraženih genov

($p < 0,05$; FDR > 0,05). Ti geni so bili obogateni v 33 poteh (Bonferroni vrednost $p < 0,05$), od tega jih je bilo kar 19 povezanih z odzivom na delovanje interferonov (IFN). Zaznali pa smo tudi obogateno pot, povezano s celično adhezijo. Med 382 spremenjeno izraženimi geni je bilo tudi 23 lncRNA. Tem lncRNA smo računali Pearsonov korelacijski koeficient, da smo določili značilne korelacije s preostalimi transkripti podatkovnega seta RNA-seq. Izdelali smo koekspresijsko mrežo mRNA-lncRNA in prepoznali transkripta lncRNA *DRAIC* in *CADM3-AS1* kot pomembni vozlišči s pretežno negativno korelacijo na izražanje ciljnih transkriptov mRNA, ki so bili obogateni v poteh, povezanih s celično adhezijo.

Z obogatitveno analizo smo integrirali set 382 genov, povezanih z endometrijsko receptivnostjo naše adenomiozne skupine, z genskimi seti, ki smo jih oblikovali na podlagi sinteze lokusov s pregleda literature in poenotenjem njihove nomenklature (adenomioza 42 genov, endometrioza 173 genov in zdrava maternica 151 genov). Osredotočili smo se na obogatene poti, kjer so anotirani geni izhajali iz vseh uporabljenih setov. Na tak način smo identificirali poti, povezane s preurejanjem zunajceličnega matriksa, s celičnim odzivom na VEGF, uravnavanjem reproduktivnega procesa in signalizacijo z IL ter IFN. Te poti in anotirane gene poleg mehanizmov odziva na IFN predlagamo kot močnejše kandidate spremenjenih procesov endometrijske receptivnosti pri adenomiozi.

Na podlagi obogatitvenih analiz setov 78, 518, 42 in 60 genov pa smo identificirali 52 močnejših kandidatnih genov patofiziologije ter 10 močnejših kandidatov spremenjene endometrijske receptivnosti pri adenomiozi.



Slika 1: Grafični povzetek raziskave doktorske disertacije. Kandidatne poti in gene spremenjene endometrijske receptivnosti pri adenomiozi smo identificirali s pristopi sistemske biologije: pregled literature za pridobivanje genetskih vzrokov spremenjenega izražanja v endometriju; bioinformacijske analize združenih list genov; analiza RNA-seq za primerjavo transkriptoma endometrija med adenomiozno in kontrolno skupino; integracija spremenjeno izraženih genov naše adenomiozne skupine s podatki iz literature. Uporabljene kratice: HGNC = Odbor za nomenlaturo genov HUGO; IL = interlevkin; IFN = interferon; LH = luteinizirajoči hormon; GO = genska ontologija; totRNA = totalna RNA; lncRNA = dolga nekodirajoča RNA

Figure 1: Graphical abstract of doctoral dissertation research. Candidate pathways and genes of altered endometrial receptivity in adenomyosis were identified using systems biology approaches: the literature mining to retrieve genetic causes associated with altered endometrial expression; bioinformatics analyses of synthesized gene lists; RNA-seq analysis to compare endometrial transcriptome between the adenomyosis group and the control group; integration of the identified differentially expressed genes of our adenomyosis group with data from the literature. Abbreviations used: HGNC = HUGO Gene Nomenclature Committee; IL = interleukin; IFN = interferon; LH = luteinising hormone; GO = Gene Ontology; totRNA = total RNA; lncRNA = long noncoding RNA.

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Molekularni podpis evtopičnega endometrija pri endometriozi na podlagi večomske integracijske sinteze

Prašnikar E., Knez J., Kovačič B., Kunej T. 2020. Molecular signature of eutopic endometrium in endometriosis based on the multi-omics integrative synthesis. Journal of Assisted Reproduction and Genetics, 37: 1593-1611

Namen: Združiti podatke omskih raziskav o molekularnem podpisu evtopičnega endometrija pri endometriozi glede na fazo menstruacijskega ciklusa. **Metode:** Iz raziskav smo pridobili podatke o genetskih vzrokih spremenjenega izražanja v endometriju žensk z endometriozo. Poimenovanje pridobljenih spremenjeno izraženih transkriptov RNA in proteinov smo poenotili po nomenklaturnem sistemu zbirke HGNC. Gene smo dalje razvrščali glede na fazo menstruacijskega ciklusa, v kateri je bila izvedena analiza endometrija v izvorni raziskavi, in sicer: M, proliferacijska (P), sekrecijska (S), ZS, SS in PS faza ter nenavedena faza (NN), če datiranje endometrija ni bilo izvedeno. Sete genov smo obogatili z bioinformacijskim orodjem DAVID. **Rezultati:** Iz literature smo pridobili 21 raziskav, kjer so poročali o molekularnih spremembah endometrija pri endometriozi. Od skupno 21 je bilo 13 raziskav opravljenih na transkriptomski, 6 na proteomski in 2 na epigenomski ravni. Pridobljene podatke smo zbrali v katalog s skupno 670 genetskimi vzroki in za 591 vzrokov pridobili uradne genske simbole. Gene smo razvrstili po fazah, tj. M = 3, P = 188, S = 81, ZS = 82, SS = 173, PS = 36 in NN = 28. Med obogatenimi potmi smo identificirali *pot signalizacije estrogena*, *organizacija zunajceličnega matriksa* in *kemotaksa endotelijskih celic*. Naša raziskava je pokazala, da je znanje biologije endometrija razdrobljeno zaradi heterogenosti objavljenih podatkov. Kljub temu smo identificirali 15 genov, ki so bili v vsaj dveh raziskavah iste faze menstruacijskega ciklusa poročani kot spremenjeno izraženi in 33 bioloških poti zbirke GO-BP ali KEGG, ki so bile značilno obogatene v več fazah ciklusa. **Zaključki:** Večomski vpogled v molekularne vzorce endometrioze bi lahko prispeval k identifikaciji patoloških mehanizmov, ki vplivajo na plodnost takšnih bolnic.

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TECHNOLOGICAL INNOVATIONS

Molecular signature of eutopic endometrium in endometriosis based on the multi-omics integrative synthesis

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Abstract

Purpose To synthesise data from genome-wide studies reporting molecular signature of eutopic endometrium through the phases of the menstrual cycle in endometriosis.

Methods Extraction of data from publications reporting genetic signatures characterising endometrium associated with endometriosis. The nomenclature of extracted differentially expressed transcripts and proteins was adopted according to the HUGO Gene Nomenclature Committee (HGNC). Loci were further sorted according to the different phases of the menstrual cycle, i.e. menstrual (M), proliferative (P), secretory (S), early-secretory (ES), mid-secretory (MS), late-secretory (LS), and not specified (N/S) if the endometrial dating was not available. Enrichment analysis was performed using the DAVID bioinformatics tool.

Results Altered molecular changes were reported by 21 studies, including 13 performed at the transcriptomic, 6 at proteomic, and 2 at epigenomic level. Extracted data resulted in a catalogue of total 670 genetic causes with available 591 official gene symbols, i.e. M = 3, P = 188, S = 81, ES = 82, MS = 173, LS = 36, and N/S = 28. Enriched pathways included *oestrogen signalling pathway*, *extracellular matrix organization*, and *endothelial cell chemotaxis*. Our study revealed that knowledge of endometrium biology in endometriosis is fragmented due to heterogeneity of published data. However, 15 genes reported as dysregulated by at least two studies within the same phase and 33 significantly enriched GO-BP terms/KEGG pathways associated with different phases of the menstrual cycle were identified.

Conclusions A multi-omics insight into molecular patterns underlying endometriosis could contribute towards identification of endometrial pathological mechanisms that impact fertility capacities of women with endometriosis.

Keywords Endometriosis · Expression signature · Gene set enrichment analysis (GSEA) · Genome-wide study · Multi-omics data integration

Abbreviations

ASRM	American Society for Reproductive Medicine
GSEA	Gene set enrichment analysis
HGNC	HUGO Gene Nomenclature Committee
NCBI	National Centre for Biotechnology Information
DAVID	Database for Annotation, Visualization and Integrated Discovery
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology
mRNA	Messenger RNA
lncRNA	Long non-coding RNA
sncRNA	Small non-coding RNA
snoRNA	Small nucleolar RNA
miRNA	Micro RNA
M	The menstrual phase of the menstrual cycle
P	The proliferative phase of the menstrual cycle
S	The secretory phase of the menstrual cycle
ES	The early-secretory phase of the menstrual cycle
MS	The mid-secretory phase of the menstrual cycle

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LS	The late-secretory phase of the menstrual cycle
WOI	Window of implantation
N/S	Not specified
ART	Assisted reproductive techniques

Introduction

Endometriosis is a common disease where tissue similar to normal endometrium (ectopic endometrium) grows outside of the uterus. Most commonly affected sites are the pelvic peritoneum, ovaries, uterosacral ligaments, pouch of Douglas, and the rectovaginal septum [1]. Endometriosis affects approximately 10% of women in their reproductive age. The condition is more common in women suffering with chronic pelvic pain and infertility. Although associated with several shortcomings, the most commonly used classification system is the revised American Society for Reproductive Medicine classification [2]. According to this, endometriosis is classified into sub-phenotypic stages from I to IV (minimal–severe), based on lesion number and size, presence of adhesions, and ovarian vs peritoneal involvement [3]. The diagnosis is made by direct surgical visualisation with histological confirmation of the endometrial tissue in biopsied lesions [4]. The nature of disease is often progressive, with gradually worsening pain which can lead to absence from social and work obligations [5]. This can result in significant burden to healthcare systems [6]. Non-invasive diagnostic testing could provide earlier diagnosis and improve disease management for these women [7]. Measurable biologic markers (biomarkers) from eutopic endometrium (an innermost lining layer of the uterus) and body fluids are currently the most promising non-invasive diagnostic approaches [8].

The most widely accepted aetiology of endometriosis is Sampson theory [9], whereby retrograde menstrual bleeding from the Fallopian tubes to the peritoneal cavity leads to migration, attachment, and growth of endometrial tissues at ectopic sites. However, retrograde menstrual bleeding by itself does not always lead to development of endometriosis, and multiple dysregulated mechanisms in the eutopic endometrium potentially govern pathophysiology in endometriosis. These include altered processes of apoptosis, immunosurveillance, adhesion, steroid responsiveness, tissue remodelling, neovascularisation, and enhanced inflammatory response [10], thus providing a source for biomarker identification [11].

The endometrium periodically undergoes morphological changes caused by fluctuations in the ovarian steroid hormones. Each menstrual cycle starts with the M-phase (days 1–4 in a normal 28-day cycle), characterised by shedding of the endometrial tissue. This is followed by the P-phase (days 4–14 in a normal 28-day cycle), during which higher levels of oestrogen stimulate the proliferation of the stroma and glands, resulting in thickening of the endometrium. After ovulation, progesterone levels start to rise in the ES-phase (days 15–20 in a normal 28-day cycle), stimulating the glands to secret glycogen and mucus. In the MS-phase (days 21–24 in a normal 28-day cycle), the window of implantation (WOI) opens, representing the optimal time for blastocyst implantation. This coincides with the differentiation of the endometrial stromal cells into decidual cells, known as decidualisation. In the absence of pregnancy, the endometrial tissue starts to degrade in the LS-phase (days 25–28 in a normal 28-day cycle), [12–14]. Therefore, changes in the endometrium through the menstrual cycle phases are reflected in the changes in transcriptome [15–17] and regulation patterns [18]. Consequently, differentially expressed transcripts or proteins may shed light on the important biological processes occurring at the time endometrial biopsy samples are collected. Integrating multi-omics data can provide insights into the underlying physiological and pathophysiological mechanisms [19].

Due to endometrial periodical transitions through the cycle, endometrial dating of tissue biopsies from women with and without endometriosis may play an important role in identification of the molecular patterns specific for endometriosis [20]. Although candidate endometrial biomarkers from genome-wide studies associated with endometriosis were reviewed [21], dysregulated molecular patterns of eutopic endometrium throughout the menstrual cycle among studies have not yet been integrated. Data synthesis of differentially expressed transcripts and proteins may provide an additional insight into endometrial molecular signature in women with endometriosis.

The aim of this study was therefore to (1) screen for published genome-wide studies reporting genetic causes distinguishing eutopic endometrium between women with and without endometriosis, (2) develop a catalogue of genetic causes (mRNAs, ncRNAs, and proteins) associated with altered molecular patterns in eutopic endometrium in women with endometriosis, (3) modify the gene nomenclature of extracted loci according to the HGNC system, (4) sort genes according to the phases of the menstrual cycle, and (5) perform gene set enrichment analysis (GSEA) associated with each phase of the menstrual cycle.

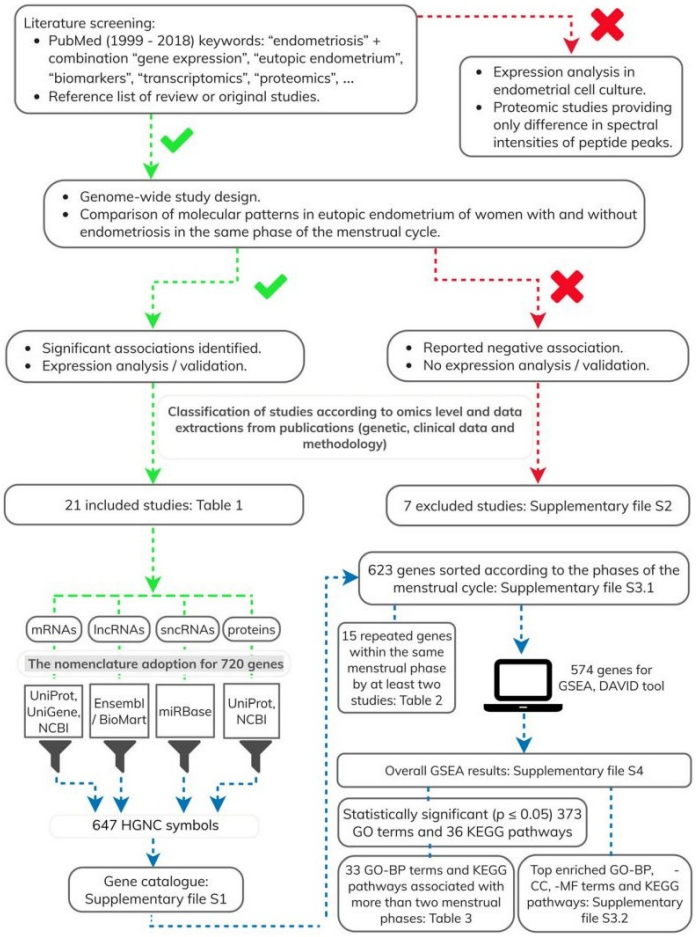
Material and methods

Workflow of the study is presented in Fig. 1.

Literature screening and data extraction

Original publications with genome-wide approach were retrieved from PubMed database [22] using keyword “endometriosis” with combination of terms including “eutopic endometrium”, “molecular dysregulation”, “gene expression”,

Fig. 1 Workflow of the study



"transcriptomics", "omics", "proteomics", "epigenomics", "genomics", and "biomarkers". The time span of the literature screening was set from 1999 to December 2018. Some studies were additionally identified using the reference list of review or retrieved articles. We performed literature screening for studies that compared eutopic endometrium of women with and without endometriosis in the same phase of the menstrual cycle. We screened for studies performed at the genomic (DNA), transcriptomic (mRNAs, miRNAs, lncRNAs), proteomic, and epigenomic levels. Proteomic studies including the measuring of different spectral intensities of the peptide peaks and the peaks which could not be associated with candidate proteins were excluded. High-throughput data obtained from in vitro experiments using treated primary human endometrial cells were also excluded. Only studies published in English language were included.

The following data was extracted from publications: indications for endometrial tissue sample collection for case and control study groups, stage and type of endometriosis and other uterine/pelvis pathologies of the case group, gynaecological condition of women in the control group, clinical symptoms of pelvic pain and/or infertility for both groups, age of participants, endometrial dating (phase of the menstrual cycle) and number of endometrial tissue samples used for genome-wide analysis, procedures for processing of endometrial tissue samples (collection, storage, and nucleic acid/protein isolation), platform used for genome-wide analysis, cut-off values for expression/methylation change identification, genetic causes associated with endometriosis, determined expression/methylation fold change of transcripts/proteins, and corresponding significance values.

Adoption of HGNC gene nomenclature for the development of the catalogue

The gene catalogue was developed from the extracted data of published studies associated with altered expression at the RNA and protein levels in eutopic endometrium of endometriosis. Up to 15 differentially expressed (either up- and down-regulated) transcripts (mRNAs, lncRNAs, and sncRNs) and proteins associated with specific phase of the menstrual cycle, fold change of expression with statistical significance value if available were extracted from publications.

The nomenclature of genes coding for transcripts and proteins reported to be dysregulated in endometriosis was adopted according to the HGNC nomenclature system (version updated February, 25th 2019). HGNC database (https://www.genenames.org/) is the resource for approved human gene nomenclature [23]. In addition, corresponding Gene ID numbers were obtained from the National Centre for Biotechnology Information (NCBI) database, release 230. NCBI is the resource that provides biological information and data [22]. Workflow of nomenclature editing for each type of extracted loci is described further. The identification name or synonym for each extracted mRNA transcript was entered into the UniProt Knowledgebase (UniProtKB), release 2019_2, and/or HGNC database. UniProtKB (https://www.uniprot.org/) is a source of sequences and annotations for over 120 million proteins [24] which also provide web links to HGNC database. Gene symbols for transcripts marked with the expression sequence tag (EST) clusters with prefix "Hs." (*Homo sapiens*) were retrieved from NCBI's UniGene database. In July 2019, UniGene web pages retired, but UniGene cluster numbers are matched with gene records. For example, NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene) provided *BPIFB1* for "Hs.65551" that was extracted from the study performed by Burney et al. [25]. To obtain gene symbols for lncRNAs, the SeqName IDs of lncRNA transcripts were uploaded into the Ensembl/BioMart tool (version 96) (https://www.ensembl.org/biomart/martview/). BioMart is a web-based tool that provides access to the gene annotation of Ensembl data [26]. For example, Ensembl/BioMart returned *MAP4K3-DT* gene for the transcript "ENST00000451547", obtained from the study performed by Wang et al. [27]. To obtain the official gene symbols for miRNAs, the MiRBase MIMAT accession number or miRNAs strand-specifying -3p and -5p suffixes of each miRNA transcript was entered into the miRBase database, release 22. The miRBase database (http://www.mirbase.org/) provides microRNA sequences and annotation [28]. In cases where the mature miRNA without an available ID number was obtained from the reference source, gene names for both stem loop sequences were included in the gene catalogue as they result in the mature miRNA with the same sequences. For example, extracted transcript "hsa-miR-138-5p" from the study performed by Zhou et al. [29] provided *MIR138-1* and *MIR138-2* genes by the miRBase. Synonyms, names, or UniProt accession IDs of proteins were entered into the UniProtKB database to retrieve the gene symbol. When GenInfo Identifier or "GI" number was available, then the NCBI protein database was used to obtain gene symbol or Gene ID number. For example, extracted "gi|825,671" from the study performed by Rai et al. [30] was entered into NCBI protein database, which further provided web link to the HGNC database from where *NPM1* gene was obtained.

Gene set enrichment analysis

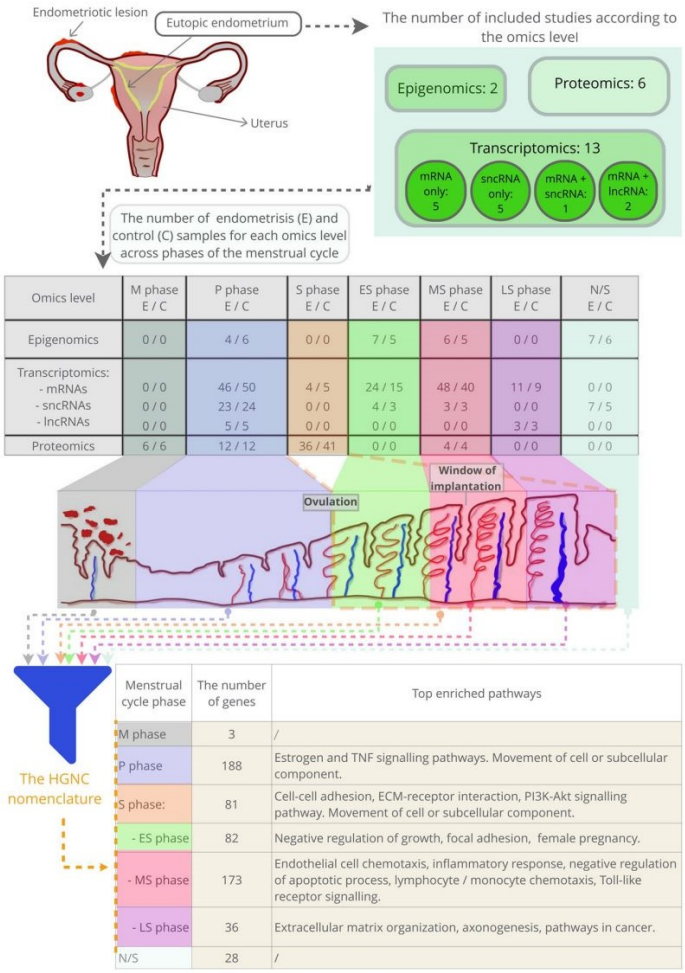
All genes from developed catalogue regardless of clinical and experimental characteristics from extracted studies were further sorted according to the phases of the menstrual cycle (M, P, S, ES, MS, and LS) and N/S, when information regarding endometrial dating was missing. Extracted transcripts and proteins with no confirmed differences in the expression levels after validation of the source references were excluded from gene sorting. Gene set enrichment analysis (also named functional enrichment analysis) was performed for each list of genes associated with specific phases of the menstrual cycle using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource (release 6.8). DAVID is a bioinformatics tool that accepts gene list and performs functional analysis using background algorithms and knowledge of annotation databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG), BioCarta, Enzyme nomenclature database and Reactome, to understand biological meaning behind the genes of interest [31]. In the present GSEA, Gene Ontology (GO) terms such as biological process (BP), cellular component (CC), molecular function (MF), and KEGG pathways specific to *Homo sapiens* with a $p \leq 0.05$ were considered as statistically significant. GO [32] and KEGG [33] are databases that collect knowledge regarding the function of gene products and their roles in the biological system and phenotypes.

Results

The main findings of the present study are overviewed in Fig. 2.

Classification of retrieved studies

The database mining (Fig. 3) provided 28 genome-wide studies that analysed the eutopic endometrium in women with and without endometriosis. The analysis of the obtained data revealed that datasets are heterogeneous and included dysregulation of expression patterns at the transcriptomics and proteomics levels. Additionally, some of the transcriptomics studies reported expression of both mRNAs and ncRNAs



Omics level	M phase E / C	P phase E / C	S phase E / C	ES phase E / C	MS phase E / C	LS phase E / C	N/S E / C
Epigenomics	0 / 0	4 / 6	0 / 0	7 / 5	6 / 5	0 / 0	7 / 6
Transcriptomics:							
- mRNAs	0 / 0	46 / 50	4 / 5	24 / 15	48 / 40	11 / 9	0 / 0
- sncRNAs	0 / 0	23 / 24	0 / 0	4 / 3	3 / 3	0 / 0	7 / 5
- lncRNAs	0 / 0	5 / 5	0 / 0	0 / 0	0 / 0	3 / 3	0 / 0
Proteomics	6 / 6	12 / 12	36 / 41	0 / 0	4 / 4	0 / 0	0 / 0

Menstrual cycle phase	The number of genes	Top enriched pathways
M phase	3	/
P phase	188	Estrogen and TNF signalling pathways. Movement of cell or subcellular component.
S phase:	81	Cell-cell adhesion, ECM-receptor interaction, PI3K-Akt signalling pathway. Movement of cell or subcellular component.
- ES phase	82	Negative regulation of growth, focal adhesion, female pregnancy.
- MS phase	173	Endothelial cell chemotaxis, inflammatory response, negative regulation of apoptotic process, lymphocyte / monocyte chemotaxis, Toll-like receptor signalling.
- LS phase	36	Extracellular matrix organization, axonogenesis, pathways in cancer.
N/S	28	/

Fig. 2 Study overview and main results. Different omics levels of studies that analysed eutopic endometrium in women with and without endometriosis. Identified enriched pathways associated with the phases of the menstrual cycle characterising eutopic endometrium of endometriosis

(sncRNAs or lncRNAs); therefore, these datasets were considered as transcriptomics and ncRNomics studies. Some retrieved studies reported dysregulation of sncRNAs only, and therefore these studies fell into both the transcriptomics and ncRNomics levels. Some studies also overlapped with epigenomics, because they reported aberrant DNA methylome and associated gene expression levels.

Out of 28, 21 studies that reported an association of altered molecular patterns related to eutopic endometrium with endometriosis were further used for the development of the catalogue. Eight out of 21 studies were performed at the transcriptomics level: five [25, 34–37] profiled only mRNAs, two [27, 38] profiled mRNAs and lncRNAs, and one [29] reported dysregulation of mRNAs and sncRNAs (miRNAs and snoRNAs). Five additional studies analysed dysregulation of sncRNAs only [39–43]. Six [30, 44–48] out of 21 studies were performed at the proteomics level. Additionally, we also included differentially expressed protein-coding genes from two epigenomics studies [49, 50], which reported altered mRNA expression levels of differentially methylated genes. Studies included in the present analysis are listed in Table 1. Figure 4 presents data extraction (differentially expressed transcripts and proteins) for multi-omics integration.

Out of 28, seven studies were not included in the catalogue development because they did not meet the inclusion criteria of the present study (no expression analysis or data not available, expression analysis performed using separated cell types, platforms with limited number of target genes, and negative association reported), [51–57]. Although not included in the developed catalogue, these studies contribute in understanding of endometrial biology in endometriosis and are therefore summarised in Supplementary file S2.

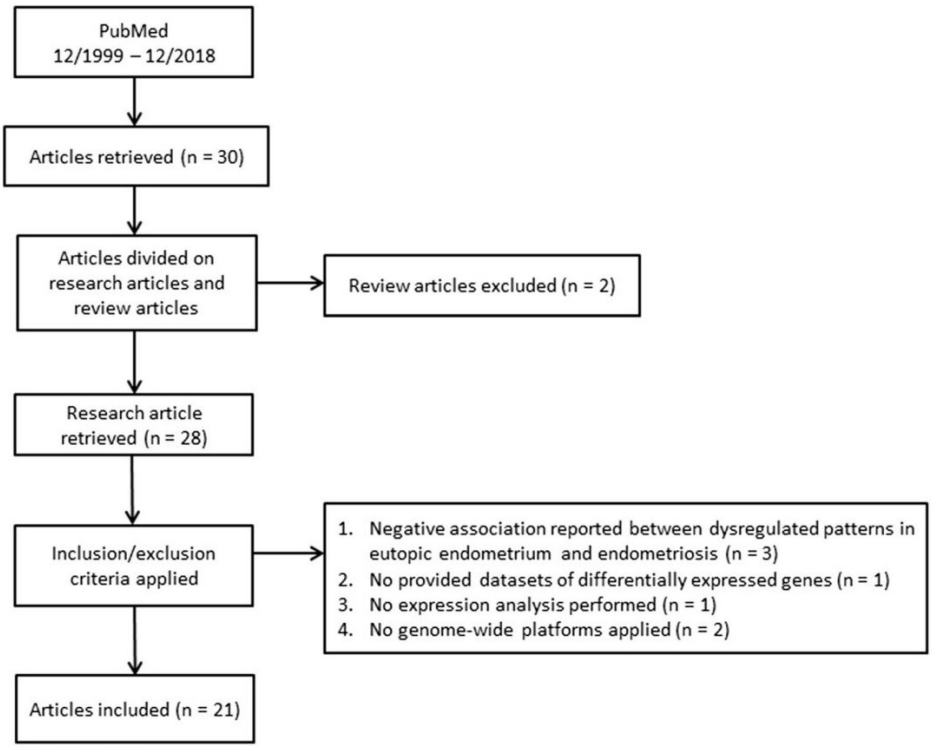


Fig. 3 Workflow of the database mining

Description of the gene catalogue

From 21 publications, 760 dysregulated transcripts (mRNAs, lncRNAs, and sncRNAs) and proteins in endometriosis were extracted and were used for the gene catalogue development. Among 760 genetic causes, 37, 425, 169, and 129 were associated with endometriosis at the epigenomics, transcriptomics, ncRNomics, and proteomics level, respectively. The gene catalogue is provided in Supplementary file S1. After nomenclature editing according to HGNC database, 647 official gene symbols were retrieved. The official gene coding for 113 dysregulated transcripts/proteins is currently not available. Among 647, 24 genes were excluded since additional validation analysis using qPCR, immunohistochemistry, or western blot did not confirm genome-wide screening findings. Therefore, the final set of genes included 623 genes: 33, 368, 103, and 119 genes associated with endometriosis at the epigenomics, transcriptomics, ncRNomics, and proteomics level, respectively. Sorting of these 623 genes according to phases of the menstrual cycle resulted in 3, 200, 87, 87, 181, 37, and 28 genes associated with M-, P-, S-, ES-, MS-, LS-phase, and N/S, respectively. Analysis of the catalogue revealed that same genes were associated with different menstrual phases or were associated with the same phase in different studies. Among 623 genes, 72 genes were repeated 2 times (55 genes) or at least 3 times (17 genes) in the same or in different phases of the menstrual cycle. For example, *FOS* was associated with the MS-phase by two different studies [29, 37] as well as with S- [34], P-, and ES-phases [37]. Eleven genes were repeated within the same menstrual phase in the same study. For example, two transcripts of *SNORD3A* gene were reported by Cui et al. [38] and two isoforms of the VIM protein were shown to be up- and down-regulated [30]. Among 72 repeated genes, there are 61 genes that were repeated in different phases of the menstrual cycle. For example, down-regulated *MIR374B* was associated with P-phase [43] and with MS-phase [29]. A list of 72 repeated genes with associated phase of the menstrual cycle, omics level, and source reference are presented in Supplementary file S5. Table 2 includes 15 genes associated with the same phase of the menstrual cycle by more than one study, for example up-regulated *CCN1* in the MS-phase [29, 37]. Removing duplicated genes from the same phase of the menstrual cycle resulted in 591 unique phase-specific genes, i.e. M = 3, P = 188, S = 81, ES = 82, MS = 173, LS = 36, and N/S = 28 (Supplementary file S3.1).

Table 1 Clinical and experimental characteristics of included studies

Reference	Indication for endometrial tissue sample collection	Number of endometriosis (E) samples used for genome-wide analysis in studied phase of the cycle. Stage and type of E	Number of control samples used for genome-wide analysis in studied phase of the cycle. Phenotype	-Endometrial tissue collection -Sample preservation -Nucleic acid/protein isolation	Platform for genome-wide analysis	Cut-off for fold change (FC) or *p* value and genetic causes associated with E
Epigenomic level						
[49]	Laparoscopy	N/S = 7 in reproductive age. Stage E: N/S	N/S = 6 in reproductive age	-N/S -RNAlater -Genomic DNA: Qiagen DNeasy Blood & Tissue Kit. Total RNA: TRIzol Reagent, purified by the Qiagen RNeasy MinElute Cleanup Kit	-Illumina Infinium Human Methylation 27 K RevB Beadchip -qRT-PCR (validation)	FC ≥ 1.5: 59 hypermeth. genes and 61 hypometh. genes $p < 0.05$ (validation)
[50]	Laparoscopy for endometriosis-related pain and/or infertility (cases). Endometrial biopsy, hysterectomy, or gynaecologic surgery for benign condition (controls)	P = 4 (39 ± 9 years), ES = 7 (35 ± 3 years), MS = 6 (34 ± 10 years). Stage IV*E	P = 6 (43 ± 1 years), ES = 5 (41 ± 3 years), MS = 5 (46 ± 1 years)	-N/S -N/S -Genomic DNA: NucleoSpin Tissue Kit. Total RNA: DNase treatment by Qiagen RNeasy Plus Kit	-Illumina Infinium Human-Methylatio-n27K -Affymetrix HU133 Plus 2.0 (validation)	$p < 0.05$: P: 58 DMCs corresponding to 58 loci. ES: 39 DMCs corresponding to 36 loci. MS: 137 DMCs corresponding to 125 loci. Spearman correlation (validation)
Transcriptomics level (only protein-coding mRNAs)						
[25]	Normally cycling women underwent laparoscopy (cases), hysterectomy, or endometrial biopsy (controls)	P = 6 (35 ± 5 years), ES = 6 (32 ± 6 years), MS = 9 (35 ± 6 years). Stage III/IV. Surgery and histologically confirmed E: ovarian/peritoneal E (n = 5), ovarian/peritoneal E+ leiomyoma (n = 2), peritoneal E (n = 1), peritoneal + liver E (n = 1), rectovaginal/ovarian/peritoneal E (n = 4), rectovaginal/ovarian/peritoneal E	P = 5 (37 ± 7 years), ES = 3 (46 ± 2 years), MS = 8 (37 ± 8 years). Uterine prolapse (n = 3), uterine leiomyomata (n = 5), pelvic pain (n = 1), and normal volunteers (n = 7). Laparoscopy proven without E	-Pipelle catheter or uterine curetting -Liquid N_2 -Total RNA: TRIzol reagent, DNase treatment, and purified by the Qiagen RNeasy Kit	Affymetrix HU133 Plus 2.0	FC > 1.5, $p < 0.05$: P: 278↑/461↓ ES: 986↑/2321↓ MS: 430↑/315↓

Table 1 (continued)

Reference	Indication for endometrial tissue sample collection	Number of endometriosis (E) samples used for genome-wide analysis in studied phase of the cycle. Stage and type of E	Number of control samples used for genome-wide analysis in studied phase of the cycle. Phenotype	-Endometrial tissue collection -Sample preservation -Nucleic acid/protein isolation	Platform for genome-wide analysis	Cut-off for fold change (FC) or *p* value and genetic causes associated with E
		+ leiomyoma (n = 1), rectovaginal/peritoneal E (n = 3), rectovaginal/peritoneal E + leiomyoma (n = 4). Many infertile with previous failed ART treatments				
[34]	Endometrial biopsy or hysterectomy	P = 6, S = 4. Aged 19–48 years. Stage: N/S	P = 5, S = 5. Aged 19–48 years.	-N/S -Collected in Moscona solution, frozen in liquid N_2 -Total RNA: a Qiagen RNeasy Kit	Affymetrix Human Genome U95A	FC ≥ 1.5: P: 29↓/9↑ S: 35↓/23↑
[36]	Normally cycling women underwent endometrial biopsy	LS = 8. Stage I/II (n = 4) and III/IV (n = 4). Laparoscopically proven endometriosis	LS = 6. Laparoscopically proven without E	-Pipelle catheter -Liquid N_2 -Total RNA: TRIzol reagent	Microarray Core Facility custom made array	FC >1.75, $p < 0.01$: 8↑ / 1↓
[37]	Archived endometrial samples provided by UCSF Human Endometrial Tissue Bank from normally cycling women underwent procedures for diagnosis and treatments of pelvic pain, infertility or benign gynaecologic conditions, and normal volunteers	Minimal/Mild E: P = 11 (37 ± 5 years), ES = 6 (37 ± 6 years), MS = 10 (36 ± 8 years). Stage I/II. Peritoneal endometriosis concurrent with fibroids (n = 7) and/or adhesions (n = 2), chronic pelvic pain (n = 21), dysmenorrhea (n = 3), and/or infertility (n = 4) Moderate/Severe E: P = 18 (36 ± 7 years), ES = 12 (35 ± 6 years), MS = 18 (34 ± 7 years),"indeterminate" cycle phase = 1. Stage III/IV. Extensive endometriosis with concurrent adhesions (n = 25), fibroids (n = 10) chronic pelvic pain (n = 36), adenomyosis (n = 1) and/or infertility (n = 6)	Group "healthy non-E": P = 20 (32 ± 5 years), ES = 6 (32 ± 3 years), MS = 8 (33 ± 4 years). Healthy with abdominal pain/pyloric stenosis (n = 2), desired (n = 9) / undesired (n = 7) future fertility, unexplained pelvic pain (n = 1) or infertility (n = 3), normal volunteer (n = 7), egg donor in natural cycle (n = 5). Group "non-E with other uterine/pelvic pathology": P = 15 (43 ± 5 years), ES = 6 (42 ± 6 years), MS = 14 (43 ± 6 years). Symptomatic uterine fibroids (n = 16), adenomyosis (n = 3), chronic pelvic pain (n = 3), uterine (n = 3)/uterovaginal (n = 1)/pelvic organ (n = 3)/prolapse, adhesions (n = 2), dysmenorrhea (n = 4), endometrial polyp (n = 1), cystocele (n = 3), stress urinary incontinence (n = 3),	-Pipelle catheter or uterine curetting -Liquid N_2 -Total RNA: TRIzol reagent	Affymetrix HU133 Plus 2.0	FC > 1.5: "Min./Mild+ Mode./Severe E" vs. healthy non-E": P: 7573↑/11,8-66↓. ES: 2905↑/796-6↓. MS: 4020↑/643-8↓. "Min./Mild+ Mode./Severe E" vs. non-E + uterine/-pelvic pathology: P: 2↑ / 85↓. ES: 12↑ / 55↓. MS: 31↑ / 74↓.

Table 1 (continued)

Reference	Indication for endometrial tissue sample collection	Number of endometriosis (E) samples used for genome-wide analysis in studied phase of the cycle. Stage and type of E	Number of control samples used for genome-wide analysis in studied phase of the cycle. Phenotype	-Endometrial tissue collection -Sample preservation -Nucleic acid/protein isolation	Platform for genome-wide analysis	Cut-off for fold change (FC) or *p* value and genetic causes associated with E
			menorrhagia ($n = 2$) or benign ovarian cyst ($n = 1$)			
[35]	Normal cycling women underwent endometrial biopsy	MS aged 28–39 years (LH + 6–LH + 10 timed to expected WOI) = 8. Stage II/III. Surgically confirmed pelvic E	MS aged 28–39 years (LH + 6–LH + 10 timed to expected WOI) = 7. Surgically confirmed without E	-Pipelle catheter -Liquid N_2 -Poly (A) + RNA: Oligotex Direct mRNA isolation kit	Affymetrix Genechip Hu95A	FC ≥ 2, $p < 0.05$: 91↑/115↓
Transcriptomics (protein-coding genes and ncRNAs)						
[27]	Normal cycling women underwent total hysterectomy	LS = 3. Stage: N/S.	LS = 3. Normal endometrium without oestrogen-dependent disease	-N/S -Snap-frozen in liquid N_2, stored at -80 °C -Total RNA: TRIzol reagent	Human lncRA Expression Microarray V3.0	FC > 2.0: 578↑/638↓ (mRNAs) 488↑/789↓ (lncRNAs)
[38]	Normal cycling women underwent eutopic endometrial samples collection	P = 5. Stage III/IV. Laparoscopically diagnosed E	P = 5. Without visible E	-N/S -N/S -RNA: TRIzol reagent	Illumina HiSeq 2500 with 150 bp paired-end reads provided 1,045,089,518 clean reads	$p < 0.05$: 753↑/475↓ (mRNAs) 33↑/53↓ (lncRNAs)
[29]	Normal cycling women underwent hysteroscopy.	MS = 3 aged 20–35 years. Stage I/II. Laparoscopically confirmed.	MS = 3 aged 20–35 years. Laparoscopically proven without E.	-N/S -Liquid N_2 -Total RNA: TRIzol reagent	-Agilent Human 4 × 44 K -miRCURY LNA microRNA array	FC > 2.0: 224↑/133↓ (mRNAs) FC ≥ 2.0: 54↑/12↓ (miRNAs)
Transcriptomics (only ncRNAs)						
[42]	Laparoscopy for non-malignant ovarian lesions (cases) or infertility work-up or removal of simple ovarian cysts (controls)	P = 10 aged 20–35 years. Stage III/IV. Ovarian E confirmed by laparoscopy and histopathology with some concurrent primary/secondary infertility	P = 10 aged 20–35 years. Primary/secondary infertility or with simple ovarian cysts	-Pipelle suction catheter -RNAlater, stored at −80 °C -Total RNA: mirVana miRNA Isolation Kit	TaqMan Array Human MicroRNA A v2.1 + B v2.0 Cards	FC ≥ 2.0 or $p \leq 0.1$: 2↑/13↓
[41]	Normal cycling women underwent laparoscopy for adnexal mass or infertility	Mean age 31.4 ± 0.9 years of a total 21 women. P = 10. Stage III/IV. Ovarian E confirmed by laparoscopy and histopathology with some concurred primary/secondary infertility	Mean age 30.7 ± 0.9 years of a total 25 women. P = 11. Primary/secondary infertility or with simple ovarian cysts	-Pipelle catheter -RNA later, stored at −80 °C -Total RNA: mirVana miRNA Isolation Kit	Exiqon's miRCURY LNA microRNA Array 7th	Adjusted *p* values < 0.05: 1198 dysregulated miRNAs
[40]	Normal cycling women underwent laparoscopy for endometriosis	ES = 4 (28 ± 7 years). Stage III/IV. E confirmed by laparoscopy and	ES = 3 (45 ± 3 years). Uterine leiomyomata, none of which	-Pipelle catheter or uterine curettage	Exiqon's miRCURY LNA array v. 10.0	FC > 1.5 and FDR < 0.05:

Table 1 (continued)

Reference	Indication for endometrial tissue sample collection	Number of endometriosis (E) samples used for genome-wide analysis in studied phase of the cycle. Stage and type of E	Number of control samples used for genome-wide analysis in studied phase of the cycle. Phenotype	-Endometrial tissue collection -Sample preservation -Nucleic acid/protein isolation	Platform for genome-wide analysis	Cut-off for fold change (FC) or *p* value and genetic causes associated with E
	treatment and hysterectomy for uterine leiomyomata	histology: rectovaginal/peritoneal E + leiomyomata (n = 1), ovarian/peritoneal/rectovaginal E (n = 2), ovarian/peritoneal E (n = 1)	submucosal in location. No pathological evidence of inflammation in sampled endometrium	-N/S -Total RNA: TRIzol reagent		6↓
[43]	Pre-menopausal women underwent laparoscopy.	P = 3 (37 ± 5 years). Stage E: N/S.	P = 3 (39 ± 5 years)	-Uterine curettage -N/S -Total RNA: TRIzol reagent	Exiqon's miRCURY LNA microRNA array v. 14.0	FC > 2.0: 36↓
[39]	Laparoscopy for endometriosis treatments due to abdominal pain (74.5%) and sterility (25%), or tubal sterilisation (controls)	N/S = 7 (aged 20–45 years). Stage E: N/S. E confirmed by laparoscopy and histology. No concurrent other pelvic pathology.	N/S = 5 (aged 27–45 years). Surgical confirmed without E or negative diagnosis of biopsies of suspicious areas of endometriosis.	-Suction cannula -Rinsed in PBS, stored in liquid N_2 -Total RNA: mirVana miRNA isolation kit	Affymetrix GeneChip miRNA 2.0. Array	p < 0.05: 11↑ / 9↓
Proteomics level						
[48]	Laparoscopy	S = 6 in reproductive age. Stage II (n = 2), stage III (n = 2), stage IV (n = 2). E confirmed by laparoscopy and pathology	S = 6 in reproductive age	-N/S -Washed in PBS, grounded into powders in liquid N_2 -Benzonase DNase for DNA and RNA removal, the homogenates centrifuged, the supernatant precipitated with acetone and re-suspended	2-DE IEF, SDS-PAGE + MALDI-TOF-MS	FC ≥ 3.0: 11 DEPs
[46]	Laparoscopic sterilisation or assessment of tubal patency	MS = 4 in reproductive age. Stage I (n = 2), stage II (n = 1), stage IV (n = 1)	MS = 4 in reproductive age. Healthy with unknown medical condition	-Uterine curettage -Snap frozen on dry ice, stored at −80 °C -Homogenised, pelleted and re-homogenised. Proteins precipitated with acetone	2D-DIGE + MALDI-TOF-MS, MS/MS	p < 0.05: 20 DEPs
[30]	Diagnostic procedure for infertility, tubal re-enastomosis, or pelvic pain	Mean age 27.5 ± 4.4 years of a total 57 women. P = 6: only stage IV. S = 18: Stage II (n = 6), stage III (n = 6), and stage IV (n = 6). Laparoscopically proven different types of E: pelvic lesions, adhesions or endometrioma	Mean age 26.7 ± 3.9 years of a total 59 women. P = 6, S = 18. Normal menstrual cycle and hormone profiles, without uterine abnormalities	-N/S -Frozen in liquid N_2, stored at −80 °C -Lysed, homogenised, and supernatant precipitated with trichloroacetic acid /acetone. Pellet re-suspended in lysis buffer	2DE-PAGE + MALDI MS and/or MS/MS	FC ≥ 1.2, p < 0.05: 48 DEPs
[47]	Routine laparoscopy for unexplained infertility, tubal re-enastomisis or pelvic pain	S = 6 (40 ± 5 years). Stage II. Laparoscopically and histologically diagnosed E for the	S = 11 (33 ± 7 years). Asherman's syndrome (n = 1), menorrhagia and fibroids (n = 1).	-Uterine curettage -Frozen in liquid N_2, stored in −80 °C.	SDS-PAGE + MALDI-TOF-MS	p < 0.05: 21 DEPs

Table 1 (continued)

Reference	Indication for endometrial tissue sample collection	Number of endometriosis (E) samples used for genome-wide analysis in studied phase of the cycle. Stage and type of E	Number of control samples used for genome-wide analysis in studied phase of the cycle. Phenotype	-Endometrial tissue collection -Sample preservation -Nucleic acid/protein isolation	Platform for genome-wide analysis	Cut-off for fold change (FC) or *p* value and genetic causes associated with E
		first time or receiving recurrent treatment for pre-existing E. Concurrent with amenorrhea and pelvic pain (n = 1), infertility and removal of uterine septum (n = 1), previous dermoid cyst (n = 1), hysterectomy due to fibroid and menorrhagia (n = 1), infertility (n = 1), infertility, bowel symptoms and dysmenorrhoea (n = 1)	dysmenorrhoea (n = 1), dermoid cyst (n = 1), intramenstrual bleeding (n = 1), pelvic pain (n = 1), and unknown symptoms (n = 5)	-Powder of tissue lysed, sonicated and centrifuged to discard the pellet. Supernatant precipitated with acetone and pellet further re-suspended		
[44]	Laparoscopy for diagnostic purpose, including infertility, treatment of endometriosis or elective tubal sterilisation	P = 6 (27 ± 3 years), S = 6 (30 ± 4 years). Stage: N/S. Laparoscopically confirmed E with no coexistent condition or endometrial pathology.	P = 6 (29 ± 5 years), S = 6 (28 ± 3 years). Healthy.	-Pipelle catheter -Washed in isotonic PBS, frozen in liquid N_2, stored in −80 °C -Lysed, sonicated, and centrifuged to remove insoluble material	2-DE-IPG, PAGE + MALDI-TOF-MS	FC ≥ 3.0: P: 38↑/13↓ S: 60↑/32↓
[45]	Collection of menstrual blood	M = 6 (aged 25–40 years). Stage: N/S. Laparoscopic diagnosis of advanced E	M = 6 (aged 25–40 years) No E, adenomyosis or leiomyoma	-Suction catheter -Cultured in DMEM and FBS for 2 days at 37 °C, 5% CO2. Washed 3-times with PBS, re-suspended in DMEM with 10% FBS, after 2 weeks collected endometrial cells -Cell lysis, centrifugation for supernatant.	2-DE + ESI-Q-TOF/MS	FC ≥ 3.0: 3↓

*Endometriosis severity stages I–IV was determined according to the revised classification by American Fertility Society (AFS) and American Society for Reproductive Medicine (ASRM)

Hypometh, hypomethylated; *Hypermeth*, hypermethylated; *DMCs*, differentially methylated CpGs; *N/S*, not specified in the source reference; ↑, up-regulated; ↓, down-regulated; N_2, nitrogen; *E*, endometriosis; *C*, controls; *P*, proliferative; *S*, secretory; *ES*, early-secretory; *MS*, mid-secretory; *LS*, late-secretory; *PBS*, phosphate-buffered saline; *MALDI-TOF-MS*, matrix-assisted laser desorption ionisation/time-of-flight-mass spectrometry; *SELDI-TOF-MS*, surface enhanced laser desorption/ionisation time-of-flight-mass spectrometry; *2D DIGE*, two dimensional-differential in gel electrophoresis; *IEF*, isoelectric focusing; *IPG*, immobilised pH gradient; *SDS-PAGE*, sodium dodecylsulfate-polyacrylamide gel electrophoresis; *DEPs*, differentially expressed proteins

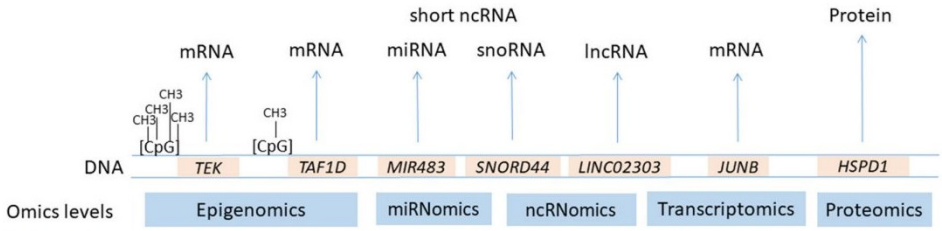


Fig. 4 Omics levels of extracted data used for the development of the gene catalogue. CpG islands present sites of methylation. mRNA, messenger RNA; miRNA, micro RNA; snoRNA, small nucleolar RNA; lncRNA, long non-coding RNA

Gene set enrichment analysis

A total of 591 unique genes sorted according to the phases of the menstrual cycle were used for GSEA. A set of 574 genes was identified by DAVID bioinformatics tool. The number of genes included in the GSEA and results of functional analysis are summarised in Table 3 (GO-BP, -CC, -MF terms, and KEGG pathways). The highest number of statistically significant enriched terms was obtained by GO-BP analysis (207), followed by GO-CC (93), GO-MF (73), and KEGG (36), however the majority of identified enriched pathways do not reach the Bonferroni correction value. The top 10 enriched GO-BP, -CC, -MF, and KEGG pathways terms with corresponding annotated genes ranked by lowest p values and Bonferroni correction for each phase of the menstrual cycle is listed in the Supplementary file S3.2, while a complete list of GSEA results is provided in the Supplementary file S4. The pathway most significantly associated with P- (p = 5.25E-07) and S- (p = 8.11E-08) phases was *movement of cell or subcellular component*. In addition, *oestrogen signalling pathway* (p = 0.03) and *TNF signalling pathway* (p = 0.04) were associated with P-phase. *PI3K-Akt signalling pathway* (p = 1.47E-05) was enriched by genes associated with the S-phase. *Negative regulation of growth* (p = 6.41E-05) was associated with the ES-phase. *Endothelial cell chemotaxis* (p = 3.85E-05), *inflammatory response* (p = 4.50E-05), and *chemokine-mediated signalling pathway* (p = 2.39E-04) were associated with the MS-phase. *Extracellular matrix organization* (p = 1.88E-04) and *pathways in cancer* (p = 0.03) were enriched by LS-phase gene list. The study also revealed that same pathways were associated with different phases of the menstrual cycle (Table 4). Among 243 statistically significant enriched GO-BP and KEGG pathways, 30 GO-BP and 3 KEGG pathways were associated with at least two phases. For example, *negative regulation of apoptotic process* was associated with four phases, i.e. P (p = 0.0004), S (p = 0.0001), ES (p = 0.0411), and MS (p = 0.0003).

Discussion

In the present study, we developed a catalogue of genes reported to have altered molecular patterns in eutopic endometrium in endometriosis. The analysis of 21 studies including 39 women with endometriosis and 236 women without endometriosis revealed that the reported datasets are heterogeneous. Obtained data from publications included dysregulated molecular patterns at diverse omics levels, i.e. transcriptomics (mRNAs, sncRNAs, and lncRNAs), proteomics, and epigenomics (dysregulated expressed genes associated with altered methylation level). The nomenclature editing of extracted data was beyond of a single omics level, therefore, the official HGNC nomenclature system for human genes was adopted which enabled downstream GSEA. Sorting of the obtained 591 unique genes resulted in 7 groups according to the phases of the menstrual cycle and further enrichment functional analysis was performed.

Table 2 Genes associated with the same phase of the menstrual cycle in at least two studies

The phase of the menstrual cycle	P-phase	S-phase	ES-phase	MS-phase	LS-phase
Repeated gene symbols and source references	*ACTB* [30, 44],	*ACTB* [30, 44],	*ANLN* [25, 37].	*CCN1* [37, 29],	/
	ANXA4 [30, 34],	*HSP90AB1* [44, 47],	/	*CRISP3* [25, 37],	/
	BPIFB1 [25, 38],	*VIM* [30, 47, 44].	/	*EGR1* [37, 29],	/
	EPHX1 [34, 37],	/	/	*FOS* [29, 37],	/
	MUC5B [34, 37, 38],	/	/	*FOSB* [29, 37],	/
	PRDX2 [30, 44],	/	/	*TRPM6* [25, 37].	/
	VIM [30, 44].	/	/	/	/

Table 3 A summary of the gene catalogue and GSEA results for each phase of the menstrual cycle

	The gene catalogue			Number of genes included in the analysis and result of total and statistically significantly ($p \leq 0.05$) enriched GO terms and KEGG pathways											
Phase of the menstrual cycle	1 All genes	2 Unique genes	3 Repeated genes in the same phase	4 Number of genes for DAVID analysis*	5 Genes included in GO-BP analysis	6 The number of enriched GO-BP terms	Significantly associated GO-BP terms ($p \leq 0.05$)	7 Genes included in GO-CC analysis	8 The number of enriched GO-CC terms	Significantly associated GO-CC terms ($p \leq 0.05$)	9 Genes included in GO-MF analysis	10 The number of enriched GO-MF analysis	Significantly associated GO-MF terms ($p \leq 0.05$)	11 Genes included in KEGG analysis	12 The number of enriched KEGG pathways	Significantly associated KEGG pathways ($p \leq 0.05$)
M-phase	3	3	0	0	0	0	0	3	3	3	0	0	0	0	0	0
P-phase	200	188	9	185	92	100	64	117	43	33	113	32	19	31	11	3
S-phase	87	81	4	77	60	81	57	72	49	39	71	38	30	30	15	13
ES-phase	87	82	5	81	40	40	23	69	11	8	18	8	3	13	4	2
MS-phase	181	173	8	170	90	88	55	114	18	5	109	27	16	35	18	15
LS-phase	37	36	1	33	13	11	8	18	7	5	21	7	5	7	5	3
N/S	28	28	0	28	0	0	0	0	0	0	0	0	0	0	0	0
Total	623	591	27	574	/	320	207	/	131	93	/	112	73	/	53	36

Column 1, the number of obtained gene symbols for extracted loci; Column 2, the number of unique gene symbols after removing of duplicates in the same phase; Column 3, the number of repeated genes in the same phase (some genes repeated in the same phase 2 or > 2 times); Column 4, the number of genes included in GSEA analysis by DAVID bioinformatics tool

*DAVID tool enabled analysis of all provided genes. Columns 5, 7, 9, and 11 refer to the number of genes included in enrichment analyses Columns 6, 8, 10, and 12 refer to the number of all identified enriched terms by DAVID bioinformatics tool

GO, Gene Ontology; *BP*, biological process; *CC*, cellular component; *MF*, molecular function; *KEGG*, Kyoto Encyclopedia of Genes and Genomes; *DAVID*, Database for Annotation, Visualization and Integrated Discovery

Table 4 Biological pathways associated with in at least two phases of the menstrual cycle. Only pathways with $p \leq 0.05$ values are shown

Enriched GO-BP and KEGG pathways term	*p* values				
	P-phase	S-phase	ES-phase	MS-phase	LS-phase
GO:0001816~cytokine production	0.0178	/	/	0.0163	/
GO:0006457~protein folding	0.0037	9.9496E-07	/	/	/
GO:0006928~movement of cell or subcellular component	5.2510E-07	8.1149E-08	/	/	/
GO:0006986~response to unfolded protein	0.0466	3.0744E-05	/	/	/
GO:0007267~cell-cell signalling	0.0186	/	/	0.0152	/
GO:0007565~female pregnancy	/	/	0.0005	0.0331	/
GO:0007568~ageing	/	/	0.0050	0.0413	/
GO:0009409~response to cold	0.0352	0.0104	/	/	/
GO:0030049~muscle filament sliding	0.0389	0.0115	/	/	/
GO:0030198~extracellular matrix organization	/	2.0573E-05	/	/	0.0002
GO:0032496~response to lipopolysaccharide	6.3165E-05	0.0334	/	/	/
GO:0032570~response to progesterone	0.0408		0.0115	0.0376	/
GO:0034599~cellular response to oxidative stress	0.0157	0.0308	/	/	/
GO:0035914~skeletal muscle cell differentiation	/	0.0187	/	0.0006	/
GO:0042060~wound healing	/	0.0462	/	0.0252	/
GO:0042493~response to drug	0.0037	0.0098	0.0003	0.0330	/
GO:0043066~negative regulation of apoptotic process	0.0004	0.0001	0.0411	0.0003	/
GO:0043154~negative regulation of cysteine-type endopeptidase activity involved in apoptotic process		0.0032	/	0.0171	/
GO:0043627~response to oestrogen	0.0164	0.0317	/	/	/
GO:0045454~cell redox homeostasis	0.0004	0.0432	/	/	/
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	0.0310	/	/	0.0007	/
GO:0050729~positive regulation of inflammatory response	0.0222	/	/	0.0003	/
GO:0050819~negative regulation of coagulation	0.0404	0.0213	/		/
GO:0050821~protein stabilisation	0.0009	0.0003	/		/
GO:0051591~response to cAMP	0.0064	0.0166	/	0.0056	/
GO:0051607~defence response to virus	0.0473	/	/	0.0413	/
GO:0051881~regulation of mitochondrial membrane potential	0.0220	0.0064	/		/
GO:0070527~platelet aggregation	0.0046	2.7906E-05	/		/
GO:0071356~cellular response to tumour necrosis factor	0.0129	/	/	0.0018	/
GO:1901998~toxin transport	0.0352	0.0104	/	/	/
hsa04668:TNF signalling pathway	0.0399	/	/	0.0083	/
hsa04915:Oestrogen signalling pathway	0.0312	0.0297	/	/	/
hsa05200:Pathways in cancer	/	0.0047	/	0.0164	0.0345

/, pathway not enriched; *P*, proliferative; *S*, secretory; *ES*, early-secretory; *MS*, mid-secretory, *LS*, late-secretory; *GO-BP*, Gene Ontology Biological Processes; *KEGG*, Kyoto Encyclopedia of Genes and Genomes

The nomenclature editing and analysis of the gene catalogue

From the literature survey, we gathered mRNA and ncRNA transcripts and proteins, and developed the catalogue by manually editing their gene nomenclature. Menstrual phase–specific sorting of 623 genes revealed that 15 genes were repeated within the same phase of the menstrual cycle since dysregulated expression was reported by at least two studies (Table 2), therefore, could present stronger candidate biomarkers associated with affected endometrial function in endometriosis. Repeated genes associated with P-phase (*ACTB*, *ANXA4*, *BPIFB1*, *EPHX1*, *MUC5B*, *PRDX2*, and *VIM*) could indicate stronger genetic causes associated with pathophysiology of endometriosis. Repeated genes from the MS-phase (*CCN1*, *CRISP3*, *EGR1*, *FOS*, *FOSB*, and *TRPM6*) could be associated with affected receptivity in endometriosis. However, independent validation studies are now needed to

verify these hypotheses. Most of the data in the catalogue included dysregulated mRNA transcripts which were supplemented with approved HGNC gene symbols. However, extracted data from publication also included proteins and genes coding for epigenetic regulators (sncRNA and lncRNAs) which were also supplemented by the corresponding HGNC gene symbols. In case of epigenetics marks (altered DNA methylation), gene symbol of the associated differentially expressed gene was added to the catalogue. Adopted gene nomenclature of reported RNA transcripts and proteins with altered expression levels in endometriosis at various omics levels enabled downstream analysis, since most bioinformatics tools require the input of official gene symbols. LncRNAs whose official gene symbols are not yet available, were also listed in the gene catalogue. Since the HGNC nomenclature is continuously updated, this catalogue will enable re-analysis of the gene list in the future.

Types of omics data in the gene catalogue

Five genes (*ANXA4*, *CDA*, *CDK10*, *DST*, and *HSP90AB1*) from the catalogue were reported to be associated with endometriosis at two omics levels, for example *ANXA4* gene at transcriptomics [34] and ANXA4 at proteomics level [30]. As reported previously, there is no complete correlation between transcriptome (expressed portion of the genome) and proteome (expressed protein set from genome). This is because post-transcriptional mechanisms such as miRNA-mediated regulation impact gene expression by degrading their target mRNAs or/and inhibiting their further translation [58]. Additionally, alternative splicing of precursor mRNA [59] and post-translational modifications [60] result in several protein isoforms. The potential role of epigenetic mechanisms in endometriosis should be further investigated, since it may provide an insight into the molecular basis of altered expression. Wang et al. [27] reported an association between the altered expression levels of AC002454.1 antisense lncRNA and target *CDK6* mRNA in endometriosis. In addition, combined analyses of both, mRNAs and ncRNAs by Zhou et al. [29] and Cui et al. [38], reported an association of differentially expressed sncRNAs and lncRNAs with their putative mRNA targets. Epigenomics studies by Naqvi et al. [49] and Houshdaran et al. [50] also provided molecular explanation for observed difference in gene expression levels due to the associated aberrant DNA methylation status.

Functional enrichment analysis by synthesised data

Functional enrichment analysis using genes sorted according to the phases of the menstrual cycle identified GO terms and KEGG pathways, potentially related with pathogenesis of endometriosis as well with affected physiological processes required for normal endometrial function and receptivity. Among top significantly enriched pathways, *oestrogen signalling pathway* was associated with P-phase. This is in correlation with Makieva et al. [14] who reviewed the important contribution of the oestradiol to the downstream pathways which enhance mitotic activity causing the thickening of the functional layer in P-phase of normo-ovulatory women [14]. Further, *negative regulation of growth* and *G1/S transition of the mitotic cell cycle* associated with the ES-phase and *MAPK* (mitogen activated protein kinase) *signalling pathway* associated by the S-phase gene set could indicate endometrial dysfunction in endometriosis. This is in accordance with the published studies [61, 62]. Velarde et al. demonstrated increased MAPK and ERK kinase 1/2 signalling cascade that inhibited cAMP-dependent cell cycle regulation in endometrial stromal fibroblasts from women with endometriosis which was further associated with potential persistence of endometrial cell proliferation from P- to S-phase [61]. Similarly, Yotova et al. have shown association of higher Ras/B-Raf/MAPK signalling activity with increased proliferation and migration rates in primary eutopic endometrial stromal cells of patients with endometriosis [62].

Extracellular matrix organization (ECM), *ECM-receptor interaction*, and *focal adhesion* pathways that were found to be associated with the S-phase gene list could also be related with pathophysiological mechanisms in endometriosis. In the literature, increased levels of metalloproteinases with a role in rearrangement of the ECM [63] and up-regulated levels of combined adhesion molecule ITGAV/ITGB3 integrin [64] were associated with greater invasiveness and susceptibility of sloughed menstrual cells being implanted at ectopic sites in endometriosis. In the present study, the *pathways in cancer* was associated with S-, MS-, and LS-phases. Although not statistically significant, the *MicroRNAs in cancer* was enriched with P-phase sub-group of genes. Sapalidies et al. reviewed genetic and epigenetic interactions that may contribute to the rare event of malignant transformation of the endometriosis lesions [65]. In the present analysis, also the *PI3K-Akt signalling pathway* was found to be associated with the S-phase group of genes. This is in accordance with the study by Kim et al. [66] who found that increased activation of AKT pathway in $Pten^{f/+}$ and $PR^{cre/+}Pten^{f/+}$ mice with autologous implantation of human endometrial tissue promoted development of ectopic lesions. Identified *antigen processing and presentation* pathway associated with the S-phase in the present study may indicate an association of endometriosis with another pathogenetic mechanisms. Matarese et al. [67] reviewed endometriosis as a chronic inflammatory disease where the immune system induces autoimmunity which favours endometrioitic lesions formation.

In the present study, *cell-cell adhesion* pathway enriched by S-phase gene list may characterise affected endometrial receptivity in endometriosis. Khorram and Lessey [68] associated decreased expression levels of ITGA5/ITGB3 integrin during the WOI in endometriosis women with an

unfavourable environment for embryo implantation. Furthermore, *endothelial cell chemotaxis*, *inflammatory response*, *monocyte chemotaxis*, and *lymphocyte chemotaxis* pathways associated with the MS-phase may also characterise affected receptivity. This is in accordance with the study by Lee et al. [69] where the role of chemokines and cytokines in recruitment of innate and adaptive immune cells for successful embryo implantation was reviewed.

Some pathways overlapped across different phases of the menstrual cycle. A total of 33 GO-BP and KEGG pathway terms (Table 4) were found to be associated in different phase-specific gene lists. For example, *oestrogen signalling pathway* was associated with P- and S-phases, *negative regulation of apoptotic process* was associated with P-, S-, ES-, and MS-phases, *cell-cell signalling* was associated with P- and MS-phases, and *female pregnancy* was associated with ES- and MS-phases. These pathways could indicate stronger potential for pathway-based identification of biomarkers associated with endometrial function in endometriosis.

The heterogeneity of retrieved studies

Studies that were included in the present integrative analysis exhibit heterogeneity at various levels, i.e. study design, recruitment criteria for participating women, sample size, procedures of endometrial tissue processing, analysed omics level, platforms for genome-wide profiling, and data presentation. Different techniques for endometrial tissue sampling, preservation, and extraction protocols were used. Also, the number of used endometrial samples and applied platforms for genome-wide profiling varies across the studies. Studies had heterogeneous enrolment criteria for participating women, i.e. different types and stages of endometriosis often with coexisted uterus/pelvic pathologies, different gynaecological conditions of control women as well as symptoms of chronic pelvic pain and/or infertility. In addition, indications for endometrium tissue sample collection were heterogeneous, including endometrial biopsy, laparoscopy, hysteroscopy, or hysterectomy.

In the present GSEA, all extracted genes across 21 studies were included, regardless of the type and stage of endometriosis, coexisting uterine/pelvic pathologies and symptoms of chronic pelvic pain and/or infertility. Therefore, identified enriched terms should be interpreted with caution. It was suggested by Painter et al. [70] that severe endometriosis may have different genetic origin compared with mild endometriosis, because most significant single nucleotide polymorphisms (SNPs) were associated with stage III/IV endometriosis in genome-wide association study (GWAS). In addition, transcriptomics studies [37, 71] distinguished endometrial expression signature between mild and severe endometriosis. Some studies recruited women with endometriosis complicated with additional uterine/pelvic pathologies and/or infertility in case group [25, 37, 41, 47]. It has been reported [72, 73] that non-endometriosis gynaecological pathologies and infertility impact endometrial expression patterns as well. Hever et al. [72] distinguished endometrial transcriptome signatures according to the presence of adenomyosis and fibroids, while Koot et al. [73] observed unique endometrium gene expression signature during the MS-phase in women with recurrent implantation failure (RIF) and associated endometrium dysfunction as one of the determining factors of infertility in reproductive technique (ART) treatments. Burney et al. [40] segregated endometriosis cases and control women with leiomyomas on the basis of endometrial miRNAs expression patterns when applied unsupervised hierarchical clustering. However, one endometriosis case was clustered together with controls since this patient had coexisting leiomyoma [40].

Some studies recruited women with leiomyomas, adenomyosis, uterine adhesions, menorrhagia, dysmenorrhoea, chronic pelvic pain, and/or infertility in the control group, which could limit identification of loci specific for endometriosis [25, 37, 45, 47, 41]. Tamaresis et al. demonstrated that different uterine/pelvic pathologies leave their own fingerprints in the endometrial transcriptome signature when comparing four different groups of women, i.e. mild-, severe endometriosis, healthy women, and women with non-endometriosis uterine/pelvic pathologies, including leiomyomas, adenomyosis, benign ovarian cysts, and endometrial polyps [37].

The catalogue developed in our study including assembled genetic data (Supplementary file S1) with characteristics of corresponding extracted studies (Table 1) now enables researchers to perform additional downstream bioinformatics analyses according to different sorting criteria (experimental characteristics and clinical data). For example, data sorting can be performed according to stage or type of endometriosis or coexisting gynaecological conditions.

Guidelines for study design harmonisation

World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) tends to overcome study design variabilities in endometriosis research. Harmonisation of standard operating procedures (SOPs) for sampling, processing, and storaging of endometrium tissue biopsies from participating women would reduce biases and measurement errors, providing detailed surgical characterisation, including determination of the menstrual phase at the time of eutopic endometrium sample collection from women with and without endometriosis, surgical and clinical phenotypic presentation of lesions [20]. In addition, documented nonsurgical aspects, including information on chronic pelvic pain, subfertility, reproductive history, menstrual history and hormone therapies used, medical and surgical history, and personal information would improve comparison across studies and enable large-scale collaborative research [74]. While WERF EPHect provides

directions for harmonised workflow across studies to identify reliable loci specific for sub-types of patients with endometriosis, it is recommended for reporting to use standardised HGNC nomenclature for gene symbols and names in publications which will facilitate data integration across studies and data exchange among researchers.

Limitations of the study

Besides contributing to the research field, the present study also has some limitations: (1) The gene catalogue was constructed from the top differentially expressed transcripts and proteins measured by high-throughput methodologies; thus, most of them were not further validated, and may therefore suffer from biases that could impact GSEA. (2) An unequal distribution of studies performed across omics levels, higher number of transcriptomics studies in comparison with epigenomics and proteomics studies. (3) An unequal distribution of studies performed across phases of the menstrual cycle is observed. For example, low ncRNomics types of studies performed through the S-phase consequently provided no enriched pathways associated with miRNA categories in specific sub-phases of S-phase. (4) The phase determination of the menstrual cycle can be subjective, which may lead to incorrect sorting into phase-specific gene lists and influence the GSEA. Endometrial dating from extracted publications was mainly determined by histological examination of an endometrial biopsy or was categorised based on self-reported date of last menstrual period and cycle day at the time of sampling. In addition, some publications provided stage of the menstrual cycle with general S-phase, while others provided specific sub-phases of S-phase (ES-, MS-, or LS-phase). (5) Variability in indications for endometrial tissue sample collection in participating women often complicated with non-endometriosis uterine/pelvic pathologies and/or infertility may impact molecular patterns in endometriosis cases. (6) Some of the articles which do not include a term "genome-wide study" in the title or keywords might have been missed from our literature screening and should be included in the next update study.

Conclusions

This study presents the first multi-omics data synthesis of reported altered molecular patterns associated with eutopic endometrium in endometriosis. Editing of heterogeneous nomenclature of reported genetic information resulted in the gene catalogue, which was further sorted according to the phases of the menstrual cycle. Functional enrichment analysis was applied to study the role of obtained genes in eutopic endometrium of endometriosis. The findings present a source of stronger candidate genes and pathways for further experiments in endometriosis. It is expected that current gene catalogue of endometriosis will expand by future studies. All reported data from additional omics levels (e.g. interactomics, microbiomics) should be captured to deepen insight into endometrial organisation in endometriosis. Identified pathophysiological and physiological mechanisms in eutopic endometrium of endometriosis could contribute to better diagnosis and treatment of women with endometriosis, and could increase the chances of successful pregnancy in infertile women seeking ART treatments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Del Frate G, Girometti R, Pittino M, Del Frate G, Bazzocchi M, Zuiani C. Deep retroperitoneal pelvic endometriosis: MR imaging appearance with laparoscopic correlation. Radiographics. 2006;26:1705–19.
2. American Society for Reproductive Medicine. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril. 1997;67:817–21.
3. Johnson NP, Hummelshoj L, Adamson GD, Keckstein J, Taylor HS, Abrao MS, et al. World endometriosis society consensus on the classification of endometriosis. Hum Reprod. 2017;32:315–24.
4. Dunselman GAJ, Vermeulen N, Becker C, Hooghe TD, De Bie B, Heikinheimo O, et al. ESHRE guideline: management of women with endometriosis. Hum Reprod. 2014;29:400–12.
5. Ballard K, Lowton K, Wright J. What's the delay? A qualitative study of women's experiences of reaching a diagnosis of endometriosis. Fertil Steril. 2006;86:1296–301.
6. Simoens S, Dunselman G, Dirksen C, Hummelshoj L, Bokor A, Brandes I, et al. The burden of endometriosis: costs and quality of life of women with endometriosis and treated in referral centres. Hum Reprod. 2012;27:1292–9.
7. Vercellini P, Giudice LC, Evers JLH, Abrao MS. Reducing low-value care in endometriosis between limited evidence and unresolved issues: a proposal. Hum Reprod. 2015;30:1996–2004.
8. Fassbender A, Vodolazkaia A, Saunders P, Lebovic D, Waelkens E, De Moor B, et al. Biomarkers of endometriosis. Fertil Steril. 2013;99:1135–45.
9. Sampson JA. Peritoneal endometriosis, due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol. 1927;14:422–69.

10. Vigano P, Parazzini F, Somigliana E, Vercellini P. Endometriosis: epidemiology and aetiological factors. Best Pract Res Clin Obstet Gynaecol. 2004;18:177–200.
11. May KE, Villar J, Kirtley S, Kennedy SH, Becker CM. Endometrial alterations in endometriosis: a systematic review of putative biomarkers. Hum Reprod Update. 2011;17:637–53.
12. Hawkins SM, Matzuk MM. Menstrual cycle: basic biology. Ann N Y Acad Sci. 2008;1135:10–8.
13. Sherman BM, Korenman SG. Hormonal characteristics of the human menstrual cycle throughout reproductive life. J Clin Invest. 1975;55:699–706.
14. Makieva S, Giacomini E, Ottolina J, Sanchez AM, Papaleo E, Vigan P. Inside the endometrial cell signaling subway: mind the gap(s). Int J Mol Sci. 2018;19:E2477.
15. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology. 2006;147:1097–121.
16. Sigurgeirsson B, Jemt A, Hanna A, Ujvari D, Westgren M, Lundeberg J, et al. Comprehensive RNA sequencing of healthy human endometrium at two time points of the menstrual cycle. Biol Reprod. 2017;96:24–33.
17. Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. Biol Reprod. 2010;82:791–801.
18. Houshdaran S, Zelenko Z, Irwin JC, Giudice LC. Human endometrial DNA methylome is cycle-dependent and is associated with gene expression regulation. Mol Endocrinol. 2014;28:1118–35.
19. Pirih N, Kunej T. Toward a taxonomy for multi-omics science? Terminology development for whole genome study approaches by omics technology and hierarchy. Omics. 2017;21:1–16.
20. Fassbender A, Rahmioglu N, Vitonis AF, Viganò P, Giudice LC, D'Hooghe TM, et al. World endometriosis research foundation endometriosis phenome and biobanking harmonisation project: IV. Tissue collection, processing, and storage in endometriosis research. Fertil Steril. 2014;102:1244–53.
21. Saare M, Peter M, Aints A, Laisk-Podar T, Salumets A, Altmäe S. OMICs studies and endometriosis biomarker identification. In: D'Hooghe T, editor. Biomarkers Endometr: Springer International Publishing; 2017. p. 227–58.
22. NCBI Resource Coordinators. Database resources of the national center for biotechnology information. Nucleic Acids Res. 2018;46:D8–13.
23. Braschi B, Denny P, Gray K, Jones T, Seal R, Tweedie S, et al. Genenames.org: the HGNC and VGNC resources in 2019. Nucleic Acids Res. 2019;47:D786–92 Oxford University Press.
24. The UniProt Consortium. UniProt: a worldwide hub of protein knowledge. Nucleic Acids Res. 2019;47:D506–15.
25. Burney RO, Talbi S, Hamilton AE, Kim CV, Nyegaard M, Nezhat CR, et al. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. Endocrinology. 2007;148:3814–26.
26. Kinsella RJ, Kähäri A, Haider S, Zamora J, Proctor G, Spudich G, et al. Ensembl BioMarts: A hub for data retrieval across taxonomic space. Database. 2011;2011:bar030.
27. Wang Y, Li Y, Yang Z, Liu K, Wang D. Genome-wide microarray analysis of long non-coding RNAs in eutopic secretory endometrium with endometriosis. Cell Physiol Biochem. 2015;37:2231–45.
28. Kozomara A, Birgaoanu M, Griffiths-Jones S. MiRBase: from microRNA sequences to function. Nucleic Acids Res. 2019;47:D155–62 Oxford University Press.
29. Zhou M, Fu J, Xiao L, Yang S, Song Y, Zhang X, et al. MiR-196a overexpression activates the MEK/ERK signal and represses the progesterone receptor and decidualization in eutopic endometrium from women with endometriosis. Hum Reprod. 2016;31:2598–608.
30. Rai P, Kota V, Deendayal M, Shivaji S. Differential proteome profiling of eutopic endometrium from women with endometriosis to understand etiology of endometriosis. J Proteome Res. 2010;9:4407–19.
31. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37:1–13.
32. Consortium TGO, Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. The Gene Ontology: tool for the unification of biology. Nat Genet. 2000;25:25–9.
33. Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 2017;45:D353–61.
34. Absenger Y, Hess-Stumpp H, Kreft B, Krätzschmar J, Haendler B, Schütze N, et al. Cyr61, a deregulated gene in endometriosis. Mol Hum Reprod. 2004;10:399–407.
35. Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, Taylor RN, et al. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. Endocrinology. 2003;144:2870–81.
36. Sherwin JRA, Sharkey AM, Mihalyi A, Simsa P, Catalano RD, D'Hooghe TM. Global gene analysis of late secretory phase, eutopic endometrium does not provide the basis for a minimally invasive test of endometriosis. Hum Reprod. 2008;23:1063–8.
37. Tamaresis JS, Irwin JC, Goldfien GA, Rabban JT, Burney RO, Nezhat C, et al. Molecular classification of endometriosis and disease stage using high-dimensional genomic data. Endocrinology. 2014;155:4986–99.
38. Cui D, Ma J, Liu Y, Lin K, Jiang X, Qu Y, et al. Analysis of long non-coding RNA expression profiles using RNA sequencing in ovarian endometriosis. Gene. 2018;673:140–8.
39. Braza-Boïls A, Marí-Alexandre J, Gilabert J, Sánchez-Izquierdo D, España F, Estellés A, et al. MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. Hum Reprod. 2014;29:978–88.
40. Burney RO, Hamilton AE, Aghajanova L, Vo KC, Nezhat CN, Lessey BA, et al. MicroRNA expression profiling of eutopic secretory endometrium in women with versus without endometriosis. Mol Hum Reprod. 2009;15:625–31.
41. Laudanski P, Charkiewicz R, Tolwinska A, Szamatowicz J, Charkiewicz A, Niklinski J. Profiling of selected microRNAs in proliferative eutopic endometrium of women with ovarian endometriosis. Biomed Res Int. 2015;2015:1–10.
42. Laudanski P, Charkiewicz R, Kuzmicki M, Szamatowicz J, Charkiewicz A, Niklinski J. MicroRNAs expression profiling of eutopic proliferative endometrium in women with ovarian endometriosis. Reprod Biol Endocrinol. 2013;11:1.
43. Shi XY, Gu L, Chen J, Guo XR, Shi YL. Downregulation of miR-183 inhibits apoptosis and enhances the invasive potential of endometrial stromal cells in endometriosis. Int J Mol Med. 2014;33:59–67.
44. Fowler PA, Tattum J, Bhattacharya S, Klonisch T, Hombach-Klonisch S, Gazvani R, et al. An investigation of the effects of endometriosis on the proteome of human eutopic endometrium: a heterogeneous tissue with a complex disease. Proteomics. 2007;7:130–42.
45. Hwang JH, Oh JJ, Wang T, Jin YC, Lee JS, Choi JR, et al. Identification of biomarkers for endometriosis in eutopic endometrial cells from patients with endometriosis using a proteomics approach. Mol Med Rep. 2013;8:183–8.
46. Stephens AN, Hannan NJ, Rainczuk A, Meehan KL, Chen J, Nicholls PK, et al. Post-translational modifications and protein-specific isoforms in endometriosis revealed by 2D DIGE. J Proteome Res. 2010;9:2438–49.
47. Ten Have S, Fraser I, Markham R, Lam A, Matsumoto I. Proteomic analysis of protein expression in the eutopic endometrium of women with endometriosis. Proteomics Clin Appl. 2007;1:1243–51.

48. Zhang H, Niu Y, Feng J, Guo H, Ye X, Cui H. Use of proteomic analysis of endometriosis to identify different protein expression in patients with endometriosis versus normal controls. Fertil Steril. 2006;86:274–82.
49. Naqvi H, Ilagan Y, Krikun G, Taylor HS. Altered genome-wide methylation in endometriosis. Reprod Sci. 2014;21:1237–43.
50. Houshdaran S, Nezhat CR, Vo KC, Zelenko Z, Irwin JC, Giudice LC. Aberrant endometrial DNA methylome and associated gene expression in women with endometriosis. Biol Reprod. 2016;95:1–16.
51. Guo SW, Wu Y, Strawn E, Basir Z, Wang Y, Halverson G, et al. Genomic alterations in the endometrium may be a proximate cause for endometriosis. Eur J Obstet Gynecol Reprod Biol. 2004;116: 89–99.
52. Li X, Zhang Y, Zhao L, Wang L, Wu Z, Mei Q, et al. Whole-exome sequencing of endometriosis identifies frequent alterations in genes involved in cell adhesion and chromatin-remodeling complexes. Hum Mol Genet. 2014;23:6008–21.
53. Saare M, Modhukur V, Suhorutshenko M, Rajashekar B, Rekker K, Sõritsa D, et al. The influence of menstrual cycle and endometriosis on endometrial methylome. Clin Epigenetics. 2016;8:1–10.
54. Rahmioglu N, Drong AW, Lockstone H, Tapmeier T, Hellner K, Saare M, et al. Variability of genome-wide DNA methylation and mRNA expression profiles in reproductive and endocrine disease related tissues. Epigenetics. 2017;12:897–908 Taylor & Francis.
55. Matsuzaki S, Canis M, Vaurs-Barrière C, Boespflug-Tanguy O, Dastugue B, Mage G. DNA microarray analysis of gene expression in eutopic endometrium from patients with deep endometriosis using laser capture microdissection. Fertil Steril. 2005;84:1180–90.
56. Ahn SH, Khalaj K, Young SL, Bruce A, Koti M, Tayade C. Immune-inflammation gene signatures in endometriosis patients. Fertil Steril. 2016;106:1420–31.
57. Fassbender A, Verbeeck N, Brnigen D, Kyama CM, Bokor A, Vodolazkaia A, et al. Combined mRNA microarray and proteomic analysis of eutopic endometrium of women with and without endometriosis. Hum Reprod. 2012;27:2020–9.
58. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97.
59. Wang Y, Liu J, Huang B, Xu Y, Li J, Huang L, et al. Mechanism of alternative splicing and its regulation. Biomed Rep. 2015;3:152–8.
60. Walsh CT, Garneau-Tsodikova S, Gatto GJ. Protein posttranslational modifications: the chemistry of proteome diversifications. Angew Chem Int Ed. 2005;44:7342–72.
61. Velarde MC, Aghajanova L, Nezhat CR, Giudice LC. Increased mitogen-activated protein kinase kinase/extracellularly regulated kinase activity in human endometrial stromal fibroblasts of women with endometriosis reduces 3′,5′-cyclic adenosine 5′-monophosphate inhibition of cyclin D1. Endocrinology. 2009;150:4701–12.
62. Yotova IY, Quan P, Leditznig N, Beer U, Wenzl R, Tschugguel W. Abnormal activation of Ras/Raf/MAPK and RhoA/ROCKII signalling pathways in eutopic endometrial stromal cells of patients with endometriosis. Hum Reprod. 2011;26:885–97.
63. Osteen KG, Yeaman GR, Bruner-Tran KL. Matrix metalloproteinases and endometriosis. Semin Reprod Med. 2003;21:155–64.
64. Kyama CM, Overbergh L, Mihalyi A, Meuleman C, Mwenda JM, Mathieu C, et al. Endometrial and peritoneal expression of aromatase, cytokines, and adhesion factors in women with endometriosis. Fertil Steril. 2008;89:301–10.
65. Sapalidis K, Machairiotis N, Zarogoulidis P, Vasilakaki S, Sardeli C, Koimtzis G, et al. Genes' interactions: a major contributor to the malignant transformation of endometriosis. Int J Mol Sci. 2019;20: E1842.
66. Kim TH, Yu Y, Luo L, Lydon JP, Jeong J, Kim JJ. Activated AKT pathway promotes establishment of endometriosis. Endocrinology. 2014;155:1921–30.
67. Matarese G, De Placido G, Nikas Y, Alviggi C. Pathogenesis of endometriosis: natural immunity dysfunction or autoimmune disease? Trends Mol Med. 2003;9:223–8.
68. Khorram O, Lessey BA. Alterations in expression of endometrial endothelial nitric oxide synthase and αvβ3integrin in women with endometriosis. Fertil Steril. 2002;78:860–4.
69. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. Clin Exp Reprod Med. 2011;38:119–25.
70. Painter JN, Anderson CA, Nyholt DR, Macgregor S, Gordon D, Wallace L, et al. Genome-wide association study identifies a locus at 7p15.2 associated with endometriosis. Nat Genet. 2011;43:51–4.
71. Aghajanova L, Giudice LC. Molecular evidence for differences in endometrium in severe versus mild endometriosis. Reprod Sci. 2011;18:229–51.
72. Hever A, Roth RB, Hevezi PA, Lee J, Willhite D, White EC, et al. Molecular characterization of human adenomyosis. Mol Hum Reprod. 2006;12:737–48.
73. Koot YEM, Van HSR, Boomsma CM, Van D, Koerkamp MJAG, Goddijn M, et al. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. Sci Rep. 2016;6:19411.
74. Vitonis AF, Vincent K, Rahmioglu N, Fassbender A, Buck Louis GM, Hummelshoj L, et al. World endometriosis research foundation endometriosis phenome and biobanking harmonization project: II. Clinical and covariate phenotype data collection in endometriosis research. Fertil Steril. 2014;102:1223–32.

Executive summary of the study

1. The impact of endometriosis on the eutopic endometrium is not well defined. It has been shown that dysregulation of eutopic endometrium can lead to the development of endometriotic lesion, which may affect endometrial function and receptivity for embryo implantation.

2. The catalogue development of 670 transcripts and proteins reported to be dysregulated in endometriosis. Data was extracted from 21 published studies, performed at transcriptomics, ncRNomics, proteomics, and epigenomics levels.

3. A total of 591 genes with available official human gene nomenclature were sorted according to the menstrual phases, i.e. M-, P-, S-, ES-, MS-, LS, and N/S.

4. The study revealed some stronger candidate loci and pathways associated with endometrial function in endometriosis. For example, six genes were reported to have altered molecular signature in the MS-phase by at least two studies indicating potential effect on receptivity in endometriosis. Additionally, some pathways (*response to progesterone*, *negative regulation of apoptotic process*, and *cell-cell adhesion*) were enriched in different phases of menstrual cycle.

5. The present study provides a multi-omics view of molecular mechanisms underlying endometriosis that can facilitate the development of novel diagnostic and therapeutic concepts.

6. The World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) guidelines for clinical performance and HGNC nomenclature should be followed for harmonisation of future multi-omics research in endometriosis.

2.1.2 Določanje molekularnega ozadja endometrijske receptivnosti pri adenomiozi

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Ozadje: Adenomioza je ginekološka patologija z omejenimi dokazi o negativnem vplivu na endometrijsko receptivnost za vgnezdenje zarodka. Pogosto se povezuje z endometriozo, za katero je bilo dokazano, da vpliva na spremenjen vzorec izražanja genov v endometriju. Posledično bi lahko kandidatni geni, ki so bili prepoznani pri endometriozi, služili kot vir za preučevanje delovanja endometrija pri adenomiozi. **Metode:** Iz pregleda literature smo pridobili transkripte RNA / proteine, ki se povezujejo z endometrijsko receptivnostjo pri ženskah z adenomiozo, ženskah z endometriozo in zdravimi ženskami. Poimenovanje lokusov smo poenotili po nomenklaturi zbirke HGNC. Pridobljene sete genov smo z aplikacijo STRING programa Cytoscape in z orodjem Reactome analizirali za obogatene biološke poti, da smo lahko izbrali kandidatne gene spremenjene endometrijske receptivnosti pri adenomiozi. Izbranim genom smo z metodo qPCR preverili stopnjo izražanja na vzorcih endometrija žensk z (n = 9) in brez (n = 13) adenomioze. **Rezultati:** Z obogatitvenima analizama zbranih 173, 42 in 151 genov, povezanih z endometriozo, adenomiozo oz. zdravo maternico, smo identificirali prekrivajoči se poti *signalizacija z interlevkini* in *signalizacija z interlevkinom-4 in interlevkinom-13*. Pripadajoče anotirane gene *LIF*, *JUNB*, *IL6*, *FOS*, *IL10* in *SOCS3* smo izbrali za kvantifikacijo. Izbrani geni so bili izraženi v zmanjšani stopnji v adenomiozni v primerjavi s kontrolno skupino, vendar je bila razlika statistično neznačilna. **Zaključki:** Izvedli smo prvo integracijsko analizo, kjer smo na podlagi podatkov endometrijske receptivnosti zdravih žensk in žensk z endometriozo predlagali kandidatne poti in gene spremenjene endometrijske receptivnosti pri ženskah z adenomiozo.

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Determining the Molecular Background of Endometrial Receptivity in Adenomyosis

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Abstract: Background: Adenomyosis is a gynaecological condition with limited evidence of negative impact to endometrial receptivity. It is commonly associated with endometriosis, which has been shown to alter endometrial expression patterns. Therefore, the candidate genes identified in endometriosis could serve as a source to study endometrial function in adenomyosis. Methods: Transcripts/proteins associated with endometrial receptivity in women with adenomyosis or endometriosis and healthy women were obtained from publications and their nomenclature was adopted according to the HUGO Gene Nomenclature Committee (HGNC). Retrieved genes were analysed for enriched pathways using Cytoscape/Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and Reactome tools to prioritise candidates for endometrial receptivity. These were used for validation on women with (n = 9) and without (n = 13) adenomyosis. Results: Functional enrichment analysis of 173, 42 and 151 genes associated with endometriosis, adenomyosis and healthy women, respectively, revealed signalling by interleukins and interleukin-4 and interleukin-13 signalling pathways, from which annotated *LIF*, *JUNB*, *IL6*, *FOS*, *IL10* and *SOCS3* were prioritised. Selected genes showed downregulated expression levels in adenomyosis compared to the control group, but without statistical significance. Conclusion: This is the first integrative study providing putative candidate genes and pathways characterising endometrial receptivity in women with adenomyosis in comparison to healthy women and women with endometriosis.

Keywords: adenomyosis; candidate genes; endometrial receptivity; endometriosis; gene expression; gene set enrichment analysis (GSEA); multi-omics; protein–protein interaction network (PPIN)

1. Introduction

Embryo implantation, characterised by synchronised interaction between vital embryo and maternal receptive endometrium [1], is restricted to the short time window of implantation (WOI), which appears in the mid-secretory (MS) phase of the menstrual cycle [2]. Endometrial-associated factor [3] could have a role in repeated embryo implantation failure (RIF), which prolongs assisted reproductive technique (ART) treatment, causing psychological trauma to infertile couples [4]. Therefore, omics technologies have been applied to identify potential biomarkers of uterine receptivity, which could

contribute to tailored infertility treatment [3]. Based on the knowledge from omics studies, the first clinical test for endometrial receptivity, the so-called "Endometrial receptivity array" (ERA, Igenomix, Spain), was developed. The ERA test is designed to evaluate a personalised WOI on a basis of transcriptomic signature of endometrial biopsy. Since WOI could be displaced during the secretory (S) phase in some infertile women, the ERA test tends to determine optimal timing of embryo transfer in ART treatments [5].

Adenomyosis is a gynaecological pathology where tissue similar to endometrium (ectopic endometrium) is located within the myometrium, the smooth musculature of the uterus [6]. Advances in high-resolution transvaginal ultrasound (TVUS) and magnetic resonance imaging (MRI) contribute to the common detection of adenomyosis in infertility diagnosis [7]. No exact criteria for sonographic features of adenomyosis are established [6], so the prevalence in a population of infertile patients is not exactly known. Puente et al. [8] diagnosed adenomyosis in 24.4% (n = 248/1015) and Hashim et al. [9] in 7.5% (n = 24/320) of women seeking ART treatment. Salim et al. [10] associated adenomyosis with a negative impact on endometrial receptivity when women underwent the first cycle of ART treatment. Lower pregnancy (22.2% vs. 47.2%) and higher miscarriage (50.0% vs. 2.8%) rates were observed in women with diagnosed adenomyosis compared to women exhibiting anovulatory menstrual cycles, endometriosis, or unexplained, tubal or male factor subfertility [10]. Higher rates of RIF (34.7% vs. 24.4%) between women with and without adenomyosis were also observed by Puente et al. [8]. In addition, a 28% decrease in the probability of clinical pregnancy in women with vs. without adenomyosis was predicted with the use of meta-analysis [11]. On the other hand, the molecular mechanism of hampered endometrial receptivity in adenomyosis is not well understood and mainly based on candidate gene/protein designed studies [12,13].

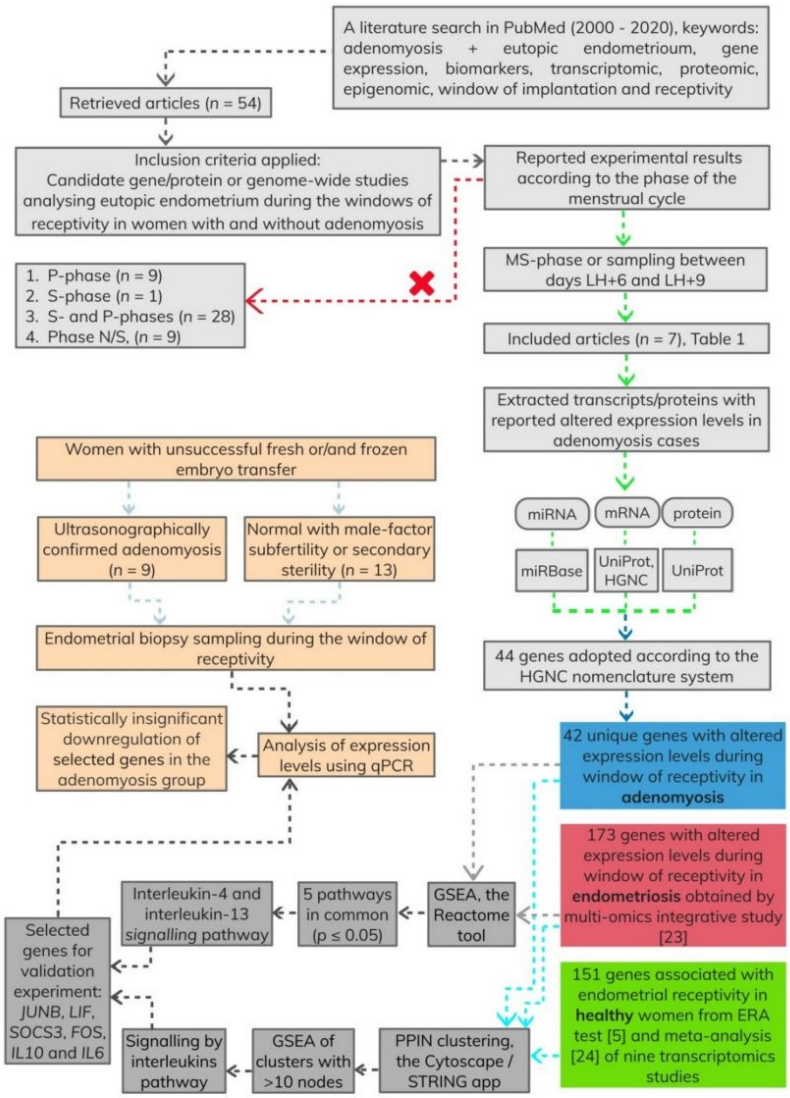
Adenomyosis is classified as "Endometriosis of uterus" according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) World Health Organisation (WHO) version for 2019 (https://icd.who.int/browse10/2019/en). Adenomyosis commonly coexists with endometriosis [14], which is characterised by ectopic endometrium located outside of the uterine cavity. The prevalence of endometriosis in infertile women with normospermic partners is estimated to be 47% (n = 104/221) [15], and it has been shown to alter the molecular patterns of endometrial receptivity. Numerous differentially expressed transcripts and proteins were identified using genome-wide profiling of endometrial samples during windows of receptivity in women with and without endometriosis [16–20]. Therefore, the reported altered endometrial signatures from pathophysiologically similar endometriosis provide a source of candidate genes to study receptivity in adenomyosis.

Biological network maps and functional enrichment analyses enable prioritisation of candidate genes for validation experiments in target tissue [21,22]. The network-based approach provides insight into the global molecular organisation of studied biological components (protein-coding or functional RNAs, proteins and metabolites). Nodes of the network present biological components, while edges present their physical relationships (protein–protein or RNA–DNA interactions, regulatory relationships between genes and transcription factors, binding, activation, inhibition, etc.) [21]. In the functional enrichment-based approach, a list of biological components is analysed by bioinformatics tools that use biological knowledge base, statistical testing, mathematical analyses and computational algorithms to recognise their relationship patterns [22]. In the present study, candidate genes for endometrial expression validation in adenomyosis were prioritised on the basis of gene set enrichment analysis (GSEA) using reported molecular signatures characterising endometrial receptivity.

Therefore, the aims of the present study were to (1) screen for reported transcripts/proteins associated with endometrial receptivity in adenomyosis, endometriosis and healthy women; (2) edit the nomenclature of extracted transcripts/proteins to develop gene lists specific for adenomyosis, endometriosis and healthy groups; (3) obtain enriched pathways associated with retrieved genes using bioinformatics network and functional enrichment-based approaches; (4) prioritise candidate genes for validation experiment; and (5) analyse expression patterns of selected genes in endometrial biopsy samples collected during expected windows of receptivity in women with and without adenomyosis.

2. Materials and Methods

The workflow of the study is presented in Scheme 1.



Scheme 1. Workflow and main results of the study. Main steps included literature mining, bioinformatics prioritisation of candidate genes and validation expression analysis of endometrial biopsy samples from women with and without adenomyosis. Legend: GSEA = gene set enrichment analysis, ERA = endometrial receptivity array, HGNC = Hugo Gene Nomenclature Committee, STRING = Search Tool for the Retrieval of Interacting Genes/Protein, P = the proliferative phase of the menstrual cycle, S = the secretory phase, MS = the mid-secretory phase, N/S = not specified, LH = luteinising hormone. LH+6 and LH+9 refer to the WOI which is between six and nine days after LH surge.

2.1. Identification of Relevant Studies for Development of Gene List

The development of the gene list associated with altered endometrial molecular patterns in adenomyosis was based on a literature search conducted in the PubMed literature database. Combinations of the keywords "adenomyosis" and "eutopic endometrium", "gene expression,"

"proteomic," "transcriptomic," "epigenomic," "window of implantation," "receptivity," and "biomarkers" were used. The search was conducted from 2000 until January 2020.

The gene list associated with endometrial receptivity in endometriosis was adopted from our previous integrative study of published genome-wide studies [23]. Briefly, top reported transcripts (protein-coding and functional RNAs) and proteins with altered expression patterns in the endometria of women with endometriosis were synthesised in the gene catalogue. Retrieved genes were further sorted according to the phases of the menstrual cycle. For the present analysis, genes from the MS-phase were extracted [23].

Gene sets characterising endometrial receptivity in healthy women were provided from two publications based on transcriptomics experiments: 238 genes from a genomic diagnostic ERA test [5] and 57 genes from a meta-analysis of nine transcriptomics studies [24].

The nomenclature of extracted transcripts and proteins from the literature survey was adopted according to the HUGO Gene Nomenclature Committee (HGNC) database (last updated 4 May 2020) [25] which is assigned to provide official symbols for human genes. Editing of the nomenclature across studies enabled downstream bioinformatics analysis for prioritisation of candidate genes for the validation experiment.

2.2. GSEA Using Retrieved Genes Associated with Adenomyosis, Endometriosis and Healthy Endometrium

The interaction of all retrieved genes was determined in the global network approach and was further clustered to distinguish molecular patterns in women with uterine disorders (endometriosis and adenomyosis) compared to healthy women. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) is a database which enables assessment and integration of protein–protein interactions of submitted biological components, such as genes [26]. The developed gene lists associated with endometrial receptivity in adenomyosis, endometriosis and healthy groups were projected into the protein–protein interaction network (PPIN) using the Cytoscape 3.8.0. software platform [27,28] and STRING app. The ClusterMaker2 app was further applied for identification of clusters with tightly connected proteins within the obtained network. The Markov cluster (MCL) algorithm was set to 2.5 for inflation value and the overall STRING confidence score was used as the array source. Obtained clusters with more than 10 nodes were analysed using STRING functional enrichment analysis (or GSEA). Obtained pathways with a false discovery rate (*FDR*) of ≤0.05 were considered for statistical significance. Annotated nodes to the top eight overrepresented terms of Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways were visualised using a split donut chart after redundant terms were removed using STRING app filtering.

2.3. GSEA Using Retrieved Genes Associated with Adenomyosis and Endometriosis Endometrium

Molecular knowledge from well-studied endometriosis was used as a model to identify putative pathways characterising endometrial receptivity in poorly studied adenomyosis. The GSEA in the Reactome bioinformatics tool version 72, released on 16 March 2020 [29] was applied for overlapping enriched pathways between retrieved genes associated with adenomyosis and endometriosis. Reactome is a database that provides a pathway over-representation (enrichment) analysis of submitted genes. The tool also provides a graphical map where known biological processes and pathways in human biology are visualised in hierarchical order [29]. Obtained enriched pathways with statistical significance ($p \leq 0.05$) were considered for overlap between the two gene lists.

2.4. Participating Women

The experimental protocol was approved by the National Ethics Committee of the Republic of Slovenia (0120-259/2018/16) and the study was conducted in accordance with the Helsinki Declaration of 175, as revised in 2013. Written informed consent was obtained from every participant prior to inclusion in the study. Participating women with unsuccessful fresh or/and frozen embryo transfer during

ART treatment were recruited from the Department of Reproductive Medicine and Gynaecological Endocrinology, University Medical Centre Maribor, Maribor, Slovenia.

Women with sonographic markers of adenomyosis according to a TVUS diagnostic infertility workup were recruited for the adenomyosis group. Women with normal TVUS findings and no history of adenomyosis, myoma, endometriosis or polycystic ovary syndrome (PCOS) who underwent ART due to male factor of subfertility or expose secondary sterility were recruited for the control group. An additional TVUS examination using Voluson E8 (GE Healthcare Austria GmbH & Co OG, Zipf, Austria) of the uterus and pelvis in all participating women was performed on the day of eutopic endometrium biopsy sample collection. Sonographic criteria for adenomyosis diagnosis were asymmetrical myometrial thickening not caused by the presence of fibroids, linear striations, parallel shadowing, myometrial cysts, hyperechoic islands and/or the presence of adenomyoma [30].

2.5. Clinical Data

Clinical data of age, body mass index (BMI), sterility (primary = never pregnant, secondary = achieved pregnancy), number of ART treatments before enrolling in the study and male factor subfertility based on evaluated spermatozoa concentration and morphology according to the WHO Laboratory Manual for the Examination and Processing of Human Semen, fifth edition [31] were obtained from the electronic medical records stored in our Meditex IVF database (Critex GmbH, Regensburg, Germany). Endometrial thickness was determined by TVUS examination prior to endometrial biopsy.

2.6. Endometrial Biopsy Sample Collection

Endometrial biopsy samples were obtained from the uterine cavity during the natural menstrual cycle using a Probet endometrial suction curette (Gynetics Medical Products N.V., Lommmel, Belgium). The luteinising hormone (LH) peak was determined by urinary LH ovulation rapid test cassettes (Hangzhou AllTest Biotech Co., Ltd, Hangzhou, P.R. China). Sampling was performed between days LH+6 and LH+9 (LH = 0 is the day of LH surge) corresponding to expected WOI. Two anovulatory women, one in the adenomyosis and second in the control group, received human chorionic gonadotropin (hCG) (Ovitrelle, Merck Europe B.V., Amsterdam, The Netherlands) to trigger ovulation. Sampling was calculated according to the day of hCG administration (hCG = 0) on day hCG+6 or hCG+7. The endometrial biopsy was immediately placed in RNAlater solution (catalog number AM7021, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) and stored overnight at +4 °C, then transferred to −80 °C until total RNA isolation.

2.7. Total RNA Isolation

Whole endometrial tissue sample was used for total RNA isolation using the miRNeasy Mini Kit (catalog number 217004, Qiagen GmbH, Hilden, Germany) according to the protocol recommended by the manufacturer. The kit also enables isolation of miRNA fraction, but that was not used in this study. Concentration and purity for each RNA sample were determined spectrophotometrically using Synergy2 (BioTek Instruments, Winooski, VT, USA). The RNA integrity number (RIN) for each sample was assessed by RNA Nano 6000 Assay Kit (catalog number 5067-1511, Agilent Technologies, Waldbronn, Germany) on the 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany).

2.8. Gene Expression Analysis by Quantitative PCR (qPCR)

Total RNA was first reverse-transcribed to 1 µg cDNA using a SuperScript IV Vilo Master Mix (catalog number 11756050, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) according to the manufacturer's instructions. Selected mRNAs, with corresponding hydrolysis probe IDs (catalog number 4331182, Life Technologies Corporation, Pleasanton, CA, USA) were: *FOS* (Hs99999140_m1), *LIF* (Hs01055668_m1), *JUNB* (Hs00357891_s1), *SOCS3* (Hs01000485_g1), *IL6* (Hs00174131_m1) and *IL10* (Hs00961622_m1). Glycealdehyde-3-phosphate dehydrogenase (*GAPDH*; Hs02786624_g1) and 18S

ribosomal RNA (*18S* rRNA; Hs99999901_s1) were used as reference genes. The final qPCR reaction mixture with 10 µL volume was as follows: 5 µL of 2× diluted TaqMan Gene Expression Master Mix (catalog number 4369016, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), 0.5 µL of 20× diluted selected hydrolysis probe assay, 2.5 µL of nuclease-free water and 2 µl of 10× diluted cDNA template. In each run, RNase-free water without cDNA (no-template controls) and mRNA added in reverse transcription reaction mix without reverse transcriptase (no reverse transcription controls) were included as negative controls. Quantification was performed by a LightCycler480 instrument (Roche, Basel, Switzerland). The qPCR protocol was as follows: uracil-N-glycosylase (UNG) incubation at 50 °C for 2 min and polymerase activation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s then annealing and extension at 60 °C for 1 min. Each sample was analysed in duplicate and average quantification cycle (Cq) values were used for calculations.

Final results were presented as fold differences in gene expression relative to the normalised calibrator, calculated by the $2^{-\Delta\Delta Cq}$ method [32]. For each studied sample, the geometrical mean of *GAPDH* and *18S* rRNA ($Cq_{sample,R}$) was used for normalisation and ΔCq values were further calculated as follows: $\Delta Cq_{sample} = Cq_{sample,gene\ of\ interest} - Cq_{sample,R}$. The value of calibrator ($\Delta Cq_{calibrator}$) for each gene of interest was determined as the average of retrieved ΔCq_{sample} values from all samples investigated with qPCR, including case and control study groups, and was used in the following formula: $\Delta\Delta Cq_{sample} = \Delta Cq_{sample} - \Delta Cq_{calibrator}$.

2.9. Statistical Analysis

Age, body mass index (BMI), endometrial thickness, number of previous ART treatments and fold change of gene expression levels are presented as medians with 95% confidence interval (CI). Non-parametric Mann–Whitney testing using SPSS 25.0 software (IBM Corporation, Armonk, NY, USA) was applied to compare study groups for statistically significant differences. The significance level was equal to ≤0.05.

3. Results

The workflow of the literature mining and the main results are presented in Scheme 1. Bioinformatics prioritisation and validation of candidate genes is visualised in graphical abstract in Figure 1. The literature search and HGNC nomenclature provided genes associated with endometrial receptivity in adenomyosis, endometriosis and healthy women. These were further used for functional enrichment analysis to prioritise candidate genes for the validation experiment. Selected genes were analysed for expression levels in endometrial biopsy samples collected during the expected window of receptivity in women with and without adenomyosis who underwent ART treatment.

3.1. Developed Gene Lists

The literature search associated with adenomyosis provided 54 studies, further divided according to the phase of the menstrual cycle (Scheme 1). Articles with candidate protein/gene and genome-wide study designs that analysed eutopic endometrium in women with and without adenomyosis during expected endometrial receptivity (LH timed to the WOI or MS-phase of the menstrual cycle) were further considered. Among 54 retrieved studies, 47 were excluded from the present analysis because they were performed in S or proliferative (P) phase, or the phase of the menstrual cycle was not specified. Among the 54 studies, six candidate gene/protein [12,13,33–36] and 1 genome-wide study [37] were included in gene list development. Extracted transcripts (mRNAs and ncRNAs) and proteins reported to be differentially expressed in adenomyosis cases are listed in Table 1, and their corresponding official humane gene symbols were provided according to the HGNC database. Of the total 44 retrieved genes, two (*LIF* and *HOXA10*) were repeated among studies. The final gene list included 42 unique genes associated with endometrial receptivity in adenomyosis. In addition, Table 1 provides information regarding composition and sample size of adenomyosis and control groups and indication of endometrial samples collection.

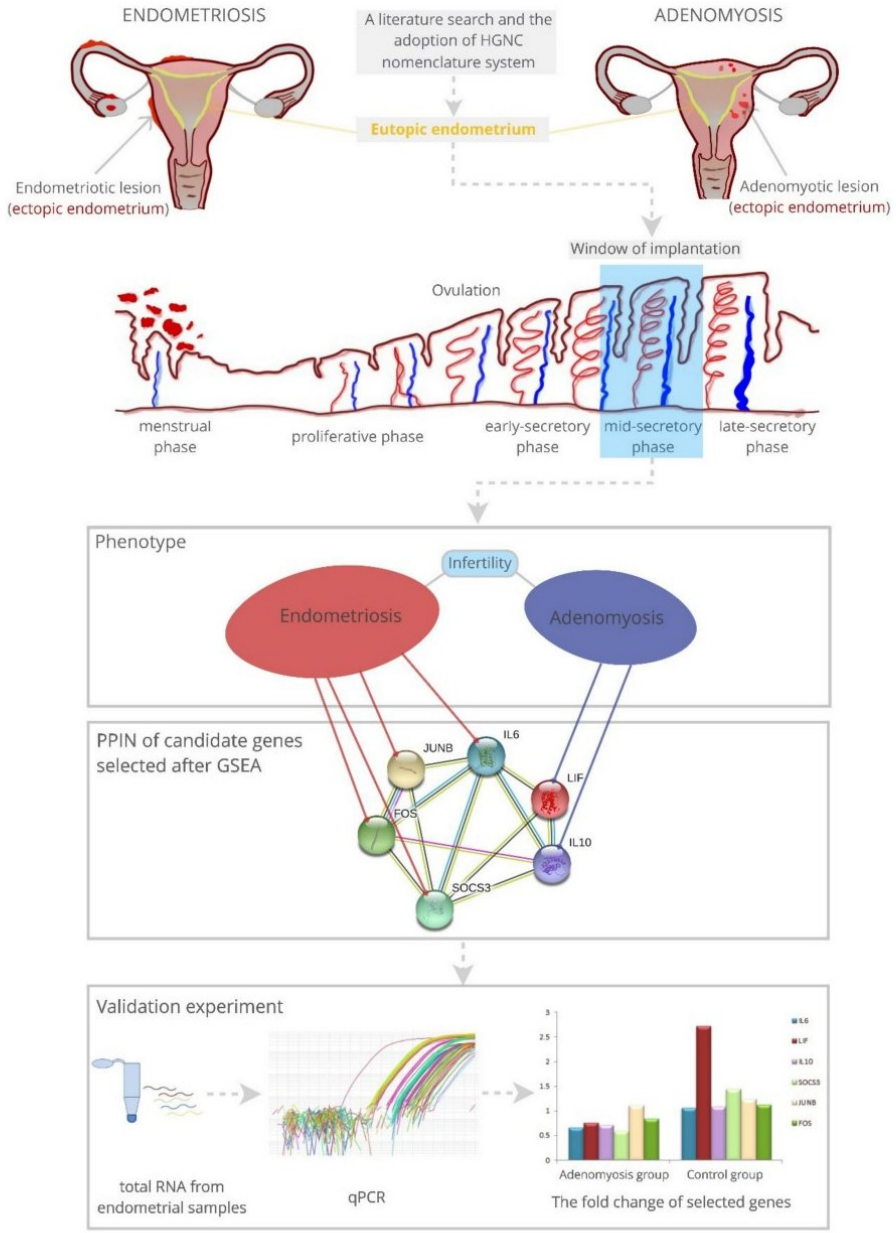


Figure 1. Graphical abstract of the study. Literature screening and HGNC nomenclature provided genes associated with endometrial receptivity that were further analysed for enriched pathways. Six candidate genes for experimental validation in adenomyosis were prioritised. Two selected genes originated from the adenomyosis group of 42 genes, and four from the endometriosis group of 173 genes. Protein–protein interaction network (PPIN) of selected genes is visualised using STRING database. Candidate genes were further validated in endometrial biopsy samples collected during window of receptivity in women with and without adenomyosis. Legend: GSEA = gene set enrichment analysis, STRING = Search Tool for the Retrieval of Interacting Genes/Protein. PPIN = Protein–protein interaction network, HGNC = Hugo Gene Nomenclature Committee.

Table 1. Retrieved studies reporting altered expression levels of transcripts/proteins in endometrial samples during frame of receptivity in women with adenomyosis. Nomenclature (gene symbol and approved gene name) of extracted data was edited according to the HUGO Gene Nomenclature Committee (HGNC) database. Letters A and C refer to adenomyosis and control women/group, respectively. Symbols ↑ and ↓ refer to reported up- and down-regulated, respectively, expression level of genes and proteins when compared adenomyosis with the control group in the source reference.

Study Design of Retrieved Studies	Biotype of Assessed Biological Entity in Source Reference	Biological Entities Assessed in Source Reference	The HGNC Gene Symbol	Approved Gene Name	Reported Up-(↑) or Down-(↓) Regulation in Adenomyosis (A) Compared to Control (C) Group, (Statistical Significance)	Indication and Procedure for Endometrial Tissue Samples Collection	Inclusion Criteria and Composition of A. Number (*n*) of Participants	Inclusion Criteria and Composition of C. *n* of Participants.	Source Reference
Candidate gene/protein	miRNA	miR-21	*MIR21*	microRNA 21	~50% ↓ ($p < 0.001$)	Endometrial biopsy between days 19 and 23 of the menstrual cycle	Confirmed 2 of 5 sonographic features of *A*. Aged 31.4 ± 0.7 years. *n* = 12	≥1 normal pregnancy and/or delivery. Aged 30.4 ± 0.6 years. *n* = 12	[36]
	protein	KLF12	*KLF12*	Kruppel like factor 12	~2-fold ↑ ($p < 0.001$)				
	protein	NR4A1	*NR4A1*	Nuclear receptor subfamily 4 group A member 1	~50% ↓ ($p < 0.001$)				
	protein	IL-10	*IL10*	Interleukin 10	40% ↓ ($p < 0.001$)	Endometrial biopsy in LH+7 using an endometrial curette	>2 of 5 sonographic features of *A*, clinical symptoms (secondary and progressive dysmenorrhea, menorrhagia and menostaxis), and clinical signs (homogenous enlargement of local uplift of the uterus, firmness and tenderness). Aged 40 years or younger. *n* = 23	Tubal factor infertility. Aged 40 years or younger. *n* = 23	[35]
	protein	HOXA10	*HOXA10*	Homeobox A10	~50% ↓ ($p < 0.001$)				
	Protein phosphorylation	p(Y705)-STAT3	*STAT3*	Signal transducer and activator of transcription 3	~50% ↓ ($p < 0.001$)				
	mRNA, protein	LIFR	*LIFR*	LIF receptor subunit alpha	0.3-fold ↓ mRNA ($p < 0.01$). Protein ↓ according to H-score: - glandular cells 43.6 in A vs. 92.3 in C ($p < 0.001$). - stromal cells: 27.3 in A vs. 57.7 in C ($p < 0.001$).	Laparoscopically assisted vaginal hysterectomy (LAVH) or myomectomy (part of C samples) between days 19 and 23 of the menstrual cycle.	Diagnosed *A* preoperatively with clinical symptoms and ultrasonogram. A confirmed postoperatively by pathologist. Aged 39.8 ± 0.8 years. - Diffuse A, *n* = 5. - Focal A, *n* = 4.	- Intramural or subserosal leiomyoma, *n* = 16. - Submucosal leiomyoma, *n* = 3. Aged 41.2 ± 0.5 years.	[13]
	mRNA, protein	LIF	*LIF*	LIF interleukin 6 family cytokine	0.15-fold ↓ mRNA ($p < 0.01$). protein ↓ according to the H-score: - glandular cells 61.5 in A vs. 231.8 in C ($p < 0.001$). - stromal cells: 40.7 in A vs. 135.4 in C ($p < 0.001$).				

Table 1. *Cont.*

Study Design of Retrieved Studies	Biotype of Assessed Biological Entity in Source Reference	Biological Entities Assessed in Source Reference	The HGNC Gene Symbol	Approved Gene Name	Reported Up-(↑) or Down-(↓) Regulation in Adenomyosis (A) Compared to Control (C) Group, (Statistical Significance)	Indication and Procedure for Endometrial Tissue Samples Collection	Inclusion Criteria and Composition of A. Number (*n*) of Participants	Inclusion Criteria and Composition of C. *n* of Participants.	Source Reference
	mRNA, protein	Integrin β3	*ITGB3*	Integrin subunit beta 3	mRNA ↓: Median value 6.2 in A vs. 21.5 in C ($p < 0.01$). protein ↓ according to the H-score: 2.0 in A vs. 2.7 in C ($p < 0.05$).	A group: hysterectomy for dysmenorrhea, hysteroscopy and diagnostic curettage for history of infertility. C group: testing for tubal patency or hysterectomy for pathological changes of the cervix. Ultrasound monitoring of menstrual cycle for endometrial sampling between days 7 and 9 following ovulation. Histologically confirmed mid-late secretory phase.	Enlarged uterus revealed by MRI and/or high level of serum CA125. Aged 40.1 ± 2.7 years. - Dysmenorrhea, *n* = 11. - History of infertility, *n* = 17.	Born at least 1 child and used some form of contraception with: - tubal factor of infertility or - pathological changes of the cervix. Aged 40.9 ± 2.1 years. *n* = 27	[34]
	mRNA, protein	OPN	*SPP1*	Secreted phosphoprotein 1	mRNA ↓: Median value 12.2 in A vs. 24.2 in C ($p < 0.01$). protein ↓ according to the H-score: 2.1 in A vs. 2.7 in C ($p < 0.05$).				
	mRNA, protein	LIF	*LIF*	LIF interleukin 6 family cytokine	Protein ↓ according to the H-score: 2.0 in A vs. 2.7 in C ($p < 0.05$).	Laparoscopy for tubal ligation, testing for tubal patency, or women without endometriosis who had hysterectomy for pathological changes of the cervix. Endometrial sampling by curettage and dating determined by histology.	Preoperative *A* diagnosis on clinical presentation, enlarged uterus determined by TVUS and/or high values of CA125. A confirmed by two histopathologists. Aged 40.3 ± 2.7 years. Total *n* = 28 with: - infertility. *n* = 21 or/and - dysmenorrhea. *n* = 11.	Fertile with: - tubal ligation - pathologic changes of the cervix Aged 40.9 ± 2.6 years. *n* = 27	[12]
	protein	HOXA10	*HOXA10*	Homeobox A10	↓ according to the H-score: 1.4 in A vs. 2.1 in C ($p < 0.001$).	A group: hysterectomy for hypermenorrhea or adnexal mass. *A* confirmed by histopathology. C group: hysterectomy for pelvic organ prolapse or benign adnexal mass. Histologically confirmed MS-phase of the menstrual cycle	Aged between 25 to 52 years old. *n* = 19	Fertile with: - pelvic organ prolapse or, - benign adnexal mass. Aged between 25 to 52 years old. Total *n* = 12	[33]

Table 1. *Cont.*

Study Design of Retrieved Studies	Biotype of Assessed Biological Entity in Source Reference	Biological Entities Assessed in Source Reference	The HGNC Gene Symbol	Approved Gene Name	Reported Up-(↑) or Down-(↓) Regulation in Adenomyosis (A) Compared to Control (C) Group, (Statistical Significance)	Indication and Procedure for Endometrial Tissue Samples Collection	Inclusion Criteria and Composition of A. Number (*n*) of Participants	Inclusion Criteria and Composition of C. *n* of Participants.	Source Reference
Genome-wide	mRNA	TMPRSS11B	*TMPRSS11B*	Transmembrane serine protease 11B	215.5 fold change (FC) ↑, (pfp* = 0.0421)	Systematic TVUS to detect ovulation. When a follicle of 17–18 mm was detected, LH peak was determined by urinary test. Endometrial biopsies sampling in LH+7 using Pipelle catheter.	A diagnosed by MRI and TVUS - Never pregnant, *n* = 2 - Two term pregnancies, *n* = 1 - A single pregnancy, *n* = 1 - Two early miscarriages, *n* = 1 - A miscarriage, *n* = 1	Young women with regular menses, no uterine or endocrine anomalies, proven fertility (previous spontaneous pregnancy at term). Normal results in a uterine ultrasonography. *n* = 6	[37]
	mRNA	CHD5	*CHD5*	Chromodomain helicase DNA binding protein 5	31.8 FC ↑ (0.00000) Confirmed by qPCR validation				
	mRNA	SST	*SST*	Somatostatin	10.6 FC ↑ (0.0350) Confirmed by qPCR validation				
	mRNA	SPBC25	*SPC25*	SPC25 component of NDC80 kinetochore complex	9.8 FC ↑ (0.0367)				
	mRNA	FLJ20105	*ERCC6L*	ERCC excision repair 6 like, spindle assembly checkpoint helicase	9.1 FC ↑ (0.0496)				
	mRNA	AKR1B10	*AKR1B10*	Aldo-keto reductase family 1 member B10	8.7 FC ↑ (0.0317) Confirmed by qPCR validation				
	mRNA	CDKN3	*CDKN3*	Cyclin dependent kinase inhibitor 3	8.0 FC ↑ (0.0406)				
	mRNA	ATP1A2	*ATP1A2*	ATPase Na+/K+ transporting subunit alpha 2	6.2 FC ↑ (0.04069 Confirmed by qPCR validation				
	mRNA	MB	*MB*	Myoglobin	5.8 FC ↑ (0.0262)				
	mRNA	KCNA4	*KCNA4*	Potassium voltage-gated channel subfamily A member 4	5.5 FC ↑ (0.0255)				
	mRNA	MMP20	*MMP20*	Matrix metallopeptidase 20	5.1 FC ↑ (0.0468)				
	mRNA	FNDC1	*FNDC1*	Fibronectin type III domain containing 1	5.0 FC ↑ (0.0464)				
	mRNA	TUBAL3	*TUBAL3*	Tubulin alpha like 3	4.9 FC ↑ (0.0425)				
	mRNA	SPINK2	*SPINK2*	Serine peptidase inhibitor Kazal type 2	4.8 FC ↑ (0.0406)				

Table 1. *Cont.*

Study Design of Retrieved Studies	Biotype of Assessed Biological Entity in Source Reference	Biological Entities Assessed in Source Reference	The HGNC Gene Symbol	Approved Gene Name	Reported Up-(↑) or Down-(↓) Regulation in Adenomyosis (A) Compared to Control (C) Group, (Statistical Significance)	Indication and Procedure for Endometrial Tissue Samples Collection	Inclusion Criteria and Composition of A. Number (*n*) of Participants	Inclusion Criteria and Composition of C. *n* of Participants.	Source Reference
	mRNA	COL11A1	*COL11A1*	Collagen type XI alpha 1 chain	4.6 FC ↑ (0.0493)				
	mRNA	LOC220115	/		4.1 FC ↑ (0.0278)				
	mRNA	LIPH	*LIPH*	Lipase H	3.9 FC ↑ (0.0356)				
	lncRNA	C21orf121	*ZNF295-AS1*	ZNF295 antisense RNA 1	3.5 FC ↑ (0.0343)				
	mRNA	PSG6	*PSG6*	Pregnancy specific beta-1-glycoprotein 6	3.3 FC ↑ (0.0280)				
	mRNA	C3orf33	*C3orf33*	Chromosome 3 open reading frame 33	3.2 FC ↑ (0.0475)				
	mRNA	MDAC1	*TMEM190*	Transmembrane protein 190	2.4 FC ↑ (0.0275)				
	mRNA	COL8A1	*COL8A1*	Collagen type VII alpha 1 chain	2.2 FC ↑ (0.0488)				
	mRNA	LTF	*LTF*	Lactotransferrin	1.9 FC ↑ (0.0340)				
	mRNA	SULT1E1	*SULT1E1*	Sulfotransferase family 1E member 1	1.8 FC ↑ (0.0410)				
	mRNA	TBX15	*TBX15*	T-box transcription factor 15	1.7 FC ↑ (0.0340)				
	mRNA	ATP12A	*ATP12A*	ATPase H+/K+ transporting non-gastric alpha2 subunit	−5.1 FC ↓ (0.0340)				
	mRNA	LOC401233	/		−3.8 FC ↓ (0.0000)				
	mRNA	CLDN4	*CLDN4*	Claudin 4	−3.7 FC ↓ (0.0488)				
	mRNA	LOC643338	*C15orf62*	Chromosome 15 open reading frame 62	−3.2 FC ↓ (0.0450)				
	mRNA	SCGB2A2	*SCGB2A2*	Secretoglobin family 2A member 2	−3.1 FC ↓ (0.0233)				
	mRNA	TCN1	*TCN1*	Transcobalamin 1	−2.6 FC ↓ (0.0220)				
	mRNA	GPR78	*GPR78*	G protein-coupled receptor 78	−1.7 ↓ (0.0175)				
	mRNA	CACNA1E	*CACNA1E*	Calcium voltage-gated channel subunit alpha1 E	−1.6 FC ↓ (0.0471)				
	mRNA	CYP3A7	*CYP3A7*	Cytochrome P450 family 3 subfamily A member 7	−1.2 FC ↓ (0.0433)				

Legend: LH+7 = the seven day after luteinising hormone (LH) surge. TVUS = transvaginal ultrasound. MRI = magnetic resonance imaging. CA125 = cancer antigen 125 biomarker. MS = the mid-secretory phase of the menstrual cycle. FC = a fold change. H-score = a method of assessing staining intensity in immunohistochemistry. miRNA = micro RNA. mRNA = messenger RNA. lncRNA = long-noncoding RNA. * pfp = statistical values presented as percentage of false positives..

The gene list associated with endometrial receptivity in endometriosis included 173 unique genes obtained from our previous multi-omics analysis [23]. Retrieved genes originated from six genome-wide studies, including 1 at the proteomics level [38], three at the transcriptomics level [16–18], one at the transcriptomics and ncRNomics level [19] and one at the epigenomics level, providing differentially expressed genes associated with altered methylation level [20].

The list associated with endometrial receptivity in healthy women included 151 unique genes. From the meta-analysis [24], 39 genes were extracted because they were experimentally validated and confirmed to exhibit altered expression patterns in endometrial samples LH+8 compared to LH+2. From the ERA test [5], 143 genes were extracted because they were validated by RT-PCR, included in a test as gold standard biomarkers of receptivity, or belonged to the endometrial receptivity transcriptomic signature. Of the total 182 genes, 31 overlapped between studies.

The retrieved 42 genes associated with endometrial receptivity in women with adenomyosis (A = 42), 173 in women with endometriosis (E = 173) and 151 in healthy women (H = 151) are listed in Table 2.

Figure 2 visualises these genes in adenomyosis, endometriosis and healthy groups connected with edges presenting the study design/omics level that was applied in the source publications that reported altered endometrial expression levels of associated genes. *SPP1*, *LIF*, *TCN1* and *CLDN4* were common to adenomyosis and healthy groups of genes, while *ANXA2*, *EDNR8*, *MMP26*, *DEPP1*, *ABCC3*, *CDA* and *SLC1A1* were common to endometriosis and healthy groups. *NR4A1* repeated in adenomyosis and endometriosis, while *SCGB2A2* was on all three genes lists.

Table 2. Gene lists associated with endometrial receptivity in women with adenomyosis or endometriosis and healthy women. Altered endometrial expression levels of genes and proteins in women with adenomyosis and endometriosis during the window of receptivity and biomarkers of uterine receptivity were retrieved from the literature survey. The gene nomenclature of extracted data was edited according to the HUGO Gene Nomenclature Committee (HGNC) database.

Group of Women	Gene List
Adenomyosis	*AKR1B10, ATP12A, ATP1A2, C15orf62, C3orf33, CACNA1E, CDKN3, CHD5, CLDN4, COL11A1, COL8A1, CYP3A7, ERCC6L, FNDC1, GPR78, HOXA10, IL10, ITGB3, KCNA4, KLF12, LIF, LIFR, LIPH, LTF, MB, MIR21, MMP20, NR4A1, PSG6, SCGB2A2, SPC25, SPINK2, SPP1, SST, STAT3, SULT1E1, TBX15, TCN1, TMEM190, TMPRSS11B, TUBAL3, ZNF295-AS1.*
Endometriosis	*ABCB11, ABCC3, ACKR1, ACO2, ADGRF1, AFF4, AGT, AIMP1, ALPI, AMY1A, AMY2A, AMY2B, ANXA2, ANXA5, AOC1, ATF3, BST2, C1QA, C1QTNF6, CA1, CA12, CASP5, CCBE1, CCL3, CCL3L1, CCL3L3, CCL8, CCN1, CCT8, CDA, CDK5R1, CELF1, COL12A1, CORO1B, CRABP1, CRISP3, CST7, CTSW, CWH43, CXCL2, CYP3A5, DDIT4L, DDX17, DEPP1, DLG5, DNAJC3, DST, EDNRB, EGR1, EGR2, EGR3, EIF1, EIF4A1, EIF4A2, ENPP3, FMN2, FOS, FOSB, GALP, GSN, GUCY1B1, GZMA, HACD1, HOXA9, HPCAL4, HSP90B1, FNA21, IL6, IMMT, JUNB, KCNK2, KRIT1, KRT18, KRT5, KRTAP19-2, LAMA3, LCK, LONRF2, LPP, LRRD1, LTB4R2, LUZP1, MALL, MAP4, MAPK8, MET, MIR135A1, MIR138-1, MIR138-2, MIR1915, MIR194-2, MIR196A1, MIR196A2, MIR219B, MIR22, MIR26B, MIR3196, MIR339, MIR365B, MIR3686, MIR374B, MIR4251, MIR4252, MIR4254, MIR4425, MIR4723, MIR505, MIR542, MIR548AA2, MIR548AP, MIR548T, MIR5585, MIR921, MMP26, MUC7, MYL12A, NCR1, NEAT1, NFAT5, NR4A1, NR4A3, PAX8, PCSK5, PCYOX1, PDHB, PER1, PITX1, PLEK, PLEKHA2, POMZP3, PRDX6, PRIM2, PRRC2C, PTAFR, RAB9BP1, RBBP4, RGS1, RIF1, RIN1, RNF150, RNH1, RSRP1, S100A3, S100A8, SAP30L, SCG2, SCGB2A2, SEMA3C, SERPINB8, SHB, SLA, SLC15A4, SLC1A1, SLC44A2, SMG1, SOCS3, SON, SP3P, TAF6L, TGFB3, THRAP3, TRIM15, TRPM6, TUBA1C, VDAC1P1, VEGFA, VHL, VIM, YBX1, YBX1P2, YWHAE, ZFP36, ZIC2.*

Table 2. *Cont.*

Group of Women	Gene List
Healthy	*ABCC3, ACADSB, ADAMTS1, ALPL, AMIGO2, ANG, ANO1, ANXA2, ANXA4, AOX1, APOD, ARG2, ARID5B, ASPM, ATP1B1, ATP6V0E2, ATP6V1A, BARD1, BCL6, BUB1B, C1R, C4BPA, CAPN6, CATSPERB, CCNB2, CDA, CDC20, CDK1, CENPE, CEP55, CFD, CLDN4, CLU, COL16A1, COMP, CP, CRABP2, CSRP2, CTNNA2, CXCL13, CXCL14, DDX52, DEFB1, DEPP1, DEPTOR, DKK1, DLGAP5, DPP4, DYNLT3, ECI2, ECM1, EDN3, EDNRB, EFNA1, ENPEP, EPHB3, FANCI, FOSL2, FXYD2, G0S2, GABARAPL1, GADD45A, GALNT12, GALNT4, GAS1, GAST, GBP2, GDF15, GPX3, GREM2, HABP2, HEY2, HLA-DOB, HPSE, ID4, IDO1, IL15, IMPA2, KCNG1, KIF11, KIF20A, KIF4A, KMO, KRT7, LAMB3, LIF, LMCD1, LMOD1, LRRC17, LYPD3, MAOA, MAP2K6, MFAP2, MFAP5, MMP26, MPPED2, MSX1, MT1G, MT1H, MT2A, MTCL1, NDC80, NDRG1, NDRG2, NNMT, NRG2, OLFM1, OLFM4, PAEP, PAQR4, PBK, PENK, PLA1A, PLAAT3, PLAAT4, PMEPA1, POLD4, POSTN, PRC1, PRKCQ, PRR15L, PRUNE2, PTPRR, RASSF2, RETREG1, RNASE4, RPRM, S100A1, S100A4, S100P, SCGB2A2, SERPINA5, SERPING1, SFRP4, SLC1A1, SNX10, SOD2, SORD, SOX17, SPDEF, SPP1, SYNE2, TACC3, TAGLN, TBC1D2, TCN1, THBD, TMSB15A, TOP2A, TRH, TSPAN8.*

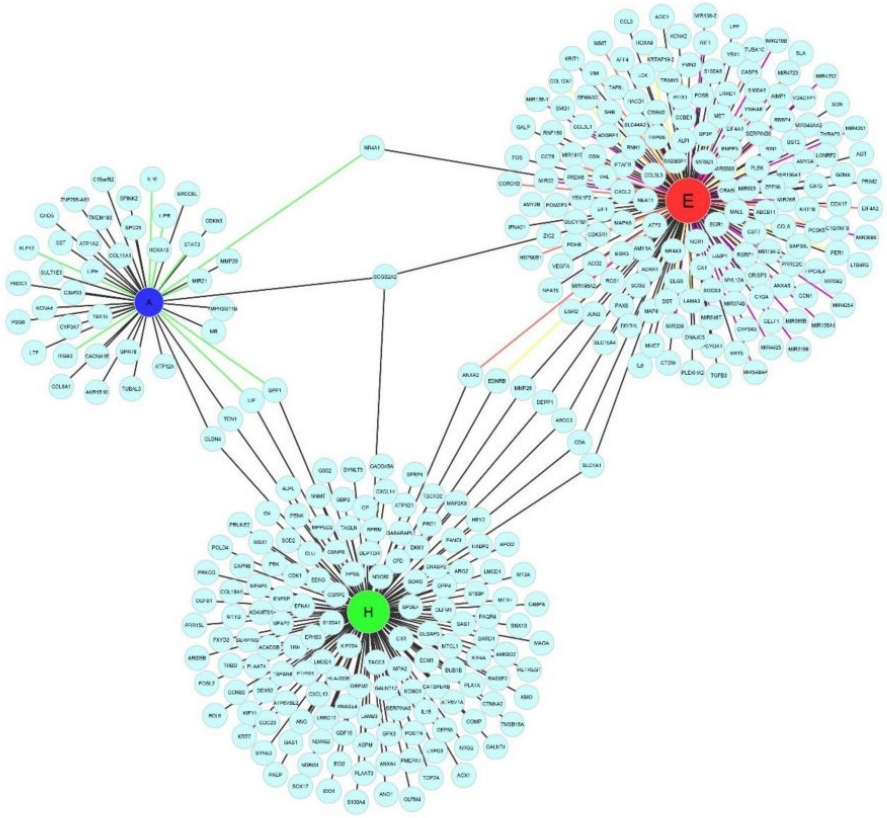


Figure 2. Retrieved genes associated with endometrial receptivity in women with adenomyosis (A = 42) or endometriosis (E = 173) and healthy women (H = 151) and overlap across groups. Colour of edges between genes represents the type of study design/omics level from which genes with reported molecular dysregulation were retrieved through literature mining: green, candidate protein/gene study design; black, transcriptomics study design; yellow, proteomics study design; red, epigenomics study design; and violet, ncRNomics study design.

3.2. Enriched Pathways after PPIN Clustering

Projection of all retrieved genes resulted in a PPIN with 315 nodes and 1130 edges. Network clustering (workflow presented in Supplementary Figure S1) resulted in three clusters containing more than 10 nodes, named cluster 1, cluster 2 and cluster 3. Figure 3 presents pathways associated with nodes in clusters 1, 2 and 3 after removal of redundant enriched terms. Cluster 1 included 210 edges and 46 nodes that were mapped to all three gene lists: adenomyosis (A = 4), endometriosis (E = 28) and healthy (H = 14). Functional enrichment analysis of cluster 1 provided 102 enriched terms of GO processes, KEGG and Reactome pathways, including response to organic substance, regulation of signalling receptor activity, inflammatory response, signalling by interleukins and cell chemotaxis. Cluster 2 included 26 nodes which were mainly mapped to the healthy gene list (H = 19) and was connected with 225 edges. Three and four nodes were mapped to adenomyosis (A = 3) and endometriosis (E = 4) gene lists, respectively. Enrichment functional analysis of cluster 2 provided 54 enriched terms of GO processes and Reactome pathways. Most enriched terms were associated with cell cycle and mitosis, including mitotic cell cycle, nuclear division, MHC class II antigen presentation and regulation of mitotic cell cycle. Cluster 3 was constructed from 11 nodes, mapped to endometriosis (E = 7) and healthy (H = 4) gene lists, and connected with 12 edges. Four enriched KEGG pathways were associated with cluster 3, including Rap1 signalling pathway and MicroRNAs in cancer. Complete GSEA results of all three clusters are presented in Supplementary Table S1.

3.3. Overlapping Enriched Pathways between Adenomyosis and Endometriosis Gene Lists

GSEA was applied to identify overlapping pathways between genes associated with endometrial receptivity in adenomyosis and endometriosis. Altogether, 56 enriched pathways associated with endometriosis and 45 with adenomyosis were provided, which are listed in Supplementary Table S2. Table 3 lists five overlapping pathways: gene and protein expression by JAK-STAT signalling after interleukin-12 stimulation, interleukin-10 signalling, interleukin-4 and interleukin-13 signalling, neutrophil degranulation and PTK6 activation of STAT3, together with annotated genes, *p*-value and *FDR*.

3.4. Prioritisation of Candidate Genes for Validation Experiment

Candidate genes were prioritised from the obtained enriched pathways with a similar biological role. Signalling by interleukins (R-HSA-449147) associated with cluster 1 (annotated as *LIF, LIFR, STAT3, IL15, JUNB, FOS, SOCS3, ANXA2, BCL6, IL6, IL10, CXCL2, CCL3* and *CCL3L3*) and interleukin-4 and interleukin-13 signalling (R-HSA-6785807) enriched by both adenomyosis and endometriosis gene lists (annotated as *IL10, STAT3, LIF, SOCS3, IL6, VIM, FOS, JUNB, VEGFA* and *HSP90B1*) were highlighted as source pathways for candidate genes selection. According to the Reactome pathway database, selected pathways are hierarchically sorted within immune response. Six annotated genes were selected for validation analysis: LIF interleukin 6 family cytokine (*LIF*) and interleukin 10 (*IL10*), originating from the adenomyosis gene list, and JunB proto-oncogene, AP-1 transcription factor subunit (*JUNB*), interleukin 6 (*IL6*), Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*) and suppressor of cytokine signalling 3 (*SOCS3*) from the endometriosis gene list (Figure 1).

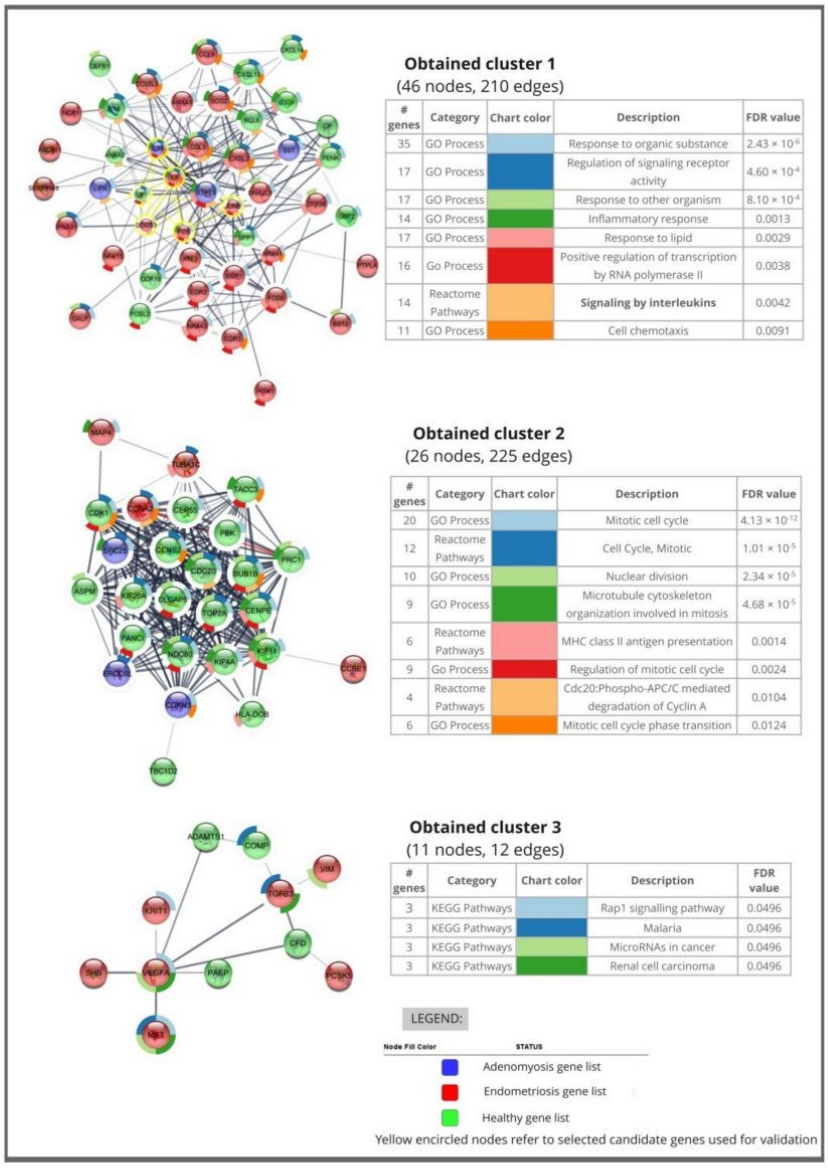


Figure 3. Clusters with more than 10 nodes obtained from PPIN associated with endometrial receptivity in women with adenomyosis or endometriosis and healthy women. GSEA was applied for enriched pathways and annotated nodes were coloured in split donut charts. Signalling by interleukins pathway (marked in bold) from cluster 1 served as a source for prioritisation of genes for validation experiment. Complete PPIN is provided in Supplementary Figure S1. STRING database projected retrieved gene symbols *ADGRF1*, *HACD1*, *GUCY1B1*, *CCN1*, *TMSB15A* and *RETREG1* in the PPIN as GPR110, PTPLA, GUCY1B3, CCNA2, TMSNB and FAM134B, respectively.

3.5. Patients

A total of 22 women between 29 and 42 years old involved in ART treatment were classified to the study groups: ultrasonographically confirmed adenomyosis group (n = 9) and control group with male factor subfertility or secondary sterility (n = 13). The most frequently observed sonographic finding in adenomyosis cases was asymmetrical myometrial thickening (88.9% of cases). One adenomyosis patient was diagnosed with coexisting myoma. Two women in the control group were diagnosed

with small myoma (7 mm) or bilateral hydrosalpinx. No differences between the composition of study groups were observed, except that endometrial thickness was statistically significantly lower in the adenomyosis group that the control group (7.3 vs. 9.2 mm (p = 0.030)). Demographic and clinical characteristics of the study groups are presented in Table 4.

3.6. Expression Patterns of Candidate Genes

Isolated total RNA from endometrial biopsy samples collected during the window of receptivity were used to verify whether selected candidate genes *LIF*, *SOCS3*, *FOS*, *JUNB*, *IL6* and *IL10* were differentially expressed between adenomyosis and control groups. The A_{260}/A_{280} ratios and RIN values of all RNA samples were above 2.0 and >8, therefore the samples were suitable for further expression analysis with qPCR. No statistically significant differences in gene expression levels between study groups were observed. However, median values (Table 5) indicate downregulation of selected genes in the adenomyosis group. Expression patterns of selected genes relative to normalised calibrator in adenomyosis and control groups are presented in Figure 4. Gene expression levels between study groups remained statistically insignificant when women with coexisting uterine pathologies from case and control groups were excluded from the calculation (data not shown).

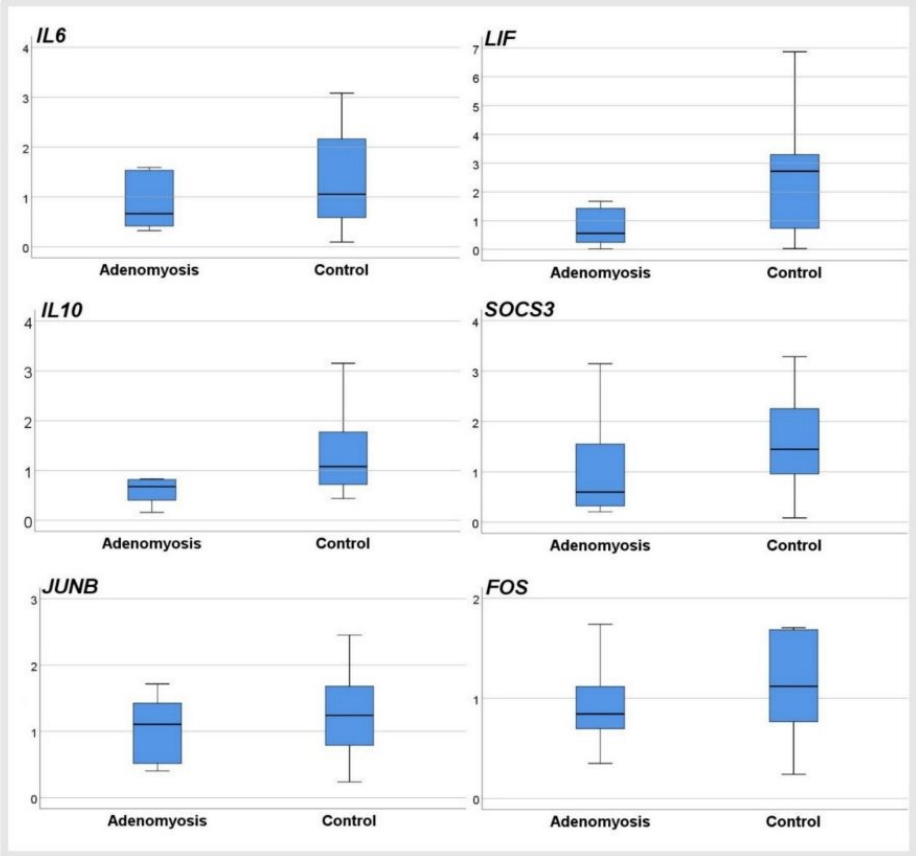


Figure 4. Relative expression levels of selected candidate genes in adenomyosis and control study groups. Selected genes were normalised to expression level of *GAPDH* and *18S* rRNA reference genes.

Table 3. Retrieved overlapping enriched pathways after gene set enrichment analysis (GSEA) between adenomyosis and endometriosis gene lists. Interleukin-4 and interleukin-13 signalling (marked in bold) served as a source for prioritisation of annotated genes for validation experiment.

Reactome Pathway ID	Number of Total Reactions	Adenomyosis				Endometriosis			
		Annotated Genes	Reaction Count	*p*-Value	FDR-Value	Annotated Genes	Reaction Count	*p*-Value	FDR-Value
R-HSA-8950505: Gene and protein expression by JAK-STAT signalling after Interleukin-12 stimulation	36	*IL10*	1	0.043	0.197	*CA1*, *ANXA2*	2	0.043	0.450
R-HSA-6783783: Interleukin-10 signalling	15	*IL10*, *STAT3*, *LIF*	15	6.26×10^{-4}	5.38×10^{-2}	*IL6*, *CCL3L1*, *CCL3L3*, *PTAFR*, *CCL3*, *CXCL2*	2	6.08×10^{-7}	2.35×10^{-4}
R-HSA-6785807: **Interleukin-4 and interleukin-13 signalling**	46	*IL10*, *STAT3*, *LIF*	19	2.60×10^{-3}	0.086	*SOCS3*, *IL6*, *VIM*, *FOS*, *JUNB*, *VEGFA*, *HSP90B1*	5	1.37×10^{-4} E-04	0.015
R-HSA-6798695: Neutrophil degranulation	10	*TCN1*, *LIPH*, *CHD5*, *CACNA1E*, *MMP20*, *LTF*	7	0.020	0.197	*CDA*, *AOC1*, *GSN*, *ANXA2*, *SLC44A2*, *CRISP3*, *PTAFR*, *CXCL2*, *PRDX6*, *CCT8*, *DNAJC3*, *BST2*, *SLC15A4*, *SCG2*, *KCNK2*, *S100A8*	9	0.014	0.360
R-HSA-8849474: PTK6 activation of STAT3	9	*STAT3*	6	0.031	0.197	*SOCS3*	3	0.007	0.281

Table 4. Comparison of study groups. Values reported as median (95% confidence interval (CI) for median).

Clinical Characteristics	Adenomyosis Group (n = 9)	Control Group (n = 13)	p-Value *
Age (years)	33 [32; 39]	36 [32; 39]	0.946
BMI (kg/m^2)	29.4 [17.8; 34.6]	22.8 [19.6; 24.0]	0.057
Endometrial thickness (mm)	7.3 [4.6; 9.7]	9.2 [7.4; 13.0]	0.030
Number of previous ART	2 [1; 3]	2 [1; 4]	0.387
Female sterility:			
primary	5	9	0.522
secondary	4	4	

* Values of statistical significance were obtained by Mann–Whitney U test.

Table 5. The fold change of selected genes in study groups. Values reported as median (95% CI for median).

Candidate Gene	Adenomyosis Group (n = 9)	Control Group (n = 13)	p-Value
IL6	0.664 [0.402; 1.589]	1.054 [0.533; 2.224]	0.333
LIF	0.761 [0.140; 4.317]	2.717 [0.423; 6.870]	0.262
IL10	0.716 [0.295; 2.080]	1.081 [0.523; 1.995]	0.193
SOCS3	0.597 [0.223; 2.780]	1.446 [0.935; 3.283]	0.301
JUNB	1.106 [0.419; 1.557]	1.242 [0.714; 1.953]	0.526
FOS	0.844 [0.420; 1.657]	1.120 [0.509; 1.705]	0.271

p-values were calculated by non-parametric Mann–Whitney U test.

4. Discussion

In the present study, prioritisation of candidate genes for an endometrial validation experiment in poorly studied adenomyosis was based on bioinformatics integration of published endometrial expression signatures associated with endometrial receptivity (Scheme 1). A literature survey and the HGNC nomenclature system provided 42 genes associated with endometrial receptivity in adenomyosis. In addition, 173 and 151 genes, respectively, were obtained from well-studied endometriosis patients and healthy women. General and more specific putative dysregulated endometrial pathways in women with adenomyosis were identified using retrieved genes and GSEA applied in Cytoscape/STRING app and Reactome tool. Six candidate genes annotated in selected pathways of immune cytokine signalling were further experimentally validated for expression patterns during the receptivity window in women with and without adenomyosis who underwent ART treatment.

4.1. Integration of Reported Signatures and Highlighted Enriched Pathways

Endometrium is highly active tissue under the control of fluctuating steroid sex hormones which enable tissue growth, shedding and regeneration in a cyclic manner [39]. The knowledge of biological events contributing to the ability of endometrium to become receptive to an embryo is limited, with an overlap of simultaneously reported up- and downregulated genes across transcriptomics studies performed in healthy women [24], and those coexisting uterine pathologies, such as endometriosis [2,23] and adenomyosis [40].

In the present study, we attempted to identify additional genes with altered expression patterns in women with adenomyosis based on published data regarding endometrial receptivity. Therefore, retrieved genes associated with reported altered molecular expression patterns during the window of endometrial receptivity in women with adenomyosis and endometriosis were integrated in the PPIN context together with biomarkers of uterine receptivity assumed to characterise healthy women. Further PPIN clustering and downstream GSEA performed by the Cytoscape/STRING app provided the signalling by interleukins pathway within cluster 1 (Figure 3). Annotated nodes in this pathway originated from gene lists associated with adenomyosis, endometriosis and healthy women as well. This could mean that the signalling by interleukins characterising endometrial receptivity in healthy women

might be dysregulated in women with endometriosis and adenomyosis. A more specific putative pathway regarding dysregulated interleukin signalling was identified based on overlapping enriched pathways between 42 and 173 genes associated with adenomyosis and better-studied endometriosis, respectively. In that way, the overlapping interleukin-4 and interleukin-13 signalling pathway was identified after GSEA was applied in the Reactome tool (Table 3).

According to the Reactome pathway database, enriched signalling by interleukins and interleukin-4 and interleukin-13 signalling pathways exposing similar biological role, i.e., immune cytokine signalling. Chaouat et al. pointed out the important role of immune cytokines in embryo implantation [41]. According to the literature, regulation of the local immune response protects the embryo from maternal immunity. During the S-phase, the decidua stimulates an influx of diverse immune cell populations in the endometrium tissue, including uterine natural killer (uNK) cells, lymphocytes and macrophages [39,42]. IL-4 and IL-13, together with IL-5, IL-9 and IL-10, belong to the type 2 cytokines, which are secreted from helper T (Th) lymphocytes. In general, these interleukins stimulate embryo implantation and trophoblast invasion, therefore, assumed to be protective for pregnancy [43]. Based on highlighted enriched pathways from the present GSEA, it was hypothesised that dysregulated patterns of affected endometrial receptivity in women with adenomyosis are associated with protective interleukins for embryo implantation.

Only published genome-wide studies were considered for present integrative analysis of genes associated with endometrial receptivity in women with endometriosis and healthy women. However, Enciso et al. [44] suggested an additional panel of 40 candidate genes as putative biomarkers for rapid determination of uterine receptivity status by qPCR. Authors discussed that only 7 out of 40 genes is in common with the ERA test [44]. To additionally verify the biomarker potential of those 40 candidate genes we performed pathway enrichment analysis using the Reactome tool. The top retrieved enriched pathway was interleukin-4 and interleukin-13 signalling with $p = 4.38 \times 10^{-9}$ and $FDR = 1.26 \times 10^{-6}$, which is in the accordance with our bioinformatics results. The interleukin-4 and interleukin-13 signalling contains 211 entities taking part in 46 reactions, according to the Reactome pathway database, meaning it could present a great pool of candidate genes for endometrial receptivity. Despite *LIF*, *IL6*, *IL10*, *JUNB*, *FOS* and *SOCS3* showing no statistically altered expression patterns between study groups, their lower expression levels were observed in the adenomyosis group. Therefore, *STAT3*, *VIM*, *VEGFA* and *HSP90B1* that were also annotated in interleukin-4 and interleukin-13 signalling in the present GSEA need to be validated to verify potential downregulation of this pathway in women with adenomyosis.

Candidate genes of the present expression analysis were prioritised on the basis of enrichment analysis using genetic loci that were gathered from different omics levels. However, limited correlation between transcriptomics and proteomics levels has been observed previously. Namely, Vogel and Marcotte [45] discussed that there is only 40% correlation between relative mRNA abundances and corresponding protein concentration in mammals as a consequence of post-transcriptional, translation and protein degradation regulation [45]. Therefore, in the future studies it is necessary to extend the research to regulatory mechanisms occurring downstream synthesised mRNA.

4.2. The Role of Selected Genes in Reproductive Biology

Prioritised candidate genes of the present study have been widely studied in the field of reproductive biology. LIF is a glycoprotein cytokine which enhances decidualisation in humans and mice. It is considered as a biomarker of endometrial receptivity [46]. When human endometrial Ishikawa cells and epithelial ECC-1 cells from endometrial adenocarcinoma were treated with LIF, higher expression levels of *ITGAV*, *ITGB3* and *ITGB5* adhesion molecules were observed; these are required for attachment of the embryo trophoblast to the receptive endometrial surface [47]. Serafini et al. reported a 6.4-fold higher chance of pregnancy in women with stronger immunohistochemical staining for LIF in secretory endometrium biopsy samples prior to ART treatment [48]. Downregulation

of endometrial *LIF* mRNA and protein observed in women with adenomyosis [12,13] and unexplained infertility [49] was associated with affected receptivity.

IL-10 is an anti-inflammatory cytokine which acts as a negative regulator of macrophage and T lymphocyte cell activation [50]. It is upregulated during early pregnancy to maintain maternal immunotolerance for the embryo [51]. Wang et al. reported downregulation of secreted IL-10 during endometrial receptivity in adenomyosis [35].

IL-6 is a pro- and anti-inflammatory cytokine involved in acute phase response, B cell maturation, macrophages and type 1/2 Th cell differentiation [52]. In regulatory menstruating women, expression of endometrial IL-6 is assumed to be low in the P- and ES-phases; then, it gradually rises in the MS-phase and reaches peak expression in the LS-phase [53]. Higher levels of endometrial IL-6 during the WOI in controlled ovarian stimulation (COS) cycles were observed in women with adenomyosis [54]. In addition, upregulation of *IL6* was observed in primary culture of endometrial stromal cells (ESCs) from women with adenomyosis after co-culture with macrophages. The findings were further associated with a potential gain of proliferative ability of ectopic endometrial implants [55]. On the other hand, its downregulation in the LS-phase was associated with its role in endometriosis pathogenesis [56].

FOS and JUNB are subunits which dimerise to form a transcriptomic factor complex, activator protein 1 (AP-1). AP-1 regulates the expression of downstream genes with roles in cell cycle regulation, including proliferation, differentiation, apoptosis and response to stress [57]. Raimundo et al. associated downregulation of the FOS-JUNB pathway with altered differentiation of smooth muscle progenitor cells and development of myomas [58]. Baiyong et al. demonstrated that dysregulation of JUNB can turn on the differentiation of Th lymphocyte populations. Naive CD4 T lymphocytes were first isolated from JUNB-positive transgenic mice and further differentiated in the Th1 cell population. However, overexpression of JUNB prioritised the synthesis of cytokine IL-4, which is normally exclusive to Th2 population cells [59]. In women with endometriosis, upregulation of *JUNB* mRNA [18,60], *FOS* mRNA [18] and FOS protein [61] in secretory endometrium was reported. On the other hand, Morsch et al. observed no difference in phosphorylated levels of FOS when comparing women with and without endometriosis [62].

SOCS3 belongs to the family of intracellular proteins that supress cytokine signalling and have a role in negative regulation of inflammatory response [63]. Dong et al. suggested that reduced SOCS3 signalling may increase inflammatory response in placental trophoblasts leading to preeclampsia. Overexpression of SOCS3 in placental JEG-3 cell culture caused enhanced secretion of pregnancy-protective IL-10 [64]. Braunschweig et al. demonstrated that silencing of *SOCS3* enhanced cytotoxicity or killing activity in NK-92 cell culture, which resembles NK cells of decidua [65].

4.3. Endometrial Tissue Variability

The difference in expression patterns between study groups was not statistically significant in the present study which might be due to whole-tissue samples used for analysis. Suhorutshenko et al. [66] demonstrated that expression contribution of low abundant cell types in heterogeneous tissue could be masked by more abundant types. They compared paired endometrium biopsy samples collected in pre-receptive (early-secretory, ES) and receptive (mid-secretory, MS) states and estimated that ES samples consisted of 65% stromal and 35% epithelial cells, while the proportion of stromal and epithelial cells in MS samples was 46% and 54%, respectively. Further computational adjustment of RNA sequencing data according to obtained proportions of cell types (deconvolution) identified non statistically significant expression for approximately 74% of a total 3591 differentially expressed transcripts that were retrieved without deconvolution, indicating on stromal and epithelial cells unique gene expression profiles [66]. Additional molecular studies, such as in situ hybridisation (ISH) or immunohistochemistry (IHC) staining are needed to localise potential expression patterns of selected candidate genes or corresponding proteins specific to endometrial cell types or cellular compartments.

Only patients treated for infertility were considered eligible for the present study. To exclude for possible confounding factors and minimise the influence of other gynaecological pathologies affecting

the endometrium, only couples with male factor subfertility were included in the control group. Our study shows that endometrial expression pattern could be disrupted during WOI in women with adenomyosis and endometriosis compared to healthy controls. This could suggest lower endometrial receptivity and embryo implantation rates in this group of women; however, this would need to be proved in future clinical trials.

Finally, observed statistically non-significant gene expression differences between study groups could also be due to the personal biological variations since small sample size in both groups was used. Additional studies with larger sample size should address these limitations to determine whether selected candidate genes are differentially expressed in women with adenomyosis.

4.4. Limitations of the Study

Despite extensive literature search and bioinformatics analysis our study has some limitations. 1. Limited published data and heterogeneity of studies. Genes used in the present integrative study were retrieved from the literature survey, focusing on heterogeneous studies with poor overlap of identified molecular signatures characterising normal or affected endometrial receptivity. 2. Prioritisation of selected genes. In the present expression analysis six candidate genes were selected, however, *STAT3*, *VIM*, *VEGFA* and *HSP90B1* annotated in the interleukin-4 and interleukin-13 signalling or *LIFR, STAT3, IL15, ANXA2, BCL6, CXCL2, CCL3* and *CCL3L3* annotated in the signalling by interleukins were not used for validation. 3. The present study included small sample size in both adenomyosis and control groups. Retrieval of endometrial samples is long-lasting process since strict inclusion criteria.

4.5. Future Directions

This was a preliminary study intended to show altered endometrial expression patterns in women with adenomyosis. As the sampling and processing of endometrial biopsies were performed in the same manner and isolated RNA samples exhibited high RIN values, genome-wide profiling could provide novel loci specific for adenomyosis. Understanding the molecular background of endometrial receptivity could help determine the impact of diagnosed adenomyosis on the fertility capacity of affected women seeking ART treatment.

5. Conclusions

The diagnosis of adenomyosis has been negatively associated with implantation rate, but this is supported by little molecular evidence of affected endometrial receptivity. Based on data integration, extensive bioinformatics analysis and preliminary validation experiment, our study contributes toward understanding of molecular background characterising endometrial receptivity in adenomyosis. Genes and pathways identified in the present enrichment analysis are a source of stronger candidate genes for further validation analysis regarding endometrial receptivity. Identifying and understanding endometrial molecular organisation could contribute to the development of new concepts for personalised endometrial preparation before embryo transfer.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/10/9/1311/s1, Figure S1: Clustering of the PPIN which was constructed from adenomyosis, endometriosis and healthy gene lists using Cytoscape/STRING App, Table S1: Complete results of GSEA applied in clusters 1, 2 and 3 following PPIN clustering., Table S2: Complete results of GSEA applied in Reactome tool for overlapping pathways between adenomyosis and endometriosis gene lists.

Author Contributions: E.P. performed literature search, bioinformatics analysis, expression validation and wrote the manuscript. J.K. performed TVUS examination, endometrial biopsy sampling and supervised the final version of the manuscript. T.K. and B.K. supervised the study and the final version of the manuscript. U.P. and K.R. provided support in experimental validation. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study.

References

1. Altmäe, S.; Reimand, J.; Hovatta, O.; Zhang, P.; Kere, J.; Laisk-Podar, T.; Saare, M.; Peters, M.; Vilo, J.; Stavreus-Evers, A.; et al. Research Resource: Interactome of Human Embryo Implantation: Identification of Gene Expression Pathways, Regulation, and Integrated Regulatory Networks. *Mol. Endocrinol.* **2011**, *26*, 203–217. [CrossRef]
2. Lessey, B.A.; Young, S.L. What Exactly is Endometrial Receptivity? *Fertil. Steril.* **2019**, *111*, 611–617. [CrossRef]
3. Edgell, T.; Rombauts, L.J.; Salamonsen, L.A. Assessing Receptivity in the Endometrium: The Need for a Rapid, Non-Invasive Test. *Reprod. Biomed. Online* **2013**, *27*, 486–496. [CrossRef]
4. Bashiri, A.; Halper, K.I.; Orvieto, R. Recurrent Implantation Failure-Update Overview on Etiology, Diagnosis, Treatment and Future Directions. *Reprod. Boil. Endocrinol.* **2018**, *16*, 121. [CrossRef]
5. Díaz-Gimeno, P.; Horcajadas, J.A.; Martinez-Conejero, J.A.; Esteban, F.J.; Alamá, P.; Pellicer, A.; Simón, C. A Genomic Diagnostic Tool for Human Endometrial Receptivity Based on the Transcriptomic Signature. *Fertil. Steril.* **2011**, *95*, 50–60. [CrossRef] [PubMed]
6. Guerriero, S.; Dueholm, M.; Leone, F.P.G.; Valentin, L.; Rasmussen, C.; Votino, A.; Van Schoubroeck, D.; Landolfo, C.; Installé, A.J.; Guerriero, S.; et al. Terms, Definitions and Measurements to Describe Sonographic Features of Myometrium and Uterine Masses: A Consensus Opinion from the Morphological Uterus Sonographic Assessment (MUSA) Group. *Ultrasound Obstet. Gynecol.* **2015**, *46*, 284–298. [CrossRef]
7. Bazot, M.; Darai, E. Role of Transvaginal Sonography and Magnetic Resonance Imaging in the Diagnosis of Uterine Adenomyosis. *Fertil. Steril.* **2018**, *109*, 389–397. [CrossRef]
8. Puente, J.M.; Fabris, A.; Patel, J.; Patel, A.; Cerrillo, M.; Requena, A.; Garcia-Velasco, J.A. Adenomyosis in Infertile Women: Prevalence and the Role of 3D Ultrasound as a Marker of Severity of the Disease. *Reprod. Boil. Endocrinol.* **2016**, *14*, 60. [CrossRef] [PubMed]
9. Abu Hashim, H.; Elaraby, S.; Fouda, A.A.; Rakhawy, M.E. The Prevalence of Adenomyosis in an Infertile Population: A Cross-Sectional Study. *Reprod. Biomed. Online* **2020**, *40*, 842–850. [CrossRef]
10. Salim, R.; Riris, S.; Saab, W.; Abramov, B.; Khadum, I.; Serhal, P. Adenomyosis Reduces Pregnancy Rates in Infertile Women Undergoing IVF. *Reprod. Biomed. Online* **2012**, *25*, 273–277. [CrossRef] [PubMed]
11. Vercellini, P.; Consonni, D.; Dridi, D.; Bracco, B.; Frattaruolo, M.P.; Somigliana, E. Uterine Adenomyosis and in vitro Fertilization Outcome: A Systematic Review and Meta-Analysis. *Hum. Reprod.* **2014**, *29*, 964–977. [CrossRef] [PubMed]
12. Xiao, Y.; Sun, X.; Yang, X.; Zhang, J.; Xue, Q.; Cai, B.; Zhou, Y. Leukemia Inhibitory Factor is Dysregulated in the Endometrium and Uterine Flushing Fluid of Patients with Adenomyosis during Implantation Window. *Fertil. Steril.* **2010**, *94*, 85–89. [CrossRef]
13. Yen, C.-F.; Liao, S.-K.; Huang, S.J.; Tabak, S.; Arcuri, F.; Lee, C.-L.; Arici, A.; Petraglia, F.; Wang, H.-S.; Kayisli, U.A. Decreased Endometrial Expression of Leukemia Inhibitory Factor Receptor Disrupts the STAT3 Signaling in Adenomyosis During the Implantation Window. *Reprod. Sci.* **2016**, *24*, 1176–1186. [CrossRef]
14. Kunz, G.; Beil, D.; Huppert, P.; Noe, M.; Kissler, S.; Leyendecker, G. Adenomyosis in Endometriosis—Prevalence and Impact on Fertility. Evidence from Magnetic Resonance Imaging. *Hum. Reprod.* **2005**, *20*, 2309–2316. [CrossRef]
15. Meuleman, C.; Vandenabeele, B.; Fieuws, S.; Spiessens, C.; Timmerman, D.; D'Hooghe, T.M. High Prevalence of Endometriosis in Infertile Women with Normal Ovulation and Normospermic Partners. *Fertil. Steril.* **2009**, *92*, 68–74. [CrossRef]
16. Kao, L.C.; Germeyer, A.; Tulac, S.; Lobo, S.; Yang, J.P.; Taylor, R.N.; Osteen, K.; Lessey, B.A.; Giudice, L.C. Expression Profiling of Endometrium from Women with Endometriosis Reveals Candidate Genes for Disease-Based Implantation Failure and Infertility. *Endocrinology* **2003**, *144*, 2870–2881. [CrossRef]
17. Burney, R.O.; Talbi, S.; Hamilton, A.E.; Vo, K.C.; Nyegaard, M.; Nezhat, C.R.; Lessey, B.A.; Giudice, L.C. Gene Expression Analysis of Endometrium Reveals Progesterone Resistance and Candidate Susceptibility Genes in Women with Endometriosis. *Endocrinology* **2007**, *148*, 3814–3826. [CrossRef]

18. Tamaresis, J.S.; Irwin, J.C.; Goldfien, G.A.; Rabban, J.T.; Burney, R.O.; Nezhat, C.; DePaolo, L.V.; Giudice, L. Molecular Classification of Endometriosis and Disease Stage Using High-Dimensional Genomic Data. *Endocrinology* **2014**, *155*, 4986–4999. [CrossRef] [PubMed]
19. Zhou, M.; Fu, J.; Xiao, L.; Yang, S.; Song, Y.; Zhang, X.; Feng, X.; Sun, H.; Xu, W.; Huang, W. miR-196a Overexpression Activates the MEK/ERK Signal and Represses the Progesterone Receptor and Decidualization in Eutopic Endometrium from Women with Endometriosis. *Hum. Reprod.* **2016**, *31*, 2598–2608. [CrossRef]
20. Houshdaran, S.; Nezhat, C.R.; Vo, K.C.; Zelenko, Z.; Irwin, J.C.; Giudice, L.C. Aberrant Endometrial DNA Methylome and Associated Gene Expression in Women with Endometriosis. *Boil. Reprod.* **2016**, *95*, 93. [CrossRef]
21. Barabasi, A.-L.; Gulbahce, N.; Loscalzo, J. Network Medicine: A Network-Based Approach to Human Disease. *Nat. Rev. Genet.* **2010**, *12*, 56–68. [CrossRef] [PubMed]
22. García-Campos, M.A.; Espinal-Enríquez, J.; Hernández-Lemus, E. Pathway Analysis: State of the Art. *Front. Physiol.* **2015**, *6*, 383. [CrossRef]
23. Prašnikar, E.; Knez, J.; Kovačič, B.; Kunej, T. Molecular signature of eutopic endometrium in endometriosis based on the multi-omics integrative synthesis. *J. Assist. Reprod. Genet.* **2020**, *37*, 1–19. [CrossRef]
24. Altmäe, S.; Koel, M.; Võsa, U.; Adler, P.; Suhorutšenko, M.; Laisk-Podar, T.; Kukushkina, V.; Saare, M.; Velthut-Meikas, A.; Krjutškov, K.; et al. Meta-Signature of Human Endometrial Receptivity: A Meta-Analysis and Validation Study of Transcriptomic Biomarkers. *Sci. Rep.* **2017**, *7*, 10077. [CrossRef]
25. Braschi, B.; Denny, P.; Gray, K.; Jones, T.E.; Seal, R.; Tweedie, S.; Yates, B.; Bruford, E.A. Genenames.org: The HGNC and VGNC Resources in 2019. *Nucleic Acids Res.* **2018**, *47*, D786–D792. [CrossRef] [PubMed]
26. Szklarczyk, D.; Gable, A.L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N.T.; Morris, J.H.; Bork, P.; et al. STRING v11: Protein–Protein Association Networks with Increased Coverage, Supporting Functional Discovery in Genome-Wide Experimental Datasets. *Nucleic Acids Res.* **2018**, *47*, D607–D613. [CrossRef]
27. Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* **2003**, *13*, 2498–2504. [CrossRef] [PubMed]
28. Wu, G.; Feng, X.; Stein, L. A Human Functional Protein Interaction Network and its Application to Cancer Data Analysis. *Genome Boil.* **2010**, *11*, R53. [CrossRef] [PubMed]
29. Fabregat, A.; Sidiropoulos, K.; Garapati, P.V.; Gillespie, M.E.; Hausmann, K.; Haw, R.A.; Jassal, B.; Jupe, S.; Korninger, F.; McKay, S.; et al. The Reactome pathway Knowledgebase. *Nucleic Acids Res.* **2015**, *44*, D481–D487. [CrossRef] [PubMed]
30. Naftalin, J.; Hoo, W.; Pateman, K.; Mavrelos, D.; Jurkovic, D.; Holland, T.K. How Common is Adenomyosis? A Prospective Study of Prevalence Using Transvaginal Ultrasound in a Gynaecology Clinic. *Hum. Reprod.* **2012**, *27*, 3432–3439. [CrossRef]
31. WHO. *Laboratory Manual for the Examination and Processing of Human Semen*, 5th ed.; Cooper, T.G., Ed.; WHO Press: Geneva, Switzerland, 2010.
32. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
33. Fischer, C.P.; Kayisili, U.; Taylor, H.S. HOXA10 Expression is Decreased in Endometrium of Women with Adenomyosis. *Fertil. Steril.* **2011**, *95*, 1133–1136. [CrossRef]
34. Xiao, Y.; Li, T.; Xia, E.; Yang, X.; Sun, X.; Zhou, Y. Expression of Integrin β3 and Osteopontin in the Eutopic Endometrium of Adenomyosis during the Implantation Window. *Eur. J. Obstet. Gynecol. Reprod. Boil.* **2013**, *170*, 419–422. [CrossRef] [PubMed]
35. Wang, J.; Huang, C.; Jiang, R.; Du, Y.; Zhou, J.; Jiang, Y.; Yan, Q.; Xing, J.; Hou, X.; Zhou, J.; et al. Decreased Endometrial IL-10 Impairs Endometrial Receptivity by Downregulating HOXA10 Expression in Women with Adenomyosis. *BioMed Res. Int.* **2018**, *2018*, 1–9. [CrossRef] [PubMed]
36. Yan, Q.; Yan, G.; Zhang, C.; Wang, Z.; Huang, C.; Wang, J.; Zhou, J.; Liu, Y.; Ding, L.; Zhang, Q.; et al. miR-21 Reverses Impaired Decidualization Through Modulation of KLF12 and NR4A1 Expression in Human Endometrial Stromal Cells. *Boil. Reprod.* **2019**, *100*, 1395–1405. [CrossRef]
37. Martinez-Conejero, J.A.; Morgan, M.; Montesinos, M.; Fortuño, S.; Meseguer, M.; Simón, C.; Horcajadas, J.A.; Pellicer, A. Adenomyosis does not Affect Implantation, but is Associated with Miscarriage in Patients Undergoing Oocyte Donation. *Fertil. Steril.* **2011**, *96*, 943–950. [CrossRef]

38. Stephens, A.N.; Hannan, N.J.; Rainczuk, A.; Meehan, K.L.; Chen, J.; Nicholls, P.K.; Rombauts, L.J.F.; Stanton, P.G.; Robertson, D.M.; Salamonsen, L.A. Post-Translational Modifications and Protein-Specific Isoforms in Endometriosis Revealed by 2D DIGE. *J. Proteome Res.* **2010**, *9*, 2438–2449. [CrossRef]
39. Okada, H.; Tsuzuki, T.; Murata, H. Decidualization of the Human Endometrium. *Reprod. Med. Boil.* **2018**, *17*, 220–227. [CrossRef]
40. Campo, S.; Campo, V.; Benagiano, G. Adenomyosis and Infertility. *Reprod. Biomed. Online* **2012**, *24*, 35–46. [CrossRef]
41. Chaouat, G.; Dubanchet, S.; Ledee, N. Cytokines: Important for implantation? *J. Assist. Reprod. Genet.* **2007**, *24*, 491–505. [CrossRef]
42. Flynn, L.; Byrne, B.; Carton, J.; O'Farrelly, C.; Kelehan, P.; O'Herlihy, C. Menstrual Cycle Dependent Fluctuations in NK and T-Lymphocyte Subsets from Non-Pregnant Human Endometrium. *Am. J. Reprod. Immunol.* **2000**, *43*, 209–217. [CrossRef] [PubMed]
43. Van Nieuwenhoven, A.V.; Heineman, M.; Faas, M. The Immunology of Successful Pregnancy. *Hum. Reprod. Updat.* **2003**, *9*, 347–357. [CrossRef] [PubMed]
44. Enciso, M.; Carrascosa, J.P.; Sarasa, J.; A Martínez-Ortiz, P.; Munné, S.; A Horcajadas, J.; Aizpurua, J. Development of a New Comprehensive and Reliable Endometrial Receptivity Map (ER Map/ER Grade) Based on RT-qPCR Gene Expression Analysis. *Hum. Reprod.* **2018**, *33*, 220–228. [CrossRef] [PubMed]
45. Vogel, C.; Marcotte, E.M. Insights into Regulation of Protein Abundance from Proteomics and Transcriptomis Analyses. *Nat. Rev. Genet.* **2013**, *13*, 227–232. [CrossRef] [PubMed]
46. Shuya, L.L.; Menkhorst, E.; Yap, J.; Li, P.; Lane, N.; Dimitriadis, E. Leukemia Inhibitory Factor Enhances Endometrial Stromal Cell Decidualization in Humans and Mice. *PLoS ONE* **2011**, *6*, e25288. [CrossRef] [PubMed]
47. Chung, T.-W.; Park, M.-J.; Kim, H.S.; Choi, H.-J.; Ha, K.-T. Integrin αVβ3 and αVβ5 are Required for Leukemia Inhibitory Factor-Mediated the Adhesion of Trophoblast Cells to the Endometrial Cells. *Biochem. Biophys. Res. Commun.* **2016**, *469*, 936–940. [CrossRef]
48. Serafini, P.; Silva, I.D.; Smith, G.D.; Motta, E.L.; Rocha, A.; Baracat, E.C. Endometrial Claudin-4 and Leukemia Inhibitory Factor are Associated with Assisted Reproduction Outcome. *Reprod. Boil. Endocrinol.* **2009**, *7*, 30. [CrossRef]
49. Franasiak, J.M.; Holoch, K.J.; Yuan, L.; Schammel, D.P.; Young, S.L.; Lessey, B.A. Prospective Assessment of Midsecretory Endometrial Leukemia Inhibitor Factor Expression Versus αvβ3 Testing in Women with Unexplained Infertility. *Fertil. Steril.* **2014**, *101*, 1724–1731. [CrossRef]
50. Kühn, R.; Löhler, J.; Rennick, D.; Rajewsky, K.; Muller, W. Interleukin-10-Deficient Mice Develop Chronic Enterocolitis. *Cell* **1993**, *75*, 263–274. [CrossRef]
51. Thaxton, J.E.; Sharma, S. Interleukin-10: A Multi-Faceted Agent of Pregnancy. *Am. J. Reprod. Immunol.* **2010**, *63*, 482–491. [CrossRef]
52. Diehl, S.A.; Rincoón, M. The Two Faces of IL-6 on Th1/Th2 Differentiation. *Mol. Immunol.* **2002**, *39*, 531–536. [CrossRef]
53. Von Wolff, M.; Thaler, C.; Strowitzki, T.; Broome, J.; Stolz, W.; Tabibzadeh, S. Regulated Expression of Cytokines in Human Endometrium Throughout the Menstrual Cycle: Dysregulation in Habitual Abortion. *Mol. Hum. Reprod.* **2000**, *6*, 627–634. [CrossRef] [PubMed]
54. Zhihong, N.; Yun, F.; Pinggui, Z.; Sulian, Z.; Zhang, A. Cytokine Profiling in the Eutopic Endometrium of Adenomyosis During the Implantation Window After Ovarian Stimulation. *Reprod. Sci.* **2015**, *23*, 124–133. [CrossRef] [PubMed]
55. Yang, J.-H.; Wu, M.-Y.; Chang, D.-Y.; Chang, C.-H.; Yang, Y.-S.; Ho, H.-N. Increased Interleukin-6 Messenger RNA Expression in Macrophage-Cocultured Endometrial Stromal Cells in Adenomyosis. *Am. J. Reprod. Immunol.* **2006**, *55*, 181–187. [CrossRef]
56. Ponce, C.; Torres, M.; Galleguillos, C.; Sovino, H.; Boric, M.A.; Fuentes, A.; Johnson, M.C. Nuclear Factor κB Pathway and Interleukin-6 are Affected in Eutopic Endometrium of Women with Endometriosis. *Reproduction* **2009**, *137*, 727–737. [CrossRef]
57. Jochum, W.; Passegué, E.; Wagner, E.F. AP-1 in Mouse Development and Tumorigenesis. *Oncogene* **2001**, *20*, 2401–2412. [CrossRef]
58. Raimundo, N.; Vanharanta, S.; Aaltonen, L.A.; Hovatta, I.; Suomalainen, A. Downregulation of SRF–FOS–JUNB Pathway in Fumarate Hydratase Deficiency and in Uterine Leiomyomas. *Oncogene* **2009**, *28*, 1261–1273. [CrossRef]
59. Li, B.; Tournier, C.; Davis, R.J.; Flavell, R.A. Regulation of IL-4 Expression by the Transcription Factor JunB during T Helper Cell Differentiation. *EMBO J.* **1999**, *18*, 420–432. [CrossRef]

60. Absenger, Y.; Hess-Stumpp, H.; Kreft, B.; Krätzschmar, J.; Haendler, B.; Schütze, N.; Regidor, P.; Winterhager, E. Cyr61, a Deregulated Gene in Endometriosis. *Mol. Hum. Reprod.* **2004**, *10*, 399–407. [CrossRef]
61. Pan, H.; Sheng, J.-Z.; Tang, L.; Zhu, R.; Zhou, T.-H.; Huang, H. Increased Expression of C-Fos Protein Associated with Increased Matrix Metalloproteinase-9 Protein Expression in the Endometrium of Endometriotic Patients. *Fertil. Steril.* **2008**, *90*, 1000–1007. [CrossRef]
62. Morsch, D.M.; Carneiro, M.M.; Lecke, S.B.; Araújo, F.C.; Camargos, A.F.; Reis, F.M.; Spritzer, P.M. C-Fos Gene and Protein Expression in Pelvic Endometriosis: A Local Marker of Estrogen Action. *J. Mol. Histol.* **2009**, *40*, 53–58. [CrossRef] [PubMed]
63. Lang, R.; Pauleau, A.-L.; Parganas, E.; Takahashi, Y.; Mages, J.; Ihle, J.N.; Rutschman, R.; Murray, P.J. SOCS3 Regulates the Plasticity of gp130 Signaling. *Nat. Immunol.* **2003**, *4*, 546–550. [CrossRef] [PubMed]
64. Dong, Q.; Fan, R.; Zhao, S.; Wang, Y. Over-expression of SOCS-3 Gene Promotes IL-10 Production by JEG-3 Trophoblast Cells. *Placenta* **2009**, *30*, 11–14. [CrossRef]
65. Braunschweig, A.; Poehlmann, T.G.; Busch, S.; Schleussner, E.; Markert, U.R. Signal Transducer and Activator of Transcription 3 (STAT3) and Suppressor of Cytokine Signaling (SOCS3) Balance Controls Cytotoxicity and IL-10 Expression in Decidual-Like Natural Killer Cell Line NK-92. *Am. J. Reprod. Immunol.* **2011**, *66*, 329–335. [CrossRef] [PubMed]
66. Suhorutshenko, M.; Kukushkina, V.; Velthut-Meikas, A.; Altmäe, S.; Peters, M.; Mägi, R.; Krjutškov, K.; Koel, M.; Codoñer, F.M.; Martinez-Blanch, J.F.; et al. Endometrial Receptivity Revisited: Endometrial Transcriptome Adjusted for Tissue Cellular Heterogeneity. *Hum. Reprod.* **2018**, *33*, 2074–2086. [CrossRef]

2.1.3 Transkriptomika receptivnega endometrija žensk s sonografskimi značilnostmi adenomioze

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Ozadje: Ženske z adenomiozo maternice se povezuje z ovirano endometrijsko receptivnostjo za vgnezdenje zarodka v postopkih OBMP. Da bi identificirali molekularne vzroke, vključene v ta proces, smo primerjali transkriptom endometrija tekom okna vgnezdenja med ženskami z in brez adenomioze. **Metode:** Ženskam z ultrazvočnimi znaki adenomioze (n = 10) in kontrolne skupine (n = 10) smo vzorčili biopsije endometrija, ki so bile na podlagi vrha LH časovno usklajene z oknom vgnezdenja. Vsak vzorec izolirane RNA endometrija smo uporabili za določevanje transkriptoma z metodo RNA-seq (NovaSeq 6000, Illumina) in za klasifikacijo receptivne faze z molekularnim orodjem za datiranje faze menstruacijskega ciklusa (beREADY®, CCHT). Analizo podatkov RNA-seq smo izvedli z uporabo paketov programa Bioconductor v programskem jeziku R, pri čemer smo upoštevali rezultate natančnega datiranja vzorcev endometrija. Za identifikacijo močnejših kandidatnih bioloških poti spremenjene endometrijske receptivnosti pri adenomiozi smo spremenjeno izražene gene naše adenomiozne skupine integrirali s 151, 173 in 42 geni, ki smo jih pridobili iz pregleda literature in se povezujejo z endometrijsko receptivnostjo pri zdravi maternici, endometriozi oz. adenomiozi. Obogatitvene analize smo opravili z uporabo aplikacij ClueGO in CluePedia v programu Cytoscape. **Rezultati:** Od skupno 20 vzorcev endometrija sta bila 2 datirana v zgodnje receptivno, 13 v receptivno in 5 v pozno receptivno fazo. S primerjavo transkriptomskih podatkov vseh 20 vzorcev smo zaznali 909 spremenjeno izraženih genov ($p < 0,05$; neznačilno po popravku vrednosti *p*) in le 4 obogatene biološke poti (Bonferroni vrednost $p < 0,05$). S ponovno analizo transkriptomskih podatkov samo 13 vzorcev, ki so bili datirani v receptivno fazo, smo zaznali 382 spremenjeno izraženih genov ($p < 0,05$; neznačilno po popravku vrednosti *p*) in 33 obogatenih poti (Bonferroni vrednost $p < 0,05$). Te poti, ki so bile že predhodno povezane z biologijo endometrija, so bile npr. *izražanje genov, induciranih z IFN* in *odziv na IFN-alfa*. Z integracijo podatkov smo identificirali poti, ki kažejo na edinstven vpliv adenomioze na molekularno organizacijo endometrija (npr. *izražanje genov, induciranih z IFN*), kot tudi motnje procesov vzpostavitve endometrijske receptivnosti (npr. *organizacija zunajceličnega matriksa* in *tvorba dejavnika tumorske nekroze*). **Zaključki:** Natančno datiranje vzorcev endometrija in analiza RNA-seq sta omogočila identifikacijo spremenjenega odziva na signalizacijo z IFN kot najbolj obetaven kandidatni mehanizem ovirane endometrijske receptivnosti pri adenomiozi.

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Transcriptomics of receptive endometrium in women with sonographic features of adenomyosis

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Abstract

Background: Women with uterine adenomyosis seeking assisted reproduction have been associated with compromised endometrial receptivity to embryo implantation. To understand the mechanisms involved in this process, we aimed to compare endometrial transcriptome profiles during the window of implantation (WOI) between women with and without adenomyosis.

Methods: We obtained endometrial biopsies LH-timed to the WOI from women with sonographic features of adenomyosis (n=10) and controls (n=10). Isolated RNA samples were subjected to RNA sequencing (RNA-seq) by the Illumina NovaSeq 6000 platform and endometrial receptivity classification with a molecular tool for menstrual cycle phase dating (beREADY®, CCHT). The program language R and Bioconductor packages were applied to analyse RNA-seq data in the setting of the result of accurate endometrial dating. To suggest robust candidate pathways, the identified differentially expressed genes (DEGs) associated with the adenomyosis group in the receptive phase were further integrated with 151, 173 and 42 extracted genes from published studies that were related to endometrial receptivity in healthy uterus, endometriosis and adenomyosis, respectively. Enrichment analyses were performed using Cytoscape ClueGO and CluePedia apps.

Results: Out of 20 endometrial samples, 2 were dated to the early receptive phase, 13 to the receptive phase and 5 to the late receptive phase. Comparison of the transcriptomics data from all 20 samples provided 909 DEGs ($p<0.05$; nonsignificant after adjusted p value) in the adenomyosis group but only 4 enriched pathways (Bonferroni p value < 0.05). The analysis of 13 samples only dated to the receptive phase provided suggestive 382 DEGs ($p<0.05$; non-significant after adjusted p value) in the adenomyosis group, leading to 33 enriched pathways (Bonferroni p value < 0.05). These included pathways were already associated with endometrial biology, such as "Expression of interferon (IFN)-induced genes" and "Response to IFN-alpha". Data integration revealed pathways indicating a unique effect of adenomyosis on endometrial molecular organization (e.g., "Expression of IFN-induced genes") and its interference with endometrial receptivity establishment (e.g., "Extracellular matrix organization" and "Tumour necrosis factor production").

Conclusions: Accurate endometrial dating and RNA-seq analysis resulted in the identification of altered response to IFN signalling as the most promising candidate of impaired uterine receptivity in adenomyosis.

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Keywords: Adenomyosis, Assisted reproductive techniques (ART), Data integration, Endometrial receptivity, Enrichment pathway analysis, Omics approaches, RNA-seq, Systems biology, Transcriptomics, Window of implantation

Executive summary of the study

1. Adenomyosis has been associated with lower pregnancy rate in infertility treatments.
2. Molecular knowledge of endometrial receptivity in women with adenomyosis is scarce and limited to studies with selected candidate genes and one genome-wide study performed using microarrays.
3. Therefore, we performed the first transcriptome sequencing of endometrial samples LH-timed to the expected WOI (LH+7 – LH+9) between women with (n = 10) and without (n = 10) sonographic features of adenomyosis.
4. Transcriptomics data comparison of 10 adenomyosis cases and 10 normal controls provided 909 DEGs ($p<0.05$; nonsignificant after adjusted p value), but functional enrichment analysis identified 4 pathways (Bonferroni p value < 0.05) that were not directly associated with endometrial biology.
5. Retrieved endometrial biopsies were applied for the external molecular tool beREADY® (CCHT, Estonia) to verify their receptivity status on the basis of the gene expression signature associated with endometrial receptivity. Out of 20 samples, 2 were classified as early receptive, 13 as receptive and 5 as late receptive.
6. Two early- and 5 late-receptive samples were excluded from the RNA-seq dataset to prevent the impact of early- and late-secretory phases of the menstrual cycle on transcriptomics analysis associated with endometrial receptivity. The RNA-seq dataset of the remaining 8 adenomyosis cases and 5 control receptive endometrial samples was reanalysed, and 382 DEGs ($p < 0.05$; nonsignificant after adjusted p value) were identified, resulting in 33 enriched pathways (Bonferroni p value < 0.05) that have already been associated with endometrial biology.
7. The 382 identified DEGs were further integrated with the most extensive set of genes from the literature associated with endometrial receptivity in the healthy uterus, endometriosis (model disease to study persistence of gynaecological pathology on endometrial molecular organization) and adenomyosis to provide candidate pathways characterizing the role of adenomyosis on endometrial molecular organization.
8. Integrative enrichment analysis provided candidate pathways that may indicate a unique effect of adenomyosis on endometrial molecular organization (e.g., "Expression of IFN-induced genes") and its interference with endometrial receptivity establishment (e.g., "Extracellular matrix organization", "Tumour necrosis factor production" and "Regulation of reproductive process").
9. Identification of robust endometrial pathways and associated genes could lead to the development of molecular tools for endometrial receptivity examination that would be specific for women with adenomyosis.
10. Accurate endometrial receptivity examination in infertile adenomyosis patients could better verify whether endometrial-associated factors are a source of recurrent implantation failures.

Background

Adenomyosis is a common acquired uterine anomaly characterized by the presence of endometrial glands and stroma within the myometrium. Advances in imaging techniques in the last decade have enabled the diagnosis of adenomyosis [1] in a large proportion of women undergoing infertility diagnostics [2, 3]. Since subtle sonographic signs of adenomyosis are becoming easier to recognize, adenomyosis is diagnosed with increasing frequency. Previous retrospective studies have shown the association between adenomyosis and lower embryo implantation rates and higher miscarriage rates [4–6].

Several functional and molecular aberrations could be responsible for altered endometrial receptivity to embryo implantation and lower fecundity in women with adenomyosis. It has been suggested that the disruption of the junctional zone architecture by adenomyosis could lead to altered contractility and interrupt endometrial receptivity [7, 8]. Other suggested causes affecting endometrial receptivity in women with adenomyosis could be increased levels of oxidative stress [9–11], abnormal endometrial vascularity [12, 13] and functional disorganization at the molecular level [14–17].

In our previous study [18], we gathered proteins, genes and functional noncoding RNAs (ncRNAs) shown to be dysregulated in the endometrium of women with adenomyosis during the expected window of implantation (WOI). Bioinformatics approaches were used to integrate

retrieved loci with endometrial receptivity genes from the literature associated with healthy (normal) uteri to identify candidate dysregulated mechanisms involved in the regulation of embryo implantation in adenomyosis. In addition, we included better characterized endometriosis as a model disorder to study the impact of gynaecological pathology on endometrial molecular organization [18]. Numerous published genome-wide studies associated with the endometrial molecular background in women with endometriosis enabled us to develop a catalogue of genes sorted according to the phases of the menstrual cycle [19]. Genes sorted in the mid-secretory phase corresponding to the appearance of the WOI were used for the integrative analysis mentioned above [18]. The identified enriched "Signalling by interleukins" and "Interleukin-4 and interleukin-13 signalling" pathways were prioritized, and the corresponding mapped *LIF*, *SOCS3*, *IL10*, *IL6*, *JUNB* and *FOS* genes were validated. Since downregulated expression levels of selected genes in adenomyosis compared to the control group showed no statistical significance, we assumed that comprehensive endometrial transcriptomics profiling would be an appropriate next step to identify adenomyosis-specific loci [18].

To date, there is only one transcriptomics study [20] profiling the endometrium in the expected WOI using microarrays, which identified 34 differentially expressed genes (DEGs) in women with adenomyosis wishing to conceive compared to healthy women [20]. The methodological improvement of transcriptome profiling from hybridization-based microarrays to next-generation sequencing (NGS) platforms provides more comprehensive insight into expression signatures and enables identification of minor differences between study groups [21]. Millions of reads generated by RNA sequencing (RNA-seq) can be aligned to a reference genome, reference transcripts or references assembled de novo for the entire transcriptome to be surveyed. Thus, additional biological constituents can be identified, and a more precise assessment of transcript expression levels can be obtained [22].

The first aim of this study was to perform RNA-seq of endometrial samples dated to the WOI between women with and without sonographic features of adenomyosis to identify DEGs. The second aim was to perform enrichment analysis of identified DEGs alone and together with endometrial receptivity genes from the literature to provide robust candidate pathways related to altered molecular background of endometrial receptivity in adenomyosis.

Methods

Study cohorts

We designed a prospective observational study including women scheduled for medically assisted reproduction at the Department of Reproductive Medicine and Gynaecological Endocrinology, University Medical Centre Maribor, Slovenia between 2018 and 2020.

The inclusion criteria were as follows: age $\leq$ 42 years, regular menstrual cycle 24 – 36 days in length, no current hormonal treatment, controlled ovarian stimulation (COS), ovulation triggering or vaginal progesterone for luteal support at least two months prior to endometrial biopsy. The exclusion criteria were anovulatory menstrual cycles, polycystic ovary syndrome (PCOS), previous surgical treatment of endometriosis or uterine surgical procedures, sonographic evidence of fibroids, endometrial polyps, hydrosalpinges, and evidence of ovarian or deep infiltrating endometriosis (unless otherwise noted in Table 1). In our clinic, all women undergoing assisted reproductive techniques (ART) have a prior transvaginal ultrasound (TVUS) examination, typically performed in the proliferative phase of the menstrual cycle. Women with echographic evidence of adenomyosis were considered eligible for the study, and the control group was composed of women with normal uteri seeking ART due to male or tubal factors of infertility.

On the day of endometrial sampling, all women underwent TVUS performed by a single expert sonographer (level 3 according to European Federation of Societies for Ultrasound in Medicine and Biology). In all women, comprehensive 2-D and 3-D ultrasound using high-range equipment was performed with a 10 MHz transvaginal transducer (Voluson E8 Expert, GE Health care, Austria GmbH & Co OG, Zipf, Austria). Diagnostic criteria for adenomyosis were based on previously published criteria [23]. The diagnosis of adenomyosis was confirmed when one of the following sonographic criteria was met: asymmetrical myometrial thickening not caused by the presence of fibroids, linear endometrial striations, irregular endometrial-myometrial junction, parallel shadowing, or the presence of myometrial cysts or hyperechoic islands [23]. Adenomyosis was classified as mild by subjective assessment, but in general, it was assessed in line with previously described principles. This was when only focal areas of adenomyosis were seen or when adenomyosis was present only in the inner third of the myometrium [24].

Demographic and clinical characteristics of participants, including age, body mass index (BMI), endometrial thickness at the time of endometrial biopsy and the number of previous ART cycles, are presented as the median (range) and were compared between study groups using the nonparametric Mann–Whitney U-test in SPSS 25.0 software (IBM Corporation, Armonk, NY, USA). Statistical significance was set at p value < 0.05.

Endometrial sample collection

Endometrial biopsy sampling was conducted in a natural menstrual cycle, and women were scheduled for

cycle monitoring by urinary luteinizing hormone (LH) tests (Hangzhou AllTest Biotech Co., Ltd, Hangzhou, P.R. China). Women were scheduled for endometrial sampling conducted by the Pipelle endometrial suction curette (the Probet, Gynetics Medical Products N.V., Lommel, Belgium) in the expected WOI on the day between LH+7 to LH+9 after a participant's LH surge determination (day LH+0). Retrieved endometrial samples were immediately placed in RNAlater solution (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), stored overnight at +4 °C and then transferred to −80 °C until RNA isolation was performed.

Total RNA isolation and quality control

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Each whole-tissue endometrial sample was first disrupted with a Bullet Blender Storm Pro homogenizer (Next Advance, Inc., Troy, NY, USA) using 1 mm zirconium oxide beads in 700 µL of QIAzol Lysis Reagent from the miRNeasy Mini Kit. After 5 min of incubation at room temperature, 140 µL of chloroform was added to the homogenate, and the solution was shaken vigorously. The sample was then centrifuged at 12 000 rfc for 15 min at 4 °C. The upper aqueous phase (approximately 300 µL) was transferred to a new Eppendorf tube, and 1.5 volumes of ethanol were added. The samples were then pipetted to RNA binding miRNeasy Mini spin columns and washed using RWT Buffer and RPE Buffer solutions of the miRNeasy Mini Kit. Total RNA was eluted in 50 µL of RNase-free H_2O.

The quantity and purity of each RNA sample were assessed with Synergy 2 spectrophotometric measurements (BioTek Instruments, Winooski, VT, USA). RNA integrity number (RIN) was estimated on the 2100 Bioanalyser system (Agilent Technologies, Waldbronn, Germany) using the RNA Nano 6000 Assay Kit (Agilent Technologies, Waldbronn, Germany). After passing those quality controls, each RNA sample was used for cDNA library construction and subsequent RNA-seq and for accurate endometrial dating of retrieved biopsies.

Accurate endometrial dating

One part of each RNA sample was shipped on dry ice to the Competence Centre on Health Technologies, CCHT, Tartu, Estonia, where endometrial receptivity testing was performed using the beREADY® test [25] (https://beready.ccht.ee/). Endometrial dating was performed according to the established protocol using targeted allele counting by sequencing (TAC-seq) methodology [26] to explore the expression levels of 57 well-described endometrial receptivity genes [27]. The results of the beREADY® test were provided in five phases: "pre-receptive", "early-receptive", "receptive", "late-receptive", and "post-receptive". The purpose of endometrial dating was to accurately classify the receptivity status of LH-timed biopsies to remove samples that could lead to possible biases in gene expression analysis associated with endometrial receptivity in adenomyosis.

Library preparation and RNA-seq

Both lncRNA and mRNA 150 bp paired-end libraries were constructed and subsequently sequenced by Novogene Bioinformatics Technology Co., Ltd. (Hong Kong, China). Briefly, a total amount of 2 µg of RNA per sample was used for cDNA sequencing library preparation. Ribosomal RNA (rRNA) was removed using the Epicentre Ribo-zeroTM rRNA Removal Kit (Epicentre, Brooklyn, NY, USA), and the remaining RNA was used for library generation by the NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA). First, rRNA-depleted RNA samples were fragmented followed by first- and second-strand cDNA synthesis. The sequencing adaptors were ligated, and library fragments were purified to obtain cDNA fragments 150~200 bp in length. Polymerase chain reaction (PCR) amplification of size-selected, adaptor-ligated cDNA was performed using universal PCR primers and index primers. Index-coded samples were clustered by Illumina TruSeq PE Cluster Kit v3-cBot-Hs. Libraries were sequenced on an Illumina NovaSeq 6000 platform, which generated 150 bp paired-end reads.

RNA-seq data alignment and identification of DEGs

Raw sequence reads were trimmed by Novogene in-house Perlscript to remove raw reads with adapter contamination and reads containing poly-N and low-quality reads. The RNA-seq data presented in this study are deposited in the Gene Expression Omnibus (GEO) database with accession number GSE185392. Provided raw fastq files were first evaluated with FastQC v.0.11.9 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to obtain a quality profile of the reads.

The statistical environment R v.4.0.2 (R Core Team 2020, Vienna, Austria) and contributed packages from the R software repository Bioconductor (http://www.bioconductor.org/) were used for high-throughput sequence data analysis. Raw paired-end reads were aligned to the UCSC Homo sapiens hg19 reference genome using the Rsubread v.2.2.4 R package [28, 29]. Properly mapped reads were sorted in files with binary alignment/map (BAM) format. Mapped reads were counted and assigned to genomic features using featureCounts [30] with the requirement that both ends should be mapped. Counts per million (CPMs) were calculated using the edgeR v.3.30.3 R package [31]. Genes expressed at low levels were filtered out based on

CPMs corresponding to read counts of 10, and retained genes were normalized using the trimmed mean of M values method (TMM) [32]. Subsequently, mean-variance modelling at the observational level transformation (VOOM) was applied [33]. Differential expression analysis of the adenomyosis group relative to the control group was determined in two RNA-seq datasets using linear models and empirical Bayes implemented in the limma v.3.44.3 R package [34]. RNA-seq datasets were composed of libraries on the basis of the results of endometrial dating of corresponding samples. The first dataset contained all LH-timed samples, while the second dataset contained only samples dated to the receptive phase. Differential expression was considered for genes with a p value < 0.05 regardless of the adjusted p value obtained after multiple testing corrections.

Integration of identified DEGs in the adenomyosis group with endometrial receptivity genes from the literature

Identified DEGs between adenomyosis cases and controls using samples dated to the receptive phase were applied for integrative bioinformatics analysis to provide robust candidate pathways associated with altered molecular background of endometrial receptivity in adenomyosis. DEGs were applied for enrichment reanalysis with lists of 42, 173 and 151 genes associated with endometrial receptivity in adenomyosis, endometriosis and healthy uterus, respectively, that were retrieved from the literature in our previous study [18]. Genes associated with endometriosis presented a model to study the impact of gynaecological pathology on endometrial molecular organization. Genes associated with a healthy uterus were used as a reference molecular background required for endometrial receptivity establishment. Two enrichment analyses were performed using two different gene lists associated with adenomyosis. The first adenomyosis gene list contained only 382 DEGs of the present sequencing experiment, while the second list combined 382 DEGs with 42 genes from the literature (in total, 424 genes). The first enrichment analysis was performed by integrating the adenomyosis gene list with 382 DEGs, the endometriosis list with 173 genes and the healthy uterus list with 151 genes. Second, enrichment analysis was performed using adenomyosis, healthy uterus and endometriosis lists with 424, 151 and 173 genes, respectively. The gene lists used are provided in Additional file 1.

Functional enrichment analyses

DEGs ($p<0.05$) that were identified by transcriptomics data comparison of endometrial samples between adenomyosis cases and controls were subjected to functional enrichment analyses using ClueGO v.2.5.8 [35] and CluePedia 1.5.8 [36] apps of Cytoscape v.3.8.2 software [37]. The same bioinformatics tools were used for enrichment analyses employing integrated gene lists associated with adenomyosis, endometriosis and healthy uterus.

When analysing identified sets of DEGs associated with the present adenomyosis groups, up- and down-regulated genes were separately uploaded as two clusters in the ClueGO app, which gave a unique colour marker to each gene set. When performing enrichment analyses of integrated gene lists associated with different gynaecological conditions, each gene list was uploaded as a cluster in the ClueGO app to distinguish study groups according to colour markers of the cluster.

Each enrichment analysis was applied by representative Gene Ontology Biological Process (GO_BP), Reactome Pathways and Reactome Reactions ontologies. Only enriched pathways (Reactome pathways/reactions and GO_BP terms) with corrected p values < 0.05 according to the Bonferroni step down test were considered. The identified pathways were sorted into groups based on their common biological role and associated genes (kappa score) and further projected into functionally organized networks. The size of nodes in the generated networks was correlated with the obtained p value. The pathway with the highest significant value was considered to be the leading term of a group and was therefore highlighted in the network by a large name label and a statistical summary. The CluePedia app was further applied to visualize shared initial genes within or between functional network groups. The proportion of visible genes mapped to each pathway was also determined. When more than 60% of mapped genes originated from one of the clusters, a pathway was shown in the network with the predefined colour of this cluster.

Results

An overview of the study is outlined in Fig. 1.

Participant characteristics

The demographic and clinical characteristics of the study cohorts are summarized in Table 1.

Total RNA quality

Total RNA was isolated from 20 endometrial samples, 10 from the adenomyosis group and 10 from the control group. The A260/A280 ratios and RIN values of all RNA samples were above 2.0 and >8.5, respectively, and were further used for endometrial dating and RNA-seq.

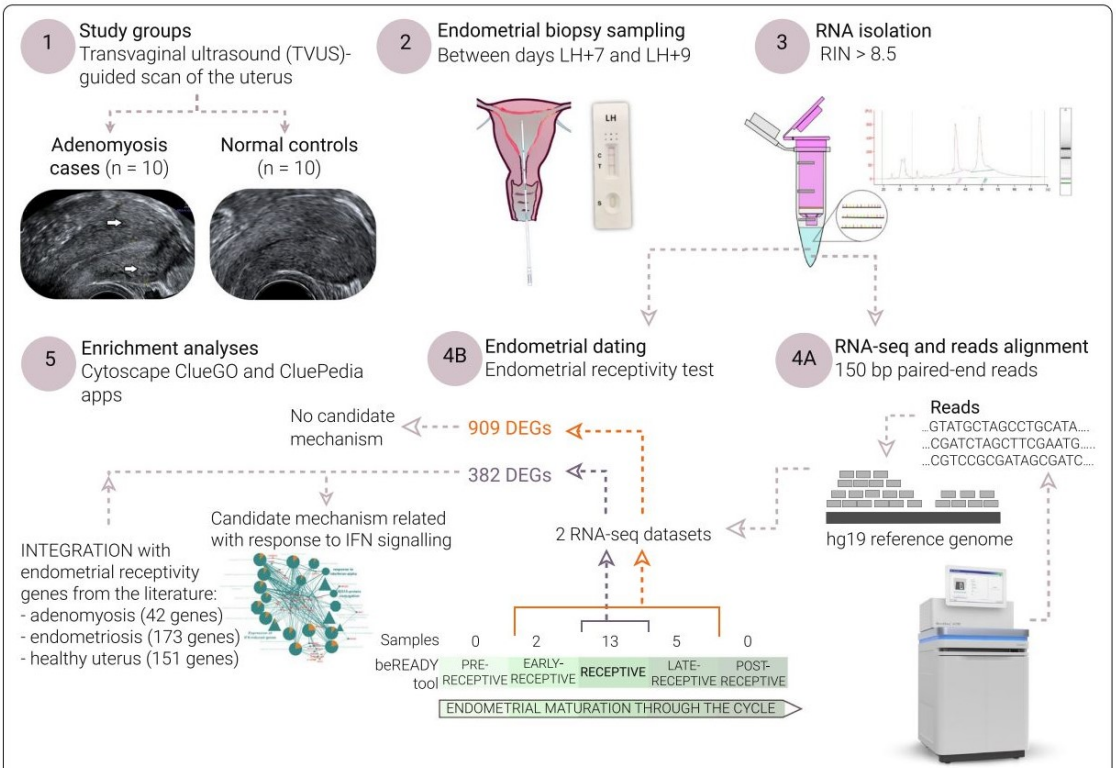

Fig. 1 The overview of the present study. Only ovulatory women with regular menstrual cycles were included. Endometrial biopsies, 10 in the adenomyosis group and 10 the in control group, were conducted between 7 and 9 days post urinary LH peak corresponding to the expected WOI. Isolated RNA samples were used for RNA-seq using the Illumina NovaSeq 6000 platform and for accurate endometrial receptivity classification using the beREADY® molecular test. When analysing transcriptomics data of all 20 LH-timed biopsies, 909 DEGs ($p < 0.05$, nonsignificant after corrected p value) were associated with the adenomyosis group. Downstream functional enrichment analysis of these genes identified no strong candidate mechanisms associated with endometrial molecular biology. According to endometrial receptivity testing, 2 out of 20 samples were classified as early-receptive, 13 as receptive and 5 as late-receptive during the menstrual cycle. To prevent early- and late-secretory phases of the menstrual cycle on the transcriptomics analysis associated with endometrial receptivity, samples dated to the early- and late receptive phases were omitted from the RNA-seq dataset. The remaining transcriptomics data of 8 adenomyosis cases and 5 control samples dated to the receptive phase were reanalysed. The 382 identified DEGs ($p < 0.05$, nonsignificant after corrected p value) in the adenomyosis group were further enriched in more robust candidate pathways, including "Expression of IFN-induced genes", "Response to interferon-alpha" and "ISG15-protein conjugation". The 382 identified DEGs were further integrated with 42, 173 and 151 genes from the literature associated with endometrial receptivity in adenomyosis, endometriosis and healthy uterus, respectively, to propose a molecular background of endometrial receptivity under adenomyosis. Abbreviations: DEGs = differentially expressed genes; LH = luteinizing hormone; RIN = RNA integrity number; WOI = window of implantation. The images of the NovaSeq 6000 Sequencing System and Gynetics suction curette were obtained from official pages Illumina.com and gyneatic.com, respectively

Endometrial receptivity classification of LH-timed biopsies

The results of endometrial receptivity testing performed on each endometrial RNA sample are provided in Table 2. According to the test, 13 out of 20 samples were classified in the receptive phase (8 adenomyosis cases and 5 controls), 2 samples in the early receptive phase, 5 samples in the late receptive phase and zero samples in the pre- or postreceptive phases.

The timing of endometrial biopsy and measured receptivity status are provided for each sample, followed by a summary of mapped RNA-seq reads and library size after filtering for low gene expression. Abbreviations "A" refer to adenomyosis and "K" to control samples. Timing of biopsy refers to the day after luteinizing hormone (LH) peak determination (LH+0) by urinary LH test.

RNA-seq outcome parameters

RNA-seq of endometrial samples utilizing the Illumina NovaSeq 6000 platform generated between 53,026,608 and

Table 1 Adenomyosis and control group characteristics

Characteristic	Adenomyosis group (N = 10)	Control group (N = 10)	p value
Age (years)	35 (30–39)	34.5 (30–42)	0.621
BMI (kg/m2)	27.2 (17.8–34.6)	21 (17.3–30.1)	0.112
Endometrial thickness (mm)	7.1 (4.6–11.2)	8 (6.2–10.1)	0.082
Number of performed ART cycles	2 (1–4)	4 (1–6)	0.353
Women sterility status:			
Primary sterility (nulligravid)	6	5	
Secondary sterility (gravida or parous)	4	5	
Factor of infertility:			
Male	5	8	
Tubal	2	1	
History of endometriosis	1	0	
Idiopathic infertility	2	1	

The median (range) is indicated for age, BMI, endometrial thickness and number of performed ART cycles (in vitro fertilization (IVF) and/or intracellular sperm injection (ICSI) treatments). P values are based on Mann–Whitney U test. Primary sterility refers to women who have never been pregnant (nulligravid), and secondary sterility refers to women who have already achieved pregnancy (gravida) or delivery (parous). Abbreviations: BMI = body mass index; ART = assisted reproductive technique

Table 2 Characteristics of endometrial RNA samples used in the study

Sample ID	Day of biopsy sampling	Endometrial dating by the beREADY® test	Number of mapped RNA-seq reads	Proportion of mapped RNA-seq reads	Library size after normalization
A10	LH+8	receptive	70,040,785	97.97%	25,194,593
A12	LH+7	receptive	71,578,907	98.25%	24,095,230
A18	LH+8	receptive	59,462,597	97.85%	19,682,295
A20	LH+7	receptive	73,911,463	97.82%	26,033,066
A21	LH+8	late-receptive	66,603,640	98.34%	24,868,485
A29	LH+7	receptive	52,378,707	98.78%	19,079,266
A31	LH+7	receptive	71,617,925	98.78%	23,269,754
A3	LH+7	receptive	60,702,730	98.69%	19,575,933
A5	LH+7	receptive	63,795,289	98.02%	21,175,249
A9	LH+7	early-receptive	64,755,616	98.12%	26,091,668
K11	LH+9	receptive	60,841,108	98.02%	22,021,130
K15	LH+8	receptive	64,147,880	98.25%	23,535,991
K17	LH+8	late-receptive	64,945,168	97.84%	21,964,495
K22	LH+9	late-receptive	66,813,165	97.91%	22,118,116
K23	LH+8	late-receptive	50,492,938	98.76%	16,086,563
K24	LH+7	receptive	58,204,527	98.72%	17,532,809
K26	LH+9	late-receptive	55,574,782	98.87%	19,198,159
K27	LH+9	receptive	64,512,592	98.81%	19,919,022
K28	LH+7	early-receptive	57,037,427	98.63%	18,335,825
K8	LH+7	receptive	66,124,560	98.08%	25360,931

75,561,205 reads per sample, with an average of 64,266,576 reads. Quality analysis of raw RNA-seq reads by FastQC revealed that each fastq file contained reads 150 base pairs (bp) in length with a mean per base sequence quality score (Phred score) of 36 and thus each file was considered for downstream bioinformatics analysis. Table 2 summarizes the number and proportion of mapped raw reads to the hg9 reference genome for each sample and obtained library sizes after filtering low-expression genes.

Identified DEGs associated with adenomyosis group

Differential expression analyses were conducted using two RNA-seq datasets constructed of samples according

to the results of endometrial receptivity testing. The first RNA-seq dataset was composed of all 20 samples: 13 receptive, 2 early- and 5 late-receptive samples. The second RNA-seq dataset was composed of 13 receptive samples only, while 2 early- and 5 late-receptive samples were omitted to exclude the influence of early- and late-secretory phases of the menstrual cycle on endometrial transcriptomic analysis associated with endometrial receptivity.

Transciptomics data comparison of 10 adenomyosis and 10 control samples resulted in 909 DEGs ($p<0.05$) associated with the adenomyosis group (the entire list of 909 DEGs is presented in Additional file 2). According to the HUGO Gene Nomenclature Committee (HGNC) (version updated March 23, 2021) nomenclature system (https://www.genenames.org/), different locus types were identified, including 829 protein-coding genes (mRNAs), 27 long noncoding RNAs (lncRNAs), 5 microRNAs (miRNAs), 5 small nucleolar RNAs, 28 pseudogenes, 1 complex locus constituent and 14 loci that were not mapped in the HGNC database. Among 909 DEGs, 487 genes (452 mRNAs, 11 lncRNAs and remaining other loci types) were upregulated, and 422 genes (376 mRNAs, 16 lncRNAs and remaining other loci types) were downregulated. However, the fold change (FC) of expression levels between study groups was nonsignificant after the application of multiple comparison correction.

Transcriptomics data comparison of 8 adenomyosis cases and 5 control endometrial samples with confirmed receptive phase provided 382 DEGs ($p < 0.05$) associated with the adenomyosis group (the entire list of 382 DEGs is presented in Additional file 3). According to the HGNC nomenclature system, 323 loci were mRNAs, 23 lncRNAs, 21 pseudogenes, 4 miRNAs, 1 complex locus constituent, 1 T cell receptor gene and 9 uncharacterized. Among 382 DEGs, there were 166 upregulated (137 mRNAs, 14 lncRNAs and remaining other loci types) and 216 downregulated (186 mRNAs, 9 lncRNAs and remaining other loci types) genes. However, there were no significant DEGs between the study groups according to the adjusted p value. Among 382 DEGs in the adenomyosis group, up to the top 10 up- and downregulated mRNAs and lncRNAs with the highest logFC values of expression levels are presented in Tables 3 and 4, respectively.

Enriched pathways associated with identified DEGs

Functional enrichment analysis of 909 DEGs associated with the adenomyosis group that were obtained from transcriptomics comparison of all 20 endometrial samples provided only 4 enriched GO terms sorted within 2 functionally organized network groups: “Intracellular

Table 3 Top upregulated mRNAs and lncRNAs

ENTREZ ID	HGNC symbol	Long name	Locus type	logFC	p value
259289	*TAS2R43*	taste 2 receptor member 43	mRNA	0.9484	0.0215
4250	*SCGB2A2*	secretoglobin family 2 A member 2	mRNA	0.9244	0.0150
1747	*DLX3*	distal-less homeobox 3	mRNA	0.9212	0.0247
54959	*ODAM*	odontogenic, ameloblast associated	mRNA	0.9184	0.0460
353091	*RAET1G*	retinoic acid early transcript 1G	mRNA	0.8803	0.0346
84072	*HORMAD1*	HORMA domain containing 1	mRNA	0.8581	0.0031
563	*AZGP1*	alpha-2-glycoprotein 1, zinc-binding	mRNA	0.8259	0.0463
100507436	*MICA*	MHC class I polypeptide-related sequence A	mRNA	0.8127	0.0203
7348	*UPK1B*	uroplakin 1B	mRNA	0.8086	0.0224
158131	*OR1Q1*	olfactory receptor family 1 subfamily Q member 1	mRNA	0.8077	0.0466
100505967	*LINC00645*	long intergenic non-protein coding RNA 645	lncRNA	2.9900	0.0056
100130231	*LINC00861*	long intergenic non-protein coding RNA 861	lncRNA	2.0003	0.0177
654412	*FAM138B*	family with sequence similarity 138 member B	lncRNA	1.8938	0.0452
100505921	*GLCCI1-DT*	GLCCI1 divergent transcript	lncRNA	1.8543	0.0323
100506334	*LINC00649*	long intergenic non-protein coding RNA 649	lncRNA	1.7910	0.0314
284578	*MFSD4A-AS1*	MFSD4A antisense RNA 1	lncRNA	1.6098	0.0022
283876	*LINC00921*	long intergenic non-protein coding RNA 921	lncRNA	1.3284	0.0443
100505625	*LINC02102*	long intergenic non-protein coding RNA 2102	lncRNA	1.3182	0.0300
100507398	*INTS6-AS1*	INTS6 antisense RNA 1	lncRNA	1.2787	0.0023
93653	*ST7-AS1*	ST7 antisense RNA 1	lncRNA	1.2489	0.0120

DEGs were insignificant after multiple testing correction of the p value. Abbreviation “FC” refers to fold change of expression levels

Table 4 Top downregulated mRNAs and lncRNAs

ENTREZ ID	HGNC symbol	Long name	Locus type	logFC	p value
169693	*TMEM252*	transmembrane protein 252	mRNA	-2.1611	0.0331
5655	*KLK10*	kallikrein related peptidase 10	mRNA	-1.7433	0.0010
5803	*PTPRZ1*	protein tyrosine phosphatase receptor type Z1	mRNA	-1.7022	0.0120
727897	*MUC5B*	mucin 5B, oligomeric mucus/gel-forming	mRNA	-1.6678	0.0421
79937	*CNTNAP3*	contactin associated protein like 3	mRNA	-1.5039	0.0096
10752	*CHL1*	cell adhesion molecule L1 like	mRNA	-1.5027	0.0391
7103	*TSPAN8*	tetraspanin 8	mRNA	-1.4535	0.0070
9723	*SEMA3E*	semaphorin 3E	mRNA	-1.4216	0.0001
10964	*IFI44 L*	interferon induced protein 44 like	mRNA	-1.3304	0.0416
5340	*PLG*	plasminogen	mRNA	-1.3219	0.0499
145837	*DRAIC*	downregulated RNA in cancer, inhibitor of cell invasion and migration	lncRNA	-1.9848	0.0392
100131825	*CADM3-AS1*	CADM3 antisense RNA 1	lncRNA	-1.6197	0.0298
100506674	*MRPS30-DT*	MRPS30 divergent transcript	lncRNA	-1.2230	0.0049
641364	*SLC7A11-AS1*	SLC7A11 antisense RNA 1	lncRNA	-0.7862	0.0302
100506305	*LINC00958*	long intergenic non-protein coding RNA 958	lncRNA	-0.6847	0.0448
100289410	*MCF2 L-AS1*	MCF2 L antisense RNA 1	lncRNA	-0.6591	0.0414
386597	*RNF144A-AS1*	RNF144A antisense RNA 1	lncRNA	-0.6095	0.0177
144481	*SOCS2-AS1*	SOCS2 antisense RNA 1	lncRNA	-0.4608	0.0205
100134229	*KDM7A-DT*	KDM7A divergent transcript	lncRNA	-0.4579	0.0344

DEGs were insignificant after multiple testing correction of the p value. Abbreviation "FC" refers to fold change of expression levels

lipid transport" (11 mapped genes, corrected p value 2.15×10^{-5}) and "Icosanoid receptor activity" (6 mapped genes, corrected p value 2.02×10^{-5}). Sorted pathways with associated genes in networks are presented in Fig. 2a. The results of the enrichment analysis are summarized in Additional file 4.

Functional enrichment analysis of 382 DEGs associated with the adenomyosis group that were obtained by transcriptomics analysis of endometrial samples in the receptive phase resulted in 33 enriched pathways, including 20 GO_BP terms, 6 Reactome pathways and 7 Reactome reactions. They were sorted into 7 network groups to remove redundancy, which is visualized in Fig. 2b. The highest proportion of enriched pathways was related to mechanisms of response to interferon (IFN) signalling, in particular antiviral response (presented in higher resolution in Fig. 2c). Most of the downregulated genes were mapped in the following network groups: "Expression of IFN-induced genes" (*BST2*, *IFI35*, *IFIT1*, *IFITM1*, *ISG15*, *MX1*, *OAS2*, *OAS3* and *STAT1* were down- and *IRF6* was upregulated, corrected p value 2.08×10^{-6}), "Response to interferon-alpha" (*BST2*, *EIF2AK2*, *IFITM1*, and *LAMP3*, corrected p value 8.75×10^{-4}), "ISG15-protein conjugation" (*ISG15*, *UBA7* and *UBE2E2*, corrected p value 3.02×10^{-5}) and "Homophilic cell adhesion via plasma membrane adhesion molecules" (*AMIGO1*, *CDH15*, *CDH24*, *CDH6*, *FAT1*, *FAT2*, *PALLD*, *PCDHA9* and *PLXNB3* were down-, while *CDHR1* and *NECTIN4* were upregulated, corrected p value 5.75×10^{-4}). Upregulated genes were mapped in the specific network group "Cysteine metabolic process" (*MPST*, *TST* and *VSIG2* were up- and *SLC7A11* was downregulated, corrected p value 5.62×10^{-4}). Nonspecific network groups characterized by equal proportions of mapped up- and downregulated genes were "Diseases associated with O-glycosylation of proteins" (*ADAMTS17*, *ADAMTS5* and *ADAMTSL2* were up-, while *ADAMTSL1*, *MUC13*, *MUC5B* and *THSD7A* were downregulated, corrected p value 4.48×10^{-4}) and "Retina homeostasis" (*AZGP1*, *CDHR1* and *NECTIN4* were up-, while *ALPK3*, *ATP1B2*, *CDH15* and *POTEJ* were downregulated, corrected p value 6.42×10^{-4}). The 33 identified enriched pathways are summarized in Additional file 5.

Enriched pathways obtained by integration of identified DEGs and endometrial receptivity genes from the literature

Only a set of 382 DEGs associated with the adenomyosis group that were identified by transcriptomics data comparison of adenomyosis case and control samples dated to the receptive phase were used for integrative enrichment analyses with endometrial receptivity genes from the literature.

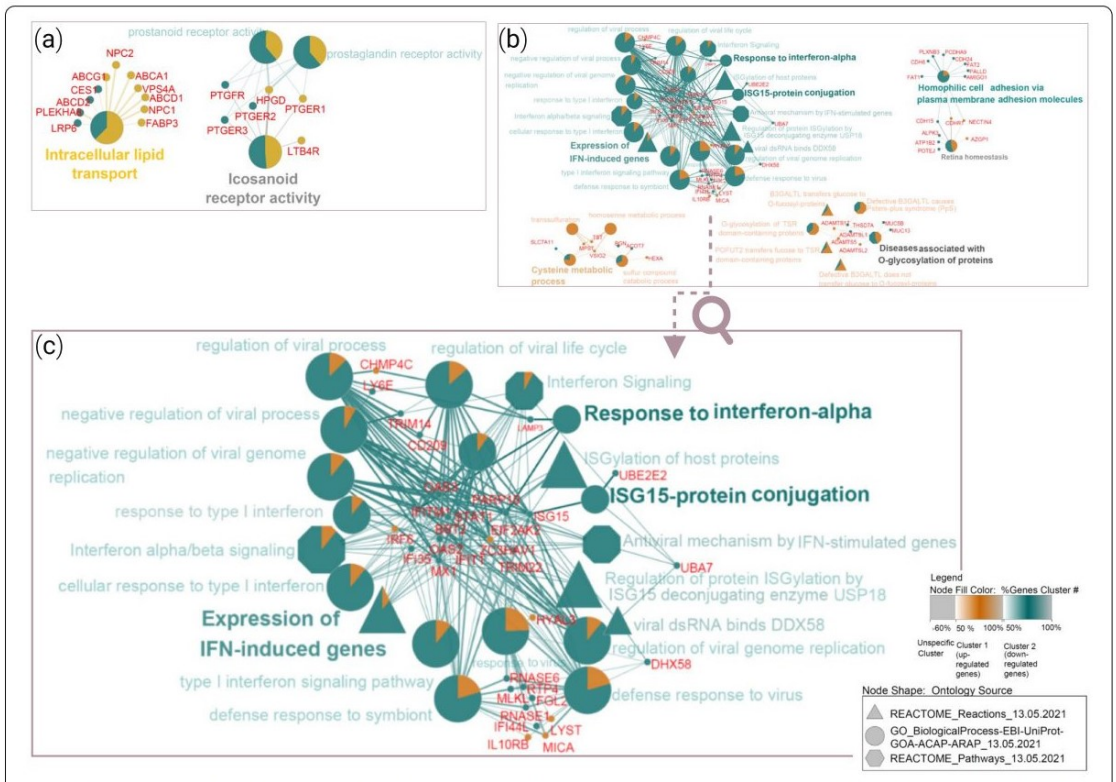

Fig. 2 Networks of enriched pathways and mapped genes associated with DEGs in the adenomyosis group. (**a**) Sorted 4 pathways in 2 network groups obtained by the enrichment analysis of 909 DEGs associated with adenomyosis group after comparing receptive, early- and late-receptive case and control samples; (**b**) Sorted 33 enriched pathways within 7 network groups identified from 382 DEGs associated with adenomyosis group after comparing only case and control samples dated to the receptive phase; (**c**) The enlargement of the connected network groups "Expression of IFN-induced genes", "Response to interferon-alpha" and "ISG15-protein conjugation" presenting candidate pathways for future studies associated with altered endometrial receptivity in adenomyosis. Each set of DEGs was uploaded in the Cytoscape ClueGO app as two separate clusters, where upregulated genes were marked with violet and downregulated genes with green colour. Shape of nodes in networks attributed to ontology sources that were applied for enrichment analysis. Enriched pathways were sorted into network groups based on their common biological role

Integration of lists with 382, 151 and 173 genes associated with adenomyosis, healthy uterus and endometriosis, respectively, provided 40 enriched pathways sorted in 11 network groups, which are presented in Fig. 3. According to the generated network, unique fingerprints of gynaecological pathologies on endometrial signatures were observed. The identified "Expression of IFN-induced genes", "Negative regulation of viral process" and "Diseases associated with O-glycosylation of proteins" network groups were specific for the adenomyosis gene list, while "Interleukin-10 signalling" and "ARC gene expression" were specific for the endometriosis gene list. In addition, nonspecific network groups, characterized by mapped genes originating from all 3 lists associated with gynaecological conditions, were identified, including "Extracellular matrix organization", "Serine-type peptidase activity", "Positive regulation of DNA-binding transcription factor activity", "Cellular response to vascular endothelial growth factor stimulus", "Response to cadmium ion" and "Regulation of reproductive process". This could indicate the interference of adenomyosis and endometriosis with molecular mechanisms required for normal endometrial receptivity. The 40 identified enriched pathways are summarized in Additional file 6.

Integration of lists with 424 (382 DEGs of the present sequencing experiment and 42 genes from the literature), 151 and 173 genes associated with adenomyosis, healthy uterus and endometriosis, respectively, provided 57 enriched pathways sorted in 18 network groups, which are presented in Fig. 4. Similar results were retrieved

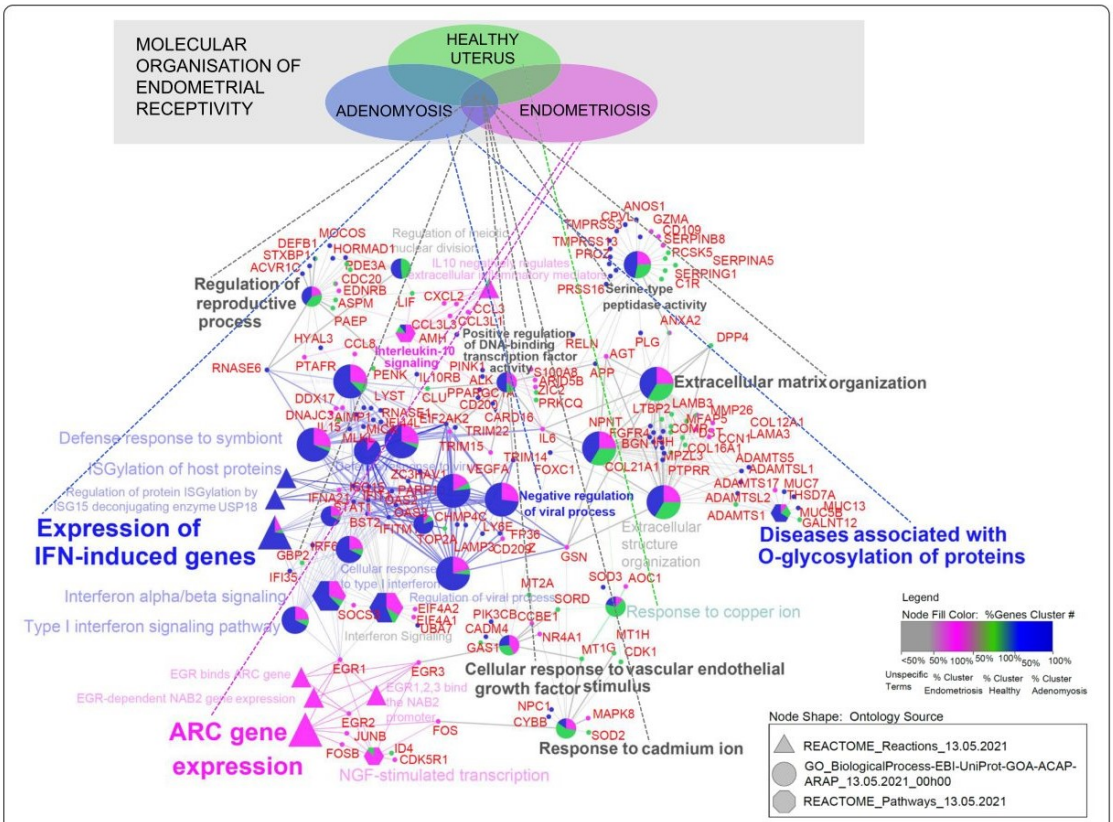

Fig. 3 Integration of 382 adenomyosis-associated DEGs with endometrial receptivity genes associated with endometriosis and healthy uterus. In total, 40 pathways sorted into 11 network groups were obtained after enrichment analysis of integrated gene lists. The adenomyosis gene list (blue colour) included 382 DEGs associated with the adenomyosis group of the present RNA-seq analysis. The healthy uterus list (green colour) and endometriosis list (pink colour) contained 151 and 173 genes, respectively, which were associated with endometrial receptivity in the literature

with the integration of the adenomyosis gene list with 382 DEGs alone. However, some additional nonspecific network groups were identified, including "Interleukin-4 and Interleukin-13 signalling", "Tumour necrosis factor production" and "Sodium ion export across plasma membrane". The 57 identified enriched pathways are summarized in Additional file 7.

Discussion

Altered endometrial molecular mechanisms obstructing successful embryo implantation in women with adenomyosis are poorly understood. The focus of the present transcriptomics analysis was to apply two novel molecular approaches to identify gene expression differences in LH-timed endometrial samples between women with and without adenomyosis: genome-wide profiling using RNA-seq and accurate classification of endometrial receptivity as assessed by the molecular tool beREADY®, measured from the same biopsy. Lists of DEGs associated with the adenomyosis group that were identified by analysing RNA-seq datasets in the setting of the endometrial dating results were applied for enrichment pathway analyses to predict their role in the context of endometrial molecular organization. In addition, a set of 382 DEGs obtained after transcriptomics data comparison of confirmed receptive samples was used for further bioinformatics analysis. They were integrated with the most extensive set of genes from the literature associated with endometrial receptivity in healthy uterus, endometriosis (model disease to study persistence of gynaecological pathology on endometrial molecular organization) and adenomyosis to predict mechanisms in which adenomyosis mediates an effect on endometrial receptivity.

Recently, Devesa-Peiro et al. [38] compared available transcriptomics data and observed a greater effect of changing phases of the menstrual cycle on the

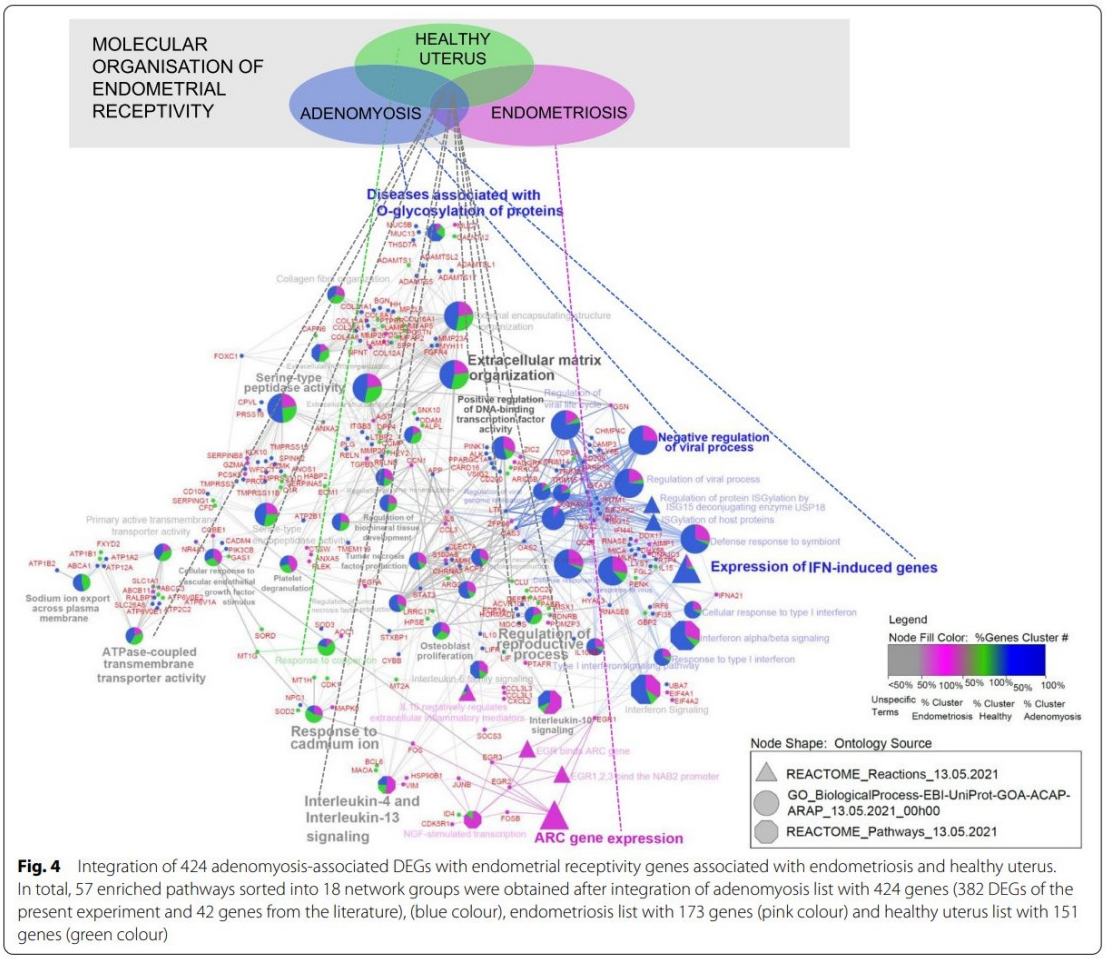

Fig. 4 Integration of 424 adenomyosis-associated DEGs with endometrial receptivity genes associated with endometriosis and healthy uterus. In total, 57 enriched pathways sorted into 18 network groups were obtained after integration of adenomyosis list with 424 genes (382 DEGs of the present experiment and 42 genes from the literature), (blue colour), endometriosis list with 173 genes (pink colour) and healthy uterus list with 151 genes (green colour)

endometrial transcriptome signature than the persistence of endometrial pathologies. Accurate dating of collected biopsies was highlighted as an important procedure when identifying endometrial biomarkers associated with uterine abnormalities [38]. In addition, displacement of the temporal appearance of the WOI has been observed in some women [39, 40], meaning that the WOI could appear earlier or later in the luteal phase, as it is generally assumed that it is constant in all women [39, 41]. In view of these data, we utilized the novel molecular beREADY® tool [25], which reliably determines endometrial dating on a transcriptomics platform, and machine-learning algorithms to assure homozygosity of LH-timed biopsies in the present study groups. Considering the results of endometrial receptivity testing, we excluded early- and late-receptive samples from the RNA-seq dataset to prevent the impact of early- and late-secretory phases associated with physiological advancement of endometrial maturation through the menstrual cycle, which could bias transcriptomics analysis associated with endometrial receptivity in adenomyosis. In that way, we identified 382 DEGs that we believe more accurately represent the effect of adenomyosis on the gene expression signature of endometrial receptivity compared to 909 DEGs associated with the adenomyosis group, which were identified by comparing transcriptomics data of samples derived from receptive, early- and late-receptive phases.

Enrichment analysis using 382 DEGs also provided a higher number of pathways tightly sorted in connected network groups compared to analysis of 909 DEGs, which were also more meaningful to relate with endometrial molecular biology (Fig. 2b). Namely, according to the results of the enrichment analysis of 382

DEGs, "Expression of IFN-induced genes", "Response to interferon-alpha" and "ISG15-protein conjugation" were sorted as connected processes (Fig. 2c). Popovici et al. [42] associated increased expression levels of genes encoding chemotactic factors, inflammatory cytokines (including type I IFN-alpha/beta) and apoptosis-inducing agents with a role in the recruitment of lymphocytes and macrophages in human endometrial decidua [42]. IFNs, as reviewed by De Veer et al. [43], are a family of multifunctional cytokines that activate the expression of many genes with antiviral, antiproliferative or immunosuppressive effects. The signal transduction pathway of IFNs is initiated upon IFN binding to specific cell surface receptors. Downstream formed complexes of phosphorylated proteins and transcription factors bind to IFN-stimulated response elements (ISREs) at the promotor region of IFN-stimulated genes (ISGs) and initiate their transcription. There are more than 300 ISGs [43]. The ubiquitin-like protein ISG15 is a posttranscriptional modifier that can be in a process termed ISGylation covalently linked to hundreds of proteins. The role of ISG15 has been associated with cellular processes such as protein translation, cytoskeleton dynamics, exosome secretion, autophagy, genome stability and cancer; therefore, it presents a potential target for therapeutic strategies [44]. ISG15 can exert functions as an intracellular and secreted protein. Intracellular expression of ISG15, which is dependent on type I IFN-alpha/beta signalling, characterizes innate immune responses to viral and microbial pathogens. Its extracellular signalling can elicit secretion of cytokine type II IFN-gamma from lymphocytes [45]. Studies in mice suggested that ISG15 plays a role in the recruitment of uterine natural killer (uNK) cells during early gestation, where it is responsible for remodelling of spiral arteries to ensure a normal blood supply to the foetus and placenta throughout pregnancy [46]. The identified enriched pathways related to the response to IFN signalling could indicate altered immune factors that have been associated with adenomyosis. Tremellen and Russell [47] associated an increased density of uNK cells and macrophages in the functional layer of late-secretory endometrium in women with severe adenomyosis experiencing implantation failures with a hostile immune environment that might interfere with successful embryo implantation [47]. In addition, Sotnikova et al. [48] reported higher levels of secreted proinflammatory cytokines (IFN-gamma, IFN-alpha, tumour necrosis factor (TNF)-alpha and interleukin (IL)-1 beta) in supernatant samples of cultured mononuclear cells obtained from late-secretory endometrium of women with adenomyosis when compared with healthy controls [48]. Another interesting enriched pathway from the 382 DEGs was related to cellular adhesion, whose importance in the process of embryo implantation has been described elsewhere [49].

The 382 Identified DEGs were also applied for the integration approach to repeat our previous enrichment pathway analysis [18] oriented to detect candidate pathways of affected endometrial receptivity in adenomyosis. Integrative enrichment analysis using the adenomyosis gene list with 382 DEGs only provided candidate pathways associated with endometrial receptivity establishment (e.g., "Extracellular matrix organization" "Cellular response to vascular endothelial growth factor stimulus" and "Regulation of reproductive process") that could be dysregulated in adenomyosis as well as in endometriosis, which is in agreement with the literature [17, 50–54]. The identified specific network group "Expression of IFN-induced genes" persisted as a unique effect of adenomyosis on endometrial molecular background after enrichment analysis using integrated gene lists. Enriched pathways related to activity-regulated cytoskeletal (ARC) gene expression were specific to the endometriosis gene list, which was used as a model to study the effect of endometrial-associated disorders. ARC is an immediate early gene involved in signal transduction. Its transcription is induced by various signalling cascades, including mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERKs) [55], which have already been associated with endometrial receptivity defects in endometriosis [56]. Integrative enrichment analysis using the adenomyosis gene list with 424 genes provided additional candidate pathways to be associated with altered cytokine responses in adenomyosis and endometriosis, including "interleukin-4 and interleukin-13 signalling", which was also identified in our previous study [18] and could be attributable to the Reactome pathway database being used as an ontology source in both studies, "regulation of TNF superfamily cytokine production" and "interleukin-10 signalling". Altered expression levels of some cytokines in the endometrium during WOI have been observed in women with adenomyosis after COS [57] and in women with endometriosis [58, 59]. Decidualization of endometrial stromal cells is characterized by a changing endometrial inflammatory environment shown as a transition from a proinflammatory to an anti-inflammatory response [60, 61]. This transition has been associated with balancing endometrial receptivity versus selectively accepting only high-quality embryos [61]. Dysregulated balance has been associated with the implantation of poor-quality embryos leading to miscarriage [62]. It could be that enriched pathways associated with the expression of IFN-induced genes indicate dysregulated endometrial selectively, which may explain the observed higher incidence of early pregnancy loss in women with adenomyosis [6, 20]. However, further

studies are needed to verify this hypothesis. The identification of robust pathways could lead to the extension of current gene sets for endometrial receptivity examination presented in a growing number of commercial molecular tests [25, 63–65] that would be specific for women with adenomyosis. Accurate endometrial receptivity examination in this group of infertile patients could better verify whether endometrial-associated factor is a source of recurrent implantation failures that prolong infertility treatments [66]. Furthermore, endometrial changes in women with adenomyosis could provide not only the relationship between pathophysiological mechanisms of adenomyosis development [17, 67, 68] but also the pathogenesis of the malignant transformation [69]. Recently, it was reported that endometrial carcinoma could co-exist or arise from adenomyosis which may be important factor in survival outcomes of the patient [70, 71].

A limitation of our study is the relatively small sample size, which prevents definitive conclusions regarding the impact of adenomyosis on the endometrial transcriptome [72]. The results could also differ if the control group is composed of women with proven fertility. Another limitation of the present study is that the diagnosis of adenomyosis could only be made noninvasively by imaging, since definitive histopathological diagnosis can only be made after hysterectomy. In genome-wide studies focusing on pathophysiological aspects of adenomyosis, the diagnosis can be based on histological examination of specimens after hysterectomy [17, 67, 68]. However, this is only possible retrospectively and is irrelevant in women who wish to preserve their fertility. In fertility-oriented transcriptomics studies [20] or studies including endometriosis [73], a diagnosis of adenomyosis was noninvasive. The diagnosis of adenomyosis by ultrasound is challenging, and there are no uniform ultrasonographic criteria for the diagnosis [74]. In the present study, TVUS of the uterus and pelvic cavity was performed by an experienced sonographer prior to each endometrial biopsy to confirm sonographic evidence of adenomyosis and to exclude other pelvic pathologies.

Conclusions

In this study, we focused on the molecular background of infertility-related adenomyosis based on our research and the available literature. We applied accurate endometrial receptivity classification of retrieved endometrial samples LH-timed to the expected WOI to avoid menstrual cycle bias in downstream transcriptomics analysis. The 382 DEGs identified in the adenomyosis group using the RNA-seq dataset of only confirmed receptive endometrial samples resulted in 33 enriched pathways further projected in the network from which "Expression of IFN-induced genes", "Response to interferon-alpha" and "ISG15-protein conjugation" were highlighted as connected processes. Additional integration of 382 DEGs with candidate genes associated with endometrial receptivity in healthy uterus, endometriosis and adenomyosis based on a literature review revealed that cytokine signalling impairments in endometrial pathologies could interfere with mechanisms of endometrial receptivity. According to our results, an altered response to IFN signalling is suggested as a candidate mechanism of impaired uterine receptivity in adenomyosis that needs to be further studied in a larger sample size.

Abbreviations

ART: Assisted reproductive technique; DEG: Differentially expressed gene; GO: Gene ontology; IFN: Interferon; LH: Luteinizing hormone; RNA-seq: RNA sequencing; TVUS: Transvaginal ultrasound ; WOI: Window of implantation.

Supplementary Information

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Additional file 1.
Additional file 2.
Additional file 3.
Additional file 4.
Additional file 5.
Additional file 6.
Additional file 7.

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Authors' contributions

EP, TK, BK and JK designed research. EP, MG, JK, TK analysed the data. EP and JK wrote the manuscript. TK, JK, BK, and UP revised the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated and analysed during this study are included in this article [and its supplementary information file]. The RNA-seq data presented in this study are deposited in the GEO database with accession number GSE185392.

Declarations

Ethics approval and consent to participate

The study was approved by the Slovenian National Medical Ethics Committee (0120–259/2018/16). Each patient signed an informed consent form before being involved in the study.

Competing interests
The authors declare that they have no competing interests.

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References

1. Andres MP, Borrelli GM, Ribeiro J, Baracat EC, Abrão MS, Kho RM. Transvaginal ultrasound for the diagnosis of adenomyosis: systematic review and meta-analysis. J Minim Invasive Gynecol [Internet]. Elsevier Inc.; 2018;25:257–64. Available from: https://doi.org/10.1016/j.jmig.2017.08.653
2. Pinzauti S, Lazzeri L, Tosti C, Centini G, Orlandini C, Luisi S, et al. Transvaginal sonographic features of diffuse adenomyosis in 18-30-year-old nulligravid women without endometriosis: association with symptoms. Ultrasound Obstet Gynecol. 2015;46:730–6.
3. Puente JM, Fabris A, Patel J, Patel A, Cerrillo M, Requena A, et al. Adenomyosis in infertile women: prevalence and the role of 3D ultrasound as a marker of severity of the disease. Reprod Biol Endocrinol [Internet]. Reproductive Biology and Endocrinology; 2016;14:60. Available from: https://doi.org/10.1186/s12958-016-0185-6
4. Sharma S, Bathwal S, Agarwal N, Chattopadhyay R, Saha I, Chakravarty B. Does presence of adenomyosis affect reproductive outcome in IVF cycles? A retrospective analysis of 973 patients. Reprod Biomed Online. Elsevier Ltd; 2019;38:13–21.
5. Thalluri V, Tremellen KP. Ultrasound diagnosed adenomyosis has a negative impact on successful implantation following GnRH antagonist IVF treatment. Hum Reprod. 2012;27:3487–92.
6. Stanekova V, Woodman RJ, Tremellen K. The rate of euploid miscarriage is increased in the setting of adenomyosis. Hum Reprod Open. 2019;2019:1–8.
7. Mehasseb MK, Bell SC, Pringle JH, Habiba MA. Uterine adenomyosis is associated with ultrastructural features of altered contractility in the inner myometrium. Fertil Steril [Internet]. Elsevier Ltd; 2010;93:2130–6. Available from: https://doi.org/10.1016/j.fertnstert.2009.01.097
8. Harada T, Khine Y, Kaponis A, Nikellis T, Decavalas G, F T. The impact od adenomyosis on women's fertility. Obstet Gynecol Surv. 2016;71:557–68.
9. Ota H, Igarashi S, Hatazawa J, Tanaka T. Endothelial nitric oxide synthase in the endometrium during the menstrual cycle in patients with endometriosis and adenomyosis. Fertil Steril. 1998;69:303–8.
10. Ota H, Igarashi S, Hatazaw J, Tanaka T. Immunohistochemical assessment of superoxide dismutase expression in the endometrium in endometriosis and adenomyosis. Fertil Steril. 1999;72:129–34.
11. Ota H, Igarashi S, Sasaki M, Tanaka T. Xanthine oxidase in eutopic and ectopic endometrium in endometriosis and adenomyosis. Hum Reprod. 2001;75:785–90.
12. Ota H, Tanaka T. Stromal vascularization in the endometrium during adenomyosis. Microsc Res Tech. 2003;449:445–9.
13. Goteri G, Lucarini G, Montik N, Zizzi A, Stramazzotti D, Fabris G, et al. Expression of vascular endothelial growth factor (VEGF), hypoxia inducible factor-1α (HIF-1α), and microvessel density in endometrial tissue in women with adenomyosis. Int J Gynecol Pathol. 2009;28:157–63.
14. Peng Y, Jin Z, Liu H, Xu C. Impaired decidualization of human endometrial stromal cells from women with adenomyosis. Biol Reprod. 2021;ioab017.
15. Wang J, Deng X, Yang Y, Yang X, Kong B, Chao L. Expression of GRIM-19 in adenomyosis and its possible role in pathogenesis. Fertil Steril. 2016;105:1093–101.
16. Campo S, Campo V, Benagiano G. Adenomyosis and infertility. Reprod Biomed Online. Reproductive Healthcare Ltd.; 2012;24:35–46.
17. Herndon CN, Aghajanova L, Bayalan S, Erikson D, Barragan F, Goldfien G, et al. Global transcriptome abnormalities of the eutopic endometrium from women with adenomyosis. Reprod Sci. 2016;23:1289–303.
18. Prašnikar E, Kunej T, Repnik K, Potočnik U, Knez J, Kovačič B. Determining the molecular background of endometrial receptivity in adenomyosis. Biomolecules. 2020;10:1–25.
19. Prašnikar E, Knez J, Kovačič B, Kunej T. Molecular signature of eutopic endometrium in endometriosis based on the multi-omics integrative synthesis. J Assist Reprod Genet. 2020;37:1593–611.
20. Martinez-Conejero JA, Morgan M, Montesinos M, Fortu S, Meseguer M, Ph D, et al. Adenomyosis does not affect implantation, but is associated with miscarriage in patients undergoing oocyte donation. Fertil Steril. 2011;96:943–51.
21. Zhao S, Fung-Leung WP, Bittner A, Ngo K, Liu X. Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. PLoS One. 2014;9:e78644.
22. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 2009;10:57–63.
23. Naftalin J, Hoo W, Pateman K, Mavrelos D, Holland T, Jurkovic D. How common is adenomyosis? A prospective study of prevalence using transvaginal ultrasound in a gynaecology clinic. Hum Reprod. 2012;27:3432–9.
24. Hulka CA, Hall DA, McCarthy K, Simeone J. Sonographic findings in patients with adenomyosis: can sonography assist in predicting extent of disease? Am J Roentgenol. 2002;179:379–83.
25. Saare M, Laisk T, Teder H, Paluoja P, Palta P, Koel M, et al. A molecular tool for menstrual cycle phase dating of endometrial samples in endometriosis transcriptome studies. Biol Reprod. 2019;101:1–3.
26. Teder H, Koel M, Paluoja P, Jatsenko T, Rekker K, Laisk-Podar T, et al. TAC-seq: targeted DNA and RNA sequencing for precise biomarker molecule counting. NPJ Genomic Med. 2018;3.
27. Altmäe S, Koel M, Võsa U, Adler P, Suhorutšenko M, Laisk-Podar T, et al. Meta-signature of human endometrial receptivity: a meta-analysis and validation study of transcriptomic biomarkers. Sci Rep [Internet]. 2017;7:10077. Available from: http://www.nature.com/articles/s41598-017-10098-3
28. Liao Y, Smyth GK, Shi W. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. Nucleic Acids Res. Oxford University Press; 2019;47.
29. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. Nucleic Acids Res. 2013;41:e108.
30. Liao Y, Smyth GK, Shi W. FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 2014;30:923–30.
31. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26:139–40.
32. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 2010;11:R25.
33. Law CW, Chen Y, Shi W, Smyth GK. Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 2014;15:R29.
34. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43:e47.
35. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: A Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics. 2009;25:1091–3.
36. Bindea G, Galon J, Mlecnik B. CluePedia Cytoscape plugin: Pathway insights using integrated experimental and in silico data. Bioinformatics. 2013;29:661–3.
37. Shannon P, Markiel A, Ozier O, Baliga SN, Wang TJ, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res [Internet]. 2003;13:2498–504. Available from: http://ci.nii.ac.jp/naid/110001910481/
38. Devesa-Peiro A, Sebastian-Leon P, Pellicer A, Diaz-Gimeno P. Guidelines for biomarker discovery in endometrium: correcting for menstrual cycle bias reveals new genes associated with uterine disorders. Mol Hum Reprod. 2021;27:1–19.

39. Ruiz-Alonso M, Blese D, Diaz-Gimeno P, Gomez E, Fernandez-Sanchez M, Carranza F, et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. Fertil Steril. 2013;100:818–24.
40. Mahajan N, Kaur S, Alonso MR. Window of implantation is significantly displaced in patients with adenomyosis with previous implantation failure as determined by endometrial receptivity assay. J Hum Reprod Sci. 2018;11:353–8.
41. Tan J, Kan A, Hitkari J, Taylor B, Tallon N, Warraich G, et al. The role of the endometrial receptivity array (ERA) in patients who have failed euploid embryo transfers. J Assist Reprod Genet. Journal of Assisted Reproduction and Genetics; 2018;35:683–92.
42. Popovici RM, Kao LC, Giudice CG. Discovery of new inducible genes in in vitro decidualized human endometrial stromal cells using microarray technology. Endocrinology. 2000;141:3510–3.
43. de Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, et al. Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol [Internet]. 2001;69:912–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11404376
44. Jiménez Fernández D, Hess S, Knobeloch KP. Strategies to target ISG15 and USP18 toward therapeutic applications. Front Chem. 2020;21:923.
45. Young Hee Choi and A-MY. Extracellular ISG15 signals cytokine secretion through the LFA-1 integrin receptor. Mollecular cell. 2017;68:581-590.e5.
46. Austin KJ, Bany BM, Belden EL, Rempel LA, Cross JC, Hansen TR. Interferon-stimulated gene-15 (Isg15) expression is up-regulated in the mouse uterus in response to the implanting conceptus. Endocrinology. 2003;144:3107–13.
47. Tremellen KP, Russell P. The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure. II: adenomyosis and macrophages. J Reprod Immunol [Internet]. Elsevier Ireland Ltd; 2012;93:58–63. Available from: https://doi.org/10.1016/j.jri.2011.12.001
48. Sotnikova N, Antsiferova I, Malyshkina A. Cytokine network of eutopic and ectopic endometrium in women with adenomyosis. Am J Reprod Immunol. 2002;47:251–5.
49. Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. Hum Reprod Update. 2006;12:731–46.
50. Matsuzaki S, Maleysson E, Darcha C. Analysis of matrix metalloproteinase-7 expression in eutopic and ectopic endometrium samples from patients with different forms of endometriosis. Hum Reprod. 2010;25:742–50.
51. Pino M, Galleguillos C, Torres M, Sovino H, Fuentes A, Boric MA, et al. Association between MMP1 and MMP9 activities and ICAM1 cleavage induced by tumor necrosis factor in stromal cell cultures from eutopic endometria of women with endometriosis. Reproduction. 2009;138:837–47.
52. Okada H, Tsuzuki T, Shindoh H, Nishigaki A, Yasuda K, Kanzaki H. Regulation of decidualization and angiogenesis in the human endometrium: mini review. J Obstet Gynaecol Res. 2014;40:1180–7.
53. Li T, Li Y-G, Pu D-M. Matrix metalloproteinase-2 and -9 expression correlated with angiogenesis in human adenomyosis. Gynecol Obstet Invest. 2006;62:229–35.
54. Di Carlo C, Bonifacio M, Tommaselli GA, Bifulco G, Guerra G, Nappi C. Metalloproteinases, vascular endothelial growth factor, and angiopoietin 1 and 2 in eutopic and ectopic endometrium. Fertil Steril [Internet]. Elsevier Ltd; 2009;91:2315–23. Available from: https://doi.org/10.1016/j.fertnstert.2008.03.079
55. Epstein I, Finkbeiner S. The Arc of cognition: signaling cascades regulating Arc and implications for cognitive function and disease. Semin Cell Dev Biol. 2018;77:63–72.
56. Lessey BA, Kim JJ. Endometrial receptivity in eutopic endometrium of women with endometriosis it is affected, let me show you why. Fertil Steril. 2017;108:19–27.
57. Zhihong N, Yun F, Pinggui Z, Sulian Z, Zhang A. Cytokine profiling in the eutopic endometrium of adenomyosis during the implantation window after ovarian stimulation. Reprod Sci. 2016;23:124–33.
58. Kharfi A, Labelle Y, Mailloux J, Akoum A. Deficient expression of tumor necrosis factor receptor type 2 in the endometrium of women with endometriosis. Am J Reprod Immunol. 2003;50:33–40.
59. Ulukus M, Ulukus EC, Seval Y, Zheng W, Arici A. Expression of interleukin-8 receptors in endometriosis. Hum Reprod [Internet]. 2005;20:794–801. Available from: https://academic.oup.com/humrep/article-lookup/doi/https://doi.org/10.1093/humrep/deh675
60. Wilczyński JR. Th1/Th2 cytokines balance - yin and yang of reproductive immunology. Eur J Obstet Gynecol Reprod Biol. 2005;122:136–43.
61. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo quality. Biol Reprod. 2014;91:1–8.
62. Quenby S, Vince G, Farquharson R, Aplin J. Recurrent miscarriage: a defect in nature's quality control? Hum Reprod. 2002;17:1959–63.
63. Díaz-Gimeno P, Horcajadas JA, Martinez-Conejero JA, Esteban FJ, Alama P, Pellicer A, et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. Fertil Steril. 2011;95:50–60.
64. Enciso M, Carrascosa JP, Sarasa J. Development of a new comprehensive and reliable endometrial receptivity map (ER Map / ER Grade) based on RT- qPCR gene expression analysis. Hum Reprod. 2018;33:220–8.
65. Haouzi D, Entezami F, Torre A, Innocenti C, Antoine Y, Mauries C, et al. Customized frozen embryo transfer after identification of the receptivity window with a transcriptomic approach improves the implantation and live birth rates in patients with repeated implantation failure. Reprod Sci. Reproductive Sciences; 2021;28:69–78.
66. Tremellen K, Russell P. Adenomyosis is a potential cause of recurrent implantation failure during IVF treatment. Aust New Zeal J Obstet Gynaecol. 2011;51:280–3.
67. Jiang JF, Sun AJ, Xue W, Deng Y, Wang YF. Aberrantly expressed long noncoding RNAs in the eutopic endometria of patients with uterine adenomyosis. Eur J Obstet Gynecol Reprod Biol [Internet]. Elsevier Ireland Ltd; 2016;199:32–7. Available from: https://doi.org/10.1016/j.ejogrb.2016.01.033
68. Xiang Y, Sun Y, Yang B, Yang Y, Zhang Y, Yu T, et al. Transcriptome sequencing of adenomyosis eutopic endometrium: a new insight into its pathophysiology. J Cell Mol Med. 2019;23:8381–91.
69. Laganà AS, Scioscia M. Endometrial cancer in women with adenomyosis: an underestimated risk? Int J Fertil Steril. 2020;14:260–1.
70. Chao X, Wu M, Ma S, Tan X, Zhong S, Bi Y, et al. The clinicopathological characteristics and survival outcomes of endometrial carcinoma coexisting with or arising in adenomyosis: a pilot study. Sci Rep. 2020;10:1–9.
71. Scioscia M, Noventa M, Laganà AS. Abnormal uterine bleeding and the risk of endometrial cancer: can subendometrial vascular ultrasound be of help to discriminate cancer from adenomyosis? Am J Obstet Gynecol [Internet]. Elsevier Inc.; 2020;223:605–6. Available from: https://doi.org/10.1016/j.ajog.2020.05.049
72. Vercellini P, Parazzini F, Oldani S, Panazza S, Bramante T, Crosignani PG. Adenomyosis at hysterectomy: a study on frequency distribution and patient characteristics. Hum Reprod. 1995;10:1160–2.
73. Dior UP, Nisbet D, Fung JN, Foster G, Healey M, Montgomery GW, et al. The association of sonographic evidence of adenomyosis with severe endometriosis and with gene expression in eutopic endometrium. J Minim Invasive Gynecol [Internet]. Elsevier Inc.; 2018;26:941–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1553465018312512%0Ahttp://www.ncbi.nlm.nih.gov/pubmed/30273686
74. Chapron C, Vannuccini S, Santulli P, Abrão MS, Carmona F, Fraser IS, et al. Diagnosing adenomyosis: an integrated clinical and imaging approach. Hum Reprod Update. 2020;26:392–411.

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2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Obogatitvene analize poročanih genov spremenjenega endometrijskega izražanja pri adenomiozi (preverjanje hipoteze 2)

2.2.1.1 Uvod

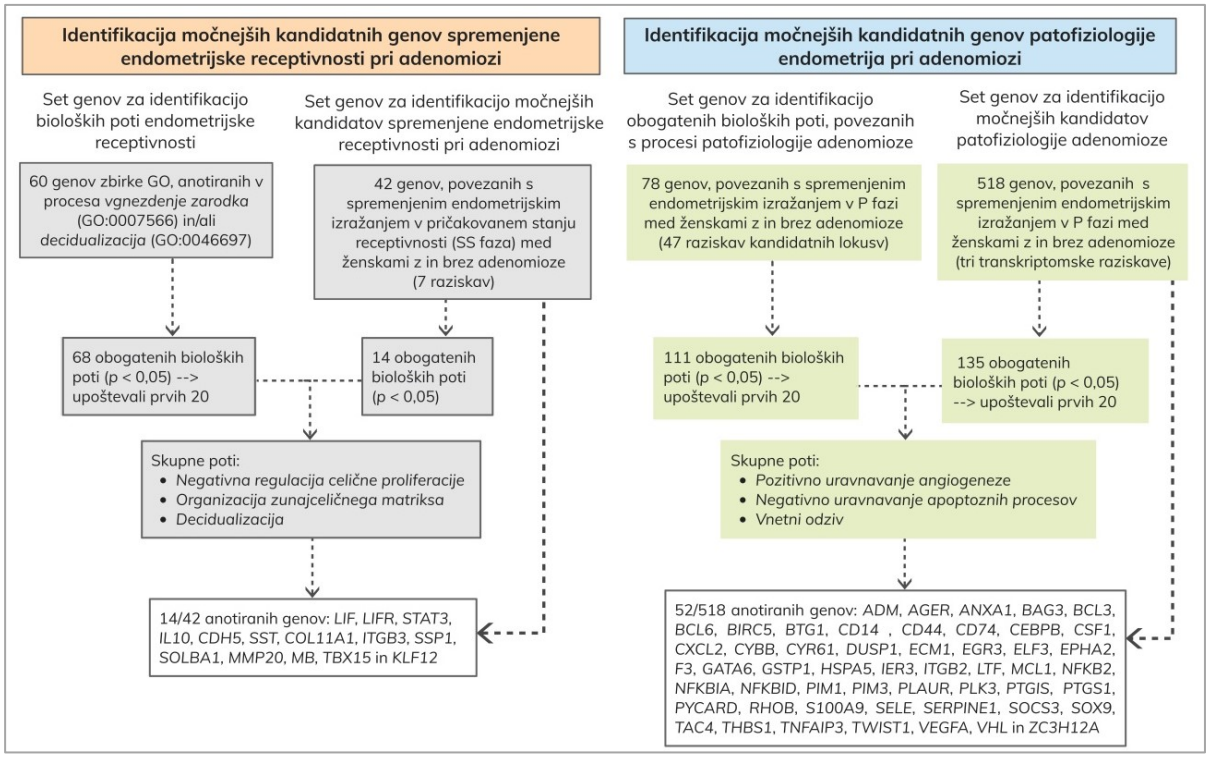
V literaturi se v povezavi z adenomiozo pogosto uporabljata izraza evtopični in ektopični endometrij. Izraz evtopični endometrij se nanaša na endometrij, ki se nahaja na pravilnem mestu v maternici, in sicer obdaja maternično votlino in predstavlja ciljno tkivo za vgnezdenje zarodka (Makieva in sod., 2018). Izraz ektopični endometrij pa se navezuje na endometriju podobno tkivo (adenomiozno tkivo ali lezije), ki se nahaja na nepravilnih mestih v maternici, in sicer znotraj miometrija. Spremenjena molekularna organizacija evtopičnega endometria pri adenomiozi (EvEA) se povezuje z dvema procesoma: vloga pri patofiziologiji, ki omogoča razvoj in napredovanje adenomioze (Vannuccini in sod., 2017) in vloga pri nepopolni endometrijski receptivnosti in decidualizaciji strome, kar ovira vgnezdenje zarodka (Campo in sod., 2012). EvEA tako predstavlja ciljno tkivo za identifikacijo molekularnih označevalcev, povezanih s to boleznijo. Intenzivno iskanje endometrijskih molekularnih označevalcev že poteka pri endometriozi (Ahn in sod., 2017).

Pri iskanju endometrijskih molekularnih označevalcev pa je pomembno datiranje menstruacijskega ciklusa vzorcev endometrija. Poročajo namreč, da imajo posamezne faze menstruacijskega ciklusa večji podpis na transkriptom endometrija kot pa patologije endometrija. Za identifikacijo endometrijskih molekularnih označevalcev patologije je pomembna analiza vzorcev, datiranih v isto fazo menstruacijskega ciklusa (Altmäe in sod., 2014; Devesa-Peiro in sod., 2021). Raziskave endometrija, ki so bile usmerjene v identifikacijo mehanizmov patofiziologije endometroze (Laudanski in sod., 2013; Shi in sod., 2014; Cui in sod., 2018) in adenomioze (Herndon in sod., 2016; Jiang J.F. in sod., 2016; Xiang in sod., 2019), so bile opravljene v P fazi menstruacijskega ciklusa. Domneva se, da so razlike v izražanju genov proliferacijskega endometrija med preiskovano in kontrolno skupino povezane s patofiziologijo bolezni, saj v tej fazi menstruacijskega ciklusa ni molekularnega odtisa endometrijske receptivnosti na celokupen transkriptom. V P fazi namreč poteka regeneracija izluščenega endometrija predhodnega ciklusa. To pa se spremeni po nastopu ovulacije, ko prične delovati hormon P4 in menstruacijski ciklus endometrija prestopi v S fazo. V tej fazi se endometrij diferencira v deciduo in v SS fazi nastopi endometrijska receptivnost za vgnezdenje zarodka (Makieva in sod., 2018). Razlike v izražanju genov endometrija SS faze med adenomiozno/endometriozno in kontrolno skupino pa se povezujejo s spremenjeno endometrijsko receptivnostjo zaradi prisotne patologije (Kao in sod., 2003a; Burney in sod., 2007; Martinez-Conejero in sod., 2011; Tamaresis in sod., 2014; Houshdaran in sod., 2016; Zhou in sod., 2016; Dior in sod., 2018).

Naš namen je bil z bioinformacijskimi analizami podatkov o poročanih genetskih vzrokih, povezanih s spremenjenim izražanjem v EvEA, identificirati močnejše kandidatne gene

patofiziologije in močnejše kandidatne gene spremenjene endometrijske receptivnosti pri adenomiozi.

2.2.1.2 Materiali in metode



Slika 2: Potek preverjanja hipoteze 2. Z orodjem DAVID smo izvedli obogatitvene analize genskih setov. Sete smo oblikovali z združevanjem podatkov (transkripti in proteini) iz pregleda literature in poenotenjem poimenovanja po genski nomenklaturi zbirke HGNC. Gene smo sortirali glede na datirano fazo menstruacijskega ciklusa endometrija v kateri je bila ugotovljena razlika v izražanju med adenomiozno in kontrolno skupino žensk. Seta 78 in 518 genov, povezana s spremenjenim izražanjem v endometriju P faze smo uporabili za identifikacijo kandidatnih genov patofiziologije adenomioze. Set 42 genov, povezan s spremenjenim izražanjem v SS fazi, pa za identifikacijo kandidatnih genov spremenjene endometrijske receptivnosti pri adenomiozi. Močnejše kandidatne gene smo prioritizirali na podlagi prekrivanja obogatenih bioloških poti. Uporabljene kratice: P faza = proliferacijska faza menstruacijskega ciklusa; SS faza = srednje sekrecijska faza menstruacijskega ciklusa; GO = genska ontologija.

Figure 2: The overview of hypothesis 2 testing. Gene set enrichment analyses were performed using the DAVID tool. Gene sets were developed by synthesising data (transcripts and proteins) from literature mining and further adopting of the HGNC gene nomenclature. Genes were sorted according to the dated phase of the menstrual cycle, in which a difference in endometrial expression levels between the adenomyosis and control group of women was reported. The 78 and 518 gene sets associated with altered expression levels in P phase endometrium were used to identify stronger candidates of pathophysiology in adenomyosis. The set of 42 genes associated with altered expression in the mid secretory phase was used to identify stronger candidates of altered endometrial receptivity in adenomyosis. The stronger candidate genes were prioritized based on the overlap of enriched pathways. Abbreviations used: P phase = the proliferative phase of the menstrual cycle; SS phase = the mid secretory phase of the menstrual cycle; GO = Gene Ontology.

Iz zbirke PubMed (PubMed, 2021) smo s ključnimi besedami »adenomyosis« in »pathogenesis« oz. »molecular studies« oz. »genetics« oz. »gene expression« oz. »endometrial differences« pridobili znanstveno literaturo. Raziskave smo v Prilogi B razvrstili glede na preučevane biološke procese. Iz vsake raziskave smo pridobili tudi podatek, če je bil le-ta podan, o datirani fazi menstruacijskega ciklusa ob času vzorčenja endometrija. Poimenovanje preučevanih transkriptom mRNA in ncRNA ter proteinov smo poenotili po genski nomenklaturi zbirke HGNC, da smo lahko glede na datirano fazo menstruacijskega ciklusa združevali podatke (gene) za obogatitvene analize.

Iz raziskav, ki so bile opravljene na svežih vzorcih endometrija (EvEA), smo zbrali gene s statistično značilnim spremenjenim izražanjem v P in/ali SS fazi menstruacijskega ciklusa pri ženskah z in brez adenomioze. Raziskave brez podatka o datiranju endometrija smo izločili iz analize. Raziskave, ki smo jih uporabili za sestavo genskih setov, so v Prilogi B označene krepko. Sestavili smo tri sete genov; dva povezana s P in enega s SS fazo. Četrti set genov, povezan s splošnimi procesi vgnezdenja zarodka oz. decidualizacijo strome (SS faza), smo pridobili iz podatkovne zbirke GO. Opis izdelave posameznega genskega seta:

- Set 60 genov, s katerim smo identificirali splošne biološke poti endometrijske receptivnosti (SS faza) za uspešno vgnezdenje zarodka. Gene smo pridobili iz zbirke GO, kjer smo se omejili na takson *homo sapiens*. Iz procesov *vgnezdenje zarodka* (GO:0007566) in *decidualizacija* (GO:0046697) smo pridobili 62 oz. 33 anotiranih genov. Po odstranitvi podvojenih genov smo pridobili unikatnih 60 genov.

- Set 42 genov, ki smo ga uporabili za identifikacijo močnejših kandidatnih genov spremenjene endometrijske receptivnosti pri adenomiozi. Gene smo pridobili z združenjem podatkov 6 raziskav kandidatnih lokusov (Xiao in sod., 2010, 2013; Fischer in sod., 2011; Yen in sod., 2016; Wang in sod., 2018; Yan in sod., 2019) in ene transkriptomske analize (Martinez-Conejero in sod., 2011), ki so primerjale izražanje v endometriju v SS faze ciklusa (pričakovan čas endometrijske receptivnosti) med ženskami z in brez adenomioze.

- Set 78 genov, s katerim smo identificirali kandidatne poti patofiziologije endometrija pri adenomiozi. Gene smo pridobili z združevanjem podatkov 47 raziskav, ki so poročale o spremenjenem izražanju kandidatnih lokusov v endometriju (EvEA) P faze med ženskami z in brez adenomioze. V zbirki DisGeNET (DisGeNET, 2021) smo sicer našli set genov, povezan z adenomiozo (poimenovano kot *endometriosis of uterus, C0341858*). Po podrobnem pregledu navedenih genov in podporne literature smo ugotovili, da so bili genski simboli pogosto dodeljeni tudi kraticam različnih uporabljenih izrazov v besedilu izvorne raziskave. V zbirki tudi ni navedeno, ali se spremenjeno izražanje navedenega gena navezuje na ektopični (EkEA) ali evtopični (EvEA) endometrij pri adenomiozi. Ker nas je zanimal patološki efekt adenomioze na ciljno tkivo vgnezdenja zarodka (EvEA), smo ročno pregledali vsako raziskavo, da smo zbrali podatke le tistih raziskav, ki so se

nanašale na spremenjeno izražanje v evtopičnem endometriju med ženskami z in brez adenomioze.

- Set 518 genov, ki smo ga uporabili za identifikacijo močnejših kandidatnih genov patofiziologije endometrija pri adenomiozi. Gene smo pridobili z združevanjem podatkov spremenjeno izraženih genov treh transkriptomskih raziskav, ki so bile izvedene na vzorcih endometrija (EvEA) v P fazi pri ženskah z in brez adenomioze (Herndon in sod., 2016; Jiang J.F. in sod., 2016; Xiang in sod., 2019).

Oblikovane genske sete smo z bioinformacijskim orodjem DAVID (Huang in sod., 2009) obogatili za ontologijo GO_BP. Iz vsakega seta smo upoštevali do 20 najbolj statistično značilnih poti ($p < 0,05$) glede na naraščajočo vrednost p. Na podlagi ujemanja obogatenih poti med setoma iste datirane faze, tj. SS (60 in 42 genov) in P (78 in 518 genov) faza, smo anotirane gene iz seta 42 genov predlagali kot močnejše kandidate spremenjene endometrijske receptivnosti in anotirane gene iz seta 518 genov kot močnejše kandidate patofiziologije. Potek identifikacije kandidatov je predstavljen na Sliki 2.

2.2.1.3 Rezultati in diskusija

Pridobili smo 110 raziskav, v katerih so preučevali endometrij pri adenomiozi. Pridobljene raziskave so bile opravljene na svežih humanih vzorcih evtopičnega (EvEA) in/ali ektopičnega (EkEA) endometrija, celičnih kulturah ali na mišjem modelu adenomioze. Pridobljene raziskave smo zbrali in predstavili v Prilogi B. Raziskave smo razvrstili glede na procese, povezane s patofiziologijo oz. patogenezo adenomioze: lokalna proizvodnja in spremenjen metabolizem E2 v maternici; hiperperistaltika maternice; proces TIAR v miometriju; kopičenje imunskih celic na mestu poškodb miometrija in v evtopičnem endometriju; hipoksija; somatske mutacije; epigenetske spremembe; polimorfizmi; aktivacija procesa EMT; zvišana celična migracija; zvišana invazivnost; spremenjena organizacija zunajceličnega matriksa; prekomerna (nevro)angiogeneza; ovirana apoptoza; prekomerna proliferacija; spremenjen vnetni odziv; spremenjene znotrajcelične signalne poti; povečan metabolizem prostih radikalov; fibroza; moten imunološki nadzor; spremenjeno izražanje integrinov in receptorjev za steroidne hormone.

Izmed 110 pridobljenih raziskav smo v sestavo genskih setov vključili 7 raziskav analize endometrija v SS fazi in 50 raziskav analize endometrija v P fazi. Geni posameznih setov so navedeni v Prilogi A. Raziskave endometrija SS faze so bile opravljena pri mlajših ženskah v njihovem reproduktivnem obdobju. V teh raziskavah je bila adenomioza diagnosticirana z neinvazivnimi metodami slikanja trebuha. Večina raziskav endometrija P faze pa je bila opravljena pri starejših ženskah z napredno obliko adenomioze. V teh raziskavah je bila diagnoza adenomioze postavljena na osnovi histoloških pregledov vzorcev histerektomije. Z rezultati obogatitvenih analiz smo med zbranimi geni identificirali močnejše kandidate z vlogo pri patofiziologiji oz. pri spremenjeni endometrijski receptivnosti.

2.2.1.3.1 Močnejši kandidatni geni spremenjene endometrijske receptivnosti pri adenomiozi

Močnejše kandidatne gene spremenjene endometrijske receptivnosti pri adenomiozi smo identificirali na podlagi treh prekrivajočih obogatenih bioloških poti, ki smo jih pridobili z analizo setov 60 in 42 genov.

Preglednica 1: 20 najznačilnejših obogatenih poti 60 genov, anotiranih v biološka procesa *vgnezdenje zarodka* in *decidualizacija* v zbirki GO. Pridobljene biološke poti so nam služile kot vir znanja o splošnih procesih endometrijske receptivnosti.
Table 1: **Top 20 enriched pathways associated with 60 genes from the GO database that were annotated to biological processes of *embryo implantation* and *decidualization*.** Retrieved biological pathways served us as a source of knowledge on the general processes of endometrial receptivity.

Biološka pot	**Vred. *p***	**Anotirani geni**
GO:0007566~embryo implantation	2.55E-83	*DDR1, HMX3, CALCA, EPO, PRDM14, STC1, FBLN1, PTGS2, PCSK5, TRO, UBE2Q1, TRIM28, STC2, BSG, SPP1, IGFBP7, H3F3B, MMP2, MST1, LIF, ACOD1, EMP2, MMP9, PRLR, TGFBR2, SOD1, VMP1, UBTFL1, POLR1B, IL1B, SCGB1A1, RPL29, FKBP4, RECK, PPARD*
GO:0046697~decidualization	2.85E-41	*GHSR, CITED2, VDR, LIF, STC1, PTGS2, EPOR, CYP27B1, GJB2, STC2, BSG, SPP1, GHRL, JUNB, DEDD, CTSB, PPARD, MEN1*
GO:0033280~response to vitamin D	2.83E-07	*CYP27B1, STC2, SPP1, STC1, PTGS2*
GO:0071456~cellular response to hypoxia	1.39E-05	*STC2, MST1, STC1, PTN, PTGS2, PPARD*
GO:0043627~response to estrogen	5.59E-05	*CYP27B1, CITED2, EPO, GHRL, TGFBR2*
GO:0008285~negative regulation of cell proliferation	2.62E-04	*DDR1, CYP27B1, VDR, IL1B, LIF, IGFBP7, PTGS2, MEN1*
GO:0007507~heart development	2.99E-04	*CITED2, PTN, PCSK5, EPOR, TGFBR2, PPARD*
GO:0032147~activation of protein kinase activity	4.05E-04	*CALCA, EPO, EMP2, TGFBR2*
GO:0030198~extracellular matrix organization	4.09E-04	*DDR1, ITGB4, BSG, SPP1, FBLN1, RECK*
hsa04668:TNF signaling pathway	8.50E-04	*IL1B, LIF, PTGS2, JUNB, MMP9*
GO:0007267~cell-cell signaling	0.0013	*GJB2, CALCA, IL1B, NDP, ASH1L, PCSK5*
GO:0010628~positive regulation of gene expression	0.0015	*CITED2, VDR, IL1B, MST1, FBLN1, PPARD*
GO:0022617~extracellular matrix disassembly	0.0019	*MMP2, BSG, SPP1, MMP9*
GO:0032496~response to lipopolysaccharide	0.0019	*CYP27B1, EPO, SCGB1A1, PTGS2, JUNB*
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	0.0020	*TRIM28, CITED2, EPO, VDR, SCGB1A1, PRDM14, JUNB, PPARD, MEN1*
GO:0050728~negative regulation of inflammatory response	0.0021	*GHSR, ACOD1, GHRL, PPARD*
hsa04060:Cytokine-cytokine receptor interaction	0.0027	*EPO, IL1B, LIF, PRLR, EPOR, TGFBR2*
GO:0042493~response to drug	0.0029	*SCGB1A1, PTN, PTGS2, JUNB, SOD1, TGFBR2*
GO:0007420~brain development	0.0032	*HMX3, H3F3B, EPOR, TGFBR2, MEN1*
GO:0030336~negative regulation of cell migration	0.0035	*CITED2, STC1, PTN, RECK*

Z obogatitvijo seta 60 genov (anotirani v biološka procesa *vgnezdenje zarodka* in *decidualizacijo* v zbirki GO) smo pridobili 68 statistično značilnih poti ($p < 0,05$), katerih prvih 20 je naštetih v Preglednici 1. Z obogatitveno analizo 42 genov (združeni podatki spremenjeno izraženih genov v endometriju SS faze med ženskami z in brez adenomioze) pa smo pridobili 14 statistično značilnih bioloških poti ($p < 0,05$), ki so navedene v Preglednici 2. Med prvimi 20 potmi obeh analiz so se prekrivale poti *negativno uravnavanje celične proliferacije*, *organizacija zunajceličnega matriksa* in *decidualizacija*. Pripadajoči anotirani geni *IL10, SST, STAT3, LIF, CHD5, CDKN3, COL8A1, COL11A1, ITGB3* in *SPP1*, ki izhajajo iz seta 42 genov, predstavljajo močnejše kandidate spremenjene molekularne organizacije receptivnega endometrija za vgnezditev zarodka pri adenomiozi maternice. Identifikacija genetskih vzrokov spremenjene endometrijske receptivnosti pri adenomiozi bi lahko vodila v razvoj molekularnega testa, ki bi na podlagi izražanja izbranih genov omogočil boljšo diagnostiko endometrija kot morebiten vzrok neplodnosti pri takšnih ženskah. Poznavanje molekularnih odstopanj pa bi lahko vodilo v razvoj ciljnih terapij za optimalno pripravo endometrija pred naslednjim prenosom zarodka v maternico, da bi zvišali stopnjo zanositve v postopkih OBMP (Revel, 2012; Altmäe in sod., 2017).

Preglednica 2: **Obogatene poti 42 genov, povezanih s spremenjenim izražanjem v endometriju v času njegove receptivnosti (SS faza) med ženskami z in brez adenomioze.** Obogatene poti, ki so se ujemale z obogatitveno analizo 60 genov zbirke GO, so označene krepko. Anotirani geni teh poti predstavljajo močnejše kandidatne gene spremenjene endometrijske receptivnosti pri adenomiozi.

Table 2: Enriched pathways using 42 genes associated with altered endometrial expression levels in the time-frame of receptivity (MS phase) between women with and without adenomyosis. Enriched pathways that matched the results of the analysis of the 60 genes from the GO database are highlighted in bold. The annotated genes in these pathways present the stronger candidate genes of altered endometrial receptivity in adenomyosis.

Biološka pot	**Vred. *p***	**Anotirani geni**
GO:0008285~**negative regulation of cell proliferation**	0.0017	***IL10*, *SST*, *STAT3*, *LIF*, *CHD5*, *CDKN3***
GO:0008202~steroid metabolic process	0.0040	*AKR1B10, SULT1E1, CYP3A7*
hsa04630:Jak-STAT signaling pathway	0.0059	*IL10, STAT3, LIF, LIFR*
GO:0030574~collagen catabolic process	0.0087	*COL11A1, COL8A1, MMP20*
GO:0048861~leukemia inhibitory factor signaling pathway	0.0088	*LIF, LIFR*
GO:0030198~**extracellular matrix organization**	0.0091	***COL11A1*, *ITGB3*, *SPP1*, *COL8A1***
GO:0001503~ossification	0.013	*COL11A1, SPP1, LTF*
hsa04512:ECM-receptor interaction	0.021	*COL11A1, ITGB3, SPP1*
GO:0036376~sodium ion export from cell	0.022	*ATP1A2, ATP12A*
GO:0030007~cellular potassium ion homeostasis	0.026	*ATP1A2, ATP12A*
GO:0010248~establishment or maintenance of transmembrane electrochemical gradient	0.028	*ATP1A2, ATP12A*
GO:0006883~cellular sodium ion homeostasis	0.041	*ATP1A2, ATP12A*
GO:0048545~response to steroid hormone	0.043	*SST, SPP1*
GO:0046697~**decidualization**	0.043	***LIF*, *SPP1***

2.2.1.3.2 Močnejši kandidatni geni patofiziologije endometrija pri adenomiozi

Močnejše kandidatne gene patofiziologije endometrija pri adenomiozi smo identificirali na podlagi treh prekrivajočih bioloških poti, ki smo jih pridobili z obogatitvijo setov 78 in 518 genov. Z analizo seta 78 genov (združeni podatki 47 raziskav posameznih lokusov, ki so nam služili za identifikacijo patoloških poti endometrija v P fazi pri adenomiozi) smo pridobili 111 statistično značilnih poti ($p < 0,05$), katerih prvih 20 je naštetih v Preglednici 3. Z obogatitvijo seta 518 genov (združeni podatki spremenjeno izraženih genov treh transkriptomskih raziskav proliferacijskega endometrija med ženskami z in brez adenomioze) pa smo pridobili 135 statistično značilnih poti ($p < 0,05$), katerih prvih 20 je navedenih v Preglednici 4. Med prvimi 20 potmi obeh analiz so se prekrivale *pozitivno uravnavanje angiogeneze*, *negativno uravnavanje apoptoznih procesov* in *vnetni odziv*. Pripadajoči anotirani geni *ADM*, *AGER*, *ANXA1*, *BAG3*, *BCL3*, *BCL6*, *BIRC5*, *BTG1*, *CD14*, *CD44*, *CD74*, *CEBPB*, *CSF1*, *CXCL2*, *CYBB*, *CYR61*, *DUSP1*, *ECM1*, *EGR3*, *ELF3*, *EPHA2*, *F3*, *GATA6*, *GSTP1*, *HSPA5*, *IER3*, *ITGB2*, *LTF*, *MCL1*, *NFKB2*, *NFKBIA*, *NFKBID*, *PIM1*, *PIM3*, *PLAUR*, *PLK3*, *PTGIS*, *PTGS1*, *PYCARD*, *RHOB*, *S100A9*, *SELE*, *SERPINE1*, *SOCS3*, *SOX9*, *TAC4*, *THBS1*, *TNFAIP3*, *TWIST1*, *VEGFA*, *VHL* in *ZC3H12A*, ki izhajajo iz seta 518 genov, predstavljajo 52 močnejših kandidatnih genov patofiziologije adenomioze.

Identificiranim prekrivajočim obogatenim potem setov P faze smo lahko pripisali podporno literaturo v povezavi s patofiziologijo adenomioze:

- *pozitivno uravnavanje angiogeneze*: (Ota in Tanaka, 2003; Li in sod., 2006; Goteri in sod., 2009; Kang in sod., 2009; Liu in sod., 2011; Wang J. in sod., 2016),
- *negativno uravnavanje apoptoznih procesov*: (Jones in sod., 1998; Yang in sod., 2007; Ren in sod., 2010; Huang in sod., 2011; Wang J. in sod., 2016; Hu in sod., 2017; Li J. in sod., 2019),
- *vnetni odziv*: (Ota in sod., 2001b; Sotnikova in sod., 2002; Ulukus E.C. in sod., 2005, 2006; Wang F. in sod., 2009, 2014, 2018; Yang in sod., 2009; Nie in sod., 2009; Huang H. in sod., 2010; Qin in sod., 2012; Zhihong in sod., 2016; Lai in sod., 2016; Park in sod., 2016; Carrarelli in sod., 2017; Jiang in sod., 2017; Jiang J.F. in sod., 2018; Li C. in sod., 2019).

Med rezultati obogatitvenih analiz genskih setov P faze pa nismo zaznali poti, ki bi neposredno kazale na mehanizme nastanka adenomioze, podrobneje opisane v uvodnem poglavju. To so poti, ki kažejo na agresivnejše lastnosti endometrijskih celic (prekinitev celičnih stikov, prehod epitelijskih celic v mezenhimske celice, višja stopnja invazije in celične migracije), da lahko migrirajo in obstanejo v spodaj ležečem miometriju.

Preglednica 3: **20 najznačilnejših obogatenih bioloških poti, povezanih s patofiziologijo endometrija pri adenomiozi (EvEA).** Poti smo pridobili z obogatitveno analizo 78 genov. Gene smo pridobili z združevanjem podatkov 47 raziskav kandidatnih lokusov, ki so na vzorcih proliferacijskega endometrija poročali o značilnem spremenjenem izražanju med ženskami z in brez adenomioze.

Table 3: Top 20 enriched biological pathways associated with the pathophysiology of endometrium in adenomyosis (EvEA). The pathways were obtained by enrichment analysis of 78 genes. Genes were obtained by data synthesis of 47 published candidate locus studies that reported altered expression levels on proliferative endometrial tissue samples between women with and without adenomyosis.

Biološka pot	**Vred. *p***	**Anotirani geni**
GO:0010628~positive regulation of gene expression	6.63E-15	*CXCL8, ROCK1, ROCK2, IL37, NOS3, HDAC1, TWIST1, IGF1, F3, RELA, VEGFA, IL6, IL1B, CRH, TLR9, PGR, VIM, TLR6, TLR4, NFE2L2*
GO:0019221~cytokine-mediated signaling pathway	4.35E-12	*CXCL8, IL37, IL10RA, MMP2, TWIST1, PTGS2, F3, MMP9, RELA, VEGFA, IL6, CD4, IL1B, BCL2, VIM*
GO:0006954~inflammatory response	5.99E-12	*CXCL8, IL37, PTGS2, RELA, NFKB1, TLR1, IL6, CNR2, IL1B, CXCR2, CRH, TLR9, TLR8, TLR6, TLR4, NFE2L2*
GO:0030335~positive regulation of cell migration	1.27E-09	*ROCK2, IL1B, MMP2, SNAI1, SNAI2, ITGA6, IGF1, F3, MMP9, RHOA, PTK2, VEGFA*
GO:0032757~positive regulation of interleukin-8 production	2.30E-09	*TLR1, IL6, IL1B, TLR9, TLR8, F3, TLR4, RELA*
GO:0007568~aging	1.68E-08	*CNR1, MMP2, DNMT3A, CAT, CD68, PTGS2, RELA, NFKB2, SOD1, NFE2L2*
GO:0071222~cellular response to lipopolysaccharide	1.84E-08	*IL6, CXCL8, IL37, IL1B, VIM, CD68, TLR4, RELA, RHOA, NFKB1*
GO:0043066~negative regulation of apoptotic process	2.96E-08	*HDAC3, HDAC1, HSPB1, TWIST1, IGF1, MMP9, RELA, PTK2, NFKB1, VEGFA, NFKBIA, IL6, CAT, BCL2*
GO:0032755~positive regulation of interleukin-6 production	6.16E-08	*TLR1, IL6, IL1B, TLR9, TWIST1, TLR8, TLR6, TLR4*
GO:0043410~positive regulation of MAPK cascade	7.53E-08	*IL6, CD4, CDH2, ROCK1, ROCK2, TLR9, IGF1, SOD1, VEGFA*
GO:0006955~immune response	7.87E-08	*CXCL8, IL37, HLA-G, TLR1, CD4, CNR2, CXCR1, IL1B, CXCR2, TLR9, TLR8, TLR6, TLR4*
GO:0003180~aortic valve morphogenesis	8.01E-08	*ROCK1, ROCK2, NOS3, SNAI1, TWIST1, SNAI2*
GO:0045766~positive regulation of angiogenesis	8.30E-08	*CXCL8, NOS3, IL1B, HSPB1, TWIST1, F3, PAK4, NFE2L2, VEGFA*
GO:0009410~response to xenobiotic stimulus	1.23E-07	*CDH1, ITGA2, MMP2, DNMT3A, CAT, BCL2, PTGS2, RHOA, DUSP6, SOD1*
GO:0048661~positive regulation of smooth muscle cell proliferation	1.27E-07	*IL6, HDAC1, ITGA2, MMP2, IGF1, PTGS2, TLR4*
GO:0007249~I-kappaB kinase/NF-kappaB signaling	1.51E-07	*NFKBIA, ROCK1, ROCK2, TLR9, TLR8, TLR4, RELA*
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	4.54E-07	*HDAC3, HDAC1, TWIST1, IGF1, RELA, ESR2, NFKB1, ACVR2A, NFKB2, VEGFA, NFKBIA, IL6, IL1B, TLR9, ITGA6, PGR, TLR4, NFE2L2*
GO:0001934~positive regulation of protein phosphorylation	5.29E-07	*CD4, HDAC3, ROCK2, IL1B, CRH, MMP9, PTK2, ACVR2A, VEGFA*
GO:0042493~response to drug	6.00E-07	*CDH1, ITGA2, DNMT3A, CAT, CRH, BCL2, RELA, RHOA, DUSP6, SOD1*

Preglednica 4: 20 najznačilnejših obogatenih bioloških poti seta 518 genov, povezanih s spremenjenim endometrijskim izražanjem P faze med ženskami z in brez adenomioze. Ujemajoče se poti obogatitvene analize seta 78 genov so označene krepko. Anotirani geni teh poti predstavljajo močnejše kandidatne gene patofiziologije adenomioze.

Table 4: Top 20 enriched biological pathways of 518 genes, associated with altered endometrial expression in P phase between women with and without adenomyosis. Overlapping enriched pathways with enrichment analysis of a set of 78 genes are highlighted in bold. The annotated genes in these pathways are stronger candidate genes of pathophysiology in adenomyosis.

Biološka pot	**Vred. *p***	**Anotirani geni**
GO:0045766~**positive regulation of angiogenesis**	1.91E-06	*ECM1, PTGIS, BTG1, ITGB2, SERPINE1, GATA6, CYBB, TWIST1, ADM, F3, THBS1, RHOB, VEGFA, ZC3H12A*
GO:0070373~negative regulation of ERK1 and ERK2 cascade	5.43E-06	*ERRFI1, DUSP1, GSTP1, SPRY4, LIF, SPRY1, KLF4, GBP1, ATF3, DUSP6*
GO:0030198~extracellular matrix organization	9.63E-06	*ELN, ITGB2, ITGA1, SERPINE1, PDGFB, TNC, THBS1, CYR61, NFKB2, VCAN, ELF3, COL4A1, BCL3, COL4A5, ITGA7, SOX9, CD44*
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	2.17E-05	*CSRNP1, KDM3A, CEBPB, CEBPD, SRF, SERPINE1, GATA6, TWIST1, DUX4, DLL1, ETS2, CYR61, GLIS1, SERTAD1, ZC3H12A, NAMPT, SOX9, CCNL1, JUNB, JUN, EGR2, LIF, NFATC2, NFATC1, KLF4, POU5F1, KLF2, NFKB2, VEGFA, NR4A2, NFKBIA, FOSL1, PER1, NR4A1, NR4A3, KLF5, RGCC, ELF3, MAFF, BCL3, FOSB, NCOA7, MZF1, ATF3*
GO:0043066~**negative regulation of apoptotic process**	3.97E-05	*GSTP1, GATA6, TWIST1, THBS1, CYR61, SOCS3, BAG3, PIM1, PIM3, VHL, SOX9, MCL1, IER3, CD74, PLK3, EGR3, ANXA1, HSPA5, DUSP1, PLAUR, VEGFA, NFKBIA, BCL3, BIRC5, CD44, LTF*
GO:0006954~**inflammatory response**	1.45E-04	*ECM1, CEBPB, ANXA1, CSF1, ITGB2, CYBB, TNFAIP3, SELE, CXCL2, AGER, THBS1, PTGS1, NFKB2, PYCARD, ELF3, BCL6, ZC3H12A, TAC4, CD14, NFKBID, S100A9, EPHA2*
GO:0008284~positive regulation of cell proliferation	1.53E-04	*CSF1, PDGFB, TNC, ADM, IRS2, THBS1, DLL1, CTGF, CDC20, ESM1, SERTAD1, NAMPT, SOX9, EDN2, LIF, VEGFA, FOSL1, GDF9, KLF5, MZB1, BIRC5, CALR, ATF3, RNF187, HBEGF*
GO:0034097~response to cytokine	1.55E-04	*FOSL1, JUN, SRF, ACP5, TYMS, JUNB, NFKB2, MCL1*
GO:0045597~positive regulation of cell differentiation	1.61E-04	*SOCS3, JUN, SRF, VHL, JUNB, CYR61, CTGF*
GO:0043124~negative regulation of I-kappaB kinase/NF-kappaB signaling	2.51E-04	*PYCARD, PER1, IL1RL1, GSTP1, ZC3H12A, TNFAIP3, NFKBID*
hsa05166:HTLV-I infection	3.11E-04	*EGR2, JUN, SRF, ITGB2, PDGFB, SLC2A1, NFATC2, NFATC1, ETS2, NFKB2, NFKBIA, FOSL1, CDC20, ZFP36, POLD2, E2F2, CALR, HLA-DOB, ATF3*
hsa04380:Osteoclast differentiation	3.33E-04	*JUN, CSF1, IFNGR1, CYBB, NFATC2, NFATC1, NFKB2, NFKBIA, FOSL1, SOCS3, FOSB, ACP5, JUNB*
GO:0006915~apoptotic process	5.00E-04	*PPP1R15A, CSRNP1, ITGB2, TNFAIP3, DUX4, PYCARD, GJA1, ZC3H12A, PIM1, PMAIP1, PIM3, CD14, PHLDA2, IER3, PLK3, GADD45B, GADD45A, GZMA, C8ORF4, RHOB, NFKBIA, NR4A1, DLC1, MZB1, BIRC5, SGK1, S100A9*
GO:0051591~response to cAMP	5.47E-04	*FOSL1, THBD, PER1, JUN, DUSP1, FOSB, JUNB*

se nadaljuje

nadaljevanje Preglednice 4

Biološka pot	**Vred. *p***	**Anotirani geni**
GO:0061469~regulation of type B pancreatic cell proliferation	5.83E-04	*NR4A1, ERRFI1, NR4A3, BIRC5*
hsa04668:TNF signaling pathway	8.91E-04	*NFKBIA, SOCS3, CEBPB, JUN, CSF1, BCL3, LIF, TNFAIP3, SELE, CXCL2, JUNB*
GO:0001938~positive regulation of endothelial cell proliferation	9.01E-04	*NR4A1, ECM1, NRP2, JUN, EGR3, PDGFB, F3, VEGFA*
GO:0032691~negative regulation of interleukin-1 beta production	0.0012	*ERRFI1, GSTP1, ACP5, TNFAIP3*
GO:0006366~transcription from RNA polymerase II promoter	0.0014	*CSRNP1, EGR2, JUN, CEBPB, CEBPD, SRF, GATA6, NFATC2, NFATC1, KLF4, OVOL1, GLIS1, POU5F1, NFKB2, FOSL1, KLF5, ELF3, POU2AF1, MAFF, FOSB, SOX9, MZF1, JUNB, ATF3*

V prihodnje bi bilo potrebno izvesti analize izražanja predlaganih kandidatnih genov, da bi preverili njihov potencial za endometrijske molekularne označevalce patofiziologije adenomioze. Identifikacija takšnih označevalcev bi lahko pripomogla k hitrejši diagnozi zgodnje adenomioze, saj njena trenutna neinvazivna diagnoza kljub visoki ločljivosti aparatur TVUZ ali MR temelji le na subjektivni oceni slik maternice (Loring in sod., 2020). Iskanje endometrijskih molekularnih označevalcev že intenzivno poteka za zgodnejše in zanesljivejše diagnosticiranje endometrioze. Zaradi nespecifičnih simptomov je namreč za kirurško diagnozo endometrioze potrebnih kar 7 let (Gupta in sod., 2016; Ahn in sod., 2017; Saare in sod., 2017: 228).

2.2.2 Koekspresijska mreža lncRNA-mRNA iz eksperimentalnih podatkov RNA-seq

2.2.2.1 Uvod

LncRNA so definirane kot transkripti RNA, daljši od 200 nukleotidov, brez protein kodirajočega potenciala. Vloga lncRNA se povezuje z regulatornimi procesi. Delujejo lahko kot epigenetski regulatorji bodisi pozitivnega ali negativnega izražanja okoliških genov. Nekatere lncRNA so prekurzorji majhnih RNA (< 200 nukleotidov) ali narekujejo procesiranje ostalih RNA preko izrezovanja in spajanja transkriptov mRNA (angl. *mRNA splicing*). LncRNA delujejo tudi kot regulatorji aktivnosti proteinov in njihovih vezavnih partnerjev ali regulirajo lokalizacijo proteinov in celičnih komponent. LncRNA lahko vodijo proteine na specifična mesta na genomu in na tak način vplivajo na stopnjo izražanja genov (Wilusz in sod., 2009).

V raziskavah proliferacijskega endometrija tako med ženskami z in brez endometrioze (Wang Y. in sod., 2015a; Cui in sod., 2018) kot tudi med ženskami z in brez adenomioze (Jiang J.F. in sod., 2016) so že poročali o spremenjeno izraženih transkriptih lncRNA, ki so jo jih povezali s patofiziologijo endometrija pri teh dveh boleznih. Delovanje transkriptov lncRNA pa je bilo povezano tudi z uravnavanjem izražanja genov celične adhezije, ki sodelujejo v procesih endometrijske receptivnosti za vezavo zarodka (Fan in sod., 2017; Li D. in sod., 2019).

S sedanjo primerjavo podatkov RNA-seq receptivnih vzorcev endometrija med adenomiozno (n = 8) in kontrolno (n = 5) skupino žensk smo med 382 spremenjeno izraženimi geni (p < 0,05; FDR > 0,05) zaznali tudi 23 transkriptov lncRNA. Tem 23 lncRNA smo s korelacijsko analizo želeli preveriti soizražanje (angl. *coexpression*) s protein kodirajočimi transkripti (mRNA) iz eksperimentalnih podatkov RNA-seq. Naš namen je bil izdelati koekspresijsko mrežo mRNA-lncRNA in identificirati kandidatne pare lncRNA-mRNA z medsebojnim vplivom na izražanje.

2.2.2.2 Materiali in metode

Eksperimentalne RNA-seq podatke 13 vzorcev endometrija, ki so bili datirani v receptivno fazo, smo uporabili za izdelavo koekspresijske mreže kodirajočih (mRNA) in nekodirajočih (lncRNA) genov (angl. *coding-noncoding gene co-expression)*. Transkriptomski podatki so se nanašali na število poravnanih RNA-seq odčitkov na posamezen transkript, ki so bili normalizirani s pretvorbo v skalo štetja na milijon (CPM).

Za izdelavo mreže lncRNA-mRNA smo uporabili aplikacijo CoExpNetViz orodja Cytoscape v3.9.1 (Tzfadia in sod., 2016). V aplikaciji smo pod izbrane gene (angl. *bait genes*) vnesli 23 lncRNA (*LINC00645*, *LINC00861*, *FAM138B*, *GLCCI1-DT*, *LINC00649*, *MFSD4A-AS1*, *LINC00921*, *LINC02102*, *INTS6-AS1*, *ST7-AS1*, *CERNA1*, *ZBTB11-AS1*, *LINC00598*, *SH3BP5-AS1*, *DRAIC*, *CADM3-AS1*, *MRPS30-DT*, *SLC7A11-AS1*, *LINC00958*, *MCF2L-AS1*,

RNF144A-AS1, *SOCS2-AS1* in *KDM7A-DT*). Te lncRNA so bile spremenjeno izražene v setu 382 genov, ki smo jih identificirali s primerjavo RNA-seq podatkov potrjeno receptivnih vzorcev endometrija med adenomiozno in kontrolno skupino žensk. Izbranim lncRNA smo računali Pearsonov korelacijski koeficient, s čimer smo lahko določili njihove značilne korelacije s preostalimi transkripti podatkovnega seta RNA-seq. Minimalna in maksimalna meja kvartilov je znašala 1 oz. 99. Iskanje pomembnih žarišč znotraj pridobljene biološke mreže lncRNA-mRNA smo izvedli s funkcijo cytoHubba (Chin in sod., 2014) in algoritmom »degree«. Iskali smo 7 najvišje uvrščenih lncRNA ($p < 0,05$) z vrednostjo korelacij, ki se je najbolj nagibala proti -1 (negativna korelacija: izražanje prvega transkripta je povišano, izražanje drugega pa znižano oz. obratno) oz. 1 (pozitivna korelacija: izražanje prvega in drugega transkripta je povišano oz. znižano). Nato smo s funkcijo »display the expanded network« pridobili mrežo korelacij 7 najvišje uvrščenih transkriptov lncRNA s transkripti mRNA.

Biološko vlogo analiziranih lncRNA smo določevali na podlagi anotacije soizraženih mRNA. Večje klastre ciljnih genov posameznih žarišč smo z bioinformacijskim orodjem DAVID (Huang in sod., 2009) analizirali za obogatene biološke poti GO_BP, KEGG in/ali Reactome ($p < 0,05$).

2.2.2.3 Rezultat in diskusija

Z računanjem Perasonovega korelacijskega koeficienta med 23 spremenjeno izraženimi lncRNA in podatki celokupnega transkriptoma endometrija v receptivni fazi smo pridobili značilnih 2648 korelacij. Na Sliki 3 je predstavljena koekspresijska mrežna mRNA-lncRNA s skupno uvrščenimi 133 geni. Glede na vrednosti korelacije so bile najvišje uvrščene lncRNA *RNF144A-AS1*, *DRAIC*, *LINC02102*, *CADM3-AS1*, *SOCS2-AS1*, *LINC00921* in *KDM7A-DT*. Med temi 7 preiskovanimi lncRNA in ostalimi 126 transkripti RNA smo zaznali 257 parov. Od tega je bilo 212 parov v negativni, 45 pa v pozitivni korelaciji.

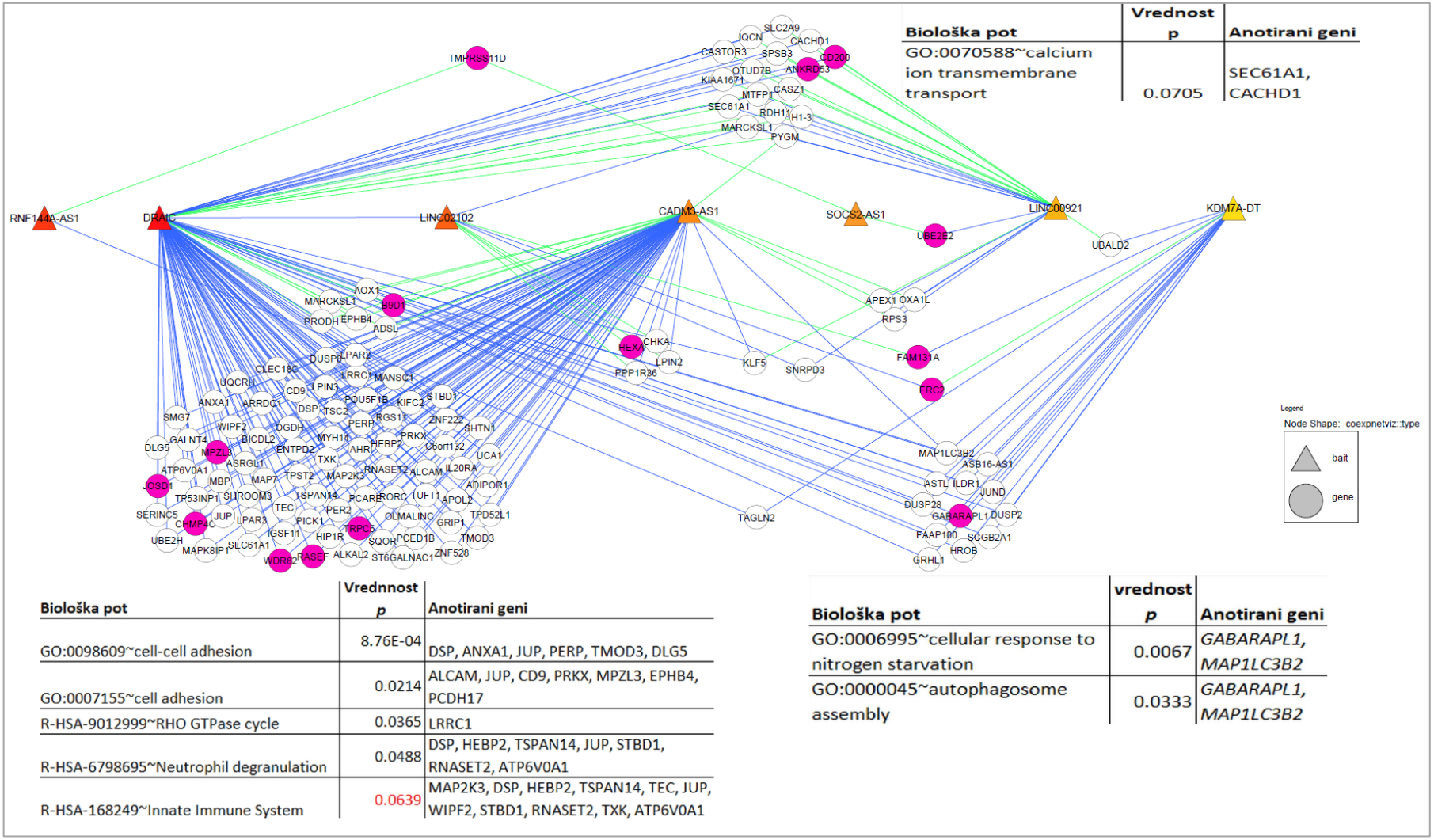
Med 126 uvrščenimi mRNA v mreži lncRNA-mRNA je bilo 15 genov, ki smo jih zaznali tudi kot spremenjeno izražene znotraj seta 382 genov naše analize RNA-seq. Npr. spremenjeno izražen gen *RASEF* iz seta 382 genov je v negativni korelaciji z izražanjem lncRNA *DRAIC* in *CADM3-AS1*. Gen *B9D1*, tudi spremenjeno izražen v setu 328 genov, pa je v pozitivni korelaciji z izražanjem *DRAIC* in *CADM3-AS1*. Identificirali smo pozitivni korelaciji med transkriptoma mRNA *UBE2E2* oz. *TMPRSS11D* in lncRNA *SOCS2-AS1*. Oba ciljna gena sta bila tudi spremenjeno izražena v setu 382 genov.

V mreži lncRNA-mRNA se kaže, da transkripta lncRNA *DRAIC* in *CADM3-AS1* predstavljata pomembni vozlišči, kar se kaže kot pretežna negativna korelacija z izražanjem ciljnih mRNA. Da bi lahko predlagali biološko vlogo omenjenih lncRNA, smo izvedli obogatitveno analizo klasta njunih ciljnih genov. Skupno smo pridobili 7 obogatenih poti GO-BP ($p < 0,05$), ki so bile povezane s celično adhezijo in organizacijo aktinskih filamentov.

Pridobili smo tudi tri obogatene poti ($p < 0,05$) zbirke Reactome, ki so bile povezane s ciklom RHO GTPaz. Pridobljeni rezultati so v skladu z literaturo iz področja endometrijske receptivnosti, v kateri so opisali vlogo lncRNA pri uravnavanju izražanja genov celične adhezije (Fan in sod., 2017; Li D. in sod., 2019). Iz zbirke Reactome pa smo z obogatitveno analizo klastra ciljnih genov transkriptov lncRNA *DRAIC* in *CADM3-AS1* identificirali tudi biološko pot *prirojen imunski sistem*, ki pa ni bila statistično značilna ($p = 0,064$). Ta pot je še posebej zanimiva, saj je bila večina 382 spremenjeno izraženih genov naše RNA-seq analize anotiranih v biološke procese, povezane z IFN, ki je del prirojenega imunskega odziva.

Z obogatitveno analizo klastra ciljnih genov transkriptov lncRNA *DRAIC* in *KDM7A-DT* smo identificirali pet obogatenih poti GO-BP ($p < 0,05$), povezanih s celičnim odzivom na pomanjkanje dušika in mitofagijo. Tudi iz zbirke KEGG smo identificirali značilno pot, povezano z mitofagijo. Z obogatitveno analizo klastra ciljnih genov transkriptov lncRNA *DRAIC* in *LINC00921* pa smo identificirali le eno neznačilno pot GO-BP *transmembranski transport kalcijevega iona*.

S sedanjo bioinformacijsko analizo smo pokazali, da bi vloga lncRNA *DRAIC* in *CADM3-AS1* pri adenomiozi lahko bila povezana s procesom adhezije. Potrebne so dodatne analize teh kandidatnih lncRNA, da bi lahko bolje ovrednotili, ali adenomioza preko epigenetskih regulatorjev vpliva na adhezivne lastnosti endometrija.



Biološka pot	Vrednost p	Anotirani geni
GO:0070588~calcium ion transmembrane transport	0.0705	SEC61A1, CACHD1

Biološka pot	Vrednnost p	Anotirani geni
GO:0098609~cell-cell adhesion	8.76E-04	DSP, ANXA1, JUP, PERP, TMOD3, DLG5
GO:0007155~cell adhesion	0.0214	ALCAM, JUP, CD9, PRKX, MPZL3, EPHB4, PCDH17
R-HSA-9012999~RHO GTPase cycle	0.0365	LRRC1
R-HSA-6798695~Neutrophil degranulation	0.0488	DSP, HEBP2, TSPAN14, JUP, STBD1, RNASET2, ATP6V0A1
R-HSA-168249~Innate Immune System	0.0639	MAP2K3, DSP, HEBP2, TSPAN14, TEC, JUP, WIPF2, STBD1, RNASET2, TXK, ATP6V0A1

Biološka pot	vrednost p	Anotirani geni
GO:0006995~cellular response to nitrogen starvation	0.0067	*GABARAPL1, MAP1LC3B2*
GO:0000045~autophagosome assembly	0.0333	*GABARAPL1, MAP1LC3B2*

Slika 3: Koekspresijska mreža sedmih izbranih transkriptov lncRNA in njihovih 126 ciljnih transkriptov na eksperimentalnih podatkih RNA-seq. Geni za lncRNA so označene s trikotniki, preostali geni pa s krogom. Glede na ocenjevalni sistem vozlišč funkcije cytoHubba so najvišje uvrščeni transkripti lncRNA obarvani z rdečo, sledijo oranžni in nato rumeni. Izmed vseh 257 pridobljenih parov lncRNA-mRNA jih je 212 v negativni (robovi mreže obarvani z modro) in 45 v pozitivni (robovi mreže obarvani z zeleno) korelaciji. Z vijolično barvo je obarvanih 15 genov, ki smo jih identificirali tudi kot spremenjeno izražene v setu 382 genov s primerjavo podatkov RNA-seq potrjeno receptivnih vzorcev endometrija med adenomiozno in kontrolno skupino.
Figure 3: The coexpression network for selected seven lncRNA transcripts and their 126 target transcripts on experimental RNA-seq data. Given genes for lncRNAs are indicated by triangles and the remaining genes by a circle. According to the node scoring system of cytoHubba function, the highest ranked lncRNA transcripts are coloured red, followed by orange and then yellow. Of the 257 lncRNA-mRNA pairs retrieved, 212 are in negative (network edges coloured with blue) and 45 in positive (network edges coloured with green) correlation. 15 genes of the network are coloured purple and were also identified as differentially expressed in the set of 382 genes by comparison of RNA-seq data of confirmed receptive endometrial samples between adenomyosis and control groups.

3 RAZPRAVA IN SKLEPI

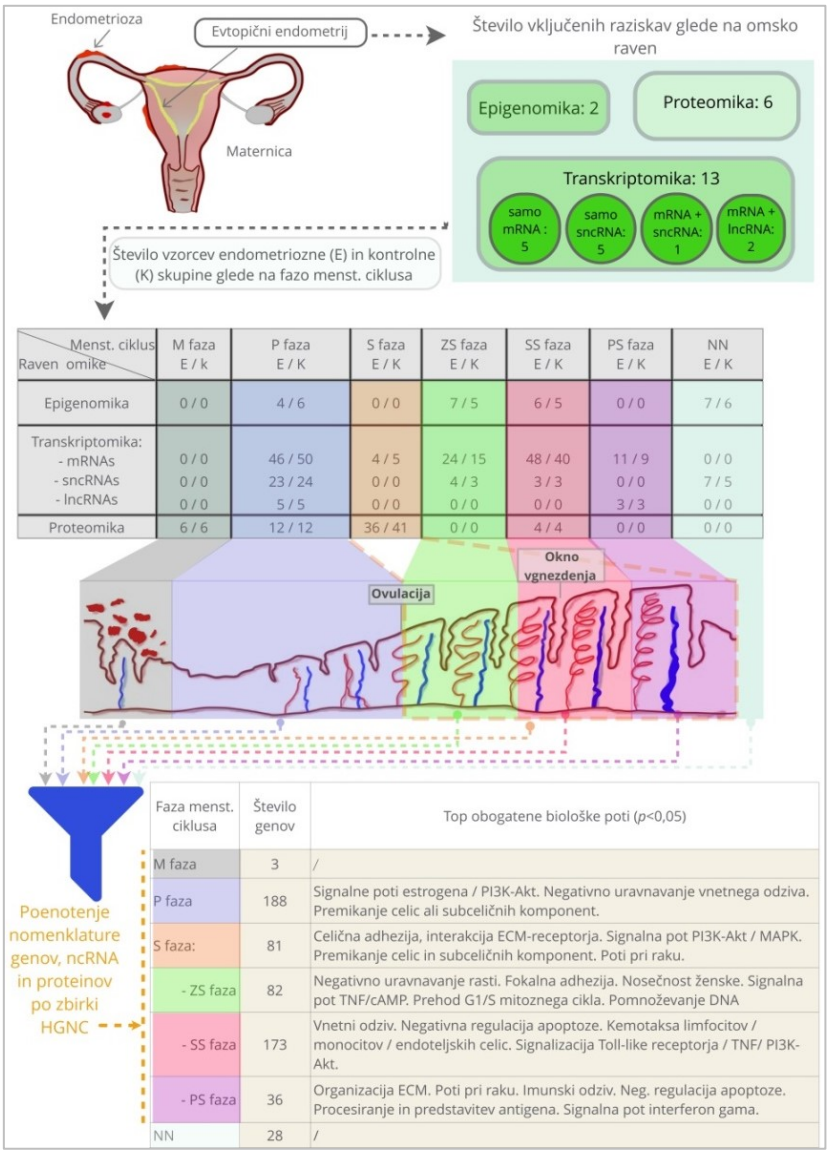
3.1 RAZPRAVA

3.1.1 Poenotenje heterogenega poimenovanja genetskih lokusov

S poenotenjem poimenovanja transkriptov RNA in proteinov, ki smo jih pridobili s pregleda literature, povezane z endometrijskim izražanjem pri različnih ginekoloških stanjih, smo lahko izvajali obogatitvene analize in predlagali močnejše kandidatne biološke poti in gene spremenjene endometrijske receptivnosti pri adenomiozi.

Najprej smo s poenotenjem genske nomenklature in obogatitvenimi analizami prikazali vpliv sorodne, a bolje raziskane endometrioze, na molekularno organizacijo endometrija po posameznih fazah menstruacijskega ciklusa (Prašnikar in sod., 2020a). Potek dela raziskave je predstavljen na Sliki 4. Iz 21 raziskav različnih omskih ravni smo zbrali genetske vzroke, ki se povezujejo z odstopanji v izražanju endometrija pri ženskah z endometriozo v primerjavi z ženskami brez endometrioze. Iz vsake raziskave smo pridobili do 15 statistično najznačilnejših znižano in/ali prekomerno izraženih mRNA, ncRNA in/ali proteinov. Skupno smo pridobili 760 lokusov, ki so bili poimenovani po različnih nomenklaturnih sistemih. Lahko so bili poimenovani s številko Gene ID zbirke NCBI (NCBI Gene, 2019), številko EST (angl. *expression sequence tag*) z oznako Hs (*homo sapiens*) zbirke NCBI UniGene (NCBI UniGene, 2019), številko SeqName ID (Ensembl BioMart. 2019), številko MIMAT zbirke MirBase (miRBase, 2019), številko proteina zbirke UniProt (The UniProt ..., 2019), številko GenInfo zbirke NCBI protein (NCBI GenBank, 2019), s starim poimenovanjem ali s sinonimom. Da smo lahko izvedli sintezo podatkov glede na datirano fazo menstruacijskega ciklusa endometrija, v kateri je bilo zaznano spremenjeno izražanje, smo morali najprej poenotiti heterogeno poimenovanje 760 lokusov. To smo storili tako, da smo jim ročno poiskali genske simbole zbirke HGNC, ki podeljuje uradne simbole humanim genom (Braschi in sod., 2019). Na tak način smo pridobili genske simbole za 623 lokusov. Po odstranitvi podvojenih genov smo unikatnih 591 genov razvrstili glede na poročano datirano fazo menstruacijskega ciklusa (M, P, S, ZS, SS in PS faza ter N/N, če podatek o datiranju ni bil naveden). S sortiranjem 623 genov smo identificirali 72 genov (med drugimi *ACTB*, *ANXA4*, *BPIFB1*, *EPHX1*, *MUC5B*, *PRDX2*, *VIM*, *HSP90AB1*, *ANLN*, *CCN1*, *CRISP3*, *EGR1*, *FOS*, *FOSB* in *TRPM6*), ki so se ponovili v več fazah ciklusa oz. v isti fazi ciklusa pri različnih raziskavah. Te gene smo izpostavili kot močnejše kandidatne za endometrijske molekularne označevalce endometrioze, kar bi v prihodnosti pripomoglo k hitrejši diagnozi te bolezni (Prašnikar in sod., 2020a). Endometriozo namreč označujejo nespecifični simptomi, kar otežuje njeno diagnozo (Gupta in sod., 2016; Ahn in sod., 2017; Saare in sod., 2017: 228). Z obogatenjem genskih setov smo identificirali poti, povezane z odstopanji v fiziološkem zorenju endometrija glede na posamezne faze menstruacijskega ciklusa. Predvsem so nas zanimale obogatene poti 173 genov SS faze, ko se pojavi stanje endometrijske receptivnosti. Identificirali smo poti *nosečnost*, *kemotaksa endotelijskih celic*, *kemotaksa monocit*,

kemotaksa limfocitov, *vnetni odziv*; in *signalna pot, vodena s kemokini*. Pridobljene poti kažejo na porušeno celično komunikacijo pri endometriozi, kar je v skladu z literaturo. Lee in sod. (2011) so namreč opisali, da je delovanje kemokinov in citokinov pomembno pri zvišanju števila celic naravne in pridobljene imunosti v endometriju, kjer so potrebne za uspešno vgnezdenje zarodka in vzdrževanje nosečnosti. Po drugi strani se porušeno delovanje citokinov in imunskega sistema povezuje s patogenezo endometrioze (Matarese in sod., 2003). Tudi z obogatitvijo seta 382 spremenjeno izraženih genov med adenomiozno in kontrolno skupino izvedene analize RNA-seq smo identificirali poti, povezane s citokini imunskega sistema (odzivi na signalizacijo z IFN) (Prašnikar in sod., 2022). Pokazali smo, da bi lahko adenomioza in endometrioza do neke mere na podoben način vplivali na biološke procese, povezane z endometrijsko receptivnostjo za vgnezdenje zarodka.

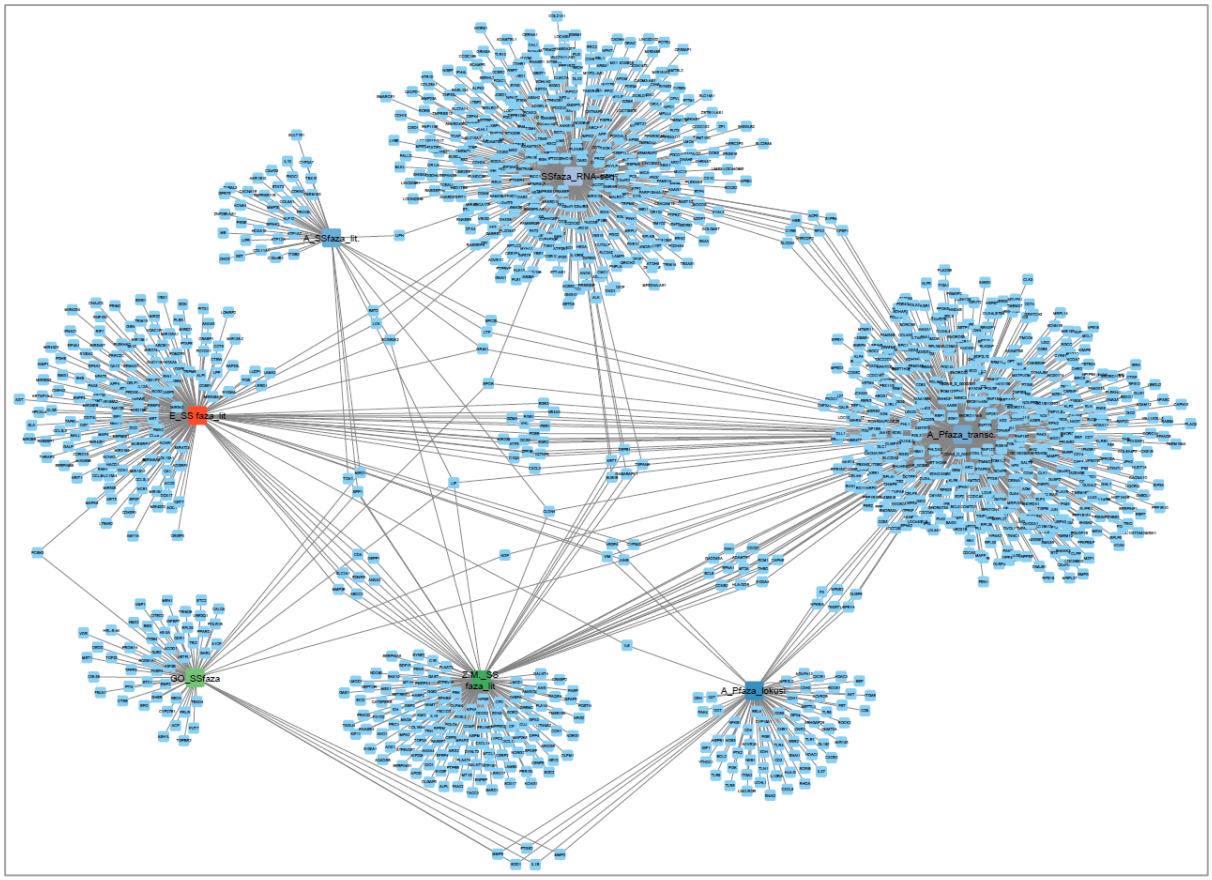


Slika 4: Potek pridobivanja znanja o molekularnih odstopanjih endometrija pri endometriozi.
Figure 4: Workflow of a study on molecular abnormalities of the endometrium in endometriosis.

Poenotenje poimenovanja po zbirki HGNC (HUGO, 2019) smo izvedli tudi za preiskovane lokuse pridobljenih 110 raziskav endometrija pri adenomiozi. Tudi tu smo pridobljene gene združevali glede na poročano datirano fazo menstruacijskega ciklusa (P in SS faza) in jih dalje obogatili. Na podlagi identificiranih poti smo predlagali 52 močnejših kandidatov patofiziologije endometrija (med drugimi *ANXA1*, *BCL3*, *BCL6*, *BIRC5*, *BTG1*, *CD14*, *CD44*, *CD74*, *CEBPB*, *CXCL2*, *CYR61*, *DUSP1*, *ECM1*, *EGR3*, *ELF3*, *EPHA2*, *HSPA5*, *IER3*, *ITGB2*, *LTF*, *MCL1*, *NFKB2*, *NFKBIA*, *NFKBID*, *RHOB*, *S100A9*, *SOCS3* in *VEGFA*) in 10 močnejših kandidatov spremenjene endometrijske receptivnosti (*IL10, SST, STAT3, LIF, CHD5, CDKN3, COL8A1, COL11A1, ITGB3* in *SPP1*) pri tej bolezni. Poznavanje vloge posameznih endometrijskih genov pri adenomiozi bi lahko vodilo v identifikacijo molekularnih označevalcev, povezanih s to boleznijo. Endometrijski molekularni označevalci patofiziologije bi lahko olajšali diagnozo zgodnje adenomioze, ki trenutno temelji na subjektivni oceni TVUZ ali MR slik maternice (Hershko-Klement in Tepper, 2016; Loring in sod., 2020). Endometrijski molekularni označevalci spremenjenega stanja receptivnosti pri adenomiozi pa bi omogočili boljšo diagnostiko endometrija kot morebiten dejavnik neplodnosti pri teh bolnicah. Nepoznavanje molekularnega ozadja endometrijske receptivnosti je dejavnik, ki omejuje uspešnost postopkov OBMP (Mahajan in sod., 2018; Sebastian-Leon in sod., 2018).

Zaradi poenotenja poimenovanja smo lahko med posameznimi oblikovanimi genskimi seti (zbrani podatki iz pregleda literature in rezultati izvedene analize RNA-seq) preverili prekrivanje genov (Slika 5). Uporabljeni geni setov doktorske disertacije so navedeni v Prilogi A. Preverili smo prekrivanje genskih setov, ki smo jih sestavili iz poročanih podatkov o endometrijski receptivnosti pri ženskah z adenomiozo (42 genov) in ženskah z endometriozo (173 genov). Oblikovali smo tudi set 151 genov, poimenovan zdrava maternica (normalni pogoji endometrijske receptivnosti), ki smo ga pridobili z združenjem seznamov genov, povezanih z endometrijsko receptivnostjo, prvega komercialnega molekularnega testa (Díaz-Gimeno in sod., 2011) in meta analize 9 transkriptomskih raziskav (Altmäe in sod., 2017). V povezavi z normalnimi pogoji endometrijske receptivnosti smo oblikovali tudi set 60 genov iz zbirke GO, ki so bili anotirani v procese vgnezdenja zarodka in/ali decidualizacijo. Vključili smo tudi seta, povezana s spremenjenim endometrijskim izražanjem v P fazi pri adenomiozi, ki smo ju oblikovali z združevanjem genov raziskav kandidatnih lokusov (78 genov) in transkriptomskih raziskav (518 genov). V primerjavo pa smo vključili še set 382 genov, ki smo jih identificirali kot spremenjeno izražene med adenomiozno in kontrolno skupino v primerjavi RNA-seq podatkov potrjeno receptivnih vzorcev. Med setoma 382 in 151 genov se je prekrivalo pet genov (*KRT7*, *DEFB1*, *TSPAN8*, *GABARAPL1* in *BUB1B*). Med setoma 328 in 173 so se prekrivali *BST2*, *LCK* in *SCGB2A2*. Slednji je bil skupen še s setom 151 genov. Setoma 382 in 518 genov je bilo skupnih 9 genov (*CPSF1*, *S1PR4*, *ACP5*, *RFX2*, *HERC2P2*, *CYBB*, *SLC2A3*, *HBB* in *EPOR*). Kar 19 genov (*ERG3*, *NR4A3*, *RGS1*, *GZMA*, *VHL*, *PER1*, *FOSB*, *MIR339*, *SOCS3*, *EGR2*, *ATF3*, *ZFP36*, *C1QA*, *C1QTNF6*, *CYP3A5*, *CXCL2*, *JUNB*, *VEGFA* in *VIM*) je bilo skupnih setoma 173 in 518

genov (oba seta sta bila oblikovana na podlagi združevanja podatkov omskih raziskav). Zanimivo je, da ni skupnega gena med setoma 60 in 151 genov, saj se oba navezujeta na procese endometrijske receptivnosti. Med setoma 151 in 518 genov je bilo skupnih 13 genov (*DKK1*, *CDC20*, *GADD45A*, *ADAMTS1*, *BCL6*, *EFNA1*, *MT2A*, *ECM1*, *CAPN6*, *THBD*, *S100A4*, *HLA-DOB* in *CCNB2*). To bi lahko nakazovalo vpliv patofizioloških procesov endometrija na njegovo stanje receptivnosti pri adenomiozi.



Slika 5: Prekrivanje genov uporabljenih setov v naši raziskavi. Posamezni seti se navezujejo na gene, pridobljene s poenotenjem HGNC genske nomenklature podatkov s pregleda literature (Set A_SSfaza_lit: 42 genov. Set A_Pfaza_transc.: 518 genov. Set A_Pfaza_lokusi: 78 genov; Set Z.M._SSfaza_lit: 151 genov. Set GO_SSfaza: 60 genov in Set E_SSfaza_lit: 173 genov) in gene, identificirane kot spremenjeno izražene s primerjavo RNA-seq podatkov potrjeno receptivnih vzorcev med adenomiozno in kontrolno skupino (Set A_SSfaza_RNA-seq: 382 genov). Uporabljene kratice: ZM. = zdrava maternica; P faza = proliferacijska faza menstruacijskega ciklusa; SS faza = srednja sekrecijska faza menstruacijskega ciklusa; lit. = literatura; transc. = transkriptomske analize; GO = genska ontologija; E = endometrioza.

Figure 5: **Gene overlap between used sets in our study**. The individual sets refer to genes obtained by adoption of the HGNC gene nomenclature for extracted data by the literature mining (Set A_SSfaza_lit: 42 genes. Set A_Pfaza_transc.: 518 genes. Set A_Pfaza_lokusi: 78 genes; Set ZM._SSfaza_lit: 151 genes. Set GO_SSfaza: 60 genes and Set E_SSfaza_lit: 173 genes) and genes identified as differentially expressed by comparing RNA-seq data of confirmed receptive endometrial samples between the adenomyosis and the control group (Set A_SSfaza_RNA-seq: 382 genes). Abbreviations used/legend: ZM. = healthy uterus; P faza = the proliferative phase of the menstrual cycle; SS faza = the mid secretory phase of the menstrual phase; lit. = literature; transc. = transcriptomics analyses; GO = Gene Ontology; E = endometriosis.

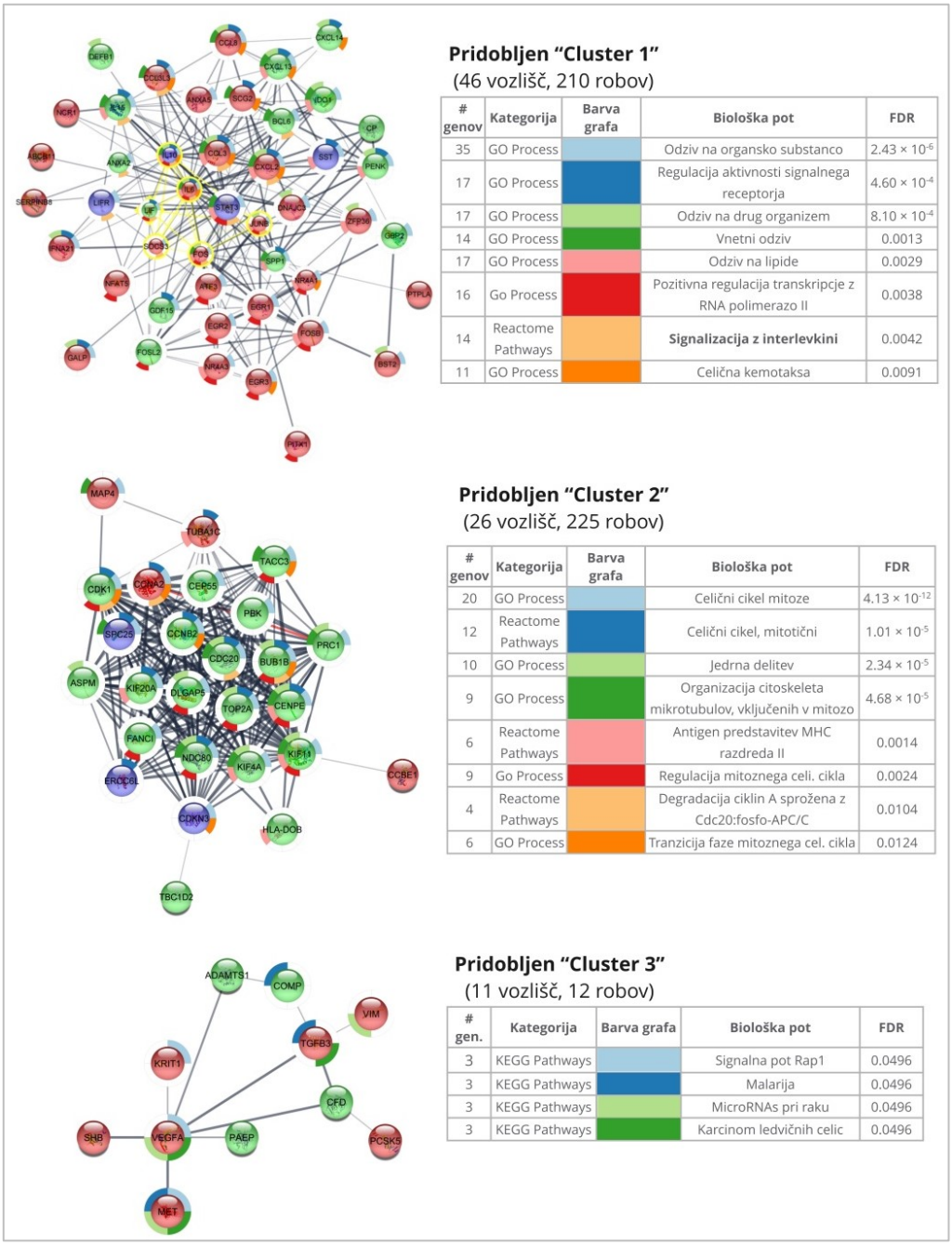
3.1.2 Identifikacija in preverjanje izražanja izbranih kandidatnih genov spremenjene endometrijske receptivnosti pri adenomiozi

V objavljeni raziskavi (Prašnikar in sod., 2020b) smo z dvema bioinformacijskima analizama predlagali 6 kandidatnih genov (*JUNB*, *IL10*, *IL6*, *SOCS3*, *FOS* in *LIF*) spremenjene endometrijske receptivnosti pri adenomiozi in dalje preverili njihovo izražanje na biopsijah endometrija žensk z in brez adenomioze. Za obogatitvene analize smo uporabili podatke iz pregleda literature, ki smo jih oblikovali v genske sete, povezane z endometrijsko receptivnostjo pri ženskah z adenomiozo (42 genov), ženskah z endometriozo (173 genov) in ženskah z zdravo maternico (151 genov).

Pri prvi bioinformacijski analizi smo v zbirki bioloških poti Reactome (Fabregat in sod., 2016) obogatili samo genska seta adenomiozne (42 genov) in endometriozne (173 genov) skupine. Pridobili smo pet prekrivajočih poti, vključno *signalizacija z IL4 in IL13*. Anotirane gene *IL10*, *STAT3*, *LIF*, *SOCS3*, *IL6*, *VIM*, *FOS*, *JUNB*, *VEGFA* in *HSP90B1* te poti smo uporabili kot vir kandidatov za analizo izražanja. Pri drugi bioinformacijski analizi pa smo poleg adenomioznega (42 genov) in endometrioznega (173 genov) seta uporabili še gene zdrave maternice (151 genov). Z aplikacijo STRING (Szklarczyk in sod., 2019) orodja Cytoscape (Shannon in sod., 2003) smo gene vseh treh setov projicirali v biološko mrežo proteinskih interakcij (angl. *protein-protein interaction network*). Tako smo pridobili mrežo s 315 vozlišči (proteini) in 1130 robovi (interakcije), ki smo jo dalje gručali in identificirali tri večje klastre, ki so prikazani na Sliki 6. Ker smo v orodju Cytoscape vsak set genov označili z drugačno barvo, smo lahko v mreži in klastrih sledili izvoru posameznih anotiranih genov. Opazili smo, da so bili v t.i. Cluster 1 anotirani geni vseh treh setov, kar smo interpretirali kot spremenjena molekularna organizacija endometrijske receptivnosti zaradi prisotne ginekološke patologije. Z obogatitveno analizo tega klastra smo identificirali poti *vnetni odziv*, *celična kemotaksa*, *odziv na druge organizme/organske substance/lipide*, *regulacija aktivnosti signalnega receptorja* in *signalizacija z IL*. Slednjo pot z anotiranimi geni *LIF*, *LIFR*, *STAT3*, *IL15*, *JUNB*, *FOS*, *SOCS3*, *ANXA2*, *BCL6*, *IL6*, *IL10*, *CXCL2*, *CCL3* in *CCL3L3* smo izbrali kot drugi vir kandidatnih genov za analizo izražanja.

Končno smo med anotiranimi geni poti *signalizacija z IL4 in IL13* in *signalizacija z IL* izbrali šest kandidatnih genov, in sicer *JUNB*, *SOCS3*, *IL6*, *LIF*, *IL10* in *SOCS3*. Izbranim genom smo s qPCR preverili stopnjo izražanja v biopsijah endometrija žensk z (n = 9) in brez (n = 13) ultrazvočnih znakov adenomioze. Biopsije endometrija smo pridobili v pričakovanem stanju receptivnosti, in sicer med 7. in 9. dnem po izmerjenem vrhu hormona LH v urinu, ki naznanja ovulacijo preiskovanke (dnevi LH+7–LH+9). Izražanje izbranih genov smo normalizirali z geometrijsko sredino izražanja referenčnih genov *GAPDH* in *18S rRNA*. Končno razliko v relativnem izražanju izbranih genov med preiskovanima skupinama smo preračunali z metodo $2^{-\Delta\Delta Cq}$ (Livak in Schmittgen, 2001). Zaznali smo znižano stopnjo

izražanja preiskovanih genov pri adenomiozni skupini (Slika 7), vendar je bila razlika neznačilna.

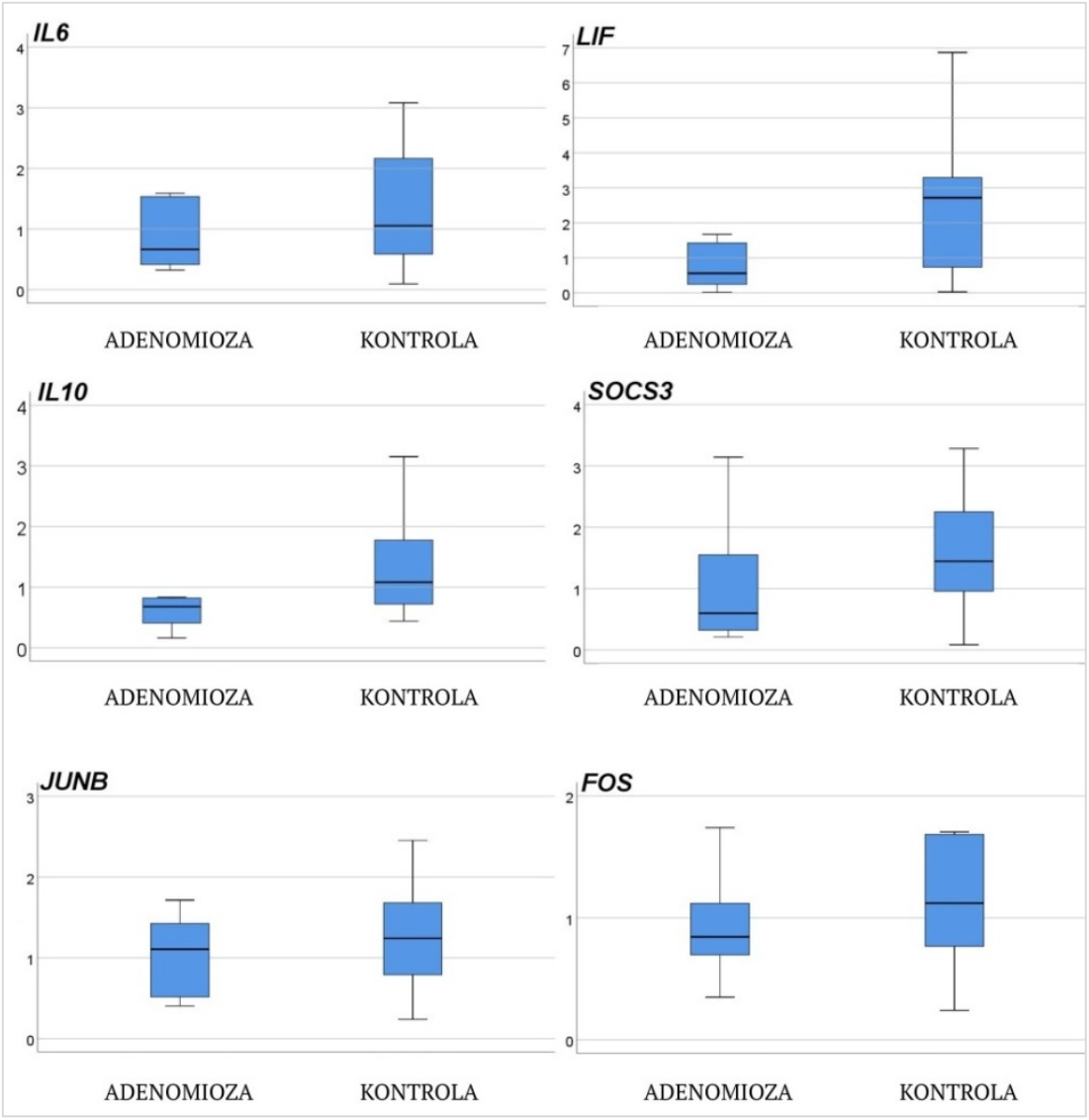


# genov	Kategorija	Barva grafa	Biološka pot	FDR
35	GO Process		Odziv na organsko substanco	2.43×10^{-6}
17	GO Process		Regulacija aktivnosti signalnega receptorja	4.60×10^{-4}
17	GO Process		Odziv na drug organizem	8.10×10^{-4}
14	GO Process		Vnetni odziv	0.0013
17	GO Process		Odziv na lipide	0.0029
16	Go Process		Pozitivna regulacija transkripcje z RNA polimerazo II	0.0038
14	Reactome Pathways		**Signalizacija z interlevkini**	0.0042
11	GO Process		Celična kemotaksa	0.0091

# genov	Kategorija	Barva grafa	Biološka pot	FDR
20	GO Process		Celični cikel mitoze	4.13×10^{-12}
12	Reactome Pathways		Celični cikel, mitotični	1.01×10^{-5}
10	GO Process		Jedrna delitev	2.34×10^{-5}
9	GO Process		Organizacija citoskeleta mikrotubulov, vključenih v mitozo	4.68×10^{-5}
6	Reactome Pathways		Antigen predstavitev MHC razredа II	0.0014
9	Go Process		Regulacija mitoznega celi. cikla	0.0024
4	Reactome Pathways		Degradacija ciklin A sprožena z Cdc20:fosfo-APC/C	0.0104
6	GO Process		Tranzicija faze mitoznega cel. cikla	0.0124

# gen.	Kategorija	Barva grafa	Biološka pot	FDR
3	KEGG Pathways		Signalna pot Rap1	0.0496
3	KEGG Pathways		Malarija	0.0496
3	KEGG Pathways		MicroRNAs pri raku	0.0496
3	KEGG Pathways		Karcinom ledvičnih celic	0.0496

Slika 6: **Identificirani klastri po gručanju mreže proteinskih interakcij.** Proteini mreže se nanašajo na zbrane gene, povezane z endometrijsko receptivnostjo pri adenomiozi (n = 42 genov; označeni z modro barvo), endometriozi (n = 173 genov; označeni z rdečo barvo) in zdravi maternici (n = 151 genov; označeni z zeleno barvo). Z rumeno barvo so v t.i. Cluster 1 označeni izbrani geni za qPCR.
Figure 6: **Identified clusters by clustering of the protein-protein interactions network.** The proteins of the network refer to the gathered genes associated with endometrial receptivity in adenomyosis (n = 42 genes; highlighted in blue), endometriosis (n = 173 genes; highlighted in red) in a healthy uterus (n = 151 genes; highlighted in green). The genes selected for qPCR are highlighted in yellow in the so-called Cluster 1.



Slika 7: **Relativno izražanje izbranih kandidatnih genov *JUNB*, *FOS*, *SOCS3*, *LIF*, *IL6* in *IL10* v endometriju pričakovanega stanja receptivnosti med ženskami z in brez adenomioze.**
Figure 7: Relative expression of selected candidate genes *JUNB*, *FOS*, *SOCS3*, *LIF*, *IL6*, and *IL10* in the endometrium of the expected receptive state between women with and without adenomyosis.

Kljub neznačilni razliki v izražanju izbranih kandidatnih genov v naši analizi, pa v literaturi citokine, kamor spadajo tudi IL, navajajo kot pomembne dejavnike uspešnega vgnezdenja zarodka (Chaouat in sod., 2007). Kot že omenjeno so citokini pomembni pri začetni adheziji zarodka, nadaljnjem preurejanju zunajceličnega matriksa decidue in pri oblikovanju ožilja za zarodek (Chaouat in sod., 2007). Citokini sodelujejo pri uravnavanju lokalnega imunskega sistema maternice, s čimer zavarujejo zarodek pred imunskim napadom matere. Med S fazo menstruacijskega ciklusa decidua stimulira pritok različnih populacij imunskih celic: uNK, limfociti in makrofagi (Flynn in sod., 2000; Okada in sod., 2018). Celice T pomagalke (angl. *helper T*, Th) izločajo citokine tipa 2 (IL-4, IL-13, IL-5, IL-9 in IL-10), ki delujejo spodbudno

na invazijo trofoblasta v deciduo (Veenstra van Nieuwenhoven in sod., 2003). Neravnovesje v izražanju citokinov naj bi vplivalo na število in delovanje imunskih celic v endometriju, kar bi lahko bil eden izmed vzrokov neplodnosti (Chaouat in sod., 2007). Za izbrane kandidatne gene naše kvantifikacijske analize smo našli veliko podporne literature v povezavi z endometrijsko receptivnostjo.

3.1.2.1 Gen *LIF*

Citokin LIF je glikoprotein, ki spodbuja decidualizacijo endometrijske strome pri človeku in miši (Shuya in sod., 2011). Chung in sod. (2016) so v kulturi EEC opazili povišano raven izražanja adhezijskih molekul *ITGAV*, *ITGB3* in *ITGB5* (potrebnih za pritrditev trofoblasta zarodka na epitelij endometrija) potem, ko so dodajali LIF (Chung in sod., 2016). Serafini in sod. (2009) so ženskam, pri katerih so v biopsijah sekrecijskega endometrija zaznali močno imunohistokemijsko barvanje za LIF, napovedali za 6,4 % višjo stopnjo zanositve v postopkih OBMP (Serafini in sod., 2009). Pri ženskah z nepojasnjeno neplodnostjo (Franasiak in sod., 2014) in pri ženskah z adenomiozo (Xiao in sod., 2010; Yen in sod., 2016) pa so že poročali o znižanih vrednostih izražanja endomertijskega *LIF* in pripadajočega proteina, kar je bilo povezano z zmanjšano endometrijsko receptivnostjo za vezavo zarodka pri teh ženskah.

3.1.2.2 Gen *IL10*

Protivnetni citokin IL-10 deluje kot negativni regulator aktivacije makrofagov in limfocitov T (Kühn in sod., 1993). V zgodnji nosečnosti se povišano izražanje IL-10 povezuje z vzdrževanjem imunotolerance matere do zarodka (Thaxton in Sharma, 2010). Wang in sod. (2018) so znižane vrednosti izražanja IL-10 v času endometrijske receptivnosti pri ženskah z adenomiozo povezali z vplivom na zmanjšano izražanje pripadajočega HOXA10, to pa na znižano stopnjo endometrijsko receptivnost pri teh ženskah. Po drugi strani pa so Wang F. in sod. (2009) poročali o prekomernem izražanju IL-10 v EEC pri adenomiozi v primerjavi s kontrolno skupino, kar so povezali z nenormalnim vnetnim odzivom in patogenezo te bolezni.

3.1.2.3 Gen *IL6*

Citokin IL-6 je vključen v akutne faze vnetnega odziva, zorenje limfocitov B, diferenciacijo makrofagov in Th tipa 1/2 (Diehl in Rincón, 2002). Domneva se, da je pri ženskah z rednimi menstruacijskimi ciklusi izražanje IL-6 najnižje v P fazi, nato pa postopoma raste z vrhom v SS fazi (Von Wolff, 2000). Zhihong in sod. (2016) so v času okna vgnezdenja po kontrolirani stimulaciji jajčnikov zaznali višje ravni izražanja endometrijskega IL-6 pri ženskah z adenomiozo v primerjavi s kontrolno skupino, kar so povezali z ovirano endometrijsko receptivnostjo. Yang in sod. (2006) so poročali o zvišani ravni izražanja *IL6* v primarni kulturi ESC žensk z adenomiozo potem, ko so jih gojili skupaj z makrofagi. Prekomerno izločanje IL-6 so avtorji povezali s povišano stopnjo proliferacije stromalnih in vaskulatornih

celic, kar spodbuja tvorbo in obstoj adenomioznih lezij na ektopičnih mestih (Yang in sod., 2006). Po drugi strani pa so Ponce in sod. (2009) pri ženskah z endometriozo zaznali zmanjšano izražanje endometrijskega *IL6* in IL6 v PS v primerjavi z ženskami brez te bolezni. To so povezali z odpornostjo endometrija na apoptozne procese, kar bi lahko pripomoglo k razvoju in napredovanju endometrioze (Ponce in sod., 2009).

3.1.2.4 Gena *FOS* in *JUNB*

Proteina FOS in JUNB sta podenoti, ki dimerizirata in tvorita kompleks prepisovalnega dejavnika, aktivator protein 1 (angl. *activator protein 1*). Ta prepisovalni dejavnik nadzira izražanje genov celičnega cikla (proliferacija, diferenciacija, apoptoza in odziv na stres), (Jochum in sod., 2001). Baiyong in sod. (1999) so pokazali, da lahko spremenjeno izražanje proteina JUNB vpliva na diferenciacijo populacij limfocitov Th v tip 1 ali v tip 2. Najprej so iz JUNB-pozitivne transgene miši izolirali naivne CD4 limfocite T in jih dalje diferencirali v populacijo celic Th1. Zaradi prekomernega izražanja JUNB pa so te celice izločale citokin IL-4, ki ga običajno izločajo celice Th2 (Baiyong in sod., 1999). V sekrecijskem endometriju žensk z endometriozo so poročali o prekomernem izražanju tako *JUNB* (Absenger in sod., 2004; Tamaresis in sod., 2014) kot tudi *FOS* (Tamaresis in sod., 2014) in FOS (Pan in sod., 2008), kar so povezali z vlogo pri patofiziologiji te bolezni. Po drugi strani pa Morsch in sod. (2009) niso opazili razlik v stopnji fosforilacije FOS med ženskami z in brez endometrioze.

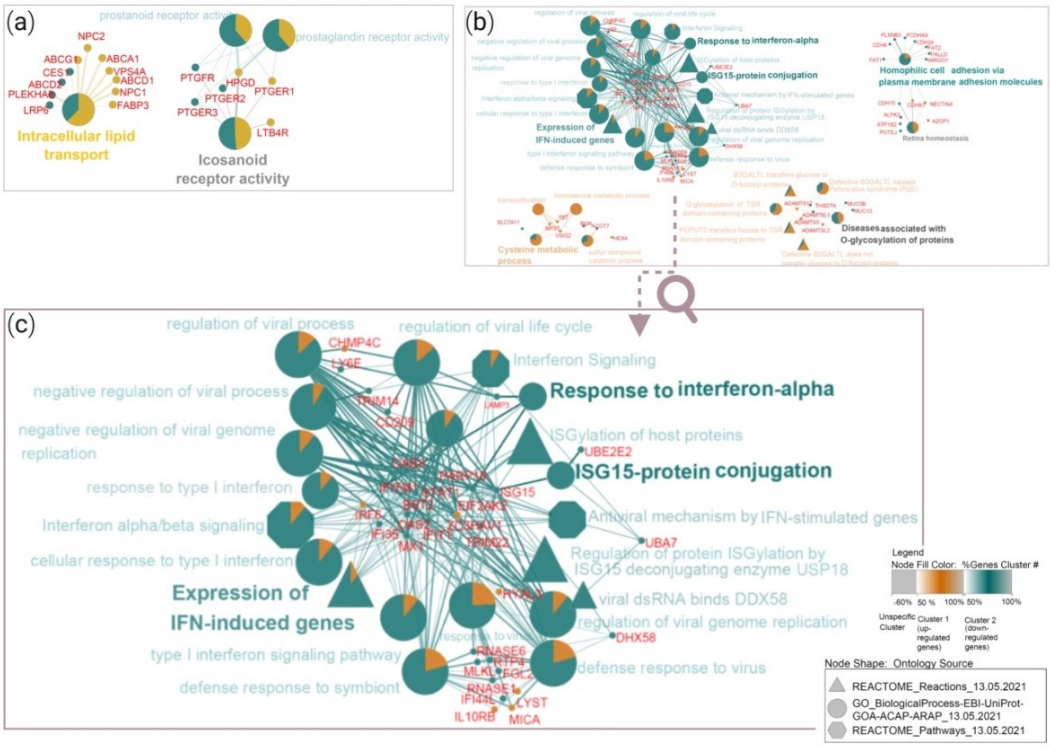
3.1.2.5 Gen *SOCS3*

Znotrajcelični protein SOCS3 sodeluje pri negativnem uravnavanju vnetnega odziva, saj zavre signalizacijo citokinov (Lang in sod., 2003). Dong in sod. (2009) so predlagali, da bi lahko zmanjšana signalizacija SOCS3 zvišala vnetni odziv na trofoblast placente (angl. *placental trophoblast*), kar vodi v preeklampsijo. Na celični kulturi trofoblasta placente JEG-3 so namreč pokazali, da je prekomerno izražanje SOCS3 vodilo v povišano stopnjo izločanja IL-10, ki deluje spodbudno za nadaljnjo nosečnost (Dong in sod., 2009). Braunschweig in sod. (2011) pa so pokazali, da utišanje *SOCS3* vodi v zvišano citotoksičnost kulture celic NK-92, ki predstavljajo celični model uNK.

3.1.3 Transkriptom endometrija v potrjeno receptivni fazi pri adenomiozi

Analizo RNA-seq sekvenciranja transkriptoma (mRNA in lncRNA) endometrija v pričakovanem stanju receptivnosti smo izvedli pri 10 ženskah z adenomiozo in 10 ženskah brez patologije rodil. Identificirali smo 909 spremenjeno izraženih genov (vrednost p (linearni model in empirični Bayes) < 0,05; vrednost p po testu FDR > 0,05). Te gene smo nato obogatili z aplikacijama ClueGO (Bindea in sod., 2009) in CluePedia (Bindea in sod., 2013) v orodju Cytoscape in pridobili štiri statistično značilne (Bonnferoni p vrednost < 0,05) biološke poti. Z aplikacijama so bile poti razvrščene v mrežo s funkcionalno urejenimi

skupinami glede na podobno biološko vlogo. S setom 909 genov smo identificirali le dve takšni skupini (Slika 8a), in sicer *znotrajcelični transport lipidov* (angl. *intracellular lipid transport*) in *aktivnost ikosanoidnega receptorja* (angl. *icosanoid receptor activity*).



Slika 8: **Mreže s funkcionalno urejenimi skupinami bioloških poti, ki so bile obogatene s setoma 909 (a) in 382 (b) spremenjeno izraženih genov med adenomiozno in kontrolno skupino v analizah podatkov RNA-seq.** a) Štiri obogatene poti, razvrščene v dve funkcionalni skupini, smo identificirali z analizo 909 genov. Set 909 genov smo identificirali s primerjavo podatkov RNA-seq vseh 20 vzorcev endometrija, ki smo jih pridobili med dnevi LH+7 in LH+9 (10 vzorcev adenomiozne in 10 vzorcev kontrolne skupine). b) Skupno 33 obogatenih poti, razvrščenih v sedem funkcionalnih skupin, smo identificirali z analizo 382 genov. Set 382 genov smo identificirali po ponovni analizi podatkov RNA-seq, kjer smo upoštevali samo potrjeno receptivne vzorce endometrija (8 iz adenomiozne in 5 iz kontrolne skupine) glede na rezultate natančnega datiranja receptivne faze. Povečava mreže (c) tesno prepletenih poti, povezanih z mehanizmi odziva na delovanje IFN. Z zeleno barvo so označene poti z anotiranimi geni zmanjšanega izražanja v adenomiozni skupini. Z oranžno barvo pa so označene poti z anotiranimi geni prekomernega izražanja v adenomiozni skupini. S sivo barvo so označene poti z enakomernim deležem anotiranih genov znižanega in genov povišanega izražanja.

Figure 8: Networks with functionally sorted groups of biological pathways enriched by 909 (a) and 382 (b) genes that were differentially expressed between the adenomyosis and the control groups in two RNA-seq data analyses. a) Four enriched pathways, sorted into two functional groups, were identified with the analysis of 909 genes. The 909 gene set was identified by comparing the RNA-seq data of all 20 endometrial samples obtained between days LH+7 and LH+9 (10 samples from the adenomyosis group and 10 samples from the control group). b) A total of 33 enriched pathways, classified into seven functional groups, were identified with the analysis of 382 genes. The set of 382 genes was identified after re-analysis of the RNA-seq data, where only confirmed receptive endometrial samples (8 from the adenomyosis group and 5 from the control group) were considered according to the results of the precise dating of the receptive phase. Magnification of the network of (c) tightly connected pathways associated with mechanisms of response to IFN action. Pathways with annotated downregulated genes in the adenomyosis group are highlighted in green. Pathways annotated with upregulated genes the adenomyosis group are highlighted in orange. Pathways with an equal proportion of annotated down- and up-regulated genes are highlighted in grey.

Identifikacija visokega števila (n = 909) spremenjeno izraženih genov, vendar nizkega števila obogatenih bioloških poti (n = 4), bi lahko bila posledica uporabe nehomogenih vzorcev glede na stadij fiziološkega razvoja endometrija ob času vzorčenja biopsije. To pomeni, da bi se lahko vzorci endometrija preiskovank v trenutku vzorčenja biopsije nahajali v različnih stadijih S faze menstruacijskega ciklusa, kljub temu, da je bilo vzorčenje izvedeno med dnevi LH+7 in LH+9, ko se pojavi pričakovani čas receptivnosti. Poročajo namreč, da časovni pojav in trajanje receptivne faze v SS fazi ni enak pri vseh ženskah, kot se je to menilo včasih. Receptivna faza se lahko pojavi prej ali kasneje od pričakovane in traja različno dolgo (Ruiz-Alonso in sod., 2013; Tan in sod., 2018). V postopkih OBMP prenos blastociste v maternico v času nereceptivnega endometrija prepreči njeno uspešno vezavo na epitelij (Sebastian-Leon in sod., 2018). Tudi pri ženskah z adenomiozo so poročali o časovnem zamiku stanja receptivnosti znotraj menstruacijskega ciklusa, kar bi lahko bila posledica nižjega deleža zanositve pri zdravljenju neplodnosti (Mahajan in sod., 2018). Dodatno, Devesa-Peiro in sod. (2021) so z analizo dostopnih transkriptomskih podatkov pokazali, da je podpis endometrijskih patologij na transkriptom endometrija zamaskiran pri primerjavi vzorcev endometrija različnih faz menstruacijskega ciklusa. Opazili so, da imajo na transkriptomsko analizo med primerjalnima skupinama večji vpliv spreminjajoče se faze menstruacijskega ciklusa, kot pa naj bi bil vpliv sopojavne patologije. Pri iskanju endometrijskih molekularnih označevalcev patologij s pomočjo visoko občutljivih omskih tehnik so avtorji poudarili pomen natančnega datiranja endometrija ob času vzorčenja (Devesa-Peiro in sod., 2021). Zaradi teh navedenih argumentov smo frakcijo vsake izolirane endometrijske RNA, ki smo jo uporabili za RNA-seq, uporabili še za personalizirano datiranje receptivne faze menstruacijskega ciklusa ob času vzorčenja biopsije. Natančna klasifikacija receptivne faze vzorcev je bila opravljena z novim molekularnim orodjem beREADY® (Saare in sod., 2019). Orodje na podlagi vzorca izražanja 57 endometrijskih genov določi stanje receptivne faze, kar pa omogoča načrtovanje optimalnega časa prenosa zarodkov v maternico s postopki OBMP (Saare in sod., 2019). Z rezultati testiranja receptivne faze smo ugotovili, da vsi vzorci niso bili pridobljeni v optimalni receptivni fazi. Od skupno 20 vzorcev jih je bilo 13 v receptivni fazi, 2 sta bila v zgodnji in 5 v pozni receptivni fazi.

Analizo pridobljenih podatkov RNA-seq smo ponovili z upoštevanjem rezultatov natančnega datiranja receptivne faze vzorcev endometrija. V ponovni analizi RNA-seq smo upoštevali le transkriptomske podatke 13 vzorcev potrjeno receptivne faze (8 iz adenomiozne in 5 iz kontrolne skupine). Transkriptomske podatke preostalih 7 mejno receptivnih vzorcev smo izključili iz analize. S tem smo preprečili vpliv ZS oz. PS faze menstruacijskega ciklusa na transkriptomsko analizo, povezano z endometrijsko receptivnostjo (SS faza). Z analizo samo potrjeno receptivnih vzorcev med adenomiozno in kontrolno skupino smo identificirali 382 spremenjeno izraženih genov (vrednost *p* (linearni model in empirični Bayes) < 0,05; vrednost *p* po testu FDR > 0,05). Ti geni so bili obogateni v statistično značilnih (Bonnferoni *p* vrednost < 0,05) 33 poteh (20 poti anotiranih v ontologijo GO_BP, 6 v zbirko Reactome Pathways in 7 v Reactome Reactions). Izmed 33 poti, se jih je kar 19 nanašalo na poti,

povezane z mehanizmi odziva na IFN (Slika 8c). Skupno 33 poti s pripadajočimi anotiranimi geni je bilo razvrščenih v mrežo s 7 funkcionalno urejenimi skupinami (Slika 8b). V aplikaciji ClueGO je možna tudi vizualizacija deleža anotiranih genov posameznega seta genov. V primeru, da več kot 60 % anotiranih genov izhaja iz posameznega genskega seta, je obogatena pot/funkcionalna skupina mreže obarvana s predhodno določeno barvo tega seta. V primeru, da anotirani geni obogatene poti/funkcionalne skupine v enakomernem deležu izhajajo iz različnih genskih setov, potem je ta pot/funkcionalna skupina obarvana sivo (Bindea in sod., 2009). Med identificiranimi 382 spremenjeno izraženimi geni izvedene analize RNA-seq smo v aplikacijo posebej vnesli zvišano (n = 166) in posebej znižano (n = 216) izražene gene v adenomiozni skupini. Od skupno 7 funkcionalnih skupin so bili v štirih večinoma anotirani geni znižanega izražanja: *izražanje genov, induciranih z IFN* (angl. *expression of IFN-induced genes*; anotirani geni *BST2*, *IFI35*, *IFIT1*, *IFITM1*, *ISG15*, *MX1*, *OAS2*, *OAS3* in *STAT1* izraženi v zmanjšani, *IRF6* pa v zvišani stopnji); *odziv na interferon alfa* (angl. *response to interferon-aplha*; anotirani *BST2*, *EIF2AK2*, *IFITM1* in *LAMP3*); *konjugacija proteina ISG15* (angl. *ISG15-protein conjugation*; anotirani *ISG15*, *UBA7* in *UBE2E2*) in *homofilna celična adhezija preko adhezivnih molekul membrane plazme* (angl. *homophilic cell adhesion via plasma membrane adhesion molecules*; anotirani *AMIGO1*, *CDH15*, *CDH24*, *CDH6*, *FAT1*, *FAT2*, *PALLD*, *PCDHA9* in *PLXNB3* izraženi v zmanjšani, *CDHR1* in *NECTIN4* v zvišani stopnji). V funkcionalno skupino *procesi metabolizma cisteina* (angl. *cysteine metabolic process*) pa so bili anotirani *MPST*, *TST* in *VSIG2* povišano izraženi, *SLC7A11* se je izražal v zmanjšani stopnji. Identificirali pa smo tudi dve funkcionalni skupini s primerljivima deležema anotiranih tako povišano kot znižano izraženih genov: *bolezni, povezane z O-glikozilacijo proteinov* (angl. *diseases associated with O-glycosylation of proteins*; anotirani *ADAMTS17*, *ADAMTS5* in *ADAMTSL2* izraženi v povišani, *ADAMTSL1*, *MUC13*, *MUC5B* in *THSD7A* v znižani stopnji) in *homeostaza retine* (angl. *retina homeostasis*; anotirani *AZGP1*, *CDHR1* in *NECTIN4* izraženi v povišani, *ALPK3*, *ATP1B2*, *CDH15* in *POTEJ* v znižani stopnji).

Identificirana obogatena pot *homofilna celična adhezija preko adhezivnih molekul membrane plazme* z večino anotiranih genov, ki so bili znižano izraženi pri adenomiozni v primerjavi s kontrolno skupino, bi lahko nakazovala na zmanjšano kapaciteto adhezivnosti endometrija pri tej bolezni. Molekule celične adhezije (integrini, kaderini, selektini in imunoglobulini) so pomembni dejavnik receptivnosti endometrija, saj zvišajo adhezivne lastnosti epitelija, kamor se lahko pritrdi blastocista (Achache in Revel, 2006).

Glede na analizo z aplikacijama ClueGO/CluePedia so funkcionalne skupine *izražanje genov, induciranih z IFN*, *odziv na interferon alfa* in *konjugacija proteina ISG15* povezani procesi, kar se znotraj biološke mreže kaže kot tesno prepletanje poti s skupnimi anotiranimi geni (Slika 8b in 8c). Te biološke poti, ki smo jih identificirali s pomočjo RNA-seq in natančnim datiranjem receptivne faze vzorcev endometrija, predlagamo kot močnejše kandidatne poti za nadaljnje raziskave endometrijske receptivnosti pri adenomiozi. Tem biološkim potem,

povezanim z IFN, smo našli podporno literaturo tako iz področja vgnezdenja zarodka kot iz patofiziologije adenomioze.

3.1.3.1 Vloga IFN pri fiziološkem razvoju endometrija

Pri procesu vgnezdenja zarodka se vloga IFN povezuje z vzdrževanjem lokalnega vnetnega okolja, ki spodbuja migracijo trofoblasta v deciduo do žilnega sistema matere (Hannan in sod., 2011; Gnainsky in sod., 2014). Popovici in sod. (2000) so povišano izražanje genov vnetnih citokinov (vključno tip I IFN alfa/beta) povezali z vključevanjem limfocitov in makrofagov v deciduo. De Veer in sod. (2001) so na podlagi analiz z mikromrežami ugotovili, da družina citokinov IFN sproži izražanje številnih genov, vključenih v protivirusne, protiproliferacijske in imunosupresijske procese. Pot prenosa signala se prične po vezavi IFN na površinske receptorje, ki sprožijo aktivacijo družine proteinov JAK (angl. *janus kinase*). Aktivirane kinaze JAK fosforilirajo družino prepisovalnih dejavnikov STAT, ti pa se dalje vežejo v homo- ali heterodimere in tvorijo komplekse z drugimi prepisovalnimi dejavniki. Skupaj se vežejo na odzivne elemente, stimulirane z IFN (angl. *IFN-stimulated response elements*, ISRE), ki se nahajajo na promotorski regiji genov, stimulirani z IFN (angl. *IFN-stimulated genes*, ISGs) in aktivirajo njihovo transkripcijo. Poznanih je več kot 300 ISGs (De Veer in sod., 2001). Ubikvintinu podoben protein ISG15 je potranskripcijski preoblikovalec, ki se lahko v procesu isgilacije (angl. *ISGylation*) kovalentno veže na različne proteine. Delovanje ISG15 se povezuje s številnimi celičnimi procesi in stanji (translacija proteinov, dinamika citoskeleta, izločanje eksosoma, avtofagija, stabilnost genoma in rak) in je močen kandidat ciljnih terapevtskih pristopov (Jiménez Fernández in sod., 2020). Protein ISG15 lahko deluje kot znotrajcelični ali kot izvencelični protein. Znotrajcelično izražanje ISG15, ki je odvisno od signalnih poti tipa 1 IFN alfa/beta, označuje prirojeni imunski odziv na virusne in mikrobne patogene. Zunajcelično izražanje ISG15 pa lahko pri limfocitih izzove izločanje IFN gama, citokina tipa 2 (Swaim in sod., 2017). Raziskave na miših so pokazale, da ima pospešeno izražanje ISG15 med zgodnjo nosečnostjo pomembno vlogo pri razvrščanju celic uNK, ki so vključene v preoblikovanje spiralnih arterij za normalen dotok krvi plodu (Austin in sod., 2003). Poročali so že o spremenjeni ravni izražanja nekaterih citokinov v pričakovanem času endometrijske receptivnosti pri ženskah z adenomiozo (Zhihong in sod., 2016) in ženskah z endometriozo (Kharfi in sod., 2003; Ulukus M. in sod., 2005). Citokini, vključeni v proces decidualizacije stromalnih celic, uravnavajo prehod iz provnetnega v protivnetni odziv (Wilczyński, 2005). Prehod v ravnovesju citokinov se povezuje tudi z uravnavanjem ravnovesja med endometrijsko receptivnostjo in selektivnostjo, s čimer se omogoči vgnezdenje samo visoko kakovostnih zarodkov (kromosomsko stabilni zarodki z dobrimi invazivnimi lastnostmi) (Macklon in Brosens, 2014). Porušeno ravnovesje med receptivnostjo in selektivnostjo endometrija pa je bilo povezano z vgnezdenjem tudi zarodkov slabše kvalitete, kar pa lahko vodi v prekinitev nosečnosti (Quenby in sod., 2002). Višjo stopnjo splavov opažajo tudi pri ženskah z adenomiozo v primerjavi z ženskami brez adenomioze (Martinez-Conejero in sod., 2011; Stanekova in sod., 2018). Tako morda

obogatene poti odziva na delovanje IFN, ki smo jih identificirali z geni zmanjšanega izražanja izvedene analize RNA-seq, kažejo na slabšo endometrijsko selektivnost žensk z adenomiozo, kar omogoča vgnezdenje tudi slabših zarodkov, a vodi v splav.

3.1.3.2 Vloga IFN pri patofiziologiji adenomioze

Sotnikova in sod. (2002) so poročali o povišanih vrednostih vnetnih citokinov IFNG, IFNA, TNF, IL1B in epidermalnega rastnega dejavnika (EGF) pri ženskah z adenomiozo v primerjavi v zdravimi ženskami, potem ko so analizirali supernatant 24 ur gojenih mononuklearnih celic (limfociti in makrofagi) endometrija PS faze. Avtorji so lokalno povišano sintezo citokinov v endometriju povezali s spodbujanjem celične proliferacije, kar vodi v razvoj adenomioze (Sotnikova in sod., 2002). Tremellen in Russell (2012) pa sta pri ženskah s hudo obliko adenomioze in ponavljajočimi neuspešnimi poskusi vgnezdenja zarodka poročala o povišanih vrednostih celic uNK in makrofagov v funkcionalni plasti endometrija PS faze, ko sta jih primerjala z ženskami z blago adenomiozo ali z zdravimi ženskami. To sta povezala z negostoljubnim imunskim okoljem endometrija zaradi bolezni, kar lahko ovira uspešno vgnezditev zarodka (Tremellen in Russell, 2012).

Na podlagi rezultatov obogatitvenih analiz setov 909 in 382 genov, ki smo ju identificirali z izvedeno analizo RNA-seq ob upoštevanju rezultatov natančnega datiranja endometrija in podporne literature sklepamo, da set 382 genov bolj reprezentativno predstavlja podpis adenomioze na transkriptom endometrija v receptivni fazi, kot pa sprva pridobljenih 909 genov. Da bi razumeli vlogo teh 382 genov v povezavi z molekularnimi procesi endometrijske receptivnosti, smo jih dalje integrirali z zbranimi podatki iz pregleda literature.

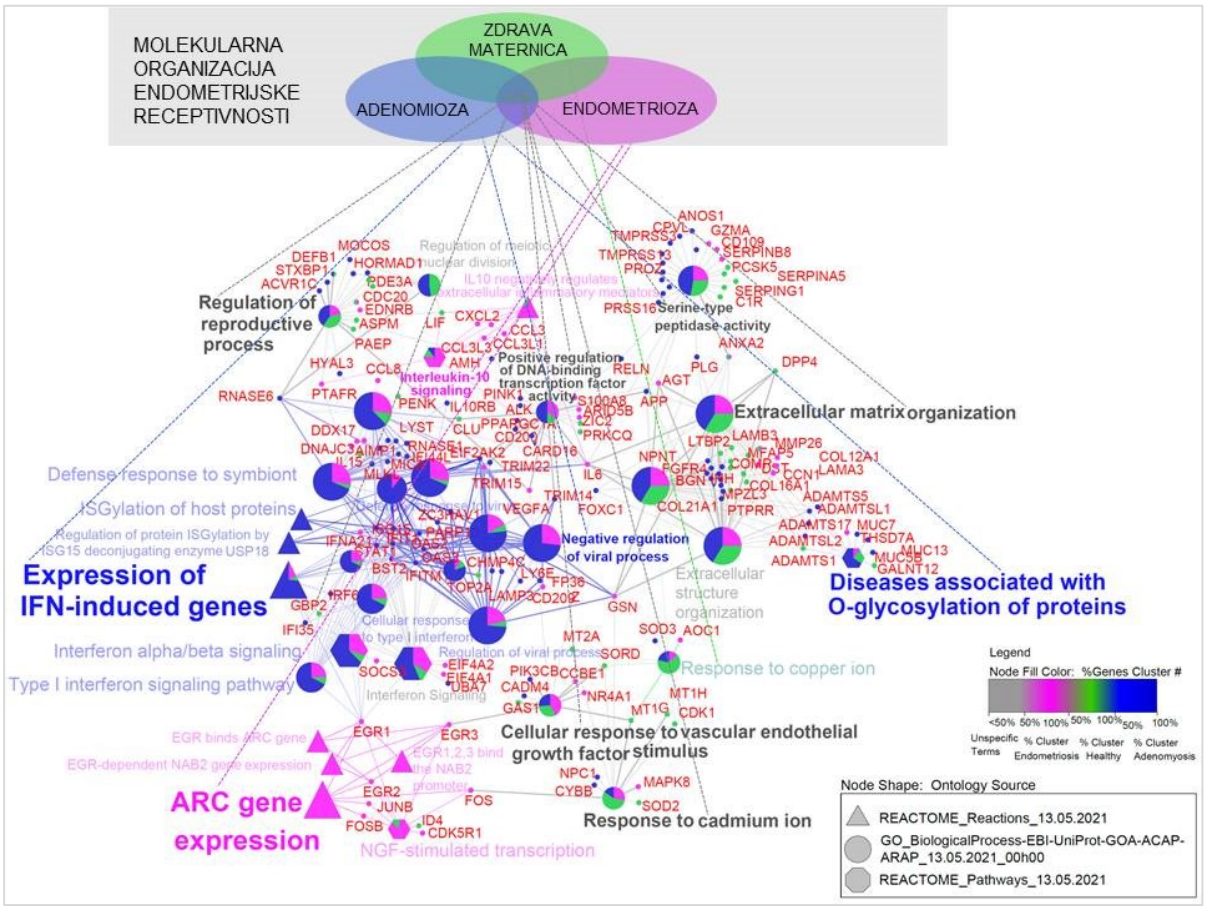
3.1.4 Integracija spremenjeno izraženih genov analize RNA-seq s podatki iz pregleda literature

Identificiranih 382 spremenjeno izraženih genov v endometriju receptivnega stanja med adenomiozno in kontrolno skupino analize RNA-seq smo integrirali z genskimi seti, ki smo jih oblikovali na podlagi podatkov iz literature. Uporabili smo tri genske sete, ki se povezujejo z endometrijsko receptivnostjo pri ženskah z adenomiozo (42 genov), ženskah z endometriozo (173 genov) in ženskah z zdravo maternico (151 genov). Integrirane genske sete smo obogatili v aplikacijah ClueGO/CluePedia v orodju Cytoscape in med pridobljenimi biološkimi potmi iskali tiste, kjer so anotirani geni izhajali iz vseh treh ginekoloških skupin (nespecifične poti). Takšne poti smo predlagali kot močnejše kandidatne biološke poti spremenjene endometrijske receptivnosti pri adenomiozi.

Integracijo identificiranih 382 genov analize RNA-seq s podatki iz pregleda literature smo izvedli z dvema ločenima obogatitvenima analizama, kjer smo spreminjali set vključenih genov adenomiozne skupine.

3.1.4.1 Integracija 382 genov adenomiozne skupine s poročanimi geni, povezanimi z endometriozo in zdravo maternico

Pri prvi obogatitveni analizi smo v set genov adenomiozne skupine vključili le 382 spremenjeno izraženih genov, ki smo jih identificirali z lastno analizo RNA-seq. Uporabili smo še seta 151 in 173 genov zdrave oz. endometriozne skupine. Po obogatitvi smo identificirali 40 bioloških poti, ki so bile urejene v 11 funkcionalnih skupin (Slika 9). Identificirali smo tri nespecifične funkcionalne skupine *organizacija zunajceličnega matriksa* (angl. *extracellular matrix organization*), *celični odziv na dražljaje vaskulatornega endotelijskega rastnega dejavnika* (angl. *cellular response to vascular endothelial growth factor (VEGF) stimulus*) in *uravnavanje procesa reprodukcije* (angl. *regulation of reproductive process*). Poti teh skupin predlagamo kot močnejše kandidate spremenjene endometrijske receptivnosti pri ženskah z adenomiozo. Predlaganim potem smo lahko tudi pripisali podporno literaturo. Preoblikovanje zunajceličnega matriksa je pomemben proces vgrezanja zarodka v deciduo (Okada in sod., 2014). Pri ženskah z adenomiozo (Li in sod., 2006; Herndon in sod., 2016) in ženskah z endometriozo (Di Carlo in sod., 2009; Pino in sod., 2009; Matsuzaki in sod., 2010) so že poročali o neravnovesju izražanja encimov, odgovornih za preoblikovanje zunajceličnega matriksa. Tudi angiogeni dejavniki (npr. *VEGF*) so pomembni mehanizmi vgnezdnja zarodka, saj omogočajo tvorbo novih krvnih žil (angiogeneza) in preoblikovanje ožilja endometrija za njegovo celično rast in diferenciacijo v deciduo (Okada in sod., 2014). Tudi pri identifikaciji močnejših kandidatnih genov patofiziologije adenomioze smo z obogatitvijo oblikovanih setov 78 in 518 genov P faze identificirali pot *pozitivno uravnavanje angiogeneze*. Veliko raziskav poroča o spremenjenem izražanju angiogenih dejavnikov (Goteri in sod., 2009; Kang in sod., 2009; Liu in sod., 2011; Wang J. in sod., 2016) in ožiljenju (Ota in Tanaka, 2003; Li in sod., 2006) endometrija pri adenomiozi. Pričakovano smo za adenomiozni set genov pridobili specifično funkcionalno skupino *izražanje genov, induciranih z IFN*. Opazili pa smo, da je bila znotraj te funkcionalne skupine tudi nespecifična pot *signalizacija IFN*, kjer so bili anotiranimi geni vseh treh uporabljenih ginekoloških skupin.



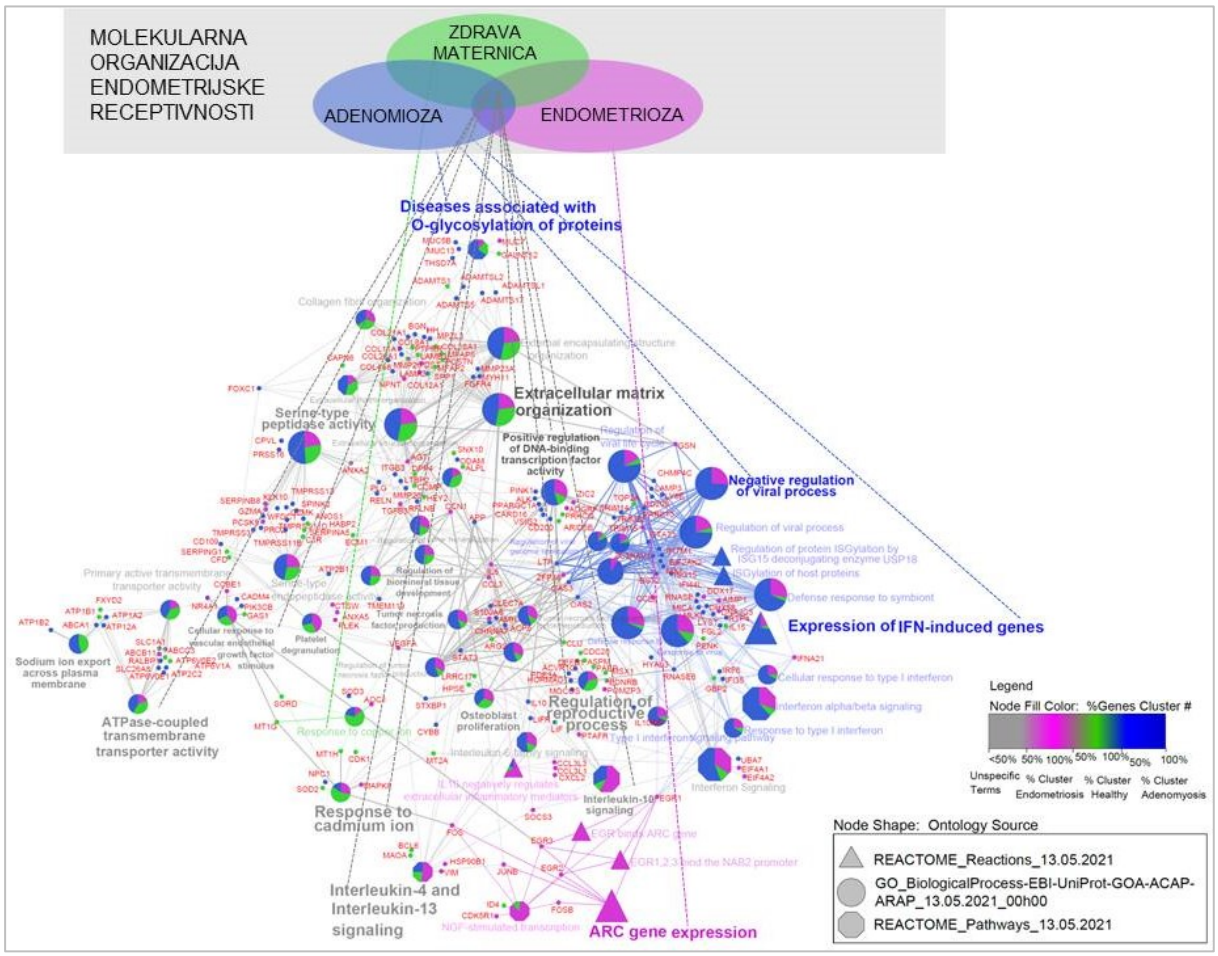
Slika 9: Mreža funkcionalno urejenih obogatenih 40 bioloških poti, pridobljenih z integracijo 382 spremenjeno izraženih genov naše adenomiozne skupine z geni, ki smo jih pridobili s pregleda literature in se povezujejo z endometrijsko receptivnostjo pri endometriozi (173 genov) in zdravi maternici (151 genov). Z modro so označene funkcionalne skupine/poti z večino anotiranih genov adenomiozne skupine. Z roza so označene funkcionalne skupine/poti anotiranih genov endometriozne skupine. Z zeleno so označene poti anotiranih genov zdrave maternice. S sivo barvo so označene nespecifične poti z enakomernim deležem anotiranih genov vseh treh ginekoloških stanj.

Figure 9: A network of functionally sorted enriched 40 biological pathways obtained by integrating 382 differentially expressed genes of our adenomyosis group with genes obtained from the literature mining that were associated with endometrial receptivity in endometriosis (173 genes) and healthy uterus (151 genes). Functional groups/pathways with the majority of annotated genes of the adenomyosis group are highlighted in blue. Functional groups/pathways of the annotated genes from the endometriosis group are highlighted in pink. Pathways annotated with genes of a healthy uterus are highlighted in green. Non-specific pathways with an equal proportion of annotated genes from all three gynaecological conditions are highlighted in grey.

3.1.4.2 Integracija 382 genov adenomiozne skupine s poročanimi geni, povezanimi z adenomiozo, endometriozo in zdravo maternico

Pri drugi analizi pa smo adenomioznemu setu s 382 geni lastne analize RNA-seq dodali še 42 genov, ki smo jih pridobili s pregledom literature in se povezujejo s spremenjeno endometrijsko receptivnostjo pri adenomiozi (skupno 424 genov). Uporabili smo še seta 173 (endometrioza) in 151 (zdrava maternica) genov. Po obogatitvi smo identificirali 57 poti, ki

so bile urejene v 18 funkcionalnih skupin (Slika 10). Identificirali smo še tri dodatne nespecifične funkcionalne poti, ki so bile povezane s signalizacijo s citokini (*signalizacija IL4 in IL13*, *uravnavanje produkcije superdružine citokinov TNF* (angl. *regulation of TNF superfamily cytokine production*) in *signalizacija IL10*).



Slika 10: **Mreža funkcionalno urejenih 57 obogatenih poti, pridobljenih z integracijo 382 spremenjeno izraženih genov naše adenomiozne skupine z oblikovanimi geni endometrijske receptivnosti pri adenomiozi (42 genov), endometriozi (173 genov) in zdravi maternici (151 genov).** Z modro so označene funkcionalne skupine/poti z večino anotiranih genov adenomiozne skupine. Z roza so označene funkcionalne skupine/poti, anotiranih genov endometriozne skupine. Z zeleno so označene poti anotiranih genov zdrave maternice. S sivo barvo so označene nespecifične poti z enakomernim deležem anotiranih genov vseh treh ginekoloških stanj.

Figure 10: Network of functionally sorted 57 enriched pathways obtained by integration of 382 differentially expressed genes of our adenomyosis group with developed gene sets associated with endometrial receptivity in adenomyosis (42 genes), endometriosis (173 genes), and healthy uterus (151 genes). Functional groups/pathways with the majority of annotated genes of the adenomyosis group are highlighted in blue. Functional groups/pathways annotated with genes from endometriosis group are highlighted in pink. Pathways annotated with genes from the healthy uterus group are highlighted in green. Grey indicates non-specific pathways with an equal proportion of annotated genes from all three gynaecological conditions.

Na podlagi integracije lastnih rezultatov analize RNA-seq in podatkov iz literature smo prišli do zaključka, da se spremenjena endometrijska receptivnost pri ženskah z adenomiozo nakazuje na ravni signalizacije citokinov imunskega sistema.

3.1.5 Pomanjkljivosti raziskave

3.1.5.1 Nizko število uporabljenih vzorcev

Transkriptomsko analizo podatkov potrjeno receptivnih vzorcev endometrija smo izvedli med 8 ženskami z adenomiozo in 5 ženskami kontrolne skupine. Nizko število uporabljenih vzorcev omejuje končne zaključke glede identificiranih 382 spremenjeno izraženih genov ($p < 0,05$), ker so bile razlike v izražanju genov statistično neznačilni po popravku vrednosti p ($FDR > 0,05$). Po izračunih z modelom negativne binomske porazdelitve, ki so jih predlagali Li X. in sod. (2019), ocenjujemo, da bi za večjo statistično moč naših rezultatov potrebovali okoli 35 vzorcev biopsij endometrija. Zaradi dolgotrajnega nabiranja biopsij endometrija, ki je bilo omejeno samo na ovulatorne ženske, mlajše od 42 let, brez pridruženih ginekoloških patologij in na točno določen stadij fiziološkega razvoja endometrija znotraj menstruacijskega ciklusa, bi bilo potrebno v prihodnje izvesti multicentrično nabiranje vzorcev. Tako bi lahko zagotovili večjo statistično moč analize RNA-seq.

3.1.5.2 Posredna diagnoza adenomioze s TVUZ

Diagnoza adenomioze pri preiskovankah naše raziskave je temeljila na pregledu rodil s TVUZ. Pri transkriptomskih raziskavah, ki so preučevale patofiziološke vidike endometrija pri adenomiozi, je zanesljiva diagnoza adenomioze lahko temeljila na histološkem pregledu vzorcev histerektomije (Herndon in sod., 2016; Jiang J.F. in sod., 2016; Xiang in sod., 2019). To pa ni mogoče pri ženskah, ki želijo ohraniti svojo reproduktivno sposobnost. V transkriptomskih raziskavah, orientiranih na plodnost pri ženskah z adenomiozo (Martinez-Conejero in sod., 2011) in na sopojavnost z endometriozo (Dior in sod., 2018), je bila možna le neinvazivna diagnoza z metodami slikanja (TVUZ ali MR). Ultrazvočna identifikacija adenomioze je zahtevna, saj še ni oblikovanih enotnih kriterijev za diagnozo (Chapron in sod., 2020). Kljub temu, pa so Andres in sod. (2018) v meta analizi 7 raziskav, ki so ocenjevale natančnost TVUZ pri diagnozi adenomioze po njeni potrditvi s histerektomijo, določili 83,8 % občutljivost in 63,9 % specifičnost metode TVUZ. Zaključili so, da je TVUZ ustrezna metoda za diagnozo adenomioze (Andres in sod., 2018). Da bi v naši raziskavi zmanjšali variabilnost ocenjevalca med opazovalci, je TVUZ maternice in medenične votline izvedel vedno isti ginekolog, ki je strokovnjak za ginekološki ultrazvok (stopnja 3 glede na evropsko zvezo društev za ultrazvok v medicini in biologiji (angl. *European Federation of Societies for Ultrasound in Medicine and Biology*)).

3.1.5.3 Blaga adenomioza

Pri sodelujočih preiskovankah smo zaznali prisotnost blage adenomioze. Rezultati RNA-seq bi lahko bili drugačni pri hudi obliki adenomioze. Pri kliničnem delu zdravljenja neplodnosti smo opazili malo žensk z napredno obliko adenomioze, ki bi izpolnjevale vključitvene kriterije (ovulatorni menstruacijski ciklus, brez sopojavnosti z endometriozo/miomov in mlajše od vključno 42 let). Rezultati RNA-seq bi lahko bili tudi drugačni, če bi kontrolno skupino sestavljale le potrjeno plodne ženske, saj velik delež preiskovank še nikoli ni bilo nosečih (primarna sterilnost). Koot in sod. (2016) so namreč poročali o molekularnih odstopanjih endometrija v stanju receptivnosti tudi pri ženskah brez patologij rodil.

3.1.5.4 Uporaba vzorcev celotnega tkiva endometrija

Za izvedbo analiz qPCR in RNA-seq smo uporabili RNA, ki je bila izolirana iz vzorcev celotnega tkiva endometrija. Endometrij je heterogeno tkivo, sestavljeno iz neenakomernih deležev epitelijskih (površinskih in žleznih), stromalnih, vaskulatornih in imunskih celic. Suhorutshenko in sod. (2018) so z računalniškim pristopom razčlenjenja (dekonvolucija) transkriptoma celotnega tkiva endometrija pokazali, da je lahko spremenjeno izražanje posameznega celičnega tipa zamaskirano z odtisom izražanja ostalih celičnih tipov (Suhorutshenko in sod., 2018). Da bi pridobili natančnejši vpogled v molekularno ozadje posameznih endometrijskih celičnih tipov v stanju receptivnosti pri adenomiozi, bi lahko izvedli metodo scRNA-seq (Tang in sod., 2010). Po drugi strani pa so poročali o tehnično zahtevni izolaciji posameznih EEC, kar je vodilo v nizko raven pridobljenih transkriptomskih podatkov in slabo pokritost referenčnega genoma (Krjutškov in sod., 2016). V literaturi je malo opravljenih omskih raziskav na posameznih celicah endometrija. Identificiranih spremenjeno izraženih genov naše analize RNA-seq ne bi mogli integrirati s podatki iz literature, ki v večini temeljijo na analizah celotnega tkiva endometrija.

3.1.5.5 Heterogenost omskih raziskav, iz katerih smo pridobivali podatke

Podatki o transkriptih RNA in proteinih spremenjenega endometrijskega izražanja pri endometriozi, ki smo jih uporabili za izdelavo kataloga, so izhajali iz heterogenih omskih raziskav. Skupno 21 raziskav se je razlikovalo po vključitvenih / izključitvenih kriterijih preiskovane in kontrolne skupine, izvedbi vzorčenja endometrija, metodi datiranja faze menstruacijskega ciklusa, protokolih shranjevanja vzorcev in izolaciji molekul, uporabljenih platformah za globalno analizo, bioinformacijskih analizah in podajanju končnih rezultatov. Posledično je med raziskavami slabo prekrivanje identificiranih spremenjeno izraženih lokusov, povezanih z endometriozno skupino. Pri raziskavi, ki so jo opravili Tamaresis in sod. (2014) smo zasledili tudi vključitev manjšega števila vzorcev endometrija žensk z adenomiozo (4 od skupno 144) v endometriozno in kontrolno skupino. Prisotnost dodatnih patologij pa bi lahko vplivalo na podpis transkriptoma endometrija (Hever in sod., 2006) Tudi

Altmäe in sod. (2017) so v meta analizi devetih transkriptomskih raziskav poročali o slabem prekrivanju identificiranih genov, povezanih z endometrijsko receptivnostjo kot posledica heterogenih zasnov raziskav.

3.1.5.6 Možna potrditvena pristranskost lokusov, pridobljenih iz literature

Gene, ki smo jih uporabili za izdelavo kataloga pri endometriozi oz. za identifikacijo močnejših kandidatov patofiziologije adenomioze, smo pridobili iz omskih raziskav, kjer pa njihovo spremenjeno izražanje večinoma ni bilo potrjeno z dodatnimi kvantifikacijskimi metodami (qPCR, prenos po western ali imunohistokemija). Vključevanje takšnih genov v sintezo podatkov pa bi lahko vplivalo na rezultate obogatitvenih analiz. Če v izvorni raziskavi niso potrdili spremenjenega izražanja izbranih lokusov z dodatnimi analizami, potem teh lokusov nismo upoštevali pri obogatitvenih analizah.

3.1.5.7 Poenotenje poimenovanja pridobljenih mRNA, ncRNA in proteinov

Poimenovanje transkriptov RNA in proteinov, ki smo jih pridobili s pregledom literature, smo poenotili po genski nomenklaturi zbirke HGNC (HUGO, 2019), da smo lahko izvajali bioinformacijske analize. Popolne povezave med transkriptomom in proteomom ni, saj se raven izražanja genov uravnava tudi s potranskripcijskimi mehanizmi. Npr. vezava molekul miRNA na ciljno mRNA vodi v njeno razgradnjo ali ovirano nadaljnjo translacijo (Bartel, 2004). Prekurzorski transkript mRNA je lahko podvržen alternativnim oblikam izrezovanja intronov in spajanja eksonov (Wang Y. in sod., 2015b) ter potranslacijskim preoblikovanjem (Walsh in sod., 2005), kar lahko vodi v več različnih izooblik proteinov.

3.1.6 Doprinos raziskave

3.1.6.1 Genski seti, povezani s spremenjenim endometrijskem izražanjem

S podatki iz pregleda literature smo razvili najobsežnejše genske sete, povezane s spremenjenim endometrijskem izražanjem pri ženskah z adenomiozo in ženskah z endometriozo. Po poenotenju heterogenega poimenovanja genetskih lokusov med objavljenimi raziskavami smo identificirali nekaj prekrivajočih se genov/obogatenih bioloških poti. Le-ti bi lahko predstavljali močnejše kandidate za endometrijske molekularne označevalce adenomioze oz. endometrioze. Poenotenje poimenovanja genetskih lokusov, ki smo ga izvedli v tej doktorski disertaciji, bo tudi olajšalo izmenjavo podatkov in informacij med raziskovalci.

S pregledom znanstvene literature in poenotenjem nomenklature genetskih lokusov smo pridobili 42 in 173 genov spremenjenega endometrijska izražanja v pričakovanem stanju receptivnosti pri ženskah z adenomiozo oz. ženskah z endometriozo. Dodatno smo s

poenotenjem poimenovanja večravninskih omskih raziskav lahko izdelali katalog 591 genov, s katerimi je mogoče preučevati vpliv endometrioze na celoten menstruacijski ciklus endometrija in iskati korelacije z vplivom adenomioze na molekularno organizacijo endometrija. Izdelan katalog z urejenimi geni glede na datirano fazo menstruacijskega ciklusa omogoča nadaljnje zbiranje podatkov. Ponovitve obogatitvenih analiz z dodatnimi poznavanji genskih interakcij, ki se zbirajo v bioloških zbirkah, pa bodo omogočile identifikacijo bolj specifičnih bioloških poti.

Po poenotenju nomenklature genetskih lokusov smo izvedli sintezo genov, povezanih s spremenjenim endometrijskim izražanjem v P in SS fazi pri adenomiozi, da smo lahko izvedli obogatitvene analize za identifikacijo močnejših kandidatov patofiziologije oz. spremenjene receptivnosti pri tej bolezni. V podatkovni zbirki DisGeNET (DisGeNET, 2021) smo sicer našli gene, povezane z adenomiozo, vendar iz zbirke ni bilo možno razbrati, ali se posamezen gen navezuje na izražanje v evtopičnem (EvEA) ali ektopičnem (EkEA) endometriju pri adenomiozi. V tej doktorski disertaciji nas je zanimal pomen spremenjeno izraženih genov le EvEA, saj predstavlja ciljno tkivo za vgnezditev zarodka. Identifikacija in poznavanje vloge posameznih genov EvEA bi lahko vodilo v izdelavo specifičnih molekularnih testov, ki bi ali olajšali zgodnjo neinvazivno diagnozo adenomioze ali omogočili diagnostiko endometrija kot možen vzrok neplodnosti pri takšnih bolnicah.

3.1.6.2 Izvedba analize RNA-seq

Analizo RNA-seq smo izvedli z vzorci endometrija brez sopojavnosti dodatnih patologij rodil tako v adenomiozni kot tudi v kontrolni skupini. Z dodatnim TVUZ pregledom rodil, ki je bil izveden tik pred vzorčenjem biopsije endometrija, smo lahko preverili morebitno sopojavnost dodatnih ginekoloških patologij pri preiskovankah. Z ultrazvočnim pregledom so bile pri eni od preiskovank adenomiozne in kontrolne skupine ugotovljene prisotnosti manjših miomov, pri eni od preiskovank kontrolne skupine pa obojestransko vnetje jajcevodov. Vzorce endometrija teh žensk smo vseeno uporabili za kvantifikacijo izbranih šestih kandidatnih genov s qPCR, saj ni bilo večjih odstopanj v izražanju z ali brez upoštevanja teh vzorcev. V analizo RNA-seq transkriptoma pa smo vključili samo tiste vzorce endometrija, kjer razen znakov adenomioze ugotovljenih drugih ultrazvočnih odstopanj rodil (v kontrolno skupino smo vključevali ženske, ki so se zdravile s postopki OBMP zaradi moškega ali tubarnega dejavnika neplodnosti). Na tak način smo preprečili podpis drugih patologij na transkriptom endometrija. V bioinformacijskih raziskavah (Prašnikar in sod., 2020a, 2020b), ki smo ju opravili s podatki iz objavljenih znanstvenih člankov, smo opazili, da so bile tako v preiskovano (endometrioza in adenomioza) kot tudi v pripadajočo kontrolno skupino pogosto vključene tudi ženske s pridruženimi patologijami maternice.

3.1.6.3 Natančno datiranje receptivne faze

Vzorcem endometrija smo natančno datirali receptivno fazo menstruacijskega ciklusa. Smo prvi, ki smo vzorce izolirane endometrijske RNA uporabili tako za RNA-seq transkriptoma, kot tudi za natančno datiranje personaliziranega stanja receptivne faze z molekularnim orodjem beREADY®. Z datiranjem smo zagotovili homogenost preiskovanih skupin v povezavi z zagotavljanjem enakega fiziološkega stadija razvoja (receptivna faza) endometrija tekom menstruacijskega ciklusa. S primerjavo podatkov RNA-seq potrjeno receptivnih vzorcev smo preprečili vpliv vzorcev zgodnje receptivne faze (ZS faza) ali pozno receptivne faze (PS faza) na podpis transkriptoma endometrija v stanju receptivnosti (SS faza). Le z upoštevanjem rezultatov datiranja endometrija pri analizi RNA-seq smo med adenomiozno in kontrolno skupino identificirali spremenjeno izražene gene, ki so bili obogateni v biološke poti, povezane z odzivom na delovanje IFN. V podobnih omskih raziskavah je bilo datiranje endometrija opravljeno z urinskimi LH testi ali s histološkim pregledom biopsije, kar se povezuje s slabo natančnostjo v primerjavi z molekularnimi testi (Coutifaris in sod., 2004; Díaz-Gimeno in sod., 2013).

3.1.6.4 Javno dostopni eksperimentalni podatki RNA-seq

Surovi podatki RNA-seq so, skupaj s podatki natančnega datiranja vsakega vzorca, javno dostopni v bazi Gene Expression Omnibus (GEO) pod številko GSE185392. Naši podatki so s tem dostopni tudi drugim raziskovalcem. Vsaka vzorčena biopsija je bila nemudoma potopljena v raztopino *RNAlater*, s čimer smo dosegli visoko stopnjo ohranjenosti izolirane RNA (številka integritete RNA (angl. *RNA integrity number*, RIN > 8,5) in nadaljnjo dobro pokritost referenčnega genoma hg19 (> 97,5 %) z odčitki RNA-seq. Z dodatnim datiranjem endometrija pa smo zagotovili transkriptomske podatke potrjeno receptivnih vzorcev endometrija, ki bodo uporabni tudi v prihodnjih integracijskih analizah.

3.1.7 Preverjanje hipotez

Hipoteza 1: Vzorca izražanja genov endometrija v stanju receptivnosti se razlikujeta med skupinama preiskovank z adenomiozo in preiskovank brez adenomioze.
Zavrnili smo hipotezo 1.
To hipotezo smo preverjali z metodama RNA-seq in qPCR. Z metodo RNA-seq smo primerjali endometrijske vzorce med skupinama 10 žensk z adenomiozo in 10 žensk brez adenomioze. Glede na rezultate datiranja receptivne faze smo mejno receptivne vzorce izključili iz seta transkriptomskih podatkov in analizo ponovili na podskupini samo receptivnih vzorcev med 8 ženskami z adenomiozo in 5 ženskami brez adenomioze. Pri prvi primerjavi smo zaznali 909, pri drugi pa 382 statistično značilno spremenjeno izraženih genov med skupinama ($p < 0,05$). Hipotezo smo zavrnili, ker je bila pri obeh primerjavah razlika v izražanju genov po popravku za testiranje vrednosti p neznačilna (FDR > 0,05). Z metodo

qPCR smo primerjali stopnjo izražanja šestih prioritiziranih kandidatnih genov *LIF*, *IL10*, *IL6*, *JUNB*, *FOS* in *SOCS3* v endometriju med 9 ženskami z in 13 ženskami brez adenomioze. Znižano izražanje izbranih genov med adenomiozno in kontrolno skupino ni bilo statistično značilno, zato smo hipotezo 1 ponovno zavrnili.

Hipoteza 2: Izražanje genov z domnevno vlogo sodelovanja pri vzpostavitvi receptivnega stanja in genov, povezanih s patofiziologijo adenomioze, je spremenjeno pri preiskovankah z adenomiozo.

Hipotezo 2 smo delno potrdili.

Hipotezo smo preverjali z bioinformacijskim orodjem DAVID, s katerim smo izvedli obogatitvene analize bioloških poti s štirimi seti genov. Sete smo oblikovali z združevanjem podatkov o genih, ncRNA in proteinih, ki se v znanstveni literaturi povezujejo s spremenjenim endometrijskim izražanjem med ženskami z in brez adenomioze.

Za preverjanje prvega dela hipoteze o vplivu adenomioze na endometrijsko receptivnost smo sestavili dva seta genov:

- 60 genov iz zbirke GO, anotiranih v biološko pot *vgnezdenje zarodka* in/ali *decidualizacija*. Z obogatitvijo seta smo pridobili biološke poti normalnega procesa endometrijske receptivnosti;
- 42 genov, ki se v dosedanji literaturi (n = 7) povezujejo o spremenjenim endometrijskim izražanjem v pričakovanem času receptivne faze (SS faza) med ženskami z in brez adenomioze. Na podlagi rezultatov obogatitvene analize smo iz tega seta identificirali močnejše kandidate spremenjene endometrijske receptivnosti pri adenomizi.

Za preverjanje drugega dela hipoteze o sodelovanju endometrija pri patofiziologiji adenomioze pa smo sestavili naslednja dva seta genov:

- 78 genov, pridobljenih iz dosedanjih raziskav kandidatnih lokusov (n = 47), v katerih so poročali o spremenjenem izražanju endometrija v P fazi med ženskami z in brez adenomioze. Vključili smo samo raziskave endometrija P faze, saj se v S fazi zaradi delovanja hormona P4 pojavi okno vgnezdenja z značilnim transkriptomskim podpisom. Razlika v izražanju v P fazi med adenomiozno in kontrolno skupino bi lahko bila zaradi patofiziologije bolezni. Z obogatenjem seta smo pridobili kandidatne poti patofiziologije evtopičnega endometrija pri adenomiozi (EvEA).
- 518 genov, pridobljenih iz transkriptomskih raziskav (n = 3) endometrija P faze med ženskami z in brez adenomioze. Na podlagi rezultatov obogatitve smo iz tega seta identificirali 52 močnejših kandidatov patofiziologije adenomioze.

Iz seta 42 genov je bilo 10 genov anotiranih v tri biološke poti (*negativna regulacija celične proliferacije*, *organizacija zunajceličnega matriksa* in *decidualizacija*), ki so bile obogatene tudi s setom 60 genov zbirke GO. Tako smo za teh 10 genov potrdili vlogo sodelovanja v procesih endometrijske receptivnosti pri adenomiozi.

Iz seta 518 genov je bilo 52 genov anotiranih v tri biološke poti (*pozitivno uravnavanje angiogeneze*, *negativno uravnavanje apoptoznih procesov* in *vnetni odziv*), ki so bile obogatene tudi s setom 78 genov. Kljub temu pa te poti niso kazale na neposredne mehanizme, ki se povezujejo z razvojem adenomioze (zvišana invazivnost, izguba celične polarnosti, zvišana motilnost in mehanizmi poškodb oz. celjenje). Hipotezo 2 smo tako le delno potrdili.

3.2 SKLEPI

V doktorski disertaciji smo preverjali dve delovni hipotezi:
1. Vzorca izražanja genov endometrija v stanju receptivnosti se razlikujeta med skupinama preiskovank z adenomiozo in preiskovank brez adenomioze;
2. Izražanje genov z domnevno vlogo sodelovanja pri vzpostavitvi receptivnega stanja in genov povezanih s patofiziologijo adenomioze je spremenjeno pri preiskovankah z adenomiozo.

Za potrjevanje hipoteze 1 smo na vzorcih endometrija v pričakovanem stanju receptivnosti pri ženskah z in brez ultrazvočnih znakov adenomioze izvedli kvantifikacijo izbranih kandidatnih genov z metodo qPCR in sekvenciranje transkriptoma z metodo RNA-seq. Za potrjevanje hipoteze 2 pa smo izvedli obogatitvene analize zbranih genov s pregleda literature, povezane s spremenjenim endometrijskim izražanjem pri adenomiozi, da bi identificirali močnejše kandidate patofiziologije endometrija oz. spremenjene endometrijske receptivnosti za vezavo zarodka pri tej bolezni.

Rezultati doktorske naloge glede potrjevanja hipoteze 1 so:

- Z RNA-seq sekvenciranjem transkriptoma (mRNA in lncRNA) endometrija pri 10 ženskah z in 10 brez adenomioze smo identificirali 909 spremenjeno izraženih genov ($p < 0,05$; FDR > 0,05), ki so bili obogateni v štirih bioloških poteh (Bonferroni $p < 0,05$), vključno v poti *znotrajceličnega transporta lipidov*.
- Kljub vzorčenju biopsij endometrija med dnevi LH+7–LH+9 je bilo z dodatnim testiranjem personalizirane endometrijske receptivnosti 13 od skupno 20 vzorcev datiranih v receptivno, dva v zgodnje receptivno in 5 v pozno receptivno fazo menstruacijskega ciklusa.
- S ponovno analizo podatkov RNA-seq samo potrjeno receptivnih vzorcev adenomiozne (n = 8) in kontrolne (n = 5) skupine smo identificirali 382 spremenjeno izraženih genov ($p < 0,05$; FDR > 0,05), ki so bili obogateni v 33 bioloških poti (Bonferroni $p < 0,05$).
- Izmed 33 obogatenih poti se jih je kar 19 navezovalo na procese odziva na signalizacijo IFN. Zaznali smo tudi obogateno pot, povezano s celično adhezijo.

- Za poti, povezane z odzivom na IFN, smo našli podporno literaturo tako v povezavi s procesi vgnezdenja zarodka, kot tudi s patofiziologijo adenomioze.
- Na koekspresijski mreži mRNA-lncRNA smo pokazali, da transkripta lncRNA *DRAIC* in *CADM3-AS1*, ki izhajata iz seta 382 spremenjeno izraženih genov analize RNA-seq, predstavljata pomembni vozlišči negativne korelacije z izražanjem ciljnih transkriptov, ki so bili obogateni v poteh, povezanih s celično adhezijo.
- Na podlagi rezultatov obogatitvenih analiz in podporne literature sklepamo, da set 382 genov bolj reprezentativno predstavlja podpis adenomioze na transkriptom endometrija v stanju receptivnosti, kot pa sprva zaznanih 909 genov. Rezultate bi bilo potrebno preveriti na večjem številu vzorcev.
- Hipotezo 1 smo zavrnili zaradi neznačilnega izražanja po popravku vrednosti *p* (test FDR) tako 909 kot tudi 382 spremenjeno izraženih genov med adenomiozno in kontrolno skupino žensk.

Rezultati doktorske naloge glede potrjevanja hipoteze 2 so:

- S poenotenjem poimenovanja transkriptov RNA in proteinov smo iz 47 raziskav kandidatnih lokusov in treh transkriptomskih raziskav pridobili 78 oz. 518 genov, povezanih s spremenjenim izražanjem v proliferacijskem endometriju pri adenomiozi.
- Na podlagi obogatitvenih analiz smo identificirali 10 močnejših kandidatnih genov spremenjene endometrijske receptivnosti pri adenomiozi (vključno *LIF*, *STAT3*, *IL10*, *CDH5*, *SSP1*, *ITGB3* in *MMP20*) in 52 močnejših kandidatov patofiziologije endometrija pri adenomiozi (vključno *ANXA1*, *BCL6*, *CXCL2*, *DUSP1*, *EPHA2*, *NFKB1A*, *PTGS1*, *S100A9*, *SOCS3* in *VEGFA*).
- Hipotezo 2 smo le delno potrdili, ker obogatene poti v povezavi s patofiziologijo, niso kazale na neposredne patološke mehanizme endometrija, ki se povezujejo z razvojem te bolezni (npr. izguba celične polarnosti, povišana agresivnost in celična migracija).

Poleg potrjevanja hipotez smo v tej doktorski disertaciji izvedli tudi analizo omskih raziskav, v katerih so poročali o genetskih vzrokih spremenjenega izražanja v endometriju žensk s sorodno, a bolje raziskano endometriozo. Geni, povezani z endometrijsko receptivnostjo pri endometriozi in zdravi maternici so nam omogočili boljše razumevanje biološkega pomena spremenjeno izraženih genov adenomiozne skupine, ki smo jih identificirali z izvedeno analizo RNA-seq. Dodatni rezultati so:

- Z združevanjem podatkov iz 21 omskih raziskav, ki so poročale o spremenjeno izraženih transkriptih in proteinih v endometriju med ženskami z in brez endometrioze, in poenotenjem genske nomenklature smo izdelali katalog s 591 geni, ki smo jih razvrstili glede na datirano fazo menstruacijskega ciklusa.

- Z obogatitveno analizo endometrioznih genskih setov, povezanih s fazami menstruacijskega ciklusa, smo identificirali poti, povezane s signalizacijo estrogenov, organizacijo zunajceličnega matriksa in celično adhezijo. Natančneje, 173 genov SS faze (pričakovani čas endometrijske receptivnosti) je bilo obogatenih v poteh, povezanih s produkcijo citokinov, vnetnim odzivom, kemotakso endotelijskih in imunskih celic ter z regulacijo apoptoze.
- Z obogatitvenimi analizami zbranih genov s pregleda literature, ki se povezuje z endometrijsko receptivnostjo pri ženskah z adenomiozo (42 genov), ženskah z endometriozo (173 genov) in zdravih ženskah (151 genov), smo identificirali močnejše kandidatne poti signalizacije z IL.
- Izbranim šestim genom *IL6*, *IL10*, *LIF*, *SOCS3*, *FOS* in *JUNB*, ki so bili anotirani v poti signalizacije z IL, smo preverili stopnjo izražanja na vzorcih endometrija pri ženskah z in brez adenomioze. Ugotovili smo nižjo stopnjo izražanja preiskovanih genov pri adenomiozni skupini, a je bila razlika neznačilna.
- V obliki mreže funkcionalno urejenih obogatenih poti smo integrirali set 382 spremenjeno izraženih genov adenomiozne skupine izvedene analize RNA-seq s seti 42, 173 in 151 genov endometrijske receptivnosti adenomioze, endometrioze oz. zdrave maternice. Identificirali smo poti *organizacija zunajceličnega matriksa*, *celični odziv na stimule z VEGF*, *signalizacija z IFN*, *signalizacija z IL* in *uravnavanje reproduktivnih procesov* z anotiranimi geni vseh uporabljenih setov. Te poti predstavljajo dodatne močnejše kandidate spremenjenih procesov receptivnosti pri adenomiozi.
- Z rezultati obogatitvenih analiz podatkov iz pregleda literature in izvedene analize RNA-seq smo pokazali na korelacije spremenjenih molekularnih procesov (produkcija citokinov, celična adhezija, organizacija zunajceličnega matriksa in ožiljenje) receptivnosti endometrija med endometriozo in adenomiozo.

Na podlagi analiz dosedanjih poročanih podatkov o molekularnem ozadju endometrijske receptivnosti in lastnih rezultatov RNA-seq sklepamo, da je endometrijska receptivnost pri ženskah z adenomiozo spremenjena na ravni signalizacije s citokini imunskega sistema. Poznavanje vloge posameznih genov v endometriju pri adenomiozi bi v prihodnje lahko vodilo v identifikacijo endometrijskih molekularnih označevalcev. Le-ti bi omogočili bodisi lažjo neinvazivno diagnozo adenomioze ali natančnejšo diagnostiko endometrijske receptivnosti za zarodek pri ženskah z adenomiozo maternice, ki se zdravijo za neplodnostjo.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Adenomiozo maternice označuje prisotnost endometriju podobnega tkiva znotraj miometrija (Van den Bosch in sod., 2015). Molekularne spremembe endometrija pri tej bolezni se povezujejo tako s patofiziologijo, ki vodijo v razvoj in/ali napredovanje adenomioze (Vannuccini in sod., 2017), kot tudi z njegovo ovirano receptivnostjo za vgnezdenje zarodka (Campo in sod., 2012). Adenomiozo so povezali z nižjo stopnjo zanositve (Salim in sod., 2012; Thalluri in Tremellen, 2012; Puente in sod., 2016; Sharma in sod., 2019). Molekularni dejavniki domnevno ovirane endometrijske receptivnosti so slabo poznani zaradi tehnoloških omejitev metode TVUZ pri neinvazivni diagnozi adenomioze v preteklosti (Campo in sod., 2012). V literaturi smo našli le šest raziskav kandidatnih lokusov (Xiao in sod., 2010, 2013; Fischer in sod., 2011; Yen in sod., 2016; Wang in sod., 2018; Yan in sod., 2019) in eno transkriptomsko (Martinez-Conejero in sod., 2011) analizo endometrija v pričakovanem stanju receptivnosti pri ženskah z adenomiozo maternice v njihovem rodnem obdobju. Zato smo v tej doktorski disertaciji uporabili pristope sistemske biologije, kjer smo na podlagi podatkov iz pregleda literature in izvedene analize RNA-seq prikazali vpliv adenomioze na molekularno organizacijo endometrijske receptivnosti.

S primerjavo podatkov RNA-seq potrjeno receptivnih vzorcev endometrija med ženskami z (n = 8) in brez (n = 5) adenomioze smo najprej identificirali 382 spremenjeno izraženih genov, ki so bili obogateni v poteh, povezanih z mehanizmi odziva na IFN. Set 382 genov smo dalje integrirali s podatki iz pregleda literature, ki so se navezovali na molekularno ozadje endometrija v stanju receptivnosti pri ženskah z adenomiozo (42 genov), ženskah s sorodno, a bolje raziskano endometriozo (173 genov) in ženskah z zdravo maternico (151 genov). Z zbranimi seti genov smo izvedli obogatitveno analizo bioloških poti in iskali poti z anotiranimi geni vseh uporabljenih setov. Na tak način smo identificirali poti *organizacija zunajceličnega matriksa*, *celični odziv na dražljaje VEGF*, *uravnavanje procesa reprodukcije*, *signalizacija z IL* in *regulacija produkcije superdružine citokinov TNF*, ki jih predlagamo kot dodatne kandidatne poti spremenjene endometrijske receptivnosti pri adenomiozi (Prašnikar in sod., 2022).

Skupno smo za sekvenciranje transkriptoma (mRNA in lncRNA) endometrija uporabili 20 vzorcev (10 v adenomiozni in 10 v kontrolni skupini). Z analizo vseh 20 vzorcev smo identificirali 909 spremenjeno izraženih genov ($p < 0,05$), ki so bili obogateni le v štirih poteh ($p < 0,05$). Zaradi njihove splošne narave (npr. pot *znotrajcelični transport lipidov*) jim nismo mogli pripisati podporne literature v povezavi z vgnezdenjem zarodka ali s patofiziologijo adenomioze. Takšni rezultati bi lahko bili posledica različne fiziološke zrelosti uporabljenih vzorcev endometrija, kljub temu, da je bila vsaka biopsija pridobljena med dnevi LH+7 in LH+9 (pričakovani čas endometrijske receptivnosti). Ker poročajo o personaliziranem pojavu in trajanju receptivne faze v menstruacijskem ciklusu (Ruiz-Alonso in sod., 2013; Mahajan in sod., 2018; Tan in sod., 2018) ter o zamaskiranem podpisu patologije na transkriptom

endometrija pri primerjavi vzorcev različnih faz ciklusa (Devesa-Peiro in sod., 2021), smo vse vzorce dodatno datirali. To smo izvedli s pomočjo novega molekularnega orodja, ki na podlagi vzorca izražanja izbranih 57 genov endometrijske receptivnosti določi personalizirano stanje receptivne faze (Saare in sod., 2019). Z rezultati testiranja smo 20 vzorcev datirali v tri skupine: v zgodnjo receptivno fazo (n = 2); receptivno fazo (n = 13); in pozno receptivno fazo (n = 5).

S ponovno analizo transkriptomskih podatkov samo potrjeno receptivnih vzorcev endometrija adenomiozne (n = 8) in kontrolne (n = 5) skupine smo identificirali že omenjenih 382 spremenjeno izraženih genov ($p < 0,05$). Ti geni so bili obogateni v 33 poteh ($p < 0,05$), od tega je bilo 19 povezanih z mehanizmi odziva na signalizacijo IFN. V povezavi z vgnezdenjem zarodka se vloga IFN povezuje z aktivacijo celic uNK, ki nadzorujejo globino vgnezdenja trofoblasta v deciduo in omogočajo preoblikovanje spiralnih arterij za dotok krvi zarodku (Austin in sod., 2003; Wilczyński, 2005; Hannan in sod., 2011). V povezavi s patofiziologijo adenomioze pa se izmerjene prekomerne vrednosti IFN endometrija povezujejo z lokalno povišano vrednostjo vnetnih citokinov v maternici, ki vodijo v razvoj lezij (Sotnikova in sod., 2002). Zaključimo lahko, da smo z RNA-seq transkriptoma in natančnim datiranjem vzorcev endometrija identificirali poti odziva na delovanje IFN, ki predstavljajo najbolj obetavne kandidate za nadaljnje raziskave endometrijske receptivnosti pri adenomiozi.

Z obogatitvijo identificiranih 382 genov analize RNA-seq smo pridobili tudi pot, povezano s celično adhezijo, kjer so bili, tako kot v poteh IFN, v večini anotirani geni zmanjšanega izražanja pri adenomiozni v primerjavi s kontrolno skupino. To bi lahko kazalo na zmanjšano receptivnost endometrija za vezavo zarodka. Rezultat je v skladu z literaturo, saj zadostna kapaciteta adhezije endometrija omogoča pritrditev blastociste na njegov epitelij (Achache in Revel, 2006). Obogatene poti, povezane s celično adhezijo, smo zaznali tudi pri analizi genov koekspresijske mreže, ki smo jo izdelali med 23 spremenjeno izraženimi lncRNA seta 382 genov in celokupnim transkriptomom. Identificirali smo namreč transkripta lncRNA *DRAIC* in *CADM3-AS1*, ki sta imela pretežno negativno korelacijo z izražanjem njunih ciljnih transkriptov, ki so bili obogateni v poteh, povezanih s celično adhezijo. Poročali so že o vplivu lncRNA na stopnjo izražanja genov, ki v endometriju kodirajo proteine adhezije (Fan in sod., 2017; Li D. in sod., 2019). Ti dve lncRNA predstavljata kandidatna dejavnika epigenetskega vpliva adenomioze na izražanje genov, povezanih z endometrijsko receptivnostjo.

Naše končne zaključke glede spremenjeno izraženih genov ($p < 0,05$) analize RNA-seq omejuje njihovo neznačilno izražanje po popravku vrednosti p (FDR > 0,05). Nizka statistična moč analize bi lahko bila posledica nizkega števila uporabljenih vzorcev. Pridobivanje biopsij endometrija je dolgotrajen proces, zato bi bilo potrebno v prihodnje izvesti multicentrično nabiranje vzorcev za dodatne analize RNA-seq.

Za izvedbo bioinformacijskih analiz podatkov o genetskih vzrokih (transkripti mRNA in ncRNA ter proteini spremenjenega endometrijskega izražanja), ki smo jih pridobili iz objavljenih raziskav, smo najprej poenotili njihovo heterogeno poimenovanje po genski nomenklaturi zbirke HGNC (HUGO, 2019). Nato smo lahko glede na poročano datirano fazo menstruacijskega ciklusa izvorne raziskave združevali gene in jih obogatili, da bi razumeli njihov biološki pomen. Na tak način smo zbrali znanje o vplivu endometrioze na molekularno organizacijo endometrija tekom menstruacijskega ciklusa (Prašnikar in sod., 2020a) in predlagali močnejše kandidatne gene patofiziologije (P faza) in spremenjene endometrijske receptivnosti (SS faza) pri adenomiozi.

Zaradi slabo raziskanega vpliva adenomioze na endometrijsko receptivnost smo pri sorodni, a bolje raziskani endometriozi (Del Frate in sod., 2006) nabirali znanje o molekularnih odstopanjih endometrija ob prisotni patologiji (Prašnikar in sod., 2020a). Iz 21 raziskav različnih omskih ravni smo združili spremenjeno izražene endometrijske lokuse ter po poenotenju nomenklature pridobili 591 genov, ki smo jih razvrstili glede na datirano fazo ciklusa. Z obogatitvijo 173 genov SS faze smo med drugimi identificirali poti, povezane s produkcijo citokinov, vnetnim odzivom in kemotakso endotelijskih in imunskih celic. Tudi IFN, čigar poti odziva na njihovo delovanje smo zaznali z obogatitvijo genov izvedene analize RNA-seq, spadajo med citokine imunskega sistema. S tem smo pokazali na možne korelacije v spremenjenih procesih endometrijske receptivnosti med adenomiozo in endometriozo. Dodatno smo z obogatitvenima analizama samo pridobljenih podatkov iz pregleda literature, tj. genov, povezanih z endometrijsko receptivnostjo pri adenomiozi (42 genov), endometriozi (173 genov) in zdravi maternici (151 genov), identificirali poti, povezane z delovanjem citokinov imunskega sistema (*signalizacija z IL* in *signalizacija z IL4 in IL13*). Izbranim šestim genom (*JUNB*, *FOS*, *SOCS3*, *IL6*, *IL10* in *LIF*), ki so bili anotirani v te dve poti, smo tudi preverili stopnjo izražanja na vzorcih endometrija ženskam z (n = 9) in brez (n = 13) adenomioze. Zaznali smo zmanjšano izražanje preiskovanih genov pri adenomiozni skupini, a je bila razlika statistično neznačilna. Raziskavo bi bilo potrebno ponoviti na večjem številu vzorcev (Prašnikar in sod., 2020b).

Za identifikacijo 52 močnejših kandidatnih genov (vključno *ANXA1*, *BCL6*, *CXCL2*, *DUSP1*, *EPHA2*, *NFKB1A*, *PTGS1*, *S100A9*, *SOCS3* in *VEGFA*) patofiziologije endometrija pri adenomioze smo združevali podatke 50 raziskav, opravljenih v P fazi. V tej fazi namreč ni delovanja hormona P4 in z receptivnostjo povezanih genov, zato razlike v endometrijskim izražanju med preiskovano in kontrolno skupino verjetneje kažejo na patofiziološke spremembe (Makieva in sod., 2018). Za identifikacijo 10 močnejših kandidatnih genov (*IL10, SST, STAT3, LIF, CHD5, CDKN3, COL8A1, COL11A1, ITGB3* in *SPP1*) spremenjene endometrijske receptivnosti pri adenomiozi pa smo združevali podatke sedmih raziskav endometrija SS faze. Poznavanje vloge posameznih genov pri adenomiozi bi lahko vodilo v identifikacijo endometrijskih molekularnih označevalcev te bolezni. Specifičen molekularni test bi lahko pripomogel k zanesljivejši diagnozi zgodnje adenomioze, ki je trenutno omejena le na visoko občutljive, vendar subjektivne metode slikanja (Hershko-Klement in Tepper,

2016). Medtem bi s specifičnim molekularnim testom za diagnostiko endometrijske receptivnosti pri adenomiozi lahko preverili, ali je endometrij dejavnik neplodnosti pri neplodnih ženskah z adenomiozo. Poznavanje patoloških procesov endometrijske receptivnosti bi lahko vodilo v razvoj novih terapevtskih pristopov priprave endometrija pred prenosom zarodka v postopkih OBMP.

4.2 SUMMARY

Uterine adenomyosis is characterized by the presence of endometrium-like tissue within the myometrium (Van den Bosch et al., 2015). Molecular alterations of the endometrium in this disease are associated both with the pathophysiology leading to the development and/or progression of adenomyosis (Vannuccini et al., 2017), as well as with its impaired receptivity for embryo implantation (Campo et al., 2012). Adenomyosis has been associated with lower pregnancy rate (Salim et al., 2012; Thalluri and Tremellen, 2012; Puente et al., 2016; Sharma et al., 2019). The molecular aspects of presumed impaired endometrial receptivity are poorly understood due to the technological limitations of the TVUS method in the non-invasive diagnosis of adenomyosis in the past (Campo et al., 2012). From the literature survey we obtained only six candidate locus studies (Xiao et al., 2010, 2013; Fischer et al., 2011; Yen et al., 2016; Wang et al., 2018; Yan et al., 2019) and one transcriptomic study (Martinez-Conejero et al., 2011) where endometrium of expected receptivity was analysed in women with uterine adenomyosis in their reproductive age. Therefore, in this doctoral dissertation, we have applied systems biology approaches to demonstrate the impact of adenomyosis on the molecular organization of endometrial receptivity based on data from a literature review and RNA-seq analysis performed.

First, we compared RNA-seq data of confirmed receptive endometrial samples between women with (n = 8) and without (n = 5) adenomyosis and identified 382 differentially expressed genes that were enriched in pathways related to IFN response mechanisms. The set of 382 genes was further integrated with data from a literature mining associated with the molecular background of the endometrial receptive state in women with adenomyosis (42 genes), women with a related but better-studied endometriosis (173 genes) and women with a healthy uterus (151 genes). We performed a biological pathway enrichment analysis with developed gene sets and searched for pathways annotated with genes from all the sets used. In this way, we identified pathways *extracellular matrix organization*, *cellular response to VEGF stimulus*, *regulation of reproductive process*, *signalling by IL* and *regulation of TNF superfamily cytokine production*, which we proposed as additional candidate pathways of altered endometrial receptivity in adenomyosis (Prašnikar et al., 2022).

In total, we used 20 endometrial samples (10 in the adenomyosis and 10 in the control group) for transcriptome (mRNA and lncRNA) sequencing. Analysis of all 20 identified 909 differentially expressed genes ($p < 0.05$) further enriched in only four pathways ($p < 0.05$).

Due to their general nature (e.g. *intracellular lipid transport*), we could not attribute to them any supporting literature in relation to the embryo implantation or to the pathophysiology of adenomyosis. Such results could be due to the different physiological maturity of used endometrial samples, despite the fact that each biopsy was retrieved between days LH+7 and LH+9 (the expected time of endometrial receptivity). As the personalized appearance and duration of the receptive phase in the menstrual cycle has been reported (Ruiz-Alonso et al., 2013; Mahajan et al., 2018; Tan et al., 2018) and a masked signature of pathology on the endometrial transcriptome when comparing samples from different phases of the cycle (Devesa-Peiro et al., 2021), we additionally performed endometrial dating of all samples. We utilized a novel molecular tool that determines a personalized receptive phase status based on the expression pattern of 57 selected endometrial receptivity genes (Saare et al., 2019). Considering the results of endometrial receptivity testing, we classified endometrial samples into three groups: early receptive phase (n = 2), receptive phase (n = 13), and late receptive phase (n = 5).

We reanalysed the RNA-seq data considering only confirmed receptive endometrial samples from adenomyosis (n = 8) in control (n = 5) groups and identified the aforementioned 382 differentially expressed genes ($p < 0.05$). These genes were enriched in 33 pathways ($p < 0.05$), 19 of which were related to IFN signalling response mechanisms. In relation to embryo implantation, the role of IFN is associated with the activation of uNK cells, which control the depth of trophoblast implantation in the decidua and support the remodelling of the spiral arteries for blood supply to the embryo (Austin et al., 2003; Wilczyński, 2005; Hannan et al., 2011). In relation to the pathophysiology of adenomyosis, upregulated levels of endometrial IFN have been associated with locally enhanced production of inflammation cytokines in the uterus leading to lesion development (Sotnikova et al., 2002). In conclusion, RNA-seq of the transcriptome and accurate dating of endometrial samples have resulted in the identification of pathways of response to IFN action that represent the most promising candidate for further investigation of impaired uterine receptivity in adenomyosis.

The enrichment of the 382 genes also identified a pathway related to cell adhesion, where, as in the IFN pathways, the majority of annotated genes were downregulated in women with adenomyosis compared to the control group. This could indicate an impaired uterine receptivity for embryo implantation in adenomyosis. This is in agreement with the literature, as sufficient endometrial adhesion capacity allows the blastocyst to attach to its epithelium (Achache and Revel, 2006). In addition, enriched pathways related to cell adhesion were also identified by analysing the genes in a co-expression network, which was constructed between 23 differentially expressed lncRNA derived from a set of 382 gene set and the whole transcriptome. Namely, we identified lncRNA transcripts *DRAIC* and *CADM3-AS1*, which had a predominantly negative correlation with the expression of their target transcripts, that were enriched in cell adhesion-related pathways. The regulatory role of lncRNA on the expression level of genes encoding adhesion proteins in the endometrium has been previously reported (Fan et al., 2017; Li et al., 2019). These two lncRNAs represent candidates factors

for the epigenetic impact of adenomyosis on the expression of genes related to endometrial receptivity.

Our final conclusions regarding the identified differentially expressed genes ($p < 0.05$) from the RNA-seq analysis are limited due to their non-significant expression level after *p* value correction (FDR > 0.05). The low statistical power of the analysis could be a consequence of small sample size. The gathering of endometrial biopsies is a time-consuming process; therefore, future multicentre sample collection for additional RNA-seq analyses should be performed.

In order to perform bioinformatics analyses of the genetic causes (mRNA and ncRNA transcripts and proteins) extracted from published studies reporting dysregulation in the endometrium, we first adopted their heterogeneous nomenclature according to the HGNC (HUGO, 2019) gene nomenclature. Then, we could sort obtained genes according to the reported menstrual cycle phase of endometrial dating in the original study for downstream enrichment analyses to understand their biological role. In this way, we provided a knowledge presenting the impact of endometriosis on endometrial molecular organisation through the menstrual cycle (Prašnikar et al., 2020a) and suggested stronger candidate genes of pathophysiology (proliferative (P) phase) and altered endometrial receptivity (mid-secretory (MS) phase) in adenomyosis.

Due to the poorly understood impact of adenomyosis on endometrial receptivity, we chose the related, but better-studied endometriosis (Del Frate et al., 2006) to gain knowledge of dysregulated endometrium when the pathology persists (Prašnikar et al., 2020a). We obtained 591 genes after pooling differentially expressed endometrial loci from 21 published studies of different omics levels and adopting their nomenclature according to the HGNC database (HUGO, 2019). The genes were further sorted according to the reported phase of the menstrual cycle. By enriching 173 genes of the MS phase, we also identified pathways related to cytokine production, inflammatory response and chemotaxis of endothelial and immune cells. On the other hand, cytokines of the immune system also include IFNs, whose response pathways to their action were identified in the enrichment analysis of identified genes from our RNA-seq analysis. In view of this, we have demonstrated possible correlations in altered endometrial receptivity processes between adenomyosis and endometriosis. Additionally, we identified pathways related to immune system cytokines (*signalling by IL* and *signalling by IL4 and IL13*) by two enrichment analyses of data retrieved only from the literature review, i.e. genes associated with endometrial receptivity in adenomyosis (42 genes), endometriosis (173 genes) and healthy uterus (151 genes). We further checked endometrial expression levels of selected six genes (*JUNB*, *FOS*, *SOCS3*, *IL6*, *IL10* and *LIF*) that were annotated in these two pathways. Endometrial samples were obtained between days LH+7–LH+9 from women with (n = 9) and without (n = 13) adenomyosis. We detected downregulation of selected genes in the adenomyosis compared to the control group, but the difference was statistically insignificant. The study should be repeated in a larger sample size (Prašnikar et al., 2020b).

Identification of 52 stronger candidate genes (including *ANXA1*, *BCL6*, *CXCL2*, *DUSP1*, *EPHA2*, *NFKB1A*, *PTGS1*, *S100A9*, *SOCS3* and *VEGFA*) of endometrial pathophysiology in adenomyosis based on data synthesis from 50 studies performed in the P phase. In this phase, progesterone hormone (P4) and receptivity-related genes are absent, so differences in endometrial expression patterns between study and control groups are more likely due to pathophysiological changes (Makieva et al., 2018). To identify 10 stronger candidate genes (*IL10, SST, STAT3, LIF, CHD5, CDKN3, COL8A1, COL11A1, ITGB3* and *SPP1*) of altered endometrial receptivity in adenomyosis, we pooled data from seven studies performed in the MS phase. Understanding the role of individual genes in adenomyosis could lead to the identification of endometrial molecular markers of this disease. A specific molecular test could contribute to a more reliable diagnosis of early adenomyosis, which is currently limited only to highly sensitive but subjective imaging methods (Hershko-Klement and Tepper, 2016). While a specific molecular test for the diagnosis of uterine receptivity in adenomyosis could better determine whether the endometrium is a factor for infertility in these women. Knowledge of the pathological processes of endometrial receptivity in adenomyosis could lead to the development of new therapeutic approaches for endometrial preparation prior the embryo transfer in assisted reproductive technology treatment.

5 VIRI

Absenger Y., Hess-Stumpp H., Kreft B., Krätzschmar J., Haendler B., Schütze N., Regidor P.A., Winterhager E. 2004. Cyr61, a deregulated gene in endometriosis. Molecular Human Reproduction, 10, 6: 399-407

Achache H., Revel A. 2006. Endometrial receptivity markers, the journey to successful embryo implantation. Human Reproduction Update, 12, 6: 731-746

Ahn S.H., Singh V., Tayade C. 2017. Biomarkers in endometriosis: challenges and opportunities. Fertility and Sterility, 107, 3: 523-532

Altmäe S., Esteban F.J., Stavreus-Evers A., Simón C., Giudice L., Lessey B.A., Horcajadas J.A., Macklon N.S., D'Hooghe T., Campoy C., Fauser B.C., Salamonsen L.A., Salumets A. 2014. Guidelines for the design, analysis and interpretation of "omics" data: focus on human endometrium. Human Reproduction Update, 20, 1: 12-28

Altmäe S., Koel M., Võsa U., Adler P., Suhorutšenko M., Laisk-Podar T., Kukushkina V., Saare M., Velthut-Meikas A., Krjutškov K., Aghajanova L., Lalitkumar P.G., Gemzell-Danielsson K., Giudice L., Simón C., Salumets A. 2017. Meta-signature of human endometrial receptivity: a meta-analysis and validation study of transcriptomic biomarkers. Scientific Reports, 7, 1: 10077, doi: 10.1038/s41598-017-10098-3: 15 str.

Altmäe S., Reimand J., Hovatta O., Zhang P., Kere J., Laisk T., Saare M., Peters M., Vilo J., Stavreus-Evers A., Salumets A. 2012. Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks. Molecular Endocrinology, 26, 1: 203-217

An M., Li D., Yuan M., Li Q., Zhang L., Wang G. 2017. Interaction of macrophages and endometrial cells induces epithelial–mesenchymal transition-like processes in adenomyosis. Biology of Reproduction, 96, 1: 46-57

Andres M.P., Borrelli G.M., Ribeiro J., Baracat E.C., Abrão M.S., Kho R.M. 2018. Transvaginal ultrasound for the diagnosis of adenomyosis: systematic review and meta-analysis. Journal of Minimally Invasive Gynecology, 25, 2: 257-264

Ashburner M., Ball C.A., Blake J.A., Botstein D., Butler H., Cherry J.M., Davis A.P., Dolinski K., Dwight S.S., Eppig J.T., Harris M.A., Hill D.P., Issel-Tarver L., Kasarskis A., Lewis S., Matese J.C., Richardson J.E., Ringwald M., Rubin G.M., Sherlock G. 2000. The Gene Ontology: tool for the unification of biology. Nature genetics, 25, 1: 25-29

Austin K.J., Bany B.M., Belden E.L., Rempel L.A., Cross J.C., Hansen T.R. 2003. Interferon-stimulated gene-15 (Isg15) expression is up-regulated in the mouse uterus in response to the implanting conceptus. Endocrinology, 144, 7: 3107-3113

Baiyong L., Tournier C., Davis R.J., Flavell R.A. 1999. Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. The EMBO Journal, 18, 2: 420-432

Bartel D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116, 2: 281-297

Bazot M., Daraï E. 2018. Role of transvaginal sonography and magnetic resonance imaging in the diagnosis of uterine adenomyosis. Fertility and Sterility, 109, 3: 389-397

Ensembl BioMart. 2019. Hinxton, EMBL's european Bioinformatics Institute http://www.ensembl.org/biomart/martview/bcec885dcf44191334b3f4cea3152994 (23. sep. 2019)

Benagiano G., Brosens I., Habiba M. 2014. Structural and molecular features of the

endomyometrium in endometriosis and adenomyosis. Human Reproduction Update, 20, 3: 386-402

Benaglia L., Cardellicchio L., Leonardi M., Faulisi S., Vercellini P., Paffoni A., Somigliana E., Fedele L. 2014. Asymptomatic adenomyosis and embryo implantation in IVF cycles. Reproductive BioMedicine Online, 29, 5: 606-611

Bindea G., Galon J., Mlecnik B. 2013. CluePedia Cytoscape plugin: Pathway insights using integrated experimental and in silico data. Bioinformatics, 29, 5: 661-663

Bindea G., Mlecnik B., Hackl H., Charoentong P., Tosolini M., Kirilovsky A., Fridman W.H., Pagès F., Trajanoski Z., Galon J. 2009. ClueGO: A Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics, 25, 8: 1091-1093

Borthwick J.M., Charnock-Jones D.S., Tom B.D., Hull M.L., Teirney R., Phillips S.C., Smith S.K. 2003. Determination of the transcript profile of human endometrium. Molecular Human Reproduction, 9, 1: 19-33

Braschi B., Denny P., Gray K., Jones T., Seal R., Tweedie S., Yates B., Bruford E. 2019. Genenames.org: the HGNC and VGNC resources in 2019. Nucleic Acids Research, 47, D1: D786-D792

Braunschweig A., Poehlmann T.G., Busch S., Schleussner E., Markert U.R. 2011. Signal transducer and activator of transcription 3 (STAT3) and suppressor of cytokine signaling (SOCS3) balance controls cytotoxicity and IL-10 expression in decidual-like natural killer cell line NK-92. American Journal of Reproductive Immunology, 66, 4: 329-335

Bulmer J.N., Jones R.K., Searle R.F. 1998. Intraepithelial leukocytes in endometriosis and adenomyosis: comparison of eutopic and ectopic endometrium with normal endometrium. Human Reproduction, 13, 10: 2910-2915

Burney R.O., Talbi S., Hamilton A.E., Kim C.V., Nyegaard M., Nezhat C.R., Lessey B.A., Giudice L.C. 2007. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. Endocrinology, 148, 8: 3814-3826

Campo S., Campo V., Benagiano G. 2012. Adenomyosis and infertility. Reproductive BioMedicine Online, 24, 1: 35-46

Carrarelli P., Yen C., Arcuri F., Funghi L., Tosti V., Wang T., Huang J.S., Petragllia F. 2015. Myostatin, follistatin and activin type II receptors are highly expressed in adenomyosis. Fertility and Sterility, 104, 3: 744-752

Carrarelli P., Yen C., Funghi L., Arcuri F., Tosti C., Bifulco G., Luddi A., Lee C., Petraglia F. 2017. Expression of inflammatory and neurogenic mediators in adenomyosis: a pathogenetic role. Reproductive Science, 24, 3: 369-375

Chaouat G., Dubanchet S., Ledée N. 2007. Cytokines: important for implantation? Journal of Assisted Reproduction and Genetics, 24, 11: 491-505

Chapron C., Tosti C., Marcellin L., Bourdon M., Lafay-Pillet M.C., Millischer A.E., Streuli I., Borghese B., Petraglia F., Santulli P. 2017. Relationship between the magnetic resonance imaging appearance of adenomyosis and endometriosis phenotypes. Human Reproduction, 32, 7: 1393-1401

Chapron C., Vannuccini S., Santulli P., Abrão M.S., Carmona F., Fraser I.S., Gordts S., Guo S.W., Just P.A., Noël J.C., Pistofidis G., Van den Bosch T., Petraglia F. 2020. Diagnosing adenomyosis: an integrated clinical and imaging approach. Human Reproduction Update, 26, 3: 392-411

Chen N., Du B., Zhou H., Shen F., Li J., Xie Z. 2017. Abnormal expression of Nrf2 may play

an important role in the pathogenesis and development of adenomyosis. PLoS ONE, 12, 8: e0182773, doi: 10.1371/journal.pone.0182773: 10 str.

Chen Y.J., Li H.Y., Huang C.H., Twu N.F., Yen M.S., Wang P.H., Chou T.Y., Liu Y.N., Chao K.C., Yang M.H. 2010. Oestrogen-induced epithelial-mesenchymal transition of endometrial epithelial cells contributes to the development of adenomyosis. Journal of Pathology, 222, 3: 261-270

Chin C.H., Chen S.H., Wu H.H., Ho C.W., Ko M.T., Lin C.Y. 2014. cytoHubba: identifying hub objects and sub-networks from complex interactome. BMC Systems Biology, 8, Suppl 4: S11, doi: 10.1186/1752-0509-8-S4-S11: 7 str.

Chung T.W., Park M.J., Kim H.S., Choi H.J., Ha K.T. 2016. Integrin αvβ3 and αvβ5 are required for leukemia inhibitory factor-mediated the adhesion of trophoblast cells to the endometrial cells. Biochemical and Biophysical Research Communications, 469, 4: 936-940

Costello M.F., Lindsay K., McNally G. 2011. The effect of adenomyosis on in vitro fertilisation and intra-cytoplasmic sperm injection treatment outcome. European Journal of Obstetrics Gynecology and Reproductive Biology, 158, 2: 229-234

Coutifaris C., Myers E.R., Guzick D.S., Diamond M.P., Carson S.A., Legro R.S., McGovern P.G., Schlaff W.D., Carr B.R., Steinkampf M.P., Silva S., Vogel D.L., Leppert P.C. 2004. Histological dating of timed endometrial biopsy tissue is not related to fertility status. Fertility and Sterility, 82, 5: 1264-1272

Cui D., Ma J., Liu Y., Lin K., Jiang X., Qu Y., Lin J., Xu K. 2018. Analysis of long non-coding RNA expression profiles using RNA sequencing in ovarian endometriosis. Gene, 673, 140-148

Cullinan E.B., Abbondanzo S.J., Andersont P.S., Pollardt J.W., Lesseyt B.A., Stewart C.L. 1996. Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. Proceedings of the National Academy of Sciences of the United States of America, 93, 7: 3115-3120

Del Frate C., Girometti R., Pittino M., Del Frate G., Bazzocchi M., Zuiani C. 2006. Deep retroperitoneal pelvic endometriosis: MR imaging appearance with laparoscopic correlation. Radiographics, 26, 6: 1705-1719

De Veer M.J., Holko M., Frevel M., Walker E., Der S., Paranjape J.M., Silverman R.H., Williams B.R. 2001. Functional classification of interferon-stimulated genes identified using microarrays. Journal of Leukocyte Biology, 69, 6: 912-920

Devesa-Peiro A., Sebastian-Leon P., Pellicer A., Diaz-Gimeno P. 2021. Guidelines for biomarker discovery in endometrium: correcting for menstrual cycle bias reveals new genes associated with uterine disorders. Molecular Human Reproduction, 27, 4: gaab011

Di Carlo C., Bonifacio M., Tommaselli G.A., Bifulco G., Guerra G., Nappi C. 2009. Metalloproteinases, vascular endothelial growth factor, and angiopoietin 1 and 2 in eutopic and ectopic endometrium. Fertility and Sterility, 91, 6: 2315-2323

Díaz-Gimeno P., Horcajadas J.A., Martinez-Conejero J.A., Esteban F.J., Alama P., Pellicer A., Simon C. 2011. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. Fertility and Sterility, 95, 1: 50-60

Díaz-Gimeno P., Ruiz-Alonso M., Blesa D., Bosch N., Martínez-Conejero J.A., Alamá P., Garrido N., Pellicer A., Simón C. 2013. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. Fertility and Sterility, 99, 2: 508-517

Diehl S., Rincón M. 2002. The two faces of IL-6 on Th1/Th2 differentiation. Molecular Immunology, 39, 9: 531-536

Dior U.P., Nisbet D., Fung J.N., Foster G., Healey M., Montgomery G.W., Rogers P.A., Holdsworth-Carson S.J., Girling J.E. 2018. The association of sonographic evidence of adenomyosis with severe endometriosis and with gene expression in eutopic endometrium. Journal of Minimally Invasive Gynecology, 26, 5: 941-948

Dong Q., Fan R., Zhao S., Wang Y. 2009. Over-expression of SOCS-3 gene promotes IL-10 production by JEG-3 trophoblast cells. Placenta, 30, 1: 11-14

DisGeNET v7.0. 2021. Endometriosis of uterus. Barcelona, Integrative Biomedical Informatics Group. https://www.disgenet.org/browser/0/1/1/C0341858/25/25/223/_a/_b./-score/ (15. jan. 2022)

Edwards R.G. 1995. Clinical approaches to increasing uterine receptivity during human implantation. Human Reproduction, 10, Suppl 2: 60-66

Enciso M., Carrascosa J.P., Sarasa J. 2018. Development of a new comprehensive and reliable endometrial receptivity map (ER Map/ER Grade) based on RT-qPCR gene expression analysis. Human Reproduction, 33, 2: 220-228

Fabregat A., Sidiropoulos K., Garapati P., Gillespie M., Hausmann K., Haw R., Jassal B., Jupe S., Korninger F., McKay S., Matthews L., May B., Milacic M., Rothfels K., Shamovsky V., Webber M., Weiser J., Williams M., Wu G., Stein L., Hermjakob H., D'Eustachio P. 2016. The Reactome pathway knowledgebase. Nucleic Acids Research, 44, D1: D481-D487. https://reactome.org/ (25. feb. 2021)

Fan L., Han H., Guan J., Zhang X., Cui Q., Shen H., Shi C. 2017. Aberrantly expressed long noncoding RNAs in recurrent implantation failure: a microarray related study. Systems Biology in Reproductive Medicine, 63, 4: 269-278

Ferenczy A. 1998. Pathophysiology of adenomyosis. Human Reproduction Update, 4, 4: 312-322

Fischer C., Kayisili U., Taylor H. 2011. HOXA10 expression is decreased in endometrium of women with adenomyosis. Fertility and Sterility, 95, 3: 1133-1136

Florio P., Rossi M., Viganò P., Luisi S., Torricelli M., Torres P.B., Maria A., Blasio D., Petraglia F. 2007. Interleukin 1beta and progesterone stimulate activin a expression and secretion from cultured human endometrial stromal cells. Reproductive sciences, 14, 1: 29-36

Flynn L., Byrne B., Carton J., Kelehan P., O'Herlihy C., O'Farrelly C. 2000. Menstrual cycle dependent fluctuations in NK and T-lymphocyte subsets from non-pregnant human endometrium. American Journal of Reproductive Immunology, 43, 4: 209-217

Franasiak J.M., Holoch K.J., Yuan L., Schammel D.P., Young S.L., Lessey B.A. 2014. Prospective assessment of midsecretory endometrial leukemia inhibitor factor expression versus αvβ3 testing in women with unexplained infertility. Fertility and Sterility, 101, 6: 1724-1731

Fusi L., Cloke B., Brosens J.J. 2006. The uterine junctional zone. Best Practice and Research: Clinical Obstetrics and Gynaecology, 20, 4: 479-491

García-Solares J., Donnez J., Donnez O., Dolmans M.M. 2018. Pathogenesis of uterine adenomyosis: invagination or metaplasia? Fertility and Sterility, 109, 3: 371-379

Gnainsky Y., Dekel N., Granot I. 2014. Implantation: mutual activity of sex steroid hormones and the immune system guarantee the maternal-embryo interaction. Seminars in Reproductive Medicine, 32, 5: 337-345

Goteri G., Lucarini G., Montik N., Zizzi A., Stramazzotti D., Fabris G., Tranquilli A.L., Ciavattini A. 2009. Expression of vascular endothelial growth factor (VEGF), hypoxia inducible factor-1α (HIF-1α), and microvessel density in endometrial tissue in women with adenomyosis. International Journal of Gynecological Pathology, 28, 2: 157-163

Greening D.W., Nguyen H.P.T., Elgass K., Simpson R.J., Salamonsen L.A. 2016. Human endometrial exosomes contain hormone-specific cargo modulating trophoblast adhesive capacity: insights into endometrial-embryo interactions. Biology of Reproduction, 94, 2: 38

Grewal S., Carver J., Ridley A.J., Mardon H.J. 2010. Human endometrial stromal cell Rho GTPases have opposing roles in regulating focal adhesion turnover and embryo invasion in vitro. Biology of Reproduction, 83, 1: 75-82

Guo J., Chen L., Luo N., Li C., Chen R., Qu X., Liu M., Kang L., Cheng Z. 2016. LPS/TLR4-mediated stromal cells acquire an invasive phenotype and are implicated in the pathogenesis of adenomyosis. Scientific Reports, 6, 21416

Guo Q., Zhang H., Zhao X., Fu Y., Zhang J., Li M. 2014. Loss of expressions of Dusp6, Sprouty4, and Sef, negative regulators of FGF2/ERK1/2 signaling, in the endometrium of women with adenomyosis. International Journal of Gynecological Pathology, 33, 3: 288-297

Guo Y., Lang X., Lu Z., Wang J., Li T., Liao Y., Jia C., Zhao W., Fang H. 2015. MiR-10b directly targets ZEB1 and PIK3CA to curb adenomyotic epithelial cell invasiveness via upregulation of E-cadherin and inhibition of Akt phosphorylation. Cellular Physiology and Biochemistry, 35, 6: 2169-2180

Gupta D., Hull M.L., Fraser I., Miller L., Bossuyt P.M.M., Johnson N., Nisenblat V. 2016. Endometrial biomarkers for the non-invasive diagnosis of endometriosis. Cochrane Database of Systematic Reviews, 4, CD012165, doi: 10.1002/14651858.CD012165: 294 str.

Halasz M., Szekeres-Bartho J. 2013. The role of progesterone in implantation and trophoblast invasion. Journal of Reproductive Immunology, 97, 1: 43-50

Hannan N.J., Evans J., Salamonsen L.A. 2011. Alternate roles for immune regulators: establishing endometrial receptivity for implantation. Expert Review of Clinical Immunology, 7, 6: 789-802

Haouzi D., Entezami F., Torre A., Innocenti C., Antoine Y., Mauries C., Vincens C., Bringer-Deutsch S., Gala A., Ferrieres-HOA A., Ohl J., Gonzalez Marti B., Brouillet S., Hamamah S. 2021. Customized frozen embryo transfer after identification of the receptivity window with a transcriptomic approach improves the implantation and live birth rates in patients with repeated implantation failure. Reproductive Sciences, 28, 1: 69-78

Hashim H.A., Elaraby S., Fouda A.A., Rakhawy M. El. 2020. The prevalence of adenomyosis in an infertile population: a cross-sectional study. Reproductive BioMedicine Online, 40, 6: 842-850

Hatok J., Zubor P., Galo S., Kirschnerova R., Dobrota D., Danko J., Racay P. 2011. Endometrial aromatase mRNA as a possible screening tool for advanced endometriosis and adenomyosis. Gynecological Endocrinology, 27, 5: 331-336

Hawkins S.M., Matzuk M.M. 2008. Menstrual cycle: basic biology. Annals of the New York Academy of Sciences, 1135: 10-18

Herndon C.N., Aghajanova L., Bayalan S., Erikson D., Barragan F., Goldfien G., Vo K.C., Hawkins S., Giudice L.C. 2016. Global transcriptome abnormalities of the eutopic

endometrium from women with adenomyosis. Reproductive Sciences, 23, 10: 1289-1303

Hershko-Klement A., Tepper R. 2016. Ultrasound in assisted reproduction: a call to fill the endometrial gap. Fertility and Sterility, 105, 6: 1394-1402.e4

Hess A.P., Hamilton A.E., Talbi S., Dosiou C., Nyegaard M., Nayak N., Genbecev-Krtolica O., Mavrogianis P., Ferrer K., Kruessel J., Fazleabas A.T., Fisher S.J., Giudice L.C. 2007. Decidual stromal cell response to paracrine signals from the trophoblast: amplification of immune and angiogenic modulators. Biology of Reproduction, 76, 1: 102-117

Hever A., Roth R.B., Hevezi P.A., Lee J., Willhite D., White E.C., Marin E.M., Herrera R., Acosta H.M., Acosta A.J., Zlotnik A. 2006. Molecular characterization of human adenomyosis. Molecular Human Reproduction, 12, 12: 737-748

Horton J., Sterrenburg M., Lane S., Maheshwari A., Li T.C., Cheong Y. 2019. Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: a systematic review and meta-analysis. Human Reproduction Update, 25, 5: 593-633

Houshdaran S., Nezhat C.R., Vo K.C., Zelenko Z., Irwin J.C., Giudice L.C. 2016. Aberrant endometrial DNA methylome and associated gene expression in women with endometriosis. Biology of Reproduction, 95, 5: 93

Hu H., Li H., He Y. 2017. MicroRNA-17 downregulates expression of the PTEN gene to promote the occurrence and development of adenomyosis. Experimental and Therapeutic Medicine, 14, 4: 3805-3811

Huang D.W., Sherman B.T., Lempicki R.A. 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research, 37, 1: 1-13. https://david.ncifcrf.gov/ (17. jan. 2022)

Huang H., Yu H., Chan S., Sc B., Lee C., Wang H., Soong Y. 2010. Eutopic endometrial interleukin-18 system mRNA and protein expression at the level of endometrial-myometrial interface in adenomyosis patients. Fertility and Sterility, 94, 1: 33-39

Huang P.C., Tsai E.M., Li W.F., Liao P.C., Chung M.C., Wang Y.H., Wang S.L. 2010. Association between phthalate exposure and glutathione S-transferase M1 polymorphism in adenomyosis, leiomyoma and endometriosis. Human Reproduction, 25, 4: 986-994

Huang T.S., Chen Y.J., Chou T.Y., Chen C.Y., Li H.Y., Huang B.S., Tsai H.W., Lan H.Y., Chang C.H., Twu N.F., Yen M.S., Wang P.H., Chao K.C., Lee C.C., Yang M.H. 2014. Oestrogen-induced angiogenesis promotes adenomyosis by activating the Slug-VEGF axis in endometrial epithelial cells. Journal of Cellular and Molecular Medicine, 18, 7: 1358-1371

Huang Y., Zheng W., Mu L., Ren Y., Chen X., Liu F. 2011. Expression of tyrosine kinase receptor B in eutopic endometrium of women with adenomyosis. Archives of Gynecology and Obstetrics, 283, 4: 775-780

HUGO Gene Nomenclature Committee. 2019. Cambridge, European Bioinformatics Institute. https://www.genenames.org/ (10. jun. 2019)

Ibrahim M.G., Chiantera V., Frangini S., Younes S., Köhler C., Taube E., Plendl J., Mechsner S. 2015. Ultramicro-trauma in the zone and pale cell migration in adenomyosis. Fertility and Sterility, 104, 6: 1475-1483

Ibrahim M.G., Sillem M., Plendl J., Chiantera V., Sehouli J., Mechsner S. 2017. Myofibroblasts are evidence of chronic tissue microtrauma at the endometrial-myometrial junctional zone in uteri with adenomyosis. Reproductive Sciences, 24, 10: 1410-1418

ICD-10. 2019. Diseases of the genitourinary system. International Statistical Classification of Diseases and Related Health Problems. Geneva, WHO. https://icd.who.int/browse10/2019/en#/N80.0 (10. apr. 2020)

Inoue S., Hirota Y., Ueno T., Fukui Y., Yoshida E., Hayashi T., Kojima S., Takeyama R., Hashimoto T., Kiyono T., Ikemura M., Taguchi A., Tanaka T., Tanaka Y., Sakata S., Takeuchi K., Muraoka A., Osuka S., Saito T., Oda K., Osuga Y., Terao Y., Kawazu M., Mano H. 2019. Uterine adenomyosis is an oligoclonal disorder associated with KRAS mutations. Nature Communications, 10, 1: 5785, doi: 10.1038/s41467-019-13708-y: 13 str.

Jiang C., Gong W., Chen R., Ke H., Qu X., Yang W., Cheng Z. 2018. RhoA/ROCK/ARHGAP26 signaling in the eutopic and ectopic endometrium is involved in clinical characteristics of adenomyosis. Journal of International Medical Research, 46, 12: 5019-5029

Jiang C., Liu C., Guo J., Chen L., Luo N., Qu X., Yang W., Ren Q., Cheng Z. 2017. The expression of Toll-like receptors in eutopic and ectopic endometrium and its implication in the inflammatory pathogenesis of adenomyosis. Scientific Reports, 7, 1: 7365, doi: 10.1038/s41598-017-07859-5: 7 str.

Jiang J.F., Sun A.J., Xue W., Deng Y., Wang Y.F. 2016. Aberrantly expressed long noncoding RNAs in the eutopic endometria of patients with uterine adenomyosis. European Journal of Obstetrics Gynecology and Reproductive Biology, 199, 32-37

Jiang J.F., Xiao S.S., Xue M. 2018. Decreased expression of interleukin-37 in the ectopic and eutopic endometria of patients with adenomyosis. Gynecological Endocrinology, 34, 1: 83-86

Jiang Y., Jiang R., Cheng X., Zhang Q., Hu Y., Zhang H., Cao Y., Zhang M., Wang J., Ding L., Diao Z., Sun H., Yan G. 2016. Decreased expression of NR4A nuclear receptors in adenomyosis impairs endometrial decidualization. Molecular Human Reproduction, 22, 9: 655-668

Jiménez Fernández D., Hess S., Knobeloch K.P. 2020. Strategies to target ISG15 and USP18 toward therapeutic applications. Frontiers in Chemistry, 21, 7: 923, doi: 10.3389/fchem.2019.00923: 12 str.

Jin Z., Liu H., Xu C. 2020. Estrogen degrades Scribble in endometrial epithelial cells through E3 ubiquitin ligase HECW1 in the development of diffuse adenomyosis. Biology of Reproduction, 102, 2: 376-387

Jochum W., Passegué E., Wagner E.F. 2001. AP-1 in mouse development and tumorigenesis. Oncogene, 20, 19: 2401-2412

Jones R.K., Searle R.F., Bulmer J.N. 1998. Apoptosis and bcl-2 expression in normal human endometrium, endometriosis and adenomyosis. Human Reproduction, 13, 12: 3496-3502

Kamada Y., Nakatsuka M., Asagiri K., Noguchi S., Habara T., Takata M., Kudo T. 2000. GnRH agonist-suppressed expression of nitric oxide synthases and generation of peroxynitrite in adenomyosis. Human Reproduction, 15, 12: 2512-2519

Kanehisa M., Furumichi M., Tanabe M., Sato Y., Morishima K. 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Research, 45, D1: D353-D361

Kang S., Zhoa J., Liu Q., Zhou R., Wang N., Li Y. 2009. Vascular endothelial growth factor gene polymorphisms are associated with the risk of developing adenomyosis. Environmental and Molecular Mutagenesis, 50, 5: 361-266

Kao L.C., Germeyer A., Tulac S., Lobo S., Yang J.P., Taylor R.N., Osteen K., Lessey B.A.,

Giudice L.C. 2003a. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. Endocrinology, 144, 7: 2870-2881

Kao L.C., Tulac S., Lobo S., Imani B., Yang J.P., Germeyer A., Osteen K., Taylor R.N., Lessey B.A., Giudice L.C. 2003b. Global gene profiling in human endometrium during the window of implantation. Endocrinology, 143, 6: 2119-2138

Khan K.N., Fujishita A., Koshiba A., Kuroboshi H., Mori T., Ogi H., Itoh K., Nakashima M., Kitawaki J. 2019. Biological differences between intrinsic and extrinsic adenomyosis with coexisting deep infiltrating endometriosis. Reproductive BioMedicine Online, 39, 2: 343-353

Khan K.N., Kitajima M., Hiraki K., Fujishita A., Nakashima M., Masuzaki H. 2015. Involvement of hepatocyte growth factor-induced epithelial-mesenchymal transition in human adenomyosis. Biology of Reproduction, 92, 2: 1-11

Kharfi A., Labelle Y., Mailloux J., Akoum A. 2003. Deficient expression of tumor necrosis factor receptor type 2 in the endometrium of women with endometriosis. American Journal of Reproductive Immunology, 50, 1: 33-40

Kim S.R., Kim S.H., Lee H.W., Chae H.D., Kim C.H., Kang B.M. 2010. Increased expression of p21-activated kinase in adenomyosis. Fertility and Sterility, 94, 3: 1125-1128

Kishi Y., Suginami H., Kuramori R., Yabuta M., Suginami R., Taniguchi F. 2012. Four subtypes of adenomyosis assessed by magnetic resonance imaging and their specification. American Journal of Obstetrics and Gynecology, 207, 2: 114.e1-114.e7, doi: 10.1016/j.ajog.2012.06.027: 6 str.

Kissler S., Zangos S., Kohl J., Wiegratz I., Rody A., Gätje R., Vogl T.J., Kunz G., Leyendecker G., Kaufmann M. 2008. Duration of dysmenorrhoea and extent of adenomyosis visualised by magnetic resonance imaging. European Journal of Obstetrics and Gynecology and Reproductive Biology, 137, 2: 204-209

Kitawaki J., Koshiba H., Ishihara H., Kusuki I., Tsukamoto K., Honjo H. 2000. Progesterone induction of 17β-hydroxysteroid dehydrogenase type 2 during the secretory phase occurs in the endometrium of estrogen-dependent benign diseases but not in normal endometrium. Journal of Clinical Endocrinology and Metabolism, 85, 9: 3292-3296

Kitawaki J., Noguchi T., Amatsu T., Maeda K., Tsukamoto K., Yamamoto T., Fushiki S., Osawa Y., Honjo H. 1997. Expression of aromatase cytochrome P450 protein and messenger ribonucleic acid in human endometriotic and adenomyotic tissues but not in normal endometrium. Biology of Reproduction, 57, 3: 514-519

Kolioulis I., Zafrakas M., Grimbizis G., Miliaras D., Timologou A., Bontis J.N., Tarlatzis B.C. 2017. Immunohistochemical expression pattern of metastasis suppressor KISS-1 protein in adenomyosis lesions and normal endometrium. European Journal of Obstetrics Gynecology and Reproductive Biology, 210: 64-68

Koot Y.E.M., Van Hooff S.R., Boomsma C.M., Van Leenen D., Koerkamp M.J.A.G., Goddijn M., Eijkemans M.J.C., Fauser B.C.J.M., Holstege F.C.P., Macklon N.S. 2016. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. Scientific Reports, 6, 19411, doi: 10.1038/srep19411: 12 str.

Krjutškov K., Katayama S., Saare M., Vera-Rodriguez M., Lubenets D., Samuel K., Laisk-Podar T., Teder H., Einarsdottir E., Salumets A., Kere J. 2016. Single-cell transcriptome analysis of endometrial tissue. Human Reproduction, 31, 4: 844-853

Kühn R., Löhler J., Rennick D., Rajewsky K., Müller W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. Cell, 75, 2: 263-274

Kunz G., Beil D., Huppert P., Noe M., Kissler S., Leyendecker G. 2005. Adenomyosis in endometriosis—prevalence and impact on fertility. Evidence from magnetic resonance imaging. Human Reproduction, 20, 8: 2309-2316

Kunz G., Herbertz M., Beil D., Huppert P., Leyendecker G. 2007. Adenomyosis as a disorder of the early and late human reproductive period. Reproductive BioMedicine Online, 15, 6: 681-685

Lai T.H., Wu P.H., Wu W. Bin. 2016. Involvement of NADPH oxidase and NF-κB activation in CXCL1 induction by vascular endothelial growth factor in human endometrial epithelial cells of patients with adenomyosis. Journal of Reproductive Immunology, 118: 61-69

Lang R., Pauleau A.L., Parganas E., Takahashi Y., Mages J., Ihle J.N., Rutschman R., Murray P.J. 2003. SOCS3 regulates the plasticity of gp130 signaling. Nature Immunology, 4, 6: 546-550

Laudanski P., Charkiewicz R., Kuzmicki M., Szamatowicz J., Charkiewicz A., Niklinski J. 2013. MicroRNAs expression profiling of eutopic proliferative endometrium in women with ovarian endometriosis. Reproductive Biology and Endocrinology, 11, 78: 1-7

Lee J.Y., Lee M., Lee S.K. 2011. Role of endometrial immune cells in implantation. Clinical and Experimental Reproductive Medicine, 38, 3: 119-125

Leyendecker G., Bilgicyildirim A., Inacker M., Stalf T., Huppert P., Mall G., Böttcher B., Wildt L. 2015. Adenomyosis and endometriosis. Re-visiting their association and further insights into the mechanisms of auto-traumatisation. An MRI study. Archives of Gynecology and Obstetrics, 291, 4: 917-932

Li B., Chen M., Liu X., Guo S.W. 2013. Constitutive and tumor necrosis factor-α-induced activation of nuclear factor-κB in adenomyosis and its inhibition by andrographolide. Fertility and Sterility, 100, 2: 568-577

Li C., Chen R., Jiang C., Chen L., Cheng Z. 2019. Correlation of LOX-5 and COX-2 expression with inflammatory pathology and clinical features of adenomyosis. Molecular Medicine Reports, 19, 1: 727-733

Li D., Jiang W., Jiang Y., Wang S., Fang J., Zhu L., Zhu Y., Yan G., Sun H., Chen L., Zhang N. 2019. Preliminary functional inquiry of lncRNA ENST00000433673 in embryo implantation using bioinformatics analysis. Systems Biology in Reproductive Medicine, 65, 2: 164-173

Li J., Yanyan M., Mu L., Chen X., Zheng W. 2019. The expression of Bcl-2 in adenomyosis and its effect on proliferation, migration, and apoptosis of endometrial stromal cells. Pathology Research and Practice, 215, 8: 152477, doi: 10.1016/j.prp.2019.152477: 6 str.

Li T., Li Y.-G., Pu D.M. 2006. Matrix metalloproteinase-2 and -9 expression correlated with angiogenesis in human adenomyosis. Gynecologic and Obstetric Investigation, 62, 4: 229-235

Li X., Wu D., Cooper N.G.F., Rai S.N. 2019. Sample size calculations for the differential expression analysis of RNA-seq data using a negative binomial regression model. Statistical Applications in Genetics and Molecular Biology, 18, 1: 20180021, doi: 10.1515/sagmb-2018-0021: 17 str.

Liu X., Guo S. 2012. Aberrant immunoreactivity of deoxyribonucleic acid methyltransferases in adenomyosis. Gynecologic and Obstetric Investigation, 74, 2: 100-108

Liu X., Nie J., Guo S.W. 2011. Elevated immunoreactivity to tissue factor and its association with dysmenorrhea severity and the amount of menses in adenomyosis. Human Reproduction, 26, 2: 337-345

Liu X., Nie J., Guo S.W. 2012. Elevated immunoreactivity against class I histone deacetylases in adenomyosis. Gynecologic and Obstetric Investigation, 74, 1: 50-55

Liu X., Shen M., Qi Q., Zhang H., Guo S. 2016. Corroborating evidence for platelet-induced epithelial-mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation in the development of adenomyosis. Human Reproduction, 31, 4: 734-749

Liu Z., Sun Z., Liu H., Niu W., Wang X., Liang N., Wang X., Wang Y., Shi Y., Xu L., Shi W. 2021. Single-cell transcriptomic analysis of eutopic endometrium and ectopic lesions of adenomyosis. Cell and Bioscience, 11, 1: 51, doi: 10.1186/s13578-021-00562-z: 17 str.

Livak K.J., Schmittgen T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods, 25, 4: 402-408

Loring M., Chen T.Y., Isaacson K.B. 2020. A systematic review of adenomyosis: it's time to reassess what we thought we knew about the disease. Journal of Minimally Invasive Gynecology, 28, 3: 644-655

Macer L.M., Taylor S.H. 2012. Endometriosis and infertility: a review of the pathogenesis and treatment of endometriosis-associated infertility. Obstet Gynecol Clin Nort Am, 39, 4: 535-549

Macklon N.S., Brosens J.J. 2014. The human endometrium as a sensor of embryo quality. Biology of Reproduction, 91, 4: 98, doi: 10.1095/biolreprod.114.122846: 8 str.

Mahajan N., Kaur S., Alonso M.R. 2018. Window of implantation is significantly displaced in patients with adenomyosis with previous implantation failure as determined by endometrial receptivity assay. Journal of Human Reproductive Science, 11, 4: 353-358

Maheshwari A., Gurunath S., Fatima F., Bhattacharya S. 2012. Adenomyosis and subfertility: a systematic review of prevalence, diagnosis, treatment and fertility outcomes. Human Reproduction Update, 18, 4: 374-392

Maia H., Casoy J., Correia T., Freitas L., Pimentel K., Athayde C., Coutinho E. 2006. Effect of the menstrual cycle and oral contraceptives on aromatase and cyclooxygenase-2 expression in adenomyosis. Gynecological Endocrinology, 22, 10: 547-551

Makieva S., Giacomini E., Ottolina J., Sanchez A.M., Papaleo E., Vigan P. 2018. Inside the endometrial cell signaling subway: mind the gap(s). International Journal of Molecular Sciences, 19, 9: 2477, doi: 10.3390/ijms19092477: 28 str.

Martinez-Conejero J.A., Morgan M., Montesinos M., Fortu S., Meseguer M., Simón C., Horajadas J.A., Pellicer A. 2011. Adenomyosis does not affect implantation, but is associated with miscarriage in patients undergoing oocyte donation. Fertility and Sterility, 96, 4: 943-951

Matarese G., De Placido G., Nikas Y., Alviggi C. 2003. Pathogenesis of endometriosis: natural immunity dysfunction or autoimmune disease? Trends in Molecular Medicine, 9, 5: 223-228

Matsuda M., Sasabe H., Adachi Y., Suzuki T., Mori T. 2001. Increased invasion activity of endometrial stromal cells and elevated expression of matrix metalloproteinase messenger RNA in the uterine tissues of mice with experimentally induced adenomyosis. American Journal of Obstetrics and Gynecology, 185, 6: 1374-1380

Matsuzaki S., Maleysson E., Darcha C. 2010. Analysis of matrix metalloproteinase-7 expression in eutopic and ectopic endometrium samples from patients with different forms of endometriosis. Human Reproduction, 25, 3: 742-750

Mehasseb M.K., Bell S.C., Pringle J.H., Habiba M.A. 2010a. Uterine adenomyosis is associated with ultrastructural features of altered contractility in the inner myometrium. Fertility and Sterility, 93, 7: 2130-2136

Mehasseb M.K., Panchal R., Taylor A.H., Brown L., Bell S.C., Path F.R.C., Habiba M. 2011. Estrogen and progesterone receptor isoform distribution through the menstrual cycle in uteri with and without adenomyosis. Fertility and Sterility, 95, 7: 2228-2235

Mehasseb M.K., Taylor A.H., Pringle J.H., Bell S.C., Habiba M. 2010b. Enhanced invasion of stromal cells from adenomyosis in a three-dimensional coculture model is augmented by the presence of myocytes from affected uteri. Fertility and Sterility, 94, 7: 2547-2551

Meuleman C., Vandenabeele B., Fieuws S., Spiessens C., Timmerman D., D'Hooghe T. 2009. High prevalence of endometriosis in infertile women with normal ovulation and normospermic partners. Fertility and Sterility, 92, 1: 68-74

Mijatovic V., Florijn E., Halim N., Schats R., Hompes P. 2010. Adenomyosis has no adverse effects on IVF/ICSI outcomes in women with endometriosis treated with long-term pituitary down-regulation before IVF/ICSI. European Journal of Obstetrics and Gynecology, 151, 1: 62-65

Miyashita M., Koga K., Takeuchi A., Makabe T., Taguchi A., Urata Y., Izumi G., Takamura M., Harada M., Hirata T., Hirota Y., Wada-Hiraike O., Yoshino O., Fujii T., Osuga Y. 2019. Expression of nerve injury-induced protein1 (Ninj1) in endometriosis. Reproductive Sciences, 26, 8: 1105-1110

Morsch D.M., Carneiro M.M., Lecke S.B., Araújo F.C., Camargos A.F., Reis F.M., Spritzer P.M. 2009. C-fos gene and protein expression in pelvic endometriosis: a local marker of estrogen action. Journal of Molecular Histology, 40, 1: 53-58

miRBase. 2019. Swindon, Biotechnology and biological Sciences Research Council. https://www.mirbase.org/ (20. sep. 2019)

Mu L.I.N., Chen W., Ma Y., Zheng W.E.I. 2015. Expression of focal adhesion kinase in the eutopic endometrium of women with adenomyosis varies with dysmenorrhea and pelvic pain. Experimental and Therapeutic Medicine, 10, 5: 1903-1907

Naftalin J., Jurkovic D. 2009. The endometrial–myometrial junction: a fresh look at a busy crossing. Ultrasound in Obstetrics and Gynecology, 34, 1: 1-11

NCBI Gene. 2019. Bethesda, National Library of Medicine. https://www.ncbi.nlm.nih.gov/gene (20. jan. 2019)

NCBI UniGene. 2019. Bethesda, National Library of Medicine. https://ncbiinsights.ncbi.nlm.nih.gov/2019/02/01/ncbi-to-retire-the-unigene-database/ (20. jan. 2019)

NCBI GenBank. 2019, Bethesda, National Library of Medicine. https://www.ncbi.nlm.nih.gov/genbank/sequenceids/ (20. sep. 2019)

Ng Y.H., Rome S., Jalabert A., Forterre A., Singh H., Hincks C.L., Salamonsen L.A. 2013. Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. PLoS ONE, 8, 3: e58502, doi: 10.1371/journal.pone.0058502: 13 str.

Nie J., Liu X., Guo S.W. 2010a. Promoter hypermethylation of progesterone receptor isoform B (PR-B) in adenomyosis and its rectification by a histone deacetylase inhibitor and a demethylation agent. Reproductive Sciences, 17, 11: 995-1005

Nie J., Liu X., Guo S.W. 2010b. Immunoreactivity of oxytocin receptor and transient receptor potential vanilloid type 1 and its correlation with dysmenorrhea in adenomyosis. American Journal of Obstetrics and Gynecology, 202, 4: 346.e1-8

Nie J., Lu Y., Liu X., Guo S. 2009. Immunoreactivity of progesterone receptor isoform B, nuclear factor k B, and IkBa in adenomyosis. Fertility and Sterility, 92, 3: 886-889

Nikas G., Makrigiannakis A. 2003. Endometrial pinopodes and uterine receptivity. Annals of

the New York Academy of Sciences, 997: 120-123
Nirgianakis K., Kalaitzopoulos D.R., Schwartz A.S.K., Spaanderman M., Kramer B.W., Mueller M.D., Mueller M. 2020. Fertility, pregnancy and neonatal outcomes of patients with adenomyosis: a systematic review and meta-analysis. Reproductive BioMedicine Online, 42, 1: 185-206
Oehler M.K., Greschik H., Fischer D.C., Tong X., Schuele R., Kieback D.G. 2004. Functional characterization of somatic point mutations of the human estrogen receptor α (hERα) in adenomyosis uteri. Molecular Human Reproduction, 10, 12: 853-860
Oh S.J., Shin J.H., Kim T.H., Lee H.S., Yoo J.Y., Ahn J.Y., Broaddus R.R., Taketo M.M., Lydon J.P., Leach R.E., Lessey B.A., Fazleabas A.T., Lim J.M., Jeong J.W. 2013. β - Catenin activation contributes to the pathogenesis of adenomyosis through epithelial-mesenchymal transition. Journal of Pathology, 231, 2: 210-222
Okada H., Tsuzuki T., Murata H. 2018. Decidualization of the human endometrium. Reproductive Medicine and Biology, 17, 3: 220-227
Okada H., Tsuzuki T., Shindoh H., Nishigaki A., Yasuda K., Kanzaki H. 2014. Regulation of decidualization and angiogenesis in the human endometrium: mini review. Journal of Obstetrics and Gynaecology Research, 40, 5: 1180-1187
Ota H., Igarashi S., Hatazaw J., Tanaka T. 1999. Immunohistochemical assessment of superoxide dismutase expression in the endometrium in endometriosis and adenomyosis. Fertility and Sterility, 72, 1: 129-134
Ota H., Igarashi S., Hatazawa J., Tanaka T. 1997. Distribution of heat shock proteins in eutopic and ectopic endometrium in endometriosis and adenomyosis. Fertility and Sterility, 68, 1: 23-28
Ota H., Igarashi S., Hatazawa J., Tanaka T. 1998a. Is adenomyosis an immune disease? Human Reproduction Update, 4, 4: 360-367
Ota H., Igarashi S., Hatazawa J., Tanaka T. 1998b. Endothelial nitric oxide synthase in the endometrium during the menstrual cycle in patients with endometriosis and adenomyosis. Fertility and Sterility, 69, 2: 303-308
Ota H., Igarashi S., Kato N., Tanaka T. 2000. Aberrant expression of glutathione peroxidase in eutopic and ectopic endometrium in endometriosis and adenomyosis. Fertility and Sterility, 74, 2: 313-318
Ota H., Igarashi S., Sasaki M., Tanaka T. 2001a. Xanthine oxidase in eutopic and ectopic endometrium in endometriosis and adenomyosis. Human Reproduction, 75, 4: 785-790
Ota H., Igarashi S., Sasaki M., Tanaka T. 2001b. Distribution of cyclooxygenase-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis. Human Reproduction, 16, 3: 561-566
Ota H., Igarashi S., Sato N., Tanaka H., Tanaka T. 2002. Involvement of catalase in the endometrium of patients with endometriosis and adenomyosis. Fertility and Sterility, 78, 4: 804-809
Ota H., Igarashi S., Tanaka T. 1996. Expression of γδT cells and adhesion molecules in endometriotic tissue in patients with endometriosis and adenomyosis. American Journal of Reproductive Immunology, 35, 5: 477-482
Ota H., Tanaka T. 1997. Integrin adhesion molecules in the endometrial glandular epithelium in patients with endometriosis or adenomyosis. The Journal of Obstretics and Gynaecology Research, 23, 5: 485-491
Ota H., Tanaka T. 2003. Stromal vascularization in the endometrium during adenomyosis. Microscopy research and technique, 60, 4: 445-449

Pan H., Sheng J.Z., Tang L., Zhu R., Zhou T.H., Huang H.F. 2008. Increased expression of c-fos protein associated with increased matrix metalloproteinase-9 protein expression in the endometrium of endometriotic patients. Fertility and Sterility, 90, 4: 1000-1007

Park H., Kim S.H., Cho Y.M., Ihm H.J., Oh Y.S., Hong S.H., Chae H.D., Kim C.H., Kang B.M. 2016. Increased expression of nuclear factor kappa-B p65 subunit in adenomyosis. Obstetrics & Gynecology Science, 59, 2: 123-129

Peng Y., Jin Z., Liu H., Xu C. 2021. Impaired decidualization of human endometrial stromal cells from women with adenomyosis. Biology of Reproduction, 104, 5: 1034-1044

Pino M., Galleguillos C., Torres M., Sovino H., Fuentes A., Boric M.A., Johnson M.C. 2009. Association between MMP1 and MMP9 activities and ICAM1 cleavage induced by tumor necrosis factor in stromal cell cultures from eutopic endometria of women with endometriosis. Reproduction, 138, 5: 837-847

Pirih N., Kunej T. 2017. Toward a taxonomy for multi-omics science? Terminology development for whole genome study approaches by omics technology and hierarchy. OMICS A Journal of Integrative Biology, 21, 1, doi: 10.1089/omi.2016.0144: 16 str.

Ponce C., Torres M., Galleguillos C., Sovino H., Boric M.A., Fuentes A., Johnson M.C. 2009. Nuclear factor κB pathway and interleukin-6 are affected in eutopic endometrium of women with endometriosis. Reproduction, 137, 4: 727-737

Popovici R.M., Kao L.C., Giudice C.G. 2000. Discovery of new inducible genes in in vitro decidualized human endometrial stromal cells using microarray technology. Endocrinology, 141, 9: 3510-3513

Prašnikar E., Knez J., Kovačič B., Kunej T. 2020a. Molecular signature of eutopic endometrium in endometriosis based on the multi-omics integrative synthesis. Journal of Assisted Reproduction and Genetics, 37, 7: 1593-1611

Prašnikar E., Kunej T., Gorenjak M., Potočnik U., Kovačič B., Knez J. 2022. Transcriptomics of receptive endometrium in women with sonographic features of adenomyosis. Reproductive Biology and Endocrinology, 20, 1: 2, doi: 10.1186/s12958-021-00871-5: 16 str.

Prašnikar E., Kunej T., Repnik K., Potočnik U., Knez J., Kovačič B. 2020b. Determining the molecular background of endometrial receptivity in adenomyosis. Biomolecules, 10, 9: 1311, doi:10.3390/biom10091311: 25 str.

PubMed. 2021. Bethesda, National Library of Medicine. https://pubmed.ncbi.nlm.nih.gov/ (20. dec.2021)

Puente J.M., Fabris A., Patel J., Patel A., Cerrillo M., Requena A., Garcia-Velasco J.A. 2016. Adenomyosis in infertile women: prevalence and the role of 3D ultrasound as a marker of severity of the disease. Reproductive Biology and Endocrinology, 14, 1: 60, doi: 10.1186/s12958-016-0185-6: 9 str.

Qi S., Zhao X., Li M., Zhang X., Lu Z., Yang C., Zhang C., Zhang H., Zhang N. 2015. Aberrant expression of Notch1/numb/snail signaling, an epithelial mesenchymal transition related pathway, in adenomyosis. Reproductive Biology and Endocrinology, 13, 96, doi: 10.1186/s12958-015-0084-2: 10 str.

Qin X., Zhang H., Wang F., Xue J., Wen Z. 2012. Expression and possible role of interleukin-10 receptors in patients with adenomyosis. European Journal of Obstetrics and Gynecology, 161, 2: 194-198

Quenby S., Vince G., Farquharson R., Aplin J. 2002. Recurrent miscarriage: a defect in nature's quality control? Human Reproduction, 17, 8: 1959-1963

Ren Y., Mu L., Ding X., Zheng W. 2010. Decreased expression of Beclin 1 in eutopic

endometrium of women with adenomyosis. Archives of Gynecology and Obstetrics, 282, 4: 401-406

Revel A. 2012. Defective endometrial receptivity. Fertility and Sterility, 97, 5: 1028-1032

Ruiz-Alonso M., Blese D., Diaz-Gimeno P., Gomez E., Fernandez-Sanchez M., Carranza F., Carrera J., Vilella F., Pellicer A., Simon C. 2013. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. Fertility and Sterility, 100, 3: 818-824

Saare M., Laisk T., Teder H., Paluoja P., Palta P., Koel M., Kirss F., Karro H., Sõritsa D., Salumets A., Krjutškov K., Peters M. 2019. A molecular tool for menstrual cycle phase dating of endometrial samples in endometriosis transcriptome studies. Biology of Reproduction, 101, 1: 1-3

Saare M., Peter M., Aints A., Laisk-Podar T., Salumets A., Altmäe S. 2017. OMICs studies and endometriosis biomarker identification. V: Biomarkers for endometriosis. D'Hooghe T. (ur.). Cham, Springer International Publishing: 227-258

Salim R., Riris S., Saab W., Abramov B., Khadum I., Serhal P. 2012. Adenomyosis reduces pregnancy rates in infertile women undergoing IVF. Reproductive BioMedicine Online, 25, 3: 273-277

Schwenke M., Knöfler M., Velicky P., Weimar C.H.E., Kruse M., Samalecos A., Wolf A., Macklon N.S., Bamberger A.M., Gellersen B. 2013. Control of human endometrial stromal cell motility by PDGF-BB, HB-EGF and trophoblast-secreted factors. PLoS ONE, 8, 1: e54336, doi: 10.1371/journal.pone.0054336: 14 str.

Sebastian-Leon P., Garrido N., Remohí J., Pellicer A., Diaz-Gimeno P. 2018. Asynchronous and pathological windows of implantation: two causes of recurrent implantation failure. Human Reproduction, 33, 4: 626-635

Serafini P.C., Silva I.D.C.G., Smith G.D., Motta E.L.A., Rocha A.M., Baracat E.C. 2009. Endometrial claudin-4 and leukemia inhibitory factor are associated with assisted reproduction outcome. Reproductive Biology and Endocrinology, 7, 30, doi: 10.1186/1477-7827-7-30: 9 str.

Shaked S., Jaffa A.J., Grisaru D., Elad D. 2015. Uterine peristalsis-induced stresses within the uterine wall may sprout adenomyosis. Biomechanics and Modeling in Mechanobiology, 14, 3: 437-444

Shan K., Lian-Fu Z., Hui D., Wei G., Na W., Xia J., Yan L. 2006. Polymorphisms in the promoter regions of the matrix metalloproteinases-7, -9 and the risk of endometriosis and adenomyosis in China. Molecular Human Reproduction, 12, 1: 35-39

Shannon P., Markiel A., Ozier O., Baliga S.N., Wang T.J., Ramage D., Amin N., Schwikowski B., Ideker T. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Research, 13, 11: 2498-2504

Sharma S., Bathwal S., Agarwal N., Chattopadhyay R., Saha I., Chakravarty B. 2019. Does presence of adenomyosis affect reproductive outcome in IVF cycles? A retrospective analysis of 973 patients. Reproductive BioMedicine Online, 38, 1: 13-21

Shen M., Liu X., Zhang H., Guo S.W. 2016. Transforming growth factor β1 signaling coincides with epithelial-mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation in the development of adenomyosis in mice. Human Reproduction, 31, 2: 355-369

Shen X., Duan H., Wang S., Gan L., Xu Q., Li J.J. 2019. Decreased expression of cannabinoid receptors in the eutopic and ectopic endometrium of patients with adenomyosis. BioMed Research International, 2019, 5468954, doi:

10.1155/2019/5468954: 8 str.
Sherman B.M., Korenman S.G. 1975. Hormonal characteristics of the human menstrual cycle throughout reproductive life. Journal of Clinical Investigation, 55, 4: 699-706
Shi B., Tu H., Sha L., Luo X., Wu W., Su Y., Yang S., Wang H. 2019. Upregulation of long noncoding RNA TUG1 by EGR1 promotes adenomyotic epithelial cell migration and invasion through recruiting EZH2 and suppressing TIMP2. Molecular Reproduction and Development, 86, 2: 239-247
Shi J.H., Jin L., Leng J.H., Lang J.H. 2016. Expression of potassium channels in uterine smooth muscle cells from patients with adenomyosis. Chinese Medical Journal, 129, 2: 200-205
Shi X.Y., Gu L., Chen J., Guo X.R., Shi Y.L. 2014. Downregulation of miR-183 inhibits apoptosis and enhances the invasive potential of endometrial stromal cells in endometriosis. International Journal of Molecular Medicine, 33, 1: 59-67
Shuya L.L., Menkhorst E.M., Yap J., Li P., Lane N., Dimitriadis E. 2011. Leukemia inhibitory factor enhances endometrial stromal cell decidualization in humans and mice. PLoS ONE, 6, 9: e25288, doi: 10.1371/journal.pone.0025288: 11 str.
Sigurgeirsson B., Jemt A., Hanna A., Ujvari D., Westgren M., Lundeberg J., Gidlof S. 2017. Comprehensive RNA sequencing of healthy human endometrium at two time points of the menstrual cycle. Biology of Reproduction, 96, 1: 24-33
Simón C., Martín J.C., Pellicer A. 2000. Paracrine regulators of implantation. Bailliere's Best Practice and Research in Clinical Obstetrics and Gynaecology, 14, 5: 815-826
Sotnikova N., Antsiferova I., Malyshkina A. 2002. Cytokine network of eutopic and ectopic endometrium in women with adenomyosis. American Journal of Reproductive Immunology, 47, 4: 251-255
Stanekova V., Woodman R.J., Tremellen K. 2018. The rate of euploid miscarriage is increased in te setting of adenomyosis. Human Reproduction Open, 2018, 3: hoy011, doi: 10.1093/cercor/bhw393: 8 str.
Stephens A.N., Hannan N.J., Rainczuk A., Meehan K.L., Chen J., Nicholls P.K., Rombauts L.J.F., Stanton P.G., Robertson D.M., Salamonsen L.A. 2010. Post-translational modifications and protein-specific isoforms in endometriosis revealed by 2D DIGE. Journal of proteome research, 9, 5: 2438-2449
Strathy J.H., Molgaard C.A., Coulam C.B., Melton L.J. 1982. Endometriosis and infertility: a laparoscopic study of endometriosis among fertile and infertile women. Fertility and Sterility, 38, 6: 667-672
Streuli I., Santulli P., Chouzenoux S. 2015. Activation of the MAPK/ERK cell-signaling pathway in uterine smooth muscle cells of women with adenomyosis. Reproductive Sciences, 22, 12: 1549-1560
Suhorutshenko M., Kukushkina V., Velthut-Meikas A., Altmäe S., Peters M., Mägi R., Krjutškov K., Koel M., Codoñer F.M., Martinez-Blanch J.F., Vilella F., Simón C., Salumets A., Laisk T. 2018. Endometrial receptivity revisited: endometrial transcriptome adjusted for tissue cellular heterogeneity. Human Reproduction, 33, 11: 2074-2086
Swaim C.D., Scott A.D., Canadeo L.A., Huibregtse J.M. 2017. Extracellular ISG15 signals cytokine secretion through the LFA-1 integrin receptor. Mollecular Cell, 68, 3: 581-590.e5., doi: 10.1016/j.molcel.2017.10.003: 31 str.
Szklarczyk D., Gable A.L., Lyon D., Junge A., Wyder S., Huerta-Cepas J., Simonovic M., Doncheva N.T., Morris J.H., Bork P., Jensen L.J., Von Mering C. 2019. STRING v11: protein-protein association networks with increased coverage, supporting functional

discovery in genome-wide experimental datasets. Nucleic Acids Research, 47, D1: D607-D613

Takahashi K., Nagata H., Kitao M. 1989. Clinical usefulness of determination of estradiol level in the menstrual blood for patients with endometriosis. Acta obstetricia et gynecologica Japan, 41, 11: 1849-1850

Tamaresis J.S., Irwin J.C., Goldfien G.A., Rabban J.T., Burney R.O., Nezhat C., DePaolo L. V., Giudice L.C. 2014. Molecular classification of endometriosis and disease stage using high-dimensional genomic data. Endocrinology, 155, 12: 4986-4999

Tan J., Kan A., Hitkari J., Taylor B., Tallon N., Warraich G., Yuzpe A., Nakhuda G. 2018. The role of the endometrial receptivity array (ERA) in patients who have failed euploid embryo transfers. Journal of Assisted Reproduction and Genetics, 35, 4: 683-692

Tang F., Barbacioru C., Nordman E., Li B., Xu N., Bashkirov V.I., Lao K., Surani M.A. 2010. RNA-Seq analysis to capture the transcriptome landscape of a single cell. Nature Protocols, 5, 3: 516-535

Thalluri V., Tremellen K.P. 2012. Ultrasound diagnosed adenomyosis has a negative impact on successful implantation following GnRH antagonist IVF treatment. Human Reproduction, 27, 12: 3487-3492

Thaxton J.E., Sharma S. 2010. Interleukin-10: a multi-faceted agent of pregnancy. American Journal of Reproductive Immunology, 63, 6: 482-491

The UniProt Consortium. 2019. UniProt: a worldwide hub of protein knowledge. Nucleic Acids Research, 47, D1: D506-D515. https://www.uniprot.org/ (10.8.2019)

Tremellen K.P., Russell P. 2012. The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure. II : adenomyosis and macrophages. Journal of Reproductive Immunology, 93, 1: 58-63

Tzfadia O., Diels T., De Meyer S., Vandepoele K., Aharoni A., Van de Peer Y.. 2016. CoExpNetViz: comparative co-expression networks construction and visualization tool. Frontiers in Plant Science, 6, 6: 1194, doi: 10.3389/fpls.2015.01194: 7 str.

Ulukus E.C., Ulukus M., Seval Y., Zheng W., Arici A. 2005. Expression of interleukin-8 and monocyte chemotactic protein-1 in adenomyosis. Human Reproduction, 20, 10: 2958-2963

Ulukus M., Ulukus E.C., Seval Y., Cınar O., Zheng W., Arici A. 2006. Expression of interleukin-8 receptors in patients with adenomyosis. Fertility and Sterility, 85, 3: 714-720

Ulukus M., Ulukus E.C., Seval Y., Zheng W., Arici A. 2005. Expression of interleukin-8 receptors in endometriosis. Human Reproduction, 20, 3: 794-801

Van den Bosch T., Dueholm M., Leone F.P.G., Valentin L., Rasmussen C.K., Votino A., Van Schoubroeck D., Landolfo C., Installé A.J.F., Guerriero S., Exacoustos C., Gordts S., Benacerraf B., D'Hooghe T., De Moor B., Brölmann H., Goldstein S., Epstein E., Bourne T., Timmerman D. 2015. Terms, definitions and measurements to describe sonographic features of myometrium and uterine masses: a consensus opinion from the Morphological Uterus Sonographic Assessment (MUSA) group. Ultrasound in Obstetrics and Gynecology, 46, 3: 284-298

Vannuccini S., Tosti C., Carmona F., Huang S.J., Chapron C., Guo S.W., Petraglia F. 2017. Pathogenesis of adenomyosis: an update on molecular mechanisms. Reproductive BioMedicine Online, 35, 5: 592-601

Veenstra van Nieuwenhoven A.L., Heineman M.J., Faas M.M. 2003. The immunology of successful pregnancy. Human Reproduction Update, 9, 4: 347-357

Vercellini P., Consonni D., Dridi D., Bracco B., Frattaruolo M.P., Somigliana E. 2014. Uterine adenomyosis and in vitro fertilization outcome: a systematic review and meta-analysis. Human Reproduction, 29, 5: 964-977

Von Wolff M.. 2000. Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. Molecular Human Reproduction, 6, 7: 627-634

Walsh C.T., Garneau-Tsodikova S., Gatto G.J. 2005. Protein posttranslational modifications: the chemistry of proteome diversifications. Angewandte Chemie - International Edition, 44, 45: 7342-7372

Wang F., Li H., Yang Z., Du X., Cui M., Wen Z. 2009. Expression of interleukin-10 in patients with adenomyosis. Fertility and Sterility, 91, 5: 1681-1685

Wang F., Shi X., Qin X., Wen Z., Zhao X., Li C. 2015. Expression of CD56 in patients with adenomyosis and its correlation with dysmenorrhea. European Journal of Obstetrics and Gynecology, 194: 101-105

Wang F., Wen Z., Li H., Yang Z., Zhao X., Yao X. 2008. Human leukocyte antigen-G is expressed by the eutopic and ectopic endometrium of adenomyosis. Fertility and Sterility, 90, 5: 1599-1604

Wang J., Deng X., Yang Y., Yang X., Kong B., Chao L. 2016. Expression of GRIM-19 in adenomyosis and its possible role in pathogenesis. Fertility and Sterility, 105, 4: 1093-1101

Wang J., Huang C., Jiang R., Du Y., Zhou J., Jiang Y., Yan Q., Xing J., Hou X., Zhou J., Sun H., Yan G. 2018. Decreased endometrial IL-10 impairs endometrial receptivity by downregulating HOXA10 expression in women with adenomyosis. BioMed Research International, 2018, 2549789, doi: 10.1155/2018/2549789: 9 str.

Wang Q., Wang L., Shao J., Wang Y., Jin L.P., Li D.J., Li M.Q. 2014. L-22 enhances the invasiveness of endometrial stromal cells of adenomyosis in an autocrine manner. International Journal of Clinical and Experimental Pathology, 7, 9: 5762-5771

Wang S., Duan H., Zhang Y., Sun F.Q. 2016. Abnormal activation of RhoA/ROCK-I signaling in junctional zone smooth muscle cells of patients with adenomyosis. Reproductive Sciences, 23, 3: 333-341

Wang Y., Duan H., Wang S., Quan Y., Huang J., Guo Z. 2021a. Talin1 induces epithelial-mesenchymal transition to facilitate endometrial cell migration and invasion in adenomyosis under the regulation of microRNA-145-5p. Reproductive Sciences, 28, 5: 1523-1539

Wang Y., Duan H., Wang S., Quan Y., Huang J., Guo Z. 2021b. Upregulated Talin1 synergistically boosts β-estradiol-induced proliferation and pro-angiogenesis of eutopic and ectopic endometrial stromal cells in adenomyosis. Reproductive Biology and Endocrinology, 19, 1: 70, doi: 10.1186/s12958-021-00756-7: 15 str.

Wang Y., Li Y., Yang Z., Liu K., Wang D. 2015a. Genome-wide microarray analysis of long non-coding RNAs in eutopic secretory endometrium with endometriosis. Cellular Physiology and Biochemistry, 37, 6: 2231-2245

Wang Y., Liu J., Huang B., Xu Y., Li J., Huang L., Lin J., Zhang J., Min Q., Yang W., Wang X. 2015b. Mechanism of alternative splicing and its regulation. Biomedical Reports, 3, 2: 152-158

Wang Z., Gerstein M., Snyder M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nature Reviews Genetics, 10, 1: 57-63

Wilczyński J.R. 2005. Th1/Th2 cytokines balance - yin and yang of reproductive

immunology. European Journal of Obstetrics and Gynecology and Reproductive Biology, 122, 2: 136-143

Wilusz J.E., Sunwoo H., Spector D.L. 2009. Long noncoding RNAs: functional surprises from the RNA world. Genes and Development, 23, 13: 1494-1504

Wu X., Zuo W., Liu H., Wang Z., Xu C. 2019. Decreased expression of cell polarity protein Scribble correlated with altered subcellular localization of the Crumbs homologue 3 protein in human adenomyotic endometrial cells. Journal of Obstetrics and Gynaecology Research, 45, 6: 1148-1159

Wyns C., Bergh C., Calhaz-Jorge C., De Geyter C., Kupka M.S., Motrenko T., Rugescu I., Smeenk J., Tandler-Schneider A., Vidakovic S., Goossens V. 2020. ART in Europe, 2016: results generated from European registries by ESHRE. Human Reproduction Open, 2020, 3: hoaa032, doi: 10.1093/hropen/hoaa032: 17 str.

Xiang Y., Sun Y., Yang B., Yang Y., Zhang Y., Yu T., Huang H., Zhang J., Xu H. 2019. Transcriptome sequencing of adenomyosis eutopic endometrium: a new insight into its pathophysiology. Journal of Cellular and Molecular Medicine, 23, 12: 8381-8391

Xiao Y., Li T., Xia E., Yang X., Sun X., Zhou Y. 2013. Expression of integrin ß3 and osteopontin in the eutopic endometrium of adenomyosis during the implantation window. European Journal of Obstetrics and Gynecology, 170, 2: 419-422

Xiao Y., Sun X., Yang X., Zhang J., Xue Q., Cai B., Zhou Y. 2010. Leukemia inhibitory factor is dysregulated in the endometrium and uterine flushing fluid of patients with adenomyosis during implantation window. Fertility and Sterility, 94, 1: 85-89

Xu X.Y., Zhang J., Qi Y.H., Kong M., Liu S.A., Hu J.J. 2018. Linc-ROR promotes endometrial cell proliferation by activating the PI3K-Akt pathway. European Review for Medical and Pharmacological Sciences, 22, 8: 2218-2225

Xue J., Zhang H., Liu W., Liu M., Shi M., Wen Z., Li C. 2013. Metformin inhibits growth of eutopic stromal cells from adenomyotic endometrium via AMPK activation and subsequent inhibition of AKT phosphorylation: a possible role in the treatment of Adenomyosis. Reproduction, 146, 4: 397-406

Yan Q., Yan G., Zhang C., Wang Z., Huang C., Wang J., Zhou J., Liu Y., Ding L., Zhang Q., Zhen X., Jiang Y., Sun H. 2019. MiR-21 reverses impaired decidualization through modulation of KLF12 and NR4A1 expression in human endometrial stromal cells. Biology of Reproduction, 100, 5: 1395-1405

Yang B., Wang L., Wan X., Li Y., Yu X., Qin Y., Luo Y., Wang F., Huang O. 2017. Elevated plasma levels of lysophosphatidic acid and aberrant expression of lysophosphatidic acid receptors in adenomyosis. BMC Women's Health, 17, 1: 118, doi: 10.1186/s12905-017-0474-z: 8 str.

Yang J.H., Wu M.Y., Chen C.D., Chen M.J., Yang Y.S., Ho H.N. 2007. Altered apoptosis and proliferation in endometrial stromal cells of women with adenomyosis. Human Reproduction, 22, 4: 945-952

Yang J.H., Wu M.Y., Chen M.J., Chen S.U., Yang Y.S., Ho H.N. 2009. Increased matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 secretion but unaffected invasiveness of endometrial stromal cells in adenomyosis. Fertility and Sterility, 91, 5 Suppl: 2193-2198

Yang J., Wu M., Chang D., Chang C., Yang Y., Ho H. 2006. Increased interleukin-6 messenger RNA expression in macrophage-cocultured endometrial stromal cells in adenomyosis. American Journal of Reproductive Immunology, 55, 3: 181-187

Yang J.H., Chen M.J., Chen H.F., Lee T.H., Ho H.N., Yang Y.S. 2004. Decreased expression

of killer cell inhibitory receptors on natural killer cells in eutopic endometrium in women with adenomyosis. Human Reproduction, 19, 9: 1974-1978

Ye H., He Y., Wang J., Song T., Lan Z., Zhao Y., Xi M. 2016. Effect of matrix metalloproteinase promoter polymorphisms on endometriosis and adenomyosis risk: evidence from a meta-analysis. Journal of Genetics, 95, 3: 611-619

Yen C., Liao S., Huang S.J., Tabak S., Arcuri F., Lee C., Arici A., Petraglia F., Wang H., Kayisli U.A. 2016. Decreased endometrial expression of leukemia inhibitory factor receptor disrupts the STAT3 signaling in adenomyosis during the implantation window. Reproductive Sciences, 24, 8: 1176-1186

Yi K.W., Kim S.H., Ihm H.J., Oh Y.S., Chae H.D., Kim C.-H., Kang B.M. 2015. Increased expression of p21-activated kinase 4 in adenomyosis and its regulation of matrix metalloproteinase-2 and -9 in endometrial cells. Fertility and Sterility, 103, 4: 1089-1097.e2

Yu S.L., Kim T.H., Han Y.H., Kang Y., Jeong D.U., Lee D., Kang J., Park S.R. 2021. Transcriptomic analysis and competing endogenous RNA network in the human endometrium between proliferative and mid-secretory phases. Experimental and Therapeutic Medicine, 21, 6: 660, doi: 10.3892/etm.2021.10092: 16 str.

Zhai J., Li S., Sen S., Opoku-Anane J., Du Y., Chen Z.J., Giudice L.C. 2020a. m6A RNA methylation regulators contribute to eutopic endometrium and myometrium dysfunction in adenomyosis. Frontiers in Genetics, 11, 716, doi: 10.3389/fgene.2020.00716: 16 str.

Zhai J., Vannuccini S., Petraglia F., Giudice L.C. 2020b. Adenomyosis: mechanisms and pathogenesis. Seminars in Reproductive Medicine, 38, 2–03: 129-143

Zhang D., Xia W., Tong T., Li C., Shi W., Yan M., Xue R., Guan X., Zhang J. 2016. Correlation between Cyr61 expression and clinicopathologic parameters in adenomyosis. Journal of Reproductive Immunology, 118: 42-49

Zhang W., Li Q., Liu H., Chen W., Zhang C., Li H., Lu X., Chen J., Li L., Wu H., Sun X. 2021. Transcriptomic analysis of endometrial receptivity for a genomic diagnostics model of Chinese women. Fertility and Sterility, 116, 1: 157-164

Zhang X., Lu B., Huang X., Xu H., Zhou C., Lin J. 2010. Innervation of endometrium and myometrium in women with painful adenomyosis and uterine fibroids. Fertility and Sterility, 94, 2: 730-737

Zhang Y., Yu P., Sun F., Li T.C., Cheng J.M., Duan H. 2015. Expression of oxytocin receptors in the uterine junctional zone in women with adenomyosis. Acta Obstetricia et Gynecologica Scandinavica, 94, 4: 412-418

Zhao L., Zhou S., Zou L., Zhao X. 2013. The expression and functionality of stromal caveolin 1 in human adenomyosis. Human Reproduction, 28, 5: 1324-1338

Zhao S., Fung-Leung W.P., Bittner A., Ngo K., Liu X. 2014. Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. PLoS ONE, 9, 1: e78644, doi: 10.1371/journal.pone.0078644: 13 str.

Zheng D., Duan H., Wang S., Xu Q., Gan L., Li J., Dong Q. 2018. FAK regulates epithelial-mesenchymal transition in adenomyosis. Molecular Medicine Reports, 18, 6: 5461-5472

Zhihong N., Yun F., Pinggui Z., Sulian Z., Zhang A. 2016. Cytokine profiling in the eutopic endometrium of adenomyosis during the implantation window after ovarian stimulation. Reproductive Sciences, 23, 1: 124-133

Zhou M., Fu J., Xiao L., Yang S., Song Y., Zhang X., Feng X., Sun H., Xu W., Huang W. 2016. MiR-196a overexpression activates the MEK/ERK signal and represses the progesterone receptor and decidualization in eutopic endometrium from women with

endometriosis. Human Reproduction, 31, 11: 2598-2608

Zhou S., Yi T., Liu R., Bian C., Qi X., He X., Wang K., Li J., Zhao X., Huang C., Wei Y. 2012. Proteomics identification of annexin A2 as a key mediator in the metastasis and proangiogenesis of endometrial cells in human adenomyosis. Molecular and Cellular Proteomics, 11, 7: M112.017988, doi: 10.1074/mcp.M112.017988: 24 str.

Zhou W., Peng Z., Zhang C., Liu S., Zhang Y. 2018. ILK-induced epithelial-mesenchymal transition promotes the invasive phenotype in adenomyosis. Biochemical and Biophysical Research Communications, 497, 4: 950-956

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PRILOGE

Priloga A. Seti genov, uporabljeni v naši raziskavi
Supplementary A. Used gene sets in our research.

Skupina	Vir (št. genov)	Geni
ADENOMIOZA (SS faza)	Lastna analiza RNA-seq (382 genov)	*TAS2R43, SCGB2A2, DLX3 ,LINC00645, ODAM, RAET1G, HORMAD1, AZGP1, MICA, UPK1B, OR1Q1, RBP7, DEFB1, APOM, ATOH8, DEFB109A, CUTALP, SYN3, OR1J4, FOXD4L4, NYAP1, LOC642846, VSIG2, AMH, LINC00861, TMPRSS13, PDCD1, MIR3124, TRGC2, GZMK, ADAMTSL2, TRPC5, CCDC168, LIMS3-LOC440895, NECTIN4, MMP23A, ABHD12B, FAM138B, GLCCI1-DT, LINC00649, HSD17B3, MFSD4A-AS1, ZP1, PTGER1, ANGPTL5, MOCOS, CEP44, DNM1P46, DNAH5, PRSS30P, ADAMTS17, RASGEF1B, HSD17B2, ANKRD40CL, ZNF600, LCK, S1PR4, PROZ, TCAP, IRF6, ANKRD53, SGSM1, MIR151A, SLC26A8, ZNF578, FAM90A25P, BMP8B, H3-4, HYAL3, ALK, LOC400464, CHMP4C, ARSA, CNKSR3, C3orf52, SYNE3, ANOS1, LINC00921, RASGRP4, HTATIP2, RASEF, NGEF, ADAMTS5, ZNF486, TGM4, CDHR1, PRSS16, LINC02102, GABARAPL1, ZC3HAV1, INTS6-AS1, GABRR2, ZNF552, HSBP1L1, MFSD4A, ST7-AS1, TOP1P1, MPZL3, SH3GLB2, ANKRD18A, CCDC152, SERHL2, SLC43A2, CARD16, ABCA1, PDLIM5, LTBP2, EPOR, CACFD1, CD200, LTB4R, CD101, GSTZ1, TST, C5orf63, MPST, CERNA1, AGTPBP1, WDR82, RFLNB, ACSS2, IMPG2, PIK3CB, ZBTB11-AS1, SPTLC3, RNF19B, LINC00598, WDR91, DTX4, HEXA, HERC2P3, DND1, ABHD15, LOC541473, UBE2H, PNPLA8, MAGI3, RALBP1, QRICH2, TRMT10C, MIGA2, NPC1, ARAP2, ATP6V0E1, JOSD1, PPTC7, IL10RB, ATP2B1, CYREN, STXBP1, FAM131A, DCBLD1, SH3BP5-AS1, PINK1, AP5B1, SFT2D2, HPS6, LYST, TAOK1, ZNF234, ZNF417, MYLIP, ABITRAM, SIDT2, SNX25, SMYD2, ZNF267, APP, SMARCE1, PCNX2, GSTO1, PARP10, CCDC82, MSI2, UBE2E2, UBA7, MRPL1, IFI35, RBL1, SOCS2, RBPMS, CMC1, MTMR9LP, CPSF1, ADGRF3, DHX58, TMEM150C, B9D1, PPIC, PALLD, PDCD2L, EPM2A, EIF2AK2, LMO4, COL4A6, LY6E, ACOT7, TRIM14, RHOJ, FAM133CP, FAT1, CRYL1, CPVL, CDH24, GNAI1, SAMD9L, CARD10, RNF113B, CADM4, CD109, SLC15A3, CISD1, RFX2, TRIM22, PCDHA9, KRT7, ABCA17P, GRIN3A, YBEY, PXYLP1, COL21A1, KDM7A-DT, SETD9, RORB, SOCS2-AS1, PTPRVP, ATP1B2, BUB1B, GOLGA8T, PDE3A, CD209, CDH6, STAT1, FGD2, RUNDC3A, NPNT, PLS1, HERC2P2, FGFR4, ADAMTSL1, ALPK3, PLXNB3, RTP4, IFITM1, ADGRL4, ACP5, RNASE6, BICC1, SUSD2, C9orf116, INKA1, CEACAM19, STK32B, DCDC2, RGL1, PPM1J, SLC46A3, CLEC7A, KCTD14, AK5, CYBB, LOC729970, LOC100134317, MIR548S, MYO7B, FGL2, SPEG, RNF144A-AS1, PPP1R27, SCAMP5, SOD3, FAM81A, MLKL, SKA3, SLC2A3, RAB39A, POTEJ, PLEKHA6, MCF2L-AS1, LAMP3, TNNI1, BGN, RNASE1, ISG15, LINC00958, FAT2, LOC644135, ZNF215, MIR181A2, FAM86B3P, CYGB, GBGT1,*

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nadaljevanje Priloge A

Skupina	Vir (št. genov)	Geni
ADENOMIOZA (SS faza)	Lastna analiza RNA-seq (382 genov)	*MYH11, SCIN, FCGR1CP, MALRD1, XG, FSD1, RELN, CCER2, SLC7A11-AS1, TMEM71, HBB, TBXAS1, C12orf75, GNRHR, CDH15, FOXC1, HTR1D, ANO4, ULK4P3, PPARGC1A, TMPRSS3, SLC7A11, FUT3, ATP2C2, ERN2, GNG10, BCRP3, PLEKHS1, SLC14A1, IHH, OAS3, THSD7A, BST2, KLHL14, SELL, WFDC1, NOSTRIN, ERC2, RGS7BP, IFIT1, CCDC198, RASL10A, CD1C, MX1, ZDHHC8P1, TCEAL5, MUC13, TLR10, PDE1C, COL26A1, AMIGO1, TRPM8, ACVR1C, KAAG1, SHISA3, CHRNA7, ASAH2, TMEM119, DOK5, OAS2, CES5AP1, SLC2A5, ANKS1B, LOC200772, LIPH, MYOM3, TMPRSS11D, SIGLEC5, OGDHL, FILIP1, CNTNAP3B, TSPAN33, MRPS30-DT, SLC4A10, SOHLH2, PLG, IFI44L, FLJ22447, SEMA3E, TSPAN8, CHL1, CNTNAP3, CADM3-AS1, MUC5B, PTPRZ1, KLK10, CES1P1, DRAIC, TMEM252, CYP4F30P.*
ADENOMIOZA (SS faza)	Literatura (42 genov)	*AKR1B10, ATP12A, ATP1A2, C15orf62, C3orf33, CACNA1E, CDKN3, CHD5, CLDN4, COL11A1, COL8A1, CYP3A7, ERCC6L, FNDC1, GPR78, HOXA10, IL10, ITGB3, KCNA4, KLF12, LIF, LIFR, LIPH, LTF, MB, MIR21, MMP20, NR4A1, PSG6, SCGB2A2, SPC25, SPINK2, SPP1, SST, STAT3, SULT1E1, TBX15, TCN1, TMEM190, TMPRSS11B, TUBAL3, ZNF295-AS1.*
ENDOMTERIOZA (SS faza)	Literatura (173 genov)	*ABCB11, ABCC3, ACKR1, ACO2, ADGRF1, AFF4, AGT, AIMP1, ALPI, AMY1A, AMY2A, AMY2B, ANXA2, ANXA5, AOC1, ATF3, BST2, C1QA, C1QTNF6, CA1, CA12, CASP5, CCBE1, CCL3, CCL3L1, CCL3L3, CCL8, CCN1, CCT8, CDA, CDK5R1, CELF1, COL12A1, CORO1B, CRABP1, CRISP3, CST7, CTSW, CWH43, CXCL2, CYP3A5, DDIT4L, DDX17, DEPP1, DLG5, DNAJC3, DST, EDNRB, EGR1, EGR2, EGR3, EIF1, EIF4A1, EIF4A2, ENPP3, FMN2, FOS, FOSB, GALP, GSN, GUCY1B1, GZMA, HACD1, HOXA9, HPCAL4, HSP90B1, FNA21, IL6, IMMT, JUNB, KCNK2, KRIT1, KRT18, KRT5, KRTAP19-2, LAMA3, LCK, LONRF2, LPP, LRRD1, LTB4R2, LUZP1, MALL, MAP4, MAPK8, MET, MIR135A1, MIR138-1, MIR138-2, MIR1915, MIR194-2, MIR196A1, MIR196A2, MIR219B, MIR22, MIR26B, MIR3196, MIR339, MIR365B, MIR3686, MIR374B, MIR4251, MIR4252, MIR4254, MIR4425, MIR4723, MIR505, MIR542, MIR548AA2, MIR548AP, MIR548T, MIR5585, MIR921, MMP26, MUC7, MYL12A, NCR1, NEAT1, NFAT5, NR4A1, NR4A3, PAX8, PCSK5, PCYOX1, PDHB, PER1, PITX1, PLEK, PLEKHA2, POMZP3, PRDX6, PRIM2, PRRC2C, PTAFR, RAB9BP1, RBBP4, RGS1, RIF1, RIN1, RNF150, RNH1, RSRP1, S100A3, S100A8, SAP30L, SCG2, SCGB2A2, SEMA3C, SERPINB8, SHB, SLA, SLC15A4, SLC1A1, SLC44A2, SMG1, SOCS3, SON, SP3P, TAF6L, TGFB3, THRAP3, TRIM15, TRPM6, TUBA1C, VDAC1P1, VEGFA, VHL, VIM, YBX1, YBX1P2, YWHAE, ZFP36, ZIC2.*
ZDRAVA MATERNICA	Literatura (151 genov)	*ABCC3, ACADSB, ADAMTS1, ALPL, AMIGO2, ANG, ANO1, ANXA2, ANXA4, AOX1, APOD, ARG2, ARID5B, ASPM, ATP1B1, ATP6V0E2, ATP6V1A, BARD1, BCL6, BUB1B, C1R, C4BPA, CAPN6, CATSPERB, CCNB2, CDA, CDC20, CDK1, CENPE, CEP55, CFD, CLDN4, CLU, COL16A1, COMP, CP, CRABP2, CSRP2, CTNNA2, CXCL13, CXCL14, DDX52, DEFB1, DEPP1, DEPTOR, DKK1, DLGAP5, DPP4, DYNLT3, ECI2, ECM1, EDN3, EDNRB, EFNA1, ENPEP, EPHB3,*

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nadaljevanje Priloge A

Skupina	Vir (št. genov)	Geni
ZDRAVA MATERNICA	Literatura (151 genov)	*FANCI, FOSL2, FXYD2, G0S2, GABARAPL1, GADD45A, GALNT12, GALNT4, GAS1, GAST, GBP2, GDF15, GPX3, GREM2, HABP2, HEY2, HLA-DOB, HPSE, ID4 , IDO1, IL15, IMPA2, KCNG1, KIF11, KIF20A, KIF4A, KMO, KRT7, LAMB3, LIF, LMCD1, LMOD1, LRRC17, LYPD3, MAOA, MAP2K6, MFAP2, MFAP5, MMP26, MPPED2, MSX1, MT1G, MT1H, MT2A, MTCL1, NDC80, NDRG1, NDRG2, NNMT, NRG2, OLFM1, OLFM4, PAEP, PAQR4, PBK, PENK, PLA1A, PLAAT3, PLAAT4, PMEPA1, POLD4, POSTN, PRC1, PRKCQ, PRR15L, PRUNE2, PTPRR, RASSF2, RETREG1, RNASE4, RPRM, S100A1, S100A4, S100P, SCGB2A2, SERPINA5, SERPING1, SFRP4, SLC1A1, SNX10, SOD2, SORD, SOX17, SPDEF, SPP1, SYNE2, TACC3, TAGLN, TBC1D2, TCN1, THBD, TMSB15A, TOP2A, TRH, TSPAN8.*
ZDRAVA MATERNICA	Poti *vgenzdnje zarodka* in *deciduali-zacija* zbirke GO (60 genov)	*ACF, MMP2, CGI-38, ITGB4, DDR1, PPARD, PCSK5, TGFBR2, HMX3, ACOD1, H3F3B, MST1, EPO, STC2, RECK, SOD1, CALCA, IL1B, UBTFL1, SPP1, SCGB1A1, MMP9, LIF, PRLR, FBLN1, PTGS2, BSG, RPL29, STC1, EMP2, H3-3A, FKBP4, FUT7, TRO, TRIM28, TEAD4, IGFBP7, UBE2Q1, VMP1, TPPP3, PRDM14, POLR1B, A1CF, HEL-S-44, MIR21, CTSB, PTN, CITED2, GJB2, MEN1, CYP27B1, DEDD, VDR, JUNB, EPOR, NDP, TCF23, GHSR, ASH1L, GHRL.*
ADENOMIOZA (P faza)	Literatura: raziskave kandidat-nih lokusov (78 genov)	*CYP19A1, CD3, CD4, CD8, CD68, VEGFA, HDAC1, HDAC3, DNMT3A, METTL3, YTHDC1, PTK2, SNAI1, SNAI2, TWIST1, CDH1, VIM, CDH2, SCRIB, TLN1, MIR145, DDT, MMP2, MMP9, F3, UCHL1, BCL2, NDUFA13, IGF1, FST, ACVR2A, ACVR2B, LINC-ROR, TLR4, IL6, IL1B, CRH, IL10RA, IL37, CXCL8, TLR1, TLR6, TLR8, TLR9, CXCR1, CXCR2, PTGS2, PI3K, AKT, CNR1, CNR2, RELA, NFKB1, PAK4, NFKBIA, NFKB2, DUSP6, SPRY4, SEF, NFE2L2, ROCK1, ROCK2, RHOA, ARHGAP26, NOS3, SOD1, GPXs, XDH, CAT, HLA-G, NKB1, GL182, HSPB1, ITGA2, ITGA6, CDH1, ESR2, PGR.*
ADENOMIOZA (P faza)	Literatura: transkript-omske raziskave (518 genov)	*LOC100293539, SNORD116-5, SNORD116-7, SNORD116-3, SNORD116-9, ND2, SNORD116-8, SNRPN, RPL13A, SNHG3, GP1BB, SNORD116-4, SNORD33, SNORA73A, SNORD41, SNORD116-1, YIPF4, DUX4L4, DUX4L6, DUX4L5, DUX4L3, DUX4L7, DUX4L2, RFC1, GP1BB, SNORD95, DUX2 SIAE, DUX4L7, DUX4, DUX2, GNB2L1, KRTAP5-1, PLCL1, TAP2, HLA-DOB, KRTAP5-1, MIR339, ZBTB34 , CXorf18, LOC100132147, SIAE, MIR326, MIR19 , SNORD55, LOC440518, MIR139, C2orf27B, HOXA11, UQCRQ, RPL35, FAM58B, POU5F1B, VHL, PGM5P2, ZNRF2, EIF3K, GDF9, TYMS, TGFBI, RPN2, ATP5I, SNRPF, ADAM12, GLIPR1, RPL38, HSPA5, EEF1A2, NDUFAB1, RPS19, UQCR11, GSTP1, C4orf46, TOMM7, C11orf10, MYL5, MT2A, KRR1, CALR, CD74, ECM1, UBE2J2, COX7C, TNC, PFDN5, RPS12, SCD, VIM, NUCKS1, POM121, RBP7, RPLP0, NDUFA1, SNORA33, VCAN, RPS25, RPS15A, RPS28, POM121C, RPS28, MMP7, GJA1, RNF113A, RBP1, RNASEK, SEC61G, RPL27, COX6C, RPL41, ZNF778, n342839, n336615, TCONS_l2_00002951, n338918,*

se nadaljuje

nadaljevanje Priloge A

Skupina	Vir (št. genov)	Geni
ADENOMIOZA (P faza)	Literatura: transkript-omske raziskave (518 genov)	*n335251, n410159, n339991, n387706, n335092, n333546, THBS2, PRUNE2, HSPB1, COL4A1, SVIL, A2M, ITGA1, CD44, RERG, MATN2, n333955, TCONS_l2_00013366, NR_002960, TCONS_00022823, n338909, n386477, n4541, n337373, n335557, n334090, HBA1, KIAA1210, HIST1H2AB, HBB, HBA2, GZMA, HIST1H3F, HIST1H3B, KLRB1, CACNA1D, CPSF1, LTF, MSRB3, ELK2AP, TAC4, MZB1, IGLL1/IGLL5, JCHAIN, S100A9, SPIB, POU2AF1, SHISA9, S1PR4, ITGB2, LOC101929567, GCHFR, LOC389033, TMEM160, F12, CIB2, GMFG, BIRC5, ABCC9, TMEM121, VAV1, TNNC1, CHCHD5, SCNN1B, LINC00506, GNLY, TMEM238, PLAC9, FUOM, CDCA5, DIO2, CYBB, SLC16A13, AP2S1, EXOSC4, GJA4, NDUFS6, POC1A, C1QTNF6, SDSL, MRPL27, NUDT18, NTHL1, FBXW9, CDT1, SPC25, ALKBH7, CD14, IFI27L2, VPS18, FAM207A, PTGS1, CORO1A, CDCA2, E2F2, SAC3D1, CKS1B, PMVK, MRPS34, MFSD5, CD7, CDKN2AIPNL, PEX11G, DPT, MRPL54, OPHN1, MRPL37, NUDT1, GPATCH3, C1QA, POLA2, RPL39L, FCGR3A/FCGR3B, ISOC2, RNF187, POLD2, EIF4EBP1, NDP, GPATCH4, CLPP, COMTD1, CDC20, SF3B5, NKG7, ZNF775, MFSD3, GPAA1, HN1, PLEKHJ1, FEN1, PYCARD, C1orf122, DCTPP1, ZNHIT2, APEH, CCNB2, USP5, ACP5, TMEM37, SDF2L1, NDUFB7, CCDC167, RRM1, S100A4, SDHAF1, BORCS6, MRPL14, VSTM4, CHMP6, B3GALT6, TIMM22 IFNGR1, RAB5A, DUSP6, RHOB, SEC31B, CLDN4, R3HDM2, SRF, MESDC1, RGL3, RNF19A, TOM1L2, PAN3, PPP1R10, LIMS3/LIMS4, NFKBID, MICAL3, WWC3, DENND4B, FAM189A2, NFKB2, SLC2A1, NRBP2, ULK3, RRN3P1, USP54, TP53INP2, PXDC1, ZSWIM6, TGIF1, KDM3A, HERC2P2, LINC00174, PDXDC2P, SLC38A2, SHANK3, TIPARP, SNX9, CNKSR1, NAMPT, RALGDS, TMCO4, SS18L1, CSF1, CLK3, MIDN, mir-8, DLC1, CELSR1, IER5, POFUT2, SUN1, SEMA3B, ERF, CAPN15, CHKA, TRIO, AGAP6 , BAG3, FHL1, MCL1, AGER, MTMR11, ZNF558, KIAA1683, AASS, CACNA1H, ZNF331, PDGFB, ZFAND5, ELMSAN1, INPP5E, VPS37B, IRS2, WEE1, IFRD1, SULT4A1, ZNF83, ITGA7, KLF5, KMT5C, TWIST1, PLA2G6, CLK1, RNF122, LLGL2, PPP1R15B, CEBPB, LOC729218, ATXN7L2, GABARAPL1, RFX2, SPRY4, DLL1, FRMD4B, TSC22D2, VEGFA, ENO2, F3, UGCG, JUN, LRP5L, ZFYVE28, CELF6, MZF1, COL4A5, ESM1, KSR1, MTRNR2L8, RGCC, CCNL1, ARID5A, ITPRIP, HERC2P9, TSPYL2, GBP1, PLIN5, FAM179A, EPHA2, ABCG1, NRP2, SPRY1, INSIG1, EPOR, BCL3, ETS2, SERTAD1, DNAJB4, NFATC2, CTGF, NCOA7, KLF2, EFNA1, COL6A4P2, RAP2C, VWA3A, SLC22A3, ANXA1, ADM, HBEGF, THBD, GADD45A, RGS1, C2CD4B, THBS1, GPRC5A, DGKD, CCDC40, RND3, BTG1, PPP1R12B, CHRD, PFKFB3, PLAUR, PIM1, JUNB, NFATC1, CACNA1C, ITPKC, MXD1, PAK3, RGS2, CAPN6, RGS16, PER2, DNAJB1, MYADM, CD69, C8orf4, NFKBIA, LINC00893, HPX ,TMEM184A, NPIPB4, CYR61, SLC2A3, DUSP1, GLIS1, ELF3, BCL6, KLF4, PTGIS, DUSP2, BRINP1, CD55, CES4A, LDLR, LOC102724428/SIK1, ELN, LOC100133331, ZC3H12A, GATA6, LOC613037, CD83, SGK1, ADAMTS8, mir-132, PMAIP1, ERRFI1, PIM3, PPP1R15A, USP43, KLF6, CEBPD, IER3, ADAMTS1, OVOL1, PLK3, TNFRSF12A, mir-614, BHLHE40, NR4A2, PHLDA2, DUSP5, LGI2, EGR2, CMYA5, CSRNP1, EDN2, DKK1, HSPB7, NR4A3, POU5F1, LIF, EMP1, GADD45B, FOSB, RERG, CNN1, SOX9, PER1, PRELP, SCGB3A1, TRIB1, JPH2, SOCS3, TNFAIP3, MAFF, ATF3, C11orf96, TNFAIP8L3, ZFP36, NR4A1, EGR3, FOSL1, DUOXA1, PDE4C, CXCL2, NFASC, SERPINE1, WHRN, PDLIM3, CYP3A5, GEM, IL1RL1, ARC, SELE, REXO1.*

Priloga B. Pregled molekularnih raziskav, povezanih z adenomiozo. Raziskave smo razvrstili glede na preiskovani mehanizem, povezan s patogenezo/patofiziologijo adenomioze. Zbrali smo tudi transkriptomske raziskave EvEA. Poimenovanje genetskih lokusov smo poenotili po zbirki HGNC. Referenčna literatura, označena s krepko, se navezuje na vključene raziskave v identifikacijo močnejših kandidatnih genov patofiziologije oz. spremenjene endometrijske receptivnosti pri adenomiozi. Uporabljene kratice: EvEA = evtopični endometrij pri adenomiozi; EkEA = ektopični endometrij pri adenomiozi; K = kontrolni endometrij iz maternice brez adenomioze; EEC = endometrijske epitelijske celice; ESC = endometrijske stromalne celice; P = proliferacijska faza; ZP = zgodnja proliferacijska faza; SP = srednja proliferacijska faza; PS = pozna proliferacijska faza; S = sekrecijska faza, ZS = zgodnja sekrecijska faza, SS = srednja sekrecijska faza, PS = pozna sekrecijska faza; E2 = estradiol; P4 = progesteron; TIAR = proces poškodbe in celjenja tkiva; EMT = prehod iz epitelijskih v mezenhimske celice; uNK = maternične celice naravne ubijalke.
Supplementary B. The overview of molecular studies associated with adenomyosis. The studies were sorted according to the observed mechanism associated with the pathogenesis/pathophysiology of adenomyosis. We also gathered transcriptomics studies of EvEA. The nomenclature of genetic loci was adopted according to the HGNC database. Reference literature highlighted in bold refers to research included in the identification of stronger candidate genes for pathophysiology or altered endometrial receptivity in adenomyosis. Used abbreviations: EvEA = eutopic endometrium of adenomyosis; EkEA = ectopic endometrium of adenomyosis; K = control endometrium from nonadenomyosis uteri; EEC = endometrial epithelial cells; ESC = endometrial stromal cells; P = the proliferative phase; ZP= the early proliferative phase; SP = the mid proliferative phase; PS = the late proliferative phase; S = the secretory phase; ZS = the early secretory phase; SS = the mid secretory phase; PS = the late secretory phase; PS = the late secretory phase; E2 = estradiol; P4 = progesterone; TIAR = tissue injury and repair; EMT = epithelial-to-mesenchymal transition; uNK = uterine natural killer.

Mehanizem patofiziologije adenomioze	Referenčna literatura	Glavni rezultat in ugotovitve raziskave
1. Sprožilci/dejavniki adenomioze		
Lokalna proizvodnja in spremenjen metabolizem E2 v maternici	(Takahashi in sod., 1989)	↑ E2 v menstrualni krvi žensk z A proti K.

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nadaljevanje Priloge B

Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
1. Sprožilci/dejavniki adenomioze		
Lokalna proizvodnja in spremenjen metabolizem E2 v maternici	(Kitawaki in sod., 1997)	↑ *CYP19A1* (encim, ki katalizira pretvorbo steroidov v estrogene) in protein v žlezah epitelija EvEA in EkEA proti K (brez navedene faze ciklusa ob vzorčenju).
	(Hatok in sod., 2011)	↑ *CYP19A1* v EvEA žensk s hudo obliko bolezni proti K, pridobljenih v ZP fazi.
	(Maia in sod., 2006)	↑ CYP19A1 v stromi EvEA proti EkEA, pridobljenih v P in S fazah → zvišana aktivnost CYP19A1 pri A bi lahko vplivala na stalno izražanje PTGS2 v S fazi.
	(Kitawaki in sod., 2000)	↑ *HSD17B2* (pretvarja E2 v biološko manj aktiven estron) v EvEA proti K, pridobljenih v S fazi → metabolizem E2 je spremenjen pri ženskah z A.
Hiperperistaltika maternice	(Leyendecker in sod., 2015)	Hiperperistaltika je glavni mehanizem samotravmatizacije maternice pri A, ki vodi v lokalno vnetje in proliferacijo bazalnega endometrija *(slikanje maternice z MRI).*
	(Zhang in sod., 2015)	↑ OXTR (receptor za oksitocin) v notranjem miometriju fundusa maternice pri A proti K, pridobljenih v P in S fazah.
	(Shi in sod., 2016)	↑ *KCNMA1* (angl. *calcium-activated potassium channel subunit alpha 1*. K+ kanalčki, ki sodelujejo pri krčenju miometrija) in *KCND2* (angl. *potassium voltage-gated channel subfamily D member 3*) v celicah gladkega mišičja miometrija A proti K.
	(Wang S. in sod., 2016)	↑ ROCK1 (protein kinaza, ki ima med drugimi tudi vlogo pri krčenju gladkih mišic) v notranjem miometriju pri A proti K, pridobljenih v P in S fazah. Brez sprememb v stopnji izražanja ROCK1 tekom menstruacijskega ciklusa pri A (pri K višje izražanje v P, kot pa v S fazi); ↑ ROCK1, RHOA in MYL (angl. *myosin light chain* (ni navedene podenote)) v kulturi celic gladkega mišičja miometrija A proti K po 24-urnem gojenju z E2 → E2 bi lahko preko ↑ signalne poti RHOA/ROCK1 vplival na krčenje notranjega miometrija pri A.
Kronično poškodovanje in celjenje notranjega miometrija (mehanizem TIAR)	(Ibrahim in sod., 2015)	↑ znaki TIAR pri A proti K: nejasna meja med bazalnim endometrijem in notranjim miometrijem; spremenjena orientiranost gladkih mišičnih vlaken notranjega miometrija in prisotnost celic gladkega mišičja znotraj strome bazalnega endometrija *(barvanje preparatov histerektomije po Van Gieson).*
	(Ibrahim in sod., 2017)	↑ ACTA2 (angl. *actin, aortic smooth muscle*: molekularni označevalec miofibroblastov, ki nakazujejo mikropoškodbo tkiva) in kolagen 1 (ni navedene natančne podenote) v notranjem miometriju pri A proti K.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
1. Sprožilci/dejavniki adenomioze		
Kronično poškodovanje in celjenje notranjega miometrija (mehanizem TIAR)	(Shaked in sod., 2015)	↑ stopnja stresa v notranjem miometriju A pri zvišani aplitudi znotrajmaternične pritiskov *(uporaba programske opreme z dvodimenzionalnim (2D) modelom maternične stene, ki je bila izpostavljena različnim frekvencam pritiskov)* → ↑ stres zaradi ↑ krčenja maternice lahko vodi v poškodbo celic notranjega miometrija, s tem pa aktivacijo mehanizmov TIAR in tvorbo lezij.
Kopičenje imunskih celic na mestu poškodb miometrija in v EvEA	**(Bulmer in sod., 1998)**	↑ število znotrajepitelnih T celic (levkociti CD3+) v P fazi EvEA in EkEA proti K; ↑ število levkocitov CD43+ v P in ES fazi EkEA proti EvEA.
	(Ota in sod., 1996)	↑ število podtipov T celic v stromi EkEA in EvEA proti K, pridobljenih v P in S fazah: - ↑ podtip gama delta (γδ) T celic (prepoznavajo antigene in proteine vročinskega šoka s čimer aktivirajo vnetne reakcije in migracijo makrofagov ter T celic); - ↑ podtip αβ T celic; - ↑ CD4+ T celic; - ↑ CD3+ T celic; - ↑ CD8+ T celic; - ↑ makrofagov. ↑ izražanje antigenov HLA, ki se nahajajo na antigen predstavitvenih celicah (HLA-ABC in HLA-DR, ki se povezuje s CD8 oz. CD4 molekulami na T celicah), in ↑ ICAM1 (adhezijski protein z vlogo pri tvorbi histokompatibilnega kompleksa) v P in S fazi žlez EkEA proti EvEA in K.
	(An in sod., 2017)	↑ število makrofagov (CD68+) v EkEA in EvEA proti K, pridobljenih v P in S fazah; ↑ CCL2 (kemokin, ki ↑ kemotakso in aktivacijo mononuklearnih fagocitov) in ↑ agregacija makrofagov v kulturi EECs iz EvEA in EkEA proti K; Polarizacija makrofagov v tip M2, ki spodbujajo obnovitev tkiva (↑ CD163, IL10 in MMP12 ter ↓ MMP9 v A proti K) ob gojenju z Ishikawa celicami (*in vitro* model A) ali s kulturo EEC iz EvEA → makrofagi lahko pri A sprožijo procese EMT.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
1. Sprožilci/dejavniki adenomioze		
Kopičenje imunskih celic na mestu poškodb miometrija in v EvEA	(Tremellen and Russell, 2012)	↑ gostota infiltriranih makrofagov (CD163+) in celic naravnih ubijalk (CD56+) v LS fazi v stromi EvEA hude oblike bolezni vs. K → imunološki mehanizmi bi lahko motili uspešno vgnezditev zarodka pri A.
	(Zhihong in sod., 2016)	↑ število makrofagov (CD68+) v epiteliju in stromi EvEA vs. K sedmi dan po injiciranju humanega horionskega gonadotropina (hCG) v procesu kontrolirane stimulacije jajčnikov → ↑ delovanje E2 v EvEA lahko vodi v ↑ vnetnih citokinov in dalje ↑ gostoto makrofagov v EvEA.
Hipoksija	**(Goteri in sod., 2009)**	↑ HIF1A (angl. *hypoxia-inducible factor 1-alpha*. Prepisovalni dejavnik, ki se veže na DNA odzivne elemente hipoksije in aktivira *VEGFA*) in VEGFA v epiteliju EkEA proti EvEA in K, pridobljenih v P in S fazi; ↑ gostota malih žilic (angl. *microvascular density*, MDV = žile/mm^2) v EkEA proti EvEA in K → hipoksija izzove izražanje *VEGFA* in tvorbo novih žil (angiogenezo) v EkEA.
Somatske mutacije	(Inoue in sod., 2019)	Mutacije onkogena *KRAS* v EkEA in EvEA *(sekvenciranje celotnega eksoma)* → mutiran KRAS ↑ invazivnost in proliferacijo endometrijskih celic na ektopičnem mestu.
	(Oehler in sod., 2004)	Mutacije *ESR1* v EkEA; ↓ zmožnost vezave mutiranega ESR1 na DNA *(in vitro test vezave na DNA)* in ↓ transaktivacijska sposobnost *(in vitro test prehodne transfekcije)* →↓ odzivnost na estrogene
Epigenetske spremembe	**(Liu in sod., 2012)**	↑ izražanje HDAC1 (angl. *histone deacetylase 1*. Uravnava deacetilacijo histonov) in HDAC3 v EkEA in EvEA proti K, pridobljenih v P in S fazah; ↑ HDAC2 v EkEA proti EvEA in K; ↑ HDAC2 v EvEA značilno pozitivno (dalje poz.) povezano s stopnjo dismenoreje.
	(Liu in Guo, 2012)	↑ izražanje DNMT1 (angl. *DNA (cytosine-5)-methyltransferase 1*. Uravnava metilacijo po podvojevanju DNA) in DNMT3B v EkEA proti EvEA in K, pridobljenih v P in S fazi; ↓ DNMT3A v EkEA in EvEA proti K; ↑ DNMT1 EvEA značilno poz. povezano s hudo menstrualno krvavitvijo; ↑ DNMT3B v EkEA značilno poz. povezano s stopnjo dismenoreje.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
1. Sprožilci/dejavniki adenomioze		
Epigenetske spremembe	(Nie in sod., 2010a)	↑ metilacija (hipermetilacija) promotorja izooblike B PGR v kulturi ESC iz EkEA proti K, pridobljenih v P in S fazi.
	(Zhai in sod., 2020a)	↓ delež N6-metiladenozina (m^6A: uravnavajo nukleacijo, alternativno izrezovanje, translacijo in stabilnost molekul mRNA) v totalni RNA EvEA proti K, pridobljenih v P fazi; ↓ izražanje m^6A regulatorjev RNA metilacije (*METTL3* in protein ter *YTHDC1*) v P fazi v EvEA proti K → spremenjeno izražanje regulatorjev m^6A RNA metilacije bi lahko bili vključeni v patogenezo A.
Polimorfizmi	(Ye in sod., 2016)	Polimorfizem -1607 1G/2G gena *MMP1* ↑ nagnjenosti za razvoj A.
	(Shan in sod., 2006)	Alel G polimorfizma -181A/G *MMP7* ↑ nagnjenost za razvoj A.
	(Kang in sod., 2009)	Genotipa G/G polimorfizma -1154G/A in C/C polimorfizma -2578C/A gena *VEGF* ↑ nagnjenost za razvoj A.
	(Huang P.C. in sod., 2010)	Ničelni genotip (ni navedene specifične lokacije) *GSTM1* (angl. *glutathione S-transferase Mu 1*. Encim detoksifikacije) in izpostavljenost ftalatom ↑ nagnjenost za razvoj A.
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Aktivacija procesa prehoda epitelija v mezenhim (EMT) Proces EMT poteka v več korakih:		
Izguba izražanja označevalcev za epitelijske celice in pridobitev izražanja mezenhimskih označevalcev	**(Zheng in sod., 2018)**	↑ *PTK2* in pripadajoči protein (angl. *focal adhesion kinase 1*. Omogoča prenos celičnega signala integrinov in drugih površinskih receptorjev. Sodeluje pri regulaciji celične adhezije, migracije, proliferacije in preživetja), *SNAI1*, *SNAI2*, *TWIST1* v EvEA proti K, pridobljenih v P in S fazah; ↓ *CDH1* in protein in ↑ *VIM* in protein ter *CDH2* (staro ime N-kaderin) v P in S fazi EvEA proti K; ↓ zmožnost migracije *(in vitro test migracije Transwell)* kulture celic EvEA po utišanju *PTK2* z malo interferenčno RNA (*PTK2*-siRNA) → ↓ VIM, SNAI1, SNAI2, TWIST1, CDH2, PI3K in AKT ter ↑ CDH1 pri A proti negativna K → signalna pot PTK2/PI3K/AKT v EvEA bi lahko vplivala na izražanje molekul, ki sodelujejo v procesu EMT.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Aktivacija procesa prehoda epitelija v mezenhim (EMT) Proces EMT poteka v več korakih:		
Izguba izražanja označevalcev za epitelijske celice in pridobitev izražanja mezenhimskih označevalcev	(Mu in sod., 2015)	↑ *PTK2* in protein v EvEA proti K (ni navedene faze ciklusa ob vzorčenju) → značilna poz. povezava med stopnjo izražanja PTK2 in dismenorejo ter bolečinami v predelu medenice pri A.
	(Chen in sod., 2010)	↑ VIM in ↓ CDH1 v epiteliju EkEA proti EvEA in K, pridobljenih v ZP in S fazah → značilna negativna (neg.) povezava med serumskimi vrednostni E2 in izražanjem CDH1 v EkEA in EvEA → E2 sproži EMT v endometrijskih celicah → A; ↑ morfološke spremembe endometrijskih epitelijskih celic v celice, podobne fibroblastom, po 24-urnem gojenju ESR+ Ishikawa celic z E2 → ↑ migracija in invazija *(in vitro test s Boydenovimi komorami)* ter ↓ *CDH1* in ↑ *SNAI2*, *VIM* in *CDH2*. Razvoj A pri SCID miših 21. dan po ksenotransplantaciji humanega EvEA ali EkEA, pridobljenih v P fazi, in izpostavljenosti E2 → E2 ↑ invazivnost in adhezijo endometrija ali A lezij.
	(Zhou in sod., 2018)	↑ *ILK* (angl. *integrin-linked protein kinase*) in protein, *CDH2* in protein ter *VIM* in ↓ *CDH1* in protein v EvEA (ne pri VIM) in EkEA proti K (ni navedene faze ciklusa ob vzorčenju) → ILK sproži EMT, kar vodi v razvoj A.
	(An in sod., 2017)	↓ CDH1 in ↑ VIM v EvEA in EkEA proti K (ni navedene faze ciklusa ob vzorčenju); ↑ CDH2 in ↓S100A4 in KRT7 (angl. *keratin, type II cytoskeletal 7*) v EkEA proti EvEA in K; ↑ stopnja EMT v EkEA in EvEA proti K *(določeno glede na razmerja med stopnjami izražanja označevalcev EMT)*; ↓ CDH1 in KRT7 ter ↑ VIM, CDH2 in S100A4 ob gojenju kulture EEC iz EvEA z makrofagi → makrofagi lahko sprožijo procese, podobne EMT.
	(Khan in sod., 2015)	↓ CDH1 v epiteliju bazalnega EvEA proti K, pridobljenih v S fazi; ↑ VIM v epiteliju bazalnega in funkcionalnega EvEA proti K; ↑ *SLUG*, *SNAI1* in *CDH2* ter ↓ *CDH1* v kulturi EEC iz EvEA in/ali Ishikawa celicah po 48-urnem gojenju s HGF (angl. *hepatocyte growth factor*) in po gojenju s HGF ob dodatku E2 → HGF in dalje E2 spodbujata procese EMT v bazalnem EvE

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Aktivacija procesa prehoda epitelija v mezenhim (EMT) Proces EMT poteka v več korakih:		
Izguba celične apikalno bazalne polarnosti	**(Wu in sod., 2019)**	↓ SCRIB (angl. *protein scribble homolog*. Evolucijsko ohranjen protein ogrodja, vključen v različne polarizirane celice) v EkEA in EvEA proti K, pridobljenih v P in S fazah → polarnost celic žleznega epitelija je spremenjena v EvEA.
	(Jin in sod., 2020)	↑ CYP19A1 in HECW1 (angl. *E3 ubiquitin-protein ligase HECW1*. Ligaza, ki sodeluje pri ubikvitinaciji proteinov) ter ↓ SCRIB v EvEA proti K (ni navedene faze ciklusa ob vzorčenju); Izgubljena apikalno bazalna polarnost epitelijskih celic na 3D kulturi primarnih celic žlez EvEA ob gojenju z E2; ↓ proteinov polarnosti (SCRIB, CRB3 in CDH1) žlez epitelija v kulturi Ishikawa celic po 24 in 48-urnem gojenju z E2; ↑ oz. ↓ SCRIB v kulturi Ishikawa celic po HECW1-siRNA oz. hiperizražanju HECW1 → E2, preko ubikvintina HECW1 spodbuja potranslacijske modifikacije, ki vodijo v degradacijo proteina SCRIB. To uniči apikalno-bazalno polarnost žleznih epitelijskih celic bazalnega endometrija, kar pripomore k razvoju difuzne A.
Zvišana celična migracija (motilnost)	(Zhou in sod., 2018)	↑ zmožnost motilnosti *(in vitro test celjenja rane)* in invazivnosti kulture ESC iz EvEA proti K.
	(Ibrahim in sod., 2015)	↑ migracija znotrajepitelijskih golih celic (niso povezane z okolico prek dezmosomov) iz žlez bazalnega endometrija v stromo EkEA *(transmisijsko elektronsko mikroskopiranje).*
	(Wang in sod., 2021a)	↑ *TLN1* (angl. *Talin-1*. Povezuje strukture citoskeleta) in ↓ MIR145 (miR-145-5p cilja regijo 3'UTR *TLN1*) v EvEA in EkEA proti K, pridobljenih v P in S fazah → izražanje *TLN1* bi lahko bilo regulirano preko miRNA; ↑ gibljivost in kapaciteta invazivnosti *(in vitro testa invazije Transwell in celjenja rane)* kulture EEC iz EvEA in EkEA pri prekomernem izražanju TLN1 oz. ↓ celična invazija po izbitju TLN1.
	(Zhai in sod., 2020a)	↑ *DDT* (angl. *D-dopachrome decarboxylase*. Označevalec celične migracije in proliferacije) v P fazi EvEA proti K → ↑ *DDT* v EvEA bi lahko, z vplivom na procese m^6A RNA metilacije, uravnaval celično migracijo.
	(Khan in sod., 2015)	↑ migracija *(in vitro test z Boydenovo komoro)* kulture EECs iz EvEA po 48-urnem gojenju samo s HGF in po gojenju s HGF in dodatkom E2 → HGF/E2 bi lahko ↑ invazivnost endometrijskih celic in njihovo migracijo proti miometriju.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Aktivacija procesa prehoda epitelija v mezenhim (EMT) Proces EMT poteka v več korakih:		
Zvišana invazivnost endometrija	(Zhou in sod., 2012)	↑ ANXA2 (angl. *annexin A2*. Na E2 odziven protein) v EkEA proti EvEA, pridobljenih v P in S fazah; Značilna poz. povezava med stopnjo izražanja ANXA2 v EkEA in hudo obliko dismenoreje; ↑ ANXA2 in proces EMT (↓ CDH1 in ↑ VIM ter SNAI2) po gojenju ESR+ Ishikawa celic z E2; ↓ rast endometrijskega tkiva, metastaz in stopnje angiogeneze v mišjem modelu A po utišanju ANXA2 → E2 bi lahko ↑ ANXA2 in sprožil proces EMT.
	(Zhao in sod., 2013)	↓ CAV1 (angl. *caveolin-1*: ogrodni protein znotraj kaveolarnih membran) in ↑ CCL5 (angl. *C-C motif chemokine 5*; pogosto uporabljen simbol RANTES) v stromi EkEA proti EvEA in K, pridobljenih v P in S fazah; ↑ kapaciteti proliferacije *(in vitro test celične proliferacije)* in metastaz *(in vitro migracijski test Transwell)* v kulturi ESC iz EvEA po utišanju s *CAV1*-siRNA → izguba izražanja CAV1 v stromi vodi v sproščanje CCL5 in s tem morebiti na razvoj A; ↑ izražanje mediatorjev provnetnega okolja (TNF, IL6, CCL2, IL10 in CCL5) in mediatorjev dismenoreje (NO in PGE2) ter aktivacija signalne poti ERK-PTK2 (se povezuje s celično motilnostjo in invazijo) v kulturi ESC iz EvEA po izbitju *CAV1* → ↓ CAV1 v endometriju bi lahko vodilo v vnetno okolje in sprožilo tvorbo NO in PGE2, kar vodi v simptom dismenoreje.
	(Kolioulis in sod., 2017)	↑ KISS1 (angl. *metastasis-suppressor KiSS-1*. Protein zaviranja metastaz) v žlezah EvEA in EkEA proti K (ni navedene faze ob vzorčenju).
	(Matsuda in sod., 2001)	↑ invazivnost *(in vitro test invazivnosti z rekonstrukcijo bazne membrane matriksa in želatine, Matrigel)* kulture ESC iz mišjega modela A proti K.
	(Qi in sod., 2015)	↑ NOTCH1 (angl. *neurigenic locus notch homolog protein 1*. Spada v družino transmembranskih receptorjev z domnevno vlogo pri vzpostavitvi procesa EMT. Vezani v kompleks lahko uravnavajo prepisovanje genov, povezanih z EMT: *SNAI1* in *SNAI2*), CDH1, SNAI1 in SNAI2 ter ↓ NUMB (angl. *protein numb homolog*, inhibitor NOTCH signalizacije) v EkEA proti EvEA, pridobljenih v P in S fazah → signalna pot NOTC1/NUMB/SNAIL ima vlogo pri patogenezi in razvoju A.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Aktivacija procesa prehoda epitelija v mezenhim (EMT) Proces EMT poteka v več korakih:		
Zvišana invazivnost endometrija	(Shi in sod., 2019)	↑ lncRNA TUG1 (angl. *taurine upregulated 1*. lncRNA, ki je ↑ pri različnih oblikah raka, kjer ↑ celično migracijo in invazijo) v EkEA proti EvEA, pridobljenih v različnih fazah ciklusa; ↑ migracija in invazija v kulturi EEC iz EkEA proti EvEA, pridobljenih v S fazi (*in vitro test Transwell*); ↓ migracija in invazija ter ↑ TIMP2 v kulturi EEC iz EkEA po TUG1-siRNA (*in vitro test Transwell*); ↓ TUG1 v kulturi EEC iz EkEA po EGR1-siRNA (angl. *early growth response protein 1*; predviden prepisovalni dejavnik, ki se veže na promotor *TUG1*) → EGR1 se veže na promotor *TUG1* in regulira njegovo prepisovanje. ↑ TUG1 se preko epigenetskega delovanja veže na ciljni EZH2 (angl. *histone-lysine N-methytransferase EZH2*), *(test imunoprecipitacije RNA-vezavnih proteinov)*, kar ↓ njegovo zmožnost vezave na promotor TIMP2 (uravnava celično migracijo in invazijo). Posledično se ↑ migracija in invazija epitelijskih celic ter pospeši razvoj A.
	(Guo in sod., 2015)	↓ MIR10B in ↑ ZEB1 (angl. *zinc finger E-box-binding homeobox 1*. Prepisovalni dejavnik, ki deluje kot zaviralec transkripcije) in PIK3CA (angl. *phospatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform*). Kodira enoto proteina PI3K v EkEA in EvEA proti K, pridobljenih iz različnih faz ciklusa; ↓ celična migracija in invazivnost (*in vitro test Transwell*) ter ↓ ZEB1 in PIK3CA v kulturi EEC iz EkEA po prekomernem izražanju miR-10b; ↑ ZEB1 in PIK3CA po transfkeciji kulture EEC iz EkEA z anti-miR-10b; ↑ CDH1 in ↓ p-AKT po ZEB1-siRNA in PIK3CA-siRNA (ciljna gena miR-10b) v kulturi EECs iz A → miR-10b cilja 3' UTR regije *ZEB1* in *PIK3C* s čimer ↑ invazivnost (↑ CDH1 in inhibicija fosforilacije AKT) epitelijskih celic pri A.
Spremembe v organizaciji zunajceličnega matriksa	(Matsuda in sod., 2001)	↑ *MMP14* v maternici mišjega modela A vs. K → ↑ invazivnost stromalnih celic v zunajcelični matriks vpliva na razvoj A.
	(Li in sod., 2006)	↑ MMP2 in -9 v EvEA in EkEA proti K, pridobljenih v P in S fazah → ↑ izražanje MMPs bi lahko vplivalo na razvoj A, preko ↑ invazije endometrijskega tkiva v miometrij in angiogenezo v A lezijah.

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2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Spremembe v organizaciji zunajceličnega matriksa	(Yang in sod., 2009)	↑ MMP2 in TIMP1 v kulturi ESC iz EvEA proti K, pridobljenih v ES ali SS fazah, po gojenju 48 ur v mediju DMEM na 37°C.
	(Yi in sod., 2015)	↓ MMP2 in -9 v kulturi ESC iz EvEA proti K (ni navedene faze ciklusa ob vzorčenju) in v kulturi Ishikawa celic vs. negativna K po transfekciji s PAK4-siRNA → ↑ PAK4, preko regulacije MMP2 in -9, ↑ invazivnost endometrijskih celic, kar vpliva na razvoj A.
	(Zhang in sod., 2016)	↑ *CCN1* in protein (angl. *CCN family member 1*. Protein zunajceličnega matriksa z vlogo spodbujanja adhezij, migracij in mitoze ter pri uravnavanju proliferacije, angiogeneze, rasti tumorjev in embriogenezi) v EkEA proti EvEA, pridobljenih v P in S fazah; ↑ *CCN1* v EkEA žensk s hudo proti blagi menoragiji in/ali dizmenoreji. ↑ *CCN1* v EvEA žensk starih 30 - 39 let proti EvEA žensk starih 40 - 49 in > 50 let.
Prekomerna neuroangiogeneza		
Angiogeneza	(Huang in sod., 2014)	↑ ožiljenje EkEA proti EvEA, pridobljenih v EP, SP in S fazah *(3D Doppler ultrazvok)*; ↑ gostota malih žil, VEGFA in SNAI2 v EkEA proti EvEA, pridobljenih v ZP, SP in S fazah; ↑ SNAI2 in ↑ pripadajočega odzivnega VEGFA v ESR+ Ishikawa celicah proti ESR- Ishikawa celicam po 24-urnem gojenju z E2; ↑ implantacija ksenotransplantiranega adenomioznega tkiva in angiogeneza pri SCID miši po 21-dnevni izpostavljenosti E2 → ↑ E2 vodi v ↑ VEGFA, kar spodbuja tvorbo žil ter v ↑ SNAI2, kar ↑ migracijo endotelijskih celic in sproži EMT *(in vitro test tvorbe kapilarne cevke)*.
	(Li in sod., 2006)	↑ VEGFA v EvEA in EkEA proti K, pridobljenih v P in S fazah → ↑ izražanje MMP2 in -9 vpliva na ↑ invazivnost endometrijskega tkiva v miometrij in ↑ angiogenezo v A lezijah.
	(Wang in sod., 2021b)	↑ žilni potencial *(in vitro test žilne cevke in tvorbe združb)* kulture ESC iz EkEA in EvEA proti K, pridobljenih v P fazi; ↑ VEGFB in ANGPTL4 ter dodatno ↑ žilni potencial kulturi ESC iz EvEA in EkEA proti K po dodatku E2.
	(Ota and Tanaka, 2003)	↑ ožiljenje v P in S fazi EvEA proti K (pri K ↑ ožilje endometrija samo v S fazi), *(morfometrične meritve kapilar ob histereskopiji)*→ nenormalno ožiljenje EvEA je povezano s simptomom hipermenoragije.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Prekomerna neuroangiogeneza		
Angiogeneza	**(Wang J. in sod., 2016)**	↑ VEGFA v žlezah epitelija EvEA in EkEA proti K, pridobljenih v P in S fazah; ↑ gostota malih žilic v EkEA proti EvEA in K → VEGFA inducira angiogenezo pri razvoju A; ↑ VEGFA in STAT3 v Ishikawa celical po transfekciji z *NDUFA13*-siRNA → spremenjeno izražanje NDUFA13 vpliva na uravnavanje apoptoze in angiogeneze pri razvoju A.
	(Goteri in sod., 2009)	↑ VEGFA v epiteliju, stromi in endoteliju EvEA proti K, pridobljenih v P in S fazi.
	(Liu in sod., 2011)	↑ F3 (angl. *tissue factor*. Spada v družino citokinskih receptorjev z vlogo pri procesu koagulacije) v žlezah epitelija EkEA in EvEA proti K, pridobljenih v P in S fazah → ↑ imunoreaktivnost F3 v poz. povezavi s stopnjo dismenoreje in količino menstrualne krvi pri A.
Tvorba živčnih vlaken	**(Zhang in sod., 2010)**	↑ UCHL1 (angl. *ubiquitin carboxyl-terminal hydrolase isozyme L1,* pogosto uporabljen sinonim PGP9.5. Povezuje se s tvorbo živčnih niti) v P in S fazah funkcionalnega EvEA žensk z v primerjavi z ženskami brez simptomov bolečin v mali medenici.
	(Carrarelli in sod., 2017)	↑ *NGF* (angl. *beta-nerve growth factor*), *SYP* (angl. *synaptophysin*), *MAP2* (angl. *microtuble associated protein 2*) v EkEA proti EvEA in K, pridobljenih v P fazi → oživčene A lezije bi lahko povzročale bolečine v predelu medenice; ↑ *NGF* v kulturi ESCs iz K po 3-urnem gojenju z UCN (urokortinom: vnetni dejavnik).
	(Wang F. in sod., 2015)	↑ NCAM1 (angl. *neural cell adhision molecule 1*, pogosto uporabljen sinonim CD56. Molekula, vključena v adhezijo nevronov) v epiteliju EkEA z dismenorejo proti EkEA brez dismenoreje, EvEA ter K → izražanje NCAM1 značilno poz. povezano s stopnjo dismenoreje. Pri A žleze epitelija ↑ izločajo NCAM1, kar stimulira rast živčnih niti v stromi in povzroča dismenorejo; ↑ NCAM1 v S proti P fazi EvEA žensk z dismenorejo (ni bilo razlik v izražanju NCAM1 med fazama v EkEA) → Spolni steroidni hormoni bi lahko uravnavali izražanje NCAM1.
	(Nie in sod., 2010b)	↑ TRPV1 (angl. *transient receptor potential cation channel subfamily V member 1*. Vlogo pri vnetni bolečini in hipealgeziji – preobčutljivost na bolečinske dražljaje) in OXTR v EkEA proti EvEA in K, pridobljenih v P in S fazah (izražanje TRPV1 tekom ciklusa se pri K ni spreminjalo, medtem ko je bilo izražanje OXTR višje v S kot pa v P fazi) → ↑ OXTR in TRPV1 v značilni poz. povezavi s stopnjo dismenoreje.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Prekomerna neuroangiogeneza		
Tvorba živčnih vlaken	(Miyashita in sod., 2019)	↑ NINJ1 (angl. *ninjurin-1*. Molekula nevronskih celic) v epiteliju EkEA proti EvEA in K (ni navedene faze ciklusa ob vzorčenju); ↑ NINJ1 v kulturi ESC, pridobljenih iz žensk z endometriomom, po 3- do 6-urnem gojenju z IL1B → vnetni dražljaji bi lahko ↑ NINJ1, kar ↑ simptom bolečine v predelu medenice.
Blokirana apoptoza	**(An in sod., 2017)**	↑ apoptoza (*pretočna citometrija*) Ishikawa celic po 48-urnem gojenju z makrofagi → makrofagi ↑ invazivnost in migracijo endometrijskih epitelijskih celic v procesu EMT.
	(Yang in sod., 2007)	↓ apoptoza (*FACS*) v kulturi ESC iz EvEA proti K po 24-urnem gojenju z ali brez vodikovega peroksida (H_2O_2).
	(Li J. in sod., 2019)	↑ *BCL2* in protein (angl. *apoptosis regulator Bcl-2.* Blokira signalne poti apoptoze in spodbuja preživetje celic) v P in S fazi EvEA proti K; ↑ stopnja apoptoze *(pretočna citometrija)* in ↓ stopnja preživetja *(kit za štetje celic 8, CCK-8 assay*) ter relativne razdalje migracije *(in vitro test celjenja rane)* v kulturi ESC iz EvEA 48 ur po transfekciji z BCL2-siRNA proti neg. K.
	(Jones in sod., 1998)	↓ BCL2 v strome EkEA proti EvEA, brez variacij v izražanju tekom menstru. ciklusa; Brez razlik v izražanju BCL2 v EvEA tekom menstr. ciklusa (pri K ↑ BCL2 v PS fazi); Nizka stopnja apoptoze v K, EkEA in EvEA *(barvanje celic z metodo TUNEL (the dUTP nick-end labelling));* BCL2+ stromalne celice identificirane kot pretežno levkociti *(dvojno imunohistokemijsko barvanje)* → povišano izražanje BCL2 v endometriju v PS fazi sovpada z značilnim ↑ številom infiltriranih levkocitov.
	(Hu in sod., 2017)	↓ *PTEN* in protein (tumor supresor, ki se povezuje s celično proliferacijo, apoptozo, invazijo in metastazo) in ↑ MIR17 v EvEA proti K (ni navedene faze ciklusa ob vzorčenju); ↑ PTEN in BAX (angl. *apoptosis regulator BAX*) ter ↓ BCL2, CCNE1 (angl. *G1/S-specific cyclin-E1*) in CCND1 v kulturi ESCs po utišanju MIR17; ↑ apoptoza *(pretočni citometer)* in ↓ živost (*test MTT: kolorimetričen test za določevanje aktivnosti celičnega metabolizma*) kulture ESCs po prekomernem izražanju PTEN ali utišanju MIR17 → miR-17 bi lahko ciljala 3'UTR regijo *PTEN.* Izražanje MIR17 pri A bi lahko vplivalo na celično apoptozo in izražanje ciklinov, ki regulirajo celični cikel.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Blokirana apoptoza	**(Wang J. in sod., 2016)**	↓ NDUFA13 (angl. *NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13*. Regulator apoptoze in angiogeneze) v EvEA in EkEA proti K, pridobljenih v P in S fazah; ↓ stopnja apoptoze EkEA in EvEA proti K (*TUNEL barvanje*) → ↓ NDUFA13 v žlezah epitelija bi lahko ↓ stopnjo apoptoze in ↑ kapaciteto infiltracije v miometrij, kar vodi v razvoj A.
	(Huang in sod., 2011)	↑ *NTRK2* (angl. *BDNF/NT-3 growth factors receptor*: tirozin kinazni receptor, ki sodeluje tudi pri odpornosti celic na apoptozo) v S fazi EvEA proti K.
• ***Avtofagija***	(Ren in sod., 2010)	↓ *BECN1* (angl. *Beclin-1*) v EvEA proti K (ni podatka o fazi ciklusa ob vzorčenju); ↓ *BECN1* in protein v kulturi ESC iz EvEA proti K; Značilna neg. korelacija v izražanju BECN1 v EvEA in serumskimi vrednostmi CA125 ter bolečinami v predelu medenice.
Prekomerna celična proliferacija	(Wang in sod., 2021b)	↑ celična proliferacija (*in vitro test tvorbe kolonij na plošči in test celične viabilnosti*) kulture ESC iz EvEA in EkEA proti K, pridobljenih v P fazi; Dodatno ↑ proliferacija (↑ PCNA (angl. *proliferating cell nucleas antigen*) in MKI67 (angl. *proliferation marker protein Ki-67*)) kulture EEC iz EkEA in EvEA proti K po 24-urnem gojenju z E2 → E2 s ↑ TLN1 bi lahko spodbujala proliferacijo stromalnih endometrijskih celic in tvorbo novih žil, kar omogoča rast in preživetje A lezij.
	(Li J. in sod., 2019)	↓ stopnja preživetja *(in vitro štetje celic)* kulture ESC iz EvEA 48 ur po transfekciji z *BCL2*-siRNA.
	(Yang in sod., 2007)	↑ MKI67 in celična proliferacija *(test celične proliferacije CellTiter 96 AQueous Nonradioactive)* kulture ESC iz EvEA proti K, pridobljenih v ZS ali SS fazah, po 48-urnem samostojnem gojenju ali ob dodajanju E2, MPA, IL6 ali IFNG → spremenjena proliferacija in apoptoza EvEA bi lahko nakazovala nekatere mehanizme patofiziologije A.
	(Zhai in sod., 2020a)	↑ *IGF1* (angl. *insulin-like growth factor*. Rastni dejavnik, ki uravnava proliferacijo epitelijskih celic in signalne poti AKT) v P fazi v EvEA proti K; Spremenjeno izražanje *IGF1* v značilni povezavi z izražanjem *METTL3* (metilira adenozinske ostanke na položaju N_6 nekaterih RNA) v EvEA *(določanje transkriptoma z mikromrežo)* → IGF1 bi lahko bil ciljni gen regulatorjev m^6A RNA metilacije.

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Mehanizem patofiziologije adenomioze	Referenčna literatura	Glavni rezultat in ugotovitve raziskave
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Prekomerna celična proliferacija	**(Carrarelli in sod., 2015)**	↑ rastnih dejavnikov proliferacije, apoptoze in angiogenezo (*INHBA* (angl. *inhibin beta A chain*, pogosto uporabljeno ime Activin A. Spada med citokine) in *MSTN* (miostatin)) v EkEA proti EvEA in K, pridobljenih v P fazi; ↑ *FST* (folistatin) v EkEA in EvEA proti K; ↑ *ACVR2A* (tip 2A receptorja za aktivin) in *ACVR2B* v EkEA in EvEA proti K → ↑ INHBA v A lezijah bi lahko vodilo v vnetje, spremenjeno celično proliferacijo, ↑ invazivnost in angiogenezo v miometriju. Spremenjeno izražanje rastnih dejavnikov v EkEA vpliva, preko avtokrinih/parakrinih efektov, na spremenjeno izražanje pripadajočih receptorjev v EvEA, kar bi lahko vplivalo na plodnost takšnih žensk.
	(Xu in sod., 2018)	↑ LINC-ROR (intergenska lncRNA) v EvEA in EkEA proti K, pridobljenih v P fazi; ↑ proliferacija *(test CCK-8)*, (↓ PTEN in ↑ p-Akt, p-PTEN in p-PDK1) kulture EEC ob prekomernem izražanju LINC-ROR oz. ↑ PTEN in ↓ p-Akt, p-PTEN ter p-PDK1 po izbitju LINC-ROR → LINC-ROR spodbuja delitev endometrijskih celic preko poti PI3K-Akt.
	(Guo in sod., 2016)	↑ *TLR4* (angl. *toll-like receptor 4*. Mediator naravne imunosti) in pripadajoč protein v EvEA in EkEA proti K, pridobljenih v ZP fazi; ↑ proliferacija *(test CCK-8)* in invazivna rast v ESC po 24-urnem gojenju z LPS (bakterijski lipopolisaharid, ki v gostitelju izzove vnetje) → aktivacija LPS/TLR4 signalne poti (↑ TLR4, LY96, (angl. *lymphocyte antigen 96*. Veže bakterijski LPS), MXD88, NFKB v kulturi ESC iz EvEA, EkEA in K → signalna pot TLR4 izzvala ↑ TGFB1, IL6, VEGF, EGF in MMP2 → LPS bi lahko stimuliral stromalne celice, da izražajo dejavnike proliferacije in invazivnosti pri A.
Spremenjeno izražanje mediatorjev vnetja (citokini, kemokini in prostaglandini)	(Zhihong in sod., 2016)	↑ IL6, IFNG in CCL2 (kemokin, ki je močan aktivator celic uNK. Visoko število uNK bi lahko bilo povezano s splavi in neplodnostjo) ter ↓ IL10 in IL17 v EvEA proti K, pridobljenih sedem dni po injeciranju hCG v procesu kontrolirane stimulacije jajčnikov.
	(Sotnikova in sod., 2002)	↑ IFNG, IFNA, TNF, IL1B in EGF ter ↓ CXCL8 (pogosto uporabljen sinonim IL8) v supernatantu 24 ur gojenih mononuklearnih celic, pridobljenih iz PS faze EvEA proti K → spremenjeno izražanje citokinov bi lahko nakazovalo aktivacijo T limfocitov. ↑ aktivnost imunskih celic bi lahko ustvarila pogoje, ki dovoljujejo infiltracijo in proliferacijo celic, s tem pa razvoj A.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Spremenjeno izražanje mediatorjev vnetja (citokini, kemokini in prostaglandini)	**(Carrarelli in sod., 2017)**	↑ *IL1B* in *CRH* (angl. *corticoliberin*. Vnetni dejavnik) v P fazi EvEA proti K; ↑ vnetnih posrednikov (*IL1B*, *CRH*, *UCN* (angl. *urocortin*)) in ↑ nevrogenih posrednikov (*NGF*, *SYP* in *MAP2*) v EkEA proti EvEA in K → A lezije so nova mesta izražanja vnetnih in nevrogenih posrednikov, ki so vpleteni v patogenezo A.
	(Yang in sod., 2006)	↑ *IL6* v primarni kulturi ESC iz EvEA proti K ob gojenju z makrofagi → Makrofagi naj bi izločali citokine, ki ↑ celično proliferacijo ektopičnih implantov pri A (čeprav so makrofagi sposobni citolize EvE in EkE, lahko z izločanjem citokinov tudi ↑ celično rast in proliferacijo → IL6 ima angiogeni in mitogeni efekt).
	(Wang F. in sod., 2009)	↑ IL10 (protivnetni citokin) v žlezah epitelija EkEA in EvEA proti K, pridobljenih v S fazi; ↑ IL10 v epiteliju EvEA v S proti P fazi → ciklično variranje izražanja IL10 v EvEA tekom faz menst. ciklusa → v EvEA in EkEA prihaja do nenormalnega vnetnega odziva.
	(Wang in sod., 2018)	↓ IL10, HOXA10 (prepisovalni dejavnik, ki sodeluje pri razvoju endometrija: decidualizacija strome, infiltracija levkocitov in razvoj pinopodov ter vpliva na izražanje drugih genov, ki sodelujejo v procesu vgnezdenja zarodka) in p-STAT3 v SS fazi v EvEA proti K; Značilna poz. povezava med stopnjo IL10 in p-STAT3; ↑ HOXA10 po 12-urnem gojenju Ishikawa celic z IL10 → ↑ stopnja vezave *in vitro* modela blastociste *(BeWo sferoidi)* na Ishikawa celice → IL10 ↑ fostofilacijo STAT3, s tem pa ↑ HOXA10, kar deluje spodbudno na proces vgnezdenja → spremenjena signalna pot IL10/STAT3/HOXA10 v EvEA bi lahko vplivala na vgnezditev zarodka ali decidualizacijo.
	(Fischer in sod., 2011)	↓ HOXA10 v SS fazi v stromi EvEA proti K → ↓ HOXA10 bi lahko vplivala na nižjo stopnjo vgnezitve zarodka pri A.
	(Qin in sod., 2012)	↑ IL10RA v epiteliju EkEA in EvEA proti K, pridobljenih v P in S fazah (višje izražanje IL10RA v S kot pa v P fazi pri K in EvEA); ↑ IL10RB v epiteliju EkEA proti EvEA in K (brez variabilnosti v izražanju med P in S fazama) → receptorji za IL10 bi lahko bili vključeni v imunotolerantne in/ali protivnetne procese pri A.
	(Jiang J.F. in sod., 2018)	↓ IL37 (protivnetni citokin) v EvEA in EkEA proti K, pridobljenih v P in S fazah → v EvEA lahko pride do nenormalnega vnetnega odziva.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Spremenjeno izražanje mediatorjev vnetja (citokini, kemokini in prostaglandini)	(Huang H. in sod., 2010)	↑ IL18R in razmerje med IL18BP in IL18 (IL18BP/IL18) v EvEA proti K (ni navedene faze ciklusa ob vzorčenju).
	(Wang in sod., 2014)	↑ IL22, IL22RA1, IL10RB v EkEA in EvEA proti K (ni navedene faze ciklusa ob vzorčenju); ↑ invazivnost *(in vitro test invazivnosti z Matrigel)*, IL22RA1, IL10RB, CCL5, CXCL8, IL6 in VEGFA ter ↓ CD82 (supresor metastazij) po 48-urnem gojenju z IL22 kulture ESC iz EvEA proti K → IL22 ima avtokrini efekt na izražanje IL22RA1 in IL10RB. ↑ izražanje IL22 vodi v ↑ izražanje z invazivnostjo povezanih molekul, kar ↑ invazivnost ESC pri razvoju A.
	(Lai in sod., 2016)	↑ CXCL1 (kemoatraktant za nevtrofilce. Avtokrino prenaša vpliv na endotelijske celice) v epiteliju EvEA proti K (ni navedene faze ciklusa ob vzorčenju); Značilna poz. povezava med konc. VEGFA in CXCL1 v epiteliju → VEGFA je močan mediator izločanja CXCL1; ↑ CXCL1 v kulturi EECs iz EvEA po 16-urnem gojenju z naraščajočimi koncentracijami VEGFA → VEGFA inducira sproščanje CXCL1 preko NADP oksidaze (↑ NCF1: encim s sodelovanjem pri tvorbi reaktivnih kisikovih spojin) in signalne poti NFKB (↑ NFKBIB, RELA in NFKB1), kar ↑ proliferacijo EEC *(in vitro test celične viabilnosti/proliferacije)* in celično migracijo vaskulatornega endotelija *(in vitro test migracije HUVEC (angl. human umbilical vein endothelial cell))*.
	(Jiang in sod., 2017)	↑ *IL6*, *CXCL8* (pogosto uporabljen simbol IL8), *TLR1, -6, -8 in -9* (angl. *Toll-like receptors.* Komponentne naravne imunosti, ki varujejo gostitelja pred vdorom bakterij in virusov) in pripadajočih proteinov v EkEA in EvEA proti K, pridobljenih v P fazi; Značilna poz. korelacija med izražanjem TLR1, -2, -4, -5 in -9 ter IL6 in CXCL8 v EkEA in EvEA → IL6 in CXCL8 bi lahko bili vključeni v vnetne procese pri A.
	(Ulukus E.C. in sod., 2005)	↓ CXCL8 (kemoatrtaktni dejavnik, ki aktivira nevrofilce in T celice, ne pa monocit) in CCL2 (pogosto uporabljen simbol MCP-1. Izzove kemotakso in aktivacijo mononuklearnih fagocitov) v S fazi v epiteliju EvEA proti K (pri K ↑ izražanje CCXL in CCL2 v S proti P fazi) → ni ciklične variacije v izražanju CXCL8 in CCL2 v epiteliju EvEA (spolni steroidni hormoni bi lahko v EvEA izzvali nenormalni vnetni odziv); ↑ CXCL8 in CCL2 v EkEA proti EvEA → v EvEA bi lahko potekal nenormalen vnetni odziv.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Spremenjeno izražanje mediatorjev vnetja (citokini, kemokini in prostaglandini)	**(Ulukus in sod., 2006)**	↑ CXCR1 in CXCR2 (receptorja za CXCL8, ki se nahajata na nevtrofilcih) v P fazi v epiteliju EvEA in EkEA proti K → receptorji za CXCL8 bi lahko bili vključeni v patogenezo A.
	(Ota in sod., 2001b)	↑ PTGS2 (angl. *prostaglandin G/H synthase 2*, pogosto uporabljen simbol COX-2. Vzdržuje celično homeostazo, izraža se tekom vnetja in celične proliferacije ter diferenciacije. Vlogo ima tudi v procesih reprodukcije) v P fazi na površini epitelija EvEA proti K (ni razlik v izražanju PTGS2 tekom menstruacijskega ciklusa v EvEA, medtem ko je pri K najnižje v ZP fazi s postopnim naraščanjem, ki ostaja visoko v S fazi) → ↑ PTGS2 in posledična ↑ produkcija prostaglandinov bi lahko vodila v nenormalne pogoje v maternici, z negativnim vplivom na procese vgnezdenja zarodka.
	(Li C. in sod., 2019)	↑ *ALOX5* (angl. *polyunsaturate fatty acid 5-lipoxygenase*. Encim, ki katalizira pretvorbo arahidonske kisline v levkotrine, ki so pomembni mediatorji vnetja) in protein, *PTGS2* in protein, *IL6* in *CXCL8* v EkEA in EvEA proti K, pridobljenih v ZP fazi; Značilna poz. povezava med stopnjami izražanja *ALOX5* in *PTGS2* z *IL6* in *CXCL8* v EvEA → A je kronična imunska vnetna bolezen.
	(Xiao in sod., 2010)	↓ *LIF* v SS fazi EvEA proti K in LIF v izpirku maternične tekočine A vs. K → A bi lahko vplivala na hormonske in imunološke pogoje, kar ↓ receptivnost endometrija za zarodek.
	(Yen in sod., 2016)	↓ *LIF* in protein, *LIFR* in protein**,** pSTAT3 in pERK v SS fazi EvEA proti K; ↑ p-STAT3 in pERK v kulturi ESC po 5 do 60 min gojenja z LIF → ovirana signalizacija LIF sovpada z ↓ stopnjo vgnezditve zarodka pri ženskah z A.
Spremenjene znotrajcelične signalne poti	(Xue in sod., 2013)	↑ z AMP aktivirana protein kinaza (AMPK) v kulturi ESC iz EvEA proti K, pridobljenih v P in S fazah; ↑ p-AMPK in ↓ signalna pot PI3K/AKT ter proliferacija po gojenju z metforminom (zdravilo, ki se uporablja za zdravljenje diabetesa tipa 2, poz. pa naj bi vplival tudi na endometrijske nepravilnosti) kulture ESC iz EvEA proti K, pridobljenih v S fazi → metformin inhibira celično rast preko aktivacije AMPK in posledičnega zaviranja signalne poti PI3K/AKT v stromi EvEA, kar je opazno predvsem v S fazi.
	(Zheng in sod., 2018)	↑ *PI3K* in protein ter *AKT* in protein v P in S fazah EvEA proti K; ↓ PI3K in AKT ter označevalci EMT po transfekciji kulture endometrijskh celic s PTK2-siRNA → Signalna pot PTK2/PI3K/AKT bi lahko sodelovala v procesu EMT endometrijskih celic pri razvoju A.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Spremenjene znotrajcelične signalne poti	(Wang in sod., 2021a)	↑ CTNNB1 (angl. *catenin beta-1*) in GSK3B (angl. *glycogen synthase kinase-3 beta*) ter ↓ AXIN1 (angl. *axin-1*) po 72-urnem gojenju kulture EEC iz EkEA in EvEA, pridobljenih v P in S fazah, s prekomernim izražanjem TLN1 → aktivacija signalne poti Wnt/beta-katenin bi lahko ↑ migracijske in invazivne lastnosti epitelija EvEA in EkEA.
	(Shen in sod., 2019)	↓ *CNR1* (angl. *cannabioid receptor 1*. Receptor za endogene kanabinoide) in protein, ter *CNR2* in protein v EvEA in EkEA proti K, pridobljenih v P in S fazah → Izguba ciklične spreminjanja izražanju CNR1 glede na menstr. ciklus pri A (pri K izražanje CNR1 in -2 višje v S kot pa v P fazi) → kanaboidni receptorji bi lahko imeli vlogo pri patogenezi A.
	(An in sod., 2017)	↑ izražanje genov signalnih poti TGFB1/SMAD3 in IL6/STAT3 po gojenju Ishikawa celic z makrofagi *(analiza globalnega transkriptoma).*
	(Nie in sod., 2009)	↓ NFKBIA (angl. *nuclear factor-kappa-B inhibitor alpha*) in ↑ RELA (NFKB podenota p65), NFKB1 (NFKB podenota 50) in NFKB2 (NFKB podenota p52) v EkEA in EvEA proti K, pridobljenih v P in S fazah → EvEA kaže stalno aktivnost signalne poti NFKB (NFKB je prepisovalni dejavnik z vlogo uravnavanja izražanja genov, vključenih v procese vnetja, proliferacije, apoptozo, invazijo in angiogenezo. Različni rastni dejavniki in oksidativni stres lahko aktivirajo NFKB), kar bi lahko ↓ izoobliko B PGR (deluje kot protivnetni dejavnik, tako da zavira aktivacijo NFKB); Značilna poz. povezava med izražanjem NFKB2 in hudo menstrualno krvavitvijo.
	(Li in sod., 2013)	↑ NFKB-DNA vezavna aktivnost (↑ RELA in NFKB1), PTGS2, VEGF in F3 (angl. *tissue factor*: vlogo pri vzpostavitvi aktivacije trombocitov in koagulacije) v stromi EkEA proti K, pridobljenih v P in S fazah → NFKB bi lahko vplival na razvoj A, saj regulira izražanje PTGS2 in genov vnetnih citokinov/kemokinov, celične proliferacije, invazije, angiogeneze ter oksidativnega stresa.
	(Park in sod., 2016)	↑ RELA v jedru in citoplazmi stromalnih celic EvEA in EkEA proti K v P, ZS (ne v EkEA proti K) in SS fazah; ↑ RELA v jedru žleznih celic EvEA in EkEA proti K v P, ZS, SS in PS fazi in v citoplazmi žleznih celic EvEA in EkEA proti K v P fazi → aktivirana signalna pot NFKB bi lahko sodelovala pri patogenezi in/ali patofiziologiji A.
	(Yi in sod., 2015)	↑ PAK4 (angl. *serine/threonine-protein kinase PAK 4*. Serin proteaza z vlogo v signalnih poteh, ki uravnavajo organizacijo citoskeletnega aktina, celično morfologijo, adhezijo in motilnost) v P, ZS, SS in PS fazah v epiteliju EvEA in EkEA proti K; ↑ PAK4 v ZS in SS fazah v stromi EvEA in EkEA (ne v ZS fazi) proti K; ↑ aktivacija NFKB (↑ RELA) in PAK4 v kulturi ESC iz EvEA po dodajanju TNF → Pot NFKB regulira izražanje PAK4 → ↑ PAK4 lahko vpliva na invazivnost endometrijskih celic preko uravnavanja aktivnosti MMP2 in -9, kar prispeva k razvoju A.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Spremenjene znotrajcelične signalne poti	(Kim in sod., 2010)	↑ PAK1 v S fazi v žlezah epitelija EkEA (in LS fazi) in EvEA proti K; ↑ PAK1 v SS fazi v stromi EvEA in EkEA (in P, ES in LS fazah) proti K → V EvEA tekom SS faza bi lahko bilo ovirano zmanjševanje izražanja PAK1, ki ga narekuje P4.
	(Streuli in sod., 2015)	↑ fosforilacija ERK1 in -2 v kulturi gladkega mišičja miometrija A proti K; ↑ stopnja proliferacije v kulturi celic gladkega mišičja miometrija A proti K → ↑ aktivacije signalne poti protein kinaze aktivirana z miotegom / kinaza regulirana z zunajceličnim signalom (MAPK/ERK) v miometriju pri A vodi v ↑ celično proliferacijo.
	(Guo in sod., 2014)	↓ DUSP6, SPRY4 in SEF (angl. *similar expression to FGF*) v P in S fazah EvEA proti K → ↓ izražanje DUSP6, SPRY4 in SEF (neg. modulatorji signalne poti FGF2/ERK1/-2) v EvEA bi lahko vplivalo na razvoj A.
	(Chen in sod., 2017)	↑ NFE2L2 (angl. *nuclear factor erythroid 2-related factor 2*. Jederni dejavnik, ki nadzira procese stresa zaradi poškodb) v EkEA in EvEA proti K, pridobljenih v P in S fazah; ↑ oz. ↓ MMP9 in migracija *(in vitro test hitrosti celjenja rane)* v kulturi EEC iz EvEA proti K po prekomernem izražanju NFE2L2 oz. transfekciji celic z NFE2L2-siRNA → NFE2L2 bi lahko preko regulacije izražanja MMP9 sprožil migracijo endometrijskih celic na ektopična mesta.
	(Jiang C. in sod., 2018)	↑ *ROCK1*, *ROCK2* in *RHOA* (povezani z organizacijo citoskeleta, celično migracijo in celičnim ciklom) in pripadajoči proteini v EkEA in EvEA proti K, pridobljenih v P fazi; ↓ *ARHGAP26* (angl. *Rho GTPase-activating protein 26*. Protein, ki aktivira GTPazo za RHOA. Ključen pri razvoju mišic, deluje tudi kot supresor tumorjev) v EvEA in EkEA proti K, pridobljenih v P fazi → aktivirana signalna pot RHOA/ROCK pri A bi lahko bila povezana z nenormalnim krčenjem miometrija, dismenorejo in s količino menstrualne krvavitve.
	(Jiang Y. in sod., 2016)	↓ *NR4A1* (angl. *nuclear receptor subfamily 4 group A member 1*), *NR4A2*, *NR4A3*, *FOXO1* in *IGFBP1* v SS fazi EvEA proti K; ↓ označevalca decidualizaacije (PRL in IGFBP1) v kulturi ESC iz EvEA proti K po 4 dnevnem gojenju z 8-Br-cAMP in MPA; ↑ oz. ↓ PRL in IGFBP1 v kulturi ESC iz EvEA po prekomernem izražanju NR4A1 oz. NR4A1-siRNA / FOXO1-siRNA → Prepisovalni dejavnik FOXO1 cilja gene receptorjev za NR4A, ki so močni regulatorji decidualizacije endometrijske strome.

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Mehanizem patofiziologije adenomioze	Referenčna literatura	Glavni rezultat in ugotovitve raziskave
2. Molekularni procesi nastanka A glede na teorijo vrivanja celic bazalnega endometrija v spodaj ležeči miometrij		
Spremenjene znotrajcelične signalne poti	**(Yang in sod., 2017)**	↑ LPAR1 (angl. *lysophosphatidic acid receptor 1*. Spada med receptorje, ki vežejo LPA: fosfolipidi, ki posredujejo signale za celično proliferacijo, diferenciacijo, preureditev citoskeleta in celične interakcije), LPAR4 in LPAR5 ter ↓ LPAR2 in LPAR3 v EkEA in EvEA proti K, pridobljenih v P fazi; ↑ CXCL8 v kulturi ESC iz K po 24- ali 48-urnem gojenju z LPA → LPA imajo vlogo pri patofiziologiji A.
	(Yan in sod., 2019)	↓ NR4A1 in MIR21 ter ↑ *KLF12* in protein v SS fazi v EvEA proti K; ↑ oz. ↓ *PRL* in *IGFBP1*, KLF12 in NR4A1 (ne pri utišanju miR-21) v kulturi ESC iz A in K pri prekomernem izražanju oz. utišanju miR-21; ↓ decidualizacija v ESC iz K po prekomerno izražanje KLF12 → miR-21 cilja 3'UTR regijo prepisovalnega dejavnika *KLF12,* s čimer ↓ *KLF12,* to pa dalje spodbuja decidualizacijo.
3. Molekularne spremembe zaradi prisotne adenomioze		
Povečan metabolizem prostih radikalov	(Ota in sod., 1998b)	↑ NOS3 (angl. *nitric oxide synthase, endothelial*) v epiteliju in stromi EvEA proti K preko celotnega menst. ciklusa (na površini in v žlezah epitelija K najšibkejše izražanje NOS3 v ZP fazi, nato narašča z vrhom v SS fazi, ko prične upadati. V stromi K je izražanje stalno) → citokini, ki jih izločajo ali stroma EvEA, ali prekomerno št. makrofagov ali T celice lahko ↑ NOS3, kar ↑ dušikov oksid (NO). S tem pa sta lahko prizadeta gibljivost spermijev in razvoj zgodnjega zarodka.
	(Kamada in sod., 2000)	↑ NOS3 v S fazi v žlezah epitelija EkEA proti EvEA in K (izražanje NOS3 v epiteliju in endoteliju žil K in EvEA šibko v P fazi, ki se okrepi v S fazi. Pri EkEA izražanje tudi v P fazi); ↑ NOS2 (angl. *nitric oxide synthase, inducible*) v stromi EkEA proti EvEA in K, pridobljenih v P fazi; ↓ NOS2 v stromi EvEA in EkEA proti K, pridobljenih v S fazi → v EkEA se tvorijo visoke doze NO in superoksida.
	(Ota in sod., 1999)	↑ Cu,Zn-SOD1 (angl. *superoxide dismutase [Cu-Zn]*. Encim z antioksidativno vlogo, ki katalizira razpad superoksidnega aniona na kisik (O_2) in H_2O_2) na površini in v žlezah epitelija EvEA proti K preko celotnega menst. ciklusa (pri K šibko izražanje v ZP in SP fazi, ki postopoma raste z vrhom v SS fazi, nato pa upada); ↑ Mangan (Mn)-SOD2 v žlezah epitelija EvEA proti K preko celotnega menst. ciklusa (pri K šibko izražanje v P fazi, ki narašča z vrhom v PS fazi) → endometrijske celice, imunske celice ali makrofagi stimulirajo ↑ SOD1 v EvEA, kar bi lahko bil vzrok neplodnosti in splavov.

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Mehanizem patofiziologije adenomioze	Referenčna literatura	Glavni rezultat in ugotovitve raziskave
3. Molekularne spremembe zaradi prisotne adenomioze		
Povečan metabolizem prostih radikalov	**(Ota in sod., 2000)**	↑ GPXs (angl. *glutathione peroxidase* (ni naveden podtip). Deluje kot koencim glutationina, s katerim katalizira razpad H_2O_2 in lipidnih peroksidov do H_2O in alkohola) na površini in v žlezah epitelija EkEA in EvEA proti K skozi celoten menst. ciklus (pri K šibko izražanje v ZP fazi, ki postopoma narašča z vrhom v ZS fazi, nato upade) → aktiviran imunski sistem in tvorba prostaglandina pri A bi lahko vodila v tvorbo reaktivnih hidroksilnih radikalov.
	(Ota in sod., 2001a)	↑ XDH (angl. *xanthine dehydrogenase/oxidase*. Katalizira oksidacijo hipoksantina in ksantina v sečno kislino, kar ob visokih koncentracijah O_2 producira superokside) na površini in v žlezah epitelija ter v stromi EvEA in EkEA preko K preko celotnega menst. ciklusa (v epiteliju K nizko izražanje v ZP fazi, ki narašča z vrhom v SS fazi, medtem ko v stromi vrh izražanja doseže v PP fazi).
	(Ota in sod., 2002)	↑ CAT (angl. *catalase*. Katalizira pretvorbo H_2O_2 v H_2O in O_2) v žlezah epitelija EvEA in EkEA proti K preko celotnega menst. ciklusa (v žlezah epitelija K nizko izražanje v ZP fazi, s postopnim naraščanjem in vrhom v PS fazi).
Moten imunološki nadzor	**(Wang in sod., 2008)**	↑ izražanje HLA-G (angl. *HLA class I histocompatibility antigen, alpha chain G*) v žlezah epitelija EvEA in EkEA proti K (stroma in epitelij K skoraj brez izražanja HLA-G) tekom menst. ciklusa; ↑ izražanje HLA-G v stromi EvEA proti EkEA → izražanje HLA-G bi lahko ektopičnim celicam omogočilo, da preživijo imunski nadzor gostitelja.
	(Yang in sod., 2004)	↓ receptorjev za inhibicijo celic ubijalk (angl. *killer cell inhibitory receptors*, KIRs - NKB1 in GL182) na površini uNK celic v EvEA proti K, pridobljenih v P in S fazah → nakazuje se ↑ citotoksičnost uNK v EvEA (KIRs na površini uNK se vežejo z MHC molekulami normalnih celic, kar inhibira delovanje uNK).
	(Ota in sod., 1997)	↑ HSPB1 (angl. *heat shock protein beta-1*. Stresni proteini, ki sodelujejo pri imunskem nadzoru preko procesiranja/predstavitve antigena ali delujejo kot molekularni šaperoni pri vezavi denaturiranih peptidov) v P in S fazah v žlezah epitelija EvEA proti K (pri K višje izražanje v S, kot pa v P fazi).
Spremenjeno izražanje integrinov	**(Ota and Tanaka, 1997)**	↑ integrinov (ITGA2 in -6 ter CDH1. Tvorijo interakcije med celicami in celicami z zunajceličnim matriksom) v P fazi žleznega epitelija EvEA proti K; ↓ ITGA4 (spodbuja interakcije med celicami) v S fazi žleznega epitelija EvEA proti K → spremenjeno izražanje integrinov in kaderina bi lahko nakazovalo na spremenjeno mikrookolje receptivnosti EvEA za zarodek.

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Mehanizem patofiziologije adenomioze	**Referenčna literatura**	**Glavni rezultat in ugotovitve raziskave**
3. Molekularne spremembe zaradi prisotne adenomioze		
Spremenjeno izražanje integrinov	**(Xiao in sod., 2013)**	↓ *ITGA3* (adhezijski receptor na površini celic, čigar izražanje se poviša v času okna vgnezdenja) in protein ter *SPP1* (ligand, ki se veže na ITGA3) in protein v SS fazi v lumnu in žlezah epitelija EvEA proti K → možna ↓ interakcija med trofoblastom in endometrijem pri A.
Spremembe v izražanju receptorjev za steroidne hormone	**(Mehasseb in sod., 2011)**	↓ ESR1 v SS fazi v žlezah epitelija in strome funkcionalne plasti EvEA proti K; ↑ ESR2 v P fazi v žlezah epitelija funkcionalne plasti EvEA in v vseh fazah menst. ciklusa v EkEA proti K; ↓ PGR (izooblika A in B) v SS in LS fazi v stromi funkcionalne plasti EvEA in EkEA proti K → ↑ ESR2 v EvEA, ki bi lahko omogočal ↑ prenos signala E2 s proliferativnim in proapoptoznim delovanjem, in istočasno ↓ izooblike B PGR, ki prevaja signal P4 (ima antiproliferativni efekt), bi lahko ustvarilo okolje, ki spodbuja razvoj A. Spremenjeno izražanje receptorjev v EvEA bi lahko vodilo v ↓ izražanje genov, povezanih z vgnezdenjem zarodka.
	(Nie in sod., 2009)	↓ izooblika B PGR v EvEA in EkEA proti K, pridobljenih v P in S fazah → EvEA bi lahko kazal znake odpornosti na P4. Odsotnost PGR v EkEA pa bi bil lahko vzrok slabe uspešnosti zdravljenja A s progestacijsko terapijo.
Fibroza adenomioznih lezij	(Shen in sod., 2016)	↑ št. trombocitov (*trikromo barvanje po Massonu, barvanje po Van Gieson)* v EkEA v miši z inducirano A proti K → EkEA so rane, ki so podvržene ponavljajočim se poškodbam in celjenju, kar vodi v fibrozo.

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Mehanizem patofiziologije adenomioze	Referenčna literatura	Glavni rezultat in ugotovitve raziskave
3. Molekularne spremembe zaradi prisotne adenomioze		
Fibroza adenomioznih lezij	(Liu in sod., 2016)	↑ stopnja kolagenskih vlaken *(trikromo barvanje po Massonu, barvanje po Van Gieson in Picrosirius rdeče barvanje)* v EkEA proti K → A vsebuje fibrozno tkivo; ↑ gostota trombocitov (↑ ITGA2B (angl. *integrin alpha-IIb*, pogost uporabljen sinonim CD41)) v stromi EkEA proti K, pridobljenih v P in S fazah; ↑ VEGF v epiteliju in CD31, ki označuje MVD, v stromi EkEA proti K; ↑ PCNA (angl. *proliferating cell nuclear antigen*) v epiteliju EkEA proti K; ↑ TGFB1 (angl. *transforming growth factor beta-1 proprotein*) in p-SMAD3 v EkEA proti K; ↓ CDH1 in ↑ VIM v EkEA proti K; ↑ ACTA2 (pogost sinonim α-SMA) in Kolagen 1 (ni podatka o podtipu) v epiteliju in stromi EkEA proti K; ↑ LOX (Označevalec za določanje obsežnosti fibroze) v epiteliju in stromi EkEA proti K; ↓ izooblika B PGR v EkEA proti K; ↑ DES (angl. *desmin*. Označevalec diferenciranih in zrelih celic gladkega mišičja), MYH11 (angl. *Myosin-11*, pogosto uporabljen sinonim SM-MHC(*smooth muscle myosin heavy chain*): specifičen označevalec celic gladkega mišičja) in OXTR v tkivu gladkega mišičja in stromi EkEA proti K → kopičenje trombocitov v EkEA ↑ TGFB1, kar ↑ mehanizme EMT, transdiferenciacijo fibroblastov v miofibroblaste (angl. *fibroblast-to-myofibroblast transdifferentiation,* FMT) in metaplazijo gladkih mišic (angl. *smooth muscle metaplasia*, SMM). Tvori se fibroza. Trombociti, bi lahko bili tudi vzrok prekomernega krčenja maternice in oživčenja miometrija.
4. Transkriptomske raziskave EvEA		
	(Martinez-Conejero in sod., 2011)	Adenomioza potrjena s TVUZ in MR; Biopsija endometrija pridobljena v SS fazi 6 ženskam z in 6 brez adenomioze; Uporabljena tehnologija mikromrež (ni naveden podatek o platformi) → 34 spremenjeno izraženih genov ($p < 0,05$).
	(Herndon in sod., 2016)	Adenomioza potrjena s histerektomijo; Vzorci endometrija pridobljeni v P fazi 3 ženskam z difuzno adenomiozo in 5 kontrolam; Uporabljena tehnologija mikromrež (Human Gene 1.0 ST, Affymetrix) → 1024 spremenjeno izraženih genov (FDR < 0,05; > 2-kratna razlika v izražanju).

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Mehanizem patofiziologije adenomioze	Referenčna literatura	Glavni rezultat in ugotovitve raziskave
4. Transkriptomske raziskave EvEA		
	(Jiang J.F. in sod., 2016)	Adenomioza potrjena s histerektomijo; Vzorci endometrija pridobljeni v P fazi 4 ženskam z adenomiozo in 4 kontrolam; Uporabljena tehnologija mikromrež (Human Transcritome Array 2.0., Affymetrix) → 165 in 612 spremenjeno izraženih lncRNA oz. mRNA (FDR < 0,05).
	(Dior in sod., 2018)	Adenomioza potrjena s TVUZ ženskam s pridruženo endometriozo; Vzorci endometrija pridobljeni v ZP, SP, ZS, SS in PS fazi ženskam z in brez adenomioze (ni navedenih številk); Uporabljena tehnologija mikromrež (Human HT-12v4.0 Beadchips, Illumina) → 0 spremenjeno izraženih genov (FDR < 0,05).
	(Xiang in sod., 2019)	Adenomioza potrjena s histerektomijo; Vzorci endometrija pridobljeni v P fazi 6 ženskam z adenomiozo in 6 kontrolam; Uporabljena tehnologija RNA-seq (Hiseq 2500, Illumina) → 373 spremenjeno izraženih genov (FDR < 0,05).
	(Liu in sod., 2021)	Adenomioza potrjena z histerektomijo; Vzorci endometrija pridobljen (ni navedenega podatka o datiranju) eni ženski z adenomiozo in eni kontroli z miomom; Uporabljena tehnologija scRNA-seq (HiSeq X Ten, Illumina) → 196 spremenjeno izraženih genov vseh celičnih tipov (p < 0,05).