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Does reproductive stage impact cardiovascular disease risk factors? Results from a population-based cohort in Lausanne (CoLaus Study)

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Short title

Cardiovascular risk across reproductive stage

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Conflicts of Interests

The authors declare no conflicts of interest.

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Data Availability Statement

The datasets generated during and/or analyzed during the current study are not publicly available. Information related to data access can be made available to interested researchers at https://www.colaus-psycolaus.ch/professionals/how-to-collaborate/.

Keywords

Female, Cardiovascular Diseases, Risk Factors, Reproduction, Cardiovascular System, Menopause

Abstract

Context

Menopause has been associated with adverse cardiovascular disease (CVD) risk profile, yet it is unclear whether the changes in CVD risk factors differ by reproductive stage independently of underlying aging trajectories.

Design

The CoLaus study is a prospective population-based cohort study in Lausanne, Switzerland.

Patients

We used data from women at baseline and follow-up (mean 5.6 ±0.5 years) from 2003 to 2012 who did not use hormone therapy. We classified women into (i) premenopausal, (ii) menopausal transition, (iii) early (≤ 5 years), and (iv) late (> 5 years) postmenopausal by comparing their menstruation status at baseline and follow-up.

Measurements

We measured fasting lipids, glucose, and cardiovascular inflammatory markers. We used repeated measures (linear mixed models) for longitudinal analysis, using premenopausal women as a reference category. We adjusted analyses for age, medications, and lifestyle factors.

Results

We used the data from 1,710 women aged 35-75 years. Longitudinal analysis showed that the changes in CVD risk factors were not different in the other three menopausal categories compared to premenopausal women. When age was used as a predictor variable and adjusted for menopause status, most CVD risk factors increased, while interleukin 6 and interleukin 1β decreased with advancing age.

Conclusion:

The current study suggests that women have a worsening cardiovascular risk profile as they age, and although menopausal women may have higher levels of cardiovascular risk factors compared to premenopausal women at any given time, the five-year changes in cardiovascular risk factors may not depend on reproductive stage.

Introduction

Natural menopause is defined as the absence of menstrual periods for 12 consecutive months for which there is no other obvious pathological or physiologic cause.[1] It is a consequence of the depleted pool of primary ovarian follicles and the termination of folliculogenesis, resulting in a decline in estrogen production and an increase in iron body stores due to the cessation of menses.[1] Although women reach menopause at the average age of 50-52 years, the menopausal transition may start several years before the last menstrual period.[2,3] The menopause transition phase is a period characterized by fluctuations in sex hormones and the presence of menopausal symptoms that could be highly troublesome, requiring hormone therapy.[4,5]

Changes in the sex hormone levels occurring around the menopause onset have been associated with metabolic changes, resulting in slower lipid metabolism, impairment of glucose tolerance, and increased body weight[6], leading to higher cardiovascular morbidity and mortality in aging women.[7-10] Further, menopausal symptoms have been associated with increased cardiovascular risk factors, increased risk of coronary heart disease (CHD), and increased all-cause and cardiovascular disease

(CVD) mortality.[8,10,11] Thus, menopause has been suggested as a risk factor for developing cardiometabolic diseases (CVD, metabolic syndrome, and type 2 diabetes).[12-14] Indeed, the incidence of CVD is increased in the postmenopausal period, thus, consensus statements from leading experts have made this a priority for cardiovascular prevention in women's health.[15,16]Cross-sectional studies have consistently reported more adverse cardiovascular risk profiles for postmenopausal women than premenopausal women.[17-24] Although the findings from longitudinal studies have been conflicting, some studies suggested increased cardiometabolic risk factors for advanced reproductive stages.[25-29] Therefore, it remains insufficiently explored whether increased cardiometabolic risk after menopause is a direct consequence of transitioning through menopause or it is a result of cumulative aging. In this context, we performed a cross-sectional and longitudinal analysis using data from a prospective cohort of Swiss women to explore the changes in CVD risk factors according to the reproductive stage and considering the role of chronological age.

Materials and Methods

Study population

The CoLaus study is a single-center, population-based cohort study in Lausanne, Switzerland established in 2003 to investigate the epidemiology of CVD and metabolic syndrome in the area.[30,31] The study enrolled adults between 35 and 75 years of age of Caucasian origin from 2003 to 2006. Caucasian origin was defined as having both parents and grandparents from a list of countries specified by the study group. The total population of adults meeting these criteria was 56,694, of which 19,830 were selected through simple random sampling by electronic draw, comprising 35% of the source population. Among these 15,109 (76.19%) responded to the invitation, for which 799 were ineligible, and 6,189 refused to participate. Thus, only 8,121 were eligible for baseline interviews. Ultimately, 6,104 adults completed the baseline interview. A follow-up visit was then scheduled between 2009 to 2012. The average time between the visits was 5.6 (±0.5) years. For the current analysis/study, we used data on baseline and first follow-up **(Online Supplement Figure S1).**

Classification of Exposure (Reproductive Stage)

The reproductive staging was based on a self-reported prospective evaluation of menstrual bleeding at baseline and follow-up. Women were asked if they had menstrual bleeding during the year preceding

the clinic visit. We classified the women into four reproductive stages based on their baseline menstruation status and follow-up. The groups were as follows: premenopause (PRE), transition or perimenopause (TRANS), early (EPOST), and late postmenopause (LPOST) **(See Figure 1).** PRE was identified as having menstrual bleeding at baseline and follow-up. TRANS were those who had menstrual bleeding at baseline but had no bleeding on follow-up (women became postmenopausal during the follow-up period), EPOST those women without menstrual bleeding for 1-5 years at baseline, and LPOST those with amenorrhea for 5 years or more at baseline. Women with ovarian cancer or any malignancy requiring ovarian surgery, hysterectomy (with or without oophorectomy), or chemotherapy were classified under post-menopause and analyzed in this group. Statistical analysis of natural menopause (those without any surgery) and those with concurrent medication use (antihypertensives, statins/antilipidemic, and oral hypoglycemics/antidiabetic drugs) are discussed in another section. *Inclusion and Exclusion Criteria*

We included all women from the CoLaus cohort (35-75 years, of Caucasian origin, with consent to participate). We restricted our analyses to all women with known menstruation status to facilitate their reproductive stage classification. Furthermore, we removed dropouts for our cross-sectional and longitudinal analysis (women with no information at follow-up).

Of 6,104 CoLaus participants, we included 3,544 women **(See Figure 1).** We removed 891 who had no available follow-up data. We also removed 95 women who did not have information on menstruation status or with inconsistent menstruation information. In detail, we used the age of last menstruation to detect inconsistent menstruation information. At baseline, 39 of 1906 women who reported no menstrual bleeding had no age of last menstrual period (2.04% erroneous response), and 41 of 1908 women who had an age of last menstrual period claimed menstrual bleeding (2.14% erroneous response). At follow-up, 59 of 1935 women with no menstrual bleeding reported no age of last menstrual period (3.04% erroneous response), and 59 of 1885 women with a reported age of last menstrual period claimed menstrual bleeding (3.12% erroneous response). Furthermore, we found 8 women who replied not having menstrual bleeding (menopause) at baseline but reported menstrual bleeding (premenopausal) on follow-up (8/2653 women with follow-up data or 0.3% erroneous response). A total of 2,558 women were eligible for analysis. For the final analysis cohort, we excluded

848 women with current and ever-use of hormone therapy. Thus, we used data on 1,710 women for cross-sectional and longitudinal analysis **(Figure 1).**

From this database, we extracted the age, incident and prevalent cardiometabolic diseases, smoking, drinking habits, physical activity, medication-use, and menstrual bleeding status of women at baseline (2003-2006) and first follow-up visit (2009-2012).

Measurement of Outcome (Cardiovascular Risk Factors)

Weight and height were measured using the same scale that underwent serial calibration during the study period. Body mass index was calculated from the standard formula (weight in kilograms divided by height in meters squared). Systolic and diastolic blood pressure were assessed in a seated position after a 10 minutes rest using Omron® HEM-907 automated oscillometric sphygmomanometer. We recorded the average of the last two measurements (out of three attempts) expressed in mmHg. We computed the 10-year cardiovascular risk of women using Framingham risk score (FRS) using a formula from a previous publication.[32] We also computed for insulin resistance index using Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) based on a previous publication.[33]

Venous blood samples (50 mL) were obtained from each participant after overnight fasting. Serum lipids and glucose assays were performed by the Centre Hospitalier Universitaire Vaudois (CHUV) Clinical Laboratory. Inflammatory markers were also measured, namely, high-sensitivity C-reactive protein (hsCRP), interleukin 6 (IL 6), interleukin 1β (IL 1β), adiponectin, leptin, and tumor necrosis factor-alpha (TNF-α). Laboratory analyses were performed within the time frame of blood sample collection.

Baseline TNF-α, IL 6, and IL 1β were measured using multiplex particle-based flow cytometric cytokine assay (Luminex®) with lowest detection limit of 0.2 pg/mL. Intra and inter-assay coefficients of variation were 12.5% and 13.5% for TNF-α, 16.9% and 16.1% for IL 6, and 15% and 16.7% for IL 1β. Leptin and adiponectin at baseline were measured using ELISA (R&D Systems, Inc, Minneapolis, USA) with maximum interassay CV of 12.8% and 8.3%, respectively. On follow-up, we used multiplexed particle-based flow cytometric cytokine assay (Luminex®) with CV 9.5%. hsCRP was measured using latex immunoassay (Roche diagnostics, Interbatch coefficient of variability, CV, 4.6%-1.3% on baseline and 8.0%-7.4% on follow-up). Leptin and adiponectin were measured using ELISA (American Laboratory

Products Co, CV 12.8% - 5.8%; and R&D Systems Inc., CV 8.3% - 8.3%). Details of other routine laboratory assays can be found in the Online Supplement.

Measurement of Covariates

Information on age, educational attainment, alcohol consumption, tobacco use, and physical activity were obtained through a self-administered questionnaire. Educational attainment was defined as "high" for those with at least a university degree, "middle" for those who finished secondary school or apprenticeship, and "low" for those who completed mandatory elementary education. Alcohol-use was obtained by asking if participants regularly consume alcohol and asking for their weekly consumption before the assessment (wine, beer, and spirits; units per week). Tobacco use was self-reported, and participants were classified as current, former smoker, or never smokers. Physical activity was elicited by asking the frequency of exercise for at least 20 mins in a day per week in each participant. Prevalent CVD, diabetes, medication-use (antihypertensive drugs, antidiabetic drugs, or statins) were collected using a recruiter administered questionnaire. Hormone therapy use was also obtained through a questionnaire (current or ever used).

Statistical analysis

We used linear regression analysis to determine the association between reproductive stages (independent variable) and cardiovascular risk factors (dependent variable). Iterative models were developed to correct for (a) outcome-specific medication-use (antilipid drugs/statins for cholesterol, triglycerides, HDL and LDL; antidiabetics/hypoglycemic drugs for insulin, fasting glucose, and insulin resistance index; and antihypertensive drugs for systolic and diastolic BP), (b) chronologic age, (c) baseline health and social factors (baseline CVD, educational attainment, alcohol consumption, tobacco use, and physical activity), (d) body mass index that was added incrementally. We used PRE as the reference group in the linear regression model. Covariates selected for adjustments were based on current literature and scientific plausibility for the association of menopause and CVD (See Online Supplement).

To explore the longitudinal changes, we examined the changes (mean difference) of cardiovascular risk factors between baseline and follow-up within each reproductive stage using paired t-test. We used a multilevel mixed-model approach fitted using a random-slope model to examine the association of

cardiovascular risk factors (dependent variable) of different reproductive stages (independent variable). The PRE was used as a reference category. Since the reproductive stage could be correlated with the time gap of observation, we included an interaction term of these two factors (reproductive stage and time gap) in our regression model.

To determine the influence of chronologic age, we iterated the cross-sectional and longitudinal analysis using age as the independent variable. We performed regression using the adjustments as previously mentioned, with the addition of menstruation status. We compared the fully adjusted models using reproductive stage and chronologic age as independent variables to determine which was more associated with longitudinal changes in cardiovascular risk factors.

In sensitivity analyses, we compared the women included in the cohort and those excluded from the analysis to investigate any selection bias in cross-sectional and longitudinal analyses. In addition, we restricted the analyses to specific women groups, namely: (i) women who reported natural menopause (removed women with ovarian surgery, hysterectomy with or without oophorectomy, and chemotherapy menopause), and (ii) women without comorbidities (no intake of medications namely, antilipids/statins, antidiabetics/oral hypoglycemics, and antihypertensive drugs, no prevalent CVD and no prevalent diabetes). We iterated for both cross-sectional and longitudinal analyses for both of these groups. We explored the use of time-varying covariates considering changes in smoking and alcohol consumption habits across time. Finally, to gain higher statistical power, we performed iteration of all mentioned models to include women with hormone therapy use (data on 2,558 women).

All analyses were performed using STATA 15.1 for Windows (STATA Corp, Texas, US). All computations were done using two-tailed tests. P-value < 0.05 was considered significant, yet we used Bonferroni correction to adjust for multiple testing. We analyzed the women with available outcomes (baseline and follow-up). We did list-wise deletion for those with incomplete outcomes.

Ethical consideration

The institutional Ethics Committee of the University of Lausanne, which afterward became the Ethics Commission of Canton Vaud (www.cer-vd.ch), approved the baseline CoLaus study (reference 16/03). The approval was renewed for the first (reference 33/09) follow-up. The study is compliant with the Swiss Human Research Act (810.30 Federal Act of 30 September 2011 on Research involving Human

Beings) and Federal Regulations on Data Protection (235.1 Federal Act of 19 June 1992 on Data Protection). The database was managed and kept at the Institute of Social and Preventive Medicine – University of Bern. Written informed consent was obtained from the participants upon their visit for clinical assessment. To maintain the anonymity of the participants, we removed all identifying information not relevant to the analysis.

Results

Baseline Characteristics

We used data from 1,710 women for both cross-sectional and longitudinal analysis. Based on menstruation status at baseline and follow-up, women were classified as premenopausal (PRE, n=696), women in menopause transition (TRANS, n=463), women in early (EPOST, n=164) and late menopause (LPOST, n=387), **Figure 1**. The baseline characteristics are summarized in **Table 1**. The clinical characteristics of excluded participants, clinical profile of included participants on follow-up, and missing data table can be found in the Online Supplement **(Table S1a-c).** The details of women with paired data used for the analysis (outcome data on baseline and follow-up) can be found in **Table S1b.** At baseline, most of the advanced reproductive stages have higher cardiovascular risk factors, with some exceptions. Insulin, leptin, TNF-α, and IL 1β were higher in PRE (insulin 7.27 ± 4.54 micro IU/mL; leptin 1.25 ± 0.90 ng/mL; TNF-α 11.4 ± 131.6 pg/mL; and IL 1β 5.64 ± 52.4 pg/mL) compared to TRANS (Insulin 7.08 ± 4.27 micro IU/mL; leptin 1.28 ± 0.96 ng/mL; and IL 1β 0.69 pg/mL IQR 2.04) **(Table 1).** The mean BMI was highest (26.8 ± 5.4 kg/m2), and hypertension (47.6%), diabetes (9.8%), CVD (9.3%) and the use of antihypertensive (25.6%) and antidiabetic (6.5%) medications were the most prevalent in LPOST group **(Table 1).** In women without a history of CVD, Framingham risk scores were also substantially higher in EPOST and LPOST (6.37 ± 4.08 and 10.92 ± 7.2) compared to PRE and TRANS (2.32 ± 1.76 and 3.94 ± 2.57).

Reproductive stage versus chronologic age: Association with cardiovascular risk factors (Cross-sectional analysis)

Using reproductive stage as independent variable (fully-adjusted model) **(Table 2 and Table S2a)**, the EPOST and LPOST groups had higher BMI (β 1.5, 95% CI 0.5, 2.4; and β 2.4, 95% CI 1.3, 3.4), total cholesterol (β 0.4, 95% CI 0.2, 0.6; and β 0.2, 95% CI 0.02, 0.5), adiponectin (β 0.2, 95% CI 0.1, 0.4; and

β 0.2, 95% CI 0.1, 0.4) and IL6 (β 0.4, 95% CI 0.1, 0.7; and β 0.5, 95% CI 0.1, 0.9) compared to PRE group **(Table 2)**. Serum triglyceride (β 0.1, 95% CI 0.02, 0.2), fasting glucose (β 0.2, 95% CI 0.03, 0.4), hsCRP (β 0.2, 95% CI 0.03, 0.3), and leptin (β 0.2, 95% CI 0.03, 0.3) was higher in LPOST compared to the reference group (PRE). The incremental addition of covariates showed that the coefficients (EPOST and LPOST) were positive for body mass index, diastolic blood pressure, total cholesterol, LDL, triglycerides, fasting glucose, insulin, insulin resistance index, leptin, and IL6 **(Table S2a,** Model 1-3**).**

Using age as the independent variable, fully adjusted models showed increasing systolic BP (β 0.67, 95% CI 0.5, 0.8), total cholesterol (β 0.03, 95% CI 0.02, 0.04), HDL (β 0.004, 95% CI 0.001, 0.007), LDL (β 0.025, 95% CI 0.02, 0.03), triglycerides (β 0.005, 95% CI 0.0008, 0.009), and IL6 (β 0-0.03, 95% CI -0.04, -0.01) with increasing age **(Table 2).**

Reproductive stage versus chronologic age: Changes in cardiovascular risk factors across time (Longitudinal analysis)

We examined the mean difference in CVD markers comparing women at baseline and follow-up in each group **(Table S3a).** Using linear mixed models for longitudinal analyses, fully corrected models did not show any statistically significant differences in five-year changes on all cardiovascular risk factors comparing TRANS, EPOST, and LPOST to PRE **(Table 3).** On the incremental addition of covariates **(Table S3b),** we found that medication-adjusted model (Model 1) showed higher cholesterol and LDL in early- and late postmenopausal women. There is also an increase in triglycerides in the menopause transition group compared to premenopausal women in medication-, age-, and lifestyle-adjusted models (Model 1-3).

Using age as the independent variable in the linear mixed models, we observed age to be associated with changes in cardiovascular risk factors. In particular, there was an increase in systolic BP (β 0.7, 95% CI 0.6, 0.8), diastolic BP (β 0.09, 95% CI 0.01, 0.16), total cholesterol (β 0.03, 95% CI 0.02, 0.04), HDL (β 0.006, 95% CI 0.003, 0.009), LDL (β 0.02, 95% CI 0.02, 0.03), triglycerides (β 0.006, 95% CI 0.003, 0.009), fasting glucose (β 0.006, 95% CI 0.002, 0.01), and adiponectin (β 0.008, 95% CI 0.003, 0.01) with increasing chronologic age **(Table 3).** Other inflammatory markers, IL 6 (β -0.02, 95% CI -0.03, -0.006) and IL 1β (β -0.02, 95% CI -0.03, -0.009), decreased with age.

Sensitivity analyses

In comparison to women who were excluded from the study **(Table S1a),** women included in our study were younger (mean age 48.6 ± 9.7 years versus 57.1 ± 9.9 years), with a higher proportion of current smokers (26.8% versus 23.0%). We also noted a lower proportion of diabetes (3.2% versus 4.7%) and hypertension (21.9% versus 37.7%). The women included in the study had lower BMI, blood pressures, lipid profiles, glucose metabolism indices, and cardiovascular/inflammatory markers compared to those excluded.

Upon restricting our analyses to specific groups of women (i.e., 1,590 women with natural menopause, and 1,213 women without comorbidities) in our cross-sectional analyses, our estimates were similar except when restricting to women without comorbidities **(Table S2b)**. For women without comorbidities, early postmenopause had higher cholesterol and LDL compared to premenopausal women. Both early and postmenopausal women without comorbidities had higher adiponectin and IL6 compared to premenopausal women. In our longitudinal analyses, early menopause women had higher total cholesterol compared to premenopausal women for those with no comorbidities (β 1.9, 95% CI 0.09, 3.9) **(Table S3c).** Furthermore, triglycerides were higher in the menopause transition group than premenopausal women upon restriction to those who had natural menopause (β 0.55, 95% CI 0.04, 1.1). Albeit, both comparisons did not reach statistical significance when adjusting for multiple testing. We also explored our models by using time-varying covariates. Our longitudinal model showed higher triglyceride levels (β 0.52, 95% CI 0.001, 1.04) for the menopause transition group than premenopausal women considering changes in smoking and alcohol intake habits over time **(Table S4).**

Finally, to explore changes in cardiovascular risk in women with larger sample size, we iterated all our models using 2,558 women, including hormone therapy users. Results were in line with our main results **(Online Supplement Tables S5-S7)**

Discussion

Women in midlife experience dramatic hormonal, physiologic, psychologic, and social changes. As women in midlife transition to menopause, previous studies have associated this critical period with increased cardiovascular risk. Aging is the strongest predictor of cardiovascular risk. However, in women, this relationship is more complex because of their transitions through reproductive stages. Since chronologic age and reproductive stage are closely related, it is challenging to determine

whether reproductive stage or cumulative aging are more relevant to increased cardiovascular risk after menopause.

In cross-sectional analyses we observed a poorer cardiovascular risk factor profile among women in advanced reproductive stage (postmenopausal). While postmenopausal women still had the worst cardiovascular profile after five years of follow-up, the longitudinal changes in cardiovascular risk factors were similar across all reproductive stages. In our sensitivity analyses, we removed women with surgical or chemotherapy-induced menopause as they carry higher cardiovascular risk due to their primary condition. The models did not result in statistically significant changes. On the other hand, age risk models showed statistically significant associations with cardiovascular risk factors even after adjusting for the reproductive stage. We surmise that age is still a stronger predictor of poorer cardiovascular risk than advancing reproductive stage.

Cross-sectional analyses investigating the role of the reproductive stage in cardiovascular risk factors have unanimously identified a poorer CVD risk profile in postmenopausal compared to premenopausal women.[19-23] Our findings reiterate these findings. CVDs result from accumulated stress on several pathways that ultimately lead to vascular damage and heart disease. However, the clinical profiles of premenopausal and postmenopausal women within the same age range are different and incomparable. Women of the same age with different reproductive stages are deemed to have different health status and lifestyle that confounds their cardiovascular risks. Also, the directionality of the variables (cause and effect) is something that the cross-sectional analysis cannot resolve. Thus, cross-sectional study designs are inherently limited in resolving this issue.

Longitudinal analyses allow one to follow through with each subject and consider variability among women with a reproductive stage, and variability between stages. More importantly, the direction of causality can only be determined through this study design. To our knowledge, only a few studies used this approach,[25-29] and far fewer were done in Europe.[27] Body composition was studied in two analyses with divergent results.[28,29] One study demonstrated an increased fat mass seen in advanced reproductive stage,[29] but no difference was seen in another study.[28] Our study was limited to body mass index, for which we observed no significant changes across different reproductive stages. Various longitudinal studies were consistent on the association of older reproductive stage and poorer lipid

profile, although there was incoherence as to which specific lipid molecule. In a women's cohort in Australia with 150 participants, the longitudinal analysis showed a difference with the older reproductive stage (postmenopause). These women underwent repeated measures across seven years of observation as they transitioned to menopause. HDL was lower in the postmenopausal phase compared to the premenopausal phase of their reproductive life.[25] In another women's cohort in the US with 1,054 participants compared across different reproductive stage groups, Matthews et. al. reported total cholesterol, apoB, and LDL were higher in the menopause transition.[26] We did not find any statistically significant difference in changes in serum lipids across different reproductive stages on our fully adjusted models. In our sensitivity analyses, we found triglycerides levels were higher for women in menopause transition in medication-, age-, and lifestyle-adjusted models than the premenopausal women **(Table S3b, Model 1-3).** Cholesterol and LDL were also higher in early and late postmenopausal group than the premenopausal group only in medication-adjusted model **(Table S3b, Model 1)**. However, owing to multiple comparisons, it is highly possible that these are merely chance findings with limited clinical significance.

Most cross-sectional data consistently showed a higher prevalence of diabetes in postmenopausal compared to the premenopausal group.[34-36] However, the aging trajectory is rarely considered or treated inappropriately in all these studies. Our findings are consistent with other menopause longitudinal studies showing no significant changes among different reproductive stages.[26,37] Thus, menopause, per se, seems to be less important, and the aging process and other lifestyle factors are still dominant factors in the development of diabetes. A systematic review has found that postmenopausal obesity influences the development of diabetes, [38] therefore, highlighting the importance of lifestyle modification during menopause transition and thereafter.

IL 6 and IL 1β are inflammatory markers that are known to be higher in the aging population.[39] Both are proinflammatory cytokines that are sometimes used as markers of chronic disease and frailty in the elderly.[40,41] Our analysis showed no significant changes in IL 6 and IL 1β according to reproductive stage but we report inverse association (decreases) with age. Changes in laboratory standards across time, selection bias towards the healthy group, and missing data could have led our results to an opposite trend. Nevertheless, these finding warrants to be investigated further.

Our study has important differences in the study design and analysis compared to the other women's health studies. We assumed a linear relationship, used two-time points with a 5.5 (±0.4) year interval, and specified premenopausal women as the comparison group. The Study of Women's Health Across the Nation (SWAN) in the US and the Melbourne Women's Midlife Health Project in Australia both used piecewise regression (and explored non-linear relationship), used multiple data points across time, and used menopause transition as a reference. These crucial differences could be the main reason for the disparity of our results to these women's cohorts.

The normal aging process in women is accompanied not only by a decline in ovarian function but also by changes in epigenetic profile, increased oxidative damages, and varied iron metabolism. All these are known to contribute to elevated cardiovascular risk profiles in aging women. For example, the changes in sex hormones accompanying menopause affect the baseline chronic inflammation, endothelial function, and serum lipid levels, which puts them at higher risk for CVD.[42] However, genetic variations and epigenetic changes that are related to early-onset menopause had been discovered to be associated with increased cardiovascular risk factors.[43] Also, aging is associated with the accumulation of oxidative stress, one of the main drivers of heightened risk, independent of menopause status. Together, all these factors make it challenging to associate whether menopause alone is an independent risk factor for CVD.

Strengths and weaknesses

The onset of menopause has been largely affected by region and ethnicity. External and other environmental factors such as diet, physical activity, smoking, mental health, and socioeconomic factors influence the menopause onset. We present one of the few studies from a cohort of women in Europe, with a growing aging population, and thus, at a high burden of cardiovascular diseases. This is with a background of varying menopausal ages and practices in hormone replacement therapy in European compared to North American women cohorts that may significantly affect the cardiovascular risk. Europeans have a better dietary pattern, higher physical activity, and better health-seeking behavior than in the US.[44,45] Women in Switzerland, and Europe in general, have higher rates of smokers compared to women in the other cohorts.[46] Our cohort had 53.9% (923/1,710) women who never smoked, which is higher compared to SWAN cohort in the US (42.7%)[26] and to Melbourne

Womens' Health in Australia (22%).[25,47,48] Also, our study is one of the largest longitudinal analyses in terms of sample size and has a lower dropout rate compared to other cohorts that have longitudinal data on reproductive stage and cardiovascular risk factors[26,49-53] Lastly, we analyzed inflammatory markers utilized in cardiovascular risk stratification, which were reported in few cohorts.[26,27]

However, our study has important limitations that merit to be discussed. First, the included participants had more favorable lifestyle and cardiovascular risk factor profiles compared to the excluded **(Table S1a).** Our study population had higher baseline physical activity levels, lower proportion of smokers and higher educational attainment. All of which could point to a bias similar to healthy users or adherers. This is a common bias in observational studies when individuals with healthier lifestyles have higher participation rates than non-participants or those lost to follow-up. A selection bias towards the healthy individuals could have been the reason for our null findings or finding no statistically significant difference among different reproductive stages across time. As a result, our results on CVD risk factors prevalence across different reproductive stages might have been underestimated. The true differences between reproductive stages may be revealed if women with poorer lifestyles were included. Second, our exposure classification (menstruation status) is based on self-reported data. Women were classified into reproductive stage groups based on the baseline questionnaire, and the status was confirmed using self-reported age at last menstruation. Also, there was no active follow-up on the women between the five-year gaps to confirm the menstrual status. We did the necessary counterchecks to confirm the menstruation status of the women in our cohort. All women with invalid responses to menopause were removed from the analysis. With the erroneous response rate (as mentioned in the methods), we surmise that up to 5% of the women may have been misclassified in our analysis and would be unlikely to change our estimates dramatically. Third, focusing on a specific population can remove other confounding factors, but this also raises the question of the generalizability of results with women of different ethnicities. Fourth, our laboratory assays were done in real-time between baseline and follow-up, and not simultaneously performed on biobank blood samples. Although all samples were processed by one laboratory (CHUV), the laboratory standards could have changed across the period, and these were not accounted for in our models. Fifth, women who underwent hysterectomy, oophorectomy, and chemotherapy were all classified as non-natural

menopause. Women who underwent hysterectomy with preserved ovaries are a particular group of women with different endocrinologic profiles than those who underwent oophorectomy (ovaries removed). Unfortunately, our data was not able to distinguish these two groups, and we iterated our models on women with natural menopause. Finally, we only compared two data points to characterize the early changes in cardiovascular risk factors. We expect a more optimized model that would likely capture the true effects by increasing the data points. Corollary to this, we also assumed that associations are mostly linear, which is different from some studies that performed a piecewise linear regression model (over multiple time points) and performed spline modeling.[26]

Future direction and outlook

Women in menopausal transition are a particular phenotype as the body begins to experience hormonal changes (reproductive stage) and advancing age (chronologic age). Studies have shown that women in the transition phase of menopause have higher cardiovascular risk factors (i.e., lipid profile, glucose, blood pressure, fat composition), and a longitudinal analysis had further strengthened the influence of the reproductive stage on lipid profile. However, women in the transition phase are not being targeted by current clinical guidelines on cardiovascular disease prevention. This is likely due to the absence of universally accepted reproductive aging criteria that are in use by healthcare providers, and thus, precluding preventive care to this group. Currently, there is no validated biomarker that aids in the classification or a widely accepted scoring system in determining those at the transition phase of menopause.[54] The *sine qua non* for the diagnosis, and the operational definition of menopause remain the absence of menstrual bleeding for a year. More research is needed to look for biomarkers in aid of classification. More longitudinal studies on biomarkers for ovarian age are still lacking.

Aside from a standard classification of reproductive stage, there is still a considerable discussion over menopause and sex hormones' effect on women's cardiovascular health. As of now, there is no large women's cohort in Europe with detailed information on their reproductive and menstrual history, combined with biomarkers and genetic information. The cohorts in North America have driven much of our current knowledge, albeit with different dietary patterns, lifestyle habits, and genetic predispositions. Lastly, there is a need for more longitudinal studies accounting for the natural aging

process and, most importantly, explain the directionality and causality of cardiovascular risk differences.

Conclusion

All women increase their cardiovascular risk as they get older, and in our study, we found no differences in cardiovascular risk changes comparing women in advanced reproductive stages with premenopausal women. This highlights the strong association between chronological age and the cumulative deleterious effects in cardiovascular disease risk for women. More longitudinal studies that use novel biomarkers for ovarian age are still needed to disentangle the association between menopause and CVD risk in postmenopausal women and women in the menopause transition. Although the proof of menopause and the menopause transition is still lacking, it would be prudent to do screening and preventive measures during menopause transition as these are also aging women with inherent cardiovascular risks. Cardiovascular preventive measures should target not only postmenopausal women, but also women in the transition phase while waiting for more conclusive evidence.

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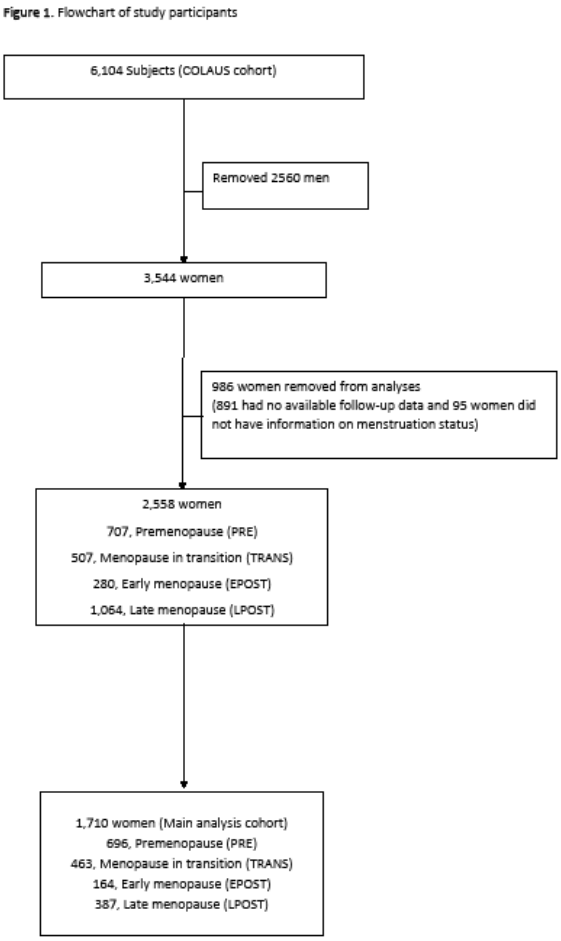

Figure 1. Flowchart of study participants

Table 1. Baseline characteristics of women across different reproductive stages

	Premenopause (n=696)	Transition (n=463)	Early Postmenopause (0-5y) n=164	Late Postmenopause (>/=5 y) N=387	p value[1]
Average follow-up time, years, mean (SD)	5.6 (0.5)	5.6 (0.5)	5.6 (0.5)	5.5 (0.4)	0.313
Demographic and Lifestyle factors					
Age, mean (SD)	40.7 (3.6)	47.2 (4.1)	53.2 (4.2)	62.5 (7.4)	<0.001
Age at menopause onset, mean (SD)	-	-	50.7 (3.9)	47.8 (5.4)	<0.001
Educational attainment (%) • High • Middle • Low	 189 (27.2) 201 (28.9) 306 (43.9)	 98 (21.2) 129 (27.9) 236 (50.9)	 27 (16.5) 42 (25.6) 95 (57.9)	 36 (9.3) 93 (24.0) 258 (66.7)	<0.001
Physical activity[2] • None • 1 per week • 2x per week • >3 per week	 197 (28.9) 91 (13.3) 383 (56.2) 11 (1.6)	 168 (36.6) 48 (10.5) 233 (50.8) 10 (2.2)	 65 (39.9) 13 (8.0) 84 (51.5) 1 (0.6)	 135 (35.6) 15 (4.0) 228 (60.2) 1 (0.2)	<0.001
Drinker (%)	449 (64.5)	300 (64.8)	97 (59.2)	230 (59.4)	0.215
• Alcohol consumption (units/week)	3.7 (5.5)	4.2 (6.3)	3.4 (4.1)	3.7 (5.2)	0.379
Smoking • Current smoker (%) • Former smoker (%) • Never (%)	 186 (26.7) 183 (26.3) 327 (47.0)	 143 (30.9) 127 (27.4) 193 (41.7)	 36 (22.0) 47 (28.7) 81 (49.3)	 93 (24.0) 104 (26.9) 190 (49.1)	0.176
Hypertension (%)	69 (9.9)	70 (15.1)	52 (31.7)	184 (47.6)	<0.001
• Use of antihypertensive	30 (4.3)	29 (6.3)	26 (15.9)	99 (25.6)	<0.001
Diabetes (%)	5 (0.7)	6 (1.3)	6 (3.7)	38 (9.8)	<0.001
• Use of antidiabetic meds (%)	2 (0.3)	4 (0.9)	5 (3.1)	25 (6.5)	<0.001
Baseline cardiovascular disease (%)	9 (1.3)	5 (1.1)	10 (6.1)	36 (9.3)	<0.001
Use of Statins (Lipid lowering drugs) (%)	5 (0.7)	7 (1.5)	8 (4.9)	56 (14.5)	<0.001
Body mass index (kg/m2)	23.8 (4.3)	24.2 (4.4)	25.6 (5.2)	26.8 (5.4)	<0.001
Blood pressure					
• Systolic blood pressure (mmHg)	114.6 (12.4)	118.9 (14.8)	123.2 (16.5)	133.1 (19.6)	<0.001
• Diastolic blood pressure (mmHg)	74.6 (10.1)	76.7 (10.9)	78.4 (11.1)	80.1 (11.0)	<0.001
Lipid profile					
• Total cholesterol (mmol/L)	5.1 (0.8)	5.3 (0.9)	5.9 (1.0)	6.0 (1.0)	<0.001
• High density lipoprotein (mmol/L)	1.8 (0.4)	1.8 (0.5)	1.8 (0.4)	1.8 (0.5)	0.029
• Low density lipoprotein (mmol/L)	2.9 (0.7)	3.0 (0.8)	3.5 (0.9)	3.5 (0.9)	<0.001
• Triglycerides (mmol/L)[3]	0.95 (0.48)	1.02 (0.53)	1.12 (0.54)	1.37 (0.72)	<0.001
Glucose metabolism					
• Fasting glucose (mmol/L)	5.09 (0.74)	5.19 (0.59)	5.31 (0.86)	5.64 (1.38)	<0.001
• Insulin	7.27 (4.54)	7.08 (4.27)	7.37 (3.78)	9.35 (6.34)	<0.001

(microIU/mL)[3]					
• Insulin resistance index [4]	1.68 (1.25)	1.66 (1.05)	1.81 (1.13)	2.48 (2.09)	<0.001
Cardiovascular/inflammatory markers					
• High sensitivity C-reactive protein (pg/mL)[3]	2.26 (3.52)	2.07 (3.09)	2.25 (3.35)	3.03 (3.94)	<0.001
• Leptin (ng/mL)[3, 5]	1.25 (0.90)	1.28 (0.96)	1.47 (0.99)	1.69 (1.10)	<0.001
• Adiponectin (ng/mL) [3,5]	1.19 (0.80)	1.17 (0.63)	1.51 (1.31)	1.38 (0.93)	<0.001
• Tumor necrosis factor alpha (pg/mL)[3]	11.4 (131.6)	4.4 (11.0)	12.8 (96.6)	4.7 (13.4)	<0.001
• Interleukin 6 (pg/mL) [3]	9.27 (65.4)	18.5 (247.1)	10.8 (43.8)	10.3 (98.2)	0.576
• Interleukin 1β (pg/mL) [3]	5.64 (52.4)	4.15 (15.5)	6.27 (25.7)	2.58 (9.2)	0.049
Framingham Risk score[6]	2.32 (1.76)	3.94 (2.57)	6.37 (4.08)	10.92 (7.2)	<0.001

[1]ANOVA for continuous variables and chi-square test for categorical variables

[2]Physical activity was obtained by self-report of doing physical activity for more than 20 mins/day per week

[3]Crude values. Transformation of values done in logarithmic scale prior to testing for statistical significance. Summary values expressed as median (interquartile range)

[4] Based on Homeostatic Model Assessment for Insulin Resistance, HOMA IR. (Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28:412-19.)

[5]Converted to standardized scores (subtracting the assay values by the mean, and divided by the standard deviation)

[6] Framingham Risk score computed only for women without a history of cardiovascular disease (PRE 687, TRANS 458, EPOST 256, LPOST 351) (Formula based on Circulation. 2008 Feb 12;117(6):743-53.)

Table 2. The association between chronologic age or reproductive stage with cardiovascular risk factors at baseline (using linear regression on fully corrected models, expressed as beta coefficients and 95% confidence interval)

Cardiovascular risk factors	Chronologic age[1]	Reproductive stage[2]			
		Premenopause (PRE)	Transition (TRANS)	Early Menopause (EPOST)	Late Menopause (LPOST)
Body mass index (kg/m2)	0.099 (-0.144, 0.343)	Ref	0.542 (-0.042, 1.128)	**1.451 (0.537, 2.365)**	**2.361 (1.277, 3.444)**
Blood pressure					
• Systolic blood pressure (mmHg)	**0.671 (0.529, 0.812)**	Ref	*-0.463 (-2.358, 1.432)*	*-2.597 (-5.563, 0.368)*	*-0.267 (-3.797, 3.262)*
• Diastolic blood pressure (mmHg)	0.043 (-0.056, 0.143)	Ref	**1.542 (0.205, 2.881)**	1.322 (-0.771, 3.416)	1.793 (-0.699, 4.285)
Lipid profile					
• Total cholesterol (mmol/L)	**0.032 (0.023, 0.041)**	Ref	0.054 (-0.066, 0.174)	**0.388 (0.201, 0.576)**	**0.248 (0.024, 0.471)**

• High density lipoprotein (mmol/L)	**0.005 (0.001, 0.009)**	Ref	0.044 (-0.005, 0.094)	**0.087 (0.010, 0.165)**	0.015 (-0.077, 0.108)
• Low density lipoprotein (mmol/L)	**0.025 (0.017, 0.033)**	Ref	*-0.003 (-0.112, 0.106)*	**0.290 (0.119, 0.461)**	0.161 (-0.041, 0.364)
• Triglycerides (mmol/L)[3]	**0.005 (0.0008, 0.009)**	Ref	0.019 (-0.037, 0.075)	0.029 (-0.058, 0.117)	**0.125 (0.021, 0.230)**
Glucose metabolism					
• Fasting glucose (mmol/L)	0.0017 (-0.005, 0.008)	Ref	0.075 (-0.016, 0.166)	0.081 (-0.061, 0.224)	**0.202 (0.032, 0.371)**
• Insulin (microIU/mL)[3]	*-0.002 (-0.007, 0.003)*	Ref	*-0.041 (-0.116, 0.033)*	*-0.052 (-0.168, 0.064)*	0.104 (-0.033, 0.241)
• Insulin resistance index[3,4]	*-0.001 (-0.007, 0.004)*	Ref	*-0.021 (-0.100, 0.056)*	*-0.036 (-0.158, 0.086)*	0.139 (-0.004, 0.283)
Cardiovascular/inflammatory markers					
• High sensitivity c-reactive protein[3]	*-0.001 (-0.011, 0.009)*	Ref	*-0.031 (-0.123, 0.060)*	*-0.023 (-0.151, 0.106)*	**0.181 (0.034, 0.329)**
• Leptin (ng/mL)[3,5]	*-0.018 (-0.052, 0.016)*	Ref	0.007 (-0.076, 0.091)	0.102 (-0.027, 0.230)	**0.184 (0.030, 0.338)**
• Adiponectin (ng/mL)[3,5]	0.004 (-0.0019, 0.0107)	Ref	0.015 (-0.070, 0.099)	**0.223 (0.094, 0.353)**	**0.213 (0.057, 0.369)**
• Tumor necrosis factor alpha (pg/mL)[3]	*-0.002 (-0.011, 0.007)*	Ref	*-0.004 (-0.130, 0.123)*	0.146 (-0.050, 0.343)	0.067 (-0.169, 0.303)
• Interleukin 6 (pg/mL)[3]	***-0.027 (-0.042, -0.012)***	Ref	**0.216 (0.008, 0.424)**	**0.410 (0.093, 0.729)**	**0.532 (0.146, 0.919)**
• Interleukin 1β (pg/mL)[3]	*-0.009 (-0.029, 0.009)*	Ref	*-0.110 (-0.363, 0.142)*	0.155 (-0.242, 0.553)	*-0.143 (-0.619, 0.333)*

[1]Beta coefficients based on multivariate linear regression on fully corrected models (*Italicized for negative beta coefficient*, ***p value < 0.05 in bold**, corrected for use of hypoglycemic drugs, statins and antihypertensive drugs, smoking history, alcohol-use, baseline physical activity, baseline cardiovascular disease, and body mass index)

[2]Beta coefficients based on multivariate linear regression on fully corrected models (*Italicized for negative beta coefficient*, ***p value < 0.05 in bold,** corrected for use of hypoglycemic drugs, statins and antihypertensive drugs, age, smoking history, alcohol-use, baseline physical activity, baseline cardiovascular disease, and body mass index)

[3]Transformation of values done in logarithmic scale prior to testing for statistical significance.

[4] Based on Homeostatic Model Assessment for Insulin Resistance, HOMA IR

[5]Converted to standardized scores (subtracting the assay values by the mean, and divided by the standard deviation)

Table 3. Changes in intermediate cardiovascular with chronologic age or reproductive stage over time (using linear mixed models regression on fully corrected models, expressed as beta coefficients and 95% confidence interval)

Cardiovascular risk factors	Chronologic age[1]	Reproductive stage[2]			
		Premenopause (PRE)	Transition (TRANS)	Early Menopause (EPOST)	Late Menopause (LPOST)
Body mass index (kg/m2)	*-0.014 (-0.049, 0.021)*	Ref	3.882 (-2.050,9.815)	1.494 (-5.604,8.591)	0.324 (-4.795,5.443)
Blood pressure					
• Systolic blood pressure (mmHg)	**0.716 (0.612, 0.820)**	Ref	*-2.472 (-20.09, 15.14)*	*-7.451 (-30.57, 15.67)*	*-6.039 (-26.74, 14.66)*
• Diastolic blood pressure (mmHg)	**0.087 (0.015, 0.159)**	Ref	1.139 (-11.07, 13.358)	*-1.107 (-17.14, 14.93)*	*-2.413 (-16.76, 11.93)*
Lipid profile					
• Total cholesterol (mmol/L)	**0.031 (0.025, 0.038)**	Ref	0.445 (-0.683, 1.594)	1.442 (-0.054, 2.939)	0.853 (-0.471, 2.179)
• High density lipoprotein (mmol/L)	**0.006 (0.003, 0.009)**	Ref	-0.231 (-0.728, 0.266)	0.009 (-0.644, 0.664)	0.0164 (-0.562, 0.595)
• Low density lipoprotein (mmol/L)	**0.022 (0.016, 0.029)**	Ref	0.425 (-0.611, 1.462)	1.215 (-0.147, 2.577)	0.756 (-0.450, 1.963)
• Triglycerides (mmol/L)[3]	**0.006 (0.003, 0.009)**	Ref	0.513 (-0.004, 1.031)	0.513 (-0.004, 1.031)	0.325 (-0.276, 0.928)
Glucose metabolism					
• Fasting glucose (mmol/L)	**0.006 (0.002, 0.011)**	Ref	0.308 (-0.455, 1.072)	*-0.251 (-1.255, 0.753)*	*-0.194 (-1.082, 0.695)*
• Insulin (microIU/mL)[3]	0.001 (-0.002, 0.005)	Ref	*-0.174 (-0.784, 0.437)*	*-0.379 (-1.176, 0.418)*	*-0.305 (-0.988, 0.378)*
• Insulin resistance index[3,4]	0.002 (-0.001 0.006)	Ref	*-0.105 (-0.753, 0.543)*	*-0.428 (-1.274, 0.418)*	*-0.320 (-1.044, 0.403)*
Cardiovascular/inflammatory markers					
• High sensitivity c-reactive protein[3]	0.0005 (-0.006, 0.007)	Ref	0.199 (-1.035, 1.435)	*-1.083 (-2.693, 0.526)*	*-0.227 (-1.662,1.206)*
• Leptin (ng/mL)[3,5]	*-0.002 (-0.008, 0.003)*	Ref	0.241 (-0.791, 1.273)	0.778 (-0.519, 2.075)	0.273 (-0.873, 1.419)
• Adiponectin (ng/mL)[3,5]	**0.008 (0.003, 0.012)**	Ref	*-0.330 (-1.007, 0.440)*	0.403 (-0.588, 1.392)	0.110 (-0.745, 0.965)
• Tumor necrosis factor alpha (pg/mL)[3]	0.0010 (-0.006, 0.008)	Ref	*-0.025 (-1.188, 1.137)*	*-0.00154 (-1.477, 1.474)*	0.461 (-0.863, 1.785)
• Interleukin 6	***-0.017 (-***	Ref	0.206 (-	*-0.511 (-*	*-1.296 (-*

(pg/mL)[3]	***0.028, -0.006)***		1.726, 2.138)	*2.958, 1.937)*	*3.494, 0.901)*
• Interleukin 1β (pg/mL)[3]	***-0.020 (-0.032, -0.009)***	Ref	0.529 (-1.412, 2.471)	*-0.502 (-3.008,2.004)*	0.481 (-1.759,2.722)

[1]Beta coefficients based on multivariate linear regression on fully corrected models (*Italicized for negative beta coefficient* ***p-value < 0.05 in bold**, corrected for reproductive stage, use of hypoglycemic drugs, statins and antihypertensive, smoking history, alcohol-use, baseline physical activity, baseline cardiovascular disease, body mass index and menopause status at baseline and follow up)

[2]Beta coefficients based on multivariate linear regression on fully corrected models (*Italicized for negative beta coefficient*, ***p-value < 0.05 in bold**, corrected for use of hypoglycemic drugs, statins and antihypertensives, age, smoking history, alcohol-use, baseline physical activity, baseline cardiovascular disease, and body mass index at baseline and follow-up)

[3]Transformation of values done in logarithmic scale prior to testing for statistical significance.

[4] Based on Homeostatic Model Assessment for Insulin Resistance, HOMA IR

[5]Converted to standardized scores (subtracting the assay values by the mean, and divided by the standard deviation)