

**Menopause Modifies the Association of Leukocyte Telomere Length with Insulin Resistance and Inflammation**

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## **Abstract**

Context: Leukocyte telomere length is inversely correlated with age, insulin resistance, serum leptin and smoking.

Objective: We explored whether menopausal status modifies the relations between leukocyte telomere length and insulin resistance. In addition, we examined the effect of menopause on the relation between leukocyte telomere length and C-reactive protein (CRP), an index of inflammation.

Design: observational cohort study

Setting: community based.

Participants: 1517 women aged 18-79 selected only for belonging to a twin pair and representative of the general population.

Main outcome measure: Leukocyte telomere restriction fragment length (TRFL).

Results: Insulin resistance, (expressed in the homeostasis model assessment), leptin and CRP were inversely correlated with leukocyte TRFL in pre-menopausal but not postmenopausal women. Insulin resistance, CRP, but not leptin independently accounted for variation in WBC TRFL in pre-menopausal women.

Conclusions: Menopausal status impacts leukocyte telomere length and its relation with insulin resistance and inflammation in women.

## **Key Words**

Aging, body mass index, smoking, inflammation, oxidative stress

## Introduction

Increased adiposity is associated with a rise in systemic inflammation (1-4) and oxidative stress (5,6). Both processes may accelerate telomere erosion in leukocytes, because inflammation enhances the turnover rate of leukocytes and oxidative stress heightens the loss of telomeric repeat per cell replication (7). Such mechanisms provide a potential explanation for findings of accelerated leukocyte telomere attrition with a rise in insulin resistance and a gain in the body mass index (BMI) in a longitudinal study (8), and the inverse correlations of leukocyte telomere length with insulin resistance (unpublished data), serum leptin and BMI (9) in cross-sectional analyses of relatively large populations. What's more, it appears that leukocyte telomere dynamics (telomere length and attrition rate) are influenced not by the body mass per se, but by mechanisms linked to obesity, expressed in elevated insulin resistance and leptin levels.

There are considerable age-dependent reconfigurations of both insulin resistance and adiposity, which, in women, are further modified by the menopausal status. For instance, glucose intolerance is increased not only with the redistribution of body fat towards more central obesity, but also due to fat accumulation in skeletal muscle and liver — a phenomenon associated with mitochondrial dysfunctions (10-14). These changes may alter insulin-dependent glucose regulation. Moreover, while insulin resistance primarily relates to visceral fat, leptin levels are predominantly a function of subcutaneous fat (15-17).

Given that the postmenopausal state is frequently marked by increased central obesity (18), the inter-relation between leukocyte telomeres dynamics with both insulin resistance and leptin may differ in postmenopausal vs. premenopausal women. Although previous works have observed that insulin resistance (8) and leptin (9) were inversely correlated with age-adjusted telomere length, the question is whether these two variables, which are linked to adiposity, independently account for variation in

leukocyte telomere length. In addition, as insulin resistance is a state of increased inflammation (1-3), we examined in this work the inter-relation between leukocyte telomere length and C-reactive protein (CRP), the plasma concentration of which increases with inflammation (19,20). Our main goal was to examine the effect of menopausal status (women  $\leq$  50 years vs. women  $>$  50 years) on the relations of leukocyte telomere length with insulin resistance, serum leptin and CRP in a large female cohort and explore which of these variables independently accounts for variation in telomere length among individuals.

## **Methods**

**Subjects:** We studied 1517 Caucasian female twins (ages 18-79) from the St Thomas' (TwinsUK) Adult Twin Registry. They were ascertained from the general population and shown to be comparable to age-matched population singletons (21,22). These unselected twins have been recruited since 1992 using twin registers and national media campaigns and used in a wide variety of studies (23). Historically the cohort is predominantly female and measurements were performed preferentially on DZ twins as part of ongoing genetic studies. All women provided informed consent approved by The St Thomas' Hospital Research Ethics Committee. A recent communication reported leukocyte telomere length in relation to the body mass BMI, leptin, and cigarette smoking in a subset of this cohort (9).

For all practical purposes, by the age of 50 years, most women have reached menopause. We therefore characterized women according to age  $\leq$  50 years as pre-menopausal, and women  $>$  50 years as postmenopausal. In this cohort, the subset of women for which age at natural menopause (at least one year since the last period) could be accurately determined (excluding surgical menopause) was 715 with, the median age at menopause was 50 years (mean=48.5, SD= 4.8).

General Considerations: Means and ranges of quantitative phenotypes in Twins UK are normally distributed and similar to the age-matched general population in the UK (22). Zygosity was determined by standardized questionnaire and confirmed by DNA fingerprinting.

Biochemical Measurements: Blood sample collection for determination of fasting insulin and glucose was as described by de Lange et al (23). Fasting insulin was determined by immunoassay (Abbott Laboratories Ltd. Maidenhead, UK) and glucose measured on Ektachem 700 multichannel analyzer, using an enzymatic colorimetric slide assay (Johnson and Johnson Clinical Diagnostic Systems, Amersham, UK). Insulin resistance was evaluated using the homeostasis model assessment (HOMA-IR), calculated as  $(\text{fasting insulin} \times \text{glucose})/22.5$  (23). Plasma leptin concentration was determined after an overnight fast using a radioimmunoassay (Linco Research, St Louis, MO, USA). C-reactive protein (CRP) assays were performed by an ELISA method, which has a lower detection limit of 0.15mg/L and a coefficient of variation of 8.7% at 0.5mg/L. Subjects with levels above 10mg/L were excluded as this indicated clinically relevant infection inflammation or malignancy.

Measurement of the mean terminal restriction fragment length (TRFL): Measurements were performed as previously described (8). Briefly, DNA samples were checked for integrity on 0.8% agarose gel. They were then digested overnight with restriction enzymes Hinf I (10 U) and Rsa I (10 U) (Roch, Indianapolis IN). DNA samples (3  $\mu\text{g}$  each) and 4 DNA ladders (1 kb DNA ladder plus  $\lambda$  DNA/Hind III fragments; Invitrogen, Carlsbad, CA) were resolved on a 0.6% agarose gel (20 cm x 20 cm) at 50V (GNA-200 Pharmacia Biotech). After 16 hours, the DNA was depurinated for 15 minutes in 0.25 N HCl, denatured 30 minutes in 0.5 mol/L NaOH/1.5 mol/L NaCl and neutralized for 30 minutes in 0.5 mol/L Tris, pH 8/1.5M NaCl. The DNA was transferred for 1 hour to a positively charged nylon membrane (Roch) using a vacuum blotter (Appligene, Oncor). The membranes were hybridized at 65°C with the telomeric probe [digoxigenin 3'-end labeled 5'-(CCTAAA)<sub>3</sub>] overnight in 5

x SSC, 0.1% Sarkosyl, 0.02% SDS and 1% blocking reagent (Roch). The membranes were washed 3 times at room temperature in 2 x SSC, 0.1% SDS each for 15 minutes and once in 2 x SSC for 15 minutes. The digoxigenin-labeled probe was detected by the digoxigenin luminescent detection procedure (Roch) and exposed on X-ray film. The autoradiographs were scanned and the TRFL signal digitized between MW of 1-20 kb. The mean TRF length was then calculated accordingly. Conversion of the OD vs. DNA migration distance to OD (adjusted for background)/MW vs. MW yielded a new histogram from which the mean TRFL was calculated. We routinely resolve each DNA sample in duplicate (on different gels). If the difference between the duplicates is > 5%, a third measurement is performed and the mean of two results < 5% apart is taken.

Statistical analysis: Univariate and multivariate analyses of variance were used to compare the characteristics of women age  $\leq$  50 years with women >50 years. Standard multiple linear regression techniques were used to assess the correlation of TRFL with the individual factors listed in table 1 including age and other covariates as indicated. Natural log transformed leptin, insulin, glucose, HOMA-IR and CRP values were used for all statistical analyses. Because twin-pair data are not independent observations, we examined the correlation between TRFL vs. the various factors taking an independent sample each time, i.e. a subset of samples composed of a random twin from each pair. This bootstrap procedure was carried out 100 times, and the mean test statistic was used to assess statistical significance where indicated. S-Plus 6.0 (Insightful Corp) software was used.

## **Results**

General Characteristics: Table 1 displays major characteristics of pre-menopausal (age  $\leq$  50), postmenopausal (age > 50) and both combined. There was approximately a 20-year difference in the mean age between postmenopausal and pre-menopausal women. The BMI, fasting insulin, glucose, HOMA-IR, leptin and CRP were all higher in postmenopausal than pre-menopausal women. Whereas

26.5% of women  $\leq 50$  smoked, only 15.2% of women  $> 50$  were still smokers. Ex-smokers comprised 49.9% of women  $\leq 50$ , and 51.0% of women  $>50$ .

TRFL Parameters: The overall yearly rate of leukocyte TRFL attrition was  $20.5 \pm 1.1$  base pairs (bp) ( $r = -0.385$ ,  $p = 1 \times 10^{-62}$ ). The yearly TRFL attrition for pre-menopausal women and postmenopausal women were respectively,  $22.1 \pm 2.9$  bp and  $20.2 \pm 4.1$  bp.

Table 2 displays the leukocyte TRFL without and with adjustment for age, smoking and BMI. These adjustments were undertaken based on our previous observations that leukocyte TRFL was inversely correlated not only with age, but also the BMI and cigarette smoking (8,9). Without adjustments, TRFL was shorter by 390 bp in postmenopausal than in pre-menopausal women, but this difference largely disappeared after adjustment for age, smoking and BMI.

Correlations Between HOMA-IR, Leptin and the BMI: For both pre-and postmenopausal women, there were strong positive correlations between ln- HOMA-IR and ln-leptin (Figure 1). Strong positive correlations were also observed for ln-HOMA-IR and ln-leptin with the BMI (Figure 1).

Correlations of CRP with HOMA-IR and Leptin: There were strong correlations of ln-CRP with both ln-HOMA-IR and ln-leptin for women  $\leq 50$ , women  $> 50$  and all women combined (Table 3).

Associations of TRFL with Indices of Insulin Resistance, Leptin and CRP: Table 4 summarizes the relations of age and smoking-adjusted TRFL with indices of glucose regulation (fasting insulin and glucose, and HOMA-IR), leptin and CRP in pre-and postmenopausal women. Age and smoking-adjusted TRFL was inversely correlated with indices of glucose regulation in pre-menopausal but not postmenopausal women. Age and smoking-adjusted TRFL was also inversely correlated with leptin

in pre-menopausal women. This relation was of borderline significance in the postmenopausal women. For the pre-menopausal women, the relations between age and smoking-adjusted TRFL and indices of glucose regulation were considerably more robust than those between TRFL and leptin. Age and smoking-adjusted TRFL was inversely correlated with CRP in pre-menopausal but not postmenopausal women.

Given the high correlations between HOMA-IR, leptin and CRP, we performed multiple stepwise regression analysis, including in the model the following independent variables: age, smoking history, ln-HOMA-IR, ln-leptin and ln-PCR (Table 5). For pre-menopausal women, age, smoking, ln-HOMA-IR, ln-PCR, but not ln-leptin, independently accounted for variation in TRFL. For postmenopausal women, age and smoking, but not ln-HOMA-IR, ln-leptin, and ln-PCR independently accounted for variation in TRFL.

To further ascertain that age or its interaction was not a confounder for the TRFL findings we also explored within twin pair differences for discordant pairs. For 204 pairs (114 aged  $\leq 50$ , 90 aged  $> 50$ ) discordant for HOMA-IR (top and bottom quartiles of the HOMA-IR distribution), the twin with low HOMA-IR had on average 183 bp longer telomeres than the twin with high HOMA-IR ( $7.01 \pm 0.08$  kb vs  $7.20 \pm 0.08$  kb  $p < 0.10$ ) among women aged  $\leq 50$ . However, among women aged  $> 50$  the twins with low HOMA-IR had telomeres 40 bp shorter ( $6.72 \pm 0.10$  kb vs  $6.67 \pm 0.10$  kb  $p < 0.77$ ) than the twin with high HOMA-IR. Although the differences are not statistically significant, the data are consistent with the results from the multiple linear regressions.

## **Discussion**

The central findings of the present work were as follows: Insulin resistance, leptin and CRP were inversely correlated with leukocyte telomere length in pre-menopausal but not postmenopausal women. What's more, the underlying mechanisms that accounted for variation in leukocyte telomere length in pre-menopausal women appeared to relate to insulin resistance and inflammation (CRP) rather than leptin. The question then is: What are the factors that might explain these enigmatic differences between pre-and-post menopausal women?

Neither insulin nor glucose is likely to be the factor that mechanistically connects insulin resistance with leukocyte telomere dynamics. Rather, the states of insulin resistance and adiposity, expressed by inflammation and perhaps oxidative stress (1-5), probably modify leukocyte telomere attrition.

One potential explanation for the altered relation between insulin resistance and leukocyte telomere length in postmenopausal women is aging itself. As women get older, the underlying reasons for their insulin resistance might be less related to the body mass and adiposity per se. Such a transformation would weaken the relation between leukocyte telomere dynamics and insulin resistance. For instance, studying lean ( $BMI < 25\text{kg/m}^2$ ) elderly and young volunteers, Petersen et al. (14) observed marked insulin resistance in the elderly, which arose from a decline in insulin-mediated glucose metabolism in skeletal muscle, associated with a considerable reduction in mitochondrial oxidative activity and phosphorylation. These findings indicate that independent of adiposity, aging itself contributes to insulin resistance, and they point to an aging-related shift in the mechanisms behind insulin resistance.

Another alternative explaining the absent association between leukocyte telomere length and insulin resistance in postmenopausal women is the dramatic decline in ovarian steroid hormones—particularly estrogen—during the postmenopausal period. Estrogen may be linked to leukocyte

telomere dynamics through its anti-inflammatory and anti-oxidant attributes and by its ability to stimulate telomerase, a reverse transcriptase that elongates telomere ends (24).

Estrogen is a potent anti-inflammatory agent, because it lowers the production of cytokines, including the pro-inflammatory, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (25-28). Depending on tissues examined, insulin resistance may arise from or be caused by oxidative stress (29-32). One of the factors that defend against oxidative stress is estrogen (32-34). The anti-oxidant activity of estrogen may also mediate its anti-diabetic property (35). Estrogen serves as an anti-oxidant by mechanisms that are not fully elucidated, but appear to be exerted via membrane/cytoplasmic receptors (36). Estrogen stimulates the mitochondrial superoxide dismutase (Mn-SOD) and glutathione peroxidase (Gpx) (32), two powerful enzymes engaged in the metabolism of reactive oxygen species. Since neither Mn-SOD nor Gpx have an estrogen responsive element in their promoter regions, a direct genomic effect in this stimulation is unlikely.

In contrast to Mn-SOD and Gpx, the catalytic subunit of telomerase (TERT) promoter possesses not only an estrogen response element (37), but also binding sites for a number of transcription factors, including nuclear factor kappa B (NF $\kappa$ B) (38). Telomerase comprises in addition to TERT, a RNA subunit (24). Whereas the RNA subunit is constitutively expressed in most cells, the activity of the enzyme correlates with the expression of TERT. Estrogen stimulates telomerase via TERT and through other post-transcriptional modifications that include Akt protein kinase, a downstream mediator of phosphoinositol 3-kinase (PI3K) (39,40). The PI3K mode of telomerase activation is particularly relevant in that estrogen stimulates the PI3K/Akt cascade and induces the association of NF $\kappa$ B with TERT in different cell types, including lymphocytes (41-43). In addition, estrogen induces the Akt-dependent endothelial nitric-oxide synthase (eNOS) (44) to increase nitric oxide (NO) production and stimulate telomerase activity (45). These effects may explain telomere dynamics in

cells possessing estrogen receptors (45,46). As estrogen-mediated upregulation of Mn-SOD and Gpx expressions is also through the NF $\kappa$ B (32), the hormone may exert some of its anti-oxidant effects and stimulate telomerase through the same cellular pathways.

The postmenopausal period would therefore dramatically recast some of the key variables that affect leukocyte telomere dynamics. Not only the drop in estrogen, but also the redistribution of body fat centrally would alter leukocyte telomere attrition because fat is the source of not only leptin but also other adipocytokines that impact both inflammation and insulin resistance (47,48). Subsequently, the changing nature of insulin resistance with age would further modify its link to leukocyte telomere dynamics. In this regard, the fact that CRP accounted independently of insulin resistance for variation in leukocyte telomere length in pre-menopausal women suggests that the effect of inflammation on leukocyte telomere dynamics goes above and beyond that of insulin resistance.

As leukocyte telomere length is a record of their replicative history and the cumulative burden of inflammation and oxidative stress over the life of the individual, menopause might alter the trajectory of telomere attrition and thereby offset the relations between telomere length and indices of insulin resistance and inflammation observed during the pre-menopausal period. This tenet can be explored by longitudinal studies of leukocyte telomere dynamics, which may enrich our understanding of the role of menopause in the biology of human aging.

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## Figure Legends

Figure 1: The relationships between ln-HOMA-IR, ln-leptin and the BMI. The  $r^2$  between ln-leptin and the BMI for all samples using the best non-linear model (ln-leptin=ln-BMI) was only slightly better (0.58) than the linear model used in the figure (0.55).

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**Table 1. Descriptive statistics of study participants.**

Parameter	All Samples (1517)		Age≤50 (833)		Age>50 (683)		P <sup>1</sup>
	mean	SD	mean	SD	mean	SD	
Age	48.03	12.68	38.31	8.28	58.96	6.18	–
BMI kg/m <sup>2</sup>	24.99	4.69	24.60	4.78	25.39	4.56	0.0004
Insulin μU/mL	7.85	6.43	7.14	4.46	8.74	8.20	2x10 <sup>-5</sup>
Glucose mmol/L	4.57	0.96	4.40	0.59	4.78	1.24	1x10 <sup>-11</sup>
HOMA-IR <sup>2</sup>	1.63	1.70	1.41	0.98	1.90	2.30	2x10 <sup>-8</sup>
leptin ng/mL	17.08	12.67	15.65	11.90	18.62	13.29	7x10 <sup>-5</sup>
CRP mg/L	2.13	2.15	1.84	2.17	2.39	2.10	4x10 <sup>-6</sup>

<sup>1</sup> comparing women aged ≤50 years with those aged >50 years

<sup>2</sup> (fasting insulin x glucose)/22.5.

9 singletons and 754 twin pairs

**Table 2. Descriptive statistics of TRFL.**

TRFL Parameters (kb)	All Samples		Age≤50		Age>50		P <sup>1</sup>
	mean	SD	mean	SD	mean	SD	
Unadjusted	7.06	0.67	7.24	0.67	6.85	0.62	1x10 <sup>-25</sup>
Age-adjusted	7.06	0.67	7.03	0.65	7.10	0.61	0.19
Age, smoking-adjusted	7.06	0.64	7.03	0.64	7.09	0.60	0.21
Age, BMI-adjusted	7.06	0.66	7.02	0.65	7.10	0.62	0.15
Age, smoking, BMI-adjusted	7.06	0.64	7.03	0.65	7.10	0.61	0.18

<sup>1</sup> comparing women aged ≤ 50 years v those aged >50 years.

**Table 3. Correlations of CRP with insulin resistance and leptin.**

Factor	All Samples		Age ≤50		Age>50	
	Correlations with ln CRP <sup>1</sup>	p <sup>2</sup>	Correlations with ln CRP <sup>1</sup>	P <sup>2</sup>	r	P <sup>2</sup>
ln-HOMA-IR <sup>1</sup>	0.2669	5.27X10 <sup>-18</sup>	0.2760	6.22X10 <sup>-11</sup>	0.2162	2.13X10 <sup>-6</sup>
ln-leptin <sup>1</sup>	0.4219	1.86X10 <sup>-44</sup>	0.4263	2.07X10 <sup>-23</sup>	0.3758	2.22X10 <sup>-11</sup>

<sup>1</sup> in the natural logarithm scale

<sup>2</sup> adjusted for non-independence between twins in pairs

**Table 4. Correlations of TRFL with insulin resistance, leptin and CRP.**

Factor	All Samples		Age ≤50		Age>50	
	Correlations with TRFL <sup>1</sup>	p <sup>2</sup>	Correlations with TRFL <sup>1</sup>	p <sup>2</sup>	Correlations with TRFL <sup>1</sup>	p <sup>2</sup>
ln-Insulin <sup>3</sup>	-0.069	0.023	-0.144	0.002	-0.003	0.51
ln-Glucose <sup>3</sup>	-0.032	0.260	-0.114	0.005	-0.010	0.50
ln-HOMA-IR <sup>3</sup>	-0.062	0.054	-0.149	0.001	0.021	0.56
ln-Leptin <sup>3</sup>	-0.069	0.021	-0.083	0.037	-0.066	0.071
ln-CRP <sup>3</sup>	-0.050	0.078	-0.110	0.017	-0.016	0.62

(<sup>1</sup>) age and smoking adjusted TRFL  
(<sup>2</sup>) adjusted for non-independence between twins in a pair  
(<sup>3</sup>) in the natural logarithm scale

**Table 5. Multiple linear regression model of TRFL.**

Factor	Age ≤50				Age>50			
	Value	Std.Error	t-value	p	Value	Std.Error	t-value	p
(Intercept)	8.415	0.189			8.639	0.303		
Age	-0.026	0.003	8.025	$6 \times 10^{-15}$	-0.025	0.005	5.362	$1 \times 10^{-7}$
Smoking History	-0.061	0.031	1.971	0.049	-0.096	0.038	2.523	0.012
ln-HOMA-IR	-0.139	0.053	2.619	0.009	0.060	0.042	1.422	0.156
ln-leptin	0.029	0.052	0.559	0.576	-0.078	0.048	1.612	0.108
ln-CRP	-0.054	0.026	-1.996	0.046	-0.047	0.032	1.490	0.137
Multiple R <sup>2</sup>	0.126	$P < 1 \times 10^{-15}$			0.075	$P < 2 \times 10^{-6}$		

