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**Common CX3CR1 alleles are associated with a reduced risk of headaches**

Short title: CX3CR1 gene polymorphisms and headaches

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## **ABSTRACT**

*Objectives:* The aim of this study was to investigate the role of the chemokine receptor CX3CR1 in headaches and migraine.

*Methods:* Distribution of two polymorphisms of the chemokine receptor CX3CR1 (V249I and T280M) was determined in a population-based sample of 1179 elderly individuals.

*Results:* Heterozygotes for both CX3CR1 polymorphisms had a reduced risk of recurrent headaches, with an odds ratio (OR) of 0.64 (95% CI=0.46-0.90) for the I249 allele and 0.55 (95% CI=0.38-0.81) for the M280 allele. Haplotype analysis showed that carriers of the rarer CX3CR1 I249-M280 haplotype had a reduced risk of recurrent headaches, with an OR of 0.57 (95% CI, 0.41 to 0.80;  $p = 0.001$ ). This association was seen for both nonmigraine headaches (OR=0.47, 95% CI, 0.28 to 0.79;  $p = 0.004$ ) and for migraine (OR=0.65, 95% CI, 0.43 to 0.98;  $p = 0.041$ ).

*Conclusions:* These results need to be replicated but suggest that the chemokine receptor CX3CR1 may play a role in recurrent headaches.

## INTRODUCTION

The pathogenesis of migraine, which is not fully elucidated (1), seems to include both neurogenic inflammation and painful vasodilation of cerebral vessels with activation of the trigeminal system (2). Chemokines released at sites of inflammation in response to proinflammatory cytokines are known to play a role in leukocyte activation and recruitment but there is growing evidence that they are involved in other biological processes, including vascular activity (3), cell survival (4) and pain (5). The chemokine CX3CL1 and its specific receptor CX3CR1 are abundantly expressed in the central nervous system (CNS) where they mediate microglia-neuron interaction under physiological and pathological conditions (6). The level of CX3CL1 in the CNS has been often found increased in association with traumatic and/or pathological events and it is considered responsible for microglial recruitment to the injured sites (7) and neuronal protection from toxic stimuli (8,9,10,11). CX3CL1 induces vascular dysfunction by stimulating vascular reactive oxygen species resulting in reduced NO bioavailability (12). Endothelium-dependent vasodilation is predominantly due to stimulation of NO release (13), which is suggested as a causative mechanism for headache pain (14). We identified two common single-nucleotide polymorphisms, known as V249I and T280M, in the open reading frame of the chemokine receptor CX3CR1 gene (15). The common CX3CR1-I249 allele and its genetically linked partner, the CX3CR1-M280 allele, are associated with a reduced risk of cardiovascular diseases (3,16,17) and reduced risk of inflammatory lung disorders (18,19). CX3CR1 is thus a plausible link between neuroinflammation, vascular activity and migraine. We hypothesized that CX3CR1-I 249 and M280 variants are associated with attenuation of neurogenic inflammation and lower migraine prevalence. We investigated the association between these polymorphisms and migraine in a population-based study of elderly individuals.

## SUBJECTS AND METHODS

*Study participants and migraine assessment.* The Epidemiology of Vascular Aging (EVA) is a longitudinal study of cognitive and vascular aging that recruited men and women born between 1922 and 1932 from the electoral rolls of the city of Nantes, France (20). The migraine study took place at the four-year follow-up between 1995 and 1997 has been described in details in a previous paper (20). Briefly, study participants were first systematically asked whether they ever had any recurrent attacks of headaches. A headache specialist then interviewed by telephone all

who answered yes to the screening question. This interview served as the basis for diagnosis of migraine and its subtypes, according to the 1988 International Headache Society (IHS) criteria (21). Special effort was made to obtain a lifetime history of migraine by asking participants questions about headaches in their young adulthood. The participation of the neurologist was limited to interviews about headaches as he was blinded to all clinical and laboratory data concerning the participants. The sample included 1,179 participants (689 women), and 233 reported a history of recurrent headaches. Migraine was diagnosed in 140 participants, including 120 women and 20 men, and recurrent nonmigraine headaches in 93. Participants who did not report ever having recurrent headaches (N = 946) were further denominated as 'no recurrent headaches' subjects. Written informed consent was obtained from all participants and the study was approved by the Ethics Committee of the Hôpital de Kremlin-Bicêtre.

*Screening for polymorphisms.* CX3CR1 polymorphisms were identified with Minor Groove Binder PCR-based amplification technology from Perkin Elmer (Applied Biosystems Division, Cheshire, UK), described elsewhere (22). Due to technical limitations, CX3CR1 polymorphisms could be identified in only 1100 participants for the V249I polymorphism and 1099 for the T280M polymorphism (93% of the sample).

*Statistical Analysis.* Allelic frequencies were calculated by gene counting. Hardy–Weinberg equilibrium was tested with the chi-square test with 1 *df*. We used the chi-square test to compare genotype distributions and allele frequencies in participants with and without headaches. Potential confounders were identified in bivariate analyses and included in multivariate models if appropriate. Associations between genotypes and headache were estimated through multiple logistic regression models, with the most common homozygotes as the reference category. All analyses were performed with the SAS® statistical package (version 9.1, SAS Institute, Inc., Cary, NC).

## RESULTS

### *General characteristics of the sample*

Table 1 summarizes the participants' general characteristics. Compared with participants without recurrent headaches, those in the migraine group were more frequently women, more often had a history of depression, and were less frequently smokers or drinkers. Those in the nonmigraine

headache group had more frequently hypertension. None of these factors however was associated with the two CX3CR1 polymorphisms studied (data not shown).

#### *Distribution of CX3CR1 polymorphisms in the EVA population*

Table 2 shows the distribution of the CX3CR1 genotypes in participants with and without migraine and nonmigraine headaches. Among participants without recurrent headaches, the frequency of the CX3CR1 I249 allele was 0.32 and of the M280 allele, 0.18. These rates are similar to the frequencies previously reported in other white populations (22). The frequencies of the CX3CR1 polymorphisms did not deviate from Hardy-Weinberg equilibrium (chi square = 2.83,  $p = 0.09$  for CX3CR1 I249 and 0.78,  $p = 0.38$  for CX3CR1 M280). The proportion of individuals carrying the CX3CR1 I249 allele was slightly lower for participants with headaches (0.27) than participants with non-recurrent headaches (0.32), but the difference was of borderline significance ( $p=0.052$ ). Carriers of the rarer CX3CR1 M280 allele were significantly less frequent among participants with headaches (0.12) than among participants without (0.18), ( $p=0.005$ ). After stratification for type of headache, participants with nonmigraine headaches carried the rare CX3CR1-I249 or the CX3CR1-M280 allele least often (Table 2,  $p = 0.01$  for both).

#### *CX3CR1 polymorphisms and risk of headache*

We then conducted multivariate analyses of the relation between the CX3CR1 polymorphisms and headaches; the reference groups comprised the subjects homozygous for the frequent allele (VV or TT) (Table 3). Heterozygosity for either or both CX3CR1 alleles was associated with a reduced risk of both migraine and nonmigraine headaches, with an OR of 0.64 (95% CI=0.46-0.90) for the I249 allele and 0.55 (95% CI=0.38-0.81) for the M280 allele. ORs were slightly lower in the nonmigraine headache group: 0.51 for heterozygosity for I249 ( $p=0.009$ ) and 0.40 for M280 ( $p=0.003$ ). Heterozygotes also had a lower risk of migraine, although the association did not reach statistical significance. Homozygosity for the rare I249 and M280 alleles was not associated with either type of headache: ORs were of the same order of magnitude as for heterozygotes. Haplotype analysis sought to identify the alleles associated with headaches (Table 3). Only the IM haplotype was associated with headaches under the recessive model (OR= 0.57; 95% CI= 0.41-0.80). After stratification by headache type, the IM haplotype was associated with

a reduced risk of both nonmigraine (OR=0.47,  $p=0.004$ ) and migraine (OR= 0.65;  $p=0.041$ ) headaches.

## DISCUSSION

These results suggest an association between CX3CR1 polymorphisms and recurrent headaches in a population-based study of elderly individuals. The rare CX3CR1 I249 and M280 alleles were less frequent in participants with non migraine headaches. Haplotype analysis revealed that only individuals carrying the rarer CX3CR1 I249M280 haplotype were at reduced risk of recurrent headaches overall.

Contrary to our primary hypothesis, CX3CR1 was apparently less strongly associated with migraine as strictly defined according to the IHS criteria than to recurrent headaches not fulfilling IHS criteria. We have no explanation for this unexpected finding which should nevertheless be considered with caution as the confidence intervals between both estimates largely overlap. Also, within the recurrent headaches group, some misclassification may have occurred as these elderly participants might not always remember some of the characteristics of their past recurrent headaches. Recall bias may also partly explain the relatively low prevalence of nonmigraine recurrent headaches in this study. Further studies will confirm, or not, whether the association is stronger in migraine or in nonmigraine recurrent headaches.

These data indicate that CX3CR1 plays a role in headache, but the precise molecular and cellular mechanism of this CX3CR1 variant in headache risk remains elusive. Functional consequences of CX3CR1 polymorphisms remain controversial. An initial study reports a global impairment of the CX3CR1-M280 cell response to soluble CX3CL1, i.e., ligand binding, calcium response, adhesive and chemotactic functions (17). However, this was not confirmed in two following studies (23,24). Indeed, we found that the number of cells adhering to CX3CL1 increases with the number of I249 alleles an individual carries (23). CX3CR1 participates in several biological responses that may affect headache pathophysiology. We anticipated that, similarly to previous studies associating common CX3CR1-I249 allele and reduced risk of inflammatory processes such as cardiovascular diseases (3,16,17) and lung disorders (18,19), variant of CX3CR1 may be associated with reduced risk of headache by dampening the inflammatory environment. Interestingly, endothelium-dependent vasodilation is greater in individuals carrying the CX3CR1-I249 allele than in individuals homozygous for the CX3CR1-V249 allele (3). Endothelium-

dependent vasodilation is predominantly due to stimulation of NO release (13), which is suggested as a causative mechanism for headache pain (14). Although leukocyte involvement in headaches is unlikely, platelet dysfunctions have been linked to headache pathophysiology. CX3CR1 is expressed on platelets, and its ligand enhances their adhesion and activation (25). The functional impact of CX3CR1 variants in headaches may thus involve a delicate balance between excessive platelet adhesion and activation. Finally, an alternative hypothesis for this association is that the CX3CR1 polymorphism is in linkage disequilibrium with one or more genes responsible for the observed effect. Because no genetic association has yet been described between CX3CR1 and its surrounding genes and because the biological role of CX3CR1 is compatible with headache etiology, the association between CX3CR1 and headaches may well be direct. An important aspect of this association is the relatively high frequency of the rare allele. For example, 32% (353/1099) of the sample carried the rare allele of the CX3CR1 M280 polymorphism. This suggests the underlying mechanism is fairly widespread. These results support the hypothesis of the pathogenic role of CX3CR1 in headache but causative role of this gene remains to be elucidated. Further clinically-based or family-based studies are needed to confirm the association between CX3CR1 polymorphisms and headaches and to clarify its mechanisms. If confirmed, these results could help to propose new hypotheses about the mechanisms of headaches and to explore new therapeutic pathways.

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**Table 1 - General characteristics of participants according to their headache status**

	<b>No recurrent headaches</b> (n=946)	<b>Nonmigraine headaches</b> (n=93)	<b>Migraine</b> (n=140)
<b>Median age, (range)</b>	65 [59 - 71]	66 [60 - 71]	65 [60 - 71]
<b>Female sex, % (n)</b>	54.2 (513)	60.2 (56)	85.7 (120) ¥
<b>Years of schooling, years (SD)</b>	10.75 (3.52)	10.48 (3.43)	10.92 (3.19)
<b>BMI, kg/m2, mean (SD)</b>	25.39 (3.7)	25.67 (3.6)	24.64 (3.8) ¥
<b>Hypertension* (%)</b>	37.95	49.46 ¥	34.29
<b>Tobacco Consumption (%)</b>			
Never	56.45	60.22	72.14 ¥
Ex-smoker	34.57	29.03	23.57
Current	8.99	10.75	4.29
<b>Alcohol (%)</b>			
No alcohol	25.38	29.67	43.07 ¥
< 20 ml	41.18	42.86	43.8
>= 20 ml	33.44	27.47	13.14
<b>Depressive symptoms* (%)</b>	11.13	15.22	15.33
<b>History of depression* (%)</b>	2.79	3.26	6.57 ¥
<b>History of coronary disease*, % (n)</b>	10.15 (96)	13.98 (13)	7.86 (11)
<b>History of stroke*, %(n)</b>	3.70 (35)	2.15 (2)	3.57 (5)

\* Defined as systolic blood pressure  $\geq 160$  mm Hg or diastolic blood pressure  $\geq 95$  mm Hg or receiving antihypertensive therapy. Hypertension was measured by the Center For Epidemiologic Studies Depression (CES-D) Scale; defined as a score  $\geq 17$  for men and  $\geq 23$  for women. Depression was defined as a history of depressive symptoms confirmed by a medical doctor and treated with antidepressant drugs. History of coronary disease and stroke were asked during the general interview. ¥ indicates  $p < 0.05$  in comparison with the no-headache group.

**Table 2- Distribution of genotypes for polymorphisms in the CX3CR1 receptor by headache status**

<b>Frequency (number) of genotype</b>	<b>V249I</b>				<b>Frequency (number) of genotype</b>	<b>T280M</b>			
	<b>No recurrent headaches (n=879)</b>	<b>All headaches (n=221)</b>	<b>Nonmigraine headaches (n=90)</b>	<b>Migraine (n=131)</b>		<b>No recurrent headaches (n=879)</b>	<b>All headaches (n=220)</b>	<b>Nonmigraine headaches (n=90)</b>	<b>Migraine (n=130)</b>
<b>VV</b>	46.9 (413)	56.1 (124)	63.3 (57)	51.1 (67)	<b>TT</b>	65.6 (577)	76.8 (169)	81.1 (73)	73.8 (96)
<b>VI</b>	42.1 (370)	34.4 (76)	30.0 (27)	37.4 (49)	<b>TM</b>	31.4 (276)	21.8 (48)	16.6 (15)	25.4 (33)
<b>II</b>	10.9 (96)	9.5 (21)	6.6 (6)	11.4 (15)	<b>MM</b>	2.9 (26)	1.3 (3)	2.2 (2)	0.8 (1)
<b>Allelic frequency of allele I</b>	0.32	0.27	0.21	0.30	<b>Allelic frequency of allele M</b>	0.18	0.12	0.10	0.13
<b><i>p</i></b>	ref	0.0515	0.0122	0.5921	<b><i>p</i></b>	ref	0.0055	0.0113	0.105

**Table 3 - Odd ratios for all headaches, nonmigraine and migraine headaches, according to CX3CR1 polymorphisms.**

<b>Genotype</b>	<b>All headaches</b>		<b>Nonmigraine headaches</b>		<b>Migraine</b>	
	OR [95% CI]	<i>p</i> *	OR [95% CI]	<i>p</i> *	OR [95% CI]	<i>p</i> *
<b>V249I</b>						
VV	1 [ref.]		1 [ref.]		1 [ref.]	
VI	0.64 (0.46-0.90)	0.010	0.51 [0.31-0.85]	0.009	0.75 [0.49-1.15]	0.18
II	0.69 (0.40-1.20)	0.19	0.47 [0.83-3.94]	0.10	0.93 [0.48-1.78]	0.82
<b>T280M</b>						
TT	1 [ref.]		1 [ref.]		1 [ref.]	
TM	0.55 [0.38-0.81]	0.002	0.40 [0.22-0.73]	0.003	0.67 (0.42-1.05)	0.08
MM	0.33 [0.10-1.15]	0.08	0.53 [0.12-2.36]	0.41	0.20 [0.03-1.53]	0.12
<b>Haplotype</b>						
VT	1 [ref.]		1 [ref.]		1 [ref.]	
IT	0.99 [0.72-1.36]	0.943	0.76 [0.46-1.25]	0.278	1.20 [0.81-1.77]	0.362
IM	0.57 [0.41-0.80]	0.001	0.47 [0.28-0.79]	0.004	0.65 [0.43-0.98]	0.041

\*Adjusted for age, sex, education, BMI, hypertension, alcohol and tobacco consumption, and history of depression and of coronary disease.