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Effects of intermittent senolytic therapy on bone metabolism in postmenopausal women: a phase 2 randomized controlled trial

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Abstract

Preclinical evidence demonstrates that senescent cells accumulate with aging and that senolytics delay multiple age-related morbidities, including bone loss. Thus, we conducted a phase 2 randomized controlled trial of intermittent administration of the senolytic combination dasatinib plus quercetin (D + Q) in postmenopausal women (n = 60 participants). The primary endpoint, percentage changes at 20 weeks in the bone resorption marker C-terminal telopeptide of type 1 collagen (CTx), did not differ between groups (median (interquartile range), D + Q −4.1% (−13.2, 2.6), control −7.7% (−20.1, 14.3); P = 0.611). The secondary endpoint, percentage changes in the bone formation marker procollagen type 1 N-terminal propeptide (P1NP), increased

significantly (relative to control) in the D + Q group at both 2 weeks (+16%, $P = 0.020$) and 4 weeks (+16%, $P = 0.024$), but was not different from control at 20 weeks (−9%, $P = 0.149$). No serious adverse events were observed. In exploratory analyses, the skeletal response to D + Q was driven principally by women with a high senescent cell burden (highest tertile for T cell *p16* (also known as *CDKN2A*) mRNA levels) in which D + Q concomitantly increased P1NP (+34%, $P = 0.035$) and reduced CTx (−11%, $P = 0.049$) at 2 weeks, and increased radius bone mineral density (+2.7%, $P = 0.004$) at 20 weeks. Thus, intermittent D + Q treatment did not reduce bone resorption in the overall group of postmenopausal women. However, our exploratory analyses indicate that further studies are needed testing the hypothesis that the underlying senescent cell burden may dictate the clinical response to senolytics. ClinicalTrials.gov identifier: NCT04313634.

Main

Cellular senescence was first described by Hayflick and Moorhead in 1961 as an in vitro phenomenon, whereby normal human fibroblasts stopped proliferating and entered a state of irreversible growth arrest[1]. Subsequently, extensive studies have demonstrated that senescence occurs not only in vitro, but also in vivo and is not limited to proliferating cells but also occurs in quiescent, terminally differentiated cells such as neurons, hepatocytes, cardiomyocytes and osteocytes[2]. These studies, done predominantly in mice, also established that accumulation of senescent cells with aging contributed to multiple age-associated comorbidities by virtue of their senescence-associated secretory phenotype (SASP), which consists of cytokines, proteases, bioactive lipids and other proinflammatory molecules[3]. Moreover, the demonstration in old mice that clearing senescent cells using a genetic approach extended health span and lifespan[4,5] generated intense interest in therapeutically targeting senescent cells in humans to prevent or delay age-related comorbidities as a group[6].

An important key development critical to the translation of these findings to humans was the identification of compounds (‘senolytics’) that targeted survival pathways unique to senescent cells, resulting in their selective killing and beneficial effects in old mice similar to those observed with genetic clearance of senescent cells[7,8]. Because some of these senolytic compounds include natural flavanols (for example, quercetin (Q) and fisetin)[9] that are readily available without US Food and Drug Administration (FDA) approval, they are already being widely used on an empiric basis by otherwise healthy aging individuals and have been touted in the lay press as being ‘a major anti-aging advance’[10].

Despite this intense public interest and potential use of these compounds, at present

there is little data on their safety or efficacy, particularly in terms of systemic administration of these drugs to otherwise healthy older individuals aged 60 years and above. In an uncontrolled study, Gonzales et al.[11] reported data on the safety and biodistribution of the senolytic cocktail, D + Q[7], in five patients with mild Alzheimer's disease. More recently, Crespo-Garcia et al.[12] demonstrated that a single intraocular administration of another senolytic compound, the BCL-xL inhibitor UBX1325, was safe and improved visual acuity in five of eight patients with diabetic macular edema 24 weeks following injection, although no formal statistical analysis for efficacy was possible because of the lack of a control group and the small number of patients. Although representing important progress in the development of senolytic approaches to treat specific diseases, neither of these studies addressed the issue of safety or efficacy of systemic administration of senolytics to otherwise healthy individuals aged >60 years.

Although preclinical studies have found beneficial effects of clearing senescent cells in aged mice on measures of frailty, cognition, glucose tolerance, cardiovascular function and others[6], we chose bone metabolism as an outcome for several reasons. First, we have previously demonstrated that both genetic and pharmacological (using D + Q) clearance of senescent cells prevented bone loss in aged mice by both increasing bone formation and reducing bone resorption[13,14]. Second, our preclinical studies demonstrated that skeletal benefits in mice were evident with intermittent administration (once monthly) of D + Q, consistent with a 'hit-and-run' mechanism, in which D + Q reduces the senescent cell burden, which then takes weeks to return to pretreatment levels, allowing for maximizing therapeutic benefit with

minimal off-target or other adverse effects[13]. Finally, unlike changes in frailty or cognition, which can be difficult to measure, may include subjective features and require large numbers of participants and prolonged interventions, bone turnover markers are simple, objective measures of a biological outcome that respond within only a few weeks to an intervention[15]. Therefore, the aim of this phase 2 RCT was to examine whether our findings in aged mice demonstrating both anabolic and antiresorptive effects of intermittent senolytic therapy on bone metabolism[13,14], were applicable to humans.

Results

p16 expression in T cells as a senescence biomarker

p16 is a key trigger of cellular senescence[16], and its expression increases with aging in several rodent and primate tissues[17,18,19], suggesting its potential use as a biomarker of mammalian aging. This concept has been translated to humans by measurement of *p16*^{Ink4a} (also known as *CDKN2A*) mRNA expression in peripheral blood T cells[20]. In addition to increasing with chronological age, T cell *p16*^{Ink4a} expression is accelerated by 'gerontogenic' behaviors such as tobacco use and physical inactivity[20]. As described in the Methods, we modified the T cell *p16*^{Ink4a} assay (which measures both *p16*^{Ink4a} (*p16_variant 1*) and a related splice variant (*p16_variant 5*)) to also measure only *p16_variant 5*. As shown in Extended Data Fig. 1, the *p16_variant 5* is transcribed from the same promoter and also encodes a functional p16 protein[21]. We then evaluated both *p16*^{Ink4a} (*p16_variant 1* + *5*) and *p16_variant 5* as potential biomarkers for predicting a response to D + Q. Note that it is not possible to design quantitative polymerase chain reaction (qPCR) primers that only measure *p16_variant 1*. In preliminary validation studies, we first directly compared the *p16_variant 1* + *5* with the *p16_variant 5* assay in a population sample of

228 women aged 23 to 88 years (Methods). As shown in Extended Data Fig. 2a,b, the *p16_variant 1 + 5* demonstrated a substantial spread even in young women (23–60 years), whereas *p16_variant 5* was extremely low in these women. Moreover, *p16_variant 1 + 5* correlated positively with age even in younger women (Extended Data Fig. 2c), whereas *p16_variant 5* did not (Extended Data Fig. 2d). By contrast, the converse was true in older women (age >60 years), where *p16_variant 5* correlated more strongly with age than *p16_variant 1 + 5* (Extended Data Fig. 3a,b). Finally, in the specific group of participants ($n = 60$) recruited and randomized into this study, *p16_variant 1 + 5* did not correlate well with age, whereas *p16_variant 5* showed a highly significant correlation ($r = 0.51$, $P < 0.001$; Extended Data Fig. 3c,d). For these reasons, we primarily used the *p16_variant 5* assay (referred to subsequently as the *p16* assay for simplicity), although all participants had basal measurements of both variants and we compared them to find which assay was more predictive of a biological response to D + Q.

Study participants and study design

We conducted a 20-week, phase 2 RCT comparing D + Q with control for improving bone turnover markers in 60 postmenopausal women. The original study design included a third arm in which participants were to be administered a flavanol with senolytic properties (fisetin)[9], but because of the COVID pandemic and cost issues, the study was modified to a two-arm study (control versus D + Q). Figure 1 shows the CONSORT flow diagram for the study. We screened 177 participants, with the first participant enrolled on 9 June 2020, and the last participant enrolled on 15 November 2022. We hypothesized that women with a high basal senescent cell burden would be more likely to respond to a senolytic intervention; thus, we selected women aged >60 years with a senescent cell burden based on *p16* mRNA expression in peripheral blood T cells greater than the 95th percentile of young control women aged 22–31 years.

Fig. 1: CONSORT flow diagram.

Flow chart showing study subject inclusion, randomization and follow-up throughout the study. ITT, intention-to-treat; PBTL, peripheral blood T lymphocytes. [a]Fisetin group was discontinued.

Source data

Table 1 shows the baseline descriptive and clinical characteristics of the study participants. The two groups were well matched for age, with a range of 62–88 years. All were vitamin D sufficient at the time of participation. Serum calcium was slightly lower in the participants assigned to D + Q.

Table 1 Descriptive and baseline clinical characteristics of the study

participants

The overall study design is shown in Fig. 2, and Supplementary Table 1 summarizes assessments at each study visit. Note that over-encapsulating all study medications to ensure double-blinding proved to be cost-prohibitive so, in this initial 'proof-of-concept' study, we elected to use an open-label design, although both the D + Q and control participants underwent exactly the same study visits and assessments.

Fig. 2: Overall study design.

Schematic of 20-week study design depicting the intermittent dosing schedule, adherence checks, blood draws and baseline/endpoint clinical assessments. D, day.

Source data

Based on our previous studies in aged mice demonstrating a reduction in bone resorption and increase in bone formation following intermittent senolytic treatment[13], our primary endpoint was the percentage changes in the bone resorption marker, serum CTx from baseline to 20 weeks. In terms of bone formation,

we recognized that the effects of bone formation-stimulating agents (for example, romosozumab[22]) may be transient, based on the underlying bone biology that is still poorly understood. Thus, we specified the key secondary endpoints as percentage changes in P1NP not only at 20 weeks, but also over intermediate time points. Bone mineral density (BMD) at the radius, femur neck and spine were additional secondary endpoints.

Primary endpoint

Figure 3a shows the percentage change from baseline in serum CTx at 20 weeks, which did not differ between the D + Q versus the control groups.

Fig. 3: Percentage changes in bone turnover markers.

a–**f**, Percentage changes from baseline in serum CTx levels at 20 weeks (**a**), 2 weeks (**b**) and 4 weeks (**c**) and in serum P1NP levels at 20 weeks (**d**), 2 weeks (**e**) and 4 weeks (**f**) in control ($n = 30$) versus D + Q ($n = 30$) treated participants. Data are shown as median (interquartile range). *P* values are based on two-sided Wilcoxon rank-sum tests.

Source data

Secondary endpoints

Figure 3b,c shows the percentage changes from baseline in serum CTx at the early time points (2 and 4 weeks). The percentage changes in CTx did not differ between groups at either time point.

Figure 3d–f shows the percentage changes from baseline in serum P1NP at 20 weeks and also at the 2- and 4-week time points. Although P1NP was no different between groups at 20 weeks, it was significantly higher (relative to control) in the D + Q group at 2 weeks (difference from control, +16%, $P = 0.020$) and 4 weeks (difference from control, +16%, $P = 0.024$). Percentage changes in radius, femur neck or lumbar spine BMD did not differ between groups (Extended Data Fig. 4).

Safety and adverse events

Extended Data Table 1 lists adverse events reported by more than one participant in each group. Unsurprisingly, given the open-label study design, most adverse events were in the D + Q group. The most prominent adverse events were headache (53%) and gastrointestinal complaints. No serious or severe adverse events related to D + Q administration occurred. One participant in the D + Q group was withdrawn because of QTc interval prolongation following D administration and was lost to follow-up after week 4; a second participant in the D + Q group withdrew from active intervention at week 9 because of fatigue and other nonspecific side effects; a third D + Q participant withdrew after week 2 because of concerns about COVID. Two participants in the control group terminated the study early (at week 17 and just before week 20). All randomized participants were analyzed in the intention-to-treat protocol (Methods).

Exploratory and post hoc analyses

To better understand our findings and potentially guide future trials of senolytics, we performed additional analyses. First, although we had hypothesized that women >60 years with a high senescent cell burden would be most likely to respond to D + Q and therefore selected these women based on T cell *p16* mRNA levels above the 95th percentile of young women, we reasoned that it was possible that we had underestimated just how high the senescent cell burden needed to be to elicit a skeletal response to D + Q. In preliminary analyses, it appeared that participants in the highest tertile (T3) of T cell *p16* mRNA levels differed in their responses compared with those in the lower two tertiles (T1/T2). Thus, we examined percentage changes in the bone turnover markers stratified by baseline T cell *p16* mRNA levels (T3 and T1/T2 combined), recognizing that in the T3 group, power would be limited because of the small sample size of ~10 per study arm (control and D + Q). Despite this limitation, as shown in Fig. 4a, serum P1NP increased at 2 weeks in the T3 D + Q group (relative to control, +34%, $P = 0.035$) with a similar magnitude of increase (+38% relative to control, but with greater variability, $P = 0.160$) at 4 weeks, although these differences disappeared by 20 weeks. Likewise, serum CTx decreased at 2 weeks in the T3 D + Q group (relative to control, −11%, $P = 0.049$) with a similar magnitude of decrease (relative to control, −18%, $P = 0.053$) at 4 weeks, and a return to baseline by 20 weeks (Fig. 4b). By contrast, neither P1NP nor CTx showed any differences between groups at any time point in the T1/T2 group (Fig. 4c,d), with the exception that P1NP was lower in the D + Q group at 20 weeks compared with control. Thus, a favorable response to D + Q in markers of bone metabolism appeared to be largely driven by individuals with the highest T cell *p16* mRNA levels (T3).

Fig. 4: Time course of changes in bone turnover markers based on T3 and

T1/T2 groups.

a,**b**, Percentage changes over time in serum P1NP (**a**) and serum CTx (**b**) in the T3 group. **c**,**d**, Percentage changes over time in serum P1NP (**c**) and serum CTx (**d**) in the T1/T2 group. $n = 21$ T3 and $n = 39$ T1/T2 at 2 weeks; $n = 20$ T3 and $n = 39$ T1/T2 at 4 weeks; $n = 18$ T3 and $n = 38$ T1/T2 at 20 weeks. Data are shown as median (interquartile range). *P* values based on two-sided Wilcoxon rank-sum tests. Horizontal dashed lines indicate zero percentage change.

Source data

This pattern was also evident for radius BMD at 20 weeks, which increased by 2.7% in response to D + Q relative to control ($P = 0.004$) in the T3 group but did not differ

between groups in the T1/T2 group (Fig. 5a). BMD changes at 20 weeks at the femur neck or lumbar spine were not significant, even in the T3 group (Fig. 5b,c).

Fig. 5: Percentage changes in BMD based on T3 and T1/T2 groups.

a–**c**, Percentage changes from baseline in radius (**a**), femur neck (**b**) and lumbar spine (**c**) BMD in the T3 and T1/T2 groups. T3: $n = 19$, 19 and 15 for radius, femur neck and lumbar spine BMD; T1/T2: $n = 36$, 38 and 31 for radius, femur neck and lumbar spine BMD. Data are shown as median (interquartile range). *P* values based on two-sided Wilcoxon rank-sum tests.

Source data

The *p16* tertiles in the above analyses were based on the *p16_variant 5* transcript. To test whether the standard assay measuring *p16_variant 1 + 5* performed similarly, we

repeated the above analyses stratifying the T3 and T1/T2 groups using the *p16_variant 1 + 5* assay. As shown in Extended Data Fig. 5a, the overall pattern for longitudinal changes in P1NP was similar when T3 was defined using the *p16_variant 1 + 5* assay; however, the individual differences did not reach statistical significance. By contrast, T3 stratification using the *p16_variant 1 + 5* assay failed to identify 'responders' to D + Q based on changes in CTx (Extended Data Fig. 5b). Consistent with the *p16_variant 5* assay, changes in the T1/T2 group in P1NP or CTx did not differ between the D + Q and control participants when stratified using the *p16_variant 1 + 5* assay (Extended Data Fig. 5c,d). Thus, the *p16_variant 5* assay appeared to more effectively identify individuals likely to have a favorable skeletal response to D + Q compared with the *p16_variant 1 + 5* assay.

Assessment of SASP factors

In additional analyses, we measured a panel of 36 SASP factors at baseline and 2 weeks postdosing. As shown in Extended Data Table 2, baseline SASP factors were generally higher in the T3 group versus the T1/T2 group, with four factors (sclerostin, Fas, MMP2 and PARC) individually significant and two factors achieving borderline significance (osteoactivin and TNFR1; Extended Data Fig. 6a–f). However, specifically focusing on the T3 group where we observed biological responses to D + Q, changes in SASP factors did not differ between the control and D + Q groups (Extended Data Table 3).

Discussion

In this phase 2 RCT, we found that intermittent D + Q, at the doses and intervals we administered, did not achieve our primary outcome, a reduction in the bone

resorption marker, serum CTx. We did, however, demonstrate effects on our secondary outcome, percentage changes in the bone formation marker, serum P1NP, at 2 and 4 weeks following D + Q dosing in the overall group of women, which returned to baseline and was not different from control by 20 weeks. We note that given that our previous studies in aged mice had demonstrated a reduction in bone resorption and increase in bone formation following senolytic treatment[13,14], the choice of whether to assign CTx or P1NP as the primary endpoint was entirely arbitrary, particularly in the absence of any previous human data on the effects of senolytics on bone turnover. Our studies do clarify this issue and indicate that in humans, senolytics (or at least, D + Q with our monthly dosing regimen) may preferentially stimulate bone formation over inhibiting bone resorption, and this will help guide primary endpoints in future studies.

Systemic intermittent D + Q administration over 20 weeks to otherwise healthy postmenopausal women was safe, with no serious adverse events reported. However, as noted earlier, given the open-label design, the majority of relatively minor adverse events were reported in the D + Q-treated women.

The development of senolytic approaches and, in particular, D + Q, may or may not be successful. However, even studies that do not achieve a prespecified primary endpoint may help inform future studies. Thus, we conducted several exploratory and post hoc analyses. One of our prespecified hypotheses was that women with a higher baseline senescent cell burden may have a better response to a senolytic intervention, which is why we selected women aged 60 years or older who had T cell *p16* mRNA levels above the 95th percentile for young women. Our analyses would indicate, however, that even this was likely not a sufficiently high threshold for senescent cell burden, at least in terms of observing a beneficial skeletal response to D + Q. Specifically, we found that even within this preselected group of women, the

observed increases in serum P1NP in the overall group of D + Q-treated participants were driven principally by women in the highest tertile (T3) of T cell *p16* mRNA levels. In addition, these women also had a significant reduction in the bone resorption marker, serum CTx, at 2 weeks, followed by an increase in radius BMD at 20 weeks with monthly intermittent D + Q treatment. Importantly, these effects of D + Q on serum P1NP, CTx and radius BMD were observed despite the limited power in this group (approximately $n = 10$ participants per study arm in the T3 group), suggesting a biologically robust signal that may achieve greater statistical significance in future studies with a larger sample size of women specifically with a higher senescent cell burden. Moreover, the observation that women in the T3 group had increases in P1NP, reductions in CTx and increases in radius BMD—with no efficacy signal detected in the T1/T2 group—suggests a biologically consistent effect rather than statistical artifact. We acknowledge, however, that exploratory analyses such as this, even if based on a plausible, prespecified hypothesis, do not definitively establish that women with high T cell *p16* mRNA levels would achieve skeletal benefits following D + Q treatment, and further studies are needed to rigorously test this hypothesis. In addition, given that D + Q represents a first-generation senolytic cocktail, an important question is whether future, more potent, senolytics will demonstrate skeletal efficacy in older women with a lower senescent cell burden than that required to elicit a favorable response to D + Q. Moreover, studies are needed to evaluate whether any potential threshold senescent cell burden is the same across tissues and/or functional outcomes (for example, prevention of bone loss versus alleviation of frailty or other endpoints). Importantly, this hypothesis, if true, also has broader implications for the use of senolytics in humans. Thus, to the extent that cells and tissues in specific disease states (diabetic or macular degeneration and diabetic kidney disease, idiopathic pulmonary fibrosis, Alzheimer's disease, among others) have a higher senescent cell burden than normal tissues[23], these diseased tissues within an individual may be

more susceptible to senolytic treatments than normal tissues. Thus, our biologically plausible exploratory analyses showing a better skeletal response in women with the highest senescent cell burden have implications not only for the use of senolytics in normal aging, but also potentially in specific disease states.

The attenuation of the bone formation response by 20 weeks we observed in the overall group of women as well as in the T3 group is most likely due to the underlying bone biology determining the response to drugs that stimulate bone formation. Specifically, the time course of changes in bone formation we observed over 20 weeks of intermittent D + Q treatment in the T3 group is virtually identical (albeit smaller in magnitude) to that observed following treatment with the FDA-approved sclerostin inhibitor, romosozumab, where serum P1NP increased rapidly over the first 1–4 weeks following dosing and then returned to baseline by 24 weeks[22]. Moreover, the simultaneous, acute increase in P1NP and reduction in CTx that we observed in the T3 group is qualitatively identical (but smaller in magnitude) to the effects of sclerostin inhibition on bone markers in humans[22], and arguably pathognomonic of the consequences of sclerostin inhibition on bone. Interestingly, *Sost* mRNA levels are reduced in osteocyte-enriched bone samples in old mice following senescent cell clearance[13,14]; moreover, in vitro studies have identified *SOST* as a legitimate SASP factor that increases following induction of senescence in nonskeletal human cells, particularly in endothelial cells[24]. These preclinical findings, combined with the remarkable similarity in the time course of changes in P1NP and CTx following D + Q treatment in the T3 group compared with romosozumab treatment[22], strongly suggest that at least part of the skeletal effect of D + Q treatment may be through a reduction in sclerostin production. However, although serum sclerostin levels were higher in the T3 group versus the T1/T2 group at baseline, we did not find reductions in circulating sclerostin levels in the T3 D + Q-treated participants. This may be

because sclerostin functions primarily as a paracrine factor[25], so local reductions in sclerostin in the bone microenvironment following senolysis may not be reflected by circulating sclerostin levels. Alternatively, it is possible that there is an acute reduction in local and systemic sclerostin levels (note that with romosozumab, serum P1NP levels increased within a week of the first dose[22]) that triggers changes in bone turnover, but perhaps because of compensatory changes, alterations in circulating sclerostin levels are no longer evident by 2 weeks following dosing, which was our time point for measuring SASP factors. It is also important to note that although these findings indicate that at least some of the effects of D + Q (and perhaps other senolytics) are mediated via modulation of sclerostin levels (or possibly other effects on Wnt signaling in bone), the advantage of senolytics would be their potential nonskeletal effects on frailty, metabolic dysfunction and other age-associated morbidities[16], in addition to their skeletal actions.

A second possibility for the attenuation of bone formation over time is that with prolonged senolytic treatment, there is an emergence of some type of senolytic 'resistance.' Arguing against this is the observation from our preclinical studies that senescent osteocytes remained reduced in mice following 4 months of monthly D + Q administration, with similar findings noted in our genetic clearance models[13,14]. Nonetheless, this is an important question that needs to be addressed in future studies. Along these lines, we used D + Q dosing every 4 weeks based on our previous studies in mice where this dosing regimen was effective in preventing age-related bone loss[13]. However, our finding that the most robust changes in P1NP and CTx in the T3 group were present at 2–4 weeks postdosing but absent following multiple dosing cycles at 20 weeks raises the possibility that perhaps even more intermittent senolytic administration (for example, every 5–6 months) may result in repeated, transient increases in bone formation and reductions in bone resorption with

minimal risk. Under this scenario, senolytic clearance every 6 months or so may result in skeletal benefits in women with a high senescent cell burden, along with potential nonskeletal benefits (for example, frailty). This intriguing possibility clearly warrants further investigation in future preclinical and clinical studies.

We were not able to demonstrate effects of D + Q on circulating SASP markers. However, there are several possible explanations for this finding, including that D + Q was developed based on targeting senescent mesenchymal and endothelial cells[7]. Thus, circulating SASP factors, which likely originate largely from immune cells unaffected by D + Q, may not reflect changes at the tissue level. Consistent with this, although we previously reported that administration of D + Q to patients with diabetic kidney disease reduced expression (using a global gene set enrichment analysis) of a senescence/SASP gene set (SenMayo) in adipose tissue biopsies, analysis of peripheral blood mononuclear cells failed to find a similar signal[26]. As such, future clinical trials of senolytics may need to obtain tissue biopsies to demonstrate treatment effects on the SASP or senescent cells in specific tissues of interest. A limitation of our study is that although we used the T cell *p16* mRNA assay to stratify participants at baseline, because of logistical and cost issues, we were not able to obtain follow-up T cell *p16* mRNA measurements in response to D + Q treatment, relying rather on the peripheral SASP measures to assess treatment response. Thus, the question of whether T cell *p16* mRNA levels are responsive to senolytic treatments needs to be addressed in future studies.

Given that we were unable to demonstrate effects of D + Q on circulating SASP markers, the evidence that the observed skeletal responses following D + Q administration reflect senescent cell clearance, rather than potentially direct effects of D + Q on osteoclasts or osteoblasts, is admittedly indirect. The fact that we found a biological signal for positive effects on bone turnover combined with improved

radius BMD exclusively in women with the highest senescent cell burden (group T3) would be consistent with effects of D + Q on senescent cells driving our findings. Furthermore, both D and Q have very short half-lives (3–4 h[27] and 3.5 h[28], respectively), so once-monthly administration of these drugs would be very unlikely to have direct effects on bone turnover weeks following administration.

We found an effect on radius BMD in the T3 group, but no detectable effects on femur neck or spine BMD. Here it is important to note that our study was relatively short in duration (20 weeks) and powered for changes in bone turnover markers, whereas changes in BMD typically require longer treatment duration and much larger numbers of participants. We observed similar findings in a previous, identically designed RCT of beta-blockers where relatively β_1-selective blockers reduced bone resorption and increased BMD at the radius, but not central sites, presumably because of the greater precision for the dual-energy X-ray absorptiometry (DXA) measurements at the radius[29].

We also recognize that our stratification for senescent cell burden relied on peripheral blood T cell *p16* levels. Importantly, there is growing support for use of this biomarker in providing an index of organismal senescence. Indeed, T cell *p16* levels have been demonstrated to increase with age[20], physical inactivity[20] and smoking[20]; to be associated with plasma interleukin-6 (IL-6) levels (a marker of human frailty)[20]; to increase following chemotherapy;[30] and to predict length of hospital stay after coronary artery bypass surgery in older adults[31]. Moreover, we also found that baseline SASP measures were generally higher in the T3 group with the highest T cell *p16* levels across our stratified groups. Thus, stratification based on T cell *p16* levels is supported by multiple lines of evidence, although additional markers quantifying total body and perhaps tissue-specific senescent cell burden are clearly needed. Along these lines, our data indicate that measurement of T cell

p16_variant 5 rather than the standard $p16^{Ink4a}$ assay (*p16_variant 1 + 5*) may have better specificity to predict biological responses to senolytics, although further studies comparing both assays are needed to evaluate this issue.

Our study represents the largest ($n = 60$) RCT of sustained intermittent administration of a senolytic regimen to generally healthy older women. Previous studies of systemic administration of senolytics have not included a control group, recruited only a limited number of patients with diabetes-associated chronic kidney disease ($n = 9$)[32], idiopathic pulmonary fibrosis ($n = 14$)[33], dementia ($n = 5$)[11] or diabetic macular edema ($n = 8$)[12] and were focused primarily on safety or biodistribution and a battery of SASP measures.

We recognize several limitations of our study, many of which have been discussed above. In addition to those already noted, we acknowledge that this study was an early phase attempt at translating key findings regarding the effects of a senolytic regimen on bone from mice to humans. Our primary outcome was not met, although as noted earlier, P1NP could also justifiably have been chosen as our primary outcome based on our animal data[13,14]. Nonetheless, this study did provide important insights that have generated hypotheses for future studies to test. We also acknowledge that we only studied women to have an adequate sample size, and future studies including both sexes and their response to senolytics need to be performed.

In summary, we found that intermittent D + Q, with the dosing regimen and intervals we administered, did not have significant effects on bone resorption in otherwise healthy postmenopausal women. In the overall group of women, we did find a stimulation of bone formation at 2–4 weeks following initiation of intermittent D + Q administration that waned by 20 weeks, as has been observed with other drugs stimulating bone formation such as romosozumab[22]. In exploratory analyses, serum

P1NP increased more robustly and CTx decreased at 2–4 weeks, followed by improved radius BMD at 20 weeks postdosing in the women with the highest senescent cell burden as assessed by the T cell *p16* assay. Collectively, our findings support the hypothesis that the skeletal, and perhaps nonskeletal, response to a senolytic intervention depends on the existing senescent cell burden. Rigorously testing this hypothesis in future studies will, however, also likely require the continued development and validation of additional biomarkers to assess senescent cell burden across tissues in humans.

Methods

Study design

All studies were performed at the Mayo Clinic outpatient Clinic Research and Trials Unit (CRTU) following IRB approval (protocol no. 18-010546; IND no. 145558). Participants were provided a compensation of US$400 for completion of the study, with a prorated amount provided for early withdrawal. This amount was approved by the IRB as compensation for time and inconvenience. The first participant enrolled on 9 June 2020, and the last participant enrolled on 15 November 2022. At screening, the nurse study coordinator explained the study, including potential benefits and risks to volunteer participants. After obtaining written informed consent and following comprehensive screening studies (including blood draw after 12-h overnight fast for the T cell *p16* assay and blood counts/clinical chemistries), participants who met eligibility criteria (as detailed below) and agreed to take the assigned therapy for the entire study duration, were randomized to one of two groups: (1) control, or (2) D (100 mg per day for two consecutive days; Sprycel, Bristol Myers Squibb) plus Q (250 mg four times a day (1,000 mg per day) for three consecutive days; Quercetin Phytosome, Thorne Research). D + Q was taken orally on an intermittent schedule

with no-therapy periods in between doses, repeated every 28 days over 20 weeks, resulting in five total dosing sessions throughout the entire intervention (Fig. 2). The first dose of D + Q in each cycle was administered under supervision in the CRTU; for subsequent doses, participants were provided with a log to fill out and asked to return the medication bottles to the study coordinator at the next visit.

Block randomization was performed by the study statistician to ensure balanced group assignments. Doses were administered onsite by the nurse study coordinator who provided eligible, randomized participants with their allocated therapy from the Mayo Clinic Research Pharmacy. Details regarding the study visits and assessments are provided in Supplementary Table 1. Symptom checks were performed by phone call the day following each visit. Throughout the study, all participants were administered 1,000 IU of vitamin D and were counseled to obtain a total daily calcium intake of ~1,200 mg. Symptom questionnaires were administered at each onsite study visit (Fig. 2 and Supplementary Table 1) to assess safety, tolerability and potential side effects; mild to serious adverse events were examined and documented. At each visit, a fasting morning blood draw was obtained. Participants were given a blank notebook for recording adverse events that the study team reviewed and documented at each visit.

Selection of study participants

We randomized 60 eligible postmenopausal women (follicle-stimulating hormone >20 IU l^{-1}; sex/gender based on self-reporting) aged 60–90 years to one of two groups: control or D + Q. Exclusion criteria were as follows: (1) a history of diabetes or hemoglobin A1c ≥6.5%; (2) any screening laboratory abnormality (as detailed below); (3) the presence of significant liver or renal disease, heart failure, malignancy, malabsorption, hypo- or hyperparathyroidism, Cushing's syndrome or

hypopituitarism; and (4) undergoing treatment with any medications that affect bone turnover, including beta-blockers, corticosteroids (oral for >3 months within the past year), anticonvulsants, supraphysiologic thyroid hormone doses, bisphosphonates (within the past 3 years), denosumab, estrogen or selective estrogen receptor modulators, or teriparatide/abaloparatide or romosozumab (within the past year). Participants with any fracture within the past 6 months were excluded (because fracture may alter bone turnover in this period)[34], but we did not specifically exclude participants with BMD values at the lumbar spine or femur neck in the osteopenic or osteoporotic range. Participants with hypovitaminosis D (25-hydroxyvitamin D (25(OH)D) <20 ng ml^{-1}) were treated with 1,000 IU d^{-1} of vitamin D for 8 weeks. If the level remained <20 ng ml^{-1}, they received a second 8-week course; if their 25(OH)D still remained <20 ng ml^{-1}, they were not enrolled but referred to their primary provider. We also obtained a screen visit electrocardiogram and excluded participants with a corrected QT (QTc) interval >450 ms because prolongation of the QTc interval has been observed in a subset of patients with chronic myelogenous leukemia taking long-term daily D[35]. As such, patients taking antiarrhythmic medications known to cause QTc prolongation or antiplatelet/coagulant medications were also excluded. Similarly, participants treated with quinolone antibiotics or drugs metabolized by the same liver enzymes as D or Q were excluded. As noted earlier, participants were only eligible if they had a T cell *p16* mRNA expression level greater than the 95th percentile of young female controls.

Screen visit

Screening included a brief history, physical examination, electrocardiogram and the following fasting blood screening tests: complete blood count, hemoglobin A1c, serum calcium, creatinine, aspartate aminotransferase, follicle-stimulating hormone and 25(OH)D.

Dosages of D + Q

D is an FDA-approved tyrosine kinase inhibitor used clinically to treat hematologic disorders with a long-standing, acceptable safety profile[36,37]. The oral dose of D (100 mg per day) selected was based on the FDA-approved dose for chronic therapy as effective for inducing apoptosis in human cancer cells. The oral dose of Q (a natural flavanol present in many fruits and vegetables)[38] selected was 1,000 mg per day for three consecutive days using the same intermittent dosing regimen as D (see below), based on a previous preliminary efficacy signal on serum SASP markers[32] and SASP genes in adipose biopsies[26] following a similar, single course of D + Q in patients with diabetic chronic kidney disease[32].

Evaluation of safety and tolerability

We monitored and rigorously documented all participants who withdrew from the study, the reasons for withdrawal and all adverse events (serious and nonserious). This information is detailed in the CONSORT flow diagram (Fig. 1) and was provided to the Data Safety Monitoring Board. The Common Terminology Criteria for Adverse Events was used for adverse event reporting in this RCT.

Study endpoints

The primary study endpoint was the percentage change in CTx from baseline over 20 weeks. Secondary endpoints included changes in serum P1NP, as well as in serum CTx at 2, 4 and 20 weeks. In addition, we assessed percentage changes in BMD by DXA (radius, femur neck and lumbar spine) at 20 weeks. Additional endpoints were safety, tolerability and circulating SASP factors.

Measurement of T cell *p16* mRNA levels

At baseline, morning fasting peripheral whole blood (10 ml) was obtained from the participants for measuring *p16* mRNA in $CD3^+$ peripheral blood T cells via qPCR. In brief, peripheral blood $CD3^+$ cells were isolated from human whole-blood samples (10 ml) using an automated magnetic cell sorting machine (AutoMACS pro separator; Miltenyi Biotec). The cells were tagged with $CD3^+$ microbeads (Miltenyi Biotec) before they were sorted via positive separation with the magnetic cell sorting machine. The isolated $CD3^+$ cells were then spun down and stored in QIAzol lysis reagent (TRIzol). Total RNA was isolated using RNeasy Mini Columns (Qiagen). DNase treatment was applied to degrade potential contaminating genomic DNA using an on-column RNase-free DNase solution (Qiagen). RNA quantity and purity were confirmed with a Nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), according to the manufacturer's instructions. PCR reactions were run using the ABI Prism 7900HT Real Time System (Applied Biosystems) with SYBR Green (Qiagen) as the detection method.

We used forward and reverse primers that measured both the *p16_variant 1* and *p16_variant 5* combined (Extended Data Fig. 1 and Extended Data Table 4; note that for a qPCR assay, it is not feasible to design primers only for *p16_variant 1*). We also designed primers specific for *p16_variant 5* (Extended Data Fig. 1 and Extended Data Table 4).

Population cohort for analyses of T cell *p16* mRNA level

Using the identical protocol as described above, morning fasting peripheral whole blood (10 ml) was obtained from a population-based cohort of 228 healthy women,

aged 23–88 years, for measuring *p16* mRNA in $CD3^+$ peripheral blood T cells via qPCR.

Serum biochemical and SASP assays

Fasting serum and EDTA plasma (10 ml each) were drawn at baseline and at 2, 4 and 20 weeks and stored at −80 °C in 1.0-ml aliquots for batched analyses of the following biochemical assays. As we have established[29,39,40], serum P1NP and CTx were measured with standard, validated assays: P1NP by radioimmunoassay (Orion Diagnostica, coefficient of variation <9%); and CTx by one-step ELISA (Nordic Bioscience Diagnostics, coefficient of variation <8%). SASP proteins were measured in patient morning fasting plasma samples obtained at baseline and at 2 weeks using commercially available multiplex magnetic bead immunoassays (R&D Systems) based on a Luminex xMAP multianalyte profiling platform. These assays were performed according to the manufacturer's protocols and analyzed on a MAGPIX System (Merck Millipore), as described previously[24].

DXA measurements

DXA scans of the lumbar spine (L1–L4), hip (femoral neck) and radius were obtained at baseline and 20 weeks (study endpoint) using the Lunar iDXA (GE Medical Systems; Encore software v.12.2) in the Mayo Clinic CRTU by a licensed X-ray technician with long-term experience[29,39,40]. Phantoms were scanned to detect potential drift and results were appended to a quality control database.

Statistical analyses

Unless otherwise indicated, all data are presented as median (interquartile range). The study endpoints were expressed as a percentage of baseline for the figures and statistical tests. Comparisons between the control and D + Q groups were made using

the Wilcoxon rank-sum test for continuous variables and the chi-squared test for categorical variables; a P value <0.05 was considered significant. Spearman correlation coefficients were used to summarize relationships with T cell *p16* mRNA levels. Sensitivity analyses were conducted using imputed values and using an analysis of covariance model with treatment as a fixed effect and the baseline value as a covariate. Using previously published data[29], power calculations were conducted, while allowing for an estimated withdrawal rate of 10%. This calculation demonstrated that 30 participants per group would provide the study with 90% power to detect a 19.8% difference in CTx between the D + Q and control groups.

Statistics and reproducibility

In this human trial, the experiments were conducted once and all relevant data are presented.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All information on materials and reagents is provided in the Methods and Supplementary Methods. Individual deidentified participant data that underlie the results reported in this article (text, tables, figures) are available as an Excel file in the Supplementary Information. The study protocol, which includes the statistical analysis plan, is also available in the Supplementary Information. No restrictions are placed on the availability of this data. Source data are provided with this paper.

Code availability

No code was used in the analyses.

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Contributions

J.N.F. and S.K. conceived and directed the project, with input from E.J.A., J.S., M.T.D., T.T., N.K.L., J.L.K., M.D., D.S. and D.G.M. T.L.V. and A.J.T. recruited the study participants and conducted the study. E.J.A. and S.J.A. performed all the statistical analyses. I.B., K.Y. and N.K.L. provided population samples for T cell *p16* assays. S.J.V. and M.R. were responsible for handling and managing all study samples. J.N.F. and S.K. wrote the manuscript, which all authors then reviewed and approved.

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Ethics declarations

Competing interests

N.K.L., T.T. and J.L.K. have a financial interest related to this research, including patents and pending patents covering senolytic drugs and their uses that are held by Mayo Clinic. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with the Mayo Clinic's conflict of interest policies. The remaining authors declare no competing interests.

Peer review

Peer review information

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Additional information

Extended data

Extended Data Fig. 1 Alternative splicing of the human *p16* proximal promoter produces two distinct variants.

Schematic representation of the proximal *p16* promoter and alternative splicing patterns of the pre-mRNA, indicating the *p16*-variant 1 mRNA (Exons 1/2/4; NM_000077) and the *p16*-variant 5 mRNA (Exons 1/2/3/4; NM_001195132). The protein products between the two variants encode identical proteins from amino acids 1-152, however the *p16*-variant 5 protein has a unique 15 amino acid C-terminal sequence in place of the 4 amino acid C-terminal sequence of the *p16*-variant 1 protein, due to the retained Exon 3 in the *p16*-variant 5 mRNA. PCR primer sequences are indicated, demonstrating that the *p16_variant 1 + 5* primer pair amplifies both variant 1 and 5 isoforms, whereas the *p16_variant 5* primer pair only amplifies *p16*-variant 5.

Source data

Extended Data Fig. 2 Distribution by age and age correlations of *p16* variants in women aged < 60 years.

Distribution by age in a cohort (n = 228, age 23-88 years) of women for (**a**) the *p16_variant 1 + 5* and (**b**) the *p16_variant 5*. Correlations with age for (**c**) *p16_variant 1 + 5* and (**d**) *p16_variant 5* in women < 60 years of age (n = 42). Spearman correlation coefficients are shown.

Source data

Extended Data Fig. 3 Age correlations of *p16* variants in women aged > 60 years.

Correlation with age for (**a**) *p16_variant 1 + 5* and (**b**) *p16_variant 5* in women > 60 years of age (n = 186). Correlations with age specifically in the study participants for (**c**) *p16_variant 1 + 5* and (**d**) *p16_variant 5* (n = 60). Spearman correlation coefficients are shown.

Source data

Extended Data Fig. 4 Percent changes in BMD.

Percent changes from baseline in (**a**) radius, (**b**) femur neck, and (**c**) lumbar spine BMD. n = 55, 57, and 46 for radius, femur neck, and lumbar spine BMD. Data are shown as Median (IQR); *P*-values based on two-sided Wilcoxon rank-sum tests.

Source data

Extended Data Fig. 5 Time course of changes in bone turnover markers based on T3 and T1/T2 tertiles derived from *p16_variant 1 + 5*.

Percent changes over time in (**a**) serum P1NP and (**b**) serum CTx in the T3 group; percentage changes over time in (**c**) serum P1NP and (**d**) serum CTx in the T1/T2

group. n = 20 T3 and 40 T1/T2 at 2 weeks; n = 19 T3 and 40 T1/T2 at 4 weeks; n = 18 T3 and 38 T1/T2 at 20 weeks. Data are shown as Median (IQR); *P*-values based on two-sided Wilcoxon rank-sum tests.

Source data

Extended Data Fig. 6 Select SASP factors in T1/T2 vs T3 groups.

(**a**) Sclerostin, (**b**) Fas, (**c**) MMP2, (**d**) PARC, (**e**) Osteoactivin, and (**f**) TNFR1 levels in the T1/T2 vs T3 groups. n = 21 T3 and 39 T1/T2 weeks. Data are shown as Median (IQR); *P*-values based on two-sided Wilcoxon rank-sum tests.

Source data

Extended Data Table 1 Adverse events in the study participants

Extended Data Table 2 Baseline circulating senescence-associated secretory phenotype (SASP) factors in the study subjects stratified by *p16* tertiles

Extended Data Table 3 Comparisons of percentage changes in circulating senescence-associated secretory phenotype (SASP) factors at 2 weeks in the T3 study subjects between the control and D + Q groups

Extended Data Table 4 Primer sequences

Supplementary information

Supplementary Information

Supplementary Table 1.

Reporting Summary

Source data

Source data

Source data for Table 1, Figs 3a-f, 4a-d, 5a-c, Extended Data Tables 2, 3 and Extended Data Figs 2a-d, 3a-d, 4a-c, 5a-d, 6a-f .

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