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Antecubital Venous Endothelial ETB Receptor Protein Expression is Preserved with Aging in Men

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ABSTRACT

Changes in endothelial function precede the development of cardiovascular disease (CVD). We have previously shown that age-related declines in endothelial function in women are due in part to a reduction in endothelial cell endothelin-B receptor (ET_BR) protein expression. However, it is not known if ET_BR protein expression changes with aging in men. The purpose of this study was to test the hypothesis that ET_BR protein expression is attenuated in older men (OM) compared to younger men (YM). Primary endothelial cells were harvested from the antecubital vein of 14 OM (60±6 yrs; 26±3 kg/m^2) and 17 YM (24±5 yrs; 24±2 kg/m^2). Cells were stained with 4',6-diamidino-2-phenylindole, vascular endothelial cadherin, and ET_BR. Images were quantified using immunocytochemistry. Endothelial function was assessed using brachial artery flow-mediated dilation (FMD). Systolic BP was similar (OM: 123±11 vs. YM: 122±10 mmHg) whereas diastolic BP was higher in OM (OM: 77±7 vs. YM: 70±6 mmHg; P<0.01). Total testosterone was lower in OM (OM: 6.28±4.21 vs. YM: 9.10 ±2.68ng/mL; P=0.03). As expected, FMD was lower in OM (OM: 3.85±1.51 vs. YM: 6.40 ±2.68%; P<0.01). However, ET_BR protein expression was similar between OM and YM (OM: 0.39±0.17 vs. YM: 0.42±0.17 AU; P=0.66). These data suggest that ET_BR protein expression is not altered with age in men. These findings are in contrast to our previous data in women, and further support sex differences in the endothelin system.

New & Noteworthy

Our laboratory has previously shown that age-related declines in endothelial function are associated with a reduction in endothelial cell ET_BR protein expression in women. However, it is unclear if endothelial cell ET_BR protein expression is reduced with aging in men. This study demonstrates that endothelial cell ET_BR protein expression is preserved with aging in men, and provides additional evidence for sex differences in the endothelin system.

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause for morbidity and mortality in men and women (25). Aging is a primary risk factor for CVD. One mechanism linking the age-related increase in CVD is impaired endothelial function (11). Endothelial cells produce and release several vasoactive substances to regulate vasodilation and vasoconstriction and maintain vascular health. Declines in endothelial function have been documented with aging in both men and women and precede the development of CVD. However, the molecular mechanisms contributing to reductions in endothelial function with aging are not fully understood.

Endothelin-1 (ET-1) is a potent vasoconstrictor that is mainly secreted by endothelial cells. ET-1 acts through two receptors: endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR). Both ET_AR and ET_BR are found on vascular smooth muscle cells and induce vasoconstriction (9), however, ET_BR are also located on endothelial cells and mediate vasodilation (6). The ET-1 system is over-active with aging in men. For example, plasma ET-1 concentration is greater in older men compared to younger men (12), and cellular protein expression of ET-1 in endothelial cells is also greater in older compared to younger men (3). This higher protein expression of ET-1 in endothelial cells correlated with lower flow-mediated dilation (FMD) – a common measure of endothelial function – suggesting the ET system is one mechanism contributing to age-related declines in endothelial function (3, 19). Concomitant with this heightened ET-1 activity is greater ET-1 vasoconstrictor tone mediated primarily by the ET_AR, which contributes in part to the age-related reduction in endothelium dependent dilation in older men (22, 26). Our laboratory has previously shown that age-related reductions in endothelial function are associated with a reduced protein expression of ET_BR on endothelial cells in women (8), however this has not yet been examined in men.

With this background in mind, the purpose of the study was to assess endothelial cell protein expression of ET_BR in young and older men. We hypothesized that ET_BR protein

expression would be lower in older men compared to younger men, and that ET_BR protein expression would be positively correlated with FMD.

METHODS

All experimental protocols were approved by the University of Delaware Institutional Review Board, and the study conformed to the standards outlined in the Declaration of Helsinki. Verbal and written consent was acquired voluntarily from all men before participation. All men completed a standard medical history questionnaire to rule out prior history of disease; men were excluded if there was a previous history of cardiovascular, metabolic, neurologic, or endocrine disease. Height, body mass, and resting blood pressure (BP) were measured in all men. Hypertensive and obese men were excluded, as well as anyone using tobacco products within the past 6 months. Eight of the 14 OM reported engaging in regular exercise, whereas 14 of the 17 YM reported regular exercise. Three YM reported using over the counter (OTC) multi-vitamins and 1 YM reported using seasonal allergy medication. Three OM reported using OTC multi-vitamins, 2 using either fish oil, vitamin C, or magnesium; 4 using vitamin D, 1 using melatonin, and 1 using Nexium. All participants were instructed to refrain from OTC supplements for 72 hours prior to the experimental protocol (below). A fasting blood sample was obtained in a subset of men (13 OM and 8 YM), which was analyzed for cholesterol and blood glucose by LabCorp.

Experimental Protocol

Men reported to the laboratory after refraining from exercise for 24 h, alcohol and caffeine for 12 h, and food for at least 4 h. All subjects laid supine for ~ 20 minutes before measurements were taken. An 18-gauge catheter was placed into an antecubital vein to obtain a blood sample and harvest endothelial cells. Approximately 30 minutes later, endothelial function was assessed via FMD in accordance with currently published guidelines (24). Briefly,

longitudinal images of the brachial artery were acquired via ultrasound (GE Logiq e, Healthcare, Wauwatosa, WI) as previously described by our laboratory (8). Images were analyzed offline using automated edge-detection commercial software (QUIPU). Images were not analyzable in 1 OM and 3 YM due to movement or inability to maintain a clear vessel throughout the protocol.

Endothelial Cell Collection and Analysis

Endothelial cells were collected using a J-wire that was threaded through an 18-gauge catheter and advanced 2-4 cm into the antecubital vein as previously described (8). The wires were cut and placed into a dissociation buffer (phosphate buffered saline, (PBS), 100ug/mL heparin, 2mMEDTA, and 0.5% BSA) in order to remove the cells from the J-wire, then centrifuged. Cells were then lysed using a red blood cell lysis buffer and centrifuged, isolating the primary endothelial cells. The cells were then fixed in 4% paraformaldehyde and washed in PBS. The endothelial cells were pipetted onto poly-l-lysine coated coverslips, dried at 50°C, and stored at -80°C until staining as previously described (8).

Prior to staining, coverslips were randomized so that the technician performing the staining, imaging, and analysis (ST) was blinded to participant ID and group (OM vs YM). The cells were washed and rehydrated with PBS (8). A blocking solution made of cold-water fish skin (2%, Cat. No. G7765, Sigma-Aldrich) and normal donkey serum (5%, Cat. No. D9663, Sigma-Aldrich) was applied (8). Afterwards, the cells were incubated with primary antibodies overnight at 4°C (8). The primary protein of interest was stained using the antibody for the ETBR (1:250, Cat. No. NBP1-30599, RRID:AB_10003663; Novus) as previously described (8). Vascular endothelial cadherin (VE Cad; 1:100, Cat. No. 14-1449-82, RRID AB_467495, Thermo Fisher Scientific) was utilized to identify endothelial cells. The next day, the cells were washed with PBS and incubated with Alexa-fluor conjugated secondary antibodies at 1:1000 dilution for 1 hour at 22℃ (donkey anti-mouse IgG, Alexa Fluor 594, Cat. No. A-21203, RRID AB_2535789, and donkey anti-rabbit IgG, Alexa Fluor 488 Cat. No. A-21206, RRID AB_2535792; Thermo

Fisher Scientific). The cells were washed again and incubated with 4',6-diamidino-2-phenylindole (DAPI; NucBlue, Cat. No. R37606; Thermo Fisher Scientific) to identify the nucleus of the cells. The coverslips were mounted onto glass slides using ProLong Diamond (Cat. No. P36966; Thermo Fisher Scientific) and set aside to harden at 22°C for at least 24 h.

Cells were imaged with a ZEISS Axio Imager A2 using a 40x objective lens and HXP 120V light source with an AxioCam MRm camera (Zeiss, Oberkichen, Germany) as previously described (8). Laser intensity and exposure time were kept consistent during imaging. Fluorescence intensity of 30 cells were measured per participant and reported as an average intensity, consistent with prior papers in the literature (3, 8, 16). Adequate cell collection was not obtained in two YM and two OM. Human umbilical vein endothelial cells (HUVECs) were stained with the primary antibody to serve as a positive control. To determine any nonspecific binding, HUVECs were also stained with the secondary antibody only, which also served as a negative control. ET_BR protein expression from the primary endothelial cells were normalized to ET_BR protein expression in HUVECs to account for any variability during staining sessions, consistent with the literature (3, 8, 16), and reported as a ratio of ET_BR/HUVEC in arbitrary units (AU).

Blood sample Analysis

A serum blood sample was collected in the morning of the study visit. After centrifugation, serum was stored at -80°C until analyzed. Total testosterone was measured in duplicate using an enzyme-linked immunoassay purchased from Crystal Chem using a BioTek Synergy 2 Multi-Detection Microplate Reader.

Statistical Analyses

Baseline characteristics, ET_BR protein expression, and FMD were compared using a two-tailed, unpaired t-test between the older and younger men. Pearson's correlations were performed to examine the relation between ET_BR protein expression, FMD, and testosterone, as

well as cholesterol and blood glucose in the subset of participants on whom we had screening blood samples. All results are reported as mean±SD.

RESULTS

Participant characteristics are shown in Table 1. By design, OM were significantly older than YM ($P< 0.001$). Total cholesterol and LDL cholesterol were higher in OM than YM ($P < 0.001$), as well has HDL cholesterol ($P=0.05$). Plasma glucose was also greater in OM compared to YM ($P< 0.01$). Additionally, BMI tended to be higher in OM compared to YM ($P = 0.07$). Systolic BP was similar between groups ($P=0.69$), however diastolic BP was higher in OM ($P< 0.01$). Total testosterone was lower in OM (n=13) compared to YM (n=17; $P = 0.03$).

As expected, OM (n=13) had lower FMD compared to YM (n=14; $P<0.01$), indicating age-related declines in endothelial function (Figure 1A). However, as shown in Figure 1B, ET_BR protein expression was not different between OM (n=12, 0.39±0.17) and YM (n=15, 0.42±0.17; $P = 0.66$). There was a trend towards a negative correlation between ET_BR protein expression and FMD (Figure 1C). ET_BR protein expression was negatively correlated with plasma glucose (r=0.49; $P=0.04$). ET_BR protein expression was not correlated with BMI, exercise frequency, any measures of cholesterol, or total testosterone (all P=NS).

DISCUSSION

Endothelial function declines with aging and precedes the development of CVD. Numerous studies have demonstrated that endothelial function is reduced in older compared to younger individuals utilizing the highly reproducible, non-invasive technique of FMD (20). However, the molecular mechanisms are not fully understood and differ based on sex. The primary finding of the current study is that although FMD is lower in older men compared to younger men, ET_BR protein expression is not different between groups. This is in contrast to previous data from our laboratory in women, where we demonstrated decreased protein

expression of ET_BR in postmenopausal women compared to young women (8). These findings provide further support for sex differences in the regulation of the endothelin system.

Endothelin is a primary mechanism contributing to declines in endothelial function with aging. For example, ET-1 vasoconstriction was greater in older compared to younger men, and blockade of the ET_AR enhanced acetylcholine-mediated vasodilation (27). In addition, protein expression of ET-1 in endothelial cells was greater in older compared to younger men (3). Taken together, these data suggest that heightened ET-1 activity and vasoconstriction via the ET_AR are a primary pathway contributing to age-related declines in endothelial function in men. The current data add to our understanding by showing that the protein expression of ET_BR on endothelial cells is not different with aging in men. Furthermore, ET_BR protein expression was not correlated with endothelial function in men, although there was a trend towards a negative correlation between ET_BR protein expression on endothelial cells and FMD. It is unclear why greater ET_BR protein expression was associated with lower FMD, but could be a compensatory mechanism in response to greater ET-1 protein expression and ET_AR mediated vasoconstriction.

Aerobic exercise is known to attenuate ET_AR vasoconstrictor activity, and is one mechanism whereby exercise improves endothelial function in men (26, 27). However, the effect of exercise on ET_BR expression or function has not been extensively studied. In the current study, exercise frequency (days per week) was self-reported as part of our general medical history form, and included both sedentary and active young and older men. While we did not observe an association between self-reported exercise frequency and FMD or ET_BR protein expression, only 30% of our sample was sedentary; future studies that are adequately powered to address the impact of exercise on ET_BR expression and function utilizing quantitative metrics of exercise (such as VO_2max) are needed in both men and women.

The findings of the current study of preserved ET_BR protein expression with aging in men are in contrast to previous data in women, where ET_BR protein expression was lower in

postmenopausal compared to young women (8). Moreover, ET_BR protein expression in women was positively correlated with FMD, suggesting that a decline in ET_BR protein expression may contribute in part to the declines in endothelial function in women after menopause. Women generally have a greater proportion of ET_BR compared to men (4), and therefore this receptor may have a greater influence on vascular health in women, whereas men have a greater reliance on ET_AR (21). It is interesting to note that when compared to our previous publication (8), YW have higher ET_BR compared to younger men, but this sex difference is no longer apparent in older adults (Fig 2). Therefore, our data further support sex differences in the ET-1 system and suggest ET_BR regulation of vascular control is a female-specific mechanism.

As men age, reductions in endothelial function may be due to decreasing concentrations of testosterone. Babcock et al. demonstrated that decreased FMD in middle aged/older men compared to younger men were largely driven by testosterone concentration (2). Middle aged/older men with lower testosterone had decreased FMD compared to men of the same age with normal testosterone, and there was a moderate positive correlation between testosterone concentration and FMD. In our study, older men had lower testosterone compared to younger men, but we did not observe a correlation between testosterone and FMD. One reason for the differences between our study and Babcock et al. may be related to sample size, as they tested 58 men whereas our sample size was not as robust. However, on an exploratory basis, we performed a median split of testosterone concentration in our older men (“high T”, n=6, 9.37±4.41 vs. “low T”, n=7, 3.62±1.19 mg/dL). Older men with higher testosterone had greater FMD compared to older men with lower testosterone (5.10±1.02 vs. 3.23±1.24 %; P=0.02), which is similar to Babcock et al. and suggests that testosterone may be a mediating factor in vascular aging in men.

Changes in testosterone concentration can also impact the ET-1 system. For example, testosterone treatment increased ET-1 expression in endothelial cells (15). In porcine coronary arteries, vasoconstrictor responses to ET1 are augmented when treated with testosterone (23).

These studies suggest that high concentration of testosterone may augment ET-1 production and vasoconstrictor activity. However, other models of hypoandrogenism also indicate that low testosterone negatively effects ET-1. For example, orchidectomy in animal models resulted in increased gene expression of ET-1 and ET_BR in portal veins of male rats but did not alter gene expression of ET_AR (18). In humans, low testosterone concentration was associated with higher plasma ET-1 in healthy adults (14) and patients with coronary artery disease (1). Ours is the first study to examine the relation between testosterone concentration and ET_BR protein expression in men, however we did not observe a significant correlation between these two variables. Furthermore, ET_BR protein expression in men with high and low T was not different (0.37±0.14 vs. 0.42±0.20 AU; P=0.63), further supporting preserved receptor expression regardless of testosterone concentration. Future studies are needed to determine if cellular expression of ET-1 is altered by testosterone in men, and whether there is a relation between free testosterone concentration and cellular changes in ET_BR and ET-1.

Traditional cardiometabolic risk factors also change with aging. Although our sample was free of overt disease, we did observe expected age-related increase in total cholesterol, LDL cholesterol, and fasting plasma glucose. In our previous study in young and postmenopausal women (8), these age-related changes were also noted, but we reported a significant inverse correlation between ET_BR and LDL cholesterol in women (8). Thus, endothelial ET_BR may be atheroprotective in women (13). Interestingly, we did not observe a significant correlation between these variables in men in the current study, but did see a significant inverse correlation between ET_BR protein expression and fasting plasma glucose. Emerging evidence suggests that the ET_BR on vascular smooth muscle cells are upregulated in response to high glucose concentrations and improve insulin sensitivity (10, 17), but whether there is a role for endothelial ET_BR regulating glucose remains unknown.

Limitations: We recognize limitations with our study. First, we assessed ET_BR protein expression in venous cells as opposed to arterial cells. However, previous studies have

demonstrated a strong correlation in protein expression of various markers related to endothelial health between venous and arterial cells (20). Protein expression of various markers from venous endothelial cells often correlate with functional outcomes of vascular health such as FMD (3, 5, 7, 8). Thus, venous cells are a useful surrogate (20) and pose less risk to participants. Second, we assessed ET_BR on endothelial cells, but they are also located on vascular smooth muscle cells along with ET_AR. The balance between ET_BR on endothelial cells vs smooth muscle cells could elicit important function changes (i.e., vasoconstriction), and the ratio of ET_BR and ET_AR may be critical in understanding age and sex differences in vascular health.

In conclusion, we show that aging does not alter endothelial cell ET_BR protein expression in men. These findings are in contrast to previous data in women (8), and further support sex differences in the endothelin system. Understanding which mechanisms do and do not contribute to endothelial function are crucial for developing strategies to prevent or offset the development of cardiovascular disease with aging.

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Disclosures

MMW is a paid consultant for Orchestra BioMed.

Author contributions

ST, ADV, AVK, AS, and MMW performed the experiments; ST, AVD, AS, and MMW analyzed the data; ST, AVK, AS, ADV, DGE and MMW interpreted the results of the experiments; ST, ADV, and MMW prepared the figures; AVK, DGE and MMW conceived and designed the study; ST and MMW drafted the manuscript; all authors critically revised and approved the final manuscript.

Figure Legends:

Figure 1: (*A*) Flow-mediated dilation (%) is lower in older men (OM, n=13) compared to young men (YM, n=14). (*B*) Endothelial-B receptor (ET_BR) protein expression expressed as arbitrary units (AU) in venous endothelial cells collected from 15 YM and 12 OM along with representative images of venous ETBR protein expression from one YM and one OM. Unpaired t-tests were used to determine differences between groups (panels A & B). *(C)* Pearson correlation showing trend towards inverse relation between flow-mediated dilation percent change (FMD %) and endothelin-B receptor (ET_BR) protein expression (in arbitrary units, AU), in young men (n=13, Closed circles) and older men (n=11, open circles).

Figure 2: Endothelial-B receptor (ET_BR) protein expression expressed as arbitrary units (AU) in venous endothelial cells collected in young and older men (current study) compared to young women (YW) and postmenopausal women (PMW) from our prior publication (redrawn from (8)). Expression of ET_BR is higher in YW compared to PMW (P=0.02), and also higher in YW compared to YM.

* $P < 0.05$.

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Figure 1A

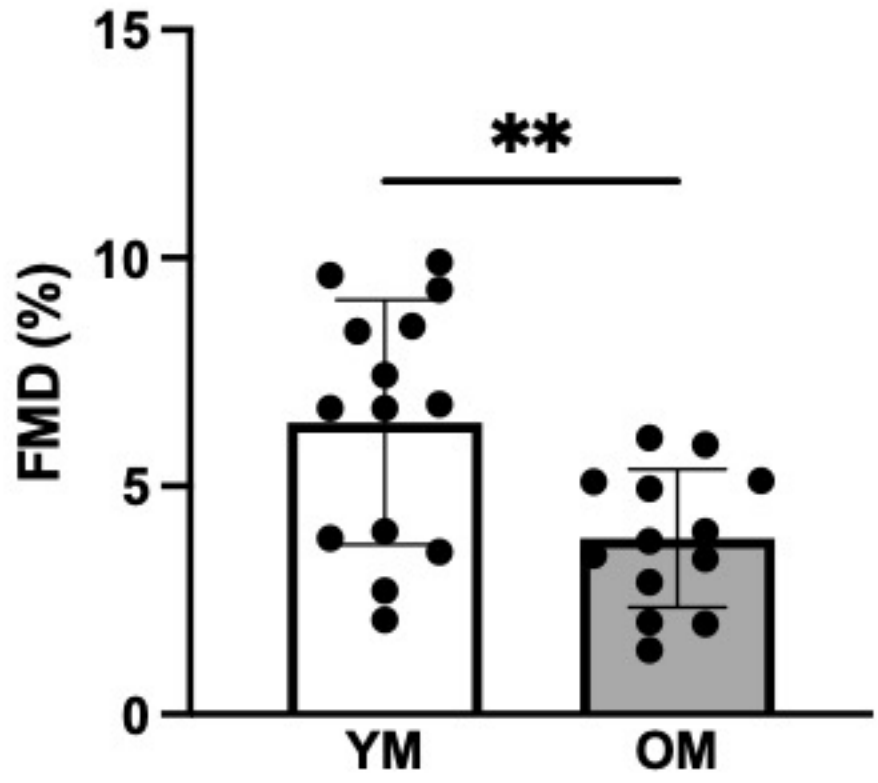

Figure 1B

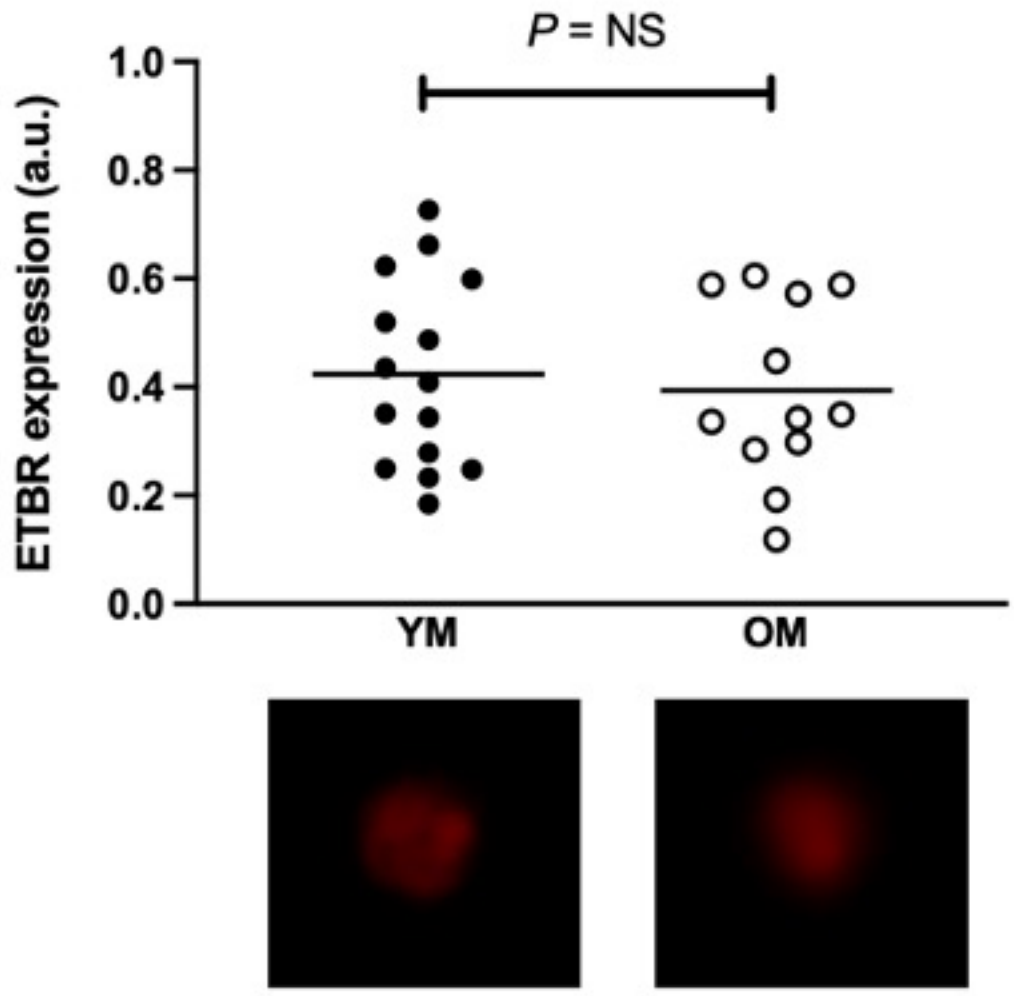

Figure 1C

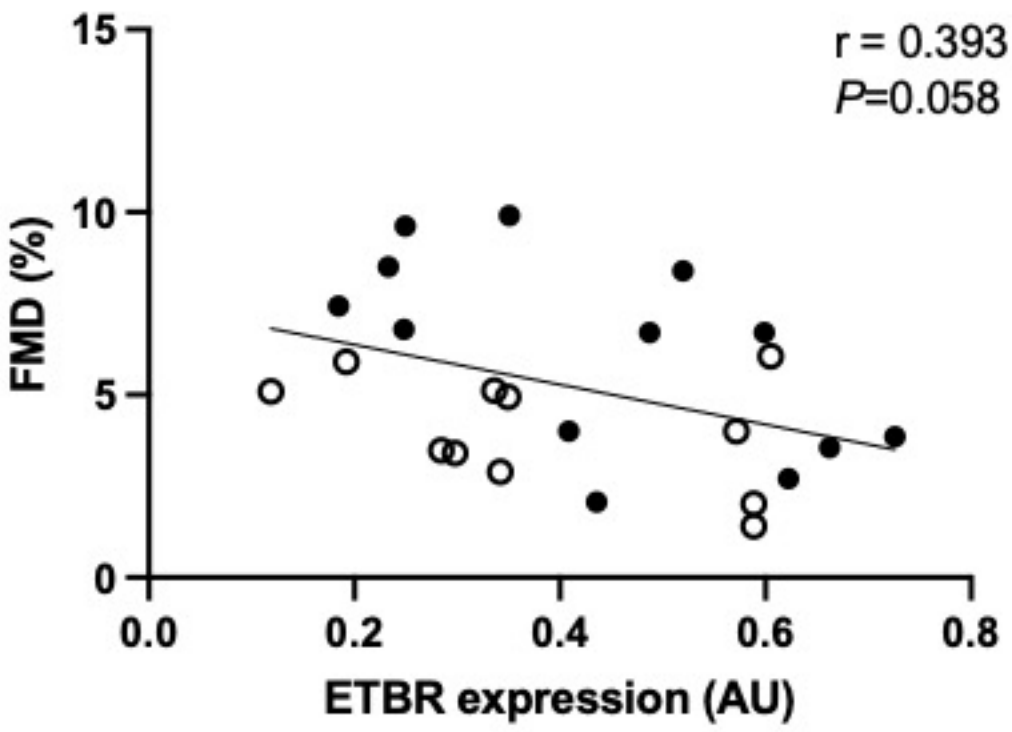

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Figure 2

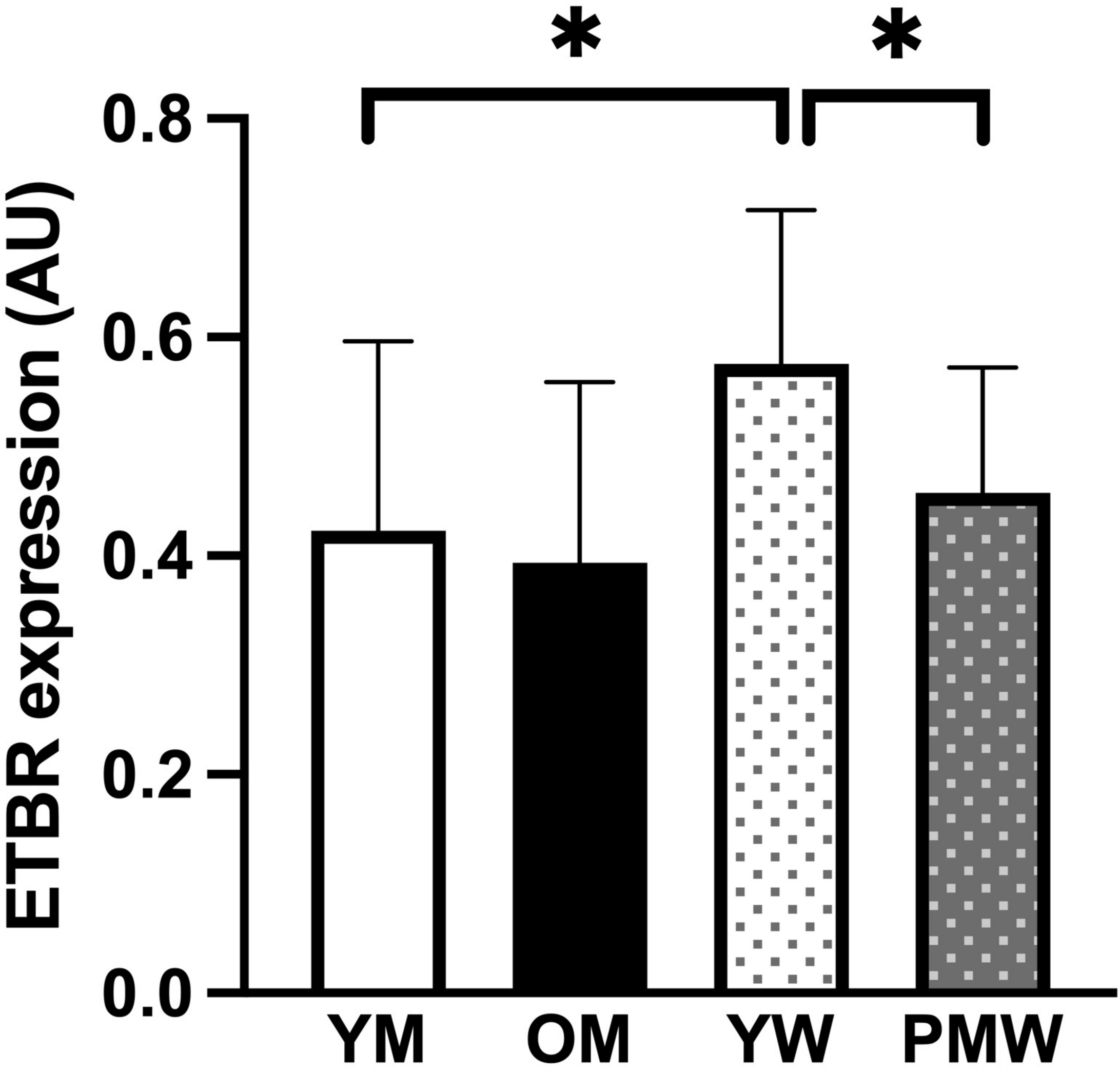

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Table 1. Participant characteristics.

	YM (n = 17)	**OM** (n = 14)
Age, yrs	24 ± 5	60 ± 6 *
BMI, kg/m^2	24 ± 2	26 ± 3
Body mass (kg)	79 ± 11	81 ± 11
Height (cm)	181 ± 6	177 ± 7
SBP, mmHg	122 ± 10	123 ± 11
DBP, mmHg	70 ± 6	77 ± 7 *
Total Cholesterol, mg/dL	133 ± 31	210 ± 34 *
HDL, mg/dL	46 ± 5	56 ± 14 *
LDL, mg/dL	73 ± 29	131 ± 28 *
Glucose, mg/dL	84 ± 5	94 ± 8 *
Total Testosterone, mg/dL	9.10 ± 2.68	6.28 ± 4.21 *

Values are mean±SD. YM, young men; OM, older men; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Total Cholesterol, HDL, LDL, and Glucose were obtained in 8YM and 13 OM. Total testosterone was measured in 17 YM and 13 OM. Groups were compared using unpaired t-tests. *P<0.05 vs. YM.