Mestrado Integrado em Medicina Dentária

O gene *P2XR7* e a suscetibilidade para a reabsorção radicular apical externa (RRAE) em pacientes sob tratamento ortodôntico

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ABSTRACT

Introduction: External apical root resorption (EARR) is a common and unpredictable side effect of orthodontic treatment. It is a complex phenotype dependent on both environment and multiple low penetrance genetic variables. The P2XR7 gene encodes a purigenic receptor involved in bone remodeling and inflammation and may be considered a candidate gene for genetic susceptibility to EARR. In our study, we analysed the contribution of gain-of-function missense variant rs1718119 to orthodontic-induce EARR.

Methods: The study sample comprised 195 orthodontic patients whom six teeth, the maxillary incisors and canines were analysed. Panoramic radiographs were used for EARR evaluation, using a specific software that allowed image processing and data calculations. The % of EARR was analysed for each tooth after introduction of a magnification correction factor. The maximum % EARR for each patient was also evaluated. Genotyping of P2XR7 was performed with a TaqMan real-time PCR assay. For statistical analysis, Chi-square and logistic regression model analysis were used.

Results: We confirmed that incisors are the most susceptible teeth to orthodontic-induced EARR. No significant association was found with rs1718119, though GG genotype showed a trend to be associated with a worse phenotype. According to logistic regression analysis, this polymorphism explained 3% of EARR variability among patients.

Conclusion: The gain of function polymorphism of P2XR7 gene, rs1718119, has a sparse effect on EARR variability. Studies with more genetic variables and larger population samples need to be performed.

KEYWORDS: Polymorphism; SNP; P2XR7; external apical root resorption; orthodontic treatment

RESUMO

Introdução: A reabsorção radicular apical externa (RRAE) é um efeito secundário e imprevisível comum do tratamento ortodôntico. É um fenótipo complexo dependente tanto do ambiente como de múltiplas variáveis genéticas de baixa penetrância. O gene P2XR7 codifica um recetor purinérgico envolvido na remodelação óssea e na inflamação, podendo ser considerado um gene candidato para a suscetibilidade genética da RRAE. No nosso estudo analisámos a contribuição do ganho de função da variante missense rs1718119 na RRAE induzida pelo tratamento ortodôntico.

Métodos: A amostra foi composta por 195 pacientes ortodonticamente tratados, cujos 6 dentes, incisivos e caninos maxilares, foram analisados. Foram utilizadas radiografias panorâmicas para avaliar a RRAE usando um software específico que permitiu o processamento da imagem e o cálculo dos dados. A % de RRAE foi analisada para cada dente após a introdução de um fator de correção da ampliação. Também foi avaliada a % de RRAE para cada paciente. A genotipagem do P2XR7 foi realizada através da análise de PCR em tempo real com sonda TaqMan. Para a análise estatística foram utilizados o Qui-quadrado e o modelo de regressão logística.

Resultados: Confirmámos que os incisivos são os dentes mais suscetíveis à RRAE induzida pelo tratamento ortodôntico. Nenhuma associação significativa foi encontrada com o rs1718119, embora o genótipo GG tenha apresentado uma tendência para ser considerado o pior fenótipo. De acordo com a análise de regressão logística, este polimorfismo explicou 3% da variabilidade da RRAE entre os pacientes.

Conclusão: O ganho de função do polimorfismo rs1718119 do gene P2XR7 tem pouco efeito sobre a variabilidade da RRAE. Estudos com mais variáveis genéticas e amostras maiores deverão ser realizados.

PALAVRAS-CHAVE: Polimorfismo; SNP; P2XR7; reabsorção radicular apical externa; tratamento ortodôntico

INTRODUCTION

External Apical Root Resorption (EARR) is a common occurrence in orthodontic treatment. It consists in a permanent reduction of the roots of the teeth, which may be seen in routine x-rays, such as panoramic and periapical radiographies (1–4). It may begin during the initial phases of orthodontic treatment (5,6). Root shortening means values range from 0.5 to 3 mm: resorption higher than 3 mm was reported to occur at a frequency of 30% and only 5% of treated individuals were found to have values higher than 5 mm (6–9). EARR may occur in any tooth but the most affected are usually the maxillary incisors (1,5,10,11).

When teeth are orthodontically moved, the periodontal ligament is submitted to tension and compression forces (1). Mechanical stress is thought to induce cells in the PDL to release biologically active mediators responsible for local activation of inflammatory cells and cells involved in alveolar bone and root remodelling (12,13). Forces are concentrated at root apex, where the cementum is cellular and dependent on irrigation and molecular microenvironment, thus, more susceptible to aggressions. The radicular resorption takes place when the cementum repairing capacity is exceeded, allowing for the multinucleate odontoclasts to degrade the root substance (14). When resorption extends into dentin, the loss of root apical material became unpredictable and irreversible (11,15). The biologic mechanisms involved in periodontal tissues interactions are complex and poorly understood. Odontoclasts, the cells resorbing dental hard tissues, have similar morphological and functional characteristics with bone-resorbing osteoclasts, and both cells share molecular pathways, raising the question of what determines EARR, absence of bone resorption or directly induction of root resorption. Accordingly, authors have put the focus of investigation either on bone or root remodeling cells and mediators (9,16–18).

The panoramic and cephalometric radiographs are usually the main diagnostic tool in orthodontics. In comparison with periapical, the panoramic radiographs allow less radiation exposure, less time-consuming for the operator, a view of the complete dentition and better patient cooperation (19). Several recent reports chosen this method (20–23). Thus, periapical radiographs, although more accurate, are often used as complementary diagnosis for specific clinical situations, as with adult patients, and in clinical investigation, its use seriously limits the number of teeth and dimension of patient sample to be studied. Panoramic films may overestimate by approximately 20% the amount of root loss (19) but this magnification factor is relatively constant in the vertical dimension (24,25) which is clinically the most important aspect in analysing

EARR (26). Moreover, mainly due to image distortion, compared panoramic films with the full-mouth periapical radiographs showed maximum differences in the lower incisors (19), but minimum in the maxillary incisors, precisely the most frequently affected teeth (27). Cone-beam computed tomography (CBCT), a new radiography method with application in several diagnosis areas, offers 3-dimensional (3D) imaging of dental structures and provides clear images of highly contrasted structures, such as bone. Compared with conventional computed tomography, CBCT technology in clinical practice has important advantages such as minimization of the radiation dose, image accuracy, rapid scan time, fewer image artifacts, chair-side image display, and real-time analysis (4). In the future, common use of these three-dimensional imaging systems will allow improving accuracy (28,29).

The etiology of EARR is multifactorial (15). Susceptible factors may be biological, related to genetic predisposition or to the effect of orthodontic forces (1,2,6,30). The risk of EARR may therefore be related to the patient or to the treatment (2). The full understanding of the contribution of these factors to orthodontic-induced EARR would provide dentists a way of predicting the occurrence of this complication and allow a more personalized treatment planning (5). Patient related factors reported in literature, include age, gender, individual genetic profile (2,5,6,20,30), systemic factors (2,5,6,20,30), medication (1,2,30), occlusion (2,30), existence of anterior open bite (14), tongue thrust (7), morphology of the root (17), previous history of radicular resorption, shape of the alveolar ridge (17), proximity of the root to the cortical bone or endodontic treatment (2,30). Treatment related factors include: duration of treatment, magnitude of force applied, direction of dental movement, amount of apical displacement and method of force (2,5,6,30).

Since mechanical forces and other environmental factors do not adequately explain the variation in the degree of EARR among orthodontic treated patients, an increased interest has focused on the role of genetic factors (14). According to Shaza *et al.,* genetic factors explain about 64% of the variation observed in EARR associated to orthodontic treatment (1,11). Familial, twins and animal models studies, also support a genetic contribution (11). Candidate gene approaches have searched for polymorphisms in genes encoding molecular mediators known to be involved in bone and root remodeling. Increased levels in crevicular fluid of orthodontic patients (12,31), in vitro studies with PDL cells (9,16,32) and knock out animal studies (11,16,33) support the role of these molecules in EARR.

The most extensively studied gene is the gene encoding interleukin 1B (IL-1B), a potent bone remodeling factor (3,7,11,21–23,34,35). According to Al-Qawasmi *et al.* studies in Caucasian patients (3,34), citosine (C) variant of a single nucleotide polymorphism (SNP) of IL-1B, rs1143634 or C3954T, significantly increases the risk of EARR. Though this is a synonymous (Phe105Phe) polymorphism, the risk variant has been associated with decreased production of IL-1B, less alveolar bone remodeling and consequently more tension imposed to dental structures during orthodontic movement (3). Other candidate genes have been proposed, such as those encoding IL-1 receptor antagonist (IL-1RN) (23), IL-1A (21–23), IL-6 (1,12), vitamin D receptor (36), or tumor necrosis factor α related proteins RANK (1,7), RANKL (1,7) and OPG (1,7). Yet, as summarized in table 1, contradictory results have frequently been reported (20,21,23,34).

Another candidate gene to be considered is P2RX7 that codifies the purinergic receptor P2X ligand-gated ion channel 7, a non-selective ion channel dependent on high levels of extracellular ATP. Involved in multiple cellular phenotypes, P2RX7 also seems to be a key mediator of inflammation and mechanically induced bone formation (16). Recent research has shown that this receptor has a major role in the metabolism of apoptotic and necrotic tissues (16,37). P2X7R gene is highly polymorphic and several non-synonymous functional SNPs are known to increase or decrease the receptor function (38). In the unique study involving P2RX7 gene and EARR, Viecilli *et al.* using a knock-out mouse model, concluded that the absence of the P2RX7 gene predisposes to EARR (16).

In our study, we analyse the role of gain-of-function variant rs1718119 (Ala348Thr; GCT>ACT) in the susceptibility to orthodontic-induced EARR in four maxillary incisors and both maxillary canines.

Tabela 1. EARR and genetic polymorphisms associated.

Authors	Gene (Polymorphism)	Sample	X-ray	Results
Al-Qawasmi 2003[a] (3)	IL-1B (rs1143634)	35 American Caucasian Families 118 subjects	P and C	Evidence of linkage disequilibrium between IL-1B polimorphism and EARR in the maxillary central incisors
Al-Qawasmi 2003[b] (34)	TNSALP TNFα, TNFRSF11A	38 American Caucasian families 124 subjects	P and C	Suggestive evidence for linkage between EARR in the maxillary central incisor and the polymorphic marker D18S64 (lies close to the candidate gene TNFRSF11A)
Lages 2009 (35)	IL-1B (rs1143634)	61 Brazilian subjects 23 patients 38 controls	Pe	Significant association
Gülden N 2008 (21)	IL-1A (rs1800587) IL-1B (rs1143634)	258 German subjects 96 patients 162 controls	P	Significant association for IL-1A polymorphism No significant association for IL-1B polymorphism
Tomoyasu Y 2009 (20)	IL-1B (rs1143634)	54 Japanese patients	P and C	No significant association
Iglesias-Linares 2012 (22)	IL-1A (rs1800587) IL-1B (rs1143634)	93 Caucasian patients (root-filled teeth)	P and C	No significant association for IL-1A polymorphism Significant association for IL-1B polymorphisms
Linhartova P 2012 (23)	IL-1A (rs1800587) IL-1B (rs1143634) IL-1RN (rs419598)	106 subjects from Czech Republic 32 patients 74 controls	P and C	No significant association for IL-1A and IL-1B polymorphisms Association with IL-1RN, especially in girls
Iglesias-Linares 2012 (39)	IL-1 (rs1800587) IL-1B (rs1143634) IL-1RN (rs419598)	54 Caucasians patients (vital teeth)	P and C	No significant association for IL-1A polymorphism Significant association for IL-1B and IL-1RN polymorphisms
Fontana M 2012 (36)	Vitamin D receptor gene (rs731236)	377 Brazilian subjects 339 patients with EARR 38 controls	Pe	Significant association for Vitamin D receptor gene polymorphism

Legend: C- Cephalometric radiograph; P- panoramic radiograph; Pe- Periapical radiograph; *TNFRSF11A*- gene encoding RANK

MATERIALS AND METHODS

Subjects

One hundred ninety five Caucasian patients, selected from the archives of two orthodontic clinics and from the Department of Orthodontics, Dentistry Area, Faculty of Medicine, University of Coimbra, were invited to participate in this study. Patients began and completed orthodontic treatment during 2000-2010.

Criteria used for patient selection were: having received comprehensive orthodontic treatment (straight-wire technique); having two high-quality panoramic radiographies (before and after treatment) and a clinical file allowing data collection (clinical patient's information). Also, maxillary incisors and canines should have completed the formation of the root at the beginning of treatment, had no previous history of dental trauma and be free of fractures, abrasion or caries on the incisal edges between measurements. Patients shouldn't have genetic craniofacial malformation or any congenitally missing, supernumerary or impacted maxillary canines or incisors. Patients with persistent periodontitis during treatment were excluded.

All patients were informed of all procedures and signed a written informed consent. This study was performed with the ethical principles governing medical research and human subjects as laid down in the Helsinki Declaration (2002 version, www.wma.net/e/policy/b3.htm) as well as the approval of the Research Ethics Commission of the Faculty of Medicine of Coimbra University.

X-ray analysis and measurements

From the sample of the 195 patients, three hundred and ninety panoramic radiographs were obtained. The radiographs were scanned (with a resolution of 300 dpi and 256 gray levels) using a scanner (Expression 1680 Pro, Epson, Suwa, Japan) and saved in TIF file format (TIFF). We analysed the four maxillary incisors and both maxillary canines using before (T-1) and after (T-2) orthodontic treatment radiographs patients. The final film was taken during the first three months after debonding. Both radiographs were performed with the same equipment. The standard quality criteria of a normal panoramic radiograph were verified.

To assure a standardized and accurate measure of EARR, a software prototype was developed (ARIAS - Apical Resorption Image Analysis System) in MATLAB version 7.12.0.635 (R2011a). This program speeds up measuring and minimizes human errors, as all considered features are automatically computed and can be saved to an individual Microsoft Excel file associated to each patient. The method included the following three steps: (1) image preprocessing to improve the potential of teeth area discrimination; (2) selection of four points on each tooth, two vertical end points positioned on root and crown respectively and two horizontal end points localized in the root, which are expected to be collinear with the intersection point of root and crown; (3) extraction of the parameters to produce a set of digital measurements of tooth length – initial root (R1), final root (R2), initial crown (C1), final crown (C2) and corrected final root (CR2).

The corrected final root results from the application of a correction or enlargement factor corresponding to the ratio between the initial and final crown lengths (C1/C2), because it is accepted that, during orthodontic treatment, the crown length does not change. Possible measurement errors due to the magnification effects associated with panoramic films were minimized by the use of the correction factor and the percentage of root length variation instead of the use of root length variation itself to evaluate EARR.

The six maxillary teeth in the study were measured using the method of Linge and Linge (40) modified by Brezniak *et al.* (41). The root and crown lengths in both T-1 and T-2 radiographic images were processed by the software to calculate the other parameters.

Mathematical formulation computed to obtain the final % EARR, is the following:

$$CF = C1/C2 \quad \text{where } CF \text{ is the correction factor}$$

$$CR2 = R2 \times CF \quad \text{where } CR2 \text{ is the corrected final root}$$

$$ratio\ CR2/R1 \quad \text{represents the ratio remained root}$$

$$\%EARR = 1 - (CR2/R1)$$

Sample collection and genotype

DNA was extracted from buccal swabs, three for each patient, using Chelex 100® and stored at -20°C until analysed. SNP rs1718119 was genotyped using *TaqMan ® Pre-Designed SNP Genotyping Assays*, ref. C_11704039_10 (Applied Biosystem, New Jersey, USA), iQ™ Supermix (Bio-Rad Laboratories) and 30 ng of DNA for each sample. Amplification was conducted in a CFX96 (Biorad) equipment as follows: 10 minutes at 95°C and 40 cycles of 15 seconds at 92°C and one minute at 60°C.

As positive controls, we used samples previously genotyped by automatic sequencing in an *AbiPrism 3130 Genetic Analyser* using *BD v1.1* (*Applied Biosystem*) and *Sequencing Analysis Software v5.2.* Primers 5'AACGCATCTATCCAAGTC 3' and 5'TCTTCCTGTAGTAGTATTCG 3', amplifying a 392 bp sequence, were used for the first PCR and sequencing amplification (gene Reference Sequence: NG_011471.2.).

Reliability of Measurement Method

To avoid inter-observator error, the same operator, specialist in orthodontics, executed all the aforementioned measurement procedures. The intra-observator error analysis on measuring panoramic radiographs consisted in a statistical evaluation of the difference between 2 measurements, taken 15 days apart, on each tooth type, of 20 randomly selected patients.

Statistical analysis

The Student test for paired samples was used for the intra-observator error analysis. A one-way repeated measurement using ANOVA with post-hoc tests was conducted to compare EARR of the select teeth. In order to analyse the EARR phenomena for the individual and not only for each separate tooth, we propose a metric based on the maximum observed EARR on the six selected teeth. A chi-square goodness of fit test was used to assess whether the distribution of the maximum observed EARR on the six selected teeth was homogeneous among the six teeth. We have used a stepwise regression model with the mixed forward/backward option which successively adds and deletes variables according to the two criteria: Prob to Enter = .05 and Prob to Removal = .10. The statistical package SPSS (version 19.0, IBM SPSS Statistics for Windows, IBM Corp.) was used to perform the statistical analysis. Hardy-Weinberg equilibrium was verified using the chi square test.

RESULTS

The intra-observational mean error for root resorption measurements ranged from 0.01 (central incisors) to 0.35 (canines). No statistically significant differences between 2 measurements were found when performed by the same operator ($P > 0.05$). For IL-1B polymorphism, the sample population was in Hardy-Weinberg equilibrium ($p > 0.05$).

The mean age of the sample was 17,24 years (± 6.8 years) and the average treatment time was of 36 months (± 10 months).

Table 2 summarises the results of the % EARR for each of the tooth analysed for the 195 individuals. On average, EARR ranged from 8.0 (tooth 13 and 23) to 11.0 (tooth 12). The tooth 12 stands out by presenting the highest values of resorption in the general tendency, with half of the 195 individuals presenting a resorption higher than 9%. For percentile 95, representing the 5% of population sample with the highest EARR, values ranged from 22.0 (tooth 13) to 29.0 (tooth 12). There was no significant difference between symmetrical teeth but incisors were significantly more affected than canines (P <0.01), reaching maximum difference between teeth 13 and 12.

For the evaluation of each individual patient, instead of calculating the average EARR of the six teeth, we considered the maximum % EARR value (% EARR max.) obtained in each patient (table 3). The results ranged from 1.9 to 49.7, with an average of 17.9 (95%CI 18,5-19.2), a median of 16.6 and a value of 36.0 for percentile 95. Table 4 depicts the distribution of teeth with EARR max. As for global EARR, the distribution of EARR max. was not homogeneous among the six teeth, with the lateral incisors (12 and 22) being the most frequent teeth involved (P< 0.01).

To analyse the association of P2XR7 genotypes with susceptibility to % EARR max., patients were divided in two groups, as having % EARR max. above or under the sample median value of 17%. The frequencies of P2XR7 genotypes in the two groups are shown in table 5. There was no statistical significant difference between groups (χ^2 = 5.13; P = 0.08), though GG genotype showed a trend to be associated with higher root resorption. Similar results were obtained using a logistic regression model (Omnibus: Chi square tests = 5.15; P = 0.076), that also showed that only 3% of % EARR max. variability was explained by P2XR7 genotype (Cox and Snell and Nagelkerke statistics).

Table 2- Results of % EARR for each tooth.

		Teeth					
%RRAE		13	12	11	21	22	23
Asymmetry		1.694	0.867	1.457	1.463	0.987	1.293
Average		0.08	0.11	0.10	0.10	0.10	0.08
SD		0.07	0.09	0.08	0.1	0.08	0.07
Maximum		0.48	0.37	0.42	0.5	0.37	0.35
Percentiles	25	0.03	0.05	0.03	0.03	0.03	0.03
	50	0.06	0.09	0.07	0.07	0.08	0.07
	75	0.11	0.17	0.14	0.14	0.15	0.11
	95	0.22	0.29	0.26	0.26	0.27	0.24

Legend: SD- standard deviation

Table 3 – Results of Maximum % EARR for each patient

%EARRmax		Statistic
Mean		0.179
95% Confidence Interval for Mean	Lower Bound	0.165
	Upper Bound	0.192
Median		0.166
Minimum		0.019
Maximum		0.497
Skewness		0.834
Percentiles 95		0.36

Table 4 – Distribution of teeth with % EARR max.

Tooth	N	(%)
13	19	9.7
12	49	25.1
11	22	11.3
21	31	15.9
22	42	21.5
23	32	16.4
Total	195	100,0

Table 5 – Distribution of *P2XR7* SNP genotypes and alleles according to % EARR max.

	%EARRmax	
	< 17%	> 17%
Genotypes	N (freq.)	N (freq.)
GG	37 (0.356)	47(0.516)
GA	54 (0.519)	35 (0.385)
AA	13 (0.125)	9 (0.099)
Total	104	91
Alleles frequency		
G	0,615	0,709
A	0,385	0,291

Legend: N- Subjects; Freq.- frequency

DISCUSSION

EARR is the most undesirable side effect of orthodontic treatment. It is a multifactorial trait, resulting from a combination of genetic and environmental risk factors (2,11,12). Non-genetic, mainly treatment-related factors, accounts for no more than 30% of EARR variability, the remaining being probably due to complex genetic profiles (6,42).

In terms of x-ray imaging and morphologically speaking, EARR is characterized by apical roundness, which can have several levels of intensity from a slightly flat or rounded vertex to a grossly reabsorbed apex (5). Radiographic evaluation of EARR took place by measuring the maxillary anterior teeth in panoramic radiographs using a specific software that allowed an improvement in the degree of accuracy and reproducibility of measurements. The intra-operator reliability of the method was confirmed.

Panoramic films may overestimate by approximately 20% the amount of root loss (19) but this magnification factor is relatively constant in the vertical dimension (24,25) which is clinically the most important aspect in analysing EARR (26). Though periapical film accuracy is higher, x-ray exposure limits, the number of teeth and the dimension of patient sample to be studied, explaining why panoramic films are still used in many recent publications (19).

Since there are variations in the population tooth and root lengths, and to minimize measurement errors, the % of EARR was evaluated instead of variation of root length itself. The maximum percentage of EARR for each patient was also analysed, which is clinically a more meaningful data to evaluate patient's need of specific treatment proceedings. As previous authors (1,6,10,11,43–45), we found that incisors were the most affected teeth, confirming the reliability of the method used for EARR evaluation. Lateral incisors have been found to resort more than the central incisors (10), but in our sample, there was no difference between symmetrical teeth. Only 5% of patients had teeth with % EARR values higher than 20%.

Orthodontic forces induce an inflammatory process in the periodontal ligament responsible by local activation of immunoinflamatory cells and release of molecular mediators that will induce alveolar bone and root remodeling (32). This process is essential for teeth movement and therapeutic success, but if disturbed, it may lead to root resorption. Functional polymorphisms in the genes encoding the molecules of the involved cellular pathways may interfere with susceptibility to EARR.

In this study, we analysed if the gain-of-function variant of P2XR7 gene, rs1718119 (Ala348Thr; **G**CT>**A**CT), was associated with susceptibility to EARR. In bone cells, the activation of P2XR7, a purinergic cell membrane receptor, is thought to have a pro-osteogenic effect, activating osteoblast function and inducing osteoclast apoptosis (46–48). It also stimulates the release of inflammatory cytokines such as IL-1B by immune cells (49,50), and *in vivo* experiments suggested a role in mechanotransduction pathways (38). Our data revealed that GG genotype was correlated with EARR max., though not reaching statistical significance (*P*=0.08). Logistic regression analysis showed that rs1718119 polymorphism accounts for 3% of EARR variability.

If confirmed, these results suggest that the variant associated with less bone-tissue formation is the risk allele. Our results are in accordance with the unique study involving P2RX7 gene and EARR, where Viecilli *et al.* (16), using a knock-out mouse model, concluded that the absence of the P2RX7 gene caused increased EARR. Yet, previous studies in EARR susceptibility showed association with an IL-1B polymorphism that is believed to reduce the levels of this interleukin in crevicular fluid (3,33), leading to decrease bone resorption and consequently, increase pressure applied to the apical root. A possible explanation may be the described role of P2XR7 in the induction of IL-1B by immune cells (49). In a context of inflammation, such as the one induced by orthodontic forces, the reduction of IL-1B due to a low active P2XR7, might be the dominant effect in bone metabolism.
Another explanation is that the role of P2RX7 in EARR is more related to its function in root remodeling cells such as odontoblasts, cementoblasts and odontoclasts than with is osteogenic effect (32).

CONCLUSION

In conclusion, our data suggest that the gain of function polymorphism of P2XR7 gene, rs1718119, has a sparse effect on EARR variability. Studies with more genetic variables and larger population samples need to be performed.

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