Running title: The epitranscriptomics of uterine fibroids.

Article title: Characterization of m^{6}A modifiers and RNA modifications in uterine fibroids

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Abstract:
Uterine leiomyoma or fibroids are the most common prevalent noncancerous tumors of the uterine muscle layer. Common symptoms associated with fibroids include pelvic pain, heavy menstrual bleeding, anemia, and pelvic pressure. These tumors are a leading cause of gynecological care but lack long-term therapy as the origin and development of fibroids are not well understood. Several next-generation sequencing technologies have been performed to identify the underlying genetic and epigenetic basis of fibroids. However, there remains a systemic gap in our understanding of molecular and biological process that define uterine fibroids. Recent epitranscriptomics studies have unraveled RNA modifications that are associated with all forms of RNA and are thought to influence both normal physiological functions and the progression of diseases. We quantified RNA expression profiles by analyzing publicly available RNA-seq data for 15 known epigenetic mediators to identify their expression profile in uterine fibroids compared to myometrium. To validate our findings, we performed RT-qPCR on a separate cohort of uterine fibroids targeting these modifiers confirming our RNA-seq data. We then examined protein profiles of key m^6A modifiers in fibroids and their matched myometrium. In concordance with our RNA expression profiles, no significant differences were observed in these proteins in uterine fibroids compared to myometrium. To determine abundance of RNA modifications, mRNA and small RNA from fibroids and matched myometrium were analyzed by UHPLC MS/MS. In addition to the prevalent N6-methyladenosine (m^6A), we identified 11 other known modifiers but did not identify any aberrant expression in fibroids. We then mined a previously published dataset and identified differential expression of m^6A modifiers that were specific to fibroid genetic sub-type. Our analysis also identified m^6A consensus motifs on genes

previously identified to be dysregulated in uterine fibroids. Overall, using state-of-the-art mass spectrometry, RNA expression and protein profiles, we characterized and identified differentially expressed m^6A modifiers in relation to driver mutations. Despite the use of several different approaches, we identified limited differential expression of RNA modifiers and associated modifications in uterine fibroids. However, considering the highly heterogenous genomic and cellular nature of fibroids, and the possible contribution of single molecule m^6A modifications to fibroid pathology, there is a need for greater in-depth characterization of m^6A marks and modifiers in a larger and varied patient cohort.

Introduction:

Uterine fibroids are steroid hormone-responsive, benign neoplasms of the uterus composed of smooth muscles, fibroblasts, and an abundance of extracellular matrix (1, 2). These benign tumors are estimated to occur in around 70% of women and clinically manifest in 30% of women by the age of 50 (3). Common clinical symptoms associated with fibroids are heavy bleeding, pain, infertility, and recurrent pregnancy loss (4, 5). Nonsteroidal anti-inflammatory drugs (NSAIDs), gonadotropin-releasing hormone agonists, elective estrogen receptor modulators, aromatase inhibitors, anti-progestins, and progesterone receptor modulators provide some relief but are only recommended for short-term use due to variable side effects and disease recurrence. (2). Accordingly, the lack of long-term therapeutic options and clinical morbidity associated with fibroids, hysterectomy remains the only option for many women (6). The cost of fibroid treatment and related health complications cost to the U.S. health care system is annually estimated to cost $5.9 to $34.4 billion (4, 7). Fibroids remain a significant burden on both health care costs and quality of life.

Several factors, including age, parity, ethnicity, and family history, are thought to act as drivers, but direct evidence identifying etiology of the disease has been difficult (4). Genome wide exome sequencing identified that 30-90% of fibroids, depending on patient ethnicity and fibroid number, contained mutations in the second exon of the mediator complex subunit 12 (*MED12*) gene (8). Additionally, chromosomal rearrangement at 12q15 and 6q21, leading to overexpression of the High Mobility Group A1/2 (*HMGA1/2*) gene, has been observed in 8-35% of fibroids (9, 10). Biallelic

inactivation of *FH* (Fumarate Hydratase), deletion of the collagen genes *COL4A5* and *COL4A6*, and mutations of the SNF2-Related CBP Activator Protein (SRCAP) complex subunits are among the rarer subtypes that have been reported in uterine fibroids (11, 12). These chromosomal events trigger sub-type specific gene expression patterns that are either shared or unique to the genetic event (12, 13).

Since its discovery in 1974, methylation of adenosine on RNA (m^6A or N^6-methyladenosine) has emerged a major post-transcriptional RNA modification (14). The past decade has seen a resurgence in examining the role of m^6A RNA modifications in regulating RNA processing, splicing, export, stability, and translation (15). Transcriptome-wide profiling of m^6A modification identified the modification as wide-spread, highly selective, and dynamic in nature, with levels varying in development and cellular stress (16, 17). Addition of m^6A sites is catalyzed by the "writer" proteins, specifically by the catalytic activity of Methyltransferase-like protein 3, METTL3, and target RNA binding activity of methyltransferase-like protein 14 (METTL14) (18). In addition to METTL3, other regulatory proteins involved in the process include methyltransferase-like protein 16 (METTL16), Wilms tumor 1-associated protein (WTAP), RNA-binding motif 15 (RBM15), Cbl proto-oncogene-like protein 1 (CBLL1), zinc-finger CCCH-type-containing 13 (ZC3H13), and Vir-like m^6A methyltransferase-associated (VIRMA) (or also known as KIAA1429) (18, 19). Following addition of m^6A modification, biological consequence is regulated by specific RNA-binding proteins, or "readers". These readers recognize and bind to the DRACH (D=A, G or U, R= G or A, and H= A, C, or U) consensus sequence of modified RNA transcripts leading to regulation of gene expression and modulation of diverse processes including splicing,

mRNA stabilization, and translation efficiency. Reader proteins include YTHD domain protein family (YTHDC1, YTHDC2, YTHDF1, YTHD2, and YTHDF3) (20). In addition, heterogenous nuclear ribonucleoprotein (HNRNP) protein, HNRNPA2B1, has been shown to regulate m^6A-modified transcript including a subset of primary miRNA (21). Two proteins, fat mass and obesity-associated protein (FTO) and alkB homologue 5 (ALKBH5) have been identified as m^6A demethylases or “erasers” due to their ability to remove m^6A marks (20). Altered expression of these eraser proteins can contribute to atypical cellular functions and physiological activity thereby promoting tumorigenesis (18, 20).

To date multiple studies carried out by us, and others have identified differential DNA methylation patterns, histone modifications, altered miRNA and long noncoding RNA expression as they relate to uterine fibroidogenesis (11-13, 22-24). However, an in-depth characterization of m^6A modifiers and RNA modifications in uterine fibroids is lacking. The goal of this study was to investigate expression patterns of the vast array of m^6A modifier proteins and RNA modifications in both mRNA and small RNA as they relate to uterine fibroids compared to myometrium.

Material and methods:

Human Tissue collection and Sample Preparation

Matched samples of human myometrium and fibroid samples were collected from pre-menopausal women undergoing hysterectomy for symptomatic uterine fibroids. Use of human tissue was approved by the University of Nebraska Medical Center (IRB# 112-21-EP) and University of Kansas Medical Center (IRB#: 5929), and all patients signed a written informed consent form to donate tissue for this study. Human samples

were processed as previously described (13). Upon arrival, samples were minced and sub-divided for a) RNA extraction and b) protein isolation for western blots and then immediately flash frozen and stored at -140 °C.

RNA extraction

Total RNA was extracted from freshly frozen samples as previously described (13). Following total RNA extraction by Trizol, mRNA from fibroids and matched myometrium (n=6) was isolated by two rounds of purification using olido-dT Dynabeads mRNA DIRECT Micro kit (Dynabeads) according to manufacturer's protocol. Depending on patient sample, 8-50 µg total RNA was used per purification column. Integrity and purity of isolated mRNA was evaluated using Fragment Analyzer Automated CE System (Advanced Analytical Technologies, Inc). RNA Integrity Numbers (RINs) were used to evaluate integrity and samples with RIN >7.0 were considered intact and used for further downstream LC-MS/MS analysis.

Small RNA (<200 bp) was isolated from fibroids and matched myometrium (n= 5) using mirVana miRNA isolation kit (Thermo Fisher Scientific) according to manufacturer's protocol. Following isolation, small RNA purity was confirmed using Fragment Analyzer Automated CE System (Advanced Analytical Technologies, Inc).

Quantitative real-time PCR (RT-qPCR)

cDNA was synthesized from 1µg total RNA using qScript cDNA synthesis kit (Quantbio, Beverly, MA). Quantitative Real rime PCR (RT-qPCR) analysis was performed on genes of interest (**Supplementary Table 1**) using SYBRGreen (BioRad). Sso Fast EvaGreen Supermix was performed to analyze gene expression on a BioRad CFX96 Real-Time System (BioRad, Hercules, CA). Relative quantification of gene of

interest was established using RPL17 as reference and calculated using the comparative Ct method.

LC-MS/MS Analysis of RNA Chemical Modifications

Measurement of the levels of RNA chemical modifications was performed using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) by a method similar as described (25-27). Briefly, Total amount of 100 ng of small RNA or mRNA was digested with a Nucleoside Digestion Mix (New England BioLabs) according to the manufacturer's instruction. The digested samples were then lyophilized and reconstituted in 100 µl of RNAse-free water, 0.01% formic acid prior to UHPLC-MS/MS analysis. The UHPLC-MS/MS analysis was accomplished on a Waters XEVO TQ-S^{TM} (Waters Corporation, USA) triple quadruple tandem mass spectrometer equipped with an electrospray source (ESI) source maintained at 150 °C and a capillary voltage of 1 kV. Nitrogen was used as the nebulizer gas, which was maintained at 7 bars pressure, flow rate of 1000 l/h and at temperature of 500°C. UHPLC-MS/MS analysis was performed in ESI positive-ion mode using multiple-reaction monitoring (MRM) from ion transitions previously individually determined for these RNA chemical modifications (28). A Waters ACQUITY UPLC^{TM} HSS T3 guard column, 2.1x 5 mm, 1.8 µm, attached to a HSS T3 column, 2.1 x50 mm, 1.7 µm was used for the separation. Mobile phases included RNAse-free water (18 MΩcm^{-1}) containing 0.01% formic acid (Buffer A) and 50% acetonitrile (v/v) in Buffer A (Buffer B). The digested nucleotides were eluted at a flow rate of 0.2 ml/min with a gradient as follows: 0-1 min, 0 %B; ramp to 0.2% B in 1.4 min; then to 0.8% in 1.4 min, 3.8-5.2 min, 0.8-1.8% B; 5.2-6.6 min,

1.8-3.2%B; 6.6-10 min, 3.2-5.0% B;10-13.5 min, 5-8%B; 13.5-18 min, 8-30%B; in 0.5 min to 100% B and kept for 1.5 min. The total run time was 25 min. The column oven temperature was kept at 25 °C and the sample injection volume was 10 µl. Three injections were performed for each sample. Data acquisition and analysis were performed with MassLynx V4.1 and TargetLynx. Calibration curves were plotted using linear regression with a weight factor of 1/x.

Western Blot

Protein was isolated from fibroids and matched myometrium by homogenization in Radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors. Following isolation, lysate protein concentration was quantified using the Pierce BCA Protein assay kit and 10 µg of protein were separated on a 10% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham). Membranes were blocked in 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature for 1 h and probed with METTL3 (1:1000; 15073-1-AP; Proteintech), METTL14 (1:1000; 26158-1-AP; Proteintech), RBM15 (1:1000; VIRMA (1:1000; 25712-1-AP; Proteintech), WTAP (1:1000; 10200-1-AP; Proteintech) , CBLL1 (1:1000; 21179-1-AP; Proteintech), FTO (1:1000; 27226-1-AP; Proteintech), ALKBH5 (11:1000; 6837-1-AP; Proteintech), and β-actin (1:5000; A5441; Sigma) antibodies at 4 °C overnight. Following washes in TBST, membranes were blocked in secondary HRP-conjugated antibodies (1:10,000) in 5% BSA in TBST for 1 h, washed, and imaged using iBright system (ThermoFisher). Densitometry analysis was performed using Image J. All protein levels were normalized to respective ACTB which served as loading control.

Statistics

RNA-seq data (13) were filtered, normalized, and converted to log2-counts per million (CPM) value per sample. The study included two factors: within-subject factor tissue (Fibroid or Normal) and the between-subject factor race (B or W). For each gene, linear mixed models were used to assess the race effect and tissue effect on the mean normalized gene expression levels (log_2CPM), accounting for correlations between observations from the same patient. We are interested in the following hypothesis testing: whether the race effect on the normalized gene expression level was significant in fibroids and normal tissues respectively, whether the tissue effect on the normalized gene expression level is significant in black and white patients respectively, and whether the tissue effect alters in black patients compared to white patients per gene. The associated p-values for each comparison on each gene were adjusted by the false discovery rate (FDR) method of Benjamini and Hochberg method (1) due to multiple hypotheses testing. Differentially expressed genes were identified as those having an FDR below 0.05.

Statistical analyses were performed using GraphPad Prism 9.0. The Student's t-test was used to compare fibroid samples to myometrium and significance level was set at $P < 0.05$. Sample numbers are indicated in all figure legends. Data presented in graphs are expressed as mean ± SEM.

Results:

Transcriptomic expression of m^6A regulators in uterine fibroids

To determine the role of m^6A modification and their modifiers, we began by analyzing transcriptomic expression levels of writers (*METTL3, METTL14, METTL4, CBLL1, VIRMA, WTAP, RBM15, ZC3H13*), readers (*HNRNPA2, YTHDF1, YTHDF2,*

YTHDC1, YTHDC2), and erasers (*FTO* and *ALKBH5*) in normal myometrium (n=9) and fibroids (n=12) [SRP166862 from our previous study (13)] (**Figure 1**). Out of these candidates, mRNA expression of writer protein *RBM15* was found to be significantly upregulated in uterine fibroids by RNA-seq (**Figure 1g**). To confirm our finding, we performed RT-qPCR on a separate set of fibroids and matched myometrium patient samples (**Suppl. Figure 1**) with an emphasis on *METTL3* due to the catalytic nature of the protein and RBM15 as it was found to be significantly induced. Overall, RT-qPCR data confirmed mRNA levels of METTL3 was not statistically significant between normal myometrium and fibroid samples (**Suppl. Figure 1a**). We confirmed that *RBM15* expression was significantly different when comparing fibroids with matched myometrium (**Suppl. Figure 1e**). Expression levels of other writer proteins METTL14, METTL16, VIRMA was unchanged (**Suppl. Figure 1b-d**). Similarly, mRNA levels of reader proteins YTHDF1, YTHDF2, YTHDF3, YTHDC1 (**Suppl. Figure 1f-i**) and erasers ALKBH5 and FTO (**Suppl Figure 1j, 1k**) were not statistically significant.

Previous studies identified differential expression of mRNA and miRNA between fibroids isolated from Black and White women indicating these candidate factors could drive racial disparity of the disease (29, 30). To determine if m^6A modifiers were differentially expressed between fibroids obtained from Black and White women, we utilized RNA expression profile obtained from our recently published RNA-seq data (13). Among the 15 epigenetic marks, we identified upregulation of *RBM15* in fibroids compared to normal myometria in White (**Suppl Figure 2a**) but not Black women (**Suppl Figure 2b**). No race specific molecular disparity within normal myometrium or fibroids, within Black and White women were identified (**Suppl Figure 3a, 3b**).

Protein expression of m^{6}A modifiers in uterine fibroids

To confirm RNA expression profile, we performed western blot analysis on key m^{6}A proteins (**Figure 2**). Specific emphasis was laid on METTL3 due to the fundamental nature of the protein in driving m^{6}A RNA methylation and RBM15 as its transcript profiles were elevated in our analysis (**Figure 1g** and **Suppl Figure 1e**). Protein levels of METTL3 were not significantly altered within normal myometrium (n= 19) and fibroids (n= 26) (**Figure 2a**, **2b**). There was no significant difference in RBM15 protein expression within fibroids (n=23) and matched myometrium (n=19) (**Figure 2a**, **2b)**. Our analysis of other core reader proteins METTL14, WTAP, CBLL1, VIRMA also did not show differential expression in fibroids and matched myometrial samples (**Figure 2A, 2B**). In concordance with our transcript analysis, eraser proteins, FTO and ALKBH5 remained statistically unchanged between myometria and fibroid patient samples (**Figure 2A, 2B**). Overall, protein expression levels of m^{6}A modifiers were in concert with the transcriptomic finding, except for RBM15, indicating a lack of differential expression in protein levels between fibroids and myometrium.

Abundance of mRNA and small RNA modifications in uterine fibroids

We next considered the possibility of differential methylase and demethylase activity in absence of significant RNA and protein expression profiles fibroids and myometrium. mRNA was isolated, concentration of modified RNA nucleosides was measured by UHPLC-MS/MS. No differential signal abundance of m^{6}A was observed between fibroids and matched myometrium (**Figure 3a**). We extended our analysis to other well characterized mRNA modifications that have been identified in eukaryotes

and known to impact various cellular functions (31) to determine if these were dysregulated in uterine fibroids. We did not identify any additional modifications (m^5C, m^7G, ac^4c, m^1A, f^5C, dA, ho^5u) to have altered abundance in our fibroid samples versus myometrium samples (**Figure 3b-h**).

In addition to mRNA, small RNAs (<200 bps) are known to harbor diverse RNA modifications that can modulate complex biological processes (32). Small RNAs have been identified to be differentially expressed in fibroids and thought to regulate multiple processes that influence uterine fibroid development and progression (30). Following isolation of small RNA from normal myometrium and matched fibroids, we measured post translational RNA modifications. We were unable to detect differential expression of m^6A levels in our fibroid samples and myometrium samples (**Suppl. Fig 4a**). We simultaneously measured other modified nucleoside abundance including, m^1A, i^6A, ac^4c, m^5C, m^3C, f^5C, m^1G, m^7G, mo^5U, ho^5U, m^5U that have been implicated to regulate translational machinery and influence physiological processes (32). Our analysis did not identify these modifications to be differentially expressed (**Suppl. Fig 4b-l**), indicating an absence of preferential small RNA modifications in fibroids.

Characterization of m6A modifiers with respect to genetic sub-type.

Uterine fibroids are driven by multiple driver mutations, including *MED12*, *HMGA1*, *HMGA2*, *FH* and the more recently characterized mutation in the SRCAP complex subunits (11-13). Multiple studies both from our lab and others have shown that normal myometrium and these driver mutations form separate transcription clusters indicating altered pathways are activated in these genetic subtypes (11-13). To define a broader clinical perspective, we mined gene expression profiles of 162 normal

myometrium and 190 fibroid samples that were recently published by Berta et al (11). These fibroid samples were divided into *MED12* (n=38), *HMGA2* (n=44), *HMGA1* (n=62), *FH* (n=15), *YEATS* (n=16) and *OM* (n=15) allowing capture of majority of fibroid subtypes as described (11). We mapped each genetic sub-type against fold change to determine if these modifiers were preferentially expressed (**Figure 4**). We identified statistical, though modest changes in majority of epigenetic regulators based on mutation status. Among the readers, *METTL3* was found to be significantly upregulated in *HMGA2* (log2- fold 0.11) and *YEATS* (log 2-fold 0.15) sub-type fibroids (**Figure 4a**). *METTL14* on the other hand was significantly downregulated in *MED12* (log 2-fold -0.16) and *HMGA2* (log 2-fold -0.08) fibroids, while upregulated in *YEATS* (log 2-fold 0.12) fibroids but was not statistically significant (**Figure 4b**). In congruence with our RNA-seq analysis (**Figure 1g**), *RBM15* was upregulated in almost all fibroid sub-types (**Figure 4g**). Among writer proteins, *YTHDF1* was upregulated in majority of fibroid sub-types (**Figure 4k**), while *YTHDC2* was significantly downregulated in *HMGA1* (log 2-fold -0.09), *FH* (log 2-fold -0.16), *YEATS* (log 2-fold -0.13) fibroid subtype (**Figure 4n**). There was elevated expression of m^6A demethylase *ALKBH5* in majority of fibroid sub-types (**Figure 4o**), while mRNA expression of *FTO* was significantly decreased in all sub-types (**Figure 4p**).

We next harnessed mammalian m^6A predictor, SRAMP (sequence-based RNA adenosine methylation site predictor)(33) to predict if key fibroid genes identified by us and others (11-13), had m^6A sites in their mRNA (**Supplementary Table 2**). We identified a collection of putative m^6A sites with varying levels of confidence in steroid hormone receptor genes, (Progesterone Receptor (*PGR)*, Estrogen Receptor (*ESR1*)),

transcription factors (Pleomorphic adenoma gene 1 (*PLAG1),* Pappalysin 2, (*PAPPA2)*, Chromobox 2 (*CBX2)*, Chromobox 4 (*CBX4)*, Chromobox 8 (*CBX8)*, SATB homeobox 2 *(SATB2)*), DNA repair protein (RAD51 Paralog B (*RAD51B)*), steroidogenic genes (Hydroxysteroid 17-beta dehydrogenase 6 (*HSD17B6)*, steroid 5 alpha-reductase 2 (*SRD5A2)*, tryptophan 2,3-dioxygenase (*TDO2)*), collagen associated genes (ADAM metallopeptidase domain 12 (*ADAM12)*, Collagen type I alpha 1 chain (*COL1A1)*, collagen type 3 alpha 1 chain (*COL3A1)*, Periostin (*POSTN)*) and growth factors Cyclin D1 (*CCND1),* vascular endothelial growth factor A *(VEGFA*)). These in silico analysis suggests that key fibroid genes are possibly susceptible to RNA modification and subsequent transcriptional regulation. However, in-depth analysis will be required to define transcriptome-wide m^6A location and efficacy of these marks in fibroid etiology.

Discussion:

Over 170 RNA modifications have been identified of which m^6A accounts for the most abundant and widespread mRNA internal modification (20, 34). The diverse distribution patterns, its dynamic nature, and ability to regulate multiple physiological processes has added another layer to post-transcriptional regulation. Multiple studies have now identified that regulation of m^6A is driven by methyltransferases, demethylases, or reader proteins and dysregulation of which is closely associated with human cancers (35). In reproductive cancers, m^6A modifiers were identified to regulate ovarian, endometrial, and cervical cancer (36, 37). However, characterization of m^6A modification proteins have not been defined in uterine fibroids. Here, for the first time, we provide an in-depth characterization of major modifiers of m^6A modification as it relates to uterine fibroids.

The m^6A methyltransferase complex is comprised of a METTL3/METTL14 heterodimer core that adds m^6A in a highly specific manner (38). Due to the enzymatic ability of METTL3 which allows addition of m^6A to nuclear RNA, we paid special attention to both its transcriptomic and protein abundance in fibroids. Our analysis did not identify differential expression in either RNA or protein of METTL3 in uterine fibroids in absence of mutation status. Lack of differential m^6A RNA modification was also observed in our LC-MS/MS data from both purified fractions of mRNA and small RNA (**Figure 3A**; Supplementary **Figure 3A**). However, increased expression of METTL3 has been reported and could be attributed to patient and fibroid heterogeneity (39, 40). When broken down by fibroid sub-type, we see mild increased expression of METTL3 mRNA in *HMGA2* and *YEATS* fibroid (**Figure 4A**). METTL3 and METTL14 form a 1:1 heterodimer and recognize the DRACH motif leading to induction of m^6A modification on mRNA (41, 42). Inactivation or deletion of METTL14 results in depletion of m^6A in mRNA, identifying it as a core regulator of m^6A addition (43). While no significant changes were observed in transcriptomic and protein levels of METTL14 in global fibroid samples, we identified changes when pared down by fibroid genetic sub-types. In addition to METTL3 and METTL14, other core components are known to mediate m^6A addition. Among these include WTAP which interacts and anchors METTL3 and METTL14 to nuclear speckles regulating gene expression and alternative splicing (31). WTAP interacts with another well-known m^6A mediator, VIRMA (43, 44). Apart from VIRMA, ZC3H13 is another WTAP interactor and shown to be required for nuclear localization of the writer complex (20, 34). CBLL1 has also been shown to couple with WTAP (20, 34) and loss of CBLL1 led to reduction of global m^6A levels, identifying it as

another writer protein. WTAP expression was found to be increased in *YEATS* fibroid and decreased in *MED12* mutants. While VIRMA and ZC3H13 were increased in *YEATS* fibroid and CBLL1 was increased only in *HMGA2* fibroids. Among writer proteins, we saw increased transcriptomic expression of RBM15 in both global (**Figure 1G**), by race (**Suppl Figure 2 a**) in multiple fibroid genetic sub-type (**Figure 4G**). However, protein levels were not significantly changed in fibroids (**Figure 2a, 2b**). RBM15 has been identified as part of m^6A writer complex and shown to bind the long non-coding RNA, *XIST*. Knocking down RBM15 decreased m^6A methylation on *XIST* RNA, leading to reduced *XIST* mediated gene silencing (45). *XIST* has been identified to regulate fibroid pathology by sponging miR-29c and miR-200c leading to increased expression of COL1A1, COL3A1, and FN1, key regulators of extracellular accumulation (46). As an RNA binding protein, in addition to its role as a m^6A modulator, RBM15 regulates splicing of key differentiation genes involved in hematopoietic stem cells quiescence (47). A hypothesis put forward to define uterine fibroid etiology is reprogramming of myometrial stem cells leading to fibroid development (48). Whether RBM15 plays a role in regulating cell fate decision of myometrial cells and if so, does it mediate its action through m^6A or alternative splicing, and its contribution to uterine fibroid pathogenesis remains to be explored.

There are five YTH domain-containing proteins (YTHDC1-2 and YTHDF1-3) that have been structurally identified to recognize m^6A through a conserved aromatic cage. YTHDF 1-3 is cytoplasmic, YTHDC1 is predominantly nuclear, while YTHDC2 can be both nuclear and cytoplasmic. YTHDF1 has been linked to enhanced translation of m^6A mRNA, while YTHDF2 binding leads to RNA degradation bought about by recruitment

of CCR4-NOT deadenylation complex. Finally, YTHDF3 cooperatively binds to YTHDF1 and YTHDF2 regulating translation and degradation thereby impacting gene expression profile of m^6A- containing mRNA (20, 49). Among YTHDF1-3, statistical significance was identified only in YTHDF1 (**Figure 4K-O**). In addition to regulating m^6A mRNA, YTHDC1 also appears to mediate function of long noncoding RNA, in particularly *XIST* and regulating transcriptional silencing of the X-chromosome (45, 50). Transcript levels of both YTHDC1 and YTHDC2 were found to decreased in some variation in all fibroid genetic subtypes.

Two m^6A demethylases, FTO and ALKBH5, have been identified that are able to convert m^6A to A and regulate global m^6A levels. FTO and ALKBH5 have been reported to be dysregulated in diverse diseases leading to m^6A demethylation, modulation of gene expression downstream and influencing biological consequence (51, 52). With relation to fibroid genetic subtype, we saw an inverse correlation with regards to transcript profiles between ALKBH5 and FTO, indicating that these demethylases are nonredundant and exhibit distinct epigenetic regulation.

There is a strong racial disparity in the disease, with Black women presenting with an earlier onset of the disease and greater severity (53). We postulated that fibroids from Black women could exhibit differential expression of m^6A modifiers when compared to White women. Transcriptomic analysis of m^6A modifiers identified increased transcript expression of RBM15 in fibroids from White women (**Suppl Figure 2**), but not Black (**Suppl Figure 2b**) indicating that there might be differential m^6A levels between fibroids obtained from White and Black women but will need confirmation in a much larger cohort of patient samples.

429 Altered levels of modifiers based on genetic sub-type, led us to explore if genes
430 known to be associated with fibroids had specific m^6A methylation patterns. As uterine
431 fibroids rely on estrogen and progesterone to grow, increased levels of ESR1 and PGR
432 may affect underlying molecular pathways driving fibroid growth and progression.
433 Traditionally anti-progestins are prescribed to reduce uterine bleeding and decrease
434 fibroid volume (54-59). In silico analysis identified multiples sites on *ESR1* and *PGR* that
435 could harbor m^6A modification with moderate and high confidence, indicating that
436 transcript abundance of these key steroidogenic receptor molecules may be regulated
437 post-transcriptionally. SRAMP analysis also predicted m^6A modification sites on several
438 transcription factors that were implicated to regulate uterine fibroids, namely, *PLAG1*,
439 *PAPPA2*, *CBX2*, *CBX4*, *CBX8*, *SATB2*. Transcript levels of *PLAG1* and *PAPPA2* were
440 identified to have elevated expression in HMGA1/2 uterine fibroids (13). While *CBX2*,
441 *CBX4*, *CBX8*, *SATB2*, have been previously implicated in uterine fibroidogenesis(11,
442 13, 60). DNA repair protein, RAD51B is upregulated in *MED12*, *HMGA1*, and *HMGA2*
443 fibroids suggesting a cell response to genomic instability and a possible “second-hit”
444 pushing normal myometrial cells to tumorigeneses (61). In addition, in silico analysis
445 identified m^6A marks on TDO2 mRNA, a key enzyme catalyzing the conversion of
446 tryptophan to kynurenine which was recently identified to be upregulated in *MED12*
447 mutant fibroids and dependent on race (62-64). TDO2 inhibitor, 680C91, reduced
448 expression of *COL1A1* and *COL3A1*, genes involved in collagen production and
449 extracellular matrix (ECM) accumulation, in primary uterine fibroid culture (62). More
450 recently, primary myometrial cells treated with mono(2-ethyl-5-hydroxyhexyl) phthalate
451 (MEHHP), increased expression of *TDO2*, promoting tryptophan metabolism. Depletion

of TDO2 reduced proliferative action of MEHHP on primary fibroid cells identifying it as pro-survival factor. Our in-silico analysis also identified m^6A marks on *COL1A1*, *COL3A1*, and *POSTN*, known structural constituents of ECM organization. Notably, in triple negative breast cancer cells, increased expression of METTL3 was negatively correlated with COL3A1 expression (65). We and others identified increased expression of POSTN and characterized its role as potential regulator of fibroidogenesis (66, 67). POSTN has also been identified to be regulated by through m^6A modification during cardiac remodeling (68). *CCND1* and *VEGFA*, other known regulators of uterine fibroids (13, 69, 70) were similarly ordained with m^6A modifications in their RNA. CCND1 has been identified to be regulated through its m^6A modification and influence hematopoietic stem/progenitor cells differentiation (71).

In summary, while global transcriptomics and protein levels were unchanged, we identified modest genetic sub-type expression of m^6A modifiers. Our analysis did identify transcript levels of *RBM15* to be consistently increased but differential expression in protein levels were not detected. While modest (1-1.4-fold), we identified statistically significant differential expression, indicating that driver mutations could regulate m^6A deposition in a fibroid specific subtype manner. Characterization of key fibroid genes identified multiple m^6A marks indicating the possibility of interplay between methylation and mRNA expression and downstream deregulation of biological processes. However, in-depth sequencing and characterization of m^6A sites will be needed to be performed to further define the possibility of m^6A in fibroid pathology. Since protein abundance can be post-transcriptionally regulated and protein levels are not always correlated with mRNA levels (72), the discovery of m^6A marks and modifiers

in uterine fibroids opens the field to additional effector molecules that were previously unappreciated in tumor formation. While our studies did not identify difference in protein expression or RNA modifications in uterine fibroids, an in-depth approach with larger patient cohorts with regards to genetic sub-types and race will be needed to define expression profiles and validate possible influence of RNA modifications on fibroid pathogenesis and progression.

Reference:

1. M. M. McWilliams, V. M. Chennathukuzhi, Recent Advances in Uterine Fibroid Etiology. *Semin Reprod Med* **35**, 181-189 (2017).
2. A. E. Commandeur, A. K. Styer, J. M. Teixeira, Epidemiological and genetic clues for molecular mechanisms involved in uterine leiomyoma development and growth. *Hum Reprod Update* **21**, 593-615 (2015).
3. S. F. Cramer, A. Patel, The frequency of uterine leiomyomas. *Am J Clin Pathol* **94**, 435-438 (1990).
4. S. E. Bulun, Uterine fibroids. *N Engl J Med* **369**, 1344-1355 (2013).
5. H. Cook, M. Ezzati, J. H. Segars, K. McCarthy, The impact of uterine leiomyomas on reproductive outcomes. *Minerva Ginecol* **62**, 225-236 (2010).
6. E. A. Stewart, L. T. Shuster, W. A. Rocca, Reassessing hysterectomy. *Minn Med* **95**, 36-39 (2012).
7. E. R. Cardozo *et al.*, The estimated annual cost of uterine leiomyomata in the United States. *Am J Obstet Gynecol* **206**, 211 e211-219 (2012).
8. N. Makinen *et al.*, MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. *Science* **334**, 252-255 (2011).
9. A. A. Sandberg, Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: leiomyoma. *Cancer Genet Cytogenet* **158**, 1-26 (2005).
10. M. Nilbert *et al.*, Characteristic chromosome abnormalities, including rearrangements of 6p, del(7q), +12, and t(12;14), in 44 uterine leiomyomas. *Hum Genet* **85**, 605-611 (1990).
11. D. G. Berta *et al.*, Deficient H2A.Z deposition is associated with genesis of uterine leiomyoma. *Nature* **596**, 398-403 (2021).
12. M. Mehine *et al.*, Integrated data analysis reveals uterine leiomyoma subtypes with distinct driver pathways and biomarkers. *Proc Natl Acad Sci U S A* **113**, 1315-1320 (2016).

13. J. W. George *et al.*, Integrated Epigenome, Exome, and Transcriptome Analyses Reveal Molecular Subtypes and Homeotic Transformation in Uterine Fibroids. *Cell Rep* **29**, 4069-4085 e4066 (2019).
14. R. Desrosiers, K. Friderici, F. Rottman, Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells. *Proceedings of the National Academy of Sciences* **71**, 3971-3975 (1974).
15. B. S. Zhao, I. A. Roundtree, C. He, Post-transcriptional gene regulation by mRNA modifications. *Nature reviews Molecular cell biology* **18**, 31-42 (2017).
16. K. D. Meyer *et al.*, Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. *Cell* **149**, 1635-1646 (2012).
17. D. Dominissini *et al.*, Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. *Nature* **485**, 201-206 (2012).
18. I. Barbieri, T. Kouzarides, Role of RNA modifications in cancer. *Nat Rev Cancer* **20**, 303-322 (2020).
19. Y. Lee, J. Choe, O. H. Park, Y. K. Kim, Molecular Mechanisms Driving mRNA Degradation by m(6)A Modification. *Trends Genet* **36**, 177-188 (2020).
20. K. D. Meyer, S. R. Jaffrey, Rethinking m(6)A Readers, Writers, and Erasers. *Annu Rev Cell Dev Biol* **33**, 319-342 (2017).
21. C. R. Alarcón *et al.*, HNRNPA2B1 is a mediator of m6A-dependent nuclear RNA processing events. *Cell* **162**, 1299-1308 (2015).
22. M. Mehine, N. Makinen, H. R. Heinonen, L. A. Aaltonen, P. Vahteristo, Genomics of uterine leiomyomas: insights from high-throughput sequencing. *Fertil Steril* **102**, 621-629 (2014).
23. T. D. Chuang, D. Quintanilla, D. Boos, O. Khorram, Differential Expression of Super-Enhancer-Associated Long Non-coding RNAs in Uterine Leiomyomas. *Reprod Sci* **29**, 2960-2976 (2022).
24. A. R. Zota *et al.*, Phthalate exposures and microRNA expression in uterine fibroids: The FORGE Study. *Epigenetics insights* **13**, 2516865720904057 (2020).
25. Y. Wu *et al.*, RNA Phosphorothioate Modification in Prokaryotes and Eukaryotes. *ACS Chem Biol* **15**, 1301-1305 (2020).
26. M. Tardu, J. D. Jones, R. T. Kennedy, Q. Lin, K. S. Koutmou, Identification and Quantification of Modified Nucleosides in Saccharomyces cerevisiae mRNAs. *ACS Chem Biol* **14**, 1403-1409 (2019).
27. J. Lorent *et al.*, Translational offsetting as a mode of estrogen receptor alpha-dependent regulation of gene expression. *EMBO J* **38**, e101323 (2019).
28. M. Basanta-Sanchez, S. Temple, S. A. Ansari, A. D'Amico, P. F. Agris, Attomole quantification and global profile of RNA modifications: Epitranscriptome of human neural stem cells. *Nucleic Acids Res* **44**, e26 (2016).
29. S. D. Peddada *et al.*, Growth of uterine leiomyomata among premenopausal black and white women. *Proc Natl Acad Sci U S A* **105**, 19887-19892 (2008).
30. T.-D. Chuang, H. Panda, X. Luo, N. Chegini, miR-200c is aberrantly expressed in leiomyomas in an ethnic-dependent manner and targets ZEBs, VEGFA, TIMP2, and FBLN5. *Endocrine-related cancer* **19**, 541 (2012).
31. S. H. Boo, Y. K. Kim, The emerging role of RNA modifications in the regulation of mRNA stability. *Experimental & Molecular Medicine* **52**, 400-408 (2020).

32. X. Zhang, A. E. Cozen, Y. Liu, Q. Chen, T. M. Lowe, Small RNA Modifications: Integral to Function and Disease. *Trends Mol Med* **22**, 1025-1034 (2016).
33. Y. Zhou, P. Zeng, Y. H. Li, Z. Zhang, Q. Cui, SRAMP: prediction of mammalian N6-methyladenosine (m6A) sites based on sequence-derived features. *Nucleic Acids Res* **44**, e91 (2016).
34. S. Murakami, S. R. Jaffrey, Hidden codes in mRNA: Control of gene expression by m6A. *Molecular Cell* **82**, 2236-2251 (2022).
35. X. Y. Chen, J. Zhang, J. S. Zhu, The role of m(6)A RNA methylation in human cancer. *Mol Cancer* **18**, 103 (2019).
36. W. Huang, F. Kong, R. Li, X. Chen, K. Wang, Emerging Roles of m6A RNA Methylation Regulators in Gynecological Cancer. *Frontiers in Oncology* **12** (2022).
37. J. Chen *et al.*, Roles of N6-methyladenosine (m6A) modifications in gynecologic cancers: mechanisms and therapeutic targeting. *Experimental Hematology & Oncology* **11**, 98 (2022).
38. J. Liu *et al.*, A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nat Chem Biol* **10**, 93-95 (2014).
39. B. M. Yang Q, He C, Boyer T, Al-Hendy A (2021) Aberrant expression of N6-Methyladenosine regulators in uterine fibroids from the Eker rat model (Abstract P-314). in *American Society for Reproductive Medicine, Baltimore, MD*.
40. S. K. Yang Q, He C, Al-Hendy A, Boyer T (2021) Pathological reprogramming of epitranscriptomics via METTL3 in uterine fibroids (Abstract W-046). in *Society for Reproductive Investigation's 68th Annual Scientific Meeting* (Boston, MA).
41. B. Linder *et al.*, Single-nucleotide-resolution mapping of m6A and m6Am throughout the transcriptome. *Nature Methods* **12**, 767-772 (2015).
42. Kate D. Meyer *et al.*, Comprehensive Analysis of mRNA Methylation Reveals Enrichment in 3′ UTRs and near Stop Codons. *Cell* **149**, 1635-1646 (2012).
43. J. Liu *et al.*, A METTL3–METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nature chemical biology* **10**, 93-95 (2014).
44. X.-L. Ping *et al.*, Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase. *Cell research* **24**, 177-189 (2014).
45. D. P. Patil *et al.*, m(6)A RNA methylation promotes XIST-mediated transcriptional repression. *Nature* **537**, 369-373 (2016).
46. T. D. Chuang, A. Rehan, O. Khorram, Functional role of the long noncoding RNA X-inactive specific transcript in leiomyoma pathogenesis. *Fertil Steril* **115**, 238-247 (2021).
47. L. Zhang *et al.*, Cross-talk between PRMT1-mediated methylation and ubiquitylation on RBM15 controls RNA splicing. *Elife* **4** (2015).
48. M. Ono *et al.*, Role of stem cells in human uterine leiomyoma growth. *PLoS One* **7**, e36935 (2012).
49. R. Shi *et al.*, Linking the YTH domain to cancer: the importance of YTH family proteins in epigenetics. *Cell Death & Disease* **12**, 346 (2021).
50. W. Xiao *et al.*, Nuclear m6A reader YTHDC1 regulates mRNA splicing. *Molecular cell* **61**, 507-519 (2016).
51. J. Qu *et al.*, RNA demethylase ALKBH5 in cancer: from mechanisms to therapeutic potential. *J Hematol Oncol* **15**, 8 (2022).

52. R. Gao *et al.*, m6A Modification: A Double-Edged Sword in Tumor Development. *Frontiers in Oncology* **11** (2021).
53. D. D. Baird, D. B. Dunson, M. C. Hill, D. Cousins, J. M. Schectman, High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. *Am J Obstet Gynecol* **188**, 100-107 (2003).
54. C. Benassayag *et al.*, Estrogen receptors (ERα/ERβ) in normal and pathological growth of the human myometrium: Pregnancy and leiomyoma. *American Journal of Physiology - Endocrinology and Metabolism* **276**, E1112-E1118 (1999).
55. K. A. Kovács, A. Oszter, P. M. Göcze, J. L. Környei, I. Szabó, Comparative analysis of cyclin D1 and oestrogen receptor (α and β) levels in human leiomyoma and adjacent myometrium. *Molecular Human Reproduction* **7**, 1085-1091 (2001).
56. H. Otsuka, M. Shinohara, M. Kashimura, K. Yoshida, Y. Okamura, A comparative study of the estrogen receptor ratio in myometrium and uterine leiomyomas. *International Journal of Gynecology & Obstetrics* **29**, 189-194 (1989).
57. M. A. Borahay *et al.*, Estrogen Receptors and Signaling in Fibroids: Role in Pathobiology and Therapeutic Implications. *Reprod Sci* **24**, 1235-1244 (2017).
58. H. Ishikawa *et al.*, Progesterone is essential for maintenance and growth of uterine leiomyoma. *Endocrinology* **151**, 2433-2442 (2010).
59. A. S. Cloud *et al.*, Loss of the repressor REST affects progesterone receptor function and promotes uterine leiomyoma pathogenesis. *Proceedings of the National Academy of Sciences* **119**, e2205524119 (2022).
60. M. Mehine *et al.*, Integrated data analysis reveals uterine leiomyoma subtypes with distinct driver pathways and biomarkers. *Proceedings of the National Academy of Sciences* **113**, 1315-1320 (2016).
61. M. Mehine *et al.*, Characterization of Uterine Leiomyomas by Whole-Genome Sequencing. *New England Journal of Medicine* **369**, 43-53 (2013).
62. T. D. Chuang, D. Quintanilla, D. Boos, O. Khorram, Tryptophan catabolism is dysregulated in leiomyomas. *Fertil Steril* **116**, 1160-1171 (2021).
63. T.-D. Chuang, D. Quintanilla, D. Boos, O. Khorram, Further characterization of tryptophan metabolism and its dysregulation in fibroids. *F&S Science* **3**, 392-400 (2022).
64. A. P. Hutchinson *et al.*, Tryptophan 2, 3-dioxygenase-2 in uterine leiomyoma: Dysregulation by MED12 mutation status. *Reproductive Sciences* **29**, 743-749 (2022).
65. Y. Shi *et al.*, Reduced Expression of METTL3 Promotes Metastasis of Triple-Negative Breast Cancer by m6A Methylation-Mediated COL3A1 Up-Regulation. *Frontiers in Oncology* **10** (2020).
66. Y. Y. Lenis *et al.*, The Effects of Periostin Expression on Fibroid-Like Transition of Myometrial Cells. *Reprod Sci* 10.1007/s43032-022-01128-1 (2022).
67. M. F. B. Jamaluddin *et al.*, Proteomic Profiling of Human Uterine Fibroids Reveals Upregulation of the Extracellular Matrix Protein Periostin. *Endocrinology* **159**, 1106-1118 (2017).
68. Y. Han *et al.*, Loss of m6A Methyltransferase METTL5 Promotes Cardiac Hypertrophy Through Epitranscriptomic Control of SUZ12 Expression. *Frontiers in Cardiovascular Medicine* **9** (2022).

69. E. N. Paul *et al.*, Transcriptome Analyses of Myometrium from Fibroid Patients Reveals Phenotypic Differences Compared to Non-Diseased Myometrium. *Int J Mol Sci* **22** (2021).
70. Y. Li *et al.*, HMGA2-mediated tumorigenesis through angiogenesis in leiomyoma. *Fertility and Sterility* **114**, 1085-1096 (2020).
71. X. Zhang *et al.*, YTHDF3 modulates hematopoietic stem cells by recognizing RNA m6A modification on Ccnd1. *Haematologica* (2020).
72. Z. Khan *et al.*, Primate transcript and protein expression levels evolve under compensatory selection pressures. *Science* **342**, 1100-1104 (2013).

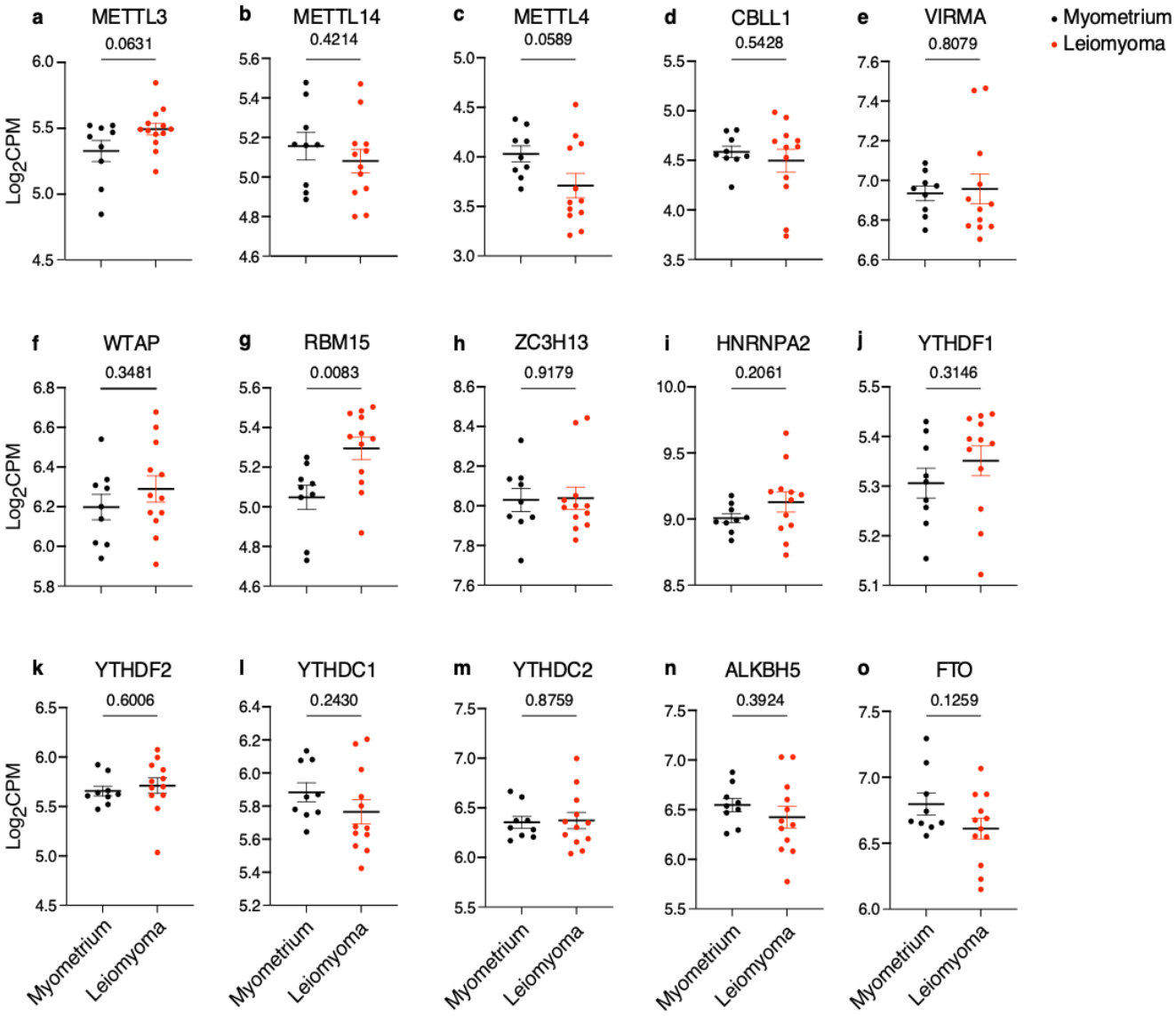
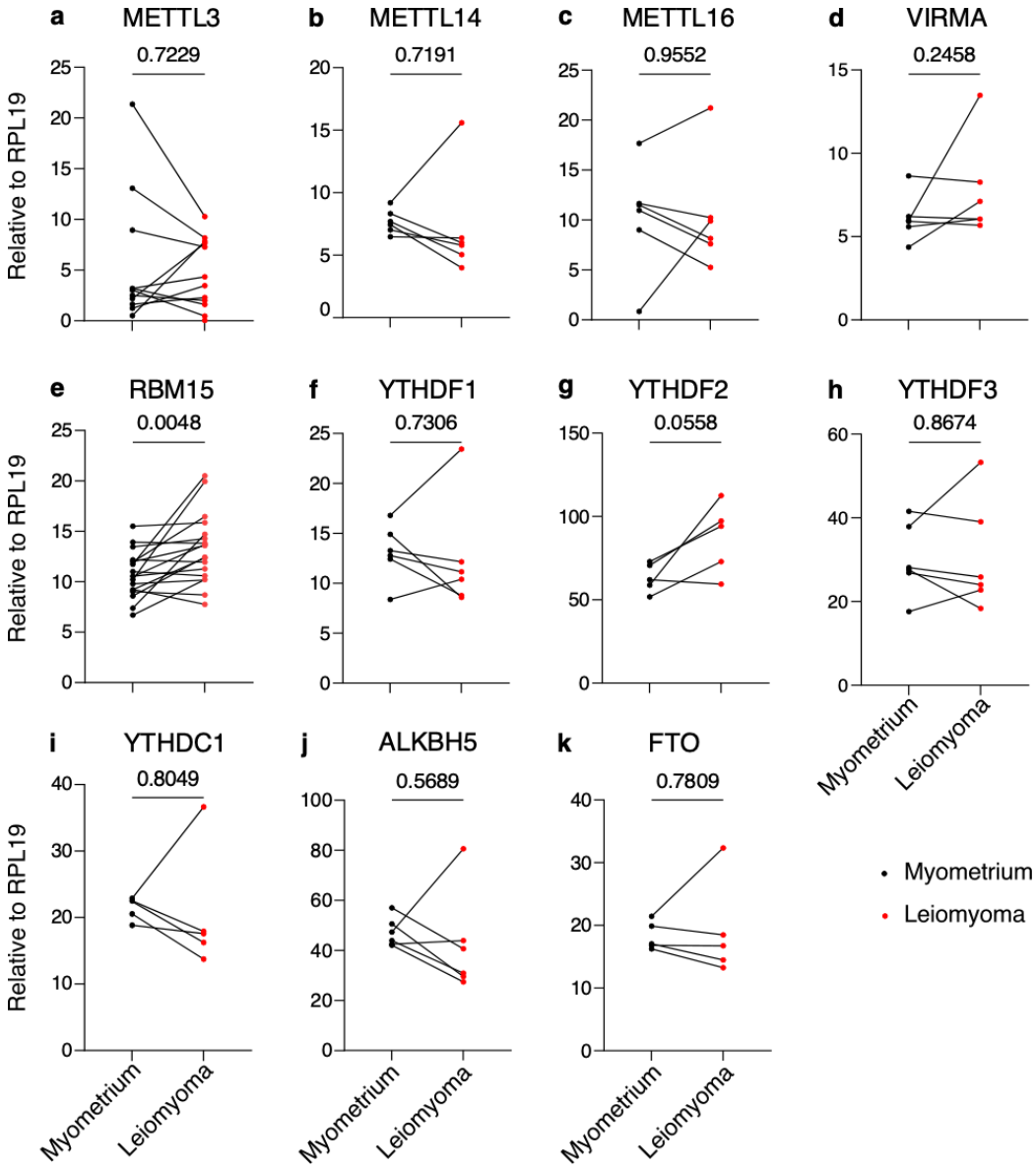
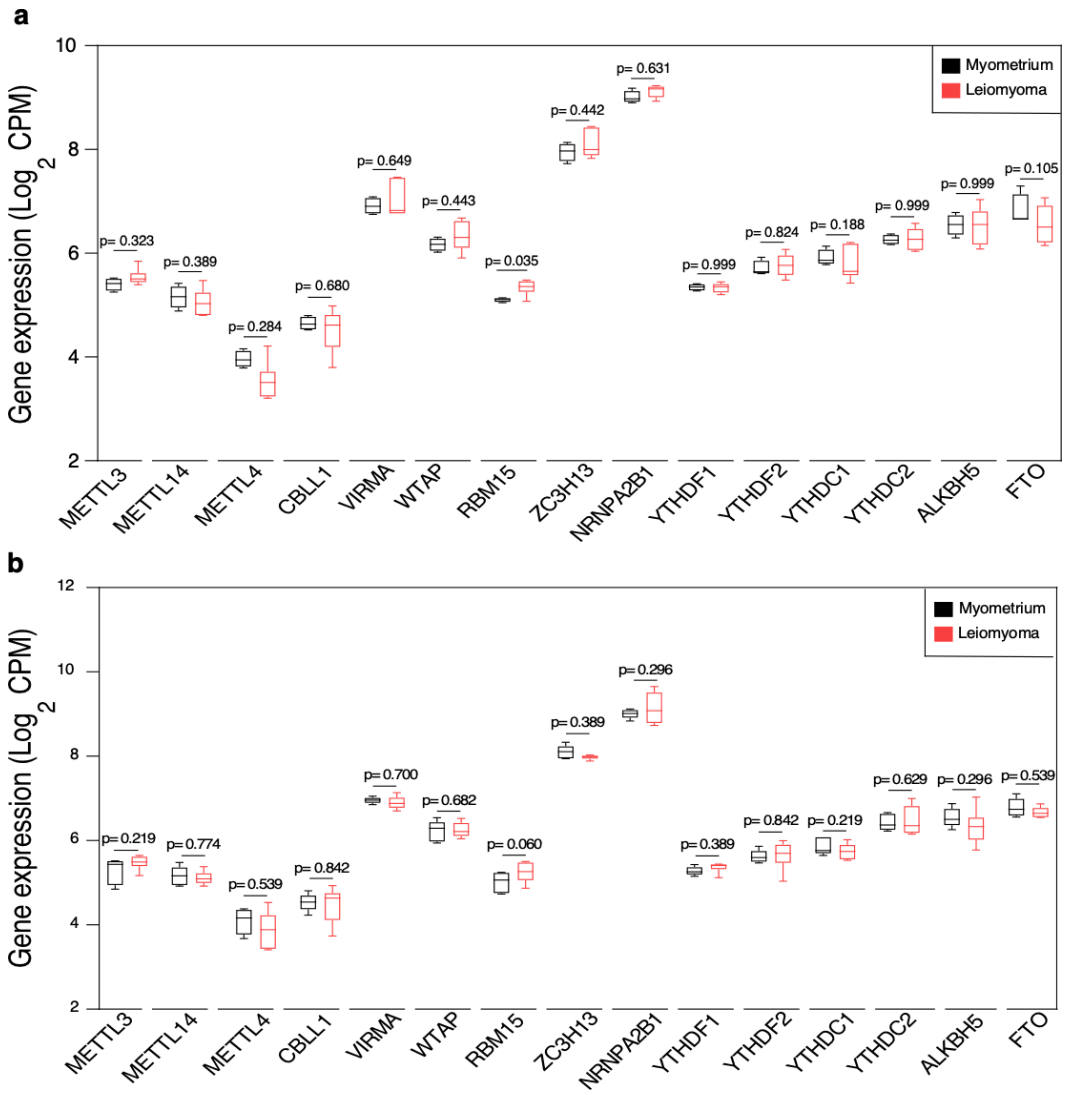


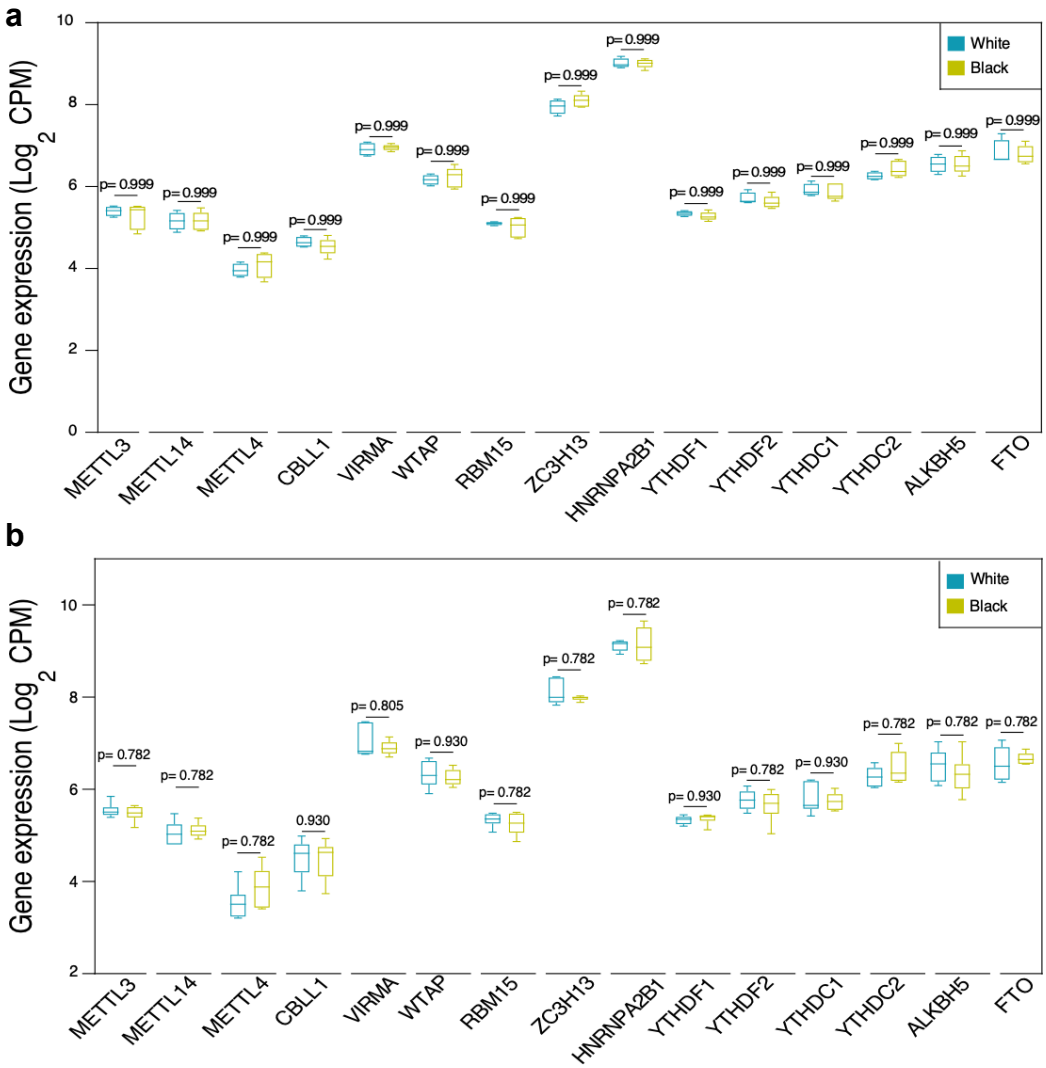
Figure 1: Transcriptomic analysis of m^{6}A modifiers in uterine fibroids. RNA-seq analysis of normalized expression of leiomyoma and matched myometrium (SRP166862). The y-axis is signal abundance quantified as log2 counts per million ($\log_2$ CPM) from myometrium (n=9) and fibroids (n=12). **a-g**. mRNA expression of m6A writers (*METTL3*, *METTL14*, *METTL4*, *CBLL1*, *VIRMA*, *WTAP*, *RBM15*). **h-m**. Readers (*ZC3H13*, *HNRNPA2*, *YTHDF1*, *YTHDF2*, *YTHDC1*, *YTHDC2*), and erasers (**n, o**), (*ALKBH5*, *FTO*). Data are represented as means ± SEM. Statistically significant differences between groups were calculated with paired student's t-test. *P*-values for each comparison is reported.



Supplementary Figure 1: RT-qPCR analysis of m^{6}A modifiers in uterine fibroids and matched myometrium. RNA levels of (**a**) *METTL3* (n=12), (**b**) *METTL14* (n=6), (**c**) *METTL16* (n=6), (**d**) *VIRMA* (n=6), (**e**) *RBM15* (n=18) (**f**) *YTHDF1* (n=6), (**g**) *YTHDF2* (n=6), (**h**) *YTHDF3* (n=6), (**i**) *YTHDC1* (n=6), (**j**) *ALKBH5* (n=6), (**k**) *FTO* (n=6). Results are presented relative to *RPL17*. Statistically significant differences between groups were calculated with paired student's t-test. *P*-values for each comparison is reported.



Supplementary Figure 2: Transcriptomic analysis of m^{6}A modifiers in leiomyoma and matched myometrium in White and Black women. RNA-seq analysis (SRP166862) of normalized expression of leiomyoma and matched myometrium (GSE120854). The y-axis is signal abundance quantified as log2 counts per million (log2 CPM). **a.** mRNA expression in normal myometrium (n=4) and leiomyoma (n=6) from white women. **b.** mRNA expression in normal myometrium (n=5) and leiomyoma (n=6) from black women. Data are represented as means ± SEM. *FDR* for each comparison is reported.



Supplementary Figure 3: Transcriptomic analysis of m^{6}A modifiers in normal myometrium and leiomyoma in White and Black women. RNA-seq analysis (SRP166862) of normalized expression of leiomyoma and matched myometrium (GSE120854). The y-axis is signal abundance quantified as log2 counts per million (log2 CPM). **a**. mRNA expression in normal myometrium from White (n=4) and Black (n=5) women. **b**. mRNA expression in leiomyoma from White (n=6) and Black (n=6). Data are represented as means ± SEM. *P*-values for each comparison is reported.

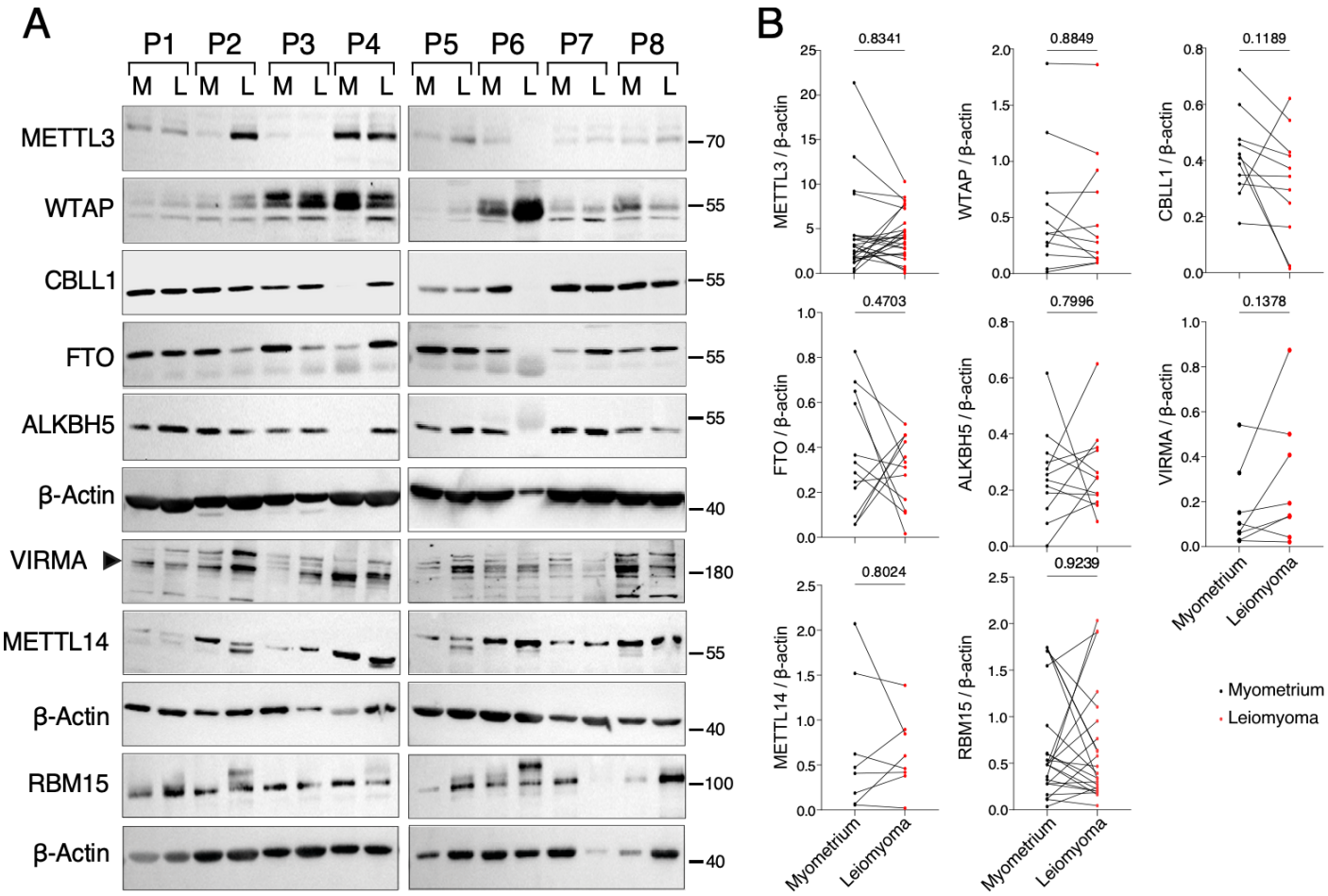


Figure 2: Western blot analysis of m^{6}A modifiers in leiomyoma. Proteins were isolated from uterine leiomyoma and matched myometrium and probed for m^{6}A modifier proteins. (**A**) Representative western blots of METTL3, WTAP, CBLL1, VIRMA, METTL14, RBM15, FTO, and ALKBH5. ACTB was used as loading control. M=Myometrium; L=Leiomyoma. P denotes individual patient samples. (**B**) Data was quantified for METTL3 (myometrium (n=19) and leiomyoma (n=26), RBM15 (myometrium (n=15) and leiomyoma (n=23), WTAP, CBLL1, VIRMA, METTL14, FTO, and ALKBH5, were quantified from 12 paired fibroids and matched myometrium patient samples. Statistically significant differences between groups were calculated with paired student's t-test. *P*-values for each comparison is reported.

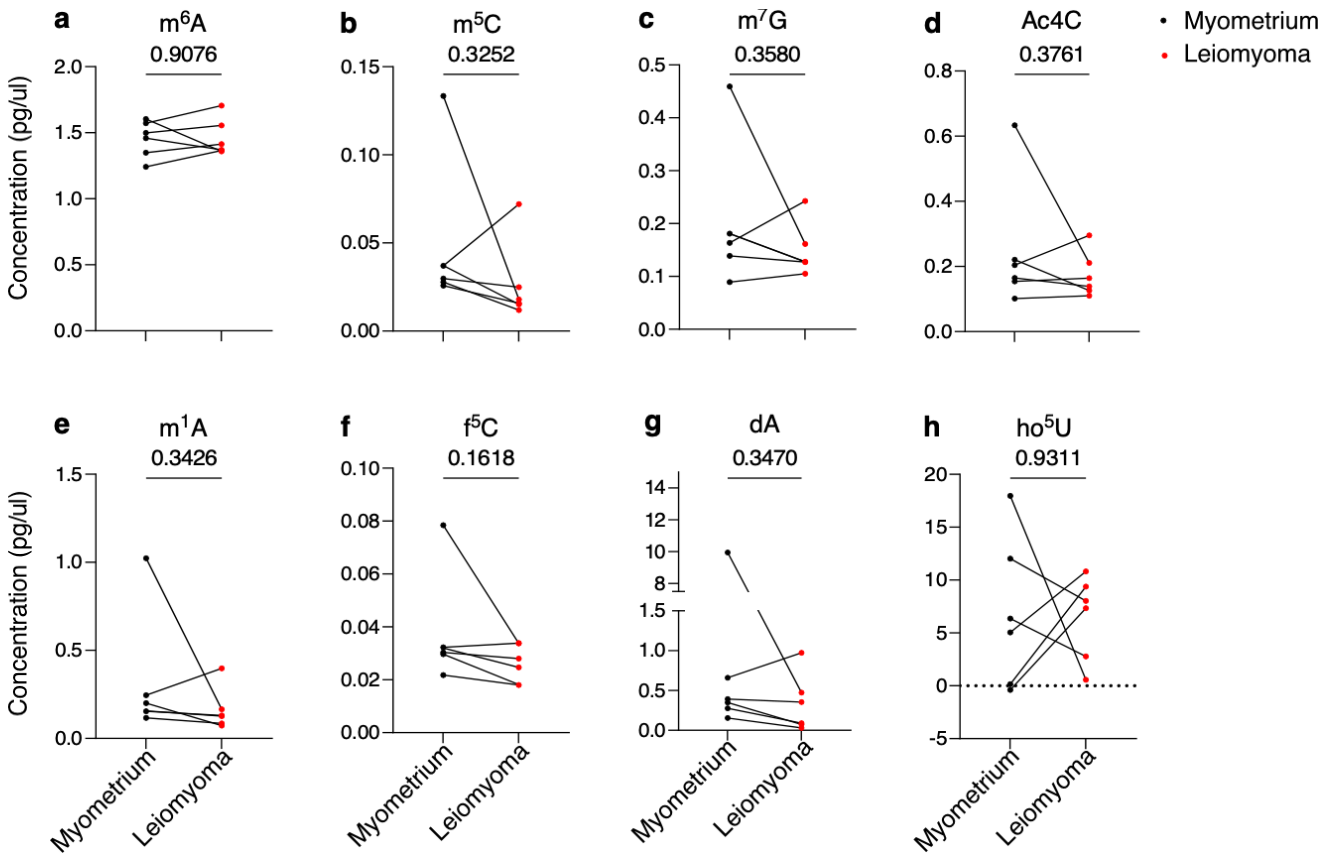
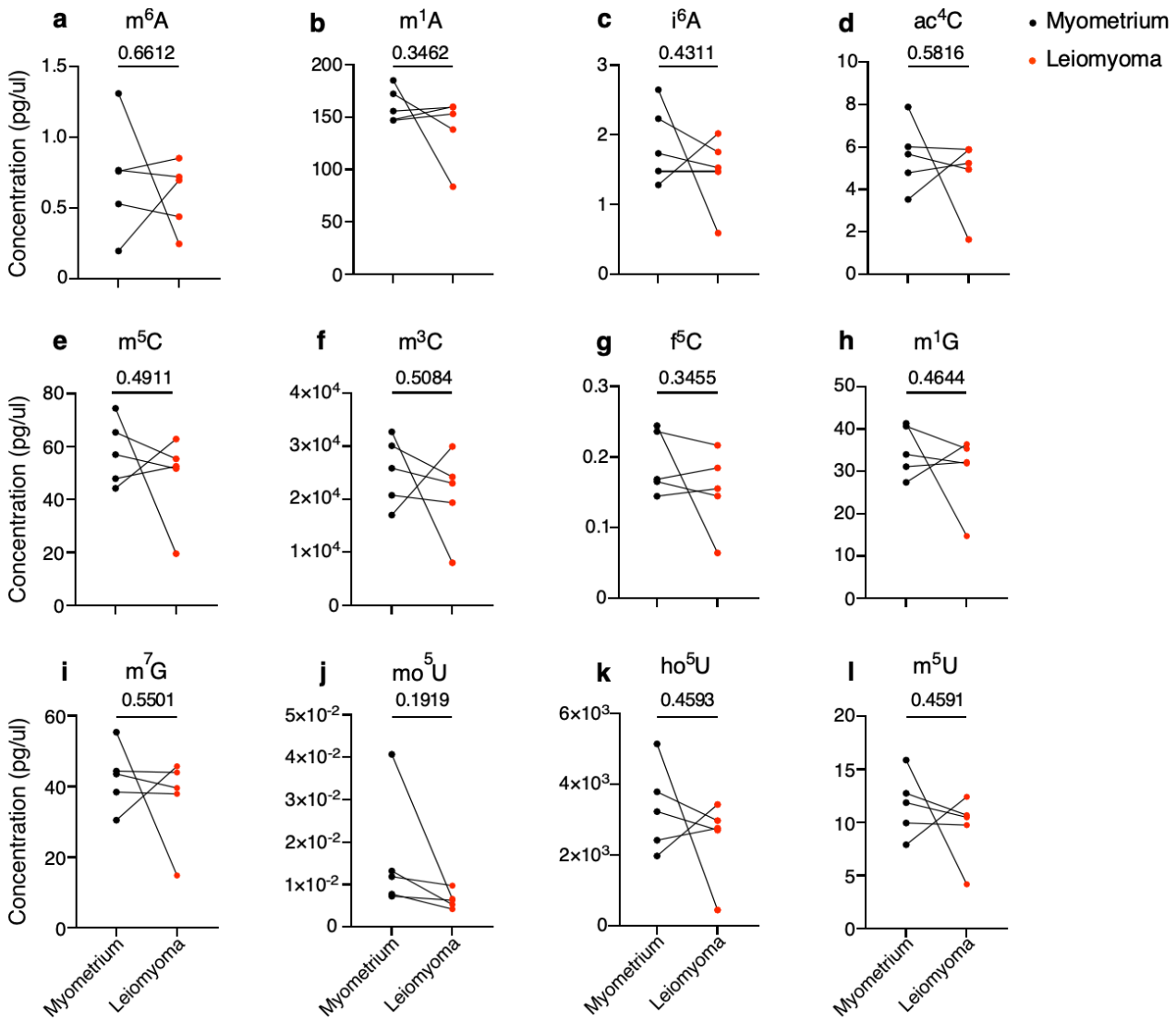


Figure 3: mRNA modifications from normal myometrium and matched fibroids. The y-axis is signal abundance quantified as pg/µl myometrium (n=6) and matched fibroids (n=6). Changes in (**a**) N^6-methyladenosine (m^6A), (**b**) 5-methylycytosine (m^5C), (**c**) N^7-methylguanosine (m^7G), (**d**) N^4-acetylcytidine (ac^4C), (**e**) N^1-methyladenosine (m^1A), (**f**) 5-Formylcytidine (f^5C), (**g**) 2'-deoxyadenosine (dA), (**h**) 5-hydroxyuridine (ho^5U) were measured. Statistically significant differences between groups were calculated with paired student's t-test. *P*-values for each comparison is reported.



Supplementary Figure 4: small RNA modifications from normal myometrium and matched fibroids. The y-axis is signal abundance quantified as pg/µl myometrium (n=5) and matched fibroids (n=5). Changes in (**a**) N^6-methyladenosine (m^6A), (**b**) N^1-methyladenosine (m^1A), (**c**) N^6-isopentenyladenosine (i6A), (**d**) N^4-acetylcytidine (ac^4C), (**e**) 5-methylycytosine (m^5C), (**f**) N^3-methylcytidine (m^3C), (**g**) 5-Formylcytidine (f^5C), (**h**) N^1 methylguanosine (m^1G), (**i**) N^7-methylguanosine (m^7G), (**j**) 5-methoxyuridine (mo^5U) (**k**) 5-hydroxyuridine (ho^5U), (**l**) 5-methyluridine (m^5U) were measured. Statistically significant differences between groups were calculated with paired student's t-test. *P*-values for each comparison is reported.

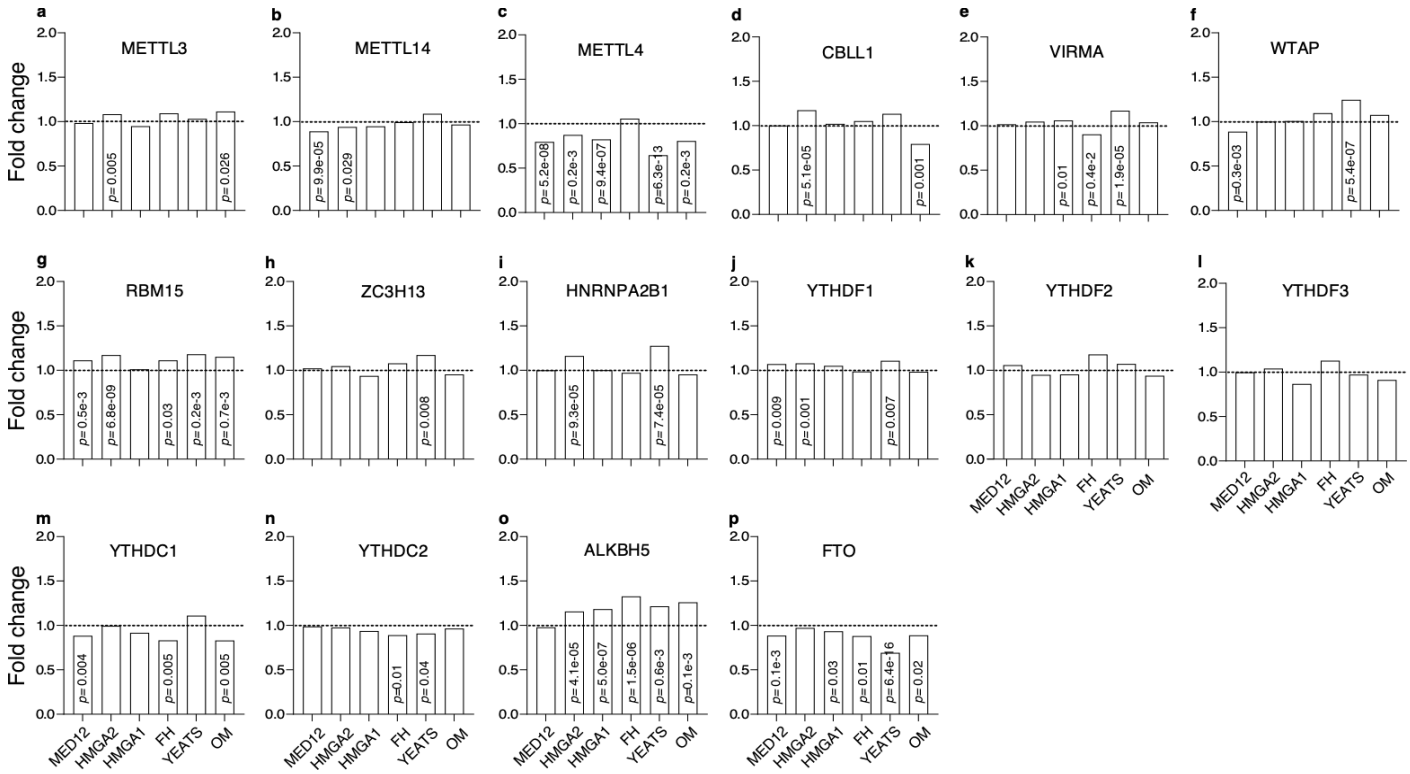


Figure 4: Expression profile of m^{6}A modifiers in relation to fibroid genetic sub-types. Gene expression of m^{6}A modifiers mapped in relation to fibroid genetic sub-types as defined by Berta et al (11). Y-axis denotes fold-change, and each genetic subtype is denoted in the X-axis. *MED12* (Mediator complex subunit 12), *HMGA1/2* (High Mobility group A1/2), *FH* (Fumarate Hydratase), *YEATS* (YEATS domain containing 4), *OM* (Other Mutations as defined by alteration of other members of the SRCAP complex subunits). *MED12* (n=38), *HMGA2* (n=44), *HMGA1* (n=62), *FH* (n=15), *YEATS* (n=16) and *OM* (n=15). Reader proteins (**a-h**), Writers (**i-n**), and Erasers (**o, p**). Statistical significance was set at FDR <0.05 and value for each comparison is reported.