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Effect of menopause and exercise training on plasma apolipoprotein M and sphingosine-1-phosphate

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Running title: Menopause, training and the apoM/S1P axis
Abstract

Objective: The axis of apolipoprotein M (apoM) and sphingosine-1-phosphate (S1P) is of importance to plasma lipid levels, endothelial function, and development of atherosclerosis. Menopause is accompanied by dyslipidemia and an increased risk of atherosclerosis, which can be lowered by exercise training. The aim of this study was to explore if effects of menopause and training are paralleled by changes in the apoM/S1P axis.

Methods: Healthy, late premenopausal (n=38, age 49.2±2) and recent postmenopausal (n=37, age 53.3±3) women from the Copenhagen Women Study participated in a three-month, aerobic high-intensity exercise intervention.

Results: Before training, plasma apoM was higher in postmenopausal (1.08±0.2 µmol/l (mean±SD)) compared to premenopausal (0.82±0.2 µmol/l) women (p<0.0001). Plasma S1P was similar in the two groups (0.44±0.1 and 0.46±0.1 µmol/l, respectively). Hence, the pre-training S1P/apoM ratio was 26% lower in postmenopausal than premenopausal women (p<0.0001). After the training program, plasma apoM increased from 0.82±0.2 to 0.90±0.3 µmol/l in premenopausal women and from 1.08±0.2 to 1.16±0.3 µmol/l in postmenopausal women (p<0.05). Plasma S1P increased from 0.44±0.1 to 0.47±0.1 µmol/l in premenopausal women and from 0.46±0.1 to 0.48±0.1 µmol/l in postmenopausal women (p<0.05).

Conclusions: The results suggest that menopause is accompanied by higher plasma apoM but not S1P concentrations, and that exercise training increases plasma apoM and S1P in healthy middle-aged women irrespective of menopausal status.

Keywords
Apolipoproteins, lipoprotein metabolism, lipids, sphingolipids, atherosclerosis, menopausal transition, cardiovascular training
The ApoM/S1P complex is involved in maintaining a healthy endothelial barrier function. Our study is the first to show how menopause affects apoM/S1P axis. The results suggest that the menopause is accompanied by higher plasma apoM but not S1P concentrations. Secondly, the study is also the first to show that exercise training increases both apoM/S1P in women irrespective of menopausal status.
Introduction

Menopause is associated with an elevated risk of developing endothelial dysfunction (33) and metabolic syndrome, including dyslipidemia and atherosclerosis (7). Long-term hormone therapy is not generally recommended for postmenopausal women due to increased risk of cancer and athero-thrombotic disease (32). Currently, there is a lack of mechanistic understanding of changes that cause dyslipidemia, atherosclerosis, and endothelial dysfunction in postmenopausal women.

Apolipoprotein M (apoM) is a lipocalin mainly bound to plasma high density lipoprotein (HDL) particles (11). ApoM has an important role in protecting the endothelial barrier function (8, 12) and affects several potential anti-atherogenic pathways, such as reverse cholesterol transport (10, 19), formation of preβ-HDL (10, 34, 47), and removal of reactive oxygen species (10, 18). Moreover, variations in the apoM gene are associated with risk of cardiovascular disease (CVD) and altered plasma lipids (5). Further investigations are however needed to clarify the exact role of apoM in atherosclerosis and dyslipidemia.

The bioactive lipid sphingosine-1-phosphate (S1P) is carried by apoM (12). ApoM-containing HDL carries ~65% of plasma S1P, while albumin carries ~35% (27, 35). Hence, apoM is a chaperone, and its biological effects are likely provided by S1P (36, 40). S1P acts through five G-protein-coupled receptors (S1P₁-₅) (24), affecting diverse processes such as protection of the endothelial barrier (8, 12, 45), regulation of angiogenesis (16), promotion of lymphocyte trafficking (39), and activation of endothelial nitric oxide synthase (eNOS) (23). Accordingly, S1P has been implicated in several diseases, including inflammatory diseases and atherosclerosis (30).

Exercise training decreases CVD risk by effects on endothelial function (21) and plasma lipids, including in postmenopausal women (4, 28, 42). This finding is of particular interest as the menopausal transition is accompanied by increased prevalence of dyslipidemia (7, 14, 15, 25), and endothelial dysfunction (33). The apoM/S1P axis is associated with endothelial function (8, 12) and plasma lipids (5), which are all influenced negatively by the menopausal transition and can be improved with exercise training. The effect of menopause and exercise training on the apoM/S1P axis itself is unknown, and further investigation is needed in order to understand if apoM and S1P are involved in menopausal and exercise-induced changes. This has prompted the present study on how plasma apoM and S1P concentrations are
affected by exercise training in early postmenopausal compared with late premenopausal women, as well as how apoM and S1P concentrations differ in pre-and postmenopausal women.
Materials and methods

The data presented in this study are part of the Copenhagen Women Study (31, 37), which is an interdisciplinary study on the effects of exercise training in the late premenopausal and early postmenopausal phase. The study was approved by the Ethics Committee in the capital region of Denmark (protocol number: H-1-2012-150) and hosted institute. All participants were given informed consent at time of inclusion, and the study was conducted in accordance with the guidelines of the Declaration of Helsinki.

Participants - In the present study, we used samples from 38 premenopausal women and 37 postmenopausal women from the Copenhagen Women Study (31, 37). All included participants were healthy non-smokers with no excessive alcohol intake and a body mass index of 18.5-30 kg/m². The premenopausal women had regular menstrual cycles and were not using hormonal contraceptives. The postmenopausal women had not experienced a menstrual cycle for at least 1 year and were not receiving hormone therapy. All women were physically active less than 2 hours per week prior to the training intervention.

Study design - As described previously (31, 37), all women underwent testing before and after three months of high-intensity exercise training on a cycling ergometer for one hour three times per week. The training sessions were supervised by instructors, and exercise intensity was monitored and increased gradually during the three-month period. As described by Nyberg et al (37), the heart rate was at 71-95% of the maximal heart rate almost 90% of the time. On test days, all women were fasting and had not exercised for 24 hours.

Plasma lipids - Blood samples taken with a BD Vacutainer system (Becton-Dickinson, Plymouth, UK) were analyzed at the Department of Clinical Biochemistry at Rigshospitalet, Denmark. Plasma aliquots were used for analysis of total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), and triglycerides with enzymatic absorption photometry (Cobas 8000, c702 module, F. Hoffmann-La Roche Ltd., Rotkreuz, Switzerland). Other blood samples for apoM and S1P measurements were centrifuged for 5 minutes at 4000g and stored at -80°C. Blood samples for S1P measurements were placed on ice immediately after collection.
Plasma apoM - ApoM was measured with ELISA as described by Bosteen et al. with intra and inter assay coefficients of variation of 3.2% and 7.9%, respectively (6). Plasma for measurement of apoM was available for 38 premenopausal women (38 pre-training samples and 36 post-training samples) and 37 postmenopausal women (37 pre- and post-training samples). For the two-way repeated measures ANOVA, we could only include subjects with paired values obtained before and after exercise training (pre-menopause n=36; postmenopause n=37).

Plasma S1P - S1P was measured with an HPLC-based method as described by Christoffersen et al. (12). Plasma for measurement of S1P was available for 37 premenopausal women (36 pre-training samples and 31 post-training samples) and 35 postmenopausal women (32 pre-training samples and 33 post-training samples). For two-way repeated measures ANOVA, we could only include subjects with paired values obtained before and after exercise training (premenopause n=30; postmenopause n=30).

Statistical Analysis - Data were analyzed using GraphPad Prism 4 software. The significance level (alpha) was set at p<0.05. The effects of menopausal status and exercise training were assessed using two-way repeated measures ANOVA; only women with measurements before and after the training intervention were included in the two-way repeated measures ANOVA. Correlations were assessed using Pearson correlation analysis. Normally distributed data are presented as mean±SD. Data that are not normally distributed are log-transformed and presented as median (25-75 percentile). After the log-transformation, all data were normally distributed.
Results

Characteristics of the participants - Before the training intervention, the premenopausal women were 49.2±2 years old. The postmenopausal women were 53.3±3 years old and 3.1±1 years past their final menstruation.

Effects of menopausal status - Before training, the plasma apoM concentration was 32% higher in the postmenopausal than in the premenopausal women (1.08±0.2 µmol/l and 0.82±0.2 µmol/l, respectively) (p<0.0001) (Figure 1A). The plasma S1P concentration was similar in the two groups (p>0.05) (Figure 1B). Hence, the S1P/apoM ratio was 26% lower in postmenopausal than premenopausal women before training (0.45±0.1 and 0.61±0.2, respectively) (p<0.001) (Figure 2A). In accordance with previous findings from the Copenhagen Women Study (40), plasma TC (p<0.0001), HDL-C (p<0.001), and LDL-C (p<0.01) concentrations were higher in this subset of postmenopausal compared with premenopausal women, while the plasma triglyceride concentration was similar in the two groups (p>0.05) (Table 1). The plasma apoM/HDL-C ratio was also similar in the two groups (p>0.05) (Figure 2B).

Effects of exercise training - The three-month high-intensity training intervention increased the plasma apoM concentration from 0.82±0.2 to 0.90±0.3 µmol/l in premenopausal women and from 1.08±0.2 to 1.16±0.3 µmol/l in postmenopausal women (p<0.05) (Figure 1A). The plasma S1P concentration increased from 0.44±0.1 to 0.47±0.1 µmol/l in premenopausal women and from 0.46±0.1 to 0.48±0.1 µmol/l in postmenopausal women (p<0.05) (Figure 1B). The plasma S1P/apoM ratio was not affected by training (p>0.05) (Figure 2A). As previously reported (40), plasma TC (p=0.01) and LDL-C (p<0.01) concentrations decreased after the training period, whereas plasma HDL-C and triglyceride concentrations did not change (p>0.05) (Table 1). The plasma apoM/HDL-C ratio was increased from 0.50±0.1 to 0.54±0.2 in premenopausal women and from 0.56±0.2 to 0.60±0.2 in postmenopausal women, which was borderline statistically significant (p=0.05) (Figure 2B).

Correlations between apoM and lipids in plasma - The plasma apoM concentration correlated positively with plasma TC, HDL-C, and LDL-C concentrations both before and after the training intervention, while no correlation was found between the plasma apoM and triglyceride concentration (Figure 3). Also, there was
no correlation between plasma apoM and S1P concentrations (p>0.05) (Figure 4). Additionally, plasma S1P concentrations did not correlate with plasma TC, HDL-C, LDL-C, or triglyceride concentrations (p>0.05) (data not shown).
There were two main findings in the present study. Firstly, recent postmenopausal women had higher plasma apoM than late premenopausal women. Secondly, exercise training increased plasma apoM and S1P in both late premenopausal and recent postmenopausal women.

Recent postmenopausal women had 32% higher plasma apoM concentrations compared with late premenopausal women before the training intervention. There are at least two possible explanations for this. The first possible explanation for the higher postmenopausal apoM concentration is a difference in lipid levels. The postmenopausal women had higher TC (by 15%), HDL-C (by 21%), and LDL-C (by 14%) compared with the premenopausal women before the training intervention. In accordance with previous findings, apoM correlated positively with plasma TC, HDL-C, and LDL-C (1). ApoM also correlates negatively with the fractional catabolic rate of LDL (9), suggesting that LDL-C should be increased when apoM is increased, which is consistent with our findings. As discussed previously (31), there is a general agreement that TC and LDL-C are elevated in postmenopausal women compared with premenopausal women, rendering a more atherogenic profile (7, 14, 25). In contrast, findings on the relationship between menopause and HDL-C and triglycerides are inconsistent (7, 14, 15, 25, 38). The Study of Women’s Health Across the Nation (SWAN) found that HDL-C was higher in recent postmenopausal women (≤24 months after the final menstrual period [FMP]), but then declined to the premenopausal level in late postmenopausal women (>24 months after the FMP) (14). In the present study, no significant correlation between lipid levels or apoM levels and age was found (data not shown). A study on a sub-cohort from SWAN also found that an increase in HDL-C over the menopausal transition was associated with a greater development of atherosclerosis (17). This is an interesting observation as HDL particles are generally considered anti-atherogenic (20). The finding that plasma apoM was higher in the postmenopausal women might be explained by a concomitant increase in HDL-C, illustrated by a stable apoM/HDL-C ratio between pre- and postmenopausal women. Thus, one may speculate that apoM-containing HDL particles may lose their endothelium-protective and anti-atherogenic potential over the menopausal transition. Nyberg et al., found that the early postmenopausal phase was associated with a marked reduction in vascular function in the Copenhagen Women Study (37), and observed that several biomarkers of vascular function were adversely
altered in a similar cohort (38). Further studies are however still needed to conclude whether apoM-containing HDL plays a role in this reduction. Second, it is possible that the higher apoM concentration can be explained by the changes in hormone levels that occur over the menopausal transition and possibly even a direct effect of sex hormones on apoM. Axler et al. found that apoM concentrations correlate positively with age for women only, with women aged 18-49 years having lower apoM concentrations than women aged 50 years or older (1). This observation does not prove a link between apoM and menopause, but the study supports the notion that apoM concentrations could be dependent on hormone levels, as the positive correlation with age is seen for women only. Few studies have addressed the effect of sex hormones on apoM, but it has been shown that estrogen upregulates apoM expression in vitro and in vivo in rats (44). This suggests that plasma apoM concentrations should be higher in pre- than postmenopausal women, which was not the case in the present study. Further, we did not find any correlation between levels of estrogen and apoM (data not shown). Thus, present findings suggest that the difference in apoM levels between groups may not be related to estrogen alone, but rather to a combination of age, hormonal status, and other unknown variables.

Plasma S1P was similar in pre- and postmenopausal women, causing the S1P/apoM ratio to be significantly lower in postmenopausal women. A recent study found that apoM without S1P did not have anti-inflammatory properties (41); this further supports the notion that the higher apoM concentrations in postmenopausal women do not necessarily provide an atheroprotective effect as the S1P concentration did not differ between the two groups. This finding is in contrast to an earlier study which found that plasma S1P in premenopausal women. The study found S1P to be negatively correlated with age in both men and women (22). A disadvantage of the study is a large age difference between the subjects (~30 years), and lack of follow up on the same subject before, during, and after postmenopausal transition. While the Copenhagen Women Study neither is a prospective study, it has the strength of a minimal age difference (~4 years) between the two groups. In the future, it would be relevant to examine the effect of menopause on the apoM/S1P axis by following the same cohort throughout the menopausal transition since the pre-menopausal women in the present study could be at varies pre-transitional ages.
The training intervention increased plasma apoM and plasma S1P. Importantly, the S1P/apoM ratio did not change in contrast to the menopause-related changes, implying that the training-induced increase is different from and possibly renders a more atheroprotective profile than the menopause-induced changes. There are at least two possible explanations for the post-training increase in apoM. First, it is possible that the exercise-induced changes can be explained by changes in plasma lipids. The general consensus is that exercise training increases HDL-C in healthy adults, providing an atheroprotective effect (13, 29). However, previous findings in postmenopausal women have shown that exercise training decreases TC and LDL-C without changing HDL-C (4), which is in accordance with the present findings. Thus, it does not seem likely that the post-training increases in apoM and S1P can be explained solely by changes in plasma lipids.

Another possible explanation could be that training also lowered plasma insulin during the oral glucose tolerance test (31) which could lead to an increase in plasma apoM as insulin inhibits the expression of apoM through a Foxa2-mediated mechanism (46). The increase in S1P with training agrees with a previous study showing that plasma S1P was 37% higher in endurance trained (average experience of 4.3±1.7 years of long distance running) than in untrained healthy, young males (2). However, another study found no difference in plasma S1P between endurance-trained athletes and obese, sedentary controls (3). The reason for this discrepancy between studies is unclear, but the variation may be due to differences in study setups, including gender, age, and duration of the training period.

Potential Clinical Value – Currently, there is a lack of mechanistic understanding of changes that occur during the menopausal transition. Also, few intervention studies have been conducted addressing the effects on plasma apoM levels in humans. One study has shown that 8 weeks of statin treatment decreases apoM by 7% (26). In the present study the range of changes are comparable observed between pre-and postmenopausal women. However, the higher apoM in postmenopausal women without a concomitant increase in S1P may contribute to understanding how previously atheroprotective apoM-containing HDL particles can lose their anti-atherogenic and endothelium-protective potential in the menopausal transition. The finding that postmenopausal women have a lower S1P/apoM ratio gives rise to the question of whether S1P analogues – which are currently released on the market for treatment of multiple sclerosis (43) – can be...
beneficial in treating risk factors for endothelial dysfunction and atherosclerosis related to the postmenopausal phase. Finally, exercise increased both plasma apoM and S1P in pre-and postmenopausal women. It is likely that an extended period of training could increase the plasma apoM/S1P levels further. To maintain a high level of apoM – and S1P containing HDL particles could be of clinical value due to its anti-atherogenic and endothelial protective value.
Acknowledgements

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**Figure legends**

**Figure 1. Effect of menopausal status and exercise training on plasma apoM and S1P concentrations.**

(A) Plasma apoM concentrations in pre- (n=36) and postmenopausal (n=37) women. Changes between groups were analyzed using Two-way repeated measures ANOVA: menopause*exercise, P>0.05; effect of menopause, P<0.0001; effect of training, P<0.05. (B) Plasma S1P concentration in pre- (n=30) and postmenopausal (n=30) women. Changes between groups were analyzed using Two-way repeated measures ANOVA: menopause*exercise, P>0.05; effect of menopause, P<0.0001; effect of training, P<0.04. Only women with measurements before and after training have been included. *P<0.05: significantly different from premenopausal. #P<0.05: significantly different from before training. ApoM, apolipoprotein M; S1P, sphingosine-1-phosphate.

**Figure 2. Effect of menopausal status and exercise training on plasma S1P/apoM and apoM/HDL-C ratios.** (A) Plasma S1P/apoM ratio in pre- (n=30) and postmenopausal (n=30) women. Changes between groups were analyzed using Two-way repeated measures ANOVA: menopause*exercise, P>0.05; effect of menopause, P<0.001, effect of training P>0.05. (B) Plasma apoM/HDL-C ratio in pre- (n=34) and postmenopausal women (n=36). Changes between groups were analyzed using Two-way repeated measures ANOVA: menopause*exercise, P>0.05; effect of menopause, P>0.05, effect of training P=0.05. Only women with measures before and after training have been included. *P<0.05: significantly different from premenopausal. ApoM, apolipoprotein; S1P, sphingosine-1-phosphate; HDL-C, high-density lipoprotein cholesterol.

**Figure 3. Linear correlation between plasma apoM and lipid concentrations.** Correlations between plasma apoM and TC (A, B), HDL-C (C, D), LDL-C (E, F), and triglyceride (G, H) concentrations in all women before training (n=73) and after training (n=72). Data were evaluated by Pearson’s correlation. ApoM, apolipoprotein; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
Figure 4. Linear correlations between plasma apoM and S1P concentrations. (A) Pre- and postmenopausal women before training (n=68). (B) Pre- and postmenopausal women after training (n=64). Data were evaluated by Pearson’s correlation. ApoM, apolipoprotein M; S1P, sphingosine-1-phosphate.
### Table 1: Lipid levels (modified from Mandrup et al. (40))

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<td>(n=36)</td>
<td>(n=36)</td>
<td>(n=37)</td>
<td>(n=36)</td>
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<td>TC (mmol/l)</td>
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<td>5.64±0.7*</td>
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<td>HDL-C (mmol/l)</td>
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<td>1.70±0.4</td>
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<td>2.03±0.4*</td>
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<tr>
<td>LDL-C (mmol/l)</td>
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<td>3.26±0.6*</td>
<td>3.17±0.6*#</td>
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<td>TRIG (mmol/l)</td>
<td>0.83 (0.6-1.1)</td>
<td>0.86 (0.7-1.1)</td>
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Parametric data are given as mean±SD, and non-parametric data are given as median (25-75 percentiles). Data are for all available measurements. Changes between groups were analyzed using Two-way repeated measures ANOVA. Only women with measurements before and after training were included in the two-way repeated measures ANOVA. No significant interactions for menopause*exercise were found (P>0.05) for any parameter. *P<0.05: significantly different from premenopausal. #P<0.05: significantly different from before training. TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TRIG, triglycerides.
Figure 1: Yafasova et al.
Figure 3, Yafasova et al.
**Figure 4. Yafasova et al.**

(A) Scatter plot showing plasma apoM (μmol/l) against plasma S1P (μmol/l) before training in premenopausal and postmenopausal women. The plot includes data points for 68 women. The correlation coefficient is $r = 0.07$. There is no significant difference in the correlation ($P > 0.05$).

(B) Scatter plot showing plasma apoM (μmol/l) against plasma S1P (μmol/l) after training in premenopausal and postmenopausal women. The plot includes data points for 64 women. The correlation coefficient is $r = 0.17$. There is no significant difference in the correlation ($P > 0.05$).