Original Article

PNPLA3 as a Genetic Determinant of Risk for and Severity of Non-alcoholic Fatty Liver Disease Spectrum

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Abstract

Background and Aims: *Patatin-like phospholipase domain protein 3* (*PNPLA3*) polymorphisms (*rs738409* C>G) are associated with non-alcoholic fatty liver disease (NAFLD). We performed a systematic review and meta-analysis to examine the association of *PNPLA3* polymorphisms with the spectrum and severity of this disease. ***Methods:*** Studies evaluating the association between the *PNPLA3* polymorphism spectrum (fatty liver, steatohepatitis, cirrhosis, and hepatocellular carcinoma) and NAFLD were included. Pooled data are reported as odds ratios (ORs) with 95% confidence intervals. ***Results:*** Of 393 potentially relevant studies, 35 on NAFLD were included in the analysis. Compared to healthy controls, the pooled ORs for *rs738409* CG and GG compared to CC among patients with non-alcoholic fatty liver (NAFL) were 1.46 (1.16–1.85) and 2.76 (2.30–3.13), and were 1.75 (1.24–2.46) and 4.44 (2.92–6.76) among patients with non-alcoholic steatohepatitis respectively. The respective ORs for CG and GG compared to the CC genotype were 2.35 (0.90–6.13) and 5.05 (1.47–17.29) when comparing non-alcoholic hepatocellular carcinoma to NAFL patients. Among the NAFLD patients, the ORs for G allele frequency when comparing steatosis grade 2–3 to grade 0–1 NAFL, when comparing the NAFLD activity score of ≥ 4 to score ≤ 3, when comparing NASH to NAFLD, when comparing the presence of lobular inflammation to absence, and when comparing the presence of hepatocyte ballooning to absence were 2.33 (1.43–3.80), 1.80 (1.36–2.37), 1.66 (1.42–1.94), 1.58 (1.19–2.10), and 2.63 (1.87–3.69) respectively. Subgroup analysis based on ethnicity showed similar results. ***Conclusions:*** *PNPLA3* polymorphisms have strong association with the risk for and severity of NAFLDs. *PNPLA3* polymorphism plays an evolving role in diagnosis and treatment decisions in patients with NAFLD.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of liver cirrhosis and end-stage liver disease in the US, behind hepatitis C virus infection and alcoholic liver disease (ALD).[1,2] Within the past few years, multiple genetic factors have been associated with the severity and predisposition to the spectrum of NAFLDs, including the associated increased risk of hepatocellular carcinoma (HCC).[3] Recently, the single nucleotide polymorphism *rs738409 C>G* (causing an isoleucine to methionine substitution at position 148, I148M) in the *patatin-like phospholipase domain containing protein 3 (PNPLA3)* gene has been shown to be associated with steatosis among NAFLD patients.[3] Since then, other genetic polymorphisms of this gene have been shown to be associated with various other liver diseases, including ALD.[4] *In vitro*, the human adiponutrin (another name for the *PNPLA3* gene product) exhibits activity as a triglyceride hydrolase and transacylase, as well as modest activity as a calcium-independent phospholipase A2. However, in the cellular context, these activities appear minor; similarly, overexpression or knockdown of adiponutrin did not affect lipolysis or the cellular TG level.[5,6]

We have shown previously that *PNPLA3* is associated with ALD and its severity spectrum (including fatty liver (FL), steatohepatitis, cirrhosis, and HCC).[4] ALD and NAFLD share several histological features and pathophysiologic mechanisms, including oxidative and endoplasmic reticulum–mediated stress, gut derived endotoxin-mediated cytokine release, and mitochondrial defects.[7] Over the last 5 years, meta-analyses of pooled data on the associations between the *PNPLA3* gene and risk of NAFLD have been reported.[8–10] However, given that these previous meta-analyses were limited and did not examine the association of this genetic polymorphism with the entire spectrum of liver disease—from FL and steatohepatitis to fibrosis, cirrhosis, and HCC—amongst NAFLD patients, we performed the meta-analysis presented herein, with the aim of examining the association of *PNPLA3* polymorphisms

Keywords: *PNPLA3*; Polymorphisms; Non-alcoholic liver disease; *rs738409* C>G.
Abbreviations: ALD, alcoholic liver disease; BMI, body mass index; CRN, clinical research network; FL, fatty liver; HCC, hepatocellular carcinoma; HWE, Hardy-Weinberg equilibrium; ICAM-1, intercellular adhesion molecule 1; MOOSE, Meta-analysis Of Observational Studies in Epidemiology; NAC, non-alcoholic cirrhosis; NAHCC, non-alcoholic hepatocellular carcinoma; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; OR, odds ratio; PNPLA3, patatin-like phospholipase domain protein 3.
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with the predisposition to and severity of the whole spectrum of liver disease among patients with NAFLDs.

Methods

Identification and retrieval of primary studies

Following the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines, we searched the literature from the PubMed/Medline, Embase and Cochrane search engines to identify full-length articles written in English that examined *PNPLA3* polymorphism in NAFLD patients.[11] The initial search terms were "non-alcoholic fatty liver disease" and "adiponutrin, human". The search was then expanded to include the terms "*rs738409* C>G" and "patatin-like phospholipase domain-containing 3 protein". All databases were searched from their inception through April 2016. Meeting abstracts from major gastroenterology conferences within the past 4 years were also searched to identify studies that were potentially missed by our database searches. Articles were selected for full-text review based on initial reading of the title and abstract. Manual search of the retrieved publications was done by three independent investigators (HS, MM, and MA) to avoid missing articles and ensure as comprehensive search of the literature as possible.

Inclusion and exclusion criteria

Studies reporting association of the *PNPLA3* variant (*rs738409 C>G*) among NAFLD patients and published as full-length articles were eligible for inclusion in this analysis. Studies on patients with other liver diseases but reporting separate data on *PNPLA3* polymorphisms for NAFLD were also eligible for inclusion. However, studies without available gene frequency data for analysis and studies including subjects with other liver diseases and without separate data on NAFLD patients were excluded.

Definitions

The disease spectrum across studies on NAFLD patients was defined as non-alcoholic fatty liver (NAFL) according to: steatosis on liver ultrasound in the absence of elevated liver enzymes and/or histologic evidence of steatohepatitis or non-alcoholic steatohepatitis (NASH); steatosis with histologic evidence of steatohepatitis or non-alcoholic cirrhosis (NAC); biopsy or clinical evaluation supported by hematological, biochemical, and/or radiologic imaging findings, and presence of non-alcoholic hepatocellular carcinoma (NAHCC); diagnostic imaging findings on triple0phase magnetic resonance imaging or computed tomography or using histological confirmation from the liver tissue. Healthy controls were defined as subjects without liver disease, for the whole spectrum of NAFLD.

Study selection and data extraction

After determining the eligibility for inclusion, three reviewers (HS, MM, and MA) independently extracted data for (a) study characteristics (author and year of publication, and study design (population-based or not, using controls or not)), (b) study population (liver disease spectrum and the sample size), (c) frequencies of *PNPLA3* polymorphism genotypes (*rs738409* CC, CG, and GG), and (d) odds ratio (OR; for association of *PNPLA3* polymorphism and the spectrum of liver disease and for severity of liver disease). Any discrepancy between the reviewers was resolved by jointly reviewing the study in question. Among studies comparing a diseased population with healthy controls, similar data was also extracted for the healthy controls.

Endpoints and outcomes

Our study endpoints for analysis on the data from included NAFLD studies were risks of (a) entire spectrum of liver disease compared to healthy controls and (b) severity of liver disease spectrum among the NAFLD diseased population.

Assessment of study quality

The quality of included studies was assessed independently by three authors (HS, MM, and MA) using the Newcastle-Ottawa Quality Assessment Scale for case-control studies.[12] This scale has one instrument for assessing case–control studies and another one for cohort studies. Each of these instruments includes measures of quality in selection, comparability, and exposure domains. While one point is granted for each of the areas measured within the selection and exposure domains, a maximum of two points can be assigned within the comparability domain with highest possible total score of nine. A score of seven or greater denotes a high-quality study. Any discrepancies between the three coauthors were addressed by a joint reevaluation of the original article.[13]

Since deviation from Hardy-Weinberg equilibrium (HWE) in healthy controls has been associated with problems in the design and conduct of genetic association studies,[14,15] we examined the studies on the gene allele frequencies of healthy controls for any deviation from HWE. HWE analysis on gene frequencies among healthy controls was examined using the chi-square test, with the value of < 3.84 indicating allele frequency to be in HWE.

Statistical analysis

The strength of association between *rs738409 C>G* and NAFLD prevalence was expressed by OR and the corresponding 95% confidence interval (CI). Random effects modeling was used for analyzing the pooled data for all the analyses.[16] Heterogeneity was measured using the I^2 statistic for inter-study variance, with the chi-square test used for statistical analysis.[17] Heterogeneity was defined with $I^2 \geq 50\%$ or chi-square $p < 0.10$.[17] At least two studies are needed to examine and report heterogeneity; in our investigations of heterogeneous data and the source of heterogeneity, sensitivity analyses were performed in a stepwise fashion for (a) study quality, (b) excluding studies on the pediatric population for NAFLD analyses, and (c) excluding studies with highest and lowest OR. Publication bias was assessed using Egger's regression and the Begg and Mazumdar's rank correlation tests.[18–20] Egger's test is a regression method that evaluates the association between effect sizes and standard error and uses actual effect size for each study.[20] Begg and Mazumdar's test is a rank correlation test that evaluates the association between effect estimates (taken as a rank and not exact effect size) and sampling variance or standard error.[19] At least three studies are needed for examining and reporting publication bias; in our analyses of publication bias, the analyses were

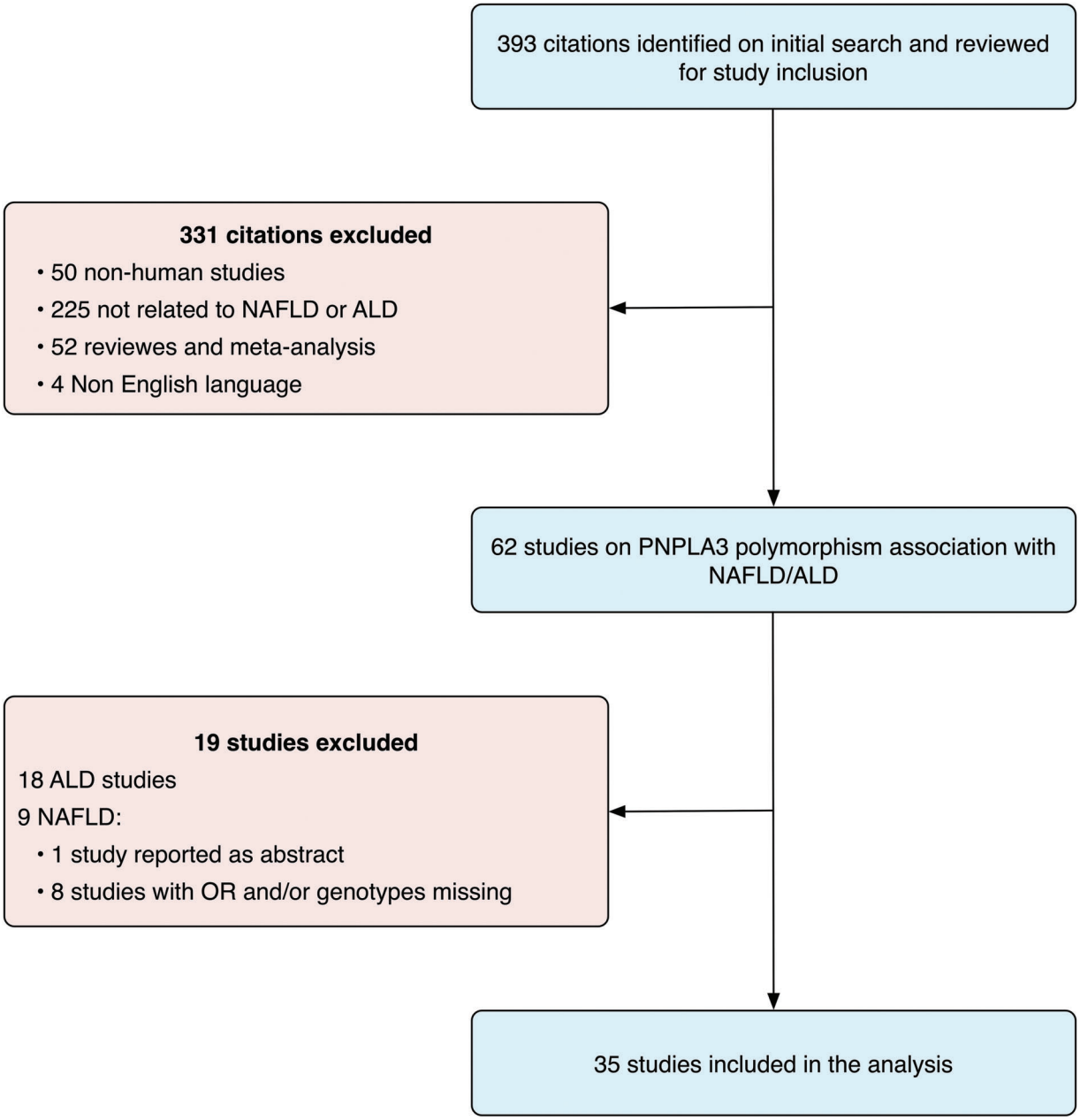

Fig. 1. Literature search and study inclusion.

repeated using the Duval and Tweedie trim-and-fill method, a nonparametric (rank-based) data augmentation technique.[21] Most extreme results on one side of the funnel plot are suppressed and this tool used to estimate the number of studies missing from a meta-analysis. Subsequently, it augments the observed data so that the funnel plot is more symmetric and re-computes the summary estimate based on the complete data.[22] This method should be regarded as a way of examining the sensitivity of the results to one particular selection mechanism rather than a way of yielding a more "valid" estimate of the overall effect or outcome. To examine the effect of ethnicity on the association of *rs738409* with NAFLD, subgroup analysis was performed by grouping studies with similar populations. All statistical analyses were performed using R software (Foundation for Statistical Computing, Stanford, CA, USA) and its associated metaphor package, or Comprehensive Meta-analysis software (Biostat, Englewood, NJ, USA).

Results

Baseline study characteristics

Of the 393 citations retrieved by the initial search, 462 were reviewed with full-text reading (Fig. 1). Among these, 27 studies were excluded for including patients with ALD (n = 18) and

Table 1. Baseline characteristics of patients from studies included in this analysis

Author (Yr)[Ref]	Study design	Controls, n	Cases								
			n	Ethnicity	% Males	Mean age	Mean BMI	*rs738409* genotype count, CC:CG:GG			
								NAFL	NASH	NAC	NAHCC
Kantartzis (2009)[31]	P#	50	330	C	39	45	29.51	188:111:31	–	–	–
Sookoian (2009)[32]	P#	94	103	Mixed	28	55	32.5	10:18:12	8:22:33	–	–
Goran (2010)[36]Ped	P	117	71	H	46	14	30.3	26:30:15	–	–	–
Hotta (2010)[40]	P#	578	253	A	48	52	27.7	19:26:19	26:85:78	–	–
Petit (2010)[33]	P	79	139	NR	47	62	34.9	70.4%:29.6%*	–	–	–
Rotman (2010)[35]	P#	336	894	Mixed	38	48	34.4	48.2%:51.8%*	50.8%:49.2%*	–	–
Speliotes (2010)[37]	P	1405	592	C	37	NR	NR	–	50%:50%*	–	–
Valenti (2010)[34]	P#	179	IC: 253	C	70	46	30.5	103:114:36	44:55:23	–	–
			UK&IC: 321		62	50	34	142:140:39	65:83:29	–	–
Valenti (2010)[38] Ped	P	NA	149	C	62	10	NR	65:61:23	2:45:23	20:29:17	–
Lin (2011)[41] Ped	P#	418	102	A	67	11	26.1	24:52:26	–	–	–
Wagenknecht (2011)[55]	P#	NA	843	H	37	43	29	324:367:152	–	–	–
			371	AA	38		30.1	248:111:12	–	–	–
Wang (2011)[39]	P#	723	156	Mixed	60	48	26.9	40:80:36	–	–	–
Kawaguchi (2012)[43]	P#	932	527	A	47	55	27.3	49:100:52	39:136:151	–	–
Li (2012)[46]	P#	202	203	A	51	47	26.6	67:85:51	–	–	–
Peng (2012)[42]	P#	553	552	A	72	45	25.4	183:276:93	–	–	–
Petta (2012)[44]	P#	NA	160	C	68	46	29.7	20:41 **	27:72 **	–	–
Zain (2012)[45]	P	198	144	A	53	51	28.7	65%:35%*	52%:48%*	–	–
Kitamoto (2013)[47]	P	1,012	564	A	52	50	27.8	96:241:227	72:183:187	–	–
Verrijken (2013)[54]	P#	NA	287	C	30	44	37.6	164:106:17	66:69:16	–	–
Guichelaar (2014)[49]	P	12	132	NR	15	48	46.6	43:13:04	36:28:08	–	–
Islek (2014)[48]	P#	303	80	C	51	NR	NR	–	23:43:14	–	–
Kanth (2014)[51]	P#	150	156	A	89	37	27	60%:40%*	–	–	–
Lin (2014)[52] Ped	P#	606	191	A	70	12	NR	58:95:38	–	–	–
Liu (2014)[53]	P	NA	375	C	57	56	33.8	125:117:33	–	–	028:043:029
Baclig (2014)[72]	P#	36	32	A	50	NA	28.5	14:18 **	–	–	–
Lee (2014)[73]	P#	184	155	NA	65.2	44.7	27.5	20:48:31	–	–	–
Park (2015)[74]	P#	NA	1363	A	100	49	24.4	172:293:137	–	–	–
Wei (2015)[75]	P#	NA	911	A	41.9	48	NA	359:550 **	–	–	–
Xia (2015)[76]	P#	2915	1385	A	38.40	62	25.9	486:684:215			

(*continued*)

Table 1. (*continued*)

Author (Yr)[Ref]	Study design	Controls, n	Cases n	Ethnicity	% Males	Mean age	Mean BMI	*rs738409* genotype count, CC:CG:GG NAFL	NASH	NAC	NAHCC
Oniki (2015)[77]	P#	472	119	A	69.70	61.2	25.6	23:69:27	–	–	–
Nishioji (2015)[78]	P#	NA	824	A	66.5	NA	NA	–	–	–	–
Akuta (2015)[56]	P#	NA	211	A	57.8	52	25.9	21:60:59	–	–	–
Tai (2015)[79]	P#	29	152	A	32	30.2	41.8	31:24:05	35:36:21	–	–
Tai (2016)[80]	P#	NA	177	A	38	29.8	42.7	77:73:27			
Honda (2016)[81] non-obese	P#	782	134	A	38.4	5.51	25.9	23:47:64			
Honda (2016)[81] - obese	P#	230	406	A	38.4	5.51	25.9	78:180:148			
Summary	–	12,595	13,817	–	51.85	53.2	28.9	–			

$ Genotype counts were reported as ratios (CC wild genotype: CG heterozygote genotype: GG homozygote genotype) or * as allele frequency percentage (C allele%: G allele%) or ** as ratios (CC wild genotype: CG and GG genotypes).

Abbreviations: A, Asian; AA, African American; BMI, body mass index; C, Caucasian; H, Hispanic; IC, Italian cohort; NA, not applicable; NAC, non-alcoholic cirrhosis; NAHCC, non-alcoholic hepatocellular carcinoma; NAFLD, non-alcoholic liver disease; NASH, non-alcoholic steatohepatitis; NR, not reported; P, prospective; Ped, pediatric studies; R, retrospective; P#, population-based studies; UK&I, United Kingdom and Italian.

other reasons (n = 9),[3,23–30] including 5 studies due to unavailability of needed data for analysis and 4 studies published as abstracts. The included 35 studies encompassed a total of 13,817 patients (mean age of 52 yrs, 53% male, mean body mass index (BMI) of 29) and reported on *PNPLA3* polymorphisms data in NAFLD (Table 1).[31–55] Based on the Newcastle-Ottawa scale, 23 NAFLD studies were of high quality, with study quality score of 7 or more, and the remaining 12 studies were of poor quality (Table 2). Twenty-five studies had control populations.[31–37,39–43,45–52] Genotype frequency (to perform HWE analysis in healthy controls) was available for only 19 of the studies (Table 3); among these, the control population in only one study deviated from HWE.[31]

PNPLA3 polymorphisms in NAFLD compared to healthy controls

NAFL

Among the 26 included studies on 10,236 NAFLD patients, the pooled ORs for *rs738409 C*> -CG and GG- genotypes compared to the CC genotype in NAFL patients were 1.46 (1.16–1.85) and 2.76 (2.30–3.13) respectively (Fig. 2A, 2B). The data were heterogeneous for the CG analysis but not for the GG analysis, with respective values of I^2 = 67.4%, $p < 0.0001$ and I^2 = 27.9%, $p = 0.16$. No publication bias was found by Egger's test ($p = 0.14$ and 0.28) or Begg and Mazumdar's test ($p = 0.63$ and 0.74). The pooled OR for G allele frequency for NAFL compared to healthy controls was 1.91 (1.64–2.21) (Fig. 2C), with heterogeneous data (I^2 = 72.6%, $p < 0.0001$) and publication bias (by Egger's test, $p = 0.0023$; by Begg and Mazumdar's test, $p = 0.009$). Sensitivity analysis using the trim-and-fill method showed homogeneous data, with similar effect size of 1.65 (1.55–1.77).

Among studies of Asian populations, the pooled ORs for the CG (8 studies with 2,610 patients) and GG (10 studies with 2,748 patients) genotypes as compared to the CC genotype were 1.50 (1.13–2.00) and 2.67 (2.16–3.31) respectively. The data were heterogeneous for the CG analysis (I^2 = 70%, $p = 0.001$) and homogeneous for the GG analysis (I^2 = 32%, $p = 0.15$). No publication bias was found by Egger's test ($p = 0.17$ and 0.59) or Begg and Mazumdar's test ($p = 0.27$ and 1.0). Similarly, among studies of Caucasian populations, the pooled ORs for the CG (2 studies with 904 patients) and GG (2 studies with 904 patients) genotypes as compared to the CC genotype were 1.29 (0.29–3.49) and 3.51 (2.00–6.14) respectively. The data were heterogeneous for both analyses (I^2 = 84%, $p = 0.01$ and I^2 = 89%, $p < 0.0001$); however, publication bias could not be assessed, as there were only two studies in each analysis. The pooled OR for the G allele frequency in 12 Asian studies with 5,319 patients was 1.78 (1.52–2.08), with heterogeneous data (I^2 = 70, $p < 0.0001$) and publication bias as assessed by Egger's test ($p = 0.04$) but not by Begg and Mazumdar's test ($p = 0.12$). Other ethnicities (Caucasians, Blacks, and Hispanics) were reported in one study each (Table 4). Combined analysis on the non-Asian population for the G allele frequency was 3.29 (1.89–5.54), with homogenous data (I^2 = 51, $p = 0.13$) and no publication bias (Egger's test, $p = 0.37$; Begg and Mazumdar's test, $p = 0.5$).

Table 2. Newcastle-Ottawa scale quality score of the included studies

	Selection				Comparability	Exposure			
Author (year)[Ref]	Independent validation	Case representation	Controls selection	Controls definition	Case and control design/ analysis	Ascertainment of exposure	Same method of ascertainment	Same response rate	Total
Kantartzis (2009)[31]	*			*	**	*	*	*	**7**
Sookoian (2009)[32]	*	*	*	*	**	*	*		8
Petit (2010)[33]	*	*		*	*	*	*		6
Valenti (2010)[34]	*	*	*	*	**	*	*		8
Rotman (2010)[35]	*		*	*	**	*	*		7
Goran (2010)[36]	*	*	*		**	*	*		7
Speliotes (2010)[37]	*	*	*		**	*	*		7
Valenti (2010)[38]	*	*			*	*	*		5
Hotta (2010)[40]	*	*	*	*	**	*	*		8
Wang (2011)[39]	*	*	*		**	*	*		7
Lin (2011)[41]	*	*	*	*	**	*	*		8
Wagenknecht (2011)[55]	*	*			**	*			5
Peng (2012)[42]	*	*	*	*	**	*	*		8
Kawaguchi (2012)[43]	*	*	*	*	**	*	*		8
Petta (2012)[44]	*	*			*	*			4
Zain (2012)[45]	*	*	*	*	**	*	*		8
Li (2012)[46]	*		*	*	**	*	*		7
Kitamoto (2013)[47]	*		*	*	**	*	*		7
Verrijken (2013)[54]	*	*			**	*			5
Islek (2014)[48]	*		*	*		*	*		5
Guichelaar (2014)[49]	*	*			**	*			5
Tai (2014/2015)[50]	*		*	*	**	*	*		7
Kanth (2014)[51]	*		*	*	**	*	*		7
Lin (2014)[52]	*	*	*	*	**	*	*		8
Liu (2014)[53]	*	*	*	*	**	*	*		8
Baclig (2014)[72]	*	*	*	*	**	*	*		**8**
Lee (2014)[73]	*	*	*	*	**	*	*		8
Park(2015)[74]	*	*			**	*	*		6
Wei (2015)[75]	*	*			**	*	*		**6**
Xia (2015)[76]	*	*	*	*	**		*		7

(*continued*)

Table 2. *(continued)*

Author (year)[Ref]	Selection				Comparability	Exposure			
	Independent validation	Case representation	Controls selection	Controls definition	Case and control design/ analysis	Ascertainment of exposure	Same method of ascertainment	Same response rate	Total
Oniki (2015)[77]	*	*	*	*	**	*	*		8
Nishioji (2015)[78]	*	*			**	*	*		6
Akuta (2015)[56]	*	*				*	*		4
Tai (2016)[80]	*	*			**	*	*		6
Honda (2016)[81]	*	*	*	*	**	*	*	*	9

NASH

For the two studies on 353 NAFLD patients, the pooled ORs for the *rs738409 C>G* -CG and GG- genotypes compared to the CC genotype for presence of NASH were 1.75 (1.24–2.46) and 4.44 (2.92–6.76) respectively (Fig. 3A, 3B). The data were very robust for both analyses, with respective values of I^2 = 0%, p = 0.55 and I^2 = 0%, p = 0.38. The Pooled OR for the G allele frequency from five studies with 1,596 NASH patients was 2.54 (2.03–3.16) (Fig. 3C). The data were heterogeneous (I^2 = 67%, p = 0.019) and there was no publication bias (Egger's test, p = 0.56; Begg and Mazumdar's test, p = 0.5). Ethnicity-based analysis among the 3 Asian studies encompassing 924 patients showed similar effect size of 2.25 (1.94–2.61), with homogeneous data (I^2 = 0%, p = 0.73). In additional, two Caucasian studies encompassing 672 patients showed an effect size was 3.20 (2.72–3.75), with homogeneous data (I^2 = 0%, p = 0.45) (Table 4).

Association of PNPLA3 polymorphisms with the severity of NAFLD

Steatosis grade 2–3 vs. 0–1

The ORs of the *rs738409 C>G* -CG and GG- genotypes compared to the CC genotype for NAFL grade 2–3 vs. grade 0–1 were 1.17 (0.35–3.92) and 2.15 (0.96–4.84) respectively (Fig. 4A, 4B). The data for the GG analysis were heterogeneous (I^2 = 67.8%, p < 0.045) and there was no publication bias (Egger's test, p = 0.61; Begg and Mazumdar's test, p = 1.0). After excluding the study with extremely high OR and wide CI effect size, the results remained similar, with OR of 1.64 (1.10–2.46) and homogenous data (I^2 = 8.9%, p = 0.30). The OR for the G allele frequency pooled from six studies encompassing 2,521 patients was 2.33 (1.43–3.80) (Fig. 4C), with heterogeneous data (I^2 = 86%, p < 0.0001) and evidence of publication bias by Egger's test (p = 0.025) but not by Begg and Mazumdar's test (p = 0.13). Ethnicity based analysis for four Caucasian studies encompassing 1,475 patients showed the OR for the G allele frequency to be 2.69 (1.27–5.69) (Table 4), with homogeneous data. However, after excluding one study on a pediatric population,[38] the pooled OR for the G allele frequency became 1.54 (1.19–1.98), with homogeneous data (I^2 = 48.9%, p = 0.1) and evidence of publication bias by the Egger's test (p = 0.019). Using the trim-and-fill method, the effect size remained unchanged at 2.33 (1.43–3.80), without any publication bias.

NASH in NAFLD

The pooled ORs for presence of NASH among NAFLD patients from four studies encompassing 1,146 patients for the CG and GG genotypes compared to the CC genotype were 1.92 (1.43–2.57) and 3.53 (2.02–6.15) respectively (Fig. 5A, 5B); the data were homogeneous, with respective values of I^2 = 0%, p = 0.51 and I^2 = 0%, p = 0.8. There was no publication bias for the CG analysis (Egger's test, p = 0.7; Begg and Mazumdar's test, p = 1.0). There was also no publication bias for either the CG or the GG analysis (Egger's test, p = 0.43 and 0.2; Begg and Mazumdar's test, p = 1.0 and 0.29). Ethnicity-based analysis was not performed, as all studies were carried out with Asian populations. The OR for the G allele frequency pooled from 10 studies encompassing

Table 3. Observed versus expected *PNPLA3 rs738409* genotype frequency in the control populations included in this meta-analysis. Expected genotype frequency is based on the Hardy-Weinberg equilibrium (HWE), which was calculated using the chi-square test. The chi-square value reflects the difference between expected and observed genotype counts. Values < 3.84 indicate that the allele frequency in the population does not deviate from HWE

Study	Observed genotype frequencies CC:CG:GG	Expected genotype frequencies CC:CG:GG	Chi-square value
Kantartzis (2009)[31]	137:70:18	131.48:81.03:12.48	4.17
Valenti (2010)[34]	118:56:5	119.08:53.83:6.08	0.29
Hotta (2010)[40]	175:296:104	181.44:283.12:110.44	1.19
Goran (2010)[36]	38:60:19	39.52:56.96:20.52	0.33
Wang (2011)[39]	269:335:119	263.53:345.94:113.53	0.72
Lin (2011)[41]	167:192:59	165.48:195.05:57.48	0.1
Li (2012)[46]	95:91:16	97.72:85.55:18.72	0.81
Kawaguchi (2012)[43]	247:468:217	248.24:465.52:218.24	0.03
Peng (2012)[42]	235:259:59	240.25:248.49:64.25	0.99
Kitamoto (2013)[47]	300:513:199	306.02:500.96:205.02	0.58
Islek (2014)[48]	156:129:18	160.46:120.07:22.46	1.67
Guichelaar (2014)[49]	9:3:0	9.19:2.63:0.19	0.25
Tai (2014)[50]	12:15:2	13.11:12.78:3.11	0.87
Lin (2014)[52]	238:293:75	243.96:281.08:80.96	1.09
Lee (2014)[73]	30:50:20	30.1:49.5:20.3	0.01
Oniki (2015)[77]	143:249:80	151.6:231.8:88.6	2.60
Park (2015)[74]	280:364:117	280.5:363.0:117.5	0.00
Xia (2015)[76]	1200:1363:352	1214.4:1334.2:366.4	1.36
Honda(2016)[81] non-obese	237:389:156	238.1:386.8:157.1	0.02
Honda (2016)[81] obese	63:124:43	67.9:114.1:47.9	1.72

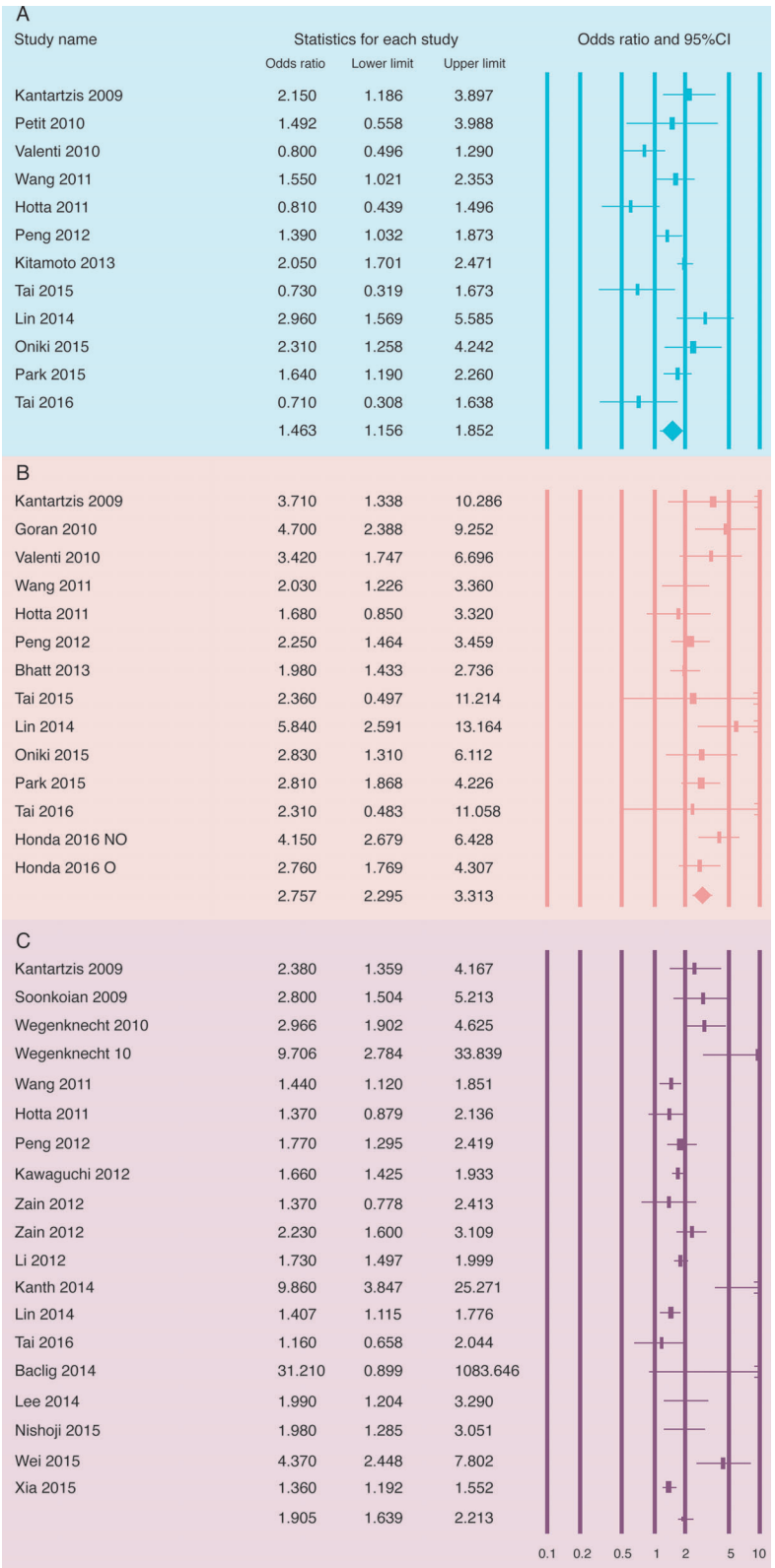

Fig. 2. Forest plots for analysis of non-alcoholic fatty liver studies comparing patients to healthy controls for association of *PNPLA3* polymorphisms with non-alcoholic fatty liver: A) CG vs. CC, B) GG vs. CC, and C) G allele. The bottom line in the "statistics for each study" represents the pooled effect size that was analyzed using the random effects model. An OR > 1 denotes risk for the respective outcome or positive association and an OR < 1 indicates a protective effect or negative association. A 95% confidence interval not crossing 1 indicates a significant association. NO, non-obese; O, obese.

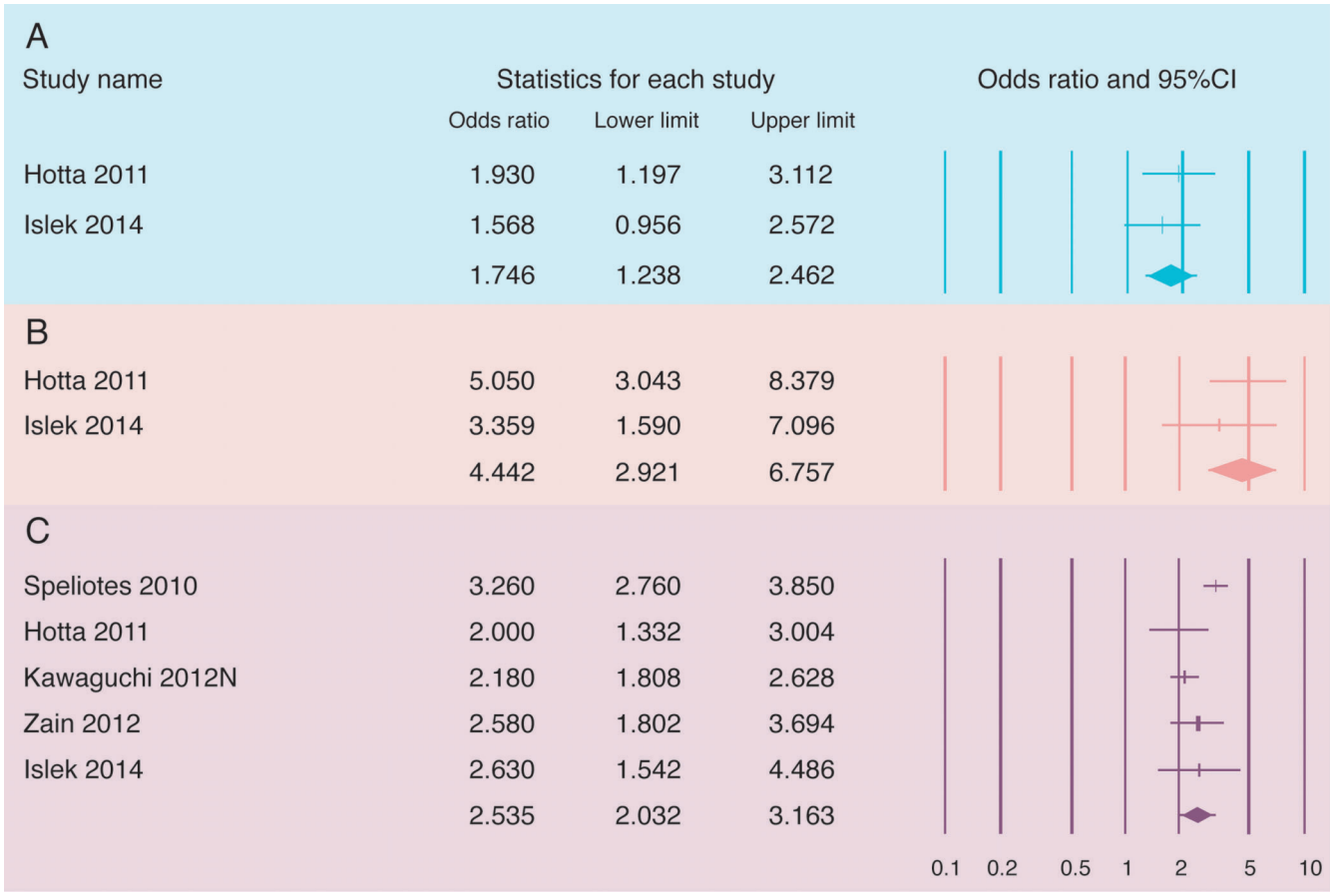

Fig. 3. Forest plots for analysis of non-alcoholic fatty liver studies comparing patients to healthy controls for association of *PNPLA3* polymorphisms with non-alcoholic steatohepatitis: A) CG vs. CC, B) GG vs. CC, and C) G allele frequency. The effect size is reported as odds ratio with 95% confidence interval. The bottom line in the "statistics for each study" represents the pooled effect size that was analyzed using the random effects model. An OR > 1 denotes risk for the respective outcome or positive association and an OR < 1 indicates a protective effect or negative association. A 95% confidence interval not crossing 1 indicates a significant association.

2,814 patients was 1.66 (1.24–1.94) (Fig. 5C). The data were homogenous (I^2 = 21.4%, p = 0.24) and there was no publication bias (Egger's test, p = 0.096; Begg and Mazumdar's test, p = 0.15). Ethnicity-based analysis showed that the OR for the G allele frequency in three Caucasian studies encompassing 1,326 patients was 1.42 (1.01–2.00) and in five Asian studies was 1.84 (1.53–2.21); the data were homogenous for both analyses, with respective values of I^2 = 54%, p = 0.11 and I^2 = 0%, p = 0.29. In addition, there was no publication bias in either analysis (Egger's test, p = 0.39 and 0.29; Begg and Mazumdar's test, p = 0.30 and 0.46). One study reported data in a population of mixed ethnicities, without separate ethnicity-based data.[32]

NAFLD activity score and histopathologic severity

NAS

The pooled OR for the G allele frequency in the presence of NAS > 4 vs. < 3 among NAFLD patients from four studies encompassing 1,586 patients was 1.80 (1.36–2.37) (Fig. 6A); the data were homogenous (I^2 = 19.8%, p = 0.12) and there was no publication bias (Egger's test, p = 0.12; Begg and Mazumdar's test, p = 0.31). However, after excluding a study that used a population of mixed ethnicities,[35] the OR from the three Asian studies, now encompassing 692 patients, was 2.05 (1.35–3.12), with no publication bias (Egger's test, p = 0.32; Begg and Mazumdar's test, p = 1.0).

Lobular Inflammation

The pooled OR for the G allele frequency for the presence of lobular inflammation in five studies encompassing 2,313 patients was 1.58 (1.19–2.10) (Fig. 6 B); the data were heterogeneous (I^2 = 56.9%, p = 0.054) and there was no publication bias (Egger's test, p = 0.46; Begg and Mazumdar's test, p = 0.46). Ethnicity-based analysis (Table 4) showed that the OR for the G allele frequency in two studies of Caucasian populations encompassing 879 patients was 1.47 (1.19–1.83) and in two studies of Asian populations encompassing 540 patients was 1.72 (0.52–5.69). The data were homogenous in the Caucasian analysis (I^2 = 0%, p = 0.40) but not in the Asian analysis (I^2 = 84%, p = 0.012). No analysis of publication bias was performed, as only two studies were included in each analysis.

Table 4. Ethnicity-based analysis on the association between *PNPLA3* and risk for and severity of disease among NAFLD studies

Comparison	Ethnicity	No. of studies	Genotypes	OR	95% CI
NAFL vs. controls	Caucasians	2	CG vs CC	1.29	0.29–3.49
	Asians	8		1.50	1.13–2.00
	Caucasians	2	GG vs CC	3.51	2.00–6.14
	Hispanics	1		4.70	2.39–9.25
	Asians	10		2.67	2.16–3.31
	Caucasians	1	G allele	2.38	1.36–4.17
	Blacks	1		9.71	2.78–33.84
	Hispanics	1		2.97	1.90–4.80
	Asians	12		1.78	1.52–2.08
NASH vs. controls	Caucasian	2	G allele	3.20	2.72–3.75
	Asian	3		2.25	1.94–2.61
NAFL 2–3 vs. NAFL 0–1	Asian	1	G allele	3.32	1.56–7.01
	Caucasian	4		2.69	1.27–5.69
NASH vs. NAFL	Caucasian	3	G allele	1.42	1.01–2.00
	Asian	5		1.84	1.53–2.21
NAFLD activity score	Mixed	1	G allele	1.56	1.12–2.17
	Asian	3		2.05	1.35–3.12
Lobular inflammation	Mixed	1	G allele	1.84	1.33–2.55
	Caucasian	2		1.47	1.19–1.83
	Asian	2		1.72	0.52–5.69
Hepatocyte ballooning	Caucasian	2	G allele	3.17	2.68–3.76
	Asian			1.81	1.07–3.03
Fibrosis/Cirrhosis	Caucasian	2	G allele	1.41	1.13–1.74
	Asian	2		2.16	1.90–2.58

Abbreviations: NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

Hepatocyte ballooning

The pooled OR for the G allele frequency for hepatocyte ballooning in four studies encompassing 1,419 patients was 2.63 (1.87–3.69) (Fig. 6C); the data were homogenous (I^2 = 43.3%, p = 0.15) and there was no publication bias (Egger's test, p = 0.24; Begg and Mazumdar's test, p = 0.31). Ethnicity-based analysis (Table 4) showed that the OR for the G allele frequency in two studies of Caucasian populations encompassing 879 patients was 3.17 (2.68–3.76) and in two studies of Asian populations encompassing 540 patients was 1.81 (1.07–3.03). The data were homogeneous in both analyses (I^2 = 0%, p = 0.66 and I^2 = 0%, p = 0.32 respectively)

Portal inflammation

Only one study encompassing 894 patients reported the OR of the G allele frequency for portal inflammation, and the value was 1.57 (1.24–1.99).

Fibrosis and cirrhosis

Among the eight studies of advanced cirrhosis and fibrosis encompassing 3,571 patients, the pooled ORs for the *rs738409* C>G CG and GG genotypes compared to the CC genotype were 18.80 (2.12–166.75) (from one study[56]) and 2.72 (0.84–8.83) respectively (Fig. 7A). The data were heterogeneous for the GG analysis (I^2 = 76.8%, p = 0.013) and there was no publication bias (Egger's test, p = 0.22; Begg and Mazumdar's test, p = 0.29). When the pediatric study[38] was excluded, the pooled OR showed a similar effect size of 1.43 (0.94–2.17). The data were homogenous (I^2 = 0%, p = 0.61). The pooled OR for the G allele frequency was 1.67 (1.37–2.03) (Fig. 7B), with heterogeneous data (I^2 = 55.9%, p = 0.045) and no publication bias (Egger's test, p = 0.61; Begg and Mazumdar's test, p = 1.0). The pooled OR for the G allele frequency after excluding the pediatric study[38] was 1.64 (1.32–2.04) (data not shown in figure form), with heterogeneous data (I^2 = 64%, p = 0.03) and no publication bias (Egger's test, p = 0.49; Begg and Mazumdar's test, p = 1.0). Ethnicity-based analysis showed that among two studies on Caucasian populations encompassing 1,166 patients the pooled OR for the G allele frequency was 1.41 (1.13–1.74), with homogeneous data (I^2 = 0%, p = 0.61), and among the two studies on Asian populations encompassing 682 patients the pooled OR for the G allele frequency was 2.16 (1.90–2.58), with homogeneous data (I^2 = 0%, p = 0.60) (Table 4).

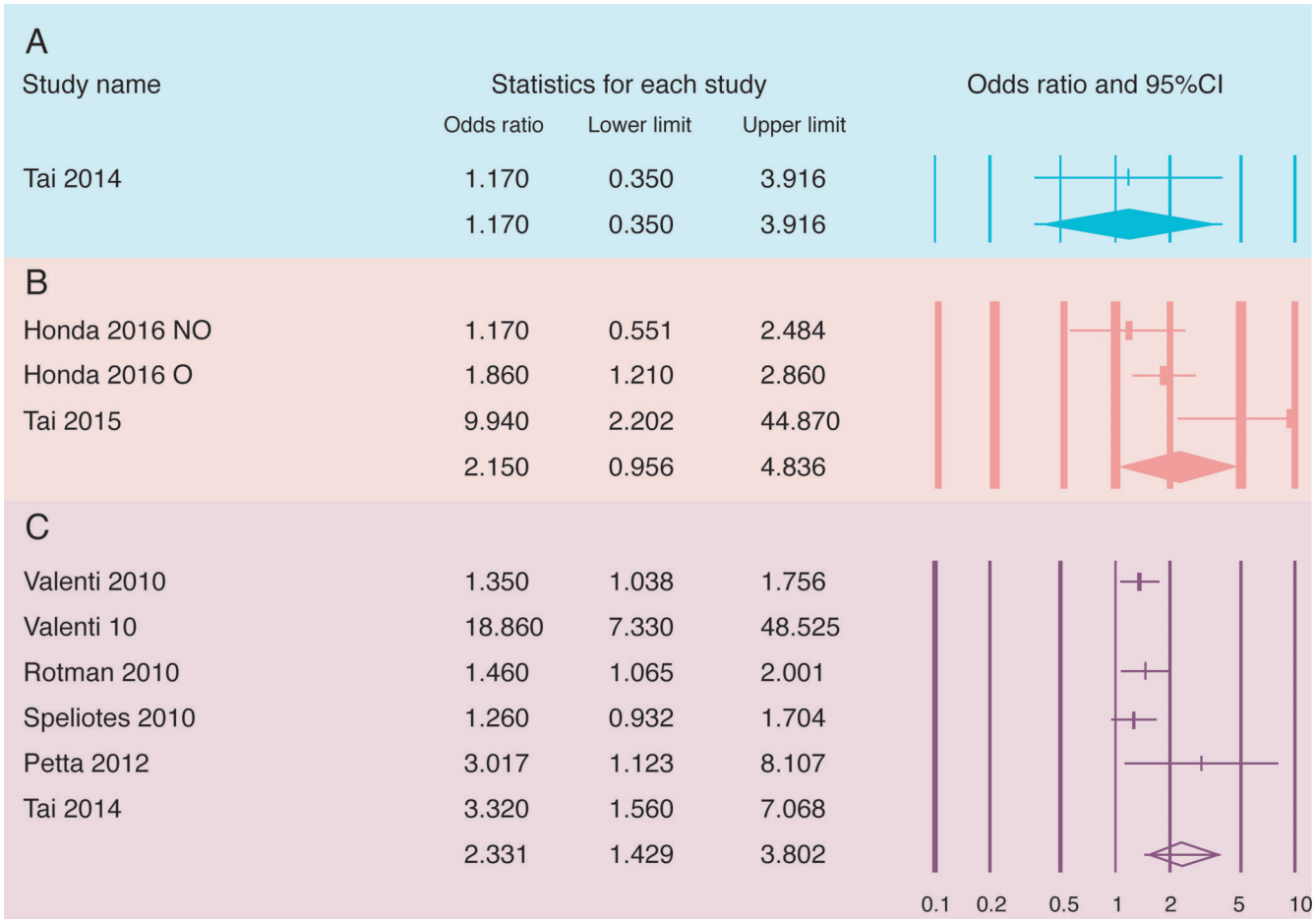

Fig. 4. Forest plots for analysis of the risk of spectrum of non-alcoholic fatty liver disease on the association of *PNPLA3* polymorphism with steatosis grades 2–3 vs. 0–1: A) CG vs. CC, B) GG vs. CC, and C) G allele frequency. The bottom line in the "statistics for each study" represents the pooled effect size that was analyzed using the random effects model. An OR > 1 denotes risk for the respective outcome or positive association and an OR < 1 indicates a protective effect or negative association. A 95% confidence interval not crossing 1 indicates a significant association. NO, non-obese; O, obese. Valenti 10 refers to the pediatric study.[38]

HCC in NAFLD

The ORs of the *rs738409 C>G* -CG and GG- genotypes as compared to the CC genotype for the risk of NAHCC among NAFL patients without cirrhosis were 2.35 (0.90–6.13) and 5.05 (1.47–17.29) respectively. The pooled OR of the *rs738409 C>G* CG as compared to the CC genotype for NAHCC among patients with NAC was 2.06 (1.07–3.94). All these analyses were reported in one study each, and the forest plots are not shown for these analyses.[53]

Discussion

This meta-analysis includes data for both adult and pediatric NAFLD, by which we demonstrate that *PNPLA3* polymorphisms are associated with a) the risk of NAFL and NASH compared to healthy controls and b) more severe disease on the spectrum of NAFLD, where the polymorphism correlated with the grade of steatosis, NAS, histopathologic findings, cirrhosis and when NASH or NAHCC were compared to NAFL.

The *PNPLA3* gene, a transmembrane protein encoding for adiponutrin in human, is highly expressed in the liver and adipose tissues. Its expression in subcutaneous and intra-abdominal adipose tissues correlates with obesity.[57] The biochemical function of adiponutrin is uncertain, but it is considered to have lipogenic transacetylase activity, likely facilitating energy mobilization and lipid storage in adipose and liver tissues.[57,58] Recently, it has been reported that the 148M adiponutrin allele is a loss-of function variant that predisposes an individual to steatosis by decreasing triglyceride hydrolysis in hepatocytes.[59] However, this genetic variant does not appear to correlate with visceral or subcutaneous fat content, insulin sensitivity, or peripheral blood lipid levels.[37,38] Consistent with the phenotype of NAFLD patients, overexpression of *PNPLA3* G allele increases the size of lipid droplets, as compared with the overexpression of *PNPLA3* C allele.[60] The results of this meta-analysis are compatible with the mechanism of action of *PNPLA3* and its presumed effect on liver toxicity. The exact mechanism by which *PNPLA3* polymorphism increases the risk of HCC is yet to be determined. *PNPLA3* was recently linked to increasing circulating levels of the intercellular adhesion molecule 1 (ICAM-1),[61] which is a proinflammatory marker and characterized as a determinant of the malignant nature of tumors;[62,63] in addition, it has been

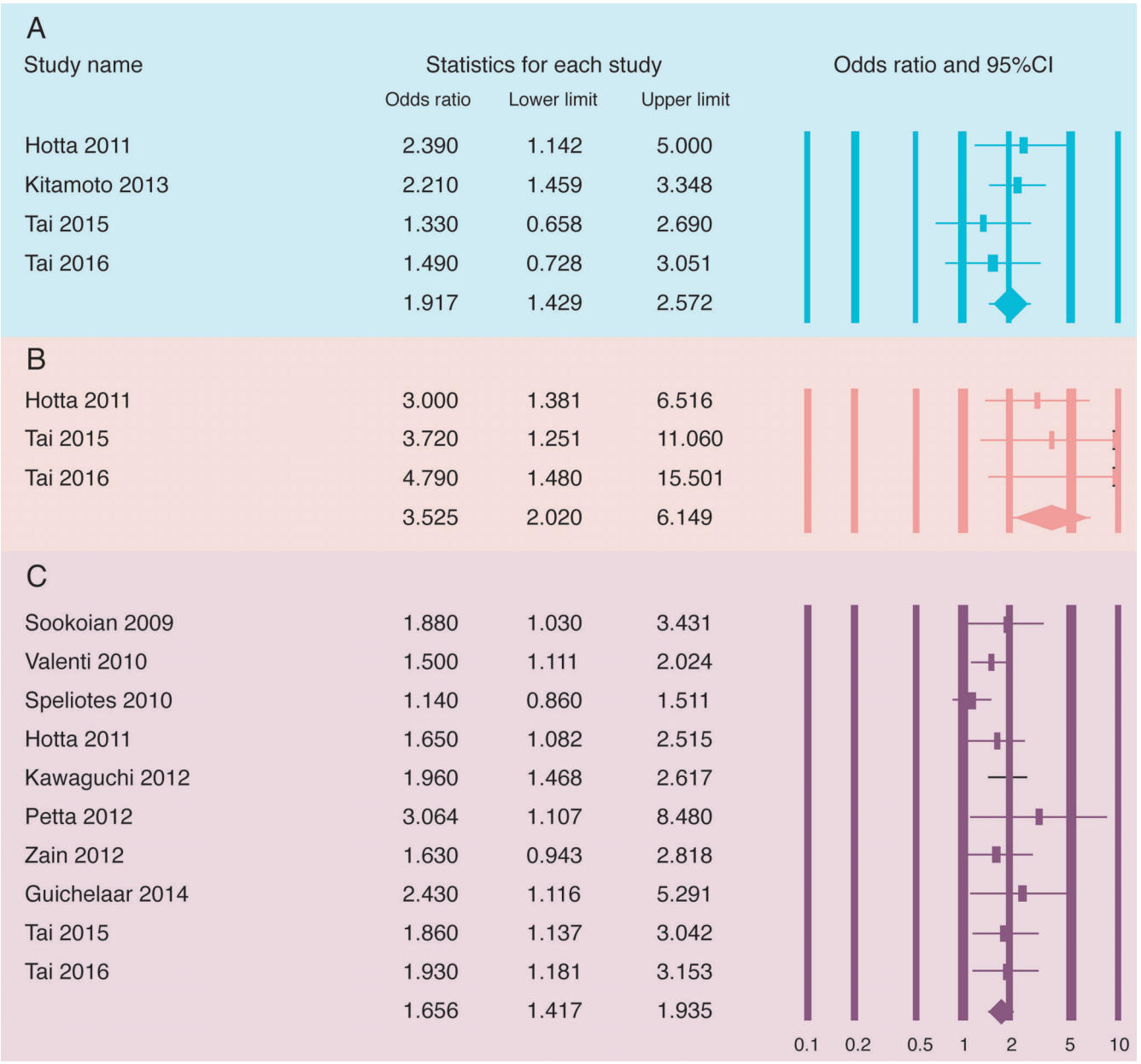

Fig. 5. Forest plots for analysis of the risk of spectrum of non-alcoholic fatty liver disease on the association of *PNPLA3* polymorphism with non-alcoholic steatohepatitis vs. non-alcoholic fatty liver: A) CG vs. CC, B) GG vs. CC, and C) G allele frequency. The bottom line in the "statistics for each study" represents the pooled effect size that was analyzed using the random effects model. An OR > 1 denotes risk for the respective outcome or positive association and an OR < 1 indicates a protective effect or negative association. A 95% confidence interval not crossing 1 indicates a significant association.

shown to be associated with decreased levels of adiponectin, a protein with oncosuppressive properties.[64]

This is the first comprehensive meta-analysis examining the association of *PNPLA3* polymorphisms with the risk for and severity of the spectrum of liver disease among NAFLD patients. The previous meta-analysis by Xu et al[9] included 23 studies and looked at the association between NAFL/NASH and *PNPLA3* polymorphism, but did not include NAHCC, NAC, steatosis grade, NAS, etc. The other most recent meta-analysis published in the literature investigated the association between NAFLD and *PNPLA3* in studies of Asian populations only.[10] Our current analysis included a total of 25 NAFLD studies and confirmed these findings, expanding our understanding of the associations with different liver disease phenotypes. In addition, we confirmed the HWE calculations when possible, looked at the study quality assessment based on the Newcastle-Ottawa scale, and performed subgroup analysis based on age and ethnicity to confirm our findings. All of these features of our study make the current analysis more comprehensive than the previous ones for gaining a more detailed understanding of the NAFLD and *PNPLA3* genetic association.

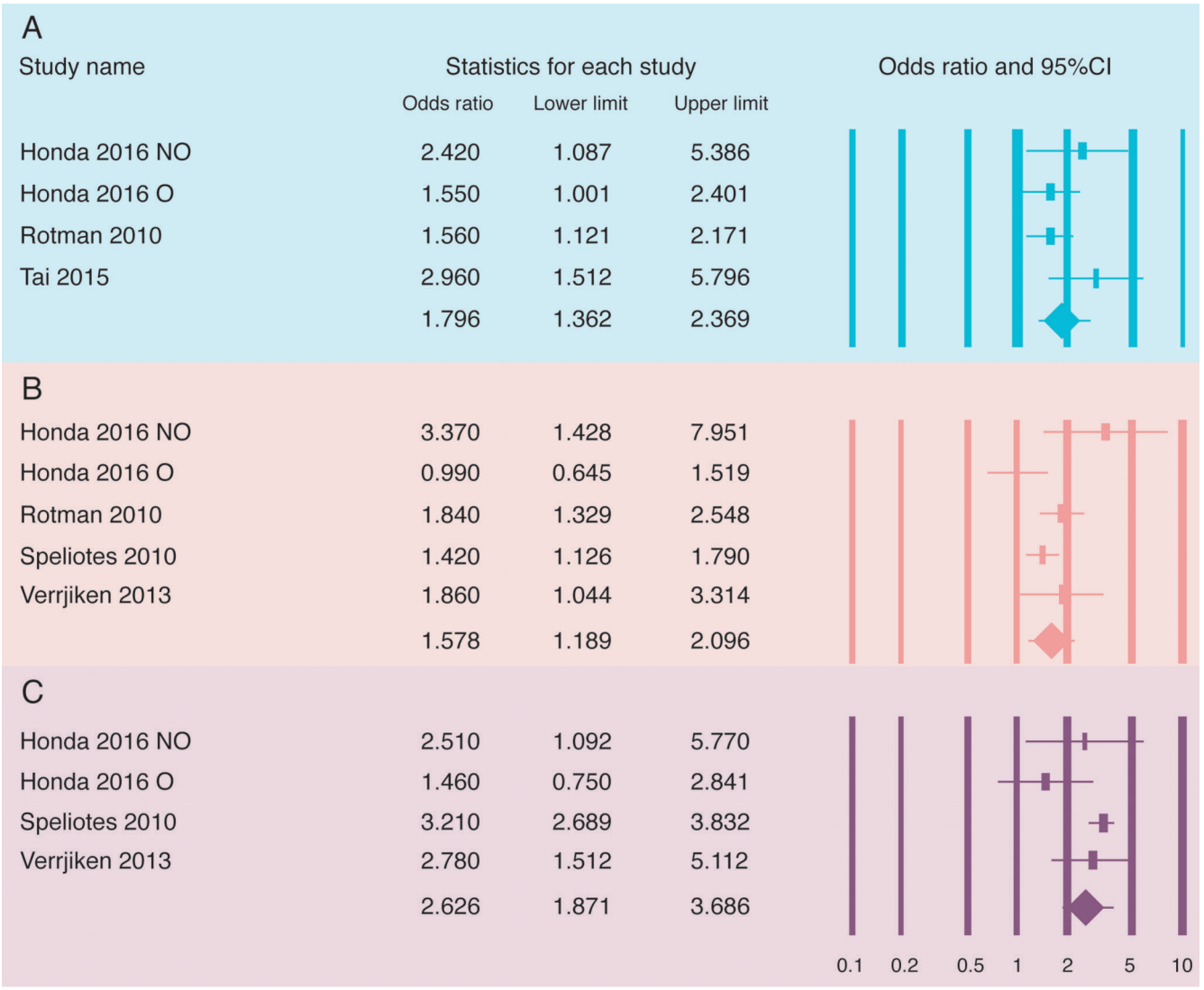

Fig. 6. Forest plots for analysis of the risk of spectrum of non-alcoholic fatty liver disease on the association of *PNPLA3* polymorphism with G allele frequency in NAFLD activity score in panel (A), in lobular inflammation in panel (B) and in hepatocyte ballooning in panel (C). The bottom line in the "statistics for each study" represents the pooled effect size that was analyzed using the random effects model. An OR > 1 denotes risk for the respective outcome or positive association and an OR < 1 indicates a protective effect or negative association. A 95% confidence interval not crossing 1 indicates a significant association. NO, non-obese; O, obese.

Herein we present results from our meta-analysis of ten ALD studies, which also included individual participant data from five studies, showing that the *PNPLA3* polymorphism was associated with predisposition to and severity of the ALD spectrum.[4] When we compared the current meta-analysis with the previous one we noted certain differences between NAFLD and the ALD disease spectrum and the association with the *PNPLA3* polymorphism. First of all, there is a stronger association amongst NAFLD, as compared to the ALD studies. This could be due to more studies published and analyzed with larger sample size for the current analyses. Secondly, the association was lacking with alcoholic FL but it was more prominent in the current study for association with NAFL. A similar prevalence of the polymorphisms in drinkers with FL and non-drinkers aligns well with the fact that the FL in alcoholics is a universal phenomenon in alcoholics and is reversible upon abstinence.[65–67]

Our study is potentially limited by the possibility of publication bias. In order to minimize this potential limitation, however, and to subsequently overestimate the true effect size due to negative studies' identification failure,[68] we combined searches from the PubMed/Medline, Embase and Cochrane databases with manual searches. Unfortunately, we could not examine the association of *PNPLA3* polymorphism with NAC and NAHCC compared to controls due to the lack of studies examining this association. Unavailability of individual patient data also precluded our ability to examine the impact of confounders such as age, sex, comorbidities, and BMI on the association with the *PNPLA3* polymorphisms.

Another limitation to our study relates to the differences in the definition of NASH used across the studies included in this analysis. Bedossa et al[69] described various features of hepatic injury in NASH, with the histological finding of lobular inflammation and hepatocyte ballooning being key findings.

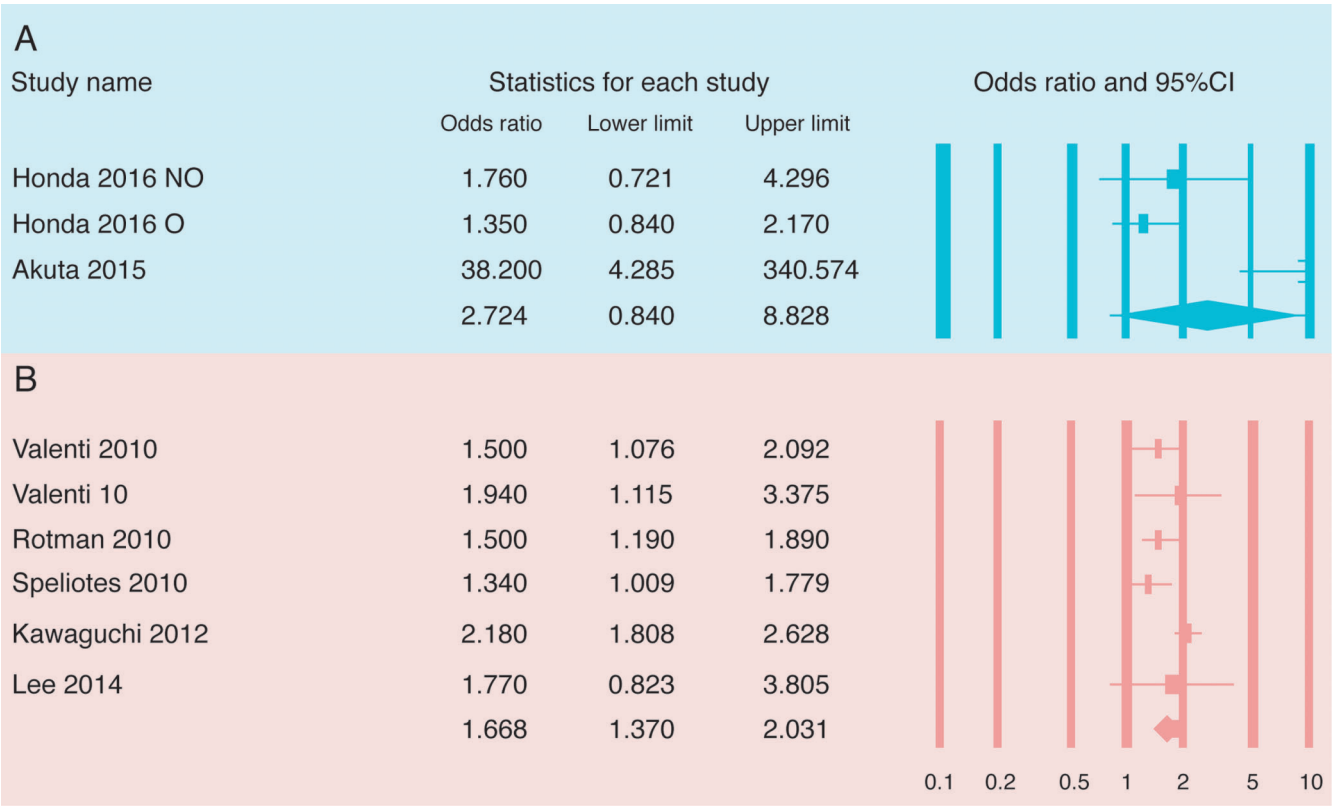

Fig. 7. Forest plots for analysis of the risk of spectrum of non-alcoholic fatty liver disease on the association of *PNPLA3* polymorphism with fibrosis/cirrhosis vs. NAFLD: A) GG vs CC, and B) G allele frequency. The bottom line in the "statistics for each study" represents the pooled effect size that was analyzed using the random effects model. An OR > 1 denotes risk for the respective outcome or positive association and an OR < 1 indicates protective effect or negative association. A 95% confidence interval not crossing 1 indicates a significant association. NO, non-obese; O, obese. Valenti 10 refers to the pediatric study.[38]

Different scoring systems were proposed, such as the Steatosis, Activity and Fibrosis system[70] and the NASH Clinical Research Network system.[71] To resolve this limitation, we grouped studies with almost similar definitions for the sake of homogeneity of the analysis.

In summary, the present study provided unequivocal evidence of *rs738409* as a strong modifier of the natural history of NAFLD in different ethnic and age populations. As such, the *PNPLA3* gene may be a potential target for therapy in NAFLD. Prospective data are now needed to further understand the association of *PNPLA3* polymorphisms, particularly related to a) a response to control of risk factors of NAFLD and b) for prediction of the natural history of the disease.

Conflict of interest

None

Author contributions

Study design (HS, AKS), data collection (HS, MAH, MM, MN, TM, AE), drafting the manuscript (HS, MAH, MM, MN, TM, AE, AKS), data analysis and interpretation (HS, AKS), critical revision and funding (AKS), statistical analysis (AKS, AE).

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