ScieRxiv
NOT PEER-REVIEWED
Original Article

# Proteomic and immunophenotypic signatures reveal immune-mediated resistance to cetuximab in metastatic colorectal cancer

**Carmen Navarrete-Sirvent[1,2,*], Aurora Rivas-Crespo[1,2], Nerea M. Herrera-Casanova[1], Ana Mantrana[3], Alejandra Díaz-Chacón[1], María Teresa Sánchez-Montero[1,2], María José Ortiz-Morales[4,5], Gema Pulido[4,5], Mª Teresa Cano[4,5], Auxiliadora Gómez-España[4,5], Rafael González-Fernández[1,6], Silvia Guil-Luna[1,2,7], Antonio Rodríguez-Ariza[1,2,5,#,*], Enrique Aranda[1,2,4,5,#]**

[1]Maimónides Biomedical Research Institute of Córdoba (IMIBIC)/University of Córdoba, Córdoba 14004, Spain.
[2]Cancer Network Biomedical Research Centre (CIBERONC), Madrid 28029, Spain.
[3]Centro Nacional de Investigaciones Oncológicas (CNIO), Madrid 28029, Spain.
[4]Department of Medicine, Faculty of Medicine, University of Córdoba, Córdoba 14004, Spain.
[5]Medical Oncology Department, Reina Sofía University Hospital, Córdoba 14004, Spain.
[6]Immunology Department, Reina Sofía University Hospital, Córdoba 14004, Spain.
[7]Veterinary Pathological Anatomy Department, University of Córdoba, Córdoba 14004, Spain.
[#]These authors share senior authorship.

***Correspondence to**: Dr. Carmen Navarrete-Sirvent, Dr. Antonio Rodríguez-Ariza, Maimónides Biomedical Research Institute of Córdoba (IMIBIC)/University of Córdoba, Córdoba 14004, Spain. Email: carmen.navarretesirvent@imibic.org; antonio.rodriguez.exts@juntadeandalucia.es

**Received: 29 July 2026 | Approved: 31 August 2026 | Online: 31 August 2026**

## Abstract

**Aim:** Metastatic colorectal cancer (mCRC) remains a major clinical challenge. Cetuximab, an anti-EGFR monoclonal antibody, is a standard first-line treatment for patients with RAS wild-type mCRC; however, many patients develop primary or acquired resistance. This study investigated immune-related mechanisms underlying cetuximab resistance, focusing on circulating biomarkers and natural killer (NK) cell reprogramming.

**Methods:** A cohort of 34 mCRC patients receiving first-line cetuximab-based therapy were prospectively enrolled. Patients were classified as responders (R) or non-responders (NR) according to time to progression. Plasma proteomics, PBMCs immunophenotyping, and data-independent acquisition (DIA) proteomic analysis of purified NK cells were performed at baseline and disease progression.

**Results:** At baseline, NR patients displayed a distinct pro-inflammatory and immunosuppressive profile characterized by increased expression of ARG1, HO-1, and TNFSF14, between others, proteins associated with impaired immune cytotoxicity and poor clinical outcomes. Conversely, R patients exhibited higher levels of IFN-γ, IL-12, and multiple chemokines. A composite immune-SCORE demonstrated strong prognostic value. Longitudinal analyses revealed extensive immune remodelling during disease progression, particularly in initial responders, with a shift toward naïve, less cytotoxic NK cell phenotypes and reduced expression of key cytotoxic receptors. NK cell proteomics identified dysregulation of TGFβ, Notch, and apical surface pathways, supporting impaired antibody-dependent cellular cytotoxicity (ADCC) as a mechanism of acquired resistance.

**Conclusion:** Immune dysfunction and NK cell reprogramming contribute to acquired cetuximab resistance. Circulating immune biomarkers showed predictive and prognostic potential, enabling early identification of patients at increased risk of treatment failure and supporting personalised therapeutic strategies in mCRC.

**Keywords:** Metastatic colorectal cancer, cetuximab, progression, immune exhaustion, TGFβ

## INTRODUCTION

Metastatic colorectal cancer (mCRC) is associated with a poor prognosis, with a 5-year survival rate of only 14%[1]. However, recent advances in cancer biology have expanded the treatment repertoire, introducing novel targeted therapies, such as monoclonal antibodies against vascular-endothelial growth factor (VEGF) and epithelial growth factor receptor (EGFR), as well as different immunotherapy approaches[2,3]. Cetuximab, the first EGFR-targeting monoclonal antibody (IgG1) approved for mCRC, prevents the binding of its ligands, EGF and TGF-α, thereby inhibiting cell proliferation, angiogenesis and tumour invasion[4]. Cetuximab has demonstrated improved survival benefit, either alone or combined with chemotherapy, in mCRC patients[5]. As a result, it has become a primary therapeutic option for EGFR-expressing *wild-type* RAS ($RAS^{wt}$) mCRC patients[6]. However, up to 65% of patients with $RAS^{wt}$ tumours exhibit resistance to this therapy[4,7]. In addition, while most cases of $RAS^{mutant}$ CRC do not respond to cetuximab, some patients may show sensitivity under certain conditions, suggesting that additional genetic changes and altered mechanisms beyond EGFR blockade could be involved[8,9].

An important feature of cetuximab is that, in addition to its primary action of EGFR blockade, it may also mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Specifically, the Fc-region of cetuximab binds to FcRγRIII receptor (CD16) on the surface of natural killer (NK) cells, leading to the targeted destruction of tumour cells[4,8,10]. In this context, NK cells act as effectors in cetuximab-mediated ADCC, and potentially play a crucial role in determining the efficacy of this therapy[10-12]. The cytotoxicity of NK cells is regulated by a series of activating or inhibitory signals, playing a crucial role in controlling tumour progression. The two major NK subpopulations are $CD56^{Bright}$ (approximately 15% of total circulating NK cells in peripheral blood), which have primarily immunoregulatory functions and are high cytokine producers, and $CD56^{Dim}$ (approximately 85% of total circulating NK cells), which are associated with a cytotoxic phenotype[13,14].

Despite significant advances in the management of mCRC, current therapeutic strategies remain effective for only a small proportion of patients, many of whom inevitably develop resistance, leading to a reduced duration of response[7]. These alterations give rise to resistant clones with distinct molecular characteristics, which reshape the tumour microenvironment (TME), promoting the upregulation of multiple inhibitory receptors and immunosuppressive factors, as well as the development of immune cell exhaustion.

This exhausted status, primarily associated with T and NK cells, is marked by a progressive decline in effector functions, including proliferation, cytokine production, and cytotoxicity[15]. Beyond the functional exhaustion, T cell maturity states are conventionally defined by the coordinated expression of CD45RA and CCR7 and classified lymphocytes into four subsets: naïve cells ($CD45RA^+$ $CCR7^+$); central memory cells ($CD45RA^-$ $CCR7^+$), which retain lymphoid homing potential; effector memory cells ($CD45RA^-$ $CCR7^-$) specialised for peripheral tissue surveillance, and terminally differentiated effector cells ($CD45RA^+$ $CCR7^-$). Distinguishing these maturation stages is critical, as the proteomic, metabolic and immune landscape of exhausted T cells often diverges significantly from these classical memory transitions during chronic antigen stimulation[16,17].

Therefore, the present study focused on elucidating the mechanisms underlying CRC progression, particularly in patients who respond to cetuximab but fail after an initial clinical benefit. Importantly, we previously demonstrated that several circulating immune mediators revealed the existence of a proinflammatory and dysfunctional immune status in patients who did not respond to therapy, which may explain their primary resistance to cetuximab. In the present study, we extend these findings by showing that those patients who initially respond to therapy, despite a strong early response, subsequently exhibit a shift toward a non-cytotoxic immune profile and upregulation of key immunosuppressive pathways, thereby promoting disease progression. Our findings provide an explanation for why some patients initially responsive to cetuximab develop secondary resistance after drug exposure, opening new avenues for addressing acquired resistance to cetuximab in mCRC.

## METHODS

### Patients and clinical-pathological data

A total of 34 patients were prospectively enrolled in the study between 2021 and 2025 according to the following inclusion criteria: (i) adult patients (aged 18 and over), (ii) diagnosed with metastatic colorectal cancer, (iii) *wild-type* RAS status, (iv) scheduled to receive cetuximab in the first-line setting and (v) signed informed consent to participate in the study. The study protocol was approved by the Ethics Committee of the Reina Sofia Hospital (COLO-NK, Committee Reference 4884, version 1.0 – 01/12/2020). Clinical data and peripheral blood samples (24 mL) were collected in ACD-B tubes (BD

Vacutainer, New Jersey, US) from each patient before cetuximab treatment (pretreatment samples, *pretto*) and at disease progression (progression/follow-up samples, *prog*). Patients were classified as *non-responders* (NR, $n$ = 15) when they progressed within 9 months from the start of cetuximab treatment, whereas those who had progressed (or not) after this time were defined as *responders* (R, $n$ = 19). The clinical-pathological variables evaluated are summarised in Supplementary Table 1.

**Plasma Olink® proteomics**

Plasma samples ($n$ = 9 NR and $n$ = 10 R) were obtained from peripheral blood using K2-EDTA tubes (Thermo Fisher Scientific, Waltham, MA, USA) after centrifugation at 3,000 × $g$ for 10 min at 4 ºC. Then, proteomic analysis was performed using the Olink® Target 96 Immuno-oncology panel (Cobiomic Bioscience, Córdoba, Spain) following the manufacturer's protocol. The Olink proteomics assay uses dual antibody-based proximity extension and DNA amplification to enable high-throughput, highly specific, and sensitive quantification of multiple proteins from minimal sample volumes. Four internal controls were included in each sample to assess both assay performance and the quality of individual samples. Protein quantification was expressed as Normalised Protein Expression (NPX) values, which were $\log_2$ transformed. Protein-protein interaction network was performed using STRING (https://string-db.org/), and GraphPad Prism 9 (v9.0.0; San Diego, California, US) was used to analyse the protein levels data.

**Immunophenotyping of peripheral blood mononuclear cells (PBMCs)**

PBMCs were isolated by Ficoll gradient (LymphoPrep, StemCell-Technologies, Vancouver, Canada). Briefly, 25 mL of a 1:1 mix of blood and PE (Phosphate Buffered Saline, PBS, supplemented with 2 mM EDTA, Sigma-Aldrich; San Luis, Missouri, US) were carefully pipetted onto 10.3 mL of Ficoll and centrifuged at 800 × $g$ (acceleration = 1 and deceleration = 0 profiles) at RT for 20 min. One sample from NR group and two samples from R groups were discarded due to poor quality. Immunophenotyping of PBMCs was performed by flow cytometry analysis on LSR-Fortessa flow cytometer (BD Biosciences, New Jersey, US) with FlowJo software version v10.5.3 (FlowJo, Ashland, OR, USA), using the antibodies listed in Supplementary Tables 2 and 3. Firstly, PBMCs were incubated with the antibody mix for 20 min at room temperature (RT) in the dark, then washed with PBS containing 2% Foetal Bovine Serum (FBS) and fixed for 20 min with CytoFix Fixation Buffer. All antibodies were titrated before use, and spectral overlap

compensation was performed automatically using the BD FACSDiva software with CompBeads Plus (BD Biosciences, New Jersey, US). 8-peaks Rainbow Compensation Particles Set (BD Biosciences, New Jersey, US) were used before each experiment for standardisation. NK cells were defined as $CD45^+$ $CD3^-$ $CD56^+$, and T cells as $CD45^+$ $CD3^+$ $CD56^-$. $CD56^{high}$ $CD16^{low/-}$ ("Bright") NK cells and $CD56^{low}$ $CD16^{high}$ ("Dim") NK cell subsets were assessed depending on their expression of CD56 and CD16 markers. In our study, Dim subset was further subdivided into two groups based on CD16 expression ($CD16^+$ and $CD16^-$), and Dim $CD16^-$ was grouped with Bright NKs.

**Data-independent acquisition (DIA) proteomic analysis**

DIA proteomic analysis was performed on highly purified NK cells from NR and R patients, including both pretreatment and progression samples ($n$ = 10 NR-pretto; $n$ = 5 NR-prog; $n$ = 9 R-pretto; $n$ = 6 R-prog). NK cells were isolated from PBMCs by negative selection using magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany), and proteins were extracted using RIPA buffer lysis (Sigma-Aldrich, San Luis, Missouri, US), following ultracentrifugation at 10,000 × g at 4 ºC for 30 min. Finally, the supernatant was frozen at -80 ºC. After thawing, proteins were precipitated with methanol:chloroform and total protein concentration was measured by using the Qubit Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were digested using the iST kit (PreOmics, Germany) according to the manufacturer's instructions. All samples were analysed by LC-MS/MS using the DIA-PASEF mode on the EvosepOne-TIMSTOF-Flex Clinical Proteomics Platform IMSMI (Bruker Daltonik GmbH, Bremen, Germany). One NR sample and two R samples failed quality control and were excluded from further analysis. Differentially expressed proteins (±2 *fold-change* and $p$-value < 0.05) between groups were identified using the *MetaboAnalyst* platform (https://www.metaboanalyst.ca/), and Gene Sets Enrichment Analysis (GSEA) was performed for functional enrichment, considering significant pathways when False Discovery Rate (FDR) ≤ 0.25. Mass spectrometry proteomic data have been deposited in the *ProteomeXchange Consortium* via the PRIDE[18] partner repository with the dataset identifier PXD057989.

**Statistical analyses**

Clinicopathological data were analysed by *Fisher's exact* test for categorical data and *t-student* test for continuous variables. Overall survival (OS) and time to progression (TTP)

were assessed using *Kaplan-Meier* curves and *log-rank* analyses, with *NR* group as reference. Receiver Operating Characteristic (ROC) curves were used to evaluate the performance of each marker in predicting response to cetuximab. *One-way ANOVA* test was used to compare between conditions (*pretto vs.. prog*) for normally distributed data, while *Mann-Whitney* and *Kruskal-Wallis* tests were used when normality assumptions were not satisfied. Statistical significance was set at $p$-value < 0.05 (two-tailed, 95% confidence interval) using GraphPad Prism 9 (software v9.0.0, San Diego, California, US).

## RESULTS

### Association between response to cetuximab and clinicopathological characteristics

Clinicopathological characteristics of the patients are summarised in Supplementary Table 1. The study cohort comprised 34 patients with mCRC, including 27 men and 7 women, with a mean age of 64 years (range, 36-85 years). Most patients have primary tumours located in the left colon (67.65%), whereas 29.41% had rectal tumours. A single patient presented with peritoneal carcinomatosis. The mean tumour size was 4.22 ±0.29 cm and the majority of tumours were of moderate *WHO* grade (67.65%). Most patients presented with unresectable disease (67.71%) and good baseline ECOG performance status (91.18%). Slightly more than half underwent primary tumour resection and presented with a single metastatic site, while approximately one-third of patients underwent metastasectomy. All patients presented *wild-type* RAS tumours and were scheduled to receive first-line cetuximab in combination with chemotherapy (5-fluorouracil, 5-FU; 5-FU and oxaliplatin, FUOX; 5-FU, oxaliplatin and folinic acid, FOLFOX; 5-FU and irinotecan, FUIRI) according to ESMO consensus guidelines [Supplementary Table 4].

Based on treatment response, 15 patients were classified as *non-responders* (NR) and 19 as *responders* (R). To assess whether the chemotherapy regimen could contribute to the differences observed between groups, we evaluated the distribution of the administered chemotherapy regimen. No significant differences were observed between chemotherapy regimen and response groups (*Fisher's exact test, $p = 0.4285$,* Supplementary Table 5).

Next, the association between response to cetuximab and clinicopathological characteristics is shown in Table 1. Only resectability ($p = 0.0297$) and the number of

cetuximab cycles received ($p$ = 0.0028) showed a significant association with the response to treatment. A trend towards younger age ($p$ = 0.0663), better ECOG ($p$ = 0.0760), and higher rate of metastasectomy ($p$ = 0.0790) was observed among R patients. Survival analysis confirmed poorer prognosis of NR patients, with significantly shorter OS (498 *vs.* 1522 days, $p < 0.0001$) and TTP (203 *vs.* 550 days, $p < 0.0001$) compared with R patients [Supplementary Figure 1]. Among NR patients, good ECOG status and rectal tumour location were significantly associated with improved OS, whereas smaller tumour size correlated with longer TTP [Table 2]. Conversely, in R patients, left-sided tumour location, a single metastatic site and metastasectomy were associated with improved TTP outcomes [Table 2].

**Table 1. Association between clinicopathological characteristics and response to cetuximab.**

| **Clinicopathological characteristics** | **Non responders (NR)** ($n$ = 15) | **Responders (R)** ($n$ = 19) | ***P* value** |
|---|---|---|---|
| **Gender** | | | |
| *Male* | 11 (73.33 %) | 16 (84.21 %) | 0.6722 |
| *Female* | 4 (26.67 %) | 3 (15.79 %) | |
| **Age** (*years*) | 67.67±2.445[a] | 61.95±2.132[a] | *0.0663* |
| **Tumour size** *(cm)* | 4.23±0.33[a] | 4.22±0.46[a] | 0.9768 |
| **Location** | | | |
| *Left colon* | 11 (73.33 %) | 13 (68.42 %) | 0.6982 |
| *Rectum* | 3 (20 %) | 6 (31.58 %) | |
| *Other** | 1 (6.67 %) | 0 (0 %) | |
| **Grade** | | | |
| *Low* | 11 (73.34 %) | 19 (100 %) | 0.1573 |
| *Moderate-High* | 2 (13.33 %) | 0 (0 %) | |
| *N/A*[b] | 2 (13.33%) | 0 (0 %) | |
| **Resectability** | | | |
| *Resectable* | 2 (13.33 %) | 9 (52.63 %) | ***0.0297***[c] |
| *Unresectable* | 13 (86.67 %) | 11 (47.37 %) | |

| | | | |
|---|---|---|---|
| **Cetuximab cycles received** | 10.00±1.238[a] | 21.00±2.852[a] | ***0.0028*** |
| **ECOG** | | | |
| *0-1* | 12 (80 %) | 19 (100 %) | *0.0760* |
| *< 2* | 3 (20 %) | 0 (0 %) | |
| **Primary tumour resection** | | | |
| *Yes* | 8 (53.33 %) | 12 (63.16 %) | 0.7282 |
| *No* | 7 (46.67 %) | 7 (36.84 %) | |
| **Number of metastases** | | | |
| *One* | 6 (40 %) | 12 (66.16 %) | 0.2998 |
| *More than one* | 9 (60 %) | 7 (36.84 %) | |
| **Metastasectomy** | | | |
| *Yes* | 3 (20 %) | 10 (52.63 %) | *0.0790* |
| *No* | 12 (80 %) | 9 (47.37 %) | |

[a]Mean ± SEM; [b]Non-available; [c]Significant *P* values are indicated in bold. NR: Non responders; R: Responders; ECOG: Eastern Cooperative Oncology Group; N/A: Not applicable.

**Table 2. Association between clinicopathological characteristics and overall survival or time to progression in *non-responders* and *responders*.**

| | **Non responders (NR)** | | | | **Responders (R)** | | | |
|---|---|---|---|---|---|---|---|---|
| | **OS[a]** | | **TTP[b]** | | **OS** | | **TTP** | |
| **Variables** | HR (95% CI) | *P*-value | HR (95% CI) | *P*-value | HR (95% CI) | *P*-value | HR (95% CI) | *P*-value |
| **Gender** | | | | | | | | |
| *Male*[R] | 0.6549 (0.151-2.841) | 0.5025 | 0.5678 (0.147-2.188) | 0.3110 | Undefined | 0.0961 | 1.238 (0.309-4.977) | 0.7765 |
| *Female* | | | | | | | | |
| **Age** (*median*) | | | | | | | | |
| *< 64 years* | 0.7044 (0.197-2.523) | 0.5441 | 0.3768 (0.080-1.780) | *0.0713* | 1.498 (0.297-7.548) | 0.6370 | 1.857 (0.642-5.372) | 0.2384 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *≥ 64 years*[R] | | | | | | | | |
| **Tumour size** | | | | | | | | |
| *< 4.2 cm* | 0.7811 (0.258-2.368) | 0.6309 | 0.3688 (0.103-1.315) | ***0.0437*** | 0.8984 (0.181-4.462) | 0.8846 | 0.7748 (0.272-2.209) | 0.6259 |
| *≥ 4.2 cm*[R] | | | | | | | | |
| **Location** | | | | | | | | |
| *Left colon* | 4.650 (0.483-44.78) | ***0.0067***[c] | 2.872 (0.448-18.40) | *0.0769* | 0.2967 (0.058-1.517) | 0.8696 | 0.3201 (0.112-0.916) | ***0.0477*** |
| *Rectum*[R] | | | | | | | | |
| **Grade** | | | | | | | | |
| *Low*[R] | 0.8780 (0.185-4.171) | 0.8700 | 0.4867 (0.140-1.691) | 0.2727 | Undefined | *0.0738* | 0.7578 (0.231-2.485) | 0.6669 |
| *Moderate-high* | | | | | | | | |
| **ECOG** | 4.544 (0.476-43.34) | ***0.0088*** | 2.027 (0.401-10.25) | 0.2477 | N/A[d] | | | |
| *0-1* | | | | | | | | |
| *≥2*[R] | | | | | | | | |
| **Surgery primary tumour** | 0.7034 (0.229-2.161) | 0.5007 | 0.5439 (0.171-1.726) | 0.2047 | 0.2991 (0.447-2.001) | *0.0881* | 0.4301 (0.127-1.457) | 0.1037 |
| *Yes*[R] | | | | | | | | |
| *No* | | | | | | | | |
| **Number of metastases** | 2.119 (0.580-7.747) | 0.1282 | 1.246 (0.401-3.873) | 0.6585 | 0.4223 (0.073-2.433) | 0.2539 | 0.3434 (0.103-1.141) | ***0.0258*** |
| *One location*[R] | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *More than one* | | | | | | | | |
| **Metastasectomy** | | | | | | | | |
| *Yes*[R] | 1.068 (0.287-3.972) | 0.9177 | 0.900 (0.261-3.119 | 0.8481 | 0.3039 (0.059-1.559) | 0.2463 | 0.3358 (0.108-1.043) | ***0.0310*** |
| *No* | | | | | | | | |

[R]: reference group; [a] *OS: overall survival;* [b] *TTP: time to progression;* [c] *significant P*-values *are indicated in bold;* [d] N/A: non-applicable, all R patients exhibited 0 or 1 ECOG status. NR: Non responders; HR: Hazard ratio; CI: Confidence interval.

**Circulating immune mediators indicate a proinflammatory and dysfunctional immune status in NR patients**

To investigate whether plasma-soluble markers reflect functional differences in the immune system prior to cetuximab therapy, we analysed 19 plasma samples (NR $n$ = 9; R $n$ = 10) using the Olink 96® Target Immuno-Oncology panel. Of the 92 proteins analysed, 82 were reliably detected with high quality. In the comparative analysis, NR patients showed seven significantly upregulated proteins: arginase I, ARG1; caspase 8, CASP8; heme oxygenase-I, HO-1/HMOX1; CD40L; endothelial growth factor, EGF; tumour necrosis factor (TNF) superfamily receptor member 12A, TNFSFR12A; and TNF superfamily member 14, TNFSF14 [Figure 1A].

Notably, protein-protein interaction network and biological process enrichment analysis showed that these proteins were associated with negative regulation of cytokine production [Figure 1B-C]. Conversely, eight proteins were found significantly upregulated in R patients: endothelial nitric oxide synthase, NOS3; chemokine C-C motif ligand 17, CCL17; interferon gamma, IFNg; C-X-C motif chemokine 5, CXCL5; CD70; interleukin (IL) 12 receptor subunit beta 1, IL12RB1; killer cell lectin-like receptor D1, KLRD1 (CD94); IL12 [Figure 2] These proteins are mainly involved in positive regulation of cell killing and cytokine-mediated signalling pathways [Figure 2B-C].

Finally, to evaluate the capacity of these markers to predict response to cetuximab, we performed ROC analysis [Figures 1D and 2D]. Importantly, 14 out of the 15 proteins analysed exhibited strong predictive power. Next, we stratified patients according to the median expression levels of the three most significant circulating biomarkers (ARG1,

HO-1 and TNFSF14), and developed a prognostic circulating immune-SCORE to stratify patients according to their risk of disease progression. As shown in Figure 3, those patients with at least two positive parameters showed significantly worse OS and TTP, highlighting the potential utility of these circulating proteins as prognostic and predictive biomarkers of disease progression in mCRC patients treated with cetuximab.

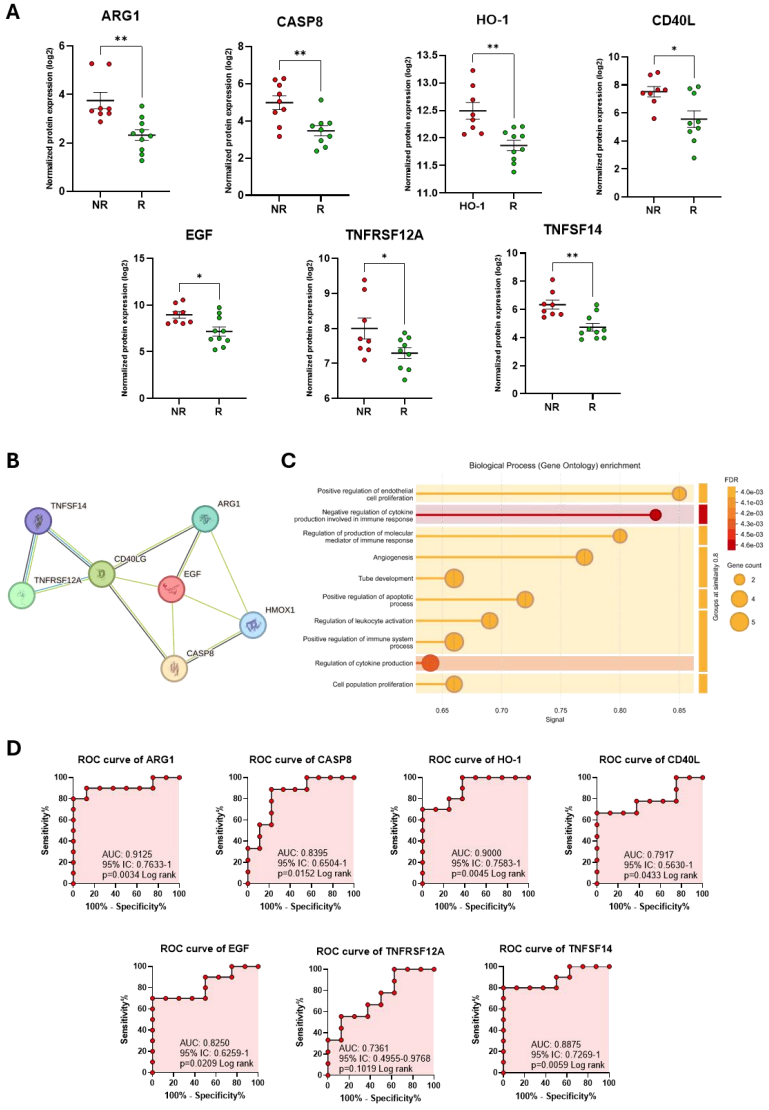


**Figure 1.** Circulating immune mediators associated with non-response to cetuximab. (A) Circulating immune mediators significantly upregulated in non-responding (NR) patients, compared to responders (R); Data are shown as mean ± SEM, and statistical significance is indicated as *$p < 0.05$; **$p < 0.01$; (B) Protein-protein interaction network of up-regulated proteins in NR patients and (C) biological process enrichment analysis; (D) ROC curve analysis evaluating the predictive performance of each protein marker.

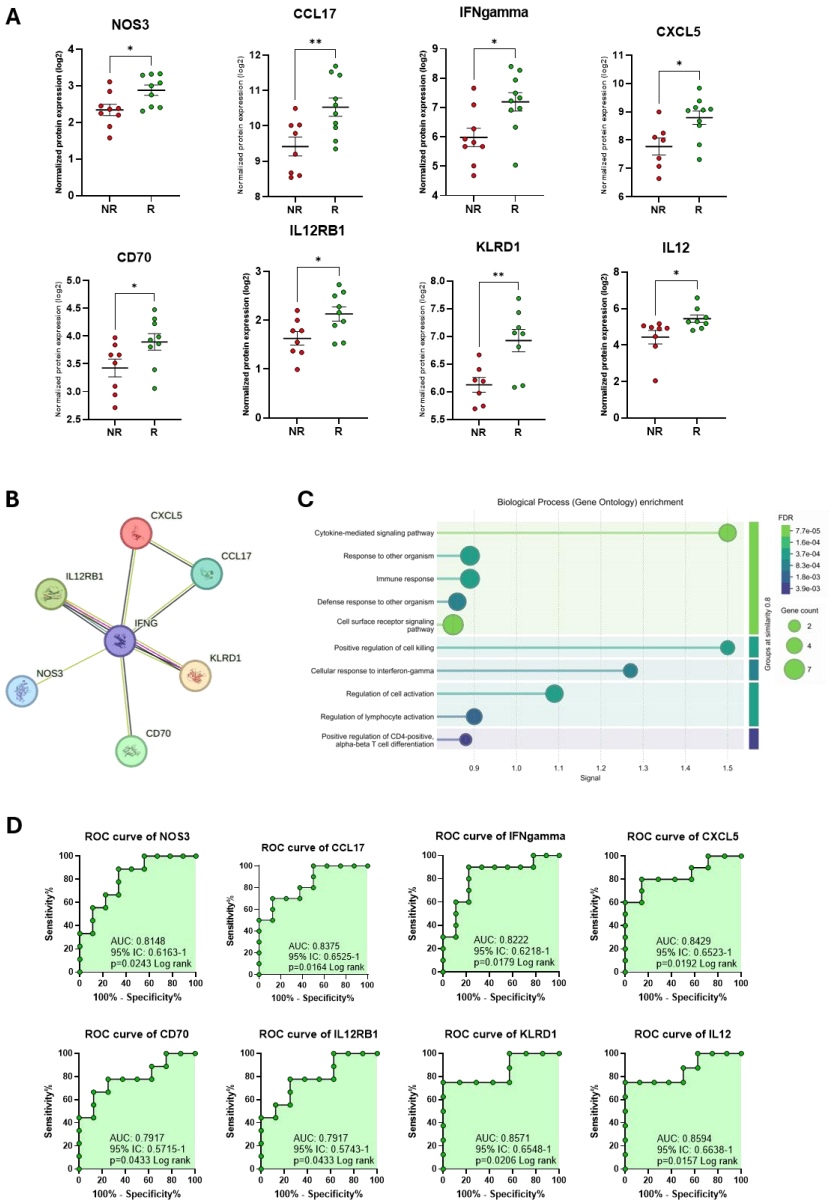


**Figure 2.** Circulating immune mediators associated with response to cetuximab. (A) Circulating immune mediators significantly upregulated in responding (R) patients, compared to non-responders (NR). Data are shown as mean ± SEM, and statistical significance is indicated as *$p$-value < 0.05; **$p$ < 0.01; (B) Protein-protein interaction network of up-regulated proteins in R patients and (C) biological process enrichment analysis; (D) ROC curve analysis evaluating the predictive performance of each protein marker.

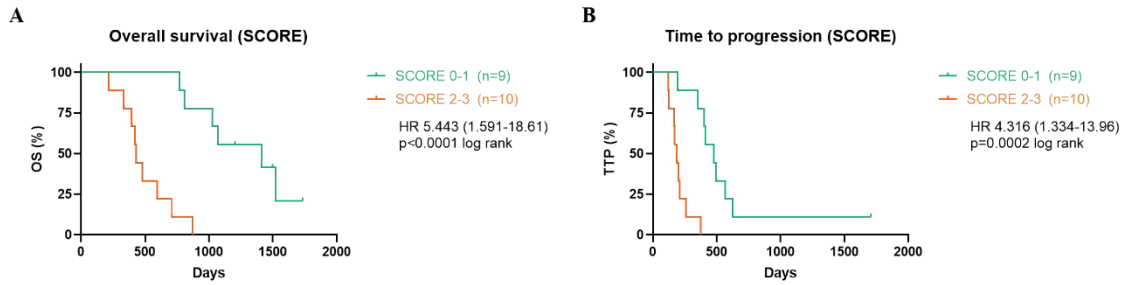


**Figure 3.** Prognostic value of a circulating immune-SCORE in mCRC patients receiving cetuximab. A composite immune-SCORE was developed based on the frequency of positivity for three circulating markers: ARG1, HO-1 and TNFSF14. (A) Kaplan-Meier curves for overall survival (OS) and (B) time to progression (TTP) in patients stratified by immune-SCORE: those with ≥ 2 positive markers (poor prognosis) versus those with 0-1 positive marker (better prognosis). Hazard ratios (HR) were calculated using ≥ 3 positive markers as the reference group using *log-rank* (Mantel-Cox) method.

**Disease progression during cetuximab treatment is associated with a loss of functional memory and reduced cytotoxicity in circulating T and NK cells**

Having demonstrated that circulating mediators can serve as biomarkers of disease progression, we next investigated phenotypic differences in circulating T and NK cells in mCRC patients receiving cetuximab by comparing pre-treatment and progression samples. In NR patients, T-cell phenotypes remained largely unchanged between the two time points [Figure 4]. In contrast, NK cells from NR (NK-NR) exhibited an increased expression of NKG2A and TIGIT along with decreased NKp30 expression [Figure 5], further supporting the involvement of these NK cell markers in the response to cetuximab, as previously reported by our group[12].

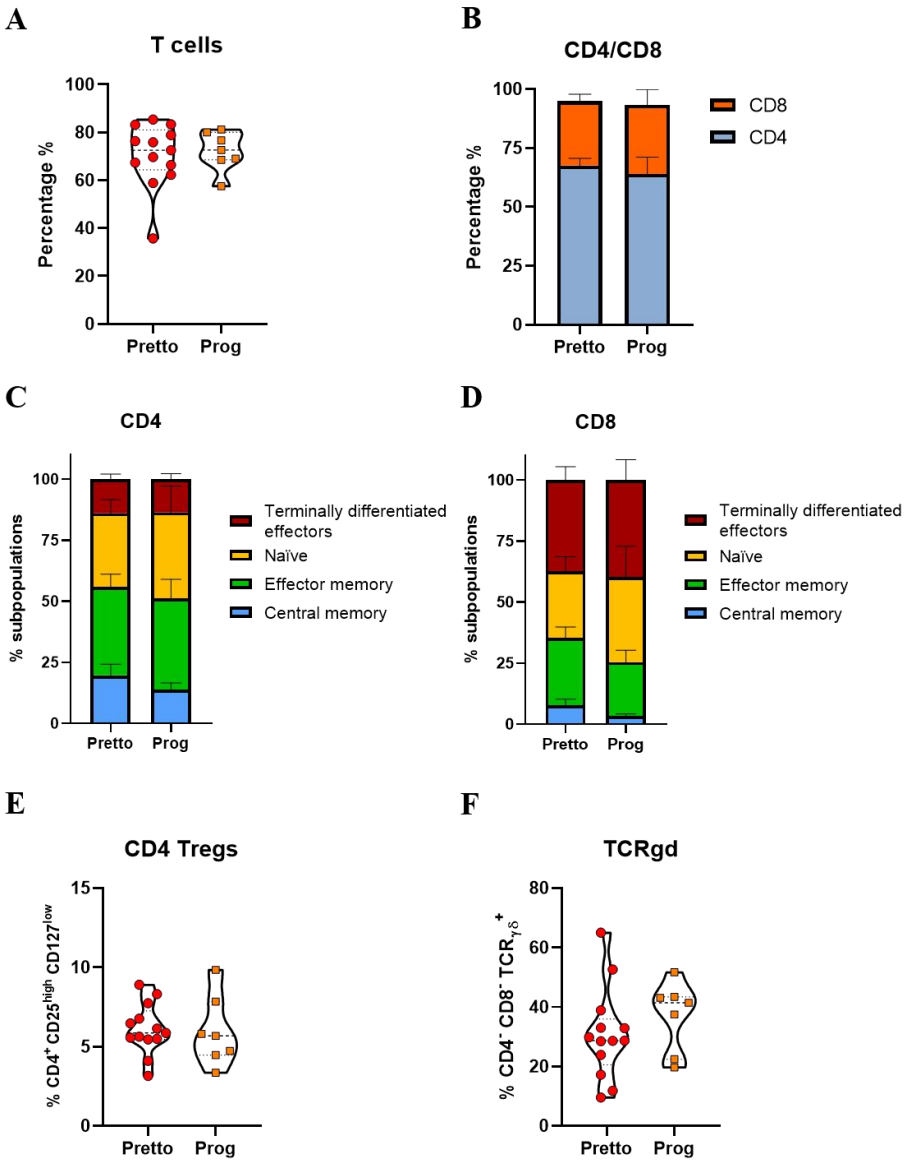


**Figure 4.** Phenotypic changes in circulating T-cell populations between pretreatment and progression in non-responding (NR) patients. Frequencies of (A) total T cells; (B) $CD4^+/CD8^+$ T cells; (C) $CD4^+$ maturation status and (D) $CD8^+$ maturation status based on CD45RA and CCR7 expression; (E) $T_{regs}$, defined as $CD3^+$ $CD4^+$ $CD25^{High}$ $CD127^{Low}$; and (F) $TCR_{\gamma\delta}$ cells as $CD3^+$ $CD4^-$ $CD8^-$ $TCR_{\gamma\delta}^+$.

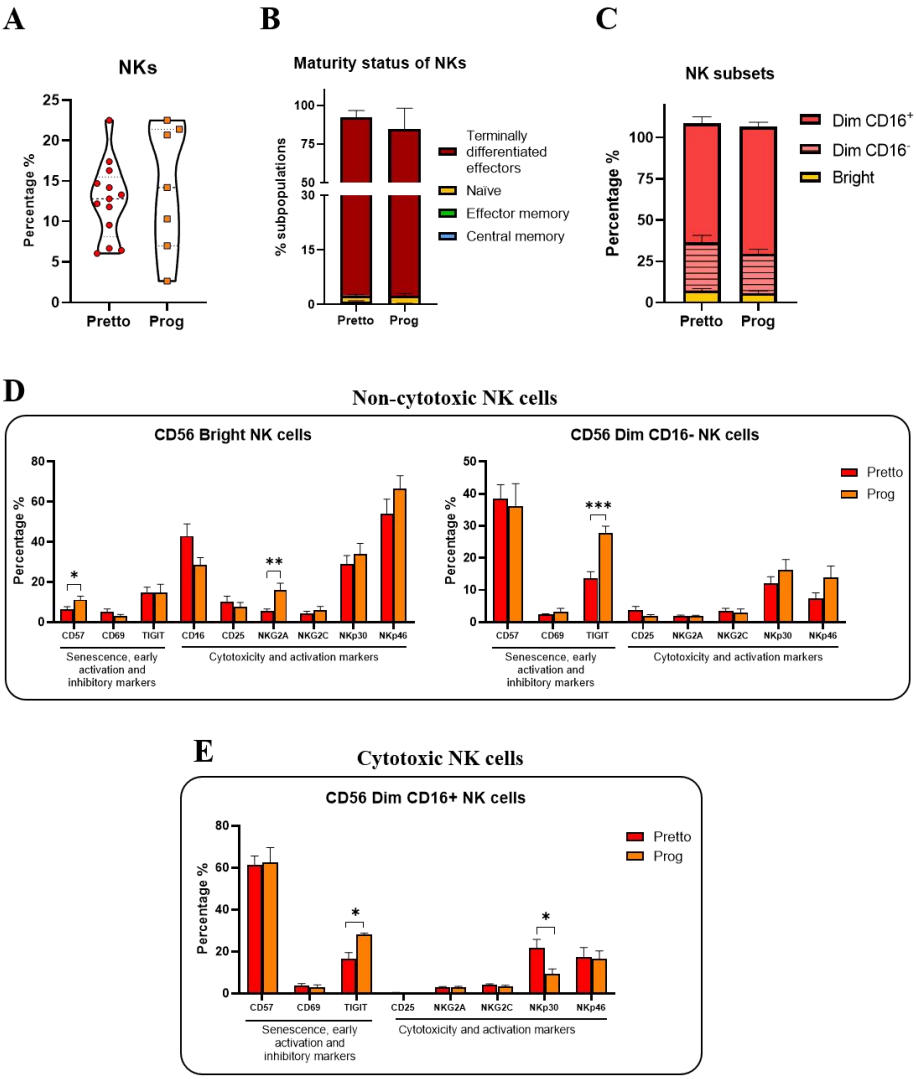


**Figure 5.** Phenotypic changes in circulating NK cell populations between pretreatment and progression samples in non-responders (NR). Frequencies of (A) total NK cells; (B) maturation status of NK cells based on CD45RA and CCR7 expression; (C) subsets of NK cells based on the expression of CD56 and CD16; (D) senescence, inhibitory and activation markers in non-cytotoxic NK cells and (E) cytotoxic NK cells.

Conversely, several phenotypic changes in T cells were observed at follow-up in R patients. Progression samples exhibited a shift towards a less cytotoxic T-cell phenotype with reduced memory function, characterized by increased proportions of naïve $CD4^+$ and $CD8^+$ T cells and decreased frequencies of effector and TCRγδ T cells [Figure 6].

Notably, the proportion of regulatory T cells, which exert immunosuppressive roles, was reduced at progression, possibly reflecting their recruitment to the tumour microenvironment [Figure 6E]. Additional alterations were also detected in NK cells

[Figure 7]. At progression, NK cells from responder patients (NK-R) displayed a reduced proportion of cytotoxic subsets, together with an increased frequency of naïve cells and decreased expression of key cytotoxicity markers, including NKp30 and NKp46. Furthermore, all NK cell subsets showed increased TIGIT expression, reduced NKG2A levels, and a trend towards higher CD57 expression, consistent with a shift towards a more exhausted and senescent phenotype. Taken together, these findings reveal substantial remodelling of both T-cell and NK-cell compartments during disease progression in cetuximab-treated patients, supporting the existence of an evolving immunosuppressive state associated with acquired resistance and warranting further investigation into the mechanisms underlying this process.

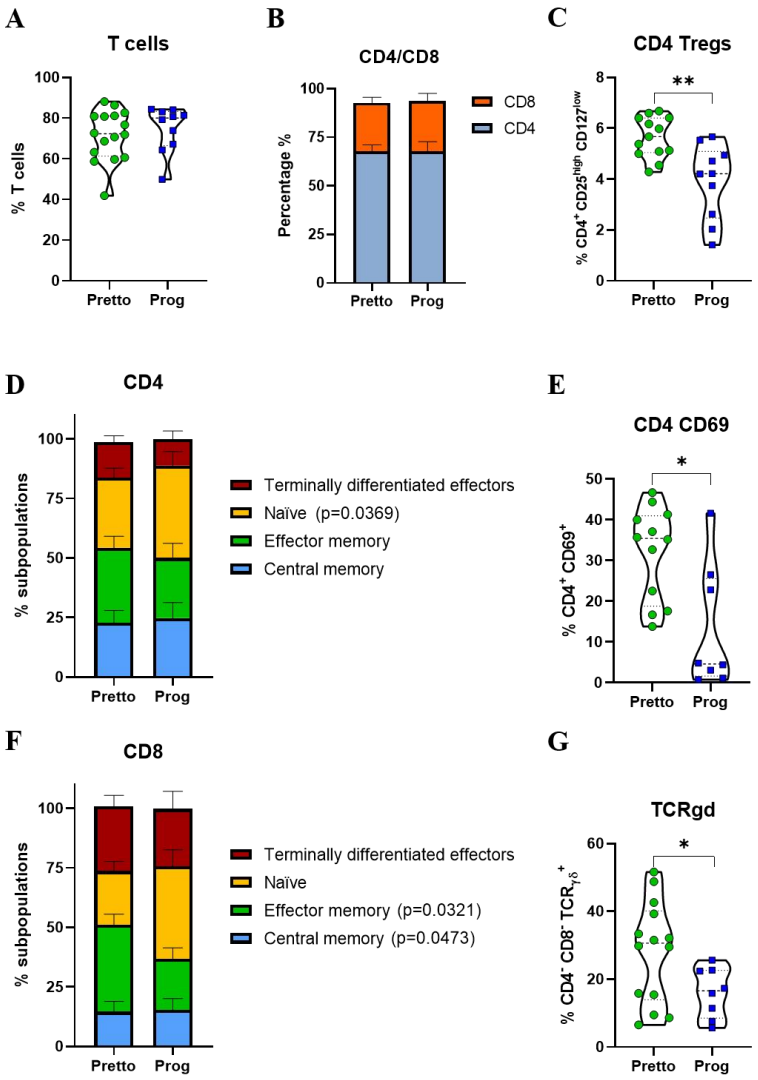


**Figure 6.** Phenotypic changes in circulating T cell populations between pretreatment and progression in responders (R). Frequencies of (A) total T cells; (B) $CD4^+/CD8^+$ T cells;

(C) $T_{regs}$, defined as $CD3^+$ $CD4^+$ $CD25^{High}$ $CD127^{Low}$; (D) $CD4^+$ maturation status (E) expression of CD69 in $CD4^+$; (F) $CD8^{++}$ maturation status based on CD45RA and CCR7 expression; (G) $TCR_{\gamma\delta}$ cells as $CD3^+$ $CD4^-$ $CD8^-$ $TCR_{\gamma\delta}^+$.

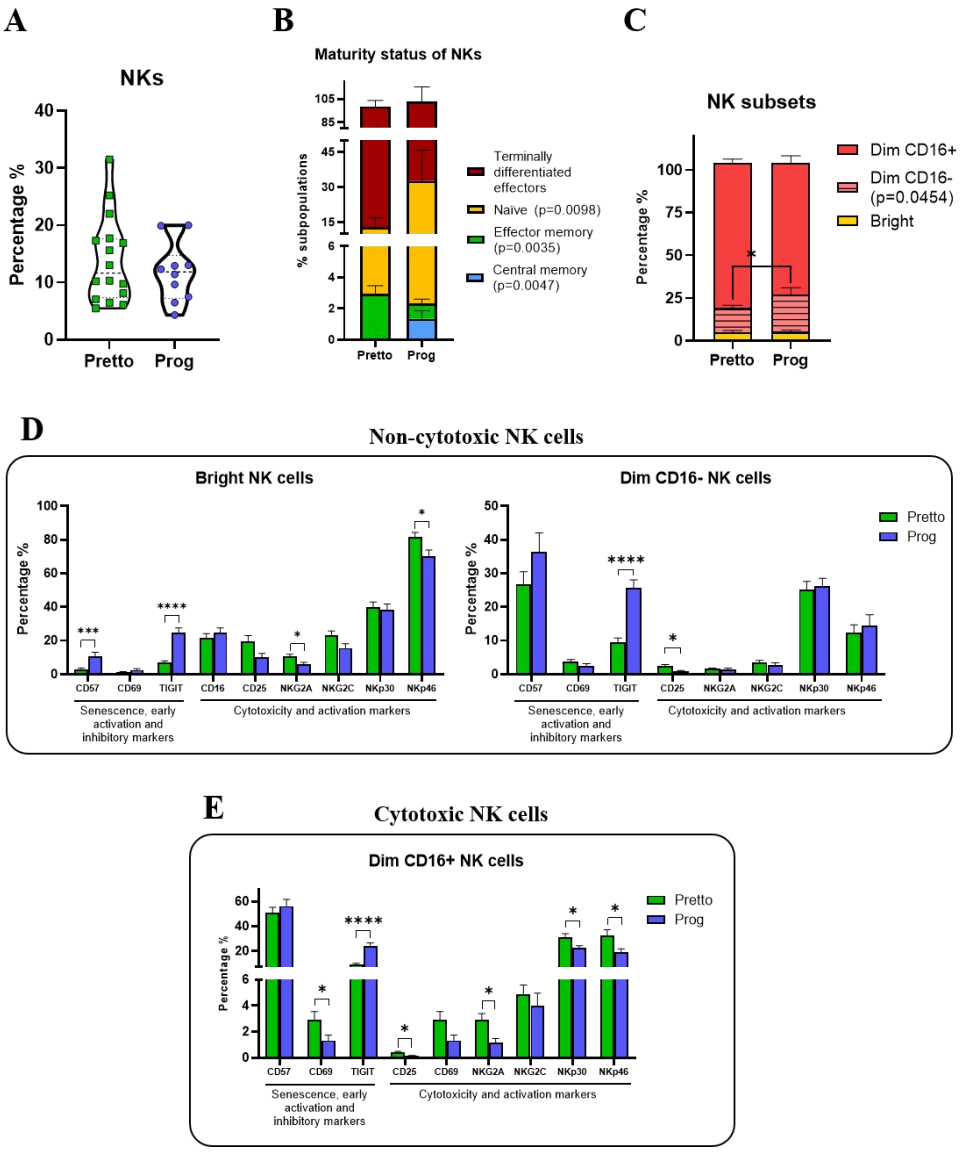


**Figure 7.** Phenotypic changes in circulating NK cell populations between pretreatment and progression samples in responders (R). Frequencies of (A) total NK cells; (B) maturation status of NK cells based on CD45RA and CCR7 expression; (C) subsets of NK cells based on the expression of CD56 and CD16; (D) senescence, inhibitory and activation markers in non-cytotoxic NK cells and (E) cytotoxic NK cells.

**Proteomic profiling of NK cells identifies signalling pathways associated with immune dysfunction during disease progression on cetuximab**

Considering the observed immune cell exhaustion at progression, we aimed to elucidate potential mechanisms underlying treatment failure, focusing primarily on NK cells, as they are the main effectors of cetuximab-mediated ADCC. To this end, we compared proteomic profiles between pretreatment and progression samples within both NR and R groups. As shown in Figures 8 and 9, PCA did not clearly separate the two time points [Figure 8A and 9A]. In contrast, partial least squares discriminant analysis (PLS-DA) [Figures 8B and 9B] and unsupervised clustering of the top 100 proteins [Figures 8C and 9C] distinguished pretreatment and progression samples in both NR and R groups, revealing a proteomic signature potentially associated with treatment adaptation or resistant mechanisms of biological relevance.

In the NR group, we identified 70 proteins significantly downregulated and 13 upregulated at progression [Figure 8D], with enrichment of the TGFβ signalling pathway (NES = 0.65, FDR = 0.051; Figure 8E). Conversely, in R patients, 27 proteins were significantly downregulated and 11 upregulated at progression [Figure 9D]. Notably, these changes were associated with enrichment of the apical surface (NES = 0.68, FDR = 0.154; Figure 9E, left), TGF-β (NES = 0.57, FDR = 0.240, Figure 9E, middle) and Notch signalling pathways (NES = 0.54, FDR = 0.221; Figure 9E, right), which may reflect impaired cytotoxic function and consequently reduced ADCC[19-21]. Altogether, these findings suggest an acquired form of resistance to cetuximab in mCRC patients and provide a potential explanation for why some eligible patients fail to respond to first-line treatment.

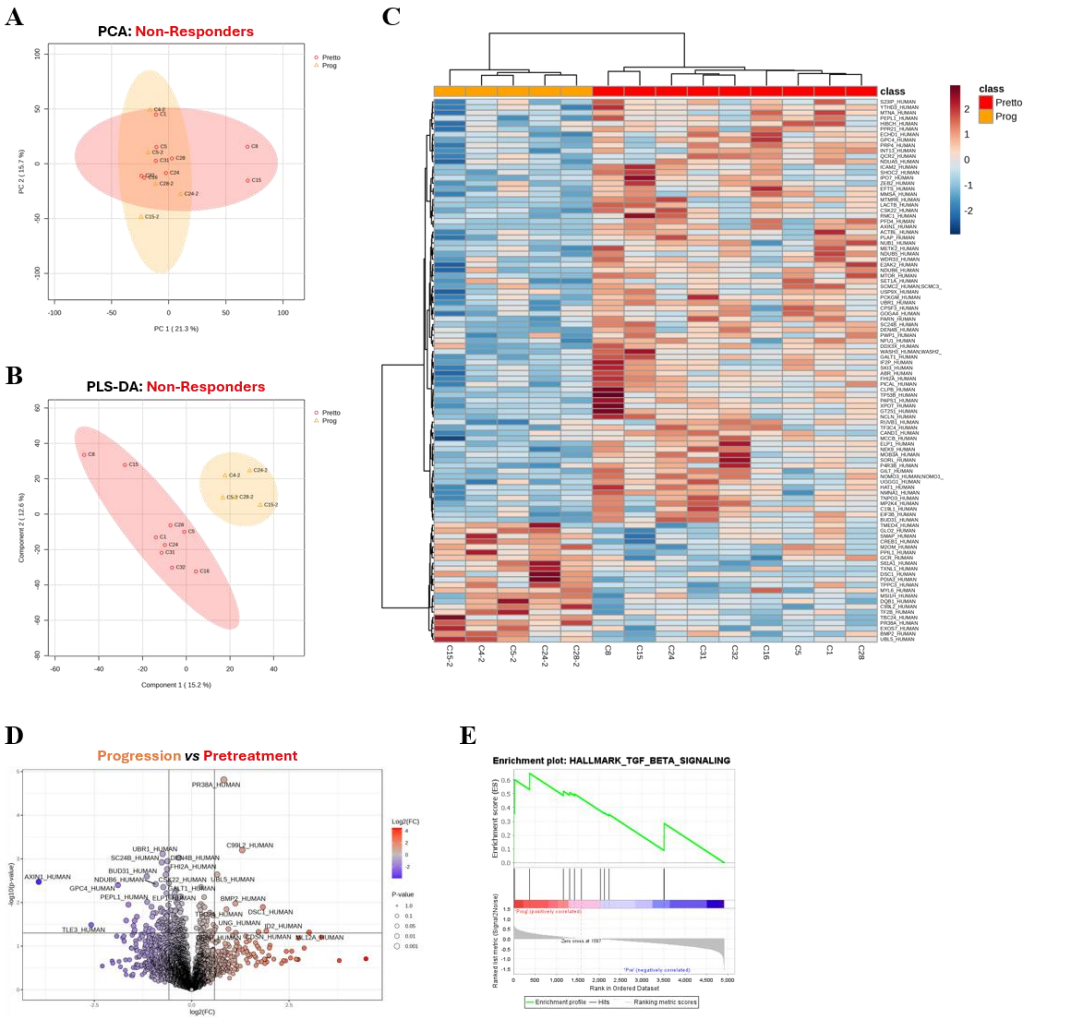


**Figure 8.** NK cell proteomic changes during progression in cetuximab non-responders highlight enrichment of TGFβ signalling. (A) Principal component analysis (PCA) and (B) partial least squares discriminant analysis (PLS-DA) of NK cell proteomes from non-responders at pretreatment and progression stages; (C) Unsupervised hierarchical clustering heatmap of the top 100 most variable proteins; (D) Volcano plot showing differentially expressed proteins between progression and pretreatment samples; (E) Gene set enrichment analysis (GSEA) highlights the TGFβ signalling pathway as significantly upregulated in NK cells at progression.

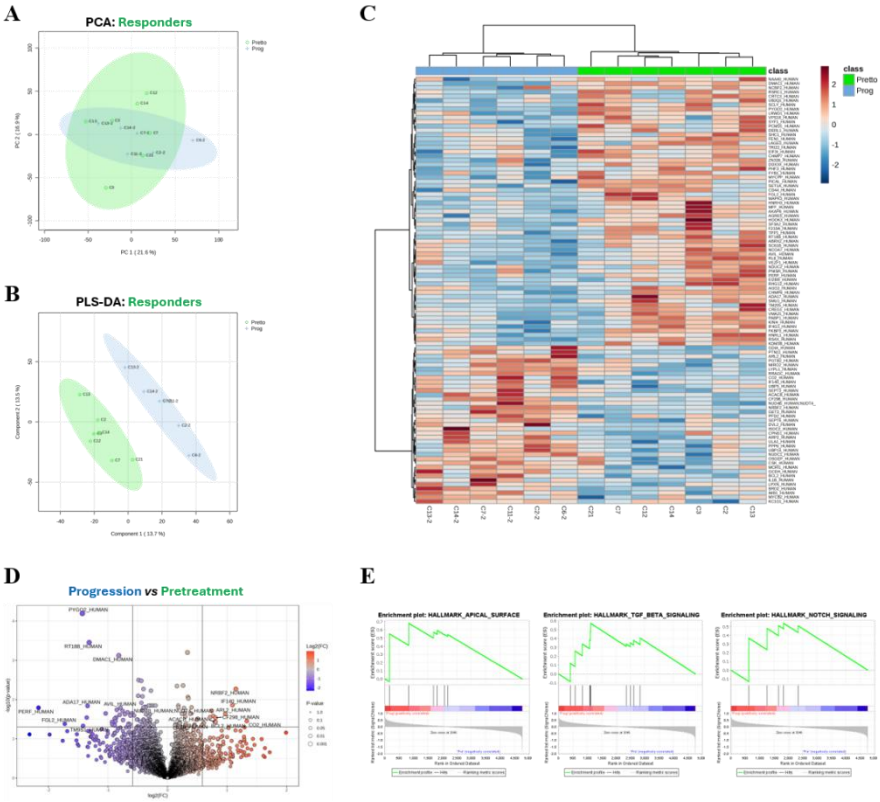


**Figure 9.** NK cell proteomic changes during progression in cetuximab responders highlight several signalling pathway alterations. Proteomic analysis of NK cells from mCRC patients responding to cetuximab, comparing pretreatment and progression samples. (A) Principal component analysis (PCA) and (B) partial least squares discriminant analysis (PLS-DA) of NK cell proteomes from responders at pretreatment and progression stages; (C) Unsupervised hierarchical clustering heatmap of the top 100 most variable proteins; (D) Volcano plot showing differentially expressed proteins between progression and pretreatment samples; (E) Gene set enrichment analysis (GSEA) highlighting upregulated signalling pathways in NK cells at progression.

## DISCUSSION

Current treatments with anti-EGFR monoclonal antibodies, such as cetuximab, have demonstrated improved clinical outcomes in patients with $RAS^{wt}$ mCRC. Despite their clinical benefit, both innate and acquired resistance to anti-EGFR therapy remain major challenges in treatment[22]. Targeted therapies often provide an initial response in mCRC patients, but continuous exposure can promote the selection of resistant clones, which may give rise to more aggressive tumour phenotypes[23,24]. This acquired resistance usually involves alterations in drug transport, secondary mutations in the EGF receptor or *RAS*-related genes, and activation of compensatory signalling pathways, among

others[24-27]. Patients may also develop multidrug resistance, which represents a major obstacle to second-line treatment strategies[24]. Recently, the concept of "immunogenicity" has gained increasing attention, as the functional status of the immune system is known to play a key role in tumour progression and development of tumours[12,28-30], and may explain why some otherwise eligible patients eventually stop responding to therapy.

In our cohort, men were more frequently represented than women, which aligns with the consistently higher incidence of left-sided tumours in males, whereas right-sided CRC is more common in females[31]. Moreover, since cetuximab is indicated for patients with RAS *wild-type*, left-sided tumours, this selection criterion likely contributes to the predominance of male patients in our study.

Regarding clinical-pathological data, no relevant differences were observed for most variables when comparing R and NR patients, except for those related to survival, with NR patients showing shorter OS and TTP compared to R patients, as previously reported[12]. Notably, patients with resectable tumours who received more cetuximab cycles were more likely to respond to treatment, suggesting that less advanced disease and longer treatment exposure may be associated with better clinical outcomes. Within the NR group, patients with smaller, rectal tumours and poorer ECOG performance status exhibited the worst prognosis. This may reflect the fact that rectal tumours are often treated with radiotherapy and/or chemotherapy before metastasis, potentially selecting for resistant clones that acquire more aggressive characteristics[32]. In contrast, R patients with left-sided tumours, a single metastatic site and those who underwent metastasectomy showed longer TTP, which may reflect an initial favourable response to treatment followed by the eventual development of acquired resistance[33].

Our analysis of baseline circulating immune mediators effectively distinguished mCRC patients according to their response to cetuximab, revealing a proinflammatory and immunosuppressive profile in non-responding patients. Notably, a subset of plasma proteins, including ARG1, HO-1, and TNFSF14, demonstrated strong predictive and prognostic value, enabling the development of a risk score associated with disease progression during cetuximab treatment. We found seven upregulated proteins at baseline in NR patients, potentially reflecting a proinflammatory state associated with poor clinical

outcomes. Hence, ARG1, which is constitutively expressed by neutrophils during inflammation, depletes extracellular L-arginine, impairing the proliferation and effector functions of T and NK cells[34]. Additionally, elevated HO-1, soluble EGF, TNFRSF12A, TNFSF14, CD40L, and CASP8 may promote cetuximab resistance and tumour progression by impairing immune cytotoxicity[35], enhancing EGFR signalling[36], regulating apoptosis, promoting angiogenesis[37-39], and facilitating immune escape and tumour cell migration[40-43]. Taken together, these alterations indicate an inflammatory and dysfunctional immune landscape that impairs NK cell–mediated ADCC, which may underlie the lack of response to cetuximab. By contrast, in responder patients, elevated NOS3, CXCL5, CCL17, IL-12/IL12RB1, IFN-γ, CD70, and KLRD1 at baseline may underlie cetuximab efficacy by shaping a supportive immune landscape. NOS3-derived nitric oxide maintains immune balance[44,45], while CXCL5 and CCL17 promote recruitment of cytotoxic lymphocytes into the tumour microenvironment[46-49]. IL-12/IL12RB1 and IFN-γ drive Th1 polarisation and activate NK and $CD8^+$ T cell responses[50,51]. Besides, soluble CD70 enhances $CD8^+$ cytotoxicity and establishes antitumour memory[52-55], while KLRD1 modulates NK cell activity[56]. Collectively, the high baseline levels of these proteins in the plasma of R patients reflect a coordinated potent antitumour immunity capacity that may contribute to the favourable response to cetuximab treatment.

We have previously reported a cetuximab resistance-associated immune signature in NR patients, characterised by high expression of TIGIT and other inhibitory receptors in circulating NK cells[12]. A previous study found a wide range of transcriptional, molecular and cellular markers of the immune exhaustion associated with high TNM-stage CRC tumours[15], identifying several proteins involved in tumour progression. However, unlike our study, treatment response was not assessed, and the analysis did not address why patients initially expected to respond ultimately fail to benefit from therapy. In our study, the immunophenotypic changes between pre-treatment and progression samples of NR patients were minimal. However, profound changes were found in responding patients. For example, T cells showed a shift toward a more naïve phenotype, along with a decrease in memory and effector functions. Moreover, several inhibitory receptors were upregulated in NK cells in progression samples, most notably TIGIT and CD57 across all NK cell populations, and reduced activation markers, driving reduced cytotoxicity and a more senescent phenotype. Notably, when we explored the mechanisms underlying

treatment failure, we found that TGFβ, Notch and apical surface signalling pathways were enriched in progression samples of patients that initially responded to cetuximab. In this regard, TGFβ downregulates CD16 on NKs, impairing ADCC[57], and this was confirmed by our finding of reduced CD16 expression in the $CD56^{Dim}$ subset, which is consistent with a shift towards a less cytotoxic phenotype[58]. Besides, the Notch signalling pathway is known to be a key regulator of NK function, and dysregulated Notch signalling in CRC promotes immune evasion and tumour progression by altering the balance and function of immune cells[59].

An important consideration is that all patients received cetuximab in combination with chemotherapy. Therefore, chemotherapy-related immune modulation, as well as potential interactions between cetuximab and the different chemotherapy backbones, cannot be completely excluded. However, in our cohort, the distribution of the administered chemotherapy agents did not differ significantly between responders and non-responders (*Fisher's exact test*, $p = 0.4285$), so we could suggest that the observed differences between conditions were due to the exposure to cetuximab.

Our findings also supported the dysregulation of the apical surface signalling pathway, which is essential for NK cell polarity and immunological synapse formation between NK cells and target cells[60,61]. Importantly, the convergence of these alterations on NK cells relevant to cetuximab activity provides a biologically mechanism to acquired resistance. Cetuximab-mediated ADCC depends on the interaction between antibody-bound tumour cells and CD16-expressing NK cells[62], so the observed reduction in CD16 expression, together with the increased inhibitory/senescent phenotype and alterations in pathways involved in NK cell cytotoxicity and immune synapse formation, is consistent with impaired cetuximab-mediated cytotoxicity. Impaired polarisation of the microtubule-organising centre and lytic granules has been reported to be responsible for the formation of unstable immune synapses[63]. Our results suggest that rearrangement of receptors, such as CD16, on the NK cell surface may underlie this dysfunction, thereby compromising cytotoxic efficacy and promoting immune escape[64]. Altogether, our findings reveal an acquired form of resistance to cetuximab not previously described in mCRC patients and offer an explanation for why some eligible patients fail to respond to first-line treatment.

## CONCLUSIONS

Our study supports that baseline circulating immune mediators can distinguish mCRC patients according to their response to cetuximab, with non-responders exhibiting a proinflammatory and immunosuppressive profile. Disease progression is associated with extensive immune remodelling, including impaired cytotoxicity, loss of memory function, and increased exhaustion of T and NK cells, providing potential novel mechanisms of acquired resistance in patients who initially benefit from cetuximab-based therapy. These findings have important clinical implications, as they may enable early identification of patients at higher risk of treatment failure, guide personalised therapeutic strategies, and support the development of robust predictive and prognostic tools to optimise cetuximab-based treatment in mCRC.

## DECLARATIONS

### Acknowledgements

The authors extend their special thanks to all the patients and their families; to E. Peralbo from the Flow Cytometry Unit and E. Chicano from the Proteomics Unit at IMIBIC, as well as C. Gutiérrez and I. Reina for their invaluable support and collaboration in this work.

### Author's contributions

Investigation, methodology, and writing-original draft: Navarrete-Sirvent C;
Methodology and investigation: Rivas-Crespo A, Mantrana A;
Formal analysis: Herrera-Casanova N.M;
Investigation: Díaz-Chacón A, Sánchez-Montero M.T;
Resources: Ortiz-Morales M.J., Pulido G, Cano M.T., Gómez-España A, González-Fernández R;
Methodology and writing—review & editing: Guil-Luna S;
Conceptualization, writing—review & editing, and supervision: Rodríguez-Ariza A, Aranda E.

### Availability of data and materials

The authors confirm that the data supporting the findings of this study are available in the PRIDE database with the project accession ID PXD057989. Other data supporting the

findings of this study are available from the corresponding authors upon reasonable request.

## AI and AI-assisted tools statement

Not applicable

## Financial support and sponsorship

Instituto de Salud Carlos III (ISCIII), “PI20/00997”, co-funded by the European Union. A.R.A. was funded with a researcher contract through the program “Nicolás Monardes” from Junta de Andalucía.

## Conflicts of interest

All authors declared that there are no conflicts of interest.

## Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Reina Sofia Hospital (COLO-NK, Committee Reference 4884, version 1.0—01/12/2020) in accordance with the World Medical Association Code of Ethics (Declaration of Helsinki, 2017). Informed consent was obtained from all the patients before their enrolment in the study.

## Consent for publication

Not applicable



## REFERENCES

[1] Siegel RL, Miller KD, Fedewa SA, et al. Colorectal cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(3):177-93.[DOI: 10.3322/caac.21395]

[2] Chen Y, Zheng X, Wu C. The Role of the Tumor Microenvironment and Treatment Strategies in Colorectal Cancer. *Front Immunol*. *Frontiers Media S.A.* 2021;12.[DOI: 10.3389/fimmu.2021.792691]

[3] Zheng E, Włodarczyk M, Węgiel A, et al. Navigating through novelties concerning mCRC treatment—the role of immunotherapy, chemotherapy, and targeted therapy in mCRC. *Front Surg*. 2024;11.[DOI: 10.3389/fsurg.2024.1398289]

[4] Zhou J, Ji Q, Li Q. Resistance to anti-EGFR therapies in metastatic colorectal cancer: underlying mechanisms and reversal strategies. *Journal of Experimental and Clinical Cancer Research*. *BioMed Central Ltd*. 2021;40(1).[DOI: 10.1186/s13046-021-02130-2]

[5] Saoudi González N, Ros J, Baraibar I, et al. Cetuximab as a Key Partner in Personalized Targeted Therapy for Metastatic Colorectal Cancer. *Cancers (Basel)*. *Multidisciplinary Digital Publishing Institute (MDPI)*. 2024;16(2).[DOI: 10.3390/cancers16020412]

[6] Sastre J, Grávalos C, Rivera F, et al. First-Line Cetuximab Plus Capecitabine in Elderly Patients with Advanced Colorectal Cancer: Clinical Outcome and Subgroup Analysis According to KRAS Status from a Spanish TTD Group Study . *Oncologist*. 2012;17(3):339-45.[DOI: 10.1634/theoncologist.2011-0406]

[7] Kocoglu H, Velibeyoglu FM, Karaca M, Tural D. Clinical efficacy and drug resistance of anti-epidermal growth factor receptor therapy in colorectal cancer. *World J Gastrointest Oncol*. 2016;8(1):1-7.[DOI: 10.4251/wjgo.v8.i1.1]

[8] Mirone G, Shukla A, Marfe G. Signaling mechanisms of resistance to EGFR- and Anti-Angiogenic Inhibitors cancer. *Crit Rev Oncol Hematol*. *Elsevier Ireland Ltd*. 2016;97:85-95.[DOI: 10.1016/j.critrevonc.2015.08.012]

[9] Cruz-Duarte R, de Almeida CR, Negrão M, et al. Predictive and Therapeutic Implications of a Novel PLCγ1/SHP2-Driven Mechanism of Cetuximab Resistance in Metastatic Colorectal Cancer. *Clinical Cancer Research*. 2022;28(6):1203-16.[DOI: 10.1158/1078-0432.CCR-21-1992]

[10] Baysal H, De Pauw I, Zaryouh H, et al. The Right Partner in Crime: Unlocking the Potential of the Anti-EGFR Antibody Cetuximab via Combination With Natural Killer Cell Chartering Immunotherapeutic Strategies. *Front Immunol*. *Frontiers Media S.A*. 2021;12.[DOI: 10.3389/fimmu.2021.737311]

[11] Chen S, Li X, Chen R, Yin M, Zheng Q. Cetuximab intensifies the ADCC activity of adoptive NK cells in a nude mouse colorectal cancer xenograft model. *Oncol Lett*. 2016;12(3):1868-76.[DOI: 10.3892/ol.2016.4835]

[12] Navarrete-Sirvent C, Mantrana A, Rivas-Crespo A, et al. Imbalanced NK cell subpopulations and TIGIT expression limit cetuximab efficacy in colorectal cancer: A

promising target for treatment enhancement. *Clin Transl Med*. 2025;15(6).[DOI: 10.1002/ctm2.70351]

[13] Caligiuri MA. Human natural killer cells. *Blood*. 2008;112(3):461-9.[DOI: 10.1182/blood-2007-09-077438]

[14] Cooper, M. A., Fehniger, T. A., & Caligiuri, M. A. (2001). The biology of human natural killer-cell subsets. *Trends in immunology*, *22*(11), 633-40.[DOI: 10.1016/s1471-4906(01)02060-9]

[15] Sorrentino C, D'Antonio L, Fieni C, Ciummo SL, Di Carlo E. Colorectal Cancer-Associated Immune Exhaustion Involves T and B Lymphocytes and Conventional NK Cells and Correlates With a Shorter Overall Survival. *Front Immunol*. 2021;12:778329.[DOI: 10.3389/fimmu.2021.778329]

[16] Kizmaz MA, Simsek A, Bozkurt T, et al. Effector memory T cell subset CD45RA−CCR7−CD27−CD28− EM3 increases in direct proportion to the disease severity of COVID-19. *Scand J Immunol*. 2023;97(1):e13217.[DOI: 10.1111/sji.13217]

[17] Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 1999 401:6754*. 1999;401(6754):708-12.[DOI: 10.1038/44385]

[18] Perez-Riverol Y, Bai J, Bandla C, et al. The PRIDE database resources in 2022: A hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res*. 2022;50(D1):D543-52.[DOI: 10.1093/nar/gkab1038]

[19] Aldeghaither DS, Zahavi DJ, Murray JC, et al. A Mechanism of Resistance to Antibody-Targeted Immune Attack. *Cancer Immunol Res*. 2018;7(2):230.[DOI: 10.1158/2326-6066.CIR-18-0266]

[20] Turlej E, Domaradzka A, Radzka J, Drulis-Fajdasz D, Kulbacka J, Gizak A. Cross-Talk Between Cancer and Its Cellular Environment—A Role in Cancer Progression. *Cells*. 2025;14(6):403.[DOI: 10.3390/CELLS14060403]

[21] Wong JKM, McCulloch TR, Alim L, et al. TGF-β signalling limits effector function capacity of NK cell anti-tumour immunity in human bladder cancer. *EBioMedicine*. 2024;104.[DOI: 10.1016/j.ebiom.2024.105176]

[22] Parseghian CM, Napolitano S, Loree JM, Kopetz S. Mechanisms of innate and acquired resistance to anti-EGFR therapy: A review of current knowledge with a focus on rechallenge therapies. *Clinical Cancer Research*. *American Association for Cancer Research Inc.* 2019;25(23):6899-908.[DOI: 10.1158/1078-0432.CCR-19-0823]

[23] Bitar S Al, El-Sabban M, Doughan S, Abou-Kheir W. Molecular mechanisms targeting drug-resistance and metastasis in colorectal cancer: Updates and beyond. *World J Gastroenterol*. 2023;29(9):1395-426.[DOI: 10.3748/wjg.v29.i9.1395]

[24] Parseghian CM, Napolitano S, Loree JM, Kopetz S. Mechanisms of innate and acquired resistance to anti-EGFR therapy: A review of current knowledge with a focus on rechallenge therapies. *Clinical Cancer Research*. 2019;25(23):6899-908.[DOI: 10.1158/1078-0432.CCR-19-0823]

[25] Wang Q, Shen X, Chen G, Du J. Drug Resistance in Colorectal Cancer: From Mechanism to Clinic. *Cancers (Basel)*. 2022;14(12):2928.[DOI: 10.3390/cancers]

[26] Albadari N, Xie Y, Li W. Deciphering treatment resistance in metastatic colorectal cancer: roles of drug transports, EGFR mutations, and HGF/c-MET signaling. *Front Pharmacol*. 2023;14.[DOI: 10.3389/fphar.2023.1340401]

[27] Zhao B, Wang L, Qiu H, et al. Mechanisms of resistance to anti-EGFR therapy in colorectal cancer. *Oncotarget.* 2017;8(3):3980-4000.[PMID: 28002810 PMCID: PMC5354808 DOI: 10.18632/oncotarget.14012]

[28] Muntasell A, Servitja S, Cabo M, et al. High numbers of circulating CD57+ NK cells associate with resistance to her2-specific therapeutic antibodies in HER2+ primary breast cancer. *Cancer Immunol Res*. 2019;7(8):1280-92.[DOI: 10.1158/2326-6066.CIR-18-0896]

[29] Muraro E, De Zorzi M, Miolo G, et al. KIR-HLA Functional Repertoire Influences Trastuzumab Efficiency in Patients With HER2-Positive Breast Cancer. *Front Immunol*. 2022;12.[DOI: 10.3389/fimmu.2021.791958]

[30] de Vries NL, Swets M, Vahrmeijer AL, Hokland M, Kuppen PJK. The Immunogenicity of Colorectal Cancer in Relation to Tumor Development and Treatment. *Int J Mol Sci*. 2016;17(7):1030.[DOI: 10.3390/ijms17071030]

[31] Joo HJ, Lee HS, Jang BI, et al. Sex-specific differences in colorectal cancer: A multicenter retrospective cohort study. *Cancer Rep (Hoboken)*. 2023;6(8).[DOI: 10.1002/CNR2.1845]

[32] Board PATE. Rectal Cancer Treatment (PDQ®). *National Cancer Institute*. Published online 2023. Accessed March 20, 2026. https://www.cancer.gov/types/colorectal/patient/rectal-treatment-pdq

[33] Van Emburgh BO, Arena S, Siravegna G, et al. Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. *Nat Commun*. 2016;7:13665.[DOI: 10.1038/ncomms13665]

[34] Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I Production in the Tumor Microenvironment by Mature Myeloid Cells Inhibits T-Cell Receptor Expression and Antigen-Specific T-Cell Responses. *Cancer Res*. 2004;64:5839-49. http://aacrjournals.org/cancerres/article-pdf/64/16/5839/2516899/zch01604005839.pdf

[35] Seo GS, Jiang WY, Chi JH, et al. Heme oxygenase-1 promotes tumor progression and metastasis of colorectal carcinoma cells by inhibiting antitumor immunity. *Oncotarget*. 2015;6(23):19792.[DOI: 10.18632/ONCOTARGET.4075]

[36] Gharib E, Robichaud GA. From Crypts to Cancer: A Holistic Perspective on Colorectal Carcinogenesis and Therapeutic Strategies. *Int J Mol Sci*. 2024;25(17):9463.[DOI: 10.3390/ijms25179463]

[37] Sun L De, Zhang LL, Wan Z, et al. TNFRSF12A expression in stomach adenocarcinoma and its preliminary role in predicting immunotherapy response. *Front Immunol*. 2025;16:1578068.[DOI: 10.3389/FIMMU.2025.1578068]

[38] Holmes TD, Wilson EB, Black EVI, et al. Licensed human natural killer cells aid dendritic cell maturation via TNFSF14/LIGHT. *Proc Natl Acad Sci U S A*. 2014;111(52):E5688-96.[DOI: 10.1073/pnas.1411072112]

[39] Han M, Sun Y, Zhao W, et al. Comprehensive characterization of TNFSF14/LIGHT with implications in prognosis and immunotherapy of human gliomas. *Front Immunol*. 2022;13.[DOI: 10.3389/fimmu.2022.1025286]

[40] Herold Z, Herold M, Herczeg G, et al. High plasma CD40 ligand level is associated with more advanced stages and worse prognosis in colorectal cancer. *World J Clin Cases*. 2022;10(13):4084.[DOI: 10.12998/WJCC.V10.I13.4084]

[41] Kostova I, Mandal R, Becker S, Strebhardt K. The role of caspase-8 in the tumor microenvironment of ovarian cancer. *Cancer Metastasis Rev*. 2020;40(1):303.[DOI: 10.1007/S10555-020-09935-1]

[42] Keller N, Ozmadenci D, Ichim G, Stupack D. Caspase-8 function, and phosphorylation, in cell migration. *Semin Cell Dev Biol*. 2018;82:105-17.[DOI: 10.1016/J.SEMCDB.2018.01.009]

[43] Barbero S, Mielgo A, Torres V, et al. Caspase-8 association with the focal adhesion complex promotes tumor cell migration and metastasis. *Cancer Res*. 2009;69(9):3755-63.[PMID: 19383910 PMCID: PMC2684981 DOI: 10.1158/0008-5472.CAN-08-3937]

[44] Cunha PP, Bargiela D, Minogue E, et al. Infiltration of Tumors Is Regulated by T cell–Intrinsic Nitric Oxide Synthesis. *Cancer Immunol Res*. 2023;11(3):351-63.[PMID: 36574610 PMCID: PMC9975666 DOI: 10.1158/2326-6066.CIR-22-0387]

[45] Ibiza S, Serrador JM. The role of nitric oxide in the regulation of adaptive immune responses. *Inmunología*. 2008;27(3):103-117.[DOI: 10.1016/S0213-9626(08)70058-1]

[46] Zajkowska M, Dulewicz M, Kulczyńska-Przybik A, et al. CXCL5 and CXCL14, but not CXCL16 as potential biomarkers of colorectal cancer. *Sci Rep*. 2023;13(1).[DOI: 10.1038/S41598-023-45093-4]

[47] Zhang W, Wang H, Sun M, et al. CXCL5/CXCR2 axis in tumor microenvironment as potential diagnostic biomarker and therapeutic target. *Cancer Commun*. 2020;40(2-3):69.[DOI: 10.1002/CAC2.12010]

[48] Li Y, Cao H, Jiang Z, et al. CCL17 acts as an antitumor chemokine in micromilieu-driven immune skewing. *Int Immunopharmacol*. 2023;118.[DOI: 10.1016/j.intimp.2023.110078]

[49] Ye T, Zhang X, Dong Y, et al. Chemokine CCL17 Affects Local Immune Infiltration Characteristics and Early Prognosis Value of Lung Adenocarcinoma. *Front Cell Dev Biol*. 2022;10.[DOI: 10.3389/fcell.2022.816927]

[50] Kumagai K, Takeda K, Hashimoto W, et al. Interleukin-12 as an inducer of cytotoxic effectors in anti-tumor immunity. *Int Rev Immunol*. 1997;14(2-3):229-56.[DOI: 10.3109/08830189709116854]

[51] Bhat AA, Nisar S, Singh M, et al. Cytokine- and chemokine-induced inflammatory colorectal tumor microenvironment: Emerging avenue for targeted therapy. *Cancer Commun*. 2022;42(8):689-715.[DOI: 10.1002/cac2.12295]

[52] Cormary C, Gonzalez R, Faye JC, Favre G, Tilkin-Mariamé AF. Induction of T-cell antitumor immunity and protection against tumor growth by secretion of soluble human CD70 molecules. *Cancer Gene Ther*. 2004;11(7):497-507.[DOI: 10.1038/SJ.CGT.7700708]

[53] Miller J, Eisele G, Tabatabai G, et al. Soluble CD70: A novel immunotherapeutic agent for experimental glioblastoma: Laboratory investigation. *J Neurosurg*. 2010;113(2):280-5.[DOI: 10.3171/2009.11.JNS09901]

[54] Jacobs J, Deschoolmeester V, Zwaenepoel K, et al. CD70: An emerging target in cancer immunotherapy. *Pharmacol Ther*. 2015;155:1-10.[DOI: 10.1016/J.PHARMTHERA.2015.07.007]

[55] Flieswasser T, Van den Eynde A, Van Audenaerde J, et al. The CD70-CD27 axis in oncology: the new kids on the block. *J Exp Clin Cancer Res*. 2022;41(1):12.[DOI: 10.1186/S13046-021-02215-Y]

[56] Dong C, Lin Z, Hu Y, Lu Q. KLRD1 (CD94): A Prognostic Biomarker and Therapeutic Candidate in Head and Neck Squamous Cell Carcinoma. *J Cancer*. 2025;16(3):982-95.[DOI: 10.7150/JCA.104762]

[57] Wong JKM, McCulloch TR, Alim L, et al. TGF-β signalling limits effector function capacity of NK cell anti-tumour immunity in human bladder cancer. *EBioMedicine*. 2024;104.[DOI: 10.1016/j.ebiom.2024.105176]

[58] Viel S, Marçais A, Guimaraes FSF, et al. TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Sci Signal*. 2016;9(415).[DOI: 10.1126/scisignal.aad1884]

[59] Sun J, Chen Y, Xu Z, Wang W, Li P. Notch signaling in the tumor immune microenvironment of colorectal cancer: mechanisms and therapeutic opportunities. *J Transl Med*. 2025;23(1):315.[DOI: 10.1186/s12967-025-06282-z]

[60] Wülfing C, Purtic B, Klem J, Schatzle JD. Stepwise cytoskeletal polarization as a series of checkpoints in innate but not adaptive cytolytic killing. *Proc Natl Acad Sci U S A*. 2003;100(13):7767-72.[PMID: 12802007 PMCID: PMC164662 DOI: 10.1073/pnas.1336920100]

[61] Chao Z, Mei Q, Yang C, et al. Immunological synapse: structures, molecular mechanisms and therapeutic implications in disease. *Signal Transduct Target Ther*. 2025;10(1):254.[DOI: 10.1038/S41392-025-02332-6]

[62] Monteverde M, Milano G, Strola G, et al. The relevance of ADCC for EGFR targeting: A review of the literature and a clinically-applicable method of assessment in patients. *Crit Rev Oncol Hematol*. 2015;95(2):179-90.[DOI: 10.1016/j.critrevonc.2015.02.014]

[63] Friedman D, Simmonds P, Hale A, et al. Natural killer cell immune synapse formation and cytotoxicity are controlled by tension of the target interface. *J Cell Sci*. 2021;134(7):jcs258570.[DOI: 10.1242/JCS.258570]

[64] Ross P, Fatima H, Leaman DP, et al. Spatial localization of CD16a at the human NK cell ADCC lytic synapse. *bioRxiv*. Published online August 10, 2024:2024.08.09.605851.[DOI: 10.1101/2024.08.09.605851]