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Title:
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Date:
2018-12-01

Citation:
Lappas, M. (2018). Expression and regulation of metallothioneins in myometrium and fetal membranes. American Journal of Reproductive Immunology, 80 (6), https://doi.org/10.1111/aji.13040.

Persistent Link:
https://hdl.handle.net/11343/284404

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Article type : Original article

Expression and regulation of metallothioneins in myometrium and fetal membranes

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Running title: Metallothioneins and human labor

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ABSTRACT

***Problem*:** Metallothioneins (MTs) play important roles in regulating oxidative stress, inflammation and hormone signalling. These processes plays a major role in labor at term and preterm. The aims of this study were to characterise (i) temporal and labor associated changes and (ii) the effect of pro-inflammatory and pro-labor insults on the expression of MT1 isoforms, MT2A, MT3 and MT4 in fetal membranes and myometrium.

Method of Study: The expression of MTs was assessed in fetal membranes and myometrium from non-laboring and laboring women at preterm and term by RT-qPCR. Tissue explants were used to assess the effect of pro-inflammatory cytokines and toll-like receptor (TLR) ligands on the expression of MTs in fetal membranes and myometrium.

Results: In fetal membranes, the expression of MT1A, MT1E, MT1F, MT1X and MT2A was higher at term compared to preterm. Preterm labor and preterm histological chorioamnionitis was associated with increased MT1A, MT1G, MT1M, MT1X, MT2A and MT3 expression. Term labor was associated with increased MT1A, MT1F, MT1X, MT2A and MT3 expression in fetal membranes and MT1A, MT1E, MT1F, MT1G, MT1M, MT1X, MT2A and MT3 expression in myometrium. Pro-inflammatory cytokines and TLR ligands increased the expression of MT1A, MT1E, MT1F, MT1G, MT1H, MT1X and MT2A in fetal membranes and myometrium.

Conclusions: Temporal, labor and infection associated increases in MT1 isoforms, MT2A and MT3 have been observed in fetal membranes and/or myometrium. Furthermore, pro-inflammatory cytokines and bacterial and viral products increased the expression of MT1 isoforms, MT2A, MT3 and MT4 mRNA expression in fetal membranes and myometrium.

Key words: metallothioneins; myometrium; fetal membranes; term labor; preterm labor; inflammation; infection

INTRODUCTION

Metallothioneins (MTs) are a family of low molecular weight (6–7 kDa) cysteine-rich, metal-binding proteins. In humans, four isoforms have been reported (MT1-4). MT1 has 8 isoforms (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1M, and MT1X), while MT2A, MT3 and MT4 are encoded by single genes [1] while in in rodents, four MT isoforms have been identified (MT1-4). MT1 and MT2 are ubiquitously expressed, MT3 is expressed mainly in the central nervous system and reproductive organs and MT4 is expressed primarily in squamous epithelia [2]. Human MTs are regulated independently of each other and stress hormones and reactive oxygen species (ROS) can induce their expression [2]. They are also robustly upregulated by toll-like receptor (TLR) ligands

such as the TLR4 ligand lipopolysaccharide and the viral dsRNA analogue and TLR3 ligand poly(I:C), and pro-inflammatory cytokines like IL1B and TNF [2-5]. Upregulated MT expression has been identified in a number of diseases including inflammatory bowel diseases, neuroinflammatory brain diseases, cancer, obesity and diabetes [2, 6-8]. Additionally, MT polymorphisms have been associated with a number of pathological processes including cancer and diabetes [9-11]. Pleiotropic roles of MTs have been demonstrated. These include a well-established role in metal homeostasis, metal detoxification and protection against oxidative stress, as well as roles in metabolic regulation, apoptosis, cell proliferation and differentiation, and inflammation [1, 12].

Little is known regarding the expression and regulation of MTs in gestational tissues. The only published studies available have used high throughput transcription screening techniques to identify changes in MT expression with pregnancy and labor. In myometrium, there is increased MT1A, MT1E, MT1F, MT1G, MT1M, MT1X, MT2A and MT3 mRNA expression with spontaneous term labor compared to term no labor [13-19]. Interestingly, increased MT1A and MT2A mRNA expression has been reported in myometrium obtained from arrest of dilatation compared to spontaneous term labor [20]. In fetal membranes, MT1A and MT2A mRNA expression is increased in the rupture site compared to non-rupture site in chorion obtained from spontaneous labor at term [21]. Further, when compared to fetal membranes obtained from non-laboring women, there is increased MT1H and MT1X expression and decreased MT2A mRNA expression with spontaneous term labor [22]; although there is no information on the sampling site. Except for MT1E in myometrium from laboring and non-laboring women [13], these results have not been validated. Furthermore, the effect of pro-inflammatory mediators on the expression of MTs has not been assessed. Therefore, the aims of this study were to use real-time quantitative PCR (RT-qPCR) to characterise 1) temporal and labor associated changes in the expression of MT1 isoforms, MT2A, MT3 and MT4 in myometrium and fetal membranes; 2) the expression of MT1-4 in myometrium from a mouse model of inflammation-induced preterm birth; and 3) the effect of pro-inflammatory insults on the expression of MT1 isoforms, MT2A, MT3 and MT4 in myometrium and fetal membranes.

METHODS

Human tissue collection

Human myometrium and fetal membranes were obtained (with institutional Research and Ethics Committee approval) from the upper margin of the lower uterine segment incision during Caesarean section. Tissues were brought to the research laboratory and processed within 15 mins of delivery. Tissues were washed in PBS to remove excess blood, cleared of serosa, fibrous or damaged tissue and visible blood vessels, and then dissected into smaller pieces. Tissues were immediately snap

frozen in liquid nitrogen and stored at -80^{o}C for expression studies, or used immediately for tissue explant experiments. Women with any underlying medical conditions such as diabetes, asthma, polycystic ovary syndrome, preeclampsia and macrovascular complications were excluded. Additionally, women with multiple pregnancies, obese women, and fetuses with chromosomal abnormalities were excluded. All preterm placentas were subject to histopathological examination and fetal membranes were swabbed for microbiological culture studies. Acute chorioamnionitis was diagnosed pathologically according to standard criteria [23].

Expression studies

Cohort 1 - To characterise the temporal associated changes in the mRNA expression of MT1 isoforms, MT2A, MT3 and MT4, myometrium and fetal membranes were obtained from women at preterm or term Caesarean section in the absence of labor (n=8-9 patients per group). In the term group, indications for Caesarean section in the absence of labor were breech presentation and/or previous Caesarean section. In the preterm group, indications for Caesarean section in the absence of labor were placenta praevia, vasa praevia, fetal growth restriction, placental abruption and antepartum haemorrhage. The relevant clinical characteristics of the patients used for Cohort 1 are described in Supplementary Table 1.

Cohort 2- To characterise labor associated changes in the mRNA expression of MT1 isoforms, MT2A, MT3 and MT4 in myometrium, samples were obtained from the upper margin of the lower uterine segment from women at term Caesarean section (i) in the absence of labor or (ii) during active spontaneous labor (n=8 patients per group). Labor was defined as the presence of regular uterine contractions (every 3–4 min) resulting in cervical effacement and dilation. None of the patients received any medications to augment or induce labor. Indications for Caesarean section in the absence of labor were breech presentation and/or previous Caesarean section. Indications for Caesarean section in the laboring samples were for fetal malpresentation, fetal distress and delayed or failure to progress. The relevant clinical characteristics of the patients used for Cohort 2 are described in Supplementary Table 2.

Cohorts 3 and 4 - To characterise labor associated changes in the mRNA expression of MT1 isoforms, MT2A, MT3 and MT4 in fetal membranes, samples were obtained from women at preterm (Cohort 3) or term (Cohort 4) (i) undergoing elective Caesarean section in the absence of labor or (ii) after spontaneous labor and vaginal delivery (n=9 patients per group). Indications for Caesarean section were breech presentation, and/or previous Caesarean section. Fetal membranes from the non-laboring group were obtained from the area overlying the cervix (i.e. supracervical

site, SCS) as previously described [24]. In the after labor group, fetal membranes were obtained from the site of membrane rupture as previously described [24]. None of the patients received any medications to augment or induce labor or had prelabor rupture of membranes. The relevant clinical characteristics of the patients used for Cohort 3 (preterm samples) are described in Supplementary Table 3. The relevant clinical characteristics of the patients used for Cohort 4 (term samples) have previously been described [25].

Cohort 5 - To characterise infection associated changes in the mRNA expression of MT1 isoforms, MT2A, MT3 and MT4, amnion was collected from women undergoing Caesarean section in the absence of labor (i) with histologically confirmed acute chorioamnionitis or (ii) without histologically confirmed acute chorioamnionitis (n=8 patients per group). As previously detailed [25], choriodecidual tissue could not be collected from the samples with histologically confirmed acute chorioamnionitis as it was degraded. The relevant clinical characteristics of the patients used for this cohort have previously been described [25].

Regulation studies

Tissue explants were performed to determine the effect pro-inflammatory insults on MT mRNA expression in fetal membranes and myometrium. Tissue explants were performed as previously described [26]. Briefly, fresh fetal membrane and myometrium were dissected and placed in DMEM at 37^{o}C in a humidified atmosphere of 8% O_2 (fetal membranes) or 21% O_2 (myometrium) for 1 h. Tissues were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (50 mg wet weight per well). The explants were incubated in 1 ml DMEM (containing 100 U/ml penicillin G and 100 µg/ml streptomycin) with or without 10 ng/ml IL1B (PeproTech; Rocky Hill, NJ, USA), 10 ng/ml TNF (PeproTech; Rocky Hill, NJ, USA), 250 ng/ml fsl-1 (InVivoGen; San Diego, California, USA), 20 µg/ml poly(I:C) (Sigma-Aldrich; St. Louis, MO, USA), 10 µg/ml LPS (derived from E. coli strain 026:B6; Sigma-Aldrich; St. Louis, MO), 1 µg/ml flagellin (purified flagellin from *Salmonella typhimurium;* InVivoGen; San Diego, California, USA). After 20 h, tissues were collected and stored at −80°C until assayed for MT mRNA expression by qRT-PCR as detailed below. Experiments were performed from fetal membranes and myometrium obtained from 6 patients.

Mice studies

Mice studies were conducted, with approval from the Austin Health's Animal Ethics Committee, as previously described [27]. Eight week old timed-pregnant C57BL/6 female mice were purchased from WEHI (Melbourne, Australia). Myometrium was obtained from 15.5 dpc timed-pregnant C57BL/6

female mice intraperitoneally injected with LPS (15 μg in 50 μl of PBS; Sigma) or sterile PBS (vehicle control) as previously described [27]. This model of preterm labor results in a high rate of preterm delivery (18-22 h post-LPS treatment) and does not cause maternal mortality. None of the vehicle-injected mice went into labor. The mice were killed on the birth of one pup, and time-matched controls were killed directly afterward. Myometrial tissue was washed in PBS, flash frozen and stored at -80°C until further analysis by RT-qPCR as detailed below.

RNA extraction and RT-qPCR

RNA extractions and RT-qPCR was performed as previously described [28]. RNA concentration and purity were measured using a NanoDrop ND1000 (Thermo Fisher Scientific; Scoresby, Vic, Australia). RNA was converted to cDNA using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific; Scoresby, Vic, Australia) according to the manufacturer's instructions. The RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories; Gladesville, NSW, Australia). For the human studies, quantification of MT isoform mRNA expression was performed using primers synthesized from Integrated DNA Technologies (IDT; Singapore Science Park II, Singapore); primer sequences are as detailed elsewhere [29]. For the mouse studies, pre-designed and validated QuantiTect primers from Qiagen (Chadstone Centre, Vic, Australia) were used (primer sequences not available). For the human samples, gene Ct values were normalised to the average YWHAZ and succinate dehydrogenase (SDHA) Ct values of the same cDNA sample. For the mouse samples, gene Ct values were normalised to the average actin and GAPDH Ct values of the same cDNA sample. Fold differences were determined using the comparative Ct method.

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Shapiro-Wilk test was used to test the normality of all the data. For Figure 1-7, an unpaired Student's t-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann-Whitney U (unpaired) test was used. For Figures 8 and 9, data were analysed by a repeated measures one-way ANOVA (with LSD post-hoc testing to discriminate among the means); non-normally distributed data were logarithmically transformed before analysis. Statistical significance was ascribed to a P value ≤ 0.05. For normally distributed data, values are expressed as mean ± SEM. For nonparametric data, values are expressed as median and interquartile ranges (IQR).

RESULTS

Temporal associated changes in MT1-4 expression in fetal membranes

The expression of MT1 isoforms, MT2A, MT3 and MT4 in fetal membranes obtained at preterm and term gestations is presented Figure 1. MT1A, MT1E, MT1F, MT1X and MT2A mRNA expression were significantly higher in fetal membranes obtained at term compared to preterm. In contrast, there was no significant difference in the expression of MT1B, MT1G, MT1H, MT1M, MT3 or MT4 between fetal membranes obtained at preterm or term.

Labor associated changes in MT1-4 expression in fetal membranes at term

Figure 2 demonstrates the expression of MT1 isoforms, MT2A, MT3 and MT4 in fetal membranes obtained at term from non-laboring and laboring women. MT1A, MT1F, MT1X, MT2A and MT3 mRNA expression were significantly upregulated in fetal membranes from laboring compared to fetal membranes obtained from non-laboring women at term. On the other hand, there was no significant difference in the expression of MT1B, MT1E, MT1G, MT1H, MT1M or MT4 between fetal membranes obtained from non-laboring and laboring women at term.

Labor associated changes in MT1-4 expression in fetal membranes at preterm

The expression of MT1 isoforms, MT2A, MT3 and MT4 in fetal membranes obtained at preterm from non-laboring and laboring women is depicted in Figure 3. MT1A, MT1G, MT1M, MT1X, MT2A and MT3 mRNA expression were significantly elevated in fetal membranes from laboring compared to fetal membranes obtained from non-laboring women at preterm. There was no significant difference in the expression of MT1B, MT1E, MT1F, MT1H or MT4 between fetal membranes obtained from non-laboring and laboring women at preterm.

Expression of MT1-4 in human fetal membranes from women with and without histological chorioamnionitis at preterm

MT1 isoforms, MT2A, MT3 and MT4 in amnion obtained at preterm from women with or without histological chorioamnionitis is shown in Figure 4. MT1A, MT1B, MT1F, MT1G, MT1H, MT1M, MT1X, MT2A and MT3 mRNA expression were significantly higher in amnion obtained from women with histological chorioamnionitis compared to amnion obtained from women without histological chorioamnionitis. Contrastingly, the expression of MT1E and MT4 was similar in amnion obtained from women with and without histological chorioamnionitis.

Temporal associated changes in MT1-4 expression in myometrium

The expression of MT1 isoforms, MT2A, MT3 and MT4 in myometrium obtained from women at the time of Caesarean section at preterm or term gestations is presented Figure 5. The mRNA

expression of all MT1 isoforms, MT2A, MT3 or MT4 were not significantly different between myometrium at preterm or term.

Labor associated changes in MT1-4expression in myometrium at term

Figure 6 illustrates the expression of MT1 isoforms, MT2A, MT3 and MT4 in myometrium obtained at the time of term Caesarean section from non-laboring and laboring women. MT1A, MT1E, MT1F, MT1G, MT1M, MT1X, MT2A and MT3 mRNA expression were significantly upregulated in myometrium from laboring compared to myometrium obtained from non-laboring women at term. On the other hand, there was no significant difference in the expression of MT1B, MT1H and MT4 between myometrium obtained from non-laboring and laboring women at term.

Expression of MT1-4 in myometrium from a mouse model of preterm birth

In order to determine whether similar increases in MT1-4 mRNA expression were associated with preterm labor in myometrium, we used a mouse model of inflammation-induced preterm birth [27]. Intraperitoneal injection of LPS into 15.5 dpc mice induced labor within 18-22 h in all of the mice [27]. LPS-treated mice were killed on the birth of one pup, and time-matched vehicle-injected mice were killed directly afterward. None of the vehicle-injected mice delivered preterm. When compared with vehicle-injected controls, LPS significantly increased MT1 (Figure 7A) and MT2 (Figure 7B) mRNA expression in the mouse myometrium. On the other hand, there was no significant difference in MT3 (Figure 7C) or MT4 (Figure 7D) mRNA expression between vehicle and LPS injected mice.

Effect of pro-inflammatory insults on MT1-4 expression in human fetal membranes and myometrium

Pro-inflammatory cytokines [30] and TLR ligands [31-34] that induce preterm birth *in vivo* can upregulate MT expression [2-5]. Thus, it was of interest to determine the effect of the pro-inflammatory cytokines and the TLR ligands on MT1-4 expression. To examine this, human fetal membranes and myometrium were treated with the pro-inflammatory cytokines IL1B, TNF, the bacterial products fsl-1 (TLR2/6 ligand), LPS (TLR4 ligand) or flagellin (TLR5 ligand) or the viral dsRNA analogue poly(IC) (TLR3 ligand) for 20 h and MT mRNA expression quantified. Figures 8 and 9 demonstrate the effect of pro-inflammatory insults on MT1 isoforms, MT2A, MT3 and MT4 mRNA expression in human fetal membranes and myometrium, respectively. In fetal membranes, treatment with IL1B, TNF, fsl-1, poly(I:C), LPS or flagellin significantly increased MT1A (Figure 8A), MT1E (Figure 8C), MT1F (Figure 8D), MT1G (Figure 8E), MT1M (Figure 8G) and MT2A (Figure 8I) mRNA expression. Furthermore, MT1H mRNA expression was significantly augmented by

incubation with IL1B, fsl-1, poly(I:C) or flagellin (Figure 8F), MT1X mRNA expression was significantly increased by treatment with IL1B, fsl-1, poly(I:C), LPS or flagellin (Figure 8H), while only LPS significantly increased MT3 mRNA expression (Figure 8J). There was, however, no effect of IL1B, TNF, fsl-1, poly(I:C), LPS or flagellin on MT1B (Figure 8B) or MT4 (Figure 8K) mRNA expression in fetal membranes. In myometrium, treatment with IL1B, TNF, fsl-1, poly(I:C), LPS or flagellin significantly upregulated MT1A (Figure 9A), MT1E (Figure 9B), MT1F (Figure 9C), MT1G (Figure 9D), MT1M (Figure 9F), MT1X (Figure 9G) and MT2A (Figure 9H) mRNA expression. On the other hand, there was no effect of IL1B, TNF, fsl-1, poly(I:C), LPS or flagellin on MT1H (Figure 9E), MT3 (Figure 9I) and MT4 (Figure 9J) mRNA expression. MT1B expression in myometrium was too low to detect (data not shown).

DISCUSSION

In this study, the temporal and labor associated changes in the mRNA expression of MT1 isoforms, MT2A, MT3 and MT4 in fetal membranes and myometrium have been characterised. In addition, the effect of pro-inflammatory insults on MT1 isoforms, MT2A, MT3 and MT4 mRNA expression in human fetal membranes and myometrium has been investigated. The findings of this study are summarised in Supplementary Tables 4 and 5.

The expression of MT1 isoforms, MT2A and MT3 in human myometrium from laboring and non-laboring women has been previously established using high throughput screening techniques [13-19]; see Supplementary Table 4 for a summary of the data. Using RT-qPCR, this study has confirmed these findings demonstrating a significant increase in MT1A, MT1E, MT1F, MT1G, MT1M, MT1X, MT2A and MT3 in myometrium from laboring women compared to myometrium from non-laboring women at term. Interestingly, in silico analysis of microarray transcriptomic data for myometrium revealed MT2A as one of the top three master regulators of labor [35]. Akin to our study, no changes in MT1B, MT1H or MT4 expression have previously been reported.

Like myometrium, the only other previous study examining labor-associated changes in MT expression in fetal membranes has used high throughput transcription screening [22]. This study found that when compared to fetal membranes obtained from non-laboring women, there is increased MT1H and MT1X expression and decreased MT2A mRNA expression with spontaneous term labor [22]; no details, however, were provided on the sampling site for fetal membranes. Fetal membranes overlying the cervix (i.e. putative rupture site) display unique biochemical and physical properties when compared to fetal membranes close to the placental edge [36-38]. Notably, MT1A and MT2A mRNA expression is increased in the rupture site compared to non-rupture site in chorion obtained

from spontaneous labor at term [21]. A strength of this study is that we have been able to characterise
282 MT expression in fetal membranes obtained from the site overlying the cervix in the absence of labor to those obtained from the rupture site from spontaneous labor. Specifically, when compared
284 to the no labor group, term labor was associated with a significant increase in MT1A, MT1F, MT1X, MT2A and MT3 mRNA expression. On the other hand, preterm labor was associated with a
286 significant increase in MT1A, MT1G, MT1M, MT1X, MT2A and MT3 mRNA expression when compared to the preterm non-laboring group. Notably, our studies confirm previous reports that
288 labor at term and preterm are different [39].

290 This study is the first study to demonstrate temporal associated changes in MTs in fetal membranes. Specifically, there was increased mRNA expression of MT1A, MT1E, MT1F, MT1X and MT2A in
292 fetal membranes at term compared to preterm. This suggests that these MTs are required for the preparation of labor in fetal membranes. Further increases of MT1A, MT1F, MT1X and MT2A in
294 fetal membranes during term labor suggests they may be involved in propagating labor. On the other hand, although various MTs were increased in myometrium during term labor, there were no
296 differences in the expression of MTs in myometrium obtained at preterm or term gestations. Collectively, this suggests that increased MT expression in myometrium is a consequence, not a
298 cause, of labor.

300 The underlying mechanism by which labor is associated with a higher expression of MTs, especially in the myometrium, is not known. However, it is possible that the inflammatory cells that
302 infiltrate intrauterine tissues during labor [40] may be responsible for the increase in MT expression. In support, a recent study demonstrated that choriodecidual leukocytes express higher mRNA levels
304 of MT1A, MT1E, MT1F, MT1G, MT1H, MT1M and MT1X when compared to maternal circulating leukocyte after spontaneous labor at term [41]. Leucocytes are a rich source of pro-
306 inflammatory cytokines such as IL1B and TNF which are increased in gestational tissues during both term and preterm labor [40, 42]. Thus, it was of interest to determine if these cytokines the
308 expression of MTs in fetal membranes and myometrium; see Supplementary Table 5 for a summary of the data. We found that treatment with IL1B or TNF significantly increased MT1A, MT1E,
310 MT1F, MT1G, MT1M, MT1X and MT2A mRNA expression in fetal membranes and myometrium, suggesting a mechanism by which these MTs may be increased during labor.

312

Preterm birth is a major obstetric concern, being associated with increased perinatal mortality and
314 morbidity [43]. Although many causes of preterm birth exist [44], one of the leading causes of early preterm birth is infection [45]. Bacterial and and/or viral pathogens can induce an inflammatory

response leading to preterm labor by binding distinct TLRs on gestational tissues [31-34]. In non-gestational tissues, MT expression is upregulated by various TLR ligands [2, 3, 5] where they have been implicated in regulating inflammatory reactions and infection [46]. To elucidate if MTs are also increased in association with preterm pregnancies complicated by bacterial infection, amnion obtained from women with or without histologically-confirmed chorioamnionitis were compared. We report a significant upregulation of MT1A, MT1G, MT1M, MT1X, MT2A and MT3 in amnion obtained from women with histological chorioamnionitis. We also used a mouse model of preterm labor induced by LPS to determine of MT1-4 may play a role in the induction of preterm labor in myometrium. We found LPS significantly up-regulated MT1 and MT2 mRNA expression in the maternal myometrium as compared with vehicle-injected controls. *In vitro*, a human fetal membrane and myometrial explant model was used to elucidate the effect of TLR ligands on the mRNA expression of MT1 isoforms, MT2A, MT3 and MT4; see Supplementary Table 5 for a summary of the data. The bacterial products fsl-1 (TLR2/6 ligand), LPS (TLR4 ligand) and flagellin (TLR5 ligand), and the viral dsRNA analogue poly(IC) (TLR3 ligand) were chosen as they have been shown to induce an inflammatory response in gestational tissues resulting in increased expression of pro-labor mediators [47, 48]. All these TLR ligands significantly upregulated MT1A, MT1E, MT1F, MT1M, MT1X and MT2A mRNA expression in fetal membranes and myometrium. Additionally, in fetal membrane, MT3 mRNA expression was significantly augmented by LPS and MT1H mRNA expression was significantly increased by poly(I:C) and flagellin. Collectively, these findings support a role for MTs in regulating the inflammatory processes that may contribute to infection-associated preterm birth.

There are a few limitation of this study that may impact on the conclusions. Firstly, specific antibodies for all MT isoforms do not exist; MT1A and MT2A antibodies are available; however, they are too small (MW~6 kD) to be analyzed by Western blotting. Thus, no protein data to show how these isoforms are regulated post translationally nor any localization data to show if these are produced by all cells or specific cell types in the fetal membranes and myometrium. Another limitation is the preterm samples. In humans, it is extremely difficult to assess non-laboring preterm samples without additional confounding pathology; a limitation not unique to this study [49]. In the absence of labor, indications for preterm delivery in this study were placenta or vasa praevia, fetal growth restriction, placental abruption and APH, which are closely related to inflammation [50]. Thus, the overall increase in inflammation associated with these preterm deliveries may have masked any further effect of labor on MT expression. Notwithstanding these limitations, a comprehensive analysis of MT mRNA expression changes in fetal membranes and myometrium in term and preterm labor as well as in response to inflammatory and infectious mediators was performed. This

study utilised a well-defined population of samples. With the exclusion of the preterm non-laboring samples, to limit factors that may influence MT expression, patient exclusion criteria involved smokers, obesity, asthma, preeclampsia, diabetes and hypertension.

The role of MT1 isoforms, MT2A and MT3 in labor and delivery are not known and an avenue for further research given that MTs can regulate many processes important in human labor including cellular homeostasis [51], inflammation [46], and progesterone signalling [52]. There is only one study of the functional role of MTs in human myometrium [35]. Using Ingenuity Pathway Analysis, it was recently reported that MT2A is a primary regulator the transcription factor NF-κB and its downstream inflammatory genes [35]. Confirming these results, in vitro suppression of MT2A resulted in downregulated a number of pro-inflammatory genes including IL1A, IL1B, IL6 and IL8 [35].

In summary, this study has identified temporal and/or labor associated increases in the expression of MT1 isoforms, MT2A, MT3 and MT4 in human fetal membranes and myometrium. Furthermore, MT1-4 are also upregulated in intrauterine tissues in association with preterm labor and preterm birth complicated by bacterial infection. Collectively, these findings suggest that MTs may contribute to the mechanisms that regulate human labor and delivery both at preterm and term. Functional knockdown or overexpression studies are required to elucidate the role of MTs in regulating oxidative stress, inflammation and progesterone signalling in fetal membranes and myometrium.

ACKNOWLEDGEMENTS

The clinical research midwives Genevieve Christophers, Gabrielle Pell, and Rachel Murdoch are gratefully acknowledged for sample collection; and the Obstetrics and Midwifery staff of the Mercy Hospital for Women for their co-operation.

FUNDING

Associate Professor Martha Lappas is supported by a Career Development Fellowship from the National Health and Medical Research Council (NHMRC; grant no. 1047025) and a Research Fellowship from The University of Melbourne. Funding for this study was provided by the NHMRC (grant no. 1058786), Norman Beischer Medical Research Foundation, the University of Melbourne and the Mercy Research Foundation.

CONFLICT OF INTEREST

The authors have nothing to declare.

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FIGURE LEGENDS

Figure 1. Temporal associated changes in MT1-4 expression in fetal membranes

Fetal membranes were obtained from women at preterm Caesarean section in the absence of labor (preterm, n=9 patients) or from women at term Caesarean section in the absence of labor (term, n=9 patients). MT1-4 mRNA expression was analysed by RT-qPCR. **(A,B,D,E,G,H,I,J,K)** Dare are displayed as median and IQR. **P*≤0.05 vs. preterm (Mann-Whitney U test). **(C,F)** Data are displayed as mean ± SEM. Data are displayed as mean ± SEM. **P*≤0.05 vs. preterm (unpaired Student's t-test).

Figure 2. Labor associated changes in MT1-4 expression in fetal membranes at term

Fetal membranes were obtained from women at term Caesarean section in the absence of labor (term no labor, n=9 patients) or from women after term spontaneous labor onset and delivery (term after labor, n=9 patients). MT1-4 mRNA expression was analysed by RT-qPCR. **(A,B,D,E,G,H,I,J,K)** Dare are displayed as median and IQR. **P*≤0.05 vs. term no labor (Mann-Whitney U test). **(C,F)** Data are displayed as mean ± SEM and data analysed by an unpaired Student's t-test.

Figure 3. Labor associated changes in MT1-4 expression in fetal membranes at preterm

Fetal membranes were obtained from women at preterm Caesarean section in the absence of labor (preterm no labor, n=9 patients) or from women after preterm spontaneous labor onset and delivery (preterm after labor, n=9 patients). MT1-4 mRNA expression was analysed by RT-qPCR. **(A,B,C,D,E,G,H,I,J)** Dare are displayed as median and IQR. **P*≤0.05 vs. preterm no labor (Mann-Whitney U test). **(F,K)** Data are displayed as mean ± SEM and data analysed by an unpaired Student's t-test.

Figure 4. Expression of MT1-4 in human fetal membranes from women with and without histological chorioamnionitis at preterm

Amnion was obtained from women at preterm Caesarean section without histological chorioamnionitis (preterm no CAM, n=8 patients) or from women at preterm Caesarean section with histological chorioamnionitis (preterm CAM, n=8 patients). MT1-4 mRNA expression was analysed by RT-qPCR. **(A-K)** Dare are displayed as median and IQR. **P*≤0.05 vs. preterm no labor (Mann-Whitney U test).

Figure 5. Temporal associated changes in MT1-4 expression in myometrium

Myometrium was obtained from women at preterm Caesarean section in the absence of labor (preterm, n=8 patients) or from women at term Caesarean section in the absence of labor (term, n=8 patients). MT1-4 mRNA expression was analysed by RT-qPCR. **(A,D-F,K)** Data are displayed as mean ± SEM. **(B,C,G-J)** Dare are displayed as median and IQR.

Figure 6. Labor associated changes in MT1-4 expression in myometrium at term

Human myometrium was obtained from women at term Caesarean section in the absence of labor (term no labor, n=8 patients) or from women at term Caesarean section during labor (term in labor, n=8 patients). MT1-4 mRNA expression was analysed by RT-qPCR. **(A,B,C,D,E,G,H,I,J,K)** Data are displayed as median and IQR. *$P \leq 0.05$ vs. term no labor (Mann-Whitney U test). **(F)** Data are displayed as mean ± SEM and data analysed by an unpaired Student's t-test.

Figure 7. Expression of MT1-4 in myometrium from a mouse model of preterm birth

Myometrium was obtained from mice injected with either LPS (15 µg) or PBS (vehicle) at 15.5 dpc. From the LPS-treated group, myometrium was obtained after the birth of one pup (n=4 dams). Myometrium was also obtained from time-matched vehicle-injected mice (n=4 dams). MT1-4 mRNA expression was analysed by RT-qPCR and the fold change was calculated relative to vehicle group. **(A-C)** Data are displayed as mean ± SEM. *$P \leq 0.05$ vs. vehicle (unpaired Student's t-test). **(D)** Dare are displayed as median and IQR and data analysed by a Mann-Whitney U test.

Figure 8. Effect of pro-inflammatory insults on MT1-4 expression in fetal membranes

Human fetal membranes were incubated in the absence or presence of 10 ng/ml IL1B, 10 ng/ml TNF, 250 ng/ml fsl-1, 20 µg/ml poly(I:C), 10 µg/ml LPS or 1 µg/ml flagellin for 20 h (n=6 patients per treatment). MT1-4 mRNA expression was analysed by RT-qPCR. Individual data points represent 6 independent experiments and displayed as median and IQR. *$P \leq 0.05$ (repeated measures one-way ANOVA).

Figure 9. Effect of pro-inflammatory insults on MT1-4 expression in myometrium

Human myometrium was incubated in the absence or presence of 10 ng/ml IL1B, 10 ng/ml TNF, 250 ng/ml fsl-1, 20 µg/ml poly(I:C), 10 µg/ml LPS or 1 µg/ml flagellin for 20 h (n=6 patients per treatment). MT1-4 mRNA expression was analysed by RT-qPCR. Individual data points represent 6 independent experiments and displayed as median and IQR. *$P \leq 0.05$ (repeated measures one-way ANOVA).

Figure 1

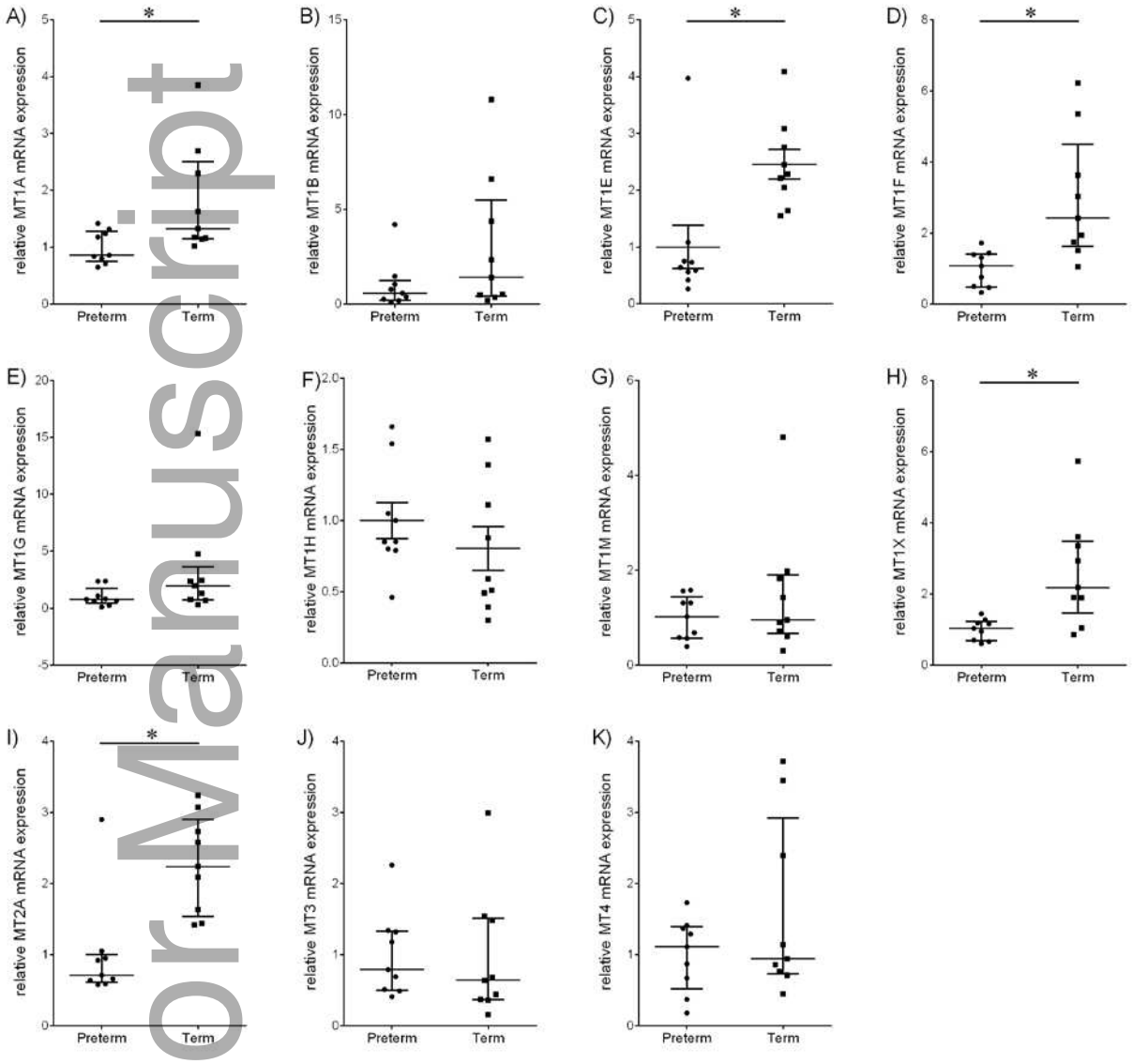
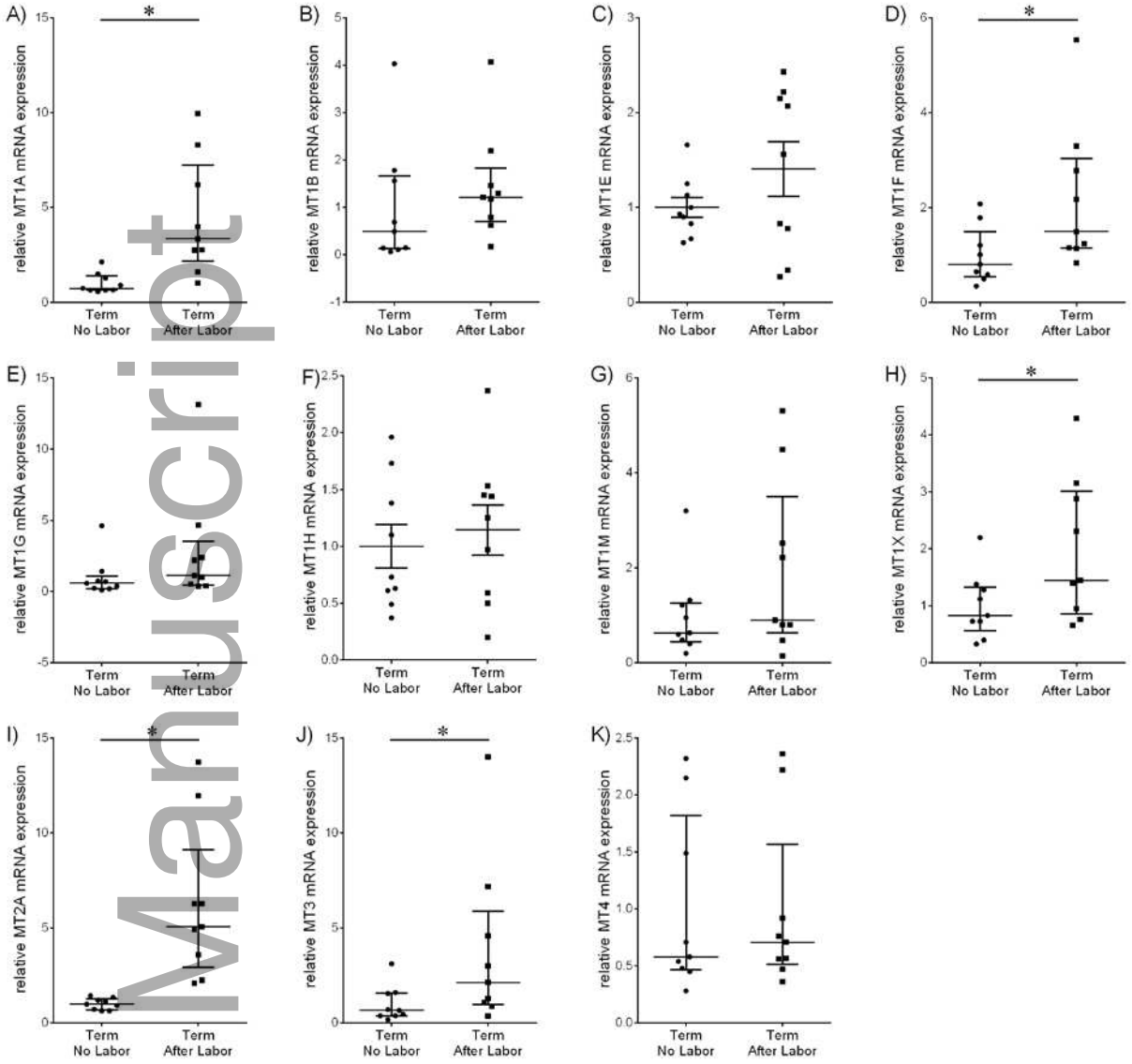


Figure 2



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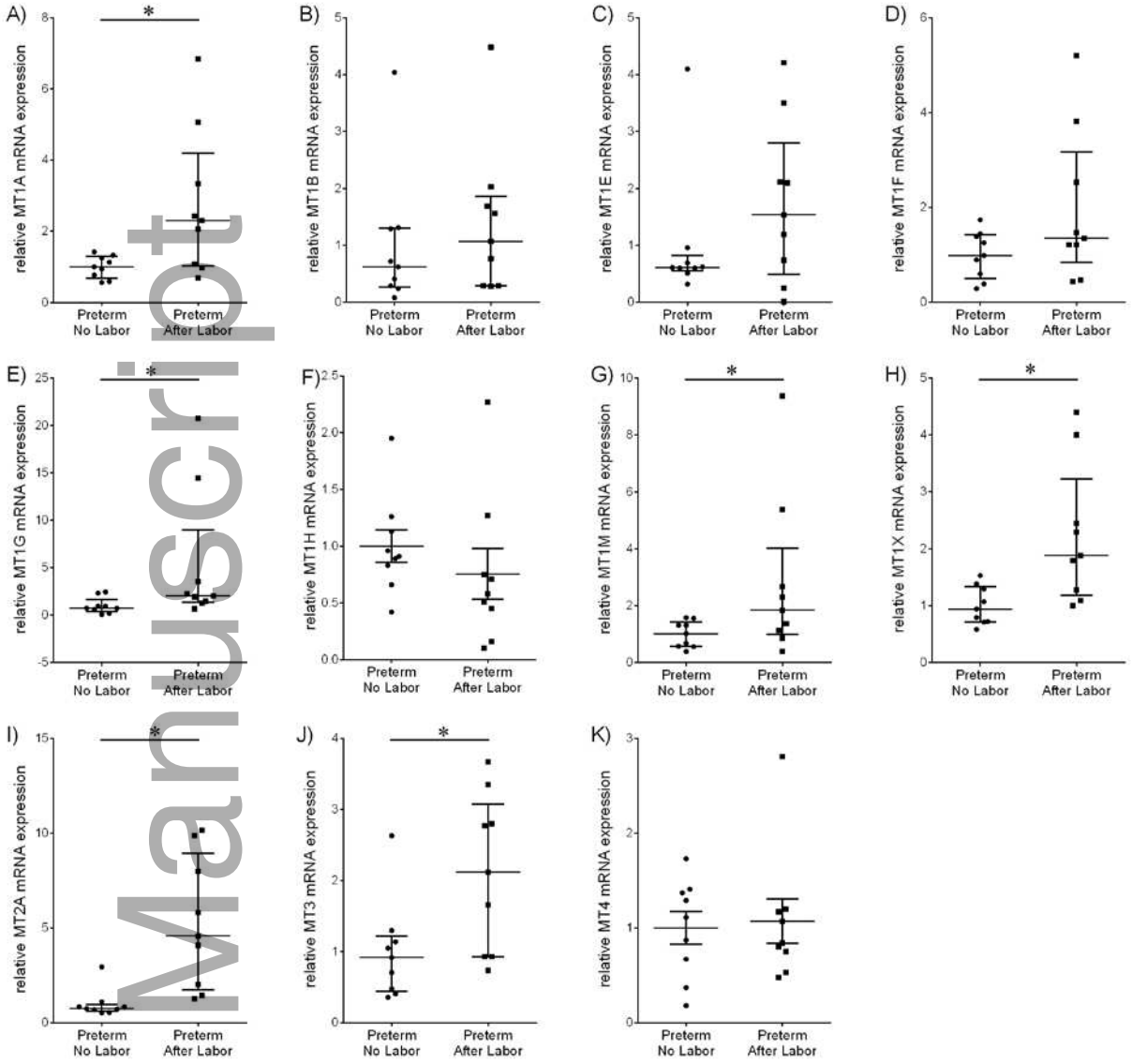

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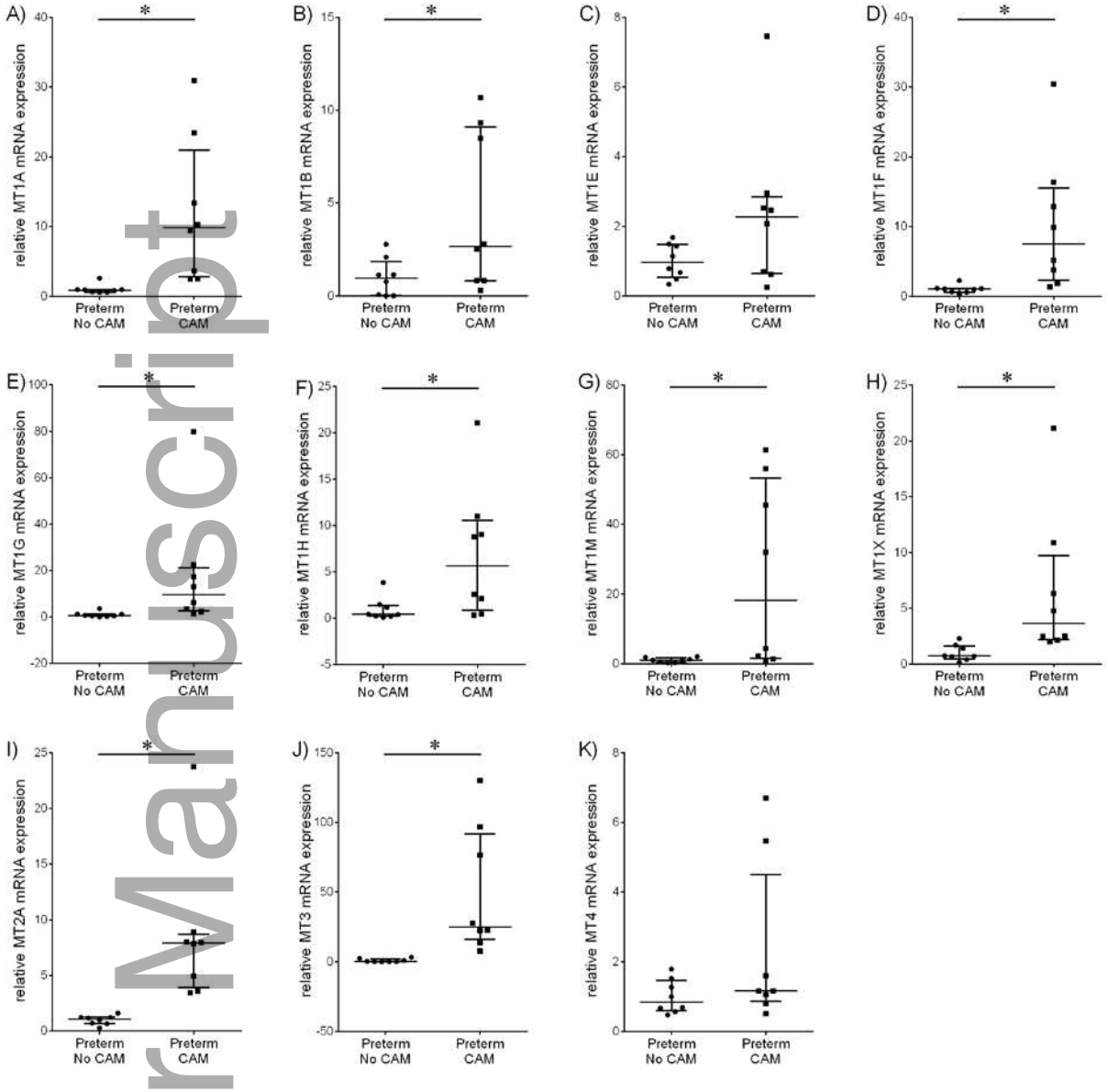

Figure 5

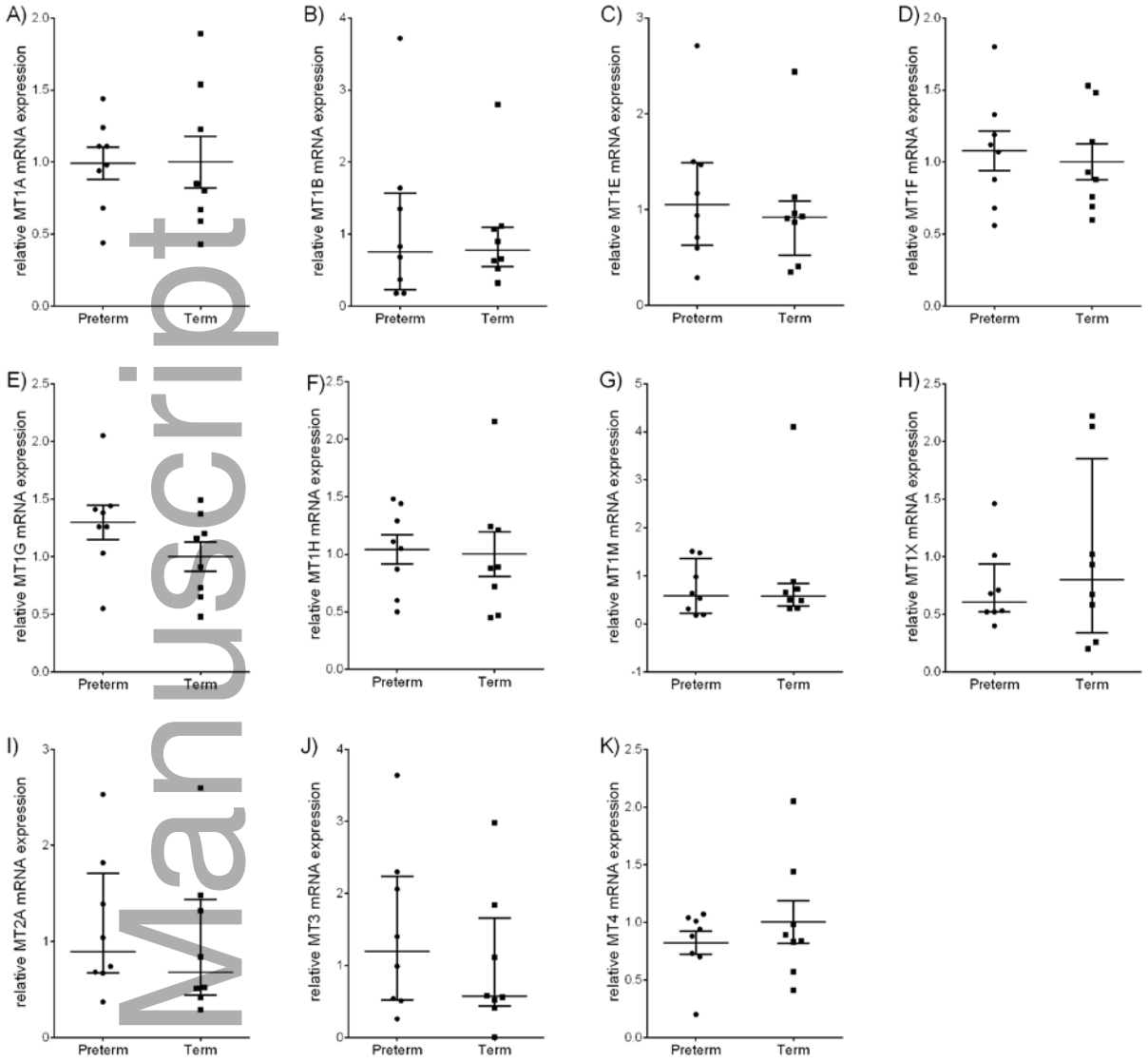

Figure 6

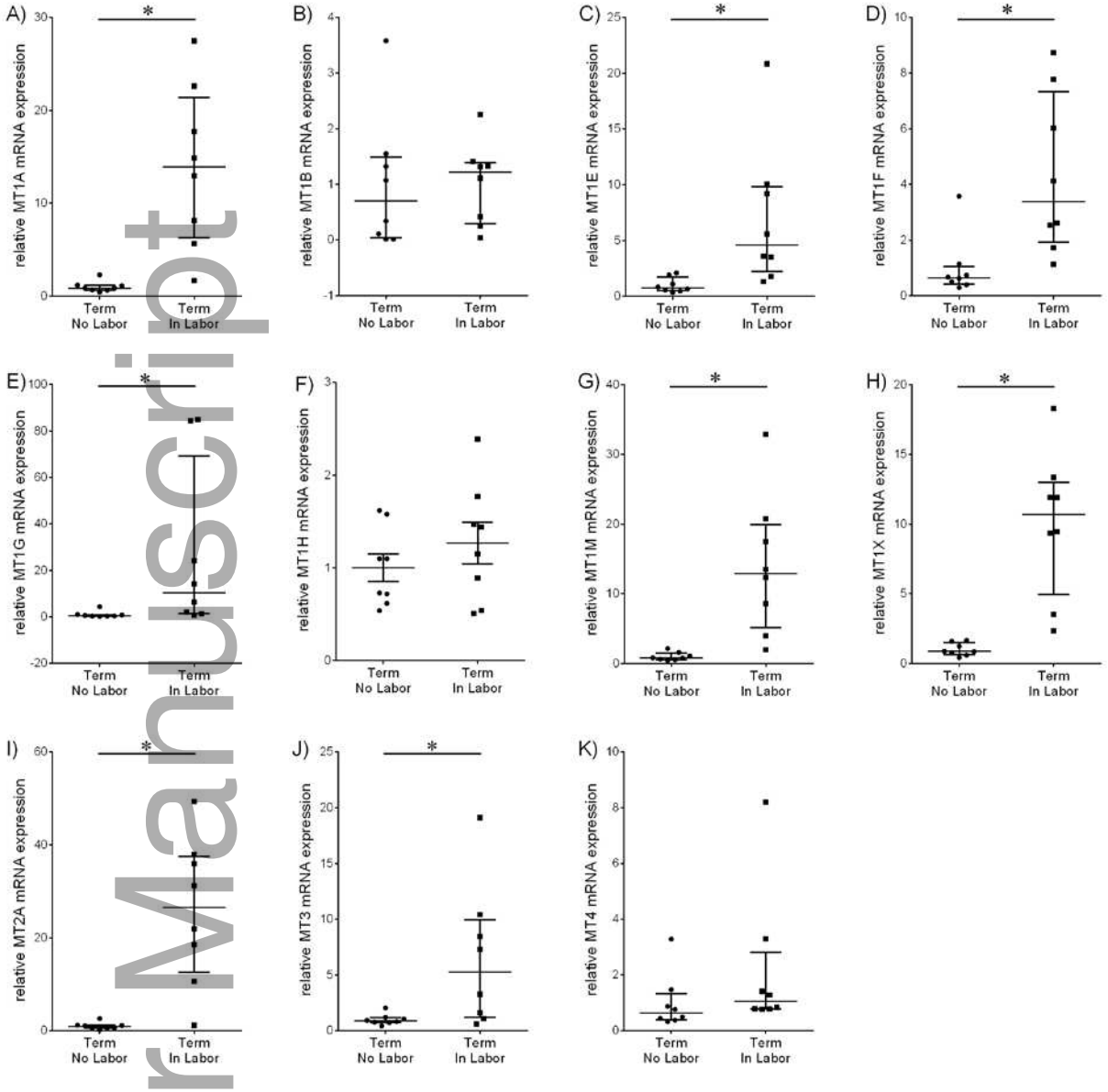

Figure 7

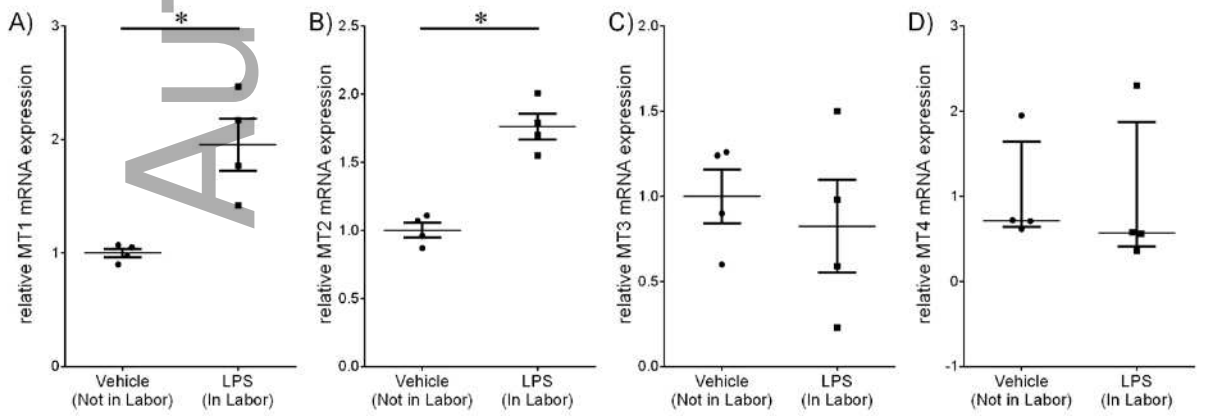

Figure 8

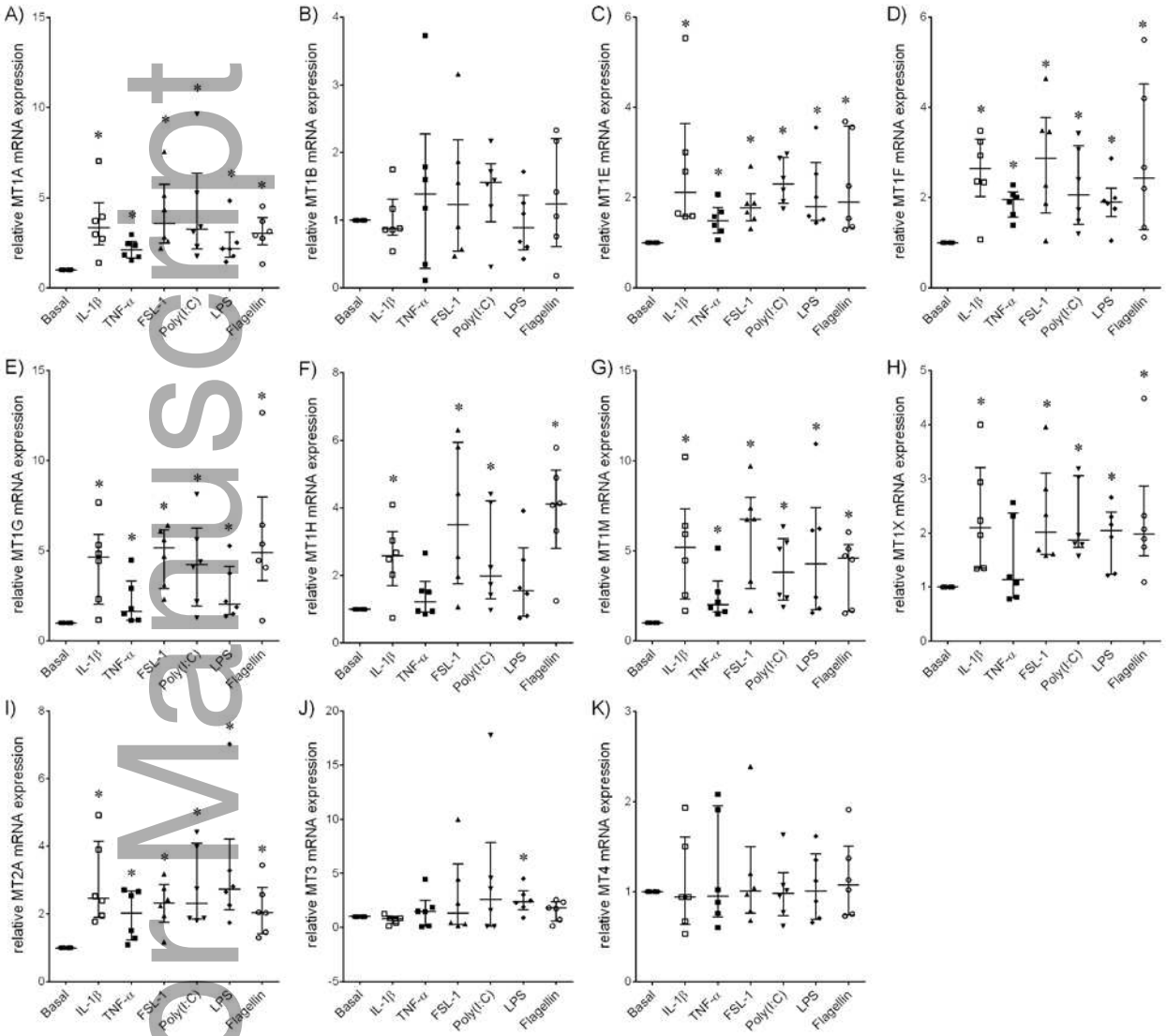

Figure 9