Research Article

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Menopausal transition alters female skeletal muscle transcriptome

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Abstract

Objectives: Although skeletal muscle is a target of hormonal regulation, the muscle transcriptome, including messenger-RNA (mRNA), long non-coding RNAs (lncRNAs), and microRNAs (miRNAs) has not previously been studied across the menopausal transition. Thus, we took a multi-RNA-omics approach to get insight into transcriptome-wide events of menopause.

Methods: We included baseline and follow-up muscle samples from seven early (EarlyMT) and 17 late perimenopausal (LateMT) women transitioning to early postmenopause during the study. Total RNA was sequenced and differential expression (DE) of the transcriptome was investigated. Gene functions were investigated with pathway analyses and protein level expression with Western Blot.

Results: We found 30 DE mRNA genes in EarlyMT and 19 in LateMT participating in pathways controlling cell death, growth, and interactions with the external environment. Lack of protein level changes may indicate a specific role of the regulatory RNAs during menopause. 10 DE lncRNA transcripts but no DE lncRNA genes were identified. No DE miRNAs were found. We identified putative regulatory networks likely to be affected by estradiol availability. Changes in gene expression were correlated with changes in body composition variables, indicating that muscularity and adiposity regulators are affected by menopausal transition. We also found correlations between gene expression and physical activity levels.

Conclusions: The observed DE genes and their regulatory networks offer novel mechanistic insights into factors affecting body composition during and after menopause. Our results imply that physiological deteriorations orchestrated by the muscle transcriptome likely depend on the magnitude of hormonal change and are influenced by physical activity.

Keywords: estrogen; menopause; muscle transcriptome; female physiology; non-coding RNA; multi-omics

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Introduction

Physical activity and exercise are well-known positive exogenous factors that impact skeletal muscle [1]. Contrarily, menopause negatively impacts skeletal muscle [2, 3], which is a critical but understudied phenomenon affecting approximately half of the global population. Despite its significance, the comprehensive transcriptome-wide changes that occur during the menopausal transition remain unclear. Muscle tissue, comprising about 40 % of body weight, is vital for movement, balance, thermoregulation, amino acid storage, metabolism, and inter-tissue signaling [4]. Protein-level contributors like myostatin, mTOR, and MyoD, and the balance

between protein synthesis and degradation influence muscle mass [5], yet the RNA-level regulatory network, especially in women during menopause, is not fully understood. Due to the pivotal metabolic role of skeletal muscle, understanding the influence of menopause on the regulatory RNA network within the tissue has the potential to impact understanding of the regulation of overall body health and metabolism [6].

Discovery of the non-coding RNAs (ncRNAs), such as long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), has widened our understanding of gene expression regulation. LncRNAs are classified as≥200 nucleotide-long oligonucleotides lacking protein-coding potential [7]. LncRNAs control gene expression through pre-transcriptional and post-transcriptional mechanisms such as regulation of chromatin accessibility and transcription factor recruitment and by affecting messenger RNA (mRNA) stability or competing with miRNAs on target mRNAs [8]. miRNAs are short ncRNA molecules known to inhibit protein translation of about 60 % of mRNA genes [9]. To date, more than 170,000 different lncRNA transcripts [10] and approximately 2,600 different mature miRNA sequences have been identified from the human genome [11]. As different RNA species have significant regulatory effects on each other, understanding more about the complicated functional RNA signaling networks in tissues is critical to understanding how they orchestrate human physiology and health.

Natural menopause at midlife results in permanently lowered female sex hormone levels, including progesterone (P4) and the biologically most potent estrogen, estradiol (E2). While E2 is known to have beneficial effects in several tissues, including skeletal muscle, the executive mechanisms have remained largely unknown. Higher systemic E2 levels and pre- or perimenopausal status have been associated with higher muscle mass [2, 12], better muscle quality and strength [3, 13], lower body fat mass [14, 15], and lower risk for metabolic syndrome [16]. Concerning muscle transcriptome, only a few cross-sectional studies examining the role of E2 and other menopause-related hormones have been conducted thus far. In postmenopausal women, E2 levels have been shown to be associated with differences in the muscle mRNA transcriptome, indicating E2-mediated regulation of muscle mass, performance and metabolism [17, 18]. In the case of small ncRNAs, E2 has been shown to regulate several growth, autophagy and glucose metabolism-linked miRNAs in human skeletal muscle [19], but to our knowledge, estrogenic regulation of lncRNAs has not been previously studied in human skeletal muscle. The few *in vivo* studies available suggest a regulatory role of female sex hormone levels in muscle lncRNA expression [20, 21].

While skeletal muscle significantly impacts human health, and menopause is known to negatively impact skeletal muscle, our understanding of the underlying mechanisms, such as how menopausal transition impacts muscle transcriptome, has remained limited. This study aimed to investigate the longitudinal associations between the menopausal transition and the expression of mRNA, lncRNA, and miRNA in skeletal muscle (Figure 1). We also investigated whether changes in the transcriptome were associated with body composition and physical activity variables.

Methods

A detailed description of the study methods is in the Supplementary Methods file. This study used the data and samples from the ERMA (Estrogenic Regulation of Muscle Apoptosis) study [22], which was approved by the ethics committee of the Central Finland Health Care District (8U/2014) and complied with the Declaration of Helsinki except that the study was not registered in advance. The participants provided informed consent.

Study design

Here we used repeated sampling of muscle biopsies taken from 25 women who were either early perimenopausal or late perimenopausal at baseline and early postmenopausal at follow-up forming two longitudinal datasets across the menopausal transition: EarlyMT (n=8) and LateMT (n=17), respectively. Total RNA from ~10–60 mg of muscle tissue was extracted with a Qiagen miRNeasy Mini kit (Hilden, Germany) according to the manufacturer's instructions. Sequencing was outsourced to Novogene (Novogene Company Limited, Cambridge, United Kingdom). Separate protocols were used for the long RNA (mRNA and lncRNA) and the small RNA (sRNA) transcripts. Briefly, for the long RNA transcript sequencing, after ribosomal RNA removal, the remaining RNA was fragmented and used for the construction of double-stranded cDNA libraries. Libraries were agarose gel purified and sequenced using Illumina NovaSeq 6,000 at Novogene to generate paired-end reads of 150 bp. The average sequencing depth was 35 M mapped reads per sample. For the sRNA transcript sequencing, libraries were generated using a NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) following the manufacturer's recommendations. Library quality was assessed on the Agilent Bioanalyzer 2,100 system using DNA High Sensitivity Chips and sequencing was performed using Illumina NovaSeq 6,000 at Novogene to generate 50 bp single-end reads. The average sequencing depth was 10.2 mapped reads per sample.

The samples of one EarlyMT participant were later excluded from the analysis due to low data quality. Thus, the final n of samples selected for further analysis was 48, comprising 24 longitudinal sample pairs (EarlyMT: n=7 sample pairs, LateMT: n=17 sample pairs).

Bioinformatics and statistics

The differential expression (DE) analysis of long and short RNAs was performed with R package *DESeq2* [23] with paired-samples design formula and default settings. Genes, transcripts, or miRNAs with log fold change (LFC) > ± 1.5 and $p_{adj}<0.05$ (p_{adj}=p-value subjected to false

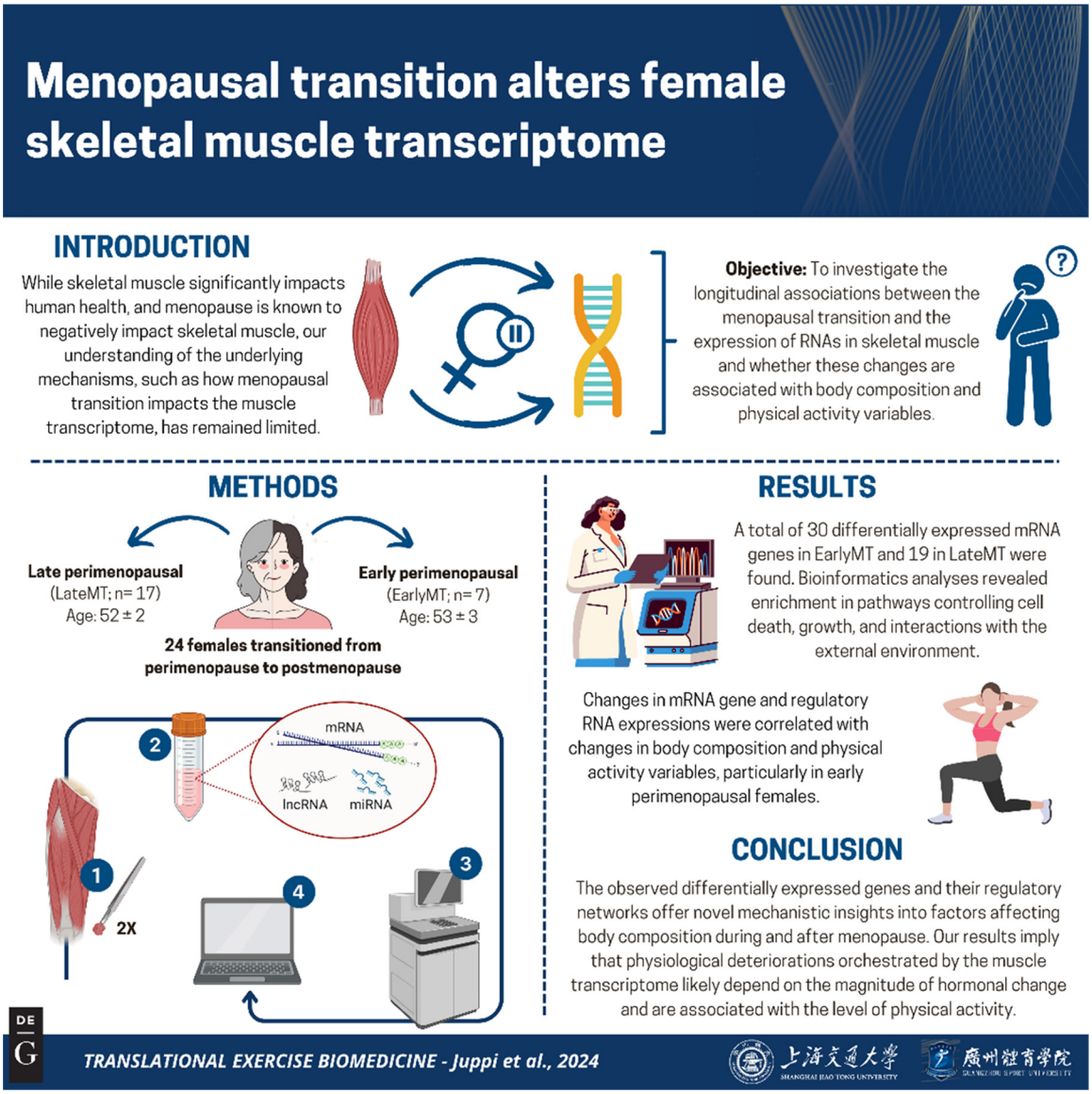

Figure 1: Graphical representation of the study. Key points: 1) being physically active is well-known to have a positive exogenous impact on skeletal muscle and whole-body composition, whereas menopause has a negative impact. The effect is likely controlled by the regulatory RNAs affecting mRNA expression and thereby molecular pathways within muscle cells. 2) A comprehensive understanding of whole transcriptome-wide longitudinal changes during the menopausal transition has been lacking. 3) Menopausal transition-associated physiological deteriorations are orchestrated by the muscle transcriptome and are likely influenced by physical activity.

discovery rate (FDR) correction) were regarded as DE. The Gene Enrichment analysis (GSEA) was performed separately for the EarlyMT (n=314 mRNA genes) and LateMTa (n=175 mRNA genes) groups using Panther 18.0 (pantherdb.org) against gene ontology (GO) Biological Processes databases. In addition, the main transcripts (highest expression level) of all the prefiltered mRNA genes (EarlyMT: n=12,328, LateMT: n=12,189) were analyzed with the *fgsea* [24] R package using t-test statistic (difference in means scaled by the standard deviation and the number of samples) as the ranking metric. Reactome and GO Biological Processes databases from the Molecular Signatures Database (MSigDB) were used as data sources. Furthermore, associations between the different RNA species were investigated with *mixOmics* [25] R package using multiblock sparse partial least squares-discriminant analysis (sPLS-DA). Datasets were constructed from normalized EarlyMT and LateMT expression data by dividing the postmenopause values by the perimenopause values for the same individual. DE genes were used as mRNA input. For the lncRNAs and miRNAs, the input data was the 20 most abundantly expressed, 20 most absolutely, and 20 most relatively differentially expressed lncRNA transcripts or miRNAs in each menopausal group. EarlyMT and LateMT datasets were compared to each other with block sPLS-DA restricting the number of variables allowed to be selected to 10 miRNAs, 10 mRNAs, and 10 lncRNAs. The resulting construction of the relevance network was plotted using a correlation cutoff value of 0.7 and re-plotted with Cytoscape. Finally, interactions between upstream regulators, mRNAs, lncRNAs, miRNAs, and downstream functions were investigated using Ingenuity Pathway Analysis

(IPA, Qiagen). For the core analysis of IPA, all RNA molecules with unadjusted p<0.05 and LFC ≥ ± 1.5 were included (EarlyMT: n=267, LateMT: n=133). For the My Pathway analysis of IPA, we selected the DE mRNA genes and the top 20 abundantly expressed (normalized base mean per group) lncRNA genes and miRNAs.

Before statistical analysis, the normality of the variables was evaluated and parametric tests were used where appropriate. Longitudinal changes in characteristics were investigated using paired T-test and Wilcoxon Signed Rank test. Differences in changes between EarlyMT and LateMT groups were investigated using independent samples T-test and Mann-Whitney *U*-test. Western blot results were investigated using the Wilcoxon Signed Rank Test. Correlations between changes in gene/transcript expression level and body composition or physical activity variables (Δ=POST minus PERI) were calculated using Spearman correlations. For mRNAs and lncRNA genes, the most abundantly expressed transcript was used in correlation analysis.

Results

Characteristics of the study participants: EarlyMT and LateMT groups differed in the rate of decline in estradiol

As the process of menopausal transition varies from one woman to another, the follow-up time was individualized and each participant's menopausal transition was monitored by checking their follicle-stimulating hormone (FSH) levels and menstrual diaries every three to six months until they were regarded as early postmenopausal [26]. The mean follow-up time was 1.5±0.9 years for the EarlyMT and 1.0±0.6 years for the LateMT group. At baseline, women in the EarlyMT group were on average 52.6±2.5 years old, and in the LateMT group 52.2±2.0 years old. In line with our earlier findings using a larger sample of this same study cohort [2, 15], lean body mass tended to decrease and fat mass to increase (Supplementary Table 1). A typical menopausal transition-associated hormonal change was observed in both groups; i.e., FHS levels increased and E2 decreased, although the E2 decrement was significantly greater in the EarlyMT than in the LateMT group (p=0.034) (Supplementary Table 1).

Over 18,000 mRNA genes were expressed in muscle including structural proteins, hormone receptors, and steroidogenic enzymes

Preprocessing of the sequencing data was done separately for sequences representing long (mRNAs + lncRNAs) and short (miRNAs) RNA classes (Figure 2a). It is well known that transcription of a single gene can result in several splice variants and all of them can be present as single RNA transcripts in the sequencing data of the long RNA. Here we investigated long RNA expression considering both the transcript and the gene levels. In the latter, transcripts resulting from the same gene were combined by *tximport* [27]. For simplicity and clarity, we use the terms transcript and gene to distinguish between the transcript or gene level, respectively.

Of the long RNA clean reads, 96 % were mapped to the reference genome. Approximately 89,000 long RNA transcripts were classified as mRNA representing 18,769 different genes (Figure 2a). The most abundant mRNA transcripts included several splice variants of typical skeletal muscle structural proteins such as *MYH7*, *MYH2*, *ACTA1, TTN,* and *NEB*. After filtering, a total of 12,503 different mRNA genes remained (for filter specifications, see Figure 2a and Supplementary methods file). Due to the study aims, we had a particular interest in inspecting gene expression levels of sex hormone receptors, steroidogenic enzymes, and proteins related to muscle function and metabolism (Supplementary Table 2). The receptor for FSH (*FSHR*) and steroidogenic enzymes *HSD3B2*, *HSD17B2*, and *CYP19A1* were detected among the sequences data but their count numbers were too low to meet the criteria to be classified as an expressed transcript or gene and were thus omitted from further analyses. Of the included mRNA genes, 314 were expressed only in EarlyMT and 175 only in LateMT (Figure 2b and Supplementary Table 3).

Overrepresentation analysis of the mRNA genes pointed toward changes in the extracellular matrix

To get an insight into the potential functional differences in the expressed mRNA genes between EarlyMT and LateMT groups, we did a focused GO analysis of the expressed mRNA genes that were not shared between EarlyMT and LateMT (Figure 2b). The 314 EarlyMT-specific genes were enriched into 35 GO terms (p_{adj}<0.05) while no statistically significant enrichment was observed for the 175 LateMT genes. The EarlyMT-specific enriched genes were connected (Supplementary Table 4), for example, to the biological processes related to immune and inflammatory responses (15 GO pathways) and the metabolism of nucleic acids and other molecules (15 GO pathways).

Next, we wanted to get overall insight into the changes in active pathways during menopausal transition. Therefore, we did GSEA using all the prefiltered mRNA

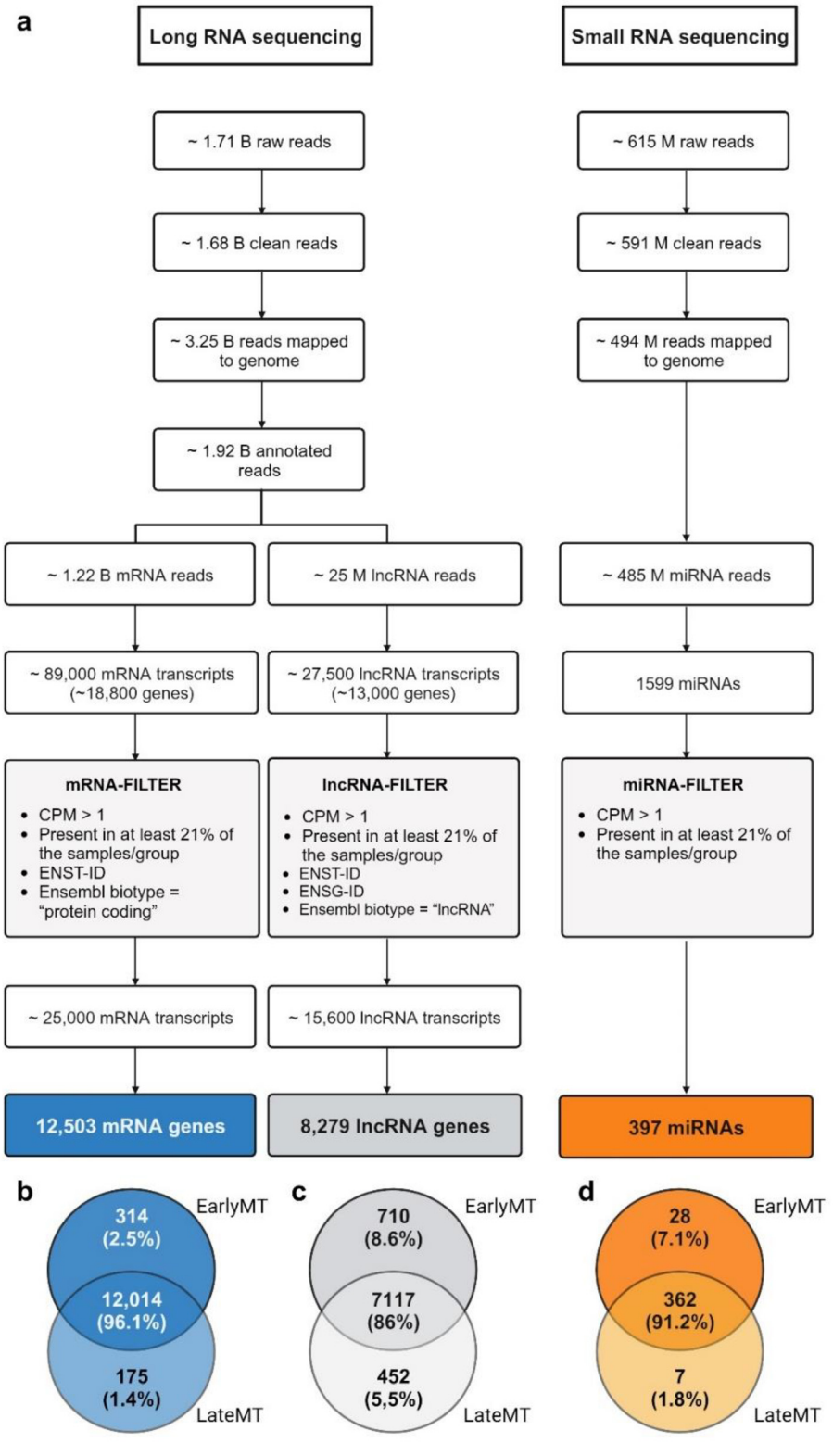

Figure 2: Preprocessing and analytical flow of the sequencing data. a) Flowchart of the data inclusion process. Venn-diagram presenting **s**hared and unshared b) mRNA genes, c) lncRNA genes and d) miRNAs in EarlyMT and LateMT. B, billion; M, million; mRNA, messenger RNA; lncRNA, long non-coding RNA; miRNA, microRNA; CPM, counts per million; ENST-ID, Ensembl transcript ID that is a unique identifier for each transcript of a gene; ENSG-ID, Ensembl gene ID that is a unique identifier for each gene; EarlyMT, a group of women transitioning from early perimenopause to early postmenopause; LateMT, a group of women transitioning from late perimenopause to early postmenopause.

Table 1: Significant results (p_{adj}<0.1) from GSEA analysis of the protein-coding genes.

EarlyMT	Hits/gene set size	p_{adj}-Value	LateMT	Hits/gene set size	p_{adj}-Value
Gene ontology: biological process					
External encapsulating structure organization	103/210	0.005	Cell substrate adhesion	80/275	0.082
Cell substrate adhesion	83/277	0.065	External encapsulating structure organization	72/210	0.082
Receptor mediated endocytosis	67/183	0.065			
Collagen biosynthetic process	18/30	0.065			
Regulation of cell substrate adhesion	36/163	0.097			
Reactome					
ECM organization	95/203	0.002	ECM organization	71/202	0.001
Interferon signaling	57/151	0.084	Syndecan interactions	11/24	0.046
Degradation of the ECM	43/83	0.084	ECM proteoglycans	18/51	0.064
Regulation of insulin-like growth factor transport and uptake by insulin-like growth factor binding proteins	29/74	0.084			

EarlyMT, a group of women transitioning from early perimenopause to early postmenopause; LateMT, a group of women transitioning from late perimenopause to early postmenopause; ECM, extracellular matrix; GSEA, gene set enrichment analysis; *Hits* refer to genes found in our dataset, while *Gene set size* refers to the gene sets used on the database.

genes (Figure 2a) expressed in EarlyMT and LateMT as input data to be compared against Reactome and GO biological processes databases. The most significant findings are shown in Table 1. As a generalization of the GSEA results, we conclude that in both EarlyMT and LateMT, the mRNA genes were enriched in cellular processes controlling cell survival and interactions with the external environment.

Human muscle expresses nearly 13,000 different lncRNA genes

Altogether ~27,500 long RNA transcripts constituting 12,942 different genes were classified as lncRNAs (Figure 2a). The most abundant lncRNA transcripts included *MALAT1*, *NORAD*, *NEAT1,* and two miRNA host genes (for miR-1 and miR-3648). After filtering, a total of 8,279 different lncRNA genes remained for further analysis. Of these, 710 were only expressed in EarlyMT while 452 were unique to LateMT (Figure 2c and Supplementary Table 5). Of the 20 most expressed genes, 19 were shared between groups (including *NORAD*, *MALAT1*, *XIST*, *NEAT1*, *MIR1-1HG*, *H19*, *SNHG14*, *KCNQ1OT1*, *FGD5-AS1*, *SNHG5*, *SNHG16*, *MIR133A1HG*, *MIR193BHG*, *LINC01405*, and five genes with currently uncharacterized function). *ZNF710-AS* was only included in the EarlyMT top 20 list, and *NUTM2A-AS1* only occurred in the LateMT top 20 list.

The top 20 miRNAs constituted the majority of all observed miRNA reads

Reference genome alignment of the clean small RNA reads was 83 %, on average (Figure 2a). Altogether 1,599 different miRNAs were sequenced. After filtering, 397 miRNAs were included in the further analysis. Of these, 362 were shared by both groups, while 28 were only expressed in EarlyMT and seven only in LateMT (Figure 2d and Supplementary Table 6). The 20 most abundant miRNAs in both groups constituted 97.2 % of all miRNA reads and were with two exceptions the same in both groups including let −7a–5p, 7f–5p, −7g–5p, −7i–5p, and miR-1-3-p, −21–5p (only present in EarlyMT top 20 list), −26a–5p, −27b–3p, −30a–5p, −30d–5p, −99a–5p, −126–3p, −133a–3p, −143–3p, −148a–3p, −206, −378a–3p, −378c, −378d, −451a, and −486–5p (only present in LateMT top 20 list). Notably, of the muscle-enriched myomiRs [28], four (miR-1-3-p, −133a–3p, −206, and −486–5p) were also present in the top 20 list.

Menopausal transition is associated with changes in mRNA gene expression

We found 30 DE mRNA genes within EarlyMT and 19 within LateMT (for all p_{adj}<0.05, LFC ≥ ± 1.5, further details in Table 2 and Supplementary Figure 1). EarlyMT DE genes included for example structural protein *ELN*, steroidogenic enzyme

SRD5A1, and apoptosis-linked *PIDD1*. LateMT DE genes included molecular switch regulator *NUDT4*, extracellular matrix component *ECM1*, and the negative regulators of apoptosis *NAA35* and *BIRC6*. In both groups, the DE genes included several regulators of transcription, such as *GTF2F2*, *E2F3*, *MAFK*, *ZEB1*, *KIAA0355/GARRE*, and zinc fingers *ZNF84* and *ZNF611*. The DE genes also included *MKNK1*, *JAK2*, and *MYD88*, which are components of the important growth, metabolism, and adaptation regulating MAPK, mTOR, and PI3K/Akt signaling pathways in the muscle.

No DE lncRNA genes or miRNAs were found. However, at the individual transcript level, we found a total of 10 DE lncRNA transcripts in the EarlyMT and LateMT groups (p_{adj}<0.05 and LFC ≥ ± 1.5, Supplementary Table 7). In EarlyMT, *OSER1-DT*, *MALAT1*, and *AC025171.1* were downregulated. In LateMT, *BAIAP2-DT* and *LINC02541* were upregulated and *AC083798.2*, *AL050309.1*, *LINC00667*, *IQCH-AS1* and *ENTPD1-AS1* were downregulated.

Correlation network and Ingenuity Pathway Analysis predicted associations between menopause-related hormones, transcriptome, and downstream functions

To inspect relationships between the three RNA classes and to identify the most significant associations, we used

Table 2: Differentially expressed protein-coding genes (POST vs. PERI) in the EarlyMT and LateMT groups.

EarlyMT									
Upregulated					Downregulated				
Gene	LFC	Basemean PERI	Basemean POST	p_{adj}-Value	Gene	LFC	Basemean PERI	Basemean POST	p_{adj}-Value
ELN	12.4	11±27	121±179	0.012	*EXTL3*	−9.6	1,123±760	282±458	0.004
GTF2F2	11.9	61±149	122±193	0.024	*KIAA0355*	−9.2	756±182	361±421	0.002
PRKCA	8.5	167±256	276±175	0.035	*VPS28*	−7.7	49±31	21±36	0.004
PYCR1	8.5	31±54	70±64	0.018	*ZNF84*	−6.6	133±72	47±65	0.005
SESN2	8.4	24±38	63±47	0.019	*PIDD1*	−5.7	44±43	1±1	0.005
INTU	6.7	57±68	120±67	0.041	*NLRC5*	−5.0	93±91	3±5	0.024
TMEM39B	6.3	22±35	77±39	0.034	*AC093512.2*	−4.9	455±867	38±59	0.004
SRD5A1	6.3	46±58	126±20	0.004	*ALMS1*	−4.2	474±377	173±374	0.004
CHPF2	6.0	63±102	146±129	0.047	*C1QTNF9*	−3.9	71±38	20±30	0.035
MTHFSD	5.5	29±47	143±60	0.004	*SGSH*	−2.9	54±26	10±10	0.024
MYD88	5.4	2±4	23±14	0.007	*MGLL*	−2.8	884±994	31±12	0.037
APC	4.7	1±1	662±771	0.010	*GBP5*	−2.6	76±81	59±130	0.037
TOM1L2	4.5	37±34	586±588	0.043	*MAFK*	−2.3	677±303	218±305	0.009
TMEM120B	4.0	178±261	643±364	0.008	*RBMS1*	−1.8	349±101	145±134	0.038
ATP5MC2	1.9	325±209	890±450	0.007					
JAK2	1.7	307±183	1,104±716	0.024					
LateMT									
NUDT4	14.6	430±984	639±1,278	0.002	*E2F3*	−12.2	82±108	32±94	0.022
SLFN11	11.1	103±180	160±167	0.005	*NAA35*	−9.6	260±228	216±324	<0.001
AL136295.3	11.0	53±116	115±125	0.005	*ZNF611*	−6.9	79±76	32±54	<0.001
MKNK1	8.9	80±111	173±122	<0.001	*SLC22A17*	−4.4	19±16	5±13	0.005
ARHGAP19	8.6	48±77	117±115	0.033	*TLK2*	−3.1	126±157	51±116	0.017
ZNF761	6.2	44±56	86±39	<0.001	*BIRC6*	−2.9	300±249	77±131	0.003
LYPLA1	5.4	53±107	130±177	0.016	*ZEB1*	−2.7	1,271±938	550±719	0.005
KANK3	5.0	29±71	75±113	0.036	*GORASP1*	−1.7	160±117	48±45	0.004
PTPN20	4.8	27±48	41±48	0.002					
ECM1	2.4	12±16	34±22	0.019					
ULK3	1.7	74±84	173±117	0.012					

EarlyMT, a group of women transitioning from early perimenopause to early postmenopause; LateMT, a group of women transitioning from late perimenopause to early postmenopause; LFC, $\log_2$ fold change; PERI, perimenopausal sample; POST, postmenopausal sample.

sPLS-DA in *mixOmics* [25]. sPLS-DA reduces the dimensionality of the multi-omics data (here mRNA, lncRNA, and miRNA expression data) by selecting the most predictive or discriminant features [29]. After applying a correlation threshold of r≥0.7, all remaining correlations between the identified key features were positive (Figure 3). Two distinct correlation networks were observed: one around mRNA genes *ALMS1* and *RBMS1*, and the other one around mRNA genes *SGSH* and *KIAA0355*. MyomiRs miR-486, miR-133a, and miR-133b were part of these networks. Although this analysis does not allow directional causal conclusions to be drawn, it is tempting to speculate that the involved regulatory RNAs (lncRNAs and miRNAs) would form a regulatory network for controlling the expression of *ALMS1*, *RBMS1*, *SGSH*, and *KIAA0355*.

Using IPA, we further explored in which cellular pathways the DE mRNA genes were enriched. The advantage of IPA is that it applies the researcher-input expression data and its curated knowledge base to compute z-scores with the predictive value of potential activation (positive) or inhibition (negative) of pathways or none indicating inconsistencies between expression data and knowledge base [30]. Analyses were done separately for the EarlyMT and LateMT groups. We present the top 15 IPA-identified muscle-related enriched ($p<0.05$) canonical pathways in Figure 4a and b. For the LateMT group, IPA was not able to provide a prediction towards pathway activation or inhibition for any of the pathways. The total list of pathways and their molecules can be found in Supplementary Tables 8 and 9. Interestingly, the identified pathways included several pathways related to hormonal regulation, e.g., androgen signaling, estrogen receptor signaling, and gonadotropin-releasing hormone (GnRH) signaling, as well as regulation of cell death and energy metabolism.

Next, we used the My Pathway analysis option in IPA to inspect whether E2, P4, or FSH, as the major female hormones subjected to change during the menopausal transition, could function as upstream regulators of the DE genes, thereby providing a mechanistic link between muscle transcriptome and the physiological decrements observed during menopause. The DE mRNA genes from Table 2 and the top 20 expressed regulatory RNAs were included. Figure 4c and d show the results of the combined analysis, where all DE mRNA genes that were associated either with upstream regulators or downstream functions are shown in the same network despite being differentially expressed only in either the EarlyMT or LateMT group. This presentation provides a scene over the whole menopausal transition period while the colors represent the transition from early (**3c**) or late (**3d**) perimenopause to early postmenopause. FSH was not found to regulate any of the observed DE genes, thus it is not included in the figures. E2 was predicted to be an upstream regulator of *APC*, *PRKCA*, *JAK2*, *E2F3*, and *MGLL*, while P4 was predicted to regulate *MYD88* and *E2F3*. E2-and P4-regulated genes were associated with several aspects of muscle function and morphology including muscle cell apoptosis, death and proliferation, muscle hypertrophy, and glucose metabolism disorder. Steroidogenesis and satellite cell function also appeared as associate pathways, but for the sake of clarity, are not shown in the figures. In EarlyMT, muscle cell apoptosis and cell death were predicted to be activated, while glucose metabolism disorder and muscle

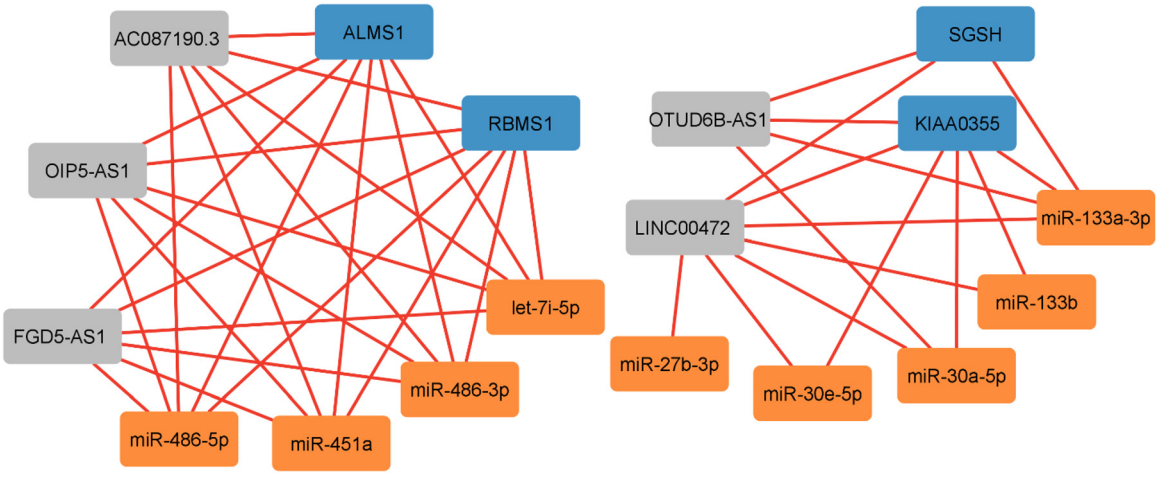

Figure 3: The multi-RNA correlation networks resulting from multiblock-analysis of the observed longitudinal changes in the expression among 24 women transitioning from early or late perimenopause to early postmenopause. Only correlations>0.7 are shown. Blue, mRNA; grey, lncRNA; orange, miRNAs.

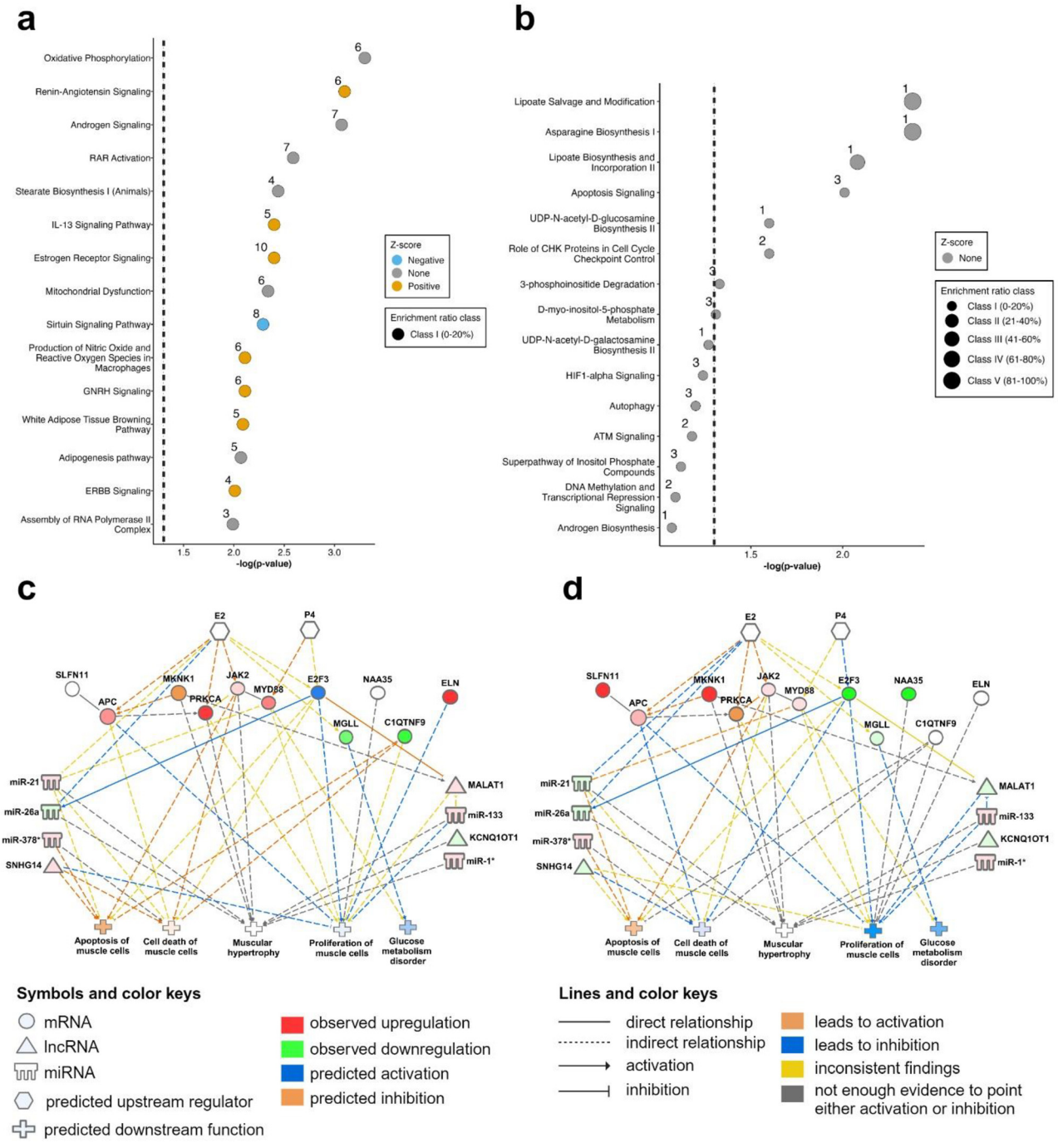

Figure 4: Results of the ingenuity pathway analysis. Top 15 most significant canonical pathways in a) EarlyMT and b) LateMT groups. Only muscle-relevant pathways are shown. In a) and b), circle size refers to the ratio class of observed genes to all genes in the pathway. The z-score of the pathway is used to predict the activation (positive, orange) or inhibition (negative, blue) of the pathway. If neither could be predicted, circle color was left grey. The number on the left side of the circle refers to the actual number of the observed genes. The dashed line indicates significance (log(p-value) > 1.3). Results of the my pathway analysis to identify potential upstream regulators and the affected downstream functions focusing on c) EarlyMT and d) LateMT groups. For both analyses, the same list of differentially expressed mRNA genes and top 20 expressed lncRNA and miRNAs were used as input while color keys represent observations originating from EarlyMT and LateMT. EarlyMT, a group of women transitioning from early perimenopause to early postmenopause; LateMT, a group of women transitioning from late perimenopause to early postmenopause.

cell proliferation were predicted to be inhibited (Figure 4c). Using expression data from LateMT, muscle cell proliferation, cell death, and glucose metabolism disorder were predicted to be inhibited, while muscle cell apoptosis was expected to be activated (Figure 4d). IPA also predicted, that within the included top 20 regulatory RNAs there were several downstream targets of estrogenic regulation as well as associations between the muscle tissue properties and regulatory RNAs.

Protein level analyses suggest regulatory steps before translation

We further wanted to validate whether some of the mRNA level differences in muscle regeneration and metabolism regulators such as ZEB1, MYD88, PRKCA, JAK2, E2F3, and APC were directly translated to the protein expression level. Unfortunately, the antibodies for JAK2, E2F3, and APC turned out to be unspecific. Western blot analyses of paired samples revealed no difference in protein levels for ZEB1 or PRKCA in either of the menopausal groups, while there was a trend for upregulation of MYD88 in LateMT (p=0.070) (Figure 5), similar to what was seen at the gene level in EarlyMT (Table 2).

Changes in mRNA gene and regulatory RNA expressions correlated with changes in body composition and physical activity variables

Next, we investigated whether changes (Δ) in muscle RNA expression correlated with measured changes in body composition (Figure 6). The potential "muscularity regulators" in EarlyMT were Δ*GTF2F2* and Δ*C1QTNF9*, which were positively correlated with lean and muscle mass variables, and Δ*TMEM39B*, which was negatively correlated. The potential "adiposity regulators" in EarlyMT were Δ*INTU*, Δ*C1QTNF9,* and Δ*PIDD1*, which were positively correlated with adiposity variables, and Δ*ALMS1*, Δ*EXTL3,* and Δ*MAFK*, which were negatively correlated. In LateMT, all correlations were rather weak, likely because the phenotype-level changes were also modest in the LateMT group.

We also found several associations between the physical activity variables. Δ*APC*, Δ*ATP5MC2*, Δ*MYD88*, Δ*ALMS1*, Δ*MAFK,* and Δ*MGLL* correlated negatively with the change in either self-reported or accelerometer-measured physical activity, while Δ*INTU* and Δ*GORASP* correlated positively with the changes. More interestingly, we noticed that in some genes, the expression change due to menopause was opposite to the change associated with physical activity. For example, in EarlyMT, *APC*, *ATP5MC2,* and *MYD88* were found to be upregulated by menopause (Table 2) while the change in their expression was negatively correlated with an increase in physical activity (Figure 6). Although we found no DE regulatory RNAs at the gene level, we nevertheless investigated whether the expression level changes in the top 20 expressed lncRNA genes and miRNAs correlated ($r > \pm 0.49$ and $p<0.05$) with body composition and physical activity variables (Supplementary Table 10).

Discussion

This study represents the first longitudinal investigation of the changes occurring in the muscle transcriptome during the menopausal transition as underlying mechanisms for the observed menopause-associated skeletal muscle deterioration. Leveraging a multi-RNA-omics approach, we identified gene expression enrichment in cellular processes controlling cell survival, growth, and cellular interactions with the external environment. Of the expressed mRNA genes, 49 were differentially expressed between peri- and postmenopausal stages. Additionally, we observed potential regulatory relationships between representatives of the three RNA classes. Furthermore, IPA predicted female hormones to be upstream regulators of some of the DE mRNAs and regulatory RNAs pointing to cell death, cell proliferation, and glucose metabolism to be affected by menopausal transition in skeletal muscle. Lastly, we investigated whether the observed changes in gene expression were linked to the observed changes in body composition and identified putative muscularity regulators, such as *GTF2F2* and *TMEM39B*, and putative adiposity regulators, such as *ALMS1* and *MAFK*. *ALMS1* and *MAFK* were also negatively associated with physical activity volume.

The DE mRNA genes that we identified were related to several important functions in the tissue, such as extracellular matrix modification, muscle cell proliferation, energy metabolism, and apoptosis signaling. These findings are partially supported by earlier *in vivo* studies reporting that loss of estrogens reduces oxidative capacity and respiratory function, and activates apoptotic signaling [31–34]. More importantly, our results share similarities with the previous cross-sectional studies conducted among postmenopausal women, which have observed responses in genes related to cellular and environmental interactions, anatomical structure, protein post-translational modifications, proteolysis, peptidolysis, and cell proliferation [17, 18]. However, we found only one common DE gene (*ZNF84*) among our and the previously reported findings, despite the similarities in the pathways. ZNF84 has been previously associated with

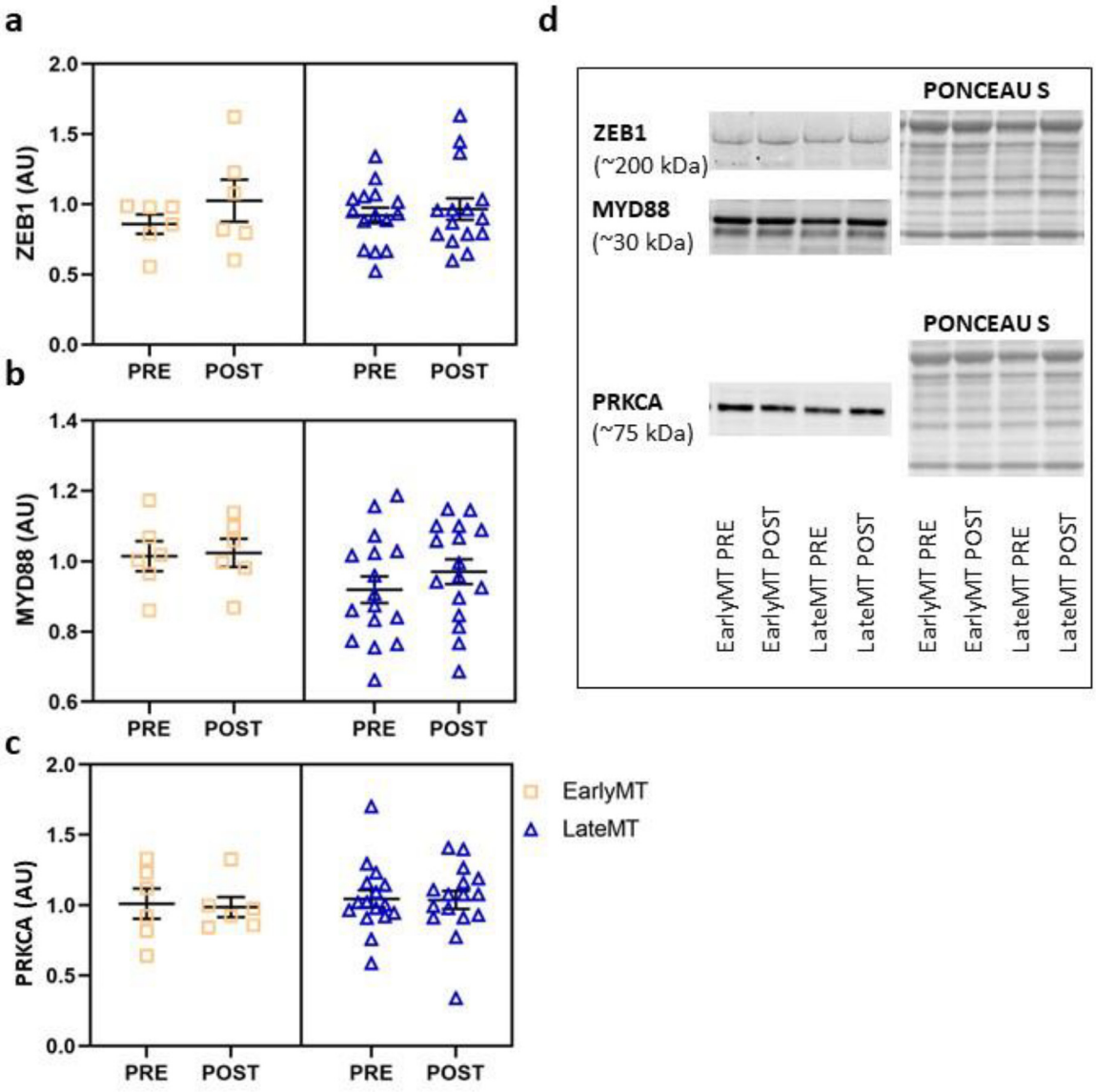

Figure 5: Western blot results for a) ZEB1, b) MYD88 and c) PRKCA with d) representative blot images. AU, arbitrary units; EarlyMT, a group of women transitioning from early perimenopause to early postmenopause; LateMT, a group of women transitioning from late perimenopause to early postmenopause; PRE, baseline sample; POST, follow-up sample.

the upregulation of cellular senescence [35]. Contrary to our observation showing *ZNF84* to be downregulated during the transition from early perimenopause to early postmenopause, Pöllänen et al. [17] instead reported an upregulation of *ZNF84* with advancing postmenopausal status. In that study, participating women were early postmenopausal at the time of baseline muscle sampling, and the follow-up samples were taken 12 months later. Therefore, it is likely that the differences between the identified DE genes, despite the similarities of the pathways found in our and other human studies, are explained by differences in menopausal status. The earlier studies have focused on postmenopause and the postmenopausal use of hormone therapy, while here we focused on the natural menopausal transition from peri-to postmenopause without the use of any sex hormone-containing medication for contraception or to treat menopausal symptoms.

Our results suggest that hormonal changes related to menopausal transition affect specifically the mRNA transcriptome of skeletal muscle. We found, for example, *E2F3* and *ZEB1* to be downregulated and *PRKCA*, *MYD88*, and *JAK2* to be upregulated during menopausal transition. Supporting the hypothesis that hormonal change might be the driving force behind this observation, earlier studies have shown,

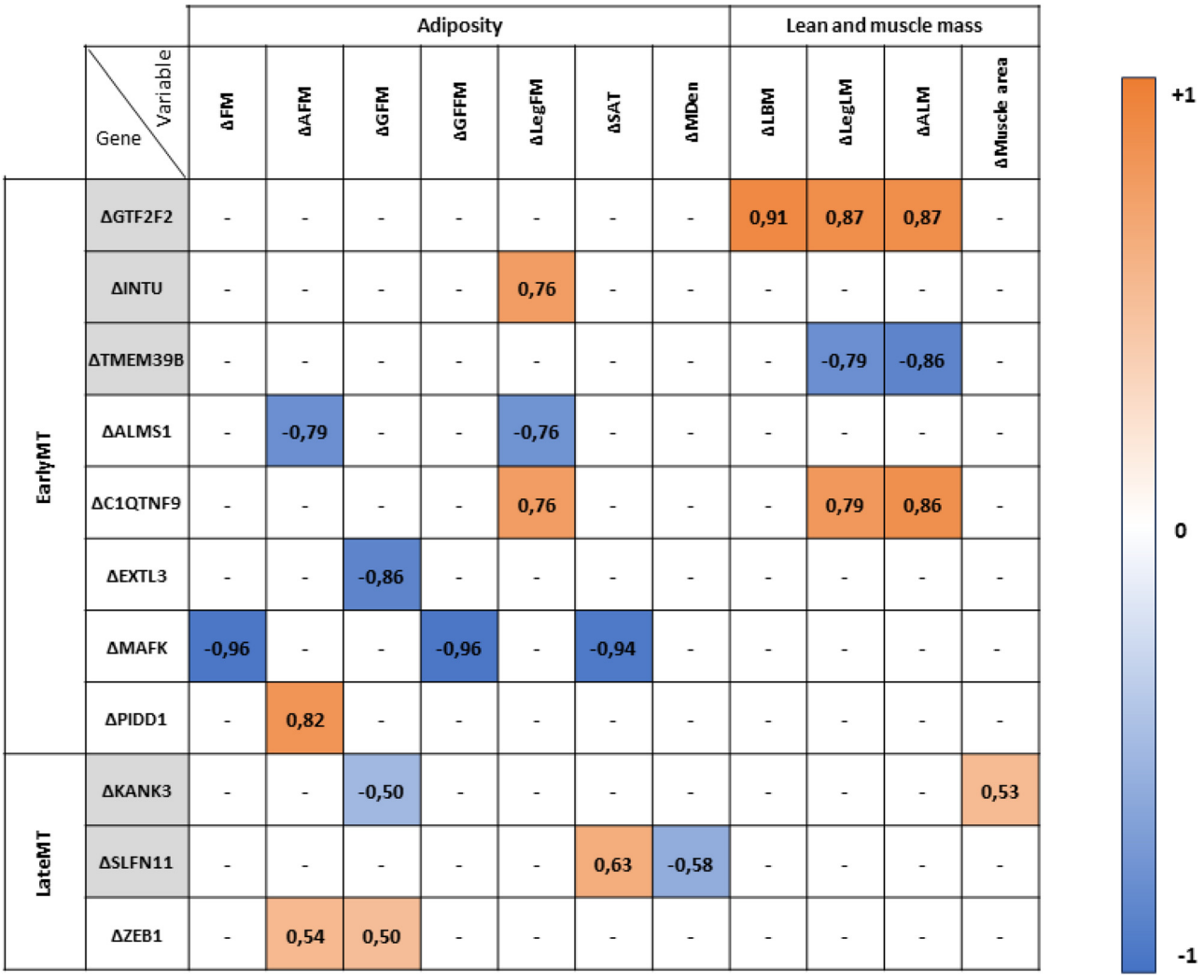

Figure 6: Significant ($p<0.05$) correlations of changes in the differentially expressed mRNA genes with body composition and physical activity variables. Grey gene, upregulated in postmenopause; white gene, downregulated in postmenopause; orange, positive correlation; blue, negative correlation. FM, total fat mass; AFM, android fat mass; GFM, gynoid fat mass; GFFM, gluteofemoral fat mass; LegFM, right leg fat mass; SAT, cross-sectional subcutaneous adipose tissue area of the thigh; MDen, muscle density; LBM, total lean body mass; LegLM, right leg lean mass; ALM, appendicular lean mass; muscle area, cross-sectional muscle area of the thigh; SR-PA, self-reported physical activity; ACC-MVPA, accelerometer measured moderate-to-vigorous physical activity; ACC-MAD accelerometer-measured physical activity in mean amplitude deviations.

and our IPA analysis suggested (except for ZEB1), them to be responsive for E2 or P4. E2F3 is a transcription factor that controls genes related to cell cycle, proliferation, and apoptosis, and has been classified as a transcriptional activator [36]. In myoblasts and *in vivo*, E2F3 depletion has been associated with reduced cell proliferation, total lean mass and muscle power [37, 38]. ZEB1 is required for skeletal muscle regeneration owing to its important role in satellite cell quiescence [39]. *E2F3* and *ZEB1* have previously been shown to be upregulated by E2 and P4 [40–42], and their downregulation due to menopause may offer a new pathway for the previously observed menopausal decrease in satellite cells and muscle mass [2, 43]. In muscle, PRKCA has been observed to regulate hypertrophy and to inhibit glucose intake [44, 45]. E2 has been found to upregulate *PRCKA* in breast cancer [46], while our results for muscle suggest the opposite. MYD88, which has a role in myoblast fusion and tissue inflammation, has also been shown to be vital for satellite cells, as its deletion results in loss of muscle mass and strength in rodents [47]. MYD88 expression is E2 and P4 responsive in monocytes and breast cancer cells [48, 49]. Another upregulated gene, JAK2, is part of the JAK/STAT signaling pathway. In muscle, JAK2 has been associated with the differentiation of satellite cells and improved energy

metabolism [50, 51]. E2 has been reported to affect JAK2 activation in a cell type-specific manner [52, 53].

E2 and P4 likely exert their effects on gene expression through their receptors. Although not differentially expressed, we observed estrogen receptor ESR1 to be more abundantly expressed than progesterone receptor PGR. Thus, the loss of E2 may be one of the strongest hormonal contributors to female muscle RNA signaling. In mice, E2 has been shown to regulate the expression of the estrogen receptors [54]. Conflicting with these rodent results, but similar to our findings, the earlier human study also reported that systemic E2 levels did not affect muscle estrogen receptor mRNA levels in postmenopausal women [17]. Why this occurs in rodents, but not in humans, merits more attention. Of the observed 49 DE genes, none were differentially expressed in both EarlyMT and LateMT groups. This further supports the notion that changes in the skeletal muscle transcriptome in menopause occur in a staged chronological order, possibly reflecting the magnitude of change in E2, which was reduced by nearly 50 % from the baseline in EarlyMT while change was negligible in LateMT. Overall, we observed more changes in mRNA gene expression during the early perimenopause to early postmenopause transition. The duration of the follow-up period did not vary significantly between the two peri-groups, thus, merely time or chronological aging are unlikely to be contributing factors.

Although we discovered several DE mRNA genes, we did not observe statistically significant changes in the levels of regulatory RNAs. Previous literature especially concerning muscle lncRNA expression is limited. To our knowledge, muscle lncRNA regulation by menopausal hormones has not been studied in humans before and the corresponding animal studies are rare. In rats, ovariectomy was found to be associated with 13 DE muscle lncRNAs [20], whereas in rainbow trout, E2 exposure was found to induce expression level changes in seven lncRNAs [21]. In human cells outside muscle tissue, E2 has been found to induce, for example, H19 expression and repress MALAT1 expression [55]. These lncRNA genes were also included in our top 20 expressed lncRNAs in both EarlyMT and LateMT and in fact, we did observe one transcript of MALAT1 (ENST00000618227) to be downregulated. MALAT1 is known to have a role as a promotor of myoblast proliferation and to be a target of myostatin [56]. MALAT1 is also a target of miR-133 and this connection has been found to regulate myocyte differentiation [57]. Overall, our results somewhat support the literature-driven hypothesis that ovarian hormones might have a regulatory role in muscle lncRNAs, although we observed only mild menopausal changes in the lncRNA gene expression levels. Plausible reasons for the difference may again include different study designs, but also differences in species-specific gene regulation.

To our surprise, we did not observe statistically significant menopausal transition-associated changes in the skeletal muscle miRNA expression. This contrasts with earlier studies in which we and others have shown E2 levels to influence skeletal muscle miRNA expression. For example, in a study with postmenopausal twins discordant for HT, we showed that the use of HT was associated with lower expression levels of miRNAs miR-142–3p, –182, and –223 in muscle [19]. These miRNAs regulate the FOXO and IGF1 pathways controlling muscle atrophy and insulin sensitivity. In animal studies, lower E2 levels have been associated with lower expression levels of apoptosis-linked miR-122–5p and –214–3p and cell proliferation- and differentiation-related miR-26b, –27a–5p, –27b and –199a–3p levels [32, 58]. In addition, other studies with *in vitro* models have found E2 and P4 to regulate the expression of several other miRNAs [59–61]. Of the mentioned miRNAs, in EarlyMT, the expression of miR-26b, –27b, –122–5p, –142–3p and –199a–3p was lower in postmenopause than in perimenopause, which is in line with previous studies. However, the expression of miR-27a–5p and –214–3p was higher, thus contradicting expression concordance in previous studies, although in our dataset none of these differences were statistically significant. Thus, our results somewhat diverge from those of previous studies, although we are aware of the uniqueness of the current dataset. One reason for the discrepancy in results may be that the women in the current study were closer to each other in their hormone levels, especially in E2 levels, and that the E2 was the body's endogenous product rather than an exogenous medical product.

To reveal new potential contributors to muscle mass and adiposity, and thereby metabolic health, we inspected whether the change in DE mRNA genes correlated with changes in muscularity or adiposity variables. Correlations with changes in several lean and muscle mass variables were found for Δ*GTF2F2*, Δ*C1QTNF9*, and Δ*TMEM39B*. GTF2F2 encodes a polypeptide that is a component of the transcription-initiating complex but has not been reported to be functional in skeletal muscle. In skeletal muscle, loss of C1QTNF9 protein leads to reduced insulin signaling and mitochondrial content (62). In cardiac myocytes and smooth muscle, it reduces apoptosis [63], but also cell proliferation [64]. Thus, although data from human skeletal muscle are not yet available, the results on other cell types suggest that the downregulation of this protein may contribute to cell loss. This notion is supported by our correlation analyses, which showed a positive association between the change in

the *C1QTNF9* level and the changes in lean mass variables. TMEM39B is a transmembrane protein, which regulates the production and secretion of procollagen and regulates endoplasmic reticulum stress [65], but is still poorly understood in skeletal muscle. Interestingly, in a study of older men and women, *TMEM39B* was found to be upregulated in older age [66], as in our data. In our study, the change in the expression of *TMEM39B* was also negatively associated with a change in leg (ΔLegLM) and appendicular lean mass (ΔALM), indicating that it may have a function in the loss of muscle mass, although this may also be related to aging. Among the adiposity variables, we found significant negative correlations for Δ*ALMS1* and Δ*MAFK*. ALMS1 has a function in cell microtubule organization and defects in *ALMS1* have been associated with young-onset obesity syndrome [67]. In epithelial cells, E2 decreases *ALMS1* mRNA expression [68], but no studies thus far have reported its role in skeletal muscle. In our study, *ALMS1* was downregulated in postmenopause, and the change correlated negatively with a change in android and leg fat mass. This might imply that in skeletal muscle, the loss of estrogen signaling decreases *ALMS1* expression, which then further contributes to increased adiposity. *MAFK*, a transcriptional repressor, is upregulated by progestogens in endometrial stromal cells [69], but information from muscle is lacking. We found *MAFK* to be downregulated in postmenopause and to be strongly negatively associated with body adiposity variables. How these two observations are connected remains to be investigated.

Physical activity has a strong influence on skeletal muscle metabolism. Thus, we also investigated whether we could see correlations between the changes in protein-coding gene expression and physical activity in our data from menopausal women. For example, we observed that decreased *MYD88* and *MGLL* were associated with increased physical activity. Earlier results regarding MYD88 vary between species. In humans, skeletal muscle *MYD88* mRNA decreased after physical inactivity [70]. One study has previously investigated the effect of physical activity on MGLL levels in muscle and found that only chronic combined exercise (aerobic and resistance) was associated with increased levels of MGLL [71]. Thus, our results contradict the previous human results. Possible contributors to the conflicting results may also be the changing hormone environment in our study, as both MYD88 and MGLL are regulated by E2 and P4 [48, 49, 72], although the exact regulatory mechanisms have not been investigated in muscle. More interestingly, we observed that the directions of some of the correlations were opposite between menopausal status and physical activity, meaning that the effect of increased physical activity was opposite to the effect of menopause. These correlations were found for *APC*, *MYD88*, *ATP5MC2*, and *GORASP*. For example, we found that an increase in physical activity was associated with decreased expression of *APC*, whereas postmenopause was associated with an upregulation of *APC*. Previously, physical activity has been found to increase *APC* expression [73]. For *ATP5MC2* (ATP synthase in mitochondria) and *GORASP*, the effects of the change in their expression levels as well as physical activity are unknown. For *MYD88* the situation might be different, as MYD88 has been proposed to be a proinflammatory molecule. This may indicate that the menopause-associated increase in *MYD88* could be counteracted by physical activity.

Overall, we conducted a thorough investigation of the effects of the menopausal transition on the skeletal muscle transcriptome. To our knowledge, this is the first study to investigate these associations. However, the study has its limitations. One limitation is the relatively small sample size and the large number of statistical tests performed. To control the possibility of false positive findings, we applied the FDR correction protocol. The repeated-sampling design helps to control random bias as in the analysis each participant is compared to her baseline value. Nevertheless, there is still a possibility of false positives, so our results should be considered as hypothesis-generating rather than a proof-of-concept. Moreover, this study focused on RNA expression and was only able to investigate the protein-level effects of three genes. The strengths of the study include the longitudinal study design and the use of gold-standard methods in menopausal status assignment and body composition measurements.

Conclusions

The menopausal transition was associated with changes in human skeletal muscle transcriptome specifically regarding mRNA gene expression. The non-protein coding regulatory RNAs, i.e., lncRNA genes or miRNAs, showed only subtle changes in their expression or higher variation between samples prohibiting statistical significance from being observed. Nevertheless, suggestive regulatory networks were identified. The DE mRNA genes identified in this study may contribute to muscle tissue homeostasis and add to the unfavorable menopausal changes in total body composition. Moreover, the observed DE mRNA genes differed between the two perimenopausal groups, which may indicate a change in the regulatory mechanisms at different stages of the menopausal transition. The observed DE mRNA genes

serve as a possible starting point for mechanistic studies aimed at a more detailed understanding of the effects of hormonal changes in female skeletal muscle.

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Research ethics: This study used the data and samples from the ERMA (Estrogenic Regulation of Muscle Apoptosis) study [22], which was approved by the ethics committee of the Central Finland Health Care District (8U/2014) and complied with the Declaration of Helsinki except that the study was not registered in advance.

Informed consent: Informed consent was obtained from all individuals included in this study.

Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission. V.K., E.K.L., and S.S. designed the original ERMA study, and U.M.K., and P.A. contributed to the planning. E.K.L. and H-K.J. were responsible for the muscle sample handling. H-K.J. did RNA and protein isolations and related work while S.K. performed the Western Blot and related analyses. H-K.J. and N.C. analyzed the DXA and CT scans. S.S. performed the CT scanning and supervised the body composition analyses. H-K.J did all correlation analyses, the majority of the pre-analysis of datafiles provided by Novogene, performed IPA, and constructed the final tables. T-M.K. performed the main bioinformatic analyses and result images, while T.S. assisted. V.K. and E.K.L. provided funding for the study. H-K.J. prepared the first version of the manuscript. All co-authors have participated in the interpretation of the results and critically commented on the manuscript during the writing process. E.K.L, H-K.J, and T-M.K prepared the final version of the manuscript, which all the authors have read and approved.

Competing interests: The authors state no conflict of interest.

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Data availability: The raw data can be obtained on request from the corresponding author. The complete individual-level data is not publicly available due to privacy or ethical restrictions. Access to the data may be granted upon justification for research purposes and as long as the request does not violate the consent provided by the study participants. Such requests should be addressed to the project leader (eija.k.laakkonen@jyu.fi) and will be evaluated with the board consisting of senior researchers involved in the ERMA study. The metadata of the ERMA study is publicly available (doi:10.17011/jyx/dataset/83491). The main code used to conduct this study is available on GitHub at LaakkonenLab/menopausal-muscle-RNA (github.com).

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