

Menopause transition timing reflects systemic biological aging

Dorota Komar

`dorota.komar@eurecat.org`

Centre for Omic Sciences (COS)

Julio Baudin

Eurecat

Andrea Costa

Eurecat

Lucía Tadeo-Orellana

Centre for Omic Sciences (COS)

Lorena Garcia

Centre for Omic Sciences (COS)

Juozas Gordevicius

Clock Foundation

Jordi Romero Giménez

Eurecat

Lorena Calderón-Pérez

Eurecat

Alícia Domingo-Tàrrach

Eurecat

Anna Crescenti

Eurecat

Helena Torrell

Centre for Omic Sciences (COS)

Antoni Caimari

Eurecat

Nuria Canela

Centre for Omic Sciences (COS)

Article

Keywords:

Posted Date: August 26th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10709927/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Natural menopause marks the end of female reproductive capacity, yet its relationship with biological aging remains unclear. Earlier menopause has been associated with reduced lifespan and increased disease risk, but whether menopause itself accelerates aging or reflects an underlying faster biological aging trajectory remains unresolved. Here, we combined animal models of menopausal transition with epigenetic analyses in postmenopausal women, to investigate this relationship. In rats, neither ovariectomy-induced menopause nor natural reproductive senescence resulted in increased epigenetic age acceleration. In postmenopausal women, accelerated epigenetic aging was observed only in individuals who reached postmenopause earlier than the cohort median, independent of metabolic health status. Exploratory differential DNA methylation analysis suggested an association between age at menopause and pathways involved in longevity, chromatin regulation, and neurodegeneration. Together, these findings support a model in which the timing of reproductive aging reflects systemic biological aging rather than driving it, positioning age at menopause as a potential biomarker of the rate of biological aging.

Introduction

While reproductive senescence - characterized by a decline in reproductive capacity with age - is widespread across mammalian species, the complete cessation of reproduction followed by a prolonged post-reproductive lifespan is relatively rare^{1,2}. Humans are among the few species in which females experience menopause and live substantial post-reproductive lives. Interestingly, while a significant lifespan extension has been observed during the last decades, the age at which women reach the final menstrual period (FMP), - age at menopause (AM, defined posteriori after 12-month period of amenorrhea required to define that the final menstrual period has occurred), did not change much: data from United States show that between 1959 and 2018 average lifespan increased by more than 8 years (more than 10% increase, from 73.3 to 81.5)³, whereas in the same time period, mean age at natural menopause increased only by 1,5 year, from 48.4 to 49.9 (merely 3%)⁴.

Primordial follicles are formed during fetal development and remain in a resting state within the ovarian cortex, constituting the ovarian reserve. From birth through the reproductive years, ovarian function undergoes progressive changes primarily driven by the gradual depletion of these follicles. The rate of follicle loss is not uniform; it accelerates from birth until around 24 years of age, after which it gradually slows down until FMP. There is considerable variability in these processes, leading to differences in the AM across the population. The FMP typically occurs between ages 45 and 55. An FMP occurring before age 45 is generally considered “early menopause”, whereas an FMP occurring before age 40 is classified as “premature menopause”. “Late menopause” is characterized by the FMP after the age of 55⁵.

AM holds intrinsic clinical and public health interest as it may be a marker of aging and health status. While later AM has been associated with reduced risk of cardiovascular disease, less bone density loss, reduced risk of osteoporosis and fracture, greater life expectancy and reduced all-cause mortality, early

menopause is correlated with adverse health outcomes, including earlier decline in cognitive function and increased risk of ischemic heart disease⁶, among others. A large Dutch study following 12,134 postmenopausal women revealed that each additional year in AM was associated with a 2% reduction in age-adjusted mortality. Furthermore, life expectancy for women who experienced FMP after age 55 was approximately 2.0 years longer than for those reached FMP before the age of 40⁷. Indirect evidence supporting this association was reported in an Israeli study in which mortality risk among parous women was lowest in those who had late-age births and remained fertile beyond the age of 45, compared with parous women whose last birth occurred before 35 years of age⁸.

Although data suggest earlier AM is associated with reduced lifespan, establishing causal relationships remains challenging. It is unclear whether menopause itself influences aging and longevity, or whether an accelerated rate of biological aging leads to an accelerated reproductive aging, greater disease burden, and reduced life expectancy.

To investigate the connection between reproductive senescence and longevity Morgan Levine⁹ and colleagues used the “epigenetic clock”, a biological age predictor based on DNA methylation patterns^{10–16}. These clocks estimate the pace of biological aging: individuals whose biological age exceeds their chronological age are considered biologically older and are therefore aging faster. The study found that AM was significantly associated with epigenetic age acceleration (defined as the difference between estimated biological age and chronological age) with greater epigenetic age acceleration observed in women who experienced FMP at a younger age. Since certain SNPs associated with earlier AM were also correlated with accelerated aging, there is a biological base for this observation. However, the direction of causality remained unclear - whether menopause itself accelerates aging, or is it accelerated biological aging that accelerates reproductive aging and AM. Women in which menopause was forced due to surgical removal of the ovaries (bilateral oophorectomy, ovariectomy) were found to be significantly biologically older than their age-matched controls, leading the authors to conclude that menopause accelerates epigenetic aging.

However, Joop Laven¹⁷ argued the opposite. Recent genome-wide association studies (GWAS) have shown that many genes associated with menopause are involved in the repair of DNA damage. For example, an analysis of the data from 132,370 women in the UK Biobank, found a correlation between rare damaging variants in *CHEK2*, *DCLRE1A*, and *HELB* with later menopause, and variants in *TOP3A* and *CLPB* with earlier menopause¹⁸. The DNA damage theory of ageing proposes that a decline in a cell's or organism's ability to maintain genomic integrity leads to cellular dysfunction, senescence, and aging. In the ovaries, these same processes may also result in accelerated depletion of the oocyte reserve and, consequently, an earlier reproductive aging^{17,18}. Together, these findings suggest that aging and menopause may share common biological mechanisms. Supporting this view, one of the two SNPs investigated in Levine's study, rs16991615, is located in the *MCM8* gene, which play an important role in DNA damage repair¹⁹. Interpretation of data from women who underwent bilateral oophorectomy may be biased, as Levine et al reported that the surgery was frequently performed due to underlying

conditions, such as fibroids, menstrual disorders, or endometriosis (or some combination of these)⁹. Some of these conditions have themselves been associated with differences in epigenetic aging²⁰ suggesting that the observed epigenetic age acceleration may reflect pre-existing pathology rather than the direct effect of menopause.

To address whether menopause accelerates biological aging, or, whether accelerated biological aging leads to earlier reproductive aging, we utilized data from two established rat models of reproductive aging and a clinical cohort of postmenopausal women. Importantly, the use of animal models allowed us to minimize key confounding factors commonly present in human clinical studies—such as variations in health status, lifestyle, and genetic background. This controlled experimental design ensured that the observed effects could be attributed more specifically to the menopause rather than to extrinsic variables.

Results

Bilateral ovariectomy does not induce epigenetic age acceleration

To investigate whether menopause accelerates biological aging, we used a rat model in which menopausal transition was induced by bilateral ovariectomy. The study included three groups: 10 rats were bilaterally ovariectomized (OVX) and received subcutaneous injections of corn oil (vehicle), 10 OVX rats received subcutaneous injections of estradiol treatment (E2), and 10 rats underwent a sham operation (SH) and received the vehicle. The experiment was performed twice using the same design: first in 12-weeks-old rats, corresponding to very early postmenopausal status (Fig. 1), and second in older, 24-weeks-old rats (Fig. 2). Epigenetic age was measured in all animals (DNA extracted from spleen for 12-week-old and PBMCs for 24-weeks-old rats). Because sacrifices were performed over a two-week period (when rats were 23-24-weeks-old in early postmenopause model and 34-35-weeks-old in older postmenopause model), resulting in slight differences in chronological age at the time of sampling, we also calculated epigenetic age acceleration, defined as the difference between epigenetic age and chronological age. In both experimental settings, bilateral ovariectomy did not accelerate epigenetic aging. No statistically significant differences in epigenetic age or epigenetic age acceleration were observed between OVX, OVX+E2, and SH animals.

No difference in epigenetic age or epigenetic age acceleration between animals of the same chronological age, but at different stages of reproductive aging

Age at natural menopause (determined retrospectively after 12 consecutive months of amenorrhea in the absence of other physiological, pathological, or clinical causes) is highly heritable, with at least 50% of the interindividual variability in menopausal age attributable to genetic effects^{21,22}. Nevertheless, variability has been observed even among monozygotic twins, with cases highly discordant for age at

menopause (≥ 10 years)²³. Similar variability is also present in inbred rodent strains^{24,25} and can be used to investigate the relationship between reproductive and biological aging.

To model natural reproductive senescence, we monitored cycle duration in 6.5-month-old Sprague-Dawley rats for 10 weeks. Based on cycle characteristics, 9-month-old animals were stratified into three groups reflecting stages of ovarian senescence, resembling the human Stages of Reproductive Aging Workshop (STRAW) classification^{26,27}: 1) REG, with regular 4–5 days cycles (corresponding to human, premenopausal stage); 2) IRREG, with irregular, 5–8 day cycles (corresponding to human perimenopausal stage); 3) AC, acyclic animals with no cycles for at least 9 days (corresponding to postmenopausal stage). 6-month-old rats served as a younger control group.

Epigenetic age was measured in liver, in young animals and in rats of the same chronological age but at different stages of reproductive aging. As expected, 6-month-old rats showed substantially lower epigenetic age across all the clocks tested. There was no difference between the 9-month-old REG, IRREG and AC groups (Supplemental Fig. 1). Similarly, no significant differences in epigenetic age acceleration were observed between REG and AC animals (Fig. 3). If anything, age acceleration appeared slightly lower in the older animals compared with the 6-month controls, which is consistent with the known logarithmic relationship between epigenetic age and chronological age, where the rate of increase slows slightly with advancing age²⁸. Overall, results from the natural reproductive senescence model do not support the hypothesis that menopause accelerates biological aging.

In postmenopausal women, epigenetic aging acceleration is observed only in women with earlier-than-median age at menopause

Since age at natural menopause has been shown to be affected by environmental, socioeconomic, reproductive, dietary, and lifestyle factors²⁹, we assessed epigenetic age acceleration in postmenopausal women. Based on metabolic biomarkers, we classified women into four health-status groups: 1) healthy – women with measurements indicating overall good metabolic health evidenced by: total cholesterol < 200 mg/dL, LDL-c < 115 mg/dL, adiponectin/leptin > 1, glucose < 100 mg/dL, HOMA-IR < 2.5, ALT < 45 U/L, AST < 43 U/L and GGT < 36 U/L; 2) Compromised hepatic function – women with elevated liver enzymes (ALT > 45 U/L or AST > 43 U/L or GGT > 36 U/L); 3) altered lipid metabolism - women with elevated total cholesterol (> 200 mg/dL) and LDL-c (> 130 mg/dL); 4) high cardiometabolic risk - women with abdominal obesity according to the International Diabetes Federation (waist circumference > 80) or with a BMI > 25, elevated total cholesterol (> 200 mg/dL), LDL-c (> 115 mg/dL), impaired glucose metabolism (glucose ≥ 100 mg/dL - prediabetes, or HOMA-IR > 2.5 - insulin resistance), and an adiponectin/leptin ratio < 0.5, a surrogate marker of adipose tissue inflammation and dysfunction.

None of the groups characterized by compromised metabolic health showed increased epigenetic age acceleration, and no changes have been observed when comparing to healthy group (Supplementary Fig. 3). Women in all four groups experienced reproductive aging at different chronological ages, and the mean AM did not differ significantly between the groups (Supplementary Fig. 4). Interestingly, the

hepatic group showed the widest ranges of AM raising the possibility that both lower and higher age when FMP is reached may be associated with hepatic health. However, when participants were stratified according to AM relative to the median value of the study cohort (< 50 vs ≥ 50 years), women who reached FMP earlier than the cohort median, exhibited epigenetic age acceleration (Fig. 4). This pattern was consistently detected across multiple epigenetic clocks. The same pattern of epigenetic age acceleration in women with earlier-than-median AM was consistently observed within each metabolic health subgroups.

Taken together, findings from animal experiments and clinical observations suggest that menopause itself does not accelerate epigenetic aging. Rather the opposite; it seems that earlier reproductive aging and an earlier age at menopause are the consequences of accelerated biological aging.

Neuronal and longevity pathways are differentially methylated between women with earlier and later than median AM

To investigate the biological consequences of differences in AM, we performed differential DNA methylation analysis comparing women who reached FMP before age 50 with those who reached FMP at age 50 or later, adjusting for chronological age at the time of sample collection. In total, 60,502 differentially methylated probes were identified (40,381 showing increased methylation and 20,121 decreased methylation), however none remained significant after correction for multiple testing (adjusted p Val = 0.05). We therefore selected probes with a methylation fold change greater than 2 (1,799 in total; 1,135 hypermethylated and 665 hypomethylated) and performed Gene Ontology (GO) and KEGG pathway enrichment analysis.

Among the 20 most enriched pathways was “hsa04213: Longevity regulating pathway” – with multiple genes (*FOXO3*, *IGF1R*, *KRAS*, *PRKACA*, *SOD1*, *SOD2*), involved not only in longevity, but also ovarian follicle maintenance highlighting the connection between systemic aging and reproductive aging^{30–33}. This observation suggests that, in addition to genetic variation, epigenetic dysregulation of longevity genes may contribute to both accelerated aging and loss of reproductive capacity.

Interestingly, the 7th most significant KEGG pathway was “hsa05012: Parkinson disease” (Fig. 5A, Supplemental Fig. 5D). While Parkinson disease (PD) is generally more common in men than in women^{34,35}, epidemiological evidence suggests that the risk profile changes among women who experience menopause at an earlier age. For example, a mendelian randomization study from 2021 analysing data from over 19,000 women reported that each additional year of later AM was associated with a 7% reduction in PD risk³⁶.

Further analysis of molecular functions associated with hypermethylated genes revealed strong enrichment for histone methyltransferase activity, with many of them having histone, mainly histone H3, methyltransferase activity (Fig. 5B, Supplemental Fig. 6A). Many epigenetic players have also been hypomethylated; cellular component analysis showed hypomethylated genes were enriched in components of chromatin remodelling complexes (Fig. 5C, Supplemental Fig. 7B).

These findings suggest a strong epigenetic component linking earlier AM with pathways involved in longevity, chromatin regulation, and neurodegeneration, supporting the concept that reproductive aging and systemic biological aging are tightly interconnected at the molecular level.

Discussion

This paper addresses a longstanding question in aging biology by distinguishing between menopause as a driver versus a biomarker of systemic aging. Our results do not support the hypothesis that menopause accelerates epigenetic aging. Instead, our findings are more consistent with the alternative hypothesis that accelerated epigenetic aging precedes FMP and contributes to earlier reproductive aging that causes earlier AM.

One of the main arguments supporting the idea that menopause accelerates epigenetic aging comes from the results showing that forced menopause, induced by bilateral oophorectomy, is associated with increased epigenetic age acceleration in both blood and saliva⁹. However, the authors of that study noted that the primary indications for oophorectomy were underlying medical conditions such as fibroids, menstrual disorders, or endometriosis (or combination). While no studies reporting epigenetic age of individuals with those conditions have been found, accelerated epigenetic aging has been documented in other chronic medical conditions³⁷. It is therefore plausible that the observed epigenetic age acceleration reflects underlying pathology rather than the direct effect of oophorectomy or menopause.

In our animal experiments, where pre-existing health conditions were not a confounding factor, bilateral ovariectomy, did not affect epigenetic aging regardless of the age at which surgery was performed (12 or 24 weeks). Similarly, no differences in epigenetic age were observed between rats at different stages of reproductive aging in the spontaneous reproductive senescence model. Importantly, these experiments were not originally designed to study the relation between reproductive and epigenetic aging - they were designed to evaluate nutritional interventions in a model of the female postmenopausal state. Consequently, different tissues were analysed across experiments. In the study using 12-week-old rats, spleen tissue, considered best proxy for blood, was analysed; in the 24-week-old rat experiment, peripheral blood mononuclear cells (PBMCs) were used to more closely resemble clinical studies, whereas liver tissue was analysed in the spontaneous reproductive aging model. Although DNA methylation patterns vary between tissues, the pan-tissue clock, which applies to all nucleated cells, was used in all three experiments. Most tissues have roughly the same age according to the pan tissue clock with some exceptions such as female breast tissue and the cerebellum^{38–40}.

Both epigenetic aging and age at menopause have been associated with health status and lifestyle factors. In our clinical study of postmenopausal women, participants were stratified into four metabolic health categories: metabolically healthy individuals, individuals with altered lipid profiles, individuals with indicators of compromised liver function, and individuals with elevated cardiovascular risk. We hypothesized suboptimal metabolic parameters would be associated with accelerated epigenetic aging

and earlier reproductive aging. However, no significant differences in epigenetic age acceleration were observed between these health groups. In contrast, when participants were stratified according to age at menopause relative to the cohort median (before vs after 50 years of age), women who experienced FMP earlier exhibited greater epigenetic age acceleration, independent of chronological age at sample collection and time since FMP. Similar observations have been reported in earlier studies. A study from the United States, that analysed 658 postmenopausal women, found that later age at menopause was associated with lower epigenetic age acceleration estimated using the GrimAge clock (a small but statistically significant effect)⁴¹. An medRxiv preprint of the study involving 770 postmenopausal women aged 50–85 investigated the connection between reproductive profiles, epigenetic aging and mortality. Within 4 groups, only those characterised by early menopause transition had accelerated epigenetic aging, calculated with DunedinPoAm⁴². Finally, a recent study analysing 1117 U.S. women aged 50 or older from NHANES 1999–2002 cohort, confirmed that later FMP and a longer reproductive lifespan were significantly associated with decelerated epigenetic aging⁴³.

We believe that, despite being limited by cross-sectional design and small sample size of clinical study and the use of different tissues in animal models, the findings suggest that epigenetic age acceleration is unlikely to be caused by menopause itself. Rather, reproductive aging may reflect the broader biological aging trajectory of the organism, with earlier menopause representing a manifestation of accelerated systemic aging. Higher prevalence of health problems and increased mortality associated with earlier menopause^{44–46}, may therefore reflect an overall higher biological age, faster “use of material”. This interpretation is consistent with genetic evidence linking reproductive aging and longevity pathways. Several genes associated with lifespan regulation also play a role maintaining ovarian follicle reserve and have been linked to age at menopause⁴⁷. Recent studies have also reported genetic links between menopausal timing and cancer risk⁴⁸. Additional evidence supporting the connection between reproductive capacity and biological aging comes from studies of fertility. Accelerated epigenetic aging has been reported in women undergoing assisted reproductive technologies (ART) (proxy for fertility problems)⁴⁹, in individuals with poor ovarian response during ART⁵⁰, and in those with reduced oocyte yield⁵¹. Accelerated epigenetic age during adolescence has been associated with advanced pubertal, neuroendocrine, psychiatric and cognitive maturity⁵². It poses a very interesting avenue for investigation, because these observations raise the possibility that female reproductive potential can be extrapolated from her epigenetic aging trajectory. This hypothesis is especially important nowadays, as the trend of individuals having children at later ages⁵³ contributes to raising globally fertility problems⁵⁴.

Menopause is a neuroendocrine process involving extensive metabolic and structural adaptations in the brain^{55,56}. Up to 62% of perimenopausal and postmenopausal women report cognitive symptoms such as memory lapses and “brain fog”, around the time of FMP. Many other symptoms of perimenopause, including hot flashes, disturbed sleep and mood changes, have neurological bases⁵⁷. Earlier menopause not only has been associated with more severe symptoms⁵⁸, but also with the increased risk of cognitive impairments and dementia later in life^{59–61}.

Consistent with these observations, pathways associated with neuronal function were among the most strongly deregulated in the DNA methylation analysis in women who reached FMP earlier compared with those who reached FMP later than study median, despite the analysis being performed in PBMCs. The Parkinson disease pathway was among the most significantly enriched KEGG pathways, suggesting that there is strong epigenetic component that links earlier AM with increased dementia risk.

Indeed, many pathways connected with histone methylation have been identified within hypermethylated regions. This finding suggests that transcriptional repression of histone methyltransferase genes may become transcriptionally inactive during menopause transition, potentially leading to reduced histone methylation levels. Indeed, in a separate study using the spontaneous reproductive aging model described here, we observed that histone H3 methylation marks (H3K4me3 and H3K27me3) in the hypothalamus and hippocampus gradually declined during menopausal transition, with the highest levels observed in animals with regular cycles and progressively lower levels in irregular and acyclic animals. A similar pattern was observed for DNA methylation in the same model in a study conducted by Roberta Brinton's team²⁷. In that work, treatment with DNA demethylating compound 5-aza-2'-deoxycytidine, caused hypomethylation and accelerated menopause transition in rats. Conversely, supplementation with methionine, a universal methyl donor involved in DNA and histone methylation pathways, delayed reproductive aging, with substantially higher proportion of animals remaining in the regular cycling stage (REG) at 10 months (86% of methionine-treated animals and 25% of control animals).

These findings raise the possibility that supplements or dietary factors influencing methionine and folic cycles, known to promote methyl group production and promoting DNA and histone methylation, can be promising intervention for perimenopausal women. These nutrients have been associated with longevity^{62,63} and may also delay reproductive aging and by that reduce the risk of health complications associated with earlier menopause.

Methods

Animal studies

Bilateral ovariectomy model

Detailed animal housing and treatment have been described elsewhere^{64,65}. Shortly, all the procedures have been approved by The Animal Ethics Committee of the Technological Unit of Nutrition and Health of EURECAT (Reus, Spain) and the Generalitat de Catalunya (protocol code 11223). The study followed the 'Principles of Laboratory Animal Care', complied with the ARRIVE guidelines and was conducted in compliance with the EU Directive 2010/63/EU for animal experiments. 12-week-old (early menopause transition model, (ENVIGO, Indianapolis, USA) or 24-week-old (later menopause transition model, JANVIER LABS, Saint-Berthevin, France) female Sprague-Dawley rats. Thirty rats at each age group were bilaterally ovariectomized (OVX), and 10 rats underwent a sham operation (SHAM) at the corresponding facility. Throughout the study, all rats were housed in pairs at 22°C on a 12 h light/dark cycle with lights

on at 9:00 am, and they had ad libitum access to food and water. After an acclimatization period of 1 week, the 30 OVX rats at each age group, were divided into three experimental groups based on the treatment they received for 8 weeks: OVX – no additional treatment, OVX-E2 – biweekly subcutaneous injections of $25 \mu\text{g kg}^{-1}$ 17 β -estradiol, OVX-MSI – treatment with nutritional supplement, not described in this study. 10 sham-operated (SHAM; $n = 10$) rats at each age group were assigned to the control group. All groups were fed a standard chow diet (Teklad Global 14% Protein Rodent Diet 2014, Harlan, Barcelona, Spain). The caloric breakdown of the diet (2.9 kcal g^{-1}) was 20% protein, 13% fat, and 67% carbohydrates. Body weight was recorded once each week, food was renewed daily, and the food intake was documented every 7 days.

Both groups were sacrificed at day 57, under anaesthesia (pentobarbital sodium, 60 mg per kg body weight) after 6 h of diurnal fasting.

Natural reproductive senescence model

Detailed animal housing and treatment have been described elsewhere⁶⁶. Shortly, all procedures were approved by the Animal Ethics Committee of the Technological Unit of Nutrition and Health of EURECAT (Reus, Spain) and the Generalitat de Catalunya (protocol code 11223). The study followed the ‘Principles of Laboratory Animal Care’, complied with the ARRIVE guidelines and was conducted in compliance with the EU Directive 2010/63/EU for animal experiments. Twelve young (3.5 months old, sexually naïve) and sixty middle-aged (8 months old) female Sprague–Dawley rats (INOTIV, Horst, Netherlands) were used at the beginning of the study. Throughout the study, all rats were housed in pairs at the animal facility of EURECAT (Reus, Spain) at 22°C on a 12 h light/dark cycle with lights on at 9:00 am, and they had ad libitum access to food and water. All animals were fed a standard chow diet (Teklad Global 14% Protein Rodent Diet 2014, Harlan, Barcelona, Spain), renewed daily. Estrous cyclicity was assessed daily according to the procedure described by McLean et al⁶⁷. To minimize stress and acclimatize the animals to handling, rats were handled for 5 min per day for one week before the start of the study. Estrous cycle monitoring was then performed by daily vaginal lavage with ultrapure distilled water. Vaginal smears were collected at 11:00am and evaluated by cytological examination, and the estrous cycle stage was determined based on the morphological characterization of uterine cells corresponding to the four stages of the cycle: estrus, metestrus, diestrus and proestrus. Animals were classified according to the regularity of the estrous cycle based on the last 14 days of monitoring. Regular animals exhibited three consecutive 4–5 day cycles, irregular animals showed two consecutive cycles lasting 6 days or longer (extended diestrus phase), and acyclic animals displayed 14 continuous days in persistent estrous. Rats presenting persistent estrus during the initial 14 days were excluded from the study. Animals were sacrificed after 4 h of diurnal fasting under anesthesia (pentobarbital sodium, 60 mg kg⁻¹ body weight). Rats were assigned to the following experimental groups: young rats with regular cycling (CTRL; $n = 10$, ~ 5 months), middle-aged rats with regular cycling (REG; $n = 7$, ~ 10 months), middle-aged rats with irregular cycling (IRREG; $n = 8$, ~ 10 months), and middle-aged rats with persistent estrous (AC; $n = 8$, ~ 10 months).

Sample Collection

For the analysis of epigenetic age in the early menopause transition model spleen has been excised, weighed, frozen in liquid nitrogen, and stored at -80°C until the analysis. In the later menopause transition model blood (3.5 ml) was collected by beheading into 15 ml tubes pretreated with heparin. Peripheral blood mononuclear cells (PBMCs) have been separated with the Ficoll method, by first mixing the blood with 1 ml of balanced salt solution (5.5 mM anhydrous d-glucose NaCl, 0.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 98 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.4 mM KCl, 0.145 M Tris mixed 1:9 with 0.14 M NaCl), slowly applying into a fresh falcon tube with 3.5 mL of Ficoll, centrifugation at 900 g for 40 min at RT, washing pelleted PBMCs with fresh balanced salt solution and removal of red blood cell with Red Blood Cell Lysis Buffer (11814389001, Roche), following manufacturers extraction. PBMCs have been flash-frozen in liquid nitrogen, and stored at -80°C until the analysis. In the natural reproductive senescence model, liver has been excised, weighed, frozen in liquid nitrogen, and stored at -80°C until the analysis.

Human study

Participant Recruitment and Sample Collection

Samples for the present study were derived from a sub-cohort of the SHE-HEALTH study (ClinicalTrials.gov Identifier: NCT05397015), an observational study with postmenopausal women who were characterized by measuring metabolic and anthropometric biomarkers, among others. Recruitment and data/sample collection were performed between 2021 and 2022.

The SHE-HEALTH study protocol included women aged 40 to 63 years who reported amenorrhea for at least 12 consecutive months. To ensure a definitive postmenopausal status, clinical reports were biochemically validated by measuring serum levels of follicle-stimulating hormone (FSH) and 17β -estradiol. Exclusion criteria included: (i) diagnosis of chronic diseases such as diabetes (fasting glucose ≥ 126 mg/dL), cardiovascular or coronary diseases, chronic kidney disease (serum creatinine ≥ 1.5 mg/dL), or chronic gastrointestinal pathologies (e.g., Celiac or Crohn's disease); (ii) use of medications for hypertension or dyslipidaemia, or intake of anti-inflammatory drugs within the week prior to enrolment; and (iii) lifestyle or physical factors, specifically a Body Mass Index (BMI) < 18.5 or ≥ 35 kg/m², current smoking habit, alcohol consumption exceeding 14 drinks per week, or adherence to a prescribed weight-loss diet or participation in another clinical trial within the previous month.

The study protocol was approved by the Clinical Research Ethical Committee of Institut d'Investigació Sanitària Pere Virgili (Ref. CEIM: 153/2020), Reus, Spain. The protocol and trial were conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed, written consent was obtained from all participants.

Blood Sample Processing

Venous blood samples were collected after an overnight fast using specific vacuum tubes for serum and plasma (EDTA) separation. For serum, samples were allowed to clot for 30 minutes at room temperature and then centrifuged at 1500g for 15 minutes at room temperature. For plasma, EDTA-containing tubes were centrifuged immediately at 1500g for 10 minutes at 4°C. For epigenomics, 2mL of blood was collected to EDTA-containing tubes, centrifuged at 900g for 10 minutes at 4°C to allow plasma from blood cell separation. Buffy coats were transferred to a fresh microcentrifuge tube using Pasteur pipette. All aliquots were immediately stored at – 80°C until analysis.

Metabolomic Health Analysis

The following circulating parameters were measured in the analytical service of the Laboratori de Referència de Catalunya using standard procedures: plasma glucose (mg/dL), total cholesterol (mg/dL), high-density lipoprotein cholesterol (HDL-C, mg/dL), gamma-glutamyl transpeptidase (GGT, U/L), alanine aminotransferase (ALT, U/L), and aspartate aminotransferase (AST, U/L). Low-density lipoprotein cholesterol (LDL-C, mg/dL) was calculated using the Friedewald formula whenever TG levels were inferior to 400 mg/dL. Circulating insulin (μ U/mL) and adiponectin (ng/mL) levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits from Mercodia (AD Bioinstrument) and Biovendor, following the manufacturers' instructions.

Subjects' insulin resistance was assessed by the homeostatic model assessment for insulin resistance (HOMA-IR). The HOMA-IR score was calculated from fasting insulin and glucose concentrations using the formula of Matthews et al⁶⁸. Furthermore, the adiponectin to leptin ratio (AL) was determined as an indicator of adipose tissue function and inflammation linked to cardiometabolic risk, as described by Frühbeck et al⁶⁹. Thus, an AL ratio ≥ 1.0 is considered normal, while a ratio between ≥ 0.5 and < 1.0 suggests moderate-to-medium increased risk, and a ratio < 0.5 indicates a severe risk of suffering from cardiometabolic diseases.

Metabolic Health Groups Definition

Participants were classified into metabolic health categories based on predefined biochemical thresholds derived from circulating metabolic and hepatic biomarkers. Four metabolic profiles were defined. The lipid alteration (lipid, n = 17) group included participants presenting dyslipidaemia, defined as total cholesterol > 200 mg/dL and/or low-density lipoprotein cholesterol (LDL) > 130 mg/dL. Women having prediabetes (glucose ≥ 100), insulin resistance (HOMA_IR > 2.5), adiponectin/leptin ratio < 0.5 , ALT > 45 U/L or AST > 43 or GGT > 36 were excluded. The hepatic alteration (hepatic, n = 14) group included participants with elevated liver enzyme levels, defined as alanine aminotransferase (ALT) > 45 U/L and/or aspartate aminotransferase (AST) > 43 U/L and/or gamma-glutamyl transferase (GGT) > 36 U/L, independently of other metabolic parameters. The high cardiometabolic risk (high cardio, n = 12) group was defined by women with abdominal obesity according to the International Diabetes Federation (waist circumference > 80) or with a BMI > 25 and the presence of multiple metabolic alterations, including total cholesterol > 200 mg/dL, LDL > 130 mg/dL, fasting glucose > 100 mg/dL, and an adiponectin-to-leptin ratio < 0.5 . The metabolically healthy (healthy, n = 7) group included participants

with all parameters within the normal range, defined as total cholesterol < 200 mg/dL, LDL < 130 mg/dL, ALT < 45 U/L, AST < 43 U/L, GGT < 36 U/L, fasting glucose < 100 mg/dL, and an AL ratio > 1.

DNA methylation analysis

DNA extraction

Spleen and liver were placed on Sterilin™ 35mm Petri dish on ice, and cut with razor blade to achieve a slice of 30mg. DNA was extracted with *Quick-DNA/RNA* Miniprep kit (D7001, Zymo) following manufacturer's instruction. DNA from rat and human PBMCs has been extracted with the use of NucleoSpin™ TriPrep Mini kit (11972392, Macherey-Nagel™) following manufacturer's instruction. Purity was determined using the NanoDrop™ 2000 spectrophotometer and quantity by Qubit 4 Fluorometer.

DNA methylation

Bisulfite conversion, DNA methylation analysis by Illumina Infinium Microarrays were performed by the Clock Foundation (<https://clockfoundation.org/>); spleen from 12-week-old bilateral ovariectomy using the mammalian methylation array (HorvathMammalMethylChip40)⁷⁰, PBMCs from 24-week-old bilateral ovariectomy and liver from natural reproductive senescence by extended HorvathMammalMethylChip320⁷¹ and human PBMCs by IlluminaHumanMethylationEPICv2.

Biological age calculations

Biological age calculations were performed by the Clock Foundation in *R*. Raw methylation signal intensities were retrieved using the function `read.metharray.exp` of the *minfi* v1.48.0 *R* package, followed by linear dye bias correction and noob background correction to account for technical variation in background fluorescence signal. β -value was calculated from the intensity of the methylated and unmethylated sites, as the ratio of fluorescent signals. Epigenetic age was calculated with linear models algorithms with the *limma* package using the formula: $\text{Clock} \sim \text{Group} + \text{Age}$. The model was fitted using the robust aggregation regression method by running *lmFit* function. Empirical Bayes statistics were estimated using *eBayes* function. Benjamini-Hochberg (BH) method was used to correct for multiple testing.

Epigenetic age acceleration

Epigenetic Age Acceleration measures biological aging dynamics. It is computed as the difference between DNA methylation age and chronological age. A positive EAA value indicates that an individual's biological age surpasses their chronological age, suggesting accelerated aging.

Differential methylation between “early” and “late” menopause clinical groups

Volunteers were divided into 2 groups: “early” menopause, if they reached final menstrual period before the median for the study population, which equals to 50, and agrees with wider populational studies in Catalonia. The remaining women, that reached final menstrual period at the age of 50, or later, were

assigned to the “late” menopause group. Raw methylation signal intensities were retrieved using the function `read.metharray.exp` of the *minfi* v 1.54.1 R package and were pre-processed with the standard Illumina method. Loci containing snps and located in Y chromosome have been removed. Differentially methylated probes between the group (early vs late) were selected with the *limma* package (v3.64.0) including chronological age as covariate using the formula: $\sim \text{Group} + \text{Age}$. Empirical Bayes statistics were estimated using *eBayes* function. Benjamini-Hochberg (BH) method was used to correct for multiple testing. Gene ontology and KEGG pathway enrichment analysis were done using *gometh* function of *missMethyl* (v1.42.0) package, bubble plots were generated with *ggplot2* (v3.5.2) package for the visualisation.

Declarations

Data availability

The datasets generated and/or analysed during the current study are available in the GEO repository, GSE338255 and supplementary information files of this article.

Code availability

The underlying code for this study is available in GitHub and can be accessed via this link <https://github.com/>.

Acknowledgements

We gratefully acknowledge the help given by Paula Cid Martínez, former technician at the Centre for Omic Sciences (Eurecat, Reus, Spain) in processing of human samples.

The research described here was financially supported by the Agency for Business Competitiveness of the Government of Catalonia (ACCIÓ/TECCT11-1-0012 and ACCIÓ Tecniospring INDUSTRY ACE003/20/000013)

Author contributions

Dorota Komar identified a research question, secured research funding, conceptualised and designed studies (at the level of research question, methods and analysis), contributed to computational calculation (epigenetic age in clinical samples) and visualisation, writing - original draft, writing - review & editing. **Julio Baudin** is a main contributor to preclinical animal experiments, animal handling and sample collection, evaluation of reproductive stage of female rats in natural senescence model. **Andrea Costa** is a main contributor to clinical trial follow up, ensuring proper sample selection, collection and analysis of anthropometric and biochemical parameters and group identification, writing - review & editing. **Lucía Tadeo-Orellana** and **Lorena Garcia** helped in clinical sample processing, **Jordi Romero Giménez** helped in preclinical animal experiments, **Lorena Calderón-Pérez**, **Alícia Domingo-Tàrrrech**, **Anna Crescenti** helped in clinical trial through the design, recruitment and sample collection. **Helena**

Torrell responsible of genomics unit, monitoring sample processing activities. **Antoni Caimari**, director of biotechnology area, conceptualization and design of both preclinical and clinical studies, funding acquisition, writing - review & editing. **Nuria Canela**, director of the Center for Omic Sciences, helped securing research funding, management of study performance, writing - review & editing. All the authors have read and agreed to the final version of the manuscript. AI (corporate ChatGTP) was used for assisted copy editing

Competing interests

Author Juozas Gordevicius is an employee of Clock Foundation, but declares no non-financial competing interests. All other authors declare no financial or non-financial competing interests.

References

1. Chapman, S. N., Ellis, S., Lahdenperä, M., Croft, D. P. & Lummaa, V. Menopause has not evolved as a general trait in mammals: A response to 'Do mammals have menopause?' 2024.02.29.582687 Preprint at <https://doi.org/10.1101/2024.02.29.582687> (2024).
2. Winkler, I. & Goncalves, A. Do mammals have menopause? *Cell* 186, 4729–4733 (2023).
3. Interstate Variations in Mortality in the United States, 1959–2018 | SOA.
<https://www.soa.org/resources/research-reports/2021/interstate-variations-in-mortality-in-the-united-states-1959-2018/>.
4. Appiah, D., Nwabuo, C. C., Ebong, I. A., Wellons, M. F. & Winters, S. J. Trends in Age at Natural Menopause and Reproductive Life Span Among US Women, 1959–2018. *JAMA* 325, 1328–1330 (2021).
5. Eckhardt, S. & Wellons, M. Defining Menopause: What Is Early, What Is Late? in *Primary Ovarian Insufficiency: A Clinical Guide to Early Menopause* (eds Santoro, N. F. & Cooper, A. R.) 1–17 (Springer International Publishing, Cham, 2016). doi:10.1007/978-3-319-22491-6_1.
6. Gold, E. B. The Timing of the Age at Which Natural Menopause Occurs. *Obstet. Gynecol. Clin. North Am.* 38, 425–440 (2011).
7. Ossewaarde, M. E. *et al.* Age at menopause, cause-specific mortality and total life expectancy. *Epidemiol. Camb. Mass* 16, 556–562 (2005).
8. Jaffe, D. *et al.* Influence of late-age births on maternal longevity. *Ann. Epidemiol.* 25, 387–391 (2015).
9. Levine, M. E. *et al.* Menopause accelerates biological aging. *Proc. Natl. Acad. Sci. U. S. A.* 113, 9327–9332 (2016).
10. Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol.* 14, 3156 (2013).
11. Field, A. E. *et al.* DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. *Mol. Cell* 71, 882–895 (2018).
12. Duan, R., Fu, Q., Sun, Y. & Li, Q. Epigenetic clock: A promising biomarker and practical tool in aging. *Ageing Res. Rev.* 81, 101743 (2022).

13. Teschendorff, A. E. & Horvath, S. Epigenetic ageing clocks: statistical methods and emerging computational challenges. *Nat. Rev. Genet.* <https://doi.org/10.1038/s41576-024-00807-w> (2025) doi:10.1038/s41576-024-00807-w.
14. Bell, C. G. et al. DNA methylation aging clocks: challenges and recommendations. *Genome Biol.* 20, 249 (2019).
15. Zhang, Q. et al. Improved precision of epigenetic clock estimates across tissues and its implication for biological ageing. *Genome Med.* 11, 54 (2019).
16. Lu, A. T. et al. DNA methylation GrimAge strongly predicts lifespan and healthspan. *Aging* 11, 303–327 (2019).
17. Early menopause results from instead of causes premature general ageing - Reproductive BioMedicine Online. [https://www.rbmojournal.com/article/S1472-6483\(22\)00220-6/fulltext](https://www.rbmojournal.com/article/S1472-6483(22)00220-6/fulltext).
18. Ward, L. D. et al. Rare coding variants in DNA damage repair genes associated with timing of natural menopause. *HGG Adv.* 3, 100079 (2022).
19. Desai, S. et al. MCM8 and MCM9 Nucleotide Variants in Women With Primary Ovarian Insufficiency. *J. Clin. Endocrinol. Metab.* 102, 576–582 (2016).
20. Leap, K., Yotova, I., Horvath, S. & Martinez-Agosto, J. A. Epigenetic age provides insight into tissue origin in endometriosis. *Sci. Rep.* 12, 21281 (2022).
21. de Bruin, J. P. et al. The role of genetic factors in age at natural menopause. *Hum. Reprod. Oxf. Engl.* 16, 2014–2018 (2001).
22. Heritability of Age at Natural Menopause in the Framingham Heart Study | The Journal of Clinical Endocrinology & Metabolism | Oxford Academic. <https://academic.oup.com/jcem/article-abstract/90/6/3427/2870578?redirectedFrom=fulltext&login=false>.
23. Gosden, R. G. et al. Prevalence of premature ovarian failure in monozygotic and dizygotic twins. *Hum. Reprod. Oxf. Engl.* 22, 610–615 (2007).
24. Gosden, R. G., Laing, S. C., Felicio, L. S., Nelson, J. F. & Finch, C. E. Imminent oocyte exhaustion and reduced follicular recruitment mark the transition to acyclicity in aging C57BL/6J mice. *Biol. Reprod.* 28, 255–260 (1983).
25. Finch, C. E. The menopause and aging, a comparative perspective. *J. Steroid Biochem. Mol. Biol.* 0, 132–141 (2014).
26. Harlow, S. D. et al. Executive summary of the Stages of Reproductive Aging Workshop + 10: addressing the unfinished agenda of staging reproductive aging. *Menopause N. Y. N* 19, 387–395 (2012).
27. Bacon, E. R. et al. Neuroendocrine aging precedes perimenopause and is regulated by DNA methylation. *Neurobiol. Aging* 74, 213–224 (2019).
28. Snir, S., Farrell, C. & Pellegrini, M. Human epigenetic ageing is logarithmic with time across the entire lifespan. *Epigenetics* 14, 912–926 (2019).

29. Sapre, S. & Thakur, R. Lifestyle and dietary factors determine age at natural menopause. *J. -Life Health* 5, 3–5 (2014).
30. Wood, M. A. & Rajkovic, A. Genomic Markers of Ovarian Reserve. *Semin. Reprod. Med.* 31, 399–415 (2013).
31. Baumgarten, S. C., Armouti, M., Ko, C. & Stocco, C. IGF1R Expression in Ovarian Granulosa Cells Is Essential for Steroidogenesis, Follicle Survival, and Fertility in Female Mice. *Endocrinology* 158, 2309–2318 (2017).
32. Mihalas, B. P., Redgrove, K. A., McLaughlin, E. A. & Nixon, B. Molecular Mechanisms Responsible for Increased Vulnerability of the Ageing Oocyte to Oxidative Damage. *Oxid. Med. Cell. Longev.* 2017, 4015874 (2017).
33. Matzuk, M. M., Dionne, L., Guo, Q., Kumar, T. R. & Lebovitz, R. M. Ovarian Function in Superoxide Dismutase 1 and 2 Knockout Mice. *Endocrinology* 139, 4008–4011 (1998).
34. Wooten, G. F., Currie, L. J., Bovbjerg, V. E., Lee, J. K. & Patrie, J. Are men at greater risk for Parkinson's disease than women? *J. Neurol. Neurosurg. Psychiatry* 75, 637–639 (2004).
35. Zirra, A. et al. Gender Differences in the Prevalence of Parkinson's Disease. *Mov. Disord. Clin. Pract.* 10, 86–93 (2023).
36. Kusters, C. D. et al. Increased menopausal age reduces the risk of Parkinson's disease: a Mendelian randomization approach. *Mov. Disord. Off. J. Mov. Disord. Soc.* 36, 2264–2272 (2021).
37. Margiotti, K., Monaco, F., Fabiani, M., Mesoraca, A. & Giorlandino, C. Epigenetic Clocks: In Aging-Related and Complex Diseases. *Cytogenet. Genome Res.* 163, 247–256 (2023).
38. Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol.* 14, 3156 (2013).
39. Sehl, M. E., Henry, J. E., Storniolo, A. M., Ganz, P. A. & Horvath, S. DNA methylation age is elevated in breast tissue of healthy women. *Breast Cancer Res. Treat.* 164, 209–219 (2017).
40. Horvath, S. et al. The cerebellum ages slowly according to the epigenetic clock. *Aging* 7, 294–306 (2015).
41. Daredia, S. et al. Timing of menarche and menopause and epigenetic aging among U.S. adults: results from the National Health and Nutrition Examination Survey 1999–2002. *Clin. Epigenetics* 17, 31 (2025).
42. Ye, Q., Ahamed, A., Shalev, I. & Etzel, L. Evaluation of reproductive profiles, epigenetic aging, and mortality in post-menopausal women. 2025.02.08.25321904 Preprint at <https://doi.org/10.1101/2025.02.08.25321904> (2025).
43. Xu, Y.-Q. et al. Reproductive life events and biological aging in women over 50: evidence from DNA methylation clocks. *Npj Aging* <https://doi.org/10.1038/s41514-026-00394-6> (2026) doi:10.1038/s41514-026-00394-6.
44. Cooper, G. S. & Sandler, D. P. Age at Natural Menopause and Mortality. *Ann. Epidemiol.* 8, 229–235 (1998).

45. Ossewaarde, M. E. *et al.* Age at Menopause, Cause-Specific Mortality and Total Life Expectancy. *Epidemiology* 16, 556 (2005).
46. Jacobsen, B. K., Heuch, I. & Kvåle, G. Age at natural menopause and all-cause mortality: a 37-year follow-up of 19,731 Norwegian women. *Am. J. Epidemiol.* 157, 923–929 (2003).
47. Laven, J. S. E. Early menopause results from instead of causes premature general ageing. *Reprod. Biomed. Online* 45, 421–424 (2022).
48. Stankovic, S. *et al.* Genetic links between ovarian ageing, cancer risk and de novo mutation rates. *Nature* 633, 608–614 (2024).
49. Lee, Y. *et al.* Associations between epigenetic age acceleration and infertility. *Hum. Reprod. Oxf. Engl.* 37, 2063–2074 (2022).
50. Hanson, B. M. *et al.* Young women with poor ovarian response exhibit epigenetic age acceleration based on evaluation of white blood cells using a DNA methylation-derived age prediction model. *Hum. Reprod. Oxf. Engl.* 35, 2579–2588 (2020).
51. Monseur, B., Murugappan, G., Bentley, J., Teng, N. & Westphal, L. Epigenetic clock measuring age acceleration via DNA methylation levels in blood is associated with decreased oocyte yield. *J. Assist. Reprod. Genet.* 37, 1097–1103 (2020).
52. Suarez, A. *et al.* The epigenetic clock and pubertal, neuroendocrine, psychiatric, and cognitive outcomes in adolescents. *Clin. Epigenetics* 10, 96 (2018).
53. Datta, J., Maxwell, K. J., Mitchell, K. R., Lewis, R. & Wellings, K. Factors shaping the timing of later entry into parenthood: Narratives of choice and constraint. *Soc. Sci. Humanit. Open* 8, 100700 (2023).
54. Ding, X., Li, H., Yang, Q. & Nawsherwan. The Trend in Delayed Childbearing Age and Its Potential Impact on Adverse Maternal-Perinatal Outcomes in Developed and Developing Countries: A Narrative Review. *Iran. J. Public Health* 54, 1–12 (2025).
55. Mosconi, L. *et al.* In vivo brain estrogen receptor density by neuroendocrine aging and relationships with cognition and symptomatology. *Sci. Rep.* 14, 12680 (2024).
56. Mosconi, L. *et al.* Menopause impacts human brain structure, connectivity, energy metabolism, and amyloid-beta deposition. *Sci. Rep.* 11, 10867 (2021).
57. Mishra, A. *et al.* A tale of two systems: Lessons learned from female mid-life aging with implications for Alzheimer's prevention & treatment. *Ageing Res. Rev.* 74, 101542 (2022).
58. Nakanishi, M. *et al.* Associations among age at menopause, depressive symptoms, and cognitive function. *Alzheimers Dement.* 21, e70063 (2025).
59. Sundermann, E. E. *et al.* Natural premature menopause relates to higher risk of mild cognitive impairment and Alzheimer's disease and related dementias in APOE-ε4 carriers only. *Alzheimers Dement.* 18, e068132 (2022).
60. Liao, H. *et al.* Association of earlier age at menopause with risk of incident dementia, brain structural indices and the potential mediators: a prospective community-based cohort study. *eClinicalMedicine*

- 60, 102033 (2023).
61. Rocca, W. A., Smith, C. Y., Gazzuola Rocca, L., Savica, R. & Mielke, M. M. Association of Premenopausal Bilateral Oophorectomy With Parkinsonism and Parkinson Disease. *JAMA Netw. Open* 5, e2238663 (2022).
62. Annibal, A. *et al.* Regulation of the one carbon folate cycle as a shared metabolic signature of longevity. *Nat. Commun.* 12, 3486 (2021).
63. Li, Y. *et al.* Association between methyl donor nutrients' dietary intake with phenotypic aging among US adults: a cross-sectional study from NHANES 2005–2018. *Sci. Rep.* 15, 12591 (2025).
64. Baudin, J. *et al.* A cocktail of histidine, carnosine, cysteine and serine reduces adiposity and improves metabolic health and adipose tissue immunometabolic function in ovariectomized rats. *Biomed. Pharmacother.* 179, 117326 (2024).
65. Baudin, J. *et al.* Combined supplementation with hesperidin, phytosterols and curcumin decreases adiposity and improves metabolic health in ovariectomized rats. *Food Funct.* 15, 4905–4924 (2024).
66. Baudin, J. *et al.* Impact of the natural female reproductive aging on the rat serum lipidome. *Clin. Sci. Lond. Engl.* 1979 139, 957–977 (2025).
67. McLean, A. C., Valenzuela, N., Fai, S. & Bennett, S. A. L. Performing Vaginal Lavage, Crystal Violet Staining, and Vaginal Cytological Evaluation for Mouse Estrous Cycle Staging Identification. *J. Vis. Exp. JoVE* 4389 (2012) doi:10.3791/4389.
68. Matthews, D. R. *et al.* Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28, 412–419 (1985).
69. Frühbeck, G., Catalán, V., Rodríguez, A. & Gómez-Ambrosi, J. Adiponectin-leptin ratio: A promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. *Adipocyte* 7, 57–62 (2018).
70. Arneson, A. *et al.* A mammalian methylation array for profiling methylation levels at conserved sequences. *Nat. Commun.* 13, 783 (2022).
71. Arneson, A. *et al.* A mammalian methylation array for profiling methylation levels at conserved sequences. *Nat. Commun.* 13, 783 (2022).

Figures

Fig.1

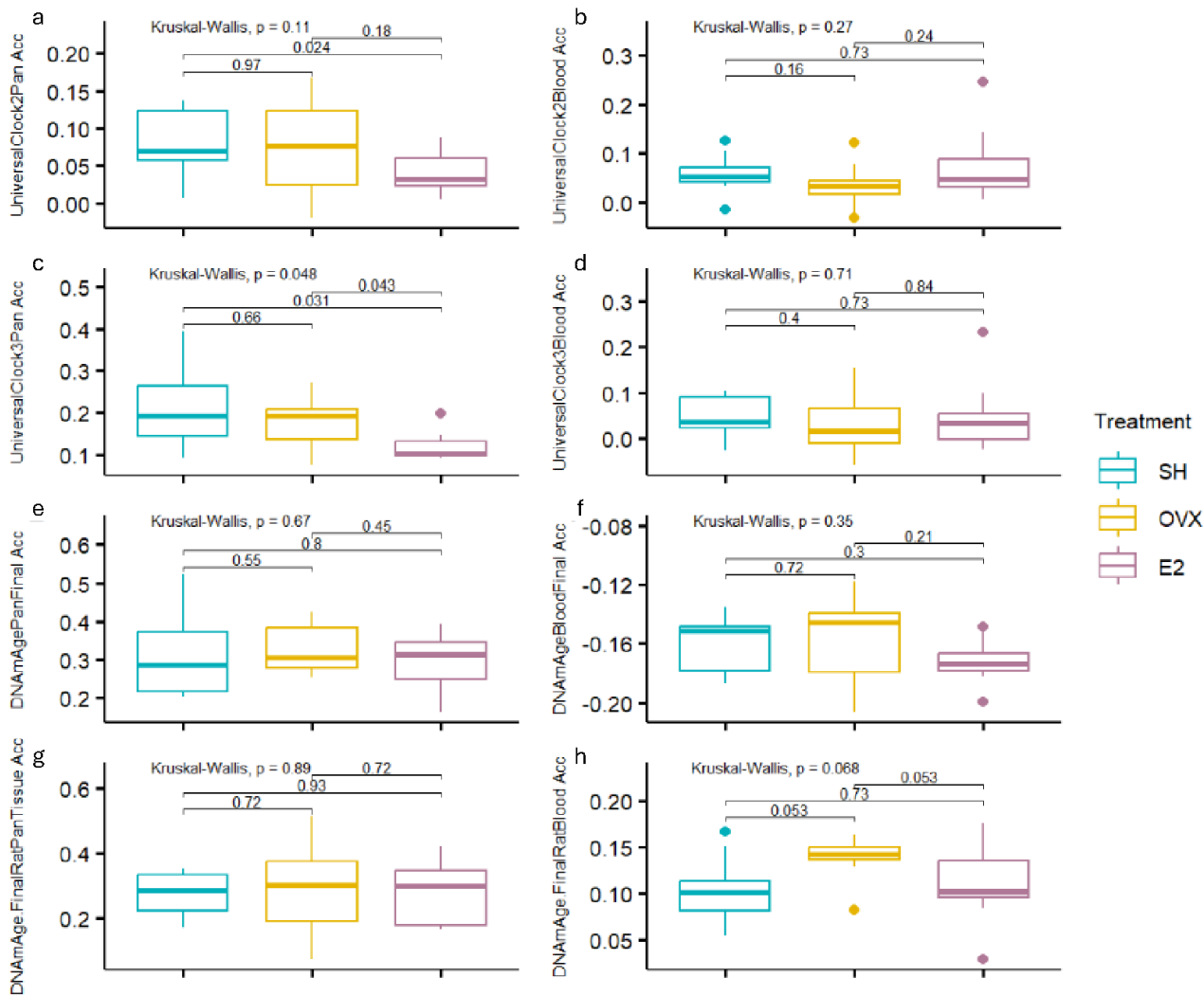


Fig.1 Epigenetic age acceleration, measured with different epigenetic clocks, comparison between sham rats (SH, n = 10), rats that undergone bilateral oophorectomy (OVX, n = 10) and rats that undergone bilateral oophorectomy, terated with estradiol (E2, n = 10). Rats have been operated when they were 12 weeks old. Epigenetic age acceleration calculated as the difference between the chronological age and: **a)** Universal Mammalian Clock 2 Pan-Tissue, **b)** Universal Mammalian Clock 2 for blood, **c)** Universal Mammalian Clock 3 Pan-Tissue, **d)** Universal Mammalian Clock 3 for blood, **e)** preliminary DNAmAge Pan-Tissue for rats, **f)** preliminary DNAmAge for rat blood, **g)** final DNAmAge Pan-Tissue for rats, **h)** final DNAmAge for rat blood.

Figure 1

See image above for figure legend.

Fig.2

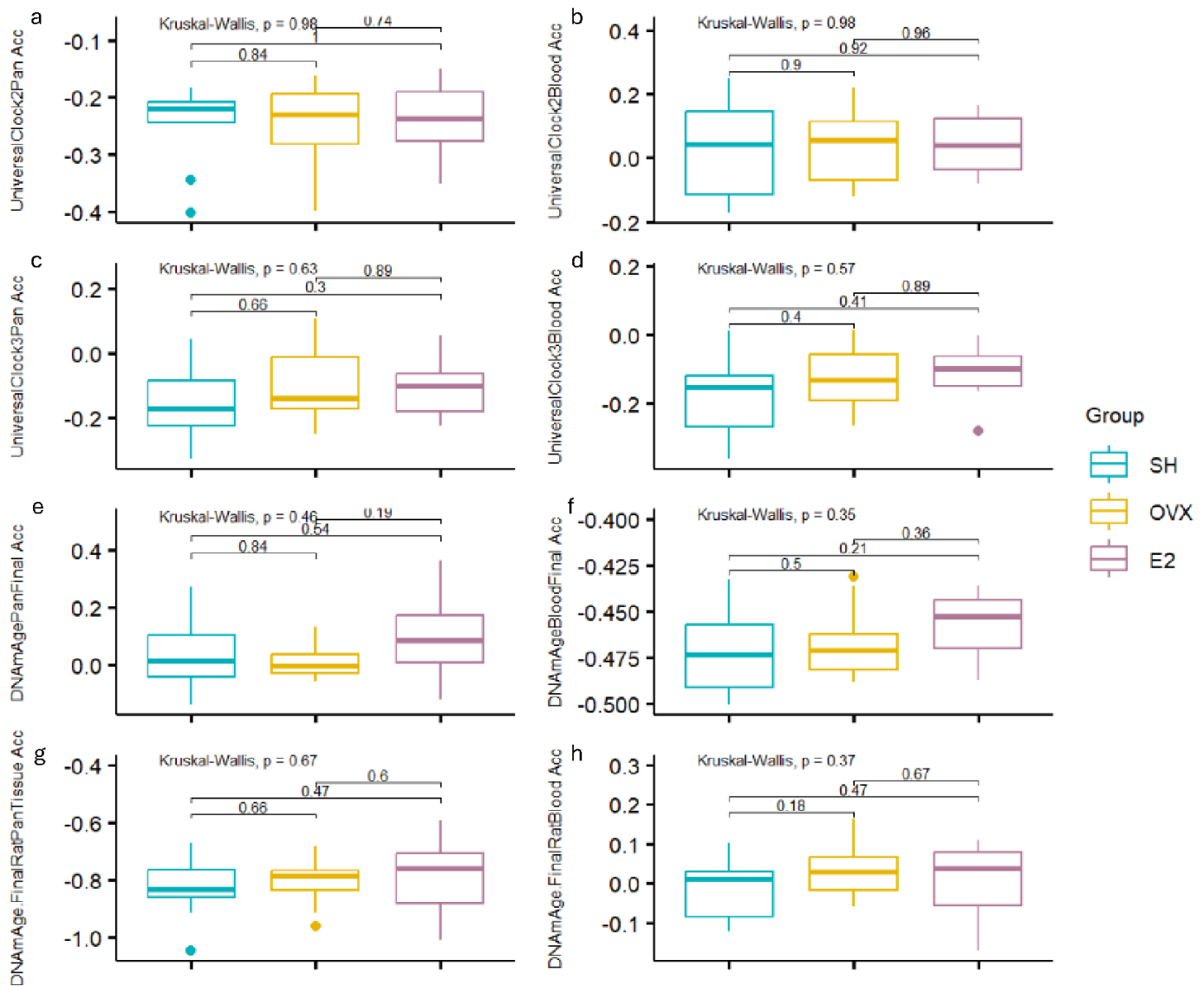


Fig.2 Epigenetic age acceleration, measured with different epigenetic clocks, comparison between sham rats (SH, $n = 10$), rats that undergone bilateral oophorectomy (OVX, $n = 10$) and rats that undergone bilateral oophorectomy, terated with estradiol (E2, $n = 10$). Rats have been operated when they were 24 weeks old. Epigenetic age acceleration calculated as the difference between the chronological age and: **a)** Universal Mammalian Clock 2 Pan-Tissue, **b)** Universal Mammalian Clock 2 for blood, **c)** Universal Mammalian Clock 3 Pan-Tissue, **d)** Universal Mammalian Clock 3 for blood, **e)** preliminary DNAmAge Pan-Tissue for rats, **f)** preliminary DNAmAge for rat blood, **g)** final DNAmAge Pan-Tissue for rats, **h)** final DNAmAge for rat blood.

Figure 2

See image above for figure legend.

Fig.3

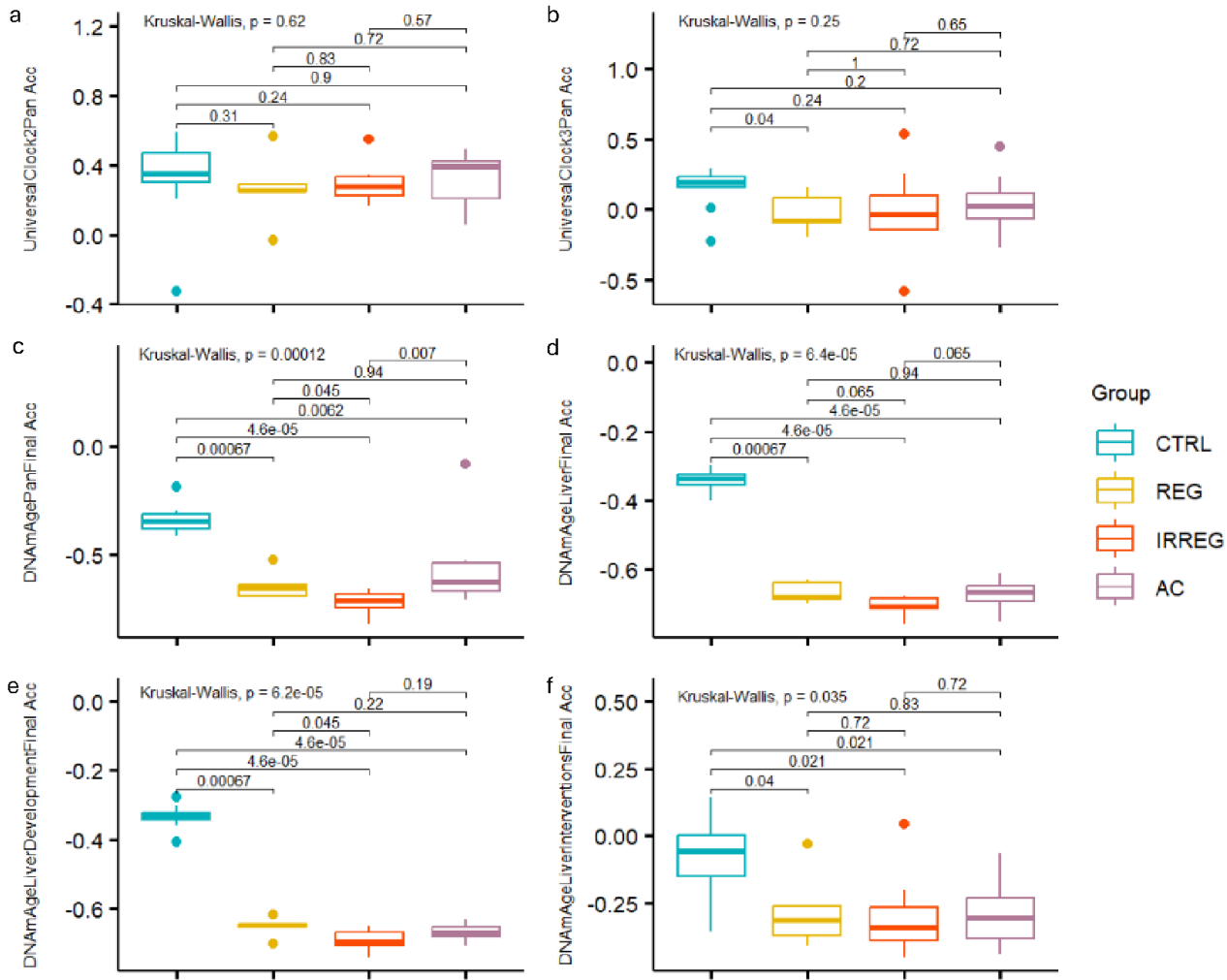


Fig.3 Epigenetic age acceleration, measured with different epigenetic clocks, compared between the rats at 4 different stages of reproductive senescence: young, 6 months old rats, that corresponds to human early premenopausal stage (CTRL, $n = 10$), 9 months old rats, regularly cycling, correspond to human premenopausal stage (REG, $n = 7$), 9 months old rats, irregularly cycling, correspond to human perimenopausal stage (IRREG, $n = 8$), 9 months old rats, after cessation of cycles, corresponding to human post-menopausal stage (AC, $n = 8$). Epigenetic age acceleration calculated as the difference between the chronological age and: **a)** Universal Mammalian Clock 2 Pan-Tissue, **b)** Universal Mammalian Clock 3 Pan-Tissue, **c)** final DNAmAge Pan-Tissue rat clock, **d)** final DNAmAge rat liver, **e)** DNAmAge rat liver for CpG that changes during development, **f)** DNAmAge rat liver for CpG that changes during anti-aging interventions.

Figure 3

See image above for figure legend.

Fig.4

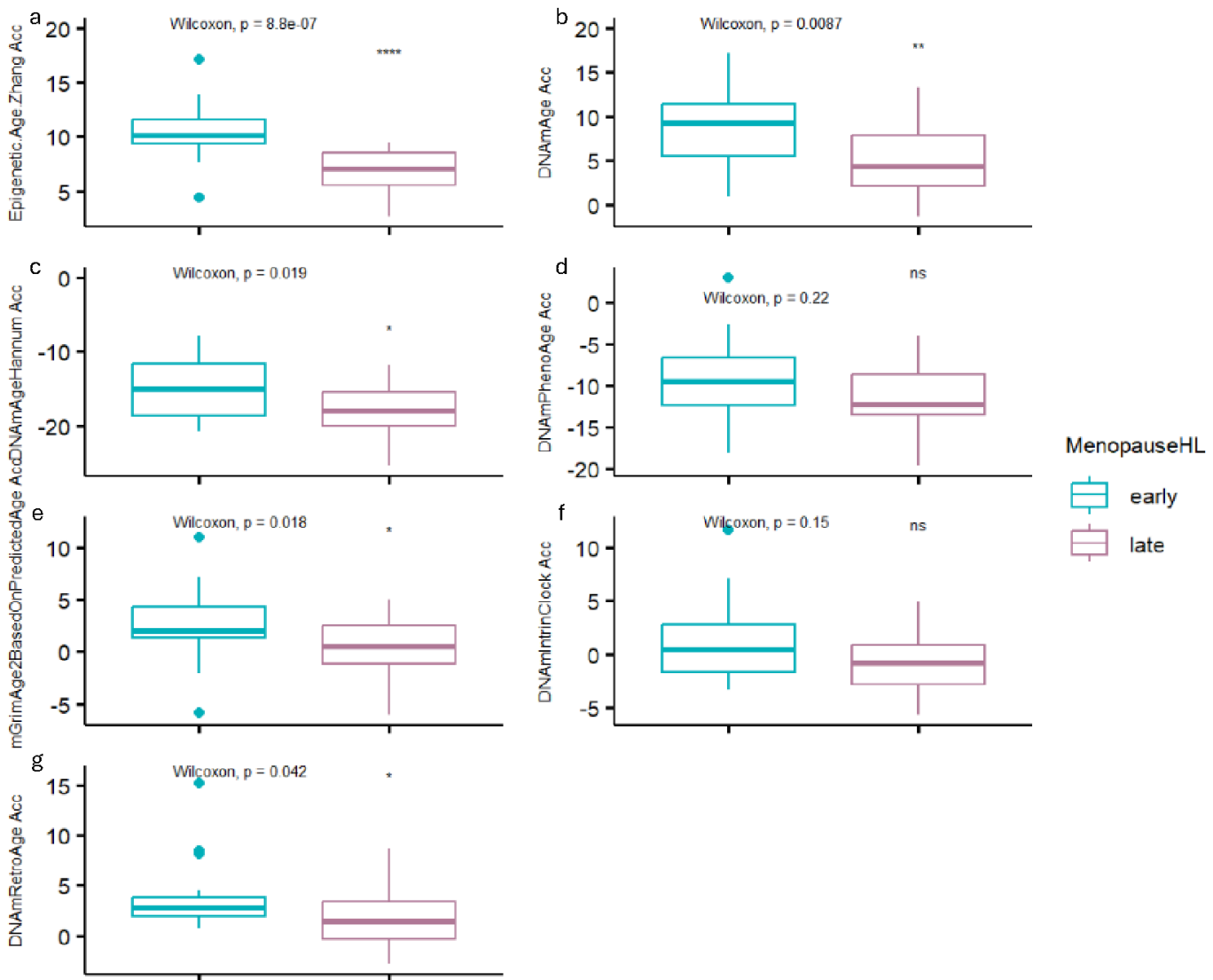


Fig.4 Epigenetic age acceleration in postmenopausal women, divided into two groups based on their age at postmenopause: early (n = 18)- postmenopause reached below age 50, late (n = 29) – postmanopause reached at 50 years old or older. Epigenetic age acceleration calculated as the difference between the chronological age and: **a)** Epigenetic Age clock developed by Zhang, **b)** DNAmAge developed by Horvath’s team, **c)** DNAmAge developed by Hannum, **d)** DNAmPhenoAge, **e)** GrimAge2, **f)** DNAmIntrin Clock, **g)** DNAmRetro Age

Figure 4

See image above for figure legend.

Fig.5

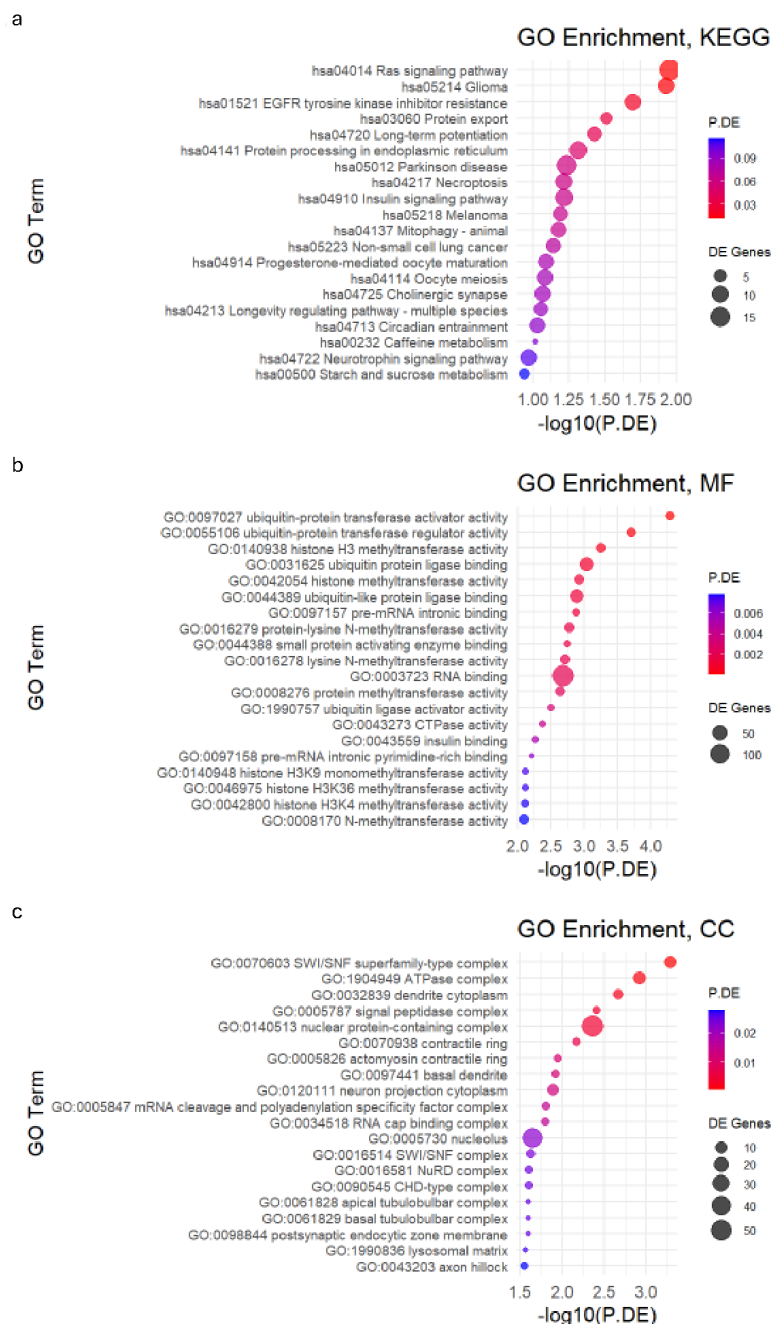


Fig.5 20 most significantly enriched GO terms of the differentially methylated genes between post-menopausal women that transitioned to menopause below the age of 50, and post-menopausal women that transitioned to menopause at 50 years old or older. a) KEGG terms from all differentially methylated genes, b) Molecular Function GO category for hypermethylated genes, c) Cellular component GO category for hypomethylated genes

Figure 5

See image above for figure legend.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryData.xlsx](#)
- [SupplementaryInformationnpjAging.docx](#)