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Functional Implications of NLRP1 Variants for Autoimmune Disease

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FUNCTIONAL IMPLICATIONS OF *NLRP1* VARIANTS FOR AUTOIMMUNE DISEASE

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ABSTRACT

FUNCTIONAL IMPLICATIONS OF *NLRP1* VARIANTS FOR AUTOIMMUNE DISEASE

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Bachelor of Science

NLRP1 is a protein-coding human gene that plays a crucial role in the NLRP1 inflammasome. Variants to the *NLRP1* gene have been associated with autoimmune and autoinflammatory diseases. We examined the effects of polymorphisms at two SNPs on cytokine levels and *NLRP1* gene expression in 50 human volunteers without diagnosed autoimmune disease. *NLRP1* was genotyped at SNPs rs2670660 and rs12150220 and individuals who were homozygous at one or more SNP were selected for further analysis. Serum IL-18 and IL-1β levels were quantified using ELISA. *NLRP1* gene expression was measured using real-time PCR. A strong linkage was found between genotypes of rs2670660 and rs12150220 ($p = 2.33 \times 10^{-13}$). Participants with an AAAA genotype (written as rs2670660 genotype + rs12150220 genotype) had significantly higher levels of IL-18 than participants with a GGTT genotype (0.439 ng/μL vs 0.152 ng/μL, $p = 0.024$). A trend towards increased *NLRP1* expression was seen in the AAAA genotype (4.392-fold increase, $p = 0.101$). It is likely that the AAAA genotype represents a combination of two homozygous autoimmunity risk variants. The AAAA genotype showed increased production of IL-18 even in non-autoimmune individuals, with a trend

towards higher gene expression, suggesting that the AA variants of rs2670660 and rs12150220 lead to increased *NLRP1* activity. Due to the linkage of rs2670660 and rs12150220, it was impossible to differentiate which SNP was responsible for the observed effects. Further studies with a larger sample size would contribute to an increased understanding of the effects of *NLRP1* variants on autoimmune disease.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Poole, for all the support, guidance, and feedback he has given to me for this project. I would like to thank my husband, Zach, for supporting me through long, hard days and convincing me to keep trying. I would like to thank everyone in the Poole Lab who has contributed their time and efforts to this research, especially Erin Gill for getting this project off the ground and maintaining it in my absence.

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BACKGROUND

Autoimmune diseases are a group of more than 80 chronic illnesses that can affect almost any system, organ, or tissue in the body. Often acquired with age and always non-contagious, these disorders are difficult to treat because they are caused by immune system attacks on the body's own tissues and cells. Of significant concern to the field of healthcare, autoimmune diseases affect between 14.7 and 23.5 million Americans [1].

The *NLRP1*, or *NALP1,* gene is a protein-coding human gene found on chromosome 17. The NLRP1 protein plays a crucial role in the nucleotide-binding domain leucine-rich repeat containing (NLR) inflammasome. NLRP1 is the sensor protein; when activated it recruits caspase-1 to initiate inflammasome assembly. Inflammasomes are multiprotein oligomers responsible for the inflammatory responses that are essential to the innate immune system [2]. Components and homologues of the NLRP1 inflammasome have been implicated in many autoinflammatory diseases, examples of which include inflammatory bowel disease, Blau syndrome, Muckle-Wells syndrome, and neonatal onset multisystem inflammatory disease [3-6]. However, another subset of research has found a strong epidemiological correlation between *NLRP1* polymorphisms and vitiligo, an autoimmune disease causing loss of skin pigmentation, especially when vitiligo is presented in concert with other autoimmune diseases including autoimmune thyroid disease, latent autoimmune diabetes in adults, rheumatoid arthritis, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease [7-9].

This connection between *NLRP1* and vitiligo-associated autoimmune disease is especially fascinating given the usual separation in the categorization of autoimmune and autoinflammatory diseases. Autoinflammatory diseases are caused by a self-induced

increase in inflammation, whereas autoimmune diseases are caused by the weaponization of the body's immune system against its own cells. The NLR pathway is an innate immune pathway, meaning it is usually associated with only autoinflammatory diseases. Thus, *NLRP1*'s association with autoimmune diseases, which are usually associated with adaptive immunity, presents a compelling research interest [10].

NLRP1 has been implicated in the secretion of interleukin 1 beta (IL-1β) and the secretion of interleukin 18 (IL-18) [11-14]. IL-1β and IL-18 are structurally and functionally similar [15]. They are produced as biologically inactive precursors, which are then cleaved and thereby activated by caspase-1 [16]. The secretion of these interleukins may be connected to the development of autoimmune diseases; for example, IL-18 has been postulated to play a role in the autoimmune disease Hashimoto's thyroiditis, and is found in higher than average levels in patients with rheumatoid arthritis and systemic lupus erythematosus, among others [17-19]. Human *NLRP1* variants associated with autoimmune diseases have been identified in meta-analysis and epidemiological studies, and the general mechanisms of the NLR pathway have been identified [2, 7, 8]. In their foundational 2007 study on *NLRP1* and vitiligo-associated autoimmune disease, Jin et al. identified 19 SNPs that were associated with autoimmune and autoinflammatory diseases, including rs12150200 and rs2670660. rs12150220 is an L115H substitution located in the protein-coding region of *NLRP1*, while rs2670660 is located in the promoter region (Figure 1) [7, 12]. The segment in which rs2670660 is located is conserved in several mammals, suggesting that it has functional importance. This area of the promoter changes predicted binding motifs for the transcriptional factors MYB and HMGA1, with MYB regulating transcription during differentiation,

proliferation, and apoptosis of lymphoid, myeloid, and erythroid cell lines [7]. Despite the interesting potential effects of variations to rs2670660, rs12150220 has been studied more extensively for its effects on autoimmune disease [11, 20, 21]. The current literature lacks consensus on the effects of rs2670660 on mRNA expression, cytokine expression, or protein function of NLRP1, and neither SNP has been explicitly tested for effects on IL-18 expression [12]. Therefore, the purpose of this study was to characterize the effects of two specific *NLRP1* gene variants, at SNPs rs2670660 and rs12150220, on *NLRP1* mRNA transcription levels and IL-1β and IL-18 secretion, to determine the differential roles of *NLRP1* polymorphisms and their implications on autoimmune disease.

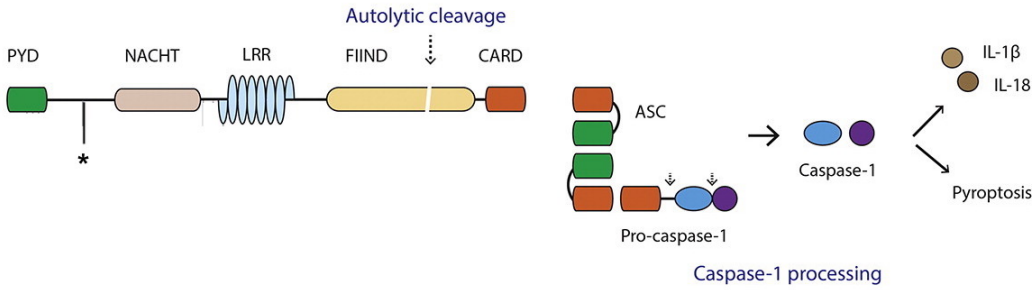


Figure 1. Visual representation of the NLRP1 inflammasome, indicating location of rs12150220. Domains of the human NLRP1 inflammasome and the eventual activation of IL-1β and IL-18 by caspase-1 are shown. The location of rs12150220 is marked (*), representing a nonsynonymous L155H substitution. The location of rs2670660 is not indicated because the architecture of the *NLRP1* promoter region is less well-characterized. Adapted from Yu et al. (2018).

MATERIALS AND METHODS

Experimental design overview

Blood samples were recruited from participants and processed to prepare for further analyses. DNA was isolated from whole blood for genotyping and ELISAs were performed using plasma. RNA was extracted from lymphocytes, used to produce cDNA, and analyzed using qPCR. All samples were genotyped at both SNPs (rs2670660 and rs12150220), but only samples with at least one homozygous locus were selected for further testing. Figure 2 provides a visual organization of the main tests performed for this analysis.

Selection of participants

The presence of homozygous genotypes at both SNPs has been shown in control populations—18% and 32% for rs2670660 and 17% and 36% for rs12150220 according to one study—despite an absence of autoimmune disease presentation [22]. For this project, we did not have access to a population of patients with vitiligo-associated autoimmune disease. As such, we analyzed the effects of risk variants in a population without diagnosed autoimmune disease, anticipating that the high presence of risk variants would still cause a change to mRNA transcription or interleukin levels. If *NLRP1* variants are only one contributing factor to autoimmune disease presentation, it is possible that functional effects of these variants can be detected in the absence of diagnosed autoimmune disease. Vitiligo and other associated autoimmune diseases tend to be polygenic, so by using participants without a diagnosed autoimmune disease we attempted to eliminate the confounding influence of other genes associated with risk variants for autoimmune disease, such as *PTPN22* in vitiligo-associated autoimmunity, or

IRF5 in systemic lupus erythematosus [9, 23]. It is, of course, possible that our participants had these or other autoimmunity risk variants in addition to *NLRP1* variants.

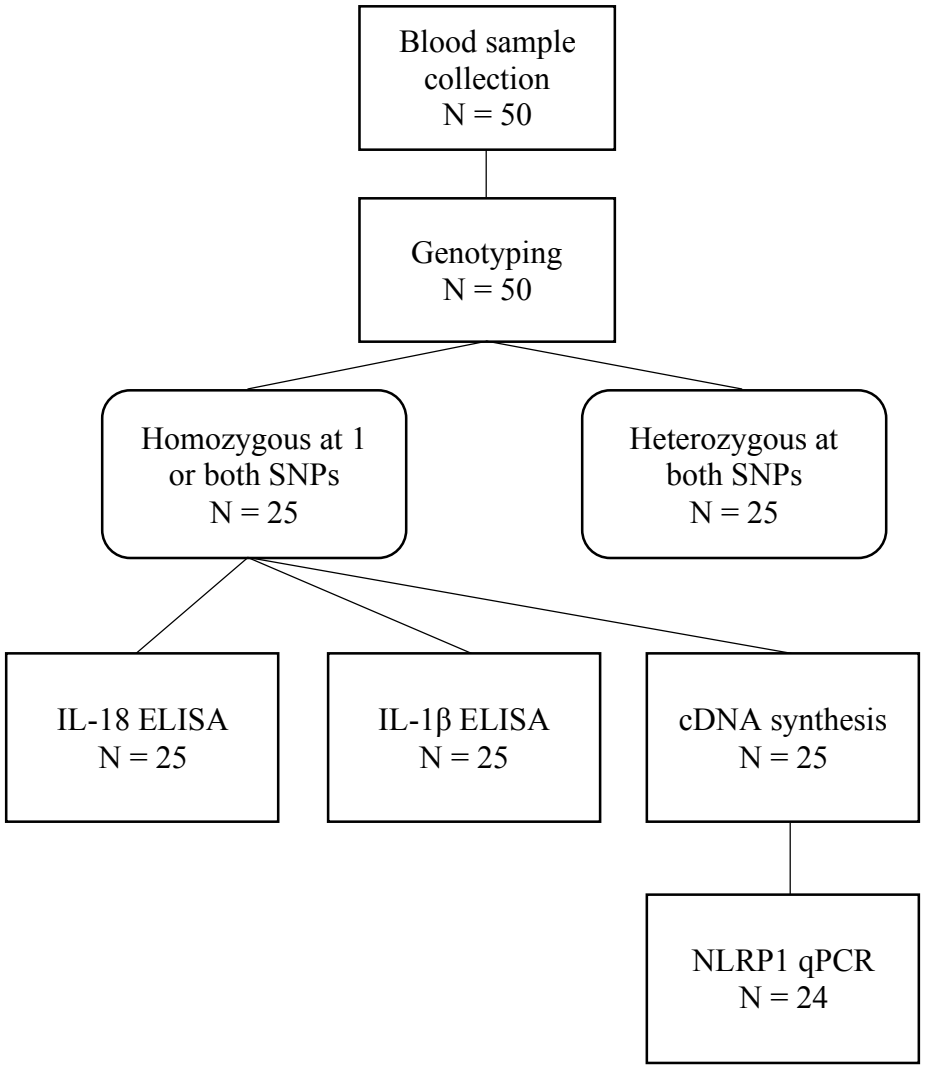


Figure 2. Visual overview of main experimental steps.

Blood collection

Participants without a diagnosed autoimmune disease were recruited by flyer and word-of-mouth and reimbursed $15 for their donation. This sample collection was approved by the IRB of Brigham Young University for the period of March 8th, 2019 to March 7th, 2020. Up to 24 mL of blood were drawn by certified phlebotomists into BD

Vacutainer™ tubes with either K_2 EDTA or ACD Solution B anti-coagulant. Blood was stored at room temperature until processed. All samples were processed within 22 hours of collection.

Blood processing

Blood samples were centrifuged at 200 rcf for 10 minutes to separate plasma from red blood cells. 1 mL of plasma was extracted from each sample and placed in a 1.5 mL microcentrifuge tube to be stored at -80° C for later cytokine analysis. Blood was then agitated and 250 μL of whole blood placed in a 1.5 mL microcentrifuge tube to be stored at -80° C for later DNA extraction. For lymphocyte extraction, 10 mL of Corning Lymphocyte Separation Medium was placed in a 50 mL conical tube. Then, 10 mL of the remaining sample blood was carefully added to form a layer on top of the separation medium. 10 m L of PBS was then added in the same manner. The 50 mL tube was centrifuged at 400 rcf for 30 minutes, adding up to 15 more minutes if a distinct lymphocyte layer was not visible after 30 minutes. As much as possible of the lymphocyte layer (usually 5-10 mL) was then extracted and moved to a new 50 mL conical tube, which was filled to 40 mL with PBS. This was centrifuged at 300 rcf for 5 minutes. The liquid was decanted and disposed of and the cell pellet maintained in the tube. 350 mL of Ambion® RNAqueous® Lysis/Binding Solution was added to the cell pellet. 500 mL of lysed cell matter was then transferred into a 1.5 mL microcentrifuge tube to be stored at -80° C for later RNA isolation.

DNA isolation

DNA was isolated from whole blood samples using Qiagen DNeasy® Blood & Tissue Kit, following the manufacturer's protocol. Quality of isolated DNA was confirmed using Thermo Scientific™ NanoDrop™ Lite Spectrophotometer.

Genotyping

Genotyping was performed with Applied Biosystems™ TaqMan® SNP Genotyping Assays for rs2670660 and rs12150220 following the manufacturer's protocol. Applied Biosystems™ MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1mL) was used with Applied Biosystems™ StepOnePlus™ Real-Time PCR System and StepOne™ Software v2.3 was used for experimental design and analysis. SNP rs2670660 has A/G variants, and SNP rs12150220 has A/T variants. For some analyses, genotypes of rs2670660 and rs12150220 are combined into one conglomerate genotype; for example, genotype AAGT represents an individual with the genotype AA at rs2670660 and the genotype GT at rs12150220.

ELISA

Plasma was used for IL-1β and IL-18 ELISA analysis. IL-18 analysis was performed using eBioscience™ Human IL-18 Platinum ELISA Kit, following the manufacturer's protocol. IL-1β analysis was performed using Invitrogen Human IL-1beta Uncoated ELISA Kit, following the manufacturer's protocol.

RNA isolation

RNA was isolated using Invitrogen RNAqueous™ Phenol-free total RNA Isolation kit following the manufacturer's protocol. RNA concentration was measured for

calibrating future cDNA synthesis using Thermo Scientific™ NanoDrop™ Lite Spectrophotometer.

cDNA synthesis

cDNA was synthesized from total RNA using Invitrogen SuperScript™ IV First-Strand Synthesis System and Eppendorf Mastercycler® Nexus thermal cycler, following the manufacturer's protocols. Oligo dT primers were selected in order to isolate mRNA.

NLRP1 qPCR

We designed custom PCR primers for *NLRP1* and ordered them from Integrated DNA Technologies. Primers spanned the exon-exon junction created by the splicing of the first intron of the *NLRP1* gene to select for the amplification of mature mRNA and exclude DNA. Forward primer sequence: CTACGTTGGCCACTTGGGAT. Reverse primer sequence: AGGTGAAGGTACGGCTATGC. Real-time PCR was performed on *NLRP1* cDNA with *GAPDH* endogenous controls using Applied Biosystems™ *Power*SYBR® Green PCR Master Mix following the manufacturer's protocol. Applied Biosystems™ MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1mL) was used with Applied Biosystems™ StepOnePlus™ Real-Time PCR System and StepOne™ Software v2.3 was used for experimental design and analysis. Significance threshold was set above the background in the linear portion of the amplification curve ($\Delta Rn = 0.048564$). Ct values denote at which cycle of the PCR procedure a sample's amplification crossed the significance threshold. Lower Ct values represent higher gene expression because a higher concentration of target RNA will begin to amplify earlier in the PCR procedure. ΔCt values are a subtraction comparison between the average Ct value of the target and the average Ct value of the endogenous control for a sample.

Statistical analysis

Demographic data was collected from participants by survey at the time of blood collection. Significance of the demonstrated tendency towards AAAA and GGTT combined genotypes was calculated by first calculating expected genotype outcomes using the observed allele frequencies and then using a chi-squared test. Originally, we intended to use paired analyses for ELISA and cDNA data, but the non-random assortment of alleles in combination with demographic mismatches (all 3 non-white homozygous participants had genotype AAAA) left too few pairs for powerful analysis, so data was analyzed in genotype aggregates. Significance in IL-18 ELISA results was analyzed using Student's t-tests. No statistical methods were used on IL-1β ELISA results due to the absence of IL-1β in the samples. Student's t-tests were also used to compare *NLRP1* Ct values in the qPCR results. All statistical tests were performed using Microsoft Excel.

RESULTS

Our sample of 50 participants included 35 females and 15 males, comprising 70% and 30% of the sample, respectively. There were 45 participants who identified as White, 2 who identified as Hispanic, and 3 who identified as Asian, comprising 90%, 4%, and 6% of the sample, respectively. There were 44 participants between the ages of 18-29, 4 between the ages of 30-39, none between the ages of 40-49, and 2 between the ages of 50-59, comprising 88%, 8%, 0%, and 4% of the sample, respectively. See Table 1.

Demographics		N = 50	(%)
Sex	Female	35	70%
	Male	15	30%
Race	White	45	90%
	Hispanic	2	4%
	Asian	3	6%
Age	18-29	44	88%
	30-39	4	8%
	50-59	2	4%

Table 1. Sample demographics. Participants were largely white, female, and between 18-29 years of age.

Genotyping

Using TaqMan genotyping we determined that at SNP rs2670660, 11 participants were homozygous AA, 27 participants were heterozygous AG, and 12 participants were homozygous GG, comprising 22%, 54%, and 24% of the sample, respectively. This resulted in allele frequencies at this SNP of 0.49 for A and 0.51 for G. Furthermore, we determined that 11 participants were homozygous AA, 28 participants were heterozygous

AT, and 11 participants were homozygous TT at SNP rs12150220, comprising 22%, 56%, and 22% of the sample, respectively. This resulted in allele frequencies at this SNP of 0.50 for A and 0.50 for G. See Table 2.

	Genotypes, n (%)			Alleles, frequency	
rs2670660	AA	AG	GG	A	G
	11 (22)	27 (54)	12 (24)	0.49	0.51
rs12150220	AA	AT	TT	A	T
	11 (22)	28 (56)	11 (22)	0.50	0.50

Table 2. Genotypes and allele frequencies for rs2670660 and rs12150220 as calculated from sample.

When consolidated into combined genotypes, the genotype results of rs2670660 and rs12150220 show very strong indications of linkage (Table 3). (Combined genotypes are written in this paper as the genotype at rs2670660 and then the genotype at rs12150220. For example, genotype GGTT has homozygous GG at rs2670660 and homozygous TT at rs12150220). Homozygous combined genotypes AAAA and GGTT are strongly favored over homozygous combined genotypes AATT or GGAA, with 10 AAAA and 10 GGTT participants and zero AATT or GGAA participants. Mixed homozygous/heterozygous combined genotypes were also rare, with 1 each of AAAT, AGAA, and AGTT genotypes, and 2 of GGAT. There were 25 participants who were heterozygous at both rs2670660 and rs12150220. If rs2670660 and rs12150220 were assorting independently with the observed allele frequencies from Table 2, the expected distribution of combined genotypes would be as follows for 50 samples: 3.001 AAAA, 6.248 AGAA, 3.251 GGAA, 6.003 AAAT, 12.495 AGAT, 6.503 GGAT, 3.001 AATT, 6.248 AGTT, and 3.251 GGTT. Comparing these expected values with the observed

genotype results via a chi-squared test strongly disproves the null hypothesis, with a p-value of 2.33×10^{-13}. Thus, it can be asserted that rs2670660 and rs12150220 are co-inherited or linked.

		rs2670660, n (%)		
		AA	AG	GG
rs12150220, n (%)	AA	10 (20)	1 (2)	0 (0)
	AT	1 (2)	25 (50)	2 (4)
	TT	0 (0)	1 (2)	10 (20)

Table 3. Combined genotypes of rs2670660/rs12150220 show strong linkage. AAAA and GGTT are the strongest homozygous combined genotypes, with no AATT or GGAA homozygous combined genotypes present. There are also more heterozygotes than would be expected in independent assortment based on the observed allele frequencies. $P = 2.33 \times 10^{-13}$.

Cytokine analysis

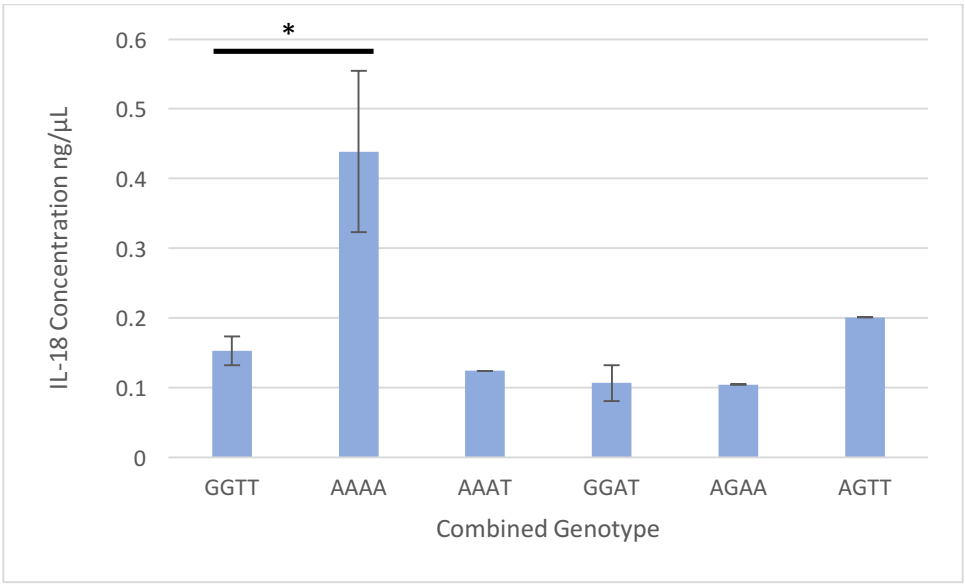
The IL-18 ELISA produced significant results in the combined genotype comparison as well as at each SNP. In the combined genotype comparison, the average concentrations for each genotype were as follows (samples were run in duplicate): GGTT = 0.152 ng/μL (SE = 0.021, n = 10); AAAA = 0.439 ng/μL (SE = 0.116, n = 10); AAAT = 0.124 ng/μL (n = 1); GGAT = 0.106 ng/μL (SE = 0.026, n = 2); AGAA = 0.104 ng/μL (n = 1); AGTT = 0.2005 ng/μL (n = 1). Only AAAA and GGTT combined genotypes were deemed to have sufficient quantity for statistical analysis, and a Student's T-test found that the concentration of IL-18 was significantly higher in the AAAA genotype than the GGTT genotype (p = 0.024). See Figure 3a.

The combined genotypes were subdivided back into SNP genotypes for comparisons between homozygous individuals at each SNP, incorporating those that had

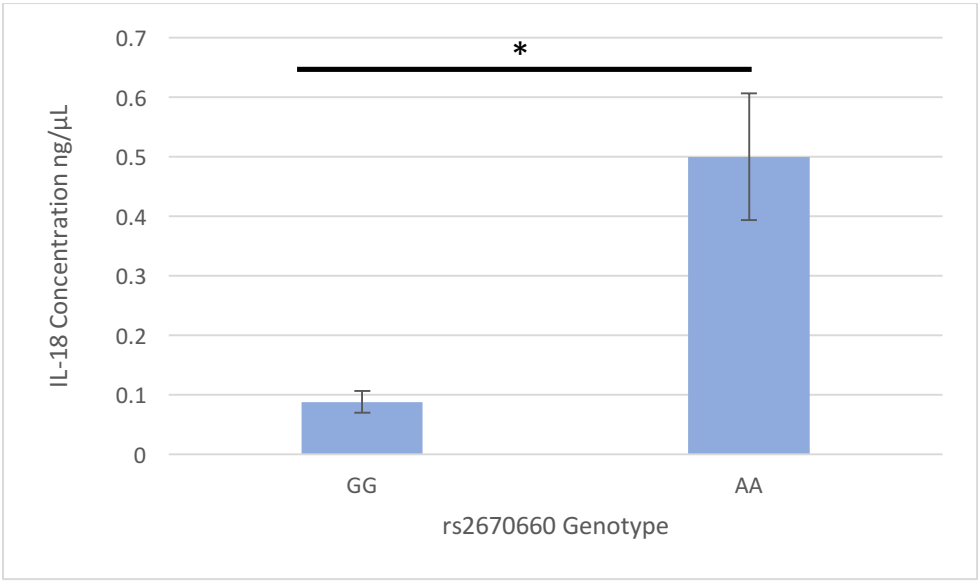
mixed combined genotypes. In the comparison for rs2670660, the average concentrations for each homozygous genotype were 0.145 ng/μL for GG (SE = 0.018, n = 24) and 0.410 ng/μL for AA (SE = 0.107, n = 22). A Student's T-test found significance at the level of p = 0.022 for this comparison. See Figure 3b.

In the comparison for rs12150220, the average concentrations for each homozygous genotype were 0.157 ng/μL for TT (SE = 0.019, n = 22) and 0.408 ng/μL for AA (SE = 0.107, n = 22). A Student's T-test found significance at the level of p = 0.030 for this comparison. See Figure 3c.

a)



b)



(Figure 3. See legend on next page.)

c)

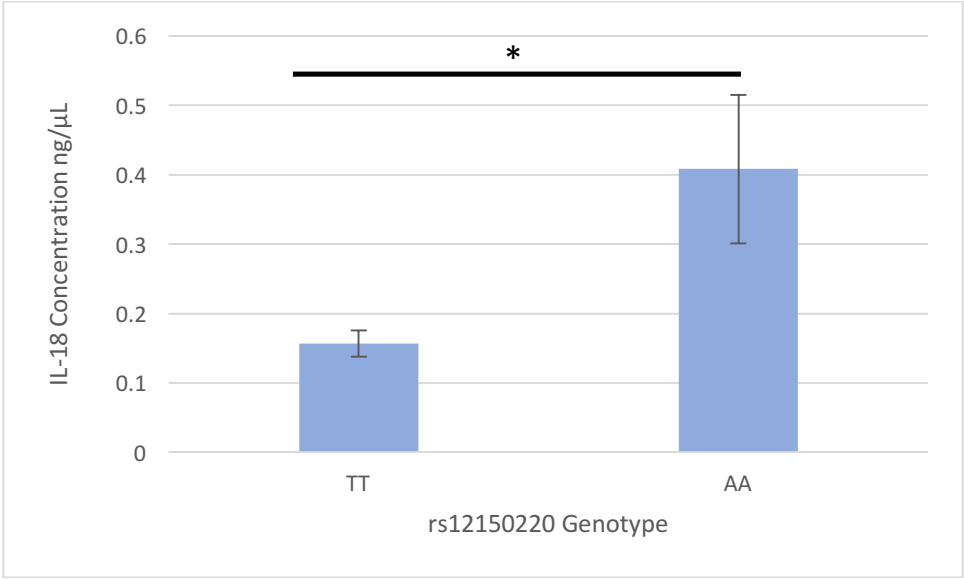


Figure 3. Significantly higher concentrations of IL-18 displayed in AAAA combined genotype and individual AA genotypes. a) Comparison of IL-18 concentrations across all present combined genotypes. *p = 0.024 for AAAA vs. GGTT. Statistical significance was not calculated for other combined genotypes due to small sample sizes. b) Comparison of IL-18 concentrations for variants of rs2670660 homozygous samples. *p = 0.022 for AA vs. GG. c) Comparison of IL-18 concentrations for variants of rs12150220 homozygous samples. *p = 0.030 for AA vs. TT.

The IL-1β ELISA did not produce any significant results for any of the samples. All sample concentrations were unmeasurable (<0.000 ng/µL) but the standards worked as expected (Figure 4), validating the sample results.

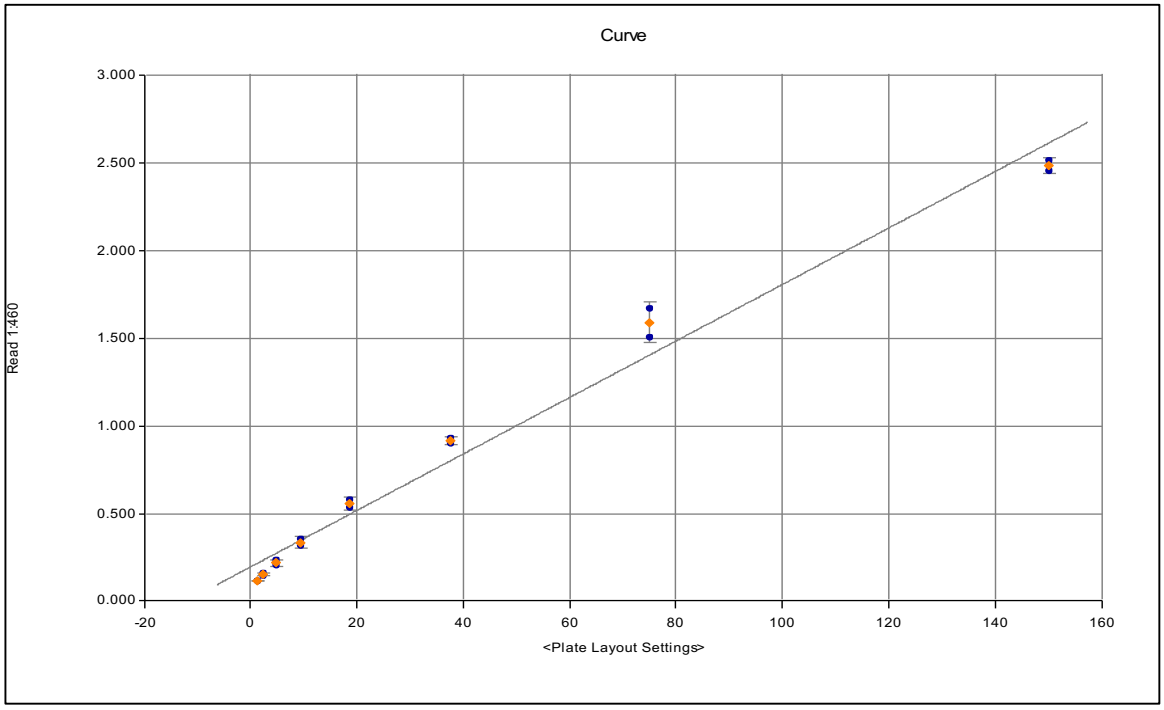


Figure 4. Standard curve of IL-1β demonstrates functional ELISA. No samples showed measurable concentrations of IL-1β. However, the standards worked as anticipated, demonstrating the accuracy of the non-measurements.

NLRP1 gene expression

Quantitative PCR was used to determine differences in *NLRP1* gene expression. The qPCR showed a trend towards earlier amplification in the AAAA genotype, with earlier amplification indicating higher gene expression (Figure 5). *GAPDH* was used as an endogenous control, but some *GAPDH* samples had unusual melt curves, perhaps indicating poor primers. The correct shape of *NLRP1* melt curves indicates that the *NLRP1* primers functioned properly (Figure 6). Since an analysis using ΔCt scores calculated with *GAPDH* did not show different trends than an analysis using only *NLRP1* Ct scores, *NLRP1* Ct scores were used for comparison between *NLRP1* combined genotypes to avoid any complications from the unusual melting of some *GAPDH* samples. One sample of redundant demographics and genotype (GGTT) was excluded from this analysis, and all other samples were run in duplicate and then averaged for each

sample. The mean *NLRP1* Ct scores were as follows for each combined genotype: AAAA = 33.237 (n = 10); GGTT = 35.382 (n = 9); AGTT = 28.735 (n = 1); AAAT = 37.651 (n = 1); AGAA = 29.876 (n = 1); GGAT = 30.031 (n = 2). There is no statistically significant difference between the means of the AAAA and GGTT genotypes (p = 0.101). However, due to the exponential nature of Ct calculations, the difference in means of 2.135 represents a 4.392-fold increase in gene expression, and the lower Ct value represents the higher gene expression. Therefore, these results demonstrate a trend towards higher gene expression in genotype AAAA when compared to genotype GGTT. Combined genotypes AGTT, AAAT, AGAA, and GGAT were not statistically analyzed due to small sample sizes (Figure 7). An analysis of homozygous genotypes for rs2670660 found the average *NLRP1* Ct score for AA to be 33.247 (n = 11) and for GG to be 34.046 (n = 11), and an analysis of homozygous genotypes for rs12150220 found the average of *NLRP1* Ct score for AA to be 32.941 (n = 11) and for TT to be 34.717 (n = 11).

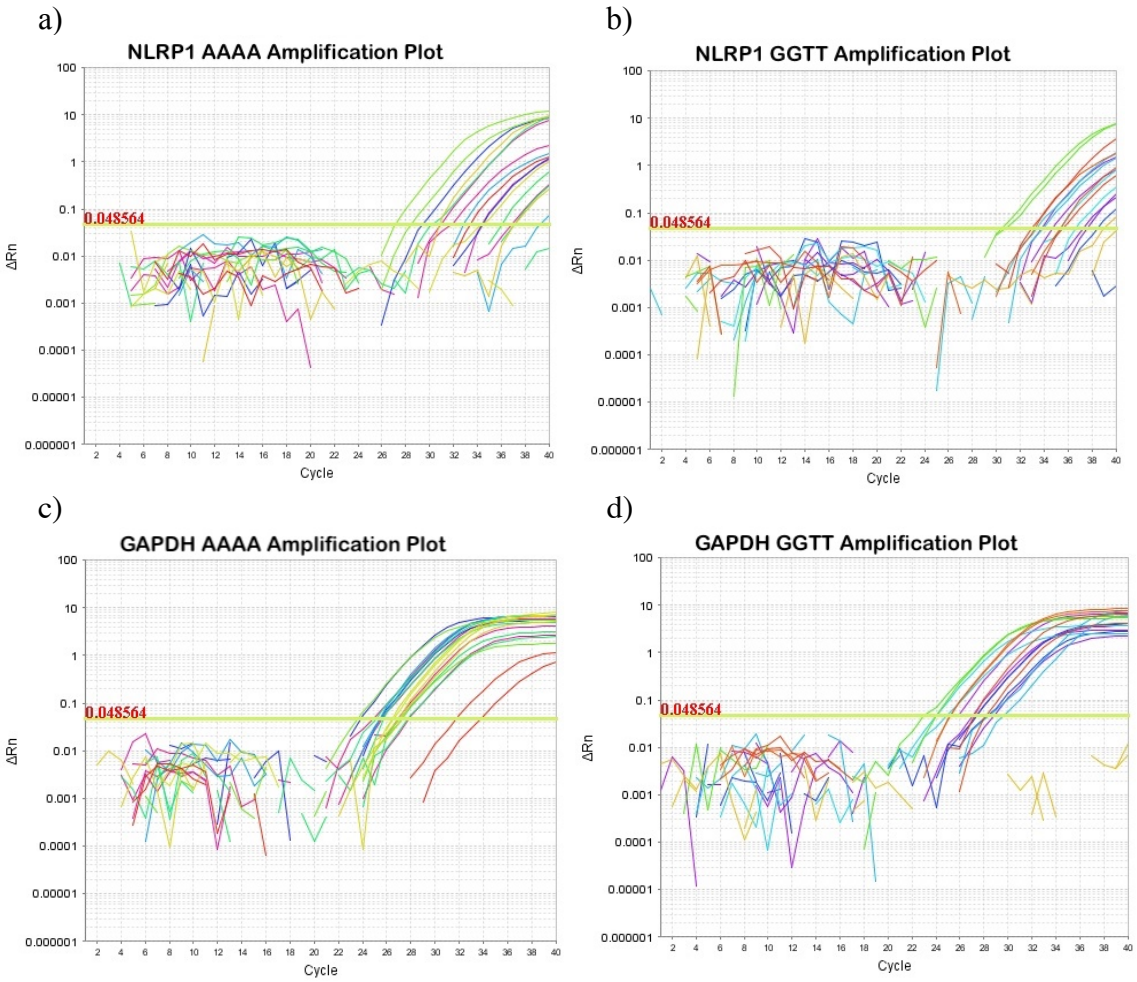


Figure 5: Amplification plots indicate a trend towards earlier amplification in *NLRP1* AAAA combined genotype. a) qPCR amplification plot of *NLRP1* AAAA genotype displays some earlier amplification compared to b) qPCR amplification plot of *NLRP1* GGTT genotype. c) *GAPDH* AAAA genotype control shows consistent amplification with two outliers. d) *GAPDH* GGTT genotype control shows consistent amplification with two outliers. Significance threshold for qPCR set to ΔRn = 0.048564.

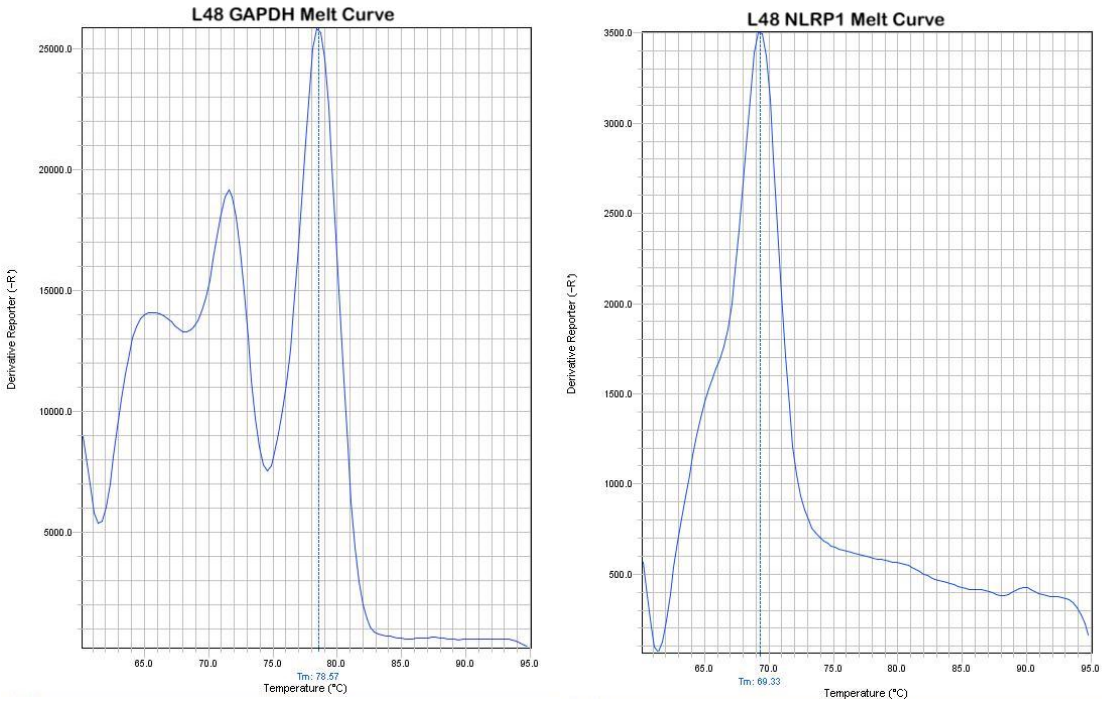


Figure 6: Example of unusual *GAPDH* melt curve in comparison with normal *NLRP1* melt curve. The qPCR melt curves of *GAPDH* and *NLRP1* are shown for one sample, number L48. A reliable melt curve should only have one major peak, as seen in the results for *NLRP1*. The variety of peaks in the results for *GAPDH* demonstrates the possibility of poor primers. These trends were typical across all samples.

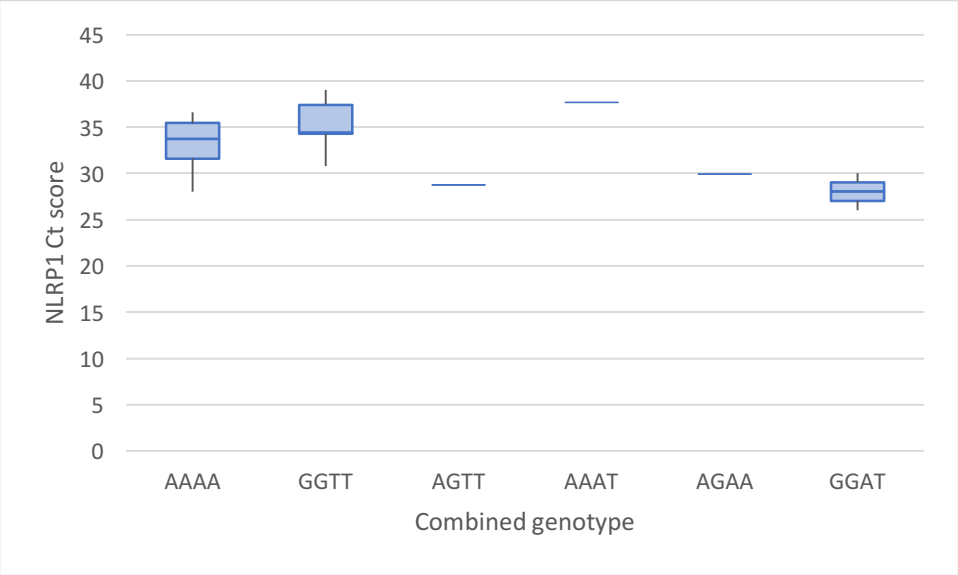


Figure 7: AAAA combined genotype trends towards higher gene expression than GGTT combined genotype in qPCR. This box and whisker plot shows the median and interquartile range of Ct scores for the *NLRP1* qPCR, with whiskers extending to the full range of collected data. The mean for genotype AAAA is 33.237 and the mean for genotype GGTT is 35.382. There is no statistically significant difference between these means ($p = 0.10$). Due to the exponential nature of Ct calculations, the difference in means of 2.135 represents a 4.392-fold increase in gene expression, and the lower Ct value represents the higher gene expression. Therefore, these results may indicate a possible trend towards higher gene expression in genotype AAAA when compared to genotype GGTT. Further analysis with more samples would be needed to provide statistical power for this conclusion. Combined genotypes AGTT, AAAT, AGAA, and GGAT were not statistically analyzed due to small sample sizes ($n = 1, 1, 1,$ and 2, respectively).

DISCUSSION

A variety of allele frequencies for rs2670660 and rs12150220 have been reported across many large-scale analyses for different racial groups [23, 24]. As our sample population was 90% White and located in the United States, comparisons to previous study results from European and American cohorts are most demographically accurate. Our allele frequencies are much closer to 0.50/0.50 at both SNPs than many of these study results, which tend to favor the A variant of both SNPs in ratios that approach 0.60/0.40 [23, 24]. For example, in an gnomAD study of Americans, rs2670660 allele frequencies were 0.57 for A and 0.43 for G [23].

There is consensus that the risk variant for autoimmune disease for rs12150220 is A, however the literature is less clear concerning the risk variant for rs2670660 [8, 20, 25]. In a European population, Magitta et al. found variant A at rs2670660 to have a trend of association with Addison's disease and type 1 diabetes compared to healthy controls. Therefore, it is possible that our AAAA combined genotype represents two homozygous risk variants, with the GGTT combined genotype representing two homozygous protective variants. The IL-18 precursor needs to be cleaved by caspase-1 to become biologically active, and the NLRP1 protein recruits caspase-1 [2, 26]. In this context, the significant increase in IL-18 concentration found in the AAAA genotype could be indicative of an association between increased autoimmune risk and increased processing of IL-18 by the NLRP1 inflammasome. The absence of results for the IL-1β ELISA are not surprising for a sample population without diagnosed autoimmune disease, as elevated levels of IL-1β production related to *NLRP1* have only been observed in autoimmune-presenting populations [11].

rs12150220 is an L115H substitution located in the protein-coding region of *NLRP1*, while rs2670660 is located in the promoter region [12]. As such, we would expect rs2670660 to have more of an influence on levels of gene expression than rs12150220. Although there was a trend towards higher *NLRP1* expression in the samples with AA genotypes at each SNP, our pPCR analysis was not sufficient to distinguish the influence of each SNP on gene expression due to the strong linkage demonstrated in our genotype analysis. If there were more intermediate combined genotypes—ex. AGAA or AAAT—it would perhaps become possible to distinguish the effects of each locus on gene expression.

Variants to several SNPs in the *NLRP1* gene have been associated with increased presentation of autoimmune disease [7]. Here we find that SNPs rs2670660 and rs12150220 are strongly linked, and that combined genotype AAAA leads to an increased presence of IL-18. Further analysis is needed to determine which *NLRP1* SNPs are more specifically implicated in an increase in IL-18 production and *NLRP1* gene expression. Several hundred samples, of both healthy participants and those with diagnosed autoimmune diseases, should be collected and analyzed for cytokine production and *NLRP1* gene expression at several more risk-associated SNPs in addition to rs2670660 and rs12150220. Understanding the mechanisms of the *NLRP1* gene and the NLRP1 inflammasome may give clues to the causes of autoimmune conditions, possibly one day leading to improved health outcomes for the millions of people affected by these conditions.

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