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VARIANTS OF THE ELN GENE AND SUSCEPTIBILITY TO INTRACRANIAL ANEURYSM: A SYNTHESIS OF GENETIC ASSOCIATION STUDIES USING A GENETIC MODEL-FREE APPROACH

A THESIS BY

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CONTENTS

Abstract

Subarachnoid hemorrhage due to intracranial aneurysm rupture is one of the most devastating conditions leading to high rates of mortality and disability. The presence of an intracranial aneurysm is thought to originate from an interaction of environmental and genetic factors. Several GWAS and GAS have suggested ELN as a candidate gene for the development of intracranial aneurysms. The variants EX201264G>A and INT20 1315T>C of the ELN have been implicated in the development of intracranial aneurysms. However, the results are contradictory and no conclusions can be drawn. In order to address this discrepancy we performed a meta-analysis using a genetic model-free approach based on ORGASMA software Electronic databases were searched until August 2015 for the variants. Five eligible studies including 828/964 cases/controls for EX20 and 920/997 cases/controls for INT20 were analyzed. From the results of this study became evident that EX20 1264G>A variant correlates with an increased risk for intracranial aneurysm (RE=1.44, FE=1.72) whereas INT20 1315T>C shows a protective effect (RE=0.66). However the heterogeneity between studies is large, and the findings should be interpret with caution. Further GWAS and GAS are needed in order to narrow down the responsible genetic factors and to help us understand the pathomechanism leading to aneurysm formation.

Keywords: ELN, elastin gene, intracranial aneurysm, subarachnoid haemorrhage, intracranial haemorrhage, intracerebral haemorrhage GAS

1. Introduction

Subarachnoid hemorrhage (SAH) accounts for approximately 5% of strokes and is frequently a catastrophic condition leading to death or severe disability in adults. (1). It occurs in 8-10 per 100.000 persons per year. The majority of SAH can be attributed to the rupture of an intracranial aneurysm (IA), present in 2-5% of the general population, with a mortality rate of 25-50% (2,3). Survivors are often left with severe disability and decreased quality of life. Most of the mortality after rupture of an IA is due to the primary brain injury from the bleeding that is irreversible, regardless the type of treatment, either medical or surgical. (4). Unruptured IAs are associated with a variable risk of aneurysmal rupture that increases with size of the aneurysms (3). Known risk causes for the formation and growth and rupture of IAs include age, and common modifiable factors such as smoking, alcohol consumption and hypertension (6,7) Other known factors are sex, with women being affected 1.6 times more often than men (2), and race with blacks suffering from SAH 2.1 times more often than whites (8). Accordingly, a WHO multinational study found that the age-adjusted average annual SAH attack rates may differ between races, with the incidence varying even 10-fold (1). Furthermore, the significance of genetic factors is increasingly recognized. Familial IAs account for 7 to 20% of patients with aneurysmal SAH and are generally not associated with any of the known heritable connective tissue disorders (9). Hence, much research is currently being directed at identifying candidate intracranial aneurysm genes.

The elastin gene (ELN), encodes the elastin protein which is an important extracellular matrix protein and has been investigated to clarify its role in the formation of IAs. ELN is a single copy gene, localized in chromosome 7 in humans and under physiological conditions is expressed by various cell types during the prenatal and neonatal stages of development (10). It is known that mutation in the ELN results in several diseases such as supravalvular aortic stenosis, William's syndrome, cutis laxa, etc. (10,11). Regarding intracranial arteries elastin it is mainly confined to the internal elastic lamina. Due to the fact that defects in the internal elastic lamina have been proved in patients with IAs, ELN it has been suggested as causative gene in their pathogenesis (12,13). However the results of subsequent GAS and GWAS studies regarding ELN were contradictory (14,15,16,17). Two common polymorphisms (variants) of the ELN gene are the ELN INT20 1315T>C and EX20 1264G>A. The ELN INT20 1315T>C variant is located in intron 20 whereas the ELN EX20 1264G>A variant is located in exon 20 and is

a non-synonymous change which results in a change in the aminoacid chain, serine instead of glycine, with no known change in the protein function (18) These two polymorphisms are implicated with the development of IA (14,15) and have already been studied with different disorders (19)

The variant ELN EX20 1264G>A has been associated with increased risk for IA (14) whereas the variant INT20 1315>C has been linked to a decreased risk of IA (20). However, other functional polymorphisms in the ELN gene may also contribute to the risk of IA formation (21-24)

The genetic association studies (GAS) that investigated the association between IA and the ELN INT20 1315T>C and EX20 1264G>A variants have produced diverging results, partly because the studies had limited sample sizes and their power was not adequate to demonstrate significant association (20,25-28) In addition, the studies involved different populations and sampling strategies making the interpretation of results difficult.

In the present study, a meticulous meta-analysis [29] was performed for GAS related the ELN INT20 1315T>C and EX20 1264G>A variants with IA. The main purpose of the meta-analysis was to provide an estimated pooled genetic risk effect with decreased uncertainty. The consistency of genetic effects across traditionally defined ethnicities was also examined.

2. Material and Methods

2.1. Identification and eligibility of relevant studies

GAS that investigated the association of the ELN INT20 1315T>C or EX20 1264G>A genetic variants with the risk of developing IA published in English before August 2015 were considered in the meta-analysis. The studies were identified by systematically searching the PubMed database and the HuGE Navigator. The following search criterion was used: (“ELN” or “elastin gene’) and (“intracranial aneurysm” or “subarachnoid hemorrhage” or “intracranial hemorrhage” or “intracerebral hemorrhage”) . Then, the abstracts were retrieved and screened to assess their appropriateness for inclusion in the meta-analysis. After the abstract screening, the articles were read in their entirety in order to assess their eligibility for the meta-analysis. Finally, all the references in the eligible articles were extensively reviewed to identify additional published articles not indexed by PubMed database or HuGE Navigator.

GAS provided the genotype distribution of INT20 1315T>C or EX20 1264G>A variants in subjects with IA and in healthy controls were eligible for inclusion in the meta-analysis. Family-based studies were not considered because of different design considerations.

2.2. Data extraction

From each study the following information was extracted: first author, journal, year of publication, ethnicity of study population, demographics and genotype distribution.

2.3. Data synthesis and analysis

The meta-analysis examined the association between each variant (INT20 1315T>C and EX20 1264G>A) and the risk of IA based on a genetic model-free approach based on the generalized odds ratio (ORG) [30]. The ORG provides an estimate of the overall risk effect by utilizing the complete genotype distribution. The ORG express the probability of a subject being with IA relative to probability of being free of disease, given that the subject with IA has a higher mutational load than the non-diseased (30).

In synthesizing the studies, the fixed effects (FE) and random effects (RE) ORG were estimated (29-31). The RE model incorporates in the estimates the between study variability [29]. The heterogeneity between studies was tested using the Q-statistic (32) and the heterogeneity was considered significant at PQ<0.10. The heterogeneity was also quantified using the I2 metric, which takes values between 0% and 100% with higher values denoting greater degree of heterogeneity (33). When the heterogeneity is not significant the RE model coincides with the FE model. In testing for potential bias (publication bias), the differential magnitude of effect in large versus small studies for the allele contrast was tested using the Harbord's test (34)

The meta-analysis consisted of the overall analysis, which includes all available data and subgroup analysis by ethnicity ("racial" descent). A sensitivity analysis, which examines the effect of excluding specific studies, was also considered (29). The distribution of the genotypes in the control group was tested for conforming to the Hardy-Weinberg equilibrium (HWE) rule using an exact test (29). Deviation from HWE indicates possible genotyping errors and/or population stratification and studies with controls deviated from HWE were subjected to a sensitivity analysis [19]. The analysis was performed using the software ORGGASMA (http://biomath.med.uth.gr) (30).

3. Results

3.1. Eligible studies and studies' characteristics

The literature review identified 27 articles in PubMed that met the search criteria. The articles identified in HuGE Navigator were already traced in PubMed Data. One more article was retrieved from the references of the eligible articles Five articles met the inclusion criteria. Figure 1 presents a flow chart of the retrieved and excluded studies with specification of reasons.

The characteristics of the individual studies included in the meta-analysis are provided in Table 1. One study dealt with the variant EX20 1264G>A and 1 with the variant INT20 1315T>C; three studies dealt with both variants. The studies provided 828/964 cases/controls for EX20 1264G>A and 920/997 cases/controls for INT20 1315T>C. The studies were published between 2003 and 2013. The studies used validated genotyping methods for the determination of the genetic variants. Studies were conducted in various populations of different ethnicity: four involved Whites and one East Asians. In all studies, for both variants, the controls conform with the HWE rule ($P \geq 0.05$).

3.3. Meta-analysis results

Figure 2 and Table 2 show the meta-analysis results for the association between the two variants of the *ELN* gene and the risk of developing IA. Regarding the variant EX20 1264G>A, the overall analysis yielded a large and significant heterogeneity between studies ($I2=91\%$, $PQ<0.01$) and non-significant association between the variant and the development of IA: RE ORG=1.41 (0.75, 2.63). However, if the heterogeneity between studies is ignored the association become significant: FE ORG=1.72 (1.44, 1.06); though, this finding can be used to draw inferences. In subgroup analysis according to ethnicity, a non-significant association was showed for Whites [FE OR=1.07 (0.84-1.37)] with the heterogeneity being non-significant ($I2=0\%$, $PQ=0.82$). However, the one study in East Asians, produced a significant association [OR=3.10 (2.36-4.06)].

For the INT20 1315T>C variant, the overall analysis produced significant heterogeneity ($I2=72\%$, $PQ=0.01$) and significant association between the variant and the development of IA [RE ORG=0.66 (0.45, 0.95)]. The interpretation of the finding is as follows: for any two subjects, diseased with IA and healthy, the probability of being diseased is 34% lower (relative to the probability of being non-diseased) given that the diseased subject

has higher mutational load for the variant INT20 1315T>C than the healthy one. Thus, an increased genetic exposure (mutational load) implies protection disease. Of course, in the analysis, it was assumed that subjects who are homozygous for C allele were considered to have the highest mutational load, those homozygous for T allele to have the lowest, and heterozygous to have an intermediate load. However, when the analysis was restricted to Whites, the analysis produced non-significant association [FE OR=1.07 (0.84-1.37)] with the heterogeneity being non-significant (I2=0%, PQ=0.84). Thus, the significant result of the overall analysis was attributable to the one study in East Asian population (ORG=0.44 (0.35-0.56).

In testing for potential bias, the test by Harbord et al. (34) for the variant EX20 1264G>A indicated that there is differential magnitude of effect in large versus small studies (P=0.02) whereas for the variant INT20 1315T>C the differential magnitude of effect in large versus small studies was not significant (P=0.12).

4. Discussion

Herein, we presented a comprehensive meta-analysis of GAS investigated the association between two variants in the *ELN* gene and IA susceptibility. The purpose of the meta-analysis was to provide an estimate of the genetic risk effect with a reduced uncertainty and to explore the trend of risk effect as evidence accumulates. The individual GAS included in the meta-analysis have relatively small sample sizes to detect the minor contribution of the mutant alleles in IA susceptibility. Typically, for a modest significant risk effect (ORG around 1.3) to be detected in single GAS, a sample size of more than 10,000 subjects is needed to achieve power >80% (35). The meta-analysis has the advantage by synthesizing data from published GAS to provide greater power to detect significant associations than an individual GAS, especially in the absence of large heterogeneity between studies (29). However, there is no established methodology for calculating the power of the meta-analysis. In addition, meta-analysis is a retrospective all inclusive synthesis of published studies and power analysis may not be applicable (29). Nevertheless, type II errors are expected to be less in a meta-analysis than in the individual GAS (29).

The associations were assessed based on the ORG. The current practice is to analyze various genetic models, such as the dominant, recessive, additive and co-dominant models; however, these models are not independent (30). In addition, there is no a priori biological justification for choice of a specific genetic model. The ORG is a metric that

quantifies the probability of disease, given that a diseased subject has a higher, or lower, mutational load than a healthy one. The use of *ORG* is a genetic-model free approach and provides an integrated way to evaluate genetic associations, by exploiting all available information in terms of disease and genotype distribution [22]. Thus, the application of ORG in analysis and meta-analysis of individual GAS may overcome the drawbacks of multiple model testing or erroneous model specification (30).

From the results of this analysis becomes evident that EX20 1264G>A variant correlates with an increased risk for IA (RE=1,41, FE=1.72) whereas INT20 1315T>C shows a protective effect for IA (RE=0.66) . However, the heterogeneity between studies is large and therefore, the findings should be interpret with caution. A lack of consistency of genetic effects across Whites and East Asians was shown indicating that significant association is restricted only to East Asians. Although this, a consistency of genetic effects does not necessarily means that "race"-specific genetic effects are exactly the same (35).

Although the meta-analysis produced non-significant associations, it still may provide useful information for the uncertainty of the estimated genetic risk. For example, regarding the association between *INT20 1315T>C* and the risk of IA, the ORG excluded with 95% certainty subjects with high mutational load would have more, or less, than 5% increased, or reduced, risk of IA.

The meta-analysis indicated the existence of differential magnitude of effect in large versus small studies ("publication bias"); however, inspection of funnel plot was avoided since its interpretation can be misleading (36). Nevertheless, the conclusions reached in the present meta-analysis were based on relatively small numbers of studies and participants and thus, any inferences have to be cautious.

The meta-analysis was based on unadjusted ORGs and possible effect modifiers (e.g. demographics and other clinical characteristics) may influence the estimates of associations. Also, sampling variability and stratification in GAS might also be a possible modifier on the effect of variants. Although, the cases and controls in each GAS were well defined, they unavoidably cover a wide spectrum of disease and demographic characteristics.

The lack of strong association between the *ELN* variants and IA might be due to other unidentified functional variants that exist in the ELN/LIMK1 pathway (21) and the ELN gene that are in linkage disequilibrium, and therefore, they may affect the susceptibility to IA. The two investigated variants are in a weak linkage disequilibrium (22,28). Moreover, the development of IA might be associated with gene-gene-

environment interactions and their effects should be considered in individual GAS and subsequent meta-analyses (37). Currently, limited information is provided by the individual GAS, and the available study-level genotype distribution was used in the meta-analysis, precluding the adjusted analysis for potential gene–gene–environment interactions (38). Thus, failure to account for these interactions may have reduced the efficiency of the genetic risk estimates, but is unlikely to inflate false-positive results.

In conclusion, intracranial aneurysms are cerebrovascular diseases that can cause disastrous subarachnoid hemorrhages. Several linkage regions and candidate genes showing association have been reported. Possibly the disease is caused by an interaction of environmental factors and multiple gene disorders, localized in more than one loci. Identification of predisposing genes would allow, instead of phenotyping an aneurysm after its rupture, to predict patients harboring or prone to develop it, thus implying the necessary measures in order to decrease the chance of a catastrophic SAH. Additional GWAS and GAS may narrow down the responsible genetic factors and may help in the understanding of the pathomechanism leading to an aneurysm formation, possibly allowing in the future to interact with these resulting in a blockage of the pathway.

ΠΕΡΙΛΗΨΗ

ΠΟΛΥΜΟΡΦΙΣΜΟΙ ΤΟΥ ΓΟΝΙΔΙΟΥ ΤΗΣ ΕΛΑΣΤΙΝΗΣ ΚΑΙ Η ΣΥΣΧΕΤΙΣΗ ΤΟΥΣ ΜΕ ΤΗΝ ΠΑΡΟΥΣΙΑ ΑΝΕΥΡΥΣΜΑΤΩΝ: ΜΙΑ ΜΕΛΕΤΗ ΓΕΝΕΤΙΚΗΣ ΣΥΣΧΕΤΙΣΗΣ ΜΕ ΤΗΝ ΕΦΑΡΜΟΓΗ ΜΟΝΤΕΛΛΟΥ ΠΟΥ ΔΕΝ ΛΑΜΒΑΝΕΙ ΥΠΟΨΗ ΤΗ ΓΕΝΕΤΙΚΗ ΚΛΗΡΟΝΟΜΙΚΟΤΗΤΑ

Η αυτόματη υπαραχνοειδής αιμορραγία εγκεφάλου αποτελεί μια από τις πλέον καταστροφικές παθήσεις με υψηλά ποσοστά θνητότητας και νοσηρότητας. Η ανάπτυξη και ρήξη ενός ενδοκρανίου ανευρύσματος θεωρείται ότι οφείλεται στην αλληλεπίδραση περιβαλλοντικών και γενετικών παραγόντων. Διάφορες μελέτες γενετικής συσχέτισης και συσχέτισης ολόκληρου του γονιδιώματος έχουν αναδείξει το γονίδιο της ελαστίνης σαν ένα πιθανό γονίδιο για την ανάπτυξη ενδοκράνιων ανευρυσμάτων. Δύο από τους πολυμορφισμούς του γονιδίου, οι EX201264G>A και INT201315T>C έχουν μελετηθεί και συσχετισθεί με την ανάπτυξη ενδοκρανίων ανευρυσμάτων. Ωστόσο, τα αποτελέσματα είναι αντικρουόμενα και δεν μπορούν να εξαχθούν ασφαλή συμπεράσματα. Προκειμένου να μελετήσουμε αυτή την αντίθεση, πραγματοποιήσαμε μια μετα-ανάλυση χρησιμοποιώντας μια προσέγγιση ελεύθερη γενετικής κληρονομικότητας με την εφαρμογή του λογισμικού ORGGASMA.

Η αναζήτηση έγινε στις ηλεκτρονικές βάσεις δεδομένων PUBMED, και HUGE Navigator μέχρι τον Αύγουστο του 2015. Αναλύθηκαν τα δεδομένα από 5 μελέτες που πληρούσαν τα κριτήρια αναζήτησης και στις οποίες συμπεριλαμβάνονταν 828 και 964 ασθενείς και υγιείς αντίστοιχα για το EX20 και 920 ασθενείς και 997 υγιείς για το INT20. Από τα αποτελέσματα της μελέτης κατέστη εμφανές ότι ο πολυμορφισμός EX20 1264G>A συσχετίζεται με αυξημένο κίνδυνο παρουσίας ενδοκρανίου ανευρύσματος (RE=1.44, FE=1.72). Αντίθετα το INT 20 1315T>G φαίνεται να έχει προστατευτική δράση (RE=0.66). Παρόλα αυτά, λόγω της μεγάλη ετερογένειας μεταξύ των μελετών, τα συμπεράσματα της παρούσας σύνθεσης, θα πρέπει να εκτιμηθούν με αρκετή επιφύλαξη. Επιπλέον μελέτες γενετικής συσχέτισης και συσχέτισης ολοκλήρου του γονιδιώματος, απαιτούνται προκειμένου να ταυτοποιήσουμε τους υπεύθυνους γενετικούς παράγοντες, καθώς και την αλληλεπίδραση μεταξύ τους, με στόχο την καλύτερη κατανόηση των παθολογικών μηχανισμών που οδηγούν στον σχηματισμό ενδοκρανίου ανευρύσματος.

Table 1 Characteristics of the studies considered in the meta-analysis.

Author	**year**	**gene variant**	**ethnicity**
Yang S et al.	2013	EX20 1264G>A and INT20 1315T>C	East Asian
Kaushal R et al.	2007	EX20 1264G>A	Caucasian
Ruigrok et al.	2004	EX20 1264G>A and INT20 1315T>C	Caucasian
Krex S et al.	2004	EX20 1264G>A and INT20 1315T>C	Caucasian
Hofer A et al	2003	INT20 1315T>C	Caucasian

Table 2. Heterogeneity results (I2, PQ-value) for the generalized odds ratio (ORG) of ELN INT20 1315T>C and EX20 1264G>A variants for testing the association with IA.

INT20 1315T>C

Study	ORG	95% LL	95% UL
1	0.4405946	0.3450351	0.5626200
2	0.7328733	0.4584610	1.171535
3	0.7460962	0.4263636	1.305598
4	0.8672775	0.5858628	1.283868

META-ANALYSIS RESULTS

Heterogeneity metrics

Q= 10.63015 P-value for Q= 1.3904035E-02

I^2(%)= 71.77838

Fixed Effects model

ORG= 0.5778065

95% Lower Limit= 0.4827697

95% Upper Limit= 0.6915521

Random Effects model

ORG= 0.6553569

95% Lower Limit= 0.4505785

95% Upper Limit= 0.9532029

EX20 1264G>A

Study	ORG	95% LL	95% UL
1	3.096566	2.363619	4.056797
2	1.006971	0.6464960	1.568440
3	1.179341	0.8046076	1.728600
4	1.005245	0.6433682	1.570667

META-ANALYSIS RESULTS

Heterogeneity metrics

Q= 33.11768 P-value for Q= 2.9802322E-07

I^2(%)= 90.94139

Fixed Effects model

ORG= 1.723087

95% Lower Limit= 1.438387

95% Upper Limit= 2.064138

Random Effects model

ORG= 1.407709

95% Lower Limit= 0.7533646

95% Upper Limit= 2.630392

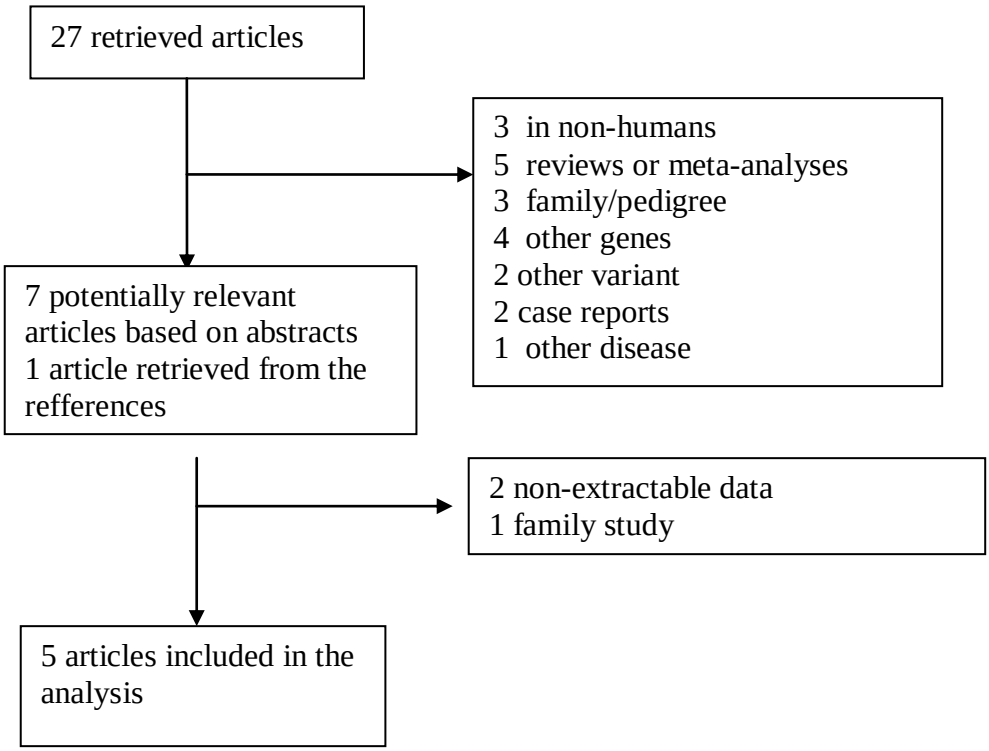
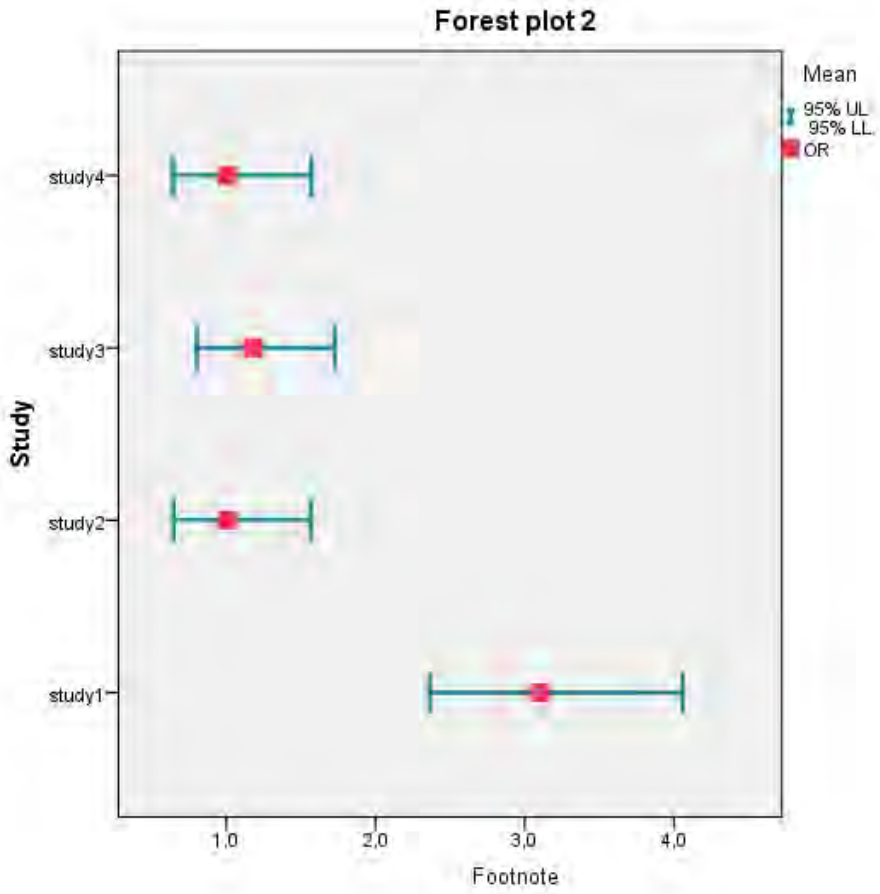


Figure 1 Flow chart of retrieved studies and studies excluded, with specification of reasons.

Figure 2. Random effects (RE) generalized odds ratio (ORG) estimates with the corresponding 95% confidence interval (CI) for the (a) ELN INT20 1315T>C and (b)ELN EX20 1264G>A variants and the risk of IA.

(a



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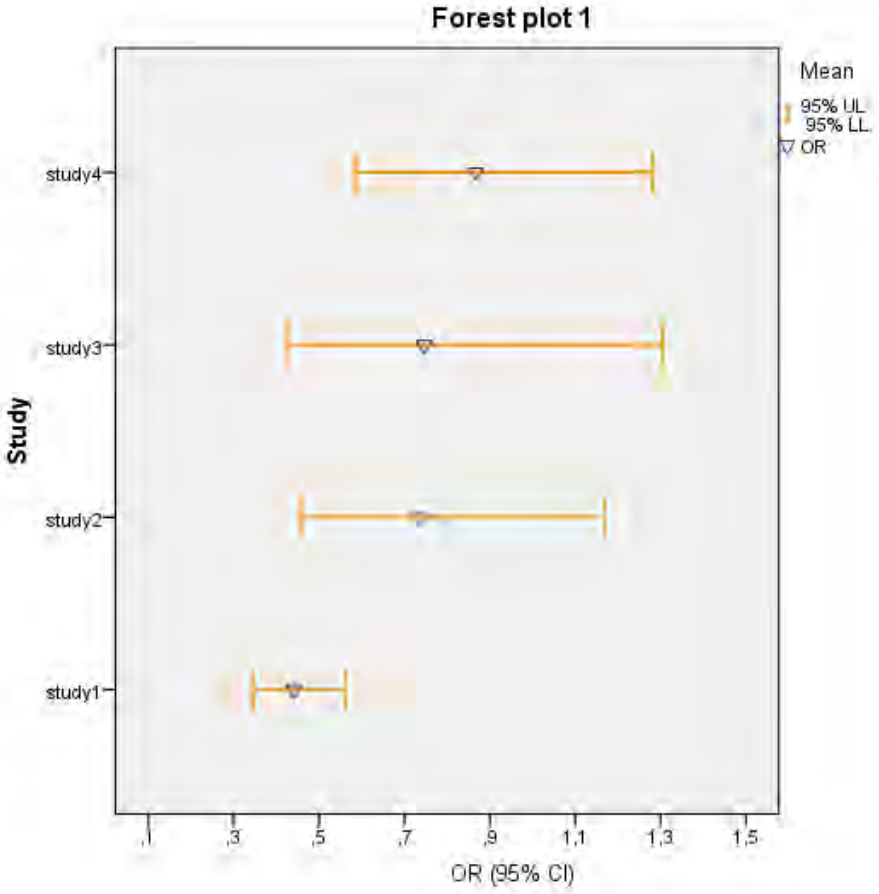

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