Circulating leukocyte telomere length and oxidative stress: A new target for statin therapy

Sebastien Saliques, Jean-Raymond Teyssier, Catherine Vergely, Luc Lorgis, Julie Lorin, Michel Farnier, Anne Donzel, Pierre Sicard, Juliane Berchoud, Anne-Cécile Lagrost, et al.

Circulating leukocyte telomere length and oxidative stress: A new target for statin therapy

Sebastien SALIQUES [1], Jean-Raymond TEYSSIER [1-2], Catherine VERGELY [1], Luc LORGIS[1-3], Julie LORIN [1], Michel FARNIER[4], Anne DONZEL [2], Pierre SICARD[1]Anne-Cécile LAGROST[3], Claude TOUZERY[3], Sylviane RAGOT [1], Yves COTTIN [1-3], Luc ROCHETTE [1], Marianne ZELLER [1]

[1]IFR Santé-STIC, Laboratoire de Physiopathologie et Pharmacologie Cardiovasculaires Expérimentales, UFR Médecine, Université de Bourgogne (EA2979)
[2]Laboratoire de Génétique Moléculaire, CHU Dijon
[3]Service de Cardiologie, CHU Dijon
[4] Le point médical, Dijon

Corresponding author :
Marianne Zeller
Laboratoire de Physiopathologie et Pharmacologie Cardiovasculaires Expérimentales,
UFR Médecine, 7Bd Jeanne d'Arc 21000 Dijon
Phone : +33380393292
Fax : +33380393293
Email : marianne.zeller@u-bourgogne.fr

Word count :

Short title : Leukocyte telomere length and statin

Key words : Leukocyte telomere length; statin; myocardial infarction; inflammation; oxidative stress

Abstract

Background. In patients with acute myocardial infarction (MI), we aimed to investigate the relationship between chronic prior HMGCoA-reductase inhibitor (statin) treatment and leukocyte telomere length (LTL) and their interaction with potential new biomarkers of oxidative DNA lesions and reactive oxygen species-induced inflammation.

Methods. From 278 consecutive patients admitted for an acute MI <24 H after symptom onset, blood samples were collected on admission. LTL was assessed by quantitative PCR , and leucocytes Finkel-Biskis-Jinkins Osteosarcoma (FOS) and 8-oxoguanine DNA glycosylase (OGG1) mRNA levels were measured by quantitative RT-PCR. Patients under prior chronic statin therapy were compared with patients without statin treatment.

Findings. Patients under statin treatment (n=73 (26%)) had a higher rate of risk factors and medications. Although older, patients under statin treatment had mean LTL strikingly increased when compared to patients not under chronic statin therapy (1.29±0.11 vs. 1.25±0.11 T/S ratio, p=0.008). In contrast, FOS and OGG1 mRNA levels were similar in the 2 groups. LTL decreased with increasing age (p=0.004), increasing FOS (p=0.039), and OGG1 mRNA levels, (p<0.001). Interestingly, neither gender nor lipid parameters, nor systemic inflammation markers including CRP, nor risk factors were associated with LTL. Statin therapy remained associated with longer LTL, even after adjustment for confounding factors (p=0.001), and in particular age in younger patients (≤64 y). Even in cohorts matched for propensity score for statin use, LTL was markedly longer in patients under statin therapy (p=0.026). The other independent determinants of LTL were FOS and OGG1 transcripts.

Interpretation. Our prospective study showed for the first time that statin treatment was associated with longer LTL. Given the compelling evidence for a causal role of telomere shortening in coronary artery disease, these data suggest opportunities for identifying new target for early primary

preventive treatment strategies. Moreover, our findings emphasize on the role of FOS and OGG1 as new relevant biomarkers of LTL.

Funding. This work was supported by the University Hospital of Dijon, the Association de Cardiologie de Bourgogne, and by grants from the Union Régionale des Caisses d'Assurance Maladie de Bourgogne (URCAM), the Agence Regionale d'Hospitalisation (ARH) de Bourgogne, the Conseil Régional de Bourgogne and the Fédération Française de Cardiologie (FFC).

Introduction

Telomeres are nucleoproteins structures localized at the ends of eukaryotic chromosomes, made up of several thousand repetitive DNA sequences (TTAGGG) coated by capping proteins. They provide protection against threats to the genome and support chromosome stability. Telomeric repeats shortens with each cell division, because of ineffective replication of the 3'-end by DNA polymerase, eventually resulting in critically short telomeres, thus prompting cellular senescence, and ultimately cell death. Thus, telomere length shortening is a potential mechanism for a biological clock that determines cellular ageing. As telomere shortening is approximately the same in different tissues, circulating leukocytes from blood cells is an easily accessible surrogate tissue for telomere length assessment in human studies analyzing the systemic effects of chronic diseases, such as cardiovascular diseases. Strikingly, individuals with shorter leukocytes telomere length (LTL) present a higher prevalence of cardiovascular risk factors (Benetos, Gardner et al. 2004, Gardner, Li et al. 2005, Valdes, Andrew et al. 2005), coronary artery lesions and higher risk of cardiovascular mortality, in particular from acute myocardial infarction (MI) (Samani, Boultby et al. 2001, Brouilette, Singh et al. 2003, Ogami, Ikura et al. 2004, Fuster and Andres 2006, Brouilette, Moore et al. 2007). This increased risk seems to be independent of the classical and novel risk factors such as C-reactive protein (CRP) (Brouilette, Moore et al. 2007), and strongly suggests a link between LTL shortening and cardiovascular diseases, however the mechanisms are poorly understood. LTL regulation is a complex mechanism involving several factors including genetic, epigenetic, environmental and metabolic determinants. Thus, a major current issue in telomere research field is to understand which factors, in addition to age, influence LTL in humans, and their implications for potential clinical relevance and therapeutics.

Oxidative stress and inflammation are major factors known to accelerate age-related telomere shortening in cell culture, leading to the hypothesis that telomere attrition could serve as a biomarker of cumulative burden of both oxidative stress and inflammation (Houben, Moonen et al.

2008, Babizhayev, Savel'yeva et al. 2010). However, the relationship between oxidative stress, inflammation and TL has been only poorly investigated in vivo. Due to their high content in guanines, telomeres are highly sensitive to Reactive Oxygen Species (ROS)-induced damage in vitro (Kawanishi and Oikawa 2004), but the mechanisms of ROS-induced TL shortening in vivo needs further investigation. 8-oxoguanine DNA glycosylase (OGG1) is an enzyme involved in base excision and repair that has been recently shown to be activated after telomeric DNA oxidative damage, and Finkel-Biskis-Jinkins Osteosarcoma (FOS) is a ROS-induced transcription factor specifically involved in atherosclerosis-associated inflammation. Recently, OGG-1 and FOS have been evoked as new biomarkers of oxidative damage and inflammation processes linked to TL shortening (Patino, Mian et al. 2005, Lu and Liu 2010, Wang, Rhee et al. 2010).

Emerging data evoke a beneficial effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) redutase inhibitors, i.e. statin, on telomere biology, possibly contributing to the beneficial effects of statin therapy in coronary artery diseases (Spyridopoulos, Haendeler et al. 2004, Brouilette, Moore et al. 2007) . Moreover, statin therapy has been shown to prevent TL erosion after ex vivo incubation of endothelial progenitor cells of healthy subjects or after 12 months of intensive statin therapy in CAD patients (Assmus, Urbich et al. 2003, Satoh, Minami et al. 2009). However, no study has specifically addressed the potential impact of statin treatment on LTL. Thus, we tested the hypothesis that leukocyte TL could be influenced by statin treatment and related to the transcripts levels of these genes (FOS and OGG1), as new biomarkers of TL associated-oxidative stress and inflammation.

In patients with acute MI, we aimed to investigate clinical, pharmacological and biological determinants of circulating leukocyte TL, including statin treatment, and the relationships between TL and the expression of genes involved in oxidative DNA lesions and ROS-induced inflammation.

Methods

Patients

All the consecutive patients aged > 18 y and hospitalized <24 hours after symptoms onset for acute MI in the coronary care unit of Dijon University Hospital between 1st March and 30th September 2009 were included. MI was defined by an increase in serum troponin I (> upper limit of the hospital normal (ULN) range) associated with symptoms of ischemia and/or characteristic ECG signs. ST segment elevation myocardial infarction (STEMI) was defined by new ST segment elevation > 1 mm or left bundle branch block on the qualifying ECG. Patients under chronic treatment with corticosteroid or anti COX drugs (?) were excluded from the study. The present study complied with the Declaration of Helsinki and was approved by the ethics committee of University Hospital of Dijon. Each patient gave written consent before participation.

Data collection.

For each patients, data on demographics, cardiovascular (CV) risk factors (history of hypertension, diabetes, hyperlipidemia, current smoking (active smoking or stop smoking <3 months), body mass index (BMI) (weight (kg)/(height (m))^2), prior MI were prospectively collected, along with time delays to admission and clinical characteristics. Patients were prospectively asked for their current use of treatments before MI, including statin therapy, type of molecule and dose. Echocardiography was performed at day 3 ± 1 by a local investigator according to the Simpson method to calculate left ventricular ejection fraction (LVEF).

Biological data

Blood samples were drawn at admission (time delay from symptom onset to blood sampling: 230 [105 to 580] min). Homocysteine concentrations were determined by chemiluminescence on an Immulite 2000 analyzer (Diagnostic Products Corporation, Los Angeles, USA), C-reactive protein (CRP), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) concentrations were measured on a Dimension analyzer (Dade Behring, Newark, NE) using immunonephelometry assay. Low-density lipoprotein cholesterol (LDL-C) level was calculated using the Friedewald formula (Warnick, Knopp et al. 1990). Plasma glucose concentrations (enzymatic

method (glucose oxidase)) and creatinine levels were measured on a Vitros 950 analyzer (Ortho Clinical Diagnostics, Rochester, NY). Glycated hemoglobin A1c (HbA1c) was measured with ion exchange HPLC (Bio-Rad Laboratories, Richmond, CA).

Telomere length assay.

Blood collection

Blood samples were collected on admission in EDTA-containing Tubes and processed within 1 hour in a lysing solution (Tris HCL pH 7.5; 50 mM; $MgCl_2$ 25 mM; NaCl 50 mM), and leukocyte DNA was extracted by Salting-out. Mean telomere length was measured from DNA by quantitative polymerase chain reaction (PCR)-based assay (Cawthon 2002). Relative ratio telomere repeat copy number (T) to single-copy gene copy number (36B4 gene, encoding ribosomal phosphoprotein PO, located on chromosome 12; S) with all samples being compared with the same reference DNA sample. The T/S ratios have been confirmed previously to be highly consistent with the classical Southern blot on terminal restriction fragments (Epel, Blackburn et al. 2004, Grabowski, Hultdin et al. 2005). For each DNA sample, we performed 6 quantitative PCR runs: 3 “T” runs and 3 “S” runs. All DNA samples were analyzed in duplicate on separates plates, but in the same well positions. Determination of T and S quantities was performed using standardized threshold and without knowledge of clinical data.

The telomere-specific primers were: forward, 5’-GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T-3’, and reverse 5’-TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA-3’. The 36B4-specific primers were: forward 5’-CAG CAA GTG GGA AGG TGT AAT CC-3’, and reverse 5’-CCC ATT CTA TCA TCA ACG GGT ACA A-3’.

mRNA extraction and Quantitative Real-Time RT-PCR.

mRNA were obtained using à TRIZOL® (Invitrogen, Paisley, UK) protocol extraction assay and optimized by a RNA isolation method for quantitative real time RT-PCR described by Damla D Bilgin (Bilgin, DeLucia et al. 2009), and reverse transcribed by using Murine Moloney Leukemia Virus

Reverse Transcriptase (Invitrogen, Paisley, UK). Primer sequences for *c-Fos* and GAPDH are provided in Table 1.

Table 1: Primer pair sequences used for quantitative RT-PCR of the various listed genes.

Gene	Forward (5'-3')	Reverse (5'-3')
c-Fos	GGAGGACCTTATCTGTGCGTGA	GAACACACTATTGCCAGGAACACA
GAPDH	CATCTCTGCCCCCTCTGCT	ACGCCTGCTTCACCACCTT

Standard quantitative RT-PCR was performed in duplicates at least two or three times using SYBR green (Quiagen, UK) and TaqMan protocols on the Rotorgene 3000 (Corbett Research, Cambridge, UK) (Cerutti, Delcelo et al. 2004). RT-PCR are normalized by measuring average cycle threshold (Ct) ratio between investigated genes and control gene, glyceraldehyde-3-phosphate deshydrogenase (GAPDH).

Group definition

Patients under chronic statin therapy, defined by a current use of any statin therapy initiated > 6 months (?) before the index event, were compared with patients not under statin therapy. Statin therapy included pravastatin (29%), simvastatin (26%), atorvastatin (26%), rosuvastatin (17%), and fluvastatin (2%). Statin were also classified according to their hydrophilic properties. In order to further explore the potential impact of statin, statin dosages and type were converted to statin equivalent dosage (Grundy, Cleeman et al. 2004).

Statistical analysis.

Continuous data are represented as median (25^{th} to 75^{th} percentile) or mean ± SE as appropriate or as proportion. For continuous variables, a Kolmogorov-Smirnov analysis was performed to test for normality. Mann-Whitney Rank Sum test or student's t test was performed to compare the data between the 2 groups. Pearson or Spearman's rank correlation was applied to test for associations between continuous variables. Dichotomic variables, expressed as numbers (%), were compared by Chi square test for the 2 groups. Backward multiple linear regression analysis was performed with the LTL as a dependent variable. Variables entered into the models were those with a significant

relationship ($p<0.05$) with the dependent variable in univariate analysis. Stratified analysis based on median age (64 y) of the study population (≤64 y or >64 y) was also performed with the model.

Because statin therapy was not randomly assigned in the study population, a propensity score was calculated to account for potentially confounding factors by using multivariate logistic regression analysis. A multivariate logistic regression model was built to predict the chronic use of statin therapy, and to calculate a propensity score for statin therapy (vs no statin). All the variables listed in table 1 were tested for their univariate relationship with chronic statin use, and were included in a backward logistic regression model on the basis of $p<0.05$. Several approaches were used to assess the goodness of fit of the logistic regression model: 1) Area Under Curve (AUC) of the Receiver-Operating Characteristic Curve, 2) the Hosmer-Lemeshow p(HL) value, and 3) -2LogLikelihood. Then, two cohorts were built, with one patient under statin matched with one patient not under statin therapy, based on the propensity score. Two-sided P values <0.05 were considered statistically significant. Analyses were performed using SPSS software version 13.0 package (SPSS Inc).

Results

Characteristics of the study population

Baseline characteristics of the study population according the use of statin are shown in Table 1. Patients on statin treatment were older, with a higher risk profile including hypertension, obesity, diabetes, hypercholesterolemia and prior MI than patients not under statin treatment. The time from symptom onset to hospital admission was slightly reduced in the statin group. Statin therapy was also more frequently associated with other CV drugs such as ACE inhibitors, beta-blocker, and aspirin. Clinical characteristics of acute MI were similar for the 2 groups.

In statin group, chronic glycemia, was less well controlled and, as expected, LDL-cholesterol and total cholesterol levels were dramatically reduced –by about 30%- when compared with patients without statin treatment ($p<0.001$). Admission glycemia, CRP, homocystein, HDL-cholesterol and triglycerides levels were similar for the 2 groups. Leukocyte count was slightly lower in statin-treated patients.

One major finding of our study, which further lend support to our hypothesis, was that, although patients under chronic statin treatment were markedly older, their mean LTL was strikingly increased when compared with patients not under chronic statin therapy (1.29±0.11 vs. 1.25±0.11 T/S ratio, p=0.008) (figure 1 A). In contrast, mRNA levels of FOS and OGG1 were similar for the 2 groups (0.91±0.10 vs. 0.91±0.11, p=0.905 and 0.86±0.14 vs. 0.87±0.15, p=0.774, respectively) (figure 1 A). Patients under hydrophilic statin (n=32 (45%)), including pravastatin and rosuvastatin, had similar LTL than lipophilic statin (1.31±0.11 vs. 1.27±0.11, p=0.161). No significant difference was noted according to equivalent statin dosages (p=0.125).

Determinants of leukocyte telomere length

All the parameters listed in table 1 were tested by univariate analysis for their relation with LTL. Only 3 variables, in addition to statin treatment, were significantly associated with LTL: age, FOS and OGG1 mRNA levels. LTL gradually decreased with increasing age (r=-0.124, p=0.004), with increasing FOS (r=-0.199, p=0.039), and OGG1 mRNA levels, which showed the strongest relationship with LTL (r=-0.235, p<0.001) (figure 2). Interestingly, neither gender, nor lipid parameters (LDL-cholesterol, nor triglyceride, nor HDL-cholesterol or total cholesterol), nor classical systemic inflammation markers (white cell count, or CRP), nor CV risk factors were significantly associated with LTL.

Another set of stratified analysis based on sex showed that men and women share the same relationship of OGG1 transcripts levels with LTL. However, FOS and statin were only associated with LTL in men, while age and creatinine clearance were only significant in women, probably due to their older mean age (women: 70±16 vs. men: 63±13 years).

By multivariate analysis, age, FOS, and OGG1 mRNAs, in addition to statin treatment, remained independent predictors of LTL (R^2=0.13), as presented in table 2. In particular, statin therapy was strongly associated with longer LTL, even after adjustment for the confounding (p=0.001) (table 2).

Given the multifactorial modulation of TL shortening, we further wondered if determinants of LTL were similar in younger vs. older patients, by performing linear regression in stratified analysis: the

study population was divided in 2 equal groups (n=139 for each group), based on the median age (=64 years). Surprisingly, correlation analysis showed differential influence of age on LTL: in younger group (mean age=53±7y), LTL failed to diminish with age (r=+0.081, p=0.340) (figure 3). By multivariate analysis, in younger patients (≤64y), statin therapy was a strong and independent factor associated with longer LTL (p=0.005) (table 2). In contrast, in older patients (mean age77±7 years), there was a significant fall in T/S ratio with increasing age (r=-0.225, p=0.008) (figure 3). In older patients (>64 y), multivariate analysis showed that age was the strongest independent determinant of LTL (table 2). In both age groups, increased OGG1 mRNA level was a major factor independently associated with reduced LTL (table 2).

Propensity score analysis

Because statin therapy was not randomly assigned in the study population, a propensity score was calculated to account for potentially confounding factors by using multivariate logistic regression analysis. Backward multivariate analyses to predict the use of statin therapy included all the variables listed in the table 1 that were univariately associated with statin therapy (i.e. smoking, diabetes, hypercholesterolemia, leukocytes count, obesity, hypertension, CV treatments, time to admission, prior MI and age). The final model showed that only age, hypercholesterolemia and prior MI were independently associated with the chronic use of statin (table 3). The goodness of fit of the model was tested and the p value for the Hosmer-Lemeshow test was >0.05, indicating that the quality of the model's performance was satisfactory (AUC: 0.85±0.03, p<0.001 and p(HL)=0.141).

In order to further limit the potential imbalances between patients receiving vs. those not receiving statin, two cohorts matched on the propensity score for statin use were built (n=48 for each group). Both cohorts had similar baseline characteristics (table 4), including demographic, clinical and risk factors. Patients on statin therapy were more often treated with ACE inhibitors or aspirin. As expected, LDL-cholesterol and total cholesterol were lower in statin treated patients.

Again, in these cohorts matched for propensity score, LTL was markedly longer in patients under statin therapy (1.29±0.10 vs. 1.24±0.11 T/S ratio, p=0.026), and no significant influence of statin treatment on both FOS (0.91±0.10 vs. 0.92±0.12, p=0.835) and OGG1 mRNA levels (0.87±0.14 vs. 0.85±0.13, p=0.477) was noted (figure 1 B).

Discussion

Statin therapy represents the mainstay of treatment for hypercholesterolemia and demonstrated its efficacy in reducing CAD mortality in primary and secondary intervention trials. Whether part of this beneficial effect could be linked to TL preservation has been previously suggested, but not clearly assessed (Brouilette, Moore et al. 2007). Further supported by multivariate adjustments including many demographic and biological variables, and confirmed by the comparison of 2 cohorts of patients matched on a propensity score for statin use, our study showed for the first time that in high risk patients, 1) statin treatment was associated with longer telomere length, an effect which was independent of systemic inflammation and oxidative stress. 2) This association, which was particularly strong in younger patients, may have major clinical impact, since these patients may extensively benefit from statin treatment. 3) Our study also extends to humans the recent findings concerning the link between DNA-oxidative damage and telomere shortening found in yeast and mice, suggesting the necessity to repair oxidative guanine damage in order to maintain telomere integrity (Lu and Liu 2010, Wang, Rhee et al. 2010).

Leukocyte telomere length and statin

Although the interaction between telomere length and HMG-CoA redutase inhibitors have not been specifically addressed in humans, recent studies, including randomized clinical trials, suggested a potential therapeutic impact of statin on telomere biology. To the best of our knowledge, only Brouillette et al, 2007, in WOSCOPS trial, have addressed the relationship between LTL and chronic

statin treatment: they found that patients with increased risk based on telomere length were more prone to benefit from statin treatment for primary prevention of CAD (Brouilette, Moore et al. 2007). Like in our study, this effect was unrelated to lipid levels (i.e. triglyceride, LDL or HDL cholesterol) or CRP as a classical marker of inflammation. Hence, our data further extends the findings from WOSCOPS trial which concerned only pravastatin at the dose of 40 mg/d, to various types and doses of statins, and in the setting of routine clinical practice. Whether this relation depends on a specific molecule, on the dose or on the lipophilic nature of the molecule, deserves further investigation. Moreover, our results identifying younger patients (≤64 y) as more specifically relevant to target for prevention therapy, are also consistent with WOSCOPS trial which included only middle-aged patients (45-64 y, mean age 55 y).

Although the underlying mechanism of statin-related telomere protection is not known, it could hence constitute a new pleiotropic effect of this class of molecules. To further support this hypothesis, a small randomized trial designed to evaluate the effects of intensive lipid lowering therapy for 12 months in CAD patients, showed that telomere erosion of endothelial progenitor cells was prevented; this beneficial effect of statin was dose-dependent in vitro (Satoh, Minami et al. 2009). In addition to reduce CV risk as a consequence of lipid lowering, statins exert multiple actions on vascular and inflammatory cells, including a reduction of inflammation and associated oxidative stress, which could contribute to the pleiotropic effects of statin. In cultured human atherosclerotic plaque VSMCs, atorvastatin treatment delayed cell senescence and inhibited telomere shortening (Mahmoudi, Gorenne et al. 2008)), independently from a reduction of oxidative stress. In our work, statin therapy had also no significant impact on leukocyte biomarkers of ROS-induced oxidative damage and inflammation; taken together, these data indicate that the effect of statin on TL is at least partly independent of inflammation and oxidative stress. Ex vivo in cultured endothelial progenitor cells, atorvastatin dose-dependently inhibited the onset of senescence , independently of ROS (Spyridopoulos, Haendeler et al. 2004). Such beneficial effects of statin are believed to arise via geranyl-geranylpyrophosphate pathways modulating mRNA and protein levels of various cell cycle-

promoting proteins, but not by changes in telomerase activity (Assmus, Urbich et al. 2003). Alternatively, statin treatment could also act by a posttranscriptional mechanism, via an increase in telomere capping protein TRF-2 (Spyridopoulos, Haendeler et al. 2004) .

Determinants of leukocyte telomere length

In our study, we did not observe significant associations of telomere length with demographic characteristics, treatments or traditional risk factors including LDL-cholesterol, white cell count, and CRP. Most primary or secondary prevention studies showed no or only modest association with classical risk factors (Brouilette, Singh et al. 2003, Bekaert, De Meyer et al. 2007, Brouilette, Moore et al. 2007). Conversely, the present study identified new major determinants of LTL, i.e. FOS and OGG1, thus providing new hypothesis on telomere regulation in high risk patients.

Previous studies in humans have demonstrated the involvement of ROS and inflammation in LTL variations. Demissie *et al*. reported that systemic oxidative stress assessed by urinary 8-epi-prostaglandin $F_{2\alpha}$ was associated with reduced leukocyte TL in hypertensive subjects (Demissie, Levy et al. 2006). Moreover, in a large population of middle-aged men and women free of overt CV disease, TL was shorter in subjects with high interleukin 6 (IL-6), a marker of oxidative stress and inflammation ($p<0.01$) (Bekaert, De Meyer et al. 2007). In agreement with previous findings in patients with type 2 diabetes, chronic obstructive pulmonary disease (COPD) or in postmenopausal women, our data confirmed the lack of independent association of LTL with plasma CRP, as a classical marker of inflammation (Aviv, Valdes et al. 2006, Sampson, Winterbone et al. 2006, Adaikalakoteswari, Balasubramanyam et al. 2007, Houben, Mercken et al. 2009). Our study confirmed these data and established that reduced LTL were significantly associated with increased levels of new biomarkers of oxidative stress and inflammation, independently of age. FOS, a ROS-induced transcription factor integrating multiple signaling pathway, that is more sensitive and specific to disease severity than CRP, has been recently localized in atherosclerotic plaques, and could play a direct role in atherosclerosis (Lavezzi, Milei et al. 2003, Patino, Mian et al. 2005).

Although the mechanisms remain to be explored, our findings importantly suggest that leukocyte FOS transcript levels is a major biomarker associated with LTL variation, with more clinical relevance than traditional inflammation marker, including CRP, for exploring the relationship between telomere and CAD. This relation is particularly true in younger patients.

One of the main finding of our study is that OGG1 mRNA level was a major and independent determinant of leukocyte telomere length. Moreover, this strong inverse relation was found whatever the age of patients. Our data confirmed and extended to MI patients some recent experimental data obtained in yeast and mice, further supporting the hypothesis that BER pathway actively participate in oxidative base repair to maintain telomere integrity (Lu and Liu 2010, Wang, Rhee et al. 2010).

Study limitation

The main strength of this study is the use of prospective design, reflecting the daily clinical practice in patients with acute MI. The adjustment for a wide range of possible confounding factors limits the risk of bias in our conclusions. This study, however, suffers the usual limitations of non randomized studies, and therefore determines correlations, rather than causal relationships. Moreover, linear regression model only explained 13% of LTL variance, suggesting that other unknown factors have influenced LTL in such patients. However, in order to limit the influence of such factors, we have developed several analytical strategies: 1) first we have performed the analysis by testing a very wide range of possible confounding factors, including demographics, risk factors and medical history, treatments, biological factors, etc. that could limit the risk of bias in our conclusions; 2) secondly, a propensity score for the use of statin has been calculated, and a matching procedure, based on the propensity score has been used, in order to further limit the potential imbalances between patients receiving statin versus not receiving statin. Hence, although we cannot exclude the impact of other unmeasured confounding factors, we may think that the observed effects of statin on LTL are robust and reliable. Also the lack of information on the duration of statin treatment, in addition to the

diversity of the doses and type of statin used have limited the interpretation of the analysis. Further longitudinal or randomized studies are needed to validate these hypotheses. As our results on telomere length were obtained from circulating leukocytes, we can only presume of the extrapolation of the findings on vascular cells. However, a strong correlation between telomeres length in leukocytes and in aortic vascular wall cells was recently established by Wilson *et al.* (Wilson, Herbert et al. 2008). Given the strength of this association, irrespective of the presence of overt vascular wall disease, we may think that the findings reported in the present study in circulating white blood cells may translate in vascular cells.

Conclusion

Our prospective study provides important information on the positive impact of chronic statin treatment on telomere biology in high risk patients, consistent with what was suggested earlier. If these data are confirmed in longitudinal and randomized studies, it could represent a new pleiotropic effect of such molecules in humans. Interestingly, the beneficial influence of statin therapy on LTL was more particularly evident in younger patients (<64 y). Given the compelling evidence for a causal role of vascular ageing for CAD, as indicated by telomere shortening, these data suggest opportunities for identifying new targets for early primary preventive treatment strategies. Moreover, our findings provides new insights on the potential mechanism influencing telomere length, and raised mRNA FOS and OGG1 as new relevant biomarkers of leukocytes telomere length in the setting of coronary artery disease.

Acknowledgement

We wish to thank Jean-Claude Beer, Edith Fusier and Martine Jeanningros for research assistance and Philip Bastable for english assistance.

Figure legend

Figure 1 : Statin treatment and levels of Leukocyte Telomere Length, FOS and OGG1 transcrits in **A**) whole study population (n=278) and **B**) population matched on propensity score for statin treatment (n=48 for each matched group).

Figure 2: Relationship between Leukocyte Telomere Length and age, FOS and OGG1 transcrits levels.

Figure 3: Relationship between Leukocyte Telomere Length and age, classified by age group (≤64y or >64y).

Reference

Adaikalakoteswari, A., M. Balasubramanyam, R. Ravikumar, R. Deepa and V. Mohan (2007). "Association of telomere shortening with impaired glucose tolerance and diabetic macroangiopathy." Atherosclerosis **195**(1): 83-89.

Assmus, B., C. Urbich, A. Aicher, W. K. Hofmann, J. Haendeler, L. Rossig, I. Spyridopoulos, A. M. Zeiher and S. Dimmeler (2003). "HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes." Circ Res **92**(9): 1049-1055.

Aviv, A., A. Valdes, J. P. Gardner, R. Swaminathan, M. Kimura and T. D. Spector (2006). "Menopause modifies the association of leukocyte telomere length with insulin resistance and inflammation." J Clin Endocrinol Metab **91**(2): 635-640.

Babizhayev, M. A., E. L. Savel'yeva, S. N. Moskvina and Y. E. Yegorov (2010). "Telomere Length is a Biomarker of Cumulative Oxidative Stress, Biologic Age, and an Independent Predictor of Survival and Therapeutic Treatment Requirement Associated With Smoking Behavior." Am J Ther.

Bekaert, S., T. De Meyer, E. R. Rietzschel, M. L. De Buyzere, D. De Bacquer, M. Langlois, P. Segers, L. Cooman, P. Van Damme, P. Cassiman, W. Van Criekinge, P. Verdonck, G. G. De Backer, T. C. Gillebert and P. Van Oostveldt (2007). "Telomere length and cardiovascular risk factors in a middle-aged population free of overt cardiovascular disease." Aging Cell **6**(5): 639-647.

Benetos, A., J. P. Gardner, M. Zureik, C. Labat, L. Xiaobin, C. Adamopoulos, M. Temmar, K. E. Bean, F. Thomas and A. Aviv (2004). "Short telomeres are associated with increased carotid atherosclerosis in hypertensive subjects." Hypertension **43**(2): 182-185.

Bilgin, D. D., E. H. DeLucia and S. J. Clough (2009). "A robust plant RNA isolation method suitable for Affymetrix GeneChip analysis and quantitative real-time RT-PCR." Nat Protoc **4**(3): 333-340.

Brouilette, S., R. K. Singh, J. R. Thompson, A. H. Goodall and N. J. Samani (2003). "White cell telomere length and risk of premature myocardial infarction." Arterioscler Thromb Vasc Biol **23**(5): 842-846.

Brouilette, S. W., J. S. Moore, A. D. McMahon, J. R. Thompson, I. Ford, J. Shepherd, C. J. Packard and N. J. Samani (2007). "Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study." Lancet **369**(9556): 107-114.

Cawthon, R. M. (2002). "Telomere measurement by quantitative PCR." Nucleic Acids Res **30**(10): e47.

Cerutti, J. M., R. Delcelo, M. J. Amadei, C. Nakabashi, R. M. Maciel, B. Peterson, J. Shoemaker and G. J. Riggins (2004). "A preoperative diagnostic test that distinguishes benign from malignant thyroid carcinoma based on gene expression." J Clin Invest **113**(8): 1234-1242.

Demissie, S., D. Levy, E. J. Benjamin, L. A. Cupples, J. P. Gardner, A. Herbert, M. Kimura, M. G. Larson, J. B. Meigs, J. F. Keaney and A. Aviv (2006). "Insulin resistance, oxidative stress, hypertension, and leukocyte telomere length in men from the Framingham Heart Study." Aging Cell **5**(4): 325-330.

Epel, E. S., E. H. Blackburn, J. Lin, F. S. Dhabhar, N. E. Adler, J. D. Morrow and R. M. Cawthon (2004). "Accelerated telomere shortening in response to life stress." Proc Natl Acad Sci U S A **101**(49): 17312-17315.

Fuster, J. J. and V. Andres (2006). "Telomere biology and cardiovascular disease." Circ Res **99**(11): 1167-1180.

Gardner, J. P., S. Li, S. R. Srinivasan, W. Chen, M. Kimura, X. Lu, G. S. Berenson and A. Aviv (2005). "Rise in insulin resistance is associated with escalated telomere attrition." Circulation **111**(17): 2171-2177.

Grabowski, P., M. Hultdin, K. Karlsson, G. Tobin, A. Aleskog, U. Thunberg, A. Laurell, C. Sundstrom, R. Rosenquist and G. Roos (2005). "Telomere length as a prognostic parameter in chronic lymphocytic leukemia with special reference to VH gene mutation status." Blood **105**(12): 4807-4812.

Grundy, S. M., J. I. Cleeman, C. N. Merz, H. B. Brewer, Jr., L. T. Clark, D. B. Hunninghake, R. C. Pasternak, S. C. Smith, Jr. and N. J. Stone (2004). "Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines." Circulation **110**(2): 227-239.

Houben, J. M., E. M. Mercken, H. B. Ketelslegers, A. Bast, E. F. Wouters, G. J. Hageman and A. M. Schols (2009). "Telomere shortening in chronic obstructive pulmonary disease." Respir Med **103**(2): 230-236.
Houben, J. M., H. J. Moonen, F. J. van Schooten and G. J. Hageman (2008). "Telomere length assessment: biomarker of chronic oxidative stress?" Free Radic Biol Med **44**(3): 235-246.
Kawanishi, S. and S. Oikawa (2004). "Mechanism of telomere shortening by oxidative stress." Ann N Y Acad Sci **1019**: 278-284.
Lavezzi, A. M., J. Milei, D. R. Grana, F. Flenda, A. Basellini and L. Matturri (2003). "Expression of c-fos, p53 and PCNA in the unstable atherosclerotic carotid plaque." Int J Cardiol **92**(1): 59-63.
Lu, J. and Y. Liu (2010). "Deletion of Ogg1 DNA glycosylase results in telomere base damage and length alteration in yeast." EMBO J **29**(2): 398-409.
Mahmoudi, M., I. Gorenne, J. Mercer, N. Figg, T. Littlewood and M. Bennett (2008). "Statins use a novel Nijmegen breakage syndrome-1-dependent pathway to accelerate DNA repair in vascular smooth muscle cells." Circ Res **103**(7): 717-725.
Ogami, M., Y. Ikura, M. Ohsawa, T. Matsuo, S. Kayo, N. Yoshimi, E. Hai, N. Shirai, S. Ehara, R. Komatsu, T. Naruko and M. Ueda (2004). "Telomere shortening in human coronary artery diseases." Arterioscler Thromb Vasc Biol **24**(3): 546-550.
Patino, W. D., O. Y. Mian, J. G. Kang, S. Matoba, L. D. Bartlett, B. Holbrook, H. H. Trout, 3rd, L. Kozloff and P. M. Hwang (2005). "Circulating transcriptome reveals markers of atherosclerosis." Proc Natl Acad Sci U S A **102**(9): 3423-3428.
Samani, N. J., R. Boultby, R. Butler, J. R. Thompson and A. H. Goodall (2001). "Telomere shortening in atherosclerosis." Lancet **358**(9280): 472-473.
Sampson, M. J., M. S. Winterbone, J. C. Hughes, N. Dozio and D. A. Hughes (2006). "Monocyte telomere shortening and oxidative DNA damage in type 2 diabetes." Diabetes Care **29**(2): 283-289.
Satoh, M., Y. Minami, Y. Takahashi, T. Tabuchi, T. Itoh and M. Nakamura (2009). "Effect of intensive lipid-lowering therapy on telomere erosion in endothelial progenitor cells obtained from patients with coronary artery disease." Clin Sci (Lond) **116**(11): 827-835.
Spyridopoulos, I., J. Haendeler, C. Urbich, T. H. Brummendorf, H. Oh, M. D. Schneider, A. M. Zeiher and S. Dimmeler (2004). "Statins enhance migratory capacity by upregulation of the telomere repeat-binding factor TRF2 in endothelial progenitor cells." Circulation **110**(19): 3136-3142.
Valdes, A. M., T. Andrew, J. P. Gardner, M. Kimura, E. Oelsner, L. F. Cherkas, A. Aviv and T. D. Spector (2005). "Obesity, cigarette smoking, and telomere length in women." Lancet **366**(9486): 662-664.
Wang, Z., D. B. Rhee, J. Lu, C. T. Bohr, F. Zhou, H. Vallabhaneni, N. C. de Souza-Pinto and Y. Liu (2010). "Characterization of oxidative Guanine damage and repair in mammalian telomeres." PLoS Genet **6**(5): e1000951.
Warnick, G. R., R. H. Knopp, V. Fitzpatrick and L. Branson (1990). "Estimating low-density lipoprotein cholesterol by the Friedewald equation is adequate for classifying patients on the basis of nationally recommended cutpoints." Clin Chem **36**(1): 15-19.
Wilson, W. R., K. E. Herbert, Y. Mistry, S. E. Stevens, H. R. Patel, R. A. Hastings, M. M. Thompson and B. Williams (2008). "Blood leucocyte telomere DNA content predicts vascular telomere DNA content in humans with and without vascular disease." Eur Heart J **29**(21): 2689-2694.