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Short running head: Telomere shortening in osteoarthritis.

Relationship between the dynamics of telomere loss in peripheral blood leukocytes from osteoarthritis patients and mitochondrial DNA haplogroup

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The authors declare that they have no conflict of interests.

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Statement of ethics and consent:

The OAI study was approved by the institutional review boards at each OAI clinical site and the coordinating center (University of California, San Francisco) and informed consent was obtained from the participants. The study was also approved by the Local Ethics Committee (*Comité de Ética de la Investigación de A Coruña- Ferrol*) with registry code 2018/129.

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ABSTRACT

Objective: The evaluation of the evolution of telomere length from peripheral blood leukocytes (PBL) in subjects from the Osteoarthritis Initiative (OAI) cohort in relation to the incidence of osteoarthritis (OA) and explore its possible interactive influence with the mitochondrial DNA (mtDNA) haplogroup.

Methods: Dynamics of telomere sequence loss was quantified in PBL from initially healthy individuals, without symptoms or radiological signs, 78 carrying the mtDNA cluster HV and 47 with cluster JT, from the OAI, during a 72-month follow-up. The incidence of knee OA during this period (n=39) was radiographically established when Kellgren-Lawrence (KL) score increased from < 2 at recruitment to ≥ 2 during 72 months of follow-up. Multivariate analysis using binary logistic regression was performed to assess PBL telomere loss and mtDNA haplogroups as associated risk factors of incidence of knee OA

Results: Carriers of cluster HV showed an OA incidence twice that of the JT carriers (n=30 vs. n=9). Rate of PBL telomere loss was higher in cluster HV carriers and in incident individuals. Multivariate analysis showed that the dynamics of PBL telomere shortening can be a consistent risk marker of knee OA incidence. Non-incidents showed a slower telomere loss than incidents, the difference being more significant in carriers of cluster JT than in HV.

Conclusions: An increased telomere loss rate in PBL may reflect a systemic accelerated senescence phenotype which could be potentiated by the mitochondrial function, increasing the susceptibility of developing OA.

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BACKGROUND

Telomeres are essential elements for maintaining genome integrity since they protect the chromosome ends from exonucleolitic degradation and end-to-end fusion (1). Yet, they progressively shorten with consecutive cell divisions. When a telomere becomes critically shortened and chromosome capping cannot be properly exerted, cell proliferation is inhibited and it may enter into apoptosis. This phenomenon is known as replicative senescence and limits cell longevity in most somatic cells (1, 2).

Because of their relationship with cellular turnover, the quantification of the telomere DNA sequences from peripheral blood leukocytes (PBL) has been found to be a potential marker of biological age and a predictor of longevity (3). Telomere size from PBL strongly correlates with that from other tissues from the same individual, thus its determination may report information about the telomere status in less accessible tissues (4, 5). Telomere size determined in PBL has been revealed to be a general associated risk factor for age-related chronic diseases, such as some types of cancer, type 2 diabetes, dementia and cardiovascular disease (2, 6, 7).

Osteoarthrithis (OA) is a common age-related chronic disease defined by confined progressive destruction of articular cartilage, leading to pain and work incapacitation (8). Due to chondrocyte dysfunction, the extracellular matrix is degraded, progressing to subchondral bone remodeling, osteophyte formation, local inflammation, degeneration of ligaments and loss of normal joint function. Chondrocytes release proinflammatory cytokines that contribute to inflammation and promote apoptosis, aggravating the progression of the disease (9). OA chondrocytes show shorter telomeres than those from healthy individuals which

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may be related to accelerated articular senescence and could contribute to the incidence and progression of OA (10).

Mitochondrial genetics also has a significant influence in the pathogenesis of OA. Several studies established associations between specific mtDNA haplogroups and longevity and particularly with disorders like hypertension, diabetes, obesity, neurodegenerative diseases and OA (11). In Caucasian populations, mtDNA variants belonging to the mtDNA haplogroup cluster JT have been associated with a reduced risk of prevalence of knee OA and hip OA and lower rates of incidence and of progression of knee OA, in comparison to subjects with the most common haplogroup H (12-14). Interestingly, individuals carrying the mtDNA haplogroup J exhibit a PBL telomere length longer than those non-J carriers (15).

Recent evaluation of subjects from the cohort study belonging to the Osteoarthritis Initiative (OAI) from the USA, indicated that PBL telomere size is shorter in patients with concurrent knee OA and with hand OA (16, 17).

The aforementioned association was found to be even stronger regarding future incidence of hand OA (17). Determination of PBL telomere size in all of these studies was cross-sectional, exclusively at the moment of recruitment of the cohorts of OA patients and controls. However, telomeres decrease progressively with time, with variant rates among individuals. Here we evaluated for the first time the evolution of PBL telomere length in subjects from the OAI cohort in relation to the incidence of OA and explore its possible interactive influence with the mtDNA haplogroups.

MATERIALS AND METHODS

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Subjects

The study included individuals from a subsample of controls at recruitment, i.e., without symptoms or radiological signs, from the OAI from the USA (n=125; 45 males and 80 females), carriers of mtDNA cluster HV (n=78) or cluster JT (n=47).The subsample was restricted to Caucasian subjects with an age range of 45-77 years (mean 57.6); Subjects carrying these mtDNA clusters were selected because they constitute the majority of the clusters in the Caucasian population and are the most studied and relevant regarding the incidence and/or progression of OA. 19.2% of subjects were hypertensive and their body mass index (BMI) was 26.57± 4.36. The Western Ontario McMaster Universities Osteoarthritis Index (WOMAC), a clinical measure of disease activity, was also recorded from the OAI database. The study was approved by the Local Ethics Committee (Comité de Ética de la Investigación de A Coruña- Ferrol) with registry code 2018/129 and informed consent was obtained from participants.

Evaluation of Knee OA

Individuals were assessed through the use of radiologic knee images, according to the Kellgren-Lawrence (KL) score, ranging from 0 to 4. Agreement between 2 readings: kappa = 0.70 to 0.80 (18). Presence of radiographic knee OA was defined when an individual had a maximum KL grade (max-KL) ≥2 considering both knees. Incident knee OA was defined when max-KL grade increased from <2 at recruitment to ≥2 during 72 months of follow-up. Baseline KL data were obtained from project 15 and KL data at 72 months were obtained from project 37 or 42 from OAI database.

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Evaluation of Telomere Sequences

DNA from PBL was extracted from bloods samples obtained at recruitment and after a 72-month follow-up. Coded DNA samples were processed by personnel blinded to the status of the subjects. The average telomere amount in PBL was measured with a standard validated quantitative PCR (qPCR) based assay as described (10). This method measures the average ratio of telomere repeat copy number to a single gene (36B4) copy number (T/S ratio) in each sample and may be considered an indirect measure of mean telomere length. DNA samples from baseline and after 72 months from the same individual were simultaneously processed in the same PCR plate to avoid possible inter-assay variability. The relative percentage of telomere loss in each individual was defined as [(T/S ratio at recruitment - T/S ratio after 72 months) / T/S ratio at recruitment] x 100.

mtDNA Haplogroups Genotyping

A combination of the single-base extension assay with the PCR-restriction fragment length polymorphism technique was employed to obtain the different single-nucleotide polymorphisms (SNPs) that characterize the mtDNA haplogroups, as previously described (19).

Statistical Analysis

Data were analyzed using SPSS software (Chicago, Ilinois, USA). Since the percentage of PBL telomere loss was not normally distributed, as ascertained by the Kolmogorov-Smirnov test, nonparametric Mann-Whitney U-test was performed for homogeneity testing. A Pearson chi-square test was used to determine

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statistical differences between percentages of incidence. Multivariate analysis using binary logistic regression was performed to assess PBL telomere loss, mtDNA haplogroups, age, sex hypertension and WOMAC, as associated risk factors of incidence of knee OA. The linearity of relative PBL telomere loss and log odds was confirmed by linear regression (R^2: 0.974). Significance was defined as $p<0.05$.

RESULTS

The study was performed in a group of 78 individual carriers of mtDNA cluster HV and 47 carrying cluster JT, all of them without radiological signs of knee OA at recruitment. Percentage of radiologic OA incidents and the relative percentage of telomere loss in PBL were evaluated after a follow-up period of 72 months. From the 125 subjects, 39 developed knee OA, i.e, incident.

Regarding mtDNA polymorphisms, individuals with incident knee OA were increased in carriers of cluster HV (38.46%), two times higher in comparison with carriers of cluster JT (19.15%) ($p=0.024$). Moreover, the relative percentage of telomere loss in PBL was significantly lower in subjects carrying cluster JT than in those carrying cluster HV ($p<0.001$) (Figure 1A). The relative PBL telomere shortening was also lower in non-incident subjects than in incident individuals ($p<0.001$) (Figure 1B, Table 1).

In the multivariate analysis, using binary logistic regression, of the joint role of both variables (mtDNA cluster and relative PBL telomere shortening), the relative PBL telomere loss remained significantly associated with incidence as a risk marker (OR: 1.062, 95% CI: 1.023-1.102, $p=0.001$), so higher PBL shortening

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rates were associated with a higher risk of knee OA incidence. The OR is per percentage unit of telomere loss. Otherwise, the mtDNA cluster lost statistical significance (OR: 0.696, 95% CI: 0.264-1.803, *p*=0.452).

When the analysis was stratified by the cluster, the relative percentage of PBL telomere shortening was significantly higher in the individuals with incident knee OA than in the stable individuals that carry the JT cluster (*p* = 0.003; OR: 1.125, 95% CI 1.037-1.220, *p*=0.005) .(Figure1C, Table 1). In carriers of the HV cluster, the differences in PBL telomere shortening rates between stable and incidents were smaller and tightly close to the limit of statistical significance (*p*=0.075; OR: 1.04, 95% CI 0.999-1.084) (Figure 1C, Table 1).

When age, sex, BMI, WOMAC and hypertension are included in the multivariate analysis, using binary logistic regression, the relative PBL telomere loss remained significantly associated with incidence (OR: 1.048, 95% CI: 1.007-1.090, *p*=0.020), together with BMI (OR: 1.257, 95% CI: 1.116-1.415, *p*<0.010) and WOMAC (OR: 1.248, 95% CI: 1.039-1.499, *p*<0.018). Age, sex and hypertension were not significant.

DISCUSSION

In previous reports, reduced PBL telomere length determined at a single time- point was associated with prevalence of knee OA (16) and with prevalence and incidence of hand OA (17). Since the telomeres evolve over time, the determination of longitudinal changes of PBL telomeres must provide a more stringent testing. The association of the dynamics of PBL telomere shortening with the risk of incidence of knee OA presented in this study confirms their relationship

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with a stronger confidence. Moreover, mitochondrial function may have a relevant role since the more significant in carriers of mtDNA cluster JT than of HV. This fact is due to the much lower PBL telomere decay in non-incident subjects with cluster JT than with HV.

The polymorphisms (SNPs) that characterize haplogroup cluster JT may decrease the efficiency of mitochondrial coupling, reducing both ATP and ROS production, as well as lowering the expression of genes related to the inflammatory response, complement and apoptosis (12, 20).

Senescent cells accumulate with age and in age-related diseases and are associated with a loss of tissue function. One main factor associated with the development of knee OA must be the progression of biological aging of the joint cartilage. Replicative senescence at the cellular level is triggered when telomeres are excessively shortened, so this mechanism would be more plausible in high-turnover tissues. Nevertheless, chondrocytes do not exhibit a high proliferation rate and their telomeres are higher than those from PBL in the same subject (10). Importantly, telomere DNA damage can occur independently of length, and this has been shown to contribute to the senescent phenotype. Telomeres may act as sensors of intrinsic and extrinsic stresses, and maintain genomic stability by limiting replication of cells that have accumulated significant genomic damage (21). The higher ROS generation in chondrocytes carrying mtDNA cluster HV may damage telomeric DNA, favoring the telomere length-independent senescence, unlike in the less inflammatory and oxidative phenotype from carriers of cluster JT.

The decay of telomeres in PBL provides information about how this systemic aging process is evolving, and subsequently how the articular

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senescence status is progressing. Knee OA is mainly developed in the group of subjects with a higher telomere loss over time, i.e. with an accelerated biological aging. The influence of the mtDNA cluster HV or JT on the risk of incidence of knee OA seems derived from the differential damaging oxidative activity in the body. But this harmful effect is being recognized in the PBL telomere shortening, as referred. This integration within the telomere behaviour could explain the loss of significance of mtDNA cluster in the multivariate analysis. Furthermore, the PBL telomere shortening rate may also collect information concerning other additional risk factors of incidence, independent of mtDNA haplogroup.

The main limitation of this study is that the results are not generalizable to non-Caucasian populations. It would be interesting to replicate in racially diverse samples. In addition, the sample size of the individuals with incident knee OA is not very high, but necessarily related to the natural evolution of the pathology itself after the selection of healthy individuals. Despite these issues, our results are remarkably consistent with previous epidemiologic studies in this field.

CONCLUSION

The slower telomere decay in PBL is associated with a lower risk of incidence of knee OA over time. Moreover, this slow telomere shortening is more significant in non-incident subjects carrying mtDNA cluster JT than those with cluster HV. The influence of the mtDNA haplogroup as a risk factor of incidence may be incorporated in the PBL telomere shortening dynamics.

ABBREVIATIONS

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PBL: Peripheral blood leukocytes; KL: Kellgren-Lawrence; mtDNA: Mitochondrial DNA; OAI: Osteoarthritis initiative; qPCR: Quantitative polymerase chain reaction; ROS: Reactive oxygen species; SNPs: Single-nucleotide polymorphisms; T/S ratio: Ratio of telomere repeat copy number to a single gene (36B4) copy number; BMI: Body mass index; WOMAC: Western Ontario McMaster Universities Osteoarthritis Index.

AUTHOR CONTRIBUTIONS

JLF and FJB designed the study and prepared the manuscript draft. RG and FO performed the experimental work and statistical procedures. AM and IR-P contributed to the design and supervised the statistical analysis. All authors approved the manuscript.

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TABLE LEGENDS

TABLE 1. Statistical Values of the Relative Percentage of Telomeres Loss in Peripheral Blood Leukocytes and Analysis of Significant Differences in Subjets with Incidence or not of Knee OA, and individuals with mtDNA Cluster HV or JT. Quartiles (Q1: lower, Q2: median, Q3: upper) are indicated.

FIGURE LEGENDS

FIGURE 1. Relative percentage of telomere loss in peripheral blood leukocytes from subjects carriers of mtDNA cluster JT and cluster HV (A); subjects non-incident and with incident knee OA (B); subjects non-incident and with incident knee OA carriers of mtDNA cluster JT and individuals non-incident and with incident knee OA carriers of cluster HV (C).

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TABLE 1. Statistical Values of the Relative Percentage of Telomeres Loss in Peripheral Blood Leukocytes and Analysis of Significant Differences in subjets with Incidence or not of Knee OA, and individuals with mtDNA Cluster HV or JT. Quartiles (Q1: lower, Q2: median, Q3: upper) are indicated.

			N	Q1	Q2	Q3	*p (Mann Whitney-U test)*
Subjects	Non-incident		86	19.39	27.79	39.31	< 0.001
	Incident		39	35.47	41.36	44.22	
mtDNA cluster	HV	Non-incident	48	23.91	32.72	44.41	0.075
		Incident	30	35.88	41.43	44.27	
	JT	Non-incident	38	16.82	21.83	27.05	0.003
		Incident	9	25.60	36.49	44.91	

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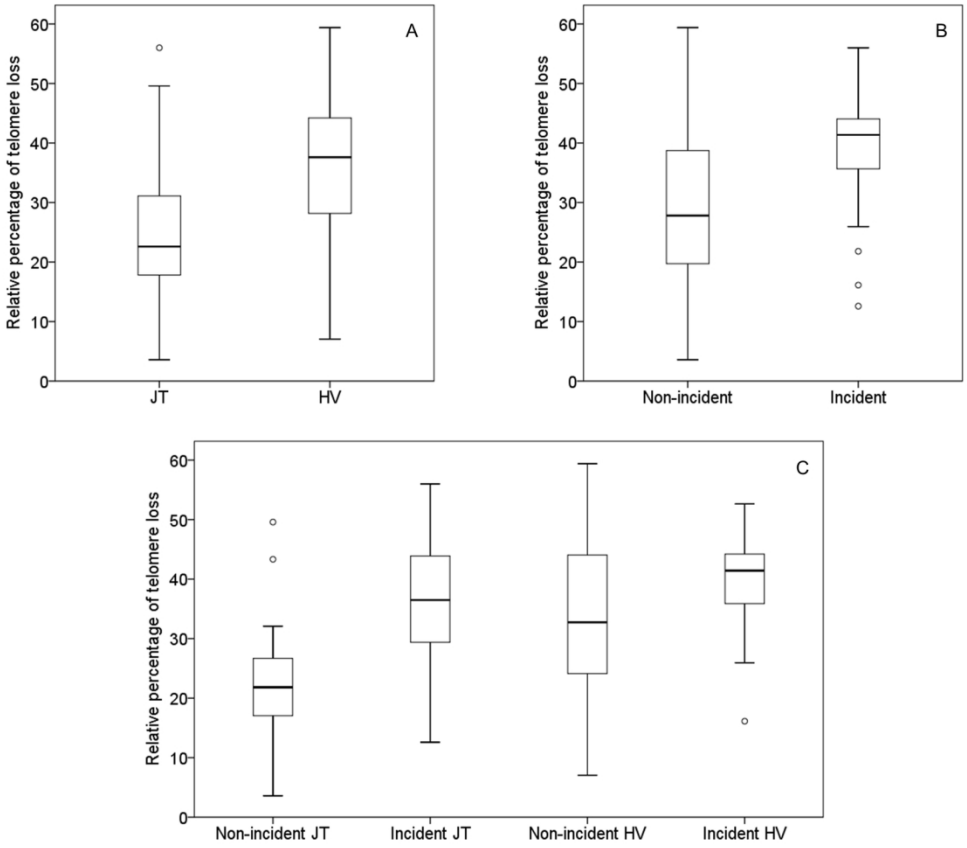

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