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ORIGINAL STUDY

Circulating cell-free DNA is elevated in postmenopausal compared with pre- and perimenopausal women

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Abstract

Objective: With the rising use of circulating cell-free DNA (cirDNA) liquid biopsies for disease screening, it is important to understand biological differences that may impact the accuracy of cirDNA-based clinical tests. Although a number of biological factors have been researched, the relationship between menopause and cirDNA has not been thoroughly investigated. We aimed to compare plasma cirDNA concentration and DNA fragment integrity in healthy women pre- and postmenopause.

Methods: Blood was collected from healthy female volunteers 40 years and older. cirDNA was extracted from plasma (n = 52) and quantified by quantitative polymerase chain reaction (n = 47; 26 premenopause, mean age—46 y; 21 postmenopause, mean age—59 y). cirDNA concentration was quantitated using an ALU repetitive sequence with a 115-base-pair (bp) product (ALU-115), and long cirDNA fragments were quantitated using an ALU repetitive sequence with a 247-bp product (ALU-247). cirDNA integrity was expressed as a ratio of ALU-247 over ALU-115. Mann-Whitney *U* test was used to compare pre- and postmenopause qPCR results, and a two-tailed, unpaired *t* test was undertaken to compare the integrity ratio between the two groups.

Results: Postmenopause plasma samples were found to have a significantly higher cirDNA concentration ($P < 0.0001$, premenopause: mean, 3.10 ± 1.84 ng/mL; median, 2.90 ng/mL; postmenopause: mean, 5.28 ± 2.76 ng/mL; median, 4.56 ng/mL) and significantly higher concentration of long-stranded cirDNA fragments ($P = 0.0033$, premenopause: mean, 1.06 ± 0.48 ng/mL; median, 0.96 ng/mL; postmenopause: mean, 1.69 ± 0.89 ng/mL; median, 1.48 ng/mL). There was no significant difference in the integrity ratio between the groups ($P = 0.1788$).

Conclusions: Plasma cirDNA concentrations are higher in postmenopausal women. This has important implications in cirDNA liquid biopsy development and screening, especially for diseases such as cancer where the majority of cases are diagnosed postmenopause.

Key Words: Biomarkers – Circulating cell-free DNA – Liquid biopsy – Menopause – Quantitative PCR.

Menopause is broadly understood as the final menstrual period and can only be determined retrospectively 12 months after the event.[1] For most women, natural menopause occurs between the ages of 45 and 55 years; however, induced menopause, which may be a result of chemotherapy or the surgical removal of the ovaries, can occur much

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earlier.[2] The menopausal transition is associated with a range of hormonal and physiological adjustments within the body that manifest physical, vasomotor, and psychological changes.[3]

Circulating cell-free DNA (cirDNA) is a focus of increasing research interest as it poses as an attractive substrate for minimally invasive screening for conditions, including cancer, fetal abnormalities, autoimmune diseases, and cardiovascular pathologies. It is important to understand biological factors that may impact cirDNA levels and thus the clinical accuracy of cirDNA-based tests. Although the relationship between cirDNA and factors such as age, body mass index, and biological sex has been heavily researched and reviewed,[4,5] the effect of menopause on cirDNA levels has not been thoroughly explored. A single ad hoc analysis reported higher cirDNA after menopause, but the study only included seven women in the postmenopausal cohort and the result has not been validated.[6]

We aimed to understand if there is any relationship between cirDNA levels and menopause status. For the purpose of our study, we have used the terminology premenopause to describe a cohort of women who are still experiencing menstruation or are on hormonal contraception, and postmenopause to describe a cohort of women who self-report as no longer menstruating.

METHODS

Ethics approval and participant recruitment

The recruitment of healthy female volunteer blood donors was approved by the University of New South Wales Human Research Ethics Committee (HC17020 and HC220198), and written informed consent was obtained from all participants. Volunteers were recruited via research advertisement flyers distributed around the University of New South Wales and shared on social media. Interested participants contacted the researchers who screened for exclusion criteria, including pregnancy, lactation, or a personal history of cancer.

Participant cohort

Blood was collected from 80 healthy female volunteers over the age of 40 as part of a larger study into biomarkers of ovarian cancer. Collection took place between 2018 and 2022. After exclusions (Fig. 1), 47 samples were analyzed for plasma cirDNA concentration, and a comparison of pre- and postmenopausal cirDNA levels was undertaken. In order to limit variability in cirDNA levels due to acute exercise,[4] all participants were asked to refrain from vigorous physical activity for at least 2 hours before blood collection, and all were seated for 15 minutes immediately before collection took place. Self-reported data on menstruation and menopause status were collected via questionnaire, with 26 of 47 volunteers reporting premenopause and 21 of 47 reporting postmenopause.

Blood collection

Peripheral blood from healthy donors was collected in 10-mL K2EDTA tubes (Becton Dickinson, Franklin Lakes, NJ). All samples were processed within 3 hours of collection. Blood tubes were centrifuged at 2500 × *g* for 10 minutes at 4°C to separate the plasma. The plasma was transferred into a new tube and centrifuged at 3500 × *g* for 10 minutes at 4°C to remove remaining cells, and was stored at −80°C with no additional freeze-thaw cycles before cirDNA extraction.

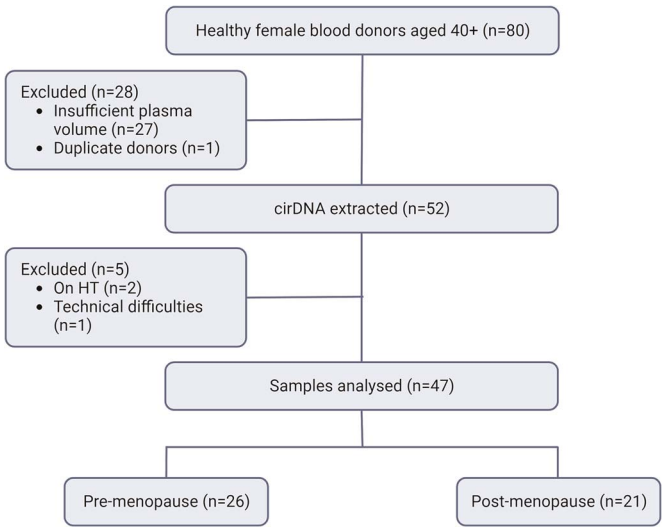

FIG. 1. Study participant recruitment—80 consecutively recruited female participants with biobanked plasma samples were considered for the study and 47 were included in the final analysis. cirDNA, circulating cell-free DNA; HT, hormone therapy. Created with BioRender.com.

cirDNA extraction

cirDNA was extracted from a total of 52 plasma samples using the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany). Protocol volumes were adjusted proportionately to account for a large plasma input volume, with all other steps carried out according to manufacturer instructions. Carrier RNA was included in the extraction protocol. All samples were extracted in duplicate 13-mL extractions carried out on separate days, except for two samples, which only underwent one extraction and were excluded from the present analysis. All 13-mL extractions were eluted in 68 µL of elution buffer and stored at −80°C until analysis.

cirDNA quantification

cirDNA concentration was measured by qPCR using an ALU repetitive sequence with a 115-base-pair (bp) product (forward 5′-CCTGAGGTCAGGAGTTCGAG-3′; reverse 5′-CCCGAGT AGCTGGGATTACA-3′) (ALU-115). Long fragments of cirDNA were quantified by amplifying an ALU repetitive sequence with a 247-bp product (forward 5′-GTGGCTCACGCCTGTTAATC-3′; reverse 5′-CAGGCTGGAGTGCAGTGG-3′) (ALU-247). Each 20-µL qPCR reaction contained 1× PCR reaction buffer (Thermo Fisher Scientific, Waltham, MA), 3 mM $MgCl_2$ (Thermo Fisher Scientific), 0.2 nM dNTP solution mix (New England Biolabs, Ipswich, MA), 0.2 µM ALU forward and reverse primers (Sigma-Aldrich, St Louis, MO), 2.5 µM Syto 9 (Thermo Fisher Scientific), 0.06 U/µL platinum Taq DNA polymerase (Thermo Fisher Scientific), and 0.067 µL of eluted cirDNA (equivalent to 0.013 mL of plasma). Commercial human genomic DNA from buffy coat (Roche, Basel, Switzerland) was serially diluted one in five for the standard curve. The qPCR began at 95°C for 10 minutes, then 40 cycles of 95°C, 60°C, and

72°C each for 30 seconds (Bio-Rad CFX 384 Touch). cirDNA concentration was expressed as nanograms of cirDNA per milliliter of plasma. The integrity of cirDNA was determined by the following:

$$\text{Ratio of ALU} : \frac{\text{cirDNA concentration (ALU}-247)}{\text{cirDNA concentration (ALU}-115)}$$

Statistical analysis

GraphPad Prism (Version 8.4.3) was used for statistical analysis. Data are expressed as mean ± standard deviation. Mann-Whitney *U* test was used to compare pre- and postmenopause qPCR results. A two-tailed, unpaired *t* test was undertaken to compare the integrity ratio between the two groups. *P* value less than 0.05 was considered significant. Dominance analysis was carried out using Stata as described by Luchman.[7]

RESULTS

Participant recruitment and cohort demographics

Participant recruitment is described in Figure 1, and cohort demographics are summarized in Table 1. Smoking status is defined as a regular smoker who smokes on average one or more cigarettes per day. Alcohol intake is defined as consuming on average at least one alcoholic drink per week. Hormonal contraception includes both oral contraception and intrauterine device.

Plasma cirDNA concentration

cirDNA concentration in plasma was measured by qPCR amplifying an ALU repetitive sequence of 115 bp (ALU-115). We analyzed 26 premenopause and 21 postmenopause samples and found cirDNA concentration to be significantly higher postmenopause ($P < 0.0001$, premenopause: mean, 3.10 ± 1.84 ng/mL plasma; median, 2.90 ng/mL plasma; postmenopause: mean, 5.28 ± 2.76 ng/mL plasma; median, 4.56 ng/mL plasma). Long fragment cirDNA concentration was measured by qPCR amplifying an ALU repetitive sequence of 247 bp (ALU-247). Postmenopause samples were similarly found to have a significantly higher concentration of long cirDNA fragments ($P = 0.0033$, premenopause: mean, 1.06 ± 0.48 ng/mL plasma; median, 0.96 ng/mL plasma; postmenopause: mean, 1.69 ± 0.89 ng/mL plasma; median, 1.48 ng/mL plasma) (Fig. 2).

Integrity ratio

The cirDNA fragment integrity is expressed as a ratio of ALU-247 to ALU-115. There was no significant difference in the integrity ratio between pre- and postmenopause groups ($P = 0.1788$) (Fig. 2).

TABLE 1. *Study cohort demographics*

	Total	Premenopause	Postmenopause
Age (y) (mean ± SD)	52.17 ± 7.57	46.46 ± 3.97	59.24 ± 4.13
BMI (kg/m^2) (mean ± SD)	25.23 ± 5.04	25.39 ± 5.01	25.04 ± 5.20
Smoking status—yes	2	1	1
Smoking status—no	45	25	20
Alcohol intake—yes	27	14	13
Alcohol intake—no	20	12	8
Hormonal contraception—yes	8	8	0
Hormonal contraception—no	39	18	21

BMI, body mass index.

Effect of age on cirDNA level

Although there was a small amount of overlap in the ages of the pre- and postmenopausal participants, the postmenopause cohort was on average 12 years older; thus, age was a possible confounding factor in determining the relationship between menopause on cirDNA levels. To observe whether age rather than menopause status was responsible for the difference in cirDNA concentration, we considered the age and cirDNA level within each cohort separately and found no relationship between the two factors within each group (premenopause $P = 0.2841$, postmenopause $P = 0.7729$) (Fig. 3).

To further examine the relationship between menopause, age, and cirDNA level, we undertook dominance analysis to test whether menopause status or age is more important in predicting cirDNA.[7,8] Dominance analysis is a statistical summary of the contribution of each of a set of predictors in all possible models predicting the outcome, with higher number indicative of higher importance. Results showed a clear dominance of menopause status over age (dominance analysis coefficients, 0.139 vs 0.057, respectively).

Adjusting for sample loss during biospecimen storage

cirDNA is known to degrade during long-term plasma storage, with a recent study reporting an annual decline of 19% when concentration was measured with the ALU-115 qPCR assay used here.[9] In our current study, 32 (67%) of the samples were collected within a 4-month interval immediately before cirDNA extraction, whereas the entire cohort was collected within approximately 52 months (see Table, Supplemental Digital Content 1, http://links.lww.com/MENO/B198, which summarizes participant cohort characteristics, blood collection and cirDNA extraction dates, and cirDNA concentrations). Adjusting the plasma cirDNA concentration to include an annual decline of 19% resulted in a greater difference in cirDNA in the postmenopausal cohort ($P = 0.0001$, premenopause: mean, 4.06 ± 2.51 ng/mL plasma; median, 3.23 ng/mL plasma; postmenopause: mean, 8.62 ± 5.71 ng/mL plasma; median, 5.94 ng/mL plasma) (see Figure, Supplemental Digital Content 2, http://links.lww.com/MENO/B199, which reports cirDNA concentration adjusted for storage time).

DISCUSSION

We compared plasma cirDNA concentration between women pre-/perimenopause and postmenopause and found the postmenopause cohort to have significantly higher levels of cirDNA. A previous analysis that stratified women based on age cutoffs that aimed to separate pre- and postmenopause participants also reported higher cirDNA in women older than 55 years (n = 7) compared with younger age groups (45-54 y, n = 13; <45 y, n = 22); however, menopause status was not directly captured in the data and the analysis was ad hoc.[6]

Higher levels of cirDNA postmenopause may be due to increased generation or slower clearance of cirDNA or both. Menopause is accompanied by hormonal changes, including a

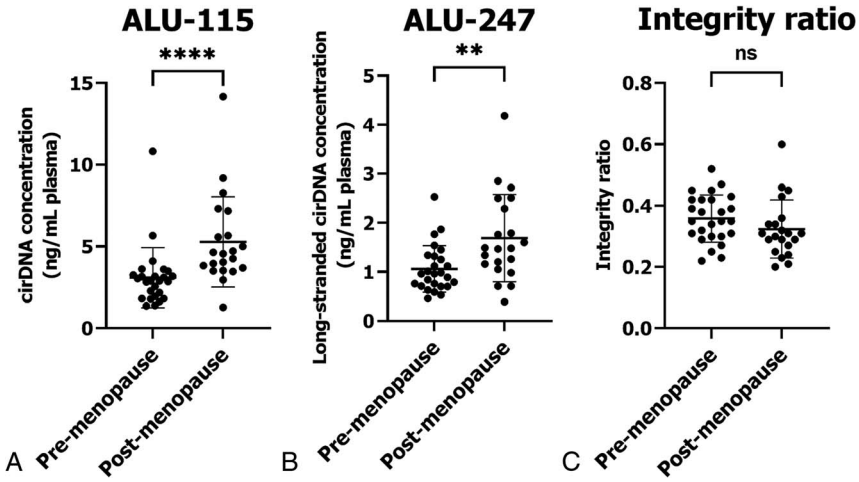

FIG. 2. cirDNA levels in premenopausal (n = 26) and postmenopausal (n = 21) participants (total n = 47). (**A**) cirDNA concentration measured using ALU-115 qPCR assay (****$P < 0.0001$). (**B**) Long-stranded cirDNA measured using ALU-247 qPCR assay (**$P = 0.0033$). (**C**) cirDNA integrity, determined as a ratio of ALU-247 qPCR target to ALU-115 qPCR target ($P = 0.1788$). Data are shown as the mean with SD as error bars. cirDNA, circulating cell-free DNA; ns, not significant.

decrease in estradiol, progesterone, and testosterone, and is generally associated with an increased propensity to inflammation, which may contribute to cirDNA levels.[10] Importantly, none of the women in our study were on hormone therapy, which may have obscured cirDNA changes associated with menopause.[11]

Clearance mechanisms of cirDNA remain poorly understood but appear to involve a combination of plasma nucleases[12] and excretion via urine. However, menopause has not been observed to affect kidney function, with changes in kidney function between pre- and postmenopausal women driven by aging rather than by the menopausal transition.[13]

It is important to consider the relationship between age and cirDNA before drawing conclusions about the impact of menopause. There is inconsistent evidence that cirDNA levels increase with age, with a comprehensive review reporting that 14 of 19 studies found no correlation between age and cirDNA levels and only 5 of 19 studies finding a positive correlation.[4] Of these five studies, three of five only saw the effect in women,[11,14,15] whereas a fourth that focused on nonagenarians had a predominantly female cohort.[16] However, Meddeb et al[6] reported an increase in cirDNA in both women and men, whereas an analysis of supplementary data of control participants from a large cancer biomarker study[17] also shows an age-related increase in both sexes. Thus, it is possible that age contributes to cirDNA increase in both sexes, with menopause an additional mechanism for higher cirDNA in women only.

A limitation of the present study is that participants in the postmenopause group are not age matched to participants in the premenopause group; indeed, their average age is 13 years apart. However, pre- and postmenopausal cohorts matched for age would of necessity be comparing participants that underwent menopause at younger and older ages, respectively, and this may also introduce artifacts, albeit different ones. Dominance analysis in our study indicated that menopause status was a more important contributor to cirDNA level than age. Future studies with larger cohorts and an age-matched study design, combined with measures to minimize fluctuations in cirDNA levels due to physical activity and variation due to extraction and storage, will clarify the relative contributions of menopause and age to cirDNA differences.

Our study included a number of measures to reduce cirDNA variability due to extraneous factors. Physical activity is the

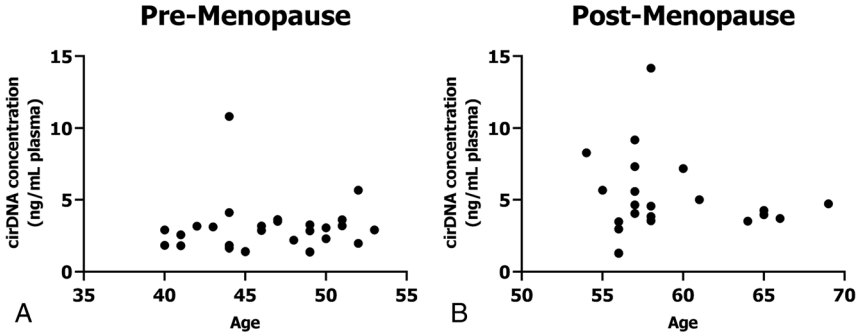

FIG. 3. Correlation between age and cirDNA concentration in (**A**) premenopause group (n = 26) and (**B**) postmenopause group (n = 21). There is no correlation between age and cirDNA concentration in each group (premenopause $P = 0.2841$, postmenopause $P = 0.7729$). cirDNA, circulating cell-free DNA.

strongest known physiological driver of cirDNA fluctuations,[4] so all participants were asked to refrain from exercise for 2 hours before the blood donation and rested sitting for 15 minutes immediately before the blood draw. Our participant cohorts were balanced for smoking status and alcohol consumption. Blood collection, processing, and storage were carried out within a single center, with a standardized operating procedure and oversight from a single investigator (K.W.). To minimize variability due to extraction efficiency, cirDNA from each sample was extracted twice, with the eluate pooled before cirDNA quantification. All samples were extracted from an identical volume of plasma and eluted in the same volume of buffer, as we have previously shown that these parameters have an impact on measured cirDNA concentrations.[9] Adjusting the measured plasma concentration to account for sample degradation during storage[9] only increased the difference between the pre- and postmenopause cohorts.

A limitation of our study is that menopause status is self-reported or based on menstruation status. Although the menstrual cycle is the principal criterion in determining reproductive age using the Stages of Reproductive Aging Workshop +10 (STRAW+10) system, there are other supportive criteria that can assist in staging.[3] These criteria such as levels of follicle-stimulating hormone, anti-Müllerian hormone, and inhibin B, as well as vasomotor symptoms, were not captured in our study. Because of the difficult nature of defining perimenopause because of its variable length, physiology, and symptoms, our premenopause cohort does not distinguish between premenopause and perimenopause stages. However, to create a cohort as uniform as possible, all women in our study were 40 years or older.

CONCLUSION

Overall, we have found a higher cirDNA concentration in postmenopausal women. This may impact the sensitivity of cirDNA-based clinical tests either by increasing the amount of target cirDNA biomarkers, which would make the test more sensitive, or by increasing the amount of background, healthy cirDNA, which may decrease the sensitivity of the test. Age-related conditions may play a role in the different cirDNA concentrations, and further study involving a larger age range should be carried out to determine the precise impact of menopause. Understanding biological influences on cirDNA levels is essential for the development of liquid biopsy tests.

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