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ORIGINAL ARTICLE

# A susceptibility putative haplotype within NLRP3 inflammasome gene influences ischaemic stroke risk in the population of Punjab, India

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## Abstract

Despite strong genetic implications of NLRP3 inflammasome, its examination as genetic determinant of ischaemic stroke (IS) remains to be done in Punjab, which has been investigated in this study. In this case control study, 400 subjects (200 IS patients, 200 stroke free controls) were included. Contributions of 5 single nucleotide polymorphisms (SNPs) including a functional SNP within NLRP3 gene (rs10754558, rs4612666, rs2027432, rs3738488 and rs1539019) for the risk of IS were investigated through genetic models after correcting the effect of significant variables. Plasma levels of three pro-inflammatory markers, that is, C-reactive protein (CRP), interleukin-1beta (IL-1$\beta$) and interleukin-18 (IL-18) were measured by enzyme-linked immunosorbent assays (ELISA). Minor alleles of 3 out of 5 SNPs (rs10754558, rs4612666 and rs1539019) exhibited association with IS risk in additive, recessive and multiplicative models. Multivariable regression analysis confirmed that higher levels of systolic blood pressure ($\beta \pm$ SE: 1.42 $\pm$ 0.57, $p = .013$), CRP ($\beta \pm$ SE: 1.22 $\pm$ 0.41, $p = .003$), IL-1$\beta$ ($\beta \pm$ SE: 1.78 $\pm$ 0.88, $p = .043$) and IL-18 ($\beta \pm$ SE: 1.13 $\pm$ 0.49, $p = .021$) were independent risk predictors for IS. Haplotype analysis revealed a susceptibility putative haplotype GTGTA, which approximately doubled the IS risk (OR: 1.98, 95% CI: 1.12–3.78, $p = .04$) in dominant mode after adjusting the effect with confounding variables. This susceptibility putative haplotype GTGTA was significantly associated with increased concentrations of CRP ($\beta = 1.21$, $p = .014$) and IL-1$\beta$ ($\beta = 1.53$, $p = .034$) in dose-dependent manner (less in carriers of 1 copy than those who had 2 copies of GTGTA).

The present study has revealed a susceptibility putative haplotype GTGTA within NLRP3 gene, carriers of which have double the risk of IS by having increased plasma levels of CRP and IL-1$\beta$ in a dose-dependent manner.

**Abbreviations:** 3′UTR, 3 prime untranslated region; AIC, Akaike information criterion; ASC, Apoptosis-associated speck-like protein containing a CARD; CE, Cardioembolism; CRP, C-reactive protein; DALY, Disability adjusted life years; DBP, Diastolic blood pressure; ELISA, Enzyme-linked immunosorbent assay; HDL, High-density lipoprotein; IL-18, Interleukin 18; IL-1$\beta$, Interleukin 1 beta; IS, Ischaemic stroke; LAA, Large artery atherosclerosis; LDL, Low-density lipoprotein; MAF, Minor allele frequency; NLRP3, Nod-like receptor family pyrin domain containing 3; PCR, Polymerase chain reaction; PRR, Pattern recognition receptor; QVSFS, Questionnaire for verifying stroke free status; $R^2h$, Stram's haplotype uncertainty measure; SBP, Systolic blood pressure; SNP, Single nucleotide polymorphism; SVO, Small vascular occlusion; TC, Total cholesterol; TG, Triglyceride; TOAST, Trial of ORG 10172 in acute stroke treatment.

KEYWORDS
dose-dependent, India, ischaemic stroke, NLRP3 gene polymorphism, pro-inflammatory variables, risk predictors

## 1 | INTRODUCTION

Ischaemic stroke (IS) is a multifactorial condition, which is manifested as a medical emergency with focal neurological dysfunction, lasting more than 24 h and confirmed by focal infarction (Hankey, 2017). It accounts for more than 87% of all strokes (Donkor, 2018). Being the third largest cause of death worldwide in the elderly population, ischaemic stroke poses a huge threat to human population (GBD 2019 Stroke Collaborators, 2021). Almost six million people die every year succumbing to the dreadfulness of IS, which is largely contributed by developing countries. In India, it is highly impinging on health statistics as early onset of age, higher case fatality rate, substantial disability adjusted life years (DALY) loss and alarming post-stroke ramifications are highly rampant (Pandian & Sudhan, 2013).

In the clinical chapters of stroke pathophysiology, atherosclerosis is the primary culprit and vascular inflammation triggers and perpetuates it (Ahmad et al., 2014; Iadecola & Anrather, 2011). The inflammatory trigger by lipid rich foam cells in atherosclerosis is already known (Libby et al., 2002). It has been observed that the micro cholesterol crystals present in atherosclerotic lesions induce danger signals, which are actually the foremost signals of inflammation (Duewell et al., 2010). These very early signals are sensed by a pattern recognition receptor (PRR) named Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome. It is a multiprotein complex which activates and initiates robust inflammatory response towards arterial occlusion and contributes to the advancement of cerebral ischaemia (Gao et al., 2017; Glass et al., 2010). In the process of inflammasome activation, NLRP3 senses several atherogenic stimuli and recruits adapter proteins; apoptosis-associated speck-like protein containing a card (ASC) and caspase-1 leading to maturation and release of pro-inflammatory cytokines; interleukin 1-beta (IL-1$\beta$) and interleukin-18 (IL-18) (Gao et al., 2017). Present abundantly in atheromatous lesions, both IL-1$\beta$ and IL-18 help in plaque development and its propagation to ischaemic stroke (Mallat et al., 2001; Varghese et al., 2016). Therefore, efforts to repress inflammasome by antagonists and inhibitors has been seen to improve neuronal health, reduction in infarct size, suppressing neuroinflammation and oedema in stroke (Glass et al., 2010; Ishrat et al., 2015; Zhu et al., 2021).

Studies have confirmed that NLRP3 inflammasome activation is genetically controlled (Hitomi et al., 2009; Tong et al., 2015). Present on 1q44 position, NLRP3 gene contains 9 exons, around 60 SNPs and encodes a protein called cryopyrin. Mainly present in chondrocytes and leukocytes, this immune regulatory protein responds to toxins, pyrogens and cellular injury because of its extra-sensitive sensing potential of very early incidents of inflammation and degeneration (Lamkanfi & Dixit, 2009). The sooner it senses cellular debris, the sooner it gets activated and starts secreting IL-1$\beta$ and IL-18. These pro-inflammatory cytokines initiate, augment and stabilize vascular inflammation leading to ischaemia (Zhu et al., 2016). It has been confirmed that minor allele (G) of rs10754558 SNP enhances 1.3-fold higher NLRP3 mRNA expression and its stability (Hitomi et al., 2009). This allele of functional SNPhas been observed to be associated with higher risk of ischaemic stroke in Swedish and Chinese populations (Cheng et al., 2018; Kastbom et al., 2015; Lv et al., 2020; Zhu et al., 2016).

Despite such a strong genetic association, the participation and contribution of those genetic variants that trigger and activate NLPR3 inflammasome in ischaemic stroke patients have not been investigated in India so far. Therefore, the objective of the study is to investigate the genetic contribution of SNPs within NLRP3 gene and their relationship with pro-inflammatory markers such as C-reactive protein (CRP), IL-1$\beta$ and IL-18 for the risk of IS in the population of Punjab, India.

## 2 | MATERIALS AND METHODS

### 2.1 | Study population

Present prospective case-control study comprises 200 ischaemic stroke patients and 200 stroke free control subjects of the same ethnicity, ranging in age from 56 to 85 years. All the patients were hospitalized in the Department of Neurology/neurosurgery in Prime Multispecialty Hospital, MK Neuro centre and Bhatia Neuro and Multispecialty Hospital, Patiala from November 2018 to November 2021. Initially, 680 subjects were screened but after applying inclusion/exclusion criteria (Figure 1), 219 subjects were enrolled for the study. Excluding some unconfirmed cases, finally, 200 confirmed ischaemic stroke patients were included after verification by the criteria of 'Trial of ORG 10172 in Acute Stroke Treatment' (TOAST) (Adams et al., 1993). All the patients had undergone magnetic resonance imaging or computed tomography. Two neurologists resolved the identification of IS subtypes independent of each other. Inter-observer reliability for differentiating stroke subtype was substantial ($\kappa$ = 0.71–0.78). For control subjects 654 individuals were screened initially. After applying inclusion/exclusion criteria (Figure 1), 200 subjects were tested and verified normal after applying Questionnaire for Verifying Stroke Free Status (QVSFS) (Jones et al., 2001). Inter-observer reliability for the identification of control subject was almost perfect ($\kappa$ = 0.87). All the subjects gave their written consent and the study was approved by Institutional Ethical Committee affiliated to Punjabi University, Patiala (IEC no. 2019/130). The present research complied strictly to the ethical guidelines prescribed in the Declaration of Helsinki.

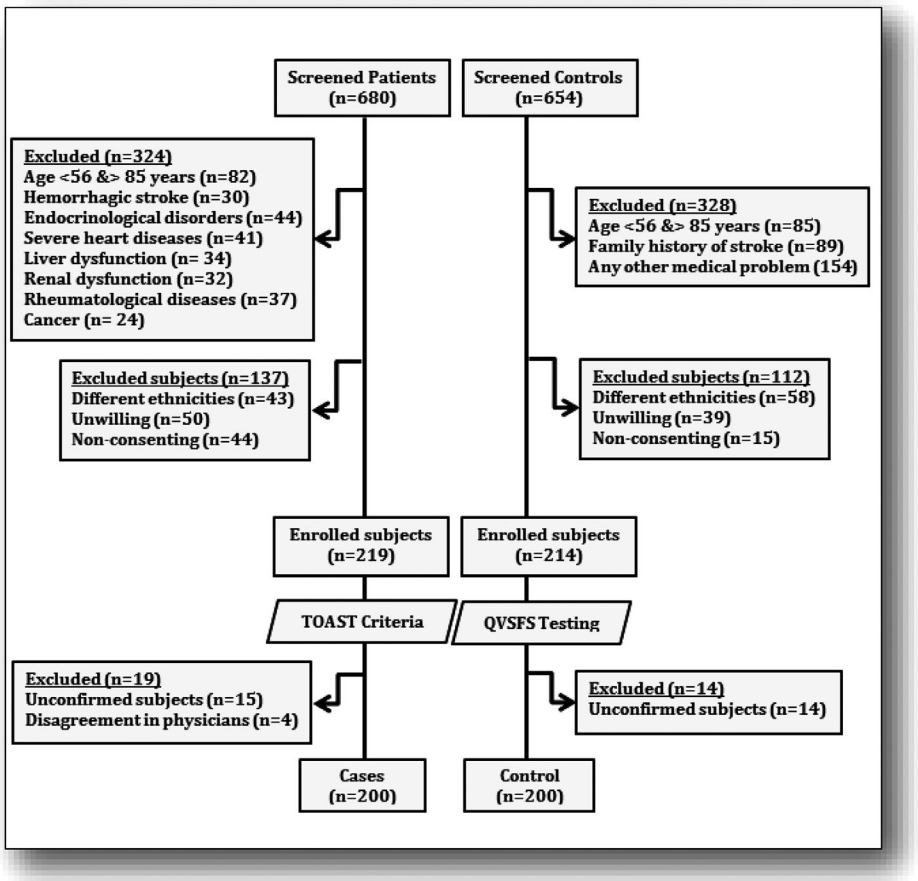


**FIGURE 1** Data collection method. TOAST: trial of ORG 10172 in acute stroke treatment, QVSFS: questionnaire for verifying stroke free status

## 2.2 | Risk variables

Demographic characteristics of age and gender along with information on other risk parameters such as smoking, alcohol drinking and physical activity was collected from the subjects through questionnaire or medical records. Arterial blood pressure was noted down at baseline. All lipid parameters were available in the medical records of ischaemic stroke patients. Plasma levels of CRP, IL-1$\beta$ and IL-18 were determined using enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, Waltham, MA, USA). All the tests were done on a microplate reader (Biotek Instruments Inc., Winooski, VT, USA) with analytical sensitivities of the kits <10, 1.2 and 6.25 pg/ml for CRP, IL-1$\beta$ and IL-18, respectively. While assaying these three parameters, coefficients of inter and intra-assay variation was observed to be less than 6%.

## 2.3 | SNP selection and genotyping

SNPs within NLRP3 gene were selected in the present study following four criteria: (i) SNP having functional effect on NLRP3 mRNA, (ii) SNP must be validated by independent submissions to the NCBI reference SNP cluster (https://www.ncbi.nlm.nih.gov/snp/), (iii) SNP should be polymorphic (having allele frequency at least 5%) and (iv) previously shown association with either NLRP3 inflammasome activation or with inflammatory disorders. In this way, 5 SNPs rs10754558 (3′UTR), rs4612666 (intron 7), rs2027432 (3661 bp upstream), rs3738448 (2667 bp upstream) and rs1539019 (intron 8) were selected and genotyped.

DNAs were extracted from whole blood with salting out procedure (Miller et al., 1988). Amplification of DNA was performed on Bio-Rad T100™ Thermal Cycler (Hercules, California, USA). Twenty-five microlitres of polymerase chain reaction (PCR) mixture contained 2 $\mu$l of DNA template, 12.5 $\mu$l of master mix, 5.5 $\mu$l of nuclease free water and 2.5 $\mu$l each of forward and reverse primers. PCR cycle conditions for the amplification of the DNA were: initial denaturation condition at 95°C for 1 min and then 30 cycles with denaturation of DNA at 94°C for 40 s, annealing at 55°C for 30 s and extension at 72°C for 30 s with final extension at 72°C for 5 min. After amplification, the products of the SNPs rs10754558, rs4612666, rs2027432, rs3738448 and rs1539019 were digested with high fidelity restriction enzymes, *Mbo*I, *Bps*I, *Bst*EII, *Tse*I and *Bse*RI, respectively (New England Biolabs Inc., Massachusetts, USA) as shown in Table 1. Digested products were analysed on 2% agarose gel having ethidium bromide to identify the presence or absence of the restriction site (Supplementary Figures). To avoid any bias, genotyping was performed by blinding the case control status of the samples. Ten per cent of the known positive/negative samples were re-analysed to confirm the internal consistency.

**TABLE 1** SNPs within NLRP3 gene showing their domain, primer sequence and restriction enzyme

| dbSNP | Domain | Alleles | Primer sequence | Restriction enzyme |
|---|---|---|---|---|
| rs10754558 | 3′ UTR Variant | C/**G** | F: 5′-CAGGAACAGCATCGGGTGTTGAT-3′<br>R: 5′-GCTGCCATAAAATTTCAACATAA-3′ | *Mbo*I |
| rs4612666 | Intron 7 Variant | C/**T** | F: 5′-TGCTTAAGGCCATTAATTGTG-3′<br>R: 5′-CTCCACCATGGACAAGGAAG-3′ | *Bps*I |
| rs2027432 | Upstream variant | G/**A** | F: 5′-CACCATACACCTTTTTTCTCGGGC-3′<br>R: 5′-GGGCCTCCATTTTCTCATCTGTG-3′ | *Bst*EII |
| rs3738448 | Upstream variant | G/**T** | F: 5′-TCTCTCTGCCTCTGCTCTGA-3′<br>R: 5′-AACCAGGACAAGTTCTGCCC-3′ | *Tse*I |
| rs1539019 | Intron 8 | C/**A** | F: 5′- ATTTCTTCTGGCGTCTCCAA-3′<br>R: 5′- CTGCTGAAGTCGTGGGTGTA-3′ | *Bse*RI |

3′UTR: 3 prime untranslated region.
*Note*: Minor allele is shown in bold face.

### 2.4 | Statistical power

Statistical power in the present study was calculated as a priori power analysis according to the method given by Cohen (1988) using software G*Power. The power ($1 - \beta$) was calculated which revealed that sample size of 400 (200 cases, 200 controls) would deliver 88% statistical power to reject correct null efficiently with Cohen's $D = 0.5$ and significance $\alpha = 0.05$ (Supplementary file).

### 2.5 | Statistical analysis

All the differences between variables were analysed by either Student's *t*-test or Mann-Whitney *U* test, if values were continuous, otherwise differences in categories were analysed by chi-square test. Allele frequencies were calculated from genotype numbers and deviation from Hardy-Weinberg equilibrium was checked using chi-square statistics. Logistic regression analysis was performed to examine the contribution of respective SNPs under unadjusted and adjusted additive, dominant, recessive and multiplicative models. Extent and degree of the independent contribution of risk variables for IS risk was assessed with univariable and multivariable regression analysis. Haplotypes were determined using genotype data in software Arlequin ver. 3.01. To identify IS risk conferred by different haplotypes, crude and adjusted odds ratio (corrected with significant risk variables) were determined using logistic regression models after taking most common haplotype as referent. Difference between haplotypes was calculated and *p* value was adjusted with Bonferroni correction. The functional effect of the susceptibility putative haplotype was further examined with Wald's statistics through dominant, recessive, multiplicative or general models. The model, which best explained its impact on the risk of IS was selected with least Akaike information criterion (AIC) value and highest haplotype uncertainty measure ($R^2h$). The association of significant risk predictors with susceptibility putative haplotype was analysed according to its dosage (0 copy, 1 copy or 2 copies) by using logistic regression analysis allowing two-tailed $p < .05$, otherwise for multiple comparisons, significance level was set at 1% ($p < .01$).

## 3 | RESULTS

### 3.1 | Baseline features of the study group

Out of 200 confirmed IS patients, 82% (164) had large artery atherosclerosis (LAA) and 7% (14) had cardioembolism (CE). Five per cent each (10) had small vascular occlusion (SVO) and stroke of determined aetiology whereas 1% (2) had stroke of undetermined aetiology. Demographic, physiological, biochemical and genetic characteristics of cases and controls revealed that both the groups were matched for age and gender ($p > .05$) as shown in Table 2. Smoking, systolic blood pressure (SBP), triglyceride (TG) and low-density lipoprotein (LDL) levels were observed to be significantly dissimilar between cases and controls ($p < .001$). No significant differences were found for alcohol drinking, diastolic blood pressure (DBP), high-density lipoprotein (HDL) and total cholesterol (TC) levels between both the groups. This would have been validated by splitting the data in different age groups, but doing so would significantly reduce the statistical power of the study and inferences would be erroneous. IS patients had significantly higher log median values of CRP, IL-1$\beta$ and IL-18 than controls. Five SNPs of NLRP3 gene were investigated, results of which exhibited that minor allele frequencies (MAFs) of rs10754558, rs4612666 and rs1539019 were significantly higher in cases than controls ($p < .05$). MAFs of rs2027432 and rs3738448 were similar in both the groups ($p > .05$).

### 3.2 | Identification of independent risk variables for ischaemic stroke

Univariable regression analysis was performed by taking IS risk as dependent variable (Table 3), which revealed that higher levels of SBP (95% CI: 0.64–3.22, $p = .004$), LDL (95% CI: 0.30–2.92, $p = .016$), CRP (95% CI: 0.17–2.37, $p = .023$), IL-1$\beta$ (95% CI: 0.37–3.63, $p = 0.016$) and IL-18 (95% CI: 0.15–2.72, $p = .029$) were associated significantly for the risk of IS. It was revealed that gender, smoking, alcohol drinking, DBP, TG, HDL and TC did not influence the IS risk ($p > .05$). All the variables that showed significant association in univariable model were included

**TABLE 2** General features of the study group

| Risk variables | Cases ($n = 200$) | Controls ($n = 200$) | 95% CI | $p$ Value |
|---|---|---|---|---|
| Age years (mean ± SD) | 70.95 ± 9.10 | 69.90 ± 9.07 | −0.73 to 2.83 | .24 |
| Gender (men/women) | 114/86 | 117/83 | 0.63 to 1.40 | .76 |
| Current smokers, *n* (%) | 59 (29.5) | 30 (15.0) | 1.45 to 3.88 | **<.001** |
| Non-smokers, *n* (%) | 141 (70.5) | 170 (85.0) | | |
| Current alcohol drinkers, *n* (%) | 113 (56.5) | 101 (50.5) | 0.86 to 1.89 | .23 |
| Non-drinkers, *n* (%) | 87 (43.5) | 99 (49.5) | | |
| Systolic blood pressure (mmHg) | 148.94 ± 12.70 | 119.74 ± 10.21 | 26.93 to 31.46 | **<.001** |
| Diastolic blood pressure (mmHg) | 82.39 ± 7.82 | 81.30 ± 6.85 | −0.35 to 2.53 | .13 |
| Lipid parameters | | | | |
| Triglycerides (mg/dl)ł | 2.25 (2.21,2.28) | 2.15 (2.10,2.20) | 0.09 to 0.11 | **<.001** |
| Low-density lipoproteins (mg/dl) | 145.02 ± 21.74 | 126.35 ± 24.08 | 14.16 to 23.17 | **<.001** |
| High-density lipoproteins (mg/dl) | 46.05 ± 8.43 | 45.23 ± 5.97 | −0.61 to 2.25 | .26 |
| Total cholesterol (mg/dl) | 181.09 ± 31.01 | 180.06 ± 16.91 | 3.88 to 5.94 | .68 |
| Inflammatory markers | | | | |
| C-reactive protein (mg/L)ł | 0.90 (0.70, 1.18) | 0.36 (0.29, 0.47) | 0.45 to 0.56 | **<.001** |
| IL-1$\beta$ (pg/ml)ł | 1.17 (1.11, 1.22) | 0.48 (0.37, 0.56) | 0.67 to 0.71 | **<.001** |
| IL-18 (pg/ml)ł | 2.52 (2.40, 2.58) | 2.25 (2.18, 2.32) | 0.23 to 0.27 | **<.001** |
| NLRP3 gene/SNPs | | | | |
| rs10754558, MAF ± SEP‡ | 0.42 ± 0.035 | 0.32 ± 0.035 | −0.003 to 0.20 | **.04** |
| rs4612666, MAF ± SEP‡ | 0.46 ± 0.035 | 0.35 ± 0.034 | 0.014 to 0.21 | **.02** |
| rs2027432, MAF ± SEP‡ | 0.10 ± 0.021 | 0.08 ± 0.019 | −0.04 to 0.08 | .48 |
| rs3738448, MAF ± SEP‡ | 0.15 ± 0.025 | 0.16 ± 0.026 | −0.08 to 0.06 | .78 |
| rs1539019, MAF ± SEP‡ | 0.37 ± 0.034 | 0.27 ± 0.031 | 0.01 to 0.19 | **.03** |

*Note*: Values are numbers, percentages or mean ± SD except łwhere values are log median (interquartile range) and ‡where values are minor allele frequencies ± standard error of proportion. $p$ Values are according to chi-square test for categorical variables, $t$ test for continuous variables and Mann-Whitney test for log-transformed median values.
Bold values indicate statistically significant difference.

in multivariable binary logistic regression analysis, which exposed that higher levels of SBP (95% CI: 0.30–2.54, $p = .013$), CRP (95% CI: 0.42–2.02, $p = .003$), IL-1$\beta$ (95% CI: 0.06–3.50, $p = .043$) and IL-18 (95% CI: 0.17–2.09, $p = .021$) were independent risk predictors for IS. A higher LDL level failed to influence IS risk in multivariable model. Analysis revealed that higher level of IL-1$\beta$ (>3 pg/ml) was the strongest risk predictor for IS ($\beta \pm$ SE: 1.78 ± 0.88) followed by higher SBP ($\beta \pm$ SE: 1.42 ± 0.57), CRP ($\beta \pm$ SE: 1.22 ± 0.41) and IL-18 ($\beta \pm$ SE: 1.13 ± 0.49).

## 3.3 | Genetic association of NLRP3 gene SNPs with IS risk through genetic models

Genetic contribution of individual SNPs within NLRP3 gene was examined based on possession of risk alleles after taking major allele as referent (Table 4). All the alleles of 5 SNPs were observed to be in Hardy-Weinberg equilibrium ($p > .05$). In adjusted model, minor allele G of SNP rs10754558 exhibited IS risk through additive in homozygous (OR: 2.16; 95% CI: 1.14–4.09, $p = .03$), dominant (OR: 1.65; 95% CI: 1.10–2.47, $p = .02$) and multiplicative(OR: 1.48; 95% CI: 1.11–1.98, $p = .01$) modes. Minor allele T of SNP rs4612666 revealed genetic association with IS risk through additive in heterozygous (OR: 1.68; 95% CI: 1.09–2.60, $p = .003$), homozygous (OR: 2.21; 95% CI: 1.14–4.28, $p = .03$), dominant (OR: 1.77; 95% CI: 1.16–2.69, $p = .01$) and multiplicative (OR: 1.46; 95% CI: 1.09–1.94, $p = .01$) modes. Similarly, minor allele T of SNP rs1539019 showed IS risk through additive in heterozygous (OR: 1.78; 95% CI: 1.16–2.71, $p = .01$), homozygous (OR: 2.11; 95% CI: 1.06–4.20, $p = .047$), dominant (OR: 1.84; 95% CI: 1.23–2.74, $p = .004$) and multiplicative (OR: 1.57; 95% CI: 1.16–2.13, $p = .004$) modes. Other SNPs, that is, rs2027432 and rs3738448 did not show any genotype-specific associations for IS risk.

## 3.4 | Haplotype association and their functional implications exhibited in best fit model

Haplotypes were generated from the genotype data in the order of rs10754558, rs4612666, rs2027432, rs3738448 and rs1539019 (Table 5). Out of expected 32 haplotypes, only 17 were evident and out of these 11 haplotypes had frequencies less than 0.05 hence, excluded from the analysis. Finally, six putative haplotypes captured 83% of genetic variation within IS patients and 68% genetic variation for

**TABLE 3** General and independent association of variables for the risk of ischaemic stroke

| | Univariable model | | | | Multivariable model | | | |
|---|---|---|---|---|---|---|---|---|
| Variables | $\beta \pm$ SE | Exp ($\beta$) | 95% CI | *p* Value | $\beta \pm$ SE | Exp ($\beta$) | 95% CI | *p* Value |
| Gender | 1.17 ± 0.65 | 3.22 | −0.102 to 2.44 | .071 | —- | —- | —- | —- |
| Smoking | 1.23 ± 0.61 | 3.42 | −0.04 to 2.50 | .058 | —- | —- | —- | —- |
| Alcohol drinking | 0.8 ± 0.56 | 2.22 | −0.30 to 1.90 | .154 | —- | —- | —- | —- |
| SBP (mmHg) | 1.93 ± 0.66 | 6.89 | 0.64 to 3.22 | **.004** | 1.42 ± 0.57 | 4.14 | 0.30–2.54 | **.013** |
| DBP (mmHg) | 0.98 ± 0.59 | 2.66 | −0.18 to 2.14 | .096 | —- | —- | —- | —- |
| TG (mg/dl) | 0.93 ± 0.63 | 2.53 | −0.30 to 2.16 | .140 | —- | —- | —- | —- |
| HDL (mg/dl) | 1.12 ± 0.61 | 3.06 | −0.08 to 2.32 | .066 | —- | —- | —- | —- |
| TC (mg/dl) | 0.83 ± 0.44 | 2.29 | −0.03 to 1.69 | .060 | —- | —- | —- | —- |
| LDL (mg/dl) | 1.61 ± 0.67 | 5.00 | 0.30 to 2.92 | **.016** | —- | —- | —- | —- |
| CRP (mg/L) | 1.27 ± 0.56 | 3.56 | 0.17 to 2.37 | **.023** | 1.22 ± 0.41 | 3.39 | 0.42–2.02 | **.003** |
| IL-1$\beta$ (pg/ml) | 2.00 ± 0.83 | 7.39 | 0.37 to 3.63 | **.016** | 1.78 ± 0.88 | 5.93 | 0.06–3.50 | **.043** |
| IL-18 (pg/ml) | 1.44 ± 0.66 | 4.22 | 0.15 to 2.72 | **.029** | 1.13 ± 0.49 | 3.09 | 0.17–2.09 | **.021** |

SBP: systolic blood pressure, DBP: diastolic blood pressure, TG: triglycerides, HDL: high-density lipoproteins, TC: total cholesterol, LDL: low-density lipoprotein, CRP: C-reactive protein, IL-1$\beta$: interleukin 1-beta, IL-18: interleukin-18.
*Note*: Groups in models are Gender: Men vs. women, Smoking: No vs. Yes, Alcohol drinking: No vs. Yes, SBP: ≤120 vs. >120 mmHg, DBP: ≤80 vs. >80 mmHg, TG: ≤150 vs. >150 mg/dl, HDL: ≥40 vs. <40 mg/dl, TC: ≤200 vs. >200 mg/dl, LDL: ≤100 vs. >100 mg/dl, Lp(a): ≤29 vs. >29 mg/dl, CRP: ≤3 vs. >3 mg/L, IL-1$\beta$: ≤3 vs. >3 pg/ml, IL-18: ≤120 vs. >120 pg/ml.
Bold values indicate statistically significant association.

control subjects. Major alleles of 4 SNPs at position 1, 2, 3 and 5 and minor allele of SNP rs3738488 at position 4 constituted a putative haplotype CCGTT, which appeared to be the most prevalent in cases and controls, hence it was considered referent for the analysis. Minor alleles of all the studied NLRP3 SNPs at all the positions except third, where major allele participated to constitute a putative haplotype GTGTA, which was observed to be risky for the IS risk (OR: 2.16; 95% CI: 1.10–4.24, $p = .03$). Inter-group comparisons of this putative haplotype after Bonferroni correction also showed significant differences ($p = .02$). Further analysis after adjusting the effect of this risky putative haplotype with significant risk variables (SBP, CRP, IL-1$\beta$ and IL-18) revealed that it was in fact a susceptibility putative haplotype, carriers of which were at two times higher risk of IS than those who did not have it (OR: 1.98; 95% CI: 1.12–3.78, $p = .04$).

In order to understand that in which best possible way of genetic model, this susceptibility putative haplotype GTGTA influenced the risk of IS, rigorous analysis was done using Wald's statistics (Table 6). Model was selected on the basis of least AIC (Akaike information criterion) and highest $R^2h$ (Stram's haplotype uncertainty measure). Analysis verified that susceptibility putative haplotype GTGTA residing within NLRP3 gene influenced the risk of IS in dominant mode ($\beta \pm$ SE: 1.95 ± 0.58, $p < .001$).

### 3.5 | Association of susceptibility putative haplotypes with independent risk predictors

Association of susceptibility putative haplotype GTGTA with independent risk predictors was analysed according to possession of no copy, one copy or two copies (Figure 2). Analysis exposed that susceptibility putative haplotype GTGTA was significantly associated with CRP ($\beta = 1.21$, $p = .014$) and IL-1$\beta$ ($\beta = 1.53$, $p = .034$) levels but not with SBP ($\beta = 0.018$, $p = .34$) and IL-18 ($\beta = 0.012$, $p = .26$). It was revealed that this putative haplotype influenced in dose-dependent manner as carriers of one copy or two copies of GTGTA had higher values of CRP and IL-1$\beta$ levels (0.8 and 0.88 mg/L) than those who did not have this putative haplotype (0.65 mg/L).

## 4 | DISCUSSION

Almost 20 years ago, Martinon et al. (2002) unwrinkled for the first time, the dilemma of inflammation activation by suggesting that a multiprotein complex exists (inflammasome), assembly of which activates Caspase-1 and triggers cascade of pro-inflammatory markers such as IL-1$\beta$ and IL-18. After 6 years, it was observed that NLRP3 inflammasome activation has some genetic implications (Kastbom et al., 2008). Consequently, it was discovered that SNP rs10754558 is functional and regulates mRNA expression of NLRP3 inflammasome (Hitomi et al., 2009). For the first time, present study has exposed that a susceptibility putative haplotype (GTGTA) within NLRP3 gene influences IS risk and correlates with dose-dependent increased concentrations of IL-1$\beta$ and IL-18 in the population of Punjab, India.

Five SNPs within NLRP3 gene (rs10754558, rs4612666, rs2027432, rs3738448 and rs1539019) have been investigated, which reveal that minor allele G of functional SNP rs10754558 associates significantly with IS risk in additive, dominant and multiplicative models after adjusting the effect of confounding variables (SBP, CRP,

**TABLE 4** Genetic association of NLRP3 gene SNPs with the risk of ischaemic stroke

| SNPs/genetic model | Cases ($n = 200$) | Controls ($n = 200$) | Unadjusted OR (95% CI) | $p$ Value | ŧAdjusted OR (95% CI) | $p$ Value |
|---|---|---|---|---|---|---|
| rs10754558 | | | | | | |
| CC | 66 (33) | 93 (46.5) | Referent | | Referent | |
| CG (additive) | 101 (50.5) | 87 (43.5) | 1.64 (1.07–2.51) | **.03** | 1.53 (1.00–2.34) | .06 |
| GG (additive) | 33 (16.5) | 20 (10) | 2.33 (1.23–4.40) | **.01** | 2.16 (1.14–4.09) | **.03** |
| CC vs. CG + GG (dominant) | 66 vs. 134 | 93 vs. 107 | 1.76 (1.18–2.65) | **.008** | 1.65 (1.10–2.47) | **.02** |
| CC + CG vs. GG (recessive) | 167 vs. 33 | 180 vs. 20 | 1.78 (0.98–3.22) | .07 | 1.71 (0.94–3.11) | .10 |
| 2CC+CG vs. CG+2GG (multiplicative) | 233 vs. 167 | 273 vs. 127 | 1.54 (1.15–2.06) | **.004** | 1.48 (1.11–1.98) | **.01** |
| rs4612666 | | | | | | |
| CC | 52 (26) | 81 (40.5) | Referent | | Referent | |
| CT (additive) | 113 (56.5) | 99 (49.5) | 1.78 (1.14–2.76) | **.01** | 1.68 (1.09–2.60) | **.03** |
| TT (additive) | 35 (19) | 20 (10) | 2.73 (1.42–5.22) | **.004** | 2.21 (1.14–4.28) | **.03** |
| CC vs. CT + TT (dominant) | 52 vs. 148 | 81 vs. 119 | 1.94 (1.27–2.96) | **.003** | 1.77 (1.16–2.69) | **.01** |
| CC + CT vs. TT (recessive) | 165 vs. 35 | 180 vs. 20 | 1.91 (1.06–3.44) | **.04** | 1.61 (0.88–2.94) | .16 |
| 2CC+CT vs. CT+2TT (multiplicative) | 217 vs. 183 | 261 vs. 139 | 1.58 (1.19–2.11) | **.002** | 1.46 (1.09–1.94) | **.01** |
| rs2027432 | | | | | | |
| GG | 165 (82.5) | 171 (85.5) | Referent | | Referent | |
| GA (additive) | 31 (15.5) | 26 (13) | 1.24 (0.69–2.17) | .55 | 1.20 (0.68–2.11) | .62 |
| AA (additive) | 4 (2) | 3 (1.5) | 1.38 (0.30–6.27) | .97 | 0.57 (0.17–2.00) | .57 |
| GG vs. GA +AA (dominant) | 165 vs. 35 | 171 vs. 29 | 1.25 (0.73–2.14) | .49 | 1.07 (0.63–1.80) | .91 |
| GG + GA vs. AA (recessive) | 196 vs. 4 | 197 vs. 3 | 1.34 (0.30–6.07) | 1.00 | 0.56 (0.16–1.94) | .54 |
| 2GG+GA vs. GA+2AA (multiplicative) | 361 vs. 39 | 368 vs. 32 | 1.24 (0.76–2.03) | .46 | 0.97 (0.61–1.54) | .98 |
| rs3738448 | | | | | | |
| GG | 147 (73.5) | 145 (72.5) | Referent | | Referent | |
| GT (additive) | 45 (22.5) | 47 (23.5) | 0.94 (0.59–1.51) | .91 | 0.87 (0.54–1.39) | .63 |
| TT (additive) | 8 (4) | 8 (4) | 0.99 (0.36–2.70) | .82 | 1.36 (0.54–3.42) | .68 |
| GG vs. GT + TT (dominant) | 147 vs. 53 | 145 vs. 55 | 0.95 (0.61–1.48) | .91 | 0.94 (0.60–1.45) | .86 |
| GG + GT vs. TT (recessive) | 192 vs. 8 | 192 vs. 8 | 1.00 (0.37–2.72) | .80 | 1.41 (0.56–3.52) | .62 |
| 2GG+GT vs. GT+2TT (multiplicative) | 339 vs. 61 | 337 vs. 63 | 0.96 (0.66–1.41) | .92 | 1.01 (0.70–1.47) | .97 |
| rs1539019 | | | | | | |
| TT | 77 (38.5) | 111 (55.5) | Referent | | Referent | |
| TA (additive) | 97 (48.5) | 71 (35.5) | 1.97 (1.29–3.00) | **.002** | 1.78 (1.16–2.71) | **.01** |
| AA (additive) | 26 (13) | 18 (9) | 2.08 (1.07–4.06) | **.04** | 2.11 (1.06–4.20) | **.047** |
| TT vs. TA + AA (dominant) | 77 vs. 123 | 111 vs. 89 | 1.99 (1.34–2.97) | **.001** | 1.84 (1.23–2.74) | **.004** |
| TT + TA vs. AA (recessive) | 174 vs. 26 | 182 vs. 18 | 1.51 (0.20–2.19) | .26 | 1.61 (0.83–3.10) | .21 |
| 2TT+TA vs. TA+2AA (multiplicative) | 251 vs. 149 | 293 vs. 107 | 1.63 (1.20–2.19) | **.002** | 1.57 (1.16–2.13) | **.004** |

*Note*: Genotype percentage is given in parenthesis. Assuming penetrance of ischaemic stroke as 1, $r$ and $r^2$ for AA, AB and BB genotypes, respectively. Additive model exhibits that risk for ischaemic stroke increases by $r$ fold for heterozygote AB and $r^2$ for homozygous BB. Dominant model: either one or two copies of allele B are required for $r$ fold increased risk. Recessive model: two copies of allele B are required for $r$ fold increased risk. Multiplicative model: each additional B allele increases $r$ fold risk. Bold values show significant associations.

ŧModel was adjusted with the values of systolic blood pressure, C-reactive protein, interleukin-1beta and interleukin-18.

IL-1$\beta$ and IL-18). This finding is in line with results of earlier studies on Chinese populations (Lv et al., 2020; Zhu et al., 2016) (Table 7). Another study on Chinese population (Cheng et al., 2018) has shown that minor allele T of SNP rs4612666 is significantly associated with higher IS risk. This inference is corroborated by the present study, whereby T allele of this SNP influences IS risk in additive (CT, TT), dominant and multiplicative modes. In addition to other genetic variants studied hitherto, present study has shown that minor allele A of SNP rs1539019 is associated with IS risk in additive, dominant and multiplicative modes. Analysis of these SNPs suggests that single copy

**TABLE 5** Putative haplotypes within NLRP3 gene and their association with the risk of ischaemic stroke

| Putative haplotype | Cases ($n = 200$) | Controls ($n = 200$) | $P^{Cor}$ | Crude OR (95% CI) | *p* Value | Adjusted OR (95% CI)‡ | *p* Value |
|---|---|---|---|---|---|---|---|
| CCGTT | 42 (0.21) | 48 (0.14) | 0.62 | 0.84 (0.53–1.350 | .55 | Referent | — |
| CCGGA | 30 (0.15) | 28 (0.14) | 1.37 | 1.08 (0.62–1.89) | .89 | 0.92 (0.76–1.44) | .75 |
| GTGGA | 28 (0.14) | 32 (0.16) | 0.86 | 0.85 (0.49–1.48) | .67 | 0.81 (0.47–1.42) | .63 |
| **GTGTA** | **28 (0.14)** | **14 (0.07)** | **0.02** | **2.16 (1.10–4.24)** | **.03** | **1.98 (1.12–3.78)** | **.04** |
| GTGGT | 23 (0.11) | 21 (0.10) | 1.30 | 1.11 (0.59–2.07) | .87 | 1.03 (0.73–1.89) | .81 |
| CTGGA | 15 (0.08) | 14 (0.07) | 1.57 | 1.08 (0.51–2.29) | 1.00 | 1.12 (0.69–2.00) | .88 |

$P^{cor}$: corrected *p* value.
*Note*: Those haplotypes which had frequencies less than 5% were excluded. SNPs in haplotypes are in the order of rs10754558, rs4612666, rs2027432, rs3738448 and rs1539019.
‡Values adjusted with systolic blood pressure, C-reactive protein, interleukin-1$\beta$ and interleukin-18.
Bold values indicate statistically significant association.

**TABLE 6** Functional effect of susceptibility putative haplotype (GTGTA) within NLRP3 gene influencing risk of ischaemic stroke in the best fit model

| Model | $\beta$ǂ | SE | Exp($\beta$) | Wald test | *P* | $R^2h$ | AIC |
|---|---|---|---|---|---|---|---|
| Dominant | **1.95** | **0.58** | **7.03** | **3.36** | **<0.001** | **1.000** | **3718.40** |
| Recessive | 0.85 | 0.44 | 1.55 | 1.93 | 0.053 | 0.7247 | 5381.12 |
| Multiplicative | −0.29 | 0.88 | 0.75 | −0.33 | 1.211 | 0.756 | 4239.33 |
| General (0 copy) | −0.12 | 0.41 | 0.89 | −0.29 | 1.190 | 0.889 | 5028.30 |
| General (1 copy) | 0.77 | 0.81 | 2.16 | 0.95 | 0.347 | 0.934 | 3935.28 |

*P*: asymptotic value, Exp($\beta$): exponentiated value of $\beta$, $R^2h$: haplotype uncertainty measure, AIC: Akaike information criterion.
*Note*: Models showing value after adjustment for risk covariates: systolic blood pressure, C-reactive protein, interleukin 1-beta and interleukin-18.
ǂEstimated haplotype effect. Values in bold face show highest $R^2h$ values and lowest AIC. Dominant: subjects having 1 copy are at the same risk as subjects having two copies.

or additional copy of minor alleles G, T and A is required to impact IS risk as these SNPs exhibit their effect in dominant and multiplicative modes.

Although effects of these SNPs have been observed after adjusting with significant variables (SBP, CRP, IL-1$\beta$ and IL-18), but individual SNP fails to capture overall effect, as these interact and collaborate, especially when SNPs are non-randomly associated with one another. Hence, haplotypes have been investigated which reveal that minor alleles G, T, T and A of SNPs rs10754558, rs4612666, rs3738448 and rs1539019 and major allele G of SNP rs2027432 collaborate and influence IS risk in the form of putative haplotype GTGTA. When the effect of this putative haplotype is adjusted with independent risk variables (SBP, CRP, IL-1$\beta$ and IL-18), it has been observed that this haplotype is a susceptibility putative haplotype, which confers approximately double the risk for IS. Cheng et al. (2018) have observed a risky haplotype TGT (in order of rs4612666, rs10754558 and rs7512998) for IS, which endorses the inference that two alleles G and T of SNP rs10754558 and rs4612666 collaborate on the position 2 and 3 in the haplotype GTGTA in the present study also. Nonetheless, present study has also explored the possible way in which this putative haplotype GTGTA exerts its impact for the risk of IS, revealing that it influences in dominant mode suggesting that even carriage of a single copy of this putative haplotype is sufficient to exacerbate IS risk. Carriers of this putative haplotype are at double the risk of IS and are more vulnerable to increased levels of pro-inflammatory markers CRP and IL-1$\beta$, which has been confirmed by the observation that possession of one copy or two copies of this putative haplotype is significantly correlated with higher levels of CRP and IL-1$\beta$ in comparison to subjects having no copy of this putative haplotype.

It is verified in this study that higher levels of SBP, CRP, IL-1$\beta$ and IL-18 are independent risk predictors for IS risk in the population of Punjab, India. Nonetheless, SBP and IL-18 have failed to associate with susceptibility putative haplotype GTGTA suggesting that although higher levels of these both variables influence IS pathology but operate independent of genetic connotation of NLRP3 inflammasome. It has been observed lately that NLRP3 inflammasome activation also participates in NETosis, a process whereby neutrophils in response to innate immunity, set the wheel of autoimmunity and inflammation within plaqued arteries (Münzer et al., 2021). It is well understood that increased CRP concentrations with 12 h of ischaemic stroke is independent prognostic marker for worst outcomes of higher Rankin Scale Scores and death (den Hertog et al., 2009). Moreover, higher CRP levels at admission and discharge are predictors for 1 year risk of either new vascular event or death (Di Napoli et al., 2001). It has been observed

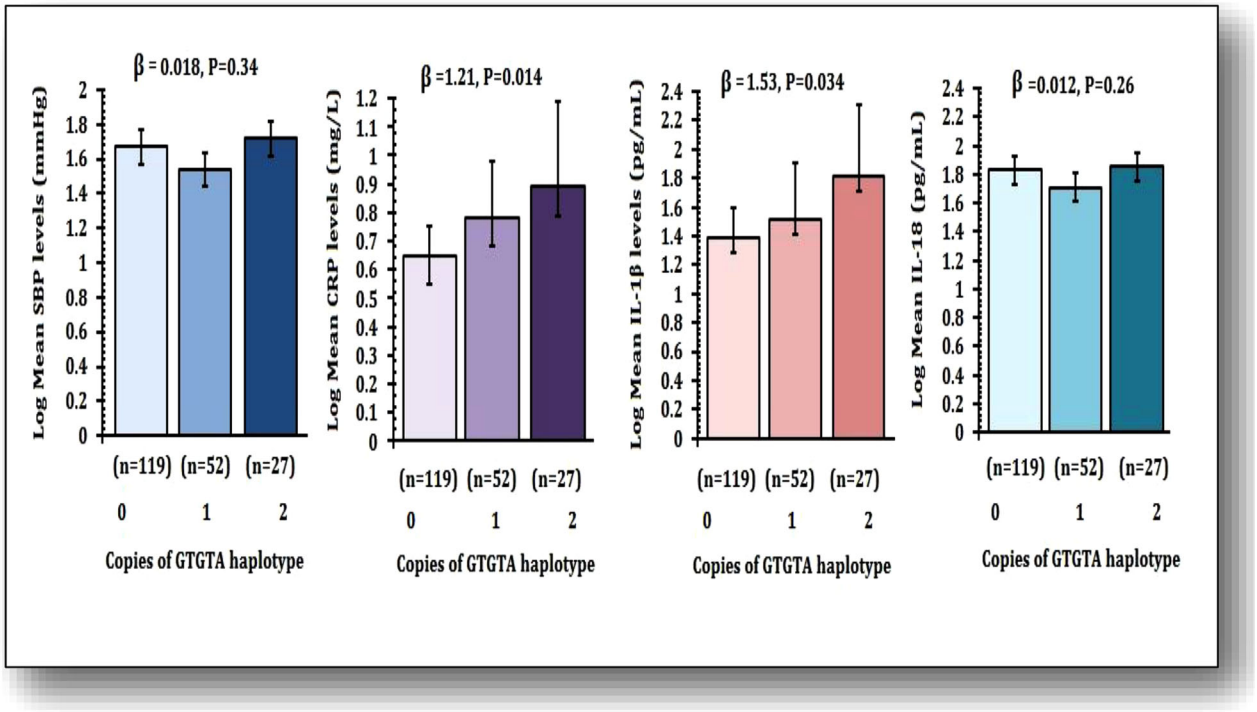


**FIGURE 2** Association of susceptibility haplotype GTGTA with independent risk predictors for ischaemic stroke. Systolic blood pressure (SBP), C-reactive protein (CRP), interleukin-1 beta (IL-1$\beta$) and interleukin-18 (IL-18) according to number of copies of haplotypes

**TABLE 7** Studies investigating NLRP3 gene polymorphism in ischaemic stroke in different populations so far

| Population | NLRP3-SNP | Design of the study | Sample size | Noticeable inference of the study | Reference |
|---|---|---|---|---|---|
| Sweden | rs35829419 (Q705K) | Case-control | CVD patients = 121<br>Controls = 401 | Minor allele carriers at higher risk of stroke/transient ischaemic attack but not with myocardial infarction/angina pectoris | Kastbom et al. (2015) |
| China | rs10754558 | Case-control | IS patients = 1102<br>Control = 1610 | Minor allele G was observed to be significantly associated with IS risk. This allele is functional as it mediates mRNA expression | Zhu et al. (2016) |
| China | rs4612666<br>rs10754558<br>rs7512998 | Case-control | LAA patients = 293<br>Controls = 265 | Minor allele T of SNP rs4612666 was associated with higher risk of large artery atherosclerosis and micro embolic signal. TGT haplotype was associated with higher risk. | Cheng et al. (2018) |
| China | rs10754558 | Case-control | IS patients = 234<br>Controls = 115 | CG heterozygotes had higher risk of IS | Lv et al. (2020) |
| India | rs10754558<br>rs4612666<br>rs2027432<br>rs3738448<br>rs1539019 | Case-control | IS patients = 200<br>Controls = 200 | Minor alleles of SNPs, rs10754558, rs4612666 and rs1539019, were observed to be associated with IS risk. A susceptibility haplotype GTGTA was observed which conferred significant IS risk in recessive mode. This haplotype influence C-reactive protein and Interleukin-1$\beta$ levels in dose-dependent manner. | Present study |

CVD: cardiovascular disorders, LAA: large artery atherosclerosis, IS: ischaemic stroke.
Bold values indicate statistically significant association.

that LDL retention in endothelial cells trigger atherosclerosis, whereby CRP plays a major role in its progression through the activation of NLRP3 inflammasome (Bian et al., 2019). Dose-dependent relationship of putative haplotype GTGTA as genetic determinant of higher CRP levels in the present study has enlightened this finding, but also instils curiosity that whether role of CRP in LDL transcytosis-induced atherosclerosis is independent or having bidirectional relationship with NLRP3 inflammasome activation.

Higher levels of pro-inflammatory cytokine IL-1$\beta$ has been strongly implicated in stroke pathology (Boutin et al., 2001). Present abundantly in atheromatous lesions in stroke patients, it participates in plaque development, its stability and progression to ischaemia (Paramel Verghese et al., 2016). Therefore, suppression of NLRP3 inflammasome-induced IL-1$\beta$ production has proved fruitful in managing the stroke volume, infarct size and post-ischaemic neuroinflammation (Dong et al., 2021). Present study has clarified for the first time that relationship of IL-1$\beta$ secretion during NLRP3 inflammasome activation is genetically mediated, as five SNPs within NLRP3 gene in the form of putative haplotype GTGTA are associated with the increased concentrations of IL-1$\beta$ in IS patients having one copy, which further increases with carriage of two copies. Albeit, GTGTA haplotype within NLRP3 gene influences the risk of IS via mediation of increased levels of CRP and IL-1$\beta$ in dose-dependent manner in the population of Punjab, but it does not defy the viewpoint that expression of genes vary according to different ethnicities (Huang et al., 2015); hence caution is urged in the interpretation of results.

## 5 | CONCLUSION

The present study has revealed that minor alleles of SNPs rs10754558, rs46124666 and rs1539019 of NLRP3 inflammasome are significantly associated with IS risk after correcting the effect of significant risk predictors. A susceptibility putative haplotype GTGTA exists within NLRP3 gene, which exacerbates IS risk in dominant genetic model. Furthermore, this putative haplotype is genetically associated with increased concentrations of pro-inflammatory markers CRP and IL-1$\beta$ in dose-dependent manner.

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### CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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