REVIEW ARTICLE

Does PNPLA3 Rs738409 Impact on NAFLD Progression?: A Systematic Review

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ABSTRACT

Background: **Non-alcoholic fatty liver disease (NAFLD) is an emerging health issue that affects 24% population world-wide, estimates about 75% of all chronic liver diseases, which results from excess accumulation of fat in the liver, producing a wide range of manifestation, simple fatty liver to non-alcoholic steatohepatitis (NASH), hepatic fibrosis, cirrhosis and even hepatocellular carcinoma (HCC). Single nucleotide polymorphism (SNP) rs738409 in the PNPLA3 C > G has been linked to NAFLD and its progression to severity, though the exact molecular mechanism and impact is not clear.**

Aim: **We aimed to explore the impact of PNPLA3 rs738409 in the NAFLD development and its progression to severe disease. In addition, would also evaluate the linkage and predisposition of PNPLA3 rs738409 risk allele (G) in NAFLD over different population and ethnicity.**

Materials and Methods: **A comprehensive search was conducted through Wiley online library, Google scholar, MEDLINE and PubMed search. Filters used were human trial, English language, time frame from 2008 to 2020 and search terms used were, "PNPLA3 + rs738409 + gene" and "non-alcoholic fatty liver disease"; (PNPLA3[All Fields] AND rs738409[All Fields]) AND ("non-alcoholic fatty liver disease" [MeSH Terms] OR ("non-alcoholic" [All Fields] AND "fatty" [All Fields] AND "liver" [All Fields] AND "disease" [All Fields]) OR "non-alcoholic fatty liver disease" [All Fields] OR "nafld" [All Fields]) AND (medline[sb] AND ("2008/01/01" [PubDate]: "2020/08/31" [PubDate]));**

Result: **The systematic review of 7 articles includes 14,154 participants of which male participants were 54.6% (39.5 to 82%) in intervention group and 41.5% (25 to 59%) in the control group. The mean age in the intervention group was 58.8 years (36.7 to 78.3) and 48.75 years (32.4 - 65.1) in the control group. Outcome of the study suggests PNPLA3 rs738409 C > G polymorphic gene is strongly linked to NAFLD and its progression to sever disease (P < 0.001, OR > 1~4 at 95% CI). The presence of PNPLA3 rs738409 (G) minor allele increases the risk of NAFLD (from 0.15 to 4-fold) (HR 1.33%. 95% CI1.15-1.53, P < 0.0001 and OR 4.0 at 95% CI).**

Conclusion: **PNPLA3 rs738409 C > G polymorphic gene is strongly linked to NAFLD and its progression to severe disease whereas, presence of G allele increases the risk of severity.**

KEY WORDS

SNP, Single Nucleotide Polymorphism, GWAS, Genome Wide Association Study, NAFLD, Non-Alcoholic Fatty Liver Disease

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an emerging health issue that affects 24% of the population world-wide, and accounts for approximately 75% of all chronic liver diseases[1,2]. About 17 - 30% of the Western population and 30 - 45% people of United States suffer from NAFLD[3-5]. NAFLD may be described as abnormal accumulation of excess fat in the liver (> 5%) without having history of significant alcohol consumption > 100 gm/week or (≥ 30 gm/day in men and 20 gm/day in women) or secondary to drug, viral infection or autoimmune insult[3,6,7]. Several studies have elicited that oxidative stress as well as metabolic syndrome and its components, insulin resistance (IR) play an important role in NAFLD and its progression to severity through an altered cellular metabolism which is thought to be modulated by environmental and genetic insult[8-10]. Though NAFLD is benign and almost reversible after removal of triggering factor, it has been observed that only 10 - 30% of NAFLD progressed in severity, NASH, fibrosis, cirrhosis even HCC, which raises a question of possible provocation factors may have behind this[11,12].

On the other hand, several studies including 'twin study' and 'familial study' have confirmed the heritable component contributing to NAFLD, estimated at 10 - 70% depending on population character, ethnicity and study methodology[13-16]. The research also observed that heritable character, distributed among monozygotic and dizygotic pairs may have been conferred through mother-daughter, sister- sister, sister-brother, father-daughter and male-female cousin pair, whereas, family study elicited NAFLD is more common in siblings (59%) and parents (78%) of children having NAFLD[14,15].

More recently, genome wide association studies (GWAS) have been identified several single nucleotide polymorphisms (SNPs)in relation of NAFLD of which Romeo and his colleague in 2008, first reported the rs738409 C > G polymorphic gene in the Patatin-like Phospholipase domain-containing 3, (PNPLA3) potentially linked to NAFLD[17].

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Table 1: Inclusion and exclusion criteria using PICO. Note: PICO, Participant, intervention, control outcome.

	Inclusion criteria	Exclusion criteria
Participant	• Patients, age > 18 years, Patients suffering from biopsy or radiology scan (USG or CT scan) proven non-alcoholic fatty liver dis- ease (NAFLD), Studies reflecting the associa- tion between PNPLA3 I148M (rs738409) gene variant. Human studies only.	• Pediatric age group. • Steatosis, secondary to alcoholism, drugs, viral infection, hepatitis B & C as well as parenteral nutrition. • Significant alcohol intake (> 100 gm/week). • Lack of available genotype data. Review articles. • Articles not clearly describe the original data.
Intervention	PNPLA3 rs738409 genotype.	Other associated genetic involvement.
Control	Non-diabetic, non-obese, healthy population.	Co-morbidity
Outcome	PNPLA3 rs738409 (I148M) is strongly associ- ated with NAFLD. PNPLA3 rs738409 (I148M) is also associated disease severity and HCC	Studies with unclear outcome, only efficacy outcome or only safety outcome.
Study design	Cross-sectional, Case controlled trial.	Case reports, Randomized controlled trial (RCT).

Table 2: The Newcastle-Ottawa quality assessment scale (NOS) [22].

Selection (Maximum 5 stars)	Representativeness of the samples	Truly representative of the average in the population. *(all subjects or random sampling Somewhat representative of the average in the target population. * Selected group of users (e.g. smokers, non-smokers) No description of the sampling strategy.
	Sample size	Justified and satisfactory. * Not justified.
	Ascertainment of exposure	Validated measurement tool. ** Non-validated measurement tool, but the tool is available or described. * No description of the measurement tool
	Non-respondent	Comparability between respondents and non-respondents characteristics is established, and the response rate is satisfactory.* The response rate is unsatisfactory, or the comparability between respon- dents and non-respondents is unsatisfactory. No description of the response rate or the characteristics of the responders and the non-responders.
Comparability (Maximum 2 stars)	Subjects in different outcome groups are comparable, based on the study design or analysis. Confounding fac- tors are controlled.	a) The study controls for the most important factor (select one, e.g. Childhood asthma). * b) The study control for any additional factor (age, gender, ethnicity). *
Outcome (Maximum 3 stars)	Assessment outcome	Independent blind assessment * Record linkage * Self report No description
	Statistical test	The statistical test used to analyze the data is clearly described and appropriate, and the measurement of the association is presented, including confi- dence intervals and the probability level (p value). * The statistical test is not appropriate, not described or incomplete.

PNPLA3 is protein in nature that possesses lipase activity and hydrolyse triglyceride and retinyl esterase to maintain lipid haemostasis mobilising fat from liver[18]. The polymorphic gene rs738409 C > G constitutes an isoleucine to methionine substitution at 148 codon of PNPLA3 resulting impairment of enzymatic activity of PNPLA3, resulting in accumulation of triglyceride (TG) in hepatic cell and lipid droplet in hepatic stellate cell, leading to fatty liver[19-21]. Though several studies have identified the significant association of PNPLA3 rs738409 polymorphic gene in NAFLD pathogenesis, the exact mechanism is still in hypothetical. NAFLD is apparently benign but could lead to severe disease, NASH, fibrosis, cirrhosis even HCC and as, till now there is no definite treatment, therefore early detection as well as modification of a provocation factors would be desirable.

Aim of the Study

This systematic review aimed to explore the impact of PNPLA3 rs738409 polymorphic gene on NAFLD and its progression to severity. In addition, we would also like to explore the predisposition and linkage of PNPLA3 rs738409 risk allele (G) in NAFLD among different populations and ethnic groups.

MATERIALS AND METHODS

Search strategy

All eligible articles used in this systemic review were selected from Wiley online library, Google scholar, Medline and PubMed search. Filters were used human trial, English language, time frame from 2008 to 2020. A computerised search was made using search keys,

"PNPLA3 + rs738409 + gen"e and "non- alcoholic fatty liver disease"; (PNPLA3[All Fields] AND rs738409[All Fields]) AND ("non-alcoholic fatty liver disease" [MeSH Terms] OR ("non-alcoholic" [All Fields] AND "fatty" [All Fields] AND "liver" [All Fields] AND "disease" [All Fields]) OR "non-alcoholic fatty liver disease" [All Fields]

Table 3: The Newcastle-Ottawa quality assessment scale (NOS) for publications, selected for systematic review.

Publications	Selection of studies (Maximum 5 stars)	Comparability (Maximum 2 stars)	Outcome (Maximum 3 stars)	Total score
Hotta., *et al*. 2010 [24]	4	1	3	8
Kawaguchi., *et al*. 2012 [25]	5	1	3	9
Peng, Wu, Lin, Lu, Hu, Lin 2012 [26]	5	1	3	9
Liu., *et al*. 2014 [27]	1	1	3	5
Mazo., *et al*. 2019 [28]	1	1	3	5
Narayanasamy., *et al*. 2020 [29]	1	1	3	5
Walker., *et al*. 2020 [30]	5	2	3	10

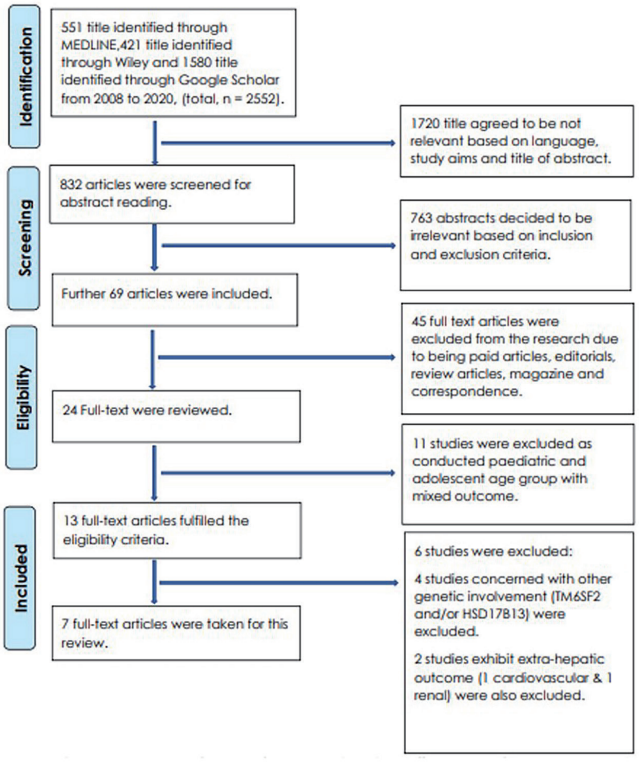

Figure 1: PRISMA chart (Adopted from Moher, Liberati, Tetslaff, Altman, 2009)

OR "nafld" [All Fields]) AND (medline [sb] AND ("2008/01/01" [PubDa te]: "2020/08/31" [PubDate])).

Selection process
Inclusion and exclusion criteria

All the participants, aged 18 years and above, histologically or radiologically diagnosed NAFLD, studies concerning association of PNPLA3 rs738409 variant gene with NAFLD as well as human studies and English language were included to evaluate the single nucleotide polymorphisms (SNPs) associated with NAFLD or NASH and its severity comparing with non-obese, non-diabetic healthy control (Table 1). Articles were screened for relevance according to the title and abstract. Editorial, magazine, correspondence and review papers were excluded. Moreover, patients in paediatric age group, significant amount of alcohol consumption (> 100 gm/week), steatosis secondary to other causes, as per say, drug, alcohol, viral infection or autoimmune insult, lack of genotype data as well as articles showing unclear outcome were excluded from this study (Table 1).

Filters used

Only English language, human trials, time frame, January 2008 and August 2020, PNPLA3 rs738409 gene and NAFLD.

Outcome

PNPLA3 rs738409 C > G genotype is strongly associated with non-alcoholic fatty liver (NAFLD) development and its progression to severe disease.

PNPLA3 G allele is significantly increase the risk associated with NAFLD and its severity than allele C.

PNPLA3 rs738409 G is also strongly associated with NAFLD carrying the gene than non-carriers.

Reviewing process

An advanced computerised search was made in Wiley online library, Google Scholar and MEDLINE data base through PubMed using relevant key words and filters. Duplication from all sources as well as articles not relevant to the title and abstract were removed. Editorial, magazine, correspondence and review papers were also excluded. Full text articles were examined from remaining studies accordance of inclusion criteria of histological or radiological diagnosis of NAFLD or NASH, provided the fatty liver or steatosis not resulting from secondary to drug, alcohol, viral infection or autoimmune insult of which most matched articles were selected for qualitative assessment.

Data extraction

All the qualitative and quantitative data, name of the articles, author, publication year, demographic data of the patient, name, age, sex, sample size study design and outcome of the study (PNPLA3 rs738409 is linked to NAFLD) (Table 4) were extracted. Results were reported using odd ratio (OR), 95% confidence interval (CI) and P values (Table 4).

Quality and risk of bias assessment

Quality of the studies and risk of bias were evaluated using Newcastle-Ottawa Quality assessment Scale[22] (Table 2 and 3). The New- castle-Ottawa Scale (NOS) is derived from the collaboration of the Universities of Newcastle, Australia and Ottawa, Canada to assess the quality of non-randomised studies as well as assessment of risk of bias in cross-sectional study in a systematic review. A 'star system' has been developed to evaluate a study on three wide perspectives: a) the selection of the study groups; b) the comparability of the groups; and c) the ascertainment of either the exposure or outcome of interest for case-control or cross-sectional studies respectively. It is notable that a study can be awarded a maximum of one star for each numbered item within the 'Selection and Exposure' categories whereas, a maximum of two stars can be given for 'Comparability'[22].

Search result

A total of 2552 articles were selected through a computerised search which was further narrowed to 832 after exclusion of irrelevance of study aim, title, abstract, language and duplication. 69 articles were matched after exclusion of paid articles, editorial, review article, magazine and correspondence. 24 full text articles were reviewed and among them 13 articles met the eligibility criteria of whom 6 articles were excluded as four of them concern with other genetic involvement (TM6SF2 and or HSD17B13), two of the articles exhibited extra-hepatic outcome (one cardiovascular and one renal). Finally, 7 articles have been selected for qualitative synthesis according to selection criteria. A PRISMA check lists have provided as supportive of this systematic

Table 4: Data extraction Impact of PNPLA3 rs738409 on NAFLD severity.

Publications	Study design	Population (N)	Intervention	Control	Outcome
1) Hotta., *et al*. 2010 [24]	Cross-sectional Japanese	831	253	578	1) Strong association of PNPLA3 rs739409 with NAFLD ($P = 9.4 \times 10^{-10}$) 2) PNPLA3 G allele frequency is higher in NAFLD than control (0.60 vs 0.44 respectively).
Kawaguchi., *et al*. 2012 [25]	Cross-sectional Japanese	1471	529	942	1) PNPLA3 rs738409 strongly associated with NAFLD histologically Type 4 (termed as Matteoni 4) ($p = 1.7610216 \times 10^{-16}$, OR = 2.18, 95%CI 1.81 - 2.63) 2) Strongly associated with progression of NASH in Japa- nese subject (p = 4.861026 x 10-6, OR 1.96, 95%CI 1.47 - 2.62)
1) Liu., *et al*. 2014 [27]	Cross-sectional European-Caucasian Multicentred	375	100	275	1) PNPLA3 rs738409 C > G strongly associated with NAFLD-related-HCC (adjusted OR 2.046, 95% CI 1.47 - 2.84], P < 0.0001) 2) G allele in PNPLA3 rs738409 bears dose dependent effect. 3) Presence of GG homozygous allele increases the risk by 3.92-fold than homozygous CC allele (OR 3.92, 95% CI 2.06-7.48).
1) Mazo., *et al*. 2019 [28]	Cross-sectional Multicentred (Brazil)	382	248	134	1) PNPLA3 rs738409 genotype GG exerts 3.29-fold (OR, 3.29,95% CI 1.504-7.225) and genotype CG exerts 1.75- fold (OR 1.75) risk of having NAFLD compared to CC genotyped. 2) PNPLA3 rs738409 genotype GG is related to higher risk of NASH with fibrosis than CC evident by elevated AST. 3) PNPLA3 rs738409 genotype CG+GG increases the risk of NAFLD in Brazilian subject.
Narayanasamy., *et al*. 2020 [29]	Case control Indian	207	105	102	Patient with PNPLA3 rs738409 4-fold increase risk of NAFLD in GG genotype (OR 4.0) than CG genotype (OR 2.881).
1) Walker., *et al*. 2020 [30]	Cross-sectional Randomised Multicentred and multi-ethnic	9822	1703	8119	1) 15%> greater risk of NAFLD (HR-1.33%,95% CI 1.15-1.53, p < 0.0001) 2) Increased risk of NAFLD (OR 1.61, 95% CI1.349- 1.73, p < 0.0001) Strongest effects on Hispanics (OR 1.43, 95% CI1.28-1.59, p < 0.0001) 3) Disease risk is associated in early age ($p < 4.95 \times 10^{-6}$)
All events	7	14154	3471	10,683	

Table 5: Data extraction Impact of PNPLA3 rs738409 on NAFLD severity (continued).

Publications	Study design	Definition of Case	Case and Control	DNA preparation and SNP genotyping
Hotta., *et al*. 2010 [24]	Cross-sectional Japanese	Histological diagnosis from liver biopsy.	Clinically non-diabetic and no-obese as well as biochemically and radiologically free from any liver pathology.	Genomic DNA was extracted from blood sample using Genomix (Talent Srl, Trieste. Italy). TaqMan probe (Applied Biosystems, Foster City, CA) was used for genotyping of rs738409.
Kawaguchi., *et al*. 2012 [25]	Cross-sectional Japanese	Histological diagnosis from liver biopsy.		DNA was extracted from blood mononuclear cells by standard Phenol-Chloroform method and for rs738409 genotyping Illumina Human 610-Quad Bead Chip on a Bead Station 500G Genotyping Sys- tem (Illumina, Inc., San Diego, CA, USA) was used.
Peng, Wu, Lin, Lu, Hu, Lin 2012 [26]	Cross-sectional Chinese	Radiological diagnosis by USG of hepato-biliary system		Genomic DNA was extracted from blood sample using standard method and MALDI-TOF mass spectrometry assay was used for rs738409 geno- typing.
Liu., *et al*. 2014 [27]	Cross-sectional European- Caucasian Multicentred	Histological diagnosis from liver biopsy.		DNA was extracted from blood lymphocyte using a perchlorate-chloroform isolation method and for rs738409 genotyping TaqMan reagents (Assay #4351379, Applied Biosystems Inc., USA) was used.
Mazo., *et al*. 2019 [28]	Cross-sectional Multicentred (Brazil)	Histological diagnosis from liver biopsy.		Genomic DNA was extracted from blood sample using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). TaqMan primers & probe (RT- PCR, Applied Biosystems, Foster City, CA) was used for genotyping of rs738409
Narayanasamy., *et al*. 2020 [29]	Case control Indian	Radiological diagnosis by USG of hepato-biliary system.		DNA was extracted from blood using Phenol- Chloroform-isoamyl alcohol, and for rs738409 genotyping PCR-RFLP was used.
Walker., *et al*. 2020 [30]	Cross-sectional Randomised	Radiological diagnosis by CT scan of hepatobiliary system		DNA was extracted from IDT xGene capture platform and for PNPLA3 rs738409 genotyping Illumina Global screening Array were used.

review (PRISMA chart) (Figure 1)[23].

Characteristics of the study

The systematic review has included in total, 14,154 participants of which 3471 participated in intervention group and 10,683 in the control group where the male participants were 54.6% (39.5 to 82%) in intervention group and 41.5% (25 to 59%) in the control group. The mean age in the intervention group was 58.8 years (36.7 to 78.3) and 48.75 years (32.4 - 65.1) in the control group. The studies included in this systematic review were cross-sectional study and one of them was case control (Table 4); among them three studies were multicentred (two from Europe and one from America) of which multi-ethnic population were included in two studies. Remaining four studies were from Asian population, two from Japanese, one Chinese and one from Indian population. The relation of PNPLA3 rs738409 gene in the development of NAFLD and its progression to severe disease has been evaluated comparing with healthy control group.

In this systematic review, intervention group is described as NAFLD diagnosed participants carrying the PNPLA3 rs738409 gene. NAFLD was diagnosed either by histological examination of liver tissues taken by liver biopsy[24,25,27,28] or by radiological scanning either by Ultrasonography[26,29] of hepatobiliary system (USG) or computer tomography (CT) scan[30]. Participants in control group were non-diabetic, non-obese and biochemically and radiologically (USG) free from any liver pathology.

DNA extraction and PNPLA3 rs738409 genotyping were done from blood sample of participants using different standard methods (Table 5). Among the 7 studies, three studies have used DNA detection Kit (Genomix, IDT xGene capture platform and QIAamp DNA blood Mini Kit)[24,28,30] whereas, three studies used Phenol- Chloroform-isoamyl alcohol to extract DNA from blood sample[25,27,29], one study did not mention the methodology[26]. For PNPLA3 rs738409 genotyping, three of the studies have used TaqMan probe manufactured by Applied Biosystems, Foster City, CA, USA[24,27,28]; whereas four of the studies have used three different methods as follows, PCR-RFLP[29], MALDI- TOF mass spectrometry[26] and Illumina Global Screening Array[30] as well as Illumina Human 610-Quad Bead Chip on a Bead station 500G Genotyping system[25].

All the studies of diverse population (Asia, Europe, America) and ethnicity have been analysed for the linkage of PNPLA3 rs738409 gene in the development of NAFLD as well as evaluate the frequency of risk allele accompanying the carrier rs738409 genotype comparing with healthy control. In addition, one study analysed the association of PNPLA3 rs738409 with histologically type 4 (Matteoni 4) NAFLD[25,31], whereas, another recent study analyses the effect of carrier gene at the age of disease onset[30]. Two studies have ob- served the impact of PNPLA3 rs738409 gene with the severity of NAFLD, of which one is related with NAFLD related HCC[27], whereas another is associated with progression to NASH with liver fibrosis[28]. Three of the study also compares the homozygous allele GG in the carrier genotype linked to NAFLD among the different population[26-28,30].

Outcome of systemic review has reported using p values, odd ratios (OR), hazard ratios (HR) and confidence interval (CI). P value < 0.001 indicates statistically significant association of the PNPLA3 rs738409 gene with NAFLD, whereas, OR indicates strength of the as- sociation, OR > 1, indicates stronger association[32,33].

Hazard ratio also indicates the risk of association, HR > 1 indicates the PNPLA3 rs738409 gene increase risk of NAFLD[34]. OR and HZ are calculated at 95% confidence interval (CI)[35].

Outcome
Primary outcome

In this systematic review out of total 14,154 participants of diverse population, 3471 diagnosed NAFLD patient carrying PNPLA3 rs738409 gene have been compared with 10,683 healthy control. The systematic review has found that PNPLA3 rs738409 polymorphic gene is strongly linked to NAFLD and its progression to severe disease, P < 0.001(P value varies from 9.4 x 10^{-10} to 1.7610^{-16} and OR >1~4 at 95% CI) than non-carriers. The review also has elicited that the presence of minor allele frequency (G) with PNPLA3 rs738409, multiply the risk of NAFLD (from 0.15 to 4-fold) (HR 1.33%. 95% CI1.15 - 1.53, P < 0.0001 and OR 4.0 at 95% CI) than (C) allele carriers (Table 4). Moreover, PNPLA3 rs738409 (GG) homozygous genotype potentiate the risk of NAFLD and its progression to NASH with liver fibrosis by 3.29-fold than (CC) homozygous (P < 0.001, OR 3.29, 95%CI 1.504 - 7.225). All the studies have shown a strong relationship of PNPLA3 rs738409 polymorphic gene in the development of NAFLD (P < 0.001 and OR > 1, varies from ~4 in presence of homozygous GG genotype, whereas ~2.88 in heterozygous CG genotype). The systemic review has also observed, PNPLA3 rs738409 G allele is strongly associated with NAFLD-related HCC (unadjusted OR 2.046, 95% CI 1.47 - 2.84, P < 0.0001), where the G allele in PNPLA3 rs738409 bears dose- dependent effect in progression to NAFLD- related HCC and the risk becomes approximately double with each copy of G allele (OR 1.95, 95% CI 1.40 - 2.70, P < 0.0001).

In addition, presence of homozygous allele GG in PNPLA3 rs738409 increases the risk to 3.92-fold (unadjusted OR 3.92, 95% CI 2.06 - 7.48, P < 0.0001) than (CC) wild-type homozygous genotype (Table 4).

Secondary outcome

The review also ascertained that the risk allele (G) frequency of PNPLA3 was higher in intervention group than control group. Moreover, it has found the PNPLA3 (G) risk allele is distributed at a higher proportion among the Hispanic/Latino (HA) community (47.16%,95% CI

47.1 - 47.2) than in European American (EA, 22.8%) and African American (AA,13.7%) (Figure 2).

DISCUSSION

In this systematic review, 7 observational studies of diverse population and ethnicity over Asia, Europe and America, have been ana- lysed to determine the linkage of PNPLA3 rs738409 polymorphic gene in the development of NAFLD, compared with healthy control. In addition, the review also evaluated the risk allele frequency of PNPLA3 rs738409 polymorphic gene among the participants that considered significant risk of NAFLD. Moreover, it also has analysed the risk allele (G) frequency of PNPLA3 rs738409 polymorphic gene in NAFLD and its progression to severity, NASH, fibrosis and NAFLD related HCC. The outcome has suggested that PNPLA3 rs738409 polymorphic gene is strongly linked to NAFLD and its progression to sever disease, P < 0.001 (P value varies from 9.4 x 10^{-10} to 1.7610^{-16}) than non-carriers, whereas, presence of minor allele frequency (G) with PNPLA3 rs738409, multiply the risk of NAFLD (from 0.15 and 4- fold) (HR 1.33%. 95% CI1.15 - 1.53, P < 0.0001 and OR 4.0 at 95% CI) than (C) allele carriers (Table 4). Moreover, PNPLA3 rs738409 (GG) homozygous genotype potentiate the risk of NAFLD and its progression to NASH with liver fibrosis by 3.29-fold than (CC) homozygous (P < 0.001, OR 3.29, 95%CI 1.504-7.225)[28]. All the studies have shown a strong relationship of PNPLA3 rs738409 polymorphic gene in the development of NAFLD (P < 0.001 and OR > 1, varies from ~4 in GG genotype whereas, ~2.88 in CG genotype) and its progression to severe disease, even NAFLD related HCC (OR, 2.046 at 95% CI 1.47-2.84, P < 0.0001), the risk of which multiply when risk allele (G) and/ or (GG) homozygous genotype accompanying with than (C) allele or (CC) wild-type homozygous genotype respectively. There is no specific treatment of NAFLD and its consequences. Therefore, it would be beneficial to detect earlier the polymorphic gene with its risk allele frequency to predict the upcoming apparently innocent NAFLD, that could lead to a more serious hepatic condition.

In this study, 7 articles including 14,154 participants of which 3,471 diagnosed case of NAFLD (either by histology or radiology proven) carrying PNPLA3 rs738409 polymorphic gene have compared with 10,683 healthy control to elicit the genetic impact in NAFLD manifestation. Among them, 3,059 participants in four studies of which 1130 participants were diagnosed case of NAFLD by histological ex- amination of tissues taken from liver biopsy[24,25,27,28]. Three studies of 11,095 participants underwent radiological intervention of which 638 participants underwent ultrasonography (USG)[26,29] and 1703 participants went for computer tomography (CT) scan for diagnosis of NAFLD[30]. All the diagnosed NAFLD carrying PNPLA3 rs738409 polymorphic gene were compared with healthy control group to analyse the evidence for linkage of PNPLA3 rs738409 polymorphic gene in the development of NAFLD. The study has found a stronger relationship of PNPLA3 rs738409 polymorphic gene in the development of NAFLD (P value < 0.001) than non-carriers[6,8,24-29,36-39]. The study also has elicited that the presence of risk allele frequency (G) along with the polymorphic genotype, multiply the risk by many folds (from 0.15 to 4-fold) (HR 1.33%. 95% CI1.15 - 1.53, P < 0.0001 and OR 4.0 at 95% CI) than the non-carriers[29,30]. Two of the studies have observed PNPLA3 rs738409 G frequency is strongly

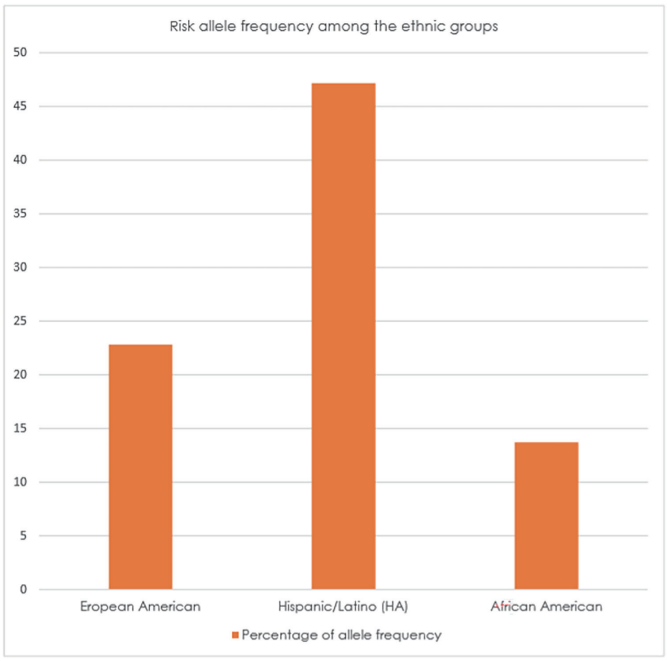

Figure 2: PNPLA3 rs738409 risk allele frequency G among the different ethnic population.
EA: European American; HA: Hispanic American; AA: African American

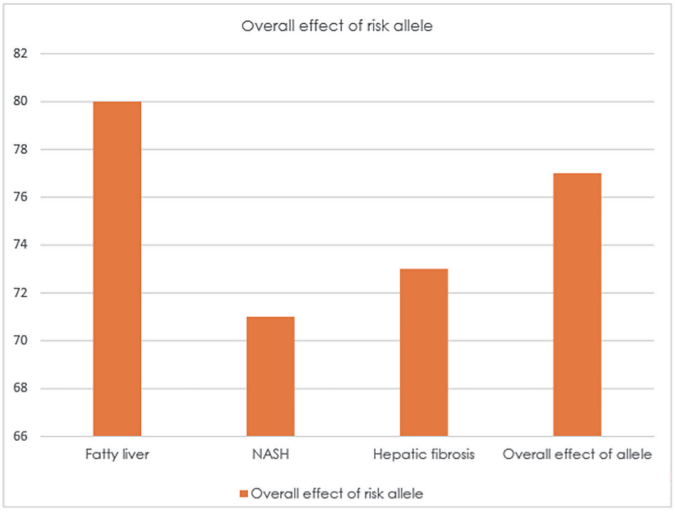

Figure 4: Overall effect or risk allele (%) non-alcoholic fatty liver disease, NASH & Fibrosis among Hispanic population (Data taken from Martínez., *et al*. 2017

related to progression to disease severity, NASH as well as liver fibrosis (P < 0.001, OR 1.96, 95% CI 1.47 - 2.62)[28,29], whereas, one study has found the PNPLA3 rs738409 C > G genotype strongly as- sociated with NAFLD-related HCC (OR, 2.046 at 95% CI 1.47 - 2.84, P < 0.0001)[27]. In addition, presence of G allele in PNPLA3 rs738409 bears dose dependent effect and doubles the risk of poorly differentiated HCC, the worst consequence of NAFLD (unadjusted OR, 1.95, 95% CI 1.40 - 2.70, P < 0.0001) of which the risk may multiply to 3,92 fold when accompanied by GG allele rather than CC allele (adjusted OR 3.92,95% CI 2.06 - 7.48, P < 0.001)[26,27]. In 2020, two separate study conducted in different population, one by Narayansamy., *et al*. (2020) in Indian population and other by Walker., *et al*. (2020) in American population, strongly supported the linkage of PNPLA3 rs738409 genotype with NAFLD[29,30]. Moreover, Narayansamy., *et al*. (2020) observed the polymorphic gene PNPLA3 rs738409 potentiate the chances of NAFLD by four-fold in presence of homozygous GG genotype (OR 4.0 at 95% CI) than heterozygous CG genotype (OR 2.881 at 95% CI)[29], whereas,

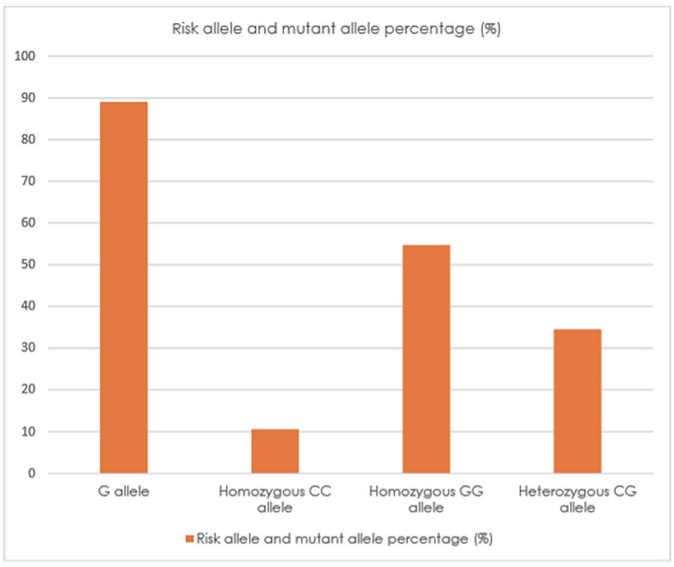

Figure 3: Frequency of risk allele and mutant allele (%) among the Hispanic population. Overall risk allele G, wild-type or homozygous mutant allele CC, Homozygous mutant allele GG and Heterozygous mutant allele GG (Data taken from Martínez., *et al*. 2017

Walker., *et al*. (2020) observed the chances of developing NAFLD increased by 15% (HR- 1.33%, 95% CI 1.15 - 1.53, p < 0.0001 and OR 4.0 at 95% CI) and could be reveal the risk of the disease earlier with the presence of the polymorphic gene[27,30] Walker., *et al*. (2020), also evaluated the presence of risk allele G frequency among the different ethnic populations and strongly supported the presence among the Hispanic/Latino (OR 1.43, 95% CI1.28 - 1.59, p < 0.0001) population[30].

PNPLA3 rs738409 G risk allele has found 3 mutant genotype, homozygous wild-type (CC), homozygous mutant (GG), and heterozygous mutant (CG) where homozygous wild-type genotype mutant (CC) is used to compare with mutant genotype, homozygous (GG) or heterozygous (CG)[40]. Several studies have demonstrated that disease severity of NAFLD is associated with dose dependent effect of homozygous GG mutant[26-28] whereas, dose dependent effect of heterozygotes CG increases the chances of hepatic fibrosis in NASH, Mazo., *et al*. 2019 noticed in a Brazilian study[28].

Another study in Hispanic population, Martínez., *et al*. in 2017, demonstrated that PNPLA3 rs738409 G variant genotype expression modulate the severity of NAFLD[41] (Figure 3 and 4). The study showed that 89% patient expressed risk allele G, of which CC-10.5%, CG-34.5%, GG-54.7% and overall allele frequency was 77%, fatty liver- 80%, NASH-71% and fibrosis-73%.

Hotta., *et al*. in 2010, compared 253 histologically diagnosed NAFLD with 578 healthy control and found a strong relation of PNPLA3 rs738409 genotype in NAFLD (P < 0.001) where risk allele frequency G found higher in NAFLD than control (0.60 vs 0.44 respectively), though he did not find the possibility of dose dependent effect of risk allele along with PNPLA3 rs738409 genotype in NAFLD development[24]. In 2012, two separate studies were conducted in two Asian countries, Japan and China by Kawaguchi., *et al*. and Peng., *et al*. respectively among their own population.

Kawaguchi., *et al*. conducted a GWAS among the Japanese population comparing 529 histologically diagnosed NAFLD with 942 healthy control and found PNPLA3 rs738409 genotype is strongly associated with genetically and clinically different subset, Matteoni type 4 NAFLD (P < 0.001, OR = 2.18, 95%CI: 1.81 - 2.63) that progress to NASH (P < 0.001, OR = 1.96, 95%CI: 1.47 - 2.62)[25,31].

Peng with his colleagues conducted a study among the Chinese population of 533 ultrasonological diagnosed NAFLD comparing with 533 healthy control and found statistically significant linkage of PNPLA3 rs738409 genotype in NAFLD (P < 0.005), whereas, risk allele frequency G with PNPLA3 rs738409 found higher in NAFLD than control (0.42 vs 0.34 respectively) which exerts dose dependent effect of risk allele on genotype[26].

In 2014, Liu., *et al*. noticed that PNPLA3 c444 C > G minor allele (G) strongly associated with NAFLD and double risk of HCC (OR 1.95,

95% CI 1.40 - 2.70, P < 0.0001), whereas, risk allele G exhibit added risk due to dose dependent effect (age and sex adjusted OR 2.046, 95% CI 1.47 - 2.84, P < 0.001), and presence of homozygous GG allele in PNPLA3 rs738409 exerts 3.92-fold higher risk than homozygous allele CC (OR 3.92, 95% CI 2.06 - 7.48), the outcome of which matched with previous meta-analysis done in 2014 by Singal., *et al*.[27,42].

The Brazilian study conducted by Mazo., et al. in 2019, and found that PNPLA3 rs738409 is strongly associated with NAFLD and its severity, where presence of G allele 2.29- fold increases the risk of NAFLD (P < 0.001, 95% CI 1.367 - 3.838). The presence of homozygous GG genotype in PNPLA3 rs738409 increases the risk by 3.29-fold of having NAFLD (OR 3.29 95% CI 1.504 - 7.225, P < 0.001) than homozygous CC. However, dose dependent heterozygotic CG mutant genotype increases the risk of hepatic fibrosis in NASH by 1.75-fold (OR 1.75 at 95% CI)[28].

The outcome of this systematic review has been matched with previously conducted studies including systematic reviews and meta- analysis[40,43,44]. One of the studies did not mention the chances of the dose-dependent effect of risk allele in SNP (PNPLA3 rs738409) in NAFLD[24]. However, the other four studies noted that risk allele G (CG or GG or both) contribute a significant dose-dependent effect with PNPLA3 genotype in NAFLD based on contemporary knowledge[26-28,30]. In this regard, we can reference the systematic review and meta-analysis conducted by Sookoian and his colleagues in 2011 over a diverse population of 5100 subject to evaluate the histological severity of the NAFLD linked to the PNPLA3 rs738409 polymorphism[43]. The study suggested that PNPLA3 rs738409, GG homozygous genotype strongly influenced hepatic fat accumulation leading to NAFLD and is 73% higher than CC homozygous allele (P < 0.001) as well as frequently provoke progression to NASH than CC homozygous (OR 3.488, 95% CI 1.859 - 6.543, $P < 2 \times 10^{-4}$).

Moreover, the presence of GG homozygous with PNPLA3 rs738409 potentiate the risk of necro-inflammatory score by 3.2-fold and liver fibrosis by 3.2- fold, evident by elevation of serum alanine aminotransferase (ALT) ($P < 1 \times 10^{-9}$).

In 2014, Singal., *et al*. conducted a large systematic review and meta-analysis of 24 studies of which 9 studies containing 2937 participants over Caucasian adult in Europe[42]. The study found PNPLA3 rs738409 genotype significantly increase the risk of HCC in cirrhotic patient (OR 1.40, 95% CI 1.12 - 1.75) of which GG homozygous allele has got more chances to develop HCC than CG or CC homozygous allele (OR 1.45, 95% CI 1.11 - 1.80).

Dai and his colleagues in 2019, conducted another systematic review and meta- analysis of 21 studies including 4,266 participants and found that PNPLA3 rs738409 genotype is strongly associated with NAFLD and its progression to severe disease evident by histological injury and fibrosis, where PNPLA3 rs738409 G allele considered a potent risk factor for NAFLD of which GG homozygous allele significantly potentiate hepatic injury in NAFLD than CC homozygous allele (OR 4.01, 95% CI 2.93-5.49)[40].

Limitations of the Study

Most of the studies selected for this systematic review have small sample size, therefore may be underpower to provide an accurate outcome. Risk of bias was also not properly evaluated. NAFLD was diagnosed by different methods, histology, ultrasonography and computer tomography, hence, there may have been human error or difference in grading of NAFLD and its progression due to variation of methods. Sensitivity and specificity may differ due to different method and equipment used to extract DNA sequence and rs738409 genotyping.

CONCLUSION

The systematic review has clearly established the linkage of the PNPLA3 rs738409 polymorphic gene in the development of NAFLD and the risk allele G, presence of which the polymorphic gene PNPLA3 rs738409, potentiate the severity of NAFLD to NASH, fibrosis, even HCC. Though the effect of the association is almost universal, amongst populations, Hispanic/Latino people are more like to suffer NAFLD. This study also elicits that the PNPLA3 rs738409 G polymorphic gene could be detected much earlier than the manifestation of disease, therefore we can detect the disease earlier which, with appropriate treatment will prevent further deterioration. However, we have been able to evaluate the linkage of PNPLA3 rs738409 polymorphic gene as well as risk allele G in NAFLD with its severity that could modulate the molecular pathogenesis of the disease but exact mechanism still is in hypothesis. Therefore, more extensive research studies would be recommended to elucidate its molecular mechanisms.

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