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ORIGINAL RESEARCH

Colonic Mucosal Gene Expression Profile in Patients with Neoplastic Progression in Longstanding Ulcerative Colitis

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Purpose: Long-standing mucosal inflammation is suspected to be one of the main drivers of colitis-associated colorectal cancer (CA-CRC), but far from all colitis patients develop cancer. Non-neoplastic mucosa located distant from neoplastic lesions may harbour early molecular events of carcinogenesis. We hypothesise that patients with UC who have progressed to neoplasia (progressors) exhibit a distinct molecular profile of mucosal inflammation from non-progressors.

Patients and Methods: We performed transcriptomic profiling of 143 mucosal biopsies from non-neoplastic colonic segments of 14 UC progressors and 30 UC non-progressors using the Agilent SurePrint G3 human gene expression 60K microarray. Subsequently, we carried out gene set ontology analyses. In addition, we assessed lymphocyte infiltration to the mucosa of biopsies taken adjacent to biopsies used for transcriptomic analysis by immunohistochemistry.

Results: Adjusting for molecular alterations associated with long disease duration, our findings revealed that UC Progressors' inflamed mucosa has a distinct gene expression profile of 529 significantly deregulated genes, eg *LTB, CXCL13, CD19, C3* and *CYP4F3*. The profile was negatively enriched for biological processes (BPs) such as adaptive immune responses and complement system activity and positively enriched for processes related to detoxification. The negatively enriched BPs were supported by the presence of fewer infiltrating B cells and less lymphoid aggregates in the microenvironment of the inflamed mucosa of progressors.

Conclusion: The inflamed colonic mucosa from UC patients who have progressed to dysplasia or cancer has a different gene expression profile than that of UC non-progressors, suggesting lower levels of lymphoid organ-initiating and immune cell-attracting signals and fewer infiltrating immune cells to the mucosa. Consequently, progressors may lack the ability to mount sufficient adaptive immune responses necessary to counteract driving forces of malignant transformation in UC.

Keywords: inflammatory bowel diseases, ulcerative colitis, colitis-associated neoplasms, gene expression profiling

Introduction

The risk of colorectal cancer (CRC) in patients with inflammatory bowel disease (IBD) is increased, with approximately 5–10% of IBD patients developing colitis-associated colorectal cancer (CA-CRC) during the course of disease.[1,2] CA-CRC differs from sporadic CRC in that it presents in young adults (median 44 years) and is associated with higher mortality.[2–4] Malignant progression in IBD is thought to occur in a stepwise manner, defined by specific morphologic changes of the mucosal cells (dysplasia).[5] To detect these morphological changes, regular colonoscopic surveillance has been advocated over the last 30 years for patients with IBD and risk factors for CA-CRC.[6] However, this strategy has not demonstrated the desired efficacy in detecting cancer early in IBD patients, nor has it enhanced their survival.[7–9] Thus, there is an urgent need to improve early diagnosis and prognosis of malignancy in these young patients.

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Many studies related to CA-CRC have focused on cancerous mucosal patient biopsies.[10–14] Thus, primarily identifying the characteristics of the established cancer mucosa and not the early molecular events driving the progression of IBD to CA-CRC. Characterisation of the early molecular changes associated with progression of UC to dysplasia and cancer in non-neoplastic mucosa of UC patients might contribute to improve detection of dysplasia and cancer in UC.

Long-standing mucosal inflammation is suspected to be a major driver of CA-CRC.[15–18] It is believed that malignant transformation of mucosal epithelial cells may arise due to mutations introduced during enhanced epithelial proliferation. This may be promoted by damage induced by molecules secreted by immune cells within the chronic inflamed mucosa.[18] Despite chronic inflammation being the histological hallmark in IBD, far from all IBD patients, who do not respond adequately to anti-inflammatory treatment and suffer from longstanding inflammation, develop dysplasia or cancer, suggesting that the inflammation in patients who develop any dysplasia or cancer (progressors) differs from the inflammation in UC patients who do not progress (non-progressors). We hypothesised that this difference would be reflected at the molecular level in inflamed colonic mucosa of progressors compared to that of non-progressors and in line with the concept of the field effect could reflect early carcinogenic processes.[19]

To test this, we generated a comprehensive biobank of biological material from progressors and non-progressors with associated clinical and histological information. We addressed our hypothesis by investigating the gene expression profiles of inflamed and non-inflamed non-neoplastic mucosal samples of UC progressors and non-progressors.

Materials and Methods

Patient Cohorts and Biological Material

Patients from the prospective Lovisenberg Diakonale Hospital (LDS) and the prospective CA-CRC cohort from Akershus University Hospital (Ahus) were included in the main study (Oslo study). Patients from the University of Washington Medical Center in Seattle (Seattle cohort) were included for partial validation of the results.

The LDS cohort was presented elsewhere.[20] Briefly, patients with UC, who were at high risk for CA-CRC eg, presenting with extension of inflammation proximal to splenic flexure (pancolitis) and at least eight years of disease duration from diagnosis of UC, were enrolled from 1999 to 2013. UC patients with primary sclerosing cholangitis (PSC), a rare complication in UC patients with particularly high risk of cancer, were also included. The Ahus CA-CRC cohort includes patients from the chromoendoscopy screening program at Ahus, enrolled from 2015 to 2021 (Crohn's and UC-pancolitis with risk factors for CA-CRC as for the LDS cohort) as well as IBD progressors that have been referred to Ahus by other hospitals in the region for assessment and treatment of neoplasia in IBD.

Sampling of biologic material of the LDS and the Ahus cohorts was similar. Dye-based chromoendoscopy was performed by experienced endoscopists. Non-targeted biopsies were taken from each segment of the colon (ascending, transverse, descending and sigmoid colon as well as rectum). From each segment, biopsies were taken for formalin fixed paraffin embedding (FFPE) by standard procedure, snap freezing in liquid nitrogen and preservation in RNA-later. All characteristics of identified lesions were recorded and evaluated before being removed and/or biopsied (FFPE, snap freezing and RNA-later). A set of biopsies were also taken within 2–4 cm of any lesions (FFPE, snap freezing and RNA-later).

Independent and blinded analyses of the FFPE biopsies were performed by two expert pathologists. Histologic degree of chronic as well as chronic active inflammation was evaluated according to Geboes index.[21] Presence of atrophy and crypt-distortion were also evaluated. Neoplasia was classified as either indefinite for dysplasia, positive for low-grade dysplasia (LGD), high-grade dysplasia (HGD), adenocarcinoma or adenomas.

Clinical data regarding age, sex assigned at birth, type of IBD diagnosis, duration of IBD, extent and severity of colitis, concomitant disease such as PSC, medication and family history of CRC were recorded at time of sampling.

Non-PSC patients with UC pancolitis and a disease duration of at least eight years were selected from the two cohorts for the analysis of gene expression and included 14 progressors (five with CA-CRC, two with HGD, seven with LGD) and 30 non-progressors. The distribution of demographic and clinical factors for these patients are shown in Table 1. There was no statistically significant difference between the groups (data not shown). In total, 143 colon biopsies (49 progressor samples and 94 non-progressor samples) were analysed covering histological non-neoplastic, non-inflamed

Table 1 Demographic and Clinical Factors of UC Patients Included in the Study

	Non-Progressors (n=30)	Progressors (n=14)
Female (Male)	12 (18)	2 (12)
Age[a] (years) median (min;max)	54 (30;69)	54 (30;75)
Age at diagnosis of IBD (years) median (min;max)	31.5 (18;50)	22 (16;54)
Duration of IBD[a] (years) median (min;max)	16.1 (8;49)	24 (8;53)
Patients with CRC in 1st degree sibling	3	1
Use of Salazopyrin/5-ASA[a]	27	8
Use of Systemic steroids[a]	1	0
Use of Azathioprine/methotrexate[a,b]	8	2
Use of Infliximab/Adalimumab[a,c]	4	1
Use of Vedolizumab[a,c]	0	2
Use of Other medications[a]	10	3

Notes: [a]at time of sampling, [b]immunomodulatory agent, [c]biologic agent.

(Geboes grade 0) as well as non-neoplastic, inflamed (Geboes grade 1–5) tissue from right and left colon and rectum. In accordance with the histology of the associated FFPE biopsies, biopsies subjected to gene expression analysis were assigned to non-inflamed and inflamed sample groups from either non-progressor or progressor patients resulting in four main sample groups (Figure 1). In addition, inflamed samples were assigned to chronic inflammation and chronic active

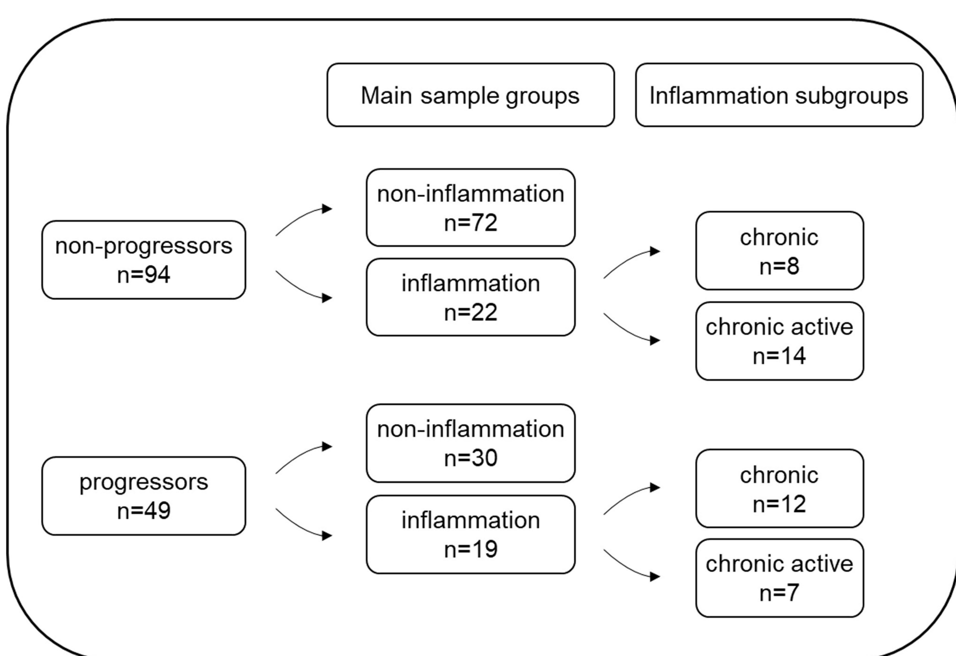


Figure 1 Overview of the study design. Colonic mucosal biopsies from non-progressors and progressors were assigned to main sample groups according to histological diagnosis of inflammation or non-inflammation. In addition, the inflamed samples were assigned to subgroups according to type of inflammation. The number of biopsies (n) is given for each group. All biopsies included in this study were histologically negative for neoplasia.

inflammation groups from either non-progressors or progressors resulting in four inflammation subgroups (Figure 1). All samples were located distant from lesions in the colon with dysplasia or cancer.

The Seattle cohort includes 96 colonic biopsies from 24 patients with UC, who underwent colonoscopic surveillance at the University of Washington Medical Center in Seattle prior to year 2000. All patients had early onset of disease (age of onset <45 years old) and had had the disease for at least 8 years. At colonoscopy, biopsies were collected in quadrants every 10–15 cm throughout the colon, fixed and embedded in paraffin blocks, and evaluated by pathologists for the presence of dysplasia. Seventeen patients were considered non-progressors because all biopsies were free of dysplasia. Seven patients were considered progressors because HGD or cancer was identified at colonoscopy or within 1–2 years after the procedure. All biopsies were inflamed. For the present study, two progressors with PSC and indefinite for dysplasia and one progressor indefinite for dysplasia were excluded to ensure comparable patient groups between the Oslo study and the Seattle cohort. In total, 86 colon biopsies (16 progressor samples and 70 non-progressor samples) were analysed covering histological non-neoplastic, inflamed tissue from right and left colon and rectum.

Processing of Biopsies and Gene Expression Profiling

Oslo cohorts: Total RNA was isolated from snap frozen mucosal biopsies, and the gene expression profile was assessed using the Agilent SurePrint G3 human gene expression 60K microarray. Seattle cohort: Total RNA was isolated from archival colonoscopy blocks containing colonic mucosa, and the expression of specific genes was assessed using a custom designed NanoString panel. For more details, see paragraph “Isolation of RNA” and “Gene expression profiling” in the Material and Methods section in the Supplementary material.

Immunohistochemistry

FFPE tissue sections (Oslo cohorts) were stained by immunohistochemistry for expression of CD3 and CD20 as described in detail in the Material and Methods in the Supplementary material.

Bioinformatic Analyses

Microarray data was processed using the limma package,[22] version 3.48.1. The processing performed follows the standard limma pipeline, where microarray files are loaded into R (version 4.1) using the read.maimages function with the “agilent” source. We then corrected the background and normalised the data using the backgroundCorrect function with the “normexp” method, and the normalizeBetweenArrays with the “quantile” method. We condensed the microarray data by using the avereps functions, which retains the average of replicate probes.

The model matrix was created based on the main sample groups (Supplementary Table 1) and inflammation subgroups (Supplementary Table 2) to be used in the differential gene expression analyses. We performed the linear model fit using the lmFit function, created the contrast, and performed the empirical Bayes Statistic using the eBayes function to obtain differentially expressed genes. Significantly differentially expressed genes (DEGs) were selected based on an adjusted p-value threshold of 0.05 and a fold change (FC) threshold of 1, where significance is associated to genes with a value greater than 1 or smaller than −1.

Gene set enrichment analysis (GSEA) was performed using the ClusterProfiler package,[23] version 4.0.0. The analysis was run for the biological process (BP) ontology. The AnnotationDbi package, version 1.54.1, was used to load the information relating to the studied organisms. The gene background of the GSEA was set using the homo sapiens organism library (org.Hs.eg.db), version 3.13.0. GSEA was performed on differential gene expression analyses, where every gene within the differential expression results, regardless of significance, was inputted for the analysis per the tools guidelines. The p-value and false discovery rate (FDR) cut-offs were set to 0.05. The semantic distance was added to the GSEA results using the function from the enrichplot package, version 1.12.1. The similarity matrix added was calculated using the “Wang” method.[24]

NanoString data was processed using DESeq2. Positive and negative controls were removed following a standard processing. Genes appearing in less than 50% of the samples, genes with 30 or fewer counts as well as genes not related to mitochondrial function were excluded from the analysis. The remaining data was loaded into DESeq2 while utilising

the 5 housekeeping genes for normalisation. We then followed the standard DESeq2 pipeline to compare the gene expression of inflamed mucosa in progressors and non-progressors.

Gene lists were analysed using the web-based g:Profiler tool (https://biit.cs.ut.ee/gprofiler/gost) version: e106_eg53_p16_65fcd97 with the organism set to human and the significant threshold to 0.05 (default).

Other Statistical Analyses

Other statistical analyses aside from bioinformatics were performed by using SPSS 28.0 and GraphPad Prism 10 for Windows. Groups were compared by using two-sided *t*-test for continuous variables. In case of categorical variables, Pearson chi-square or Fisher's Exact test was applied. Differences with a p-value < 0.05 were considered statistically significant.

Results

Characteristic Gene Expression Profile Associated with Inflammation in Progressors

Non-inflamed and inflamed tissue located distant from neoplastic lesions in the colon of progressors and non-progressors of the Oslo cohorts were analysed to identify a transcriptomic profile associated with the progression from UC to dysplasia and cancer (Figure 1).

Comparison of the gene expression profiles of inflamed tissue obtained from non-progressors and progressors revealed a large number of significantly differentially expressed genes (Figure 2a, Supplementary Table 1 and 3). In contrast, only a small number of genes were significantly differentially expressed in non-inflamed tissue between the two patient groups (Figure 2b, Supplementary Table 1 and 3). Furthermore, comparison of gene expression profiles of inflamed and non-inflamed tissue of non-progressors identified many significantly differentially expressed genes (Figure 2c, Supplementary Table 1 and 3). The equivalent comparison in progressors showed a small number of significantly deregulated genes, suggesting that the transcriptional response associated with the inflammation process in progressors is attenuated (Figure 2d, Supplementary Table 1 and 3).

For deeper exploration of the gene expression profile associated with inflammation, the inflamed samples were divided into subgroups of histologically chronic and chronic active inflammation (Figure 1). Comparison of the inflammation subgroups showed that the major differences in gene expression were found between progressors and non-progressors irrespective of the type of inflammation (Supplementary Figure 1a–d, Supplementary Table 2 and 3).

Altered Biological Processes in Inflamed Mucosa Associated with Progression of UC

Before focusing on single deregulated genes, we assessed the biological processes (BPs) associated with the differentially expressed genes in the inflamed tissue (Figure 2a, Supplementary Table 3). We performed a gene set enrichment analysis (GSEA) of all differentially expressed genes of the inflammation (non-progressor – progressor) comparison. The top 20 positively enriched BPs in the progressor inflamed tissue were related to mitochondrial activity and various metabolic pathways, whereas the top 20 negatively enriched BPs were related to immune responses and vasculature development (Figure 3a and b). Consistent with an attenuated transcriptional response to inflammation in progressors, there is an inverse direction of the enrichment for most of these BPs in inflamed tissue when compared to non-inflamed tissue for progressors and non-progressor (Figure 4). Inflamed tissue of progressors primarily shows negative enrichment of BPs related to immune response, whereas the inflamed tissue of non-progressors shows a positive enrichment. Moreover, the inflamed tissue of progressors shows either positive or no enrichment of metabolic related BPs in contrast to the non-progressors, where the inflamed tissue shows negative enrichment. Collectively, this suggests that progressors and non-progressors have characteristic differences in inflammation-related BPs, which may be associated with progression of UC to dysplasia and cancer.

The interconnection of the BPs of the inflammation (non-progressor – progressor) comparison were visualised in gene concept network (cnet) plots (Supplementary Figure 2a and b). This showed that the positively enriched BPs organised in hubs representing mitochondrial activity and a larger more heterogeneous cluster representing other metabolic pathways including xenobiotic-, carbohydrate- and lipid metabolism (Supplementary Figure 2a). The negatively enriched BPs

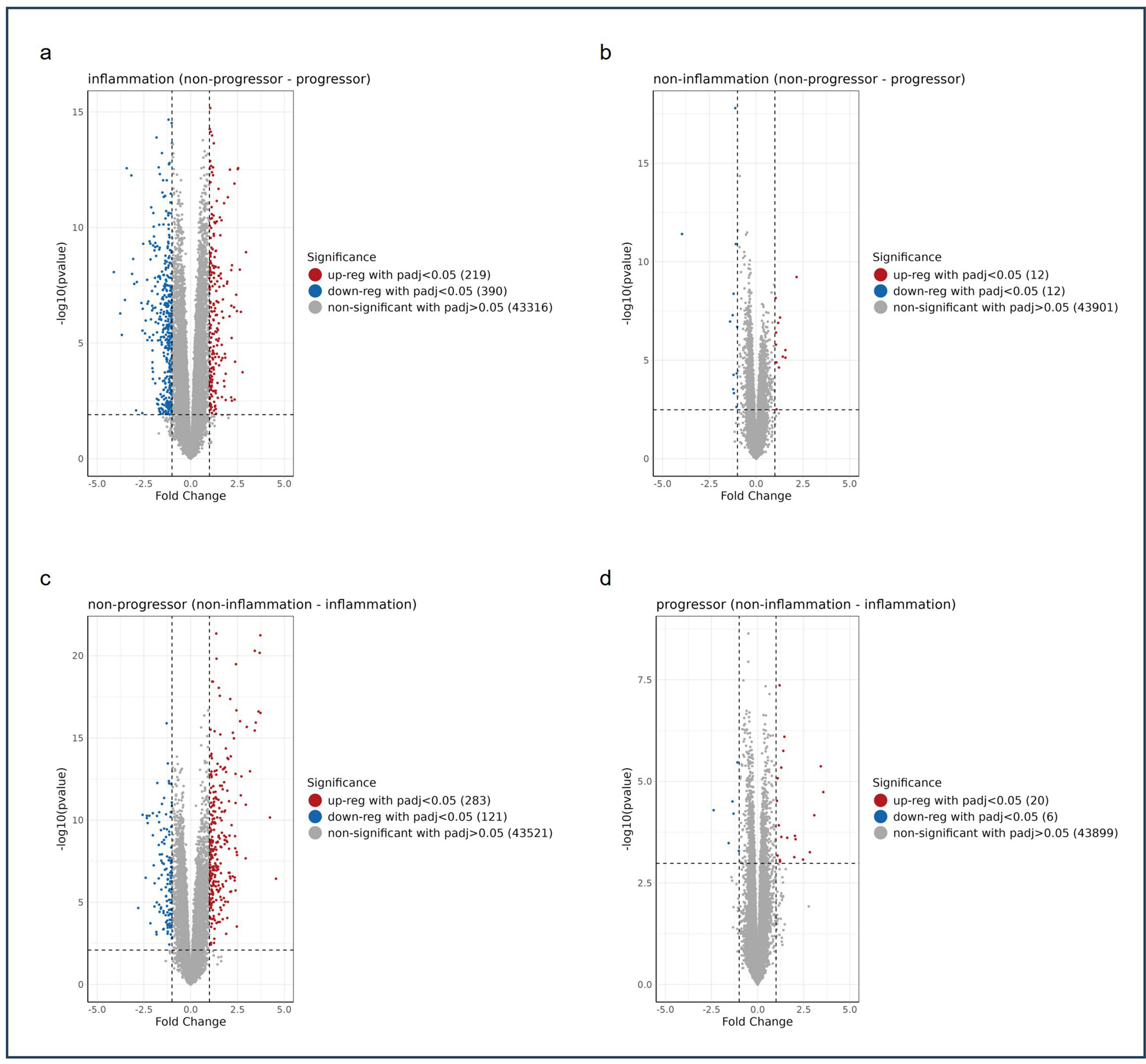
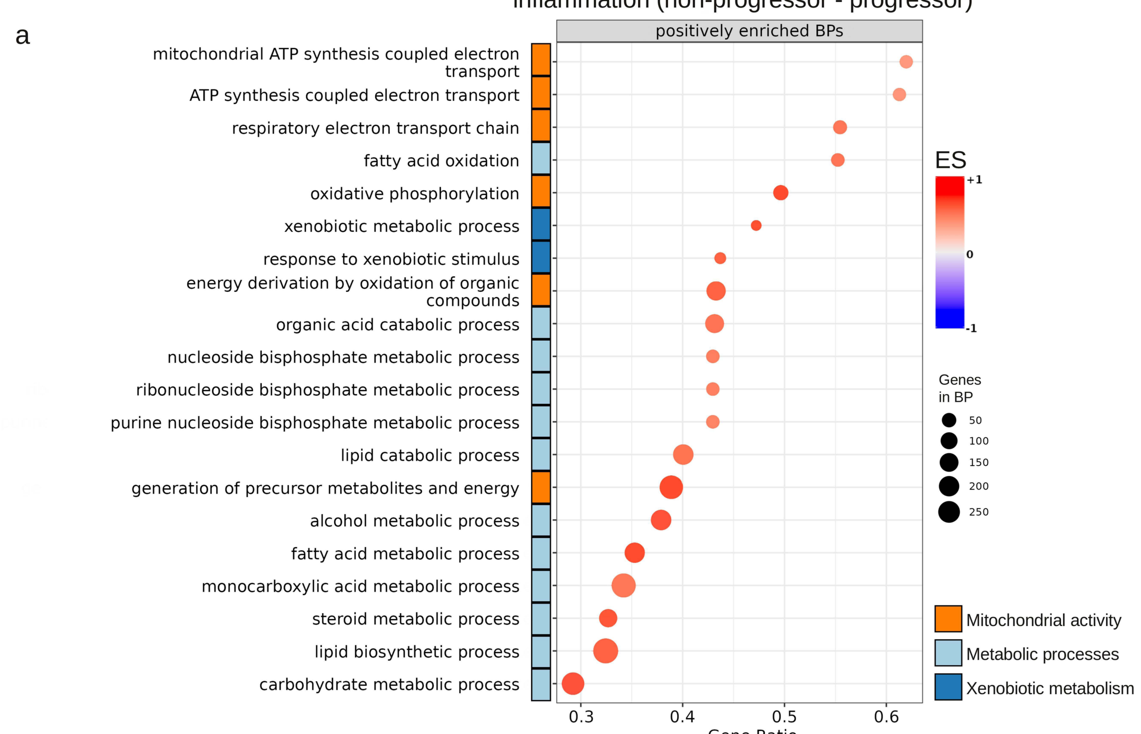


Figure 2 Prominent gene expression differences between progressors and non-progressors in the inflamed tissue. Volcano plots presenting all genes differentially expressed for a given comparison of sample groups: **a**) inflammation (non-progressor – progressor) **b**) non-inflammation (non-progressor – progressor) **c**) non-progressor (non-inflammation – inflammation) **d**) progressor (non-inflammation – inflammation). Each dot represents one differentially expressed gene, and it is plotted according to the given fold change of expression and the adjusted p-value. Each gene shows the value resulting from the differential gene expression for the given group, in the case of plot a) a gene (dot) with a fold change of 2 will be expressed two times more in inflamed progressors samples when compared to inflamed non-progressor samples. Red dots represent significantly up-regulated genes with a fold change ≥ 1 and an adjusted p-value < 0.05. Blue dots represent significantly down-regulated genes with a fold change ≤ −1 and an adjusted p-value < 0.05. Gray dots represent non-significant genes. The reference group of a comparison is the first sample group within the comparison name.

organised in hubs representing vasculature development and various aspects of innate and adaptive immune response (Supplementary Figure 2b).

We then continued our analysis by focusing on single gene regulations in representative BPs of each of the hubs. The directionality of gene regulation was shown in heatmaps (Figure 5a and b, Supplementary Table 3 and Supplementary Table 4 (genes are listed in the order they appear in the heatmap within each BP)). In general, most genes in the positively enriched BPs were upregulated in the inflamed progressor tissue compared to the non-progressor inflamed tissue and for some of the genes the expression was significantly upregulated (Figure 5a). Interestingly, many of these significantly upregulated genes, eg, several Cytochrome P450 (CYP) and solute carrier (SLC) encoding genes were



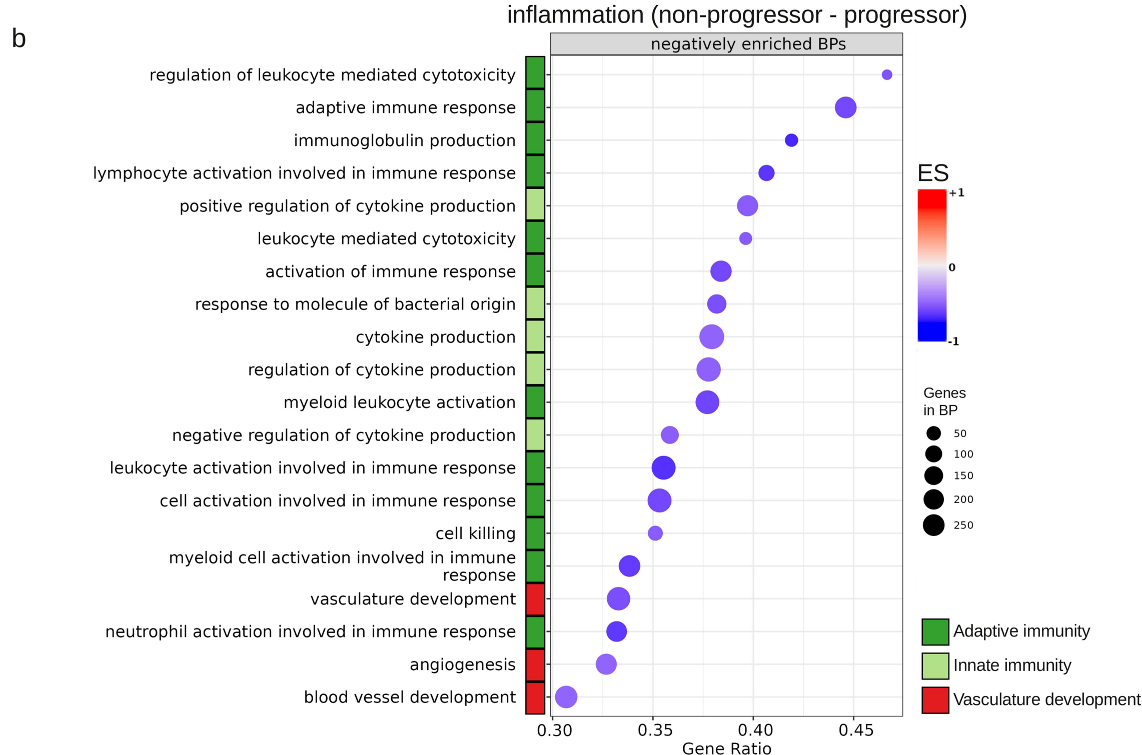


Figure 3 Altered biological processes in inflamed mucosa associated with progression of UC. Dotplots showing the top 20 positively (**a**) and negatively (**b**) enriched biological processes (BPs) within the gene set enrichment analysis (GSEA) of all differentially expressed genes of the inflammation (non-progressor – progressor) comparison. The reference group of the comparison is the first sample group within the comparison name. The name of the BPs is given on the left side of the dotplots. Coloured bars on the left side of the dotplots indicate the main category of processes a BP associates with. The colour of the dot indicates the enrichment score (ES) for a given BP. A positive ES indicates that most of the genes for that BP are upregulated (ie positive fold change) and a negative ES indicates that most of these genes would be downregulated (ie negative fold change). The size of a dot indicates the number of genes assigned to the BP and the Gene Ratio indicates the fraction of genes in a BP deregulated in our comparison.

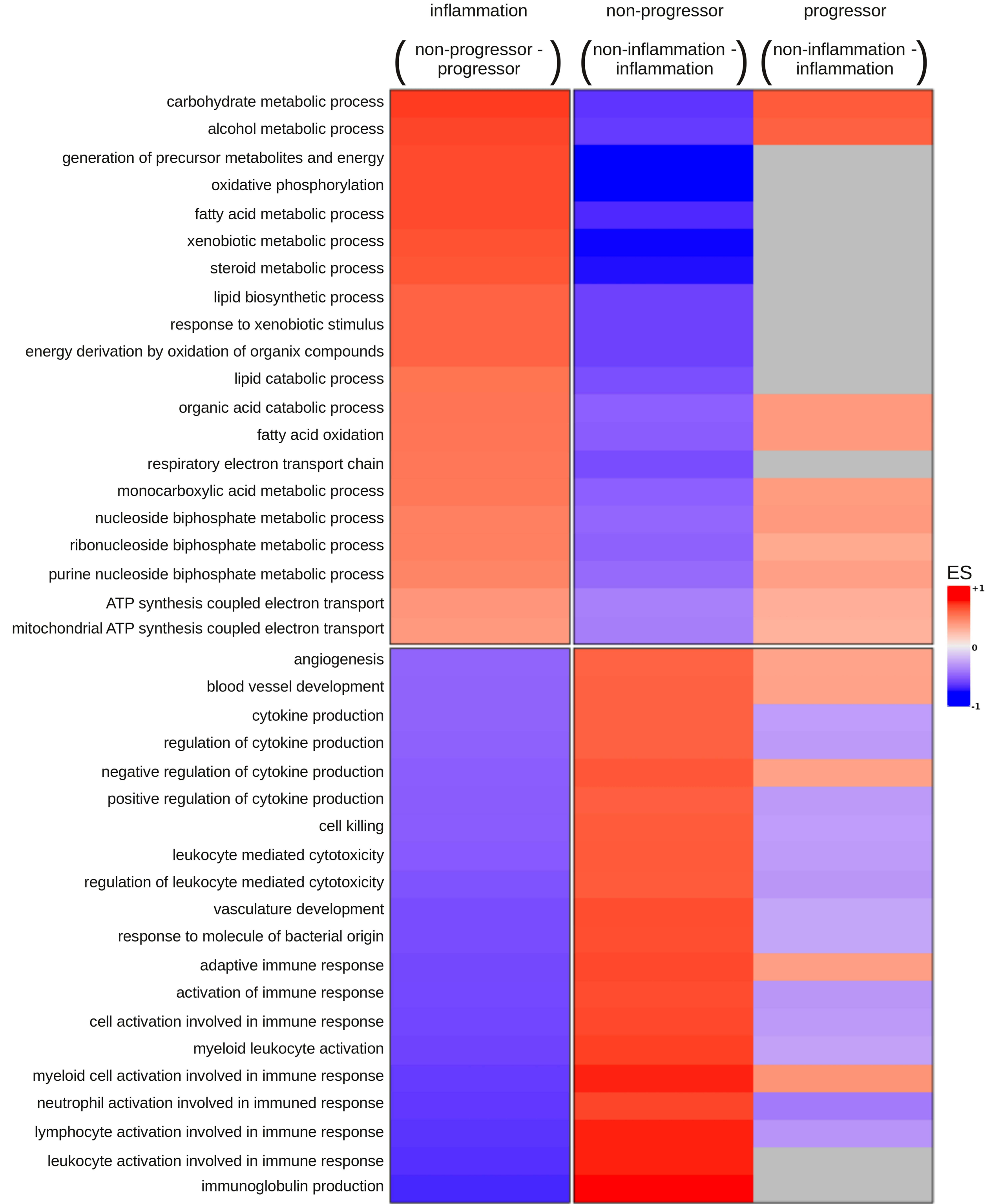


Figure 4 Inverse enrichment of inflammation associated biological processes in progressors and non-progressors. Heatmap presenting the top 20 biological processes (BPs) of both positive and negative enrichment scores (ES) identified in the gene set enrichment analysis (GSEA) of all differentially expressed genes of the inflammation (non-progressor – progressor) comparison (Figure 3). GSEAs were similarly performed for all differentially expressed genes of the non-inflammation – inflammation comparisons for non-progressors and progressors, respectively, to assess how the given BPs were enriched in these comparisons. The reference group of a comparison is the first sample group within the comparison name. The name of the BPs is given on the left side of the heatmap. The colour indicates the ES for the given BP. A positive ES indicates that most of the genes for that BP are upregulated (ie positive fold change) and a negative ES indicates that most of these genes would be downregulated (ie negative fold change). BPs appearing as gray indicate that there were not enough genes with a significant change in expression for that BP to be identified as relevant by the GSEA method for that comparison.

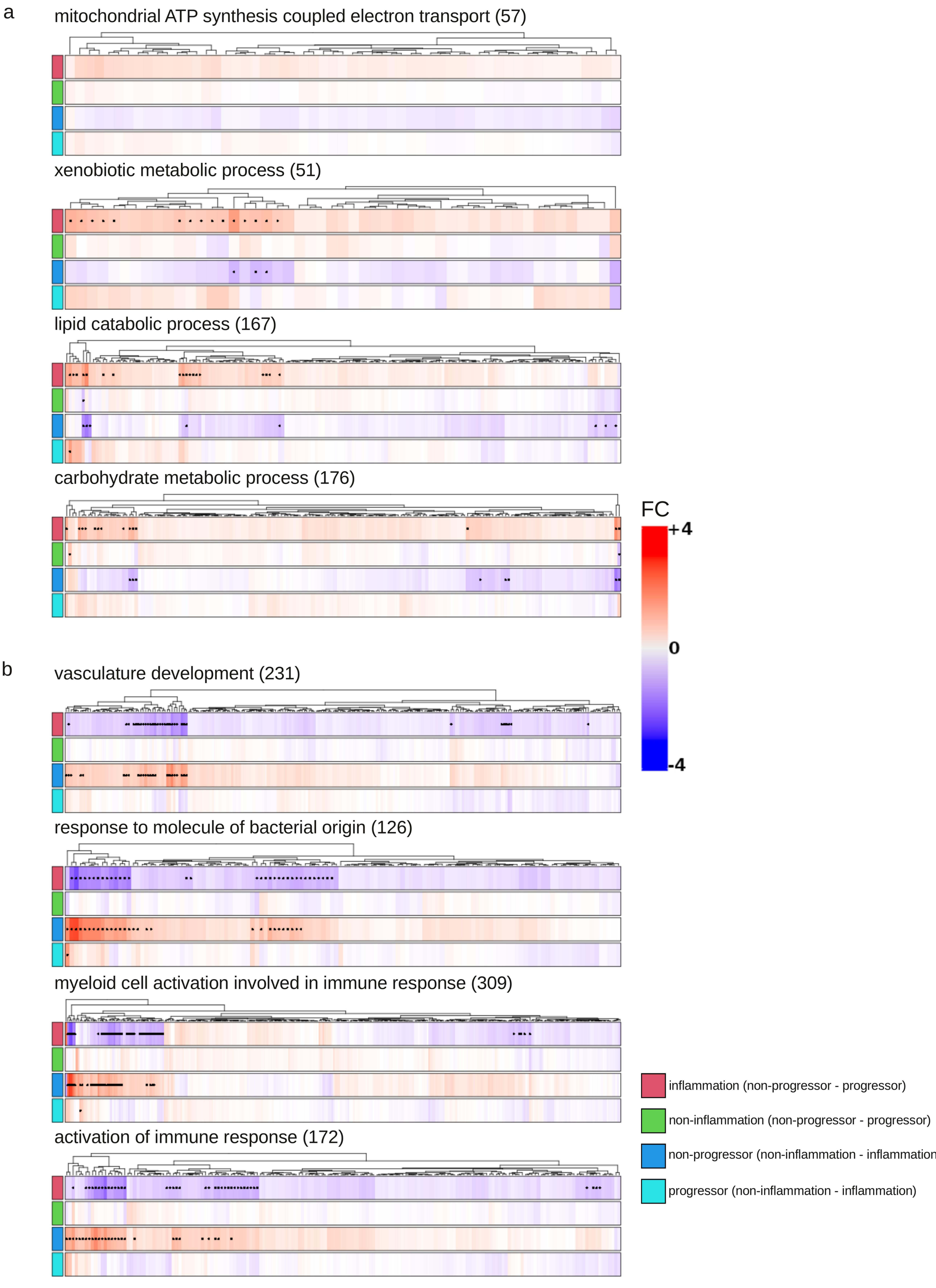


Figure 5 Inflamed tissue of progressors is associated with altered gene expression of genes primarily in immune related BPs. Heat maps showing gene regulations in fold change (FC) of representative biological processes (BPs) of the top 20 positively (**a**) and negatively (**b**) enriched BPs in the inflammation (non-progressor - progressor) comparison (top row of the heatmap). In addition, the deregulation of the given genes is shown for the three other comparisons of the main sample groups: non-inflammation (non-progressor – progressor) (second row from the top), non-progressor (non-inflammation – inflammation) (third row from the top) and progressor (non-inflammation – inflammation) (bottom row of the heatmap). To the left of each row in each heatmap, the identity of the comparison is indicated by a coloured bar. The colour association is shown on the right side of the heatmaps for all comparisons. Red: inflammation (non-progressor - progressor), green: non-inflammation (non-progressor – progressor), blue: non-progressor (non-inflammation – inflammation), turquoise: progressor (non-inflammation – inflammation). The reference group of a comparison is the first sample group within the comparison name. Significantly differentially expressed genes are marked with an asterisk and have FC ≥ 1 or ≤−1 and an adjusted p-value threshold of 0.05. The number of genes (n) within a BP follows the name of the BP.

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upregulated in inflamed tissue compared to non-inflamed tissue in progressors, whereas they were downregulated in non-progressors. Moreover, the expression levels of genes in these metabolic BPs did not differ markedly when comparing the non-inflamed tissue of progressors and non-progressors, in agreement with the fewer number of significantly deregulated genes in this comparison.

Most genes of representative negatively enriched BPs were downregulated in the inflamed tissue of progressors compared to non-progressors and a substantial number of these genes were significantly downregulated (Figure 5b). Many of these immune-associated genes such as *IL1B, CD19* and *CXCL13* were significantly upregulated in inflamed tissue compared to non-inflamed tissue of non-progressors, whereas there were no significant differences in expression levels between inflamed and non-inflamed tissue of the progressors. Further, no significant differences were found in gene expression between non-inflamed tissue of progressors and non-progressors for genes belonging to the presented BPs except for *S100P* belonging to "myeloid cell activation in immune response", which was expressed at a higher level in progressors.

A recent study identified a gene set characteristic for inflamed mucosa associated with long duration of UC.[25] Of this profile, 81 genes were significantly deregulated in the inflammation (non-progressor – progressor) comparison in the Oslo study and the genes had a similar direction of deregulation (Supplementary Table 5, duration-associated genes). Interestingly, the deregulation of these genes seems to be associated with duration of UC disease.

After, subtraction of the duration-associated genes from our list of significantly deregulated genes, we identified 529 genes that are likely to be specifically associated with progression of UC to dysplasia and cancer (Supplementary Table 5, progression-associated genes).

The differential expressed genes associated with duration of disease and progression of disease, respectively (Supplementary Table 5), were analysed applying the web-based g:Profiler tool on the gene lists to identify the related BPs. The duration-associated genes, eg, *PCK1, GBA3, S100A8* and *S100A9* were primarily associated with carbohydrate and lipid metabolism as well as innate immune responses, which corresponds well with the findings by Low and colleagues.[25] The progression-associated genes such as *CD19, CXCR5, C3* and *CYP*s were primarily associated with adaptive immune responses but also with the complement system and detoxification processes, suggesting that the activity of these processes in the inflamed mucosa of UC patients may play an important role in the progression of UC to dysplasia and cancer.

Considering that UC is an inflammatory disease, it was surprising that immune-related genes were downregulated in the inflamed tissue of progressors compared to non-progressors. Moreover, this downregulation seemed to be associated with progression of UC to dysplasia and cancer as opposed to duration of disease. Not surprisingly, many of the genes encoding, eg, chemokine ligands and receptors, lymphotoxin-beta, CD19 and CD20 are specifically expressed in immune cells or in other cells within lymphoid microenvironments in the mucosa of the colon and encode proteins important for initiating the formation of secondary and tertiary lymphoid organs and for mounting full immune responses (Table 2).[26–35] Downregulation of immune-related genes suggests less immune activity in the inflamed mucosa of progressors.

Enzymes of the Cytochrome P450 super family encoded by the *CYP* genes are central to detoxification processes.[36] Several *CYP* genes were found to have upregulated expression in the inflamed mucosa of progressors compared to non-progressors and most of these were associated with progression of UC (Table 3). The genes encode enzymes of the CYP2, CYP3 and CYP4 families, which participate in drug, steroid and fatty acid metabolism. They are generally expressed in the liver, kidney and the cardiovascular system, however they are also expressed in other tissues such as the gastro intestinal tract.[36] Upregulated expression of these *CYP* genes suggests an increased metabolic activity in the inflamed mucosa of progressors.

These findings suggest that progression of UC to dysplasia and cancer is associated with negative enrichment of immune responses including complement system activity and positive enrichment of detoxification processes in the inflamed mucosa as opposed to long duration of UC. Moreover, the differentially expressed genes are primarily expressed in various immune cells in lymphoid organs of the colon.

Table 2 Examples of Significantly Deregulated Genes Encoding Proteins Involved in Immune Response Processes in Inflamed Mucosa of UC Patients

Gene Name	Protein Name	FC[a]	Adjusted p-value
LAMP3	Lysosome-associated membrane glycoprotein 3 (LAMP-3)	−1,98	2,75E-08
LTB	Lymphotoxin-beta (LT-β)	−2,54	9,51E-08
CD83	CD83 antigen (CD83)	−2,01	1,08E-07
CXCR5	C-X-C chemokine receptor type 5 (CXCR-5)	−1,74	1,10E-06
CXCL13	C-X-C motif chemokine ligand 13 (CXCL-13)	−2,33	1,38E-06
MS4A1	B-lymphocyte antigen CD20 (CD20)	−1,76	1,60E-06
CD19	B-lymphocyte antigen CD19 (CD19)	−2,63	7,91E-06
C1R	Complement C1r	−1,13	8,76E-05
CCR7	C-C chemokine receptor type 7 (CCR-7)	−2,20	1,21E-04
C3	Complement C3	−1,74	1,56E-04
IL1B	Interleukin-1 beta (IL-1β)	−2,04	8,80E-04
CCL19	C-C motif chemokine 19 (CCL-19)	−2,02	1,86E-03
C4B	Complement C4B	−1,12	3,90E-03

Notes: [a]Fold Change (FC) for inflammation (non-progressor – progressor) comparison.

Table 3 Examples of Significantly Deregulated Genes Encoding Proteins Involved in Detoxification Processes in Inflamed Mucosa of UC Patients

Gene Name	Protein Name	FC[a]	Adjusted p-value
CYP4F3	Cytochrome P450 4F3	1,72	5,98E-06
CYP3A4	Cytochrome P450 3A4	1,43	4,42E-03
CYP2C9	Cytochrome P450 2C9	1,24	8,36E-03

Notes: [a]Fold Change (FC) inflammation (non-progressor – progressor) comparison.

The Progressor Gene Expression Profile in Inflamed Mucosa Is Independent of Type of Inflammation

Patients suffering from UC present with histological chronic inflammation and flares of chronic active inflammation in their colon. The histology of chronic inflammation is dominated by lymphocytes infiltrating the lamina propria. During active flares of the disease, however, the lamina propria will also be infiltrated by neutrophils, and the flares will often be accompanied by ulceration of the mucosa. Thus, the different types of inflammation represent different phases of an inflammatory response and may be characterised by different biological processes. We therefore analysed gene expression profiles with respect to type of inflammation.

Few deregulated genes were found comparing chronic and chronic active inflammation in progressors or non-progressors (Supplementary Figure 1c and d). In contrast, there were clear differences in gene regulation when comparing the specific type of inflammation between progressors and non-progressors (Supplementary Figure 1a and b). Positively enriched BPs associated with differentially expressed genes of chronic inflamed tissue between progressors and non-progressors were related to metabolic processes and chromatin organisation (Supplementary Figure 3a). The similar comparison for chronic active inflammation exclusively found BPs related to metabolic processes being positively enriched (Supplementary Figure 3b). Both comparisons found exclusively innate and adaptive immune response-related BPs negatively enriched (Supplementary Figure 3c and d).

Stratifying for type of inflammation did not reveal additional differences, suggesting that the differences found for mucosa of progressors applied for inflamed mucosa irrespective of the type of inflammation. However, it does suggest that chronic inflamed mucosa of progressors, in addition to metabolic processes, may be characterised by enhanced chromatin organisation processes.

Validation of Positively Enriched Mitochondrial Activity of Inflamed Mucosa in Progressors

In the Oslo cohorts, we found a positive enrichment of mitochondrial activity in inflamed tissue of progressors compared to non-progressors (Figure 3a). As the custom designed NanoString panel used for gene expression analysis of the Seattle cohort covered several genes related to mitochondrial function (Supplementary Table 6), we decided to explore if this data set could confirm the directionality of the gene expression changes of mitochondrial genes found in the Oslo cohorts. A comparison of the gene expression changes of genes related to mitochondrial function for the Seattle and Oslo data sets showed that most of the genes were regulated in the same direction in the two data sets (Supplementary Figure 4), confirming our finding of positively enriched mitochondrial activity in the inflamed tissue of progressors.

Inflamed Mucosa of Progressors Is Characterised by Fewer B Cells and Lymphoid Aggregates

FFPE biopsies taken adjacent to the biopsies subjected to gene expression profiling were stained by IHC for expression of the B cell marker CD20 and the T cell marker CD3 to examine the presence of central adaptive immune cells in the inflamed colonic mucosa. Inflamed mucosa of the progressors showed significantly fewer B cells (p=0.0104) compared to non-progressors, whereas there was no significant difference in T cells (p=0.0620) (Figure 6a and b). When analysing the CD20 and CD3 stains, lymphoid aggregates appeared to be less prevalent in the inflamed mucosa of progressors compared to non-progressors. Lymphoid aggregates may represent precursors of secondary and tertiary lymphoid organs, which are sites of mucosal adaptive immune responses in the colon (Figure 7).[32,33] We thus determined the distribution of lymphoid aggregates in the inflamed samples of progressors and non-progressors. Only 50% of the progressor samples but 90% of the non-progressor samples had lymphoid aggregates and that difference was statistically significant (p=0.0367), (Figure 6c). Furthermore, the number of lymphoid aggregates per sample was significantly fewer in progressors compared to non-progressors (p=0.0061), (Figure 6d). This difference was not significant when differentiating between chronic and chronic active inflammation (p=0.0546 and p=0.3040) (Figure 6e and f).

Taken together, our immunohistochemistry results suggest that the microenvironment in the inflamed mucosa of progressors is characterised by reduced infiltration of B cells and fewer lymphoid aggregates. This agrees with the progressor gene expression profile, which suggests a negatively enrichment of immune responses in inflamed tissue of progressors.

Controlling for Immunosuppressive Treatment Did Not Alter the Inflammation Gene Expression Profile

The use of immunomodulators and biologics of patients included in this study was equally distributed in non-progressors and progressors ($p<0.05$) except for two progressors, who received Vedolizumab at the time of sampling (Table 1). As Vedolizumab is an integrin blocker, eg, blocks influx of lymphocytes from the blood vessels into the mucosa, we examined if this had had an impact on our results. With respect to the inflammation comparison, only one of the Vedolizumab-treated patients had inflamed samples included in the analysis. These three samples were excluded and the comparison of gene expression in inflamed mucosa between non-progressors and progressors was repeated (non-vedo inflammation (non-progressor - progressor)) and showed that the two analyses did not differ (Supplementary Figure 5a). Similarly, the gene expression comparison between progressors and non-progressors of non-inflamed tissue was repeated excluding non-inflamed samples from the two Vedolizumab-treated progressors (five samples) and showed that the two analyses did not differ (Supplementary Figure 5b).

Discussion

In this cross-sectional analysis, we demonstrated that the gene expression profile in inflamed mucosa of UC patients, who have progressed to dysplasia or cancer, differs substantially from that of UC patients, who remained free of neoplasia at least eight years after diagnosis. Genes related to metabolic processes such as xenobiotic, carbohydrate, and lipid metabolism were upregulated in inflamed mucosa of progressors compared to non-progressors, whereas genes related

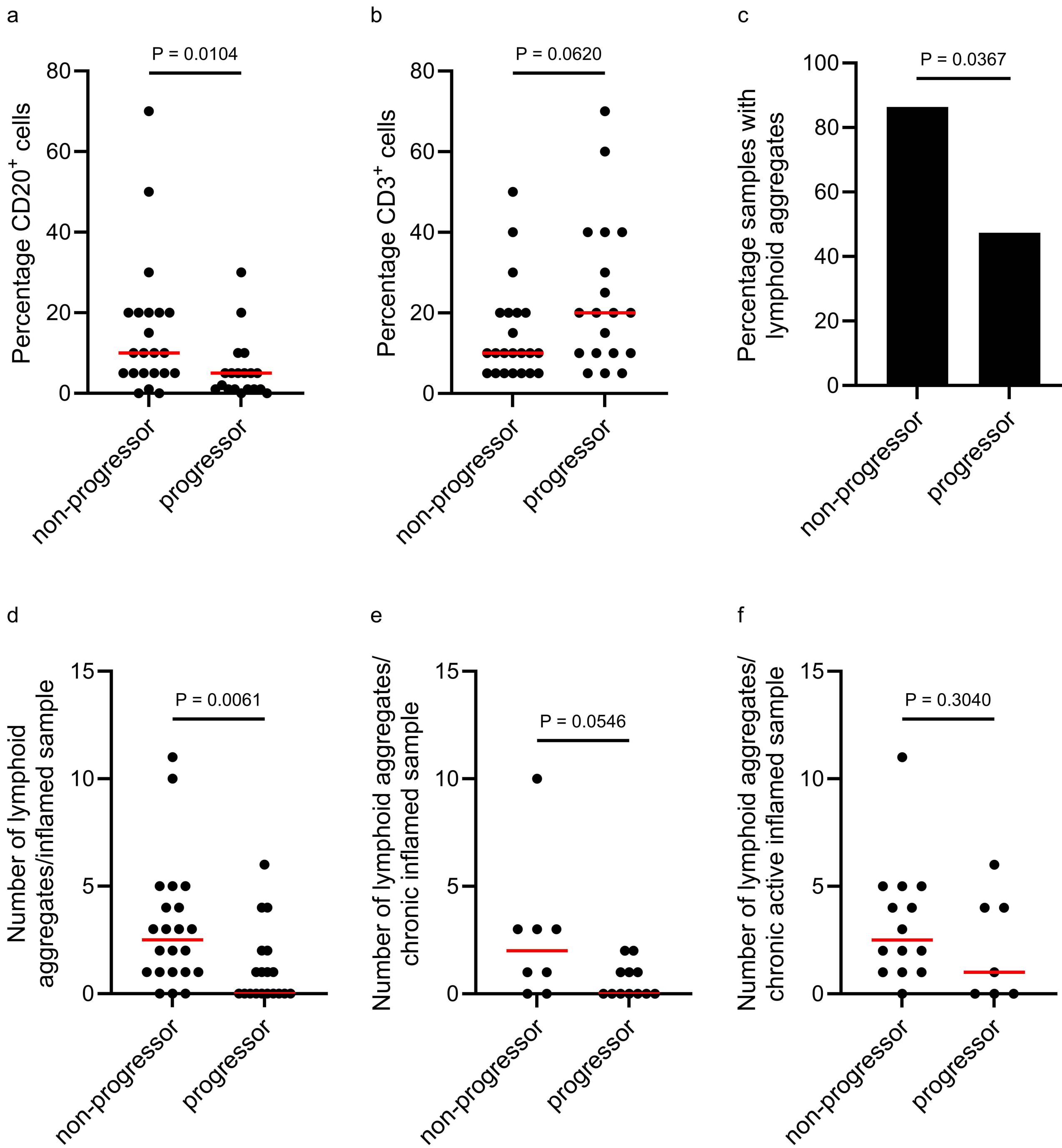

Figure 6 Inflamed mucosa of progressors is characterised by fewer B cells and lymphoid aggregates. (**a**) and **b**) Scatter plots showing the percentage of CD20 positive cells and CD3 positive cells, respectively, in inflamed samples of non-progressors and progressors assessed by IHC. (**c**) Column bar plot showing the percentage of inflamed samples with lymphoid aggregates in the mucosa of non-progressors and progressors. (**d**) Scatter plot showing the number of lymphoid aggregates per inflamed sample of non-progressors and progressors. (**e**) and (**f**) Scatter plot showing the number of lymphoid aggregates per chronic and chronic inflamed sample, respectively, of non-progressors and progressors. In (**a,b,d,e** and **f**) the median for each group is indicated by a red line. A two-tailed *t*-test was performed for comparison of two groups in (**a,b, d,e** and **f**) and a two-sided Fisher's Exact test was performed for c). P-values below 0.05 were considered significant.

to innate and adaptive immune responses were downregulated. These characteristics were not influenced by type of inflammation but present in both chronic and chronic active inflammation.

In accordance with UC being an inflammatory disease involving mucosal immunity and in line with findings in other cohorts of non-neoplastic IBD patients,[37,38] our gene expression analysis showed upregulation of many genes of innate

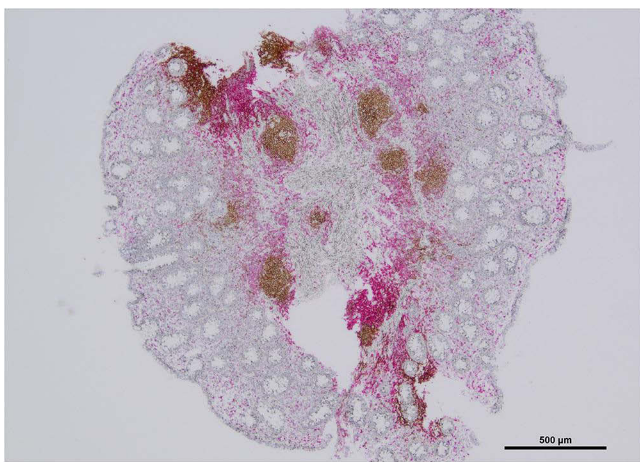

Figure 7 Example of inflamed colonic mucosa containing several lymphoid aggregates. Inflamed colonic mucosa from a non-progressor stained by immunohistochemistry for the presence of CD20$^+$ cells (brown stain) and of CD3$^+$ cells (red stain) identifying B cells and T cells, respectively. For this study lymphoid aggregates are defined as aggregates of B cells with a diameter of at least 100 μm.

and adaptive immune response in the inflamed compared to the non-inflamed mucosa within the non-progressors. This contrasts with the results we found in the progressors, where most innate and adaptive immune-related genes had minor gene expression changes, and many were downregulated. Importantly, this difference between non-progressors and progressors was not caused by an already upregulated expression in the non-inflamed mucosa of progressors as the non-inflammation (non-progressor–progressor) comparison for these genes did not show any significantly differentially expressed genes. This surprising and to our knowledge new result suggests that UC patients, who have progressed to dysplasia or cancer, have an attenuated inflammatory response in the colonic mucosa.

Many gene expression studies focusing on UC progression to CA-CRC have compared non-neoplastic biopsies with cancerous mucosal samples in CA-CRC patients.[10–12,39,40] This approach may primarily identify the characteristics of the established cancer mucosa but not the early molecular events driving the progression of UC to CA-CRC. Low and colleagues had an alternative approach comparing long-duration with short-duration inflamed mucosa of UC patients hypothesising that the long-duration UC could reveal molecular characteristics related to neoplastic progression in UC as the risk of progression to neoplasia increases with the duration of UC.[25] However, as a minority of patients in the long-duration UC group would be expected to transform to a progressor, it is more likely that the difference between long-duration and short-duration UC primarily reflects molecular events related to long duration of disease and not progression to dysplasia or cancer. As our progressor group had an eight year longer median duration of UC than the non-progressors, we suspected that some of the deregulated genes in inflamed mucosa could be associated with long duration of UC. Consequently, we used the findings by Low and colleagues to identify gene regulations associated with duration of UC in our inflamed tissue of progressors and assigned the residual gene regulations to be associated with progression of UC to dysplasia and cancer. The resulting biological processes affected by the progression-associated gene regulations were related to adaptive immune responses, the complement system and detoxification processes.

The downregulation of immune-related genes in the inflamed mucosa of the progressors such as *IL-1B*, encoding the inflammatory mediator IL-1β, and genes encoding members of the complement system suggested that inflammatory signals were reduced, which was surprising as UC is a chronic inflammatory disease and chronic inflammation is a major risk factor for progression of UC to dysplasia and cancer. Moreover, in accordance with the downregulation of *CD19* and *MS4A1*, encoding B cell specific proteins CD19 and CD20, respectively, inflamed mucosa of progressors had reduced level of infiltrating B cells compared to non-progressors. Importantly, B cells are central for the adaptive immune response in lymphoid organs.[32,33] In addition, the presence of lymphoid aggregates, which may represent central lymphoid organs in mucosal adaptive immune responses, were reduced in the inflamed samples of progressors. We found that several of the downregulated genes in inflamed tissue of progressors are specifically expressed in various immune cells and other cells of the mucosal lymphoid microenvironment, which could impact the formation of lymphoid

organs in the mucosa. *LTB*, encoding Lymphotoxin β (LTβ), is expressed by lymphoid tissue inducer (LTi) cells and other infiltrating immune cells of lymphoid organs. LTβ is part of Lymphotoxin αβ (LTαβ)[28] and binding of LTαβ to the receptor LTβR on either lymphoid tissue organiser (LTo) cells or on stromal cells are important for initiation of lymphoid organs in the colonic mucosa and for the differentiation of follicular dendritic cells (FDCs) and formation of high endothelial venules (HEVs).[28,29] Chemokines encoded by the genes *CXCL13* and *CCL19* are expressed by LTo cells and stromal cells.[28,29] Moreover, CXCL13 is produced by FDCs in B cell compartments.[29] CXCL13 attracts LTo cells, B cells and follicular T helper (Tfh) cells to lymphoid organs by binding to the chemokine receptor CXCR5 on these cells.[28,29] Similarly, CCL19 binds the chemokine receptor, CCR7, which is expressed on DCs and B and T cells and thereby attracts these cells to lymphoid organs.[29] In addition, the gene *LAMP3* is specifically expressed in mature dendritic cells (DCs)[30] and CD83 is a marker of activated immune cells.[31] In summary, this implies that attraction of immune cells to the mucosa and initiation, organisation and formation of lymphoid organs may be impaired.[28,29] Consequently, we speculate that this could lead to a reduced ability to mount adaptive immune responses against malignant transformation in inflamed mucosa of progressors.

Metabolic steroid products formed by Cytochrome P450 super family proteins have been found to have inhibitory effects on immune responses at high levels.[41] Thus, the upregulated expression of *CYP* genes, which are related to detoxification processes, may enhance the lack of ability of progressors to mount immune responses in the inflamed mucosa.

In summary, the activity status of adaptive immune responses, the complement system and detoxification processes in inflamed mucosa of UC patients may have a potential to distinguish progressors from non-progressors at an early stage of the disease course.

Due to unavailability of expression data sets that correspond to our patient categories, eg, the data set published by Low et al did not contain progressor samples,[25] we were not able to corroborate our findings in an independent cohort. We therefore used the Seattle cohort, which matched our patient groups. Even though signal intensities obtained from a NanoString analysis is not directly comparable to microarray signal intensities, it is reasonable to compare the direction of gene expression regulation. The custom-made panel of genes included in the NanoString analysis of the Seattle cohort focused mainly on genes encoding proteins with mitochondrial function. This allowed validating our finding that mitochondrial activity is positively enriched in progressor inflamed mucosa in the Seattle data set. Interestingly, the Seattle cohort confirmed the direction of regulation for most of the genes.

The present study holds both strengths and weaknesses. A main strength of the study is the selection of UC cases that had no PSC and with disease duration of eight years or longer. Concomitant PSC is recognised as a high-risk factor for dysplasia and cancer probably related to distinctive neoplastic pathways.[42] In addition, dysplasia and cancer in UC that develops early during course of disease (prior to eight years from diagnosis of UC) seem to follow a different neoplastic transformation compared to dysplasia and cancer after long duration of UC (more than eight years from UC diagnosis). The former is characterised by uni-focal neoplasia and diagnosis of UC at an older age, while the latter occurs more often and usually presents in a neoplastic field with synchronous lesions in patients with onset of UC at younger age.[7,43] CRC in Crohn's disease shows similar incidence and poor survival compared to UC-CRC, but Crohn's disease undoubtedly involves different inflammatory mechanisms than UC.[44–46] Thus, by focusing on a homogeneous group of patients with non-PSC and UC duration of eight years or longer, we were able to elucidate meaningful associations regarding the risk of neoplasia in IBD. Possible effects of medical treatment with Vedolizumab were adjusted for by analysing the data with and without the patients, who received this treatment. Furthermore, selection bias was minimised as the patients were recruited consecutively from two different prospective cohorts of surveillance in UC. On the other hand, the low number of included patients in the study is a weakness. However, considering the low incidence of CA-CRC and the selection stringency of patients included in the analysis, the sample sizes are still comparable to other equivalent studies.[10–12]

Conclusion

Over the last decades, research has focused on identification of gene expression signatures associated with progression of UC to neoplasia. Here, we have focused on the molecular changes related to inflammation in UC patients, a research area that requires further exploration to better understand early mechanisms of carcinogenesis in these patients.

We found evidence supporting the hypothesis that inflammation of UC patients, who have progressed to dysplasia or cancer, has a different gene expression profile than that of UC non-progressors. Importantly, this profile was associated with progression of disease as opposed to duration of disease. The transcriptional response to inflammation suggests that the inflamed mucosa of UC progressors has reduced levels of lymphoid organ-initiating and immune cell-attracting signals as well as reduced infiltration of immune cells to the mucosa, all of which are important factors for initiating, organising and forming lymphoid organs in the colonic mucosa. Our study suggests that the inflamed mucosa of the progressors may lack the ability to mount the required adaptive immune responses to counteract the driving forces of malignant transformation in UC. These findings, present in biopsies taken distant from the neoplastic lesions, along with the established field effect, likely represent the early events in the development of neoplasia in UC. Currently, there are no biomarkers, which distinguish UC patients at risk of developing CRC. However, they are needed to personalise surveillance for neoplasia in these patients. This study may serve as a step towards the identification of such biomarkers.

Data Sharing Statement

The raw gene expression data are available at the NCBI, Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), GEO accession number GSE236055.

Ethical Approvals

The Oslo cohorts have been approved by the Regional Committees for Medical and Health Research Ethics in Norway (2010/1093, 2014/722 and 2015/664). The Seattle cohort was approved by the Human Subjects Review Board at the University of Washington (IRB# 38186 and 36651).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

SAB is shareholder of Medevice AS, Norway and has received personal fees for participation in advisory board meetings from Abbvie, Takeda and Genetic Analysis. None of these were related to the submitted work. RAR is a consultant and equity holder at TwinStrand Biosciences Inc. and an equity holder at NanoString Technologies Inc. RAR is named inventor on patents owned by the University of Washington and licensed to TwinStrand Biosciences Inc. RAR received research funding from a joined research grant with TwinStrand Biosciences Inc. and Ovartec GmbH. HLN is co-owner of

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