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Multi-locus analysis of NLRP3 inflammasome gene polymorphisms (rs4612666, rs3806268, rs10754558) in relation to *NLRP3* expression and intestinal permeability markers in Iraqi Arab children with autism spectrum disorder: a case-control study

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Abstract

Background The NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome generates interleukin (IL)-1β and IL-18 and is hyperactivated in cells from children with autism spectrum disorder (ASD), but *NLRP3* variation has not been examined in ASD. We related three *NLRP3* polymorphisms to ASD susceptibility, *NLRP3* expression and intestinal permeability markers in Iraqi Arab children.

Methods and results Children with ASD (n = 180) and age- and sex-matched controls (n = 180) were genotyped for rs4612666, rs3806268 and rs10754558 by TaqMan assays, with haplotype and linkage disequilibrium (LD) analysis (Bonferroni $\alpha = 0.0167$). Peripheral blood mononuclear cell *NLRP3* mRNA was quantified by reverse transcription quantitative polymerase chain reaction, and serum NLRP3, IL-1β, IL-18, zonulin and lipopolysaccharide-binding protein by enzyme-linked immunosorbent assay. The rs10754558 G allele was associated with ASD (odds ratio [OR] = 1.54, 95% confidence interval [CI]: 1.15–2.08, p = 0.004), and the GG genotype remained associated after adjustment (adjusted OR = 2.21, 95% CI: 1.19–4.10, p = 0.012). rs4612666 showed a nominal association (p = 0.018), and rs3806268 none. The T-G-G haplotype was more frequent in patients (p = 0.047), and rs4612666 and rs10754558 were in strong LD ($D' = 0.85$). *NLRP3* mRNA was 2.3-fold higher in patients and increased across rs10754558 genotypes (both $p < 0.001$), paralleled by serum NLRP3, IL-1β and IL-18. Zonulin correlated with *NLRP3* mRNA (Spearman $\rho = 0.41$) and autism severity score ($\rho = 0.29$).

Conclusions *NLRP3* rs10754558 may contribute to ASD susceptibility in Iraqi Arab children with genotype-dependent *NLRP3* expression; haplotype and LD findings are exploratory.

Keywords NLRP3 · Inflammasome · Autism spectrum disorder · Polymorphism · Gene expression · Zonulin

Abbreviations

3′-UTR 3′-untranslated region
ASC Apoptosis-associated speck-like protein containing a caspase recruitment domain
ASD Autism spectrum disorder
AUC Area under the curve
BMI Body mass index
CARS-2 Childhood Autism Rating Scale, Second Edition
CI Confidence interval
Ct Cycle threshold
DSM-5 Diagnostic and Statistical Manual of Mental Disorders, fifth edition
EDTA Ethylenediaminetetraacetic acid
ELISA Enzyme-linked immunosorbent assay
eQTL Expression quantitative trait locus
GTEx Genotype-Tissue Expression
HWE Hardy–Weinberg equilibrium
IL Interleukin
LBP Lipopolysaccharide-binding protein
LD Linkage disequilibrium
LPS Lipopolysaccharide
MIQE Minimum Information for Publication of Quantitative Real-Time PCR Experiments
NLRP3 NOD-, LRR- and pyrin domain-containing protein 3
OR Odds ratio
PBMC Peripheral blood mononuclear cell
ROC Receiver operating characteristic
RT-qPCR Reverse transcription quantitative polymerase chain reaction
SD Standard deviation

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterised by persistent deficits in social communication and interaction together with restricted, repetitive patterns of behaviour, interests or activities [1]. Its global prevalence approximates one in one hundred children [2]. Heritability is high, yet no single genetic lesion explains more than a

small proportion of cases, and current aetiological models emphasise interactions between common genetic variants and environmental and immunological exposures during critical windows of neurodevelopment [3].

Immune dysregulation is among the most consistently replicated findings in ASD. Circulating interleukin (IL)-1β, IL-6 and tumour necrosis factor-α are elevated in affected individuals [4], and higher plasma cytokine concentrations are associated with poorer behavioural outcomes [5]. Neuropathological studies have documented microglial and astroglial activation in the ASD brain [6], and the evidence implicates innate rather than adaptive immunity as the principal locus of dysregulation [7], placing cytosolic pattern-recognition pathways at the centre of the mechanistic question.

The NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome is the best characterised of these pathways. It comprises the sensor NLRP3, the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1 [8]. Assembly requires a nuclear factor-κB-dependent priming signal that induces *NLRP3* and pro-IL-1β transcription, and an activating signal such as extracellular adenosine triphosphate, potassium efflux, reactive oxygen species or bacterial lipopolysaccharide (LPS) [9, 10]. Activated caspase-1 then cleaves pro-IL-1β and pro-IL-18 into their mature forms. Because IL-1β and IL-18 regulate microglial reactivity, synaptic pruning and blood–brain barrier permeability, the NLRP3 axis is a plausible upstream node for the cytokine abnormalities observed in ASD [11]. Supporting this, Vallese et al. [12] demonstrated NLRP3 inflammasome hyperactivation, with increased ASC specks, active caspase-1 and IL-1β, in dermal fibroblasts from children with ASD, coupled to mitochondrial dysfunction that improved with the NLRP3 inhibitor MCC950. Whether this hyperactivity is genetically determined, and whether it is detectable in accessible peripheral tissue, remains unknown.

The human *NLRP3* gene maps to chromosome 1q44, and gain-of-function mutations in it cause the cryopyrin-associated periodic syndromes [13]. The 3′-untranslated region (3′-UTR) variant rs10754558 alters transcript stability and *NLRP3* expression [14]; it has been associated with type 2 diabetes mellitus [15], coronary artery disease [16] and ischaemic stroke [17], and has been reported to modify the interaction of the transcript with miR-146a (reviewed in [18]). The intronic enhancer variant rs4612666 has been associated with large-artery atherosclerotic ischaemic stroke [19] and, in Iraqi Arab adults, with rheumatoid arthritis and serum NLRP3 concentration [20]. The exonic variant rs3806268 has been associated with primary gout [21] and with expression of the inflammasome adaptor gene *PYCARD* [18]. Meta-analyses confirm that these variants modulate the risk of inflammatory disease, with effects that vary by ethnicity [22, 23], and selective NLRP3 inhibitors have entered clinical development [24, 25].

Impaired intestinal barrier function offers a candidate endogenous priming stimulus. Zonulin is the principal physiological modulator of enterocyte tight junctions, and elevated serum zonulin is an accepted surrogate of increased intestinal permeability [26]. Increased permeability has been documented in children with ASD and their first-degree relatives [27], together with concurrent alterations of the intestinal and blood–brain barriers [28], low-grade endotoxaemia [29] and serum zonulin elevations that correlate with symptom severity [30], although the causal direction remains unresolved [31]. Children with ASD and gastrointestinal symptoms also show distinct immune and microbiota profiles [32]. Because translocated LPS is a canonical NLRP3 priming signal, barrier failure could supply the priming stimulus while host *NLRP3* genotype determines the amplitude of the inflammasome response.

Iraq and the wider Middle East and North Africa region have high rates of consanguineous marriage [33], which contributes to the burden of inherited and immune-related disorders [34]

and must be measured and adjusted for in genetic association studies in this setting. Genetic data on ASD in Iraqi Arab children remain sparse; we previously reported vitamin D receptor polymorphisms in this population [35], but the *NLRP3* locus has not been examined. We therefore determined the genotype, allele and haplotype frequencies of three functional *NLRP3* polymorphisms in Iraqi Arab children with ASD and matched controls, characterised linkage disequilibrium (LD) at the locus, and related genotype to *NLRP3* mRNA and protein expression, downstream cytokines, intestinal permeability markers and clinical severity. To our knowledge, this is the first study to evaluate *NLRP3* polymorphisms, haplotype structure and gene expression together in a paediatric ASD cohort.

Materials and methods

Study design and participants

This hospital-based case-control study was conducted at the Department of Laboratory Investigations, Faculty of Science, University of Kufa, in collaboration with the Autism Diagnostic and Treatment Centre of Najaf Regional Children's Hospital, Al-Najaf, Iraq. Cases and controls were enrolled consecutively and in parallel over twelve months, so that seasonal variation in immune and barrier markers affected both groups equally.

Cases were children aged 3–12 years with ASD diagnosed independently by two child psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) [1] and confirmed with the Childhood Autism Rating Scale, Second Edition (CARS-2) [36]. Severity was recorded as the continuous CARS-2 total score and, descriptively, as mild-to-moderate versus severe. Controls were typically developing children recruited from the same hospital, matched to cases by age (within one year), sex and province of residence, with no personal or first-degree family history of neurodevelopmental, autoimmune or autoinflammatory disease.

Exclusion criteria for both groups were active infection or febrile illness within the preceding four weeks; autoinflammatory, autoimmune, inflammatory bowel or coeliac disease; use of corticosteroids, non-steroidal anti-inflammatory drugs, immunomodulators, probiotics or antibiotics within three months; a monogenic or syndromic cause of ASD, including fragile X syndrome, tuberous sclerosis and Rett syndrome; pharmacologically treated epilepsy; hepatic or renal failure; and body mass index (BMI) above the 95th percentile for age and sex, because adiposity independently increases inflammasome activity. Age, sex, BMI z-score, parental consanguinity, family history of ASD or autoimmune disease, gestational age, birth weight, breastfeeding duration, dietary restriction and a structured gastrointestinal symptom score were recorded by interview with the accompanying parent.

Sample size

Power for the allelic test was estimated with the Genetic Power Calculator [37]. Assuming a minor allele frequency of 0.30 in controls, a log-additive model, a two-sided α of 0.05 and a 1:1 case-to-control ratio, 180 cases and 180 controls provide approximately 80% power to detect a per-allele odds ratio (OR) of 1.55 and 97% power (92% at the Bonferroni-corrected α of 0.0167) to detect an OR of 1.8. The design also provides more than 95% power to detect a 0.4 standard-deviation difference in log-transformed biomarker concentrations.

Sample collection and processing

Five millilitres of venous blood were obtained from each participant. Two millilitres were collected into ethylenediaminetetraacetic acid (EDTA) tubes for DNA extraction and stored at −20 °C, and two millilitres into EDTA tubes that were processed within two hours for isolation of peripheral blood mononuclear cells (PBMCs). The remainder was allowed to clot for 30–60 min and centrifuged at 3000 rpm for 10 min, and the serum was aliquoted and stored at −80 °C without repeated freeze–thaw cycles. Each analyte was assayed in a single

batch, with cases and controls randomly interleaved on each plate, by personnel blinded to case-control status.

Genotyping

Genomic DNA was extracted with a commercial spin-column kit; only samples with an A260/A280 ratio of 1.7–1.9 and intact DNA on agarose gel electrophoresis were used, at a working dilution of 5 ng/µL. The three polymorphisms were genotyped by TaqMan 5′-nuclease allelic discrimination using pre-designed TaqMan SNP Genotyping Assays (Thermo Fisher Scientific, Waltham, MA, USA; rs4612666, C__28048739_10; rs3806268, C___2447990_10; rs10754558, C__26052028_10), each with VIC- and FAM-labelled minor groove binder probes. Variant locations, allele designations on the dbSNP forward strand (GRCh38.p14) and the rationale for variant selection are given in Online Resource 1 (Table S1). Reactions of 10 µL contained 1× TaqMan Genotyping Master Mix, 1× assay and approximately 10 ng of DNA. Cycling comprised a 60 °C pre-read for 30 s, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s, and a 60 °C post-read for 30 s. Genotypes were called automatically from end-point fluorescence and reviewed manually, and samples outside a defined cluster were repeated once. Each plate included six no-template controls, a per-locus call-rate threshold of 95% was set a priori, and a random 10% of samples were re-genotyped on an independent plate by an operator blinded to the original calls. Allelic discrimination plots and quality-control metrics are presented in Online Resource 1 (Fig. S1; Table S2).

RNA extraction and quantification of NLRP3 expression

PBMCs were isolated by density gradient centrifugation, and total RNA was extracted with a column-based kit incorporating on-column DNase I treatment; only samples with an A260/A280 ratio of 1.9–2.1 were used. Complementary DNA was synthesised from a fixed RNA input with a commercial reverse transcription kit. *NLRP3* expression was quantified by SYBR Green reverse transcription quantitative polymerase chain reaction (RT-qPCR) using

pre-validated PrimePCR assays (Bio-Rad Laboratories, Hercules, CA, USA), and the experiments are reported in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [38]. Assay details are given in Online Resource 1 (Table S3).

Four candidate reference genes (*GAPDH*, *HPRT1*, *RPLP0* and *B2M*) were evaluated on a pilot set of 20 samples from both groups with the geNorm and NormFinder algorithms [39], and the two most stable genes were used for normalisation (Online Resource 1, Table S4). Relative expression was calculated by the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) against their geometric mean [40]. Amplification efficiency was determined for every assay from a five-point serial-dilution standard curve, specificity was confirmed by a single melt-curve peak, and every run included no-template and no-reverse-transcription controls. Samples were assayed in triplicate, and reactions with a triplicate standard deviation (SD) above 0.5 Ct were repeated.

Serum protein measurements

Serum NLRP3, IL-1β, IL-18, zonulin and lipopolysaccharide-binding protein (LBP) were measured by commercial double-antibody sandwich enzyme-linked immunosorbent assays (ELISAs) according to the manufacturers' instructions. Before the main analytical run, each kit was qualified on 20 pilot samples for intra- and inter-assay coefficients of variation (acceptance thresholds of 10% and 15%, respectively), spike recovery and dilutional linearity (Online Resource 1, Table S5). Absorbance was read at 450 nm, and concentrations were interpolated from four-parameter logistic standard curves, with duplicate standards and internal quality-control sera on each plate. Because NLRP3 and ASC are released into the extracellular space as particulate oligomers after inflammasome activation and pyroptotic cell death [41], and serum NLRP3 has previously been quantified by ELISA in Iraqi Arab adults

[20], serum NLRP3 was interpreted as a marker of released inflammasome material rather than of intracellular NLRP3 abundance.

In silico functional annotation

Each variant was annotated for regulatory potential with RegulomeDB [42] and HaploReg [43], and for expression quantitative trait locus (eQTL) effects in whole blood and brain with the Genotype-Tissue Expression (GTEx) portal [44]. Allele-specific microRNA binding at rs10754558 was predicted with miRNASNP-v3 and TargetScan Human 8.0 (Online Resource 1, Table S6).

Statistical analysis

Analyses were performed in SPSS version 26 (IBM Corp., Armonk, NY, USA) and R. Normality was assessed by the Shapiro–Wilk test; groups were compared by the t-test or Mann–Whitney U test for continuous variables and by the χ^2 or Fisher's exact test for categorical variables. Hardy–Weinberg equilibrium (HWE) was tested in controls by the χ^2 goodness-of-fit test. ORs with 95% confidence intervals (CIs) were estimated in SNPStats [45] under codominant, dominant, recessive, overdominant and allelic models, and adjusted for age, sex, BMI z-score, consanguinity and family history by multivariable logistic regression. Haplotypes were estimated by the expectation-maximisation algorithm (frequency < 3% pooled), and pairwise LD (D', r^2) was computed in Haploview [46]. Expression and protein levels were compared by one-way analysis of variance (ANOVA) with Tukey correction or the Kruskal–Wallis test with Dunn correction, correlations by Spearman's ρ, and diagnostic performance by receiver operating characteristic (ROC) analysis (area under the curve [AUC]; Youden-optimal cut-off). Bonferroni correction for three loci gave $\alpha = 0.0167$, with Benjamini–Hochberg false discovery rate control reported alongside; haplotype and LD analyses were exploratory and uncorrected [47]. Reporting follows the Strengthening the Reporting of Genetic Association Studies (STREGA) guidelines [48].

Ethics approval and consent

The protocol was approved by the Research Ethics Committee of the Faculty of Science, University of Kufa (approval ID UOK-FS-218; 12 January 2024), and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from a parent or legal guardian of every participant, and assent from children older than seven years who were able to provide it. Data were pseudonymised at collection. Individual genetic results were not returned to families because none of the variants has established predictive value at the individual level, and this was explained during the consent process.

Results

Genotyping performance

Call rates were 98.9%, 99.2% and 99.4% for rs4612666, rs3806268 and rs10754558, respectively, all above the pre-specified 95% threshold. Allelic discrimination plots showed three well-separated genotype clusters at each locus, with all no-template controls at the origin, and blind re-genotyping of 36 samples (10%) gave 100% concordance with the original calls (Online Resource 1, Fig. S1; Table S2). No sample was excluded for genotyping failure.

Demographic and clinical characteristics

The study included 180 children with ASD and 180 controls (Table 1). Age (6.9 ± 3.1 versus 7.2 ± 3.0 years, $p = 0.35$), sex (78.9% versus 75.6% male, $p = 0.45$) and BMI z-score ($p = 0.52$) did not differ between groups. First-cousin consanguinity (39.4% versus 26.7%, $p = 0.010$), a family history of ASD (13.3% versus 3.3%, $p < 0.001$) and gastrointestinal symptom scores (4.6 ± 2.7 versus 2.1 ± 1.8, $p < 0.001$) were higher in patients. Within the ASD group, 112 children (62.2%) had mild-to-moderate and 68 (37.8%) severe ASD, with a mean CARS-2 total score of 37.4 ± 6.8.

Table 1 Demographic and clinical characteristics of the study groups

Variable	Controls (n = 180)	ASD (n = 180)	p-value
Age, years, mean ± SD	7.2 ± 3.0	6.9 ± 3.1	0.35
Age 3–6 years, n (%)	84 (46.7)	92 (51.1)	0.40
Age 7–12 years, n (%)	96 (53.3)	88 (48.9)	
Male sex, n (%)	136 (75.6)	142 (78.9)	0.45
BMI z-score, mean ± SD	0.14 ± 0.98	0.21 ± 1.10	0.52
Consanguinity, first cousin, n (%)	48 (26.7)	71 (39.4)	0.010
Consanguinity, second cousin, n (%)	31 (17.2)	38 (21.1)	0.35
Family history of ASD, n (%)	6 (3.3)	24 (13.3)	< 0.001
Gastrointestinal symptom score, mean ± SD	2.1 ± 1.8	4.6 ± 2.7	< 0.001
CARS-2 total score, mean ± SD	—	37.4 ± 6.8	—
Severity, mild-moderate / severe, n (%)	—	112 (62.2) / 68 (37.8)	—

SD, standard deviation; BMI, body mass index; CARS-2, Childhood Autism Rating Scale Second Edition.

Hardy–Weinberg equilibrium

Genotype distributions in controls conformed to HWE at all three loci (p = 0.67, 0.71 and 0.73 for rs4612666, rs3806268 and rs10754558, respectively), providing no evidence of genotyping error or population substructure; case distributions likewise showed no significant departure (Online Resource 1, Table S7).

Single-locus association analysis

For rs10754558, the G allele was more frequent in patients than in controls (47.8% versus 37.2%; OR = 1.54, 95% CI: 1.15–2.08, p = 0.004) (Table 2). Under the codominant model, the GG genotype was associated with ASD relative to CC (24.4% versus 14.4%; OR = 2.34, 95% CI: 1.28–4.28, p = 0.006), whereas the CG genotype was not (OR = 1.42, 95% CI: 0.89–2.27, p = 0.14). Associations were also detected under the dominant (OR = 1.64, 95% CI: 1.06–2.55, p = 0.027) and recessive (OR = 1.92, 95% CI: 1.12–3.28, p = 0.018) models, but not under the overdominant model (p = 0.83). Because the heterozygote OR lies between those of the CC and GG genotypes and CG carriers showed intermediate *NLRP3* expression (see below), the data are most consistent with an allele-dose (additive) effect, with limited power to resolve the heterozygote contrast.

For rs4612666, the T allele was more frequent in patients (43.3% versus 34.7%; OR = 1.44, 95% CI: 1.06–1.94, p = 0.018), with nominal codominant (TT: OR = 1.99, 95% CI: 1.06–3.73, p = 0.032) and dominant (OR = 1.61, 95% CI: 1.05–2.47, p = 0.030) associations. rs3806268 was not associated with ASD under any genetic model.

After Bonferroni correction, only the rs10754558 allelic and codominant (GG) associations remained significant; the recessive-model result (p = 0.018) fell marginally above the corrected threshold, and the rs4612666 associations were nominal. Benjamini–Hochberg q-values for the allelic tests were 0.012 for rs10754558, 0.027 for rs4612666 and 0.76 for rs3806268.

Table 2 Genotype and allele frequencies of the three *NLRP3* polymorphisms in ASD patients and controls under five genetic models

Genotype / allele	Controls n (%)	ASD n (%)	OR	95% CI	p-value
rs4612666 (C > T)					
Codominant CC	78 (43.3)	58 (32.2)	Reference		
CT	79 (43.9)	88 (48.9)	1.50	0.95–2.36	0.082
TT	23 (12.8)	34 (18.9)	1.99	1.06–3.73	0.032
Dominant CT + TT	102 (56.7)	122 (67.8)	1.61	1.05–2.47	0.030
Recessive TT	23 (12.8)	34 (18.9)	1.59	0.89–2.83	0.114
Overdominant CT	79 (43.9)	88 (48.9)	1.22	0.81–1.85	0.34
Allele C	235 (65.3)	204 (56.7)	Reference		
Allele T	125 (34.7)	156 (43.3)	1.44	1.06–1.94	0.018
rs3806268 (A > G)					
Codominant AA	66 (36.7)	62 (34.4)	Reference		
AG	88 (48.9)	92 (51.1)	1.11	0.71–1.75	0.64
GG	26 (14.4)	26 (14.4)	1.06	0.56–2.03	0.85
Dominant AG + GG	114 (63.3)	118 (65.6)	1.10	0.72–1.70	0.66
Recessive GG	26 (14.4)	26 (14.4)	1.00	0.56–1.79	1.00
Overdominant AG	88 (48.9)	92 (51.1)	1.09	0.72–1.65	0.67
Allele A	220 (61.1)	216 (60.0)	Reference		
Allele G	140 (38.9)	144 (40.0)	1.05	0.78–1.41	0.76
rs10754558 (C > G)					
Codominant CC	72 (40.0)	52 (28.9)	Reference		
CG	82 (45.6)	84 (46.7)	1.42	0.89–2.27	0.14
GG	26 (14.4)	44 (24.4)	2.34	1.28–4.28	0.006*
Dominant CG + GG	108 (60.0)	128 (71.1)	1.64	1.06–2.55	0.027
Recessive GG	26 (14.4)	44 (24.4)	1.92	1.12–3.28	0.018
Overdominant CG	82 (45.6)	84 (46.7)	1.05	0.69–1.58	0.83
Allele C	226 (62.8)	188 (52.2)	Reference		
Allele G	134 (37.2)	172 (47.8)	1.54	1.15–2.08	0.004*

OR, odds ratio; CI, confidence interval. Asterisk denotes significance after Bonferroni correction (α = 0.0167).

Multivariable analysis

After adjustment for age, sex, BMI z-score, consanguinity and family history, the rs10754558 GG genotype remained independently associated with ASD (adjusted OR = 2.21, 95% CI: 1.19–4.10, p = 0.012), whereas the rs4612666 dominant-model effect was attenuated (adjusted OR = 1.52, 95% CI: 0.98–2.36, p = 0.062) and rs3806268 showed no effect (Table 3). First-cousin consanguinity (adjusted OR = 1.61, 95% CI: 1.02–2.54, p = 0.041) and family history of ASD (adjusted OR = 2.34, 95% CI: 1.11–4.93, p = 0.026) were also independently associated with case status (Nagelkerke $R^2 = 0.11$).

Table 3 Multivariable logistic regression of *NLRP3* genotypes and covariates on ASD risk

Predictor	Adjusted OR	95% CI	p-value
rs10754558 GG vs. CC	2.21	1.19–4.10	0.012
rs10754558 CG vs. CC	1.33	0.82–2.16	0.25
rs4612666 CT + TT vs. CC	1.52	0.98–2.36	0.062
rs3806268 AG + GG vs. AA	1.08	0.69–1.69	0.73
Consanguinity, first cousin vs. unrelated	1.61	1.02–2.54	0.041
Family history of ASD	2.34	1.11–4.93	0.026
Male sex	1.19	0.72–1.97	0.50
Age, per year	0.98	0.93–1.04	0.52
BMI z-score, per unit	1.06	0.88–1.28	0.53

Binary logistic regression with all predictors entered simultaneously; Nagelkerke $R^2 = 0.11$.

Haplotype and linkage disequilibrium analysis

Five haplotypes (rs4612666-rs3806268-rs10754558) exceeded a frequency of 3% and accounted for 96.4% of control and 96.9% of case chromosomes; the haplotype-derived allele counts reproduced the single-locus allele frequencies exactly. Relative to the most frequent haplotype, C-A-C, the T-G-G haplotype, which carries the variant allele at both regulatory loci, was more frequent in patients (35.8% versus 27.5%; OR = 1.41, 95% CI: 1.00–1.97, p = 0.047), whereas the C-G-C haplotype was less frequent (OR = 0.41, 95% CI: 0.21–0.78, p = 0.007) (Online Resource 1, Table S8). With five haplotype comparisons (Bonferroni α = 0.01), only the C-G-C estimate would remain significant.

LD was strongest between rs4612666 and rs10754558 ($D' = 0.85$, $r^2 = 0.63$), intermediate between rs4612666 and rs3806268 ($D' = 0.72$, $r^2 = 0.51$) and weakest between rs3806268 and rs10754558 ($D' = 0.66$, $r^2 = 0.38$) (Fig. 1; Online Resource 1, Table S9). Because the three markers are in appreciable LD, their association signals are not statistically independent, and the Bonferroni threshold applied here is conservative.

NLRP3 expression and inflammasome output

NLRP3 mRNA in PBMCs was 2.3-fold higher in children with ASD than in controls (2.41 ± 0.92 versus 1.03 ± 0.38 relative units, $p < 0.001$). Within the ASD group, expression increased across rs10754558 genotypes (CC 1.92 ± 0.71; CG 2.38 ± 0.84; GG 3.02 ± 1.06; ANOVA $p < 0.001$), with significant Tukey contrasts for GG versus CC ($p < 0.001$), GG versus CG ($p = 0.002$) and CG versus CC ($p = 0.012$) (Table 4; Fig. 2). This stepwise, allele-dose-dependent pattern is the expected signature of a cis-acting regulatory variant and provides internal functional support for the genetic association. Parallel gradients were observed for serum NLRP3 (CC 3.51 ± 1.22; CG 4.12 ± 1.35; GG 5.02 ± 1.58 ng/mL, $p < 0.001$), IL-1β ($p = 0.001$) and IL-18 ($p = 0.002$). A weaker gradient was detected across rs4612666 genotypes ($p = 0.048$), and none across rs3806268 genotypes ($p = 0.71$).

Table 4 *NLRP3* mRNA expression and serum inflammasome markers by group and by rs10754558 genotype within the ASD group

Marker	Controls (n = 180)	ASD (n = 180)	ASD CC (n = 52)	ASD CG (n = 84)	ASD GG (n = 44)
NLRP3 mRNA, relative units	1.03 ± 0.38	2.41 ± 0.92	1.92 ± 0.71	2.38 ± 0.84	3.02 ± 1.06
Serum NLRP3, ng/mL	2.36 ± 0.89	4.18 ± 1.47	3.51 ± 1.22	4.12 ± 1.35	5.02 ± 1.58
Serum IL-1β, pg/mL	9.2 ± 3.6	18.6 ± 7.4	15.8 ± 6.2	18.2 ± 7.0	22.4 ± 8.1
Serum IL-18, pg/mL	168 ± 61	312 ± 118	268 ± 96	305 ± 110	372 ± 134
p, ASD vs. controls	< 0.001 for all four markers				
p, across genotypes (ANOVA)	mRNA < 0.001; NLRP3 < 0.001; IL-1β = 0.001; IL-18 = 0.002				

Values are mean ± standard deviation. Group comparison by independent-samples t-test on log-transformed values; genotype comparison by one-way ANOVA with Tukey post-hoc correction.

Intestinal permeability markers and correlation analysis

Serum zonulin (42.6 ± 15.3 versus 24.1 ± 8.7 ng/mL) and LBP (18.9 ± 6.7 versus 12.4 ± 4.5 µg/mL) were higher in patients than in controls (both $p < 0.001$) (Table 5). Within the ASD group, zonulin correlated with *NLRP3* mRNA ($\rho = 0.41$, $p < 0.001$), serum IL-18 ($\rho = 0.38$, $p < 0.001$) and CARS-2 total score ($\rho = 0.29$, $p = 0.001$), and LBP correlated with serum IL-1β ($\rho = 0.34$, $p < 0.001$). *NLRP3* mRNA correlated with serum NLRP3 ($\rho = 0.52$, $p < 0.001$) and with CARS-2 total score ($\rho = 0.31$, $p < 0.001$), linking the molecular measures to the clinical phenotype.

Table 5 Intestinal permeability markers and Spearman rank correlations within the ASD group

Comparison	**Controls**	**ASD**	**p-value**
Serum zonulin, ng/mL, mean ± SD	24.1 ± 8.7	42.6 ± 15.3	< 0.001
Serum LBP, µg/mL, mean ± SD	12.4 ± 4.5	18.9 ± 6.7	< 0.001
Correlation within the ASD group	**ρ**		**p-value**
Zonulin vs. *NLRP3* mRNA	0.41		< 0.001
Zonulin vs. serum IL-18	0.38		< 0.001
Zonulin vs. CARS-2 total score	0.29		0.001
LBP vs. serum IL-1β	0.34		< 0.001
NLRP3 mRNA vs. serum NLRP3	0.52		< 0.001
NLRP3 mRNA vs. CARS-2 total score	0.31		< 0.001

SD, standard deviation; LBP, lipopolysaccharide-binding protein; ρ, Spearman correlation coefficient.

Diagnostic performance

NLRP3 mRNA discriminated cases from controls with an AUC of 0.89 (95% CI: 0.85–0.92); at the Youden-optimal cut-off of 1.58 relative units, sensitivity was 83.3% and specificity 82.2%. Serum IL-18, serum NLRP3 and zonulin gave AUCs of 0.86, 0.84 and 0.83, respectively, and a combined panel of *NLRP3* mRNA, IL-18 and zonulin reached an AUC of 0.93 (95% CI: 0.90–0.96), with 88.9% sensitivity and 85.6% specificity (Fig. 3; Online Resource 1, Table S10). These values describe discrimination between an established clinical group and healthy controls and do not indicate screening utility in an unselected population.

Discussion

To our knowledge, this is the first study of *NLRP3* polymorphisms in ASD and the first characterisation of the *NLRP3* locus in Iraqi Arab children. Of three functionally distinct variants, spanning a regulatory intron, a coding exon and the 3′-UTR, the 3′-UTR variant rs10754558 was associated with ASD; the association survived adjustment for demographic and familial covariates and correction for multiple testing, and carriers of the risk genotype showed higher *NLRP3* transcript and protein levels and higher IL-1β and IL-18 concentrations.

The direction of effect is concordant with the broader *NLRP3* literature. The rs10754558 G allele modifies susceptibility to autoimmune and inflammatory conditions [22, 23] and alters transcript stability and *NLRP3* expression [14], and it has been associated with type 2 diabetes mellitus, coronary artery disease and ischaemic stroke in independent cohorts [15–17]. Because *NLRP3* variation has not previously been examined in ASD, direct comparison is not possible, and the present finding requires independent replication. An allelic OR below two is typical of common variants in complex neurodevelopmental phenotypes and should not be interpreted as clinically predictive at the individual level.

The genotype-dependent gradient in *NLRP3* mRNA offers a mechanistic account of the association and is, in our view, the more informative of the two principal results. An OR without a functional correlate leaves open the possibility that the association reflects LD with an untyped causal variant or residual confounding. Here, the stepwise increase in transcript abundance across CC, CG and GG genotypes, mirrored by serum NLRP3, IL-1β and IL-18, is consistent with a cis-acting regulatory effect at the 3′-UTR and with the reported role of this region in transcript stability and microRNA interaction. The elevated inflammasome output in the ASD group agrees with the hyperactivation reported in ASD-derived fibroblasts [12] and

extends that observation to an accessible peripheral compartment in a substantially larger sample.

Serum zonulin was elevated in the ASD group and correlated with both inflammasome activity and symptom severity, in agreement with earlier reports of increased intestinal permeability in children with ASD [27, 28] and with the elevated zonulin concentrations described by Esnafoglu et al. [30]. Because translocated LPS is a canonical NLRP3 priming signal, these data are compatible with a model in which barrier failure supplies the priming stimulus while host genotype determines the amplitude of the response. The cross-sectional design, however, cannot establish the direction of this relationship, and the alternative explanation, in which systemic inflammation compromises barrier integrity, remains equally tenable [31]. Restrictive feeding patterns and the altered microbiota described in children with gastrointestinal symptoms [32] are further candidate mediators that were not measured here.

rs3806268 showed point estimates close to unity under every genetic model and is the marker least correlated with rs10754558, so its null result is unlikely to be explained solely by marker redundancy. The most parsimonious interpretation is that this synonymous variant does not materially alter NLRP3 function in this population, consistent with the absence of an expression gradient across its genotypes; in mild cognitive impairment, rs3806268 was associated with expression of *PYCARD* rather than *NLRP3* [18], and *PYCARD* was not measured here. The rs4612666 signal was attenuated after multivariable adjustment and did not survive Bonferroni correction, and its presence in the risk-associated haplotype most plausibly reflects LD with rs10754558. Nevertheless, given its association with rheumatoid arthritis and serum NLRP3 in Iraqi Arab adults [20], a modest independent contribution cannot be excluded and warrants examination in larger samples.

The haplotype and LD results are exploratory. The T-G-G association was of borderline significance and was not corrected for the number of haplotypes tested, and the C-G-C signal

rests on small counts (14 case and 37 control chromosomes); such secondary analyses are prone to false-positive inference [47]. Because the haplotypes are reconstructed from the same genotypes that generated the single-locus results, they add mechanistic context rather than independent evidence.

Methodologically, restriction-based assays for these variants generate fragments that differ by as little as 25 base pairs and are difficult to resolve by agarose electrophoresis, and departures from HWE have been reported in restriction-based *NLRP3* studies [15]. We therefore used TaqMan allelic discrimination with a pre-specified call-rate threshold, no-template controls on every plate and blind re-genotyping of a random 10% of samples, and we recommend an equivalent standard for future *NLRP3* association studies.

The higher rate of first-cousin marriage among cases, which persisted as an independent predictor, is consistent with the documented contribution of consanguinity to inherited and immune-related disease in Middle Eastern and North African populations [33, 34] and supports its routine adjustment in association studies in this region. Because consanguinity increases genome-wide homozygosity, it could inflate associations driven by homozygous genotypes such as rs10754558 GG; however, case genotypes conformed to HWE at all three loci and the GG association persisted after adjustment for consanguinity (Table 3), which argues against inbreeding as the principal explanation.

From a translational standpoint, NLRP3 is druggable, and selective small-molecule inhibitors have progressed into clinical development [24, 25]. The present data do not justify any therapeutic inference, but they suggest that a genetically defined subgroup with elevated inflammasome activity could be identified for future mechanistic or interventional study, and that barrier-directed interventions warrant parallel consideration.

Limitations

This study has several limitations. First, the single-centre sample was adequately powered for the primary single-locus comparisons but not for the haplotype and LD analyses or for small genetic effects. Second, inflammasome activity was measured in PBMCs, an accepted and ethically feasible proxy that may not fully reflect microglial activity. Third, no functional experiments, such as reporter or allele-specific expression assays, were performed, so the mechanism underlying the genotype–expression relationship cannot be established from these data alone. Fourth, the cross-sectional design precludes causal inference, particularly between barrier markers and inflammasome activity. Fifth, human leukocyte antigen genotyping and *NLRP3* sequencing were not performed, so causal variants in LD with the typed markers cannot be excluded, and residual population stratification remains possible despite single-province recruitment and adjustment for consanguinity. Sixth, commercial zonulin immunoassays may recognise zonulin-family proteins such as properdin rather than pre-haptoglobin 2 alone [49], so serum values should be interpreted as zonulin-family immunoreactivity. Finally, diet, microbiota composition and antibiotic exposure history were not characterised in detail.

Conclusions

In Iraqi Arab children, the *NLRP3* 3′-UTR polymorphism rs10754558 was associated with ASD susceptibility independently of demographic and familial covariates and after correction for multiple testing. The association was accompanied by allele-dose-related increases in *NLRP3* transcript, serum NLRP3 and downstream inflammasome cytokines, providing internal functional support for the genetic finding. Intestinal permeability markers were elevated in patients and correlated with inflammasome activity and symptom severity, whereas the haplotype and LD findings are exploratory and require validation. The NLRP3

inflammasome is a candidate axis in ASD that merits replication in independent populations and investigation with functional methods.

Supplementary Information

The online version contains supplementary material available as Online Resource 1, comprising Fig. S1, TaqMan allelic discrimination plots for the three *NLRP3* loci; Table S1, The three *NLRP3* polymorphisms genotyped, with genomic context, functional rationale and assay details; Table S2, Genotyping quality-control metrics; Table S3, RT-qPCR assays, amplicon sizes and amplification efficiencies; Table S4, Reference gene stability analysis; Table S5, ELISA assay validation data; Table S6, In silico functional annotation of the three variants; Table S7, HWE testing at the three *NLRP3* loci; Table S8, *NLRP3* haplotype frequencies in ASD patients and controls; Table S9, Pairwise LD between the three *NLRP3* loci; Table S10, ROC analysis of candidate markers.

Statements and Declarations

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Author contributions K.S.Z.: conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, visualization, supervision, project administration and writing – original draft. N.A.H.: investigation and data curation. M.O.: validation and formal analysis. All authors reviewed and edited the manuscript, and read and approved the final version.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Research Ethics Committee of the Faculty of Science, University of Kufa (date: 12 January 2024; approval ID: UOK-FS-218).

Consent to participate Written informed consent was obtained from the parents or legal guardians of all individual participants included in the study, and assent was obtained from children older than seven years who were able to provide it.

Consent to publish Not applicable; the manuscript contains no individually identifiable data, images or videos.

Data availability The data supporting the findings of this study are not publicly available because they comprise genetic and clinical data from children and are subject to confidentiality and ethical restrictions; de-identified data are available from the corresponding author upon reasonable request.

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Figure legends

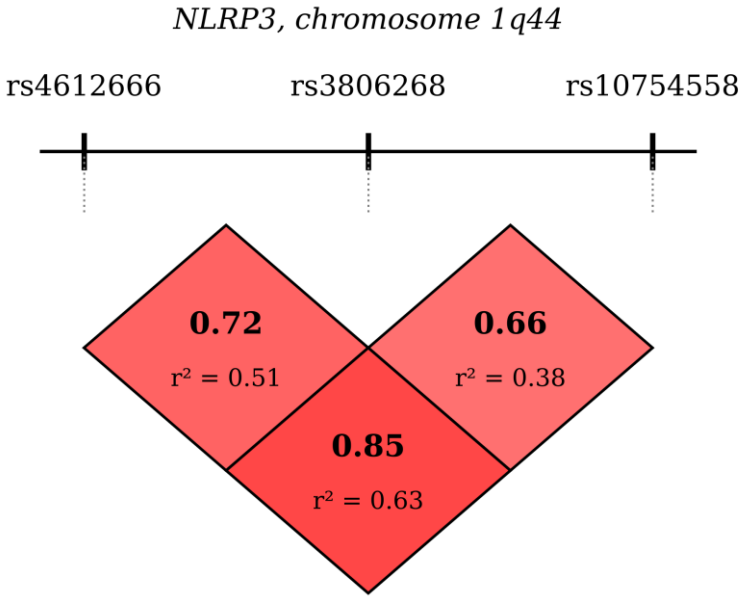


Fig. 1 Linkage disequilibrium across the *NLRP3* locus. Pairwise linkage disequilibrium between rs4612666, rs3806268 and rs10754558, displayed in the conventional triangular format. Cell shading is proportional to D′; the upper value in each cell is D′ and the lower value is r². All pairwise comparisons were significant at $p < 0.001$

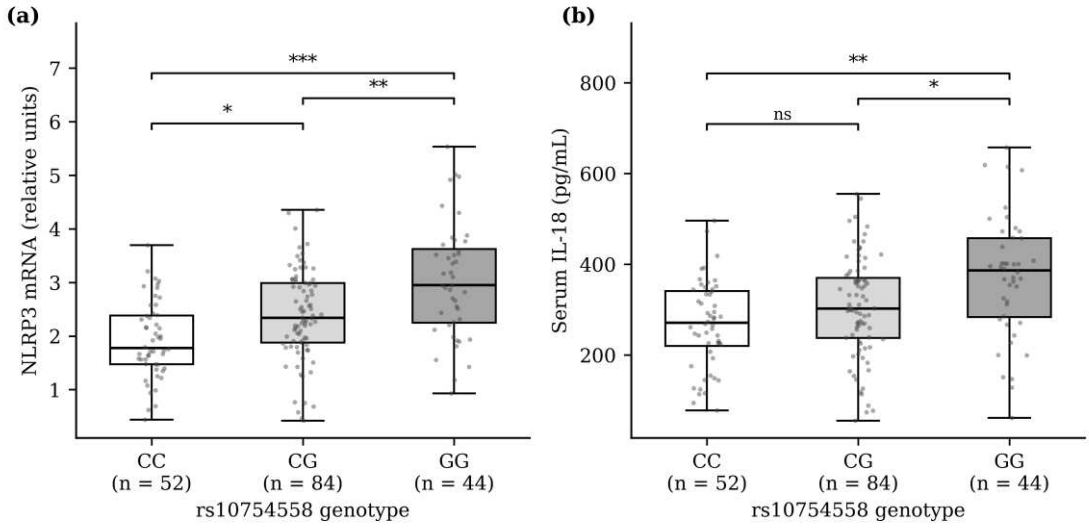


Fig. 2 *NLRP3* transcript abundance and serum IL-18 by rs10754558 genotype within the ASD group. (a) *NLRP3* mRNA expression in peripheral blood mononuclear cells, expressed in relative units. (b) Serum IL-18 concentration. Boxes show the median and interquartile range,

whiskers the 5th to 95th percentiles, and points individual participants. One-way analysis of variance with Tukey post-hoc correction: *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant

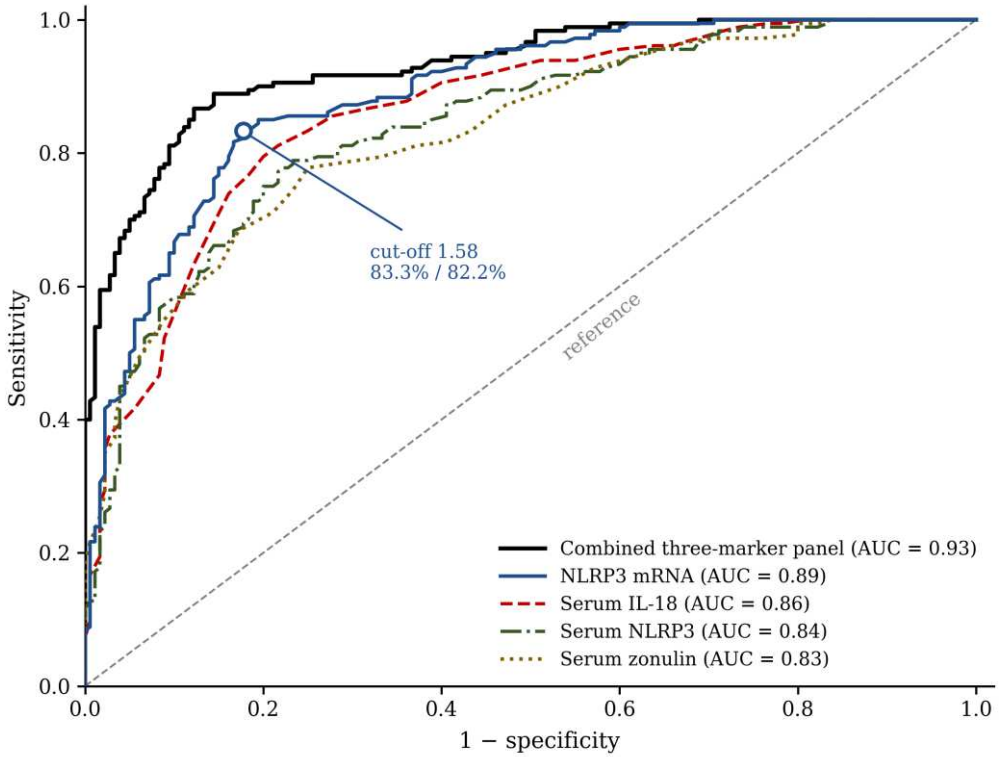


Fig. 3 Receiver operating characteristic curves for candidate markers discriminating ASD cases from controls. Curves are shown for *NLRP3* mRNA, serum IL-18, serum NLRP3, serum zonulin and the combined three-marker panel, with the reference diagonal indicated. The marked point is the Youden-optimal operating point for *NLRP3* mRNA, with the corresponding sensitivity and specificity. Curves are non-parametric (empirical) ROC curves derived from 180 cases and 180 controls; the panel curve uses predicted probabilities from logistic regression of the three markers