

# Deleterious Genetic Variation Across the NOD Signaling Pathway Is Associated With Reduced *NFKB* Signaling Transcription and Upregulation of Alternative Inflammatory Transcripts in Pediatric Inflammatory Bowel Disease

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**Background.** Inflammatory bowel disease may arise with inadequate immune response to intestinal bacteria. *NOD2* is an established gene in Crohn's disease pathogenesis, with deleterious variation associated with reduced *NFKB* signaling. We hypothesized that deleterious variation across the *NOD2* signaling pathway impacts on transcription.

**Methods.** Treatment-naïve pediatric inflammatory bowel disease patients had ileal biopsies for targeted autoimmune RNA-sequencing and blood for whole exome sequencing collected at diagnostic endoscopy. Utilizing GenePy, a per-individual, per-gene score, genes within the *NOD* signaling pathway were assigned a quantitative score representing total variant burden. Where multiple genes formed complexes, GenePy scores were summed to create a "complex" score. Normalized transcript expression of 95 genes within this pathway was retrieved. Regression analysis was performed to determine the impact of genomic variation on gene transcription.

**Results.** Thirty-nine patients were included. Limited clustering of patients based on *NOD* signaling transcripts was related to underlying genomic variation. Patients harboring deleterious variation in *NOD2* had reduced *NOD2* ( $\beta = -0.702$ ,  $P = 4.3 \times 10^{-5}$ ) and increased *NFKBIA* ( $\beta = 0.486$ ,  $P = .001$ ), reflecting reduced *NFKB* signal activation. Deleterious variation in the *NOD2-RIPK2* complex was associated with increased *NLRP3* ( $\beta = 0.8$ ,  $P = 3.1475 \times 10^{-6}$ ) and *TXN* ( $\beta = -0.417$ ,  $P = 8.4 \times 10^{-5}$ ) transcription, components of the *NLRP3* inflammasome. Deleterious variation in the *TAK1-TAB* complex resulted in reduced *MAPK14* transcription ( $\beta = -0.677$ ,  $P = 1.7 \times 10^{-5}$ ), a key signal transduction protein in the *NOD2* signaling cascade and increased *IFNA1* ( $\beta = 0.479$ ,  $P = .001$ ), indicating reduced transcription of *NFKB* activators and alternative interferon transcription in these patients.

**Conclusions.** Data integration identified perturbation of *NOD2* signaling transcription correlated with genomic variation. A hypoimmune *NFKB* signaling transcription response was observed. Alternative inflammatory pathways were activated and may represent therapeutic targets in specific patients.

**Key Words:** IBD, Crohn's disease, *NOD2*, WES, transcriptome

## Introduction

Pediatric inflammatory bowel disease (IBD) has a complex pathogenesis, reflecting genetic and environmental influences on disease etiology. Contemporary genomics has pointed to the molecular cause of disease being specific to an individual, or family. Despite this, the resulting immune dysfunction frequently converges on common inflammatory pathways such as tumor necrosis factor  $\alpha$  production and interleukin (IL)-17 signaling.<sup>1</sup> Identification of the precise cause of disease within an individual patient is challenging,

and integration of multiomic data to assess the impact of genetic variation on gene expression and immune function and the relationship with intestinal microbiota remains difficult.<sup>2</sup> Typically, functional assessment of deleterious genomic variation in an individual is through quantification of downstream cytokine levels.<sup>3</sup> Stimulation allows assessment of the impact of a mutation on downstream immune function.<sup>3</sup> Analysis may focus on transfection of cell lines with the mutation of interest, although accurate reflection of in vivo response is difficult to achieve when multiple

variants are contributing to a complex disease phenotype. While these techniques are effective in Mendelian disease, in polygenic IBD, in which multiple genetic variants appear to result in the same phenotype, establishing the effect of multiple mutations is challenging.

Expression quantitative trait loci have been identified that impact on *NOD2* expression.<sup>4</sup> Consolidation of genetic variation across a protein complex or pathway would allow grouping of patients with deleterious variation in several interacting genes. Consolidation can occur through a mathematical model based on a whole gene pathogenicity score, which sums the variation within genes, taking into account the key variation metrics.<sup>5</sup> This score reflects a patient's burden of variant pathogenicity within genes of interest.

It is recognized that monogenic disease with highly similar phenotypes can result from deleterious variation in different genes, the occurrence of polygenic conditions, such as typical forms of IBD, are also likely to have contribution from variation in a number of related genes within an individual.<sup>6</sup> The NOD signaling pathway, an innate immune signaling response, is highly implicated in Crohn's disease pathogenesis, both through genomic and transcriptomic analysis.<sup>7,8</sup> *NOD2* is the most implicated gene in the Crohn's disease pathogenesis and is a central player in the pathway, acting as an intracellular pattern recognition receptor for bacterial components, specifically muramyl dipeptide (MDP) (Figure 1).<sup>8</sup> Variation within other genes within this pathway, including *XIAP*, *ATG16L1*, *CARD9*, and *RIPK2*, has been implicated in both monogenic and polygenic forms of IBD.<sup>9</sup>

We hypothesize that genomic variation across the NOD signaling pathway will have direct impact on transcription of related genes within the pathway. Therefore, we undertook systematic analysis of genes and complexes along the NOD pathway to identify individual contributions to dysregulation of transcriptional programs. We assess whether variation in single genes, and across protein complexes, is associated with altered gene expression within the NOD signaling pathway to identify patterns in immune pathway transcription across a cohort.

## Methods

Pediatric IBD patients were recruited through from the Paediatric Gastroenterology service at Southampton Children's Hospital. Patients were recruited prior to diagnostic endoscopy and are termed treatment-naïve IBD patients. All patients had a terminal ileal biopsy and blood for whole exome sequencing data taken at the time of endoscopy. These analyses included patients with a subsequent diagnosis of Crohn's disease, ulcerative colitis, or IBD-unclassified. No patients with monogenic IBD were included. While *NOD2* variation is associated with Crohn's disease, ulcerative colitis and IBD-unclassified patients were included to ensure a spectrum of genomic mutation burden.

### Whole Exome Sequencing Analysis

Patient DNA was extracted from peripheral venous blood samples collected in EDTA using the salting-out method, or from saliva, as previously described.<sup>10</sup> DNA concentration was estimated using the Qubit 2.0 Fluorometer (Life

Technologies, Carlsbad, CA, USA). Approximately 20 µg of each patient's DNA was extracted for next-generation sequencing.

Raw FASTQ sequencing data from patients with treatment-naïve IBD were processed using our in-house pipeline (<https://github.com/UoS-HGIG>). VerifyBamID was utilized to check the presence of DNA contamination across the cohort.<sup>11</sup> Alignment was performed against the human reference genome (hg38 assembly) using BWA-mem (version 0.7.15).<sup>12</sup> Aligned BAM files were sorted, and duplicate reads were marked using Picard Tools (version 2.9.2; Broad Institute of MIT and Harvard, Cambridge, MA, USA). Following GATK v3.8<sup>13</sup> best practice recommendations,<sup>14</sup> variants were called using GATK HaplotypeCaller to produce a gVCF file for each sample and later jointly genotyped. HaplotypeCaller default settings were utilized corresponding to variants with a minimum Phred base quality score of 20 being called.

Annotation of this composite file for variant-based analysis applied Annovar v2018Apr16 using default databases refSeq gene transcripts (refGene), deleteriousness scores databases (dbnsfp35c, CADD v.1.5 and DANN), dbSNP147, and the human genetic mutation database (HGMD Pro 2018) flat file.<sup>15</sup> Variant allele frequencies were sourced through the Genome Aggregation Database (gnomAD) v2.1.1.<sup>16,17</sup>

### Application of GenePy

Whole exome sequencing data were transformed in a per gene, per individual GenePy score for integration with transcriptomic analysis.<sup>5</sup> Before GenePy scores were calculated, the joint cohort variant call format (VCF) was quality controlled by implementing methods from Carson et al.<sup>17</sup> The VCF was then annotated with the databases refGene and gnomAD exome v2.1.117, using Annovar v2018Apr16. The VCF was additionally annotated with deleteriousness metric CADD (v.1.6).<sup>18</sup> The GenePy score algorithm was then applied to the annotated cohort VCF (<https://github.com/UoS-HGIG/GenePy>). Demonstration of the contributions of individual variants to GenePy scores can be seen in [Supplementary Data 1](#).

### Normalization and Application of LOEUF Score to GenePy

Some genes can accrue very high GenePy scores (owing to length or mutability), and when summing GenePy scores across a complex, we first needed to normalize the values in order to make each gene comparable. We normalized all GenePy scores between 0 and 1, where 0 represented the lowest GenePy score for that gene across all patients in the analysis and 1 represented the highest score for that gene across all patients in the analysis.

The LOEUF (loss of function observed/expected upper bound fraction) score is a metric developed as part of the gnomAD that assigns a score to each gene based on the gene's intolerance to inactivation.<sup>19</sup> This score can then be applied to determine which genes are able to accrue variation while maintaining activity, compared with those in which variation will be highly damaging. Higher LOEUF scores are associated with increased tolerance to variation. Genes within the NOD pathway that are highly conserved and key to multiple inflammatory processes, such as *RIPK2* or *TAK1*, have very

low LOEUF scores, whereas genes in which variation is more commonly seen, such as *NOD2*, have higher scores.

We integrated the LOEUF score into the GenePy score in order to upweight the importance of variation in genes predicted to be intolerant to inactivation. LOEUF scores for genes in this analysis can be seen in [Supplementary Data 2](#). The normalized GenePy score was divided by the respective LOEUF score for each gene.

### Ileal Biopsy RNA Extraction

Terminal ileal biopsies were obtained during routine endoscopy and frozen at -80 in 1 mL of RNAlater (Sigma-Aldrich, St Louis, MO, USA) <30 minutes from collection. RNA was extracted from ileal biopsies using Maxwell processing, as previously described.<sup>20</sup> A single biopsy was used for each patient.

### Targeted RNA Sequencing

The HTG EdgeSeq Autoimmune Panel (HTG Molecular Diagnostics, Tucson, AZ, USA) was used to measure messenger RNA expression levels in 2002 genes associated with autoimmune disease, including IBD. The targeted sequencing was performed as previously described.<sup>20</sup> Briefly, an RNA sample for each patient was thawed, diluted, and loaded onto the HTG EdgeSeq instrument. Following a standardized polymerase chain reaction clean-up procedure, and quantification, each sample underwent a dilution-based normalization process. Individual libraries were pool at equimolar quantities into the final library for sequencing. The library was denatured and prepared for sequencing in line with Illumina (San Diego, CA, USA) and HTG practice guidelines. Sequencing was performed on the Illumina NextSeq platform.

### RNA Data Processing

Output files from the NextSeq run were converted from BCL format to FASTQ files. The barcode mismatch filter was set to 0 to ensure that reads were linked to the correct patient. A gene expression count matrix was constructed for each gene and each patient. These were merged to form a single output file containing all genes and all counts. Quality control was performed as previously described, in line with HTG recommendations.<sup>20</sup> Gene transcript counts were normalized using quantile normalization for downstream integration with genomic data. Gene expression using the targeted RNA panel has previously been validated utilizing single-cell RNA sequencing.<sup>20</sup> We determined if histological inflammation, regardless of underlying genomic variation, was associated with differential gene expression using DEseq2.<sup>21</sup>

### Key Genes and Protein Complexes Within the NOD signaling Pathway

We proposed that summing variation across key protein complexes within the NOD signaling pathway would enable us to discern the impact of underlying genomic variation on gene expression. We utilized a precollated list of 95 genes in the NOD signaling pathway, produced by HTG as part of their autoimmune panel product.<sup>22</sup>

The *NOD* signaling pathway genes were cross-referenced with genes known to be implicated in IBD either by genome-wide association study or as a monogenic IBD gene.<sup>22,23</sup> Invariant genes or complexes were not assessed. Single genes within the pathway, and implicated in IBD, were included

in the analysis. Molecular complexes in the NOD signaling pathway, with 2 or more constituent proteins, containing genes with variation, were included in the analysis. Molecular complexes activated because of multiple inflammatory pathways, including the IKK complex and MAPK complexes, were excluded due to the lack of specificity to *NOD* signaling.

### Summing GenePy Scores for Protein Complexes

We summed GenePy scores across the genes in key protein complexes. For each gene within a molecular complex, the LOEUF-corrected, normalized GenePy scores for each gene were summed to create a GenePy score for the molecular complex ([Supplementary Data 2](#)). This provides a quantitative score reflecting the cumulative sum of variation within that complex.

### Genomic and Transcriptomic Integration

We utilized a stepwise linear regression model to determine the impact of genomic variation on transcription. The analyses were conducted to determine whether genomic variation within the NOD signaling pathway impacted on gene expression within the same pathway. We utilized the GenePy scores for an individual gene, or summed scores for a complex, as the dependent variable. The quantile normalized (QN) expression values for the 95 NOD signaling genes were entered as independent variables. These data assume an additive model of deleteriousness.

To assess whether patients with higher burden of variation within key *NOD2* pathway genes and complexes exhibited similar expression patterns across all NOD signaling genes, we performed principal component analysis (PCA) and hierarchical clustering (HC). Patients within the top 10% of GenePy scores for *NOD2*, *NOD2-RIPK2*, and *TAK1-TAB* were annotated within the analyses, and the top 10% of patients were chosen as per previous evidence.<sup>23</sup> These 12 patients were also utilized to determine if high GenePy scores were driving inflammation within the terminal ileum, and therefore altered transcription related to inflammation, rather than altering pathway transcription per se.

### Statistical Analysis

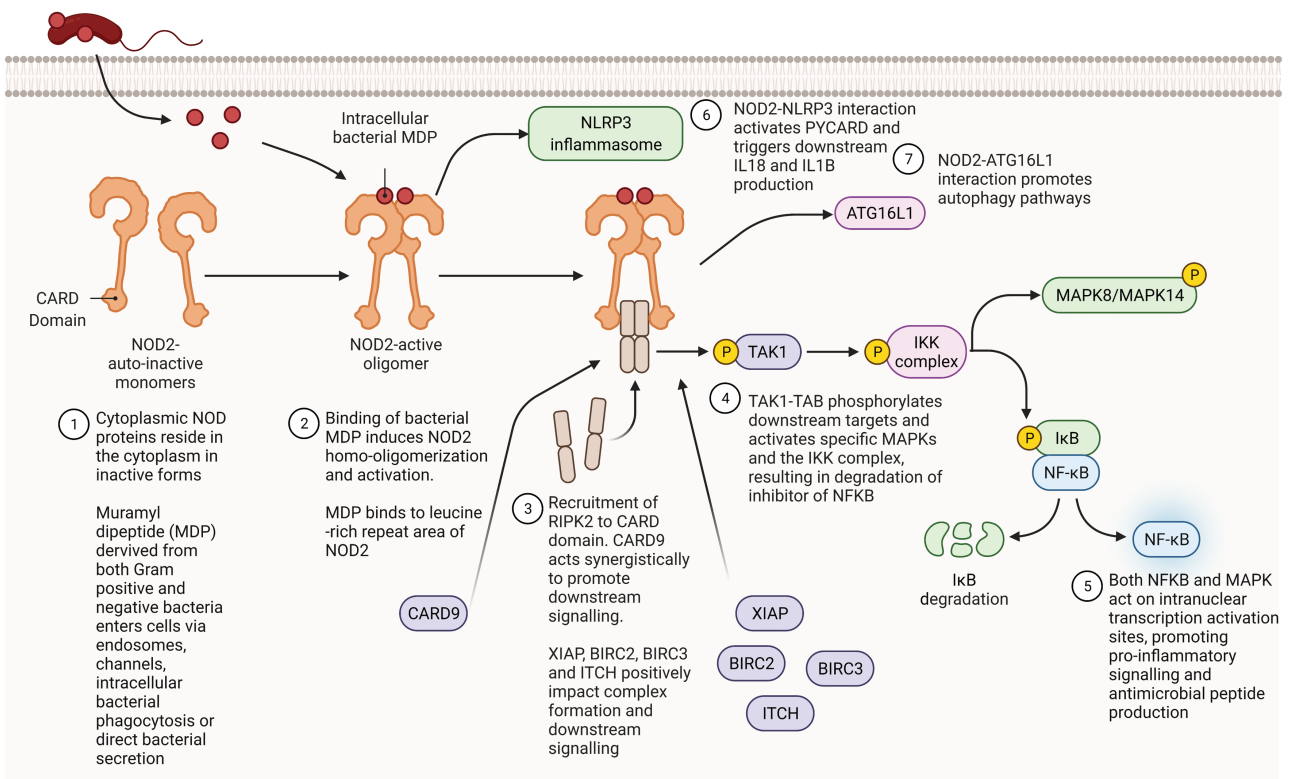
Statistical analysis was performed using SPSS (version 25; IBM, Armonk, NY, USA). PCA and HC (Euclidean distance, average clustering) were performed using Reveal (version 3.1; HTG Molecular Diagnostics). Weighted gene coexpression network analysis was performed as previously described using the WGCNA R package.<sup>20,24</sup>

### Ethical Approval

The study has category A ERGO II ethics approval (30630) and a REC approval from Southampton and South West Hampshire Research Ethics Committee (09/H0504/125). All patients and families provided informed consent at recruitment.

### Results

Thirty-nine patients were included in the analysis; 27 had a diagnosis of Crohn's disease, 9 had a diagnosis of ulcerative colitis, and 3 had a diagnosis of IBD-unclassified. The mean age at diagnosis was 13.2 years (range, 2.9-16.8 years). All patients were naïve to any immunomodulating,



**Figure 1.** Schematic representation of *NOD2* signaling cascade and directly related inflammatory signaling pathways. Adapted from “Detection of Bacterial Peptidoglycan by NOD Receptors,” by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>. IL, interleukin; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kappa B.

**Table 1.** Genes and complexes to be entered as dependent variables in regression analysis and the constituent proteins (genes)

Gene or Complex	Proteins Comprising Complex
<i>NOD2</i>	<i>NOD2</i>
<i>ATG16L1</i>	<i>ATG16L1</i>
<i>CARD9</i>	<i>CARD9</i>
<i>NOD2-RIPK2</i> complex	<i>RIPK2, NOD2, XIAP, BIRC2, BIRC3, ITCH</i>
<i>TAK1-TAB</i> complex	<i>TAK1, TAB2, TAB3</i>
<i>IRAK-TRAF6</i> complex	<i>IRAK1, IRAK2, IRAK4, TRAF6, MYD88</i>

All genes' GenePy scores are scaled to between 0 and 1 and corrected by LOEUF (loss of function observed/expected upper bound fraction) score prior to being summed to form the complex's GenePy score.

anti-inflammatory, or monoclonal treatment at the time of biopsy acquisition. The core NOD signaling pathway was interrogated (Figure 1). Three genes and 3 sets of genes encoding protein complexes were selected for analysis based on the criteria described previously (Table 1). The IRAK-TRAF6 complex acts synergistically with the core NOD2 signaling pathway and was included, in addition to the core NOD2-RIPK2 and TAK1-TAB complexes. The IKK complex, consisting of IKKα, IKKβ, and NEMO, was excluded, as it is the target of multiple activators including tumor necrosis factor α and IL-1 signaling, and is not

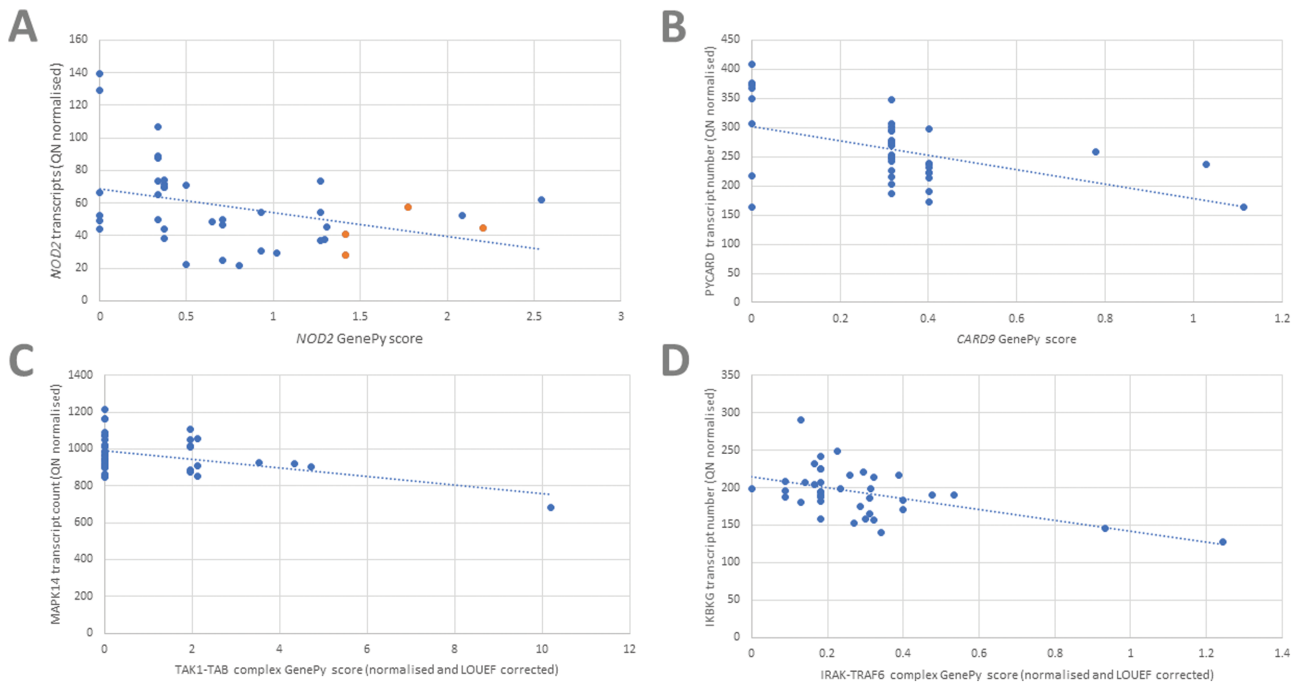
specific for NOD-dependent bacterial recognition and response.<sup>25</sup> Similarly, MAPK complexes were excluded due to the large number of MAPK genes that may be included and the lack of specificity to NOD signaling (Supplementary Figure 1A). Further data can be seen in the Supplementary Results, including the phenotype-*NOD2* genotype correlation (Supplementary Table 2).

### **NOD2 GenePy Score Is Not Associated With Specific Gene Expression Modules Across All Autoimmune Genes**

Given the established role of *NOD2* in Crohn's disease pathogenesis, we hypothesized that subgroup(s) of patients with accumulation of pathogenic *NOD2* variation would be characterized by similar gene expression. To test this hypothesis, we determined gene coexpression modules, identified through WGCNA of all 39 patients, and correlated these modules with *NOD2* GenePy score. Patients were clustered by the similarity of gene expression for all 2002 autoimmune gene transcripts (Supplementary Figure 2). *NOD2* GenePy scores were entered as a continuous variable. Hierarchical clustering did not demonstrate patients with similar *NOD2* GenePy scores in the same clusters.

### **Gene Coexpression Modules in Treatment-Naïve Patients**

Expression modules were identified within the autoimmune transcripts, based on the 39 treatment-naïve patients. Two large coexpression signatures emerged, the turquoise and



**Figure 2.** A, Relationship between quantile-normalized (QN) *NOD2* transcript levels and *NOD2* GenePy score. Four patients harboring the 1007fs variant are seen in orange. B, Relationship between QN *PYCARD* transcript levels and *CARD9* complex GenePy score. C, Relationship between QN *MAPK14* transcript levels and *TAK1-TAB* complex GenePy score. D, Relationship between QN *IKBK* transcript levels and *IRAK-TRAF6* complex GenePy score. LOEUF, loss of function observed/expected upper bound fraction.

blue modules, alongside several smaller coexpression modules (Supplementary Figure 3). In order to determine whether specific sets of coexpressed genes were associated genomic variation in *NOD2*, we analyzed whether the coexpression modules were correlated with *NOD2* GenePy score. None of the 8 modules was significantly correlated with *NOD2* (Supplementary Figure 4).

### Patients Harboring Deleterious *NOD2* Gene Variation Have Reduced *NOD2* Gene Expression and Increased Expression of Nuclear Factor Kappa B Inhibitor $\alpha$

We expected *NOD2* (Figure 1.1) gene variation to impact on downstream gene expression, specifically within the signaling pathway. We examined the effect of variation in *NOD2* through stepwise linear regression, with *NOD2* GenePy score as the dependent variable and all 95 gene transcript levels as the independent variables. Increased *NOD2* GenePy scores, reflecting increased deleterious variation, were associated with a decrease in *NOD2* transcripts (Figure 2A) and increased expression of *NFKBIA*, encoding a key inhibitory protein preventing nuclear factor kappa B (NFkB) signaling (Table 2). The effect appears to reflect a decrease in NFkB signaling as a result of deleterious *NOD2* variation (Supplementary Figure 1B). *CCL5*, a T cell chemokine, was also downregulated in patients with high variant deleteriousness in the *NOD2* gene.

### The 1007fs *NOD2* Variant Impacts Transcription But Is Not the Only Driver of Reduced Expression

*NOD2* harbors a protein-truncating variant, 1007fs, commonly seen in Crohn's disease patients. We assessed whether

this specific nonsense variant within *NOD2* was driving the inverse relationship between the GenePy score and transcript number. Four of the 39 patients were heterozygous for the 1007fs *NOD2* variant. A *t* test demonstrated that there was no significant difference in the *NOD2* expression level between those with the 1007fs variant (mean QN transcripts 42.9) and those without (mean QN transcripts 59.5) ( $P = .12$ ), although only 4 patients with the frameshift variant were seen in the patient group (Figure 2A). No other protein-truncating variants within *NOD2* were identified in the 39 patients.

We assessed if *NOD2* transcription was impacted by any of the 3 common variants associated with Crohn's disease: 1007fs (4 patients = heterozygote), R702W (1 patient = heterozygote), and G908R (5 patients = heterozygote). Comparing these 10 patients with the remaining 29 patients, who did not harbor any of these variants, demonstrated a significantly reduced *NOD2* transcript count (45.9 vs 61.8;  $P = .049$ ).

### Deleterious Variation in *CARD9* Is Associated With Decreased Expression of *PYCARD* and *TANK*

*CARD9* interacts directly with *NOD2* (Figure 1.3), resulting in activation of downstream proinflammatory signaling through mitogen-activated protein kinase (MAPK) activation alongside activation of the *NLRP3* inflammasome. It also functions independently as a signal transduction complex alongside *BCL10* and *MALT1*, largely in response to fungal infection, which then activates the IKK complex, triggering NFkB activation, or through *PYCARD*, activating the *NLRP3* inflammasome.<sup>26</sup> Regression analysis with the *CARD9* GenePy score as the dependent variable demonstrated that increased deleterious variation in *CARD9* was associated with a decrease in *PYCARD*, an upstream activator of the

**Table 2.** Linear regression results of genes and complexes and the impact on NOD signaling transcription

Dependent Variable: Gene/Complex	Gene/Complex	Independent Variable: Quantile-Normalized Gene Expression	$\beta$ Coefficient	$R^2$ Value for Linear Regression Model	P Value
NOD2		NOD2	-0.702	0.460	.000043
		NFKBIA	0.486		.001
ATG16L1		CCL5	-0.414		.008
		IKBKB	0.504	0.403	.001
		IRAK4	-0.498		.001
		CYBA	-0.288		.041
CARD9		PYCARD	-0.559	0.510	.0007
		IFNB1	0.333		.006
		TRAF2	0.371		.003
		TANK	-0.294		.025
NOD2-RIPK2 complex (RIPK2, NOD2, XIAP, BIRC2, BIRC3, ITCH)		BIRC2	0.800	0.687	$3.1475 \times 10^{-8}$
		TXN	0.417		.000084
		NLRP3	0.245		.014
		PYCARD	-0.278		.014
		IRAK4	-0.345		.001
		UBA52	-0.224		.033
		MAPK14	-0.677	0.438	.000017
		IFNA1	0.479		.001
		BIRC3	-0.375		.008
		IKBKG	-0.508	0.258	.001
TAK1-TAB complex (TAK1, TAB2, TAB3)		MAPK14	-0.677	0.438	.000017
		IFNA1	0.479		.001
IRAK-TRAF6 complex (IRAK1, IRAK2, IRAK4, TRAF6, MYD88)		BIRC3	-0.375		.008
		IKBKG	-0.508	0.258	.001

*NLRP3* inflammasome (Figure 2B, Table 2). Additionally, we observe an increase in increased transcription of *TRAF2*, and a decrease in *TANK*, the TRAF family member–associated NFKB activator. *TANK* directly inhibits *TRAF2*, and these data reflect a potential reduced ability to inhibit downstream NFKB signaling, through *TRAF2* and *IKK*, within the NOD signaling cascade.<sup>27</sup>

### Deleterious Variation in the *NOD2-RIPK2* Complex Is Associated With Increased Expression of *BIRC2*, *TXN*, and *NLRP3*

Following activation by MDP, *NOD2* forms a complex with *RIPK2* (Figure 1.3). *XIAP*, *BIRC2*, *BIRC3*, and *ITCH* all positively associate with the complex promoting downstream *RIPK2* kinase activity. Regression analysis, using the summed *NOD2-RIPK2* complex as the dependent variable, revealed that increased deleterious variation within the complex was related to an increased expression of the *NLRP3* and *TXN* (Table 2). Both of these genes are involved in the proinflammatory *NLRP3* inflammasome; however, we also observed a decrease in *PYCARD* expression, encoding a key protein in the inflammasome activation pathway (Figure 1.6). *BIRC2* was highly significantly upregulated in patients harboring deleterious variation in this complex, which includes *BIRC2*. The impact can be seen in Supplementary Figure 1C.

### Genomic Variation Within the *TAK1-TAB* Complex Leads to Reduced *MAPK14* Expression

The *TAK1-TAB* complex, including *TAK1*, *TAB2*, and *TAB3*, is a key signal transducer, both from the *NOD2-RIPK2* complex and Toll-like receptors (TLRs) (Figure 1.4). Summed GenePy scores for the *TAK1-TAB* complex were used as the dependent variable. Expression of *MAPK14* and *BIRC3* was reduced in the presence of deleterious variation within the *TAK1-TAB* complex. There was also increased expression of *IFNA1*, reflecting activation of a type I interferon response pathway (Figure 2C, Table 2). *MAPK14* autophosphorylates in the presence of the *TAK1-TAB* complex, leading to downstream activation of proinflammatory and antimicrobial gene expression (Supplementary Figure 1D).

### Deleterious Variation in *ATG16L1* Increases Expression of *IKKBK*

*ATG16L1* encodes for a protein key in autophagy pathways. Activated *NOD2* works synergistically with *ATG16L1* to promote autophagy (Figure 1.7). Variation within *ATG16L1* is an established risk for Crohn's disease development. Regression analysis utilizing *ATG16L1* GenePy score as the dependent variable demonstrated that an increased *ATG16L1* GenePy score was associated with an increase in *IKKBK*, an activator of NFKB signaling (Table 2). Autophagy represents a different pathway to core *NOD2* signaling, and variation in *ATG16L1* may be proinflammatory via NFKB signaling, while reducing autophagy.

### Variation in the *IRAK-TRAF6* Complex, Within the TLR Signaling Pathway, Results in Decreased Expression of the NFKB Activating Protein *IKKBK* (*NEMO*)

The *IRAK-TRAF6* complex (*IRAK1*, *IRAK2*, *IRAK4*, *TRAF6*, *MYD88*) plays a key role in signal transduction from TLRs through interaction with *MYD88*. It acts synergistically

with *NOD2* activation and one result of *IRAK-TRAF6* activation is downstream activation of the *TAK1-TAB* complex and subsequently activation of NFKB signaling. Variation in the *IRAK-TRAF6* complex was associated with a decrease in a single gene transcript, *IKKBK*, a component of the NFKB activating complex (Figure 2D).

### Patients With Deleterious Variants in *NOD2*, *RIPK2*, and *TAK1-TAB* Have Variable NOD Signaling Expression Clustering

Patients with high burden of variation (top 10% of GenePy scores) in the central *NOD2* signaling complexes were assessed for similarity of all NOD signaling gene expression. PCA revealed variable clustering (Figure 3A). The 4 patients with highest burden variation in the *NOD2-RIPK2* complex did cluster together, along with a patient with high mutation burden in *NOD2* and a patient with high burden in the *TAK1-TAB* complex.

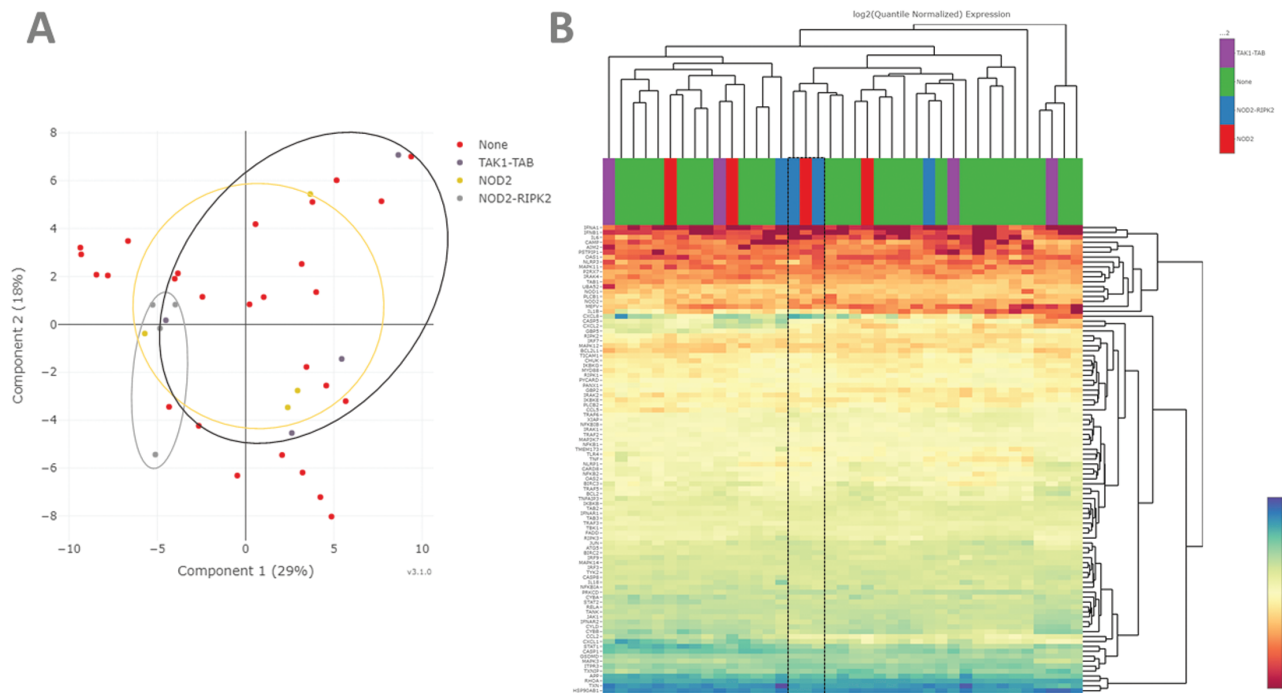
Using HC we were able to discern that several of the patients with high *NOD2-RIPK* or *NOD2* mutation were characterized by increased expression of *CXCL8* (encoding for the IL-8 neutrophil chemokine) and low expression of *IFNA1* (Figure 3B). Interestingly, while deleterious variation in the *NOD2-RIPK* complex appears to negatively impact on downstream NFKB signaling, there is still an overall inflammatory response, mediated through alternative inflammatory pathways. Further analysis of the patients with high genomic burden in *NOD2*, *NOD2-RIPK2*, and *TAK1-TAB* revealed that of the 12 patients with the highest burden, 7 had histological inflammation, while 5 were noninflamed. This indicates that transcription differences noted through regression are not solely driven by inflammation status of the biopsy.

### Differential Gene Expression Within the NOD Signaling Pathway Is Observed Between Inflamed and Noninflamed Tissue

Within the NOD signaling pathway, there were 17 genes with significant differential expression (corrected  $P < .05$ ): 8 were upregulated in inflamed biopsies and 9 were downregulated. Further analysis of the patients with the top 10% of genomic burden in *NOD2*, *NOD2-RIPK2*, and *TAK1-TAB* revealed that of the 12 patients with the highest burden, 7 had histological inflammation, while 5 were noninflamed. This indicates that transcription differences noted through regression are not solely driven by inflammation status of the biopsy, but rather by altered transcription related to underlying genomic differences.

## Discussion

The impact of genomic variation across key genes, and complexes, within the NOD signaling pathway appears to be associated with a hypoinflammatory response, with reduced activation (or increased inhibition) of NFKB signaling or reduced upstream activation of proinflammatory signaling. Variation in *NOD2*, and directly related complexes, appears to act synergistically to reduce *NOD2* transcription, while simultaneously increasing transcription of alternative inflammatory pathways including the *NLRP3* inflammasome and interferon signaling. We identify variation across the *TAK1-TAB* complex directly correlated with reduced *MAPK14* transcription. Variation in *NOD2*-synergistic activators of



**Figure 3.** **A**, Principal component analysis using quantile-normalized data from 95 NOD signaling genes demonstrating clustering of patients with the top 10% (4 patients) of deleterious genetic variation within *NOD2-RIPK2* but with less defined grouping for *NOD2* and *TAK1-TAB*. **B**, Heatmap analysis using quantile-normalized data from 95 NOD signaling genes and average linkage clustering demonstrates highly variable clustering of patients. Two patients with top 10% of deleterious genetic variation in *NOD2* and 1 with top 10% in *NOD2-RIPK2* do cluster (boxed area), with high transcription of *CXCL8* (interleukin-8) and low expression of *TFNA1*.

autophagy (*ATG16L1*), or NOD signaling (*CARD9*), impact on downstream transcription, either increasing or decreasing NFKB signaling, respectively.

Previous data have pointed to a hypoimmune response in both Crohn's disease patients and murine models harboring deleterious *NOD2* variants.<sup>3,8</sup> *NOD2* variants are thought to be loss of function, leading to impaired *NOD2* activation.<sup>28</sup> Studies detailing the direct effect of deleterious *NOD2* variants repeatedly identify a decrease in proinflammatory cytokine response after MDP stimulation in peripheral blood mononuclear cells, specifically reduced NFKB production.<sup>8</sup> Overall, the mechanism whereby *NOD2* variants increase susceptibility to Crohn's disease appears to be through impaired bacterial recognition or response leading to reduced bacterial clearance and increased chronic inflammation through non-*NOD2* proinflammatory pathways.<sup>29</sup> In addition to *NOD2*, several additional risk susceptibility genes, or monogenic IBD genes, lie within the NOD signaling pathway including *XIAP*, *CARD9*, and *TAB2*.<sup>1</sup> Loss-of-function variants within these genes are associated with severe monogenic forms of Crohn's-like IBD (*XIAP*, *CARD9*), or increased risk of "classical" Crohn's disease (*TAB2*). Several studies have described the function of these genes in downstream NFKB signaling, including variants in *XIAP*<sup>30</sup> and *CARD9*<sup>31</sup> leading to reduced NFKB production. While the mechanism by which these genes leads to disease may not have the evidence base seen with *NOD2*, it appears that a hypo-inflammatory response is implicated, potentially alongside activation of additional aberrant pathways. It was important to include patients with ulcerative colitis in this analysis, as ulcerative colitis patients should have no impact of *NOD2* variation on transcription, and it is therefore vital to include these patients to demonstrate a

full spectrum of genomic variation and transcription levels. In addition, variation in the wider *NOD2* signaling pathway may impact ulcerative colitis patients, and moving IBD toward a precise molecular diagnosis would equate to patients' being categorized by molecular perturbation, rather than by endoscopic or histological disease location.

Within this study, we hypothesized that variation across the NOD signaling pathway, with a focus on *NOD2* signaling, would result in transcription-level defects associated with a hypo-inflammatory response. Previous data have inferred that disruption at any step on the *NOD2* signaling cascade will result in decreased downstream NFKB or MAPK activation, although direct impact of genetic variation at each step has not been previously identified.<sup>32</sup> Through single gene, and whole complex, deleteriousness scoring, we identify a consistent pattern of defects associated with decreased transcription of downstream *NFKB* or *MAPK* genes. Importantly, by summing deleteriousness across a complex, we were able to observe cohort-level effects that may be missed if assessing a single variant or a single gene within an individual patient. As individual variant effects on gene transcription are likely to be very mild, or private to an individual, the ability to sum the effects of interacting genes allows a statistical association to emerge across a cohort. It is clear that for most patients with IBD, the effect of multiple genomic variants, rather than a strong effect from a single gene, leads to disease.<sup>23</sup>

At an individual gene level, we reveal a striking decrease in *NOD2* transcripts associated with increased *NOD2* deleteriousness. This is driven not only by patients harboring the nonsense 1007fs variant, with only 4 of the 39 patients being heterozygote. No patients were homozygote for this protein-truncating variant. It is not possible to determine



whether several of the more common variants harbored by patients with low *NOD2* transcript levels are in linkage disequilibrium with noncoding variants in the promoter region of *NOD2*. *ATG16L1* synergistically acts with *NOD2* to promote antibacterial autophagy and also has a role in negative regulation of proinflammatory MAPK and NF $\kappa$ B activation cascades.<sup>33</sup> Our results demonstrate the direct impact of this additional *ATG16L1* role, with *ATG16L1* variation leading to an increase in the NF $\kappa$ B activation transcript *IKBK*. Where deleterious variation in the *NOD2* canonical pathway appears to lead to reduced NF $\kappa$ B or MAPK activation of downstream inflammatory signaling, variation in *ATG16L1* may lead to increased inflammation through impaired autophagy, or directly through the inability to negatively regulate *NOD2* activity. Interestingly, recent ileal transcriptomic data integrating with *NOD2* genotyping of the patients have identified an activated and dysregulated fibroblast cell signature in those with higher mutation burden.<sup>34</sup> Within the article the authors identify an apparent *STAT3*-driven regulation of downstream transcription, specifically related to mutant *NOD2* alleles.

We identify summed variation in the *TAK1-TAB* complex directly associated with reduced *MAPK14* transcription. Alongside this, we observe an increase in gene transcription associated with alternative inflammatory pathways (*TXN* and *NLRP3*) seen with *NOD2-RIPK2* complex variation. Previous data have indicated a key role for *TAK1* in MDP-stimulated *NOD2* signaling, with absence of *TAK1* completely admonishing downstream NF $\kappa$ B and MAPK signaling.<sup>35</sup> Variation in the Toll-like receptor transduction complex, *IRAK-TRAF6*, was associated with a decrease in *IKBK* transcription, a potent activator of NF $\kappa$ B signaling. This membrane receptor-triggered pathway acts in parallel to intracellular *NOD2* signaling and also leads to NF $\kappa$ B activation in response to bacterial recognition and response. These data imply that variation across this related complex also impairs antimicrobial response. The activation of the *NLRP3* inflammasome has been reported as the key inflammatory pathway leading to colitis in *NOD2* knockout murine models.<sup>36</sup> Here, we present data suggesting that transcripts in the pathway are upregulated in IBD patients with genomic variation in a number of *NOD2* signaling genes. In contrast, we identify variation in *CARD9* resulting in a decrease in *PYCARD*, a key signal transduction protein in the *NLRP3* inflammasome, also leading to caspase-1 activation and IL-1 $\beta$ /IL-18 processing. It is possible that reduced *NLRP3* activity is the key hypimmune response for a subset of individuals, as observed in murine models, with concurrent increase in NF $\kappa$ B signaling in these patients.<sup>37</sup> Previous data have indicated both a protective and an antagonistic role for *NLRP3*, with the potential for related molecular variation within related pathways leading to activation or suppression in some patients, with a subsequent hyper- or hypoinflammatory response leading to disease.<sup>38</sup>

We hypothesize that for many patients, IBD appears to arise due to multiple “hits” across complexes or genes contributing to impairment of inflammatory pathways. These patients then fail to clear bacteria, allowing invasion and chronic inflammation to develop due to perturbation of a number of innate immune responses. The precise immune impairment within an individual is likely to lead to commonality between subgroups of patients, with many Crohn’s disease patients having disease attributable to impaired *NOD2*

signaling.<sup>3</sup> This provides an opportunity to target novel molecules with therapeutics, including the potential for *NOD2* activators. Primarily these data provide the framework for molecular profiling of patients. Long-term follow-up data for these individuals will allow integration of treatment response and disease course and may allow prediction of outcomes at the point of diagnosis.

This study has several strengths. We provide a level of validation of genomic findings without the need for time-consuming functional assays, although caution should be exercised in interpretation of these results. Through use of targeted sequencing, we enable identification of lowly expressed transcripts, which are key in many of these analyses. The Supplementary Results indicate that *NOD2* does not drive a specific gene expression signature across all genes targeted by the artificial intelligence panel, with a more select analysis revealing transcription differences. Additionally, while inflamed and noninflamed tissue demonstrated a degree of differential gene expression, the main analyses included patients with high variant burden but no ileal inflammation, demonstrating that genetic variation may be driving altered gene transcription independent of inflammation status. Additionally, we have previously determined that ileal transcriptomic profiles are not driven by inflammatory status.<sup>20</sup>

We acknowledge several limitations of this study. The curated list of *NOD* signaling genes could include either fewer or more genes. This gene list was specifically designed for use with the targeted autoimmune gene panel, which itself has the potential to limit wider findings related to WGCNA. We chose to not expand the list to retain statistical power to determine the impact of genomic variation on the most closely related transcripts. In addition, the inability of whole exome sequencing to capture promoter or regulatory variation and the dependence of GenePy on in silico deleteriousness metrics and inability to determine compound heterozygosity. Our group has previously studied cytokine induction in the context of *NOD2* variation, focused on MDP-induced responses.<sup>3</sup> Within this study, we did not study cytokine levels in relation to *NOD* signaling gene variation. Based on previous data, we hypothesize that these effector cytokines (IL-18, IL-1, tumor necrosis factor) are the common endpoint of numerous immune pathways, and the hypimmune response seen in specific IBD-related pathways, such as *NOD* signaling, is overwhelmed by alternative activation leading to high levels of effector cytokines. Finally, while the gene transcript number is frequently related to immune function, there is also posttranslational regulation of signaling molecules, meaning that inference of functional consequences of altered transcription must be cautious.

## Conclusions

These data demonstrate a pathway-wide genomic variation in *NOD* signaling genes, associated with reduced proinflammatory gene transcription within this pathway. Integration of genomic and transcriptomic data allows for statistical association of genomic variation with downstream transcription. We observe variation at each stage of the *NOD2* signaling pathway resulting in broadly reduced NF $\kappa$ B signaling, with frequent upregulation of other inflammatory genes including *NLRP3* and interferons. Expanding these analyses to additional pathways implicated in IBD may

allow for precise “immunotyping” of patients, identifying defects in specific immune pathways and paving the way for personalized therapy.

## Supplementary Data

Supplementary data is available at *Inflammatory Bowel Diseases* online.

Supplementary data 2 can be found at the following link: <https://www.dropbox.com/s/mr7xwgow3q5mac/Supplementary%20data%20-%20Genomic%20and%20RNA%20integration%20GenePy%20scores%20QN%20transcripts.xlsx?dl=0>

## Author Contributions

J.J.A., R.M.B., and S.E. conceived the study. Patients were recruited by J.J.A. and R.H. Patient samples were acquired by J.J.A., R.H., T.A.C., A.B., N.A.A., and R.M.B. Samples were processed and sequenced by J.J.A., K.B., I.S.S., and G.C. Analyses were performed by J.J.A., K.B., J.D., I.S.S., and G.C. under the guidance of M.P., A.W., and S.E. B.V. performed histological analysis for all patients. J.J.A. wrote the manuscript with help from all authors. All authors approved the final manuscript prior to submission.

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## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability

The RNA sequencing data underlying this article are available in Gene Expression Omnibus repository and can be accessed with accession number GSE153974. The exome sequencing data underlying this article cannot be shared publicly due to ethical considerations. The data will be shared on reasonable request to the corresponding author.

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