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DNA Methylation of CD4+ T-cells Reveals Association of ITGB7 in Pediatric Crohn's Disease

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B.S., Howard University, May 2015

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Abstract

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Crohn's Disease (CD) is a remitting and relapsing chronic inflammatory disorder of the gastrointestinal tract. Recently, we showed that peripheral blood cells of CD patients have distinct DNA methylation (DNAm) patterns related to inflammatory status. However, that study controlled for differences in cell composition between CD cases and controls, which prevented it from identifying cell type-specific changes. Mapping DNAm signatures to specific cell types during CD is fundamental in understanding the role of epigenetics in the onset and progression of disease. Therefore, we sought to distinguish cell type-specific composition and DNA methylation signatures during CD from bulk DNA that was isolated from blood samples and ileal biopsies obtained from CD pediatric patients and non-IBD controls (RISK cohort). Genome-wide DNAm was profiled using the MethylationEPIC array and TOols for the Analysis of heterogeneouS Tissues (TOAST) was used to test for cell-type specific DNAm differences in blood and ileal biopsies that associated with CD. The statistically significant sites were identified after multiple test correction with a false discovery rate of <0.05. In blood, CD cases had a higher proportion of neutrophils and a lower proportion of CD4+ T-cells relative to non-IBD controls. In CD4+ T-cells, methylation of cg04972065, in the intronic region of ITGB7, was lower in CD cases when compared to non-IBD controls. In ileal biopsies, we observed decreased expression of ITGB7 in cases when compared to non-IBD controls. Epigenetic regulation of CD4+ T-cells during CD leads to lower methylation of ITGB7, likely decreasing ITGB7 expression and regulating lymphocyte trafficking from the blood to and from gut-associated lymphoid tissues. Therefore, dysregulation of epigenetic factors affecting CD4+ T cell function may be a contributor to CD-associated gut inflammation.

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DNA Methylation of CD4+ T-cells Reveals Association of *ITGB7* in Pediatric Crohn's Disease

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INTRODUCTION

An estimated 1.5 million North Americans have inflammatory bowel disease (IBD), and an annual cost of IBD related cost exceeding millions of dollars with the majority of health-care costs driven by medication expenses. In general, the gastrointestinal (GI) tract plays an important role in maintaining immune homeostasis, while also protecting the host against pathogenic organisms by inducing an inflammatory response (1). IBD is classified as a chronic inflammatory, incurable, relapsing disorder of the GI tract consisting of two major subtypes, Crohn's disease (CD) and ulcerative colitis (UC). The exact cause of IBD is unknown, but IBD is thought to be the result of an overactive immune response in the gut from a leaky epithelial barrier (2). Thus, in IBD, the immune system often becomes activated by environmental triggers that can cause inflammation of the gastrointestinal tract. The human body relies on two arms of the immune system for protection against invading pathogens; the innate and adaptive immune response. Innate immunity is known as the first line of defense and is activated immediately after contact with microorganisms. The adaptive arm of the immune system is composed of specialized, systemic cells and processes that eliminate the spread of pathogens, but takes longer, on the order of days, to mount an effective response. Within the adaptive arm are the B cells and T cells. B cells produce antibodies and T cells amplify and differentiate into different subsets such as CD4+ and CD8+ to eliminate pathogens inside cells, protect human body from harmful parasites and adjust allergic reactions, remove extracellular bacteria and fungi, and promote tissue repair. However, increased T cell response can lead to excessive releases of cytokines and chemokines within the body that leads to inflammation (3). T cells are known to play a role in the progression of CD, but the mechanisms remain unknown. They proliferate in the peripheral blood and differentiate when they are stimulated by the presence of antigens (4). CD4+ T-cells play an important role in maintaining peripheral tolerance and are involved in the pathogenesis

of various clinical disorders. In the gut, regulatory T cells have also been observed to be a primary mediator in maintaining the immune homeostasis. The innate and adaptive arms of the immune system maintain intestinal homeostasis to prevent the inappropriate immune responses. (5).

Underlying mechanisms that promote disease remission, exacerbation or progression may be related to distinct cell type-specific changes in epigenetic states and/or levels of gene expression. In recent epigenome-wide association studies (EWAS), adjusting for cell composition has become the standard procedure, but this has also produced biased results that may over look cell-specific changes (6). Nonetheless, we recently observed that peripheral blood cells of CD patients having distinct DNA methylation (DNAm) patterns that coincided with inflammatory status observed at diagnosis reverted back to patterns observed in controls after inflammation was mitigated by treatment. We also found that DNAm patterns revert toward levels seen in non-IBD controls despite still having IBD (7). However, this study adjusted for differences in cell composition between CD cases and non-IBD controls, and it did not identify differences in specific blood cell types contributing to the signal. In order to better understand the role of epigenetics in the onset and progression of CD, subsequent analysis should map disease-specific DNAm signatures to specific cell types. Therefore, we sought to distinguish cell-specific methylation signatures to identify immune cell type-specific DNAm differences during CD. Since differences in immune blood cell types such as neutrophils, monocytes, B-Cells, T-Cells (CD4+ AND CD8+), and natural killer cells are an important biological difference between IBD cases and non-IBD controls, we propose herein to test the overarching hypothesis that cell-specific methylation signatures can identify novel DNAm differences indicative of the genes and pathways whose regulation distinguishes IBD cases from non-IBD controls. In this study, we will use human patient blood and ileal biopsy samples to determine if changes in epigenetic

differences specific to immune cell types can provide insight into the biological pathways underlying IBD.

METHODS

RISK Cohort

Samples from patients younger than 18 years old were obtained from the Risk Stratification and Identification of Immunogenetic and Microbial Markers of Rapid Disease Progression in Children with Crohn's Disease (RISK) study (8), the largest pediatric CD inception cohort recruited from 28 sites in the United States and Canada identifying genetic, clinical, and immunological factors that contribute to progression to complicated disease in Crohn's Disease (7). Patients who were newly diagnosed with Crohn's disease were considered cases. Patients with no bowel pathology upon colonoscopy, negative gut inflammation, and continued presentation as asymptomatic for IBD during follow-up were considered as non-IBD controls.

Samples for whom DNAm data was available, as described in our previous study (7), were included in this study. In total, we evaluated peripheral blood DNA samples from 164 pediatric patients with newly-diagnosed Crohn's disease (cases) at two time-points (diagnosis and 3-year follow-up) as well as 74 non-IBD pediatric patients without intestinal inflammation or symptoms (controls) at baseline only. Ileal bulk-biopsies were obtained from newly diagnosed CD patients by colonoscopy in the same cohort. Gene expression from baseline Ileal mucosal samples at diagnosis were available from for 308 CD cases and 66 non-IBD controls.

DNA methylation

Genome-wide DNA methylation for these samples was profiled at single-base resolution using the Illumina MethylationEPIC BeadChip. Quality control for the blood DNA methylation was performed with CpGassoc (9). CpG sites with low signal or low confidence level (detection

P> 0.05) or samples with greater than 10% of their data missing were removed, and samples with data missing or with a low confidence level for greater than 10% of CpG sites were removed. Probes mapping to multiple locations were also removed (7). After quality control, 807,511 probes and 238 samples (164 cases and 74 non-IBD controls) remained. For each CpG site, beta values were calculated as the ratio of methylated to methylated and unmethylated signal. Using the module beta-mixture quantile dilation (BMIQ), signal intensities were normalized to account for the probe design bias in the EPIC array data and these signal intensities were used to perform principal component analysis to identify sample outliers (10). We applied the same QC protocol on the ileal biopsies. Estimated cell counts for CD4+ T-cells, CD8+ T cells, natural killer cells, B cells, monocytes, and neutrophils were calculated for each subject using Houseman's approach (11).

Gene expression/RNA-Sequencing

We also had ileal biopsy gene expression profiles for a subset of RISK subjects at diagnosis (n=313) and processed as described (12) Briefly, RNA from ileal biopsies was extracted with NEBNext Ultra RNA Library Prep Kit used for Illumina RNA sequencing library preparations by following set manufacturer's recommendations (NEB, Ipswich, MA, USA). Reads were quantified with 150 base pairs in length and aligned with reference genome HG38 (Gencode v28) using STAR package (13). The quantity and quality of actual read counts were used for the quality control protocol. In total, 13 samples with low read counts (<20M) and aligned with only <60% of the reference genome were excluded. Similarly, genes with < 10 read counts in at least 95% of the samples were removed. As a total, 300 samples and ~14000 genes were used in the downstream analysis.

Statistical Analysis

TOols for the Analysis of heterogeneouS Tissues (TOAST), a method used to identify features showing distinct profiles among different cell types was used to test for cell-type specific DNAm differences in neutrophils, monocytes, B-Cells, T-Cells (CD4+ AND CD8+), and natural killer cells that associated with CD (6). The statistically significant sites were identified after multiple test correction with a false discovery rate of <0.05.

In order to evaluate the correlation of methylation and expression in blood, we used gene expression samples from an independent cohort, the Grady Trauma Project, as described by Kennedy and colleagues (14). Then, to test if gene expression from RISK ileal samples associated with IBD disease status in *ITGB7*, a linear regression controlling for age, sex, and race was used.

RESULTS

Changes in cell-type proportion and DNA methylation patterns in blood

The RISK cohort contains blood and ileal samples from pediatric CD patients and non-IBD controls. There are no significant differences in age, race, or sex between cases and controls for blood or ileal samples. However, CD cases had significant differences in multiple blood cell types relative to non-IBD controls, including a higher proportion of neutrophils (P<2.2x10-16) and a lower proportion of CD4+ T-cells (P<2.2x10-16; Figure 1).

Samples were tested for interactions between cell composition and CD status to predict methylation of each CpG. After controlling for multiple comparisons in all 6 immune cell types, cell-specific associations between DNA methylation and CD were identified in CD4+ T-cells and NK cells. In CD4+ T-cells, methylation of cg04972065, an intronic region of *ITGB7* at chr12:53591766, was lower in CD cases when compared to non-IBD controls ($p=5.4x10^{-8}$;

Figure 2A). In NK cells, methylation of cg13321967, a CpG at chr4: 53617679 that was not annotated to any known gene, was lower in CD cases when compared to non-IBD controls (P= 2.22 x10-3).

To evaluate the regulatory significance of the CD-associated CpG sites, we examined their correlation with expression levels in blood from a non-IBD cohort. We noted a positive correlation between cg04972065 and *ITGB7* expression (R^2 = 0.46; P = 1.5x10-13; Figure 2B). We did not observe an association of cg13321967 with expression of any gene expressed in blood. Thus, we concentrated our subsequent analyses on cg04972065 (*ITGB7*).

ITGB7 (cg04972065) methylation changes in response to CD treatment

In our previous study (7), DNA methylation levels changed in response to treatment that reduced inflammation so we wanted to evaluate whether methylation of cg04972065 also reverts over time in response to treatment. Methylation of cg04972065 was lower in CD cases at baseline when compared to non-IBD controls, but, in the 36-month follow-up, we observed that methylation of CD cases increases almost to the level of non-IBD controls (Figure 3).

ITGB7 Expression in Ileum

We next sought to characterize the degree to which expression of *ITGB7* associates with CD in ileal tissue, the primary tissue of interest for CD. In 308 CD pediatric patients and 66 non-IBD controls from RISK with gene expression data from their baseline visit, we noted lower expression of *ITGB7* in CD cases (P < 1.70 x 10-3) when compared to non-IBD controls (Figure 4).

DISCUSSION

The destruction of the gastrointestinal tract during CD is known to be associated with changes in the function of the immune system in response to a number of intrinsic and

environment cues (15). A hallmark of IBD is the rapid recruitment and persistence of multiple bacterial, immune, and non-immune cell types to the site of inflammation, which is a main contributor to the disease. Recent advancements in our understanding of epigenetics have continued to shed new light on several biological processes and how they affect disease. Therefore, we sought to further define epigenetic contributions to CD. In this study, using bulk DNA methylation, we identified cell-type specific DNA methylation differences in the CpG site encoding *ITGB7* in CD4+ T-cells that associated with CD. We also found corresponding differences in immune cell types in peripheral blood between cases and controls. In particular, lower CD4+ T-cell levels in CD, a subset of T-cells, are key in mediating the host protective and homeostatic responses (16). When homeostasis is disrupted, it's often accompanied by inflammation, a main contributor of CD symptoms. Inflammation activates the innate immune system leading to higher proportions of neutrophils and monocytes and lower proportions of adaptive immune cells such as CD4+ T, CD8+ T, and B cells (17).

Within the adaptive arm are T cells and within T-cells are subsets of cells that are defined based on their function: helper CD4+ and cytotoxic CD8+. When CD4+ T-cells are activated, they rapidly divide and secrete cytokines that assist immune response. Our results suggest that methylation of cg04972065 from CD4+ T-cells are lower in CD samples when compared to non-IBD controls at diagnosis. This CpG site is located in an intronic region of integrin α4β7 (*ITGB7*), an important adhesion molecule that mediates lymphocyte trafficking to gut-associated lymphoid tissues via binding to its ligand. Methylation of CpGs downstream of the promoter and in the transcribed region is often correlated with expression (18). DNA methylation in intronic regions can play a role in splicing or help to regulate transcriptional enhancers or repressors (19). Though this explanation would help to explain the positive relationship between cg04972065 methylation and *ITGB7* expression, we were unable to evaluate transcript variants of *ITGB7*. In

our results, we observed a lower proportion of methylation in cases compared to non-IBD controls, which is consistent with lower *ITGB7* expression, suggesting reduced function of *ITGB7* and potential suppression of lymphocyte migration to the gut. We also observed in blood and ileum samples that when there is lower proportion of methylation, there is lower *ITGB7* expression.

Studies have demonstrated in mouse models, that loss of integrin β7 function causes T cell depletion and intensifies dextran sulfate sodium (DSS) colitis. This study concluded that integrin β7-mediated T-cells recruitment to gut-associated lymphoid tissue is essential to suppress inflammation in DSS-induced acute colitis in mice models (20). Another study, observed in their mice model that when there is a loss *ITGB7* function, there's increased intestinal inflammation (15).

In our follow-up methylation results, we saw that methylation proportions of CD samples exposed to treatment overtime revert toward levels seen in non-IBD controls during the course of treatment, potentially increasing expression of *ITGB7* and lymphocyte trafficking in the gut. A drug used to treat IBD, Vedolizumab, blocks *ITGB7* function and maintains clinical remission in some IBD patients. A study showed that excessive inhibition of *ITGB7* function by high dose of the known IBD drug could excessively suppress trafficking of immune cells into the gut, which might have an effect on the maintenance of IBD (21). Future studies should examine *ITGB7* function and trafficking of immune cells over the course of CD in patients treated with Vedolizumab.

Our study various limitations. One of the limitations of our study is the lack of gene expression from blood samples in RISK. Nevertheless, we used data from blood samples from an independent cohort to examine the correlation of DNAm and gene expression of *ITGB7*. This analysis showed that in blood samples when there is a lower proportion of methylation, there is

lower gene expression in *ITGB7*. Another limitation is that we were not able to evaluate DNA methylation and gene expression in ileal biopsies from the same subjects. Also, available biopsies were taken at diagnosis so we could not evaluate changes in ileal tissues over time or in response to CD treatment. Finally, the number of cell types that could be evaluated in this study was limited by the available reference panels for immune cell types. As more detailed reference panels or single cell methods become available, future studies could evaluate other T cell subsets relevant to CD.

In conclusion, our study suggests that β7 integrins are essential for lymphocyte recruitment in IBD. Epigenetic regulation of CD4+ T-cells during CD leads to lower methylation of *ITGB7*, likely decreasing *ITGB7* expression and down regulating lymphocyte trafficking to/from gut-associated lymphoid tissues. Therefore, our results are providing the further evidence that the dysregulation of epigenetic factors affecting CD4+ T cell function may be a contributor to CD-associated gut inflammation. Overall, understanding the role of immune cell types in the biological mechanisms underlying IBD is important and can be leveraged to an improved targeted diagnosis, treatments, and outcomes.

Figure 1: Differences in immune cell types. In the RISK cohort, Crohn's disease (grey) and non-IBD (red) samples have 6 immune cell types in peripheral blood. B cells, CD4+ T-cells, CD8+ T cells, neutrophils, monocytes, and natural killer cells. The proportion is measured (y-axis) in each type cell type (x-axis) for CD and non-IBD controls. The proportion is lower in CD samples in B cell, CD4+ T, CD8+ T, and natural killer, but higher in neutrophils and monocytes. The opposite is seen in non-IBD controls. (error bars represent standard deviation)

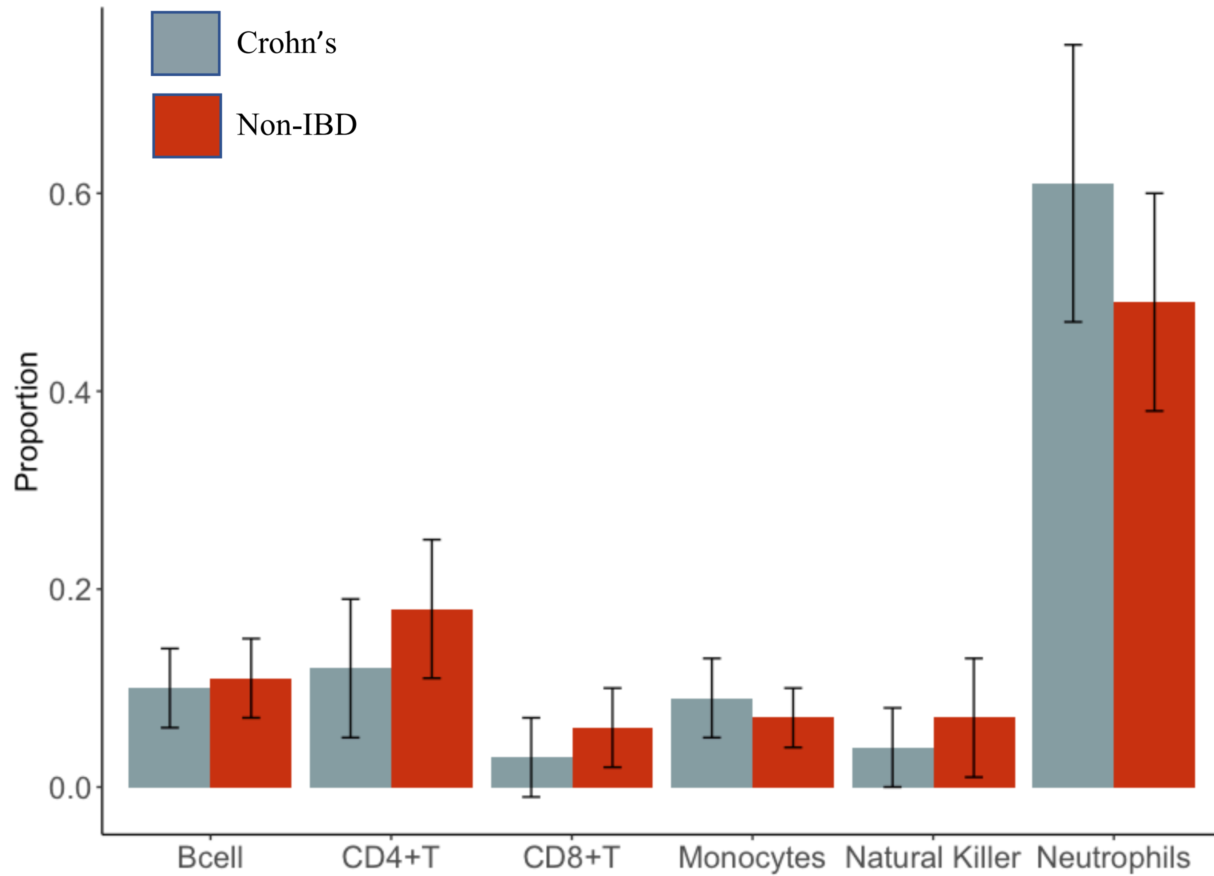


Figure 2A: Differences in methylation of cg04972065 in cases and controls. The boxplot depicts methylation differences is cg04972065 between CD cases and non-IBD control. Methylation proportion (y-axis) of cg04972065 was lower in CD cases (grey) when compared to non-IBD controls (red) (x-axis). Each line in the box plot represents the median and the whiskers represents the maximum and minimum proportions. **Figure 2B. The association of methylation and expression in blood.** The methylation of cg04972065 (x-axis) from whole blood associates with expression of ITGB7 in GTP cohort (y-axis). The red line represents confidence interval of the data. The grey shaded represents the 95% confidence interval. The correlation between methylation of cg04972065 and ITGB7 expression in blood is positive. (R = 0.46; P = 1.5x10-13).

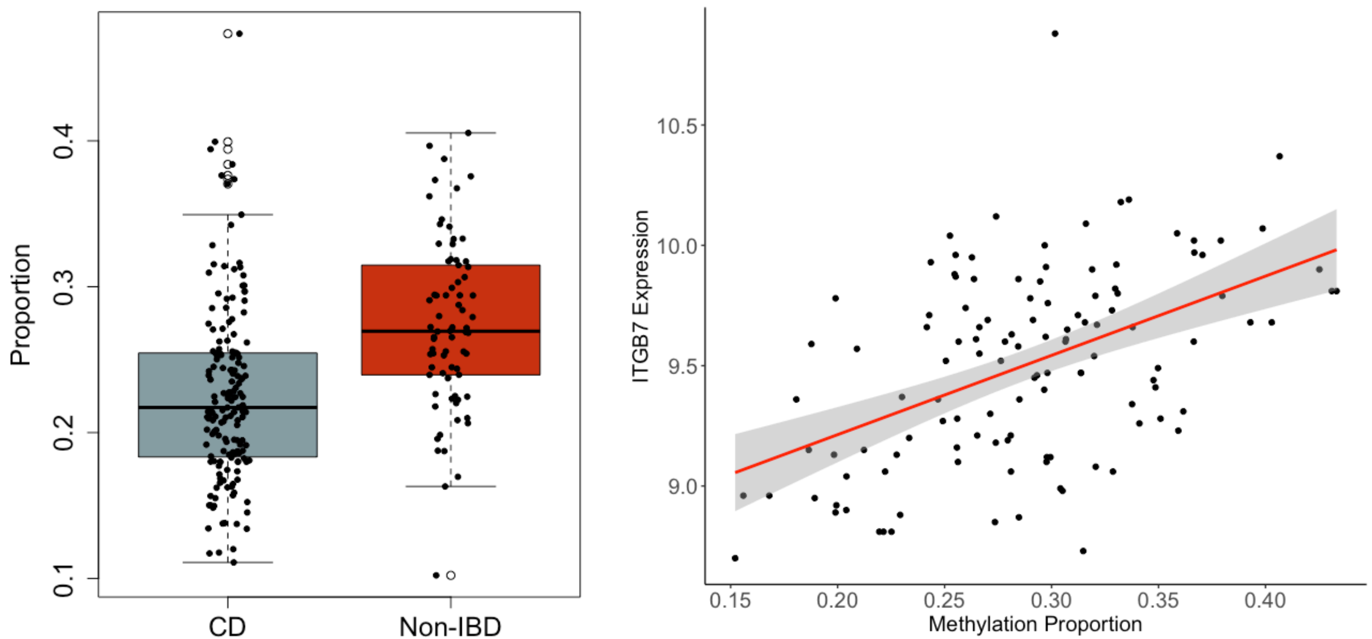


Figure 3: ITGB7 methylation changes in response to treatment in CD samples. The boxplot depicts methylation differences is cg04972065 among CD cases (baseline and 36-month follow up) and non-IBD controls. Methylation proportion (y-axis) of cg04972065 was lower in CD cases at baseline (red) when compared to non-IBD controls (red); but at 36-month follow up (white) methylation increased close to the level of non-IBD controls (x-axis). Each line in the box plot represents the median and the whiskers represents the maximum and minimum proportions.

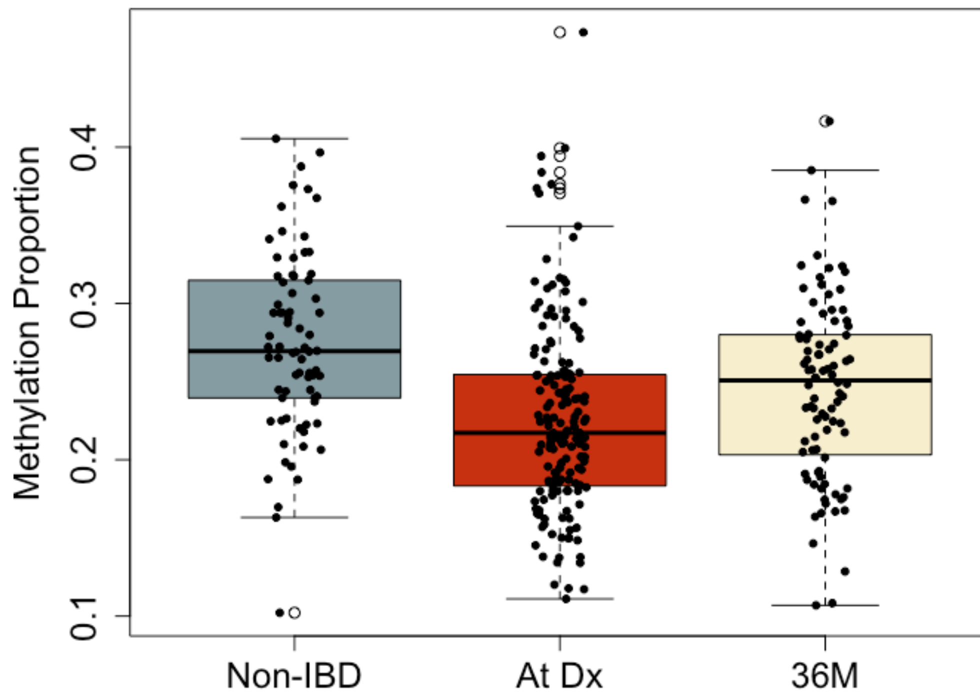
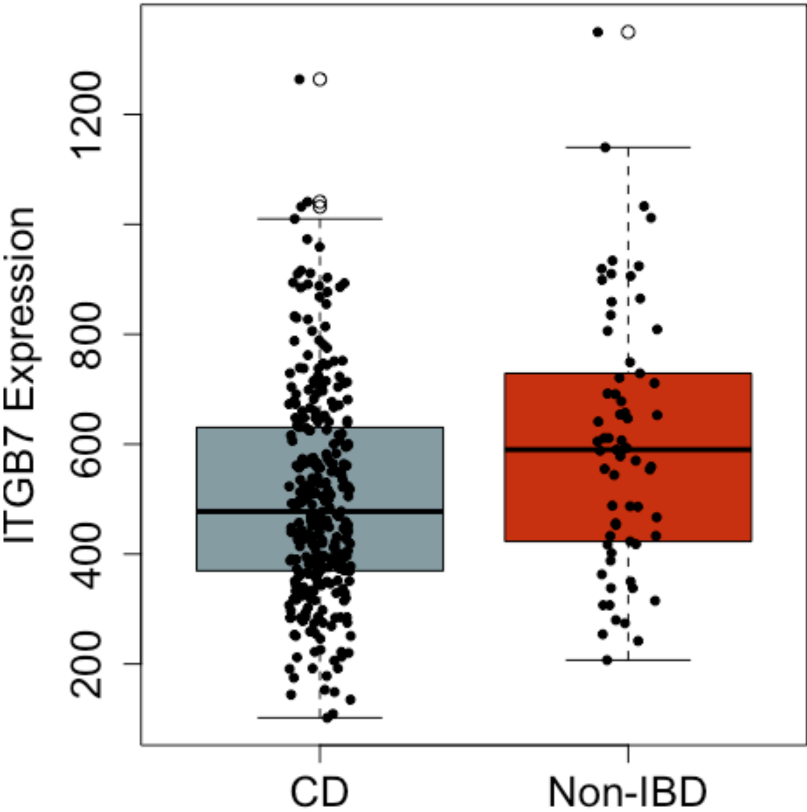


Figure 4: The differences of ITGB7 expression in ileal biopsy cases vs. controls. Expression (y-axis) of the gene *ITGB7* was lower in CD cases (grey) ($P < 1.70 \times 10\text{-}3$) when compared to non-IBD controls (red) (x-axis).



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