

EXTENDED REPORT

Patients with gout have short telomeres compared with healthy participants: association of telomere length with flare frequency and cardiovascular disease in gout

N Vazirpanah,^{1,2} L B E Kienhorst,³ E Van Lochem,⁴ C Wichers,^{1,2} M Rossato,^{1,2} P G Shiels,⁵ N Dalbeth,⁶ L K Stamp,⁷ T R Merriman,⁸ M Janssen,⁴ T R D J Radstake,^{1,2} J CA Broen^{1,2}

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For numbered affiliations see end of article.

Correspondence to

J CA Broen, WKZ Lundlaan 6 KC 02.085.2 P.O. Box 85090, Utrecht 3508 AB, The Netherlands; j.c.a.broen@umcutrecht.nl

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ABSTRACT

Aim and background Chronic inflammation associates with increased senescence, which is a strong predictor for cardiovascular disease. We hypothesised that inflammation accelerates senescence and thereby enhances the risk of cardiovascular disease in gout.

Methods We assessed replicative senescence by quantifying telomere length (TL) in a discovery cohort of 145 Dutch patients with gout and 273 healthy individuals and validated our results in 474 patients with gout and 293 healthy participants from New Zealand. Subsequently, we investigated the effect of cardiovascular disease on TL of all participants. Also, we measured TL of CD4⁺ and CD8⁺ T lymphocytes, B lymphocytes, monocytes, natural killer cells and plasmacytoid dendritic cells. Additionally, we assessed the potential temporal difference in TL and telomerase activity.

Results TL in PBMCs of healthy donors decreased over time, reflecting normal ageing. Patients with gout demonstrated shorter telomeres ($p=0.001$, $R^2=0.01873$). In fact, the extent of telomere erosion in patients with gout was higher at any age compared with healthy counterparts at any age ($p<0.0001$, $R^2=0.02847$). Patients with gout with cardiovascular disease had the shortest telomeres and TL was an independent risk factor for cardiovascular disease in patients with gout ($p=0.001$). TL was inversely associated with the number of gouty flares ($p=0.005$).

Conclusions Patients with gout have shorter telomeres than healthy participants, reflecting increased cellular senescence. Telomere shortening was associated with the number of flares and with cardiovascular disease in people with gout.

INTRODUCTION

Gout is a rheumatic disease characterised by deposition of monosodium urate crystals in and around the joints, associated with elevated serum urate levels. Gout is the most common inflammatory arthritis in men older than 40 years and it affects 1–5% of adults in the Western populations.^{1–5}

Patients experience recurrent episodes of acute joint inflammation and, in the presence of persistent hyperuricaemia, may also develop chronic joint inflammation. We recently demonstrated that patients with gout display high levels of

inflammatory mediators such as interleukin (IL)-8 even though these patients did not have clinically apparent inflammatory disease at the time of sample collection.⁶

Chronic and recurrent inflammation activates the immune system, elevating the demand for immune cells, damaging tissues and forcing cell replication. This can ultimately eventuate a state of replicative senescence in most cells. Replicative senescence is when cells can no longer divide to form new cells. These senescent cells secrete pro-inflammatory markers such as IL-6 and IL-1a.⁷ The most commonly used marker for replicative senescence is telomere length (TL). Telomeres function as a non-coding protective end region of chromosomes. Due to the DNA end-replication inefficiency of polymerases, chromosomes shorten every cell division. After a certain number of divisions, the threshold of attrition is reached, cell-cycle arrest occurs and eventuates the senescent state. Telomeres therefore are indicative of the number of divisions the cell lineage has undergone and are a measure of replicative senescence.

Since recurrent and chronic inflammation leads to a pro-inflammatory environment, measuring replicative senescence by TL has been a subject of scrutiny in inflammatory diseases.⁸ Telomere shortening has been repeatedly associated with multiple inflammatory diseases. Previously, TL has been associated with lifespan, although this remains controversial, and with cardiovascular disease.⁹ The latter association has been recently substantiated in a large meta-analysis including >50 000 individuals.¹⁰ This study showed a significantly increased relative risk for developing cardiovascular and cerebrovascular disease in the tertile of the population with the shortest telomeres. Causality between senescence and cardiovascular diseases is supported by the presence of a high number of senescent cells in atherosclerotic plaques, enhancing inflammation and decreasing recovery potential. Endothelial cells, vascular smooth muscle cells (VSMCs) and immune cells are involved. The reduced proliferative capacity of endothelial cells and VSMCs, together with the pro-inflammatory environment, leads to plaque instability, precluding vascular events.^{11–13}



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Gout is often accompanied by cardiovascular comorbid conditions including myocardial infarction (MI) and cerebrovascular events, which lead to an increased morbidity and decreased life span.^{14 15} Gout is also associated with metabolic syndrome, diabetes and renal failure, which in turn are predictors for cardiovascular disease. However, patients with gout remain at higher risk for developing cardiovascular disease after correction for these variables.¹⁶

We hypothesise that chronic and recurrent acute inflammation in gout accelerates replicative senescence, increasing the risk of cardiovascular disease. That is, patients with gout with the highest burden of replicative senescence will also have the highest burden of cardiovascular disease. To test our hypothesis, we investigated the most studied hallmark of replicative senescence, TL of PBMCs in two independent cohorts consisting of patients with gout and healthy individuals, both with and without cardiovascular disease. Also, we investigated telomere dynamics in various immune cell subsets, scrutinised TL in a follow-up cohort and quantified human telomerase reverse transcriptase (*hTERT*) (encoding human telomerase reverse transcriptase) gene expression.

METHODS

Discovery cohort and validation cohort

In our discovery cohort, we compared TL of PBMCs in 145 patients with gout with 273 Dutch participants (see online supplementary table S1). In our validation cohort, we assessed TL in PBMCs collected from 474 Caucasian patients with gout and 293 healthy participants participating from New Zealand (see online supplementary table S1).

This study was performed according to the guidelines of the Declaration of Helsinki and meets the ethical approval of the Ethical and Review committee of the University Medical Centre of Utrecht, Rijnstate Hospital, in Arnhem (both the Netherlands) and the Multi-Region Ethics Committee in New Zealand. All the participants in this study gave their informed consent to participate.

Isolation of mononuclear immune cell subsets

Mononuclear cell subsets were sorted from total PBMCs isolated by Ficoll (Ficoll-Paque Plus, GE Healthcare) from peripheral blood of 10 Dutch patients with gout and 11 healthy participants (see online supplementary table S3). Sorting the mononuclear cell subsets was performed using fluorescence-activated cell sorting (FACSARIA_III, BD Biosciences) to separate the cells according to the expression of cellular surface molecules; CD3⁺/CD56⁻/CD4⁺ for T helper lymphocytes, CD3⁺/CD56⁻/CD8⁺ for cytotoxic T lymphocytes, CD19⁺/CD20⁺ for B lymphocytes, CD14⁺ monocytes, CD3⁻/CD56⁺ natural killer (NK) cells and CD123 (IL3RA)⁺/CD304 (BDCA4)⁺ plasmacytoid dendritic cells (pDCs).

TL measurements

DNA of PBMCs was extracted using a standard salting-out protocol. Absolute TL quantification¹⁷ modified with synthetic standards as described by O'Connell *et al*¹⁸ was performed using the quantitative PCR method.

hTERT gene expression

hTERT gene expression level was quantified using synthesised cDNA (Biorad iScript kit) from RNA that was extracted from the PBMCs. Patients with gout (N=64) and healthy participants (N=89) from the Dutch cohort were randomly chosen. Quantstudio QPCR apparatus with TaqMan assay (Applied

Biosystems) were used to perform QPCR under conditions as specified by the manufacturer. To normalise the *hTERT* gene expression, the housekeeping *GUSB* and *GAPDH* genes were included. A similar method was used to assess *hTERT* gene expression in 10 patients with gout and 11 healthy participants immune cell subtypes separately.

Statistical analyses

For statistical analysis, IBM SPSS Statistics V.23 (SPSS, Chicago, Illinois 60606 USA) and GraphPad Prism V.6 (GraphPad Software, San Diego, California, USA) applications were applied. TL (in kilobase pairs) of patients with gout and healthy participants were log-transformed to normalise the distribution. A two-tailed t-test was used when appropriate with Welch's correction for comparing the unequal variances between the groups. Three independent linear regression models were applied to separately estimate (a) the interpolation between TL and age, gender, smoking, cardiovascular disease, heart failure, stroke (non-fatal), MI (non-fatal), angina pectoris, systolic and diastolic blood pressure, body mass index (BMI) (kg/m²), serum UA level (mmol/L) and creatinine level (μmol/L) in patients and healthy individuals; (b) the association between TL and the presence of cardiovascular events (heart failure, stroke (non-fatal), MI (non-fatal), angina pectoris and age) severally in individuals with gout; and (C) the relation between TL and gout hallmarks including attack frequency, age at the first attack, disease duration, serum UA level (mmol/L) and creatinine level (μmol/L) severally in patients group (table 2).

In addition, the difference in TL between gout patients with or without cardiovascular events (a) and separately the association between flare frequency and cardiovascular events (angina pectoris, heart failure, MI (non-fatal) and stroke (non-fatal)) (b) is represented in online supplementary table S4. Univariate analysis was performed to examine the effect of variables such as gender, age, smoking, BMI and creatinine levels on TL (see online supplementary table 5). Where appropriate, two-tailed Pearson's or Spearman's bivariate correlations were applied to assess the correlation between the variables (p<0.05). The Bonferroni correction was applied to account for multiple testing.

RESULTS

Demographics

Baseline characteristics of participants from the Netherlands and New Zealand are summarised in table 1.

PBMCs from patients with gout have shorter telomeres compared with their healthy counterparts in two unrelated cohorts

Within our Dutch discovery cohort, we analysed the TL of PBMCs of 273 healthy participants and 145 patients with gout and correlated the results with their age in year. The TL in patients with gout was significantly shorter than healthy participants (see online supplementary table S1). In patients with gout, linear regression analyses demonstrated no association between age and TL (p>0.05, R²=0.002853) (telomere shortening=0.002082*age+4.01). This was in contrast to the significant inverse/negative correlation between TL and age in healthy participants (p<0.0001, R²=0.3721) (telomere shortening=-0.02180*age+5.31) (see online supplementary figure S1).

Next our results in the independent cohort of New Zealand Caucasian showed that TL was significantly shorter in patients with gout (N=474) compared with healthy participants (N=293) (see online supplementary table S2). Moreover, we

Table 1 Baseline characteristics of Dutch and New Zealand patients and healthy participants

	Gout (n=619)	Healthy participants (n=566)	p Value
Male, n (%)	489 (79.00)	398 (70.32)	0.247
Age (mean±SD)	62.8±14.15	59.60±13.67	0.0001
Age at the first flare (mean±SD)	51.12±16.75	–	–
Disease duration (year) (mean±SD)	12.90±12.28	–	–
Colchicine (yes), n (%)	254 (50.50)	1 (0.17)	0.247
Non-steroidal anti-inflammatory drug (yes), n (%)	353 (75.50)	2 (0.35)	0.433
Allopurinol (yes), n (%) (mean 200 mg/day)	337 (67.10)	1 (0.17)	0.0001
Corticosteroids (yes) n (%)	197 (40.20)	1 (0.17)	0.643
CVD (yes/no) n (%)	270 (43.62)	43 (7.59)	0.0001
Diabetes (type 2) (yes), n (%)	87 (14.02)	19 (3.36)	0.0001
Stroke (yes/no), n (%)	43 (6.95)	67 (11.83)	0.0001
Myocardial infarction (non-fatal) (yes), n (%)	70 (8.60)	66 (11.60)	0.054
Heart failure (yes), n (%)	70 (11.31)	64 (11.31)	0.270
Angina pectoris (yes), n (%)	37 (5.98)	7 (1.24)	0.0001
Creatinine level (μmol/L) (mean±SD)	105.73 (±39.38)	93.44 (±30.29)	0.0001
Body mass index (kg/m ²) (mean±SD)	29.73 (±5.60)	28 (±6.37)	0.0001
Smoking (yes) n (%)	51 (8.24)	5 (0.90)	0.0001
Serum urate (mmol/L) (mean±SD)	0.43 (±0.13)	0.35 (±0.10)	0.0001
Total number of flares per year (mean±SD)	8.75±(12.67)	–	–
Presence of tophi (yes/no), n (%)	116 (21.40)	–	–
Systolic blood pressure (mm Hg) (mean±SD)	139.90 (±20.55)	136 (±17.64)	0.039
Diastolic blood pressure (mm Hg) (mean±SD)	80.40 (±11.83)	80.00 (±9.62)	0.559
Telomere length (total leucocytes) (mean±SD)	5924.90 (±8617.76)	8242.70 (±8482.00)	0.0001

The data are presented as mean±SD. The significance of the association between the two classified subgroups of gout and healthy participants was tested using Fisher's exact test (categorical values) and Mann-Whitney U test (non-parametrical continues values) (p<0.05).

found a negative association between TL and age in patients with gout ($p=0.0014$, $R^2=0.02276$) (telomere erosion= $-0.004926 \times \text{age} + 3.51$). In healthy participants, we found a negative association with age ($p<0.0001$, $R^2=0.111$) (telomere erosion= $-0.01089 \times \text{age} + 3.94$) (see online supplementary table S2 and figure S2).

Given the similarities in TL of PBMCs in both the discovery and replication cohort, we pooled the data (figure 1). When corrected for age, patients with gout have significantly shorter telomeres in PBMCs compared with healthy participants ($p<0.0001$). TL correlates negatively with age in patients with gout ($p=0.0010$, $R^2=0.01873$) (TL= $-0.006084 \times X + 3.79$). Similarly, PBMCs TL in healthy participants were inversely correlated with age ($p<0.0001$, $R^2=0.02847$) (TL= $-0.006349 \times X + 3.99$).

Finally, we had access to follow-up data on TL from nine patients with gout involved in the Dutch cohort of whom 88.90% were male 46–87 years old with a median age of 63 years. The time period between the inclusion date and the follow-up at the second time point was from 1 to 11 months with an average of 4.33 months. There was no ($p=0.34$) change in TL of the same patients with gout measured at two different time points (T1=4.20 kbp and T2=4.29 kbp).

Gout and cardiovascular disease have an independent and cumulative effect on telomere attrition

Since gout is strongly associated with cardiovascular disease, and cardiovascular disease has been associated with shorter telomeres, we investigated their separate effects. We included both patients with gout and participants with chronic heart disease, non-fatal MI, non-fatal stroke and angina pectoris (figure 2). Consistent with previous reports, healthy participants without cardiovascular disease had longer telomeres ($p=0.04$) compared

with healthy participants with cardiovascular disorders. The presence of cardiovascular disease in patients with gout resulted in significantly shorter telomeres ($p=0.0001$) compared with individuals with gout only. Moreover, TL in patients with gout without cardiovascular disease was shorter ($p=0.0037$) compared with matched healthy individuals (figure 2). Consequently, both gout and cardiovascular disease eventuated even shorter telomeres compared with healthy participants ($p=0.005$). Patients with cardiovascular disease concomitant with gout demonstrated shorter telomeres ($p<0.0001$) compared with healthy individuals without any comorbidities.

In the linear regression model involving both patients with gout and healthy individuals, gout ($p=0.008$), cardiovascular disease ($p=0.023$) and heart failure ($p=0.01$) were independently influencing TL (table 2A). Of note, although all the continuous variables mentioned in table 2 were included in this model, gout, cardiovascular disease and heart failure remained in the model as significantly associated with TL. Moreover, in a linear regression model, cardiovascular disease ($p=0.001$) and MI ($p=0.016$) influenced telomere attrition in the gout population (table 2B). Additionally, in a separately performed independent linear regression model, the age at the first gout event ($p=0.04$), frequency of gouty flares ($p=0.005$) and the disease duration ($p=0.035$) were the most significant hallmarks of gout associating with shorter telomeres (table 2C).

Furthermore, analysis revealed significantly shorter telomeres in patients with gout and cardiovascular events compared with gout patients without cardiovascular events (see online supplementary table S4A). According to the linear regression model, however, there was no association between the flare frequency and cardiovascular events in patients with gout (see online supplementary table S4B).

Table 2 Multiple linear regression models for telomere length (TL) measurements in patients and healthy participants

	Number of participants	Standardised β	SE	p Value
A				
Gout	619	-0.23	0.01	0.008
Cardiovascular disease	313	-0.19	0.02	0.023
Heart failure	134	-0.22	0.01	0.010
B				
Cardiovascular disease	270	-0.55	0.02	0.001
Myocardial infarction (non-fatal)	70	-0.40	0.01	0.016
	Mean \pm SD	Standardised β	SE	p Value
C				
Multiple number of flares	8.75 \pm 12.67	-0.30	2.02	0.005
Age at the first attack	51.12 \pm 16.75	-0.05	0.71	0.040
Disease duration	12.90 \pm 12.28	-0.22	0.52	0.035

(A) demonstrates the variables significantly associated with TL involving patients with gout and healthy individuals assessed using multiple linear regression model. Age, gender, smoking, cardiovascular disease, heart failure, stroke (non-fatal), myocardial infarction (non-fatal), angina pectoris, systolic and diastolic blood pressure, body mass index (kg/m²), serum UA level (mmol/L) and creatinine level (μ mol/L) were included in the model as independent variables ($p < 0.05$). (B) includes the results of multiple linear regression analysis associating TL with cardiovascular events (heart failure, stroke (non-fatal), myocardial infarction (non-fatal), angina pectoris and age) only in individuals with gout ($p < 0.05$). (C) represents multiple linear regression analysis results where the association between TL and gout hallmarks (attack frequency, age at the first attack, disease duration, serum UA level (mmol/L), creatinine level (μ mol/L)) was tested ($p < 0.05$).

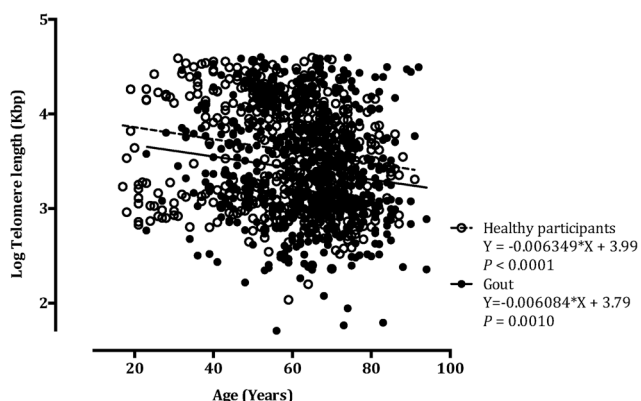


Figure 1 Log-transformed telomere length (kbp) of PBMCs of patients with gout and healthy participants consistent with age (years). Patients with gout (filled dots) (N=619) have shorter telomeres at young age and the telomere length remains short in older patients with gout ($p = 0.001$). Young healthy participants (empty dots) (N=566) represent longer telomere compared with older individuals ($p < 0.0001$). Overall telomere length of patients with gout is significantly shorter compared with healthy participants ($p < 0.0001$). Statistical analysis between the two groups was performed using two-tailed Student's t-test (Mann-Whitney correction). Correlation between telomere length and age was tested through linear regression model.

Treatment might inhibit inflammation-induced telomere erosion in immune cells of patients with gout

Since the majority of patients with gout were receiving anti-inflammatory and/or urate-lowering treatment, we tested if any treatment had any effect on the extent of telomeres. The treatment included allopurinol (mean 200 mg/day), colchicine, corticosteroids, non-steroidal anti-inflammatory drugs or a combination of these drugs. There was no significant difference between TL of

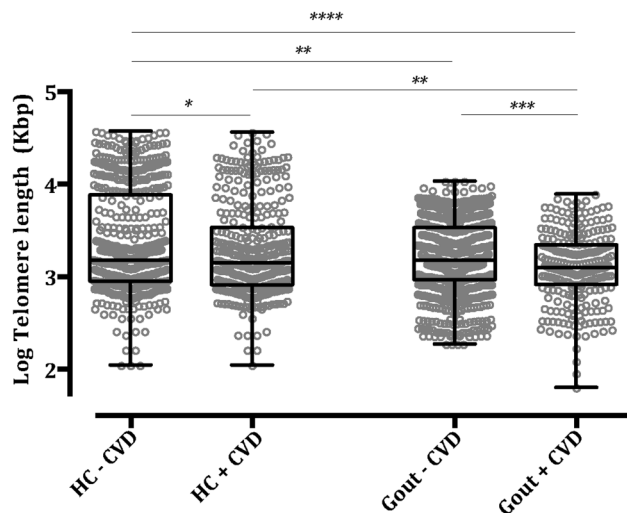


Figure 2 Log-transformed leucocytes' telomere length (kbp) of pooled Dutch and New Zealand patients with gout and healthy participants stratified according to (A) cardiovascular disease (CVD) (yes/no) and (B) the level of creatinine (μ mol/L). (A) Leucocytes' telomere length of patients with gout (N=325) and healthy participants (N=183) without (-) or with (+) (N gout=294) (N healthy participant=383) CVD (chronic heart disease, non-fatal stroke, non-fatal myocardial infarction and angina pectoris) is represented. (B) Leucocytes' telomere length of patients with gout and healthy participants stratified according to the plasma level of creatinine (μ mol/L). The threshold of plasma creatinine was set at 100 μ mol/L and below the threshold, creatinine levels of patients with gout and healthy participants are represented as normal (filled/empty bullets) and subsequently above the threshold are represented as high. (N gout normal creatinine=219) (N gout high creatinine =214) (N HC normal creatinine =208) (N HC high creatinine =92). Box plot is represented with minimum and maximum range. Statistical differences were tested using Mann-Whitney U test (* $p < 0.05$ was considered as significant).

treated (TL(\pm SD)=2301 \pm 2333.79) and untreated patients (TL (\pm SD)=2998 \pm 5166.92) (N=23) ($p = 0.584$). The insignificant difference in TL might be due to low number of untreated patients. Since the patients were treated with more than one drug, any drug specific effect on the extent of TL was intractable.

Telomere shortening is not specific for certain immune cell subsets

To identify immune cells with a dominant effect on the complications associated with senescence, we investigated the percentage and TL of mononuclear cell subsets of 10 healthy participants and 10 patients with gout (see online supplementary table S3).

In patients with gout, there were no significant differences in CD4⁺ T lymphocytes (24.42%), CD8⁺ cytotoxic T lymphocytes (6.74%), CD19⁺ B lymphocytes (7.02%), CD14⁺ monocytes (7.75%), NK cells (7.92%) and CD123⁺/CD304⁺ pDCs (0.02%) of patients with gout compared with healthy participants' cell subsets that were 33.19% ($p = 0.11$), 8.98% ($p = 0.061$), 10.07% ($p = 0.157$), 6.28% ($p = 0.90$), 7.38% ($p = 0.52$) and 0.11% ($p = 0.8$) respectively (Mann-Whitney U test, $p < 0.05$).

Moreover, CD19⁺ B lymphocytes of patients with gout had the longest telomeres and CD123⁺/CD304⁺ pDCs the shortest.

The longest and shortest telomeres in healthy participants were also respectively in CD19⁺ B lymphocytes and CD123⁺/CD304⁺ pDCs. In direct comparison between cell subsets from patients with gout and healthy individuals, we did not observe a significant difference in TL.

Telomerase activity is similar in patients with gout and healthy participants

There was no correlation between *hTERT* gene expression level and TL in total PBMCs. There was no significant difference in the mean fold change of *hTERT* expression level of patients with gout ($p=0.921$, $R^2=0.013$) and healthy participants ($p=0.087$, $R^2=-0.264$) (figure 3). Similarly, there was no correlation between *hTERT* gene expression level and TL of the immune cell subset (see online supplementary table S3).

DISCUSSION

In this study, we show that patients with gout have shorter telomeres than healthy participants, reflecting increased cellular senescence. Telomere shortening was strongly associated with the number of gouty flares. Both gout and cardiovascular disease were independently associated with shorter telomeres. This is to our knowledge the first study to show telomere erosion in patients with gout compared with healthy participants.

There are several strengths of this study that minimise the risk of chance observation or population bias. We exploited an independent replication cohort where DNA material was isolated and measured using a similar protocol. We observed intriguing TL differences in participants of both Dutch and New Zealand cohorts. It has been described previously that there are large differences in TL between Caucasians from different European descent. For instance, telomeres of inhabitants from Belgium have been shown to be sixfold longer compared with inhabitants from Italy and are approximately twice as long as inhabitants from the UK, Ireland and Scotland.^{19 20} Since the Caucasians from New Zealand have ancestors primarily from Britain and the Dutch are more closely related to the Belgians, our findings are most likely caused by an ancestral difference.^{20–22}

To rule out an effect of differences in telomere maintenance between patients with gout and healthy participants, we tested *hTERT* gene expression, which has been shown to be a reliable proxy marker to measure telomerase activity.²² We assessed *hTERT* gene expression in PBMCs and cell subsets of patients

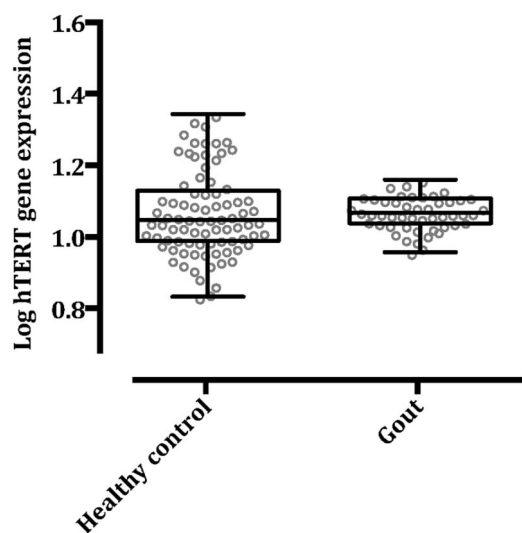


Figure 3 Log human telomerase reverse transcriptase (*hTERT*) gene expression level (FC) of Dutch patients with gout, measured using PBMCs' RNA and quantitative PCR method. *hTERT* gene expression levels between patients with gout (N=64) and healthy participants (N=89) are not significant (statistical differences were tested using Mann-Whitney U test) (* $p<0.05$ was considered as significant). Error bars represent box plot with minimum and maximum range.

with gout and healthy individuals. We did not observe any difference in *hTERT* gene expression level that underscores any bias due to telomerase activity as very unlikely. To minimise technical issues, we used the same protocols and same machines to measure TL.

To elucidate more details on underlying facets contributing to telomere attrition in gout, we quantified absolute TL in CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, CD14⁺ monocytes, NK cells and pDCs in patients with gout and matched healthy participants. TL of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, CD14⁺ monocytes, NK cells and pDCs showed no significant difference. In immune cell-subtypes of patients with gout, telomere shortening showed a slightly declining trend compared with healthy participants. However, the number of included participants is insufficient to empower us to draw definite conclusions from these results. Multiple research groups have corroborated a strong link between chronic inflammation and decrease of TL in cell subsets. For instance, studies on HIV elucidated the link between chronic infection and telomere attrition in CD4⁺ and CD8⁺ T cells.^{23 24} The replicative senescence of T lymphocytes is not limited to HIV since it is broadly investigated in association with malignancies,²⁵ autoimmune disorders²⁶ and environmental mediators.^{27 28} In autoimmune diseases, telomere erosion in lymphocytes has been less well delineated. Telomere erosion in CD14⁺ monocytes, however, was shown to be significantly associated with pro-inflammatory behaviour and, of high relevance for our findings, thereby accelerated atherosclerotic plaque formation.²⁹ In the elderly, diminished NK cell function was significantly associated with the extent of telomere erosion.^{30 31} The literature describing telomere attrition in pDCs is rather poor and requires additional investigations. Taken together, it is known from the literature that TL of various immune cell subsets can be impacted when chronic or recurrent inflammation is present. In our study, we could not pinpoint a single cell subset responsible for the shortening of telomeres as observed in the PBMCs from the gout population. Since the effects on TL difference in PBMCs only came apparent in a large cohort with hundreds of patients, we believe that our lack of findings is most likely caused by a lack of power in the subset analyses. On the other hand, since we observe such similar findings between subsets, it seems to indicate that there is not one single cell subset responsible for a large effect on TL in the whole PBMC population. The latter might point to a more generalisable effect in the cell subset populations. Additionally, the shifting of naive T lymphocytes towards a memory-like subset remains to be delineated whereby the CD28 marker repertoire might be indicative of chronic inflammation-induced activation in T lymphocytes.³²

We conclude that the shorter telomeres observed in the gout population are most likely caused by increased PBMCs turnover due to ongoing or frequent inflammatory episodes. This is consistent with association of reduced TL with increased flare frequency. In various tissues and cell-subtypes, accelerated cell turnover is inversely associated with replicative senescence. In disorders involving accelerated oxidative injury and chronic immune system activation as the main manifestation, such as systemic lupus erythematosus,³³ rheumatoid arthritis³² and systemic sclerosis,³⁴ enhanced cell turnover and telomere erosion has been extensively described. Furthermore, we observed no significant TL difference in treated patients with gout compared with patients without treatment. In addition, patients with the most frequent flares had the shortest telomeres. This might indirectly imply a modulating effect of inflammation on TL and thereby replicative senescence.

The results of our study underline the strong association between inflammation, senescence and cardiovascular disease, as suggested previously.³⁵ Recently, it has become apparent that **rheumatic diseases confer risk of cardiovascular disease development**. In rheumatoid arthritis, this risk approaches the risk of patients with diabetes.³⁶ The pathogenesis of this increased risk is largely enigmatic, but is proposed to arise from a chronic inflammatory environment, causing vascular damage and plaque formation.^{37–38} The occurrence of atherosclerotic plaques has been related to an increasing chronological age.¹¹ On the other hand, there is a large role for biological ageing as well, which has been demonstrated by the **influx of pro-inflammatory senescent immune cells in the plaque contributing to plaque instability and rupture and eventually cardiovascular and cerebrovascular events**.³⁹ The connection between replicative senescence and the presence of cardiovascular disease has been described before in the literature.⁴⁰ Likewise, the prevalence of MI in the elderly with shorter leucocytes' TL is about three times higher.^{40–42} A recent systemic review confirmed this association by combining the telomere data and vascular morbidity of >50 000 individuals.¹⁰

In summary, we present decreased TL as a feature of gout and confirm TL as an independent factor associated with flare frequency and cardiovascular disease. These data suggest that **intensive treatment of gout may prevent replicative senescence and subsequently reduce the associated cardiovascular risk**. However, large prospective studies are needed to test this possibility.

Author affiliations

¹Department of Rheumatology and Clinical Immunology, University Medical Centre Utrecht, Utrecht, The Netherlands

²Laboratory of Translational Immunology, University Medical Centre Utrecht, Utrecht, The Netherlands

³Department of Dermatology, University Medical Centre Utrecht, Utrecht, The Netherlands

⁴Department of Medical Microbiology and Immunology, Rijnstate Hospital, Arnhem, The Netherlands

⁵University of Glasgow, Institute of Cancer Sciences, Wolfson-Wohl Translational Cancer Research Centre, Glasgow, UK

⁶University of Auckland, Auckland, New Zealand

⁷University of Otago, Christchurch, New Zealand

⁸University of Otago, Dunedin, New Zealand

Contributors NV, LBEK, TRDJR and JCAB contributed equally to the article. NV contributed to research design, performed experiments, analysed data and contributed to writing the paper. LBEK, EVL, ND, LKS, TRM, MJ, contributed to patients' recruitment, interpreted data and contributed to writing the manuscript. CW performed experiments and contributed to data analysis. MR and PGS interpreted data and contributed to writing the manuscript. JCAB and TRDJR designed research, interpreted data and contributed to writing the manuscript. All authors approved the final version after being involved in drafting and revising the article for important intellectual content. JCAB had full access to the data and takes responsibility for the accuracy of the performed analysis and the integrity of the data.

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Competing interests JCAB is supported by a VENI Award from the Netherlands Organization for Scientific Research (N.W.O. project number 91614041).

Patient consent Obtained.

Ethics approval This study was performed according to the guidelines of the Declaration of Helsinki and meets the ethical approval of the Ethical and Review committee of the University Medical Centre of Utrecht, Rijnstate Hospital, in Arnhem (both Netherlands) and the Multi-Region Ethics Committee in New Zealand.

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Patients with gout have short telomeres compared with healthy participants: association of telomere length with flare frequency and cardiovascular disease in gout

N Vazirpanah, L B E Kienhorst, E Van Lochem, C Wichers, M Rossato, P G Shiels, N Dalbeth, L K Stamp, T R Merriman, M Janssen, T R D J Radstake and J CA Broen

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Supplementary figures and tables

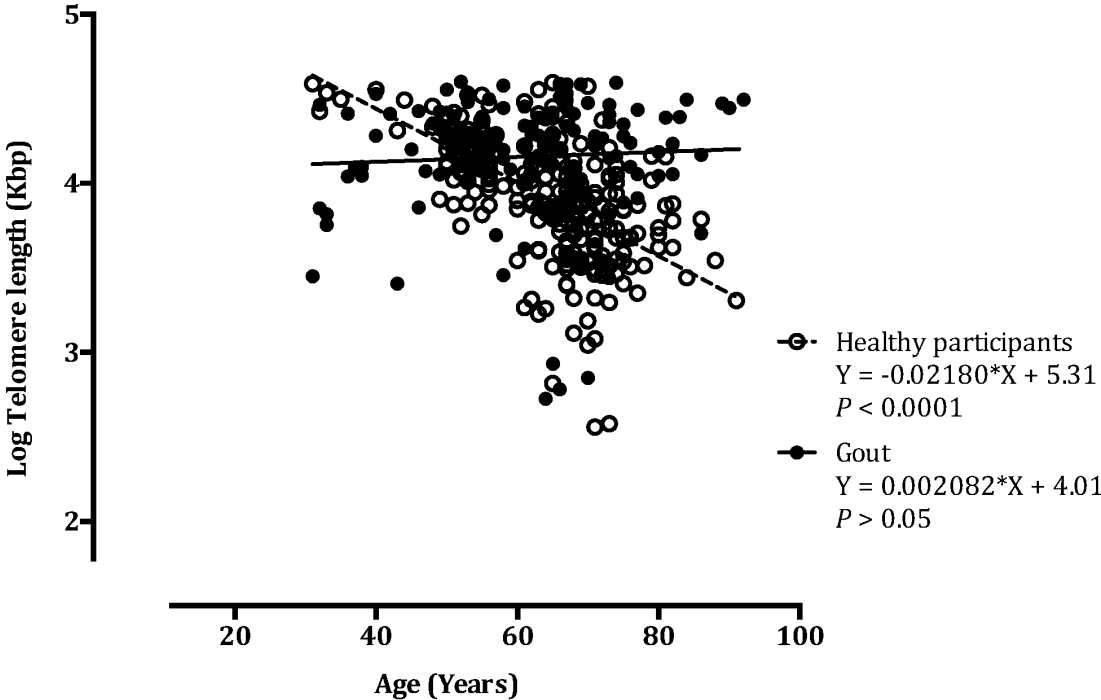


Figure 1. Log transformed telomere length (kbp) of PBMCs of patients with gout and healthy individuals participating in Dutch cohort consistent with age (years). Patients with gout (N=145) have shorter telomeres at young age and the TL remains short in older patients with gout ($P>0.05$). Young healthy participants (N=273) represent longer telomere as compared to older individuals ($P<0.0001$). Overall TL of patients with gout is significantly shorter as compared to healthy participants ($P<0.0001$). Statistical analysis between the two groups was performed using two tailed Student T-test (Mann-Whitney correction). Correlation between TL and age was tested through linear regression model.

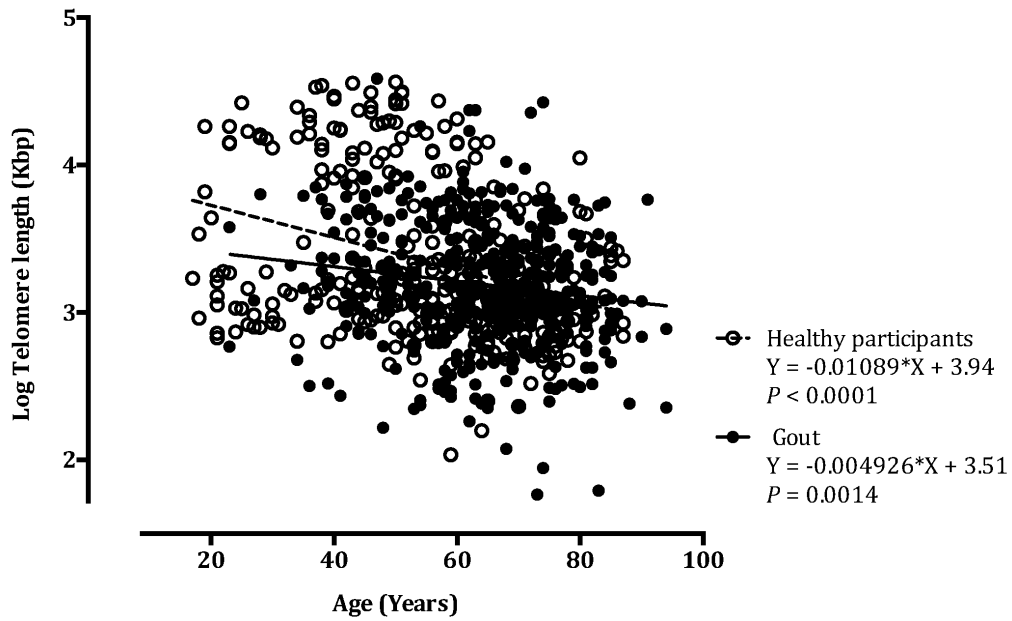


Figure 2. Log transformed telomere length (kbp) of PBMCs of patients with gout and healthy individuals participating in New Zealand cohort consistent with age (years). Patients with gout (N=474) have shorter telomeres at young age and the TL remains short in older patients with gout ($P=0.001$). Young healthy participants (N=293) represent longer telomere as compared to older individuals ($P<0.0001$). Overall TL of patients with gout is significantly shorter as compared to healthy participants ($P<0.0001$). Statistical analysis between the two groups was performed using two tailed Student T-test (Mann-Whitney correction). Correlation between TL and age was tested through linear regression model.

Supplementary files, table 1. Baseline characteristics of patients with gout and healthy participants in the Dutch cohort.

	Gout (n=145)	Healthy participants (n=273)	<i>P</i> Value
Male n (%)	91 (62.76)	190 (69.70)	<i>0.0001</i>
Age	63 ± 9.85	62 ± 14.11	<i>0.0001</i>
Telomere length (total PBMC)	11215.33 ± 8042.54	18014.77 ± 9548.66	<i>0.0001</i>

The data are presented as mean ± SD. The significance of the association between the 2 classified subgroups of patients with gout and healthy individuals was tested using Fisher's exact test (categorical values) and Mann-Whitney *U* test (non-parametrical continues values) (*P*<0.05).

Supplementary files, table 2. Baseline characteristics of patients with gout and healthy participants in the New Zealand cohort.

	Gout (n=474)	Healthy participants (n=293)	P Value
Male n (%)	388 (81.86)	233 (85.35)	0.0001
Age	64 ± 12.82	55 ± 16.59	0.0001
Telomere length (total PBMC)	2524.55 ± 3478.42	5151.93 ± 7674.79	0.0001

The data are presented as mean ± SD. The significance of the association between the 2 classified subgroups of patients with gout and healthy individuals was tested using Fisher's exact test (categorical values) and Mann-Whitney *U* test (non-parametrical continues values) ($P < 0.05$).

Supplementary files, table 3. Baseline characteristics of patients with gout and healthy participants in immune cell-subsets.

	Gout (n=10)	Healthy participants (n=11)	<i>P Value</i>
Male n (%)	8 (80)	10 (91)	>0.05
Age (mean ± SD)	59 ± 16.27	45 ± 12.88	>0.05
Age at the first flare (mean ± SD)	53 ± 5.76	-	-
Disease duration (year) (mean ± SD)	5.70 ± 2.77	-	-
Colchicine (yes) n (%)	2 (20)	N.A.	-
NSAID (yes) n (%)	1 (10)	N.A.	-
Allopurinol (yes) (mean 200mg/day) n (%)	3 (30)	N.A.	-
Corticosteroids(yes) n (%)	2 (20)	N.A.	-
CVD (yes/no) n (%)	6 (60)	N.D.	-
Diabetes (type 2) (yes) n (%)	6 (60)	N.D.	-
Hypertension (yes) n (%)	4 (40)	N.D.	-
Creatinine level (µmol/L) (mean ± SD)	109.00 (± 25.09)	N.D.	-
BMI (kg/m²) mean (SD) (mean ± SD)	32.58 (± 8.51)	N.D.	-
Smoking (yes) n (%)	1 (10.00)	N.D.	-
Serum urate (mmol/L) mean (SD)	0.54 (±0.14)	N.D.	-
Number of flares (mean ± SD)	19.70 ± 6.63	N.A.	-
Presence of tophi (yes) n (%)	4 (40)	N.A.	-
Systolic blood pressure mean (mm Hg) (SD)	121.00 (±21.81)	N.D.	-
Diastolic blood pressure mean (mm Hg) (SD)	70.20 (±12.04)	N.D.	-
Telomere length CD3⁺/CD4⁺ (mean ± SD)	15247.68 ± 13956.01	7339.70 ± 5063.97	>0.05

Telomere length CD3⁺/CD8⁺ (mean ± SD)	13036.82 ± 7838.27	6987.34 ± 5666.97	>0.05
Telomere length CD19⁺/CD20⁻ (mean ± SD)	23374.06 ± 25676.88	8620.14 ± 6476.89	>0.05
Telomere length CD14⁺/CD16⁻ (mean ± SD)	10988.64 ± 4523.27	6864.43 ± 5103.57	>0.05
Telomere length CD3⁻/CD56⁺ (mean ± SD)	12599.09 ± 27954.54	5310.30 ± 1747.49	>0.05
Telomere length CD123⁺/CD304⁺ (mean ± SD)	2804.35 ± 2536.15	4052.95 ± 4776.86	>0.05
hTERT CD3⁺/CD4⁺ (mean ± SD)	0.00112 ± 0.000240	0.00068 ± 0.000130	>0.05
hTERT CD3⁺/CD8⁺ (mean ± SD)	0.00028 ± 0.000040	0.00036 ± 0.000100	>0.05
hTERT CD19⁺/CD20⁻ (mean ± SD)	0.00039 ± 0.000054	0.00139 ± 0.001120	>0.05
hTERT CD14⁺/CD16⁻ (mean ± SD)	0.00018 ± 0.000041	0.00004 ± 0.000020	>0.05
hTERT CD3⁻/CD56⁺ (mean ± SD)	0.00030 ± 0.000001	0.00012 ± 0.000080	>0.05
hTERT CD123⁺/CD304⁺ (mean ± SD)	N.D.	N.D.	-

The data are presented as mean ± SD. The significance of the association between the 2 classified subgroups of patients with gout and healthy participants was tested using Fisher's exact test (categorical values) and Mann-Whitney *U* test (non-parametrical continues values) ($P < 0.05$) (N.D.=not defined, N.A.=not applicable).

Supplementary files, table 4. Telomere length differences between gout participants with or without cardiovascular disease (A) and the association of cardiovascular disease with the flare frequency in the gout population (B).

	A) Telomere length (bp) in gout population with or without cardiovascular disease			B) Multivariate linear regression on flare frequency and cardiovascular diseases in gout population		
	Yes (SD)	No (SD)	P Value	Standardized β	Standard error	P Value
Cardiovascular disease	2187 (± 1934.10)	6154 (± 8938.23)	<i>0.0001</i>	-1.35	1.82	<i>0.459</i>
Angina pectoris	2223 (± 2625.17)	5527 (± 7805.88)	<i>0.0001</i>	-2.94	3.07	<i>0.340</i>
Heart failure	4656 (± 28288.80)	13712 (± 28141.32)	<i>0.015</i>	-3.28	2.57	<i>0.203</i>
Myocardial infarction	2165 (± 2397.07)	7272 (± 10916.47)	<i>0.0001</i>	-4.50	2.62	<i>0.063</i>
Stroke	82 (± 2378.78)	4784 (± 8980.58)	<i>0.0001</i>	-3.32	2.83	<i>0.242</i>

Telomere length (SD) in patients with gout with cardiovascular disease, angina pectoris, heart failure, myocardial infarction (non-fatal) and stroke (non-fatal) as concomitant disorders. **A)** The significance of the association between the 2 classified subgroups of gout participants was tested using Mann-Whitney *U* test. **B)** The association between the same variables (cardiovascular comorbidities) and the flare frequency was tested in a multiple variable linear regression model ($P < 0.05$).

Supplementary files, table 5. The effect of additional co-variables on telomere length of patients with gout and healthy participants separately.

	Gout		Healthy participant	
	Correlation Coefficient (R²)	P value	Correlation Coefficient (R²)	P value
Gender	-0.023	<i>0.574</i>	0.173	<i>0.001</i>
Age (years)	-0.102	<i>0.014</i>	-0.315	<i>0.0001</i>
Smoking (yes/no)	0.418	<i>0.0001</i>	-0.041	<i>0.669</i>
BMI	0.031	<i>0.459</i>	0.080	<i>0.175</i>
Creatinine (μmol/L)	-0.205	<i>0.0002</i>	-0.203	<i>0.001</i>

The statistical significance of the data is tested using two-tailed Spearman's bivariate correlation ($P < 0.05$).