

Molecular pathways driving clarithromycin benefit in community-acquired pneumonia: analysis of the ACCESS randomised trial



Emmanouil Stylianakis,^{a,b,c,s} Nikolaos Kakavoulis,^{d,s} Spyros Foutadakis,^b Panagiotis Koufargyris,^{a,b} George Niotis,^e Karolina Akinosoglou,^f Konstantina Iliopoulou,^g Antigone Kotsaki,^a Marios Chatzopoulos,^h Lamprini Skorda,ⁱ Vassiliki Tzavara,^j George Chrysos,^k Styliani Gerakari,^l Paraskevi Katsaounou,^m Theano Kontopoulou,ⁿ George N. Dalekos,^o Vinod Kumar,^c Mihai G. Netea,^{c,p} Antonio Torres,^q Michael Niederman,^r and Evangelos J. Giamarellos-Bourboulis^{a,b,*}



^a4th Department of Internal Medicine, National and Kapodistrian University of Athens, Medical School, Athens, Greece

^bHellenic Institute for the Study of Sepsis, Athens, Greece

^cDepartment of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, the Netherlands

^d1st Department of Internal Medicine, Thrasio General Hospital of Eleusis, Athens, Greece

^e2nd Department of Propedeutic Medicine, National and Kapodistrian University of Athens, Medical School, Athens, Greece

^fDepartment of Internal Medicine, University of Patras, Rion, Greece

^g2nd Department of Internal Medicine, Thrasio General Hospital of Eleusis, Athens, Greece

^h1st Department of Internal Medicine, G. Gennimatas General Hospital of Athens, Athens, Greece

ⁱ3rd Department of Internal Medicine and Infectious Diseases Unit, Korgialeneion-Benakeion General Hospital, Athens, Greece

^j1st Department of Internal Medicine, Korgialeneion-Benakeion General Hospital, Athens, Greece

^k2nd Department of Internal Medicine, Tzaneio General Hospital, Piraeus, Greece

^lEmergency Department, Tzaneio General Hospital, Piraeus, Greece

^m1st Department of Critical Care and Pulmonary Medicine, National and Kapodistrian University of Athens, Medical School, Athens, Greece

ⁿ1st Department of Internal Medicine, Evangelismos Athens General Hospital, Athens, Greece

^oDepartment of Medicine and Research Laboratory of Internal Medicine, National Expertise Center of Greece in Autoimmune Liver Diseases, Full Member of the European Reference Network on Hepatological Diseases (ERN RARE-LIVER), General University Hospital of Larissa, 41110 Larissa, Greece

^pDepartment of Immunology and Metabolism, Life and Medical Sciences Institute, University of Bonn, Bonn, Germany

^qDepartment of Critical Care Medicine, University of Barcelona, Spain

^rDivision Pulmonary and Critical Care Medicine, Weill Cornell Medicine, New York Presbyterian/ Weill Cornell Medical Center, USA

Summary

Background The double-blind randomised controlled ACCESS trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT04724044) NCT04724044), conducted in hospitalised patients with community-acquired pneumonia (CAP), showed that the addition of clarithromycin to the standard-of-care (SoC) provided earlier resolution of symptoms during the first 72 h and prevented progression to respiratory failure and secondary sepsis. The molecular pathways underpinning these favourable effects of action of clarithromycin were investigated in this research.

Methods Gene expression was compared between treatment arms and within each arm between baseline and 72 h using DESeq2. Reactome pathway and Gene Ontology analyses were followed. Cytokine stimulation data of peripheral blood mononuclear cells (PBMCs) from the same time points were also analysed.

Findings Trajectory analysis showed that the unique upregulated genes in the clarithromycin group were mainly involved in pathways of T-cell activation and positive regulation of cytokine production. The expression of genes encoding for the major histocompatibility complex II was upregulated; genes encoding for the receptors of interleukin (IL)-1 and for neutrophil degranulation were downregulated. The production of cytokines of the IL-1 cluster was positively associated with progression to respiratory failure; fewer patients treated with clarithromycin experienced increases in production of IL-1 cytokines (odds ratio 0.47; 95% confidence intervals 0.23–0.96; $p = 0.038$). The production of monocyte-derived pro-inflammatory cytokines and chemokines (other than the IL-cytokines) was positively associated with attainment of the primary endpoint; more patients treated with

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*Corresponding author. 4th Department of Internal Medicine, ATTIKON University Hospital, 1 Rimini Street, 124 62, Athens, Greece.

E-mail address: egiamarel@med.uoa.gr (E.J. Giamarellos-Bourboulis).

^sEqual contribution.

clarithromycin exhibited increases in production of monocyte-derived cytokines and chemokines (odds ratio 1.87; 95% confidence intervals 1.05–3.35; $p = 0.035$). Production of anti-inflammatory cytokines by PBMCs was also attenuated in clarithromycin-treated patients.

Interpretation Treatment with clarithromycin attenuates the IL-1 pathway, increases production of other monocyte-derived pro-inflammatory cytokines and chemokines, improves antigen presentation and decreases neutrophil degranulation. These effects may explain the clinical benefit of clarithromycin in hospitalised patients with CAP.

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Keywords: Pneumonia; Clarithromycin; Early response; Neutrophils; Cytokines; Chemokines

Research in context

Evidence before this study

The ACCESS randomised controlled trial showed clinical benefit from the addition of macrolide to standard of care antimicrobial therapy in patients hospitalised with community-acquired pneumonia. Addition of clarithromycin led to early improvement of the clinical signs of pneumonia in the first 72 h, accompanied by a decrease of the level of organ dysfunction and favourable procalcitonin kinetics.

Added value of this study

The molecular pathways of action of clarithromycin in ACCESS trial were investigated by RNA sequencing and by cytokine stimulation from isolated peripheral blood mononuclear cells before the start of the study drug and at 72 h. Both approaches identified effects aligned towards

three main pathways: i) attenuation of the interleukin-1 cascade, ii) increased production of monocyte-derived pro-inflammatory cytokines and chemokines coupled with improvement of antigen presentation, and iii) reduction of neutrophil degranulation.

Implications of all the available evidence

ACCESS provides evidence from a prospective, randomised controlled trial of how trajectories of immune function from baseline to the censoring of the primary endpoint are associated with clinical benefits. This current study links trajectory data from RNA sequencing to data from cytokine stimulation of circulating immune cells with clinical benefit in the field of infectious diseases.

Introduction

Community-acquired pneumonia (CAP) is one of the leading causes of morbidity and mortality.¹ The major complication of severe CAP is respiratory failure (RF) necessitating mechanical ventilation (MV) and admission to the Intensive Care Unit. Current guidelines by the European Respiratory Society, the European Society of Clinical Microbiology and Infectious Diseases, the European Society of Intensive Care Medicine and the Latin Thoracic Society suggest the co-administration of β -lactams and macrolides for the treatment of severe CAP.² That position is mainly based on meta-analysis of prospective non-randomised studies showing mortality benefit from macrolide treatment but has recently been enhanced by the results of the ACCESS randomised double-blind controlled trial, in which hospitalised patients with CAP were assigned to standard-of-care (SoC) and placebo or SoC and clarithromycin.³ The primary endpoint of the ACCESS trial was early clinical and anti-inflammatory response censored after the first 72 h. This composite outcome, incorporating an at least 50% decrease of the respiratory severity symptom (RSS) score and improvement of organ dysfunction or

systemic inflammation, was attained in 38.3% of patients of the SoC and placebo arm and in 67.9% of patients of the SoC and clarithromycin arm ($p < 0.0001$). This benefit with clarithromycin was associated with a decrease of early mortality by the end-of-treatment (8% in the clarithromycin arm versus 17% in the placebo arm) and less progression to RF necessitating MV (6.0% versus 17.3%).

Related findings pointed towards reversal of CAP-associated immunoparalysis as the most probable explanation of clarithromycin benefit; there was increased production of tumour necrosis factor- α (TNF α) by blood mononuclear cells during the first 72 h; and the blood levels of the anti-inflammatory interleukin (IL)-10 decreased. This was accompanied by a reduction in the incidence of secondary sepsis, which is the clinical hallmark of immunoparalysis.⁴

Results from several previous studies, conducted mostly in patients with chronic lung disorders, suggest that macrolides attenuate host pro-inflammatory responses.^{5–7} The striking clinical benefits of adding clarithromycin to SoC in ACCESS highlight the need for a better understanding of the effect of treatment on

the inflammatory host response. To this end, we performed time-course transcriptomics analysis using RNA sequencing (RNA-seq) in both treatment arms, coupled with a comprehensive panel of cytokine-release assays. Data collection was conducted before the start of the study drug and on day 4, when the primary endpoint was censored. Our aim was to elucidate whether and how the early transcriptomic trajectory and the early changes of cytokine production capacity are linked to the achievement of the primary endpoint and the risk for progression to RF.

Methods

Ethics

ACCESS was a prospective, double-blind, randomised clinical study conducted in 18 internal medicine departments of 18 public Greek hospitals between January 2021 and April 2023, covering the major geographic regions of the country (Alexandroupolis, Attiki, Ioannina, Kerkyra, Larissa and Patras). The protocol was licenced by the National Ethics Committee of Greece (approval 122/20) and the National Organisation for Medicines of Greece (approval IS113/20) (EudraCT number 2020-004452-15; [ClinicalTrials.gov](#) NCT04724044; available as an [Appendix](#)) before the inclusion of the first patient. Written informed consent was provided by the patients or their legal representative. This sub-study is reported according to the CONSORT 2025 checklist.

Trial synopsis

Inclusion criteria for the ACCESS trial were: a) adult patients (≥ 18 years) of either sex; b) new radiological consolidation in patients; c) at least two symptoms of cough, pleuritic chest pain, sputum production and shortness of breath; d) at least two signs of the systemic inflammatory response syndrome; e) total sequential organ failure assessment (SOFA) score ≥ 2 ; and f) blood procalcitonin (PCT) ≥ 0.25 ng/mL. PCT was used to select for patients with bacterial CAP necessitating antibiotics.³ Main exclusion criteria were: intake of any macrolide for the current episode of CAP; oral or intravenous intake of corticosteroids in the last 15 days; infection by the SARS-CoV-2 virus; known infection by the human immunodeficiency virus; neutropenia; any chronic anti-cytokine treatment; hospitalisation for more than 2 consecutive days in the preceding 90 days; QT_c interval on resting electrocardiogram (ECG) of 500 msec or more; history of known congenital long QT syndrome; and pregnancy or lactation.

Patients were double-blind randomised in a 1:1 ratio to treatment with SoC and placebo or SoC and oral clarithromycin; no stratification variables were applied. Study medication was given orally every 12 h for 7 consecutive days unless a patient was discharged earlier. Each oral dose of clarithromycin was 500 mg.

The primary endpoint was evaluated on day 4 (i.e. 72 h from the start of the study drug) and was a composite of a) at least 50% decrease of the baseline RSS score; and b) at least 30% decrease of the baseline SOFA score or favourable PCT kinetics. Favourable PCT kinetics were defined as at least 80% decrease of baseline PCT or blood PCT less than 0.25 ng/mL. Patients were followed up for progression to RF for 90 days.

Transcriptomic analysis

A total of 2.5 mL of whole blood was collected after venipuncture of one forearm vein before the start of the study drug and on day 4 and placed into PAXgene® Blood RNA Tubes (Vacutainer, BD). Total RNA was isolated using the QIASymphony PAXgene® Blood RNA kit. Sequencing libraries were prepared using the NebNext Ultra II Directional RNA kit. Library quality was examined using an Agilent 2100 Bioanalyzer and libraries were quantified with a Qubit fluorometer. Samples were sequenced in single-end mode using a NovaSeq 6000 sequencer at the Greek Genome Center situated at the Biomedical Research Foundation Academy of Athens (BRFAA). The quality of sequencing reads was examined using the FastQC algorithm. Sequencing reads were mapped to the human genome (hg19 version) using HISAT2 with the reverse strand option. Transcript integrity was assessed using the Transcript Integrity Number score from RSeQC.^{8,9} Reads within genes were calculated with HT-Seq count using the reverse stranded option and union mode.¹⁰

Differential expression analysis was performed using DESeq2 after filtering out lowly expressed genes, i.e. genes that had fewer than 50 counts in 20% of the examined samples. Gene expression was compared for each day between the treatment arms and within each arm of treatment across the two time points. Differentially expressed genes (DEGs) were obtained based on a False Discovery Rate (FDR)-adjusted p-value of less than 0.05 and an absolute log₂FoldChange greater than 0.5. Pathway and Gene Ontology (GO) analyses were followed using these genes as an input in the Reactome and GO Biological Processes 2023 databases for humans. Pathways and ontologies with FDR p-values less than 0.05 were considered significant. ReactomePA and EnrichR packages were used for these analyses. All the above computational analyses were implemented using the R programming language (v4.4.2) in RStudio or the Galaxy Suite.¹¹

A similar analysis was followed to identify pathways associated with favourable outcomes among clarithromycin-treated patients. As the number of patients for whom RNA-seq data were available at both timepoints was 86 (45 in the placebo group and 41 in the clarithromycin group) it was decided to define patients as having a favourable outcome if they satisfied all the elements of the primary endpoint, plus lack of

progression to RF and survival to day 8. Patients who did not meet all three conditions were classified as having an unfavourable outcome. The trajectories of DEGs that were uniquely associated with favourable and unfavourable outcomes were compared.

PBMC preparation and cytokine measurements

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 mL of heparin-coated blood collected before the start of the study drug and on day 4 after gradient centrifugation over Ficoll-Hypaque density gradient (Biochrom, Berlin, Germany) at 1400 g at 4 °C for 25 min. After three washings in ice-cold PBS (phosphate buffered saline, pH: 7.2, Biowest, Nuaille, France), PBMCs were counted using a Neubauer plate with trypan blue exclusion of dead cells. PBMCs were incubated in RPMI 1640 (Biochrom, Berlin, Germany) enriched with 100 U/mL of penicillin G and 0.1 mg/mL of streptomycin and distributed on 96-well plates at final volume of 200 µL and density of 5×10^6 /mL. Cells were incubated at 37 °C and 5% CO₂ for 24 h either without/with 10 ng/mL lipopolysaccharide (LPS) of *Escherichia coli* O55:B5 (Sigma, St. Louis, USA), or 5 µg/mL phytohaemagglutinin (PHA) for 48 h (Biowest, Nuaille, France). Cells were also incubated under the same conditions for five days with the culture medium containing 10% v/v foetal bovine serum (Biowest, Nuaille, France) without/with 5×10^6 cfu/mL heat-killed *Candida albicans* (HKCA). After incubation, the supernatants were collected and stored at -80 °C.

For the cytokine stimulation analysis, measured cytokines were grouped into six major groups: a) interleukin (IL)-1 group of cytokines, comprising IL-1β, IL-1 receptor antagonist (IL-1ra), IL-18, IL-36α, IL-36β and IL-36γ produced after stimulation of PBMCs with bacterial lipopolysaccharide (LPS) (even if IL-1ra antagonises the function of the other cytokines, its *ex vivo* production is an indicator of the activation of the overall IL-1 cascade¹²); b) monocyte-derived pro-inflammatory cytokines and chemokines (calprotectin, CXCL8, CXCL9, IFNγ, CXCL10, GC-CSF, IL-6, IL-23 and TNFα) produced after stimulation of PBMCs with bacterial LPS or heat-killed *Candida albicans*; c) anti-inflammatory cytokines (IL-10, IL-1ra, sTNFRI and sTNFRII) produced after stimulation of PBMCs with bacterial LPS or heat-killed *C. albicans*; d) Th1 cytokines (IL-2, IL-12p70, IFNγ and TNFα) produced after stimulation of PBMCs with PHA; e) Th2 cytokines (IL-4 and IL-10) produced after stimulation of PBMCs with PHA; and f) T17 cytokines (IL-17A and IL-22) produced after stimulation of PBMCs with PHA.

The limits of detection and the manufacturer of the assay kit for each cytokine were: IL-1β (5.6 pg/mL; Diaclone, Besançon, France), IL-1 receptor antagonist (IL-1ra, 31.25 pg/mL, Diaclone), IL-18 (125 pg/mL, Diaclone) IL-36α (7.82 pg/mL, Diaclone), IL-36β (7.82 pg/mL, Diaclone) and IL-36γ (15.63 pg/mL,

Diaclone), Calprotectin (S100A8/A9, 31.25 pg/mL, ELK Biotechnology, Wuhan, China), Monokine induced by gamma interferon/Chemokine (C-X-C motif) ligand 9 (mIg/CXCL9, 31.25 pg/mL, ELK Biotechnology), IL-6 (6.25 pg/mL, Diaclone), IL-8 (31.25 pg/mL, Diaclone), IL-23 (156.25 pg/mL, Diaclone), interferon (IFN)γ (12.5 pg/mL, Diaclone), Granulocyte-macrophage colony-stimulating factor (GM-CSF, 15.625 pg/mL, Diaclone), IFNγ-induced protein 10 (IP-10, 6.25 pg/mL, Diaclone), Soluble TNF receptor superfamily member 1A (TNFRSF1A, 15.63 pg/mL, ELK Biotechnology), soluble TNF receptor superfamily member 1B (TNFRSF1B, 0.16 ng/mL, ELK Biotechnology), IL-10 (6.25 pg/mL, Diaclone), IL-2 (31.25 pg/mL, Diaclone), IL-12p70 (6.25 pg/mL, Diaclone), IFNγ (12.5 pg/mL, Diaclone), tumour necrosis factor (TNF) alpha (12.5 pg/mL, Diaclone), IL-4 (1.1 pg/mL, Diaclone), IL-10 (6.25 pg/mL, Diaclone), IL-17A (15.6 pg/mL, Diaclone), and IL-22 (31.25 pg/mL, Diaclone).

Endpoints

This study reports on three secondary endpoints of the ACCESS trial: a) change of function of monocytes, Th1, Th2, and T17; b) pathway analysis of gene expressions; and c) changes of the genes involved in the cholesterol homeostasis pathway, namely genes *MVK*, *SC5D*, *MVD*, *STARD4* and *SQLE*.

Statistics

The study power was calculated for the ACCESS primary endpoint.³ In this secondary analysis, all patients with available RNA-seq and cytokine stimulation data from PBMCs on days 1 and 4 participated. Demographic and clinical parameters were summarised using descriptive statistics. Continuous variables were reported as mean with standard deviation (SD) or median and interquartile range (IQR). Group comparisons for continuous or ordinal variables were conducted using either Student's t-test or the Mann-Whitney U test, as appropriate. Categorical variables were expressed as frequencies and percentages (%), and group comparisons were done using Fisher's exact test. Odds ratios (ORs) with 95% confidence intervals (CIs) were estimated using the Mantel-Haenszel method. A sensitivity power analysis using a Minimum Detectable Effect approach was conducted based on *a priori* assumptions from the parent randomised clinical trial (control response rate 38%, two-sided $\alpha = 0.05$, target power 80%, and the conservative subgroup sample size).

The relative changes of production of each cytokine by the PBMCs from baseline to day 4 was calculated and plotted as heatmaps for each group of treatment and outcome. For cytokines of groups (a) and (b) the impact of the number of increased cytokines on the achievement of the primary endpoint, early death and progression to RF was investigated after logistic

regression analysis calculating the odds ratio (OR) and 95% confidence intervals (CIs) with the endpoint as the dependent variable and the number of cytokines, the severity baseline scores, and baseline values of absolute white blood cell count, absolute monocyte count, and C-reactive protein as the independent variables. Where the analysis showed a significant association between the number of the cytokines and the endpoint, the cut-off of the number of cytokines associated with the endpoint was defined as the Youden index of the designed ROC (receiver operating characteristic) curve, which represents the point maximising the sum of sensitivity and specificity. Comparison of patients in each group above this cut-off was performed using Fisher exact test; ORs and 95% CIs were calculated with the Mantel and Haenszel test. For the cytokines of groups (c), (d), (e) and (f) comparisons were done by ordinal regression analysis calculating ORs and 95% CIs.

Analyses were performed with the software IBM SPSS version 26.0. Any value of *p* less than 0.05 was considered statistically significant.

Role of the funding source

The trial was sponsored by the Hellenic Institute for the Study of Sepsis (HISS) and funded by Abbott Products Operations (Abbott). HISS was responsible for the design of the study, study conduct, analysis and interpretation of data, and decision to publish. The funding body had no role in the design, conduct, analysis and interpretation of data and the decision to publish. Hughes associates, Oxford, UK, assisted with publication administration.

Results

Study population

The intent-to-treat population of the ACCESS trial was 267 patients, with 133 patients allocated to treatment with SoC and placebo and 134 patients to treatment with SoC and clarithromycin. The study populations with analyses from day 1 (before start of the study drug) and day 4 (72 h after the start of treatment) were 99 and 103 patients, respectively, for cytokine production by stimulated PBMCs; and 45 and 41 patients respectively for transcriptomic analysis (Fig. 1). The main reasons for samples from day 1 and day 4 not being available for the entire ITT population were: death before day 4; discharge before day 4; and lack of adequate quality in isolation. No patient was lost to follow-up. Baseline characteristics of patients of the two groups included in the analyses were similar (Supplementary Tables S1 and S2).

Transcriptomic analysis

After quality control, RNA-seq samples from 86 patients with repeated measures at both timepoints were

kept for downstream analysis; 45 of these patients were randomised to the SoC and placebo arm and 41 patients to the SoC and clarithromycin arm (Fig. 1). Twenty (44.4%) and 31 (75.6%) of these patients attained the study primary endpoint respectively (OR 3.87; 95% CIs 1.54–9.76; *p* = 0.004). The sensitivity power analysis, based on the conservative subgroup sample size (*n* = 41), indicated that the minimum detectable treatment response rate was 70.7%. Differential expression analysis showed no significant differences between treatment groups at baseline (Supplementary Figure S1). This lack of difference remained after adjustment for baseline values of ferritin and for medical history of chronic heart failure (data not shown). Trajectories between baseline and day 4 indicated 77 uniquely upregulated genes on day 4 in the placebo group, 112 in the clarithromycin group and 52 in both groups; 48, 388 and 89 DEGs respectively were downregulated on day 4 (Fig. 2A–D). GO analysis showed that the unique upregulated genes in the clarithromycin group were mainly involved in Positive Regulation of Cytokine Production (GO:0001819) and in T Cell Activation (GO:0042110) (Fig. 2E, Supplementary Table S3). Upregulated genes in the placebo group did not identify any specific ontology after correction for multiple testing. Downregulated and upregulated ontologies between the two groups of treatment were different. The most common downregulated ontologies in the placebo group were negative regulation of bone resorption (GO:0045779) and negative regulation in bone remodelling (GO:0046851) (Fig. 2F). The most common downregulated ontologies in the clarithromycin group were negative regulation of viral genome replication (GO:0045071), negative regulation of viral process (GO:0048525) and defence response to symbiont (GO:0140546) (Fig. 2G).

The major pathway which was upregulated in the placebo group based on the Reactome pathway analysis was mitotic transition (Supplementary Table S4). Upregulation of the differentiation of keratinocytes and of developmental cell lineages was found in the clarithromycin arm (Supplementary Table S5). The major downregulated pathways based on the Reactome pathway analysis in the placebo group were interferon-signalling, Toll-like receptors (TLR)–2, –4, –8, and –9 and the intracellular pathway of MyD88 (Supplementary Table S6). Interestingly, neutrophil degranulation was strongly downregulated in the clarithromycin group, contrary to the placebo group (Supplementary Table S7).

RNA-seq analysis of the INCLASS trial conducted in patients with sepsis and acute respiratory distress syndrome (ARDS)¹³ showed that the expression of five genes implicated in the cholesterol biosynthesis pathway was upregulated after four days of treatment with intravenous clarithromycin, namely *MVD*, *MVK*, *SC5DL*, *SQLE* and *STARD4*. We therefore compared

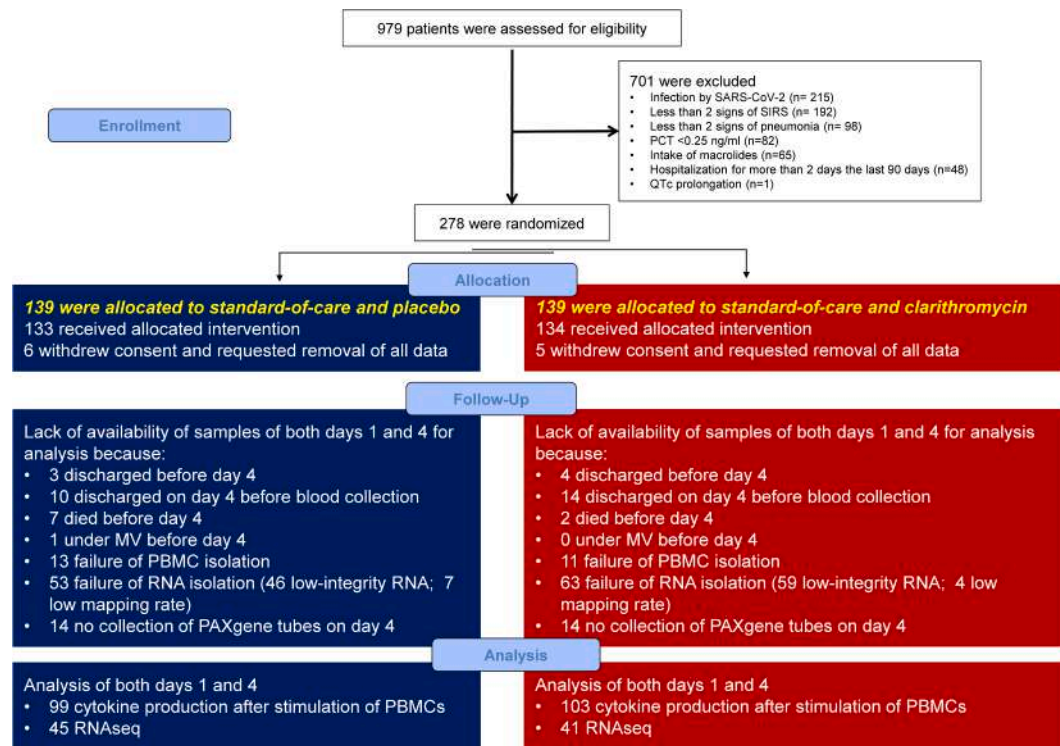


Fig. 1: Study flow illustrating sample availability of both days 1 and 4 for analysis. Abbreviations: MV, mechanical ventilation; PBMCs, peripheral blood mononuclear cells; PCT, procalcitonin; SIRS, systematic inflammatory response syndrome.

the differences in the expression of these five genes between baseline and day 4, in the two treatment assignments of the ACCESS study. On day 4, only the expression of *MVK* was upregulated in the placebo group; the expression levels of *MVD*, *MVK* and *SC5DL* were upregulated in the clarithromycin group (Supplementary Figure S2).

Among DEGs between the two groups of treatment and between the two outcomes, 27 genes were uniquely upregulated on day 4 from baseline in the SoC and clarithromycin group and associated with favourable outcome (Fig. 3A–D). These genes (Supplementary Table S8) framed two upregulated pathways; stem-cell development (GO:0048864) and Neural crest cell differentiation (GO:0014033) (Fig. 3E). Another 55 genes were uniquely downregulated on day 4 from baseline in the SoC and clarithromycin group and associated with favourable outcome (Fig. 3F). These genes (Supplementary Table S9) framed three main down-regulated pathways: positive regulation of Th1 type immune response (GO:0002827); positive regulation of Th1 cell cytokine production (GO:2000556); and regulation of Th1 cell cytokine production (GO:2000554) (Fig. 3G). It should be noted that *IL-1R1*, *IL-1R2* and *IL-18R1* implicated in the activation of the IL-1 pathway and *SOCS3* involved in cytokine regulation were among

genes decreased in expression with clarithromycin treatment.

As shown in Fig. 3A and B, *MPO*, encoding for myeloperoxidase, was upregulated and *CD177*, a marker of maturity of neutrophils that has been linked to pathology such as inflammatory bowel disease,^{14,15} was downregulated in both placebo-treated and clarithromycin-treated patients. This contrasts the findings of the Reactome analysis supporting down-regulation of neutrophil degranulation in the clarithromycin group. Comparisons of all 52 analysed gene transcripts of day 4 involved in the pathway of neutrophil degranulation indicated that the expression of vacuolar *PADI2* involved in the digestion of *Pseudomonas aeruginosa*,¹⁶ was significantly downregulated only among patients treated with clarithromycin and experiencing favourable outcome (Supplementary Figure S3).

All DEGs in association with outcomes are reported in Supplementary Tables S10 and S11.

Cytokine stimulation by PBMCs

Logistic regression analysis showed that the number of IL-1 group of cytokines which were increased from baseline to day 4 was positively associated with progression to RF (OR 1.82; 95% CIs 1.18–2.82; p = 0.007)

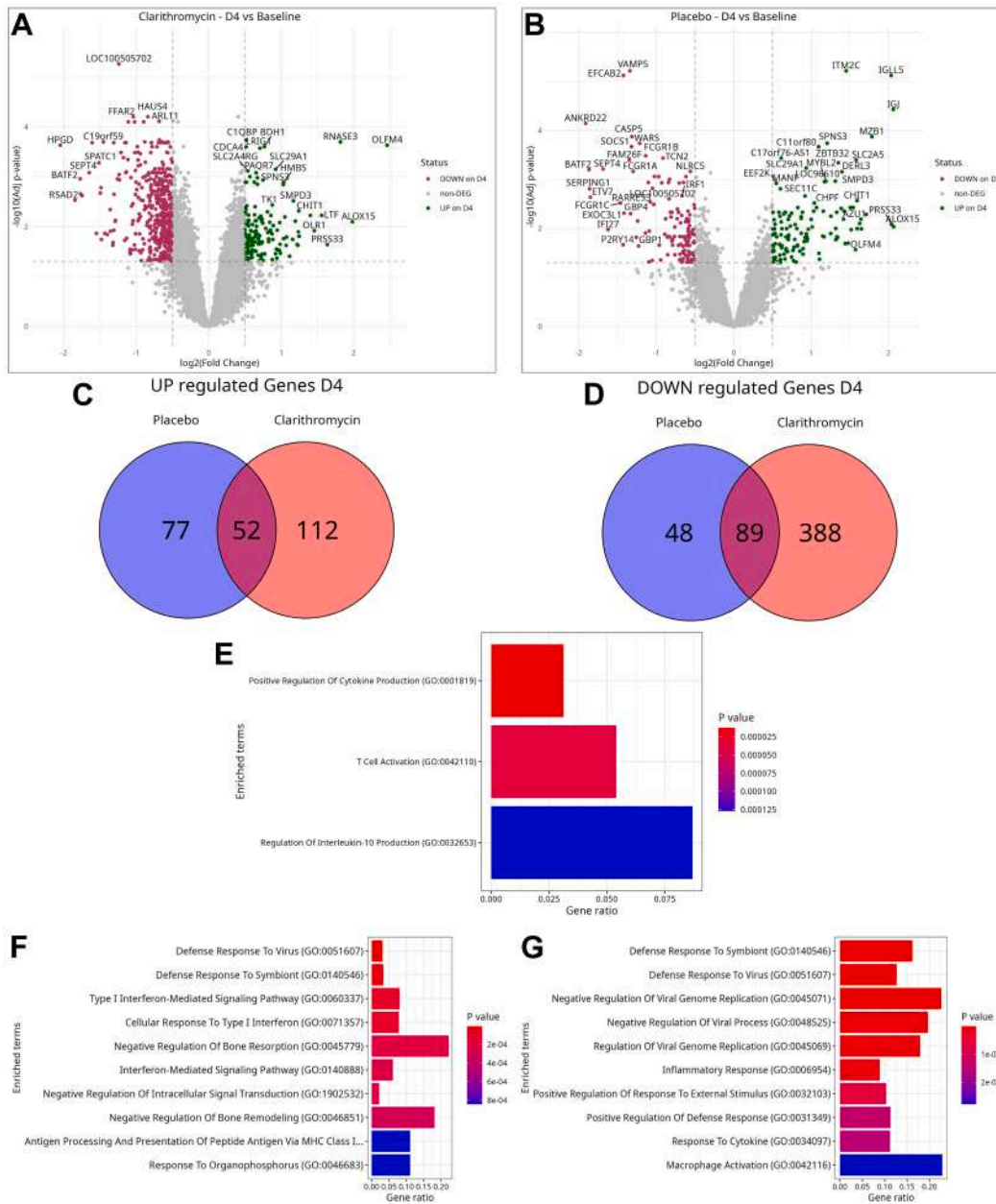


Fig. 2: Trajectory of gene expression between baseline and day 4 in each group of treatment. A) Differentially expressed genes in patients treated with clarithromycin between day 1 (before the start of the study drug) and day 4. B) Differentially expressed genes in patients treated with placebo between day 1 (before the start of the study drug) and day 4. C) Venn diagram depicting the unique and common UP regulated genes on day 4 compared to day 1 per treatment group. D) Venn diagram depicting the unique and common DOWN regulated genes on day 4 compared to day 1 per treatment group. E) Gene Ontology (GO) analysis showed that the unique upregulated genes at day four in the clarithromycin group were mainly involved in Positive Regulation Of Cytokine Production (GO:0001819) and in T Cell Activation (GO:0042110). Upregulated genes in the placebo group did not frame any specific ontology after correction for multiple testing. F) Top 10 downregulated ontologies in the SoC and placebo group. G) Top 10 downregulated ontologies in the SoC and clarithromycin group. Abbreviations: SoC, standard-of-care.

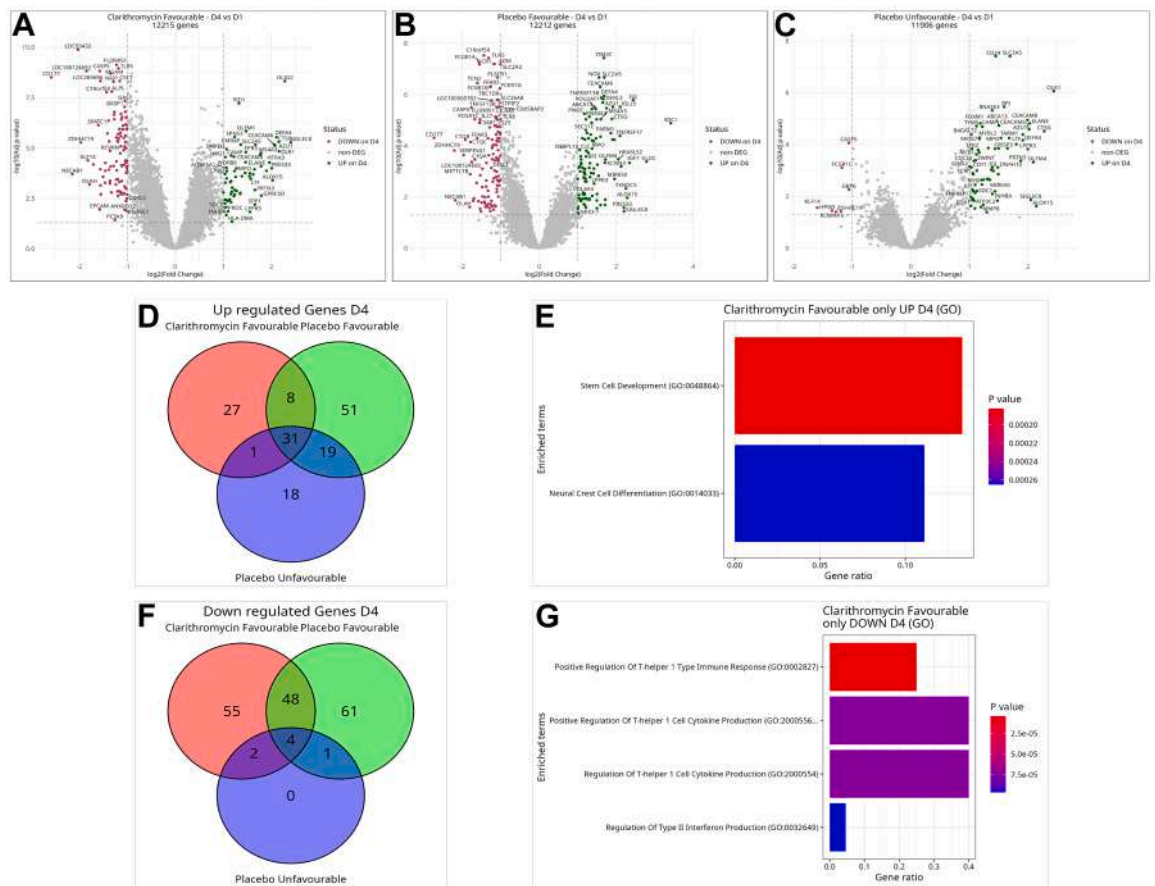


Fig. 3: Differentially expressed genes (DEGs) associated with favourable outcomes in clarithromycin-treated patients. A) DEGs between day 1 (before the start of the study drug) and day 4 (72 h after the start of the study drug) of 26 patients treated with SoC and clarithromycin and experiencing favourable outcome. B) DEGs between day 1 (before the start of the study drug) and day 4 (72 h after the start of the study drug) of 17 patients treated with SoC and placebo and experiencing favourable outcome. C) DEGs between day 1 (before the start of the study drug) and day 4 (72 h after the start of the study drug) of 28 patients treated with SoC and placebo and experiencing unfavourable outcome. D) Venn diagram of upregulated DEGs on day 4 between the SoC and clarithromycin group with favourable outcomes, the SoC and placebo group with favourable outcomes and the SoC and placebo group with unfavourable outcomes; 27 DEGs are unique in the SoC and clarithromycin group. E) Gene ontology analysis of genes uniquely upregulated in the SoC and clarithromycin group with favourable outcomes. F) Venn diagram of downregulated DEGs on day 4 between the SoC and clarithromycin group with favourable outcomes, the SoC and placebo group with favourable outcomes and the SoC and placebo group with unfavourable outcomes; 55 DEGs are unique in the SoC and clarithromycin group. G) Gene ontology analysis of genes uniquely downregulated in the SoC and clarithromycin group with favourable outcomes. Favourable outcomes are defined for patients meeting all of the following: attainment of the ACCESS primary endpoint, no progression to RF, and survival until day 8. Abbreviations: DEG, differentially expressed gene; GO, gene ontology; RF, respiratory failure; SoC, standard-of-care.

and early mortality (OR 1.83; 95% CIs 1.10–3.03; $p = 0.020$) (Supplementary Tables S12–S15). The same logistic regression model did not show any effect of the number of increased IL-1 cytokines with the study primary endpoint (data not shown). The Youden index of the ROC defined that increases of at least 4 cytokines of the IL-1 group were associated with progression to RF. The likelihood for such an increase was lower in the SoC and clarithromycin group (Fig. 4A). The heatmap also showed that the relative increase of each of the individual six cytokines of the IL-1 group on day 4 in the clarithromycin group was less than the placebo group (Fig. 4B).

Logistic regression analysis showed that the number of monocyte-derived pro-inflammatory cytokines/chemokines (other than IL-1 family cytokines) increased from baseline to day 4 was positively associated with the achievement of the primary endpoint (OR 1.16; 95% CIs 1.00–1.35; $p = 0.043$) (Supplementary Table S16). The same logistic regression model did not show any effect of the number of pro-inflammatory cytokines/chemokines with progression to RF or early death (data not shown). The Youden index of the ROC determined that increase of at least 5 cytokines/chemokines was associated with achievement of the primary endpoint. The likelihood for such an increase was higher in the

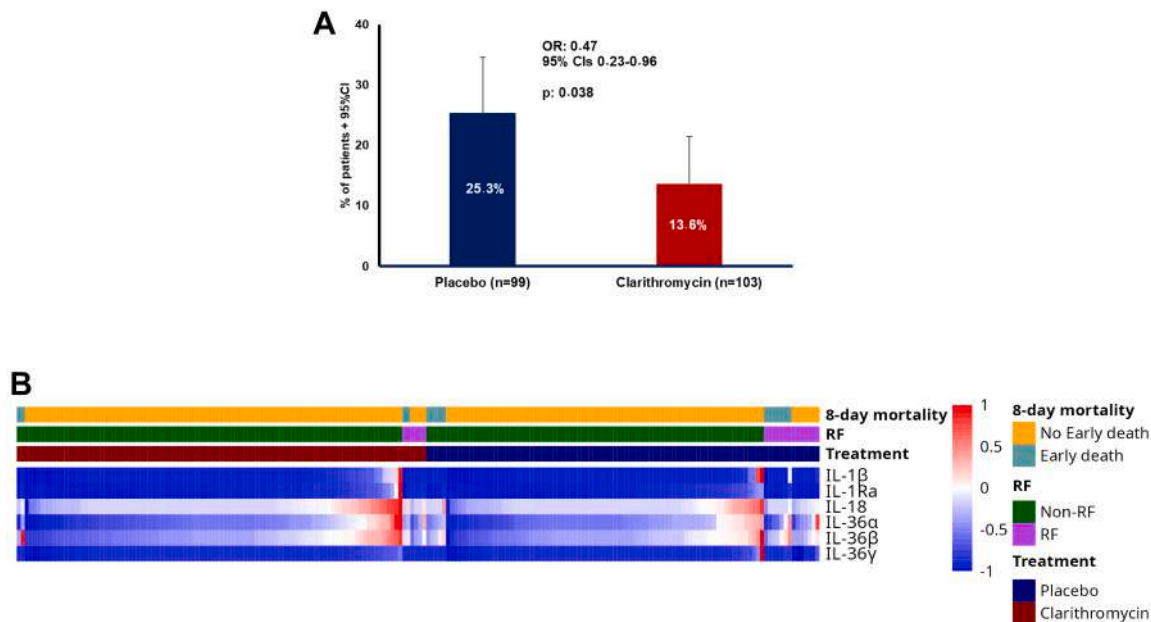


Fig. 4: Decrease of the production of cytokines of the interleukin (IL)-1 group under clarithromycin treatment on day 4 compared to baseline. A) Patients experiencing increase in production of at least 4 of the IL-1 group cytokines (IL-1 β , IL-1ra, IL-18, IL-36 α , IL-36 β and IL-36 γ) by the PBMCs of patients under stimulation by LPS. Comparison is done using Fischer's exact test. B) Heatmap of the change of the production of each of the six cytokines of the IL-1 group from each studied patient according to treatment assignment (placebo/clarithromycin), progression to RF or not and early death or not. Abbreviations: CI, confidence interval; IL, interleukin; LPS, lipopolysaccharide; n, number of patients; OR, odds ratio; PBMCs, peripheral blood mononuclear cells; RF, respiratory failure.

SoC and clarithromycin group than in the SoC plus placebo group (Fig. 5A). The heatmap also showed that the relative increases of the production of each of the individual cytokines/chemokines on day 4 in the clarithromycin group were higher than the placebo group (Fig. 5B). Patients in the clarithromycin arm with increases of at least 5 cytokines/chemokines showed also decreases in five genes involved in the neutrophil degranulation pathway, namely *CD55*, *FCAR*, *GCCR2A*, *MAPK14* and *VNN1*; this difference did not exist in the placebo arm (Supplementary Figure S4).

The number of anti-inflammatory cytokines increased from baseline to day 4 was lower with clarithromycin treatment than in the placebo group (OR 0.60; 95% CIs 0.36–0.99; $p = 0.049$) (Fig. 6). The two groups of treatment did not differ in the increases of the number of Th1 cytokines, Th2 cytokines and T17 cytokines from baseline to day 4 (Supplementary Figure S5).

Discussion

In the ACCESS randomized controlled trial, the addition of oral clarithromycin for seven days to the antimicrobial SoC of hospitalised CAP patients was associated with early anti-inflammatory responses observed as part of a composite primary endpoint of improvement of clinical signs of pneumonia,

improvement of organ dysfunction and decrease of the level of inflammation. The attainment of that endpoint was accompanied by decreased risk for progression to RF and need for MV, decreased incidence of secondary sepsis and earlier alive hospital discharge.³

The present analysis investigated the molecular pathways behind the clinical benefit of clarithromycin treatment using an integrated approach of large-scale transcriptomics and cytokine release assays and investigating the trajectory of patients from baseline to the first 72 h, when the primary endpoint was censored. Both approaches provided similar results and identified an effect of clarithromycin that converged on three points of action: i) downregulation of the deleterious effect of the IL-1 cascade; ii) upregulation of the production of other monocyte-derived pro-inflammatory cytokines and chemokines and improvement of antigen presentation by monocytes; and iii) decreased neutrophil degranulation. How might these effects interface with the clinical benefit of clarithromycin treatment? IL-1 activation and neutrophil degranulation are prominent in the pathogenesis of ARDS.¹⁷ Exhaustion of the innate immune system for cytokine and chemokine production is the hallmark of sepsis-induced immunoparalysis (SII) leading to secondary infections.¹⁸ These immune defects were restored with clarithromycin treatment.

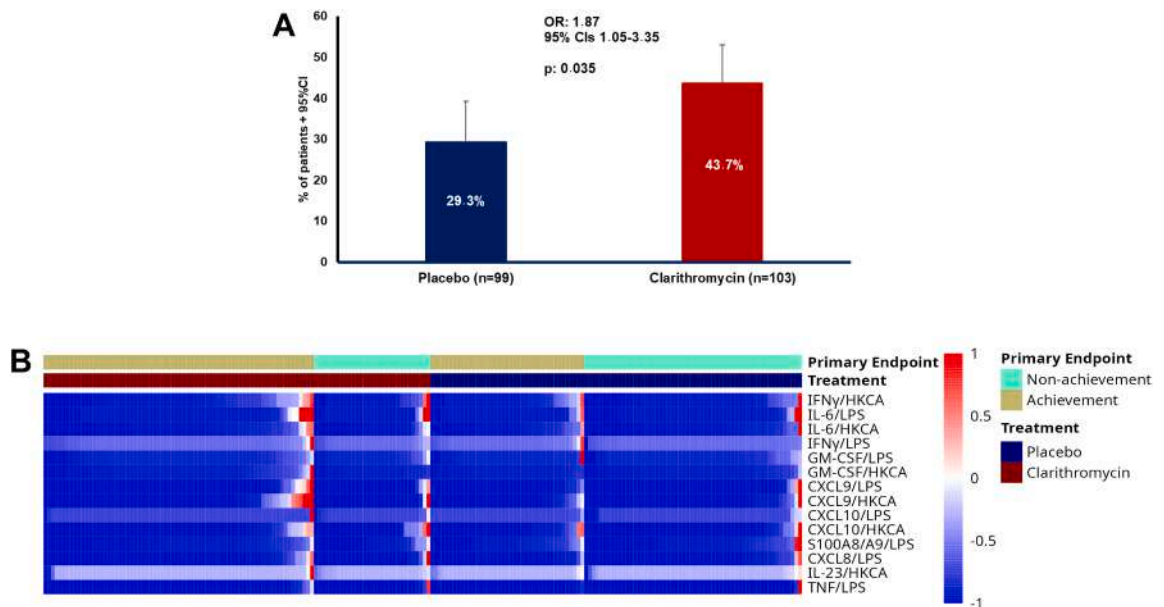


Fig. 5: Increase of the production of cytokines/chemokines indicative of monocyte activation under clarithromycin treatment on day 4 compared to baseline. A) Patients experiencing increases in production of at least 5 of the cytokines/chemokines indicative of monocyte activation (IFN γ , CXCL8, CXCL9, CXCL10, GC-CSF, S100A8/A9, IL-6, IL-23) by PBMCs in response to stimulation by LPS or HKCA. Comparison is done using Fischer's exact test. B) Heatmap of the change of the production of each of the cytokines/chemokines from each studied patient according to the allocated group of treatment (placebo/clarithromycin), and the achievement of the ACCESS primary endpoint or not. The applied stimuli are also indicated. Abbreviations: CI, confidence interval; GC-CSF, granulocyte colony stimulating factor; HKCA, heat-killed *Candida albicans*; IFN, interferon, IL, interleukin; IFN γ -induced protein-10; n, number of patients OR, odds ratio; PBMCs, peripheral blood mononuclear cells.

Observations from the COVID-19 pandemic indicate that early activation of the IL-1 cascade is a major driver of RF.¹⁹ Indeed, early treatment with the IL-1 receptor antagonist anakinra decreased the progression to RF and the need for invasive ventilation.²⁰ In the ACCESS trial, the increased production of six cytokines of the IL-1 family by circulating PBMCs was an independent marker for RF. The level of IL-1 activation, expressed by the number of over-produced IL-1 cytokines, was lower with clarithromycin treatment than in the placebo group. Clarithromycin treatment led to the downregulation of the expression of three genes encoding for the IL-1 cytokine receptors, namely *IL1R1*, *IL-1R2* and *IL-18R1* (Supplementary Table S9). In parallel, *CNR2* encoding for the cannabinoid receptor 2 was upregulated in clarithromycin-treated patients (Supplementary Table S8). *CNR2* is downregulated in acute lung injury (ALI); drugs which reverse downregulation of *CNR2* improve the level of maturation of dendritic cells and are suggested as promising candidates for the therapy of ALI.²¹

Increase of the production of monocyte-derived pro-inflammatory cytokines and chemokines other than the IL-1 family was associated with attainment of the primary endpoint; the increase in those factors was greater among clarithromycin-treated patients than in the

placebo group. GO ontology analysis showed upregulation of the cytokine production pathway with clarithromycin, whereas expression of Suppressor of Cytokine Stimulator 3 (*SOCS3*) was decreased (Supplementary Table S9). *SOCS3* inhibits the universal production of cytokines through an effect on Janus kinase. When *SOCS3* is downregulated the production of cytokines and chemokines by PBMCs increases.²² The expression levels of *WARS*, encoding for TTS (tryptophanyl-tRNA synthetase) were sustained during treatment in the clarithromycin arm, in contrast to its downregulation in the placebo arm at day 4. TTS regulates protein synthesis and acts as an innate immune cell activator through binding to TLRs.^{23,24} We conjecture that the maintenance of TTS expression levels during treatment with clarithromycin, in contrast to its downregulation in the placebo arm, could indicate both stability in global protein synthesis as well as sustained immune cell activation.

The level of antigen presentation was also improved in clarithromycin-treated patients, as shown by the upregulation of *HLA-DMA* and this can be expected to prime the upregulation of T cell activation identified by the GO pathway analysis. However, no changes of the expression of genes encoding for the co-stimulatory molecules CD28, CD86, or CTLA-4 were found

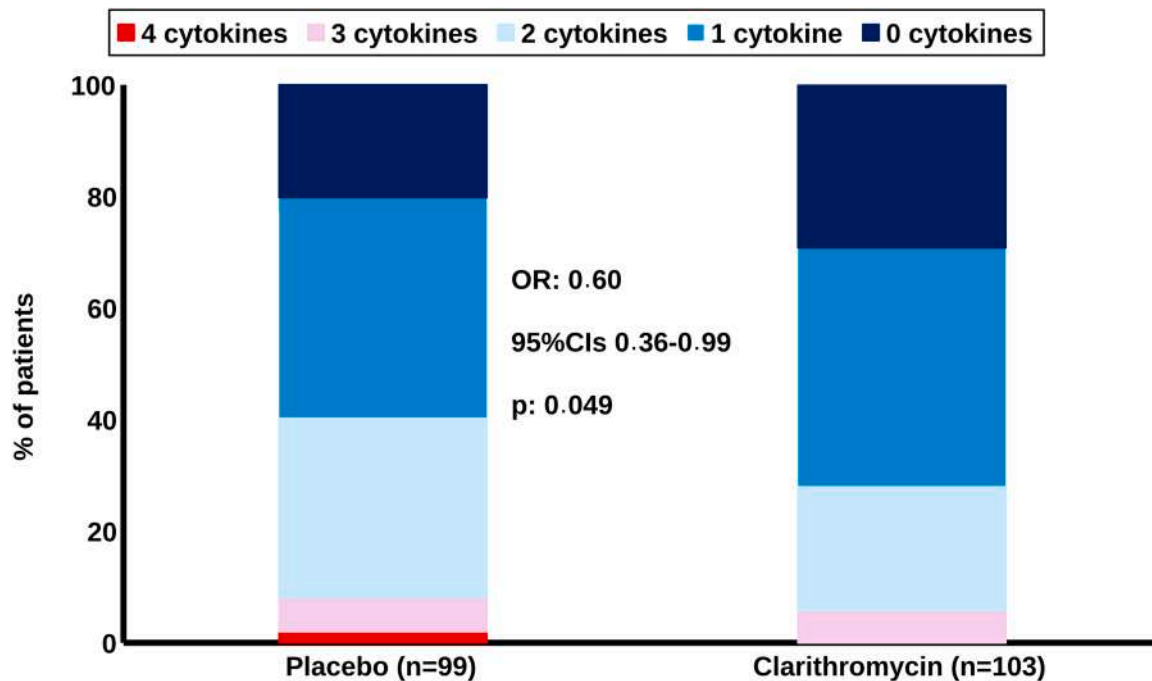


Fig. 6: Increase of the production of anti-inflammatory cytokines by PBMCs from baseline day 1 before the start of the study drug to day 4. PBMCs were stimulated with purified bacterial LPS and heat-killed *Candida albicans* and the measured cytokines were IL-1ra, IL-10, sTNFR1 and sTNFR2. Each band refers to the number of anti-inflammatory cytokines which were increased from baseline to day 4. Comparison is done by ordinal regression analysis. Abbreviations: CI, confidence interval; IL, interleukin; IL-1ra, IL-1 receptor antagonist; LPS, lipopolysaccharide; n, number of patients; OR, odds ratio; PBMCs, peripheral blood mononuclear cells; sTNFR, soluble receptor of tumour necrosis factor.

between the two groups. By contrast that analysis showed that recovery of antigen presentation was lower with placebo treatment. Decreased expression of HLA-DR molecules on circulating monocytes is the salient feature of SII, a phenomenon characterised by excess production of anti-inflammatory cytokines and susceptibility to secondary infections.^{4,18} The downregulation of antigen presentation in the placebo group and the upregulation of T cell activation and *HLA-DMA* expression in the clarithromycin group, accompanied by decreased production of anti-inflammatory cytokines, point towards a reversal of immunoparalysis in response to clarithromycin; this is mechanistically consistent with the decrease of the incidence of secondary sepsis with clarithromycin treatment described in the ACCESS trial.

These observations of reversal of immunoparalysis in response to clarithromycin corroborate and amplify previous findings in patients with sepsis and ventilator-associated pneumonia²⁵ and in patients with sepsis and ARDS¹³ who received intravenous clarithromycin. Genes of the cholesterol homeostasis pathway were upregulated with clarithromycin treatment in patients with ARDS indicating that modulation of the cholesterol synthesis pathway might contribute to the

immunomodulatory effect of clarithromycin. Several of these genes were also upregulated in the ACCESS trial. The use of statins probably does not confound this result since their administration was prohibited during the ACCESS trial.³

Spontaneous neutrophil degranulation is associated with the extracellular release of toxic compounds like oxygen radicals and metalloproteinases and is associated with progression to RF.²⁶ Using RNA-seq, we found transcriptional profiles associated with decreased neutrophil degranulation and decreased production of PADI2 with clarithromycin treatment. Our findings also suggest some moderate association between the upregulation of the production of monocyte-derived pro-inflammatory cytokines and chemokines and the downregulation of the neutrophil degranulation pathway in the clarithromycin arm.

An issue for consideration is whether the pathways of action of clarithromycin discerned in this research are universal for all cases of CAP. One reason we think it may, is that the alterations we described in placebo-treated patients in ACCESS have been repeated in other cohorts of CAP patients. For instance, in a microarray study of peripheral blood leukocytes from 38 patients the gene trajectory during the first five days

was similar to that described for placebo-treated ACCESS patients²⁷ and some downregulated genes of the placebo group of the ACCESS trial like *UBE2L6* and *NLRC5* have been associated with unfavourable outcome of CAP.²⁸

Two main strengths of the study should be noted: a) both Gram-positive cocci and Gram-negative bacteria were reported as pathogens³ and results of this secondary analysis can be extrapolated for all probable pathogens. Moreover, it is reported that the transcriptomic profile of CAP caused by Gram-positive cocci and Gram-negative bacteria is similar²⁹; and b) the pathways associated with clinical benefit of clarithromycin treatment are similar in both experimental approaches i.e. transcriptomic analysis and cytokine stimulation by the PBMCs. Five main limitations of this study should be acknowledged: a) the lack of use of lipoteichoic acid (LTA) for the ex vivo stimulation of PBMCs. LTA is a constituent of the cell wall of Gram-positive cocci targeting TLR2. However, part of the activity of HKCA used in the current study is exerted through TLR2 activation; b) direct measurements of IL-1alpha (which remains bound to cell membranes) following the stimulation of PBMCs were not done. Instead, we relied on measurement of anti-inflammatory IL-1ra as a proxy for IL-1alpha production capacity. For that reason, we have construed IL-1ra as one of the IL-1 pathway cytokines for the purposes of presenting our findings; c) results cannot be extrapolated to immunocompromised populations, such as individuals receiving corticosteroids, cytotoxic chemotherapy, or other immunosuppressive therapies, since our findings emerged from a cohort of immunocompetent hospitalised patients; d) the advanced age of the study population is associated with immunosenescence which may affect the immune functions of the study participants, albeit the age distribution was similar in both treatment groups of; and e) the trial took place in Greece which is a territory of high antimicrobial resistance. Macrolide resistance was not studied since most pathogens were detected by molecular methods. However, this does not necessarily restrict the generalisability of the findings since high rates of macrolide resistance are reported from other parts of the world.³⁰

The described modulation of the immune profile provides a mechanistic explanation for the clinical benefit coming from clarithromycin treatment in the ACCESS trial. Downregulation of IL-1 cytokines and of neutrophil degranulation are viable pathways for the prevention of progression to RF; increase of monocyte-derived pro-inflammatory cytokines and chemokines coupled with upregulation of antigen presentation explains the attainment of the primary endpoint and the prevention of secondary sepsis. These findings are supported by both RNA-seq analysis and cytokine stimulation by the PBMCs. All these observations are

strongly supportive of the use of clarithromycin for hospitalised adult CAP patients.

Contributors

ES and SF performed transcriptomic analysis, drafted the first version of the manuscript and gave approval for the final version to be submitted.

VK performed transcriptomic analysis, revised the manuscript for intellectual content and gave approval for the final version to be submitted.

NK and PK performed cell isolation and cytokine measurements, revised the manuscript for intellectual content and gave approval for the final version to be submitted.

GN, KA, KI, MC, LS, VT, GC, SG, PK, TK, and GND participated in the collection of clinical data, revised the manuscript for intellectual content and gave approval for the final version to be submitted.

MGN, AT, and MSN analysed the data, revised the manuscript for intellectual content and gave approval for the final version to be submitted.

EJG-B designed the study, analysed the data, drafted the manuscript, and gave approval for the final version to be submitted. EJG-B and ES have accessed and verified the data.

All authors read and approved the final version of the manuscript.

Data sharing statement

Raw data are available for download from the server of the Hellenic Institute for the Study of Sepsis (<https://wshiss.com/>). Username and Password are provided upon request to the corresponding author at egiamarel@med.uoa.gr.

Declaration of interests

GND has received research grants from Gilead and Ipsen, has served as advisor and/or lecturer for Ipsen, Gilead, Genesis, Pfizer, Sanofi, and Sobi and as PI for Amyndas Pharmaceuticals, Intercept Pharma, CymaBay Therapeutics, Genkyotex, Novo Nordisk, Pfizer, Regulus Therapeutics, Sobi, and Tiziana Life Sciences.

MGN is a scientific founder of Biotrip, Lemba, TTxD, and Salvina.

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The other authors do not have any conflict of interest to report.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2026.106240>.

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