**Prediction of Crohn’s disease stricturing phenotype using a *NOD2-*derivedgenomic biomarker**

James J Ashton PhD1,2, Guo Cheng PhD 1,3, Imogen S Stafford\* MSci1,4, Melina Kellermann1, Eleanor G. Seaby BMBS1, JR Fraser Cummings5, Tracy AF Coelho PhD 2, Akshay Batra MD2, Nadeem A Afzal MD2, R Mark Beattie FRCPCH2, and Sarah Ennis PhD 1

1. Department of Human Genetics and Genomic Medicine, University of Southampton, Southampton, UK
2. Department of Paediatric Gastroenterology, Southampton Children’s Hospital, Southampton, UK
3. NIHR Southampton Biomedical Research Centre, University Hospital Southampton, Southampton, UK
4. Institute for Life Sciences, University of Southampton, Southampton, UK
5. Department of Gastroenterology, University Hospital Southampton, Southampton, UK

Correspondence to-

Professor Sarah Ennis

Human Genetics & Genomic Medicine

University of Southampton

Duthie Building (Mailpoint 808)

Southampton General Hospital

Southampton

SO16 6YD

UK

Tel: +44 (0)23 8079 8614

s.ennis@soton.ac.uk

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**Conflicts of interest**

FC has served as consultant, advisory board member, or speaker for AbbVie, Amgen, Celltrion, Falk, Ferring, Gilead, Janssen, MSD, Napp Pharmaceuticals, Pfizer, Pharmacosmos, Sandoz, Biogen, Samsung, Tillotts and Takeda. He has received research funding from Biogen, Amgen, Hospira/Pfizer, Celltrion, Takeda, Janssen, GSK and AstraZeneca. No other authors declare any conflicts of interest.

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**Data Availability Statement**

Whole exome sequencing (WES) data will be available through collaborative agreement. Due to consent signed by participants WES data cannot be deposited within a public repository.

**Author contributions**

JJA, RMB and SE conceived the study. Analyses were performed by JJA, ISS and GC, under the guidance of SE. JJA wrote the manuscript with help from all authors. All authors approved the final manuscript prior to submission.

**Key words:** *NOD2*; Crohn’s disease; stricturing; personalised; prediction

**Abstract**

Background-Crohn’s disease (CD) is highly heterogenous and may be complicated by stricturing behaviour. Personalised prediction of stricturing will inform management. We aimed to create a stricturing risk stratification model using genomic/clinical data.

Methods-Exome sequencing was performed on CD patients, and phenotype data retrieved. Biallelic variants in *NOD2* were identified. *NOD2* was converted into a *per-patient* deleteriousness metric (‘GenePy’). Using training data, patients were stratified into risk groups for fibrotic stricturing using *NOD2*. Findings were validated in a testing dataset. Models were modified to include disease location at diagnosis. Cox-proportional hazards (CPH) assessed performance.

Results-645 patients were included (373 children and 272 adults), 48 patients fulfilled criteria for ‘monogenic’ *NOD2*-related disease (7.4%) of whom 24 had strictures.

*NOD2* GenePy scores stratified patients in training data into two risk-groups. Within testing data, 30/161 patients (18.6%) were classified as high-risk based on the *NOD2* biomarker, with stricturing in 17/30 (56.7%). In the low-risk group 28/131 (21.4%) had stricturing behaviour. CPH using the *NOD2* risk-groups, demonstrated a hazard ratio (HR) of 2.092, p=2.4x10-5, between risk groups. Limiting analysis to patients diagnosed aged <18-years improved performance (HR-3.164, p=1x10-6).

Models were modified to include disease location- terminal ileal (TI) disease, or not. Inclusion of *NOD2*-risk groups added significant additional utility to prediction models. ‘High-risk group’ paediatric patients, presenting with TI disease had a HR-4.89, p=2.3x10-5, compared to ‘low-risk group’ patients without TI disease.

Conclusions-A *NOD2* genomic biomarker predicts stricturing risk, with prognostic power improved in paediatric-onset CD. Implementation into a clinical setting can help personalise management.

**Summary**

*NOD2* is a well-established risk gene for development of Crohn’s disease and stricturing behaviour. Here we demonstrate *NOD2* can be utilised as a genomic biomarker, stratifying patients into two stricturing risk-groups. Further refinement using disease location at diagnosis improved risk-stratification.

**Key messages**

What is already known?

* *NOD2* is highly implicated in Crohn’s disease and has been linked to a stricturing phenotype

What is new here?

* Using *NOD2* as a genomic biomarker we are able to predict high-risk stricturing patients, including disease location data further improved prediction
* In those diagnosed <18 years of age the high-risk group had a 5x increased risk of stricturing compared to low-risk patients

How can this study help patient care?

* Routine utilisation of *NOD2* as a genomic biomarker may allow risk stratification of Crohn’s disease patients at diagnosis
* Personalising management, based stricturing risk, may be possible.
* Stratified randomised trials of high-risk patients will be important

**Introduction**

Crohn’s disease is a chronic, relapsing, and remitting condition, characterised by inflammatory change throughout the gastrointestinal tract, commonly seen in the terminal ileum. Prediction of disease severity and behaviour are extremely challenging at the point of diagnosis. Differentiating between patients who will develop inflammatory, penetrating and stricturing phenotypes could potentially enable targeted therapy to impact on disease course1. The interplay between genetic risk and environmental exposure leads to disease pathogenesis, something which appears increasingly likely to be specific to a patient or family 2. Specific disease traits and responses to therapy have been linked to genetic defects, gene expression modules or microbiome profiles 3–5. Previous attempts to translate molecular data to predict clinical outcomes have produced promising results, although no testing has routinely entered clinical practice, to date 6,7.

*NOD2* is the best characterised risk gene for development of Crohn’s disease, coding for a vital intracellular microbial pattern recognition and response protein, triggering downstream innate immune response 8,9. Recent data have pointed towards a potential monogenic role for *NOD2* in a subset of Crohn’s disease patients who appear to be at high risk of developing a stricturing disease phenotype 10,11. Despite this, *NOD2* is largely viewed as a risk-gene without a clear clinical role for routine genotyping 12. Previous studies have largely focused on the three most common risk variants within *NOD2* - R702W, G908R and 1007fs - and have failed to account for the role of rarer variation, epistasis, or accumulation of multiple variants with modest deleteriousness 13. Data from our group, utilising ‘GenePy’, a contemporary *in silico* mutational burden tool across the whole gene, has demonstrated additional *NOD2* variation playing a role in Crohn’s disease phenotype 11. Accounting for cumulative burden of pathogenic variation within genes is likely to have a discovery uplift when considering non-Mendelian complex disease14. The role of pathogenic variation throughout *NOD2* as a single-gene contributor to adult on-set disease is also poorly elucidated. Whilst *NOD2* is the strongest genetic signal for Crohn’s disease and stricturing disease, additional genetic risk loci have been identified for fibro-stenotic disease, including genes within the *NOD2*-signalling pathway such as *ATG16L1* 15. It increasingly appears that the role of *NOD2* in Crohn’s disease is not yet fully understood.

Utilising genomic biomarkers - measurable genomic characteristics that predict a specific clinical outcome or response to treatment - is an exciting avenue of personalised medicine. This study aimed to develop and optimise *NOD2* as a genomic biomarker capable of stratifying Crohn’s disease patients into high and low risk groups for development of fibrotic stricturing disease, providing a tool for translation into clinical practice. Additionally, we aimed to characterise *NOD2* genotypes in both paediatric and adult patients and determine the prevalence of deleterious variants across the age spectrum.

**Methods**

Recruitment

Patients were included from the Wessex regional paediatric inflammatory bowel disease (IBD) service at Southampton Children’s Hospital and the adult IBD service at University Hospital Southampton. Patients were recruited from 2010 to present. All patients within the cohort had a confirmed histological diagnosis of either Crohn’s disease, ulcerative colitis or IBD-unclassified, in line with the Porto criteria or British Society of Gastroenterology guidelines 16. Patients with Crohn’s disease were extracted for this analysis. There are no exclusion criteria if a patient has a conformed diagnosis of IBD and are able to give informed consent.

Longitudinal data collection

Endoscopy, small bowel magnetic resonance imaging (MRI), abdominal ultrasound, and CT abdomen scan reports were retrieved from the University Hospital Southampton electronic patient record (EHR). These records, including clinic letters, imaging reports and endoscopy reports, were searched for stricturing keywords (Fibrosis, Fibrotic, Stricture, Stricturing, Narrowing, Narrowed, Pre-stenotic dilatation, Stenotic, Reduced diameter) to reduce the number of reports requiring clinical curation. Records without key words were recorded as a not-stricturing phenotype, and the remaining were manually checked by two clinicians (JJA and MK) to assign patients as having stricturing or non-stricturing phenotypes. Where there was uncertainty a further clinician (RMB) was consulted to give a final classification. As this study was focused on fibrotic, or predominately fibrotic disease, strictures which resolved without surgery or dilatation were presumed to be purely inflammatory and these patients were assigned as a non-stricturing phenotype. As described previously we used the strict definition of fibrotic stricturing as ‘histologically proven, or narrowing demonstrable on two consecutive MRIs, with prestenotic dilatation’ to define a specific disease phenotype11. Date of stricturing was recorded and time from diagnosis to stricture was calculated. Duration of follow-up was calculated for all patients. During the follow-up period (diagnosis to most recent clinical contact) all patients were assessed for occurrence of fibrotic strictures if they had narrowing initially assigned as inflammatory and grouped accordingly. Presence of terminal ileal disease at diagnosis was retrieved from endoscopy and imaging reports.

Whole exome sequencing data processing

DNA was extracted from blood samples collected in EDTA using the salting-out method, or from saliva, as previously described17. An estimated 20ug of DNA was used for whole exome sequencing.

Raw fastq sequencing data from patients in the cohort were processed using our in-house pipeline in line with the Genome Analysis Tool Kit 4 (GATK 4) best practice (https://github.com/UoS-HGIG/WES\_multicalling\_pipeline\_2020) 18. Alignment was performed against the human reference genome (GRCh38 assembly with decoy HLA regions) using BWA-mem (version 0.7.15)19. Joint variant calling of all samples in the cohort was restricted to the 150bp padded union of the Agilent SureSelect All Exon V5 and V6 capture kits.

VerifyBamID was utilised to check the presence of DNA contamination across the cohort20. We applied our in-house fingerprint panel to confirm sample identity and provenance21. In addition, following the GATK built-in Variant Quality Score Recalibration, data were assessed for sequencing depth, genotyping quality, and variant allele frequency (AF).

Variant called format (VCF) file annotation was performed using Ensembl-VEP (v.103)22, using default databases, deleteriousness scores databases (dbnsfp35c, CADD v.1.6)23, dbSNP147 and the human genetic mutation database (HGMD Pro 2021)24. Variant allele frequencies were sourced through the genome aggregation exome database (gnomAD)25, v2.1.1. We referred to the canonical *NOD2* transcript ENST00000300589 (GENCODE) for the annotation of coding variants unless otherwise specified.

Lollipop plot of *NOD2* coding variants was generated using the MutationMapper tool of cBioPortal 26 and variants were mapped to the Pfam domains of *NOD2* (Figure 1) to visualise the distribution of variants with respect to deleteriousness metrics (CADD v.1.6) and allele frequency (gnomAD) within our cohort.

*Application of GenePy*

GenePy provides a *per gene, per individual* single metric of deleteriousness, facilitating genes to be incorporated into downstream risk stratification modelling. Whole exome sequencing data were transformed into GenePy scores for patient stratification (https://github.com/UoS-HGIG/GenePy-1.4) 14. Firstly, the joint called aggregated cohort VCF underwent recommended quality control filtration steps27, such that only good quality, biallelic variants were retained in a VCF for annotation as described above. The GenePy score algorithm was applied to exonic variants, with a CADD Phred score > 15 (as per developer guidance for determining deleterious variants) and GenePy scores were retrieved for analysis 23.

Monogenic *NOD2* disease

To stratify the risk of stricturing in individuals based on *NOD2* genotype, all patients were screened for biallelic *NOD2* variants. Variants were initially annotated with functional evidence from the literature, as previously described 11. Variants that were functionally demonstrated to impact on NOD2 function, including reduced/absent protein function, impact on downstream signalling, nonsense mediated decay or deletions, were included in line with American College of Medical Genetics guidelines (ACMG) for ‘pathogenic’ or ‘likely pathogenic’ variants 28. Patients who were homozygous, or had two or more heterozygous variants, were denoted ‘*NOD2*-related disease’.

We determined the number of patients with putative deleterious *NOD2* variation but without functional evidence to meet ACMG criteria, as ‘*in silico* *NOD2*-related disease’. These patients were homozygous, or had two or more heterozygous variants, where the variants met ‘*in silico* criteria’ for deleteriousness- allele frequency (gnomAD) <0.05 and a CADD-PHRED score of >15 23.

We have previously demonstrated that all potential *NOD2* compound heterozygous variants in paediatric-onset patients, had confirmed variant segregation and were biallelic [10]. In this study it was not possible to perform segregation analysis in patients with adult-onset disease due to lack of parental DNA.

Incidence of stricturing disease in patients was retrieved and we assessed enrichment for a stricturing vs non-stricturing phenotype in each group through a ꭓ2 test. Data were visualised using a dumbbell plot.

Stricturing disease prediction modelling

A summary of the methodology can be seen in supplementary figure 1.

*Receiver operator curve analysis*

To determine the stricturing disease classification ability of *NOD2* mutation burden we performed an area under receiver operator curve (AUROC) analysis (SPSS, IBM v27). *NOD2* GenePy score was the test variable. The analyses were performed on all patients, and then separately on the subgroup of patients diagnosed <18 years of age.

*Group optimisation- training and testing datasets*

Patients were split into training and testing (validation) sets for risk-group determination utilising the caTools ‘R’ package (training proportion = 0.75). Utilising Cutoff Finder29, a biomarker optimisation software, an iterative Fisher’s exact test was used to determine the optimal number of risk groups and *NOD2* GenePy score boundaries. All groups and boundaries were initially determined on the training data, with assessment of model performance on the testing data using a ꭓ2 test.

*Survival analysis and model performance*

Following confirmation of valid group boundaries all data were combined to determine model performance metrics. To account for variable follow-up duration between patients, survival analysis was performed using a Cox proportional hazards (CPH) model to give final model performance metrics. Survival analysis was performed on all patients. Additionally, analysis was performed on patients diagnosed <18 years of age to determine if prediction was improved in patients with a presumed higher heritable component to their disease. All statistical analyses were performed in SPSS (IBM v27).

Inclusion of disease location data

*NOD2* variants are known to predispose to terminal ileal (TI) inflammation. To assess the independent role of *NOD2* in the prediction of stricturing disease we determined whether adding the presence or absence of TI inflammation as a variable to the risk stratification model improved or negated the predictive ability of *NOD2* risk groups. Previously determined *NOD2*-risk groups were further stratified into group 1- low-risk *NOD2* group + no TI disease, group 2- low-risk *NOD2* group + TI disease, group 3- high-risk *NOD2* group + no TI disease and group 4- high-risk *NOD2* group + TI disease. Survival modelling was performed on these groups, including separate analysis for paediatric-onset patients.

Non-*NOD2* genetic determinants of stricturing disease

We hypothesised that in patients who developed strictures in the absence of significant *NOD2* variation, an alternative genetic driver may be identified to further stratify these individuals. In patients stratified to a low-risk group based on *NOD2* as a biomarker we performed a logistic regression with the GenePy scores (calculated for each gene, as described above) of *ATG16L1* and 15 additional genes identified through literature review to impact ‘stricturing disease risk’ (*CX3CR1, FUT2, IL12B, IL23R, JAK2, MAGI1, MMP3, TGFB1, SLC22A4, ICAM1, SELP, SELL, IL10, TNFSF15* and *WWOX),* as independent variables, and stricturing disease status as the dependant variable.

Patient and public involvement

Patients and families were involved in the design and conduct of this research. Patient priorities for research have determined priority analyses and dissemination.

Ethics

This study has category A ERGO II ethics approval (30630) and REC approval from Southampton and South West Hampshire Research Ethics Committee (09/H0504/125).

**Results**

Six-hundred and forty-five patients with a confirmed diagnosis of Crohn’s disease were included. Of these 373 were diagnosed <18 years of age and 272 were diagnosed as adults.

Within the cohort we identified 112 distinct variants within *NOD2*. Of these variants, 15 had functional evidence impacting on protein function or downstream signalling, identified through review of the literature, supplementary data 1. There were 11 *NOD2* variants with functional evidence and a further 32 variants fulfilling ‘*in silico* criteria’ for deleteriousness (CADD >15 and AF) <0.05). Characteristics of these variants are summarised in supplementary data 1.

Variant location within the *NOD2* gene were assessed and visualised, figure 1. ‘Pathogenic’, ‘likely pathogenic’ and ‘*in silico’* deleterious variants were present throughout the gene, apart from a 171 amino acid region (positions 441-612, GENCODE transcript ENST00000300589) within the nucleotide binding domain (NOD) in which no deleterious variants were found. Variants were observed in both caspase recruitment domains and throughout the remaining NOD and leucine rich repeat domains.

Monogenic *NOD2*-related disease

In adult-onset patients we were unable to segregate variants due to lack of parental DNA, however all potential compound heterozygote *NOD2* paediatric-onset patients have previously been confirmed to be biallelic 11. We treated all potential compound heterozygote variants in the cohort as presumed compound heterozygote.

*ACMG pathogenic or likely pathogenic criteria for ‘NOD2*-related disease’

To stratify patient risk of stricturing we considered *NOD2* as an autosomal recessive cause of Crohn’s disease. We identified patients who fulfilled ACMG criteria for harbouring causative variants (‘pathogenic’ or ‘likely pathogenic’). Across the entire cohort, 48 patients (7.4%) fulfilled ACMG criteria for ‘*NOD2*-related disease’ including 19 patients who were homozygote and 29 patients who were (presumed) compound heterozygote for a variant with functional evidence. Table 1.

We stratified patients by age at diagnosis. Of those aged <18 at diagnosis 30/373 patients (8%) had ‘*NOD2*-related disease’, compared to 18/272 patients (6.6%) diagnosed ≥18 years of age, p=0.5.

*‘In silico NOD2-related disease’*

We characterised patients with deleterious variation within *NOD2,* but insufficient functional evidence to fulfil ACMG ‘pathogenic’ or ‘likely pathogenic’ criteria. No patients were homozygous for deleterious ‘*in silico NOD2* variants’. We identified 24 patients (3.7%) with either one variant with published evidence for functional impact on *NOD2* function and one variant assessed to have *in silico* evidence of potential functional impact (an AF (gnomAD) <0.05 and a CADD PHRED score of >15), or two variants with an AF (gnomAD) <0.05 and a CADD PHRED score of >15. Table 1.

In patients aged <18 at diagnosis, 15/373 patients (4%) had *in silico* *NOD2*-related disease, compared to 9/272 patients (3.3%) diagnosed ≥18 years of age, p=0.64.

*Stricturing phenotype-genotype assessment*

We assessed the relationship between *NOD2*-genotype and stricturing phenotype, table 1. In patients fulfilling ACMG ‘pathogenic’ or ‘likely pathogenic’ criteria, 24/48 patients (50%) had strictures, compared to 156/597 patients (26.1%) not fulfilling these criteria, p=0.0004. When considering the *in silico* *NOD2*-genotype group, 10/24 patients (41.7%) had strictures.

Combining the two groups (‘*NOD2*-related disease’ and ‘*in silico* *NOD2-*related disease’) demonstrated 34/70 (48.6%) of patients had developed stricturing disease, compared to 147/575 (25.5%) of patients not fulfilling either criterion for a deleterious *NOD2*-genotype, p=0.00005.

*NOD2* as a genomic biomarker for stricturing phenotype

We assessed the ability of *NOD2* GenePy score to classify all patients by stricturing outcome using an AUROC analysis. For all patients (n=645), *NOD2* showed modest power to discriminate stricturing disease behaviour, AUROC 0.586, p=0.001, supplementary figure 2A. Performance improved when considering only patients diagnosed <18 years of age (n=373), AUROC 0.654, p=0.000024, supplementary figure 2B.

To better utilise *NOD2* as a genomic biomarker in a clinical setting we stratified patients into high and low risk groups for stricturing disease using an easily automatable bioinformatic process.

*Group number and cut-off optimisation*

To determine optimal risk groups, patients were split into training (484 patients) and testing (161 patients) sets. The training and testing datasets were balanced according to the number of stricturing patients (the minority class). We employed an iterative Fisher’s exact test within the training set to determine the number of risk groups and GenePy score cut-off values.

This analysis identified two risk groups derived from the training data, table 2. The absolute GenePy cut-off values were then applied to the testing set of patients, where ≥1.078 indicated high-risk and <1.078 indicated low-risk for stricturing disease. Within the testing set of patients, the high-risk group demonstrated a 56.7% stricturing rate, compared to 21.4% stricturing risk in the low-risk group, p=0.0001.

*Survival analysis*

To assess model performance, all patients were combined for survival modelling. We employed a CPH model to account for variable follow-up duration.

Considering all patients, the risk groups demonstrated ability to stratify patients by stricturing risk, based only on *NOD2* genomic data, figure 2A. Patients in the high-risk group (n=89), as determined by *NOD2*, had higher rates of stricturing at all timepoints from diagnosis, with 44 patients stricturing over this time (49.4%), β=2.092, p=0.000024. At maximal follow-up, over 80% of ‘high-risk group’ patients had stricturing disease, compared to less than 60% of ‘low-risk group’ patients.

*Paediatric-onset patients*

We hypothesised that genetic determinants of disease would be more prominent in patients with younger age of onset. Survival modelling using only patients diagnosed <18 years (n=373) was performed. Analysis demonstrated improved performance, with 27/57 (47.4%) patients in the high-risk group having stricturing disease, compared to only 53/315 (16.8%) of patients in the low-risk group, β=3.164, p=0.000001. Figure 2B. At maximal follow-up, nearly 80% of high-risk patients had strictures, compared to an estimated 35% of low-risk patients.

Refinement of prediction using disease location data

Disease location data, at the point of diagnosis, were available for 585 patients including 340 paediatric-onset individuals. Patients were split into those with TI disease and those without. As expected, presence of TI disease at diagnosis was associated with stricturing phenotype, odds ratio 2.5, p=0.00018.

To determine the impact of *NOD2*-risk group combined with disease location, we performed a CPH survival model. Patients were stratified into combined *NOD2* and disease location risk groups (group 1-4). Considering patients diagnosed as adults and children, the addition of *NOD2*-risk group, derived from whole exome sequencing data, to disease location resulted in a significant increase in predictive ability. When compared to group 1 (low-risk NOD2 and no TI disease), the hazard ratio (HR) increased from 1.66, p=0.028 for group 2 (low-risk *NOD2* + TI disease) to 3.19, p=0.00001 for group 4 (TI disease plus *NOD2* high risk group). Figure 3A. Only a small number of patients were in group 3- high risk-*NOD2* group and had no TI disease involvement (n=7).

We performed the same analysis for patients diagnosed <18 years of age, given the previous data indicating a stronger predictive value of *NOD2* in younger patients. There was further improvement in predictive ability. There was no significant difference between group 1 and 2 (HR 1.67, p=0.146). However, comparing group 1 to group 4 the HR was 4.89, p=0.000023. Figure 3B. Again, only a very small number of patients were in group 3- high risk-*NOD2* group and had no TI disease involvement (n=6).

Identification of additional genomic factors implicated in stricturing disease

We attempted to determine whether patients who did not harbour a high burden of *NOD2* variants, but still had stricturing disease, had an alternative genetic driver of this disease behaviour. Patients defined as high-risk of stricturing according to GenePy *NOD2* biomarker stratification were excluded from further analysis, leaving 556 patients defined as ‘low-risk’ for stricturing according to *NOD2*. Of these patients 136 (24.5%) still developed stricturing disease.

All 556 patients were included in a logistic regression model, which did not reveal any significant relationships between the GenePy score of any gene previously implicated in development of stricturing phenotype by literature review, supplementary table 1. Despite this, *ATG16L1* approached statistical significance for a positive association with stricturing phenotype, β= 3.434, p=0.064.

**Discussion**

These data demonstrate the potential utility of *NOD2* as a genomic biomarker for the prediction of stricturing phenotype in patients with Crohn’s disease. We were able to stratify patients into highly significant, high- and low-risk groups for development of stricturing disease. This could be a clinically useful tool that complements clinician decision making, for individual patient management. When combined with disease location data we are further able to refine predictive ability, whilst demonstrating the additional utility of the *NOD2* biomarker. Whilst *NOD2* is a well-established risk locus for Crohn’s disease, a patient’s distinct genetic variation in this gene is not currently utilised in the clinical setting. These data also add additional weight to the hypothesis that for some patients there is an autosomal recessive inheritance of *NOD2* variants, leading to disease 10,11.

Recent functional work has pointed to a mechanism by which impaired *NOD2* function may lead directly to fibrotic disease 30. Our data provides a bridge between this elegant functional work and a clinically applicable tool that can be used to stratify patients at diagnosis, by the risk of stricturing disease. Additionally, contemporary data has pointed towards the importance of genetic variation within the wider *NOD*-signalling pathway, with direct impact on transcription levels in patients with paediatric-onset inflammatory bowel 31. There is the possibility that in some patients, a stricturing disease phenotypes will be associated with rare genetic defects across the *NOD*-signalling pathway and further refinement of predictive models may be possible with future integration. Data from GWAS has pointed towards stricturing disease in relation to *NOD2* purely being a function of its predisposition to trigger terminal ileal disease 32. However, these analyses fail to account for any rare variation and are limited to association with phenotype derived from SNP data. Newer evidence from whole exome sequencing would appear to suggest that *NOD2* leads to stricturing disease regardless of disease location at diagnosis 10,11. Furthermore, *NOD2* genomic data alone still predicts stricturing disease occurrence at the point of diagnosis, regardless of any clinical features at diagnosis. It appears increasingly likely that the full role of *NOD2* in the development of Crohn’s disease is not yet understood.

Construction of predictive models for complicated Crohn’s disease have proven challenging, Kugathasan and colleagues previously detailed a joint model for stricturing and penetrating complications, performing with borderline significance (specificity 63%, sensitivity 66%) 7. Interestingly a *NOD2* genotype (analysis of the 3 common variants only- rs2066844, rs2066845, and rs2066847) was not a significant predictor in the competing-risk model, whereas CBir1 seropositivity and an extracellular matrix gene expression signature were positively associated with a B2 disease phenotype. Our data point to the importance of analysis of deleterious variation across the whole *NOD2* gene, rather than limiting this to specific, more frequent variants. Additional predictive models for Crohn’s disease have taken alternative approaches, including T-cell specific transcription and microbiome signatures 5,33. However, whilst yielding impressive results in a research setting, these data have not yet moved into routine practice. Furthermore, the prediction of long-term disease activity is heavily restricted by the lack of a longitudinal disease activity metric.

Long-range sequencing of *NOD2* presents an opportunity to refine any predictive model, providing additional data on regulatory, promotor and intronic regions. It is possible that some of the missing predictive power for stricturing disease will be accounted for within these *NOD2* non-coding regions. Refinement of our predictive model was possible by limiting to patients diagnosed <18 year of age. Previous data has pointed to higher heritability in patients diagnosed as children 34. However, the long-term phenotype of Crohn’s disease does not appear to significantly differ between adults and children 35. This points to additional, yet unknown, factors in the development of stricturing disease, with *NOD2* variation being the most common in early-onset disease.

Translating genomic data into clinical practice within IBD has huge potential. The importance of integration of genomic data with longitudinal and well phenotyped datasets, such as our own, has recently been highlighted, specifically in relation to preventing tissue remodelling and fibrosis 36. To date the use of next generation sequencing has yielded significant advances in the diagnosis and management of patients with monogenic forms of IBD, however these only account for a very small number of the total number of patients 37. Our data point to the importance of inclusion of clinical data to aid refinement of genomic prediction tools. Utilising these technologies for wider patient benefit has lagged significantly behind. Limited pharmacogenomic testing is now starting to emerge, with the opportunity for routine screening of *TPMT* 38, *NUDT15* 39 and *HLA-DQA*1\*05 40, to prevent thiopurine toxicity, myelosuppression, and formation of anti-TNF antibodies, respectively. Our model requires either targeted *NOD2* sequencing or whole exome or whole genome sequencing, which would incur an additional cost. However, preventing complications, or treating them early, is likely to have a net saving over the lifetime of a patient. We envisage that our prediction model could enable routine monitoring through regular small bowel imaging, alongside the potential for pre-emptive monoclonal therapy and randomised control trials of established and new therapies, based on genomic risk stratification.

To better utilise genomic data in clinical practice, there is likely to be an increased need to employ powerful machine learning algorithms to make sense of genomic and ‘big clinical data’ 41. These tools have already been widely employed for autoimmune diseases, including IBD, however the results are variable 42,43. Recent studies seem to indicate a shift in applications from diagnostics to prediction of outcomes. There is an accompanying increase in the number of studies being published but the clinical translation of these data, including from complex algorithms such as neural networks, is currently limited41.

The study has several key strengths. The genomic data are high quality with stringent quality control processing. Longitudinal phenotyping data is extracted and processed in a standardised fashion from a single institution’s electronic health record. The integration of data follows a novel methodology, revealing new predictive power of genomic data. We acknowledge limitations in this work. There was a necessity for retrospective phenotyping of patients, although this was performed in a systematic way with structured clinician validation. Due to the strict definition of fibrotic stricturing disease it is possible that some patients may be misassigned to a non-stricturing category if they were early in their disease course. Is appears likely that with improved follow-up duration the performance of the model in predicting stricturing disease would improve. In patients diagnosed as adults we were unable to perform segregation analysis to determine phase of variants, or analyse non-coding variants, both areas that would be bolstered by long-range sequencing. We accept that whilst patients in the high-risk *NOD2* group are at up to a 3-fold increased risk of developing fibrostenotic stricturing disease, this group only accounts for 25% of all stricturing cases. We hypothesise that additional factors, including genetic and environmental variables are contributing to the development of strictures in the remaining patients, which we are currently unable to integrate into the model to improve its performance. Future research must focus on identification of risk and protective factors that can be used to further refine these models, including extended sequencing of the *NOD2* region to determine the impact of non-coding sequences. Whether *NOD2* variants predispose to fibrostenotic stricturing, or whether variants predispose to ileal inflammation, leading to strictures, has been raised as a potential confounder of utilising *NOD2* as a predictive tool44. Data present here and previously published from our group indicates that *NOD2* is significantly associated with stricturing disease, even when accounting for presence (or absence) of ileal inflammation11. Utilisation of *NOD2* as a predictive biomarker has the additional advantage that it is not limited by technical difficulties at endoscopy, or limitations of imaging, to identify disease location. Combining genomic and clinical predictors appears to be a highly useful strategy for future modelling.

Conclusion

*NOD2* is a powerful predictive tool for stratification of patients by their risk of stricturing disease. Further refinement of the model through addition of disease location improved performance. Future improvement may be possible with the addition of long-range phased sequencing data. The next steps are construction of predictive tools for additional complications and diseases behaviours. Translation of these methods into a clinical application and accessible tool is an important step towards personalised medicine in IBD.

**Tables and Figures**

**Figure 1-** Lollipop plots showing homozygous variants in *NOD2* identified in our cohort (**1A**), all variants identified in the cohort (**1B**) and variants with CADD scores >15 (deleterious variants) (**1C**) within the cohort. Within figure 1C an area of 171 amino acids in the NOD domain are free from variants predicted to be deleterious. In all figures variants are shown by position within *NOD2* (x-axis) and frequency (y-axis).

**Figure 2A-** Cox-proportional hazard survival plot for all patients. Patients are divided into high-risk of stricturing (group 1) and low risk of stricturing (group 2). High-risk compared to low risk, hazard ratio of 2.092. **2B-** Restriction of survival analysis to patients diagnosed <18 years of age. High-risk compared to low risk, hazard ratio 3.164.

**Figure 3A-** Cox-proportional hazard survival plot for all patients. Patients are divided into groups 1-4. When comparing the lowest risk patients in group 1 to both group 2 and group 4 there are significant increases in the risk of developing stricturing disease. **3B-** Restriction of survival analysis to patients diagnosed <18 years of age. The highest risk of stricturing disease occurred in group 4, with a hazard ratio of 4.89, p=0.000023, compared to group 1.

**Supplementary figure 1-** Pathway for analysis of patients

**Supplementary figure 2A-** Receiver operator curve for all Crohn’s disease patients (n=645) utilising NOD2 GenePy score (CADD 1.6 >15) as the test variable, predicting stricturing disease, **2B-** Receiver operator curve for Crohn’s disease patients diagnosed <18 years of age (n=373) utilising NOD2 GenePy score (CADD 1.6 >15) as the test variable, predicting stricturing disease.

**Table 1-** Number of patients with harbouring *NOD2* variants fulfilling American College of Medical Genetics criteria for ‘Likely Pathogenic’ or ‘Pathogenic’, and variants predicted to be deleterious but with no functional evidence. For functional evidence refer to supplementary data 1. Prevalence of stricturing disease within each group is reported.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **All patients (n=645)** | **Patients diagnosed <18 years only (n=373)** | **Patients diagnosed ≥18 years only (n=272)** |
|  | ***NOD2*-related disease\*** **(number with stricturing phenotype, %)** | ***in silico* *NOD2*-related disease\*\* (number with stricturing phenotype, %)** | ***NOD2*-related disease\*** **(number with stricturing phenotype, %)** | ***in silico* *NOD2*-related disease\*\* (number with stricturing phenotype, %)** | ***NOD2*-related disease\*** **(number with stricturing phenotype, %)** | ***in silico* *NOD2*-related disease\*\* (number with stricturing phenotype, %)** |
| **Homozygote**  | 19 (9 patients, 47.4%) | 0 (0 patients) | 13 (5 patients, 38.5%) | 0 (0 patients) | 6 (4 patients, 66.7%) | 0 (0 patients) |
| **Presumed compound heterozygote**  | 29 (15 patients, 51.7%) | 15 ¥ (5 patients, 33.3%)  | 17 (9 patients, 52.9%) | 10 ¥ (3 patients, 30%)  | 12 (6 patients, 50%) | 5 ¥ (2 patients, 40%) |
| 9 ∞ (5 patients, 55.6%) | 5 ∞ (3 patients, 60%) | 4 ∞ (2 patients, 50%) |
| **Total patients** | 48 (24 patients, 50%) | 24 (10 patients, 41.7%) | 30 (14 patients, 46.7%) | 15 (6 patients, 40%) | 18 (10 patients, 55.6%) | 9 (4 patients, 44.4%) |

\* Two or more variants that functionally impact on *NOD2* function, including reduced/absent protein function, impact on downstream signalling, nonsense mediated decay or deletions, in line with American College of Medical Genetics guidelines

\*\* **Either**, one variants that functionally impacts on *NOD2* function, including reduced/absent protein function, impact on downstream signalling, nonsense mediated decay or deletions, in line with American College of Medical Genetics guidelines AND one variant had a minor allele frequency (MAF) (gnomAD\_AF) <0.05 and a CADD-PHRED 1.6 score of >15, OR two variants had a MAF (gnomAD\_AF) <0.05 and a CADD-PHRED 1.6 score of >15

¥ patients with one variant that functionally impacts on *NOD2* function, including reduced/absent protein function, impact on downstream signalling, nonsense mediated decay or deletions, in line with American College of Medical Genetics guidelines AND one variant with a MAF (gnomAD\_AF) <0.05 and a CADD-PHRED 1.6 score of >15

∞ patients with two variants of a MAF (gnomAD\_AF) <0.05 and a CADD-PHRED 1.6 score of >15

**Table 2-** Training and testing data for identification of predictive risk groups for stricturing disease using summed deleteriousness across the whole gene (GenePy score). Performance of the cut-offs derived from training data is shown in the test data.

|  |  |  |
| --- | --- | --- |
| Groups\* | ***NOD2* CADD>15 variants (training set = 484)** | ***NOD2* CADD>15 variants (testing set = 161)** |
|  | Absolute cut off value of GenePy score\* | Number of patients in group (%) | Number of stricturing patients in group | Absolute cut off values | Number of patients in group (%) | Number of stricturing patients in group |
| **Group 1 (high risk)** | ≥1.078 | 59 (12.2%) | 27 (45.7%) | ≥1.078 | 30 (18.6%) | 17 (56.7%) |
| **Group 2 (low risk)** | <1.078 | 425 (87.8%) | 108 (25.4%) | <1.078 | 131 (81.4%) | 28 (21.4%) |

\*Groups determined by Fisher’s exact test

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