

## Time will tell: Circadian clock dysregulation in triple negative breast cancer

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### TABLE OF CONTENTS

1. Abstract
2. Introduction
  - 2.1. Circadian Rhythms and physiological regulation
  - 2.2. Circadian rhythms, shift work, and racial disparity in breast cancer
  - 2.3. Triple-Negative Breast Cancer: features and molecular subtypes
  - 2.4. Around the clock: Molecular mechanism of the circadian clock
  - 2.5. Cell cycle regulation by circadian clock genes and breast cancer
3. Circadian clock genes show distinct patterns of mis-expression in TNBC
4. Some circadian clock genes are prognostic in TNBC
5. TNBC molecular subtypes exhibit distinct patterns of clock gene expression
6. Looking ahead in the nick of time
7. References

### 1. ABSTRACT

Growing evidence now links circadian disruption (CD) to increased risk of developing multiple types of cancer, including breast cancer (BC). In the US, African-American (AA) BC patients have a higher mortality rate than European-Americans (EAs) with BC, and a prime suspect in this racially disparate burden has been the greater incidence of an aggressive and highly heterogeneous BC subtype called triple-negative BC (TNBC), among AAs. AAs are also more prone to CD as larger proportions of AAs engage in night shift work than EAs, and the chronotype of AAs makes it harder for them to adapt to CD than EAs. Although clock gene dysregulation has been shown to perturb transactivation of key cell cycle and apoptosis regulators, little is known about how clock gene mis-expression affects TNBC outcomes. This review examines the prognostic value of clock genes in TNBC, and evaluates patterns of clock gene dysregulation in the individual TNBC molecular subtypes. Better understanding of how CD contributes to TNBC biology may illuminate new paths to improving disease outcomes and reducing BC-related racial disparities.

### 2. INTRODUCTION

#### 2.1. Circadian Rhythms and physiological regulation

In human beings, the 24-hour day-night periodicity of the circadian rhythm synchronizes internal physiology with visual information about ambient light

(1,2). Light first enters the retinal ganglion cells in the eye; axons of these cells enter the retinohypothalamic tract and carry the light information from the environment to the suprachiasmatic nucleus (SCN) of the hypothalamus (3,4). The sensory information is integrated in the SCN and then sent to the pineal gland that transduces the neural information into the synthesis of an indole amine (N-acetyl-5-methoxytryptamine) hormonal message called melatonin during the dark phase (5,6,7). Melatonin production peaks between 2:00 am and 4:00 am; however, low basal secretion occurs constantly, including during the day (8). By directing periodic production of melatonin, the SCN serves as the central circadian pacemaker that controls peripheral cell autonomous circadian clocks present within each somatic cell (9). The light-dark pattern incident on the retina is the primary synchronizing stimulus that constantly resets the pineal gland's timing of melatonin production to ensure that cellular circadian clocks throughout the body remain attuned to the local environment and oscillate coherently with the SCN pacemaker. If the period of the light-dark pattern is too long or too short, or if the light and dark exposures become aperiodic, the SCN master clock becomes asynchronous with peripheral circadian clocks.

Circadian molecular clocks in each somatic cell operate through an interlocked transcription-translation feedback-based circuitry involving, at minimum, twelve

core “clock” genes. This molecular clock involves the rhythmic production, followed by the tightly controlled degradation, of protein complexes that can negatively affect their own production (10,11). The circadian clock directly influences expression of ~10-15% of all mammalian genes (12). Even though circadian clock genes are expressed ubiquitously, they regulate gene expression in a tissue-specific and heterogenous manner (13). Maintaining the synchrony between cellular pathways and the ambient light is crucial for coordinated functions throughout the human body; the human body is a multi-oscillator system, wherein circadian rhythms regulate numerous body functions including sleep, blood pressure, core temperature, production of hormones, and immune activity (14). At a cellular level, circadian oscillations also direct several key processes such as mitosis and DNA damage response (15,16). Circadian disturbance results in severe dysfunctions and pathologies, including disruption in cellular proliferation and tumor suppression. More recently, a growing body of evidence has linked (a) cancer to circadian disruption in peripheral tissues, and (b) the efficacy of anti-cancer treatments to the intactness of the circadian molecular clock (14,17).

## 2.2. Circadian rhythms, shift work, and racial disparity in breast cancer

Circadian disruption (CD) results from environmental disruptions in one's sleeping schedule such as jet lag and mistimed sleep from late-night shift work, and/or genetic mutations that can also throw the circadian cycle off kilter; these perturbations result in an abnormally low level of serum melatonin. Conditions which favor melatonin suppression by light during the night, resulting in CD, are known to be a possible cause underlying several health conditions, including hypertension (18), cardiovascular disease (19), and several types of cancer (20), including breast cancer or BC (21,22). Elevated BC risk also occurs in women exposed to high levels of ambient light at night (23). In fact, the World Health Organization and International Agency for Research on Cancer (IARC) have long identified rotating-shift work as a probable cause of cancer (24,25). Irregular shift work refers to working a mix of nights and days during a week. In these individuals, the circadian clock has too little time to entrain to the new shift pattern before it is changed again. Epidemiological studies have shown that nurses who work on rotating-shifts and thus experience a lack of synchrony between their activity-rest patterns and light-dark cycles are at higher risk of developing BC compared to day-shift nurses. According to a longitudinal study, nurses who engaged in night-shift work for 1-29 years displayed an 8% increase in relative risk of BC, and nurses who worked the night shift for over 30 years showed a 36% increase (26). One study found this increase to occur predominantly in a particularly aggressive form of BC called triple negative BC or TNBC (27).

In the US, African Americans (AAs) work more non-standard shifts than European Americans (EAs) do after adjusting for occupation, and this difference is expected to grow in the future (28). Despite similar BC incidence rates among AAs and EAs, AA women with BC have a 40% higher mortality rate than EAs with the disease, and one reason for this racially disparate burden is that AA women are 2-3X more likely than EA women to get the aggressive BC subtype called TNBC (29). Many studies also report that AA women with TNBC have poorer prognosis than EA TNBC patients and the etiology of this disparity is likely to be multifactorial. Studies also demonstrate that the free-running circadian period in AAs is significantly shorter than in EAs (30,31). A longer free-running circadian period is associated with faster adjustment to CD, which means that EAs adjust to CD more easily than AAs do. The staggeringly high numbers of AA women performing shift-work suggests that CD could be a significant contributor to BC-related racial disparity in the US.

## 2.3. Triple-Negative Breast Cancer: features and molecular subtypes

TNBC is a BC subtype characterized by the absence of the three most commonly assessed drug targets, namely, the estrogen (ER), progesterone (PR), and human epidermal growth factor (Her2) receptors. 15-20% of all BCs are TNBCs (32). TNBCs generally have an aggressive clinical course and display high rates of visceral and brain metastases (32). In cases where long-range metastasis occurs, the survival rate for TNBC patients is extremely low (33). It is now becoming well appreciated that TNBC, which continues to be defined by the biomarkers it lacks, is not one disease but a constellation of molecularly, morphologically, and behaviorally diverse entities. In 2011, Lehmann *et al.* categorized TNBC into six distinct molecular subtypes, as well as one unstable subtype (34). These included two basal-like subtypes (BL1 and BL2), one immunomodulatory class (IM), one mesenchymal class (M), one mesenchymal stem cell class (MSC), and one luminal androgen receptor (LAR) class. Importantly, AA TNBCs have approximately six times higher odds of belonging to one of the two basal-like molecular subtypes compared to EA TNBCs (35,36). However, in 2015, Burstein *et al.* reported testing 198 previously uncharacterized TNBC cases using mRNA expression and DNA profiling, and subsequently identified four stable molecular subtypes (two of which are in common with Lehmann *et al.*): Luminal Androgen Receptor (LAR), mesenchymal (MES), basal-like immunosuppressed (BLIS), and basal-like immune activated (BLIA). Moreover, these subtypes were identified based on genes/biomarkers that were expressed in each subtype (as opposed to the biomarkers that were not expressed), with the hope of identifying therapeutically actionable pathways in each molecular subtype (37). Of these prognostically-

distinct subtypes, BLIA tends to have the best prognosis, whereas BLIS is known to have the poorest prognosis. LAR and MES tumors tend to downregulate cell-cycle regulators and DNA repair genes, whereas MES and BLIA tumors upregulate immune signaling and immune-related death pathways; in addition to this, BLIS and BLIA have been shown to lack p53-dependent gene activation, and BLIA tumors express STAT genes very highly (37). Given this staggering genomic and transcriptomic heterogeneity of TNBC, these TNBC molecular subtypes can be construed as distinct disease entities whose treatments must necessarily be tailored to their unique biology to improve patient outcomes.

Given (a) the large proportion of shift workers that are AA, (b) the fact that the chronotype of AAs makes it harder for AAs compared to EAs to adapt to CD, (c) that AAs are much more strongly predisposed to developing TNBC than EAs, and (d) AA TNBCs suffer worse outcomes than EA TNBCs, it is reasonable to hypothesize that CD may be a risk factor for TNBC and/or for poor prognosis among AA TNBC patients. This review therefore investigates if circadian clock genes have any prognostic role in TNBC and examines the patterns of circadian clock gene dysregulation among the four TNBC molecular subtypes defined by Burstein *et al.* Thus, we aim to cast light upon the how clock gene mis-expression may shape the biology of TNBC and contribute to racial disparity in BC outcomes. Ultimately, a better understanding of these nuances may pave the way for development of improved and customized treatment of the individual TNBC molecular subtypes.

#### 2.4. Around the clock: Molecular mechanism of the circadian clock

Both central (SCN) and peripheral (in each somatic cell) cellular circadian clocks are composed of core transcription factors that regulate the temporal expression of clock-controlled genes (14). In particular, the basic helix-loop-helix/PAS domain-containing transcription factors (CLOCK, ARNTL/BMAL1, ARNTL2/BMAL2 AND NPAS2), activate transcription of downstream periodic genes (including PER1, PER2 and PER3) and cryptochrome genes (including CRY1 and CRY2). To start off the primary cycle, CLOCK (Circadian Locomotor Output Cycles Kaput) forms a heterodimer with ARNTL/BMAL1 (Brain and Muscle ARNT-Like 1) or ARNTL2/BMAL2; this dimer binds to the E-box sequences in the promoters of target genes (PER1-3, CRY1-2) and induces their transcription (38). NPAS2 also heterodimerizes with ARNTL/BMAL1 and activates transcription of target genes by binding to their E-box elements (39). After translation, PER and CRY proteins translocate into the nucleus and exist in a dynamic equilibrium with PER-CRY complexes; solitary CRY and PER proteins as well as the CRY-PER

complexes inhibit NPAS2/CLOCK-ARNTL/BMAL1 mediated transcription through multiple mechanisms (40), thereby closing the negative feedback loop. This feedback circuitry results in a cycle that runs over a 24-hour period in which the activity of transcription factors such as CLOCK/NPAS2 and ARNTL/BMAL1 peaks during the day and the transcription inhibitory activity of CRY and PER proteins is highest at night (41).

The protein levels of CRYs and PERs are, in turn, strictly regulated by posttranslational modifications such as phosphorylation and ubiquitination (42,43,44,45). CRY and PER proteins are phosphorylated by kinases such as CSNK1D and CSNK1E which trigger their degradation unless they are in the protein complex; by doing so, CSNK1D/CSNK1E primarily regulate the accumulating phase of the PER-CRY repressive complex. An F-box ubiquitin E3 ligase, FBXL3, ubiquitinates CRY1 and CRY2, leading to their proteasomal degradation (46,47,48). FBXL21, the closest paralog of FBXL3, also ubiquitinates CRY proteins but stabilizes them by doing so (49,50); it appears that FBXL21 antagonizes FBXL3, though the mechanism through which this occurs is currently unclear. Ubiquitin-specific protease 7 (USP7) is a USP-family de-ubiquitinating enzyme that stabilizes CRY proteins, thus regulating circadian oscillation (51). TarDNA protein 43 (TDP43) is an RNA-binding protein responsible for mRNA metabolism, but studies have uncovered that it also plays a role in stabilizing CRY proteins by interfering with FBXL3 function (49). Thus, the protein network regulating the stability of CRYs plays a crucial role in determining the period of the circadian clock.

The basic helix-loop-helix transcription factors BHLHE40/DEC1 and BHLHE41/DEC2 repress transcription of PER genes (52). They interfere with the activity of the CLOCK-ARNTL/BMAL1 complex, although how they do this is not clear. It is currently unknown whether BHLHE40/41 proteins repress other genes such as CRY, but studies have demonstrated that the transcription of BHLHE40 and BHLHE41 is activated by CLOCK and ARNTL/BMAL1 (52).

Other components involved in regulating the core feedback loop mechanism include the secondary feedback loops of the Retinoic acid receptor-related Orphan Receptors (RORA, RORB, RORC), that stimulate ARNTL/BMAL1 transcription, and the REVERBs (REVERB-alpha/NR1D1, REVERB-beta/NR1D2) that suppress ARNTL/BMAL1 transcription (53,54,55,56,57,58,59). Interestingly, RORA and REVERB-alpha also regulate the expression of NPAS2 (60). NFIL3 (Nuclear Factor Interleukin 3 regulated) is a transcriptional regulator that binds as a homodimer to activating transcription factor (ATF) sites in promoters of PER1 and PER2 and represses their expression. NFIL3 repressor acts in an anti-phase manner with

respect to DBP (a transcriptional activator named for the albumin gene D-site binding protein). DBP belongs to the PAR (proline and acidic amino acid-rich) basic leucine zipper transcription factor family (61). NFIL3 and DBP compete for access to D box elements, exerting opposite effects on target genes. Thus, NFIL3, when abundant, suppresses the transcription of target genes, while DBP activates them at a different time of day. Transcription of DBP is activated by CLOCK/BMAL1, and suppressed by PER and CRY proteins through E-box motifs located in the introns of the DBP gene (62,63).

Another component of the circadian clock mechanism is TIMELESS (TIM), which helps stabilize replication forks during DNA replication and plays critical roles in DNA damage repair, activation of the S phase checkpoint, proper establishment of sister chromatid cohesion and coordination of mitotic kinase activation with termination of DNA replication. Although the precise molecular role of TIM in the mammalian circadian clock is unclear, TIM's function is essential for maintenance of circadian rhythms (64). The N-terminus of TIM interacts with CRY1 and CHK1 and plays a role in homodimerization, and the C-terminus is necessary for TIM's nuclear localization. Evidence suggests that PER2 competes with CRY1 to bind to TIM; this dynamic interaction between TIM, CRY1 and PER2 appears to be important for the clock's pace and adaption to external stimuli, such as DNA damage and/or light.

## 2.5. Cell cycle regulation by circadian clock genes and breast cancer

Circadian clock components influence cell growth and transformation in a cell-autonomous manner. Furthermore, clock proteins participate in sensing DNA damage, modulating DNA repair, and influencing the ubiquitination and degradation of key oncoproteins and tumor suppressors. For example, circadian clock genes regulate the rhythmic transcription of many cell cycle genes including c-MYC (G0/G1 transition), P21, CYCLIN D1 (G1/S transition), GADD45, CYCLIN B1 and WEE1 (G2/M transition), and thus play key roles in tumorigenesis. PER2 functions as a tumor suppressor as evidenced by PER2 mutations that result in neoplastic growth and radiation-induced DNA damage (14). Several genes involved in cell cycle regulation and tumor suppression are deregulated in PER2-deficient mice, including CYCLIN D1, CYCLIN A, and MDM2 (65,66). PER and CRY proteins modulate post-translational regulation of P53 and c-MYC; PER2 blocks MDM2-mediated ubiquitination of P53, while CRY2 stimulates ubiquitination of c-MYC by SCF (FBXL3). USP7 removes polyubiquitin chains from CRY1 as well as from P53. Like PER2, PER1 also has tumor suppressor properties (67). PER1 influences the transcription of WEE1 and CYCLIN

B1 by a p53-dependent mechanism and of p21 via a p53-independent pathway, possibly by stabilizing c-MYC (66). Both PER1 and PER2 also promote apoptosis and ectopic PER2 expression in cancer cells leads to growth inhibition, cell cycle arrest and apoptosis (66,68). Per1 may also play an important role in regulating DNA damage response because it activates the ATM checkpoint pathway (69). Mutations in PER1 and PER2 have also been found in hormone responsive cancer, particularly BC (70,67). CRY proteins regulate the G1/S phase transition checkpoint through their interactions with TIM. These interactions are dependent on the time of day and respond to changes in ambient light. CRY1 and CRY2 evolved from bacterial UV-activated DNA repair enzymes, and several studies suggest that they retain a functional role in genome protection (71,72). PER genes and TIM also modulate the G2/M phase transition (73,74). TIM upregulates the expression and transcription regulatory activities of c-MYC (75). Importantly, PER1 interacts with checkpoint proteins ATM and CHK2, whereas TIM interacts with both CRY1 and cell cycle checkpoint proteins CHK1 and the ATR-ATRIP complex (73,76). Thus, the circadian clock is intimately coupled to the cell cycle.

Disruption of the circadian rhythm has been strongly linked to increased risk of BC in women. Studies have shown that over 590 genes show circadian expression (~24 hour period) in the epithelium of the mouse mammary gland (77). Thus, CD could potentially impact numerous cellular pathways. In women, melatonin exerts strong anticancer effects via signaling pathways that control development of normal breast epithelium and BC. Melatonin entrains peripheral clocks in organs that express the receptors melatonin 1 (MT1) and melatonin 2 (MT2) to the SCN (78). Melatonin has oncostatic activity in experimental animals with mammary tumors, and also in cultured human BC cells (79). Melatonin also promotes genomic stability and may have anti-metastatic activity (80). Importantly, AA TNBCs have been reported to show a higher incidence of MT1-negative tumors compared to EA TNBCs (81). MT1 positivity in TNBC was associated with a lower stage and a smaller tumor size at time of diagnosis. In multivariable survival analysis, MT1-negative TNBC showed a significantly higher hazard ratio for disease progression, shorter progression-free survival, and shorter overall survival. These results suggest that melatonin or a melatonin receptor agonist may be useful biologic additions in the treatment of TNBC in AAs.

Mis-expression of circadian genes accelerates breast epithelial stem-cell proliferation and increases spontaneous mammary cancer development in rodents (14). In a study looking at the expression of the PER genes in BC cases, PER genes were found to have a differential expression pattern in malignant cells in



96% of the cases, compared with cells from paired normal adjacent tissues (82). Furthermore, differential expression of the PER proteins in ~50% of the cases was explained by methylation of PER gene promoters, specifically PER1 promoter methylation (82). Intratumor heterogeneity in PER gene expression patterns were also found within the BC samples, suggesting rampant asynchronization of circadian clocks within each neoplasm (82). PER 1/2 are estrogen-and androgen-responsive genes suggesting a direct link between the circadian clock and ER/AR signaling pathways in the breast (66). Increased BC risk is also associated with single nucleotide polymorphisms (SNPs) in ARNTL, ARNTL2, NPAS2, CRY1, CRY2, PER1, PER3, TIMELESS and CLOCK (83,84). Interestingly, many ethnic group-specific non-synonymous polymorphisms have been identified in the coding regions of circadian clock proteins which could potentially affect their function in circadian cycle regulation and merit further study (85). Epigenetic regulation of CLOCK, ARNTL, CRY1 and PER1 may also contribute to increased risk of BC in shift workers (86).

In addition to CD being associated with an increased incidence of BC, a key study found that loss of function of certain clock genes is associated with worse prognosis in BC (87). The study also found that coordinated co-expression of clock genes, indicative of a functional circadian clock, was maintained in ER+, HER2-, low-grade and non-metastasizing BCs but was compromised in more aggressive carcinomas, including TNBCs. Loss of expression of the PER3 gene was associated with ER-negativity, high histological grade and higher probability of BC recurrence (88) and the loss of PER3 and CRY2 co-expression was associated with a higher risk of BC metastasis (87). Altered levels of clock proteins may also influence epithelial–mesenchymal transition (EMT) and thereby facilitate invasion and metastasis. For example, loss of PER2 could directly drive EMT through OCT1. Under normal conditions, PER2 recruits transcriptional co-repressors to OCT1-binding promoters of EMT genes *Twist1*, *Slug* and *Snail*. However, PER2 becomes deregulated in hypoxic, tumor-like conditions, allowing EMT gene expression to be activated (89). In sum, dysfunction of key circadian regulators appears to impact BC progression and outcomes.

### 3. Circadian clock genes show distinct patterns of mis-expression in TNBC

Circadian clock gene mis-expression has been widely reported in BCs. For example, clinical data indicate that healthy breast tissues display a lower level of CLOCK gene expression than breast tissue from BC patients (90). Further, CLOCK gene expression is significantly higher in patients with ER-negative BC, than in patients with ER-positive BC (90). Given that higher incidence of TNBC among AAs

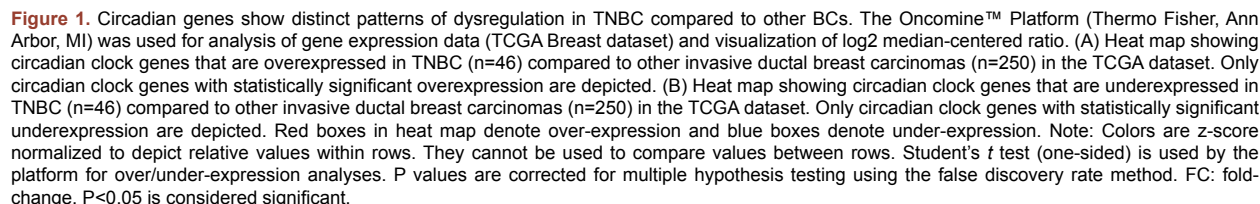
contributes to BC-related racial disparity, we evaluated if clock genes are dysregulated differently in TNBC compared to other BC subtypes. In the TCGA dataset, we found that CLOCK was significantly overexpressed in TNBC patients compared to patients with other BC subtypes (Figure 1A). Reports indicate that NPAS2 (neuronal PAS domain protein 2) overexpression seems to decrease the risk of BC (69) and cells with normal NPAS2 expression are usually found in G1 or G2 phases of the cell cycle when DNA damage repair occurs (91), rather than in S phase. Additionally, when NPAS2 binds to ARNTL/BMAL1, the resulting complex represses the transcription of the oncogene *c-MYC* (65). For this reason, NPAS2 is generally considered a tumor suppressor gene (69). Intriguingly, in the TCGA dataset, NPAS2 was significantly overexpressed in TNBC patients compared to other BC patients (Figure 1A). More research is needed to understand how NPAS2 overexpression impacts TNBC tumor biology.

ARNTL (aryl hydrocarbon receptor nuclear translocator-like) and ARNTL2 encode bHLH transcription factors, which heterodimerize with CLOCK. ARNTL is slightly underexpressed in TNBCs (Figure 1B) compared to other subtypes of BC. ARNTL2 has been identified as a metastasis susceptibility gene associated with progression in ER-negative BC patients (92). In the TCGA dataset, ARNTL2 is significantly overexpressed in TNBC patients compared to patients with other BC subtypes (Figure 1A), with a fold change of 3.18.

According to multiple clinical studies, the expression of PER1-3 (period circadian regulators 1-3) genes, which are believed to be tumor suppressors, is deregulated in BC patients (69). Our analysis uncovered that PER1 is slightly overexpressed in TNBCs (Figure 1A) but PER2 and PER3 are significantly underexpressed in TNBC patients in the TCGA dataset compared to patients with other BC subtypes (Figure 1B).

CRY genes encode flavin adenine dinucleotide-binding proteins (11). CRY2 is generally underexpressed in BC compared to adjacent normal tissues and low CRY2 expression was associated with ER-negative BCs, higher tumor grade and shorter OS in BC patients (93). We found that CRY1 was overexpressed in TNBC patients compared to patients with other BC subtypes (Figure 1A), whereas CRY2 was underexpressed in TNBC patients relative to other BC subtypes (Figure 1B). This finding is consistent with previous reports suggesting that CRY1 and 2 have antagonistic clock-adjusting functions, but more research is needed to understand their individual roles in TNBC (94).

CSNK1D (casein kinase 1 delta) is a conserved serine/threonine kinase that plays key



BHLHE40 (basic helix-loop-helix family member e40) represses activation of Per1 and controls circadian clock cycles as well as cell differentiation. BHLHE40 can negatively affect the

The RAR-related orphan receptors RORA/NR1F1, RORB/NR1F2 and RORC/NR1F3 encode members of the NR1 subfamily of nuclear hormone receptors that regulate transcription of genes involved in circadian rhythms, and cellular differentiation (97,98). Our analysis of the TCGA dataset revealed that RORB and RORC are significantly underexpressed in TNBCs compared to other BC subtypes (Figure 1B).

NFIL3 (nuclear factor interleukin 3-regulated; also commonly known as E4BP4, or E4 promoter-binding protein 4) suppresses the expression of Per1-3 and plays a role in the control of immune responses (55). NFIL3 was significantly overexpressed in TNBC patients compared to patients with other BC subtypes (Figure 1A), with a fold change of 2.64. Since PER genes are tumor suppressors, overexpression of NFIL3 may contribute to cancer growth by suppression

of PER gene expression. DBP and NFIL3 form a reciprocal pair of transcription factors that antagonize each other's expression. Indeed, in contrast to NFIL3, DBP was found to be significantly underexpressed in TNBC patients in the TCGA dataset compared to patients with other BC subtypes (Figure 1B). Thus, TNBCs are characterized by distinct patterns of circadian gene mis-expression compared to other BC subtypes.

#### 4. SOME CIRCADIAN CLOCK GENES ARE PROGNOSTIC IN TNBC

To better understand if expression levels of circadian clock genes have any potential bearing on clinical outcomes of TNBC, we evaluated the prognostic value of clock genes using the Kaplan-Meier Plotter (Breast Cancer) online tool (99). Among the circadian clock genes tested, only 10 genes were significantly prognostic in TNBC patients. The genes whose above-threshold expression was associated with significantly poorer RFS were BHLHE40 (DEC1), CLOCK, CRY1, CSNK1E, NR1D2, and NFIL3 (Figure 2). The genes whose above-threshold expression was associated with significantly better RFS were ARNTL2, CSNK1D, DBP, and TIMELESS (Figure 2). Tumors have been shown to even affect circadian rhythms in distant organs within the patient (100) and cancer patients often report disruption of sleep-wake cycles and other systemic circadian rhythms (101). Therefore, our findings regarding the potential prognostic value of clock genes in TNBC provide impetus to further explore the causes and consequences of intratumoral circadian clock gene dysregulation in TNBC patients.

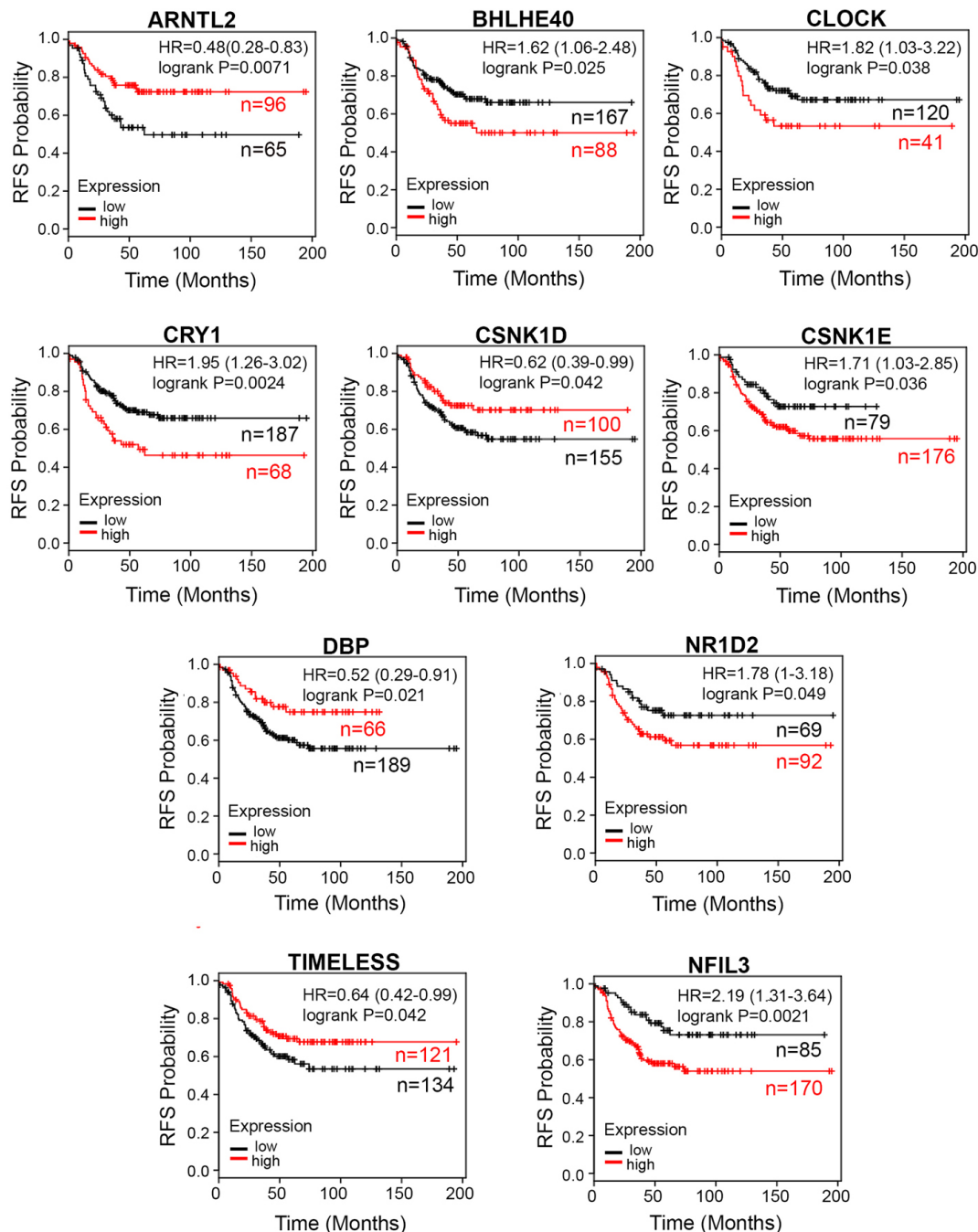
#### 5. TNBC MOLECULAR SUBTYPES EXHIBIT DISTINCT PATTERNS OF CLOCK GENE EXPRESSION

Given the inter-patient transcriptomic heterogeneity of TNBCs and the identification of 4 stable and prognostically-distinct TNBC molecular subtypes (Burststein *et al.*, 2015), we evaluated if the molecular subtypes show differences in the nature and extent of circadian clock dysregulation. Interestingly, we found that the TNBC molecular subtypes differ substantially in their patterns of intratumoral clock gene dysregulation. In the LAR subtype, which is characterized by high levels of Androgen Receptor (AR) signaling, we found overexpression of BHLHE40, RORC, PER2, CLOCK, CRY2 and DBP, and underexpression of NFIL3, ARNTL2, CSNK1E and NR1D2 (Figure 3A). We also found that the expression of BHLHE40 was significantly and positively correlated with expression of AR ( $r=0.23$ ) among TNBCs in general. The expression of RORC, PER2, CLOCK, and CRY2 were similarly correlated positively and significantly with expression

of AR (Figure 3B, top panel), with  $r$  values ranging between 0.2 (CLOCK) and 0.33 (RORC). These data suggest that the expression of BHLHE40, RORC, PER2, CLOCK, and CRY2 may be regulated by AR, and warrant further study. In contrast, NFIL3 and ARNTL2 were moderately and negatively correlated with AR expression ( $r=-0.38$  and  $r=-0.28$ , respectively), again suggesting that AR may regulate the expression of these genes (Figure 3B, bottom panel). In the MES TNBC subtype, we found that RORA, PER3, BHLHE41, CRY2, NFIL3, and PER1 are overexpressed whereas TIMELESS, RORC, and CSNK1D are significantly underexpressed (Figure 3A). In the BLIS subtype (known to have the worst prognosis in TNBC patients), CSNK1E and TIMELESS were heavily overexpressed, whereas BHLHE40 (DEC1), RORA, CRY2, PER1, DBP, CLOCK, PER2, and RORC were underexpressed (Figure 3A). In the BLIA subtype (known to have the best prognosis in TNBC patients), ARNTL2, TIMELESS, NFIL3, and CSNK1D were overexpressed compared to other subtypes, whereas PER2, CRY2, BHLHE41, DBP, and RORA were underexpressed (Figure 3A). In sum, the intratumoral expression of circadian clock genes differs substantially between the TNBC molecular subtypes, and the circadian biology of TNBCs tumors in AAs (who are much more likely than EAs to belong to one of the two basal-like subtypes) are likely to be substantially different from that of EAs.

#### 6. LOOKING AHEAD IN THE NICK OF TIME

The critical importance of good quality, restorative sleep and optimal sleep-wake schedules for human health, have recently come to the fore. In the US, AAs experience a disproportionate risk of both CD and mortality from BC. The etiology of BC-related racial disparities is multifactorial and the weight of evidence implicating CD and exposure to light at night in elevating the risk of developing BC or suffering from poorer outcomes when afflicted with BC, is growing. While CD can lead to deregulated cell division and carcinogenesis, evidence also suggests that malignant transformation may interfere not only with intratumoral molecular clock function but also generate systemic imbalances in circadian rhythms as the tumor grows. As a first step towards uncovering possible links between CD and BC disparities and advancing our understanding of modifiable risk factors from a more holistic biopsychosocial perspective, this review examined (a) clock gene expression specifically in TNBC, (b) associations between clock gene expression and outcomes in TNBC, and (c) differences in clock gene expression among TNBC molecular subtypes. Our data suggest that each TNBC molecular subtype has a unique clock gene fingerprint which could have unique effects on outcomes of those patients. Rigorous longitudinal and population-based studies are needed to better understand how clock

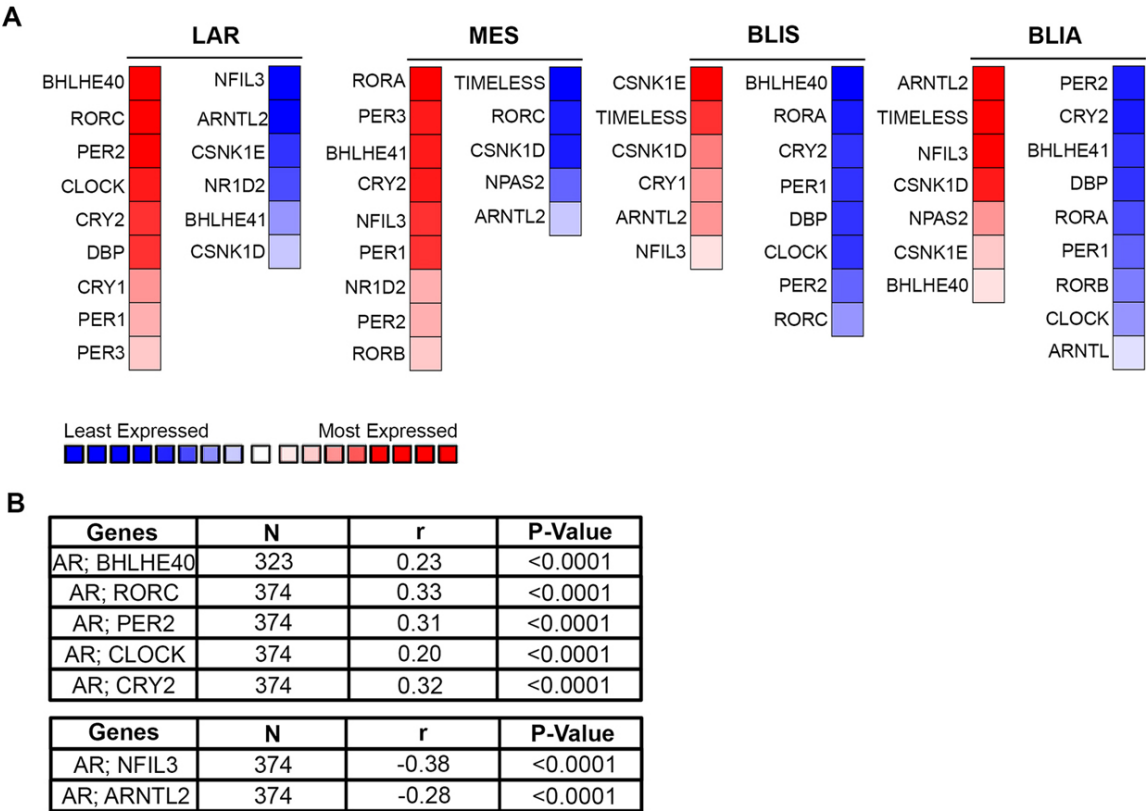


**Figure 2.** Association of circadian clock gene expression with recurrence-free survival in TNBC. The Kaplan-Meier Plotter (Breast Cancer) online tool was used for analysis and visualization. Logrank test was used to assess significance of survival differences between groups of TNBC patients that show high vs. low expression of the circadian clock genes. KM curves are only presented for those circadian genes with logrank  $p < 0.05$ . High expression of ARNTL2 (223586\_at), CSNK1D (208774\_at), DBP (201413\_at), TIMELESS (215455\_at) are associated with longer RFS, whereas high BHLHE40 (201170\_s\_at), CLOCK (227531\_at), CRY1 (209674\_at), CSNK1E (223332\_at), NR1D2 (225768\_at) and NFIL3 (203574\_at) expression are associated with shorter RFS.

gene mis-expression affects the biology of individual TNBC molecular subtypes. Given our large knowledge gaps in this domain, more multi-disciplinary studies are needed to objectively and comprehensively assess if and how CD may be an upstream risk factor for TNBC

in different racial groups, and whether CD affects treatment responses and outcomes in racially-distinct TNBC patients. Research into the intricate connections between intratumoral clock gene expression, tumor biology, immune/cellular/endocrine function, sleep





**Figure 3.** TNBCs show subtype-specific differences in circadian gene expression. (A) Differentially expressed genes in the 4 TNBC molecular subtypes (identified by Burstein *et al.*, 2015) in the Burstein dataset were analyzed to determine the percentile ranking (among 18,209 genes) of circadian clock genes (the combined rank of the discovery and validation set clusters was used). Dark red denotes that the gene's expression level lies within the top 10th percentile of dysregulated genes in the indicated molecular subtype, and that the gene is overexpressed in the indicated subtype relative to its expression in other subtypes. Dark blue denotes that the gene's expression level lies within the top 10th percentile of dysregulated genes in the indicated molecular subtype, and that the gene is underexpressed in the indicated subtype relative to the other subtypes. (B) To examine Pearson's pairwise correlations between expression of the gene encoding Androgen Receptor (AR) and circadian clock genes among BC cases defined as TNBC by immunohistochemistry, the "Targeted Correlation Module" in the bc-GenExMiner v3.0 (Breast Cancer Gene-Expression Miner v3.0) online dataset (<http://bcgenex.centregauducheau.fr>) was used. Only statistically significant pairwise correlations ( $P < 0.05$ ) are shown. LAR: Luminal Androgen Receptor; MES: Mesenchymal; BLIS: Basal-like Immunosuppressed; BLIA: Basal-like Immune-Activated.

deficiencies and responses to treatment, could enable identification of new opportunities for BC prevention, new targets for intervention and BC treatment, and perhaps even individualized chronotherapy.

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