

Research Article

Association of Selected Cell-Cycle Regulatory Gene Expression and Clinicopathological Characteristics in Nigerian Women with Breast Cancer

Mieiwari Ibifubara Jumbo^{1*}, Ebirien-Agana Samuel Bartimaeus², Evelyn Mgbeoma Eze ¹ and Zaccheaus Awortu Jeremiah¹

¹Department of Haematology and Blood Transfusion Science, Faculty of Medical Laboratory Science, Rivers State University, Nkpulu-Oroworukwo, Port Harcourt, Rivers State, Nigeria.

²Department of Clinical Chemistry, Faculty of Medical Laboratory Science, Rivers State University, Nkpulu-Oroworukwo, Port Harcourt, Rivers State, Nigeria.

*Corresponding author: mieiwari.jumbo@ust.edu.ng

Article Info

Keywords: Breast cancer, CCNA2, CCND1, E2F1, RB1, Gene expression, Nigeria.

Received: 26.08.2026;

Accepted: 16.10.2026;

Published: 23.10.2026

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Abstract

Background: Breast cancer is biologically heterogeneous, and altered cell-cycle control contributes to tumour development, proliferation and therapeutic response. Cell-cycle gene expression data from Nigerian women are limited, particularly in relation to treatment status and clinicopathological characteristics. **Aim:** To determine the expression of cyclin A (CCNA2), cyclin D1 (CCND1), E2F transcription factor 1 (E2F1) and retinoblastoma 1 (RB1), and to examine their associations with demographic, reproductive, treatment-related and clinicopathological characteristics in Nigerian women with breast cancer. **Methods:** A hospital-based comparative cross-sectional case-control study at the University of Port Harcourt Teaching Hospital included 120 women: 40 treatment-exposed (TG), 40 newly diagnosed untreated (NG) and 40 healthy controls. RNA from peripheral whole-blood-derived material was analysed by two-step RT-qPCR with ABL1 as reference gene, and relative expression was calculated by the 2^{−ΔΔCt} method with gene-specific healthy-control calibrators. Groups were compared by one-way ANOVA with post-hoc testing. Within TG and NG, normalised expression was compared across subgroups by t-test or ANOVA. Significance was $p < 0.05$, and subgroup p values were not adjusted for multiple testing. **Results:** CCNA2, CCND1 and E2F1 did not differ among groups ($p = 0.1774, 0.1201, 0.3444$). RB1 differed ($F = 5.8276, p = 0.0039$): it was lower in TG (0.28 ± 0.08) than NG (1.15 ± 0.25 ; adjusted $p = 0.032$), with controls intermediate (0.83 ± 0.18). Age, breast density and immunohistochemical subtype were not associated with expression. Of 60 subgroup comparisons, 12 were nominally significant. These were menstrual history (TG CCNA2, CCND1, E2F1), tumour type (TG E2F1), clinical stage (RB1 in TG, $p = 0.0112$; in NG, $p = 0.0006$) and histological grade (TG CCNA2, E2F1, RB1). In NG, prior breast procedure was associated with CCND1 and E2F1, and treatment status with CCND1. Only the NG RB1-stage and TG E2F1-grade associations remained below a Bonferroni threshold. **Conclusion:** RB1 was the clearest between-group discriminator, and the TG-NG difference is unlikely to be explained by age, since the two groups were of similar age. The subgroup associations are exploratory because of small subgroups and multiple testing. Protein-level, tumour-tissue and longitudinal studies are needed.

1. Introduction

Breast cancer is the most frequently diagnosed malignancy among women worldwide [1]. It is characterised by substantial biological heterogeneity in proliferation, molecular phenotype, treatment response and clinical behaviour. Conventional characteristics (stage, grade, tumour size, nodal involvement, hormone-receptor status, human epidermal growth factor receptor 2 (HER2) status and molecular subtype) remain central to classification and treatment, but they do not fully account for variation in behaviour and outcome [2]. Deregulated cell-cycle control is a recognised hallmark of malignant transformation, so the processes regulating proliferation remain important for understanding differences between breast cancer phenotypes [2, 3]. Cell-cycle progression is coordinated by cyclins, cyclin-dependent kinases (CDKs), tumour-suppressor proteins and transcription factors. Within this network, cyclin D1 (CCND1), the retinoblastoma protein (pRB), E2F1 and cyclin A (CCNA2) occupy key positions. pRB regulates the G1/S transition through E2F transcription factors [4]. CCND1 acts through CDK4/6, and phosphorylation of pRB releases E2F-dependent transcription and permits progression towards S phase [5, 6]. E2F1 regulates genes involved in DNA synthesis, cell-cycle progression, DNA-damage responses and apoptosis [7]. Cyclin A contributes to DNA replication and the G2/M transition [8, 9]. Alterations in these components have been linked to proliferative activity, tumour characteristics and therapeutic response, which justifies examining them together.

The clinical importance of these pathways is reflected in treatments directed at cell-cycle regulation. CDK4/6 inhibition acts through the cyclin D-CDK4/6-RB pathway and is established for selected hormone-receptor-positive cancers [6, 10]. RB1 functional status is particularly relevant to response, with genomic or functional RB alterations implicated in resistance [10–13]. Transcript-level measurements therefore require interpretation within the wider regulatory system.

Nigerian studies show substantial clinicopathological and molecular heterogeneity, including considerable proportions of triple-negative breast cancer and frequent presentation at advanced stages [14–18]. Most investigations have used tumour tissue, and information on the combined expression of E2F1, cyclin A, cyclin D1 and RB1 in peripheral blood of Nigerian patients is limited. Peripheral blood is a less invasive source and was used here. It was hypothesised that expression of the selected genes would differ among newly diagnosed, treatment-exposed and healthy women, and would be associated with demographic, reproductive, treatment-related and clinicopathological characteristics. The objective was to quantify and compare CCND1, RB1, E2F1 and CCNA2 expression among the groups, and to determine their associations with age, menstrual history, previous breast procedure, treatment status, tumour type, clinical stage, histological grade, breast density and immunohistochemical category in women with breast cancer.

2. Methods

2.1. Study design and setting

A hospital-based analytical comparative cross-sectional case-control study was conducted at the University of Port Harcourt Teaching Hospital (UPTH), Rivers State, Nigeria, a tertiary referral and specialised oncology centre. Recruitment was through the Breast and General Surgery Units. The study compared treatment-exposed breast cancer participants, newly diagnosed untreated participants and apparently healthy female controls at recruitment.

2.2. Study population and sampling

The study population comprised 120 women aged 20–80 years: 40 treatment-exposed (TG), 40 newly diagnosed untreated (NG) and 40 apparently healthy controls. Breast cancer was confirmed histologically on needle biopsy or mastectomy material. Controls were healthy women without clinical or radiological history of breast cancer, recruited from hospital staff and the hospital community. Purposive sampling was used. The minimum sample size, estimated in $G \times \text{Power}$ 3.1.9.2 for one-way ANOVA (effect size 0.4, $\alpha = 0.05$, power 0.95, three groups), was 102. Recruitment was completed at 120, with 40 per group.

2.3. Eligibility

Cases were females aged ≥ 20 years with histologically confirmed breast cancer, in the newly diagnosed untreated or treatment-exposed group, with complete clinicopathological information (stage, grade, hormone-receptor and HER2 status) and written informed consent. Controls were healthy females aged ≥ 20 years with no history of breast cancer or other malignancy and no known chronic inflammatory, autoimmune or haematological disorder. Exclusions were refusal of consent, previous other malignancy, documented haematological disorder, acute infection, autoimmune or chronic inflammatory disease, pregnancy or lactation, incomplete records, and inadequate or haemolysed specimens. Controls were also excluded for a family history suggestive of hereditary cancer syndromes and for immunosuppressive or chemotherapy exposure.

2.4. Ethics Approval

In line with the Helsinki Declaration, approval was obtained from the UPTH Research Ethics Committee (UPTH/ADM/90/SII/VOL.XI/1235). Written informed consent was obtained from all participants, and confidentiality was maintained using identification codes.

2.5. Clinical and clinicopathological data

Information came from clinical records, pathology reports and a structured questionnaire (age, menstrual and reproductive history, breast density, previous breast procedures and treatment status). Tumour type, grade and stage followed WHO classification and AJCC staging. Oestrogen receptor (ER), progesterone receptor (PR) and HER2 were assessed by immunohistochemistry on formalin-fixed paraffin-embedded sections (streptavidin-biotin-peroxidase method, heat-induced epitope retrieval, ASCO/CAP scoring). Triple-negative status denoted absence of ER, PR and HER2. The dependent variables were the normalised expression of CCNA2, CCND1, E2F1 and RB1. Serum

CA 15-3 and haematological parameters were measured in the same cohort and are outside the scope of this report.

2.6. RNA extraction and cDNA synthesis

Venous blood (5 mL or 2×5 mL) was collected aseptically. White cells were harvested by ammonium chloride red-cell lysis and lysed in guanidinium isothiocyanate buffer activated with β -mercaptoethanol. Total RNA was extracted by chloroform phase separation, isopropanol precipitation and ethanol washing, with column purification including DNase I treatment. RNA was stored at -80°C . Purity was assessed spectrophotometrically, and A260/A280 of 1.8–2.0 was acceptable. RNA was reverse-transcribed (42°C for about 60 min, then 85°C for 10 min), and cDNA was used immediately or stored at -80°C .

2.7. RT-qPCR

CCNA2, CCND1, E2F1 and RB1 were quantified by two-step RT-qPCR with EvaGreen chemistry, with reference to MIQE principles [19]. Each 25 μL reaction contained 12.5 μL $2 \times$ EvaGreen master mix (Solis BioDyne), 7.5 μL gene-specific primer mix (IDT Europe; final concentration 300 nM) and 5 μL cDNA. Samples and controls were run in duplicate with no-template controls in each run. Cycling was 95°C for 10 min, then 40 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 45 s, with a final extension at 72°C for 5 min. Melt-curve analysis assessed specificity. An Applied Biosystems StepOnePlus system was used.

2.8. Normalisation

Abelson tyrosine-protein kinase 1 (ABL1) was the endogenous reference. ΔCt was target Ct minus ABL1 Ct. Gene-specific healthy-control mean ΔCt values were calibrators (CCNA2 4.228; CCND1 5.641; E2F1 4.534; RB1 1.769). Relative expression was $2^{-\Delta\Delta\text{Ct}}$, so values > 1 indicate relative upregulation and < 1 relative downregulation. ABL1 Ct did not differ among groups (Kruskal-Wallis $H = 1.402$, $p = 0.496$).

2.9. Statistical analysis

Analysis used SAS 9.4 (SAS Institute, Cary, NC, USA). Data are mean $\pm$ SEM (mean $\pm$ SD where stated) or n (%). Between-group comparisons used one-way ANOVA with Tukey post-hoc testing, or Kruskal-Wallis with Dunn-Holm post-hoc comparisons for non-normal data; variance homogeneity was checked by Levene's test. Within TG and NG separately, normalised expression was compared across age category, menstrual history, breast density, previous breast procedure, treatment status, tumour type, clinical stage, histological grade and immunohistochemical category using independent t-tests (two categories) or ANOVA with post-hoc testing (more than two). Categorical variables used chi-square tests. Significance was $p < 0.05$. No correction for multiple comparisons was applied to the subgroup analyses. Sixty subgroup tests were performed, so their p values are exploratory; a Bonferroni threshold ($0.05/60 \approx 0.0008$) is reported for reference in the Discussion.

3. Results

3.1. Participant characteristics and clinicopathological profile

Mean ages were 47.15 ± 11.00 years (TG), 46.65 ± 11.48 years (NG) and 35.10 ± 10.01 years (controls), a significant difference ($\chi^2 = 21.915$, $p = 0.0013$; $F = 15.8005$, $p < 0.0001$). The two patient groups were of similar age. Of 80 breast cancer participants, 44 (55.0%) were Stage III, 32 (40.0%) Stage II, 2 (2.5%) Stage I and 2 (2.5%) Stage IV. Grade was 1 in 36 (45.0%), 2 in 42 (52.5%) and 3 in 2 (2.5%). Median stage was 3.0 (2.0–4.0) in TG and 2.5 (1.0–3.0) in NG ($p = 0.301$) Table 1.

Table 1: Clinicopathological characteristics of breast cancer participants

Characteristic	All (n=80)	TG (n=40)	NG (n=40)
Clinical stage, n (%)			
I	2 (2.5)	0	2 (5.0)
II	32 (40.0)	14 (35.0)	18 (45.0)
III	44 (55.0)	24 (60.0)	20 (50.0)
IV	2 (2.5)	2 (5.0)	0
Histological grade (SBR), n (%)			
1	36 (45.0)	20 (50.0)	16 (40.0)
2	42 (52.5)	20 (50.0)	22 (55.0)
3	2 (2.5)	0	2 (5.0)

TG, treatment-exposed; NG, newly diagnosed; SBR, Scarff-Bloom-Richardson

3.2. Between-group gene expression

ABL1 Ct was 20.73 ± 0.30 (TG), 20.93 ± 0.23 (NG) and 20.39 ± 0.23 (controls), with no significant difference ($F = 1.1747$, $p = 0.3125$). CCNA2 relative expression was 0.70 ± 0.20 , 0.39 ± 0.20 and 0.16 ± 0.02 ($F = 1.7551$, $p = 0.1774$). CCND1 was 0.20 ± 0.07 , 0.18 ± 0.05 and 0.06 ± 0.02 ($F = 2.1585$, $p = 0.1201$). E2F1 was 0.17 ± 0.04 , 0.17 ± 0.04 and 0.10 ± 0.02 ($F = 1.0759$, $p = 0.3444$).

RB1 alone differed among groups, at 0.28 ± 0.08 (TG), 1.15 ± 0.25 (NG) and 0.83 ± 0.18 (controls) ($F = 5.8276$, $p = 0.0039$; Table 2). Fold-change values showed the same pattern (0.95 ± 0.29 , 3.93 ± 0.85 , 2.83 ± 0.60 ; $F = 5.8563$, $p = 0.0038$). RB1 was significantly lower in

TG than NG (Dunn-Holm adjusted $p = 0.032$). TG versus controls (adjusted $p = 0.073$) and NG versus controls (adjusted $p = 0.644$) were not significant. Median RB1 fold change was 1.998 (NG), 0.989 (controls) and 0.285 (TG)

Table 2: Relative expression of selected cell-cycle regulatory genes across study groups

Parameter	Treatment-exposed (n=40)	Newly diagnosed (n=40)	Healthy control (n=40)	F	p
ABL1 Ct	20.73 ± 0.30	20.93 ± 0.23	20.39 ± 0.23	1.1747	0.3125
CCNA2 relative expression (NR)	0.70 ± 0.20	0.39 ± 0.20	0.16 ± 0.02	1.7551	0.1774
CCNA2 2 ⁻ ΔΔCt fold change	13.04 ± 6.19	7.32 ± 2.02	3.09 ± 0.68	1.7471	0.1788
CCND1 relative expression (NR)	0.20 ± 0.07	0.18 ± 0.05	0.06 ± 0.02	2.1585	0.1201
CCND1 2 ⁻ ΔΔCt fold change	10.02 ± 3.61	9.09 ± 2.30	3.19 ± 0.97	2.1365	0.227
E2F1 relative expression (NR)	0.17 ± 0.04	0.17 ± 0.04	0.10 ± 0.02	1.0759	0.3444
E2F1 2 ⁻ ΔΔCt fold change	3.91 ± 0.95	3.83 ± 0.96	2.35 ± 0.54	1.0941	0.3382
RB1 relative expression (NR)	0.28 ± 0.08 ^a	1.15 ± 0.25 ^b	0.83 ± 0.18 ^{ab}	5.8276	0.0039
RB1 2 ⁻ ΔΔCt fold change	0.95 ± 0.29 ^b	3.93 ± 0.85 ^a	2.83 ± 0.60 ^{ab}	5.8563	0.0038

Mean ± SEM. NR, normalised ratio. Means sharing a superscript letter do not differ significantly ($p < 0.05$).

The measured quantity is RB1 transcript; pRB protein was not measured.

3.3. Associations with demographic, reproductive, treatment and clinicopathological characteristics

Table 3 presents normalised expression within TG and NG. Most characteristics were not associated with expression ($p > 0.05$).

Age category was not associated with CCNA2, CCND1, E2F1 or RB1 expression in either patient group Table 3.

In TG, menstrual history was associated with CCNA2 ($p = 0.0460$), CCND1 ($p = 0.0241$) and E2F1 ($p = 0.0404$): women with ongoing menses had higher means (1.93 ± 0.57 , 0.50 ± 0.22 and 0.32 ± 0.07 , respectively) than women whose menses had stopped (0.15 ± 0.40 , 0.07 ± 0.01 and 0.11 ± 0.05). No association was found in NG (all $p > 0.05$).

In NG, a previous breast procedure was associated with lower CCND1 (0.13 ± 0.04 vs 0.34 ± 0.12 ; $p = 0.0403$) and E2F1 (0.09 ± 0.02 vs 0.38 ± 0.13 ; $p = 0.0015$). All TG participants had undergone a procedure, so no within-TG comparison was possible.

In NG, CCND1 differed by treatment status ($p = 0.0137$), at 0.52 ± 0.14 in the small subgroup recorded as receiving treatment and 0.15 ± 0.05 in those not receiving treatment. All TG participants were on treatment, so no within-TG comparison was possible.

E2F1 expression differed by tumour type in TG ($p = 0.0013$), being highest in ductal carcinoma (0.78 ± 0.16), followed by invasive ductal carcinoma (0.14 ± 0.04) and infiltrative ductal carcinoma (0.09 ± 0.16); the non-invasive and infiltrative categories held very few participants. The other genes did not differ by tumour type, and the NG group comprised only invasive ductal carcinoma.

RB1 was associated with clinical stage in both patient groups. In TG ($p = 0.0112$), RB1 was highest in Stage II (0.61 ± 0.22) and lower in Stage III (0.10 ± 0.02) and Stage IV (0.13); in NG ($p = 0.0006$), it was highest in Stage I (4.24 ± 0.94), followed by Stage III (1.53 ± 0.30) and Stage II (0.40 ± 0.31). CCNA2, CCND1 and E2F1 showed no stage association in either group.

In TG, CCNA2 ($p = 0.0149$), E2F1 ($p < 0.0001$) and RB1 ($p = 0.0013$) were each associated with histological grade. All three were highest in Grade 1 (CCNA2 2.56 ± 0.68 ; E2F1 0.52 ± 0.07 ; RB1 0.86 ± 0.16), with lower values in Grade 3 for E2F1 and RB1 (0.10 ± 0.04 ; 0.16 ± 0.10) and in Grade 2 (E2F1 0.04 ± 0.06 ; RB1 0.09 ± 0.13). No grade association was observed in NG, and CCND1 showed no grade association in either group.

Neither immunohistochemical subtype nor breast density was associated with the expression of any gene (all $p > 0.05$).

4. Discussion

The present analysis revealed three principal patterns that together frame the interpretation of the study. In the between-group comparison, only RB1 differed: expression was lower in treatment-exposed than in newly diagnosed women, with controls intermediate, whereas CCNA2, CCND1 and E2F1 did not differ. Second, within the cancer groups, expression was not associated with age, breast density or immunohistochemical subtype. Third, nominal associations were confined to menstrual history, previous breast procedure, treatment status, tumour type, clinical stage and histological grade, and RB1 with clinical stage was the only association observed in both patient groups. Taken together, these findings point to the RB axis, which links CDK activity to E2F-driven transcription [4, 10], as the most consistent signal in the dataset, and they provide no support for uniform elevation of all four transcripts in peripheral blood. All measurements represent peripheral-blood transcripts, and none of the associations can be read as evidence of causation.

Clinicopathological profile of the cohort

Advanced-stage presentation dominated the cohort, with 95% of patients at Stage II-III, in keeping with Nigerian reports of late presentation and aggressive disease biology [14, 17, 18]. Grade 1-2 tumours predominated (97.5%). This distribution is unexpected in a population in which triple-negative disease is widely reported to be over-represented, since triple-negative tumours are generally high-grade [15, 20]. Several non-exclusive explanations deserve consideration. Inter-observer variability in Scarff-Bloom-Richardson grading is well recognised and may have shifted tumours between grade categories, and the small overall sample restricts the precision of the grade distribution. The comparator Nigerian series are themselves heterogeneous in design and setting: Haruna and colleagues [15] deliberately enriched for triple-negative cases, Wemimo and colleagues [16] examined consecutive invasive carcinomas, and Ugiagbe and Owolabi [17] analysed a single tertiary-centre series, so subtype proportions across these reports are not directly poolable. Because receptor-defined subtypes were unevenly represented in the present cohort, inference about subtype composition must rest on this wider Nigerian literature [15, 16] rather than on internal counts.

Table 3: Normalised expression of cell-cycle regulatory genes by age, menstrual history, breast density, previous breast procedure, treatment status and clinicopathological parameters (mean $\pm$ SEM)

Characteristic	TG CCNA2	TG CCND1	TG E2F1	TG RB1	NG CCNA2	NG CCND1	NG E2F1	NG RB1
Age (years)								
20-39	0.39 $\pm$ 0.59	0.47 $\pm$ 0.13	0.20 $\pm$ 0.07	0.11 $\pm$ 0.03	0.62 $\pm$ 0.19	0.13 $\pm$ 0.05	0.31 $\pm$ 0.11	1.88 $\pm$ 0.45
40-49	1.72 $\pm$ 0.59	0.12 $\pm$ 0.13	0.17 $\pm$ 0.07	0.59 $\pm$ 0.26	0.07 $\pm$ 0.24	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01	0.50 $\pm$ 0.55
50-59	0.16 $\pm$ 0.73	0.07 $\pm$ 0.15	0.27 $\pm$ 0.09	0.21 $\pm$ 0.08	0.50 $\pm$ 0.18	0.24 $\pm$ 0.08	0.15 $\pm$ 0.05	1.19 $\pm$ 0.41
≥ 60	0.16 $\pm$ 0.73	0.05 $\pm$ 0.15	0.01 $\pm$ 0.09	0.12 $\pm$ 0.04	0.11 $\pm$ 0.27	0.35 $\pm$ 0.21	0.09 $\pm$ 0.05	0.51 $\pm$ 0.63
p	0.2442	0.1034	0.2328	0.1076	0.2222	0.2102	0.1264	0.1816
Menstrual history								
Menses stopped	0.15 $\pm$ 0.40 ^a	0.07 $\pm$ 0.01 ^a	0.11 $\pm$ 0.05 ^a	0.21 $\pm$ 0.11	0.20 $\pm$ 0.07	0.26 $\pm$ 0.09	0.09 $\pm$ 0.06	1.13 $\pm$ 0.42
Ongoing	1.93 $\pm$ 0.57 ^b	0.50 $\pm$ 0.22	0.32 $\pm$ 0.07 ^b	0.50 $\pm$ 0.15	0.59 $\pm$ 0.20	0.12 $\pm$ 0.03	0.25 $\pm$ 0.06	1.26 $\pm$ 0.34
Irregular	0.30 $\pm$ 0.99 ^c	0.10 $\pm$ 0.02	0.05 $\pm$ 0.12 ^c	0.02 $\pm$ 0.26	0.08 $\pm$ 0.00	0.03 $\pm$ 0.00	0.02 $\pm$ 0.18	0.33 $\pm$ 0.00
p	0.0460	0.0241	0.0404	0.1917	0.1637	0.2585	0.1028	0.7379
Dense breast								
No	0.26 $\pm$ 0.07	0.05 $\pm$ 0.01	0.16 $\pm$ 0.08	0.28 $\pm$ 0.11	0.47 $\pm$ 0.13	0.22 $\pm$ 0.06	0.20 $\pm$ 0.05	1.15 $\pm$ 0.27
Yes	0.88 $\pm$ 0.47	0.27 $\pm$ 0.10	0.17 $\pm$ 0.05	0.28 $\pm$ 0.11	0.05 $\pm$ 0.02	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	1.18 $\pm$ 0.67
p	0.3958	0.1600	0.8656	0.9819	0.1174	0.1310	0.1147	0.9602
Previous breast procedure								
No	-	-	-	-	0.71 $\pm$ 0.27	0.34 $\pm$ 0.12	0.38 $\pm$ 0.13	1.49 $\pm$ 0.44
Yes	0.70 $\pm$ 0.33	0.20 $\pm$ 0.07	0.17 $\pm$ 0.04	0.28 $\pm$ 0.08	0.28 $\pm$ 0.11	0.13 $\pm$ 0.04	0.09 $\pm$ 0.02	1.04 $\pm$ 0.30
p	-	-	-	-	0.0869	0.0403	0.0015	0.4491
On cancer treatment								
No	-	-	-	-	0.38 $\pm$ 0.12	0.15 $\pm$ 0.05	0.18 $\pm$ 0.04	1.20 $\pm$ 0.27
Yes	0.70 $\pm$ 0.33	0.20 $\pm$ 0.07	0.17 $\pm$ 0.04	0.28 $\pm$ 0.08	0.47 $\pm$ 0.35	0.52 $\pm$ 0.14	0.02 $\pm$ 0.13	0.73 $\pm$ 0.80
p	-	-	-	-	0.8193	0.0137	0.2446	0.5787
Tumour type								
Invasive ductal	0.77 $\pm$ 0.36	0.22 $\pm$ 0.08	0.14 $\pm$ 0.04 ^a	0.30 $\pm$ 0.09	0.39 $\pm$ 0.11	0.18 $\pm$ 0.05	0.17 $\pm$ 0.04	1.15 $\pm$ 0.25
Infiltrative ductal	0.01 $\pm$ 1.51	0.09 $\pm$ 0.33	0.09 $\pm$ 0.16 ^b	0.14 $\pm$ 0.39	-	-	-	-
Ductal carcinoma	0.00 $\pm$ 1.51	0.01 $\pm$ 0.33	0.78 $\pm$ 0.16 ^c	0.02 $\pm$ 0.39	-	-	-	-
p	0.7926	0.7815	0.0013	0.7305	-	-	-	-
Clinical stage								
I	-	-	-	-	0.10 $\pm$ 0.00	0.11 $\pm$ 0.00	0.07 $\pm$ 0.00	4.24 $\pm$ 0.94 ^a
II	1.59 $\pm$ 0.91	0.17 $\pm$ 0.04	0.22 $\pm$ 0.08	0.61 $\pm$ 0.22 ^a	0.43 $\pm$ 0.17	0.15 $\pm$ 0.07	0.09 $\pm$ 0.04	0.40 $\pm$ 0.31 ^b
III	0.21 $\pm$ 0.04	0.23 $\pm$ 0.12	0.15 $\pm$ 0.05	0.10 $\pm$ 0.02 ^b	0.38 $\pm$ 0.15	0.21 $\pm$ 0.07	0.24 $\pm$ 0.07	1.53 $\pm$ 0.30 ^c
IV	1.28 $\pm$ 0.00	0.04 $\pm$ 0.33	0.01 $\pm$ 0.33	0.13 $\pm$ 0.00 ^b	-	-	-	-
p	0.1411	0.8109	0.4842	0.0112	0.8097	0.7670	0.1718	0.0006
Histological grade								
1	2.56 $\pm$ 0.68 ^a	0.19 $\pm$ 0.16	0.52 $\pm$ 0.07 ^a	0.86 $\pm$ 0.16 ^a	0.37 $\pm$ 0.25	0.27 $\pm$ 0.10	0.05 $\pm$ 0.01	0.71 $\pm$ 0.57
2	0.17 $\pm$ 0.55 ^b	0.10 $\pm$ 0.13	0.04 $\pm$ 0.06 ^b	0.09 $\pm$ 0.13 ^b	0.31 $\pm$ 0.16	0.09 $\pm$ 0.07	0.12 $\pm$ 0.04	1.25 $\pm$ 0.38
3	0.27 $\pm$ 0.43 ^{c†}	0.27 $\pm$ 0.10 [†]	0.10 $\pm$ 0.04 ^{c†}	0.16 $\pm$ 0.10 ^{c†}	0.51 $\pm$ 0.19	0.26 $\pm$ 0.08	0.29 $\pm$ 0.10	1.29 $\pm$ 0.43
P	0.0149	0.6099	<0.0001	0.0013	0.7142	0.1661	0.0598	0.6840
IHC subtype								
ER+	0.01 $\pm$ 0.00	0.09 $\pm$ 0.33	0.09 $\pm$ 0.19	0.14 $\pm$ 0.39	-	-	-	-
HER2+	0.56 $\pm$ 0.00	0.24 $\pm$ 0.33	0.24 $\pm$ 0.19	0.57 $\pm$ 0.39	0.05 $\pm$ 0.49	0.03 $\pm$ 0.21	0.01 $\pm$ 0.19	0.07 $\pm$ 1.12
HER2+/PR-/ER-	0.03 $\pm$ 0.00	0.08 $\pm$ 0.33	0.03 $\pm$ 0.19	0.06 $\pm$ 0.39	0.02 $\pm$ 0.49	0.01 $\pm$ 0.21	0.01 $\pm$ 0.19	0.13 $\pm$ 1.12
TNBC	0.78 $\pm$ 0.39	0.21 $\pm$ 0.08	0.18 $\pm$ 0.05	0.28 $\pm$ 0.09	0.43 $\pm$ 0.12	0.20 $\pm$ 0.05	0.18 $\pm$ 0.04	1.27 $\pm$ 0.26
P	0.9277	0.9644	0.8305	0.8002	0.5615	0.5125	0.4654	0.3829

TG, treatment-exposed; NG, newly diagnosed; IHC, immunohistochemical; TNBC, triple-negative breast cancer. Bold, $p < 0.05$ (unadjusted). Different superscript letters in a block indicate post-hoc differences. Dashes indicate no comparison.

Several categories comprised one or two participants, and the NG subgroup recorded as receiving treatment was very small.

Between-group expression and the RB1-E2F pathway

Within the cyclin-CDK-RB-E2F axis, pRB functions as the gatekeeper of the G1/S transition, and its biological output depends on phosphorylation status, protein stability and interaction with E2F [4, 10, 21]. In tumour tissue, Herschkowitz and colleagues [22] reported low RB1 mRNA in basal-like and luminal B cancers accompanied by frequent RB1 loss of heterozygosity (39% overall, 72% in basal-like tumours); critically, however, that genomic loss did not translate reliably into protein loss on immunohistochemistry. That discordance carries two lessons for the present data. First, transcript abundance can diverge from protein function, so the lower RB1 in treatment-exposed women cannot be taken as evidence of RB inactivation. Second, that evidence base derives from tumour epithelium, whereas the measurements reported here derive from circulating leucocytes, a compartment whose RB1 transcription reflects haematopoietic rather than epithelial biology; the tissue literature therefore cannot be mapped directly onto these findings.

Reduced RB function is an established route to resistance against CDK4/6 inhibitors [11–13], and Rb degradation with sequential E2F activation has been described as a mechanism of escape under CDK4/6 blockade [23, 24]. Those studies, however, concern acquired resistance in largely hormone-receptor-positive disease, demonstrated in cell lines, xenografts or metastatic biopsies under defined CDK4/6-inhibitor regimens [11, 24]; the present cohort was predominantly triple-negative and received heterogeneous, unrecorded treatments, so these mechanisms offer a mechanistic precedent rather than a direct explanation. Notably, the healthy controls occupied an intermediate position between the two patient groups, which argues against a simple mapping of peripheral RB1 transcript onto either disease presence or treatment exposure. The observation that TG and NG were of similar age (47.2 and 46.7 years) further indicates that the headline RB1 difference is unlikely to be a demographic artefact. The cross-sectional design nevertheless leaves treatment effect, treatment selection and underlying disease heterogeneity as competing explanations that cannot be disentangled here.

Menstrual history

Within TG, CCNA2, CCND1 and E2F1 were higher in women still menstruating than in women whose menses had stopped, a pattern with a plausible hormonal substrate. Oestrogen induces CCND1 transcription in oestrogen-receptor-positive breast cancer cells through an ER α -FoxA1 enhancer, and oestrogen-driven cyclin D1 feeds the CDK4/6-RB-E2F axis [5, 7, 25]. A critical reading, however, substantially narrows this interpretation. The enhancer mechanism was defined in ER-positive tumour-cell chromatin [25], whereas the present patients were predominantly triple-negative and the RNA analysed was extracted from blood leucocytes, a compartment in which this regulatory architecture has not been demonstrated. Menstrual status also functions as a proxy for age and menopausal transition, and the patient groups were approximately 12 years older than the controls, so residual age confounding cannot be excluded even within TG. The wide standard errors (for example, CCNA2 1.93 ± 0.57 vs 0.15 ± 0.40) and the complete absence of a comparable association in NG are both more consistent with a context-dependent or chance finding than with a robust hormonal effect. This association is therefore best regarded as hypothesis-generating.

Previous breast procedure

In NG, a previous breast procedure was associated with lower CCND1 and E2F1 expression. The literature reviewed for this study contains no direct comparison of circulating cell-cycle transcripts before and after breast biopsy or surgery, so this finding cannot be benchmarked externally. Two mechanisms merit consideration. Biopsy and surgery provoke tissue injury and an inflammatory response, and inflammatory signalling is a recognised modifier of both tumour biology and the circulating leucocyte transcriptome [2]; alternatively, a procedure may simply mark a more advanced diagnostic pathway, in which clinical rather than biological factors generate the association. The interval between procedure and blood sampling was not recorded, so neither explanation can be tested, and the analysis cannot separate procedure effects from confounding by indication. Because every TG participant had undergone a procedure, no within-TG comparison was possible. The result is hypothesis-generating and requires prospective replication with documented timing.

Treatment status

Within NG, CCND1 was higher in the small subgroup recorded as receiving treatment (0.52 ± 0.14 vs 0.15 ± 0.05). This comparison is analytically fragile. By definition, the NG group comprised newly diagnosed untreated women, so the presence of a treated subgroup indicates ambiguity in the data definition, possibly reflecting women who commenced treatment around the time of sampling. Confounding by indication is therefore unavoidable, and no information on regimen, dose or timing was available. The literature on therapy-induced rewiring of cell-cycle control is instructive but not directly applicable: documented pathway adaptation, including RB-axis changes under CDK4/6 inhibition [10, 23, 24], has been characterised in tumour cells under defined regimens, whereas the present comparison involves an ambiguous exposure measured in blood. The comparison consequently supports no inference about treatment-induced modulation of cyclin D1 and should be treated as hypothesis-generating at most, with prospective recording of regimen and timing in future studies.

Tumour type

E2F1 differed by tumour type within TG, highest in ductal carcinoma (0.78 ± 0.16). E2F1 is overexpressed in breast cancer relative to normal tissue in large transcriptomic datasets [26] and functions dually in proliferation and apoptosis depending on context [7], yet neither body of literature describes histological-type-specific expression, so no external support exists for this association. The internal evidence is weaker still: the taxonomy captured here (ductal, invasive ductal, infiltrative ductal) reflects overlapping pathology labels rather than established WHO categories, and the non-invasive and infiltrative strata contained very few women, meaning one or two observations could account for the entire effect. Worku and colleagues [27], examining primary breast tumours, reported no histological-type difference in E2F1 expression. The association is therefore best treated as a signal for replication rather than as evidence of a tumour-type-specific E2F1 programme.

Clinical stage

RB1 was associated with clinical stage in both patient groups, the most consistent subgroup finding in the dataset. The pattern was non-monotonic: in NG, Stage I was highest, followed by Stage III and then Stage II, while in TG Stage II was highest. The published RB1 literature is organised around molecular subtype and genomic loss [22] rather than stage, and no stage-stratified comparator for circulating RB1 transcripts was identified; for E2F1, Worku and colleagues [27] reported mRNA falling with advancing stage in 127 primary breast cancers, although that trend was not statistically significant, whereas E2F1 showed no stage association here. Several non-exclusive explanations could account for a stage-RB1 relationship in blood, including stage-related differences in leucocyte composition, tumour-burden effects and greater treatment exposure at advanced stages. The Stage I (NG) and Stage IV (TG) categories each contained one or two women Table 1, so the anchor points of the non-monotonic pattern carry little weight. The defensible conclusion is that stage-related heterogeneity in RB1 was detected and merits replication in a stage-balanced design, not that RB1 tracks disease extent. This NG RB1-stage association ($p = 0.0006$) was one of two results that remained below the Bonferroni threshold of approximately 0.0008.

Histological grade

In TG, CCNA2, E2F1 and RB1 were highest in Grade 1 and lower in higher grades, a direction opposite to the expectation that proliferation-associated genes rise with grade, given that cyclin A is associated with aggressive tumour biology and prognosis [8] and that E2F1 transcriptionally regulates CCNA2 in triple-negative breast cancer [28]. The direction is, however, broadly similar to the tumour-tissue data of Worku and colleagues [27], who found E2F1 mRNA lower in higher-grade tumours, albeit non-significantly and with small numbers per grade. That concordance should not be over-weighted: it derives from tumour epithelium under a different measurement and normalisation framework, whereas the present measurements derive from leucocytes. The co-variation of CCNA2 and E2F1 observed here would be compatible with the E2F1-CCNA2 coupling reported in triple-negative disease [28], but that coupling was established in tumour cells, not circulating blood cells. Methodological caution is also warranted: SBR grading is subject to inter-observer variability, the Grade 3 stratum was very small, and the TG E2F1-grade association ($p < 0.0001$), although the only grade result below the Bonferroni threshold, rests on that sparse stratum and therefore requires independent replication before any biological interpretation.

Immunohistochemical subtype and breast density

Neither immunohistochemical subtype nor breast density was associated with the expression of any gene, and these null results should be read as a failure to demonstrate association rather than as evidence of equivalence. The non-triple-negative categories each held one or two women, so the analysis had effectively no power to detect subtype differences, and triple-negative cancer is itself biologically heterogeneous [20]. The Nigerian literature reinforces this caution: reported subtype distributions vary considerably across centres and study designs [15–18], so subtype-transcript relationships observed elsewhere may not be detectable in an unbalanced single-centre cohort. A balanced multicentre cohort is required before concluding that these transcripts lack subtype relevance.

Age

Age category was not associated with the expression of any gene within either patient group. Age nonetheless cannot be excluded as a confounder of the comparisons involving controls, who were approximately 12 years younger, or of menstrual status, which tracks age and menopausal transition; hormonal status is itself mechanistically linked to cyclin D1 regulation [5, 25]. Nigerian series commonly report breast cancer diagnosis in the mid-forties [14–17], and the age structure of the present patient groups is consistent with that pattern, but the control group was not age-matched. This limitation does not affect the TG-NG RB1 comparison, because those two groups were of similar age.

Interpretation of the non-significant between-group findings

The absence of between-group differences in CCNA2, CCND1 and E2F1 should not be read as evidence that these genes are uninvolved in breast cancer biology. Cyclins are regulated at multiple levels beyond steady-state transcript abundance, including gene amplification, protein stability and post-translational modification [5, 8], and CCND1 amplification is associated with clinicopathological features and outcome particularly in oestrogen-receptor-positive disease [29]. E2F1 is overexpressed in breast tumour tissue relative to normal tissue [26], but that evidence derives from tumour mRNA datasets, whereas the present measurements are whole-blood transcripts reflecting a mixed leucocyte population. A stable circulating transcript can therefore coexist with substantial dysregulation within tumour cells; conversely, tumour-derived signals may be diluted below detection in peripheral blood. The between-group null findings are accordingly specific to this biospecimen and cannot be generalised to tumour biology.

Multiplicity and robustness

Sixty subgroup tests produced 12 nominally significant results, whereas approximately three would be expected by chance at $p < 0.05$. Only two results remained below a Bonferroni threshold of approximately 0.0008: TG E2F1 by histological grade ($p < 0.0001$) and NG RB1 by clinical stage ($p = 0.0006$). Both are biologically coherent with the RB-E2F literature — the stage association sits at the centre of a pathway whose dysregulation is documented across breast cancer subtypes [4, 21, 22], and the grade association echoes the E2F1-CCNA2 axis described in triple-negative disease [28] — yet both depend on sparsely populated strata and therefore require replication. The remaining associations, including menstrual history, previous breast procedure, treatment status and tumour type, should be regarded as exploratory.

Methodological considerations and strengths

Several features strengthen the study. Three equal-sized groups permitted direct comparison; four genes from a single regulatory pathway were assessed together rather than in isolation; the endogenous reference gene was empirically verified as stable across groups [19]; and gene-specific healthy-control calibrators anchored the $2^{-\Delta\Delta C_t}$ calculations. Blood-based molecular sampling is increasingly explored as a less invasive alternative to tissue sampling across disease settings [30], although the present study does not constitute a validated liquid-biopsy assay. Quantification followed the comparative $2^{-\Delta\Delta C_t}$ framework, and current MIQE guidance emphasises efficiency assessment and validated normalisation [19]; because amplicon sizes and amplification efficiencies were not available, the assumption of comparable efficiencies between target and reference could not be formally verified, which remains a residual analytical uncertainty.

Limitations

The design imposes the principal constraints. The cross-sectional, single-centre, purposively sampled framework precludes causal inference, limits generalisability, and leaves treatment effect, treatment selection and disease heterogeneity entangled within the TG-NG RB1 difference; treatment regimen, dose and timing were not recorded, and cases were approximately 12 years older than the controls. The biospecimen imposes further boundaries: whole-blood transcripts do not measure tumour-tissue expression, total or phosphorylated pRB protein, or RB1 genomic status, all of which are required to infer pathway activity [22, 26]. Analytically, 60 subgroup comparisons were conducted without multiplicity correction, several categories contained one or two participants, and the definition of the small NG subgroup recorded as receiving treatment is ambiguous. The cohort's subtype imbalance, with triple-negative disease predominating, mirrored but also limited the subtype comparison [15–18, 20], and the unavailability of amplicon sizes and amplification efficiencies falls short of full MIQE compliance [19].

Recommendations

Future work should pair longitudinal blood sampling with tumour tissue obtained at the same time points, and should measure RB1 concurrently at transcript, protein (total and phosphorylated pRB) and genomic levels to resolve the transcript-function discordance documented in tissue studies [22]. Treatment regimen, dose and timing should be recorded prospectively so that therapy-related pathway adaptation can be examined within a defined treatment context [10, 23, 24].

5. Conclusion

CCNA2, CCND1 and E2F1 did not differ among treatment-exposed, newly diagnosed and healthy women, whereas RB1 was lower in treatment-exposed than newly diagnosed women, a difference not explained by age. Within the cancer groups, expression was unrelated to age, breast density and immunohistochemical subtype. Nominal associations with menstrual history, previous breast procedure, treatment status, tumour type, stage and grade are exploratory; only the NG RB1-stage and TG E2F1-grade associations remained significant after Bonferroni correction, and both require replication. Confirmation requires larger, longitudinal studies with paired tumour tissue and functional protein measurement.

Article Information

Ethical Approval: In line with the Helsinki Declaration, approval was obtained from the UPTH Research Ethics Committee (UPTH/ADM/90/SII/VOL.XI/1235).

Informed Consent: Written informed consent was obtained from all participants, and confidentiality was maintained using identification codes.

Consent for Publication: Written informed consent was obtained from the participant(s) for publication of the relevant information.

Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Clinical Trial Registration: Not applicable.

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: No external funding was received for this study.

Author Contributions: M.I.J. - Writing – Original Draft, Conceptualization, Methodology; E.S.B. - Writing – Review & editing, Supervision; E.M.E. - Writing – Review & editing, Supervision; Z.A.J. - Writing – Review & editing, Supervision.

Acknowledgments: The authors have no acknowledgments to declare.

AI Use Disclosure: No generative artificial intelligence tools were used in the preparation of this manuscript.

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