Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Dhanish Revanth Rangaswamy Nandakumar Date

Approval Sheet

Evaluating association between epigenetic clocks and cancer survival among Black women diagnosed with high grade serous ovarian cancer

By

Dhanish Revanth Rangaswamy Nandakumar
Degree to be awarded: Master of Public Health

Epidemiology

_____

Joellen M. Schildkraut, PhD., MPH
Committee Chair

Evaluating association between epigenetic clocks and cancer survival among Black women diagnosed with high grade serous ovarian cancer

By

Dhanish Revanth Rangaswamy Nandakumar

Bachelor of Technology, Biotechnology
Vellore Institute of Technology
2018

Thesis Committee Chair: Joellen M. Schildkraut, PhD., MPH

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Epidemiology
2023

Abstract

Evaluating association between epigenetic clocks and cancer survival among Black women diagnosed with high grade serous ovarian cancer.

By Dhanish Revanth Rangaswamy Nandakumar

Background: Epigenetic clocks are tools used to estimate a person's epigenetic age based on DNA methylation levels, and numerous studies have demonstrated their effectiveness in predicting the risk of cancer mortality and progression. Despite their proven efficacy, the association between epigenetic clocks and mortality in Black women with high-grade serous ovarian cancer remains largely unexplored.

Methods: We conducted a study on 202 Black women with High Grade Serous Ovarian Cancer (HGSOC) from the AACES and NCOCS study cohorts. We calculated the mitotic age using Epitoc2 and MiAge and categorized these measures as dichotomous and trichotomous exposure variables. We also analyzed the correlation between the mitotic ages calculated from the clocks and chronological age and used ANOVA to investigate any differences in the mitotic ages across the risk factors. To evaluate the association between mitotic age and survival, we fit Cox proportional hazard models for different sets of covariates.

Results: The cohort had a median age of 57 years and median follow up of 4.4 years and 110 subjects had died. The median mitotic age using Epitoc and MiAge was 64.4 with annual cell turnover rate and 820.9 relative mitotic age, respectively. We observed that mitotic age obtained from Epitoc decreased with increasing chronological age and had a higher value for localized stage, whereas MiAge-derived mitotic age did not vary with age but had a higher value for localized stage. Cox proportional hazard models between MiAge-derived mitotic age and survival did not show a significant association. However, for Epitoc-derived mitotic age categorized into tertiles, we found a significant difference in survival between the Q2 and Q1 tertiles in all models (HR = 0.61, [95% CI: 0.35, 1.05; adjusting for age, stage, and debulking status], HR = 0.50 [95% CI: 0.29, 0.88; additionally adjusted for hormone use], HR = 0.51 [95% CI: 0.31, 0.84; adjusting for age and stage], and HR = 0.57 [95% CI: 0.33, 0.98; adjusting for age and debulking status].

Conclusion: The association between mitotic age and survival is not linear. Epitoc2 appears to be a more useful clock in risk stratification. A larger study is required to identify the threshold and evaluate the true relation between mitotic age and survival.

Evaluating association between epigenetic clocks and cancer survival among Black women diagnosed with high grade serous ovarian cancer

By

Dhanish Revanth Rangaswamy Nandakumar

Bachelor of Technology, Biotechnology
Vellore Institute of Technology
2018

Thesis Committee Chair: Joellen M. Schildkraut, PhD., MPH

A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Epidemiology
2023

Table of Contents

1.0 Introduction

1.1 Descriptive Epidemiology:

Ovarian cancer is a rare cancer and as per 2017, there were approximately 239,000 newly diagnosed ovarian cancer cases worldwide and the mortality was estimated to be 152,000 deaths annually. The incidence rate varies across the continents; it is the lowest in Asia and Africa and highest in Northern and central parts of Europe followed by North America. In the United States, ovarian cancer ranks 11th in incidence among women with 11.2 new diagnoses per 100,000 women (all races) while ranking 5th in mortality with a rate of 6.7 deaths per 100,000 women (all races). From 2014 to 2018, the survival rates for ovarian cancer have improved slightly by 2.4%, while the incident rates have decreased by 1.6% [1]. While ovarian cancer accounts for a relatively small percentage of all new cancer cases (1.6%), it is responsible for a disproportionately high percentage (5%) of cancer deaths [2]. The reason for this disparity between incidence and mortality is that ovarian cancer is often not detected until it has reached an advanced stage, when it is more difficult to treat. At an advanced stage the 5-year survival rate is as low as 29%, but 5-year survival can be as high as 92% when diagnosed at stage one [3]. The American Cancer Society estimates that in 2022, the lifetime chances of getting ovarian cancer and dying due to ovarian cancer is approximately 1 in 78 and 1 in 108 respectively.

Ovarian cancer incidence rates and mortality rates differ across racial and ethnic groups [4]. The age adjusted incidence rate, mortality rate and survival rate by race and ethnicity for 2013-2017 is tabulated in Table 1.

Table 1: Incidence and mortality rate across different racial groups in the US. [3, 5]

Measure of Frequency	Non-Hispanic white (NHW)	Non-Hispanic Black (NHB)	Non-Hispanic Asian and Pacific Islanders (NHA)	Hispanic	Non-Hispanic American Indians (NHAI)
Incidence rate[a]	11.6	9.2	9.4	10.1	11.1
Mortality rate[b]	7.1	6.0	4.4	5.1	6.8
Survival rate[c]	48.5%	41%	58%	53.8%	43.5%

[a] Rates per 100,000 people for the year 2013-2017 [5]
[b] Rates are per 100,000 people for the year 2014-2018 [5]
[c] r year survival rate calculated from 2012-2018 [3]

Lowest survival rate is observed among the non-Hispanic Black (NHB) women in Table 1. This is followed by Non-Hispanic American Indians, and then non-Hispanic White women (NHW). Hispanic and Non-Hispanic Asian/Pacific Islanders (NHA) display more than 50% 5-year survival. This difference in survival between the NHW women and NHB women is also found to be significant and consistent across different age groups except for women aged 75 years and older [6]. In addition to it, NHB women are diagnosed at younger ages compared to NHW but diagnosed at a later stage than NHA and Hispanic women [6]. Despite being diagnosed at an earlier age compared to the non-Hispanic White women, the NHB women have a lower survival rate [6].

1.2 Pathological Classification of Ovarian Cancer:

Ovarian cancer is a heterogeneous cancer that can be classified into 3 subtypes based on the type of cell it originates from – Epithelial-stromal (~60 % of all ovarian tumors and 90% of malignant ovarian tumors), Sex cord-stromal (~8% of all ovarian tumors and 7% of all malignant ovarian tumors) and Germ cells (~25% of all ovarian tumors and 3-7% of malignant ovarian

tumors). Based on the level of invasiveness, tumors are classified as benign, borderline and malignant. There can also be a combination of two or more histological subtypes, which are usually less common. [7]

Epithelial Ovarian Tumors:

Among the three subtypes, EOC (Epithelial Ovarian Cancer) is the most common, accounting for approximately 90% of all malignant ovarian cancer. Common epithelial histotypes include serous, mucinous, endometrioid, and clear cell tumors.

Serous tumors are further classified into high-grade and low-grade, based on their differences in modes of carcinogenesis, distinct molecular-genetic features as well as differences in risk factors [8,9]. However, both` high-grade and low-grade serous cancer arise in the fallopian tubes. Endometrioid and Clear cell OC are associated with endometriosis (cells like those found in uterus and endometrium growing outside uterus) and are more prevalent in Asian countries [10]. Mucinous ovarian cancer is a distinct type of cancer compared to the other three, comprising of mucus secreting cells that line the stomach, endocervix and intestine [11].

This study is focused on survival among self-identified Black women diagnosed with high grade serous ovarian tumors, the most common and aggressive histotype of epithelial ovarian cancer. HGSOC (High-Grade Serous Ovarian Cancer) are typically diagnosed at advanced stages (III and IV) and account for majority of the EOC cases. HGSOCs spread by direct extension to other organs or through the cellular detachment from the primary tumor. Somatic TP53 mutations, germline BRCA1 or BRCA2 mutation or point mutations at oncogenes or tumor suppressor genes is a characteristic feature of HGSOC [8]. Age and stage are the major risk factor for survival from HGSOC, with 63 being the median age of diagnosis. 10- year survival rate of women diagnosed with HGSOC at an early stage (Stage I and II) is 55% and for those diagnosed at an advanced stage (Stage III and IV) is as low as 15% [7, 8].

Table 2: Characteristics of the most common histotypes of EOC:

Five most common histotypes of EOC	Characteristics	Proportion of total cases of EOC
High Grade Serous [8]	• Most frequently diagnosed EOC • Aggressive patterns, wide dissemination, and rapid development • Usually have a high mitotic index • Genetically unstable, TP53 mutation and defect in DNA repair mechanism.	70-80%
Low Grade Serous [7, 8]	• Less aggressive and develop from pre-malignant or borderline lesions like other epithelial cancers. • More genetically stable and usually do not have TP53 mutation.	<5%
Mucinous [7, 11]	• Mucinous tumors are formed by cells that resemble those found in the intestinal and endocervical epithelium. • Prevalent in women below 40 years and smoking is associated with increased risk • Most common mutation – KRAS	3%
Endometrioid [7, 10]	• It is the second most common EOC histotype and ~84-95% are of grade I or II • Their origin is hypothesized to be through the entry of shed endometrium retrogradely into the peritoneal cavity. • Increased risk among those with lynch syndrome and progression to carcinoma is characterized by mutation to ARID1A gene	10%
Clear Cell [12]	• They are usually rare and are formed by clear peg like cells. • The most common mutation is the ARID1A and PIK3CA activating mutation	10%

While comparing the 10-year overall survival at an early stage (localized and regional) HGSOC patients have a similar survival to that of clear cell and mucinous while patients with Endometrioid and LGSOC have a better overall survival [13]. Similarly, the 10-year overall survival of LGSOC and Endometrioid types had a better overall survival while Mucinous type

had the worst survival at distant stage. HGSOC and clear cell had a similar survival for distant stage [13].

1.3 Factors associated with survival:

Not all risk factors associated with EOC, including demographic factors, reproductive factors such as menstrual-related factors, age of menarche and menopause, gynecological characteristics, hormonal factors, genetic factors, and lifestyle, are associated with overall survival. The major factors associated with survival from ovarian cancer are age, cancer stage, access to care and treatment [14]. All these factors have been found to be significantly associated with overall survival among women with ovarian cancer, along with some clinical factors, including optimal debulking, histologic subtype, and volume of disease [14].

Reproductive factors that are associated with an increased risk of OC, including parity, breastfeeding, and menopause status, have been found to have no effect on survival [15]. Other gynecological factors, such as pelvic inflammatory disease, that influence the onset of epithelial ovarian cancer also do not affect overall survival [14].

Studies on post-menopausal hormone use and survival in women with ovarian cancer have shown mixed results. However, a recent nationwide cohort study showed a significantly better survival among women who reported post-menopausal hormone use for more than 5 years compared to those used hormone for less than 0.5 years [41]. Therefore, post-menopausal hormone use might lead to an increased survival rate in ovarian cancer patients.

Demographics characteristics:

Age is one of the most important prognostic factors for ovarian cancer, and the prognosis is better if diagnosed at a younger age [16]. This trend is mostly similar across all racial and ethnic groups (NHW – Non-Hispanic White, NHB – Non-Hispanic Black, Hispanic, and Asian

and Pacific Islanders) [17]. However, overall survival is worst among the NHB group in all age categories compared to other racial and ethnic groups, as stated earlier, even after early diagnosis [17].

Clinical and pathological characteristics:

Stage at diagnosis and residual disease after debulking surgery are important predictors of survival for HGSOC. Like other diseases, the more advanced the stage of cancer, the less likely the probability of survival. According to the American Cancer Society, the 5-year relative survival rates for localized and regional ovarian cancer are more than 90%, while the rate for distant-stage ovarian cancer is around 30%.

Optimal debulking is another important prognostic factor that affects the overall survival among EOC patients. Optimal debulking is defined as surgery involved in the removal of the tumor, leaving only residual tumor less than 1 cm. Suboptimal debulking leads to surgical complications and delay in chemotherapy and thus leaves a larger residual tumor, which decreases survival rate. Various studies were able to confirm the impact of debulking status on overall survival, with optimal debulking leading to better overall survival compared to suboptimal debulking [18].

Family history and Genetic mutations:

BRCA1/BRCA2 mutations are not associated with long-term survival in EOC patients. However, a significant difference in survival between BRCA mutation carriers and non-carriers was observed after the 10-year mark, with non-carriers having a significantly better survival rate among those with no residual disease at an advanced stage [19]. BRCA1/BRCA2 mutations were also found to be associated with short-term survival (3 years). This association between overall survival among carriers and non-carriers was not different in the group that had residual disease. Similarly, among serous ovarian cancer patients with no residual tumor, the 10-year survival

among the non-carriers was higher compared to the carriers of BRCA mutation [19]. Thus, this study identified residual disease to be a more important prognostic factor for survival than BRCA1/BRCA2 mutation.

Lifestyle factors:

Various lifestyle factors also affect the risk of mortality among patients with ovarian cancer. An increase in body mass index (BMI) is found to be associated with decreased survival, along with reduced physical activity [20]. In addition, current smokers have a higher risk of mortality compared to former and never smokers [20]. Smoking was also found to be associated with mortality among only serous and mucinous histotypes of ovarian cancer in one study [21]. The effect was stronger among the mucinous subtype, with current smokers having 91% worse survival compared to the never smokers' group. Among the HGSOC and LGSOC subtypes, former smokers had a higher risk of mortality compared to the never smokers and current smokers [21].

Socio-economic status (SES) also plays a major role in healthcare and treatment access and is one of the main factors where racial health disparities arise [4]. The lower the SES, the less likely someone will receive an early diagnosis, leading to a later stage presentation of the disease [4]. Lower poverty is also associated with a higher 5-year and 10-year relative survival rate. This disparity mostly overlaps with race, where white females tend to be in the low poverty group and Blacks in the high poverty group. In addition, access to treatment also plays a significant role in the disparity in survival rates [22].

1.4 Epigenetics and Cancer:

The term "Epigenetics" is currently defined as the study of all the heritable changes in gene expression levels that are not dependent on the primary DNA sequence [33]. Various

modifications that mediate epigenetics or are collectively called the epigenome are methylation of cytosine bases in the DNA, post-translational modifications of histone proteins, and positioning of nucleosomes along the DNA [23]. Epigenetics is necessary for the maintenance of cell division and gene expression levels, and any change in its pattern (loss of function or activation) could lead to various complications [24]. The epigenome of an individual is mainly affected by lifestyle patterns and environmental factors in addition to heredity, nutrition and diet, physical activity and obesity, smoking, alcohol consumption, exposure to toxins, and stress. [25] The connection between epigenetic pathways and cancer was initially studied in the early 2000s. While most of these early studies were correlative, there has been increasing evidence of stronger associations of the epigenetic markers with the disease outcome in recent years. This is attributed to technological advances in sequencing and a reduction in the cost to sequence a sample. Out of all the different modifications that contribute to the epigenome, DNA methylation (DNAm) is the most extensively studied due to ease in sequencing and better stability compared to other modifications, making it an ideal marker, especially in FFPE (Formalin Fixed Paraffin Embedded) tissues. DNAm is denoted as 5mC; addition of a methyl group on the 5` carbon on cytosine in the CpG (Cytosine-phosphate-Guanine) dinucleotides [24].

DNAm can be measured using a wide range of techniques, from calculating the methylation level of the whole genome to restricting it to a specific set of CpG sites. The former is conducted using an array or bead hybridization, which saves time and money and provides information on the methylation levels at particular sites of the genome. These sites are usually the promoter regions, 3’ and 5’ UTR, coding regions, and island shores of known genes. DNAm is identified using bisulfite conversion, which is the “gold standard” technique where DNA is treated with bisulphite. This leads to the conversion of unmethylated cytosine to uracil, and the

converted residues will be read as thymine by the sequencer. However, the methylated cytosine is resistant to this change and remains as cytosine [26].

The methylation level is calculated as the ratio of the methylated DNA (intensity of fluorescence measured) over the total, i.e., M/(M+U), where M and U represent methylated and unmethylated intensities. A lot of older epigenetic studies used the Infinium HumanMethylation450 Bead Chip array to quantify DNA methylation covering 485,000 CpG sites. Currently, an upgraded version of the Infinium HumanMethylation450 Bead Chip array is available called the Methylation Epic BeadChip array (850K) that covers 863,904 CpG sites. It covers >90% of the CpGs targeted by the 450K and an additional 333,365 CpGs in the enhancer region [28].

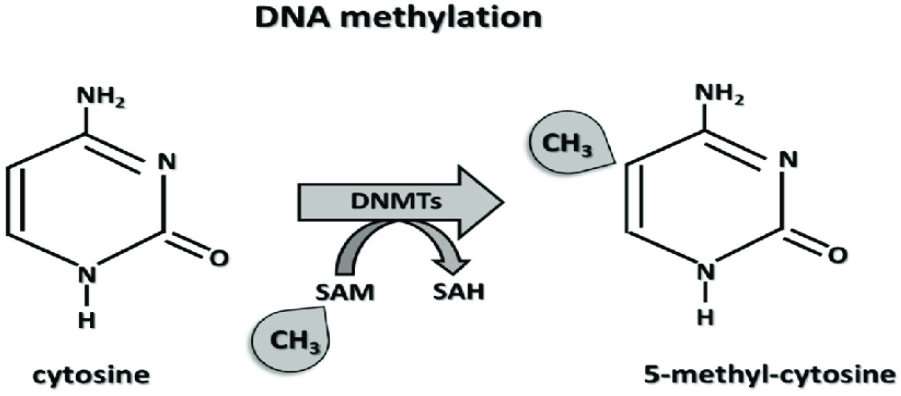

Fig 1: DNA methylation of cytosine [27]

The association between DNA methylation in cancer are characterized by two major events – Global hypomethylation and site specific hypermethylation. Global hypomethylation refers to the loss of methyl group, occurs in various regions of the genome namely, repetitive elements, retrotransposons, CpG poor promoters, introns and gene deserts. Global hypomethylation at retrotransposons which are genetic materials that copy and paste themselves to different regions of the genome, lead to their translocation to other genes. Thus, global

hypomethylation activates the proto-oncogenes and induces instability of the genome. In contrast, hypermethylation refers to the addition of a methyl group at specific CpG sites (usually the promoter region) which suppresses or inactivates a gene. In cancer the hypermethylation of tumor suppressor genes are well established, but how these genes are targeted remains unclear [24].

The use of DNA methylation information has been increasingly used in epidemiologic studies because of several reasons.

1. Explains the phenotypic variability due to differential gene expression even when the DNA sequence remains the same. [29]
2. Since DNA is more stable compared to RNA and DNAm more stable than other epigenetic modifications that include miRNA and histone modification, it is useful in large observational studies where the biospecimen is collected and stored decades ago.
3. DNA methylation is modifiable, which can be used to provide precise treatments which target the associated epigenetic markers. [27]

1.5 Epigenetic age and clocks:

Epigenetic age is a biomarker that is a combined measure of DNA methylation levels along with chronological age and calculation of epigenetic age can be used as a predictor of disease in various fields of research and treatment. [30]. Epigenetic clocks are biological clocks that measure the rate of epigenetic aging. The first multi tissue epigenetic clock was developed by Horvath where he identified 353 CpGs that could predict the epigenetic age. The clock highly correlated with most tissue types, and hence is recognized by Horvath as a pan tissue clock. However, the clock was poorly calibrated in tissues that reflect hormonal exposure and menstruation cycle like the breast and the uterine endometrium. Following Horvath's pan tissue

clock, there were many more epigenetic clocks that were developed, namely, Hannum clock, Levine clock (PhenoAge), Lu clock (GrimAge) all of which were based on whole blood. Interestingly, the DNAm age acceleration calculated from all the clocks are found to be associated with increased risk and mortality of cancer [31]. One major limitation of these clocks is that they only calculate DNAm age and DNAm age is not the same as mitotic age; it is highly correlative with cells that are non-proliferative hence this age is not correlated highly with cancer tissues. [30]

Mitotic age can be defined as the cumulative number of cell division within an individual's stem cell pool [32]. Mitotic age clock has been increasingly studied in cancer research due to high correlation with cancer progression. The clock tracks the cumulative number of DNAm errors that arise during cell division. Currently, EpiTOC (Epigenetic timer of cancer), HypoClock, MiAge and EpiTOC2, an improved version of EpiTOC, are some of the mitotic clocks that have been developed to predict cancer risk and mortality.

In Table 2, the different mitotic clocks are compared except for the HypoClock due to a few limitations. HypoClock is based on hypomethylation at a few specific WCGWs (isolated CpGs dinucleotides that are immediately flanked by adenine or thymine) which are usually methylated in the fetal stage and gradually lose methylation [32]. The reason why HypoClock was not used in this study is because HypoClock predicts the risk of cancer progression well only when the cells are under stress and that the model itself has a considerable confounding due to cell type heterogeneity which is addressed in both MiAge and EpiToc2 [32, 33]. EpiToc was also not used in this study because of the availability of a new, updated version of EpiToc (EpiToc2).

1.6 Rationale for using mitotic clocks in HGSOC:

For this study, we plan to evaluate the association of mitotic clocks – miAge and EpiTOC2 along with chronologic age for survival after HGSOC diagnosis among Black women. PRC2 complex on which the EpiTOC2 is based on, is overexpressed in ovarian cancers and miAge is tissue independent, making these clocks applicable for ovarian cancer tissues. [35]. There is no study that has evaluated these mitotic clocks as a prognostic indicator for survival in ovarian cancer. Since, NHB women are diagnosed with HGSOC at an early age compared to NHW and have a lower survival rate, chronological age alone cannot be considered as an accurate prognosis factor for survival. Therefore, it makes us wonder if there is any difference in the biological age/epigenetic age which could better explain the progression of cancer among the NHB women.

As NHB women with ovarian cancer have a relatively worse overall survival and poor prognosis compared to other racial and ethnic groups; in this study we aim to use the mitotic age to differentiate the high-risk group from the lower risk group using the above-mentioned mitotic age calculators.

Table 2: A comparison between the three different mitotic clocks is shown below:

	Mi-Age [33]	EpiToc [34]	EpiToc2 [32]
Description of the output	Mitotic Age calculator – Calculates total number of lifetime cell division	Mitotic age – *pctgAge* – Average age cumulative DNAm increase across the 385 CpG sites	Mitotic Age calculator – Calculates the intrinsic rate of stem cell division
Assumption	1. The probability of failure of maintenance in the first cell generation is the same as the denovo methylation probability for all tissue samples of the same type. 2. All cells undergo same level of mutation and the methylation of one CpG site is independent of the other	1. Assumes that the DNAm errors occur in the stem cell pool and there is no change in DNAm levels after mitosis. 2. Assumes similar stem cell numbers for all individuals 3. Extrinsic factors and intrinsic factors depend on the tissue type	1. Site specific denovo methylation and ground state methylation values are assumed to be independent of tissue type. 2. Stem cell rate is constant throughout life.
CpG Sites	286 CpG sites- increasing methylated sites – neither tissue related not tumor related	385 CpG sites 1. CpGs that are unmethylated in the fetal stage 2. CpGs that are marked by PRC2 complex (Polycomb Repressive Complex) 3. CpGs whose DNAm levels increase with age	163 CpG sites Polycomb Repressive Complex (PRC-2) which are generally unmethylated across fetal tissue types.
Instrument used	Illumina 450K	Illumina 450K	Illumina 450K
Tissue dependence	No	Yes	Yes

2.0 Methods

2.1 Data source

The study population comprises of self-reported NHB (non-Hispanic Black) women diagnosed with ovarian cancer enrolled in two case-control studies – AACES (African American Cancer Epidemiology Study) and NCOCS (North Carolina Ovarian Cancer Study).

AACES is a multi-center population-based case control study of Black and African American women who were diagnosed with histologically confirmed invasive EOC since December 2010 and residents of 11 geographical locations in the United States. Most of these regions were in the South (Alabama, Georgia, Louisiana, North and South Carolina and Tennessee), Texas from south-west, Michigan and Ohio from the Midwest and New Jersey from the mid-Atlantic region. Participants were eligible if they were between the age of 20-79 years and belonged to this geographical location. AACES had an additional inclusion criterion of including only Black women which were self-reported. Cases were identified through rapid case ascertainment systems through cancer registries, SEER registries or individual hospitals from 2011 to 2016. Passive consent was obtained from the respective physicians to contact the patients. Patients were then contacted and interviewed through telephone to obtain information on their demographic characteristics, reproductive, gynecological and medical history, external hormone use, oral contraceptives and lifestyle characteristics like smoking, alcohol consumption etc.

The NCOCS is a population-based case control study from the North Carolina region with women diagnosed with epithelial ovarian cancer who were identified through the North Carolina cancer registry from 1999 to 2008. This study included those with an incident EOC and within 20–74 years old. Unlike the AACES study where the subjects were interviewed over

phone, data on cancer risk factors was collected through an in-person interview after obtaining the informed consent from the study participants.

In the present study, we included women with HGSOC from AACES and NCOCS who self-reported Black race and had a biospecimen available and who did not have a neoadjuvant chemotherapy.

Patient level information like family history of either breast or ovarian cancer, smoking status, external hormone uses, and other epidemiological variables that were collected through surveys administered for each study (AACES (telephone interview) and NCOCS (in-person interview)), were harmonized together.

For both studies, formalin-fixed paraffin-embedded tissue blocks of the primary tumor were procured from the hospitals performing the diagnosing surgery for each participant after receiving consent.

2.2 Methylation data quality control:

A tissue release form was obtained from study participants and tissue samples were collected from the patients from their primary cancer site. DNA methylation quantification in the tissue was done using Illumina's Infinium MethylationEPIC array (EPIC Bead chip 850K) in three batches. A multiple dimensional scaling plot of the 1000 most variable CpG sites showed no significant differences between the batches and no clustering by batch was observed. Some of the quality control checks done on the DNA methylation data included the generation of density plots which showed an expected bimodal distribution representing the methylation and unmethylation signals. Cleaning and normalization steps were performed using the minfi package in R which involved determining Cholesky residuals [36], background correction and normalization.

2.3 Exposure, Outcome and covariates

The primary exposure in this study is the mitotic age calculated from the two mitotic clocks – EpiToc2 and MiAge and the outcome of interest is overall survival truncated at five years. Although the product obtained from EpiToc2 is not exactly the mitotic age but rather the cell turnover rate or IRS (Intrinsic rate of stem cell division), we would refer to it as mitotic age for convenience throughout this paper. The exposure was analyzed as a categorical variable; dichotomous and trichotomous, where the lower quantile or the tertile was our low-risk group and used as the referent group for all the analyses. In our study, we identified chronological age, smoking, family history of breast or ovarian cancer in a first-degree relative, stage and debulking status to be *a priori* confounders. We restricted the follow-up time to 5 years as we found a lot of the participants were not followed up longer than 5 years, thus preventing any bias due to follow-up time.

Age is defined as the age at diagnosis and smoking status is categorized based on if the person has ever smoked or never smoked. The cancer stage at diagnosis was categorized as localized, regional and distant where localized and regional were lumped together in one category during the analysis phase due to sample size considerations. Similarly, family history of ovarian or breast cancer was combined to form a two-level category for the family history variable – family history of either breast and/or ovarian cancer and no family history of breast or ovarian cancer. Other variables including debulking status, cancer stage, any female hormone use (pill patch or injection), and comorbidities were also considered in the multivariable analysis.

2.4 Mitotic age calculators

To calculate the mitotic age, we used two mitotic age calculators- MiAge and EpiTOC2. The function used to calculate the mitotic age by the MiAge calculator is as shown below:

$$f=\sum i\sum j(E(X_{nj,i})-\beta_{i,j})^2$$

This is the sum of squares of the differences between the observed and the expected methylation levels, which is summed over all the samples at CpG i and summed over all CpGs.

$E(X_{nj,i})$ – Expected methylation levels

$\beta i,j$ – Observed methylation level for a CpG for a sample j

Epitoc2 is a more robust mitotic age calculator which has lesser assumptions and is an improved version of EpiTOC. The output is the intrinsic rate of stem cell division – IRS which is based on the total number of stem cell division divided by the age of the person.

$$TNSC(s) = \frac{2}{n}\sum_{i=1}^{n}\frac{\beta_{is}-\beta_{i0}}{\delta_i(1-\beta_{i0})}$$

$$\mathrm{IRS(x)} = \langle TNSC(s,x)/\mathrm{A(s)}\rangle_s$$

Where <>s stands for average or median over the samples, s stands for samples, x stands for the tissue type. i denotes the CpG site, therefore β_{is} would be the methylation level at CpG site i in sample s and β_{i0} would be the methylation level at CpG site i in a fetal tissue. δ_i is the denovo methylation probability at CpG site i.

The mitotic age calculations using EpiToc2 and MiAge were performed in R version 4.2.1 with the help of the codes available in github.

2.5 Statistical Analysis:

Two sample t-test and chi square test were used to see if there was any statistically significant difference in patient characteristics by vital status (dead and alive). Normality was checked for continuous variables prior to conducting the t-test and no gross violation was identified for age. In case of BMI, Mann Whitney U test was performed due to suspected non-normal distribution. We looked at how the mitotic age calculated from the two mitotic clocks correlated with the chronologic age at the time of diagnosis using Pearson's correlation coefficient. The mitotic age obtained from both the calculators were log transformed to obtain a normal distribution prior to this analysis.

We then compared the mitotic age acceleration in different age groups, smoking status, stages and debulking status. We split the age groups into tertiles so that we have similar sample size in each group.

Univariate and multivariable survival analysis was performed using Kaplan Meier and Cox-proportional hazard regression. Five covariates were identified based on the *a priori* criteria (age, smoking status and family history of either breast or ovarian cancer, debulking status and cancer stage). We performed both graphical tests and goodness of fit test (including the global test) to test the PH (proportional hazard) assumption. Confounding was assessed through the evaluation of the best model after identifying the apriori confounders.

We initially adjusted for only chronological age and stage (Model I) and then for chronological age and debulking status (Model II). We then adjusted for all three variables – chronological age, stage and debulking status. Our final model consisted of the exposure (mitotic age – dichotomous and trichotomous), age at diagnosis, debulking status and stage of cancer. We also added post hormone replacement therapy use in our model to see if there is any effect on

survival (Model IV). In order to test for any effect due to hormone use, we restricted the study subject age at 50 years and performed the cox-proportional regression.

We also tested if the model was sensitive to study site (NCOCS and AACES) since both the study cohorts belonged to a different period. We restricted the study only from the AACES cohort and performed the Cox Proportional regression, after checking the ph assumptions. Significance level of 0.05 was used for all the tests and all the statistical analyses were performed in R version 4.2.1.

3.0 Results

3.1 Descriptive Statistics

The total study population consists of 202 patients for which methylation using FFPE ovarian tissue was determined: 161 from the AACES study and 41 from the NCOCS study. The median follow-up time in this cohort is 4.4 years, which was defined as days between diagnosis and last follow up. Out of the 202 patients in the study, 92 were alive after 5 years follow up from the time of diagnosis, while 110 died. The overall mean age of the cohort was 57.4 years and the mean age among those who died was not statistically different from those who were alive (mean age of people who died = 58.5 years vs those who were alive after 5 years = 56.1 years, p = 0.09). Similarly, the average BMI in the alive group was 31.3 kg/m^2 while the average BMI in the dead group was only a little higher with 33 kg/m^2 (p = 0.15) **(Table 3).**

A higher proportion of the patients had distant stage disease; 91.8% among the deceased and 67.4% among those who were alive. NCOCS had a higher proportion of people who died after 5 years of follow up compared to the AACES site. A possible explanation for this could be that NCOCS was conducted from 1999-2008 while AACES was conducted from 2010-2015. The

difference in survival could be attributed to difference in standards of care for the two cohorts. Patients who had sub-optimal debulking had a higher proportion of people who died compared to those who received an optimal debulking surgery. In both the deceased and alive groups, the people who did not have family history had a higher number of people who died and there was no difference between the two groups (p value = 0.37). Similarly, there is no difference between the proportions of people who died among the ever smokers and never smokers (p = 0.96) and female hormone users and never users (p value = 1).

3.2 Correlation between chronologic age and mitotic age:

After quality control, we had information on 151 of the 163 CpG sites used for the EpiTOC and 286 of 286 CpG sites used for MiAge. The mitotic age obtained from EpiToc2 had a weak negative correlation with the chronological age at the time of diagnosis ($r^2 = 0.16$) and this correlation was significant $p < 0.001$. The correlation between chronologic age at the time of diagnosis and mitotic age obtained from MiAge calculator was relatively weaker but was also negatively correlated ($r^2 = 0.015$). However, there was a strong positive correlation between both the log transformed mitotic ages with an r^2 of 0.55 measured by EpiToc2 and MiAge and this correlation was significant $p < 0.001$ (**Fig 2)**.

3.3 Mitotic age acceleration between different risk factors:

Table 4 shows the difference between mitotic age calculated from both the clocks by age, smoking status, and stage. While comparing the mitotic ages calculated from Epitoc2 and MiAge, a significant overall difference was found between the groups for mitotic age from Epitoc2 ($p<0.001$) but not for MiAge calculated mitotic age (p = 0.34) (Fig 3a).

Table 3: Description of the study population (significance at $p < 0.05$)

	Dead (N=110)	Alive (N=92)	Overall (N=202)	P value
Age (years)				0.09
Mean (SD)	58.5 (9.94)	56.1 (10.1)	57.4 (10.0)	
BMI (kg/m^2)				0.15
Mean (SD)	33.0 (8.74)	31.3 (8.40)	32.2 (8.60)	
Missing	3	0	3	
Stage				<0.0001
Localized	3 (2.7%)	13 (14.1%)	16 (7.9%)	
Regional	5 (4.5%)	15 (16.3%)	20 (9.9%)	
Distant	101 (91.8%)	62 (67.4%)	163 (80.7%)	
Missing	1	2	3	
Site				0.0012
AACES	78 (70.9%)	83 (90.2%)	161 (79.7%)	
NCOS	32 (29.1%)	9 (9.8%)	41 (20.3%)	
Debulking status				<0.0001
Optimal debulking	44 (40.0%)	70 (76.1%)	114 (56.4%)	
Suboptimal debulking	47 (42.7%)	15 (16.3%)	62 (30.7%)	
Missing	19	7	26	
Family history				0.37
No history of Breast or Ovarian cancer	64 (58.2%)	58 (63.0%)	122 (60.4%)	
Family history of either breast or ovarian cancer	41 (37.3%)	27 (29.3%)	68 (33.7%)	
Missing	5	7	12	
Ever smoked				0.96
No	53 (48.2%)	46 (50.0%)	99 (49.0%)	
Yes	56 (50.9%)	46 (50.0%)	102 (50.5%)	
Missing	1	0	1	
Ever used any female hormones				1.0
No	90 (81.8%)	76 (82.6%)	166 (82.2%)	
Yes	20 (18.2%)	16 (17.4%)	36 (17.8%)	

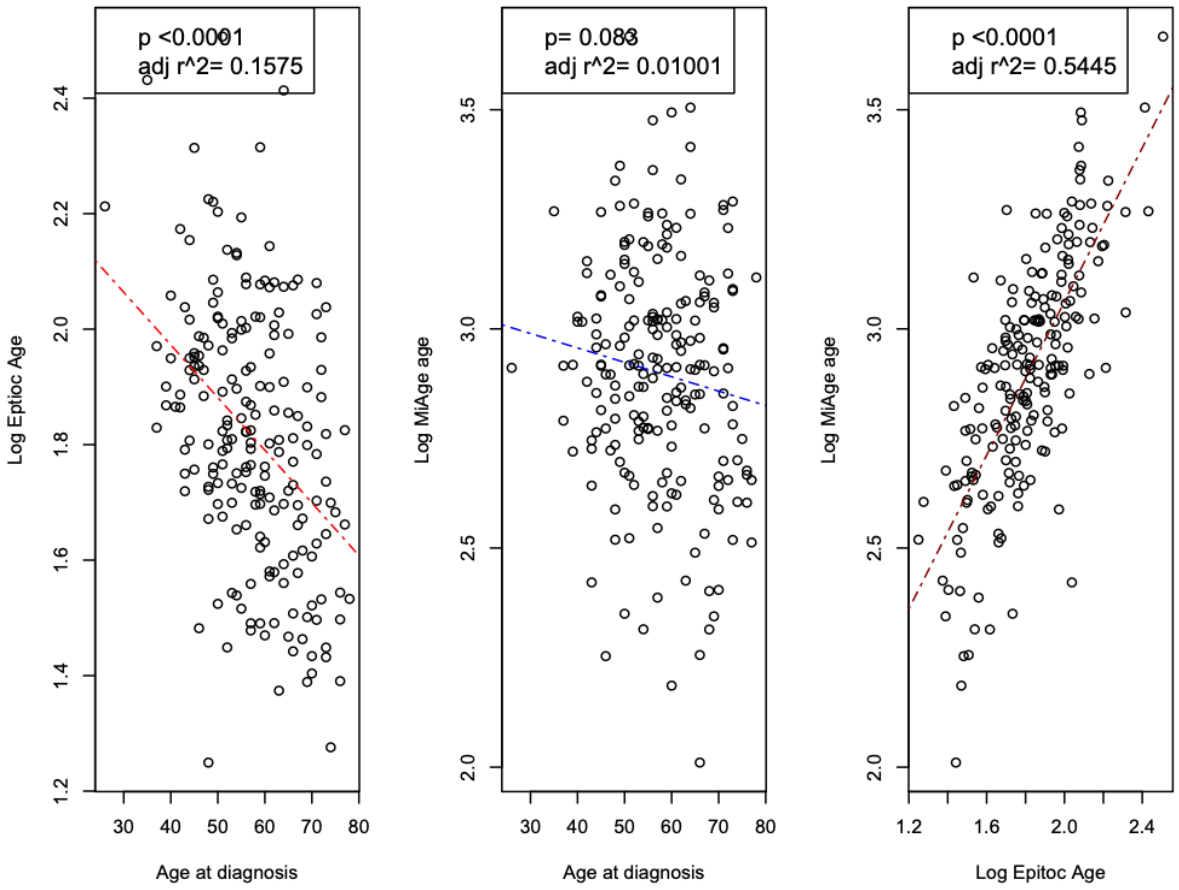

Fig 2: From the left: Correlation graph between Age at diagnosis and log transformed mitotic age obtained from EpiToc2, Correlation graph between Age at diagnosis and log transformed mitotic age obtained from MiAge and Correlation graph between log transformed mitotic age obtained from EpiToc2 and MiAge.

The Epitoc2 calculated mitotic age showed a decreasing trend with an increase in the age tertiles while no clear trend was observed for the mitotic age calculated using MiAge. No difference between the subjects' mitotic age was observed when stratified based on smoking status. Upon comparing between the stages, significant differences were observed for an overall difference between both the clocks. Both the clocks showed a similar trend in mitotic age across the stages with a high mitotic age in the localized stage as compared to the other two (Fig 3b).

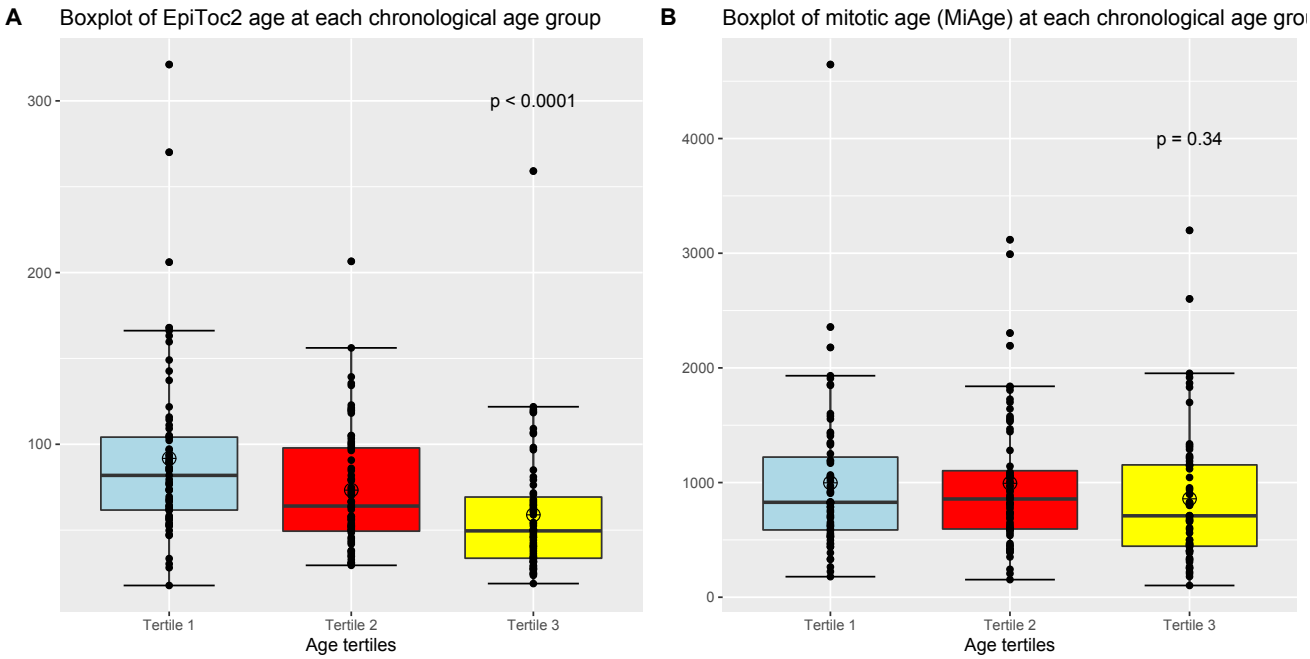

Fig 3a: Boxplot showing the difference in the mitotic age between different chronological age tertiles between the two mitotic clocks with p value for an overall difference.

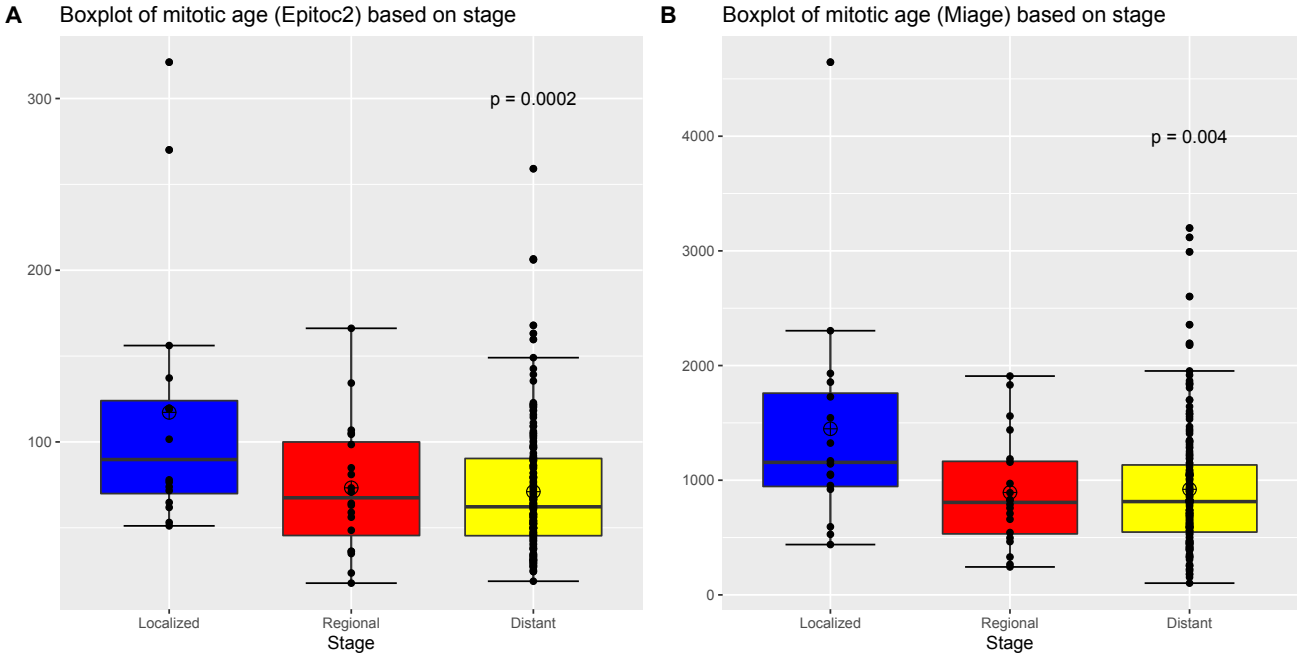

Fig 3b Boxplot showing the difference in the mitotic age between different stages between the two mitotic clocks with the p value for an overall difference

Table 4: Comparison of the mitotic age acceleration between age groups, smoking groups and stage for the two mitotic clocks:

Variables	Group comparison		Epitoc difference	95% CI	P (adj)	Miage difference	95% CI	p (adj)
Age	53 – 62 years	26-52 years	-18.5	(-35.3, -1.7)	0.027	-4.1	(-250.4, 242.3)	0.999
	63 -77 years	26-52 years	-32.9	(-50.3, 15.5)	<0.0001	-139.4	(-394, 115.3)	0.401
	63 -77 years	53 – 62 years	-14.4	(-31.5, 2.7)	0.118	-135.3	(-385.7, 115.1)	0.410
Smoking status	Former Smoker	Current Smoker	0.6	(-25.9, 27.0)	0.990	61.5	(-308.4, 431.3)	0.920
	Never Smoker	Current Smoker	1.8	(-24.2, 27.8)	0.980	-23.5	(-308.4 431.3)	0.990
	Never Smoker	Former Smoker	1.2	(-14.2, 16.7)	0.990	-85.0	(-301.4, 131.5)	0.620
Stage	Regional	Localized	-43.9	(-77.6, -10.3)	0.007	-555.4	(-1031.8, -78.9)	0.018
	Distant	Localized	-46.2	(-72.4, -19.9)	0.0001	-527.8	(-899.9, -155.6)	0.003
	Distant	Regional	-2.2	(-26.0, 21.5)	0.970	27.6	(-308.9, 364.2)	0.980

3.4 Univariate Kaplan Meier Analysis:

The median chronological age was 57 years, median value of the mitotic age from EpiToc2 was 64.4 (annual cell turnover rate) and the median mitotic age from MiAge was 820.89 (relative biological age). The mitotic age obtained from the MiAge calculator is relatively large because it is not an absolute value, but a relative value of a participant compared to the others in the study sample. The univariate survival analysis showed a difference in the survival rate by median chronological age; those who were diagnosed at or above the age of 57 years had a worse survival rate. However, this difference was not statistically significant (**Fig 4**).

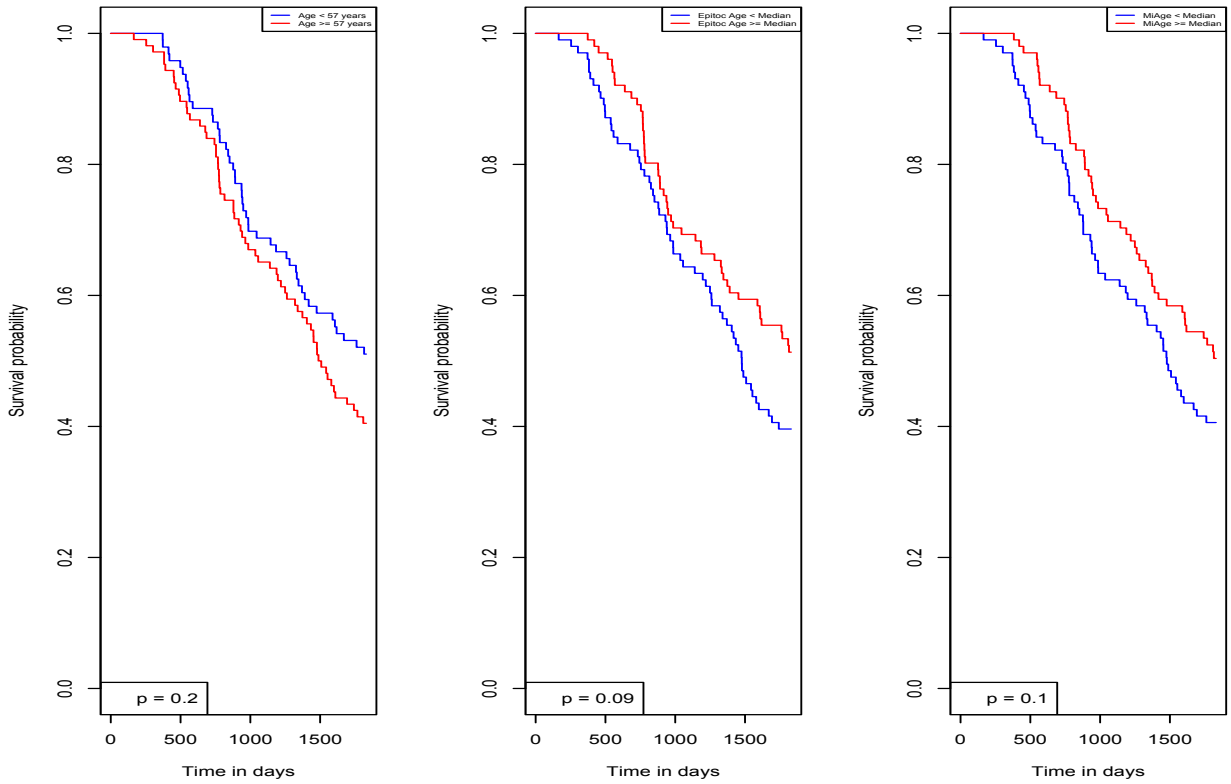

Fig 4: From left: Univariate Kaplan-meier curve based on the age at the time of diagnosis; based on mitotic age calculated from EpiToc2 and based on mitotic age calculated from MiAge.

The Kaplan Meier curves for the groups stratified based on the median value of Epitoc2 age showed a worse survival trend in those with a lower mitotic age as calculated from EpiToc2 compared to those higher than median. However, this difference was not statistically significant; p value = 0.09.

The trend was similar between the groups created based on the median mitotic age calculated from MiAge and this difference was again, not statistically significant p value = 0.1.

3.5 Multivariable Survival Analysis (Cox-Proportional Hazard):

We found no statistically significant interaction between any of the five covariates – age at diagnosis, smoking status, family history, debulking status and stage. Our final model

contained age at diagnosis, stage and debulking status along with our exposure of interest – mitotic age.

3.4.1 Mitotic age as a dichotomous variable:

HR estimated for the higher Epitoc group (those who had mitotic age calculated from EpiToc2 above the median value of 64.4 cell turnover rate) was 8% more compared to the lower Epitoc group (HR: 1.08, 95% CI: 0.67, 1.72; p = 0.76) while the lower Miage group had a higher HR by 9% compared to the higher quantile (HR: 0.91, 95% CI: 0.59, 1.4; p = 0.67) after adjusting for age at diagnosis, stage and debulking status. However, none of them were statistically significant and the 95% confidence interval contained null (**Table 5a**, **Fig 5**). Hence, there was no difference in survival between the two groups after adjusting for age, debulking status and stage. The association remained null when debulking status was removed from the model (Model I), when stage was removed from the model (Model II) and when hormone use was added to the model (Model IV) (Table 5).

3.4.2 Mitotic age as a trichotomous variable:

For comparing the tertiles, the reference group was the lower tertile q1 for both the clocks. Higher HR estimates were observed for the q3 tertile (Upper) compared to the reference and lower HR estimated for the q2 tertile (Middle) compared to the reference. This trend was similar when across both the clocks and different models (I – IV). Interaction by stage and debulking status were not statistically significant and therefore were removed from the model.

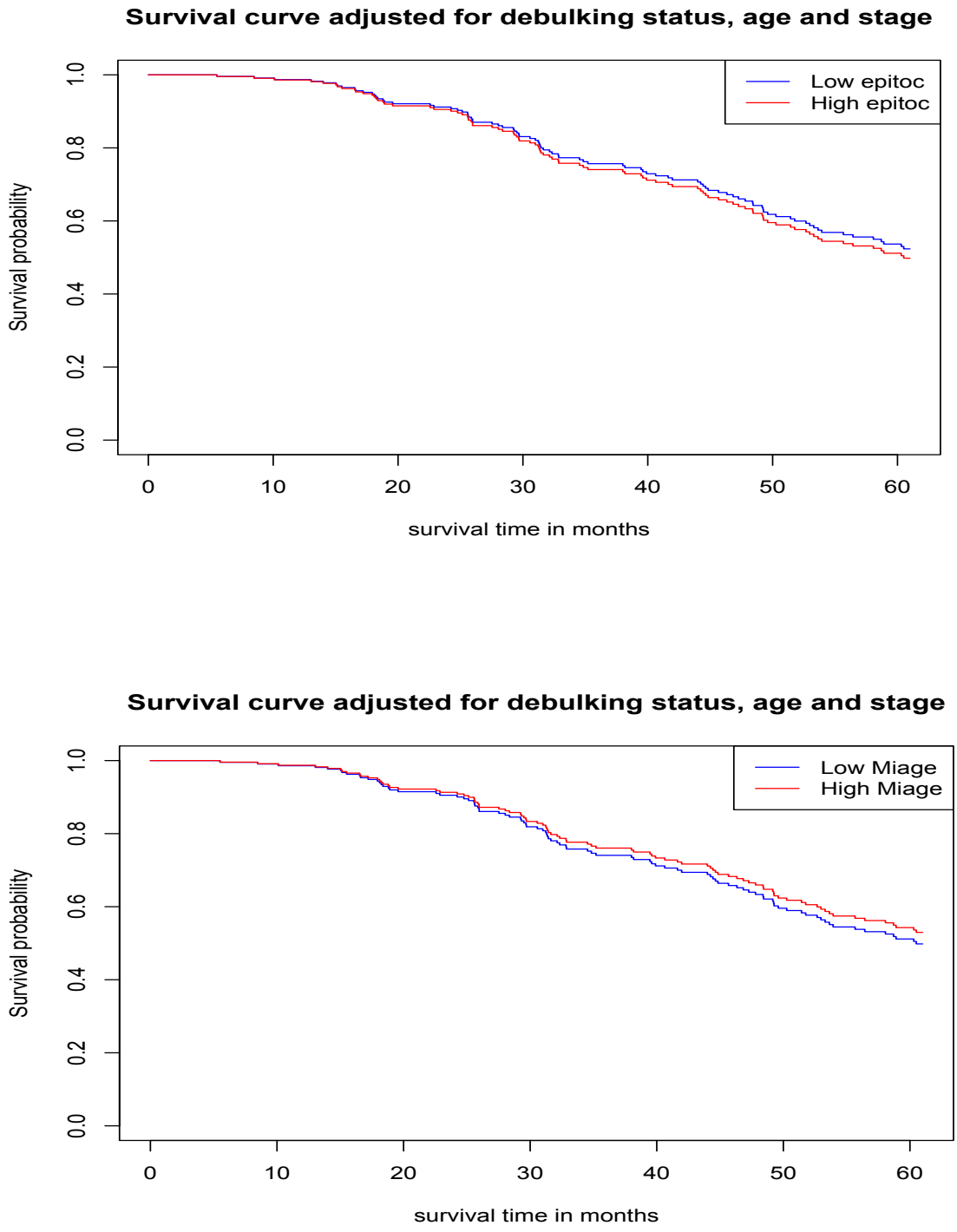

Fig 5: Top: Adjusted survival curve showing no difference in the 5-year survival between the two EpiToc groups. Bottom: Adjusted survival curve showing no difference in the 5-year survival between the two MiAge groups

For the model I, where we adjusted for only chronological age and stage and Epitoc as the exposure, we observed a better survival in the Q2 tertile compared to the reference (HR: 0.51, 95% CI: 0.31,0.84; p = 0.009). While the HR estimate was also lower for Q3 tertile compared to Q1, the confidence interval contained null and hence there was no difference in survival (HR: 0.8, 95% CI: 0.48, 1.32; p = 0.37). While comparing the survival between the tertiles categorized based on MiAge calculated mitotic age, we did not observe any difference between the groups. (Table 5).

Similarly, model II with EpiToc2 calculated mitotic age as exposure, showed a better survival in Q2 tertile compared to Q1 tertile, where we adjusted for chronological age and debulking status only (HR: 0.57; 95% CI: 0.33, 0.98; p = 0.04). However, there was no difference in survival between Q3 tertile and Q1 tertile (HR: 0.94; 95% CI: 0.53, 1.65). In contrast, no difference in survival was observed between the tertiles categorized based on MiAge calculated mitotic age (Table 5).

Model III for Epitoc2 calculated mitotic age, which looked at survival between the tertiles adjusting for chronological age, stage and debulking status, Q3 tertile had a higher HR estimate compared to Q1 tertile (HR: 1.04, 95% CI: 0.58, 1.84; p = 0.9), but the difference in survival was not significant. While comparing Q2 tertile to Q1 we observed lower HR estimate but no significant difference in survival (HR: 0.61, 95% CI: 0.35, 1.05; p = 0.07). Similarly, between the MiAge tertiles, no difference in survival was observed between the groups (**Table 5, Fig 6**). Models I and II conserve on power compared to model III due to, missing values for debulking status and stage.

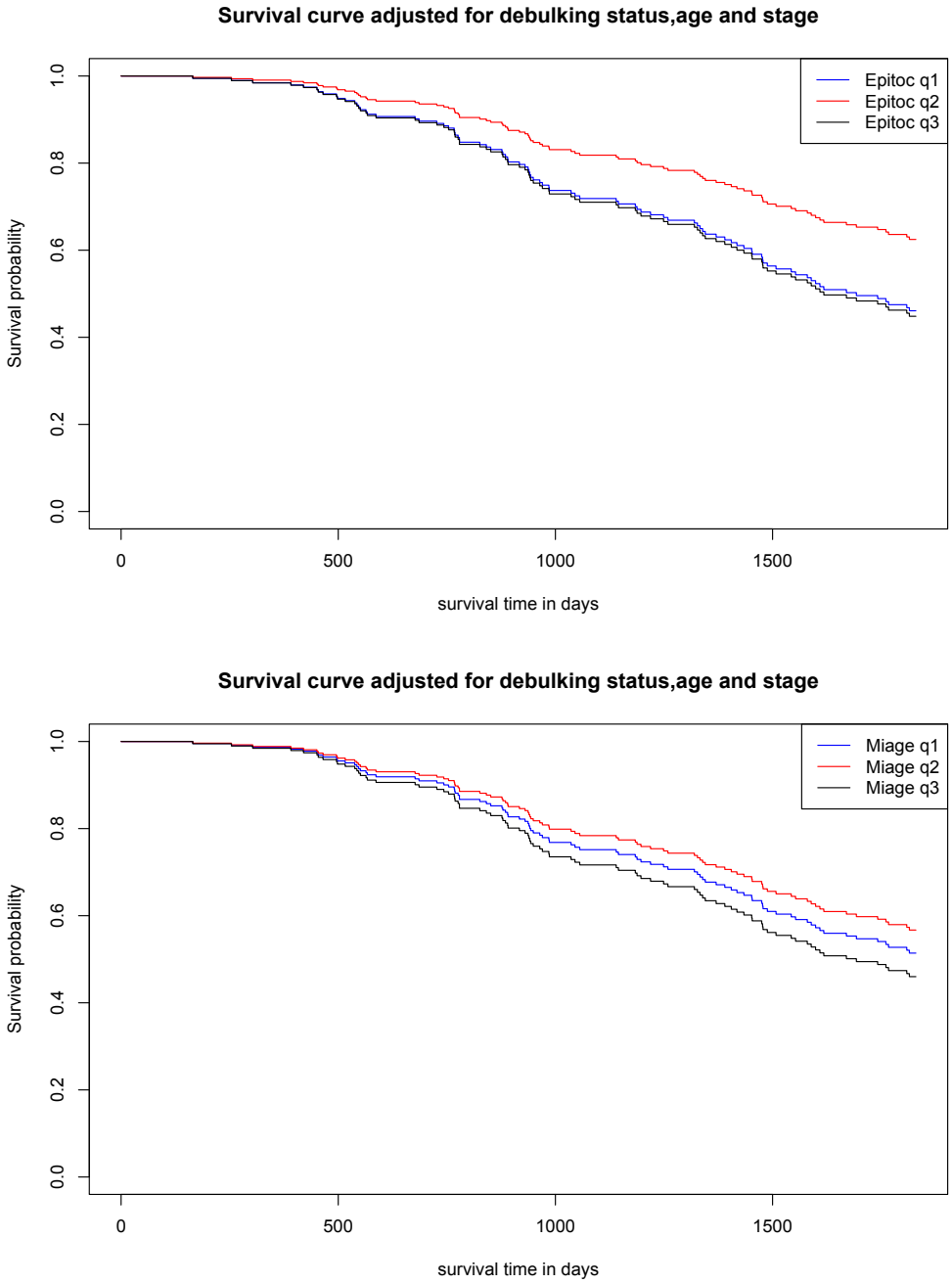

Fig 6: Top: Adjusted survival curve showing the difference in the 5-year survival between the three EpiToc based tertiles after adjusting for debulking status, age and stage.
Bottom: Adjusted survival curve showing the difference in the 5-year survival between the three MiAge based tertiles after adjusting for debulking status, age and stage

However, this did not improve the model much, though as expected the confidence became narrower.

We also looked at the influence of menopausal hormone use on the exposure and outcome. by restricting the study population in this analysis to women who were at least the age of 50. Thus, maximizing the likelihood that subjects in this sample are either postmenopausal or approaching menopausal status and eligible for hormone use. We added hormone use to our model (Model IV) after confirming that the ph assumptions were met. With the dichotomous exposure, the lower quantile Q1 (reference) had a HR estimate for both Epitoc and Miage calculated mitotic age (HR = 0.88; 95% CI: 0.53, 1.46, p = 0.6221 and HR = 0.75 (95% CI 0.46, 1.22, p = 0.25) respectively, after adjusting for debulking status, age, hormone use and stage. However, we found no significant difference in survival between the two groups.

Examination of tertiles with EpiToc as the exposure showed that the lower Q1 tertile (reference), had a worse survival compared to Q2 tertile (HR = 0.5 95% CI: 0.29, 0.88, p = 0.017) while no difference in survival between Q3 and Q1 tertiles was observed (HR = 0.86, 95% CI: 0.47, 1.58; p = 0.63) (**Table 5b, Fig 7**). Similar to the other models for MiAge, no difference in survival was observed between the groups. (Table 5).

We explored additional variables that are associated with risk of mortality in cancer patients including BMI, smoking status and diabetes status. However, the results did not change with addition of BMI, smoking status or diabetes status in the model

We looked at the trend in the HR between both the exposure variables with different models as shown in **Fig 8**. We observed a V or a U trend between the tertiles and this trend was consistent in almost all the models. The tertile Q2 consistently had a lower HR across all the models and this difference in HR between Q1 and Q2 was significant with the Epitoc2 calculated mitotic age as the variable for risk stratification.

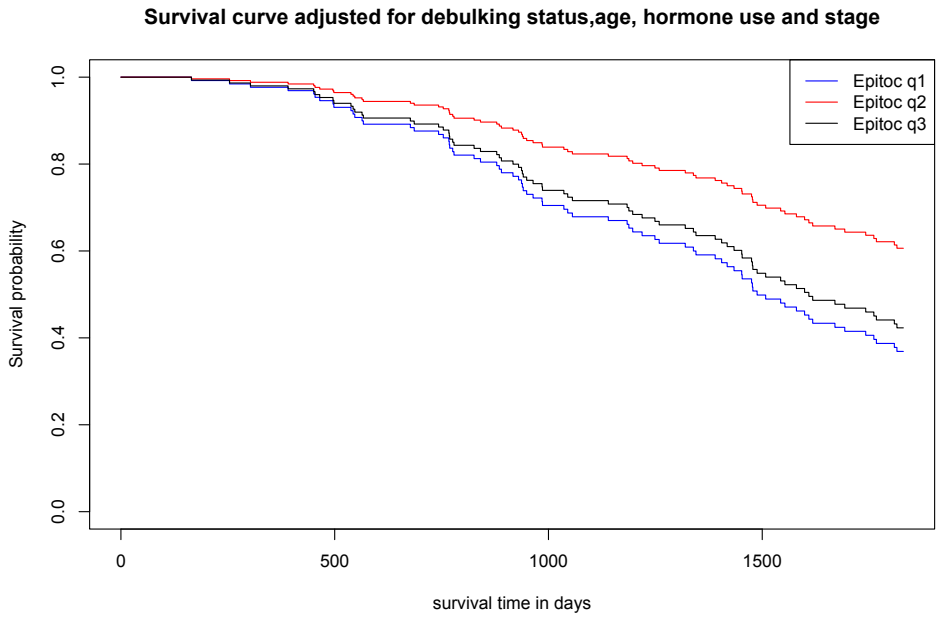

Fig 7: Adjusted survival curve showing the difference in the 5-year survival between the three EpiToc2 based tertiles after adjusting for debulking status, age, hormone use and stage. (Q2 vs Q1, p = 0.017)

Table 5: Cox proportional hazard ratio, 95% confidence interval and their p value are tabulated below for the models along with mitotic age calculated using EpiToc2 and MiAge: a) Exposure as a dichotomous variable and b) exposure as a trichotomous variable.

a)

	Reference group Q1	Model I (Age, stage)		Model II (Age, debulking status)		Model III (Age, stage, debulking status)		Model IV (Age, stage, debulking status, hormone use)	
	Q1 is < median Q2 is >= median	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
Miage	Q2	0.82 (0.56, 1.21)	0.33	0.84 (0.55, 1.28)	0.41	0.91 (0.59, 1.4)	0.67	0.75 (0.46,1.22)	0.25
EpiToc2	Q2	0.83 (0.55, 1.26)	0.39	0.989 (0.62, 1.57)	0.96	1.077 (0.67, 1.72)	0.76	0.88 (0.53, 1.46)	0.62

b)

	Reference group Q	Model I (Age, stage)		Model II (Age, debulking status)		Model III (Age, stage, debulking status)		Model IV (Age, stage, debulking status, hormone use)	
		HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
Miage	Q2	0.79 (0.49, 1.27)	0.33	0.87 (0.53, 1.44)	0.59	0.853 (0.51, 1.42)	0.54	0.83 (0.48, 1.45)	0.52
	Q3	1.08 (0.690, 1.70)	0.73	1.06 (0.64, 1.75)	0.82	1.17 (0.71, 1.93)	0.55	0.9 (0.50, 1.64)	0.74
EpiToc2	Q2	0.51 (0.31, 0.84)	0.009	0.57 (0.33, 0.98)	0.043	0.61 (0.35, 1.05)	0.07	0.5 (0.29, 0.88)	0.017
	Q3	0.8 (0.48, 1.32)	0.37	0.94 (0.53, 1.65)	0.82	1.04 (0.58, 1.84)	0.9	0.86 (0.47, 1.58)	0.63

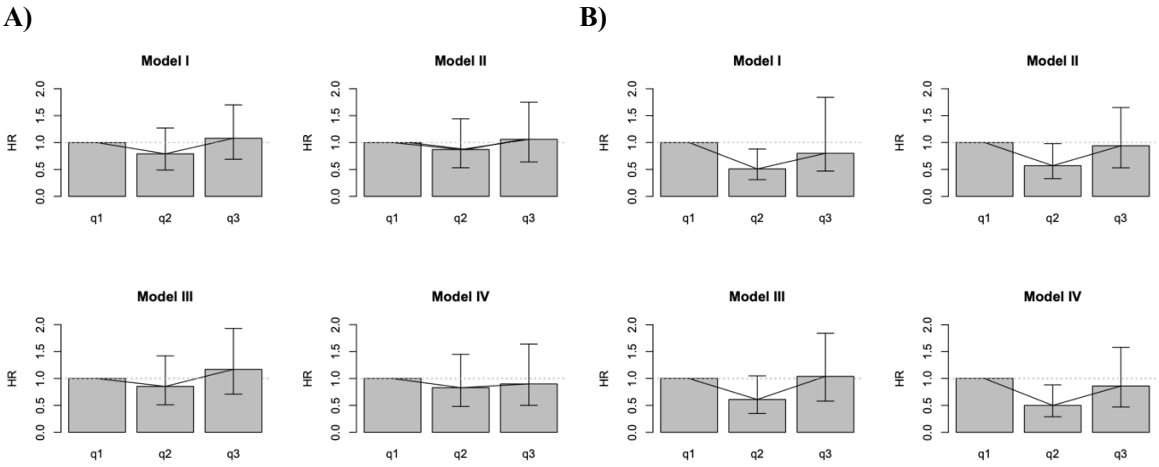

Fig 8: **Comparing the HR trend obtained for each exposure group across the different models. A: MiAge calculated mitotic age, B: Epitoc calculated mitotic age**

3.6 Sensitivity Analysis:

As NCOCS was conducted during an earlier period (1999 to 2008) than AACES, we performed a sensitivity analysis to remove NCOCS subjects from analysis for model I (the primary model) to assess any potential impact on our findings. We obtained similar estimates for both Miage and Epitoc2 based tertiles compared to when we had samples from both NCOCS and AACES cohorts.

Table 6: Comparing the HR estimates with only AACES samples and with both AACES and NCOCS samples.

Exposure tertile (Q1, Q2, Q3)	Reference group - Q1	Model adjusting for age, stage, and debulking status (Only AACES)		Model adjusting for age, stage, and debulking status (AACES and NCOCS)	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Miage	Q2	0.88 (0.51, 1.51)	0.63	0.853 (0.51, 1.42)	0.54
	Q3	1.12 (0.65, 1.95)	0.68	1.17 (0.71, 1.93)	0.55
EpiToc2	Q2	0.64 (0.36, 1.16)	0.14	0.61 (0.35, 1.05)	0.07
	Q3	1.11 (0.59, 2.10)	0.75	1.04 (0.58, 1.84)	0.9

4.0 Discussion:

We observed that both the mitotic ages (Epitoc2 and MiAge) were moderately and weakly correlated with chronological age, respectively, and the slope was negative. While comparing the mitotic age across chronological age, stage and smoking status, we obtained a higher mitotic age calculated using Epitoc2 in lower age group (26-52 years). The mitotic age calculated from Epitoc2 and MiAge were both higher for the localized stage. The survival analysis performed in this study by stratifying the mitotic age as a dichotomous and trichotomous variable did not provide significant result nor conclusive evidence that mitotic age could be used for risk stratification and a tool to indicate HGSOC cancer prognosis in Black women. However, we found a significant difference in the risk of mortality between tertile Q1 and tertile Q2 when EpiToc2 was used as the exposure, across almost all the models when categorized based on EpiToc2 calculated mitotic age.

The adjusted survival curves were interesting because the relationship between the HRs and the tertiles was not linear but rather a V or U shaped across all the models for both Epitoc2 and MiAge.

The weak correlation between mitotic age calculated from Epitoc and MiAge is expected as per the results obtained by Horvath [30] where the correlation between the DNAm and chronological age was found to be very weak in uterine and breast tissue. The high rate in error could be due to hormonal effects, menstrual cycle and concomitant increase in cell proliferation. We likely observed a better correlation of EpiToc2 calculated mitotic age with chronological age compared to MiAge because age is used in the calculation of the intrinsic stem cell rate (IRS = Total stem cell division/chronological age). Even, MiAge which is not tissue specific had a very

weak correlation with chronological age, which might be because ovarian tissue was not used in the development and validation of MiAge clock. In contrast, the correlation between the mitotic clocks was strong and positive as expected.

The increase in mitotic age calculated from Epitoc with decrease in chronological age was consistent with another study that showed acceleration in age for the different age groups with CRC (Colorectal cancer). This study also observed an age acceleration in the lower age group compared to older subjects [37]. This could be because cell division rate in general decreases with increasing age and hence the age acceleration was highest in the youngest group coupled with cancer progression. Another possible explanation for this could be that during pre-menopausal years, ovarian epithelium undergoes cell proliferation during menstrual cycle that could increase the cell turnover rate. EpiToc2 calculated mitotic age was found to be more accurate in differentiating precancerous lesions from cancerous which could also be the reason of a higher mitotic age in the localized stage [32]. Thus, we suspect that a higher mitotic age in the localized stage and among younger age group could have also contributed to the U or V shaped trend in survival.

We hypothesized an increase in the risk of mortality with an increase in the mitotic age since mitotic age tell us the rate at which cells divide and indicates cancer progression. In addition to it a pooled prospective cohort study was able to observe a decreasing survival rate with an increase in the quartiles using Horvath and Hannum clock for multiple cancers [38]. However, in our study, the middle group had the best survival among Epitoc tertiles, while the upper tertile and the lower tertile had worse survival, although the difference between the latter groups were not significant.

This is also the first study known to evaluate an association between mitotic age and survival among patients with ovarian cancer. Epigenetic age has been also associated with an increased risk of incidence cancer in a few studies but their association with mortality and survival is not very clear [38, 39]. Most of the studies that have used epigenetic clocks (Hannum, Horvath, GrimAge and PhenoAge) to evaluate their association with survival did not find an association [39]. Moreover, these studies did not include ovarian cancer in their analysis, therefore, it is difficult to understand the relationship of ovarian cancer and mitotic age given the increased rate of error in DNAm [38][39]. The pooled prospective cohort study by Dugue et al., was conducted in an Australian cohort and did not involve ovarian cancer patients and used different epigenetic clocks which could lead to the heterogeneity in the findings. One study found a higher risk in mortality among lung cancer cases with increase in epigenetic age/score (based on PhenoAge, GrimAge and MRscore) but an inverse association was obtained for breast cancer [40].

Furthermore, EpiToc2 and MiAge clocks did not use the ovarian tissue to validate their model, which adds to uncertainty in using them for ovarian cancer, which could be reason for not being able to obtain clear association for survival. However, we selected these because the CpG sites used to calculate EpiToc2 age lies in the Polycomb Repressive Complex (PRC-2) which is usually overexpressed in ovarian cancer [32]. In addition to it, MiAge is a tissue independent clock and uses global methylation levels for calculating mitotic age and has been shown to be a predictor of mortality in other cancers [33]. Between the two mitotic clocks, Epitoc based stratification led to determine association between mitotic age and age groups and stage in our study. In addition, it also helped in identification of a group (Q2) with better survival. However, Miage based stratification did not provide any conclusive results.

There are several limitations in the study; firstly, our sample size was likely not large enough to have adequate power to detect an association between the mitotic age and overall survival. However, since this is the first study on this topic, we used a well-established cohort comprising a relatively large population of Black women with HGSOC. Secondly, we only had 151 of the 163 CpGs for the calculation of IRS (Epitoc2 age) which could be due removal of certain CpGs after quality control. Thirdly, the categorization of the comparison groups was done based on quantiles (dichotomous and trichotomous) which might not have been an accurate measure of risk stratification. The reason for using quantiles and tertiles in this study to obtain the comparison groups was because a) we only had cases, so we could not determine the stem cell rate for a non-cancerous tissue to be able to determine the normal turnover rate, b) we could not determine the information on the normal turnover rate of ovarian tissue in any other published literature. Fourthly, we also have quite a few subjects missing their debulking status and stage. Therefore, we created two additional models leaving out debulking status and stage and the estimates were not very different from the model containing these variables. Finally, the tissues samples were collected and stored for a long time and even though DNA is comparatively stable than other specimen, a long-term storage could lead to some denaturation of it and thus impacting the results.

Our study also has a lot of strengths; firstly, the study population included only Black women with high grade serous histotype, so race and histotype were controlled by design. Even though we had a small sample size, this is a comparatively large sample after restricting race and histotype. Secondly, this is also the first study that evaluated the association between mitotic age and HGSOC survival in Black women and thus can be used as a baseline study for future studies. Thirdly, the study design is also prospective, collection of biospecimen was done prior to

observing the outcome and the patients were also not been treated with neoadjuvant chemotherapy. Thus, we can be sure that there is no effect of treatment on the DNA methylation levels that could affect its association with survival.

5.0 Conclusion

From our study we conclude that the association between mitotic age and survival after HGSOC diagnosis in Black women is not linear but a V or U shaped. The mitotic age is higher at a younger age and at localized stage and the risk of mortality based on risk stratification using mitotic clocks – EpiToc2 and MiAge was not significant, although Epitoc2 did identify Q2 tertile as a group with better survival.

Therefore, this topic requires more in-depth research to understand the differences between different cancer in relation to epigenetic age. Further research is needed on how methylation related biomarkers can be used to determine overall survival among cancers that interact with hormones and involving the menstrual cycle. Given the importance of epigenetic age and mitotic age in predicting cancer progression in current research, knowledge of the biological mechanism and basis to an increased mitotic age at early stages and younger age could help in better interpreting results obtained from epidemiologic studies. These could help identify the underlying type of cell proliferation that leads to an increased mitotic index. While it can be protective in some cases (immune cells) but also harmful in other cases (cancer cells). Further studies will need to be done to accurately determine the threshold for stratifying based on mitotic age, which could be obtained from a control group, or a non-cancerous ovarian tissue will be required from each subject. A more general population (not restricting on histotype and race) could provide more power and help in obtaining a much precise estimate.

Although our study lacked solid evidence of association between mitotic age calculated from Epitoc2 and MiAge, we were able to identify how mitotic age varied between different factors associated with survival. In addition to it, we have established baseline research in this field, identifying the limitations of such study that could be improved upon in future studies.

6.0 References:

1. Reid, B. M., Permuth, J. B., & Sellers, T. A. (2017). Epidemiology of ovarian cancer: a review. Cancer Biology & Medicine, 14(1), 9–32. https://doi.org/10.20892/j.issn.2095-3941.2016.0084
2. Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2022). Cancer statistics, 2022. CA: A Cancer Journal for Clinicians, 72(1), 7–33. https://doi.org/10.3322/caac.21708
3. SEER*Explorer: An interactive website for SEER cancer statistics [Internet]. Surveillance Research Program, National Cancer Institute. [Cited 2021 September 27]. Available from https://seer.cancer.gov/statistics-network/explorer/
4. Momenimovahed, Z., Tiznobaik, A., Taheri, S., & Salehiniya, H. (2019). Ovarian cancer in the world: epidemiology and risk factors. International Journal of Women's Health, 11, 287–299. https://doi.org/10.2147/IJWH.S197604
5. Islami, F., Ward, E. M., Sung, H., Cronin, K. A., Tangka, F. K. L., Sherman, R. L., Zhao, J., Anderson, R. N., Henley, S. J., Yabroff, K. R., Jemal, A., & Benard, V. B. (2021). Annual Report to the Nation on the Status of Cancer, Part 1: National Cancer Statistics. JNCI: Journal of the National Cancer Institute, 113(12), 1648–1669. https://doi.org/10.1093/jnci/djab131
6. Stenzel, A. E., Buas, M. F., & Moysich, K. B. (2019). Survival disparities among racial/ethnic groups of women with ovarian cancer: An update on data from the Surveillance, Epidemiology and End Results (SEER) registry. Cancer Epidemiology, 62, 101580. https://doi.org/10.1016/j.canep.2019.101580
7. Chen, V. W., Ruiz, B., Killeen, J. L., Coté, T. R., Wu, X. C., & Correa, C. N. (2003). Pathology and classification of ovarian tumors. Cancer, 97(10 Suppl), 2631–2642. https://doi.org/10.1002/cncr.11345
8. Lisio, M.-A., Fu, L., Goyeneche, A., Gao, Z., & Telleria, C. (2019). High-Grade Serous Ovarian Cancer: Basic Sciences, Clinical and Therapeutic Standpoints. International Journal of Molecular Sciences, 20(4), 952. https://doi.org/10.3390/ijms20040952
9. Kurman, R. J., & Shih, I.-M. (2010). The Origin and Pathogenesis of Epithelial Ovarian Cancer: A Proposed Unifying Theory. American Journal of Surgical Pathology, 34(3), 433–443. https://doi.org/10.1097/PAS.0b013e3181cf3d79
10. Chen, S., Li, Y., Qian, L., Deng, S., Liu, L., Xiao, W., & Zhou, Y. (2021). A Review of the Clinical Characteristics and Novel Molecular Subtypes of Endometrioid Ovarian Cancer. Frontiers in Oncology, 11. https://doi.org/10.3389/fonc.2021.668151
11. Babaier, A., & Ghatage, P. (2020). Mucinous Cancer of the Ovary: Overview and Current Status. Diagnostics, 10(1), 52. https://doi.org/10.3390/diagnostics10010052
12. Gadducci, A., Multinu, F., Cosio, S., Carinelli, S., Ghioni, M., & Aletti, G. D. (2021). Clear cell carcinoma of the ovary: Epidemiology, pathological and biological features, treatment options and clinical outcomes. Gynecologic Oncology, 162(3), 741–750. https://doi.org/10.1016/j.ygyno.2021.06.033
13. Peres, L. C., Cushing-Haugen, K. L., Köbel, M., Harris, H. R., Berchuck, A., Rossing, M. A., Schildkraut, J. M., & Doherty, J. A. (2019). Invasive Epithelial Ovarian Cancer Survival by Histotype and Disease Stage. JNCI: Journal of the National Cancer Institute, 111(1), 60–68. https://doi.org/10.1093/jnci/djy071
14. Kim, S. J., Rosen, B., Fan, I., Ivanova, A., McLaughlin, J. R., Risch, H., Narod, S. A., & Kotsopoulos, J. (2017). Epidemiologic factors that predict long-term survival following a diagnosis of epithelial ovarian cancer. British Journal of Cancer, 116(7), 964–971. https://doi.org/10.1038/bjc.2017.35
15. Phung, M. T., Alimujiang, A., Berchuck, A., Anton-Culver, H., Schildkraut, J. M., Bandera, E. v., Chang-Claude, J., Chase, A., Doherty, J. A., Grout, B., Goodman, M. T., Hanley, G. E., Lee, A. W., McKinnon Deurloo, C., Menon, U., Modugno, F., Pharoah, P. D. P., Pike, M. C., Richardson, J., … Pearce, C. L. (2022). Reproductive Factors Do Not Influence Survival with Ovarian Cancer. Cancer Epidemiology, Biomarkers & Prevention, 31(4), 909–913. https://doi.org/10.1158/1055-9965.EPI-21-1091
16. Stenzel, A. E., Buas, M. F., & Moysich, K. B. (2019). Survival disparities among racial/ethnic groups of women with ovarian cancer: An update on data from the Surveillance, Epidemiology and End Results (SEER) registry. Cancer Epidemiology, 62, 101580. https://doi.org/10.1016/j.canep.2019.101580 `
17. Peres, L. C., Risch, H., Terry, K. L., Webb, P. M., Goodman, M. T., Wu, A. H., Alberg, A. J., Bandera, E. v, Barnholtz-Sloan, J., Bondy, M. L., Cote, M. L., Funkhouser, E., Moorman, P. G., Peters, E. S., Schwartz, A. G., Terry, P. D., Manichaikul, A., Abbott, S. E., Camacho, F., … Schildkraut, J. M. (2018). Racial/ethnic differences in the epidemiology of ovarian cancer: a pooled analysis of 12 case-control studies. International Journal of Epidemiology, 47(2), 460–472. https://doi.org/10.1093/ije/dyx252
18. Arab, M., Jamdar, F., Sadat Hosseini, M., Ghodssi- Ghasemabadi, R., Farzaneh, F., & Ashrafganjoei, T. (2018). Model for Prediction of Optimal Debulking of Epithelial Ovarian Cancer. Asian Pacific Journal of Cancer Prevention : APJCP, 19(5), 1319–1324. https://doi.org/10.22034/APJCP.2018.19.5.1319
19. Kotsopoulos, J., Rosen, B., Fan, I., Moody, J., McLaughlin, J. R., Risch, H., May, T., Sun, P., & Narod, S. A. (2016). Ten-year survival after epithelial ovarian cancer is not associated with BRCA mutation status. Gynecologic Oncology, 140(1), 42–47. https://doi.org/10.1016/j.ygyno.2015.11.009
20. Brieger, K. K., Phung, M. T., Mukherjee, B., Bakulski, K. M., Anton-Culver, H., Bandera, E. v., Bowtell, D. D. L., Cramer, D. W., DeFazio, A., Doherty, J. A., Fereday, S., Fortner, R. T., Gentry-Maharaj, A., Goode, E. L., Goodman, M. T., Harris, H. R., Matsuo, K., Menon, U., Modugno, F., … Pearce, C. L. (2022). High Prediagnosis Inflammation-Related Risk Score Associated with Decreased Ovarian Cancer Survival. Cancer Epidemiology, Biomarkers & Prevention, 31(2), 443–452. https://doi.org/10.1158/1055-9965.EPI-21-0977
21. Præstegaard, C., Jensen, A., Jensen, S. M., Nielsen, T. S. S., Webb, P. M., Nagle, C. M., DeFazio, A., Høgdall, E., Rossing, M. A., Doherty, J. A., Wicklund, K. G., Goodman, M. T., Modugno, F., Moysich, K., Ness, R. B., Edwards, R., Matsuo, K., Hosono, S., Goode, E. L., … Kjaer, S. K. (2017). Cigarette smoking is associated with adverse survival among women with ovarian cancer: Results from a pooled analysis of 19 studies. International Journal of Cancer, 140(11), 2422–2435. https://doi.org/10.1002/ijc.30600
22. Wu, J., Sun, H., Yang, L., Deng, Y., Yan, Y., Wang, S., Yang, G., & Ma, H. (2018). Improved survival in ovarian cancer, with widening survival gaps of races and socioeconomic status: a period analysis, 1983-2012. Journal of Cancer, 9(19), 3548–3556. https://doi.org/10.7150/jca.26300

23. Sharma, S., Kelly, T. K., & Jones, P. A. (2010). Epigenetics in cancer. Carcinogenesis, 31(1), 27–36. https://doi.org/10.1093/carcin/bgp220
24. Dawson, M. A., & Kouzarides, T. (2012). Cancer Epigenetics: From Mechanism to Therapy. Cell, 150(1), 12–27. https://doi.org/10.1016/j.cell.2012.06.013
25. Alegría-Torres, J. A., Baccarelli, A., & Bollati, V. (2011). Epigenetics and lifestyle. Epigenomics, 3(3), 267–277. https://doi.org/10.2217/epi.11.22
26. Kurdyukov, S., & Bullock, M. (2016). DNA Methylation Analysis: Choosing the Right Method. Biology, 5(1), 3. https://doi.org/10.3390/biology5010003
27. Ciechomska, M., Roszkowski, L., & Maslinski, W. (2019). DNA Methylation as a Future Therapeutic and Diagnostic Target in Rheumatoid Arthritis. Cells, 8(9), 953. https://doi.org/10.3390/cells8090953
28. Moran, S., Arribas, C., & Esteller, M. (2016). Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. Epigenomics, 8(3), 389–399. https://doi.org/10.2217/epi.15.114
29. Jaffe, A. E., Murakami, P., Lee, H., Leek, J. T., Fallin, M. D., Feinberg, A. P., & Irizarry, R. A. (2012). Bump hunting to identify differentially methylated regions in epigenetic epidemiology studies. International Journal of Epidemiology, 41(1), 200–209. https://doi.org/10.1093/ije/dyr238
30. Horvath, S. (2013). DNA methylation age of human tissues and cell types. Genome Biology, 14(10), R115. https://doi.org/10.1186/gb-2013-14-10-r115
31. Salameh, Y., Bejaoui, Y., & el Hajj, N. (2020). DNA Methylation Biomarkers in Aging and Age-Related Diseases. Frontiers in Genetics, 11. https://doi.org/10.3389/fgene.2020.00171
32. Teschendorff, A. E. (2020). A comparison of epigenetic mitotic-like clocks for cancer risk prediction. Genome Medicine, 12(1), 56. https://doi.org/10.1186/s13073-020-00752-3
33. Youn, A., & Wang, S. (2018). The MiAge Calculator: a DNA methylation-based mitotic age calculator of human tissue types. Epigenetics, 13(2), 192–206. https://doi.org/10.1080/15592294.2017.1389361
34. Yang, Z., Wong, A., Kuh, D., Paul, D. S., Rakyan, V. K., Leslie, R. D., Zheng, S. C., Widschwendter, M., Beck, S., & Teschendorff, A. E. (2016). Correlation of an epigenetic mitotic clock with cancer risk. Genome Biology, 17(1), 205. https://doi.org/10.1186/s13059-016-1064-3
35. Jones, B. A., Varambally, S., & Arend, R. C. (2018). Histone Methyltransferase EZH2: A Therapeutic Target for Ovarian Cancer. Molecular Cancer Therapeutics, 17(3), 591–602. https://doi.org/10.1158/1535-7163.MCT-17-0437
36. Houseman, E. A., Ryan, L. M., & Coull, B. A. (2004). Cholesky Residuals for Assessing Normal Errors in a Linear Model With Correlated Outcomes. Journal of the American Statistical Association, 99(466), 383–394. https://doi.org/10.1198/016214504000000040
37. Joo, J. E., Clendenning, M., Wong, E. M., Rosty, C., Mahmood, K., Georgeson, P., Winship, I. M., Preston, S. G., Win, A. K., Dugué, P.-A., Jayasekara, H., English, D., Macrae, F. A., Hopper, J. L., Jenkins, M. A., Milne, R. L., Giles, G. G., Southey, M. C., & Buchanan, D. D. (2021). DNA Methylation Signatures and the Contribution of Age-Associated Methylomic Drift to Carcinogenesis in Early-Onset Colorectal Cancer. Cancers, 13(11), 2589. https://doi.org/10.3390/cancers13112589
38. Dugué, P.-A., Bassett, J. K., Joo, J. E., Jung, C.-H., Ming Wong, E., Moreno-Betancur, M., Schmidt, D., Makalic, E., Li, S., Severi, G., Hodge, A. M., Buchanan, D. D., English, D. R., Hopper, J. L., Southey, M. C., Giles, G. G., & Milne, R. L. (2018). DNA methylation-based biological aging and cancer risk and survival: Pooled analysis of seven prospective studies. International Journal of Cancer, 142(8), 1611–1619. https://doi.org/10.1002/ijc.31189
39. Wang, C., Ni, W., Yao, Y., Just, A., Heiss, J., Wei, Y., Gao, X., Coull, B. A., Kosheleva, A., Baccarelli, A. A., Peters, A., & Schwartz, J. D. (2021). DNA methylation-based biomarkers of age acceleration and all-cause death, myocardial infarction, stroke, and cancer in two cohorts: The NAS, and KORA F4. EBioMedicine, 63, 103151. https://doi.org/10.1016/j.ebiom.2020.103151
40. Li, X., Schöttker, B., Holleczek, B., & Brenner, H. (2022). Associations of DNA methylation algorithms of aging and cancer risk: Results from a prospective cohort study. EBioMedicine, 81, 104083. https://doi.org/10.1016/j.ebiom.2022.104083
41. Ji, E., Kim, K., Lee, B., Hwang, S. O., Lee, H. J., Lee, K., Lee, M., & Kim, Y. B. (2022). Postoperative Hormone Replacement Therapy and Survival in Women with Ovarian Cancer. Cancers, 14(13), 3090. https://doi.org/10.3390/cancers14133090