

# Are the leukocyte telomere length attrition and telomerase activity alteration potential predictor biomarkers for sporadic TAA in aged individuals?

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**Abstract** A large variability in occurrence, complications, and age/gender manifestations characterizes individual susceptibility of sporadic thoracic aortic aneurysms (TAA), even in subjects with the same risk factor profiles. The reasons are poorly understood. On the other hand, TAA pathophysiology mechanisms remain unclear than those involved in abdominal aorta aneurysms. However, recent evidence is suggesting a crucial role of biological ageing in inter-individual risk variation of cardiovascular diseases, including sporadic TAA. Biological age rather than chronological age is a better predictor of vascular risk. Relevant assumptions support this concept. In confirming this evidence and our preliminary data, the mean of blood leukocyte telomere length, through use of terminal restriction fragment assay and in blood samples from sporadic TAA patients and controls, was examined. Telomerase activity was also analyzed in two groups. In addition, we verified the weight of genetic inflammatory variants and the major TAA risk factors in telomere/telomerase impairment. Aorta histopathological abnormalities and systemic inflammatory mediators were ultimately

correlated with telomere/telomerase impairment. Data obtained demonstrated shorter telomeres and a reduced telomerase activity in TAA patients significantly associated with a genetic inflammatory risk profile, age, gender, smoking, hypertension, a histopathological phenotype, and higher levels of systemic inflammatory mediators than controls. In conclusion, telomere and telomerase activity's detection might be used as predictor biomarkers of sporadic TAA. Their impairment also suggests a strong role of vascular ageing in sporadic TAA, evoked by both environmental and genetic inflammatory factors.

**Keywords** Sporadic TAA · Biological ageing · Leukocyte telomere length attrition · Telomere activity alteration · Predictor TAA biomarkers

## Introduction

The medical research has as recent major objective to determine the improving of life quality of people, and particularly of aged individuals given the continuous augment of ageing population phenomenon. This trend determines several medical, economic, and social problems because of dramatic increase in the number of not autonomous individuals affected by various pathologies (Balistreri et al. 2013a). Among these, cardiovascular diseases (CVD) have a disproportionate prevalence with advancing age (Centers for Disease Control and Prevention; National Center for Injury Prevention Control. Available at: <http://webappa.cdc.gov/cgi-bin/>

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**broker.exe.** Accessed January, 2014Thus, the research efforts are centered in reducing and/or retarding the onset and progression of these diseases by searching new strategies for early prevention and diagnosis, such as appropriate (molecular and genetic) biomarkers and new and more efficient therapeutic treatments, i.e., the personalized therapies (Ouzounian et al. 2007). This purpose seems to be very ambitious, since these pathologies are characterized by a complex pathophysiology. In addition, their mechanisms are not completely clear and prevalently articulated in multistep clinical events, whose individual probability, realization, and timing vary not only with risk factors, but prevalently with the intrinsic ability of self-artery-repair. Their progression is generally assumed as irreversible and one-directional as a function of time. However, for each step, a small reverse probability does exist (Goldschmidt-Clermont et al. 2012). Some individuals remain sheltered from the consequences of vascular alterations, even in the presence of potent risk factors (Goldschmidt-Clermont et al. 2012). This might be attributed to a substantial ability genetically determined for self-repair of their arteries. As result, a large individual variability characterizes the susceptibility of all CVD diseases, such as sporadic thoracic aortic aneurysms (TAA). In the case of sporadic TAA, a silent illness and insidious in its onset and progression, several cardiovascular risk factors (i.e., age, gender, hypertension, oxidative stress, smoking, etc.) seem to explain a large amount of its susceptibility in the general population (Ince and Nienaber 2007; El-Hamamsy and Yacoub 2009; Elefteriades and Farkas 2010). At an individual level, there is a wide variation in both its occurrence and complications (i.e., dissection and rupture) and age/gender related manifestations, even in individuals with the same risk factor profiles (Ince and Nienaber 2007). Recent lines of evidence sustain that the rate of biological age may be the principal cause of inter-individual CVD risk variation. Biological age rather than chronological age is a better predictor of vascular risk (Epel et al. 2008; Huzen et al. 2010; Mainous and Diaz 2010; Saliques et al. 2010; De Meyer et al. 2011; Fitzpatrick et al. 2011; Fyrquist et al. 2013). This assumption is based on the relevant data of Wilson and colleagues (Wilson et al. 2008). They evidenced as the telomere content (the TTAGGG DNA repeats at the ends of chromosomes (Blackburn 2010)) in circulating blood leukocytes accurately reflects the biological age of the vascular wall. Consistent with these observations, a series of recent studies demonstrated significant associations between the reduction of telomere leukocyte length and

the risk of several CVDs, such as aorta aneurysms. In particular, Atturu and colleagues observed that short leukocyte telomere length is associated with abdominal aortic aneurysm, by examining a European population and evaluating the mean terminal restriction fragment (TRF) length, a marker of telomere length (Atturu et al. 2010). Yan and colleagues demonstrated a significant shorter leukocyte telomere length in a Chinese population affected by aortic dissection (Yan et al. 2011). Precisely, they detected a decreased telomere length ratio (T/S ratio) in 72 cases vs. 72 controls, by using a quantitative PCR method. No association was evidenced for multiple aneurysms in another European study (Björck et al. 2011). In previous studies, we recently obtained promising results by comparing the mean of TRF length in cases affected by sporadic TAA and controls. In particular, shorter telomeres were detected in cases than in controls (Balistreri et al. 2012; Ruvolo et al. 2014). Subsequently to our study, only the group of Salonurmi analyzed the telomere length in TAA aneurysms, surprisingly demonstrating opposite results. Precisely, they evaluated the relative lengths of telomeres by using the Cawthon's quantitative PCR method in a Finnish population, and they observed longer telomeres in the cases than healthy age matched controls (Huusko et al. 2012).

Recently, an impairment of telomerase activity has been also assessed in aorta aneurysms by two recent studies (Dimitroulis et al. 2011; Findeisen et al. 2011).

In contrast, until now there are no reports in the literature about the biological effects mediated by genetic and environmental factors in the impairment of the telomere/telomerase system in aorta aneurysms, and particularly in sporadic TAA. This is a very interesting topic. It is well recognized that the rate of vascular ageing is the result of a complex interaction between genetic and environmental factors (O'Rourke et al. 2011; Laurent 2012; Nilsson 2012; Bachschmid et al. 2013). On the other hand, in all CVDs (TAAs included), genetic component seems to play a key role. In the recent years of post-human genome sequencing project, this is confirmed by non-biased techniques (i.e., genome-wide association studies). In particular, they have discovered CVD-relevant genes, which are not associated with previously suspected pathways of lipid metabolism, diabetes, or hypertension. Their major number is prevalently associated with inflammation (such as genes related to TLR4-mediated signaling pathway) and stem cell biology (i.e., that of endothelial progenitor cells). Only a minority of implicated CVD-

genes are linked to lipid and other classical risk factor pathways (Incalcaterra et al. 2013). This underlines the crucial influence of inflammation on the rate of biological vascular ageing and its complications, such as vascular remodeling and medial degeneration, the typical pathological entities of several aorta diseases, including sporadic TAA. Among the genes able to modulate inflammation, genes related to TLR4-mediated signaling pathway have been associated with the susceptibility for several CVDs (Balistreri et al. 2004, 2009, 2013b, c; Frantz et al. 2007; Ionita et al. 2010; Hofmann et al. 2011; Navi et al. 2013; Incalcaterra et al. 2013). In our recent study (Ruvolo et al. 2014), we have also demonstrated their role in sporadic TAA. This is in agreement with current evidence, which points to the functional importance of TLR4-mediated signaling pathway in mediating physiological aorta homeostasis and the maintaining of protection against pathogens and damage cell molecules, as well as in inducing pathological aorta phenotypes. On the other hand, it is increasing the opinion to consider aortic aneurysms, and particularly the sporadic forms of TAA, as inflammatory/immune diseases with a strong genetic component (Kuivaniemi et al. 2008).

Based on these observations and our preliminary and promising results (Balistreri et al. 2012; Ruvolo et al. 2014), we detected the eventual telomere/telomerase system's impairment in a larger number of cases affected by sporadic TAA and age/gender matched controls. We also analyzed the potential influence of ten polymorphisms related to TLR4-mediated signaling pathway and major TAA risk factors in telomere/telomerase impairment. Aorta histopathological abnormalities and systemic inflammatory mediators were ultimately correlated with telomere/telomerase impairment.

## Materials and methods

### Patients and controls

Our study included a group of 80 patients [55 men (69 %) and 25 (31 %) women; mean age  $63 \pm 11.6$ ] affected by sporadic TAA, as confirmed by histopathological analyses and exclusion criteria for syndromic and familial forms (e.g., Marfan and Ehler's Danlos syndromes) and autoimmune connective tissue disorders. They randomly were selected by a cohort of 161 individuals [127 men (78 %) and 34 (22 %) women;

mean age  $63 \pm 10.7$ ] from Western Sicily, previously enrolled and analyzed in our recent studies (Pisano et al. 2012a, b; Balistreri et al. 2013b, c; Ruvolo et al. 2014). The sporadic TAA diagnosis was performed through imaging technologies (i.e., ECHO, CT, and MRI). The aneurysm's localization essentially was in the ascending aorta (precisely in the aortic sinus and tubular portion and sometimes only in the tubular portion) and aortic bulb, or both.

Medical histories pertinent to aortic disease were obtained from patient's medical records. Thus, demographic and clinical features, comorbidity conditions, and pharmacological treatments were collected (Table 1).

An equal number of controls [ $N=80$ ; 55 men (69 %) and 25 (31 %) women; mean age  $61 \pm 8.5$ ] was included in this study, randomly selected by a cohort of healthy controls previously enrolled and studied in our previous studies, as the case's group (Pisano et al. 2012a, b; Balistreri et al. 2013b, c, 2014a, b; Ruvolo et al. 2014) (Table 1).

Patients and controls belonged to same ethnic group, since their parents and grandparents were born in Sicily. No statistically significant differences were observed analyzing the case and control features as summarized in Table 1, with the exception of smoking and hypertension (42 vs. 18,  $p=0.00008$ , and 48 vs. 18,  $p=0.000001$ , by  $\chi^2$  test, respectively). In particular, hypertension characterized the 60 % of patients opportunely treated with medications like ACE inhibitors and beta-blocker etc. during the follow-up and after surgery.

Our study received approval from the local ethics committees, and all participants gave their informed consent. Data were encoded to ensure the anonymity of patients in the study and control groups. All measurements were performed without knowledge of the nature of the study.

### Telomere length assay

DNA samples of 80 matched controls and 80 cases were extracted from peripheral blood samples collected in tripotassium EDTA and purified by using a QIAamp Blood DNA Maxi kit (Qiagen, Dusseldorf, Germany). The mean terminal restriction fragment (TRF) length, a marker of telomere length, was measured through a chemiluminescence technique and using the TeloTAGGG telomere length assay kit (Roche Diagnostics, Indianapolis, USA), according to the

**Table 1** Demographic and clinical characteristics, comorbidity conditions, and pharmacological treatment of 80 patients affected by sporadic TAA, 80 control subjects

Variables	Patients N=80	Controls N=80	P1 (cases vs. controls)
Demographic characteristics			
Age, mean (SD)	63 (11.6)	61 (8.5)	NS
Body mass index, mean (SD)	26.9 (3.9)	26.7 (1.8)	NS
Size and location			
Size (mm), mean (SD)	53.5	0 (0)	
Location, no. (%):		0 (0)	
Ascending aorta	62 (85)		
Aortic bulb	12 (15)		
Comorbidity conditions, no. (%)			
Smoking	42 (53)	18 (23)	<b>0.00008</b>
Hypertension	48 (60)	18 (23)	<b>0.00001</b>
Dislipidemia	12 (15)	8 (10)	NS
Diabetes mellitus	10 (13)	8 (10)	NS
Renal failure	5 (6)	0 (0)	NS

**Bold entries** The unique features of patients and controls, which are significantly different, are smoking and hypertension

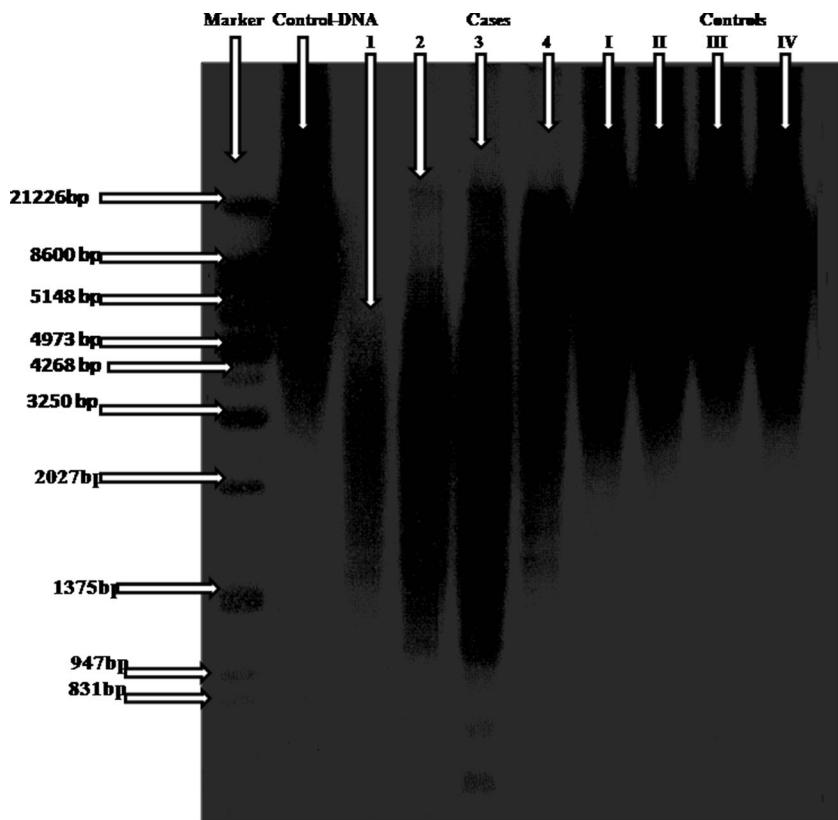
manufacture's protocol. In brief, 25 µl of each DNA sample (75 ng/µl) was digested with 10 U of Rsa I (Invitrogen Ltd, UK) and 10 U of Hinf I (Invitrogen Ltd, UK) at 37 °C for two and a half hours. The DNA fragments were separated by electrophoresis on 0.6 % agarose gels (20×20 cm) at 150 V for 1 h and 50 V for 18 h. The DNA was then depurinated with 0.25 % HCl for 20 min, denatured with 0.5 mol/L NaOH/1.5 mol/L NaCl for 30 min, and neutralized with 0.5 mol/L Tris/1.5 mol/L NaCl, pH 8 for 30 min. The DNA was transferred overnight onto a positively charged nylon membrane (Roche Diagnostics, Indianapolis, USA) using a southern blot technique and fixed with ultraviolet light. The membrane was then hybridized with a telomere repeat-specific digoxigen (DIG)-labeled probe overnight at 42 °C. The membrane was washed three times with 2× saline sodium citrate (SSC)/0.1 % sodium dodecyl sulfate (SDS) followed by 0.2×SSC/0.1 % SDS. The membrane was then incubated with a DIG-specific antibody covalently coupled to alkaline phosphatase. The immobilized telomere probe was then visualized using CDP star, a highly chemiluminescent substrate for alkaline phosphate. Exposing the membrane to an autoradiograph film (Roche Diagnostics, Indianapolis, USA) revealed the telomere smears. The mean terminal restriction fragment of each sample was calculated using the formula  $\text{mean TRF} = \text{TRF} = (\sum(i\text{OD}) / (\sum(i\text{OD}/L_i))$ , where  $\text{OD}_i$  is the optical density at a given position on the gel and  $L_i$  is the molecular weight at that

position. An internal control with a known mean TRF length (DNA extracted from HUVEC cells) was used to study the inter-gel and intra-gel variation in mean TRF length (data not shown). The mean TRF length of samples from cases and controls were adjusted to the standardized internal control to account for the inter-gel and intra-gel variation (see Fig. 1).

#### Detection of telomerase activity by TRAP assay

For quantitative analysis of telomerase activity, a Telomeric Repeat Amplification Protocol (TRAP) (Kim and Wu 1997) and a photometric enzyme immunoassay were performed using TeloTAGGG Telomerase PCR Elisa<sup>Plus</sup> kit (Roche Diagnostics, Indianapolis, USA), according to the manufacturer's protocol. This precisely involved elongation and amplification of telomerase reaction products to allow highly sensitive detection of telomerase activity by a photometric enzyme immunoassay. Briefly, we firstly obtained pellets of blood peripheral leukocytes. They were lysed directly in sterile reaction tubes using the lysis buffer provided in the kit. Protein lysate was kept on ice for 30 min and centrifuged at 16,000g for 20 min at 2–8 °C. Protein concentration was measured by standard methods. Subsequently, the supernatants obtained were utilized in quantity of 0.5–10 µg total protein for the TRAP reaction, having the assurance to prepare for each sample a negative

**Fig. 1** A film of chemiluminescence detection of the mean leukocyte TFR length



control by heat inactivation of its aliquot at 85 °C for 20 min. In performing the TRAP reaction, high control template (concentration 0.1 amol/μl; quantity used for each reaction 1 μl), a reaction mixture (25 μl for each sample), and an internal standard (IS; 5 μl for each sample) provided in the kit were also utilized. Thus, sterile tubes (each containing a total of 30 μl of the master mix-25 μl of reaction mixture and 5 μl of IS and a suitable volume of each negative or positive sample or 1 μl of control template) were transferred to a thermal cycler (MyCycler, Biorad). A combined primer elongation/amplification reaction was performed by the following protocol: primer elongation 10–30 min at 25 °C 1 cycle; telomerase inactivation 5 min at 94 °C 1 cycle; amplification (denaturation 30 s at 94 °C; annealing 30 s at 50 °C; polymerization 90 s at 72 °C) for 30 cycles; 10 min at 72 °C for 1 cycle; hold at 4 °C. During the reaction, telomerase add telomeric repeats (TTAG GG) to the 3' end of the biotin-labeled primer. The elongation products, as well as the IS included in the same reaction tube, are then amplified. The PCR products were split into two aliquots, denatured,

bound to a streptavidin-coated 96-well plate, and hybridized to a digoxigenin (DIG)-labeled telomeric repeat-specific probes, specific for the telomeric repeats and IS. The resulting products were immobilized via the biotin label to streptavidin-coated 96-well microplate. Immobilized amplicons were then detected with an antibody against digoxigenin that is conjugated to horseradish peroxidase (anti-DIG-HRP) and the sensitive peroxidase substrate TMB. Sample absorbance was measured at 450 nm (reference wavelength 690 nm) using an ELISA plate reader within 30 min after the addition of the stop reagent. Absorbance values were then utilized to calculate the relative telomerase activity (RTA) of each sample using an appropriate formula provided in the kit's protocol.

Aortic specimens, histopathological and immunohistochemical assays, TUNEL testing, and genotyping

Aorta samples as well as DNA samples of individuals selected in our study were collected, obtained, and

analyzed in our previous studies (Pisano et al. 2012a, b; Balistreri et al. 2013b, c, 2014a, b; Ruvolo et al. 2014). The procedures, criteria, definitions, and grading systems for tissue sample collection, staining, histopathological and immunohistochemical assessment, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) testing, and genotyping of ten polymorphisms related to TLR4-mediated signaling pathway were previously described in our studies (Pisano et al. 2012a, b; Balistreri et al. 2013b, c, 2014a, b; Ruvolo et al. 2014). Thus, data previously obtained were compared and correlated with telomere/telomerase impairment between cases and controls.

### Statistical analysis

All analyses were performed with R and Microsoft Excel software. Significant differences between qualitative variables were calculated by using Pearson  $\chi^2$  test. Furthermore, odds ratios (OR) with 95 % confidence intervals (CI) and their significance were calculated. The difference in mean TRF length and the mean values of RTA between cases and controls was analyzed using the unpaired *t* test with Welch correction. The difference in mean TRF length and the mean values of RTA in subjects with different risk factors was analyzed using bivariate correlation for continuous variables and an independent samples *t* test for categorical variables. The differences in systemic inflammatory mediators between the two groups were also detected by the unpaired *t* test with Welch correction. The independent effect of the risk factors on the mean TRF length and the mean values of RTA was analyzed using a linear regression model controlling for case-control status. To identify possible correlations, a non-parametrical Spearman correlation test was also used.

Allele and genotype frequencies were evaluated by gene count. Data were tested for goodness of fit between observed and expected genotype frequencies according to the Hardy-Weinberg equilibrium, by  $\chi^2$  tests. Significant differences in frequencies among groups were calculated by using  $\chi^2$  test and appropriate tables ( $2 \times 2$  and  $3 \times 2$  tables etc. where appropriate).

Analysis of variance (ANOVA) test (corrected by Bonferroni) was also utilized to compare positive inflammatory/immune cells between case and control aorta samples.

## Results

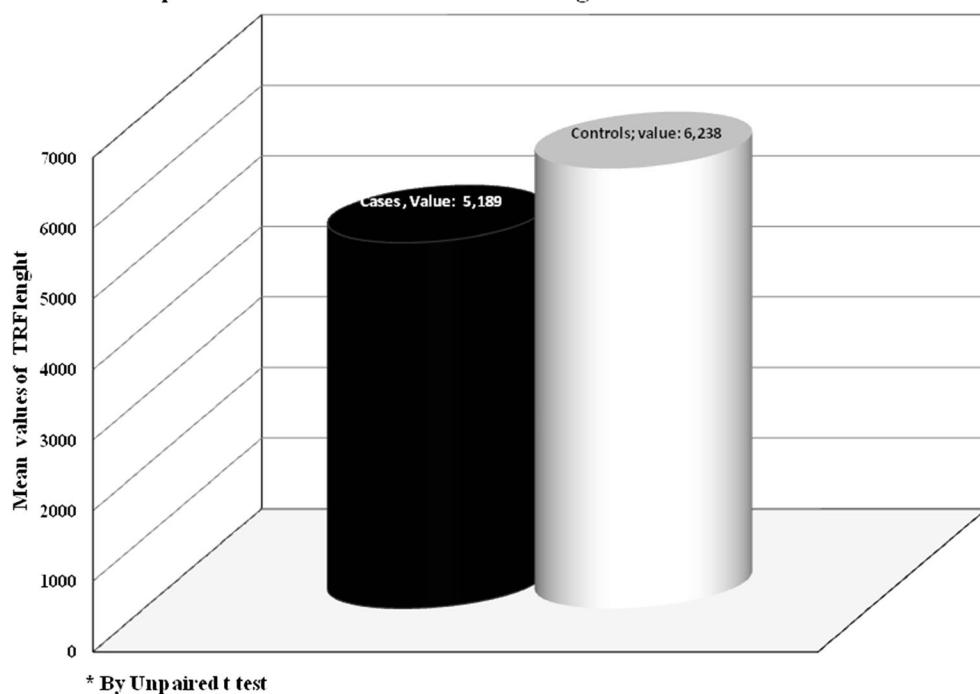
Evaluation of the mean TRF length in cases and controls, correlation with risk factors, and detection of independent effects of risk factors on telomere impairment

The evaluation of the mean TRF length in the two groups and their analysis demonstrated that the mean TRF length of the case's group (5,189, standard deviation [SD]=0.367 kbp) was significantly lower than that observed in the control group (6,238, SD=0.128 kbp). A difference of 1,049 bp was observed between cases and controls (95 % confidence interval [CI] 37–107 bp,  $p<0.0001$ , by *t* test) (Fig. 2). In addition, correlations were detected between the mean TRF length and age in both patients and controls (2.6 bp decrease per year,  $SD=2.1$ ;  $p=0.03$ ) and between the mean TRF length and aneurysm size ( $p=0.045$ ) among the cases. The comparisons of mean TRF length values between male and female patients demonstrated a significant reduction in woman (5,337,  $SD=0.119$  kbp vs. 5,012,  $SD=0.239$ ;  $p=0.0001$  by *t* test Welch corrected). Significant differences were also observed in mean TRF length between male patients and male controls (5,337,  $SD=0.119$  kbp vs. 6,402,  $SD=0.130$  kbp;  $p<0.0001$  by *t* test Welch corrected). A significant decrease was also assessed in female cases vs. female controls (5,012,  $SD=0.239$  vs. 6,202,  $SD=0.345$  kbp;  $p<0.0001$  by *t* test Welch corrected). Furthermore, we analyzed the independent effects of risk factors including age, gender, smoking, hypertension, diabetes, dislipidemia, and renal failure on the mean TRF length among the subjects. As independent risk factors, age, smoking, and hypertension in cases were the unique risk factors significantly associated with the mean TRF length ( $p=0.02$ ,  $p=0.002$ , and  $p=0.001$ , respectively). The case's subjects with smoking and hypertension histories had a significantly shorter mean TRF length (5,089,  $SD=0.089$ ) compared to subjects without these risk factors (5,521,  $SD=0.112$ ) (mean difference 432 bp, CI 8–228 bp,  $p=0.01$ ) Fig. 3.

Relative telomerase activity in cases and controls, correlation with risk factors, and detection of independent effects of risk factors on telomerase activity

The analysis of the mean values of RTA in the cases and controls demonstrated significant differences ( $7.8 \pm 2.6$

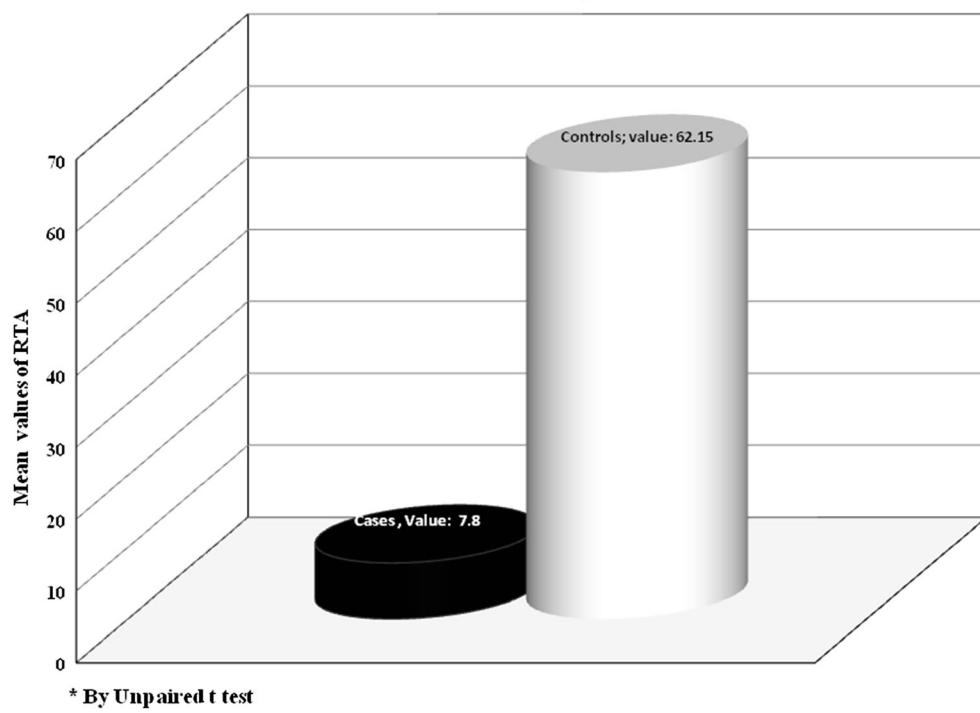
### Comparisons of mean values of TRF lenght in cases and controls



\* By Unpaired t test

**Fig. 2** The mean terminal restriction fragment (*TFR*) length in the two cohorts. *In black* sporadic TAA cases; *in white* controls

### Relative telomerase activity in cases and controls



\* By Unpaired t test

**Fig. 3** The mean RTA values in the two groups. *In black* sporadic TAA cases; *in white* controls

vs.  $62.15 \pm 8.9$ ,  $p < 0.0001$  by  $t$  test Welch corrected). In addition, correlations were assessed between the RTA mean values and age in both patients and controls ( $p = 0.038$ ), and between the RTA mean values and aneurysm size ( $p = 0.03$ ) among the cases. The comparisons of RTA mean values between male and female patients demonstrated a weak decrease in women ( $6.9 \pm 9.1$  vs.  $5.3 \pm 13.2$ ). Significant differences were observed in RTA mean values between male patients and male controls ( $6.9 \pm 9.1$  vs.  $61.8 \pm 4.8$ ;  $p < 0.0001$  by  $t$  test Welch corrected), and in female cases vs. female controls ( $5.3 \pm 13.2$  vs.  $60.5 \pm 6.7$ ;  $p < 0.0001$  by  $t$  test Welch corrected). Furthermore, the analysis of the independent effects of risk factors including age, gender, smoking, hypertension, diabetes, dislipidemia, and renal failure on RTA mean values among the subjects demonstrated that age, smoking, and hypertension in cases were the unique risk factors significantly associated with RTA mean values ( $p = 0.032$ ,  $p = 0.02$ , and  $p = 0.001$ , respectively).

#### Assessment of weight of biological effects mediated of inflammatory genetic factors in the impairment of telomere/telomerase system in cases and controls

As above reported, the (80 cases and 80 controls) subjects in study were selected by 161 cases and 128 controls previously genotyped for ten TLR4 pathway-related genetic variants (see Ruvolo et al. 2014). By analyzing the genotype distribution and allele frequencies of these variants in 80 cases and 80 controls, significant associations were found for the rs4986790 (+896A/G; Asp289Gly) TLR4, overrepresented in the cases than controls (Table 2). No significant differences were also detected stratifying the frequencies for gender (data not shown). However, in order to identify a

possible combined genotype as risk profile, the differences in the frequencies of all possible combinations of combined genotypes of these variants were assessed. Interestingly, we detected that the +896ATLR4/DACE/-1562TMMP-9/-735TMMP-2 genotype, comparing it with the frequency of other combinations, was the unique combined genotype significantly represented in cases than controls (Table 3). This combined genotype (defined in our previous study as high responder genotype, given the biological effects of these alleles; see Ruvolo et al. 2014) was found in 25 % of cases, while only 10 % of controls had this combined genotype ( $p = 0.01$  by  $\chi^2$  test, 2×2 table; Table 3). Cases bearing high responder genotype had a mean TRF length and mean RTA values significantly reduced than controls, and compared to those of both controls and patients with other genotypes (see Table 4). In addition, they showed higher levels of plasma inflammatory mediators than controls and patients without or with high responder genotype (Table 4). The patients with high responder genotype also presented a higher amount of CD3+CD4+CD8+CD68+CD20+ cells than patients with other genotypes and control aortas (Fig. 4) Furthermore, positive correlation was also identified between the reduction of mean TRF length observed from samples of patients bearing high responder genotype and the histological abnormalities observed through histopathological and immunohistochemical assays and Tunel testing (Table 5). In particular, the data obtained underlined that patients with high responder genotype showed a higher severity of histological abnormalities with the reduction of mean TRF length. Their grading and their morphological and histological entity characterized a typical tissue aorta phenotype which we defined in a previous study as phenotype III.

**Table 2** Genotype distributions and allele frequencies of +896A/G TLR4 SNP in 80 S-TAA patients and 80 matched controls (2×2 comparisons between the different groups with odd ratio (OR) and 95 % confidence interval)

Candidate gene	Reference SNP number	Candidate SNPs	Patients (N=80)		Matched controls (N=80)		$p$ (3×2, 2×2 tables)	OR (95 % CI)
TLR4	rs4986790	+896A/A	78	97.5 %	70	87 %	0.04	6.4 (1.4–29.1) $p=0.005$
		+896A/G	2	2.5 %	8	10 %		
		+896G/G	0	0 %	2	3 %		
		+896A	158	99 %	148	92.5 %	0.01	
		+896G	2	1 %	12	7.5 %		

All genotypes were in Hardy-Weinberg equilibrium

**Table 3** Frequency of +896ATLR4/DACE/-1562TMMP-9/-735TMMP-2 genotype between patients and controls ( $2 \times 2$  comparisons between the different groups with odd ratio (OR) and 95 % confidence interval)

Patients (N=80)	+896ATLR4/DACE/-1562TMMP-9/-735TMMP-2	Other genotypes	p (2×2 Table)	OR (95 % CI) (1.2–7.2)
	20 (25 %)	60 (75 %)		
Controls (N=80)	8 (10 %)	72 (90 %)	p=0.01	p=0.01

## Discussion

Ongoing and recent evidence is considering biological age as a principal risk factor for individual CVD susceptibility, even if the related molecular and cellular mechanisms remain unclear (Epel et al. 2008; Huzen et al. 2010; Mainous and Diaz 2010; Saliques et al. 2010; De Meyer et al. 2011; Fitzpatrick et al. 2011; Fyhrquist et al. 2013). Cardiovascular dysfunction and its complications, i.e., the onset of different CVDs (sporadic TAA included), are associating with the senescence of cardiovascular tissues as result of telomere shortening, DNA damage, and genomic instability (Minamino and Komuro 2008). On the other hand, the replicative ability of cardiovascular cells, particularly stem cells and/or progenitor cells, has been shown to decline with age (Minamino and Komuro 2008). Accordingly, telomere attrition, recognized as the best ageing biomarker, is now assuming as predictor for CVDs, including sporadic TAA (Epel et al. 2008; Huzen et al. 2010; Mainous and Diaz 2010; Saliques et al. 2010; De Meyer et al. 2011; Fitzpatrick et al. 2011;

Fyhrquist et al. 2013). This concept is supported by key assumptions. It has been demonstrated by Wilson and colleagues (2008) that the telomere content in peripheral blood leukocytes accurately reflects that of the vascular wall, and its decrease is associated with premature vascular disease. Based on these observations, we investigated whether blood leukocyte telomere content and telomerase activity might also be considered as predictor biomarkers for sporadic TAA. Interesting results were obtained. In particular, we detected a shorter leukocyte TRF mean length and reduced mean RTA values in cases than controls. Stratifying the data for gender, male and female patients showed a significant increased telomere/telomerase system impairment when compared to controls. A raised telomere attrition was also assessed between female and male patients. In addition, the comparisons of RTA mean values between male and female patients demonstrated a weak decrease in women. Correlations were also detected between the telomere/telomerase system impairment and age, and aneurysm size in cases. Age as independent risk factor was also associated with telomere/telomerase

**Table 4** Mean TRF length, mean values of RTA, systemic plasma mediator's levels in patients, and controls with high responder genotype or other genotypes

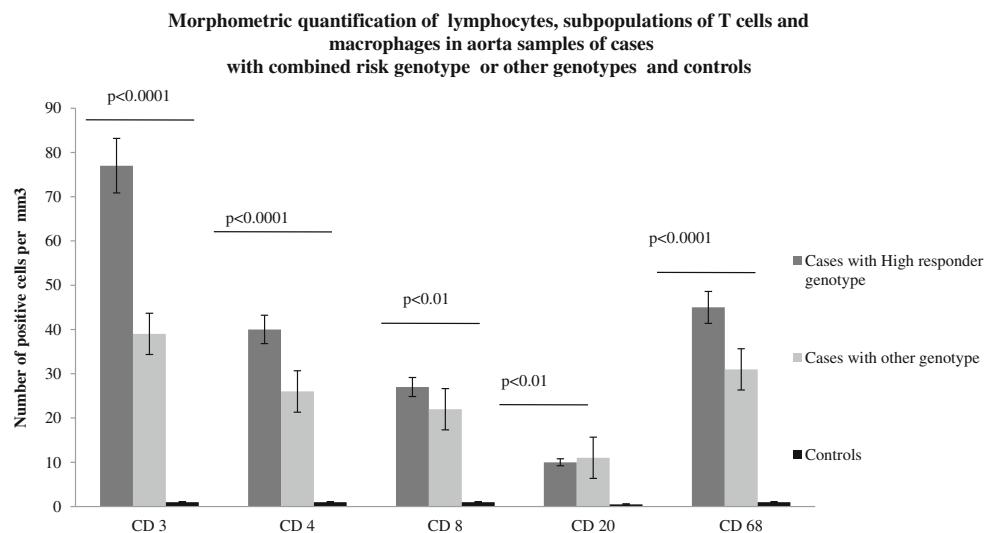
Evaluations	Patients with high responder genotype (N=20)	Controls with high responder genotype (N=8)	Patients with other genotypes (N=60)	Controls with other genotypes (N=72)	P1 values*	P2 values**	P3 values***
Mean TRF length	5,106±0.125 bp	6,210±0.345 bp	5,290±0.195 bp	6,790±0.635 bp	<0.0001	<0.0001	<0.0001
Mean RTA values	6.3±4.8	58.2±10.7	7.1±1.9	62.5±9.2	0.005	<0.0001	<0.0001
IL-6 (pg/ml)	13.8±1.9	9.1±2.9	10.1±2.9	6.3±1.2	0.01	<0.0001	0.002
TNF-α (pg/ml)	16.1±1.8	8.1±3.4	12.6±1.7	6.9±2.1	<0.0001	<0.0001	<0.0001
CRP (mg/l)	16.8±5.4	4.3±1.6	12.1±2.5	3.6±1.8	<0.0001	<0.0001	<0.0001
MMP-2 (ng/ml)	58.9±1.9	10.6±1.5	51.7±4.9	8.5±1.7	<0.0001	<0.0001	<0.0001
MMP-9 (ng/ml)	59.1±4.9	10.25±1.8	56.1±1.5	9.5±4.2	<0.0001	<0.0001	<0.0001

By unpaired *t* test with Welch correction

\*P1 values obtained comparing patients with high responder genotype vs. controls with high responder genotype

\*\*P2 values obtained comparing patients with high responder genotype vs. controls with other genotypes

\*\*\*P3 values obtained comparing patients with high responder genotype vs. patients with other genotypes



**Fig. 4** Morphometric quantization of lymphocytes, T cell subpopulations, and macrophages in aorta samples of cases with high responder genotype, other genotypes, and controls. CD3, CD4, CD8, CD20, and CD68 positive cells in media and adventitia in 10 contiguous high-power fields (magnification  $\times 400$ ) were counted

by two independent observers. Significant increased amounts of CD3+CD4+CD8+CD68+CD20+ cells were observed by comparing their values among the three groups (by ANOVA test). In particular, cases with high responder genotype had higher numbers of these cells than controls and cases with other genotypes

impairment. This might emphasize that with advancing age, the rate of biological age and consequently vascular ageing increase. In addition, we demonstrated that smoking and hypertension, other crucial risk factors, are independent factors significantly associated with the reduction of both leukocyte TRF length and RTA. These findings are in agreement with the relevant data recently published by Huzen and colleagues. They measured leukocyte telomere length by quantitative polymerase chain reaction in 8,074 participants from the Prevention of Renal and Vascular End-stage Disease Study (Huzen et al. 2014). Their relevant data evidenced that the major independent factors of telomere attrition in humans were smoking and metabolic syndrome.

Confirmation of these data derives by further studies of Chinese, Indian, and Finnish researchers that evidenced as hypertension is a significant contributor in telomere attrition (Yang et al. 2009; Fyhrquist et al. 2011; Bhupatiraju et al. 2012). Thus, these data encourage to propose as the complex interplay between the environmental and genetic factors can determine the senescence of cardiovascular cells and the onset of CVDs, i.e., sporadic TAA. In particular, it is also possible to speculate that the senescence of cardiovascular cells might also be the result of the combined action of two mechanisms genetically acquired: genetically substantial inability in self-repair of the cardiovascular system and a genetically acquired ability to have a sustained

**Table 5** Correlations between the reduction of mean TRF length observed from samples of patients bearing high responder genotype and the severity of histological abnormalities observed through histopathological and immunohistochemical assays and Tunel testing

Variables	Correlations	P values <sup>a</sup>
Mediolarcrosis of grade III	0.218	0.002
Cystic-medial change of grade III	0.227	0.001
Elastic fragmentation of grade III	0.356	0.0001
Plurifocal medial apoptosis	0.117	0.001
Elevated MMP-9 amounts	0.263	0.001

<sup>a</sup> by non-parametrical Spearman correlation test

inflammatory response. Reciprocally, a strong genetically acquired ability in self-repair of the cardiovascular system and a blunted inflammatory response genetically induced might justify the individual probability to remain sheltered from the consequences of vascular alterations and the large individual variability in the susceptibility of all CVD diseases, such as sporadic TAA, even in the presence of potent risk factors. In particular, these suggestions underlined as inflammation can influence both the rate of biological vascular ageing and its complications, such as vascular remodeling and medial degeneration, the typical features of several aorta diseases, including sporadic TAA (Phan et al. 2008; Fulop et al. 2010; Wadley et al. 2013). On the other hand, it is increasing the opinion to consider aortic aneurysms, and particularly the sporadic forms, as inflammatory/immune diseases with a strong genetic component (Kuivaniemi et al. 2008). Accordingly, we also evaluated the weight of genetic variants in inflammatory genes in influencing telomere/telomerase impairment. In particular, we focused our attention on ten variants in genes related to TLR4-mediated signaling pathway (Balistreri et al. 2009; Ruvolo et al. 2014), since in a previous study we have observed a significant association between a combined risk (+896ATL4/DACE/-1562TMMP-9/-735TMMP-2) genotype and sporadic TAA. It was also associated with an increased systemic and local inflammation and aorta histopathological abnormalities. On the other hand, an increasing number of studies underlines the involvement of this signaling pathway in several CVDs (Balistreri et al. 2004, 2009, 2013b, c; Frantz et al. 2007; Ionita et al. 2010; Hofmann et al. 2011; Navi et al. 2013; Incalcaterra et al. 2013). Its role in age-related aorta dysfunction and the aneurysm formation and related complications (dissection or rupture) recently is emerging (Pryshchep et al. 2008; Eissler et al. 2011; Li et al. 2011; Song et al. 2012; González-Ramos et al. 2013; Navi et al. 2013; Ruvolo et al. 2014). A current report demonstrated its expression in all cells of the arterial wall, and particularly in the endothelium and vascular smooth cells, by suggesting its functional importance in mediating physiological homeostasis and maintaining protection, but also in inducing pathological aorta phenotypes (Pryshchep et al. 2008). Recent experimental investigations in animal and ex vivo models also emphasize its role in the vascular aorta alterations (i.e., vascular remodeling and medial degeneration) and their complications, such as sporadic TAA (Pryshchep et al. 2008; Eissler et al. 2011; Li et al. 2011; Song et al. 2012; González-Ramos et al. 2013; Navi et al. 2013; Ruvolo et al. 2014).

Among the ten genetic variants related to TLR4-mediated signaling pathway, only the rs4986790 (+896A/G) TLR4 variant was overrepresented in the cases than controls. No significant differences were also detected stratifying the frequencies for gender. Furthermore, we identified a combined +896ATL4/DACE/-1562TMMP-9/-735TMMP-2 genotype as risk profile, significantly represented in cases than controls. Cases bearing this combined genotype (defined in our previous study as high responder genotype, given the biological effects of these alleles; see Ruvolo et al. 2014) had a mean TRF length and mean RTA values significantly reduced than those of controls, and respect to those of both controls and patients with other genotypes. In addition, they showed higher levels of systemic inflammatory mediators, a higher amount of CD3+CD4+CD8+CD68+CD20+ cells, positive correlation between the reduction of mean TRF length and the histological abnormalities (including elevated cystic medial degeneration, elevated elastic fragmentation, plurifocal medial apoptosis, and increased amounts of metalloproteinase-9) of a characteristic phenotype defined in our previous studies as phenotype III, and associated with an increased risk of rupture or dissection, independently of aneurism size. Thus, our findings seem to suggest the relevant role of the genetic inflammatory variants of TLR4 pathway in contributing to induced senescence of cardiovascular cells and, as consequence, vascular aorta ageing and alterations associated with the onset of CVDs, and, in the case specific, with sporadic TAA. Certainly, their interplay with environmental factors, such as principally smoking and life-factors influencing hypertension, i.e., diet and reduced physical exercise, plays a crucial role. On the other hand, the biological effects of functional genetic variants depend on the presence of one or different environmental causes. Because of this relationship between genetic and environmental factors, a genetic variant might never manifest itself phenotypically, if the carrier is never exposed to a specific trigger. In addition, different alleles might differently respond to the same environmental condition. Consequently, each exposed individual's risk of a given disease phenotype is determined by the particular allele of an individual carrier. Thus, further and larger investigations are certainly needed. For example, genomic, transcriptomic, and epigenomic investigations may eventually lead to a better understanding of the role of this pathway and inflammation in vascular aorta ageing and sporadic TAA.

In the complex, our results, although based on a relatively reduced number of cases and controls, seem to

suggest that the mean of blood leukocyte TFR telomere length and mean RTA values might be the predictor biomarkers for sporadic TAA. These data are in agreement with the results of other studies performed in aorta diseases, i.e., particularly in abdominal aorta aneurysms (Atturu et al. 2010; Yan et al. 2011; Björck et al. 2011). However, they are opposite to those of Saloñurmi's group study performed in a population affected by ascending aorta aneurysms (Huusko et al. 2012). In particular, a longer mean relative telomere length was observed in cases when compared to that of controls (Huusko et al. 2012). These discordances might be due to different factors. Differently to cases selected in the study of Saloñurmi's group (Huusko et al. 2012), our patients were older and characterized to have significant associations with a typical systemic inflammatory phenotype of senescence cells, *the aorta associated senescence secretory phenotype (AASSP)*, fruit of the probable activation of the inflammatory pathways (Balistreri et al. 2013d; Sikora et al. 2013; Ruvolo et al. 2014), including particularly the TLR4-mediated signaling pathway (Balistreri et al. 2009, 2014a, b; Ruvolo et al. 2014). They also showed a significant overrepresentation of some genetic variants of this pathway as indicated in this and previous studies (Balistreri et al. 2013b, c, 2014a, b; Ruvolo et al. 2014). In addition, they had different clinical conditions and aorta abnormalities than those analyzed in Saloñurmi's group study (Huusko et al. 2012; Balistreri et al. 2014a, b; Ruvolo et al. 2014). In particular, the phenotype III with elevated cystic medial degeneration, elevated elastic fragmentation, plurifocal medial apoptosis, and increased amounts of metalloproteinase-9 prevalently characterized the aorta tissue samples of our cases (Balistreri et al. 2014a, b; Ruvolo et al. 2014). In contrast, the Saloñurmi's study provided no information about genetic, systemic, and histological aorta tissue characteristics of cases (Huusko et al. 2012). Furthermore, we and the Saloñurmi's group used different methodologies and samples for detecting telomere length and telomerase activity (as mentioned in the **Introduction** section). This probably might further justify the discordant results obtained. As a consequence, it should be imperative to consider the problem of different methodologies available to determine leukocyte telomere length and telomerase activity to increase the valence of the investigations on telomere and telomerase activity in aorta aneurysms in order to identify them as possible predictor biomarkers of sporadic TAA. This problem represents one of the major consequences of the large heterogeneity existing in the

literature data on this topic. Thus, a standardization is much needed as well as the necessity for longitudinal and larger studies to elucidate these complicated relationships between genetic background and environmental factors in affecting telomere and telomerase activity system and inducing senescence of cardiovascular aorta cells and onset of sporadic TAA.

As above underlined, we propose to perform future and larger studies, which will consent to confirm and validate our data and to suggest leukocyte telomere length and telomerase activity as predictor biomarkers of sporadic TAA. Cotemporally, our findings also encourage in performing further and larger investigations to validate the key role of the variants related to TLR4 inflammatory pathway in both telomere/telomerase impairment. This might permit us to consider the TLR4 pathway as a target for the development of personalized therapies based, in our case, on the presence of high responder genotype in individuals affected by sporadic TAA. On the other hand, it is well recognized that the TLR4 pathway responds to a variety of endogenous as well as exogenous ligands (Balistreri et al. 2009). By responding to endogenous ligands that are exposed during cellular damage, TLR4 have been implicated in a range of pathological conditions associated with cardiovascular dysfunction (Pryshchep et al. 2008; Balistreri et al. 2009, 2014a, b; Eissler et al. 2011; Li et al. 2011; Song et al. 2012; González-Ramos et al. 2013; Navi et al. 2013). Increasing the knowledge on the mechanisms involved in TLR4 signaling has encouraged the exploration of therapeutic pharmacological modulation of TLR4 activation in different models of cardiovascular diseases, essentially using agonists or antagonists (Navi et al. 2013). The confirmation of the role of this pathway and related genetic variants in the pathophysiology of sporadic TAA might certainly consent to translate the data in personalized therapies.

**Author's contribution** Dr Balistreri was involved in conception and study design. Prof Ruvolo and Dr Candore were involved in the support of study materials/patients. Dr Pisano collected and assembled the clinical data of the study population. Dr Balistreri performed the experimental assays with the support in some telomerase activity's evaluations of Dr Martorana. Dr Balistreri acquired the results obtained and performed their analysis. Drs Balistreri and Pisano were involved in the data interpretation and their translation in clinical suggestions. Dr Balistreri was involved in drafting the manuscript. Dr Balistreri and Prof Ruvolo contributed in the critical revision of the text of manuscript. Dr Balistreri and Prof Ruvolo contributed in the study supervision. Dr Balistreri gave the final approval of the version to be published. All authors participated in the study, and they read and approved the final manuscript.

**Competing interests** All authors declare to have no competing interests.

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