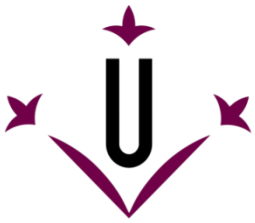

Title:

Distribution of functional polymorphic variants of inflammation-related genes *RANTES* and *CCR5* in long-lived individuals

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The authors declare that they have no proprietary, financial, professional or other personal interest of any nature or kind that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated in this manuscript.

Abstract

Although persistent inflammation has been related to unsuccessful aging, a pro-inflammatory status is the common phenotype in older people. To assess for a genetic component in the inflammatory status of the oldest we studied the distribution of two polymorphic chemokine pathway genes, *RANTES* and *CCR5*, in elderly. *RANTES -403G/A* and *RANTES Int1.1T/C* polymorphisms and *CCR5Δ32* polymorphism were genotyped in 104 elderly and 110 controls. *RANTES -403A* and *RANTES Int1.1C* alleles have been associated with pro-inflammatory and anti-inflammatory status, respectively. *CCR5Δ32* abrogates functional receptor expression of the pro-inflammatory CCR5-mediated action. Prevalence of *RANTES -403G* allele, associated in other studies with high RANTES production, was reduced in elderly males, compared with controls. In addition, *RANTES* pro-inflammatory haplotype *-403A-Int1.1T* was overrepresented in elderly males, while *RANTES* anti-inflammatory haplotype *-403G-Int1.1C* was overrepresented in elderly females. Our results suggest a sex-specific *RANTES* inflammatory genetic determinant that could contribute to the known sex-related differences in aging.

Keywords: (6) *RANTES*, *CCR5*, polymorphism, inflammation, longevity, aging

Introduction

The immune system's positive protective effect against pathogens and the spread of tumour cells can also become a detrimental chronic inflammation affecting longevity. Studies on the genetic component of longevity have tended to evaluate the role of the immune system genes, especially those involved in inflammation. Results obtained reveal that persistent activation of inflammatory status is related to unsuccessful aging and that avoiding a persistent inflammation contributes to healthy aging. However, a pro-inflammatory status is the common phenotype in older people [1].

Inflammatory response could be affected by changes in chemokine gene transcription and receptor expression levels due to polymorphisms at the corresponding genes. Previous studies of chemokine factors related to age-related disease and longevity revealed attrition or change in frequency of chemokine alleles with ageing [2, 3]. Chemokines are small chemotactic cytokines (C-C) with a pivotal role in leukocyte trafficking and activation. Their biological effects are exerted via interaction with G protein-coupled receptors that mediate intracellular signals. C-C chemokine receptor type 5 (CCR5) is the co-receptor for macrophages and dual (T cell and macrophage) tropic immunodeficiency viruses and is involved in the migration of monocytes, NK cells and Th1 cells toward an increasing concentration of β-chemokines, which results in recruitment of leukocytes to the inflammation site. Natural CCR5 ligands are the C-C motif chemokine ligands CCL3, CCL4 and CCL5, which is also known as RANTES (regulated upon activation normal T-cell expressed and secreted). Two polymorphisms have been described in the *RANTES* gene, a promoter polymorphism (*-403G/A*, *rs2107538*) whose *-403A* allele increases the promoter transcriptional activity, which results in high serum RANTES levels

favouring a pro-inflammatory status [4], and an intronic polymorphism (*Int1.1T/C*, *rs2280789*) whose *Int1.1*C variant has been associated with low RANTES expression and an anti-inflammatory status [5]. A 32pb insertion/deletion polymorphism has been described in exon 3 of the *CCR5* gene (*CCR5Δ32*, *rs333*) that abrogates functional CCR5 receptor expression, weakening the pro-inflammatory CCR5-mediated action [6]. The *CCR5Δ32* allele reaches appreciable frequencies only in Europe, following a latitudinal cline distribution that is higher in the north and lower in the south. Studies have demonstrated that the *CCR5Δ32* allele is related to reduction of portal inflammation during hepatitis C (HCV) infection, delayed progression in multiple sclerosis, protection against cardiovascular diseases (acute myocardial infarction and severe coronary heart disease), and attenuation of the inflammatory response that delays progression of atherosclerotic lesions [7].

To assess the possibility of a genetic component of inflammatory status in the elderly, we studied the distribution of functional polymorphic variants of two genes from the chemokine inflammatory pathway, *RANTES* and *CCR5*, in long-lived individuals.

Methods

RANTES -403G/A and *Int1.1T/C* and *CCR5Δ32* polymorphisms were genotyped by standard procedures (Supplementary Table 1) in a cohort of 214 subjects. A detailed description of cohorts can be found in [8], briefly: 110 sex-matched healthy young individuals as a control group, 75 women (mean age 28±5.8; range 17-39) and 35 men (mean age 29±8.6; range 17-40), and 104 elderly subjects (>85 years), 72 women (mean age 89.4±3.6; range 85-100) and 32 men (mean age 89.4±3.2; range 85-97). Subjects were enrolled from the city of Lleida in northeast Spain, between January and June 2004. Elderly subjects were recruited from attendants to Primary Care Services (25%) and Nursing Home residents (75%). A comprehensive medical history was obtained from each elderly subject. Most frequent co-morbidities were hypertension (9.6%), stroke (7.7%), chronic obstructive pulmonary disease (4.8%) and heart disease (2.9%). A written informed consent for enrolling in the study and for personal data management was obtained from all subjects (elderly and controls) in accordance with Spanish laws. The Ethics Committee of our institution approved the study.

Results & Discussion

Genotype and allele distributions of *RANTES -403G/A* and *CCR5Δ32* polymorphisms conform to Hardy-Weinberg equilibrium in both the control and elderly cohorts. In contrast, *RANTES Int1.1T/C* departs from Hardy-Weinberg equilibrium in the elderly cohort. Comparisons of genotype and allele distributions between controls and elderly were done by contingency table analysis performed by the OpenEpi web resource (http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm). Differences were evaluated considering all subjects and by sex between cohorts. For comparisons including zero values, odds ratio and 95%CI were calculated based on the Peto odds ratio method by using the on-line calculator http://www.hutchon.net/ConfidORnulhypo.htm. This approach has the advantage of accepting zero results without generating infinity. P values <0.05 were considered statistically significant. In addition, a modified Bonferroni-corrected nominal threshold of $P = 0.05/N$ was used, where N is the "effective number of independent marker *loci*" after consideration of linkage disequilibrium between markers. N was calculated using the SNP (single nucleotide polymorphism) spectral decomposition approach web-based program *SNPSpD* (http://gump.qimr.edu.au/general/daleN/SNPSpD/). This yields an experiment-wide significance threshold of 0.02 to keep Type I error rate at 5%.

Genotype distribution differences between elderly and controls showed statistical significance for *Int1.1T/C* polymorphism (P=0.03), but not for *CCR5Δ32* or *-403G/A* polymorphisms (Table 1). Sex-based differences between cohorts for the *CCR5Δ32* polymorphism were not significant. Nevertheless, differences between elderly and control males were observed for the *-403G/A* genotype (P=0.029) and allele distribution (OR=3.2; 95%CI=1.2-

8.5; *P*=0.012) (Table 1). In addition, differences were observed when *-403A* allele was modelled as dominant (OR=4; 95%CI=1.4-11.8; *P*=0.01). We also found a genotype association for *Int1.1C* allele under recessive model (Peto OR=8.8; 95%CI=1.7-44.7; *P*=0.008) that was mainly contributed by differences between elderly and control females (Peto OR=8.4; 95%CI=1.4-49.9; *P*=0.02).

Haplotypes for *RANTES* gene markers were inferred by pLink software (pLink web resource available at http://pngu.mgh.harvard.edu/~purcell/plink/). Haplotype distribution between elderly and controls in all subjects, males and females, showed significant differences (Omnibus test *P*=0.02, *P*=0.05 and *P*=0.04, respectively) (Table 1).

According to the inflammation-linked effect attributed to both *RANTES -403G/A* and *Int1.1T/C* polymorphisms, we can presume an anti-inflammatory effect for the *-403G-Int1.1C* (*GC*) haplotype and the opposite pro-inflammatory effect for the *-403A-Int1.1T* (*AT*) haplotype. Haplotype-specific test showed that anti-inflammatory *GC* haplotype was overrepresented in the elderly cohort (*P*=0.04), mainly due to the overrepresentation in females (Peto OR=8.3; 95%CI=2-34; *P*=0.005). In contrast, pro-inflammatory *AT* haplotype was overrepresented in elderly males (OR=4.9; 95%CI=0.97-24.7; *P*=0.03).

Carrier distribution in males and females of pro- and anti-inflammatory haplotypes is shown in Figure 1. As revealed by haplotype-specific test, elderly males were carriers of the pro-inflammatory *AT* haplotype, which was more prevalent in elderly than in controls (26% and 6%, respectively; OR= 5.6; 95%CI=1.06-29.7; *P*=0.038), while carriers of the AT haplotype were equally distributed in elderly and control females (10% and 13%, respectively). In contrast, elderly females were carriers of the anti-inflammatory *GC* haplotype

that was absent in control females (11.4% *vs*. 0%, respectively; Peto OR= 8.8; 95%CI=2.1-36.5; *P*=0.0024).

Our genetic study of Spanish elderly does not reveal any significant association between the *CCR5Δ32* polymorphism and longevity. The role of CCR5 in age-related diseases has been largely reviewed [9]. Assuming the hypothesis that alleles favouring inflammation-related diseases are detrimental for longevity and considering the inflammatory CCR5-mediated action, we could expect an increased prevalence of defective *CCR5Δ32* alleles, as well as *CCR5Δ32* carriers, in long-lived individuals. The *CCR5Δ32* polymorphism has been studied in relation to diseases reducing life expectancy, such as acute myocardial infarction and prostate cancer using centenarians as the reference population [10,11]. An overrepresentation of the *CCR5Δ32* allele was reported in centenarians compared to cases, assigning to this polymorphism a role in promoting longevity. In these studies the comparison of extreme phenotype populations (centenarians *vs*. affected cases) probably confers an increased power to detect association. However, the *CCR5Δ32* prevalence in centenarians does not differ from that observed in the healthy control population which agrees with results obtained in our study. This could reflect a weak association of *CCR5* with longevity that needs a more highly powered test to be established.

The association of *RANTES* polymorphisms with longevity has not yet been described. Our results denote a sex-specific association with functional *RANTES* polymorphisms. Pro-inflammatory *RANTES* alleles/haplotypes were positively associated with elderly males. In contrast, anti-inflammatory *RANTES* alleles/haplotypes showed positive association with elderly females.

Elevation of circulating levels of RANTES has been described in unstable angina pectoris patients as predictive of future cardiovascular adverse events [9]. Furthermore, a significant elevation of major C-C chemokines, including RANTES, has been described during the course of acute myocardial infarction that actively contributes to the pathophysiology of the disease. Finally, association studies of functional *RANTES* polymorphisms have been performed highlighting the role of RANTES in age-related diseases [4].

Our findings on *RANTES* allele/haplotype distribution among elderly suggest a *RANTES* genetic determinant that is prone to a pro-inflammatory phenotype in males and an anti-inflammatory phenotype in females, which could contribute to the known sex-specific differences in aging. Sexual dimorphism in immune responsiveness has been reported with respect to genetic background. Some evidence of an increase in frequency of the IL-10 anti-inflammatory cytokine gene in long-lived males has been reported [12] and early work suggested that sexual dimorphism existed in immune responsiveness in the HLA supratype A1B8Cw7DR3 [13].

It has been previously described that the inflammation index, calculated as the sum of positive indicators such as C-reactive protein, plasma fibrinogen and urinary albumin, shows sex differences and varies with age [14]. Women have a higher inflammation index during all life stages compared with men but slower rates of increase in inflammation with age. This can contribute to the decrease in the inflammation differences between the sexes in the elderly population (>80 year-old) [14].

Moreover, it has been suggested that the female sex hormone estrogen may increase the expression of antioxidant enzymes, playing a protective role that

improves host resistance to degenerative diseases, and may reduce the risk of cardiovascular diseases (CVD) by modulation of fibrinolytic factors [15]. Therefore, women have a greater capacity to recover from an inflammatory status, probably due to better regulation of the inflammatory response, which, as our data points out, can be enabled by the genetic component. The better control of inflammation in elderly women might be linked to the higher life expectancy.

We acknowledge that our study have a number of potential limitations. The relatively small number of subjects in the elderly cohort, due to the difficulty to find older subjects fitting our inclusion criteria, could be considered as an important limitation of the study. Some statistical outcomes are based in comparisons performed with small numbers or zero subjects that do not pass statistical threshold when corrected for multiple testing. Despite having used appropriated statistical methods (i.e. Peto odds ratio and Bonferroni-corrected SNP spectral decomposition approach) revealing significant results, the mentioned limitations require us to consider conclusions as suggestive rather than a clear finding.

The role of sex differences in age trajectories is still unresolved. Our data shows that the *RANTES* genetic component differs between males and females and could contribute to their differences in aging trajectories. The appropriate regulation of the inflammatory response by a proper balance of pro- and anti-inflammatory cytokines could be an important factor in reaching extreme longevity. Assuming all the limitations previously mentioned our results encourage further studies to replicate our findings and to search for additional inflammation-related genes that could define an inflammation-related genetic component contributing to sex differences in longevity.

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Figure legend

Figure 1.- Prevalence of carriers of *RANTES* anti-inflammatory *-403G-Int1.1C* (*GC*) and pro-inflammatory *-403A-Int1.1T* (*AT*) haplotypes between elderly and controls in males (left) and females (right). The numbers within each haplotype combination for *RANTES* genotypes are shown at the top of each bar.

Table(s)

Tables

Table 1. Allele, genotype and haplotype distribution of *CCR5Δ32, RANTES -403G/A and RANTES Int1.1T/C* polymorphisms in Spanish controls and elderly subjects.

rs code, (alias)	Genotype Allele Haplotype	Controls			Elderly		
		All subjects n (%)	Males n (%)	Females n (%)	All subjects n (%)	Males n (%)	Females n (%)
rs333, *(CCR5Δ32)*							
Genotypes	wtwt	88 (80)	29 (82.9)	59 (78.7)	79 (76)	22 (68.8)	57 (79.2)
	wtΔ32	22 (20)	6 (17.1)	16 (21.3)	21 (20.2)	9 (28.1)	12 (16.7)
	Δ32Δ32	0 (0)	0 (0)	0 (0)	3 (2.9)	1 (3.1)	2 (2.8)
Alleles	wt	198 (90)	64 (91.4)	134 (89.3)	179 (86.1)	53 (82.8)	126 (87.5)
	Δ32	22 (10)	6 (8.6)	16 (10.7)	27 (13)	11 (17.2)	16 (11.1)
rs2107538 *(RANTES-403G/A)*							
Genotypes	GG	75 (68.2)	28 (80)	47 (62.7)	66 (63.5)	16 (50)[a]	50 (69.4)
	GA	34 (30.9)	7 (20)	27 (36)	35 (33.7)	15 (46.8)	20 (27.8)
	AA	1 (0.9)	0 (0)	1 (1.3)	3 (2.9)	1 (3.1)	2 (2.8)
	A carriers	35 (31.8)	7(20)	28 (37.3)	38 (36.6)	16 (49.9)[a]	22 (30.6)
Alleles	G	184 (83.6)	63 (90)	121 (80.7)	167 (80.3)	47 (73.4)[a]	120 (83.3)
	A	36 (16.4)	7 (10)	29 (19.3)	41 (19.7)	17 (26.6)	24 (16.7)
rs2280789 (*RANTES Int1.1T/C*)							
Genotypes	TT	86 (78.9)	29 (85.3)	57 (76)	71 (73.2)[b]	21 (77.8)	50 (71.4)
	TC	23 (21.1)	5 (14.7)	18 (24)	20 (20.6)	5 (18.5)	15 (21.4)
	CC	0 (0)	0 (0)	0 (0)	6 (6.2)[b]	1 (3.7)	5 (7.2)[b]
Alleles	T	195 (89.5)	63 (92.7)	132 (88)	162 (83.5)	47 (87)	115 (82.1)
	C	23 (10.5)	5 (7.3)	18 (12)	32 (16.5)	7 (13)	25 (17.9)
rs2107538 & rs2280789 (*RANTES* haplotype) [c]							
Haplotype counts							
	GT	182 (83.3)	61 (89.6)	121 (80.5)	148 (76.2)	40 (74)[d]	108 (77)
	AC	23 (10.4)	5 (7.3)	18 (11.9)	24 (12.3)	7 (12.9)	17 (12.1)
	AT	13 (6.1)	2 (3.1)	11 (7.6)	14 (7.3)	7 (13.1)[e]	7 (5.1)
	GC	0 (0)	0 (0)	0 (0)	8 (4.2)[f]	0 (0)	8 (5.8)[f]

[a] Statistical differences between elderly and control males for global genotype distribution (*P*=0.029); *RANTES-403A* modelled as dominant (Odds Ratio (OR)=4.0; 95% confidence interval (CI)=1.4-11.8; *P*=0.01) and allele distribution (OR= 3.2; 95%CI=1.2-8.5; *P*=0.014).

[b] Statistical differences between elderly and controls for global genotype distribution (*P*=0.03) and *RANTES Int1.1C* under recessive model (Peto OR=8.8; 95%CI=1.7-44.7; *P*=0.008), mainly attributable to an overrepresentation in females (Peto OR=8.4; 95%CI=1.4-49.9; *P*=0.02).

[c] Statistical differences between elderly and controls for global haplotype distribution for all subjects (*P*=0.02), males (*P*=0.05) and females (*P*=0.04).

[d] GT haplotype was underrepresented in elderly males (OR=0.3; 95%CI=0.12-0.88; *P*=0.02).

[e] AT haplotype was overrepresented in elderly males (OR=4.9; 95%CI=0.97-24.7; *P*=0.03).

[f] GC haplotype was overrepresented in elderly subjects (P=0.04) due to the overrepresentation in females (Peto OR=8.3; 95%CI=2-34; *P*=0.005)

Figure(s)

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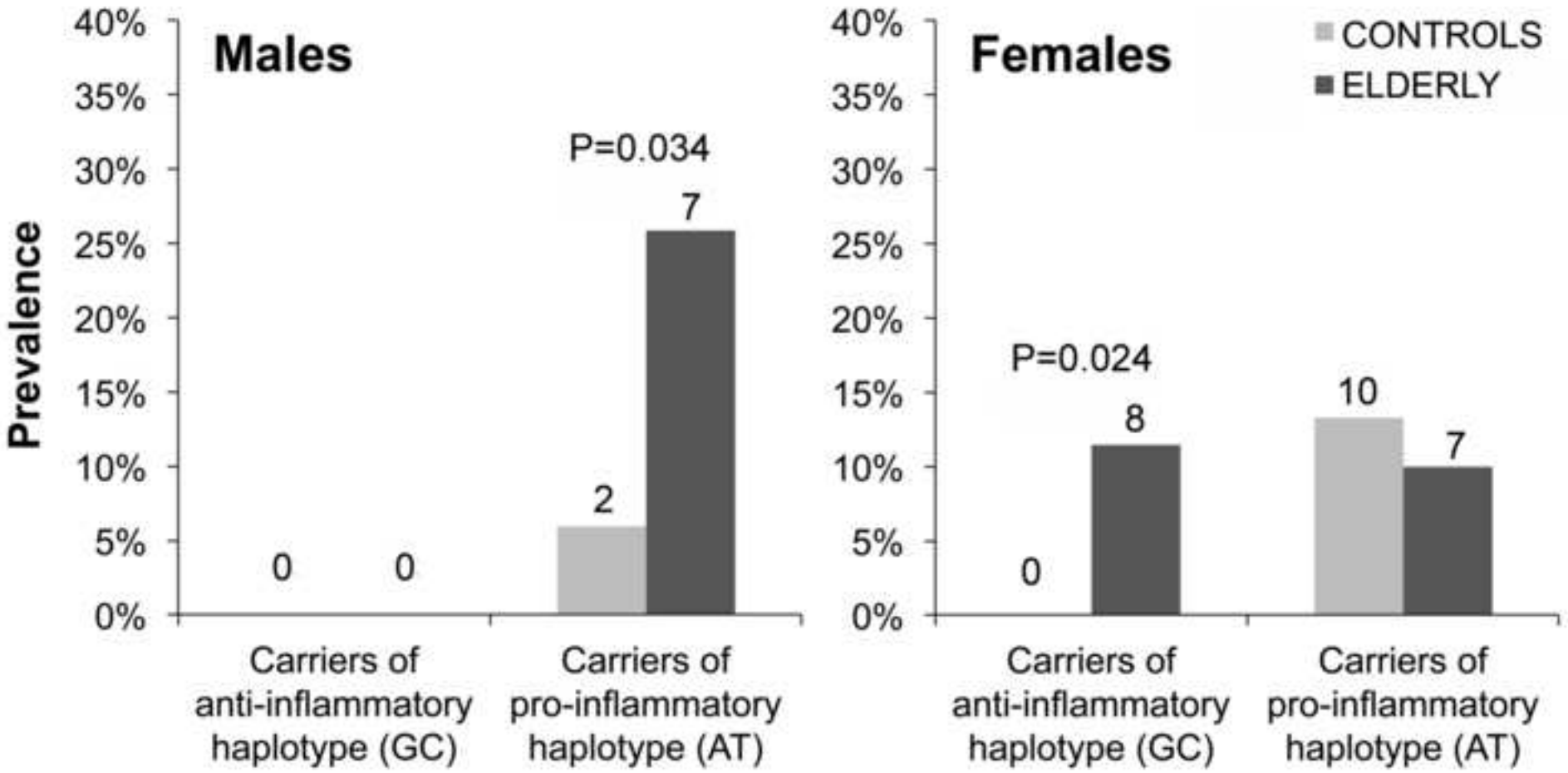

Supplementary Material

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