Assessment of cell cycle regulators in human peripheral blood cells as markers of cellular senescence

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1. Introduction

Aging is the result of a gradual functional decline at the cellular and organismal level, resulting in the development of chronic age-related diseases such as cancer, diabetes, heart disease and stroke (Partridge et al., 2020). Hayflick and Moorhead (1961) first described aging at the cellular level by demonstrating that mammalian somatic cells have a finite number of cell divisions resulting in stable growth arrest termed cellular senescence (Hayflick and Moorhead, 1961). The number of senescent cells in vivo is higher in individuals being chronologically older (Tuttle et al., 2020) and biologically older (Waaijer et al., 2012), and suffering from age-related diseases (Tuttle et al., 2021) compared to younger and healthy individuals.

Cellular senescence has been associated with the up-regulation of well-established tumor suppressors and cell cycle regulators p53, p21CIP1/WAF1 (p21) and p16INK4a (p16) (Munoz-Espin and Serrano, 2012). Senescent cells have frequently been studied in the peripheral blood of humans due to its accessibility. This review summarizes ex vivo studies describing cell cycle regulators as markers of senescence in human peripheral blood cells, along with detection methodologies and associative studies examining demographic and clinical characteristics. The utility of techniques such as the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), microarray, RNA sequencing and nCounter technologies for detection at the transcriptional level, along with Western blotting, enzyme-linked immunosorbent assay and flow cytometry at the translational level, will be brought up at salient points throughout this review. Notably, housekeeping genes or proteins serving as controls such as GAPDH and β-Actin, were found not to be stably expressed in some contexts. As such, optimization and validation of such genes during experimental design were recommended. In addition, the expression of cell cycle regulators was found to vary not only between different types of blood cells such as T cells and B cells but also between stages of cellular differentiation such as naïve T cells and highly differentiated T cells. On the other hand, the associations of the presence of cell cycle regulators with demographics (age, gender, ethnicity, and socioeconomic status), clinical characteristics (body mass index, specific diseases, disease-related parameters) and lifestyle vary in groups of participants. One envisions that increased understanding and insights into the assessment of cell cycle regulators as markers of senescence in human peripheral blood cells will help inform prognostication and clinical intervention in elderly individuals.
The master cell cycle regulator p53, a transcription factor, primarily mediates genomic damage-induced senescence which can be triggered by telomere erosion (Herbig et al., 2004; Saretzki et al., 1999), ionizing radiation (Jones et al., 2005) and chemotherapeutic drugs (Ewald et al., 2010). One of the transcription targets of p53, p21 (Di Leonardo et al., 1994; Fischer, 2017; Jackson and Pereira-Smith, 2006), inhibits cyclin-dependent kinases (CDKs) to maintain the retinoblastoma protein (pRB) in a hypophosphorylated and inactivated state, thereby halting cell cycle progression (Sherr et al., 1999). Interestingly, p21 itself is capable of inducing senescence in a p53 independent manner (Mcip et al., 2002). The cell cycle regulator p16 originating from the INK4a/ARF locus is another upstream regulator of pRB (Munoz-Espin and Serrano, 2014), which mainly mediates cellular senescence induced via hypermitogenic (Larsson, 2011), oxidative (Ksiazek et al., 2006; Parrinello et al., 2003) and endoplasmic reticulum (ER) stressors (Liu et al., 2015). While the p53/p21 pathway is important for senescence initiation, the p16 pathway is additionally associated with maintenance of the senescence-enforced cell cycle arrest (Kailman et al., 2010; Rayes et al., 2012).

Since senescent cells are terminally growth-arrested, cell cycle regulators, such as p53, p16 and p21, have been routinely employed in the detection of senescence. Senescent cells characterized by these cell cycle regulators are more prevalent in pathological tissue samples from humans and animals with higher chronological age (Tuttle et al., 2020). Compared to tissue samples, the utility of peripheral blood is valuable in research into human aging and age-related diseases (Childs et al., 2015; Tuttle et al., 2021). A variety of molecular techniques have been established to detect genes at the transcriptional level, such as quantitative reverse transcription-polymerase chain reaction (qRT-PCR), DNA microarrays and ribonucleic acid (RNA) sequencing, as well as at the translational level, such as Western blotting (WB), enzyme-linked immunosorbent assay (ELISA) and flow cytometry.

In this review, we summarize methods used to detect cell cycle regulators as senescence biomarkers in human peripheral blood cells and the associations of these cell cycle regulators with demographic, clinical characteristics and lifestyle in human participants. We focus on ex vivo studies that demonstrate direct detection of gene and protein expression of cell cycle regulators in human peripheral blood cells without any interventions including cell culture.

### 2. Detection methods of cell cycle regulators in human peripheral blood cells

In this section, we highlight a few methodologies important for

| Technique | Subtype | Pros | Cons | Cell cycle regulator detected
|---|---|---|---|---|
| Transcriptional level | qRT-PCR | -Dye | Easier to conduct; low cost; fast | Detect a limited number of transcripts at a time; susceptible to contamination of DNA; no standard protocol across labs | p16, p53, p21, p14 |
| | DNA Microarray | -One channel | Detect thousands of genes simultaneously | Cross-hybridization; non-specific hybridization; high variance of low expressed genes; limited known probes; cannot detect structural variations or isoforms | p16, p53 |
| | RNA sequencing | -Multiple channels | Sensitive; not require prior sequence knowledge; able to investigate novel genes or structural variations | Expensive; complex data analysis; time-consuming | p16 |
| | nCounter | | | | |
| | Northern blot | | | | |
| | ELISA | -RPA | Tolerate partially degraded RNA; distinguish transcripts from a multi-gene family | Incomplete nuclease digestion leads to excessive background | |
| | Protein array | -FPPA | Provide visual information about location and abundance of mRNA in a cell; no RNA isolation or electrophoretic separation required | Limited known probes | |
| Translational level | Western blotting | -One-step probing | Widely accepted; separate proteins based on molecular weight | Not amenable to a large number of samples; time-consuming | p16, p53, p21, p38 |
| | ELISA | -Protein-probing | Fast; high throughput; high specificity | Short-time readout; false positive/negative results might occur due to insufficient blocking of untargeted antigen | p53, p21 |
| | Flow cytometry | -mRNA | Detect proteins or mRNA in situ at the single cell level; no protein or RNA isolation required; detect multiple proteins or mRNA simultaneously | Costly; extensive optimization of the protocol | p16, p53, p21, p38, p27 |
| | ICC | -mRNA | Provide visual information about location and abundance of protein in a cell | Limited options of direct fluorescence-labeled primary antibody on the market; non-specific binding of secondary antibody leads to background signal | |
| | Protein array | -FPPA | FPPA-detect multiple proteins in one sample; RPPA-available for a large number of samples | Validation of highly specific antibody required before analysis to avoid cross-reactivity | |
| | MS-based proteomics | -2-DE | Detect thousands of proteins simultaneously; no antibody binding required | Complicated sample preparation | |
| Abbreviations: qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction; FISH: fluorescence in situ hybridization; NPA: Nuclease protection assay; RPA: Ribonuclease protection assay; SNA: S1 nuclease assay; ELISA: Enzyme linked immunosorbent assay; ICC: Immunocytochemistry; FPPA: Forward phase protein array; RPPA: Reverse phase protein array; MS: Mass spectrometry; 2-DE: Two-dimensional electrophoresis; ICAT: Isotope coded S1 nuclease assays affinity tag. |
discussion regarding detection utility as well as shared versus idiosyncratic suitability of detection methods within various biological contexts. Facile access to blood sampling from human participants presents peripheral blood cells as a reliable specimen of choice for the assessment of cell cycle regulators. Indeed, cell cycle regulators have been measured in human peripheral blood via a myriad of established molecular techniques. Table 1 and Fig. 1 present an overview of available techniques that were used to detect genes at the transcriptional and translational levels, respective advantages and disadvantages, as well as their utility in conjunction with cell cycle regulators as senescence markers in human peripheral blood cells.

A blood sample consists of several cell types with each having its own genetic profile (Palmer et al., 2006) and requiring proper processing (Betsou et al., 2019). Analyzing the whole blood sample can lead to a heterogeneous senescence profile as the expression of cell cycle regulators varies not just between blood cell populations but also at different cell differentiation stages (Frasca et al., 2017; Liu et al., 2009; Onyema et al., 2012, 2015). Peripheral blood mononuclear cells (PBMCs), T lymphocytes, B lymphocytes, monocytes, natural killer (NK) cells, granulocytes from healthy adults were shown to have different levels of p16 mRNA, with higher expression in T cells than in other blood cell populations (Liu et al., 2009). Higher p16 and p21 protein expressions were observed in later differentiated T cells (Onyema et al., 2012, 2015) and B cells (Frasca et al., 2017) compared to naïve T and B cells. For visualization of expression of cell cycle regulators of non-adherent cells, such as granulocytes and lymphocytes, cytocentrifuge or gravity sedimentation should be performed to enrich cells on microscope slides or coverslips for subsequent use in immunostaining (Martinez-Zamudio et al., 2021; Tan et al., 2012; Tsang et al., 2017).

The techniques qRT-PCR, microarray, RNA sequencing and nCounter technology have been used to detect cell cycle regulators in human peripheral blood cells at the transcriptional level (Table 2). Although microarray, RNA sequencing and nCounter technology are more sensitive than qRT-PCR, the qRT-PCR is the most widely used method in detecting cell cycle regulators in blood cells with the advantages of lower cost, faster analysis, easier operation and application in a larger sample size (up to 384 wells per assay) in comparison with the other three techniques (Narrandes and Xu, 2018). A smaller number of participants could be analyzed by microarray (up to 12 samples per assay) and RNA sequencing (up to 96 samples per assay). Microarray and RNA sequencing techniques are more suitable for genome-wide transcriptome profiling and identifying differentially expressed genes (van der Kloet et al., 2020). The qRT-PCR is likely to be used to confirm results obtained from microarray and RNA sequencing (Coenye, 2021). However, qRT-PCR, microarray and RNA sequencing techniques make use of PCR-based methodologies to generate cDNA or cRNA samples,
Table 2
Articles describing cell cycle regulators detected at the transcriptional level in human peripheral blood cells and characteristics of participants, stratified by methods.

| First author (year) | Population | (F/) N# | Age, years | Cell cycle regulator | Cells | Subtype | Housekeeping gene | Quantification | Main findings |
|---------------------|------------|---------|------------|----------------------|-------|---------|------------------|----------------|---------------|
| qRT-PCR Maas (2005) | Healthy control | 4a | 22–55 | p53 | PBMCs | SYBR | GAPDH | NR | p53↓ in rheumatoid arthritis |
| | Rheumatoid arthritis | 5a | 22–55 | | | | | | |
| Damjanac (2009) | Healthy control | 39a | 80 (66–90) | p53 | PBMCs | GoTaq Flexi | GAPDH | Gel | p53↓ in Alzheimer’s disease |
| | Alzheimer’s disease | 46a | 83 (63–93) | | | | | | |
| Liu (2009) | Healthy people-exploration | 42/ 80 | 47 (18–76) | p16, p14 | T cells | TaqMan | 18 S rRNA | β2-M Log2 | p16↑ with age/smoking/less exercise, p14 NC with age |
| | Healthy people-validation | 64/ 90 | 43 (18–80) | p53 | T subsets | Amplify in PCR machine | 18 S rRNA | cDNA copy number | p53↓ in CD4+CD45RO- and CD45RA- T from rheumatoid arthritis |
| Shao (2009) | Healthy control | 27a | 46.1 ± 12.8 | p53 | T subsets | Amplify in PCR machine | 18 S rRNA | cDNA copy number | p53↓ in CD4+CD45RO- and CD45RA- T from rheumatoid arthritis |
| | Rheumatoid arthritis | 27a | 48.6 ± 11.6 | | | | | | |
| Song (2010) | Healthy control | 86/ 136 | 45.2 (18–80) | p16 | T cells | TaqMan | 18 S rRNA | β2-M Log2 | p16↓ with age/smoking/less exercise, p16 NC with BMI |
| | Healthy people-validation | 16/ 16 | 37.6 (19–54) | p16 | Leuko | SYBR | GAPDH | 2^-ΔΔCt | p16↑ in HIV+ART- than HIV- or HIV+ART+ |
| Li (2011) | Healthy control | 18/ 54 | 48 (25–68) | p16, p53, p21 | T subsets | TaqMan | β-actin | NR | p16↑, p53/p21 NC in HIV-CD8 T from progressors |
| Lichterfeld (2012) | HIV controllers | 2/11 | 45 (34–64) | p16, p53, p21 | T subsets | TaqMan | β-actin | NR | p16↑, p53/p21 NC in HIV-CD8 T from progressors |
| | HIV progressors | 6/13 | 51.5 (39–72) | | | | | | |
| Nelson (2012) | HIV- | 166 | NR | p16 | T cells | TaqMan | 18 S rRNA | Log2 | p16↑ in HIV+ART- than HIV- or HIV+ART+ |
| | HIV+ART- | 23 | NR | p16 | T cells | SYBR | GAPDH | 2^-ΔΔCt | p16↑ in depression |
| | HIV+ART+ | 16/ 16 | 37.6 (23–45) | p16 | Leuko | SYBR | GAPDH | 2^-ΔΔCt | p16↑ in depression |
| | Healthy control | 17/ 17 | 39.5 (22–54) | | | | | | |
| Mandal (2013) | Healthy control | 6a | 21–74 | Δ133p53, p53β | T subsets | SYBR TaqMan | β2-M | ΔΔCt | Δ133p53 NC, p53β↑ in CD8-CD57+ T cells p16↑ in HIV+ |
| Pathai (2013) | HIV- | 176/ 250 | 40 [35–49] | | | | | | |
| | HIV+ | 190/ 236 | 39 [35–46] | | | | | | |
| | HIV+ART- | 26/ 26 | 49 [32–69] | | | | | | |
| | HIV+ART+ | 176/ 176 | 67 [50–93] | | | | | | |
| Sanoff (2014) | Breast cancer on CT | 49 | 26 | p16, p14 | T cells | TaqMan | 18 S rRNA | Log2 | p16↑ after CT |
| | Breast cancer survivor | 59 | 50 | | | | | | |
| de Kreutzeneberg (2015) | Prediabetes on placebo | 4/19 | 59, 2 | p53 | PBMCs | NR | β-actin | RPLP0 | p53 NC |
| | Prediabetes on metformin | 4/19 | 61, 1 | | | | | | |
| Onyema (2015) | Old healthy adults | 5/11 | 78 ± 4.9 | p16, p53, p21 | T subsets | T cells | KAPA2G Robust TaqMan | 18 S rRNA | β2-M Log2 | p16↑ in MM with HSCT than healthy control |
| Rosko (2015) | Healthy control | 17 | 60 (35–82) | | | | | | |
| | Newly diagnosed MM MM with HSCT | 5/11 | 70 (51–84) | | | | | | |
| | Older adults with PSD | 15/ 29 | 71 ± 7.5 | p16 | PBMCs | NR | NR | NR | p16 NC |
| | Healthy control | 10a | 55 (48–75) | p16 | Lympho | SYBR | GAPDH | NR | p16↑ in dialysis and kidney transplantation (continued on next page) |
| First author (year) | Population | (F/) | Age, years | Cell cycle regulator | Cells Subtype | Housekeeping gene | Quantification | Main findings |
|---------------------|------------|------|------------|----------------------|----------------|-------------------|----------------|--------------|
| Hemodialyzed (2016) | Kidney transplant recipients | 9/20 | 58.5 (32–80) | NR | p16 | T cells | TaqMan | YWHAZ | Log2 | p16↑ in patients experienced fatigue |
| Demaria (2016) | Breast cancer on CT + HIV+ | 89/89 | 48 (22–73) | p16 | T cells | TaqMan | 18 S rRNA | Log2 | p16↑ NC with IL-6 |
| Maas (2016) | CAB surgery | 47 | 63.5 (56–81) | p16 | T cells | TaqMan | 18 S rRNA | β2-M | Log2 | p16↑ with age/IL-6, NC with smoking/exercise/LOS/frailty |
| Rutten (2016) | Non-smoker | 15/38 | 59 ± 8 | p16, p21 | Leuko | SYBR | HPRT | ΔΔCt | p16↑ in all B subsets from old especially late memory |
| Smoker | 41/82 | 62 ± 6 | | | | | | |
| COPD | 69/160 | 62 ± 7 | | | | | | |
| Wood (2016) | Autologous HSCT | 11/26 | 59 ± 8.6 | p16 | T cells | TaqMan | YWHAZ | Log2 | p16↑ after HSCT or CT |
| Allogeneic HSCT | 13/37 | 54.2 ± 11.4 | | | | | | |
| Frasca (2017) | Young healthy adults | 3/6 | 25–55 | p16 | B subsets | TaqMan | GAPDH | ΔΔCt | p16↑ in exposed victims |
| Old healthy adults | 2/6 | ≥ 65 | | | | | | |
| Barryka (2018) | Healthy control | 307 | 45.2 ± 4.3 | p16, p53, p21 | PBMCs | TaqMan | 18 S rRNA | ΔΔCt | p16/p21↑, p53↑ in exposed workers |
| Radiation exposure | 157 | 48.9 ± 4.3 | | | | | | |
| Healthy control | 0/53 | 45.5 ± 8.4 | | p16 | Leuko | EvaGreen | PGK | ΔΔCt | p16↑ in exposed victims |
| Behboudi (2018) | SM exposure | 0/215 | 44 ± 8 | p16, p53, p21 | PBMCs | SYBR | β-actin | 2^-ΔΔCt | p16/p53/p21↑ in diabetes |
| Sathishkumar (2018) | Normal glucose tolerance | 14/32 | 46 ± 8 | | | | | | |
| Diabetes | 12/30 | 48 ± 3 | | | | | | |
| Diabetes on placebo | 18/33 | 56 ± 7 | | p16 | PBMCs | SYBR | GAPDH | Pfaff equation | p16↑ in diabetes on fish oil |
| Diabetes on fish oil | 16/34 | 55.9 ± 7.8 | | | | | | |
| Lye (2019) | Community dwelling older adults | 197 | NR | p16, p14, p21b | Whole blood | TaqMan | USP1 | | ΔΔCt | p16↑ in diabetes than obese, p53↑ in Parkinson’s disease |
| Soundararajan (2019) | Normal glucose tolerance | 15/30 | 43.5 ± 6.1 | p16, p53, p21 | PBMCs | SYBR | β-actin | 2^-ΔΔCt | p16/p21↑ in mild cognitive decline |
| Diabetes | 14/30 | 48.5 ± 6.7 | | | | | | |
| Bourlon (2020) | Healthy control | 0/16 | NR | p16 | T cells | TaqMan | 18 S rRNA | 2^-ΔΔCt | p16↑ in testicular cancer survivors |
| Testicular cancer survivors | 0/16 | 27 (24–54) | | p16 | PBMCs | SYBR | | |
| Hagman (2020) | Young untrained control | 0/35 | 18–30 | p16 | PBMCs | SYBR | 18 S rRNA | 2^-ΔΔCt | p53↑ in old untrained control than the other three groups |
| Young footballers | 0/35 | 18–30 | | | | | | |
| Old untrained control | 0/35 | 65–80 | | | | | | |
| Martin-Ruiz (2020) | Matched control | 0/35 | 65–80 | p16, p21 | Whole blood | SYBR | PGK1 | 2^-ΔΔCt | p16↑ with slower cognitive and motor decline; p21↑ in Parkinson’s disease |
| Matched control | 45/99 | 68 (63–82) | | | | | | |
| Al Dubayee (2021) | Parkinson’s disease Lean | 54/154 | 67 (60–82) | p16, p53, p21 | PBMCs | SYBR | UBC | 2^-ΔΔCt | p16/p53/p21↑ in diabetes |
| Parkinson’s disease Obese | 12/30 | 25.7, 1.1 | | | | | | |
(continued on next page)
which increases the risk of inefficient reverse transcription, RNA degradation and PCR cross-contamination (Bustin and Mueller, 2005). Although the nCounter technology does not require PCR, it is not suitable for large sample sizes (up to 12 samples per assay). Additionally, the cost is higher if multiple assays are utilized for these three techniques compared to qRT-PCR (Narrandes and Xu, 2018).

Accurate normalization of qRT-PCR data using suitable housekeeping genes is crucial (Gonzalez-Bermudez et al., 2019). The expression of housekeeping genes could vary among different biological samples, experimental conditions, or peripheral blood cell sub-populations (O’Connell et al., 2017; Rebouças et al., 2013). The commonly used housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was shown not to be a suitable reference gene in blood cells from various populations (Dheda et al., 2004; Oturai et al., 2016; Stamova et al., 2009). The widely used reference gene β-actin (ACTB), a cytoskeletal structural protein, has also reported to be unstable in senescent cells most likely due to the morphology change of senescent cells (Su et al., 2016). Glucuronidase beta (GUSB) was found to be an ideal reference gene for accurate qRT-PCR data normalization not only in PBMCs from young and old humans but also in white blood cells from ageing mice (Sieber et al., 2010; Zampieri et al., 2010), while another commonly used housekeeping gene, the ribosomal subunit 18S ribosomal RNA (rRNA), was shown to be the least suitable reference gene in PBMCs as rRNA decreases with ageing (Strehler et al., 1979; Zampieri et al., 2010). Moreover, the expressions of ubiquitin C (UBC), tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide (YWHAZ), TATA box binding protein (TBP) and succinate dehydrogenase complex, subunit A (SDHA) were shown to be stable in leukocytes from healthy individuals (Lcederoere et al., 2011; Vandesompele et al., 2002). There are no universal housekeeping genes that are stably expressed in every condition. According to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR experiments) guidelines (Bustin et al., 2009), validation of the appropriate number and choice of reference genes is highly recommended for particular experimental designs by use of at least one algorithm, such as geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfafl et al., 2004). The reported primer and probe sequences of cell cycle regulators and housekeeping genes used in human peripheral blood cells are listed in Table 3.

The protein expression of cell cycle regulators could be detected by WB and ELISA in total protein samples extracted from PBMCs or isolated specific blood cell subpopulations (Table 4). WB is the most widely used semi-quantitative technique to identify a protein of interest based on the molecular weight from a mixture of proteins. A limited number of samples could be loaded on an electrophoretic gel for WB analysis (up to 26 wells per assay). Each gel should include lanes with molecular weight markers and positive and negative controls (Jensen, 2012). The selection of internal loading control is essential to normalize the result of

### Table 2 (continued)

| First author | Population | (F/ N) | Age, years | Cell cycle regulator | Cells | Subtype | Housekeeping gene | Quantification | Main findings |
|--------------|------------|--------|------------|---------------------|-------|---------|------------------|---------------|---------------|
| Kouli (2021) | Matched control | 31/ 63 | 67.5 ± 7.2 | p16, p21 | T subsets | SYBR | PGK1 | 2^-ΔΔCt | p16; p21 NC in CD8 + T from Parkinson’s disease |
| Microarray | Healthy control | 9* | 22–55 | p53 | PBMCs | Research Genetics GF-211 membrane | p53 | in both rheumatoid arthritis groups |
| Wood (2016) | Patients on CT | 5 | NR | p16 | Leuko | Illumina Human HT-12 v4 BeadArrays | p16 | after PSD |
| RNA sequence | Pre-transplant MM | 40/ 100 | 59.5 (36–75) | p16 | T cells | Nanostring | GUSB, HPRT1, PGK1, UBC, YWAZ | p16 | with self-reported fatigue, p16 NC with LOS/ readmission |

#: (F/N): Number of female within the sample size/sample size. The number of females was not reported where there is only sample size number in this column. 
#1: Age is presented as range, mean/median (range), mean/median ± standard deviation, median [interquartile range] and mean, standard error. 
Abbreviations: qRT-PCR: Quantitative reverse transcription polymerase chain reaction; ART: Antiretroviral therapy; CT: Chemotherapy; MM: Multiple myeloma; HSCCT: Hematopoietic stem cell transplantation; PSD: Partial sleep deprivation; CAB: Coronary artery bypass; COPD: Chronic obstructive pulmonary disease; SM: Sulfur mustard; PBMCs: Peripheral blood mononuclear cells; Leuko: Leukocytes; Lympho: Lymphocytes; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; 18 S rRNA: 18 S ribosomal RNA; β2M: β-2-microglobulin; HPRT: Hypoxanthine phosphoribosyl-transferase; RPLP0: Ribosomal protein large P0; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; PGK: Phosphoglycerate kinase; GUSB: Glucuronidase beta; PPJA: Peptidylprolyl isomerase A; UBC: ubiquitin C; RPL13: Ribosomal protein L13; BMI: Body mass index; IL-6: Interleukin-6; LOS: length of stay. 
↑: Increased; ↓: Decreased; NC: Not changed; /: Or; NR: Not reported. 
* Sample size of subgroup is reported but age is reported for each total group only. 

| First author | Population | (F/ N) | Age, years | Cell cycle regulator | Cells | Subtype | Housekeeping gene | Quantification | Main findings |
|--------------|------------|--------|------------|---------------------|-------|---------|------------------|---------------|---------------|
| Maas (2005) | Healthy control | 9* | 22–55 | p53 | PBMCs | Research Genetics GF-211 membrane | p53 | in both rheumatoid arthritis groups |
| Carroll (2016) | Older adults with PSD | 15/ 29 | 71 ± 7.5 | p16 | PBMCs | Illumina Human HT-12 v4 BeadArrays | p16 | after PSD |
| Rentjescher (2019) | Psychosocial stress | 40/ 73 | 43.0 ± 7.2 | p16 | Leuko | Illumina Human HT-12 v4 BeadArrays | p16 | with stress |
| nCounter | Patients on CT | 5 | NR | p16 | T cells | Illumina HiSeq2000 | p16 | after CT |
| Rosko (2019) | Pre-transplant MM | 40/ 100 | 59.5 (36–75) | p16 | T cells | Nanostring | GUSB, HPRT1, PGK1, UBC, YWAZ | p16 | with self-reported fatigue, p16 NC with LOS/ readmission |

| First author | Population | (F/ N) | Age, years | Cell cycle regulator | Cells | Subtype | Housekeeping gene | Quantification | Main findings |
|--------------|------------|--------|------------|---------------------|-------|---------|------------------|---------------|---------------|
| Obese | 19/ 30 | 35.1, 2.3 | | | | | | |
| Diabetes | 5/ 20 | 48.4, 3.0 | | | | | | |
| Diabetes on metformin | 18/ 30 | 47.1, 2.0 | | | | | | |

| First author | Population | (F/ N) | Age, years | Cell cycle regulator | Cells | Subtype | Housekeeping gene | Quantification | Main findings |
|--------------|------------|--------|------------|---------------------|-------|---------|------------------|---------------|---------------|
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target protein accurately. It could be a single housekeeping protein or total cellular protein (Aldridge et al., 2008). The reported changes in protein levels of commonly used housekeeping protein GAPDH, β-actin and α-tubulin under certain cell culture conditions or age-associated human muscle samples questioned their usage as internal loading control in WB analysis (Greer et al., 2010; Vigelso et al., 2015). Validation of an effective control is needed for a particular experimental design (Pilai-Kastoori et al., 2020). Choosing a primary antibody with high specificity is crucial to ensure accurate and reproducible results.

### Table 3

List of reported primer sequences that have been used in qRT-PCR method to detect the transcriptional level of cell cycle regulators.

| First Author (year) | qRT-PCR Subtype | Forward primer (5′−3′) | Reverse primer (5′−3′) |
|---------------------|-----------------|------------------------|------------------------|
| Shao (2009)         | Amplify in PCR machine | p53: CATGTCCTCAAAGCTGGGCC | p53: GGAGCTTACTGGAACTGG |
| Damjanac (2009)     | GoTaq Flexi      | 18 S rRNA: AGGAATCTCCAGGAACTGG | 18 S rRNA: GCCACTTAACCACCTGATC |
| Li (2011)           | SYBR            | p16: TCTTCCGACGACAGTCGTTG | p16: TCTTCCGACGACAGTCGTTG |
| Teysni (2012)       | SYBR            | p16: GCCAAGAAGCAAGAATAGTTAG | p16: GCCAAGAAGCAAGAATAGTTAG |
| Liu (2009); Song (2010); Nelson (2012); Sanoff (2014); Rosko (2015); Maas (2016); Puntavoituan (2016); Wood (2016) | TaqMan | Probe-p16: 5′-FAM-CCTGGATCCGGTCCACG-MGB-3′ | p16: CTCATGACTCACGAGTTCA |
|                     |                 | p16: CTTCACACATCACTCGAG | p16: CTCATGACTCACGAGTTCA |
|                     |                 | p16: CAACGACACACGGAGTACATAG | p16: CAACGACACACGGAGTACATAG |
| Pathai (2013)       | TaqMan          | p16: CATAGATGCGCCGGAAGG | p16: CTCAGGAGTCTTCACAGAC |
| Mondal (2013)       | TaqMan          | p16: CCAAGCAGGAAGAATGATTAG | p16: CCAAGCAGGAAGAATGATTAG |
| Onyema (2015)       | KAPA2G Robust   | p16: AGCATGAGGCTCCGCTGGA | p16: CTCATGACTCACGAGTTCA |
| de Kreutzenberg (2015) | NR              | p16: AGCATGAGGCTCCGCTGGA | p16: CTCATGACTCACGAGTTCA |
| Chebel (2016)       | SYBR            | p16: TATCACTAGCTGGTCTGTGG | p16: CTCATGACTCACGAGTTCA |
| Rutten (2016)       | SYBR            | p16: GTGAGAGGCTGGAGGTGG | p16: CTCATGACTCACGAGTTCA |
| Demaria (2016)      | TaqMan          | p16: AGCTTCCGACGGAGGAGGACG | p16: GTGAGAGGCTGGAGGTGG |
| Behboudi (2018)     | EvaGreen        | p16: GGGGACACGACGGAGCAGGT | p16: GTGAGAGGCTGGAGGTGG |
| Touphcian (2018)    | SYBR            | p16: CTCGCTACACGGAGCTGGAGG | p16: GTGAGAGGCTGGAGGTGG |
| Soundararajan (2019) | SYBR           | p16: CTCGCTACACGGAGCTGGAGG | p16: GTGAGAGGCTGGAGGTGG |
| Hagman (2020)       | SYBR            | p16: TTGTTTCACATCATCGTTGGT | p16: GTGAGAGGCTGGAGGTGG |
| Martin-Ruiz (2020)  | SYBR            | p16: GGGGACACGACGGAGGAGGACG | p16: GTGAGAGGCTGGAGGTGG |
| Al Dubayee (2021)   | SYBR            | p16: GGGGACACGACGGAGGAGGACG | p16: GTGAGAGGCTGGAGGTGG |
| Kouli (2021)        | SYBR            | p16: PrimePCR SYBR Assay | p16: PrimePCR SYBR Assay |

**Abbreviations:** qRT-PCR: Quantitative reverse transcription polymerase chain reaction; 18 S rRNA: 18 S ribosomal RNA; β2M: β-2-microglobulin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HPRT: Hypoxanthine phosphoribosyl-transferase; PGK: Phosphoglycerate kinase; UBC: ubiquitin C; RPLP0: Ribosomal protein large P0; RPL13: Ribosomal protein L13; NR: Not reported.
### Table 4
Articles describing cell cycle regulators detected at the translational level in human peripheral blood cells and characteristics of participants, stratified by methods.

| First author (year) | Population | (F/| N) | Age, years# | Cell cycle regulator | Cells | Antibody | Housekeeping genes | Visualization | Main findings |
|---------------------|------------|------|-------------|----------------------|-------|----------|---------------------|--------------|---------------|
| **Western Blotting** |            |      |             |                      |       |          |                     |              |               |
| Dorszewska (2005)   | Matched control | 31/44 | 42.9 ± 16.5 | p53               | PBMCs | Mouse mono IgG2a-p53 | NR | Colorimetric | p53↑ in Alzheimer’s disease |
|                     | Alzheimer’s disease | 22/34 | 65.8 ± 12.5 |                      |       |           |                     |              |               |
| Maas (2005)         | Healthy control | 9* | 22-55       | p53               | PBMCs | Mouse-p53 | β-actin | Chemiluminescence | p53↑ in rheumatoid arthritis |
|                     | Rheumatoid arthritis | 10* | 22-55       |                      |       |           |                     |              |               |
| Damjanac (2009)     | Healthy control | 80 (66-90) | p53, pp53 (Ser15) | PBMCs | Rabbit mono-p53, rabbit poly-pp53 | β-actin | Chemiluminescence | pp53 to p53 ratio↑ in Alzheimer’s disease |
|                     | Alzheimer’s disease | 83 (63-93) |                      |       |           |                     |              |               |
| Liu (2009)          | Healthy people | 5* | 47 (18-76)  | p16               | T cells | Mouse IgG-p16 (G175-1239), AF680-fluorescent secondary antibody | Tubulin | Fluorescence | p16↑ with age |
| Werner (2009)       | Young healthy control | 11/26 | 21.8, 0.5 | p16, p53        | PBMCs | Mouse mono IgG16-p16 (F-12), rabbit poly IgG-p53 (FL-393) | GAPDH | Chemiluminescence | p16↑ in old athletes than old control, p53↑ in old control than young control |
|                     | Young athletes | 7/32 | 20.4, 0.6  |                      |       |           |                     |              |               |
|                     | Old healthy control | 7/21 | 50.9, 1.6  |                      |       |           |                     |              |               |
|                     | Old athletes | 6/25 | 51.1, 1.6  |                      |       |           |                     |              |               |
| Dezor (2011)        | Young matched control | 18/29 | 35.8 ± 14 | p53               | PBMCs | Mouse mono IgG2a-p53 | NR | Colorimetric | p53↑ trend in old than young control, p53 NC in Alzheimer’s disease |
|                     | Old matched control | 13/22 | 71.2 ± 5.5 |                      |       |           |                     |              |               |
|                     | Alzheimer’s disease | 25/41 | 72.5 ± 11 |                      |       |           |                     |              |               |
|                     |              | 25/41 | 72 ± 4.4  |                      |       |           |                     |              |               |
|                     | Tan (2012)   | Healthy control | 5/10 | 72.6 ± 4.8 | p53, p21, pp53 (Ser15), pp21 (Thr145) | PBMCs | Mouse mono-p53, rabbit poly-p21/pp53/pp21 | β-actin | Chemiluminescence | p53/pp53/pp21↑, p21↑ in Alzheimer’s disease |
|                     | Alzheimer’s disease | 6/10 | 21-74 |                      |       |           |                     |              |               |
|                     |              | 6/10 | 40-65 |                      |       |           |                     |              |               |
|                     | Mondal (2013) | Healthy control | 3* | 21-74 | p38, pp38 (Tyr232) | T subsets | Rabbit poly Δ133p53 (MAP4), p53Δ (TLQ9), p53 (DO-1, CM1) | β-actin | Chemiluminescence | Δ133p53↑↑ in CD28-CD57 + T |
|                     | Lanna (2014) | Healthy control | 3* | 59.2 | p35 | T subsets | Rabbit mono-p38, rabbit poly-pp38 | GAPDH | Chemiluminescence | pp38↑ in CD27-CD28↑- than CD27 + CD28↑ + T |
|                     | de Kreutzenberg (2015) | 4/19 | 61, 1 |                      |       |           |                     |              |               |
|                     | Prediabetes on placebo | 2/6 | ≥ 65 |                      |       |           |                     |              |               |
|                     | Prediabetes on met | 19/19 |                      |       |           |                     |              |               |
|                     | Frasca (2017) | Old healthy adults | 2/6 | 72.2 ± 6.3 | p53, p21 | Monocytes | Rabbit mono-p21, mouse mono-p53 | NR | p21-chromogen, p53-TMB | p53 undetectable, p21↑ in Alzheimer’s disease, p21 NC in MCI |
|                     | ELISA Hochstrasser (2011) | 21/40 | 72.2 ± 6.3 | p53, p21 | Monocytes | Rabbit mono-p21, mouse mono-p53 | NR | p21-chromogen, p53-TMB | p53 undetectable, p21↑ in Alzheimer’s disease, p21 NC in MCI |
|                     | MCI | 44/67 | 73.8 ± 7.1 |                      |       |           |                     |              |               |
|                     | Alzheimer’s disease | 71/92 | 78.8 ± 7.1 |                      |       |           |                     |              |               |
|                     | Tan (2012)   | Healthy control | 40/75 | 76.9 ± 5.2 | p53, p21, pp53 (Ser15), pp21 (Thr145) | PBMCs | Mouse mono-p53/p21/pp53/pp21 | β-actin | TMB | p53↑ in Alzheimer’s disease, p53↑ in vascular dementia, markers NC in Parkinson’s disease |
|                     | Alzheimer’s disease | 42/74 | 77.6 ± 7.1 |                      |       |           |                     |              |               |

(continued on next page)
| First author (year) | Population | (F/) | Age, years | Cell cycle regulator | Cells | Antibody | Housekeeping genes | Visualization | Main findings |
|---------------------|------------|------|------------|----------------------|-------|----------|---------------------|--------------|--------------|
| Parkinson’s disease | 4/12       | 66.8 ± 5.2 | p53 | PBMCs | FITC-p53 | NA | Percentage | p53↑ in PBMCs from hemodialysis patients |
| Vascular dementia   | 7/15       | 75.7 ± 6.2 | T subsets | PE/FITC-p16, PE-p53 | NR fluorophores-CD66 | NA | Cytoplasm | 16/p53↑ in CD56 + T cells from old; p16/p53 negligible in young |
| Flow cytometry      | Ramirez (2005) Healthy control | 7/15 | 26-68 | p53 | PE-CD3, PE-CD4, PE-CD8, PE-CD27, PE-CD45RA, PE-CD127, PE-CD28, PE-CD95 | NA | MFI | 27 NC among CD27/CD28 labelled CD8 T cells |
| Hemodialysis        | 6/15       | 23-69 | T subsets | PE-CD3, PE-CD4, PE-CD8 | PE-CD27, PE-CD45RA, PE-CD127, PE-CD28, PE-CD95 | NA | MFI | Percentage | p53↑ in CD45RA+ CD4 T from rheumatoid arthritis |
| Lemster (2008) Young adults | 115 | 18-69 | p16, p53 | T subsets | PE/CD4, PE/CD8, PE/CD27, PE/CD45RA, PE/CD127, PE/CD28, PE/CD95 | NA | Cytoplasm | Percentage |
| Old adults | 43 | 70-93 | T subsets | PE-CD3, PE-CD4, PE-CD8 | PE-CD27, PE-CD45RA, PE-CD127, PE-CD28, PE-CD95 | NA | MFI | Percentage | p53↑ in CD45RA+ CD4 T from rheumatoid arthritis |
| Henson (2009) Young healthy control | 7/15 | 20-35 | p27 | T subsets | AF488-p27 | NA | MFI | Percentage | p53↑ in CD45RA+ CD4 T from rheumatoid arthritis |
| Older healthy control | NR | ≥ 65 | T subsets | PerCP-CD8, FITC-CD27, APC-CD28, PE-CD45RA | NA | MFI | Percentage | p53↑ in CD45RA+ CD4 T from rheumatoid arthritis |
| Shao (2009) Healthy control | 10a | 46.1 ± 12.8 | p53 | T subsets | PE-p53 (PAb1620), FITC-pp53 | NA | MFI | Percentage | p53↑ in CD45RA+ CD4 T from rheumatoid arthritis |
| Rheumatoid arthritis | 10a | 48.6 ± 11.6 | p16, p21 | T subsets | PE-CD27, FITC-CD28 | NA | MFI | Percentage | p53↑ in CD45RA+ CD4 T from rheumatoid arthritis |
| Shao (2010) Healthy control | 6a | 46.8 ± 10.4 | p53 | T subsets | AF488-p53 | NA | MFI | Percentage | p53↑ in CD45RA+ CD4 T from rheumatoid arthritis |
| Rheumatoid arthritis | 6a | 49.5 ± 13.6 | p53 | T subsets | AF488-p53 | NA | MFI | Percentage | p53↑ in CD45RA+ CD4 T from rheumatoid arthritis |
| Di Mitri (2011) Healthy control | 17a | 26-60 | p38, pp38 (Thr180/Tyr182) | T subsets | AF488-p38/pp38 | PE-Cy7-CD4, APC-CD45RA, PE-CD27 | NA | Relative MFI | Percentage | p38/pp38↑ in CD27+CD45RA+ CD4 T cells |
| Onyema (2012) Young healthy adults | 5/11 | 24.6, 0.7 | p16, p21 | T subsets | FITC-p16 (F-12), FITC-p21 (F-5) | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| Old healthy adults | 5/11 | 82.0, 0.8 | T subsets | PE-cy7-CD3, PE-cy5-CD8, PE/ECD-CD28, PE-CD57 | NA | MFI | Percentage | pp38↑ in CD27-CD45RA- than CD27+CD28+ T subset |
| Lanna (2014) Healthy control | 9 | 40-65 | pp38 (Thr180/Tyr182) | T subsets | PE-pp38 | PE-CD27, FITC-CD28 | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| Henson (2015) Young healthy adults | 9 | 32 (20-35) | pp38 (Thr180/Tyr182) | T subsets | AF488-p38 | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| Old healthy adults | 9 | 72 (65-82) | T subsets | AF700-CD8, V500-CD27, BV610-CD45RA | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| Onyema (2015) Young healthy adults | 5/11 | 24 ± 2.3 | p16, p21 | T subsets | PE-cy7-CD3, PE-cy5-CD8, PE/ECD-CD28, PE-CD57 | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| Old healthy adults | 5/11 | 84 ± 1.8 | T subsets | AF488-p38 | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| Ribeiro (2016) HIV- | 19 | 49 (45-53) | p16 | T subsets | PE-p16 (G175-1239) | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| HIV+ controllers | 29 | 48 (39-54) | T subsets | PE-p16 (G175-1239) | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| HIV+ non-controllers | 28 | 43 (34-49) | T subsets | PE-p16 (G175-1239) | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| HIV+ progressors | 29 | 42 (36-49) | T subsets | PE-p16 (G175-1239) | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| HIV+ART+ | 27 | 45 (38-54) | T subsets | PE-p16 (G175-1239) | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| Suen (2016) Multiple myeloma with T cell clone | 13+ | 62 (27-86) | p16, p21, p38, pp38 | T subsets | NR fluorophores - cell cycle regulators | APC-H7-CD8, V450-CD57, PE/FTC-TCR-Vβ | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |
| Healthy control | 9 | 32-55 | pp53 (Ser15) | T subsets | AF647-pp53 (16G8) | NA | MFI | Percentage | 16/p21↑ in CD28 + CD57 + CD8 T cells from old adults |

(continued on next page)
### Table 4 (continued)

| First author | Population (F/♂) | Age, years | Cell cycle regulator | Main findings | Housekeeping genes | Visualization | Main findings |
|--------------|------------------|------------|---------------------|--------------|--------------------|---------------|--------------|
| Callender    | 125 F/100 ♂       | Mean/median (range), mean/median standard deviation, median [interquartile range] and mean, standard error. | p16, p53, p21 | Increased expression of p16, p53 and p21 with chronological age at either transcriptional level measured by qRT-PCR and microarray or translational level measured by WB and flow cytometry was found in relatively healthy populations (Dezor et al., 2011; Hagman et al., 2020; Liu et al., 2009; Pustavoitau et al., 2016; Song et al., 2010; Werner et al., 2009), further corroborated a meta-analysis study showing an association between age and cell cycle regulators in blood cells (Table 4). It is more widely used for antigens or antibodies in biological fluids such as plasma, serum, or supernatant of cell culture (Gorovits et al., 2018; Sullivan et al., 2000).

Cell sorting and protein extraction are required prior to carrying out WB or ELISA if a specific blood cell subpopulation is of interest. Flow cytometry allows for simultaneous detection of protein levels of cell cycle regulators and distinction of different specific blood cells. It could be carried out in flow cytometry tubes or 96-well plates. Unlike WB and ELISA in which expression of target protein is detected in a total protein extract after cell lysis, flow cytometry detects target protein in intact cells by intracellular staining after fixation and permeabilization steps. However, it was reported that the higher p53 protein after UV irradiation was revealed by WB but not flow cytometry, probably due to the low accessibility of nuclear-localized protein (Zamai et al., 2002). Thus, fixation and permeabilization steps are essential for detecting intracellular cell cycle regulators and need to be optimized for blood cells (Viryasova et al., 2019). Variation of immunostaining could be caused by the usage of different antibodies. The clone BP54.12 FITC- and G59–12 PE-conjugated monoclonal antibodies displayed higher sensitivity and specificity independent of fixation and permeabilization reagent compared to DO-7-FITC antibody when applying flow cytometry for detection of p53 protein in human leukemic cell lines (Zamai et al., 2002). Furthermore, stained with 53 (DO-7) and p21 (12D1) antibodies conjugated with metal instead of fluorophores, p53 and p21 protein expressions could be evaluated at a single cell level using mass cytometry, an advanced multi-parameter flow cytometry allowing for high-dimensional data analysis in blood. Antibodies conjugated with metal overcome the problems of signal overlap and auto-fluorescence–G59–12 PE-conjugated monoclonal antibodies detected expression but also mRNAs of interest on a single cell level using mass cytometry. It could also be carried out in flow cytometry tubes or 96-well plates. Unlike WB or ELISA if a specific blood cell subpopulation is of interest. Flow cytometry allows for simultaneous detection of protein levels of cell cycle regulators and distinction of different specific blood cells. It could be carried out in flow cytometry tubes or 96-well plates. Unlike WB and ELISA in which expression of target protein is detected in a total protein extract after cell lysis, flow cytometry detects target protein in intact cells by intracellular staining after fixation and permeabilization steps. However, it was reported that the higher p53 protein after UV irradiation was revealed by WB but not flow cytometry, probably due to the low accessibility of nuclear-localized protein (Zamai et al., 2002). Thus, fixation and permeabilization steps are essential for detecting intracellular cell cycle regulators and need to be optimized for blood cells (Viryasova et al., 2019). Variation of immunostaining could be caused by the usage of different antibodies. The clone BP54.12 FITC- and G59–12 PE-conjugated monoclonal antibodies displayed higher sensitivity and specificity independent of fixation and permeabilization reagent compared to DO-7-FITC antibody when applying flow cytometry for detection of p53 protein in human leukemic cell lines (Zamai et al., 2002). Furthermore, stained with 53 (DO-7) and p21 (12D1) antibodies conjugated with metal instead of fluorophores, p53 and p21 protein expressions could be evaluated at a single cell level using mass cytometry, an advanced multi-parameter flow cytometry allowing for high-dimensional data analysis in blood. Antibodies conjugated with metal overcome the problems of signal overlap and auto-fluorescence.

3. Association with demographic and clinical characteristics

3.1. Association with age, sex and ethnicity

Increased expression of p16, p53 and p21 with chronological age at either transcriptional level measured by qRT-PCR and microarray or translational level measured by WB and flow cytometry was found in relatively healthy populations (Dezor et al., 2011; Hageman et al., 2020; Liu et al., 2009; Pustavoitau et al., 2016; Song et al., 2010; Werner et al., 2009), further corroborated a meta-analysis study showing an association between age and cell cycle regulators in blood (Turtle et al., 2020). However, such an association was not consistent across all groups in some studies. The up-regulation of cell cycle regulators with older chronological age was observed in the control group not in a relatively unhealthy group. For example, increased p16 expression with age was observed in breast cancer survivors not in breast cancer patients (Sanoff et al., 2014), in HIV negative controls or treated-HIV positive controls.
not in untreated-HIV positive patients ( Nelson et al., 2012; Pathai et al., 2013; Ribeiro et al., 2016 ), in healthy controls not in multiple myeloma patients ( Rosko et al., 2015 ). Consistently, the lack of association of p21 and p16 with age in was also found in other patient cohorts; examples of these include patients with chronic obstructive pulmonary disease ( COPD ) ( Runten et al., 2016 ), and patients suffering from psychosocial stress ( Rentscher et al., 2019 ), as well as those under treatment conditions such as hemodialysis, kidney transplantation ( Chebel et al., 2016 ) and hematopoietic stem cell transplantation ( HSCT ) ( Wood et al., 2016 ) irrespective of the type of blood cells used such as leukocytes, lymphocytes, and T cells. As these disease contexts and treatments were found to induce the elevation of cell cycle regulators, the association between age and cell cycle regulators in peripheral blood cells is likely to be attenuated in the presence of other senescence inducers. The association between age and cell cycle regulators was also found to vary among different stages of immune cell differentiation. For example, higher protein expressions of p16 and p21 as measured by flow cytometry were observed in highly differentiated T cells from healthy older adults compared to younger adults ( Onyema et al., 2012, 2015 ). Furthermore, p16 mRNA detected by qRT-PCR has been shown to be higher in memory B cell subsets, especially in late/exhausted memory B cells, not in naïve B cells from healthy older adults compared to younger adults ( Frasca et al., 2017 ).

No significant relationship of p16 and p53 mRNA detected by qRT-PCR or microarray in leukocytes, PBMCs or T cells was found with gender and ethnicity in healthy or diseased populations ( Al Dubayee et al., 2021; Liu et al., 2009; Nelson et al., 2012; Pathai et al., 2013; Rentscher et al., 2019; Sanoff et al., 2014 ). However, a higher p21 mRNA level as measured by qRT-PCR in leukocytes was observed in females than males ( Ruten et al., 2016 ).

3.2. Association with socioeconomic status

The p16 mRNA expression detected by qRT-PCR in leukocytes was found to be higher in HIV-negative participants with lower incomes compared to higher incomes, unlike in HIV-positive patients ( Pathai et al., 2013 ). The p16 mRNA expression in leukocytes was not associated with education level in HIV infected patients or participants under psychosocial stress ( Pathai et al., 2013; Rentscher et al., 2019 ). It was reported that those who have relatively lower incomes are likely to have poor quality diets, resulting in acceleration of biological ageing as revealed by telomere attrition in leukocytes ( Shiels et al., 2011 ).

3.3. Association with disease

Different disease populations have different senescent profiles which might diverge across cell types. The p16 mRNA level measured by qRT-PCR was consistently shown to be higher in leukocytes, T cells and HIV-1-specific CD8 + T cell subset from HIV patients compared to healthy controls ( Lichterfeld et al., 2012; Nelson et al., 2012; Pathai et al., 2013 ), while the p53 and p21 mRNA levels in the same HIV-1-specific CD8 + T cell subset from HIV patients were similar to healthy controls ( Lichterfeld et al., 2012 ). A higher p16 protein level measured by flow cytometry was also observed in T cell subsets from HIV patients compared to healthy controls ( Ribeiro et al., 2016 ). Cell cycle regulators might play different roles in neurodegenerative diseases. The p16 mRNA expression detected by qRT-PCR or nCounter technology in leukocytes or T cells was not associated with cognitive impairment assessed in community-dwelling older adults and patients with multiple myeloma ( Lye et al. 2019; Rosko et al., 2019 ), although p16 protein was shown to be higher in cortex astrocytes from AD patients ( Bhut et al., 2012 ). By using WB and ELISA techniques, p53 protein expression was found to be higher, whereas p21 protein expression was lower in PBMCs from patients with Alzheimer’s disease ( AD ) compared to healthy controls ( Damjanac et al., 2009; Dezor et al., 2011; Dorszewskas et al., 2005; Tan et al., 2012 ). Lower p21 protein level measured by ELISA was consistently observed in isolated monocytes from AD patients than controls, whereas p53 protein in monocytes was undetectable ( Hochstrasser et al., 2011 ). Lower p21 mRNA level in leukocytes measured by qRT-PCR was also observed in newly diagnosed Parkinson’s disease ( PD ) patients than in healthy controls ( Martin-Ruiz et al., 2020 ), while its mRNA level in CD8 + T cells measured by qRT-PCR in newly diagnosed PD patients and protein level in PBMCs measured by ELISA in PD patients was similar to healthy controls in other studies ( Kouli et al., 2021; Tan et al., 2012 ). Additionally, p16 mRNA in CD8 + T cells was found to be lower in newly diagnosed PD patients compared to age- and sex-matched controls ( Kouli et al., 2021 ), and lower baseline p16 mRNA in leukocytes from newly diagnosed PD patients was associated with faster rates of motor and cognitive decline over three years ( Martin-Ruiz et al., 2020 ). Among patients with rheumatoid arthritis ( RA ), p53 and its phosphorylated form were repressed in naïve CD4 +/CD45RA + T cells at both transcriptional level detected by qRT-PCR or microarray and translational level detected by WB or flow cytometry due to the deficiency of DNA repair ( Maas et al., 2005; Shao et al., 2009, 2010 ). This finding was confirmed in another study showing that lower p53 mRNA, while higher p21, p21 mRNA levels were observed in naïve CD4 +/CD45RA + T cells from patients with RA after stimulation in vitro ( Li et al., 2016 ). The higher p21 rather than p16 in leukocytes at the transcriptional level measured by qRT-PCR was observed in COPD ( Runten et al., 2016 ). Furthermore, higher p16 mRNA detected by qRT-PCR and microarray was found in leukocytes from participants suffering from anxiety, depression ( Teyssier et al., 2012 ) and psychosocial stress ( Rentscher et al., 2019 ). The associations of p16, p53 and p21 mRNA expressions measured by qRT-PCR in PBMCs from patients with diabetes were controversial ( Al Dubayee et al., 2021; Sathishkumar et al., 2018; Soundararajan et al., 2019 ). Whether higher cell cycle regulators in human peripheral blood cells are related to the onset and/or progression of diseases is inconclusive. For example, lower levels of cell cycle regulators were observed in blood cells from patients with newly diagnosed PD and diabetes compared to healthy controls possibly due to the migration of senescent cells into pathological sites ( Al Dubayee et al., 2021; Kouli et al., 2021; Ramello et al., 2021 ). Nevertheless, higher levels of senescence markers including cell cycle regulators were observed in age-related diseases ( Tuttle et al., 2021 ).

3.4. Association with conditions and related parameters

p16 mRNA expression is higher in T cells from individuals with obesity compared to individuals with normal body mass index ( BMI ) ( Liu et al., 2009; Sanoff et al., 2014 ), although p16 mRNA expression measured by microarray or qRT-PCR in leukocytes or T cells was not associated with BMI as a continuous parameter ( Liu et al., 2009; Rentscher et al., 2019; Song et al., 2010; Teyssier et al., 2012 ). A higher mRNA expression of p16 measured by qRT-PCR in leukocytes and T cells from healthy older adults ( Liu et al., 2009 ), patients with depression ( Teyssier et al., 2012 ), or underwent coronary artery bypass surgery ( Pustavoitau et al., 2016 ) was associated with higher inflammatory marker IL-6 which is part of senescence-associated secretory phenotype ( SASP ) ( Coppe et al., 2010 ). However, the overall inflammatory status could be suppressed by antiretroviral therapy ( Li et al., 2015 ), which subsequently abrogated the relationship between p16 and IL-6 in HIV patients undergoing therapy ( Maas et al., 2016 ). Higher IL-6 has been found in frail older adults ( Marcos-Perez et al., 2018 ). Consistently, p16 mRNA expression measured by nCounter technology in T cells was positively associated with a frailty scale in patients with multiple myeloma ( Rosko et al., 2019 ). However, another study did not show this association between p16 mRNA measured by qRT-PCR in T cells and frailty in patients who underwent bypass surgery ( Pustavoitau et al., 2016 ). The difference in frailty assessment might account for this inconsistency. The frailty scale in the former study was assessed by the self-reported Brief Fatigue Inventory ( BFI ) which was developed for cancer-related fatigue ( Mendoza et al., 1999 ). The positive association
of p16 mRNA measured by qRT-PCR in T cells with fatigue has been validated in breast cancer patients (Demaria et al., 2017). The frailty status in patients who underwent coronary bypass surgery was assessed by Fried’s frailty phenotype which includes physical function components (Fried et al., 2001; Pustavoitau et al., 2016). Indeed, the p16 mRNA level measured by nCounter technology in T cells was shown to be not associated with physical function (Rosko et al., 2019). In addition, the p16 mRNA measured by qRT-PCR and nCounter technology in T cells failed to predict the length of stay in hospital which is an outcome relying on multiple clinical variables (Pustavoitau et al., 2016; Rosko et al., 2019). Furthermore, the p16 mRNA expression measured by qRT-PCR in leukocytes or T cells was not associated with comorbid conditions in patients with HIV infections, breast cancer and HSCT (Pathai et al., 2013; Sanoff et al., 2014; Wood et al., 2016).

3.5. Association with immunosenescence

Immunosenescence, referred to as age-related decline of the immune system, has been associated with higher susceptibility to infections and poor response to vaccinations in older adults (Crooke et al., 2019). Flow cytometry is broadly used to identify and sort immune cells with different differentiation stages by staining various cell surface markers (Xu and Larbi, 2017). The highly differentiated T cells that are identified by loss of CD27 and CD28 expressions, gain of KLRG1 and CD57 expressions and re-expression of CD45RA display some senescent phenotypes such as poor proliferative ability, DNA damage, and short telomere length (Akbar et al., 2016; Strioga et al., 2011). However, the senescence phenotype could also be found in less differentiated T cells. Higher p16 and p21 protein expressions measured by flow cytometry were observed in intermediate differentiated CD28+CD57−T cells compared to highly differentiated CD28−CD57−T cells from healthy younger and older adults (Onyema et al., 2012, 2015). The expression of p53 mRNA did not differ among CD8+ T cells subsets defined by CD28 and CD57 in healthy older adults (Onyema et al., 2015). However, p53 isoform p53β which accelerates replicative senescence, showed higher mRNA and protein levels in late differentiated CD8+CD28−CD57−T cells compared to early differentiated CD8+CD28+CD57−T cells from healthy adults as revealed by qRT-PCR and WB. Consistently, the other isoform Δ133p53 which inhibits replicative senescence, showed a lower protein level without change of mRNA when using the same techniques (Mondal et al., 2013). Additionally, higher phosphorylated p53 (Ser15) expression measured by flow cytometry was observed in terminally differentiated CD8+CD27−CD45RA−T cells compared to other T cell subsets from healthy adults (Callender et al., 2018). It has been demonstrated that p16 mRNA expression measured by qRT-PCR is upregulated in late/exhausted memory B cells defined as IgD−CD27− but not in naïve CD27+IgD+ B cells from both healthy young and older adults (Frasca et al., 2017). Highly differentiated immune cells have senescence-like phenotypes with a robust pro-inflammatory response, which is associated with various diseases such as RA, vascular and inflammatory bowel disease (Vallejo et al., 2004; Xu and Larbi, 2017).

3.6. Association with lifestyle

Higher p16 mRNA levels measured by qRT-PCR in PBMCs and T cells or protein expression measured by WB in PBMCs were associated with smoking and less exercise in healthy participants (Hagman et al., 2020; Liu et al., 2009; Song et al., 2010; Werner et al., 2009) while the levels of p53 protein in PBMCs measured by WB (Werner et al., 2009) and p21 mRNA in leukocytes measured by qRT-PCR were not (Butten et al., 2016). The association of p16 mRNA in leukocytes or T cells measured by microarray or qRT-PCR with an unhealthy lifestyle is likely to be weakened in populations with diseases such as HIV infection, cardiovascular diseases, breast cancer, and depression or under psychosocial stress (Nelson et al., 2012; Pathai et al., 2013; Pustavoitau et al., 2016; Rentscher et al., 2019; Sanoff et al., 2014; Teyssier et al., 2012).

3.7. Association with external stress

Chronic γ-ray radiation exposure induced down-regulation of p16 and p21 mRNA expressions, but up-regulation of p53 mRNA expression measured by qRT-PCR in PBMCs from workers with occupational radiation (Bazyka et al., 2018). Radiation could cause DNA damage in blood cells and trigger a p53-mediated DNA damage response (Lee et al., 2013). Another passive exposure to sulfur mustard could cause oxidative stress which is associated with premature senescence and result in up-regulation of p16 mRNA measured by qRT-PCR in leukocytes (Behboudi et al., 2018). Clinical treatments such as hemodialysis and transplantation have been shown to induce metabolic disturbance and cause oxidative stress (Nafar et al., 2011). Higher p53 protein level measured by flow cytometry in PBMCs was found in patients after hemodialysis (Ramirez et al., 2009), and higher p16 mRNA measured by qRT-PCR or RNA sequencing in lymphocytes or T cells was found in patients after kidney transplantation (Chebel et al., 2016; Li et al., 2011) and HSCT (Rosko et al., 2015; Wood et al., 2016). The effect of chemotherapy exposure on p16 is dependent on the type of chemotherapy. A higher p16 mRNA level measured by qRT-PCR in T cells was more likely to be induced by cytotoxic chemotherapy such as alkylators, paclitaxel but not non-cytotoxic chemotherapy such as immunomodulatory drugs (Bourlon et al., 2020; Rosko et al., 2015; Sanoff et al., 2014; Wood et al., 2016). The antiretroviral therapy was shown not to have an impact on the p16 mRNA expression as measured by qRT-PCR in leukocytes (Pathai et al., 2013) and T cells (Nelson et al., 2012) from HIV-infected patients. Furthermore, after metformin treatment, the p53 protein level measured by WB did not change in PBMCs from pre-diabetic subjects (de Kreutzenberg et al., 2015), while its mRNA level measured by qRT-PCR was higher in diabetic patients than in untreated diabetic patients (Al Dubayee et al., 2021). Although metformin has been reported to have anti-aging effects in animal studies (Kulkarni et al., 2020), it might not influence longevity by p53, unlike other effectors such as sirtuin-1 (SIRT1) (Cuyás et al., 2018). The supplementation of DHA-enriched fish oil induced higher I6 mRNA expression measured by qRT-PCR in PBMCs from diabetic patients (Touphian et al., 2018).

4. Other cell cycle regulators

The cell cycle regulator p14ARF (p14) is an alternative variant spliced from INK4a/ARF locus. One of the pathways for p14 involved in cell cycle control is to stabilize p53 via inactivation of mouse double minute 2 (MDM2) which negatively regulates p53 (Kim and Sharpless, 2006). A higher p14 mRNA levels detected by qRT-PCR in T cells was associated with higher chronological age in breast cancer survivors (Sanoff et al., 2014), not in healthy participants (Liu et al., 2009). A higher p14 mRNA expression was found in T cells sampled from an African-American cohort compared to a Caucasian cohort (Sanoff et al., 2014). No associations of p14 mRNA levels in T cells with BMI and lifestyle were found (Sanoff et al., 2014). Higher p14 mRNA levels measured by qRT-PCR in leukocytes from community-dwelling older adults were associated with mild cognitive decline (Lye et al., 2019), which might partly explain the up-regulation of p53 in patients with cognitive impairment (Djamjanac et al., 2009; Tan et al., 2012). The p14 mRNA was higher in T cells after treatment of cytotoxic chemotherapy (Sanoff et al., 2014).

Another factor that should also be considered is the signaling molecule p38 mitogen-activated protein kinase (MAPK) involved in establishing cellular senescence induced by oncogene activation or chemotherapy (Munoz-Espin and Serrano, 2014; Spallarossa et al., 2010; Sun et al., 2007). The p38 MAPK signaling plays a critical role in the regulation of p16 and p53 in cellular senescence (Hongo et al., 2017; Sun et al., 2007). Blockade of p38 MAPK decreases p16 expression in aged muscle stem cells and restores their proliferation ability (Cosgrove et al., 2014). Activated p38 MAPK is expressed as a phosphorylated form, thus, p38 MAPK is widely measured by flow cytometry and WB at
the protein level, not at the transcriptional level in human blood cells (Table 4). The spontaneous activation of p38 MAPK was found in terminally differentiated T cells (CD27-CD28-) (Lanna et al., 2014), effector memory T cells that re-express CD45RA (CD27-CD45RA+) (Di Mitri et al., 2011; Henson et al., 2015) and late/exhausted memory B cells (IgD-CD27-) (Frasca et al., 2017) from healthy donors and responsible for the senescent phenotype of these cells, indicating that p38 MAPK signaling is engaged in cellular senescence and the immune cell differentiation status matters the expression of p38 MAPK.

5. Concluding remarks

Research into the role of cellular senescence in aging and age-related diseases has progressed tremendously in recent years. However, the heterogeneous nature of senescence-associated traits makes the utility of markers to detect senescence challenging (Gonzalez-Gualda et al., 2021). Cell cycle regulators play a crucial role in mediating cell growth arrest which is the hallmark of cellular senescence. These could be detected in peripheral blood which is a minimally invasive and amenable specimen for sampling clinically. Cell cycle regulators display different mRNA and protein levels in different blood cell types, especially in a cross-sectional comparison of differentiation stages of specific blood immune cells such as T cells and B cells. Thus, depending on the blood cell type used, various associations of cell cycle regulators with demographics and clinical characteristics are found. Factors such as age, socioeconomic status, chronic diseases, body composition, lifestyle and clinical treatments (e.g. chemotherapy, transplantation) should be taken into account when clinical analyses of cell cycle regulators as senescence markers in human peripheral blood cells are performed.

The choice of the technique to be used depends on the study goals and experimental design. Identification of suitable reference genes for a particular design and validation of results across multiple platforms are recommended to obtain an accurate and thorough analysis of gene and protein expression. Techniques with high sensitivity are certainly recommended, but allowing for cost and convenience, using qRT-PCR might be a good start for detecting senescence phenotype characterized by cell cycle regulators relevant to aging and age-related diseases. In terms of the choice of blood cells, although T cells were widely used as target cells from blood specimens, it is valuable to explore the expressions of cell cycle regulators in other blood cells such as subtypes of granulocytes and monocytes. In addition to cell cycle regulators (p16, p53, p21, p14, p38 MAPK), more senescence markers such as the DNA damage marker γH2AX, proliferation marker Ki67, SASP and senescence-associated β galactosidase (SA-β-gal) should be considered to further define the senescent state of blood cells (Kohli et al., 2021). Collectively, further research is needed to better understand the biological functions of senescent blood cells and determine how they contribute to immune response and disease pathology.

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Declaration of competing interest

The authors declare no conflict of interest.

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