

## Blood gene expression biomarkers of response to anti-TNF drugs in pediatric inflammatory bowel diseases before initiation of treatment

Sara Salvador-Martín<sup>a,1</sup>, Gianluca Rubbini<sup>a,1</sup>, Perceval Velloso<sup>a,b,1</sup>, Paula Zapata-Cobo<sup>a</sup>, Marta Velasco<sup>c</sup>, Laura M. Palomino<sup>c</sup>, Susana Clemente<sup>d</sup>, Oscar Segarra<sup>d</sup>, Ana Moreno-Álvarez<sup>e</sup>, Ana Fernández-Lorenzo<sup>e</sup>, Begoña Pérez-Moneo<sup>f</sup>, Montserrat Montraveta<sup>g</sup>, Cesar Sánchez<sup>a</sup>, Mar Tolín<sup>a</sup>, Inés Loverdos<sup>h</sup>, María José Fobelo<sup>i</sup>, Victor Manuel Navas-López<sup>j</sup>, Lorena Magallares<sup>k</sup>, Ruth García-Romero<sup>l</sup>, Ricardo Torres-Peral<sup>m</sup>, Alejandro Rodríguez<sup>n</sup>, Ferrán Bossacoma<sup>o</sup>, Vicente Merino-Bohórquez<sup>p</sup>, Enrique Salcedo<sup>q</sup>, Rebeca Álvarez<sup>r</sup>, Ana Dopazo<sup>r</sup>, María Sanjurjo-Sáez<sup>a</sup>, Luis A. López-Fernández<sup>a,\*</sup>

<sup>a</sup> Instituto de Investigación Sanitaria Gregorio Marañón, Hospital General Universitario Gregorio Marañón, Madrid, Spain

<sup>b</sup> Unidad de Investigación Materno Infantil Fundación Familia Alonso (UDIMIFFA), Spain

<sup>c</sup> Hospital Universitario Infantil Niño Jesús, Madrid, Spain

<sup>d</sup> Hospital Vall d'Hebrón, Barcelona, Spain

<sup>e</sup> Hospital Universitario A Coruña, A Coruña, Spain

<sup>f</sup> Hospital Universitario Infanta Leonor, Madrid, Spain

<sup>g</sup> Hospital Universitario Germans Trias i Pujol, Badalona, Spain

<sup>h</sup> Hospital Universitario Parc Taulí, Sabadell, Spain

<sup>i</sup> Hospital Universitario Virgen de Valme, Universidad de Sevilla, Sevilla, Spain

<sup>j</sup> Hospital Regional Universitario de Málaga, Spain

<sup>k</sup> Hospital Universitario la Paz, Madrid, Spain

<sup>l</sup> Hospital Universitario Miguel Servet, Zaragoza, Spain

<sup>m</sup> Complejo Asistencial Universitario de Salamanca, Salamanca, Spain

<sup>n</sup> Hospital Universitario Virgen del Rocío, Sevilla, Spain

<sup>o</sup> Servicio de Gastroenterología, Hepatología y Nutrición Pediátrica, Hospital Sant Joan de Déu, Barcelona, Spain

<sup>p</sup> Hospital Universitario Virgen de la Macarena, Universidad de Sevilla, Sevilla, Spain

<sup>q</sup> Hospital Universitario Doce de Octubre, Madrid, Spain

<sup>r</sup> Genomics Unit, Spanish National Center for Cardiovascular Disease (CNIC), Madrid 28029, Spain

### ARTICLE INFO

#### Keywords:

RNA-seq

Pharmacogenomics

Inflammatory bowel disease

Anti-TNF drugs

Pediatrics

### ABSTRACT

**Background/aims:** Changes in gene expression profiles among individuals with inflammatory bowel diseases (IBDs) could potentially influence the responsiveness to anti-TNF treatment. The aim of this study was to identify genes that could serve as predictors of early response to anti-TNF therapies in pediatric IBD patients prior to the initiation of treatment.

\* Corresponding author.

**E-mail addresses:** [sara.salvador@iisgm.com](mailto:sara.salvador@iisgm.com) (S. Salvador-Martín), [gianluca.rubbini@unimi.it](mailto:gianluca.rubbini@unimi.it) (G. Rubbini), [perceval.vellosillo@iisgm.com](mailto:perceval.vellosillo@iisgm.com) (P. Velloso), [paula.zapata@iisgm.com](mailto:paula.zapata@iisgm.com) (P. Zapata-Cobo), [mvelasco@salud.madrid.org](mailto:mvelasco@salud.madrid.org) (M. Velasco), [lauramaria.palomino@salud.madrid.org](mailto:lauramaria.palomino@salud.madrid.org) (L.M. Palomino), [susana.clemente@vallhebron.cat](mailto:susana.clemente@vallhebron.cat) (S. Clemente), [oscar.segarra@vallhebron.cat](mailto:oscar.segarra@vallhebron.cat) (O. Segarra), [ana.moreno.alvarez@sergas.es](mailto:ana.moreno.alvarez@sergas.es) (A. Moreno-Álvarez), [ana.estefania.fernandez.lorenzo@sergas.es](mailto:ana.estefania.fernandez.lorenzo@sergas.es) (A. Fernández-Lorenzo), [begona.perez@salud.madrid.org](mailto:begona.perez@salud.madrid.org) (B. Pérez-Moneo), [mmonstraveta.germantrias@gencat.cat](mailto:mmonstraveta.germantrias@gencat.cat) (M. Montraveta), [cesar.sanchez.sanchez@salud.madrid.org](mailto:cesar.sanchez.sanchez@salud.madrid.org) (C. Sánchez), [mariamar.tolin@salud.madrid.org](mailto:mariamar.tolin@salud.madrid.org) (M. Tolín), [iloverdos@tauli.cat](mailto:iloverdos@tauli.cat) (I. Loverdos), [mariaj.fobelo.sspa@juntadeandalucia.es](mailto:mariaj.fobelo.sspa@juntadeandalucia.es) (M.J. Fobelo), [victorm.navas.sspa@juntadeandalucia.es](mailto:victorm.navas.sspa@juntadeandalucia.es) (V.M. Navas-López), [Lorena.magallares@salud.madrid.org](mailto:Lorena.magallares@salud.madrid.org) (L. Magallares), [rgarciarom@salud.aragon.es](mailto:rgarciarom@salud.aragon.es) (R. García-Romero), [rtorres@saludcastillayleon.es](mailto:rtorres@saludcastillayleon.es) (R. Torres-Peral), [alejandro.rodriguez.m.sspa@juntadeandalucia.es](mailto:alejandro.rodriguez.m.sspa@juntadeandalucia.es) (A. Rodríguez), [ferran.bossacoma@sjd.es](mailto:ferran.bossacoma@sjd.es) (F. Bossacoma), [vicente.merino.sspa@juntadeandalucia.es](mailto:vicente.merino.sspa@juntadeandalucia.es) (V. Merino-Bohórquez), [enrique.salcedo@salud.madrid.org](mailto:enrique.salcedo@salud.madrid.org) (E. Salcedo), [ralvarez@cnic.es](mailto:ralvarez@cnic.es) (R. Álvarez), [adopazo@cnic.es](mailto:adopazo@cnic.es) (A. Dopazo), [maria.sanjurjo@salud.madrid.org](mailto:maria.sanjurjo@salud.madrid.org) (M. Sanjurjo-Sáez), [Luis.lopez@iisgm.com](mailto:Luis.lopez@iisgm.com) (L.A. López-Fernández).

<sup>1</sup> These authors contributed equally to this work.

<https://doi.org/10.1016/j.bioph.2024.116299>

Received 11 November 2023; Received in revised form 6 February 2024; Accepted 17 February 2024

Available online 23 February 2024

0753-3322/© 2024 The Authors.

Published by Elsevier Masson SAS. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

**Methods:** We conducted a prospective, longitudinal, and multicenter study, enrolling 24 pediatric IBD patients aged less than 18 years who were initiating treatment with either infliximab or adalimumab. RNA-seq from blood samples was analyzed using the DESeq2 library by comparing responders and non-responders to anti-TNF drugs. **Results:** Bioinformatic analyses unveiled 102 differentially expressed genes, with 99 genes exhibiting higher expression in responders compared to non-responders prior to the initiation of anti-TNF therapy. Functional enrichment analyses highlighted defense response to Gram-negative bacteria (FDR =  $2.3 \times 10^{-7}$ ) as the most significant biological processes, and hemoglobin binding (FDR = 0.002), as the most significant molecular function. Gene Set Enrichment Analysis (GSEA) revealed notable enrichment in transcriptional misregulation in cancer (FDR = 0.016). Notably, 13 genes (*CEACAM8*, *CEACAM6*, *CILP2*, *COL17A1*, *OLFM4*, *INHBA*, *LCN2*, *LTF*, *MMP8*, *DEFA4*, *PRTN3*, *AZU1*, and *ELANE*) were selected for validation, and a consistent trend of increased expression in responders prior to drug administration was observed for most of these genes, with findings for 4 of them being statistically significant (*CEACAM8*, *LCN2*, *LTF2*, and *PRTN3*).

**Conclusions:** We identified 102 differentially expressed genes involved in the response to anti-TNF drugs in children with IBDs and validated *CEACAM8*, *LCN2*, *LTF2*, and *PRTN3*. Genes participating in defense response to Gram-negative bacterium, serine-type endopeptidase activity, and transcriptional misregulation in cancer are good candidates for anticipating the response to anti-TNF drugs in children with IBDs.

## 1. Introduction

Inflammatory bowel diseases (IBDs) comprise a family of inflammatory, chronic, and autoimmune diseases of unknown origin that affect the gastrointestinal tract and are clinically differentiated into Crohn's disease (CD), ulcerative colitis (UC), and IBD unclassified (IBD-U). The incidence and prevalence of IBDs increased dramatically in the second half of the last century [1]. In 25% of cases, IBDs develop during childhood or adolescence [2].

Genetic factors play an important role in the pathogenesis of IBDs, with 5%–10% of patients reporting a positive family history [3,4]. Analysis of the genes and genetic loci identified in IBDs indicates that intestinal homeostasis depends on a series of pathways, including epithelial barrier function, innate and adaptive mucosal defense, cell migration, autophagy, adaptive immunity and metabolic pathways associated with cellular homeostasis [5,6].

In children, the manifestations of IBDs differ from those seen in adults. Consequently, genetic alterations and effects involved in normal development and growth require specific investigation and a different therapeutic approach [7]. Anti-tumor necrosis factor (TNF) drugs, such as infliximab and adalimumab, function in IBDs by inhibiting TNF, thereby reducing inflammation in the gastrointestinal tract [8]. In pediatrics, anti-TNFs are also used for conditions such as juvenile idiopathic arthritis, which can co-occur with certain types of IBD. In some cases, these conditions may also be associated with uveitis, necessitating a similar biological treatment approach [9].

Younger people respond better to anti-TNF therapy [10]. However, since children have to live with this chronic disease for longer than adults, it is of paramount importance to identify strategies that can optimize pharmacological treatments with the aim of inducing remission of acute phases and maintaining an acceptable state. Therapy with anti-TNFs is recommended to induce and maintain remission in children with IBDs. In 10%–40% of cases, these therapies are characterized by a clinically irrelevant response or primary failure to infliximab or adalimumab [11,12]. These percentages depend on the age, sex, and anti-TNF drug and increase with time [13–15].

The expression of specific genes in RNA in blood can impact the way patients respond to anti-TNF drugs [16–19]. We previously used RNA-seq to analyze gene expression in a small cohort of children with IBDs receiving anti-TNF drugs and identified differentially expressed genes in children who are more likely to respond to anti-TNF therapy and in those who are less likely to benefit after 2 weeks of treatment [18]. On completion of the study, we continued to recruit patients, making it possible to perform a more complete and precise study.

The aim of this work was to identify new pharmacogenomic biomarkers that can predict early response in a larger cohort of pediatric IBD patients treated with anti-TNFs, with emphasis on the period prior to initiation of anti-TNF treatment. By analyzing the expression of RNA

in children with IBDs, healthcare providers might be able to identify those who are more likely to respond well to anti-TNF therapy and those who are less likely to benefit.

## 2. Material and methods

### 2.1. Study design

We performed a prospective, longitudinal, and multicenter study.

### 2.2. Patients

Participants were recruited from the pediatric gastroenterology units of 17 hospitals (Suppl. Table S1) in Spain between May 9, 2017 and January 29, 2022. The inclusion criteria were age < 18 years, diagnosis of IBD (CD, UC, IBD-U), and initiation of treatment with infliximab or adalimumab. The exclusion criterion was not meeting any of the inclusion criteria.

The results of RNA-seq experiments for 12 patients (6 responders and 6 non-responders) included in our cohort have been published elsewhere and are freely available (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159034>). We collected these data and added 12 new patients to the RNA-seq analysis to increase the statistical power of the comparison. These 12 new patients were selected from the cohort to ensure that responder and non-responder data were similar, after adjusting for sex, age, type of anti-TNF drug, and type of IBD. A further 2 non-responders and 16 new responders were selected for validation for reverse transcription real-time PCR (RT-PCR).

### 2.3. Variables and data collection

Demographic and clinical variables (sex, age at diagnosis and at start of treatment, type of IBD, anti-TNF drug, first or second anti-TNF, months until treatment, concomitant immunomodulator) were collected from the clinical records. At the start of treatment, we recorded the Pediatric Crohn's Disease Activity Index (PCDAI) for CD and the Pediatric Ulcerative Colitis Activity Index (PUCAI) for UC, as well as C-reactive protein (CRP) and fecal calprotectin (FC). The response to the anti-TNF drugs was defined as a decrease of at least 15 points in the PCDAI or PUCAI from the start of treatment to weeks 14 (infliximab) and 26 (adalimumab). Data were collected using the data capture tool (REDCap) [20].

### 2.4. Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Hospital General Universitario Gregorio Marañón (protocol code LAL-TNF-2019-01/FG-

2019–01, 23 September 2019). The study was validated at all the participating centers and approved by their respective ethics committees with the same code between October 2019 and December 2020. Written informed consent to perform this research was obtained from the patients and/or their legal guardians. Participants aged >12 years signed an assent form. The approved study authorized the use of samples and clinical data from a repository (C.0003459, Instituto de Salud Carlos III) from a previous project (protocol code LAL-TNF-2016–01, approved on 17 March 2016 by the same ethics committee). In the informed consent, the legal representatives agreed to the transfer of samples and data for future research in digestive diseases.

## 2.5. Sample collection and RNA isolation

Before initiation of anti-TNF therapy, blood samples were collected and preserved in PAXgene tubes (BD Biosciences, Franklin Lakes, NJ, USA). The tubes were received at the processing laboratory and maintained at room temperature no later than 24 hours after extraction. All the tubes were then preserved at  $-80^{\circ}\text{C}$  until processing for RNA isolation using the PAXgene blood RNA kit (Qiagen, Venlo, Netherlands) following the manufacturer's instructions. Ratio  $A_{260}/A_{280}$  of isolated RNAs were measured using the Quawell-5000 spectrophotometer (Quawell, Quebec, CA, USA). The quality of RNAs was measured using the Agilent RNA6000 kit in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with an RNA integrity number (RIN) > 8 were used.

## 2.6. RNA sequencing

RNA-seq processing of the 12 new samples was performed as with the previous 12 samples [18] (GSM481800, GSM481802, GSM481804, GSM481806, GSM481808, GSM481810, GSM481812, GSM481814, GSM481816, GSM481818, GSM481820, and GSM481822). Sample selection was based on similarity between responders and non-responders in terms of sex, treatment, and age.

Complete transcriptome sequencing was performed at the Spanish Cardiovascular Disease Research Center based in Madrid (Centro Nacional de Investigaciones Cardiovasculares [CNIC]).

The RNA-seq data of the new 12 samples have been deposited with the accession number GSE247146 in the Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE247146>) [21].

## 2.7. Differential expression (DE) analysis from RNA-seq data

Raw data per-gene counts were analyzed using the DESeq2 library [22] combined with a non-parametrical procedure to identify differential gene-expression from RNA-seq data. After estimating size factors and gene dispersions by local fit type [23], a negative binomial generalized linear model (GLM, negative binomial Wald test [22]) was used to obtain the log<sub>2</sub> fold changes (log<sub>2</sub>FC) for each gene by comparing 2 states between samples: responders and non-responders to anti-TNF drugs. The differential gene-expression log<sub>2</sub>FC data were obtained by considering non-responder patients as a basal gene-expression reference performed using DESeq2 [22]. This protocol was generated to test the effect of the treatment. The *p* values provided for each gene by the Wald test were adjusted using the Benjamini-Hochberg false discovery rate (FDR) [24]. Genes were considered significant when the FDR was < 0.05. A log<sub>2</sub>FC greater than 0.6 or less than  $-0.6$  were chosen to consider those genes as over-expressed or under-expressed respectively.

## 2.8. Functional characterization of significant DE genes from RNA-seq experiments based on the fold change values

With the aim of interpreting profile expression from RNA-seq data at the functional level, we initially performed a gene set enrichment

analysis (GSEA) [25] to provide the subset of genes that contribute to the enrichment scores based on log<sub>2</sub>FC values of a list of genes. We used Gene Ontology (GO) [26] and KEGG [27] resources for the analysis. Of note, the complete analysis—performed using GO resources—took into account GO annotations based on experimental evidence codes.

Functional enrichment analysis of DE genes with FDR < 0.05 was performed by applying the ClusterProfiler R package [28] using GO biological processes [26] and KEGG pathways [27]. *p* Values were adjusted using the Benjamini-Hochberg FDR [24] and an FDR < 0.05 was considered as significant. A simplified method provided by the ClusterProfiler package was included to remove GO functional redundancy with a cutoff value of 0.5.

The genes involved in the enriched KEGG pathways were mapped through the pathview R library [29], rendering the differential gene expression as log<sub>2</sub>FC within each pathway from the pathway database.

## 2.9. cDNA synthesis and real-time PCR validation

Complementary DNAs (cDNAs) were synthesized from 1 µg of total RNA using the high-capacity cDNA archive kit (Applied Biosystems, Waltham, MA, USA) in a volume of 20 µl following the manufacturer's instructions. For all samples, a 1/20 dilution was used as the template for the real-time PCRs.

Pairs of oligonucleotides specific for each of the selected genes were selected using the KiCqStart® web from Sigma Aldrich (<https://www.sigmaaldrich.com/ES/es/semi-configurators/kicqstartPrimers?activeLink=selectprimers>) and synthesized (Merck, NJ, USA) (Table 1).

Real-time PCRs quantification was performed for *CEACAM8*, *CEACAM6*, *CILP2*, *COL17A1*, *OLFM4*, *INHBA*, *LCN2*, *LTF*, *MMP8*, and *DEFA4* in a final volume of 10 µl (5 µl qPCR BIO SyGreen Mix 2X [PCRBiosystems, London, UK], 2 µl cDNA, and 1 µM of each primer) using the following amplification parameters: 95°C for 3 min.; 40 cycles of 95°C 15 sec., and 60°C 30 sec. Relative gene expression of *PRTN3*, *AZU1*, and *ELANE* was performed in 10 µl (5 µl FastStart Universal SYBR Green Master [RoX] [Roche Diagnostics, Basel, Switzerland], 2 µl cDNA, 1 µM of each primer) using the following amplification parameters: 95°C for 10 min., 40 cycles of 95°C 15 sec., and 60°C 60 sec. After amplification, the specificity of the PCR products was analyzed using melting curves. The *ACTB* and *GAPDH* genes were used for normalization. PCR efficiency was calculated from slope. Real-time PCRs were performed in a QuantStudio 3 (Life Technologies, Carlsbad, CA, USA). Relative quantification of gene expression was analyzed using the  $2^{-\Delta\Delta\text{Ct}}$  method and QuantStudio and Design analysis software (Life Technologies).

**Table 1**  
Oligonucleotide sequences used to quantify gene expression in RT-PCR.

Gene Symbol	Forward (5'–3')	Reverse (5'–3')
<i>CEACAM8</i>	ATGTGAAATACAGAACCAG	CATTGACAGACCAAGAATACTG
<i>CEACAM6</i>	AACTCTGGTATTACCTCC	TGAGTTTGTAAATCCAGCC
<i>CILP2</i>	ACTCAGAGTCAGACAAGAAC	CAAATCCATTCCATACCAC
<i>COL17A1</i>	ATGGACAGAATTGGATGATG	CTGTCACAATTTTGGTCTCC
<i>INHBA</i>	GAGAATTTGCTGAAGAGGAG	CAAAAGTTGTTGTGATTGCC
<i>LCN2</i>	GGAAAAGAAGTGTGACTACTG	GTAACCTCTTAATGTTGCCAG
<i>LTF</i>	CTGGCAGAGAATACAAATC	AAGATATCCTCCACAGGTC
<i>MMP8</i>	TGACAGAGACCTCATTTTCC	TATCCTTGGGATAACCTTTC
<i>OLFM4</i>	CCCAGTTGTTTCCAATTTT	CTTCTGAGAAAGAACATGAGC
<i>AZU1</i>	GCTGCTTCAAAGCCAGA	GCTGCTGATGAAAACGTC
<i>DEFA4</i>	TGCCTAGCTTGAGGATCTGTG	CATCACCTCTGCCTGGAGT
<i>ELANE</i>	TTCTCGCCTGTGTCCTG	CTGCAGGGACACCATGAA
<i>PRTN3</i>	ACGGGAGAAACAACTGAAC	ACTGGCTGTCTGCTGTG
<i>ACTB</i>	AGCCACATCGCTCAGACAC	GCCCAATACGACCAATATCC
<i>GAPDH</i>	CTGTGCTGTGGAAGCTAAGT	GATGTCACGTCACACTTCA

## 2.10. Statistical analysis

Qualitative variables were expressed as frequency and percent. Differences between qualitative variables were calculated using the Mann-Whitney test. Quantitative continuous variables were expressed as median and interquartile range (IQR). Differences between quantitative variables were calculated using the chi-squared test or Fisher's exact test (when  $n < 30$ ), respectively. Normality of distribution was evaluated using Kolmogorov-Smirnov test or Shapiro Wilk (when  $n < 30$ ) and excluded when  $p$  value  $< 0.05$ . The statistical analysis was performed using IBM SPSS Statistics, Version 26.0 (IBM Corp., Armonk, NY, USA).

Gene expression was quantified using ExpressionSuite v1.1 (Applied Biosystems, Foster City, CA, USA) with the non-responder sample (GM008) as a reference, and data were reported as the mean relative quantification and maximum and minimum relative quantification; all the assays were run in triplicate. Efficiency was calculated for each primer pair probe and used for correction.

The statistical analyses and graphs were performed using GraphPad Prism v5.1 (Dotmatrix, Boston, MA, USA). Differences in gene expression between responders and non-responders were tested using the Mann-Whitney test, with a confidence level of 95%. For all tests, a  $p$  value  $< 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Patients

A total of 129 children meeting the inclusion criteria were recruited and gave their written informed consent. Complete data for evaluation of the primary response were collected from the clinical records of 124 patients. Characteristics of all recruited patients prior to the initiation of anti-TNF therapy are summarized in Table 2. Twenty-one were classified as non-responders (14 known at the time of the validation experiments) and 103 as responders to anti-TNF drugs. For the RNA-seq study, 12 responders and 12 non-responders were included. The cohort used in this study included up to 12 patients analyzed elsewhere [18]. The demographic characteristics prior to the initiation of anti-TNF therapy of the discovery cohort ( $n = 24$ ) are summarized in Table 3. Most patients were female (58.3%), with a median age at diagnosis of 10.2 years, and had been diagnosed with CD (66.7%) and treated with infliximab (62.5%). The only statistically significant difference between the 2 groups was in months from diagnosis until the start of treatment (1.9 in responders versus 16.3 in non-responders).

For the validation study, we used all the non-responders known at that time ( $n = 14$ ) and a cohort of similar size ( $n = 16$ ) of responders adjusted for sex, age, type of IBD, and anti-TNF drug. These patients had not been included in the previous RNA-seq experiments. The demographic characteristics of this validation cohort prior to the initiation of anti-TNF therapy are summarized in Table 4. Statistically significant differences between the groups were recorded for 2 parameters: months from diagnosis until the start of treatment (2.1 in responders versus 16.3 in non-responders) and PCDAI at the start of treatment (36.3 in responders versus 17.5 in non-responders).

PCDAI or PUCAI, CRP and FC are shown at the time of response definition (14 weeks infliximab, 26 weeks adalimumab) (Table 5). All of these parameters are increased in non-responders compared to responders.

### 3.2. Genes associated with response to anti-TNF drugs

A total of 102 genes were differentially expressed ( $FC > 0.6$  or  $< -0.6$ ) between responders and non-responders after the bioinformatic analysis ( $p$  value adjusted by FDR  $< 0.05$ ). Of these, 99 genes were over-expressed in responders versus non-responders, while only 3 were downregulated (Suppl. Table S2). Among the top 10 we found *DEFA3*, *MMP8*, *COL17A1*, *AP002990.1*, *OLFM4*, *INHBA*, *CEACAM6*, *LCN2*, *LTF*

**Table 2**  
Characteristics of 124 patients.

Characteristic	Overall (n = 124)	Responders (n = 103)	Non-Responders (n = 21)	p Value
<b>Gender</b>				
Male, n (%)	69 (55.6%)	60 (58.3%)	9 (42.9%)	0.23
Female, n (%)	55 (44.4%)	43 (41.7%)	12 (57.1%)	
<b>Age (years)</b>				
At diagnosis, median (IQR, range)	11.9 (3.9, 1–17)	12 (4, 1–17)	11.5 (6.5, 1–15)	0.26
At start of treatment, median (IQR, range)	12.6 (4.5, 1.1–17.3)	12.7 (4.7, 1.4–17.3)	12 (4.6, 1.1–16.3)	0.43
<b>Type of IBD</b>				
CD, n (%)	97 (78.2%)	83 (80.6%)	14 (66.7%)	
UC, n (%)	24 (19.4%)	18 (17.5%)	6 (28.6%)	0.21
Indet, n (%)	3 (2.4%)	2 (1.9%)	1 (4.8%)	
<b>Type of Anti-TNF</b>				
Infliximab, n (%)	77 (62.1%)	64 (62.1%)	13 (61.9%)	1
Adalimumab, n (%)	47 (37.9)	39 (37.9%)	8 (38.1%)	0.02*
<b>Line treatment</b>	117	100 (97.1%)	17 (81%)	0.03*
1st	117 (94.4%)	3 (2.9%)	4 (19%)	
2nd	7 (5.6%)	5.3 (12.5, 0–96.1)	7.4 (19.4, 0.1–55.5)	
<b>Months until treatment</b>	6.1 (14.4, 0–96.1)			
PCDAI at start of treatment, median (IQR, range)	32.5 (27.5, 0–75)	35 (27.5, 0–75)	20 (13.7, 8–63)	0.04*
PUCAI at start of treatment, median (IQR, range)	47.5 (28.8, 0–75)	50 (23.8, 5–65)	40 (45, 0–75)	0.45
CRP at start of treatment, median (IQR, range)	14.5 (30.4, 0–130)	16.5 (32.6, 0–130)	10.8 (22.1, 1–127)	0.98
FC at start of treatment, median (IQR, range)	1800 (2307, 27–9543)	2000 (2248, 27–9543)	1175 (1837.5, 288–7595)	0.30
<b>Concomitant immunomodulator at start of treatment</b>				
Azathioprine, n (%)	91 (76.5%)	75 (75.8%)	16 (80%)	0.44
Methotrexate, n (%)	6 (5%)	3 (2.9%)	3 (15%)	
None, n (%)	22 (18.5%)	21 (21.2%)	1 (5%)	

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IQR, interquartile range; PCDAI, Pediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index; CRP, C-reactive protein; FC, fecal calprotectin. \*  $p$  value  $< 0.05$ , \*\*  $p$  value  $< 0.01$ . The available  $n$  is not always 124 since data for some variables are not available for all patients

and *CILP2* (Table 6), all of which were over-expressed in responders.

### 3.3. Functional analysis of differentially expressed genes

Functional enrichment analyses were performed on the 102 differentially expressed genes (FDR  $< 0.05$ ,  $\text{Log}_2\text{FC} > 0.6$ , and  $\text{log}_2\text{FC} < -0.6$ ). The heatmap in Fig. 1 displays, for each gene (x-axis) involved in the respective enriched biological processes from the Gene Ontology database (GO) (y-axis), the resulting differential expression values obtained through the DESeq2 pipeline (defined by  $\text{Log}_2$  FoldChange values represented by the color gradient on the right side of the figure). The GO biological process enrichment results corresponding to 52 over- and under-expressed genes included 28 entities, while the GO molecular functions included 6 entities (Suppl. Table S3). As seen in the heatmap, some of these genes, namely, *AHSA*, *INHBA*, *DEFA3*, *LTF*, *PRTN3*, *MMP8*, *BPI*, and *ELANE*, have high differential expression values ( $\text{Log}_2\text{FoldChange}$  values ranging between 2 and 6). Additionally, Suppl. Fig. 1 shows the number of significant DE genes (x-axis) involved in the

**Table 3**  
Characteristics of patients in RNA-seq.

Characteristic	Overall (n = 24)	Responders (n = 12)	Non-Responders (n = 12)	p Value
<b>Sex</b>				
Male, n (%)	10 (41.7%)	5 (41.7%)	5 (41.7%)	1
Female, n (%)	14 (58.3%)	7 (58.3%)	7 (58.3%)	
<b>Age (years)</b>				
At diagnosis, median (IQR, range)	10.2 (3.33, 1–16)	10.2 (5.4, 8–16)	10.1 (5.4, 1–15)	0.55
At start of treatment, median (IQR, range)	11.4 (4.7, 1.1–16.3)	10.3 (5.2, 7.9–15.9)	12 (4.8, 1.1–16.3)	0.76
<b>Type of IBD</b>				
CD, n (%)	16 (66.7%)	8 (66.7%)	8 (66.7%)	1
UC, n (%)	7 (29.2%)	4 (33.3%)	3 (25%)	
Unclassified, n (%)	1 (4.2%)	0	1 (8.3%)	
<b>Type of Anti-TNF</b>				
Infliximab, n (%)	15 (62.5%)	7 (58.3%)	8 (66.7%)	1
Adalimumab, n (%)	9 (37.5%)	5 (41.7%)	4 (33.3%)	0.22
<b>Line of treatment</b>				
1st anti-TNF, n (%)	21 (87.5%)	12 (100%)	9 (75%)	0.002**
2nd anti-TNF, n (%)	3 (12.5%)	0	3 (25%)	
Months until treatment	5.2 (15.8, 0.1–55.5)	1.9 (5, 0.1–12.2)	16.3 (19.8, 0.1–55.5)	
<b>PCDAI at start of treatment, median (IQR, range)</b>				
	26.3 (21.3, 0–63)	30 (15, 0–53)	17.5 (15, 8–63)	0.26
<b>PUCAI at start of treatment, median (IQR, range)</b>				
	55 (20, 40–75)	55 (21.3, 40–65)	55 (na, 45–75)	0.86
<b>CRP at start of treatment, median (IQR, range)</b>				
	17.4 (19.8, 0–54)	19 (34.3, 0–52)	9.3 (12.3, 5–54)	0.88
<b>FC at start of treatment, median (IQR, range)</b>				
	2464 (3831.5, 148–7595)	3136 (3509.9, 148–6000)	2027.2 (3305, 288–7595)	0.35 0.09
<b>Concomitant immunomodulator at start of treatment</b>				
Yes, n (%)	20 (83.3%)	8 (66.7%)	12 (100%)	
No, n (%)	4 (16.7%)	4 (33.3%)	0	
<b>Type of immunomodulator</b>				
Azathioprine, n (%)	18 (75%)	8 (66.7%)	10 (83.3%)	0.19
Methotrexate, n (%)	2 (8.3%)	0	2 (16.7%)	
None, n (%)	4 (16.7%)	4 (33.3%)	0	

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; Undet, IBD-unclassified; IQR, interquartile range; PCDAI, Pediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index; CRP, C-reactive protein; FC, fecal calprotectin. na, not applicable. \* p value < 0.05, \*\* p value < 0.01.

enriched GO biological processes obtained (y-axis). The color gradient displays the FDR values for each GO term, with the functions at the bottom of the figure being the most significant (lowest FDR values). Furthermore, the size of the dots represents the ratio of significant DE genes associated with the corresponding function to the total number of significant DE genes obtained from the differential expression analysis performed using the DESeq2 workflow. [Suppl. Fig. 1](#) and [Suppl. Table S3](#) show how the most significant biological processes are associated with a higher number of DE genes. However, other processes, such as positive regulation of cell adhesion, are represented by as many genes as the most significant processes ([Suppl. Fig. 1](#)). The most significant biological processes were defense response to Gram-negative bacterium (FDR =  $2.3 \times 10^{-7}$ ), erythrocyte development (FDR =  $2.3 \times 10^{-7}$ ), myeloid cell differentiation (FDR =  $2.3 \times 10^{-7}$ ), and neuroinflammatory response (FDR = 0.0126) ([Suppl. Table S3](#)).

The same analysis was performed on GO molecular functions. The most significant molecular functions were hemoglobin binding (FDR = 0.002) and serine-type endopeptidase activity (FDR = 0.003) ([Fig. 2](#), [Suppl. Table S3](#)). The proportion of differentially expressed genes from the total number of genes for each molecular function included the same

**Table 4**  
Characteristics of patients included in RT-PCR validation.

Characteristic	Overall (n = 30)	Responders (n = 16)	Non-Responders (n = 14)	p Value
<b>Gender</b>				
Male, n (%)	14 (46.7%)	9 (56.3%)	5 (35.7%)	0.30
Female, n (%)	16 (53.3%)	7 (43.8%)	9 (64.3%)	
<b>Age (years)</b>				
At diagnosis, median (IQR, range)	10.5 (5.2, 1–16)	10.6 (5.61, 2–16)	10.14 (5.77, 1–15)	0.42
At start of treatment, median (IQR, range)	11.9 (5, 1.1–16.3)	12.2 (5.5, 2–15.9)	11.9 (51.1–16.3)	0.82
<b>Type of IBD</b>				
CD, n (%)	23 (76.7%)	13 (81.3%)	10 (71.4%)	0.50
UC, n (%)	6 (20%)	3 (18.8%)	3 (21.4%)	
Undet, n (%)	1 (3.3%)	0	1 (7.1%)	
<b>Type of Anti-TNF</b>				
Infliximab, n (%)	23 (76.7%)	13 (81.3%)	11 (78.6%)	0.68
Adalimumab, n (%)	7 (23.3%)	3 (18.8%)	7 (21.4%)	
<b>Line treatment</b>				
1st	27 (90%)	16 (100%)	11 (78.6%)	0.09
2nd	3 (10%)	0	3 (28.6%)	0.01*
Months until treatment	5.7 (20.6, 0.1–55.5)	2.1 (11.5, 0.1–31.2)	16.3 (19.7, 0.1–55.5)	
<b>PCDAI at start of treatment, median (IQR, range)</b>				
	25 (31.25, 8–65)	36.3 (25, 18–65)	17.5 (12.5, 8–63)	0.007**
<b>PUCAI at start of treatment, median (IQR, range)</b>				
	55 (27.5, 40–75)	52.5 (na, 40–65)	55 (na, 45–75)	0.80
<b>CRP at start of treatment, median (IQR, range)</b>				
	11 (12.3, 0–88)	12.5 (15.8, 0–88)	9.3 (12.1, 5–54)	0.92
<b>FC at start of treatment, median (IQR, range)</b>				
	1594.5 (2048.5, 176–7595)	1662.5 (2055.6, 126–6000)	905.5 (2469.5, 288–7595)	0.98 0.14
<b>Concomitant immunomodulator at start of treatment</b>				
Yes	27 (90%)	13 (81.3%)	14 (100%)	
No	3 (10%)	3 (18.8%)	0	
<b>Type of immunomodulator</b>				
Azathioprine, n (%)	25 (83.3%)	13 (81.3%)	12 (85.7%)	0.40
Methotrexate, n (%)	2 (6.7%)	0	2 (14.3%)	
None, n (%)	3 (10%)	3 (18.8%)	0	

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IQR, interquartile range; PCDAI, Pediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index; CRP, C-reactive protein; FC, fecal calprotectin. na, not applicable. \* p value < 0.05, \*\* p value < 0.01.

processes ([Suppl. Fig. 2](#)).

The KEGG pathways enrichment results for the same differentially expressed genes showed a unique entity, transcriptional misregulation in cancer (FDR  $6.83 \times 10^{-4}$ ) ([Fig. 3](#)). The gradient color represents the expression value (Log2FoldChange values) corresponding to the significant DE genes obtained using the DESeq2 pipeline. We mapped DE genes in some cancer types, such as Ewing's sarcoma, epithelial cancer, and/or T/B lymphoblastic leukemias. In summary, the pathway yielded 8 DE genes, some of which were highly overexpressed, for example, defensins (*DEFA1B*, *DEFA3*, and *DEFA4*), ETS transcription factor (*ERG*), and neutrophil elastase (*ELANE*).

GSEA results from a pre-ranked list of genes based on log2FC values showed 9 enriched gene sets for specific GO biological processes ([Suppl. Table S4](#)). The most significant processes were defense response to Gram-positive bacterium (FDR =  $1.40 \times 10^{-5}$ ), antibacterial humoral response (FDR = 0.002), and innate immune response in mucosa (FDR = 0.004).

GSEA results for enriched gene sets for specific KEGG pathways also showed a significant result for transcriptional misregulation in cancer (FDR = 0.016).

[Suppl. Fig. S3](#) shows statistically significant GSEA results based on a

**Table 5**

Clinical characteristics of patients after 14 weeks (infliximab) or 26 weeks (adalimumab) of treatment.

RNA-seq	Overall (n = 24)	Responders (n = 12)	Non-Responders (n = 12)	p Value
PCDAI at 14 or 26 weeks, median (IQR, range)	10 (16.3, 0–38)	5 (10, 0–15)	18.8 (22.5, 8–38)	0.01*
PUCAI at 14 or 26 weeks, median (IQR, range)	10 (30, 0–40)	10 (na, 0–10)	40 (0, 40–40)	0.16
CRP at 14 or 26 weeks, median (IQR, range)	0.9 (8.2, 0–11)	0.4 (0.8, 0–3)	8.7 (4.5, 1–11)	0.003**
FC at 14 or 26 weeks, median (IQR, range)	325 (615.6, 32–1838)	264 (583, 32–1178)	326 (na, 324–1838)	0.31
qPCR validation	Overall (n = 30)	Responders (n = 16)	Non-Responders (n = 14)	p Value
PCDAI at 14 or 26 weeks, median (IQR, range)	8.8 (15, 0–38)	5 (9.4, 0–20)	20 (16.3, 8–38)	0.001**
PUCAI at 14 or 26 weeks, median (IQR, range)	10 (na, 0–40)	5 (na, 0–10)	40 (0, 40–40)	0.22
CRP at 14 or 26 weeks, median (IQR, range)	1.2 (7.1, 0–11)	0.7 (1.6, 0–4)	8.7 (4.5, 1–11)	0.003**
FC at 14 or 26 weeks, median (IQR, range)	310.6 (456, 37–1838)	171.5 (371, 37–613)	717 (1311, 324–1838)	0.04*
All	Overall (n = 124)	Responders (n = 103)	Non-Responders (n = 21)	p Value
PCDAI at 14 or 26 weeks, median (IQR, range)	2.50 (10, 0–48)	0 (7.5, 0–48)	20 (12.5, 8–38)	0.00002**
PUCAI at 14 or 26 weeks, median (IQR, range)	5 (20, 0–40)	0 (10, 0–30)	30 (na, 20–40)	0.01*
CRP at 14 or 26 weeks, median (IQR, range)	1 (5.4, 0–100)	0.9 (3.7, 0–55)	9.5 (10.3, 1–100)	0.0003**
FC at 14 or 26 weeks, median (IQR, range)	293 (522.1, 10–6685)	205 (491, 10–5100)	847 (1514, 85–6685)	0.04*

IQR, interquartile range; PCDAI, Pediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index; CRP, C-reactive protein; FC, fecal calprotectin. na, not applicable; \* p value < 0.05; \*\* p value < 0.01.

pre-ranked list of log2FC values for all genes showing enriched gene sets for specific GO biological processes and specific KEGG pathways among significant log2FC values.

### 3.4. Validation of RNA-seq by semiquantitative real-time PCR

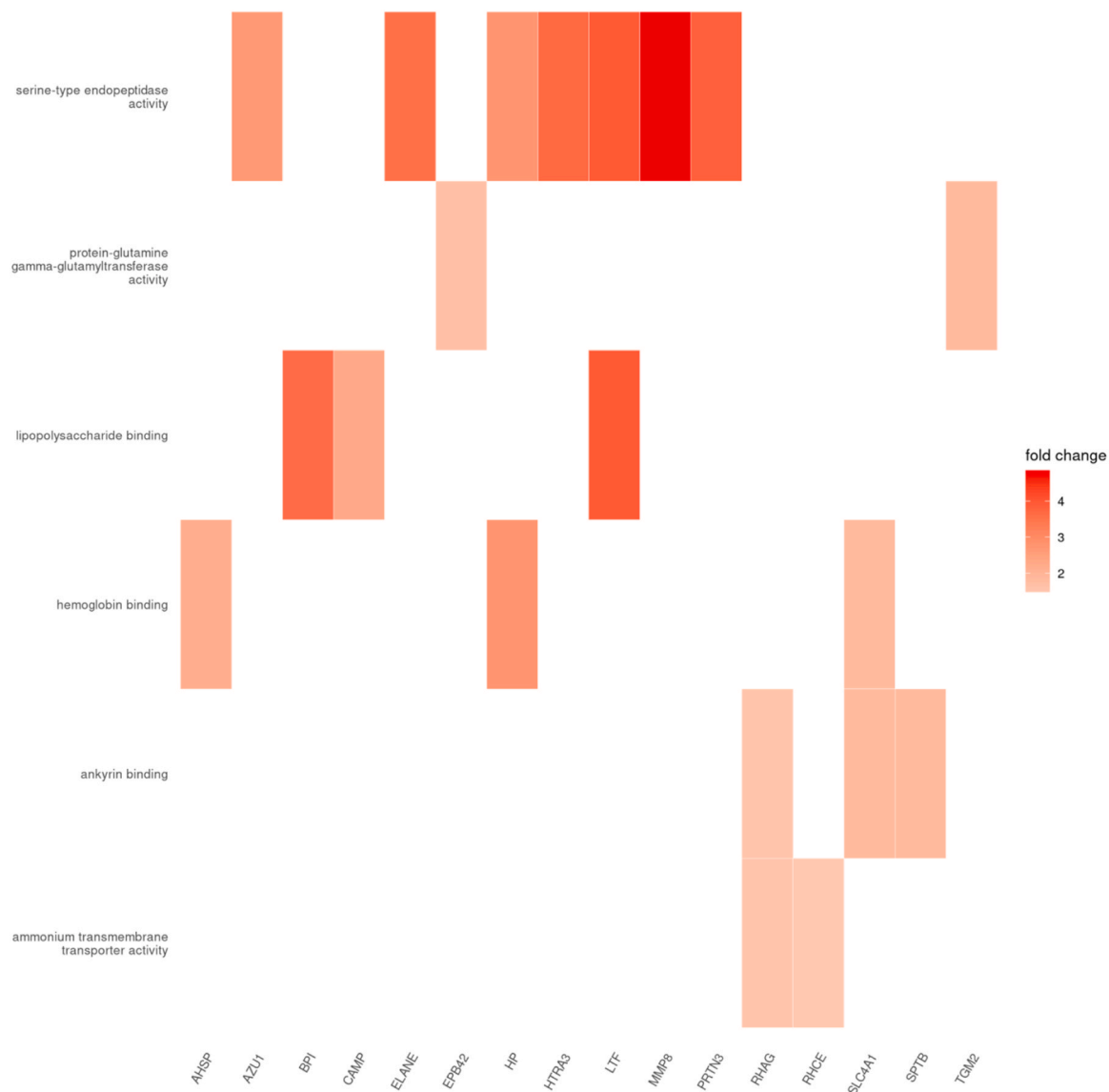
The 12 most differentially expressed genes were selected for validation using RT-PCR with KiCqStart® oligonucleotides in a new cohort of responder patients. *DEFA3* and *APO02990.1* were ruled out because no oligonucleotides were available. In addition, *DEFA4*, *ELANE*, and *AZU1* were selected, since they were found to be differentially expressed

**Table 6**

Top 35 differentially expressed genes according to response to anti-TNF drugs in children prior to initiation of treatment.

Gene Symbol	Ensembl Gene	Base Mean	Log2FC	adj p value	Validation
<i>DEFA3</i>	ENSG00000239839	7793	6.39	5.33 ×10 <sup>-7</sup>	
<i>MMP8</i>	ENSG00000118113	824	4.85	1.29 ×10 <sup>-4</sup>	YES
<i>COL17A1</i>	ENSG00000065618	41	4.69	4.44 ×10 <sup>-4</sup>	YES
<i>APO02990.1</i>	ENSG00000255508	675	4.63	8.43 ×10 <sup>-4</sup>	
<i>OLFM4</i>	ENSG00000102837	701	4.57	2.94 ×10 <sup>-3</sup>	YES
<i>INHBA</i>	ENSG00000122641	13	4.29	2.00 ×10 <sup>-3</sup>	YES
<i>CEACAM6</i>	ENSG00000086548	152	4.27	3.44 ×10 <sup>-3</sup>	YES
<i>LCN2</i>	ENSG00000148346	1064	4.15	1.30 ×10 <sup>-4</sup>	YES
<i>LTF</i>	ENSG00000012223	1136	3.96	2.43 ×10 <sup>-3</sup>	YES
<i>CILP2</i>	ENSG00000160161	2	3.95	2.55 ×10 <sup>-2</sup>	YES
<i>CEACAM8</i>	ENSG00000124469	221	3.91	2.00 ×10 <sup>-3</sup>	YES
<i>PRTN3</i>	ENSG00000196415	61	3.84	2.69 ×10 <sup>-2</sup>	YES
<i>HTRA3</i>	ENSG00000170801	15	3.71	4.61 ×10 <sup>-3</sup>	
<i>LINC02009</i>	ENSG00000283646	45	3.69	8.87 ×10 <sup>-3</sup>	
<i>BPI</i>	ENSG00000101425	519	3.64	8.44 ×10 <sup>-4</sup>	
<i>SCGB1C1</i>	ENSG00000188076	2	3.59	4.31 ×10 <sup>-2</sup>	
<i>AC104232.1</i>	ENSG00000254006	7	3.59	4.72 ×10 <sup>-2</sup>	
<i>ATP2C2</i>	ENSG00000064270	70	3.56	4.12 ×10 <sup>-3</sup>	
<i>ELANE</i>	ENSG00000197561	101	3.55	2.72 ×10 <sup>-3</sup>	YES
<i>PCOLCE2</i>	ENSG00000163710	11	3.38	3.74 ×10 <sup>-2</sup>	
<i>CRISP3</i>	ENSG00000096006	232	3.31	4.56 ×10 <sup>-3</sup>	
<i>CHIT1</i>	ENSG00000133063	101	3.27	1.04 ×10 <sup>-2</sup>	
<i>DEFA4</i>	ENSG00000164821	189	3.17	1.98 ×10 <sup>-2</sup>	YES
<i>DEFA1B</i>	ENSG00000240247	8110	3.13	3.28 ×10 <sup>-2</sup>	
<i>TARM1</i>	ENSG00000248385	9	2.95	1.04 ×10 <sup>-2</sup>	
<i>MS4A3</i>	ENSG00000149516	241	2.92	1.20 ×10 <sup>-2</sup>	
<i>RETN</i>	ENSG00000104918	199	2.92	4.61 ×10 <sup>-3</sup>	
<i>HP</i>	ENSG00000257017	717	2.75	9.11 ×10 <sup>-3</sup>	
<i>PDGFRA</i>	ENSG00000134853	2	-2.73	2.17 ×10 <sup>-2</sup>	
<i>AZU1</i>	ENSG00000172232	98	2.69	1.33 ×10 <sup>-2</sup>	YES
<i>ABCA13</i>	ENSG00000179869	30	2.64	1.87 ×10 <sup>-2</sup>	
<i>AC016526.3</i>	ENSG00000280078	2	2.64	8.87 ×10 <sup>-3</sup>	
<i>TCN1</i>	ENSG00000134827	283	2.58	1.22 ×10 <sup>-2</sup>	
<i>AC100835.2</i>	ENSG00000275527	37	2.54	5.84 ×10 <sup>-3</sup>	
<i>CA1</i>	ENSG00000133742	843	2.50	1.80 ×10 <sup>-2</sup>	





**Fig. 2.** Enriched GO terms for DE genes. Enriched Gene Ontology molecular functions (FDR < 0.05) in over-expressed and under-expressed genes ( $\log_2FC > 0.6$  and  $\log_2FC < -0.6$ , respectively). The number of genes involved in the set of enriched biological process is shown within the heatmap, and the gradient color defines the  $\log_2FC$  for each DE gene.

*CRIP2*, *CXCL13*, and *MAPKAPK2*) [31]. A recent study using 5 Gene Expression Omnibus datasets from biopsies of patients with IBDs showed overexpression of *SELE*, *TREM1*, *AQP9*, *FPR2*, and *HCAR3* in those who responded to infliximab [32]. Another study in UC patients revealed a different signature (*S100A8*, *S100A9*, *TREM1*, *TLR2*, *IL1B*, and *FPR1*) [33]. This diversity of results gives us an idea of the difficulty in finding a small set of DE genes of response to anti-TNF drugs. None of these genes was identified in blood from children with IBDs in our study. This fact may reflect strong differences in gene expression biomarkers between tissues, type of IBD, and the patient's age.

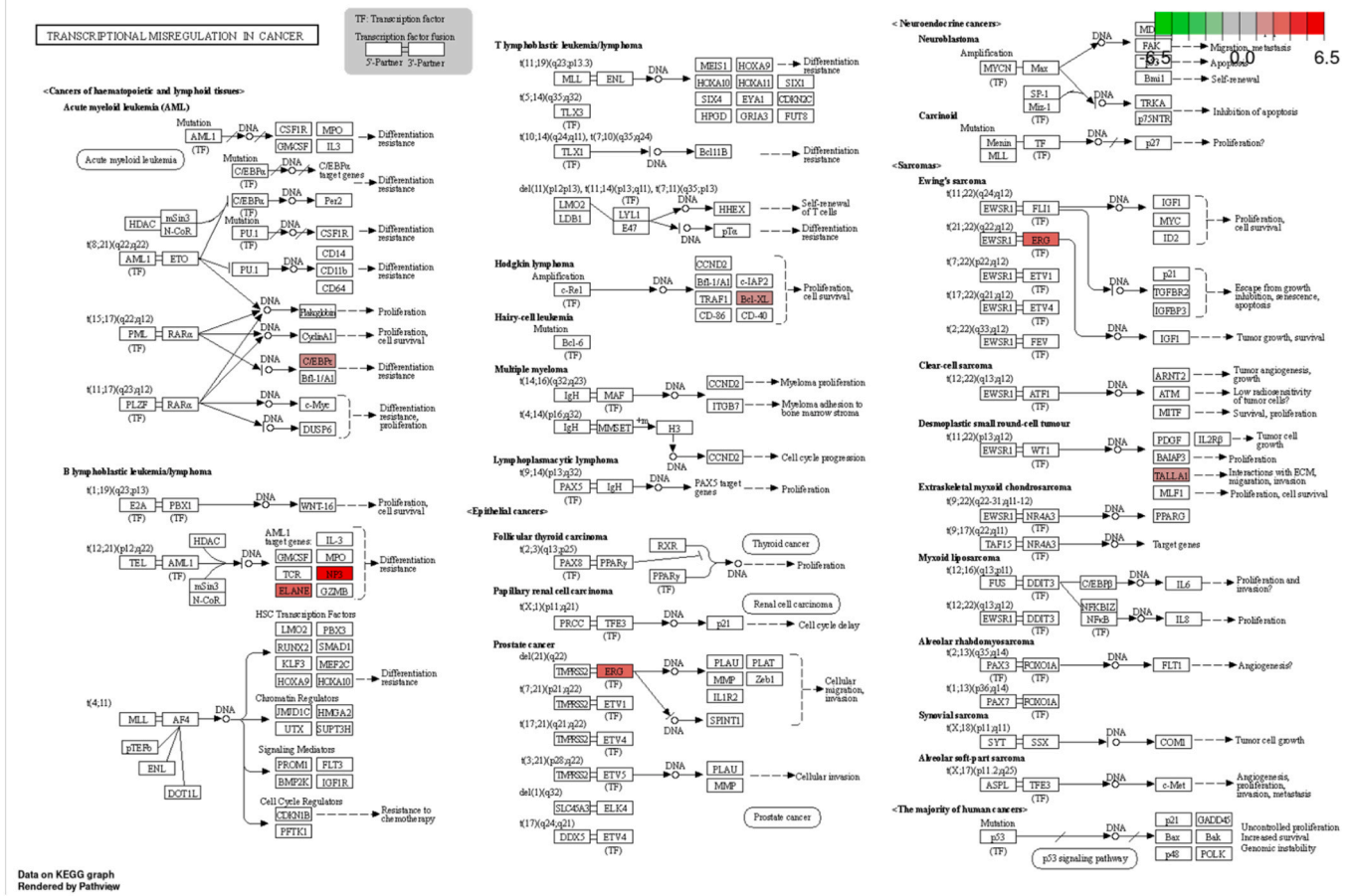
In this study, we identified 102 genes that were differentially expressed in blood from children with IBDs who responded or not to anti-TNF therapy before the initiation of treatment. Testing blood rather than tissue constitutes an extraordinary advantage, because it is less invasive. The PANTS study (324 patients) did not identify genes regulated in response to anti-TNF drugs in the blood of patients aged  $\geq 6$  years with CD at week 0. The main difference between our study and the PANTS study was the age of the participants, since PANTS also included adults. Similarly, in our previous study, which was based on half the number of the current sample, we did not identify any DE gene with an

FDR > 0.05 [18].

The 4 genes validated in our cohort, namely, *PRTN3*, *LTF*, *LCN*, and *CEACAM8*, show the importance of neutrophils in the response to anti-TNF therapy. *PRTN3* was one of the 4 genes validated in our study. A relationship has been identified between IBDs and the generation of antineutrophil cytoplasmic antibodies, such as those against PRTN3 [34,35]. This relationship is even useful for distinguishing UC from CD in children [36,37]. Measurement of proteinase 3 antineutrophil cytoplasmic antibody (PR3-ANCA) is useful not only for diagnosing UC, but also for evaluating disease severity and extension and predicting clinical course. PR3-ANCA  $\geq 3.5$  U/mL demonstrated 44.5% sensitivity and 95.6% specificity for the diagnosis of UC and 75% sensitivity and 69.0% specificity for steroid requirements in UC patients [38]. Stool levels of myeloperoxidase, another component of azurophil granules, increased significantly in active IBD patients, correlating with laboratory parameters and endoscopic grade of inflammation [39]. In role in autoimmune diseases, it seems to modify adaptive immune responses and induce vascular permeability [40].

*LTF* codes for lactoferrin and was another of the validated genes overexpressed in responders to anti-TNF drugs. Fecal lactoferrin (FL)





**Fig. 3.** Mapping of DE genes in transcriptional misregulation in cancer. Overview of differential expression within the transcriptional misregulation in cancer enriched pathway associated with the KEGG database. The DE genes with  $FDR < 0.05$  belonging to the pathway are highlighted in red or green depending on their degree of over-expression or under-expression, respectively.

was recently shown to be a useful biomarker of response in IBDs. Patients with FL levels  $>84.5 \mu\text{g/mL}$  at week 4 presented better clinical remission, endoscopic improvement, and remission (OR 0.43, 0.46, and 0.40 respectively) [41]. This finding supports the higher expression of *LTF* in blood we observed in children who responded to anti-TNF therapy before starting treatment and its potential use as a biomarker of response.

*LCN2* codes for lipocalin-2, a protein contained in neutrophilic granules, and exerts a protective effect against pathogens in intestinal inflammation. It is upregulated in serum and biopsies from colonoscopy in patients diagnosed with IBDs [42]. A 62.1% decrease in urinary levels of *LCN2* was observed in CD patients after treatment with infliximab [43]. Our results suggest that individuals with higher *LCN2* expression in blood benefit more from anti-TNF therapy.

*CEACAM8*, also known as *CD66b*, was upregulated in children with IBDs who responded to anti-TNF therapy. The expression of *CD66b* in neutrophils from mucosa, but not blood, correlated with the severity of CD [44].

The functional analysis revealed defense response to Gram-negative bacterium to be the main biological process enriched in the set of genes evaluated, while hemoglobin binding and serine-type endopeptidase activity were included in the most significant molecular functions. In our study, the genes that were more highly expressed in children with IBDs who responded to anti-TNF drugs than in those who did not and contributed more to defense response to Gram-negative bacterium were *LTF*, *BPI*, *OPTN*, *CAMP*, *DEFA4*, *AZU1*, *ELANE*, *DEFA3*, and *DEFA1B*, with most of them also being serine-type endopeptidases (*LTF*, *MMP8*, *HTRA3*, *AZU1*, *PRTN3*, *ELANE*, and *HP*). *AZU1*, *PRTN3*, *ELANE* and

*DEFA4* have been associated with methylprednisolone in relapsing-remitting multiple sclerosis [45], and in sepsis [46]. *AZU1*, *ELANE*, and *PRTN3* are expressed coordinately, and are present, together with *DEFA4*, in azurophilic granules, which are specialized lysosomes of the neutrophil for killing of microorganisms [47]. However, they are also involved in the formation of neutrophil extracellular traps, which, in addition to their role in defense against microorganisms, have also been implicated in cancer and autoimmune processes [47]. An association between neutrophil azurophilic granule exocytosis and TNF-alpha has also been described [48].

According to GO, hemoglobin binding proved to be the most significant molecular function in our study population. A relationship between indicators of iron status and indicators of inflammation has been described in children and adolescents with IBDs [49]. The authors found lower hemoglobin levels and iron-binding capacity in children with IBDs after 2–6 months of treatment with infliximab than in healthy children. Similarly, the presence of anemia is a biomarker of poor response to infliximab [50]. The higher expression of genes involved in hemoglobin binding might explain an improvement in anemia values and contribute to an improvement in IBDs in children receiving anti-TNF therapy.

Functional analysis of DE genes between children who respond and do not respond to anti-TNF drugs reveals a transcriptional misregulation in cancer due mainly to the inclusion of defensin genes. Regulation of gene expression plays an essential role in IBD-associated colorectal cancer, as shown in preclinical animal models [51]. The association between chronic diseases and increased risk of cancer is well known [52]. For example, colorectal cancer is 1.5–2 times more common in IBD patients than in the general population and is associated with up to 15%

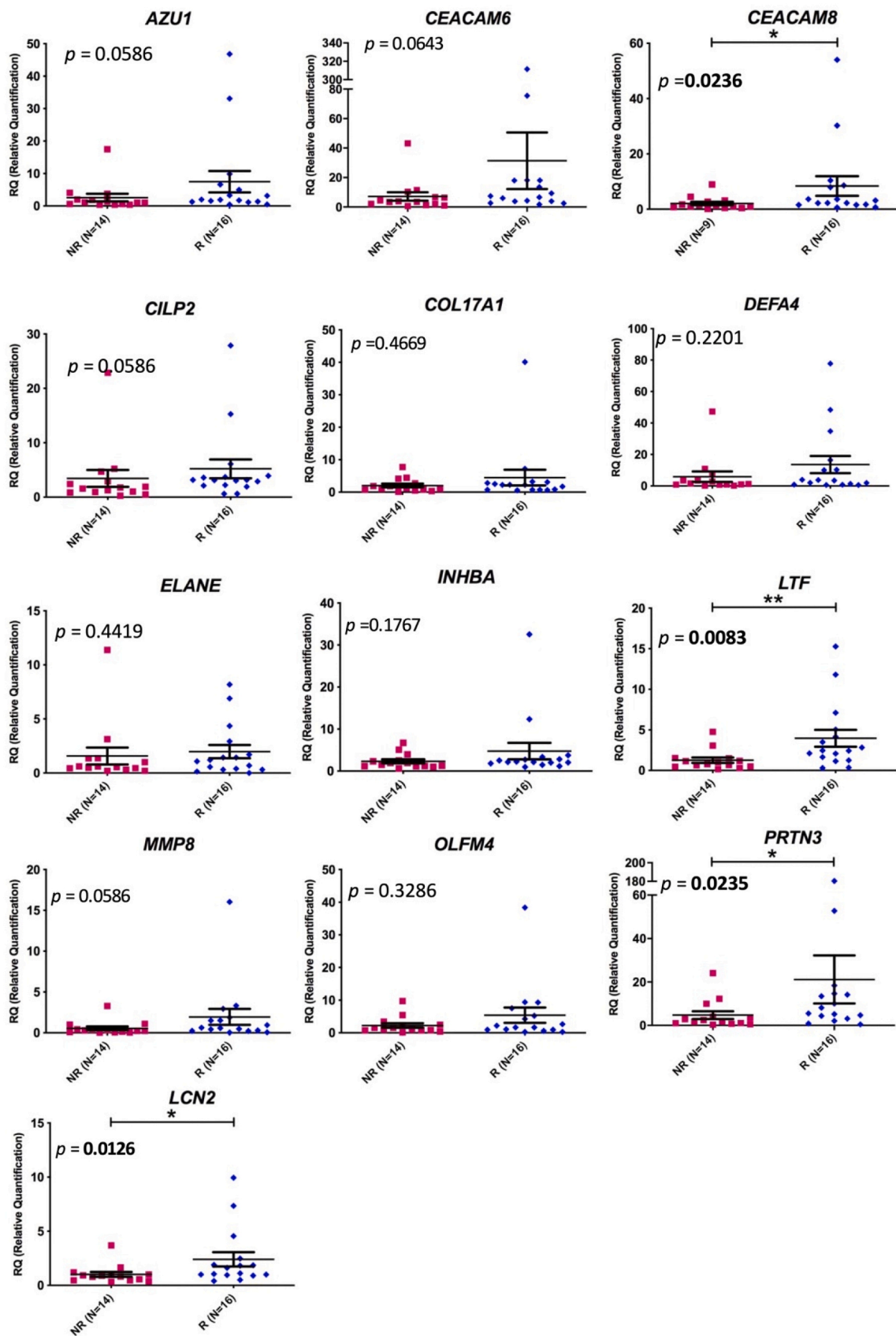


Fig. 4. Relative expression of validated genes. Relative quantification (RQ) of 13 selected genes in responders (R, blue) and non-responders (NR, red). \*  $p$  value < 0.05.

of deaths in these patients [53]. The association between TNF and cancer was also recently illustrated in a study that found lower rates of colorectal cancer in patients with IBDs treated with anti-TNF agents [54]. As children live longer with IBDs than adults, an optimal treatment response with anti-TNF drugs for their immune-mediated disease would increase the chance of not developing cancer.

CCAAT enhancer binding protein epsilon (CEBPE) is an overexpressed protein associated with acute myeloid leukemia within transcriptional misregulation in the cancer pathway. It is correlated with an increase in TNF- $\alpha$  levels and a decrease in IL-1 levels. Overexpression of the enhancer binding protein results in abnormalities of neutrophil migration and, consequently, of neutrophil cytokine expression [55]. Other subunits of CCAAT enhancer binding protein, such as beta (CEBPB), are linked to cytokine and interleukin production through the TNF-signaling pathway [56].

Since infliximab and adalimumab are anti-TNF drugs, the DE genes evaluated in our study were strongly associated with TNF. MMP8 plays a key role in neuroinflammatory processes by modulating activation of TNF- $\alpha$  [57]. INHBA is associated with the content of TNF- $\alpha$  in various anti-tumor immune responses. Upregulation of ELANE leads to TNFR2 signaling, which in turn triggers CCR5+ neutrophils and induces pro-neutrophil extracellular traps [58]. CCR5+ neutrophils are found in the lamina propria of UC patients.

Some of the significantly overexpressed genes within the pathway are directly associated with TNF-related processes. ERG transcription factor, which is associated with survival and tumor growth, inhibits vascular inflammation by repressing NF- $\kappa$ B activation and proinflammatory gene expression in endothelial cells [13], thus demonstrating that overexpression of ERG inhibits some TNF- $\alpha$ -dependent processes. Regarding IBDs, the repression of specific TNF- $\alpha$ -related processes generates underexpression of intercellular adhesion molecule-1 (ICAM-1) and interleukin 8 (IL-8) [13]. Increased levels of ICAM-1 and IL-8 are closely related to UC [14].

TNF mRNA expression was not identified among the 102 DE genes in our study. This fact is coherent with recent results indicating that, although higher levels of TNF mRNA were found in lamina propria samples from adults who did not respond to anti-TNF therapy, they were not observed in children [59].

The present findings differ from those of our earlier study, in that none of the 102 differentially expressed genes identified in the 24 patients in the present study coincided with the 22 genes identified in the 12 patients from the previous study [18]. There are several explanations for this observation. First, the bioinformatic process for identifying differentially expressed genes was different. Some studies have demonstrated differences between transcriptome analysis procedures [60,61] depending on GLMs and on other statistical, normalization, and filtering strategies. In contrast to the software used in our previous study [18], we used the DESeq2 workflow to analyze RNA-seq data. DESeq2 is optimized for this sample of 24 patients. It is designed to make an appropriate estimation of dispersion and uses GLMs based on Bayesian hierarchical models [62] and edgeR methods [63]. Both these mathematical approaches improve the over-dispersion across genes in comparison with other models and methods. For this analysis, DESeq2 relies on the negative binomial distribution to make estimates and perform statistical inference on differences and can detect outliers [64].

Second, in the first study, a non-adjusted  $p$  value was used to select genes because no statistically significant values were revealed by FDR. And third, the variability of the samples was high and the sample small. The new bioinformatic approach combined with the increase in simple size ensured that the results of this study were much more reliable.

RNA-seq analysis is not often validated in NGS experiments [65,66]. However, we aimed to validate the NGS data as thoroughly as possible. Our results revealed the same trend of over-expression in responders versus non-responders for the genes analyzed using RT-PCR as for those analyzed using NGS. However, only 4 out of 13 genes (*LTF*, *LCN2*, *CEACAM8*, and *PRNT3*) were statistically validated. Other studies fail to

validate the differentially expressed genes identified using RNA-seq [67], and some authors even suggest that independent verification of RNA-seq results is not required [68].

The main limitation of our study is the sample size and the number of variables. The limitation of sample size concerning non-responders to anti-TNF is inherent to the improvement in the management of these patients in recent years [12]. Although we analyzed twice as many patients as in our previous study, many questions remain unresolved, such as differences in anti-TNF drug (infliximab or adalimumab), differences between patients aged < 6 years and  $\geq$  7 years, and type of IBD (CD or UC). However, our main goal, improved response to anti-TNF therapy, was attained with the analysis method applied. The sample size also limited the possibility of validating DE genes in a completely new cohort of patients who did not respond to anti-TNF drugs.

## 5. Conclusion

RNA-seq identified 102 genes associated with response to anti-TNF drugs in children with IBDs. Four of these genes, *CEACAM8*, *LCN2*, *LTF*, and *PRNT3*, were fully validated by RT-PCR. Genes participating in defense response to Gram-negative bacterium, serine-type endopeptidase activity, and transcriptional misregulation in cancer are good candidates for anticipating the response to anti-TNF drugs in children with IBDs.

## Funding

Instituto de Salud Carlos III (ISCIII), "PI19/00792 and PI22/00584", co-funded by the European Union (L.L-F); Instituto de Investigación Sanitaria Gregorio Marañón (IiSGM), grant number 2021-II-postdoc-01 (S.S-M.), and by Consejería de Educación, Ciencia y Universidades Comunidad de Madrid, grant number PEJ-2021-AI/BMD-21866 and PIPF2022/SAL-GL-24790 (P.Z.C.).

## CRedit authorship contribution statement

**Victor Manuel Navas-López:** Writing – review & editing, Data curation. **Lorena Magallares:** Writing – review & editing, Data curation. **Inés Loverdos:** Writing – review & editing, Data curation. **María José Fobelo:** Writing – review & editing, Data curation. **Ruth García-Romero:** Writing – review & editing, Data curation. **Ana Fernández-Lorenzo:** Writing – review & editing, Data curation. **Cesar Sánchez:** Writing – review & editing, Data curation. **Mar Tolín:** Writing – review & editing, Data curation. **Begoña Pérez-Moneo:** Writing – review & editing, Data curation. **Montserrat Montraveta:** Writing – review & editing, Data curation. **Luis A. López-Fernández:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Laura M. Palomino:** Writing – review & editing, Data curation. **Susana Clemente:** Writing – review & editing, Data curation. **Ana Dopazo:** Writing – review & editing, Formal analysis. **Paula Zapata-Cobo:** Methodology, Investigation, Formal analysis, Data curation. **María Sanjurjo-Sáez:** Writing – review & editing, Data curation. **Marta Velasco:** Writing – review & editing, Data curation. **Oscar Segarra:** Writing – review & editing, Data curation. **Ana Moreno-Álvarez:** Writing – review & editing, Data curation. **Ricardo Torres-Peral:** Writing – review & editing, Data curation. **Alejandro Rodríguez:** Writing – review & editing, Data curation. **Enrique Salcedo:** Writing – review & editing, Data curation. **Gianluca Rubbini:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Rebeca Álvarez:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Perceval Vellosillo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ferrán Bossacoma:** Writing – review & editing, Data curation. **Vicente Merino-Bohórquez:** Writing – review & editing, Data curation. **Sara Salvador-Martín:** Writing – review & editing, Writing – original draft,

Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

RNA-seq data are uploaded to GEO.

### Acknowledgments

We would like to thank the patients and all the collaborators of the participating hospitals. Paula Zapata-Cobo is a PhD student of the “Programa de doctorado en investigación Biomédica” at Complutense University (Madrid, Spain).

### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2024.116299](https://doi.org/10.1016/j.biopha.2024.116299).

### References

- [1] E. Szigethy, L. McLafferty, A. Goyal, Inflammatory bowel disease, *Child Adolesc. Psychiatr. Clin. N. Am.* 19 (2010) 301–318, <https://doi.org/10.1016/j.chc.2010.01.007>.
- [2] S.C. Ng, H.Y. Shi, N. Hamidi, F.E. Underwood, W. Tang, E.I. Benchimol, R. Panaccione, S. Ghosh, J.C.Y. Wu, F.K.L. Chan, J.J.Y. Sung, G.G. Kaplan, Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies, *Lancet* 390 (2017) 2769–2778, [https://doi.org/10.1016/S0140-6736\(17\)32448-0](https://doi.org/10.1016/S0140-6736(17)32448-0).
- [3] D.K. Bonen, J.H. Cho, The genetics of inflammatory bowel disease, *Gastroenterology* 124 (2003) 521–536, <https://doi.org/10.1053/gast.2003.50045>.
- [4] G. Bamias, M.R. Nyce, S.A. De La Rue, F. Cominelli, New concepts in the pathophysiology of inflammatory bowel disease, *Ann. Intern. Med.* 143 (2005) 895–904, <https://doi.org/10.7326/0003-4819-143-12-200512200-00007>.
- [5] M.U. Mirkov, B. Verstockt, I. Cleynen, Genetics of inflammatory bowel disease: beyond NOD2, *Lancet Gastroenterol. Hepatol.* 2 (2017) 224–234, [https://doi.org/10.1016/S2468-1253\(16\)30111-X](https://doi.org/10.1016/S2468-1253(16)30111-X).
- [6] L.J. Cohen, J.H. Cho, D. Gevers, H. Chu, Genetic factors and the intestinal microbiome guide development of microbe-based therapies for inflammatory bowel diseases, *Gastroenterology* 156 (2019) 2174–2189, <https://doi.org/10.1053/j.gastro.2019.03.017>.
- [7] S. Salvador-Martín, G. Pujol-Muncunill, F. Bossacoma, V.M. Navas-López, C. Gallego-Fernández, O. Segarra, R. Clemente, R. Muñoz-Codoceo, J. Viada, L. Magallares, E. Martínez-Ojinaga, A. Moreno-Álvarez, A. Solar-Boga, I. Loverdos, V. Merino-Bohórquez, M.J. Balboa-Vega, A. Rodríguez-Martínez, C. Alvarez-Vayo, C. Sanchez, M. Tolin, J.A. Blanca-García, R. García-Romero, F.J. Eizaguirre, J. G. Sánchez-Hernandez, R.G. de Caldas, A. Millán-Jimenez, E. Aznal, J. Abarca-Zabalía, M. Sanjurjo-Sáez, L.A. López-Fernández, pharmacogenetics of trough serum anti-TNF levels in paediatric inflammatory bowel disease, *Br. J. Clin. Pharmacol.* 87 (2021) 447–457, <https://doi.org/10.1111/bcp.14400>.
- [8] P.L. Kozuch, S.B. Hanauer, General principles and pharmacology of biologics in inflammatory bowel disease, *Gastroenterol. Clin. N. Am.* 35 (2006) 757–773, <https://doi.org/10.1016/j.gtc.2006.09.005>.
- [9] D. Poddighe, M. Romano, M. Gattinara, V. Gerloni, Biologics for the treatment of juvenile idiopathic arthritis, *Curr. Med. Chem.* 25 (2018) 5860–5893, <https://doi.org/10.2174/0929867325666180522085716>.
- [10] S. Porcari, A. Viola, A. Orlando, A.C. Privitera, C. Ferracane, M. Cappello, A. Vitello, S. Siringo, G. Insera, A. Magnano, F. Mocciano, R. Di Mitri, N. Belluardo, O. Fidanza, S. Garufi, G. Magri, C. Bertolami, A. Carroccio, F. S. Macaluso, S. Renna, M. Ventimiglia, A. Alibrandi, M. Cottone, V. Fries, Persistence on anti-tumour necrosis factor therapy in older patients with inflammatory bowel disease compared with younger patients: data from the sicilian network for inflammatory bowel diseases (SN-IBD), *Drugs Aging* 37 (2020) 383–392, <https://doi.org/10.1007/s40266-020-00744-3>.
- [11] J.S. Hyams, A. Griffiths, J. Markowitz, R.N. Baldassano, W.A.J. Faubion, R. B. Colletti, M. Dubinsky, J. Kierkus, J. Rosh, Y. Wang, B. Huang, B. Bittle, M. Marshall, A. Lazar, Safety and efficacy of adalimumab for moderate to severe Crohn’s disease in children, *e2, Gastroenterology* 143 (2012) 365–374, <https://doi.org/10.1053/j.gastro.2012.04.046>.
- [12] B. Rodríguez Azor, R. Martín-Masot, A. Dayaldasani Khialani, J.M. Fernández-Martín, C. Gallego Fernández, V.M. Navas-López, Proactive monitoring of anti-TNF agents improves follow-up of paediatric patients with Crohn disease, *An. Pediatr.* 98 (2023) 165–174, <https://doi.org/10.1016/j.anpede.2023.01.007>.
- [13] M. Bramuzzo, S. Arrigo, C. Romano, M.C. Filardi, P. Lionetti, A. Agrusti, V. Dipasquale, M. Paci, G. Zuin, M. Aloï, C. Strisciuglio, E. Miele, M. Pastore, S. Martellosi, P. Alvisi, Efficacy and safety of infliximab in very early onset inflammatory bowel disease: a national comparative retrospective study, *U. Eur. Gastroenterol. J.* 7 (2019) 759–766, <https://doi.org/10.1177/2050640619847592>.
- [14] J. Hyams, W. Crandall, S. Kugathasan, A. Griffiths, A. Olson, J. Johanns, G. Liu, S. Travers, R. Heuschkel, J. Markowitz, S. Cohen, H. Winter, G. Veereman-Wauters, G. Ferry, R. Baldassano, REACH study group, induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn’s disease in children, *Gastroenterology* 132 (2007) 863–873, <https://doi.org/10.1053/j.gastro.2006.12.003>.
- [15] M. Fumery, C. Dupont, D. Ley, G. Savoye, V. Bertrand, N. Guillon, P. Wils, C. Gower-Rousseau, H. Sarter, D. Turck, A. Leroyer, Long-term effectiveness and safety of anti-TNF in pediatric-onset inflammatory bowel diseases: a population-based study, *Dig. Liver Dis. J. Ital. Soc. Gastroenterol. Ital. Assoc. Study Liver* 56 (2024) 21–28, <https://doi.org/10.1016/j.dld.2023.04.017>.
- [16] B. Verstockt, S. Verstockt, J. Dehairs, V. Ballet, H. Blevi, W.-J. Wollants, C. Breynaert, G. Van Assche, S. Vermeire, M. Ferrante, Low TREM1 expression in whole blood predicts anti-TNF response in inflammatory bowel disease, *EBioMedicine* (2019), <https://doi.org/10.1016/j.ebiom.2019.01.027>.
- [17] N. Mishra, K. Aden, J.I. Blase, N. Baran, D. Bordoni, F. Tran, C. Conrad, D. Avalos, C. Jaeckel, M. Scherer, S.B. Sørensen, S.H. Overgaard, B. Schulte, S. Nikolaus, G. Rey, G. Gasparoni, P.A. Lyons, J.L. Schultze, J. Walter, V. Andersen, E. T. Dermitzakis, S. Schreiber, P. Rosenstiel, Longitudinal multi-omics analysis identifies early blood-based predictors of anti-TNF therapy response in inflammatory bowel disease, *Genome Med.* 14 (2022) 110, <https://doi.org/10.1186/s13073-022-01112-z>.
- [18] S. Salvador-Martín, B. Kaczmarczyk, R. Álvarez, V.M. Navas-López, C. Gallego-Fernández, A. Moreno-Álvarez, A. Solar-Boga, C. Sánchez, M. Tolin, M. Velasco, R. Muñoz-Codoceo, A. Rodríguez-Martínez, C.A. Vayo, F. Bossacoma, G. Pujol-Muncunill, M.J. Fobelo, A. Millán-Jiménez, L. Magallares, E. Martínez-Ojinaga, I. Loverdos, F.J. Eizaguirre, J.A. Blanca-García, S. Clemente, R. García-Romero, V. Merino-Bohórquez, R. González de Caldas, E. Vázquez, A. Dopazo, M. Sanjurjo-Sáez, L.A. López-Fernández, Whole transcription profile of responders to anti-tnf drugs in pediatric inflammatory bowel disease, *Pharmaceutics* 13 (2021), <https://doi.org/10.3390/pharmaceutics13010077>.
- [19] N. Planell, M.C. Masamunt, R.F. Leal, L. Rodríguez, M. Esteller, J.J. Lozano, A. Ramírez, M. de L.S. Ayrizono, C.S.R. Coy, I. Alfaro, I. Ordás, S. Visvanathan, E. Ricart, J. Guardiola, J. Panés, A. Salas, Usefulness of transcriptional blood biomarkers as a non-invasive surrogate marker of mucosal healing and endoscopic response in ulcerative colitis, *J. Crohn’s Colitis* 11 (2017) 1335–1346, <https://doi.org/10.1093/ecco-jcc/jjx091>.
- [20] P.A. Harris, R. Taylor, B.L. Minor, V. Elliott, M. Fernandez, L. O’Neal, L. McLeod, G. Delacqua, F. Delacqua, J. Kirby, S.N. Duda, The REDCap consortium: building an international community of software platform partners, *J. Biomed. Inform.* 95 (2019) 103308, <https://doi.org/10.1016/j.jbi.2019.103208>.
- [21] E. Clough, T. Barrett, The gene expression omnibus database, *Methods Mol. Biol.* 1418 (2016) 93–110, [https://doi.org/10.1007/978-1-4939-3578-9\\_5](https://doi.org/10.1007/978-1-4939-3578-9_5).
- [22] M.I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2, *Genome Biol.* 15 (2014) 550, <https://doi.org/10.1186/s13059-014-0550-8>.
- [23] H. Wu, C. Wang, Z. Wu, A new shrinkage estimator for dispersion improves differential expression detection in RNA-seq data, *Biostatistics* 14 (2013) 232–243, <https://doi.org/10.1093/biostatistics/kxs033>.
- [24] X. Chen, False discovery rate control for multiple testing based on discrete p-values, *Biom. J.* 62 (2020) 1060–1079, <https://doi.org/10.1002/bimj.201900163>.
- [25] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, J.P. Mesirov, Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, *Proc. Natl. Acad. Sci. USA* 102 (2005) 15545–15550, <https://doi.org/10.1073/pnas.0506580102>.
- [26] M.A. Harris, J. Clark, A. Ireland, J. Lomax, M. Ashburner, R. Foulger, K. Eilbeck, S. Lewis, B. Marshall, C. Mungall, J. Richter, G.M. Rubin, J.A. Blake, C. Bult, M. Dolan, H. Drabkin, J.T. Eppig, D.P. Hill, L. Ni, M. Ringwald, R. Balakrishnan, J. M. Cherry, K.R. Christie, M.C. Costanzo, S.S. Dwight, S. Engel, D.G. Fisk, J. E. Hirschman, E.L. Hong, R.S. Nash, A. Sethuraman, C.L. Theesfeld, D. Botstein, K. Dolinski, B. Feierbach, T. Berardini, S. Mundodi, S.Y. Rhee, R. Apweiler, D. Barrell, E. Camon, E. Dimmer, V. Lee, R. Chisholm, P. Gaudet, W. Kibbe, R. Kishore, E.M. Schwarz, P. Sternberg, M. Gwinn, L. Hannick, J. Wortman, M. Berriman, V. Wood, N. de la Cruz, P. Tonellato, P. Jaiswal, T. Seigfried, R. White, The Gene Ontology (GO) database and informatics resource, *Nucleic Acids Res.* 32 (2004) D258–D261, <https://doi.org/10.1093/nar/gkh036>.
- [27] M. Kanehisa, M. Furumichi, M. Tanabe, Y. Sato, K. Morishima, KEGG: new perspectives on genomes, pathways, diseases and drugs, *Nucleic Acids Res.* 45 (2017) D353–D361, <https://doi.org/10.1093/nar/gkw1092>.
- [28] T. Wu, E. Hu, S. Xu, M. Chen, P. Guo, Z. Dai, T. Feng, L. Zhou, W. Tang, L. Zhan, X. Fu, S. Liu, X. Bo, G. Yu, clusterProfiler 4.0: a universal enrichment tool for interpreting omics data, *Innovation* 2 (2021) 100141, <https://doi.org/10.1016/j.xinn.2021.100141>.
- [29] W. Luo, C. Brouwer, Pathview: an R/bioconductor package for pathway-based data integration and visualization, *Bioinformatics* 29 (2013) 1830–1831, <https://doi.org/10.1093/bioinformatics/btt285>.

- [30] J. Abarca-Zabalía, M.I. García, A.L. Ros, I. Marín-Jiménez, M.L. Martínez-Ginés, B. López-Cauce, M.L. Martín-Barbero, S. Salvador-Martín, M. Sanjurjo-Saez, J. M. García-Domínguez, L.A.L. Fernández, Differential expression of SMAD genes and S1PR1 on circulating CD4+ T cells in multiple sclerosis and Crohn's disease, *Int. J. Mol. Sci.* 21 (2020) 676, <https://doi.org/10.3390/ijms21020676>.
- [31] M. Iacucci, L. Jeffery, A. Acharjee, E. Grisan, A. Buda, O.M. Nardone, S.C.L. Smith, N. Labarile, D. Zardo, B. Ungar, S. Hunter, R. Mao, R. Cannatelli, U.N. Shivaji, T. L. Parigi, G.M. Reynolds, G.V. Gkoutos, S. Ghosh, Computer-aided imaging analysis of probe-based confocal laser endomicroscopy with molecular labeling and gene expression identifies markers of response to biological therapy in IBD patients: the endo-omics study, *Inflamm. Bowel Dis.* 29 (2023) 1409–1420, <https://doi.org/10.1093/ibd/izac233>.
- [32] R. Kaddoura, H. Ghelani, F. Alqutami, H. Altaher, M. Hachim, R.K. Jan, Identification of specific biomarkers and pathways in the treatment response of infliximab for inflammatory bowel disease: in-silico analysis, *Life* 13 (2023), <https://doi.org/10.3390/life13030680>.
- [33] J. Zhang, X. Wu, S. Wei, C. Liu, X. Wang, W. Dong, Identified potential biomarkers may predict primary nonresponse to infliximab in patients with ulcerative colitis, *Autoimmunity* 55 (2022) 538–548, <https://doi.org/10.1080/08916934.2022.2103803>.
- [34] M.T. Arias-Loste, G. Bonilla, I. Moraleja, M. Mahler, M.A. Mieses, B. Castro, M. Rivero, J. Crespo, M. López-Hoyos, Presence of anti-proteinase 3 antineutrophil cytoplasmic antibodies (anti-PR3 ANCA) as serologic markers in inflammatory bowel disease, *Clin. Rev. Allergy Immunol.* 45 (2013) 109–116, <https://doi.org/10.1007/s12016-012-8349-4>.
- [35] U. Jerke, S.F. Marino, O. Daumke, R. Ketritz, Characterization of the CD177 interaction with the ANCA antigen proteinase 3, *Sci. Rep.* 7 (2017) 43328, <https://doi.org/10.1038/srep43328>.
- [36] M.P. Horn, A.M. Peter, F. Righini Grunder, A.B. Leichtle, J. Spalinger, S. Schibli, C. Sokollik, PR3-ANCA and panel diagnostics in pediatric inflammatory bowel disease to distinguish ulcerative colitis from Crohn's disease, *PLoS One* 13 (2018) e0208974, <https://doi.org/10.1371/journal.pone.0208974>.
- [37] M.W. Laass, J. Ziesmann, J. de Laffolie, N. Röber, K. Conrad, Anti-proteinase 3 antibodies as a biomarker for ulcerative colitis and primary sclerosing cholangitis in children, *J. Pediatr. Gastroenterol. Nutr.* 74 (2022) 463–470, <https://doi.org/10.1097/MPG.0000000000003359>.
- [38] S. Imakiire, H. Takedatsu, K. Mitsuyama, H. Sakisaka, K. Tsuruta, M. Morita, N. Kuno, K. Abe, S. Funakoshi, H. Ishibashi, S. Yoshioka, T. Torimura, F. Hirai, Role of serum proteinase 3 antineutrophil cytoplasmic antibodies in the diagnosis, evaluation of disease severity, and clinical course of ulcerative colitis, *Gut Liver* 16 (2022) 92–100, <https://doi.org/10.5009/gnl210211>.
- [39] T. Saiki, Myeloperoxidase concentrations in the stool as a new parameter of inflammatory bowel disease, *Kurum Med. J.* 45 (1998) 69–73, <https://doi.org/10.2739/kurumemedj.45.69>.
- [40] A. Strzepa, K.A. Pritchard, B.N. Dittel, Myeloperoxidase: a new player in autoimmunity, *Cell. Immunol.* 317 (2017) 1–8, <https://doi.org/10.1016/j.cellimm.2017.05.002>.
- [41] R. Chen, Y. Tie, X. Zhang, L. Li, M. Chen, S. Zhang, Fecal lactoferrin early predicts long-term outcomes in ulcerative colitis: a post-hoc analysis of the UNIFI and PURSUIT trials, *U. Eur. Gastroenterol. J.* 11 (2023) 542–550, <https://doi.org/10.1002/ueg2.12431>.
- [42] M. Reghefaoui, T.S. Peresuodei, M.S. Saavedra Palacios, A. Gill, C. Orji, T. Reghefaoui, J. Mostafa, The role of serological markers in the prediction of disease course and response to therapy in inflammatory bowel disease, *Cureus* 15 (2023) e48442, <https://doi.org/10.7759/cureus.48442>.
- [43] D. Bolignano, A. Della Torre, A. Lacquaniti, G. Costantino, W. Fries, M. Buemi, Neutrophil gelatinase-associated lipocalin levels in patients with Crohn's disease undergoing treatment with infliximab, *J. Investig. Med. Publ. Am. Fed. Clin. Res.* 58 (2010) 569–571, doi:10.2313/JIM.0b013e3181ccc20c.
- [44] A. Therrien, L. Chapuy, M. Bsat, M. Rubio, G. Bernard, E. Arslanian, K. Orlicka, A. Weber, B.-P. Panzini, J. Dorais, E.-J. Bernard, G. Soucy, M. Bouin, M. Sarfati, Recruitment of activated neutrophils correlates with disease severity in adult Crohn's disease, *Clin. Exp. Immunol.* 195 (2019) 251–264, <https://doi.org/10.1111/cei.13226>.
- [45] C. De Andres, M.I. García, H. Goicoechea, M.L. Martínez-Ginés, J.M. García-Domínguez, M.L. Martín, F. Romero-Delgado, A. Benguría, M. Sanjurjo, L.A. López-Fernández, Genes differentially expressed by methylprednisolone in vivo in CD4 T lymphocytes from multiple sclerosis patients: potential biomarkers, *Pharm. J.* 18 (2018) 98–105, <https://doi.org/10.1038/tpj.2016.71>.
- [46] S. Zhang, N. Li, W. Chen, Q. Fu, Y. Liu, Time series gene expression profiles analysis identified several potential biomarkers for sepsis, *DNA Cell Biol.* 39 (2020) 1862–1871, <https://doi.org/10.1089/dna.2020.5383>.
- [47] M. Herre, J. Cedervall, N. Mackman, A.-K. Olsson, Neutrophil extracellular traps in the pathology of cancer and other inflammatory diseases, *Physiol. Rev.* 103 (2023) 277–312, <https://doi.org/10.1152/physrev.00062.2021>.
- [48] R.M. Potera, M.J. Jensen, B.M. Hilkin, G.K. South, J.S. Hook, E.A. Gross, J. G. Moreland, Neutrophil azurophilic granule exocytosis is primed by TNF- $\alpha$  and partially regulated by NADPH oxidase, *Innate Immun.* 22 (2016) 635–646, <https://doi.org/10.1177/1753425916668980>.
- [49] F.F. Corrêa, V.L. Sdepanian, Body iron status indicators and inflammation indicators during inflammatory bowel disease therapy in children and adolescents, *Arq. Gastroenterol.* 58 (2021) 48–54, <https://doi.org/10.1590/S0004-2803.202100000-09>.
- [50] F. Magro, M.M. Estevinho, G. Catalano, M. Patita, B. Arroja, P. Lago, I. Rosa, H. Tavares de Sousa, P. Ministro, I. Mocanu, A. Vieira, J. Castela, J. Moleiro, J. Roseira, E. Cancela, P. Sousa, F. Portela, L. Correia, P. Moreira, M. Santiago, S. Dias, J. Afonso, S. Danese, L. Peyrin-Biroulet, C.C. Dias, How many biomarker measurements are needed to predict prognosis in Crohn's disease patients under infliximab? A prospective study, *U. Eur. Gastroenterol. J.* 11 (2023) 531–541, <https://doi.org/10.1002/ueg2.12420>.
- [51] K. Suri, J.A. Bubier, M.V. Wiles, L.D. Shultz, M.M. Amiji, V. Hosur, Role of MicroRNA in Inflammatory Bowel Disease: clinical evidence and the development of preclinical animal models, *Cells* 10 (2021), <https://doi.org/10.3390/cells10092204>.
- [52] A. Bardelčíková, J. Šoltys, J. Mojžiš, Oxidative stress, inflammation and colorectal cancer: an overview, *Antioxidants* 12 (2023) <https://doi.org/10.3390/antiox12040901>.
- [53] J.W. Windsor, M.E. Kuenzig, S.K. Murthy, A. Bitton, C.N. Bernstein, J.L. Jones, K. Lee, L.E. Targownik, J.N. Peña-Sánchez, N. Rohatinsky, S. Ghandeharian, J.H. B. Im, T. Davis, J. Weinstein, Q. Goddard, E.I. Benchimol, G.G. Kaplan, The 2023 impact of inflammatory bowel disease in Canada: executive summary, *J. Can. Assoc. Gastroenterol.* 6 (2023) S83–S96, <https://doi.org/10.1093/jcag/gwad003>.
- [54] M. Alkhayyat, M. Abureesh, A. Gill, G. Khoudari, M. Abou Saleh, E. Mansoor, M. Regueiro, Lower rates of colorectal cancer in patients with inflammatory bowel disease using anti-tnf therapy, *Inflamm. Bowel Dis.* 27 (2021) 1052–1060, <https://doi.org/10.1093/ibd/izaa252>.
- [55] J. Lektstrom-Himes, K.G. Xanthopoulos, CCAAT/enhancer binding protein epsilon is critical for effective neutrophil-mediated response to inflammatory challenge, *Blood* 93 (1999) 3096–3105.
- [56] J. Ma, X. Yang, X. Chen, C/EBP $\beta$  is a key transcription factor of ox-LDL inducing THP-1 cells to release multiple pro-inflammatory cytokines, *Inflamm. Res. J. Eur. Histamine Res. Soc.* 70 (2021) 1191–1199, <https://doi.org/10.1007/s00011-021-01509-3>.
- [57] E.-J. Lee, J.E. Han, M.-S. Woo, J.A. Shin, E.-M. Park, J.L. Kang, P.G. Moon, M.-C. Baek, W.-S. Son, Y.T. Ko, J.W. Choi, H.-S. Kim, Matrix metalloproteinase-8 plays a pivotal role in neuroinflammation by modulating TNF- $\alpha$  activation, *J. Immunol.* 193 (2014) 2384–2393, <https://doi.org/10.1093/jimmunol.1303240>.
- [58] F. Neuenfeldt, J.C. Schumacher, R. Grieshaber-Bouyer, J. Habicht, J. Schröder-Braunstein, A. Gauss, U. Merle, B. Niesler, N. Heineken, A. Dalpke, M.M. Gaida, T. Giese, S. Meuer, Y. Samstag, G. Wabnitz, Inflammation induces pro-NETotic neutrophils via TNFR2 signaling, *Cell Rep.* 39 (2022) 110710, <https://doi.org/10.1016/j.celrep.2022.110710>.
- [59] J.P. James, B.S. Nielsen, E. Langholz, M. Malham, E. Høgdal, L.B. Riis, Estimating tissue-specific TNF mRNA levels prior to anti-TNF $\alpha$  treatment may support therapeutic optimisation in IBD patients, *Scand. J. Gastroenterol.* 58 (2023) 1237–1245, <https://doi.org/10.1080/00365521.2023.2217313>.
- [60] A.T. Ku, S. Wilkinson, A.G. Sowsalsky, Comparison of approaches to transcriptomic analysis in multi-sampled tumors, *Brief. Bioinform.* 22 (2021), <https://doi.org/10.1093/bib/bbab337>.
- [61] X. Liu, J. Zhao, L. Xue, T. Zhao, W. Ding, Y. Han, H. Ye, A comparison of transcriptome analysis methods with reference genome, *BMC Genom.* 23 (2022) 232, <https://doi.org/10.1186/s12864-022-08465-0>.
- [62] H. Feng, K.N. Conneely, H. Wu, A Bayesian hierarchical model to detect differentially methylated loci from single nucleotide resolution sequencing data, *Nucleic Acids Res.* 42 (2014) e69, <https://doi.org/10.1093/nar/gku154>.
- [63] M.D. Robinson, D.J. McCarthy, G.K. Smyth, edgeR: a bioconductor package for differential expression analysis of digital gene expression data, *Bioinformatics* 26 (2010) 139–140, <https://doi.org/10.1093/bioinformatics/btp616>.
- [64] S. Mangiola, E.A. Thomas, M. Modrák, A. Vehtari, A.T. Papenfuss, Probabilistic outlier identification for RNA sequencing generalized linear models, *NAR Genom. Bioinform.* 3 (2021) lqab005, <https://doi.org/10.1093/nargab/lqab005>.
- [65] A. Nabbi, P. Beck, A. Delaidelli, D.A. Oldridge, S. Sudhaman, K. Zhu, S.Y.C. Yang, D.T. Mulder, J.P. Bruce, J.N. Paulson, P. Raman, Y. Zhu, A.C. Resnick, P. H. Sorensen, M. Sill, S. Brabetz, S. Lambo, D. Malkin, P.D. Johann, M. Kool, D.T. W. Jones, S.M. Pfister, N. Jäger, T.J. Pugh, Transcriptional immunogenomic analysis reveals distinct immunological clusters in paediatric nervous system tumours, *Genome Med.* 15 (2023) 67, <https://doi.org/10.1186/s13073-023-01219-x>.
- [66] Y. Ye, K.J. Maroney, H.W. Wiener, O.A. Mamaeva, A.D. Junkins, G.A. Burkholder, S.L. Sudenga, M. Khushman, S. Al Diffalha, A. Bansal, S. Shrestha, RNA-seq analysis identifies transcriptomic profiles associated with anal cancer recurrence among people living with HIV, *Ann. Med.* 55 (2023) 2199366, <https://doi.org/10.1080/07853890.2023.2199366>.
- [67] K. Patil, A. Naigaonkar, I. Hinduja, S. Mukherjee, Transcriptomic profile of GLCs of PCOS women highlights metabolic dysregulation as a plausible contributor to PCOS pathophysiology, *Reprod. Biol.* 23 (2023) 100787, <https://doi.org/10.1016/j.repbio.2023.100787>.
- [68] T. Coenye, Do results obtained with RNA-sequencing require independent verification? *Biofilm* 3 (2021) 100043 <https://doi.org/10.1016/j.biofilm.2021.100043>.