**Circulating amyloid beta 1-40 is associated with increased rate of progression of
atherosclerosis in menopause: A prospective cohort study**

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ABSTRACT

Background. Accumulating evidence suggests that circulating amyloid-beta 1-40 (A β 1-40), a pro-atherogenic aging peptide, may serve as a novel biomarker in cardiovascular disease (CVD). We aimed to explore the role of plasma A β 1-40 and its patterns of change over time in atherosclerosis progression in postmenopausal women, a population with substantial unrecognized CVD risk beyond traditional risk factors (TRFs).

Methods. In this prospective study, A β 1-40 was measured in plasma by ELISA and atherosclerosis was assessed using carotid high-resolution ultrasonography at baseline and after a median follow-up (FU) of 28.2 months in 152 postmenopausal women without history or symptoms of CVD.

Results. At baseline, high A β 1-40 was independently associated with higher carotid bulb intima-media thickness (cbIMT) and the sum of maximal wall thickness in all carotid sites (sumWT) ($p<0.05$). A β 1-40 levels increased over time and were associated with decreasing renal function ($p<0.05$ for both). Women with a pattern of increasing or persistently high A β 1-40 levels, presented accelerated progression of cbIMT, maximum carotid wall thickness and sumWT ($p<0.05$ for all) after adjustment for baseline A β 1-40 levels, TRFs and renal function.

Conclusions. In postmenopausal women, a pattern of increasing or persistently high A β 1-40 was associated with the rate of progression of subclinical atherosclerosis irrespective of its baseline levels. These findings provide novel insights into a link between A β 1-40 and atherosclerosis progression in menopause and warrant further research to clarify the clinical value of monitoring its circulating levels as an atherosclerosis biomarker in women without clinically overt CVD.

Keywords: amyloid, atherosclerosis, menopause, aging, women's health

Summary table
What is known on this topic?
A β 1-40 is involved in multiple stages of atherogenesis
Prevalence of atherosclerosis is unexpectedly high in menopause
What does this paper add?
A β 1-40 is associated with the presence of atherosclerosis in menopause
A pattern of increasing or persistently high A β 1-40 levels is associated with the rate of progression of atherosclerosis in menopause

Introduction

Despite continuously evolving algorithms aiming to accurately estimate risk in primary cardiovascular disease (CVD) prevention ¹⁻⁴, there is still considerable unrecognized CVD risk beyond traditional risk factors (TRFs) and TRF-based risk scores ³⁻¹⁰. In postmenopausal women there is a clear increase in the incidence of adverse CV events ¹¹. Beyond aging, the alternating hormonal profile and the apparent androgenicity following the time of ovarian senescence¹², may also contribute to this augmented incidence ^{11,13-15}. We and others have previously shown that postmenopausal women classified at low or moderate risk using TRF-based risk scores such as the HeartScore or the Framingham risk score, have unexpectedly high prevalence of subclinical atherosclerosis ^{5,6,10,16} similar to that of age and risk factor-matched men ¹⁷. Thus, there is need for recognition of new biomarkers in postmenopausal women that would improve accuracy of estimated CVD risk in this population. Amyloid-beta peptides are proteolytic fragments of the amyloid precursor protein (APP) cleaved by β - and γ -secretases ¹⁸ involved in aging ¹⁹ with pro-inflammatory and pro-atherosclerotic properties ^{18,20}. We have previously demonstrated that circulating levels of amyloid-beta 1-40 (A β 1-40) are associated with arterial stiffening in young adults ²¹ and with atherosclerosis in the general population and in patients with coronary heart disease (CHD) ^{21,22}. Importantly, circulating levels of A β 1-40 are associated with increased mortality identifying residual risk beyond established TRFs in patients with stable CHD ²¹ and with non-ST-segment elevation acute coronary syndrome (NSTE-ACS) ²³. These data indicate that A β 1-40 is involved in the continuum of atherosclerotic disease and vascular aging and support a role of this peptide as a possible risk biomarker in patients with established CHD. However, its role in individuals without established CVD needs further investigation. More importantly, as an aging peptide ¹⁸ higher circulating A β 1-40 levels are observed with increasing age, but the clinical relevance of patterns of A β 1-40 change over time in CVD is unknown. In specific, the value of measuring and longitudinally monitoring A β 1-40 plasma levels in postmenopausal women, a population with inherent difficulties in the precise estimation of CVD risk partly due to unrecognized yet menopause-specific risk factors, has not been explored. Therefore, we hypothesized that increased baseline plasma levels of A β 1-40

and a pattern of increasing or persistently high A β 1-40 levels would be associated with the extent and the rate of progression of carotid atherosclerosis in postmenopausal women without established CVD.

Materials and methods

Participants

In this retrospectively designed study, postmenopausal women without clinically overt CVD were recruited from the Menopause Clinic of the Aretaieion Hospital of the National and Kapodistrian University of Athens. The population of this study is part of an on-going registry in which we evaluate clinical history, metabolic, hormonal and vascular characteristics using non-invasive vascular studies at baseline and at follow-up visits every 1 to 3 years thereafter, as previously described^{12,24,25}. Clinically overt CVD was defined as previous ACS, coronary revascularization, other arterial revascularization procedures, stroke and transient ischemic attack, aortic aneurysm and peripheral artery disease¹. All subjects (n=152) who provided written consent for baseline and follow-up measurements regarding ultrasonography measurements and venipuncture were eligible for participation. Median follow-up was 28.2 months, with minimum follow-up time 12 months. Prior to recruitment, patients were evaluated according to the standard protocol in the Menopause clinic, including mammography, PAP smear and transvaginal sonography, thyroid, liver and renal function, blood coagulation tests and bone densitometry. Inclusion and exclusion criteria and the detailed protocol of assessments are described in detail in the supplementary file²⁶. Study procedures were identical in both baseline and follow-up visits. The current study was conducted according to the principles of the Declaration of Helsinki. The Local Ethics Committee of Aretaieio University Hospital approved the study's protocol.

Carotid Ultrasonography

IMT was measured at three paired segments, in the right and left common carotid artery (cc), carotid bifurcation (cb), and internal carotid (ic) artery from 3 transducer angles using B-mode ultrasound imaging (14.0-MHz multifrequency linear array probe, Vivid 7 Pro, GE Healthcare, Chicago, IL). Three measurements of the maximal IMT in the far wall of each segment were averaged and the average IMT of the three segments was calculated for each of the two carotid arteries. The average value of right and left carotid IMT from all segments was defined as avgIMT. The scans were digitally recorded for offline analysis, which was performed by a single operator blinded to the CV risk profiles of the participants. Coefficient of variation for avgIMT for two consecutive measurements in the same person was 6.5%. Plaques were defined as a clearly identified area of focally increased IMT greater than 1.5 mm or a focal structure encroaching into the carotid lumen 50% of the surrounding IMT value ²⁷. The maximum height of plaques was measured and recorded. Carotid atherosclerotic burden was assessed using the following markers: 1. AvgIMT, 2. Maximal wall thickness from all six carotid sites being either an atherosclerotic plaque or IMT (maxWT) and 3. Sum of wall thickness (sumWT) defined the algebraic sum of maximum wall thickness from all 6 carotid sites.

Laboratory variables and Biomarker Testing

Fasting blood samples were acquired with venipuncture. Plasma and serum samples were stored at -80°C until procession. Concentrations of A β 1-40 were measured in ethylenediaminetetraacetic acid (EDTA)-plasma samples. A well-characterized enzyme-linked immunosorbent assay (ELISA) (Biosource/Invitrogen, California, USA) was used, as previously described ²⁸. The intra- and inter-assay coefficient of variance of the ELISA measurements was reported to be less than 8% ²⁹ and the minimum detectable concentration of human A β 1-40 was <6 pg/ml (Biosource/Invitrogen, California, USA). All measurements were performed by experienced personnel who were blinded to patients' characteristics. A uniform algorithm was followed for blood collection and plasma preparation for all patients in previously unfrozen samples. In order to minimize systematic error

between the two time-points, baseline and follow-up samples from each participant were analyzed simultaneously in pairs in the same ELISA plate. Mean storage time at -80°C for the plasma probes used in this study to measure Aβ1-40 was 77.2±31.4 (SD) months. Aβ1-40 is a stable molecule in long-term storage settings³⁰⁻³². Routine biochemistry and hormone methods are described in the supplementary file.

Statistical Analysis

We used multivariable logistic regression analysis to examine the independent association of increased carotid wall markers with circulating levels of Aβ1-40 at baseline after controlling for a pre-specified set of biologically plausible confounders. Subsequently, we employed two-level linear mixed models with random effects for each participant and unstructured variance-covariance matrix to assess the effect of fluctuations in Aβ1-40 levels between baseline and follow up on longitudinal changes in carotid indices. To ensure normal distribution of dependent outcomes in the linear mixed model analysis, we employed a natural log transformation³³. Missing values in parameters used in these models are summarized in **Supplementary Table S1**. An interaction term between group classification of Aβ1-40 changes and time was included in the linear mixed models to capture a differential effect of each trajectory of Aβ1-40 changes on the progression of carotid atherosclerosis. Group classification was defined as previously described^{34,35}: 1. Increasing or persistently high Aβ1-40 group: those who increased tertile of Aβ1-40 or their levels remained stable in the highest tertile, 2. Stable low-intermediate Aβ1-40 group: Those who remained stable at low or middle tertile and 3. Decreased Aβ1-40 group: Those who decreased their Aβ1-40 levels to a lower tertile. Statistical analysis was conducted with STATA package, version 12.1 (StataCorp, College Station, Texas USA). Details about the statistical analysis as well as power calculations are provided in the Supplementary file.

Results

Population characteristics

As shown in **Table 1**, BMI, waist circumference, glomerular filtration rate (GFR), prevalence of hypertension and hyperlipidemia increased at follow-up. A β 1-40 plasma levels also significantly increased at follow-up ($p<0.001$). No difference was observed in drug treatment during follow-up (**Supplementary Table S2**). Regarding carotid atherosclerosis, all markers of carotid wall thickness increased between the two visits (**Supplementary Table S3**).

Determinants of baseline and longitudinal changes of A β 1-40 plasma levels

A β 1-40 at baseline was independently associated with dehydroepiandrosterone-sulfate (DHEAS) serum levels as shown in **Supplementary Tables S4, S5**. In regard to longitudinal changes of A β 1-40, among changes in several possible confounders recorded at baseline and follow-up, aging of the population (as reflected by increased duration of follow-up) and worsening renal function were the only independent predictors of changes in A β 1-40 levels over time (**Table 2**).

A β 1-40 plasma levels are associated with carotid atherosclerosis at baseline

Baseline A β 1-40 as a continuous variable was associated with baseline sumWT (adjusted association OR=1.68, per SD increase of A β 1-40, 95% CI 1.11-2.46, $p=0.014$), baseline carotid bulb IMT (adjusted association OR=1.52 per SD increase, 95% CI 1.11-2.15, $p=0.039$) and marginally with avgIMT (OR=1.52 per SD increase, 95% CI 0.989- 2.12, $p=0.056$ for multi-adjusted model).

Furthermore, patients with A β 1-40 plasma levels at the highest tertile had significantly higher cbIMT (OR=2.62, 95% CI 1.12-6.15, $p=0.027$), avgIMT (OR=2.41, 95% CI 1.03-5.68, $p=0.044$) and sumWT (OR=2.68, 95% CI 1.15-6.29, $p=0.023$) (**Table 3**). These associations remained statistically significant after adjustment for age, smoking, hyperlipidemia, hypertension, diabetes mellitus (DM) and GFR (**Table 3**). Similar results were observed in an alternate model including adjustment for SBP and use of anti-hypertensive drugs in baseline (presented in **Supplementary Table S2**) instead of hypertension. Moreover, in the subgroup of women with hormone measurements, additional

adjustment for estradiol (E2), sex hormone binding globulin (SHBG), testosterone or DHEAS did not alter the observed associations between A β 1-40 and markers of atherosclerosis (**Supplementary Table S6**).

A β 1-40 plasma levels and progression of carotid atherosclerosis.

Figure 1A depicts dynamic changes in plasma A β 1-40 levels over time according to the 3 pre-specified groups as described in the statistical methods section. Women with the increasing or persistently high pattern of A β 1-40 presented accelerated progression in cbIMT ($p=0.019$) (**Figure 1B**), adjusted for baseline age, duration of follow-up, change in smoking status, hypertension, hyperlipidemia, DM and GFR and baseline A β 1-40 (core model, **Table 4**). Similarly, an increasing or persistently high pattern of A β 1-40 was independently associated with higher rate of progression of maxWT (**Figure 1C**) and sumWT (**Figure 1D**) using the core model (**Table 4**). A relative increase in A β 1-40 plasma levels by 10% from baseline to follow-up (calculated as the ratio follow-up - baseline levels / baseline levels), sumWT increased by 0.1mm (95% CI 0.01-0.17mm, $p=0.047$). Replacing hypertension with SBP and use of anti-hypertensive drugs in baseline and follow-up (presented in **Supplementary Table S2**) in the multivariable core model did not substantially change these results except of the maxWT association which shifted to marginal significance ($p=0.073$). Adjustment for sex hormones (testosterone, SHBG or E2) or DHEAS did not alter these associations (**Supplementary table S7**). Finally, A β 1-40 plasma levels at baseline were associated with the rate of change in maxWT (by linear mixed models analysis, mean rate of increase 0.586mm, 95% CI 0.055-1.11, $p=0.047$, per SD increase of baseline A β 1-40).

Discussion

In the current study, we investigated the role of A β 1-40 plasma levels in menopause showing that A β 1-40 was associated with increased prevalence, extent, and progression of carotid atherosclerosis irrespective of women's hormone profile. This is the first evidence indicating that a pattern of increasing or persistently high A β 1-40 plasma levels is associated with accelerated rate of progression

of atherosclerosis over time in humans independently of its baseline levels, supporting the hypothesis of its pivotal role in the continuum of human atherosclerotic disease¹⁸. Thus, these findings provide proof of concept that this peptide is involved in atherosclerosis and its prognostic value in postmenopausal women, a population with substantial unrecognized CVD risk beyond TRFs, should therefore be further investigated. Menopause is associated with increased incidence of atherosclerosis and adverse CV events^{10,11,13-15,17}. However, by using only total CV risk scores based on TRFs, risk may be underestimated in postmenopausal women^{5,6,10,15-17,36-38}. This may be attributed to menopause-specific risk factors and differential associations of TRFs with triggering of adverse events^{15,39,40}. To that end, the prognostic value of novel risk factors such as hsCRP has been tested in modified risk scores in women leading to improved risk stratification³⁶. Despite these efforts, no widely accepted risk factor profile accurately predicting risk in postmenopausal women, has been established to date^{1,2}. Thus, recognition of novel pathways mediating atherosclerotic disease and of associated molecules that could serve as risk biomarkers in postmenopausal populations is clinically required to improve discrimination of high-risk women who are in need for aggressive preventive measures. A β 1-40 is a peptide initially recognized as the main molecule involved in the pathophysiology of cerebral amyloid angiopathy (CAA) and in conjunction with A β 1-42 as a key player in Alzheimer's disease¹⁸. Interestingly, A β 1-40 is adversely associated with aging, CAA and arterial disease¹⁸. A β 1-40 is implicated in multiple processes of the atherosclerotic disease including endothelial cell activation, vascular smooth muscle cell contractility, monocyte adhesion, migration and transformation, platelet activation and aggregation and plaque destabilization and rupture¹⁸ indicating that this peptide is implicated in multiple steps of the continuum of atherosclerosis. The role of A β 1-40 in arterial disease has been recently highlighted in clinical studies from our group and others⁴¹, showing that A β 1-40 plasma levels are linked with the presence and extent of atherosclerosis in the general population and in CAD patients²¹⁻²³. The results of these studies imply that A β 1-40 may play a critical role in the atherosclerosis continuum also in humans, starting from early functional and structural changes such as increased IMT and arterial stiffening^{21,22} to advanced atherosclerotic disease and their clinical sequelae^{21,23}. However, the association of A β 1-40 with atherosclerosis progression over time, which would provide a more solid translational support of the experimental evidence, was unknown.

In specific, although the regulation of A β 1-40 metabolism is multi-factorial, including degradation enzyme activity, renal and hepatic function, apolipoprotein E, drug treatment and sleep deprivation aging is a major determinant of its circulating levels¹⁸. Consequently, on average A β 1-40 plasma levels increase over time^{21,42,43} in humans, but the clinical relevance of A β 1-40 patterns of change was not explored. Herein we demonstrate that a pattern of increasing or persistently high levels of A β 1-40 over time is associated with accelerated progression of carotid atherosclerosis. Importantly, this association was independent of baseline A β 1-40 which in turn presented less strong associations with atherosclerosis progression. Thus, this finding provides a possible new clinical role for monitoring A β 1-40 plasma levels, suggesting that A β 1-40 is actively implicated in the atherosclerotic sequel. Further, we demonstrate that the main determinants of A β 1-40 plasma levels in these women is aging of the cohort and declining renal function. Of interest, DHEAS serum levels, a weak androgen produced mainly by the adrenals and considered a marker of senescence^{44,45}, were also independently associated with A β 1-40 supporting the properties of this peptide as a surrogate marker of systematic aging in menopause. The finding regarding renal function is in agreement with well-established evidence indicating that A β 1-40 clearance is largely dependent on renal dysfunction^{18,21} as well as previously reported associations between A β 1-40 circulating levels and renal function⁴⁶⁻⁴⁸ in other populations^{18,21-23}. These findings suggest that A β 1-40 might serve as a marker of declining renal function in postmenopausal women, warranting further investigation to confirm this hypothesis. On the other hand, a role of renal dysfunction as a contributor to the association of A β 1-40 with accelerated atherosclerosis was not evident in our population since our findings were independent of GFR. This is in accordance to previous findings linking A β 1-40 with markers of subclinical cardiac²² and vascular dysfunction²¹ as well as with mortality^{21,23}, irrespective of renal function, supporting a direct role of A β 1-40 in CVD. Of note, the association of A β 1-40 with atherosclerosis progression was not altered after adjusting for baseline E2, SHBG or testosterone, suggesting that this association is not driven by the hormonal milieu of menopause. However, given that a limited number of hormone level measurements was available, the results of this specific analysis should be further validated. Overall, our findings provide novel insight on a link between A β 1-40 and accelerated atherosclerosis in postmenopausal women, which was not confounded by baseline menopause-specific characteristics or

by aging or renal function known to affect its metabolism. Importantly, several interventions can affect A β 1-40 and its metabolism such as lifestyle modifications, off-target effects of routinely prescribed cardiovascular drugs¹⁸ as well as drugs specifically targeting A β 1-40⁴⁹. Thus, these results may serve as proof of concept to further assess the clinical utility of A β 1-40 plasma levels as a cardiovascular risk biomarker in this population.

Some limitations should be acknowledged. The design of this study being a non-interventional observational study does not permit inference of causal relationships. Furthermore, in accordance to previous study designs^{34,35} we assessed change of A β 1-40 at two time points. However, the inclusion of only one follow-up visit may have limited the availability of some time-dependent data. Finally, the strengths and limitations of using carotid atherosclerosis markers as surrogate CVD outcomes in this study should be discussed. Assessing subclinical atherosclerosis provides several advantages including direct *in vivo* mechanistic insight on the process of atherosclerosis and an indirect prognostic estimation of cardiovascular risk⁴. Of note, we evaluated the combination of carotid IMT and atheromatous plaques at all sites instead of common carotid IMT⁵⁰ alone, since it reflects total carotid atherosclerotic burden, is incrementally associated with CV events^{51,52}, jointly improves risk stratification over the Framingham risk score⁵³⁻⁵⁵ and assessment of carotid plaque burden may guide correct statin allocation⁵⁶. Thus, our findings suggest that patterns of increasing or persistently high A β 1-40 in menopause may be also associated with adverse cardiovascular events. However, surrogate markers cannot replace prospective analyses of adverse cardiovascular outcomes and therefore epidemiological studies are warranted to further assess this hypothesis in postmenopausal women.

In conclusion, the present study demonstrates that a pattern of increasing or persistently high A β 1-40 levels is associated with the presence, extent, and rate of progression of subclinical atherosclerosis in postmenopausal women. Considering that postmenopausal women remain a population with substantial unrecognized CVD risk beyond TRFs, and that circulating A β 1-40 provides prognostic and reclassification value in other populations, these findings suggest that A β 1-40 is implicated in the atherosclerosis continuum in menopause and justify further research for its clinical prognostic role in this population.

Conflict of interest statement

Nothing to declare.

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Figure 1. Changes of A β 1-40 plasma levels by their patterns of change from baseline to follow-up (A). P-value is derived from linear mixed model analysis for changes in A β 1-40 levels after controlling for age, changes in hypertension, hyperlipidemia, smoking, DM, GFR and duration of follow-up. Women with the increasing or persistently high A β 1-40 pattern presented higher rate of progression of carotid bulb IMT (B), maximal wall thickness (C) and sum of maximal wall thickness (D) compared to women with stable or decreasing levels of A β 1-40. P-value in B,C and D is derived from linear mixed model analysis for association of alterations in A β 1-40 levels with carotid indices' changes across follow up after controlling for age, changes in hypertension, hyperlipidemia, smoking, DM, GFR, duration of follow-up and baseline A β 1-40. Fu=follow-up; IMT=intima media thickness; Cb=carotid bulb; Max=maximal WT from all 6 carotid sites; Sum WT=sum of all max wall thickness from all 6 sites; GFR=glomerular filtration rate; DM=diabetes mellitus.

Table 1. Descriptive characteristics of the study's population

	Baseline	Follow-up	p-value
Age	56 (5.3)	58.3 (5.5)	<0.001
Years of menopause	8.0 (6.0)	10.68 (7.7)	<0.001
Smoking	39 (25.6)	38 (25)	0.999
BMI (kg/m²)	26.7 (4.3)	27.2 (4.7)	0.037
Diabetes Mellitus	4 (2.6)	9 (5.9)	0.063
Hypertension	30 (19.7)	44 (28.9)	<0.001
Hyperlipidemia	69 (45.3)	96 (63.1)	<0.001
Waist circumference (cm)	87.9 (11.1)	91.1 (11.4)	<0.001
Systolic BP (mmHg)	120.3 (17.6)	119.3 (17.2)	0.39
Diastolic BP(mmHg)	73.9 (9.1)	72.1 (10.9)	0.052
Total cholesterol (mg/mL)	218.5 (40.5)	214.4 (33.4)	0.161
LDL-C (mg/mL)	135.5 (36.2)	134.8 (31.3)	0.926
Triglycerides (mg/mL)	92.9 (36.9)	90.8 (33.8)	0.552
HDL-C (mg/mL)	61.9 (15.4)	61.8 (13.8)	0.933
Glucose (mg/dL)	93.4 (8.5)	93.0 (9.7)	0.564
Insulin (μIU/mL)	7.1 (4.8)	7.7 (4.3)	0.334
HbA1c (%)	5.5 (0.5)	5.6 (0.5)	0.391
CRP (mg/L)	0.3 (0.4)	0.3 (0.6)	0.663
GFR (mL/min/m²)	87 (13.7)	89.4 (14.0)	0.038
SHBG (nmol/L)	68.9 (34.1)		
Testosterone (ng/mL)	0.42 (0.3)		
DHEAS (μg/dl)	247.7 (343.2)		
E2 (pg/mL)	22.5 (13.0)		
Aβ1-40 (pg/mL)	17.2 (10.7)	23.4 (15.8)	<0.001

BMI=body mass index; BP=blood pressure; LDL-C=low-density lipoprotein cholesterol; HDL-C=high-density lipoprotein cholesterol; HbA1c=glycated hemoglobin; CRP=C-reactive protein; GFR= glomerular filtration rate; SHBG=sex hormone-binding globulin, n=114; testosterone n=125; DHEAS=dehydroepiandrosterone-sulfate, n=110; E2=estradiol, n=90. Continuous variables are presented as mean (SD) and nominal as count (absolute percentages), p-values are derived from paired samples Student's T Test or the Wilcoxon Rank Sum Test for continuous variables and the McNemar's chi squared test for categorical variables.

Table 2. Univariable and multivariable logistic regression analysis showing determinants of A β 1-40 changes.

	UNIVARIABLE		MULTIVARIABLE	
	OR (95% CI)	P-Value	OR (95% CI)	P-Value
<u>Age (years)</u>	0.90 (0.72/1.11)	0.392	0.87 (0.63/1.19)	0.366
<u>Years of menopause (years)</u>	1.0 (0.75/1.34)	0.932	1.38(0.93/2.09)	0.111
<u>Duration follow-up (months)</u>	1.22 (0.85/1.48)	0.38	1.45 (1.02/2.17)	0.044
<u>New-onset Smoking</u>	1.81 (0.615/5.33)	0.281	1.79 (0.551/5.79)	0.334
<u>New-onset Hyperlipidemia</u>	1.36 (0.615/3.02)	0.446	1.57 (0.656-3.76)	0.311
<u>New-onset Hypertension</u>	0.965 (0.348/2.68)	0.945	1.42 (0.428-4.69)	0.568
<u>New-onset Diabetes mellitus</u>	1.08 (0.219/5.29)	0.927	0.903 (0.173-4.71)	0.903
<u>Δ GFR (ml/min/m²)</u>	0.65 (0.47/0.92)	0.0118	0.60 (0.42-0.86)	0.005

Odds ratios refer to the probability to be categorized at the pattern of increasing or persistently high A β 1-40 group per SD increase for continuous variables or for new onset of a cardiovascular risk factor. Bold indicates CI=confidence intervals. GFR=glomerular filtration rate; Δ GFR was calculated as GFR at follow-up – GFR at baseline.

Table 3. Associations between baseline Aβ1-40 levels and carotid disease indices				
	cbIMT (mm)	avgIMT (mm)	sumWT (mm)	maxWT (mm)
	OR (95% CI)			
Highest tertile of baseline Aβ1-40 (univariable)	2.62 (1.12/6.15) p=0.027	2.41 (1.03/5.68) p=0.044	2.68 (1.15/6.29) p=0.023	1.75 (0.72-4.28) p=0.23
	OR (95% CI)			
*Highest tertile of baseline Aβ1-40 (multivariable)	3.01 (1.18-7.63) p=0.021	2.65 (1.05-6.72) p=0.039	3.52 (1.35-9.17) p=0.001	1.85 (0.70-4.89) p=0.22
<p>ORs represent the odds for a woman who is classified at the highest tertile of Aβ1-40 to have increased carotid wall thickness (ie at the highest tertile)</p> <p>*Adjusted for age, smoking, hypertension, hyperlipidemia, diabetes mellitus and glomerular filtration rate. Bold indicated statistical significance, which was set at the level of p-value <0.05.</p> <p>All carotid indices refer to highest tertile. IMT=intima media thickness; Cb=carotid bulb; Avg=average carotid IMT from all 6 sites; WT=wall thickness; Max=maximal WT from all 6 carotid sites; Sum WT= sum of all max WT from all 6 sites. Significant associations between highest tertile of Aβ1-40 and increased carotid indices are highlighted in bold</p>				

Table 4. Linear mixed model analysis showing the association between patterns of change in A β 1-40 levels with changes in carotid indices across follow-up

	cbIMT (mm)	avgIMT (mm)	sumWT (mm)	maxWT (mm)
Coefficient (95% CI)				
Increasing or persistently high A β 1-40 pattern*time (univariable)	0.09 (0.001/0.2) p=0.047	0.03 (-0.03/0.08) p=0.317	0.136 (0.05/0.22) p=0.001	0.098 (0.0001/0.2) p=0.0497
*Coefficient (95% CI)				
* Increasing or persistently high A β 1-40 pattern*time (multivariable)	0.12 (0.02/0.21) P=0.019	0.04 (-0.02/0.10) P=0.146	0.147 (0.058/0.236) P=0.001	0.112 (0.006/0.22) P=0.039
Coefficient and P-value correspond to the interaction term of group classification (increased tertile at follow-up or <u>persistently</u> at high tertile of A β 1-40 versus <u>decreasing or stable at lower</u> tertiles A β 1-40 pattern at follow up)*time				
Coefficients are derived after natural log transformation of the outcome variable (<u>carotid indices</u>)				
*Adjusted for age, changes in hypertension, hyperlipidemia, smoking, diabetes mellitus, glomerular filtration rate, duration of follow-up and baseline A β 1-40. Bold indicate statistical significance, which was set at the level of p-value <0.05.				
All carotid indices refer to highest tertile. IMT=intima media thickness; Cb=carotid bulb; Avg=average carotid IMT from all 6 sites; WT=wall thickness; Max=maximal WT from all 6 carotid sites; Sum WT= sum of all max WT from all 6 sites.				

Supplementary material

Circulating amyloid beta 1-40 is associated with increased rate of progression of atherosclerosis in menopause: A prospective cohort study

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Methods

Participants

Inclusion criteria was endometrial thickness of ≤ 5 mm (sonographically assessed). Exclusion criteria were surgical menopause and current or previous exposure to hormone therapy, gynecological malignancy, premature ovarian failure, thromboembolism, untreated thyroid dysfunction and women with adherence and retention concerns (e.g. alcoholism, IV drug users). The menopausal status was defined as FSH > 25 mIU/mL and estradiol < 50 pg/mL after 12 consecutive months without menses, as previously described [1].

Protocol procedures

Study procedures were identical in both baseline and follow-up visits. A detailed medical history was recorded for every subject using case-report forms, regarding demographics and lifestyle parameters, cardiovascular risk factors (CVRF), obstetrical and gynecological history. Anthropometric measurements included body weight, height, waist and hip circumference as well as calculated body-mass index (BMI). Waist circumference was measured at the midpoint between the bottom of the rib cage and the top of the lateral border of the iliac crest during minimal respiration. Hip circumference was measured approximately 10 cm below the lateral border of the iliac crest. CVRFs were defined as follows:

Hypertension, systolic blood pressure (SBP) ≥ 140 mmHg and or diastolic blood pressure (DBP) ≥ 90 mmHg, after two office measurements 2 minutes apart during both of the women's visits at the Menopause clinic and Vascular Laboratory or history of treatment for hypertension. Hyperlipidemia, according to previous definition for this population, as history of treatment for hyperlipidemia or total blood cholesterol level > 200 mg/dL or LDL-C > 160 mg/dL in women with < 2 CVRFs or LDL-C > 130 mg/dL in women with ≥ 2 CVRFs [2].

Diabetes mellitus (DM) was defined as fasting glucose > 126 mg/dl in 2 successive measurements or HbA1C > 6.5 % or reported history or treatment of DM. Current smoking or

cessation <6 months ago were considered as smokers. Carotid ultrasonography was performed in one session, in a quiet, temperature-controlled room, in the supine position, with the head turned 45 degrees from the site being scanned.

Laboratory variables and Biomarker Testing

Serum total cholesterol, triglycerides and HDL-cholesterol (HDL-C) were assessed enzymatically by an autoanalyzer (COBAS-MIRA, Roche Diagnostics Limited, Lewes, East Sussex, UK). The Friedewald equation was used to estimate LDL-cholesterol (LDL-C), ($\text{LDL-C} = \text{total cholesterol} - \text{triglycerides}/5 - \text{HDL-C}$). In a subgroup of women (n=90) sex hormones as follows: estradiol (E2) with Architect i1000 analyzer (Abbott Ireland, Diagnostics Division, Lisnamuck, Longford, Ireland), with an analytical sensitivity of 10 pg/mL and total coefficient of variation (CV %) ranged from 1.9% to 7.1%, Δ^4 -androstenedione (D4) with the enzyme-linked immunosorbent assay kit (IBL “Androstendione ELISA”; IBL, Hamburg, Germany), (CV% 4.7% to 9.7%, analytical sensitivity 2.9ng/dl), total testosterone with the Abbott Architect i1000 analyzer (total CV% 3.1% to 8.0%, analytical sensitivity 0.08 ng/mL), dehydroepiandrosterone (DHEAS) by immunoenzymatic assay (Elecsys 2010 Systems, Roche Diagnostics, Monza, Italy) (CV% 6%), sex hormone-binding globulin (SHBG) concentrations were measured with electrochemiluminescence immunoassay on a Cobas e-411 analyzer (Roche Diagnostics, Mannheim, Germany), (total CV% 2.6% to 5.6%, analytical sensitivity of the assay 0.35nmol/L). These techniques have been previously used by our group [1].

Statistical Analysis

Continuous variables are presented as mean $\pm$ standard deviation or median (interquartile range) for variables not following normal distribution. Nominal variables are expressed as absolute count and percentage values of valid cases (%). Normal distribution of all continuous variables was graphically assessed by histograms and P-P plots. Correlation between A β 1-40 levels and indices of carotid atherosclerosis was evaluated by unadjusted logistic regression.

Differences between the two visits were examined with the paired samples Student's T Test or the Wilcoxon signed-rank test for continuous variables and the McNemar's chi squared test for categorical variables. In the multivariable logistic regression models used to examine the independent association of increased carotid wall markers with circulating levels of A β 1-40 at baseline, the set of biologically plausible confounders were age, smoking, DM, hypertension, hyperlipidemia and glomerular filtration rate (GFR). Simulated data across 500 replications for our sample size (152 women) yielded more than 85% power to detect an avgIMT increase equal or greater than 0.2mm at the end of the follow-up period for the high group as compared with women in the low A β 1-40 group by linear mixed model analysis. Type I error was predefined at 0.05 for power considerations. The STATA module "ipdpower" was used in power calculations. Level of statistical significance was set at $p < 0.05$.

References

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Supplementary Table S1. Absolute number and Percentage of missing values in baseline and follow-up

	Baseline	Follow-up
Age	0 (0%)	0 (0%)
Hypertension	0 (0%)	0 (0%)
Hyperlipidemia	0 (0%)	0 (0%)
Smoking status	0 (0%)	1 (0.6%)
Diabetes mellitus	0 (0%)	0 (0%)
Systolic BP	1 (0.6%)	2 (1.2%)
Diastolic BP	1 (0.6%)	2 (1.2%)
Glucose	6 (3.9%)	18 (11.8%)
GFR	8 (5.2%)	12 (7.8%)
LCCA IMT	0 (0%)	1 (0.6%)
LCB IMT	1 (0.6%)	2 (1.3%)
LICA IMT	0 (0%)	1 (0.6%)
RCCA IMT	0 (0%)	1 (0.6%)
RCB IMT	2 (1.3%)	3 (1.9%)
RICA IMT	1 (0.6%)	1 (0.6%)
Average IMT	1 (0.6%)	1 (0.6%)
Max wall thickness	1 (0.6%)	2 (1.3%)
Sum of max wall thickness	1 (0.6%)	2 (1.3%)
A β 1-40	0 (0%)	0 (0%)

BMI=body mass index; BP=blood pressure; LDL-C=low density lipoprotein cholesterol; GFR= glomerular filtration rate; IMT=intima media thickness; LCCA=left common carotid artery; LCB= left carotid bifurcation; LICA= left internal carotid artery; RCCA=right common carotid artery; RCB= right carotid bifurcation; RICA= right internal carotid artery

Supplementary Table S2. Changes in medical treatments over time

	Baseline	Follow-up	p-value
Statins	29 (19.1)	30 (19.7)	0.999
B-blockers	9 (5.9)	8 (5.2)	0.999
CCBs	3 (1.9)	4 (2.6)	0.999
ACEi	7 (4.6)	7 (4.6)	0.999
ARBs	16 (10.5)	19 (12.5)	0.453
Diuretics (thiazides)	5 (3.2)	7 (4.6)	0.687

CCB=calcium channel blockers; ACEi=angiotensin converting enzyme inhibitors; ARB=angiotensin receptor blockers; Nominal variables are presented as absolute count (valid percentages). P-values are derived from the McNemar's chi-squared test

Supplementary Table S3. Changes in vascular indices over time

	Baseline	Follow-up	p-value
ccIMT (<u>mm</u>)	0.69 (0.12)	0.74 (0.13)	<0.001
cbIMT (<u>mm</u>)	0.83 (0.19)	0.88 (0.19)	0.03
icIMT (<u>mm</u>)	0.65 (0.16)	0.70 (0.17)	0.001
avgIMT (<u>mm</u>)	0.73 (0.12)	0.77 (0.12)	<0.01
maxWT (<u>mm</u>)	1.0 (0.29)	1.1 (0.41)	<0.001
sumWT(<u>mm</u>)	4.4 (0.85)	4.7 (1.0)	<0.001

IMT=intima media thickness; Cc=common carotid; Cb=carotid bulb; ic=internal carotid; Avg=average carotid IMT from all 6 sites; WT=wall thickness; Max=maximal WT from all 6 carotid sites; Sum WT= sum of all max WT from all 6 sites. Continuous variables are presented as mean (SD). P-value derived from Paired Samples T-Student's t-test. Significant (P<0.05) changes in vascular indices are denoted in bold.

Supplementary table S4. Univariable logistic regression analysis of baseline A β 1-40 tertiles (i.e. highest vs lower tertiles) on possible baseline determinants in various baseline parameters

	OR (95%CI)	P-Value
Age (years)	<u>0.95 (0.76/1.24)</u>	0.73
Years of menopause	1.0 (0.94/1.05)	0.90
Hypertension	0.85 (0.36/2.01)	0.71
Hyperlipidemia	0.83 (0.41/1.61)	0.55
Smoking	0.88 (0.40/1.92)	0.74
Diabetes mellitus	2.06 (0.28/15.1)	0.48
GFR (mL/min/m ²)	1.0 (0.76/1.49)	0.94
BMI (kg/m ²)	0.92 (0.65/1.31)	0.65
E2 (pg/mL)	0.83 (0.48/1.29)	0.53
SHBG (nmol/L)	0.71 (0.66/1.40)	0.84
DHEAS (μ g/mL)	0.512 (0.18/1)	0.03
Testosterone (ng/mL)	0.97 (0.67/1.41)	0.87

Odds ratio per comparison of highest versus lower tertiles of A β 1-40 per SD increase for continuous, as contrasted to the reference category for the dichotomous variables. CI=confidence intervals; BMI=body mass index; GFR=glomerular filtration rate; TSH=thyroid stimulating hormone; fT3=free T3; fT4=free T4; E2=estradiol; SHBG=sex hormone-binding globulin; DHEAS=dehydroepiandrosterone-sulfate. P-values are derived from univariable logistic regression analysis. Significant associations (P<0.05) between tertiles of A β 1-40 and possible baseline determinants are highlighted in bold

Supplementary table S5. Multivariable regression analysis of baseline A β 1-40 tertiles (i.e. highest vs lower) on possible baseline determinants

	OR	95% CI		p-value
DHEAS (ng/mL)	0.996	0.993	0.999	0.008
Age	0.91	0.65	1.29	0.608
Smoking	2.807	0.898	8.770	0.076
Hypertension	1.659	0.470	5.853	0.431
Hyperlipidemia	1.493	0.574	3.890	0.412
GFR (mL/min/m ²)	0.83	0.53	1.29	0.399
BMI (kg/m ²)	0.87	0.52	1.43	0.576
DM	14.479	0.547	382.950	0.11

OR= Odds ratio for high baseline (i.e. highest versus lower tertiles) A β 1-40 per SD increase (continuous determinants) or as contrasted to the reference category (dichotomous determinants). CI=confidence intervals; DHEAS= dehydroepiandrosterone-sulfate; GFR=glomerular filtration rate; BMI=body mass index; DM=diabetes mellitus. Significant associations (P<0.05) between baseline tertiles of A β 1-40 and possible baseline determinants are highlighted in bold

Supplementary Table 6. Associations between baseline carotid disease indices and baseline A β 1-40 levels in women with available sex hormones and DHEAS

	cbIMT(mm)	avgIMT(mm)	maxWT(mm)	sumWT(mm)
Analysis in women with available estradiol levels (n=90), OR (95%CI)				
Univariable	4.06 (1.36-12.2)	3.01 (1.01-9.02)	3.2 (0.99-10.3)	4.49 (1.41-14.3)
	p=0.012	p=0.048	p=0.051	p=0.011
Multivariable*	4.03 (1.34-12.1)	3.01 (1.0-9.0)	3.1 (0.96-1.01)	4.42 (1.38-14.2)
	p=0.013	p=0.048	p=0.059	p=0.012
Analysis in women with available testosterone levels (n=125), OR (95%CI)				
Univariable	2.73 (1.10-6.8)	2.81 (1.12-7.12)	2.25 (0.83-6.1)	3.63 (1.41-9.39)
	p=0.031	p=0.03	p=0.11	p=0.008
Multivariable*	2.76 (1.1-6.89)	2.9 (1.35-7.35)	2.28 (0.83-6.25)	3.74 (1.43-9.75)
	p=0.03	p=0.026	p=0.10	p=0.007
Analysis in women with SHBG levels (n=114), OR (95%CI)				
Univariable	3.69 (1.37-9.92)	3.35 (1.24-9.01)	2.37 (0.83-6.7)	4.5 (1.16-12.4)
	p=0.01	p=0.001	p=0.104	p=0.004
Multivariable*	3.61 (1.33-9.78)	3.53 (1.29-9.63)	2.35 (0.82-6.71)	4.63 (1.65-13.0)
	p=0.011	p=0.014	p=0.109	p=0.004
Analysis in women with available DHEAS (n=110)				
Univariable	4.14 (1.48-11.6)	3.75 (1.33-10.5)	2.23 (0.78-6.38)	5.2 (1.77-15.1)
	p=0.007	p=0.012	p=0.134	p=0.003
Multivariable*	4.34 (1.49-12.6)	4.16 (1.42-12.2)	2.28 (0.77-6.71)	4.53 (1.53-13.4)
	p=0.007	p=0.01	p=0.136	p=0.006
Odds ratio for high carotid indices (i.e. highest tertile) per comparison of highest to lower tertiles of baseline A β 1-40 plasma levels				
**Adjusted for each sex hormone level (estradiol, testosterone, SHBG) and DHEAS.				
IMT=intima media thickness; Cb=carotid bulb; Avg=average carotid IMT from all 6 sites; WT=wall thickness; Max=maximal WT from all 6 carotid sites; Sum WT= sum of all max WT from all 6 sites. SHBG=sex hormone binding globulin. DHEAS= dehydroepiandrosterone-sulfate.				

Supplementary Table 7. Linear mixed model analysis for the association of alterations in A β 1-40 levels with carotid indices' changes across follow-up in women with available sex hormones and DHEAS

	cbIMT(mm)	avgIMT(mm)	maxWT(mm)	sumWT(mm)
Analysis in women with available estradiol levels (n=90), % change (95% CI)				
Univariable	2.8 (-8.1/14.9)	2.9 (-3.7/9.8)	0.16 (0.01/0.31)	8 (0.6/16)
	p=0.627	p=0.39	p=0.033	p=0.033
Multivariable*	2.8 (-8.1/14.9)	2.9 (-5.7/9.8)	0.16 (0.01/0.31)	4 (-1/8)
	p=0.627	p=0.389	P=0.033	P=0.084
Analysis in women with available testosterone levels (n=125), % change (95% CI)				
Univariable	2.5 (-6.1/11.9)	3.6 (-1.8/9.4)	0.13 (0.01/0.24)	8.6 (2.6/15)
	P=0.572	P=0.189	P=0.031	P=0.005
Multivariable*	2.5 (-6.1/11.9)	3.6 (-1.8/9.4)	0.13 (0.01/0.24)	8.7 (2.5/15)
	p=0.575	P=0.189	P=0.031	P=0.005
Analysis in women with SHBG levels (n=114), % change (95% CI)				
Univariable	4.2 (-5/14.2)	2.7 (-2.7/8.4)	0.08 (-0.03/0.19)	8.0 (2.1/14.2)
	p=0.378	p=0.322	p=0.138	p=0.007
Multivariable*	4.2 (-5/14.3)	2.7 (2.7/8.4)	0.08 (-0.03/0.19)	8.0 (2.1/14.2)
	p=0.379	p=0.322	p=0.138	p=0.007
Analysis in women with available DHEAS (n=110), % change (95% CI)				
Univariable	3.8 (-5.6/14.2)	2.6 (-2.9/8.5)	0.07 (-0.03/0.19)	8.1 (2.1/14.5)
	P=0.436	P=0.351	P=0.168	P=0.008
Multivariable*	3.8 (-5.6/14.2)	2.7 (-2.9/8.5)	0.07 (-0.03/0.19)	8.1 (2.1/14.5)
	P=0.434	P=0.348	P=0.168	P=0.008

Coefficients represent the percentage change in carotid atherosclerosis progression in women classified in the increasing or persistently high A β 1-40 pattern in comparison to women classified in stable/decreasing A β 1-40 group at follow-up.

Coefficients are derived after natural log transformation of the outcome variable (carotid indices)

Coefficients are derived after natural log transformation of the outcome variable

*Adjusted for each sex hormone levels (estradiol, testosterone, SHBG) and DHEAS.

Bold indicated statistical significance, which was set at the level of p-value <0.05

IMT=intima media thickness; Cb=carotid bulb; Avg=average carotid IMT from all 6 sites; WT=wall thickness; Max=maximal WT from all 6 carotid sites; Sum WT= sum of all max WT from all 6 sites. SHBG=sex hormone binding globulin. DHEAS= dehydroepiandrosterone-sulfate.