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*Research Article*

# Alkylresorcinols as a New Type of Gut Microbiota Regulators Influencing Immune Therapy Efficiency in Lung Cancer Treatment

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*Background*. Alkylresorcinols (ARs) are polyphenolic compounds of microbial origin with a wide spectrum of biological activities and are potentially involved in host immune functioning. The present study is aimed at evaluating alterations in AR content in blood serum and faeces from healthy donors and patients with lung cancer in connection with response to immune checkpoint inhibitor (ICI) therapy to estimate the regulatory potential of AR. *Methods*. Quantitative analysis of AR levels, as well as other microbial metabolites in blood serum and faeces, was performed using gas chromatography with mass spectrometric detection; estimation of lymphocyte subsets was performed by flow cytometry; faecal microbiota transplantation (FMT) from lung cancer patients after ICI therapy to germ-free mice was performed to explore whether the intestinal microbiota could produce AR molecules. *Results*. AR concentrations in both faeces and serum differ dramatically between healthy and lung cancer donors. The significant increase in AR concentrations in mouse faeces after FMT points to the microbial origin of ARs. For several ARs, there were strong positive and negative correlations in both faeces and serum with immune cells and these interrelationships differed between the therapy-responsive and nonresponsive groups. *Conclusions*. The content of ARs may influence the response to ICI therapy in lung cancer patients. ARs may be considered regulatory molecules that determine the functioning of antitumor immunity.

## 1. Introduction

Despite knowledge of lung cancer risk, development, diagnostics, and treatment options has advanced, lung cancer still accounts for the majority of cancer deaths globally [1]. While tobacco use is the main cause of lung cancer, there are other risk factors as well, such as exposure to the environment and at work, lung infections, chronic lung disease, and lifestyle choices that can promote malignant conversion and aberrant cell growth [2]. Prevention measures against lung cancer focus on smoking cessation and avoidance, healthy diet choices, and maintaining a physically active lifestyle [3]. In addition, a whole grain diet is considered one of the most promising cancer preventive means due to its beneficial influence on human metabolism and immunity [4].

On the other hand, we have seen a rise in interest in the field of "microbiota-host organism" relationship research in recent years. The microbiota is a group of different microorganisms (primarily, bacteria) that typically live in host tissues [5]. The majority of the microbiota community lives in the gastrointestinal tract, where it functions as a barrier, regulates immunity, and influences the operation of other systems; this is confirmed by associations found between intestinal dysbiosis and specific inflammatory and metabolic disease states, including cancer [6]. Microbiome dysbiosis is believed to increase cancer susceptibility at multiple levels. At first, in conditions of dysbiosis, the barrier function of the microbiota is disrupted, leading to pathological interactions between the microbiota and epithelial cells. Furthermore, the balance between "good" and "bad" players in the microbiota community is changed in the way the population of commensal microbes is reduced and the number of inflammation-inducing bacteria increases. Therefore, bacterial toxins, as well as reactive oxygen species (ROS), gut-derived carcinogens, such as acetaldehyde, and different microbiome metabolites, rush to host cells, promoting DNA damage, chronic inflammation, and metabolic disturbances [7]. For instance, the tryptophan (Trp) metabolites produced by bacteria influence the activity of the enzyme indoleamine 2,3-dioxygenase (IDO), which is involved in the metabolism of tryptophan in the host (kynurenine pathway). It is linked to a poor prognosis for treatment effectiveness and is expressed in over 58% of human tumour tissues [8].

Thus, a number of diseases, including cancer, are linked to bacterial metabolites as causative or preventive factors [9]. Extensive controlled experiments performed in mouse models containing one or more targeted microorganisms have provided evidence in favor of this theory. Furthermore, novel approaches to treating cancer and other diseases that involve alterations to the gut microbiota are emerging [10]. For example, faecal microbiota transplantation (FMT) from healthy donors to patients is successfully used to treat *Clostridium difficile* infection, especially after antimicrobial, immunosuppressive, or cytostatic therapeutic procedures in cancer patients [11], inflammatory bowel diseases and intractable functional constipation [12], and acute intestinal graft versus host disease in patients with hematologic cancers after stem cell transplantation [13].

The composition of the microbiome and the state of dysbiosis can also influence the response to anticancer therapy. Cancer immunotherapy is successfully applied against a variety of solid and hematologic metastatic malignancies [14, 15]. Administration of immune checkpoint inhibitors (ICIs) to suppress the interaction of T cell inhibitory receptors with their cognate ligands on tumour or stromal cells allows the activation of T cell-mediated immune responses. One of the most effective clinically used checkpoint inhibitors is monoclonal antibodies targeting programmed cell death protein 1 (PD-1) and its PD-L1 ligand. PD-1 blockade is highly effective against advanced melanoma, non-small-cell lung cancer (NSCLC), and renal cell carcinoma (RCC) [16]. As became clear in recent years, the primary resistance to ICIs observed in some cancer patients can be attributed to the abnormal composition of the gut microbiome [17]. In addition, antibiotics were shown to inhibit the clinical benefit of ICIs in patients with advanced cancer. According to the study by Routy et al. [17], FMT of cancer patients who responded to ICI in germ-free or antibiotic-treated mice improved the antitumor effects of PD-1 blockade, while FMT of nonresponding patients did not. The analysis of the metagenome of patient stool samples revealed correlations between clinical responses to ICI and the relative abundance of *Akkermansia muciniphila*. Interestingly, the efficacy of PD-1 blockade restored after oral supplementation with *A. muciniphila* and T lymphocytes was shown to be recruited into tumours. Apparently, there exist complex interactions between microbiome metabolic profiles, environmental factors, in particular, diet, and host metabolism.

Alkylresorcinols (ARs) can be considered as a link between the diet, intestinal microbiome, and host organism interrelationship, as well as potential *quorum sensing* (QS) molecules. ARs are highly lipophilic polyphenols (Figure 1) synthesised by bacteria, fungi, some animals, and higher plants [18].

Humans receive ARs mainly from whole grain wheat, rye, and barley products due to the dominant content of ARs in the grain kernels. In addition, resorcinols enter the body as components of food preservatives, drugs, and cosmetic components [19]. Furthermore, ARs may be synthesised by microbiome, although, to our knowledge, there is no strict evidence of the bacterial origin of ARs in humans. It was shown that bacteria (e.g., *Actinobacteria*; *alpha*, *delta*, and *gamma proteobacteria*; and *Cyanobacteria*) were shown to be capable of producing different monoalkylresorcinols [20–23] with anticancer, antifungal, anti-inflammatory, antimicrobial, antiparasitics, antioxidant, and genotoxic activities [24]. Furthermore, as reported by Martins et al. [23], phylogenetic analysis of genes that code for type III polyketide synthases (PKS) responsible for AR synthesis revealed a large abundance of such sequences between different members of the *Cyanobacteria* phylum. Many Gram-positive and Gram-negative bacteria were found to have srs-like operon structures coding for type III PKS in the study of Funabashi [25], suggesting that AR production is a common trait of various prokaryotes. Additionally, published data indicates that the majority of dietary polyphenols undergo transformation by the intestinal microbiota in the colon

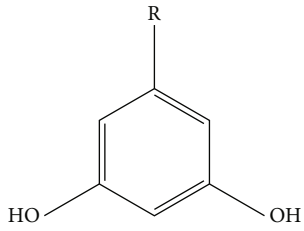



Figure 1: AR general structure. R-alkyl or alkenyl side chain; oxygenated R is also possible. The R position may be changed; the presence of additional polyphenolic rings is possible depending on the type of organism that synthesises ARs.

prior to absorption. This conversion controls the biological activity of these dietary compounds and is frequently necessary for absorption [26]. Glycosides, glucuronides, sulphates, amides, esters, and lactones can all be hydrolyzed by gut bacteria. Glycosides, glucuronides, sulphates, amides, esters, and lactones can all be hydrolyzed by gut bacteria [27–29]. The diversity of species found in the gut microbiome, such as *Lactobacillus*, *Eubacterium*, *Clostridium*, and *Bifidobacterium* spp., was thoroughly reviewed by Selma et al.; v noted how these species could metabolize various polyphenolic structures to produce less absorbable and physiologically active substances [30]. ARs with intricate structures that start in the gut may be broken down into simpler metabolites that are absorbed and affect host metabolism [31]. Conversely, phenolic compounds can influence the microbial population of the gastrointestinal tract (GI) and impact the metabolism of phenolic compounds found in food by interacting with the gut microbiota and possessing antimicrobial qualities of their own. For instance, the study by Nikolaev et al. demonstrated that AR, and specifically 4-hexylresorcinol, in combination with antibiotics significantly reduced the number of germinating *Bacillus cereus* spores in both liquid medium and agar medium compared to treatment with antibiotics alone [32]. This observation aligns with other research demonstrating ARs' capacity for autoregulation [33]. According to Oishi et al.'s findings [34], ARs markedly elevated the quantity of *Prevotella* and decreased the quantity of *Enterococcus* in the mouse faecal microbiota. Moreover, certain polyphenols found in drinks, fruits, and vegetables have the ability to either stimulate or suppress the growth of gut bacteria [35].

In recent decades, several biological properties of ARs have been discovered, including anti-inflammatory and anticancer functions. ARs were revealed to have antifungal, antimicrobial, antiparasitic, and genotoxic activities [24] and can thus be used as adjuvant agents for antibiotic or chemotherapeutic treatment. *In vitro* and *in vivo* studies have shown that ARs regulate the activity of sirtuin (SIRT) proteins and have antiageing potential [36]. ARs may prevent muscle and nerve tissue degeneration [37]. Studies of the biological properties of AR in mice demonstrate that ARs of whole grains suppress hyperinsulinemia and hyperleptinemia under the conditions of a high-fat-high-sucrose (FS) diet [34]. Furthermore, ARs significantly reduce fasting blood glucose concentrations and suppress glucose intolerance, as well as insulin resistance induced by the FS diet. ARs significantly induce cholesterol excretion with faeces along with decreasing in total circulating cholesterol [34]. Furthermore, it is shown that nonsmokers have 30% higher median AR metabolite concentration than smokers, and men of normal weight have 16% higher concentration of that than obese men. There is no difference in metabolite concentration by alcohol consumption, physical activity, or diabetes status at baseline. The concentration of AR metabolites is positively correlated with fibre intake ($r = 0.27$; $p < 0.001$) and whole grain intake ($r = 0.31$; $p < 0.001$) [38]. Epidemiological studies have shown that a whole grain diet rich in AR is associated with a lower risk of prostate, breast, and colon cancer [39, 40]. These data are in agreement with *in vitro* investigations of the antitumor properties of ARs in human hepatocarcinoma, ovarian, cervical, colon, lung, central nervous system, and breast cancer cell lines [39, 41]. The results of the studies are perfectly reviewed by Kruk et al. [42]. Briefly, ARs are responsible for inhibition of cancer cell growth because of their cytotoxic and genotoxic activities. However, another large observational study demonstrates that very high levels of plasma AR metabolites are associated with an increased risk of prostate cancer [38].

Therefore, many biological activities of ARs are known today. The most intriguing one is that ARs may have therapeutic potential in the treatment of such chronic diseases as cancer, diabetes, obesity, etc. On the other hand, associations between the quantitative and qualitative composition of ARs and the carcinogenesis process are also of interest. The present study is aimed at assessing the alterations in the content of ARs in the blood serum and faeces of healthy donors and patients with lung cancer, as well as the profile of ARs in the groups of patients administered checkpoint inhibitors in connection with response to ICI therapy to estimate the regulatory potential of ARs and their contribution to the QS process of the microbiome.

## 2. Materials and Methods

*2.1. Characteristics of the Investigated Cohorts.* The criteria for the inclusion of lung cancer patients in the study were the absence of antibiotics, prebiotic, and probiotic drug uptake for 3 months prior to the study. All the patients signed informed consent to participate in the survey. The excluded criteria for healthy participants were severe somatic diseases, any disease of the gastrointestinal tract, any acute condition, depression, alcoholism, smoking, pregnancy, and lactation. For AR profiling analysis, two groups of individuals were formed. The first group consisted of 100 healthy adults (18% men, 79% women; mean age $33.8 \pm 4.2$ years) without malignant or other chronic diseases. In the second group, 100 individuals (70% men, 30% women; mean age $61.2 \pm 6.9$ years) with diagnosed lung cancer of different stages of morphology and TNM were included, 10% of whom were diagnosed with stage I lung cancer, 14% with stage II, 50% with stage III, and 26% with stage IV.

Among lung cancer patients, 21 patients received immunotherapy with PD-1/PD-L1 inhibitors: 9 patients received atezolizumab (43%), 9 (43%) received pembrolizumab, and

Table 1: Effect of immunotherapy in lung cancer patients.

| $n$ (total) | Effect of immunotherapy | | | | |
|---|---|---|---|---|---|
| | Positive dynamics | | Stable | Non-negative dynamics (positive and stable) | Negative dynamics (progression) |
| | Complete | Partial | | | |
| 21 | 2 (9.6%) | 6 (28.8%) | 4 (19%) | 12 (57.3%) | 8 (38%) |

3 (14%) received nivolumab. The patients were subdivided according to the response to therapy (Table 1).

Complete response was observed in 2 (9.6%) of the subjects (positive dynamics), partial response was demonstrated in 6 (28.5%) (positive dynamics), 4 (19%) patients remained stable, and 8 subjects (38%) developed progression (negative dynamics). The groups of patients that demonstrated positive and stable dynamics were considered non-negative. One of the patients (4.8%) developed immune-mediated complications (the Guillain-Barré syndrome), so his treatment was cancelled.

For each of the individuals included in the study, a clinical examination, an anthropometric assessment, and a questionnaire survey were performed, as well as blood serum and stool samples for laboratory research. Blood serum samples were collected in BD Vacutainer tubes and stored in Biobank at -20°C until testing. Faeces samples were collected in ethanol and stored in the Biobank at -80°C until testing.

*2.2. Quantitative Analysis of ARs.* Quantitative analysis of AR levels in blood serum and urine was performed using gas chromatography with mass spectrometric detection (GC-MS) as previously described [43]. For quantitative determination of the compounds, a calibration was performed using an artificial matrix containing bovine serum albumin and sodium chloride. The calibration was based on the response of the internal standard (4-(benzyloxy)-phenol) introduced into the test samples in order to normalise the derivatization processes and smooth out the errors in determining the concentrations of the test substances. The study was carried out using an Agilent 6890 gas chromatograph (Agilent Inc., United States) with an automatic sample introduction system coupled to an Agilent 5850 mass spectrometric detector (Agilent Inc., United States) with electron impact ionisation. Chromatography was performed using the Restek Rtx 5 Sil-MS column (Restek Corporation, Bellefonte, USA) with the following column parameters: length, 30 m; cross-sectional diameter, 250 $\mu$m; and particle size, 0.25 $\mu$m. The chromatography parameters were as follows: sample injection with a flow division of 5 : 1, carrier gas, helium, gas supply mode, constant flow, flow rate, 1 ml/min, and input temperature, 290° C. The thermometer was set at the initial temperature of 80°C, holding time of 1 min, and then increasing temperature to 320°C at a rate of 25°C/min, holding time of 4 min.

*2.3. Sample Preparation.* The preparation of the samples was carried out using the technique described in the work [43]. For the preparation of blood serum samples, the internal standard (4-(benzyloxy)-phenol) and 200 $\mu$l of diethyl ether were added to 100 $\mu$l of serum, and the mixture was stirred on a shaker and then centrifuged at 2000 rpm. The urine samples were lyophilised; then, the internal standard (4-(benzyloxy)-phenol) and 300 $\mu$l of diethyl ether were added to approximately 5 mg of the sample, and the mixture was stirred on a shaker and then centrifuged at 2000 rpm. Liquid-liquid extraction was repeated twice. The organic phase was transferred to disposable tubes and evaporated in a stream of nitrogen, followed by derivatization carried out with a silylating agent, methyl-silyl-trifluoroacetamide (MSTFA), for 30 min at 60°C. After the derivatization procedure, the samples were analysed by GC-MS. The technique was validated in terms of selectivity, linearity, accuracy, reproducibility, matrix effect, and analyte stability. Validation was carried out in accordance with the FDA Bioanalytical Method Validation Guidelines.

*2.4. Quantitative Analysis of Cell-Mediated Immunity.* Estimation of lymphocyte subsets was performed by flow cytometry on BD FACSCanto™ II with 2 diode lasers (488 and 633 nm). EDTA-treated blood samples were stained with fluorochrome-labeled monoclonal antibodies according to the manufacturer's protocol (Becton Dickinson, USA). The applied antibodies are listed below (catalogue numbers are given in brackets): CD45 APC-Cy7 (557833), CD25 APC-Cy7 (557753), TCR$\alpha\beta$FITC (555547), TCR$\gamma\delta$PE (555717), CD127 PE (IM1980U), CD4 FITC/CD38 PE/CD3 PerCP-Cy5.5/anti-HLA-DRAPC (340573), CD4 FITC (340133), CD3 FITC/CD16+56 PE/CD45 PerCP-Cy5.5/CD4 PE-Cy7/CD19 APC/CD8 APC-Cy7 (644611), CD3 PerCP-Cy5.5 (345766), CD3 APC (555342), CD8 PE-Cy7 (557746), CD19 FITC (340409), CD14 APC (555399), CD45 PerCP-Cy5.5 (564105), CD86 PE (IM2729U), CD95 APC (558814), CD45RAFITC/CD45R0 PE/CD3 PerCP-Cy5.5/CD8 APC (340574), CD45RAFITC/CD62LPE/CD3 PerCP-Cy5.5/CD8 APC (340978), CD 288 (TLR8) PE (MA5-16194), CD282 (TLR2) FITC (11-9922-42), CD283 (TLR3) PE (12-9039-82), CD284 (TLR4) PE-Cy7 (25-9917-42), CD289 (TLR9) FITC (11-9093-82), CD279 (PD-1) FITC (329904), and CD274 (B7-H1, PD-L1) PerCP-Cy5.5 (329738).

The prepared samples were processed in BD FACSDiva software. Analysis was performed in ≥50 000 cells of each sample. The results for T-B and NK cells were counted as a percentage of total lymphocyte numbers and subsets as a percentage of T-B and NK populations. $T_{reg}$ were phenotyped as $CD4^+CD25^+CD127^{dim}$, the expression of PD-1/PDL-1 (CD279 and CD274, respectively) was assessed in T cell population, and expression of TLR 2, 3, 4, 8, and 9 was estimated in granulocytes, monocytes, and lymphocytes.

*2.5. Faecal Microbiota Transplantation.* To explore whether the intestinal microbiota has the ability to produce AR molecules, thus affecting the response to ICI therapy in lung cancer patients, we colonized germ-free Balb/c mice (8 to 10 weeks old) obtained from "Taconic Biosciences," New

Table 2: Characteristics of groups of germ-free mice and donors of stool samples for faecal microbiota transplantation.

| Group of animals | Number of animals | Material for transplantation | Route of administration | Donor of microbiota |
|---|---|---|---|---|
| 1 (control) | 10 | 0.9% NaCl solution | Intragastric | N/A |
| 2 | 10 | Faecal microbiota sample from donor 1 | Intragastric | Donor 1: female, 48 yo; lung adenocarcinoma T4N1M1a, stIV; PFS, 49 months after the ICI therapy |
| 3 | 10 | Faecal microbiota sample from donor 2 | Intragastric | Donor 2: male, 71 yo; lung adenocarcinoma T2bN3M1b, stIV; PFS, 5 months after the ICI therapy |
| 4 | 10 | Faecal microbiota sample from donor 3 | Intragastric | Donor 3: male, 73 yo; lung adenocarcinoma T2bN0M1, stIV; PFS, 7 months after the ICI therapy |

ICIs: immune checkpoint inhibitors; PFS: progression-free survival; N/A: not applicable; yo: years old.

York, USA ($n = 60$), with faecal microbiota from lung cancer patients after ICI therapy ($n = 3$) (see Table 2). For the purpose of bacterial colonization, mice of both sexes were chosen at random, with the male-to-female ratio being uniformly distributed among the groups. Three times a day (100 $\mu$l/day), mice were gavaged with either 100 $\mu$l of microorganism culture suspension or 100 $\mu$l of faecal microbiota suspension from lung cancer donors. In order to get ready for faecal microbiota transplants, frozen stool samples from patients were homogenized (0.1 g of faeces to 1000 $\mu$l of saline solution) and filtered through paper filters to create a chip. In order to get ready for faecal microbiota transplants, frozen stool samples from patients were homogenized (0.1 g of faeces to 1000 $\mu$l of saline solution) and filtered through paper filters to create a chip. Three times a day, 100 $\mu$l of 0.9 percent NaCl solution (100 $\mu$l/day) was gavaged into mice in the control group. The acclimatisation period of the animals was at least 4 days. The mice were kept in groups of five animals per cage in sterile, ventilated cages in the SPF area of the Testing Laboratory Center for two weeks following transplantation. The cages were set up on a 12-hour light/12-hour dark cycle, with free access to food and water at a temperature of 20 to 23°C and a humidity of 35 to 75%. The animals were weighed and given a clinical examination prior to the experiment commencing. All experiments were approved by the Ethics Council of the National Medical Research Centre for Oncology (protocol code № 44, date of approval 20.12.2019).

*2.6. Quantitative Analysis of Tryptophan Metabolites in Blood and Faeces.* Quantitative analysis of Trp metabolites in blood serum and faeces was carried out by high-performance liquid chromatography with mass spectrometric detection (HPLC-MS/MS) using Agilent 1200 liquid chromatograph (Agilent Inc., USA). Chromatographic separation was performed using the Discovery PFP HS F5 analytical column (2.1*150 mm; 3 $\mu$m). For detection, a mass spectrometric detector based on an Agilent 6460 triple quadrupole (Agilent Inc., USA) MRM and electrospray ionisation was used. The resulting signal was processed using MassHunter software (Agilent Inc., USA). The concentration of metabolites was calculated using the internal standard method (2-hydroxynicotinic acid). Analytical standards were prepared using an artificial matrix containing bovine serum albumin and sodium chloride. The studied metabolites were added to the matrix and prepared according to the analysis method. For the preparation of blood serum sample, the internal standard (2-hydroxynicotinic acid) was added to 100 $\mu$l of serum, proteins were precipitated with acetonitrile, and the supernatant was evaporated and redissolved in water with the addition of 10% methanol and ascorbic acid to prevent analyte oxidation. Faeces samples were lyophilised, and approximately 5 mg was extracted with 50% methanol in water with the addition of an internal standard and ascorbic acid. The samples were analysed by HPLC-MS/MS following centrifugation.

*2.7. Statistical Data Analysis.* The statistical software GraphPad Prism 10 v10.0.2 (171) was utilized to process the data using nonparametric statistics. The mean and standard deviation were used to present all data. All experimental data were analysed using Welch's one-way analysis of variance (ANOVA) or multiple Mann–Whitney tests with the two-stage step-up method (Benjamini, Krieger, and Yekutieli) (false discovery rate $Q = 5 – 20$ percent). Statistical significance was defined as $p$ values <0.05 ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$). Spearman's correlation analysis was carried out, and the statistical significance of the correlation coefficient was evaluated.

## 3. Results

*3.1. AR Profiling in Faeces Samples.* For AR representation analysis, we chose resorcinol molecules with different alkyl side chains: resorcinol (without alkyl radical, C0), methylresorcinol (C1), ethylresorcinol (C2), propylresorcinol (C3), penthylresorcinol (C5), hexylresorcinol (C6), dodecylresorcinol (C12), and pentadecylresorcinol (C15). According to data available in the literature, plant ARs predominantly have long alkyl side chains (C15-C25) [44], while the structures of microbial ARs vary greatly depending on the type of organism. Large and complex polyphenolic compounds cannot be absorbed but can be metabolized by the microbiota to more simple structures [45]. Profiling of ARs with diverse structures could shed light on the contribution of microbial and diet ARs to the regulation of metabolic processes and the development of disease states such as cancer.

In the first step, we analysed plasma and faecal AR concentrations in 100 healthy donors and 100 lung cancer donors. The results of the analyses are presented in

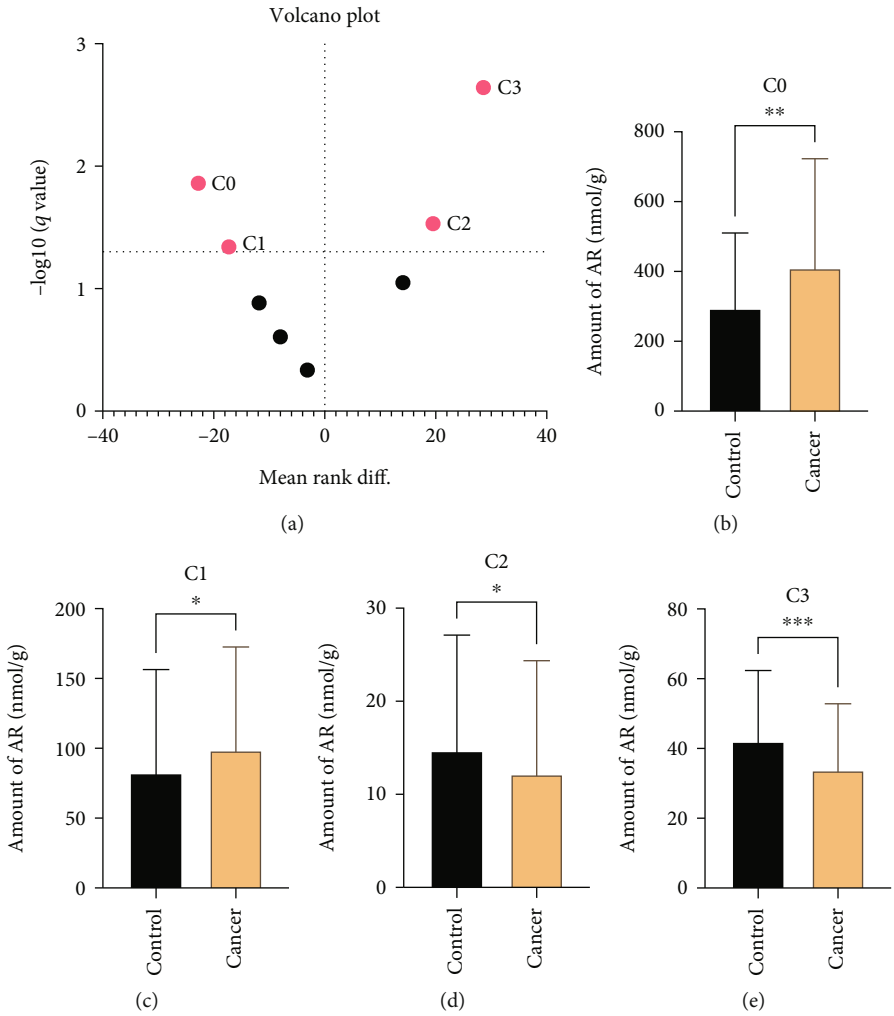



Figure 2: The representation of AR content in the faeces of healthy people and lung cancer patients. (a) A volcano plot based on multiple Mann–Whitney tests of AR content in the faeces of healthy people and lung cancer patients; the volcano graph represents the changes in AR levels between different cohorts of faeces' donors (the $Q$ value reflects a false discovery rate of 5%); the mean rank difference values reflect the direction of changes in the AR level (values below zero indicate an increase in the AR level in the faeces, while values above zero indicate a decrease in the AR level in the faeces of human donors); statistically significant values are marked with red dots. Differences in AR content in stool samples from healthy people and lung cancer patients according to unpaired Mann–Whitney test: (b) C0, resorcinol; (c) C1, methylresorcinol; (d) C2, ethylresorcinol; and (e) C3, propylresorcinol ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). Black bars indicate AR value (nmol/g) in the faeces of healthy donors; orange bars indicate AR value (nmol/g) in the faeces of lung cancer patients.

Figures 2 and 3. Statistically significant differences were observed in AR concentrations ($p < 0.05$) between lung cancer and healthy donors for AR C0, C1, C2, and C3 in faeces samples (Figures 1(a)–1(e)). Furthermore, the concentrations of ARs were significantly higher in most of the faeces samples compared to the serum samples. The highest concentration of all ARs for resorcinol was observed both in faeces and serum. It is worth noting that the amount of C0 in lung cancer patients was higher in faeces but not in blood compared to healthy donors. This high concentration of resorcinol compared to other homologs can be explained by the metabolic activity of gut bacteria that convert different polyphenolic compounds to resorcinol, which is concentrated in faeces.

For further investigation of the dynamics of AR concentrations in biosamples, we selected a group of lung cancer patients ($n = 21$) who were recommended to take ICI (anti-PD/PD-L1) and measured AR concentrations in faeces and blood serum before and after ICI therapy. According to the results of comparative analysis, there were no differences between AR amount before and after therapy.

According to the response to therapy, the patients were split into four groups with positive, negative (further cancer progression), stable (no improvement and no cancer progression), and non-negative response (combined positive + stable groups) (Table 1). In each of the groups, AR concentrations along with other serum and faecal indicators were measured and the correlation coefficients between them were counted (Supplementary Table 1). However, when analysed differentially according to immunotherapy response, changes in AR concentrations demonstrated multidirectional dynamics (Figures 4 and 5).

For example, C5 (Figure 4(b)) and C15 (Figure 4(c)) amounts that did not differ significantly in serum before

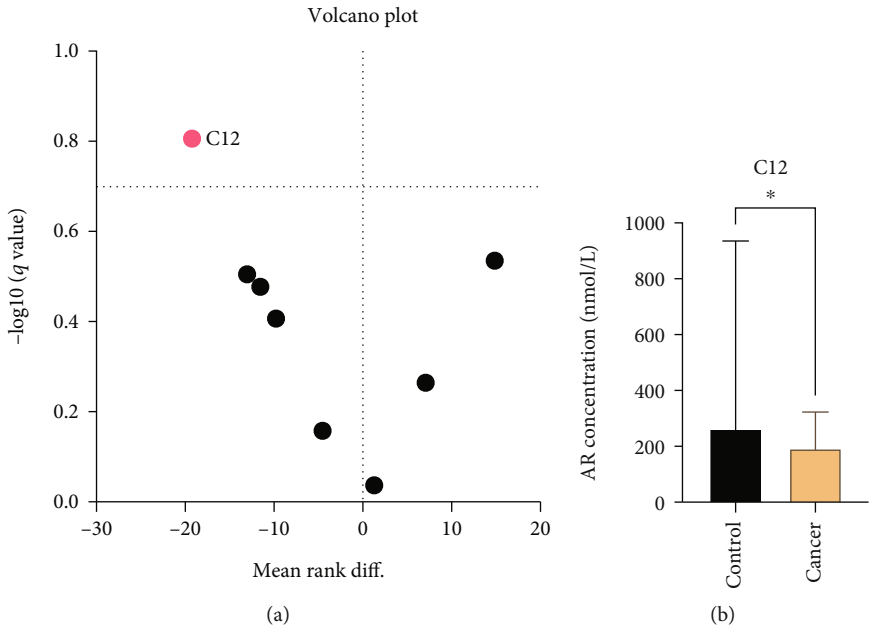



Figure 3: The representation of AR content in serum of healthy people and lung cancer patients. (a) A volcano plot based on multiple Mann–Whitney tests of AR content in serum of healthy people and lung cancer patients; the volcano graph represents the changes in AR levels between different cohorts of serum donors (the $Q$ value reflects a false discovery rate of 20%); the mean rank difference values reflect the direction of changes in the AR level (values below zero indicate an increase in the AR level in serum, while values above zero indicate a decrease in the AR level in serum of human donors); statistically significant values are marked with red dots. (b) Differences in C12 (dodecylresorcinol) content in serum samples from healthy people and lung cancer patients according to unpaired Mann–Whitney test ($^*p < 0.05$). Black bar indicates AR value (nmol/l) in serum of healthy donors; orange bar indicates AR value (nmol/l) in serum of lung cancer patients.

and after ICI therapy showed a statistically significant increase in therapy-responsive groups after therapy. At the same time, in the faeces, we observed an increase in C2 concentrations (Figure 5(b)) after ICI therapy in the therapy-responsive groups and no statistically significant changes were detected in nonresponsive groups.

*3.2. A Correlation Analysis of AR Concentrations in Blood and Faeces before and after Therapy with Faecal Microbial Metabolites.* To investigate whether these changes in AR content after therapy are related to microbiome metabolic perturbations, we conducted a correlation analysis of AR concentrations in blood and stool before and after therapy with faecal microbial metabolites. Therefore, many correlations were detected between AR concentrations and faecal microbial metabolite concentrations. The complete results of the correlation analysis are presented in the Supplementary Table 2, and some of them are discussed below.

In both blood and faeces samples, we observed a strong positive correlation ($r > 0.8$, $p < 0.02$) of C0 concentrations and tryptamine concentrations before immunotherapy for the group with positive response to therapy, while this correlation disappeared after therapy. At the same time, it is known that tryptamine has an exclusively bacterial origin and has a regulatory effect on the host organism increasing the secretion of anions and fluids in the proximal part of the colon and participating in the immune function [36]. In contrast, in the negative response group, we observed a strong positive correlation of C0 in blood ($r = 0.762$, $p < 0.05$) with xanthurenic acid in faeces and a negative correlation of C0 in faeces ($r = -0{,}738$, $p < 0.05$) with xanthurenic acid in faeces after therapy, but not before it. For C5, we observed a strong correlation with anthranilic acid in the group with a negative response before therapy ($r = 0.824$, $p < 0.05$ for blood C5, $r = 0.633$, $p < 0.07$ for faecal C5) that disappeared after ICI treatment. For C15, we observed correlations with faecal 5-HIAA in groups with positive ($r = 0.843$, $p < 0.05$ in blood, $r = -0.829$, $p < 0.05$ in faeces) and non-negative response ($r = 0.588$, $p < 0.07$ for blood C15, $r = -0.661$, $p < 0.05$) only after immunotherapy. Furthermore, correlations between different tryptophan metabolites (indole, indole-3-acetic acid, indole-3-propionic acid, indole-3-lactic acid, indole-3-acrylic acid, indole-3-butyric acid, indole-3-carboxaldehyde, kynurenic acid, and quinolinic acid) and AR homologs were found in faeces of different groups of patients. All these and other data presented in Supplementary Table 2 show the dependence of AR concentrations on the metabolic activity of the gut microbiome, although the exact role of gut bacteria in the synthesis and metabolism of ARs is not known so far.

*3.3. Profile of AR Profiling after Microbiota Transplantation.* To investigate whether the ARs examined have microbiotic origin or come from diet, we carried out faecal microbiota transplantation (FMT) from three lung cancer donors after the ICI therapy to germ-free mice of the Balb/c lineage. A significant increase in individual members of the homologous series of ARs was revealed after FMT was revealed (Figure 6).

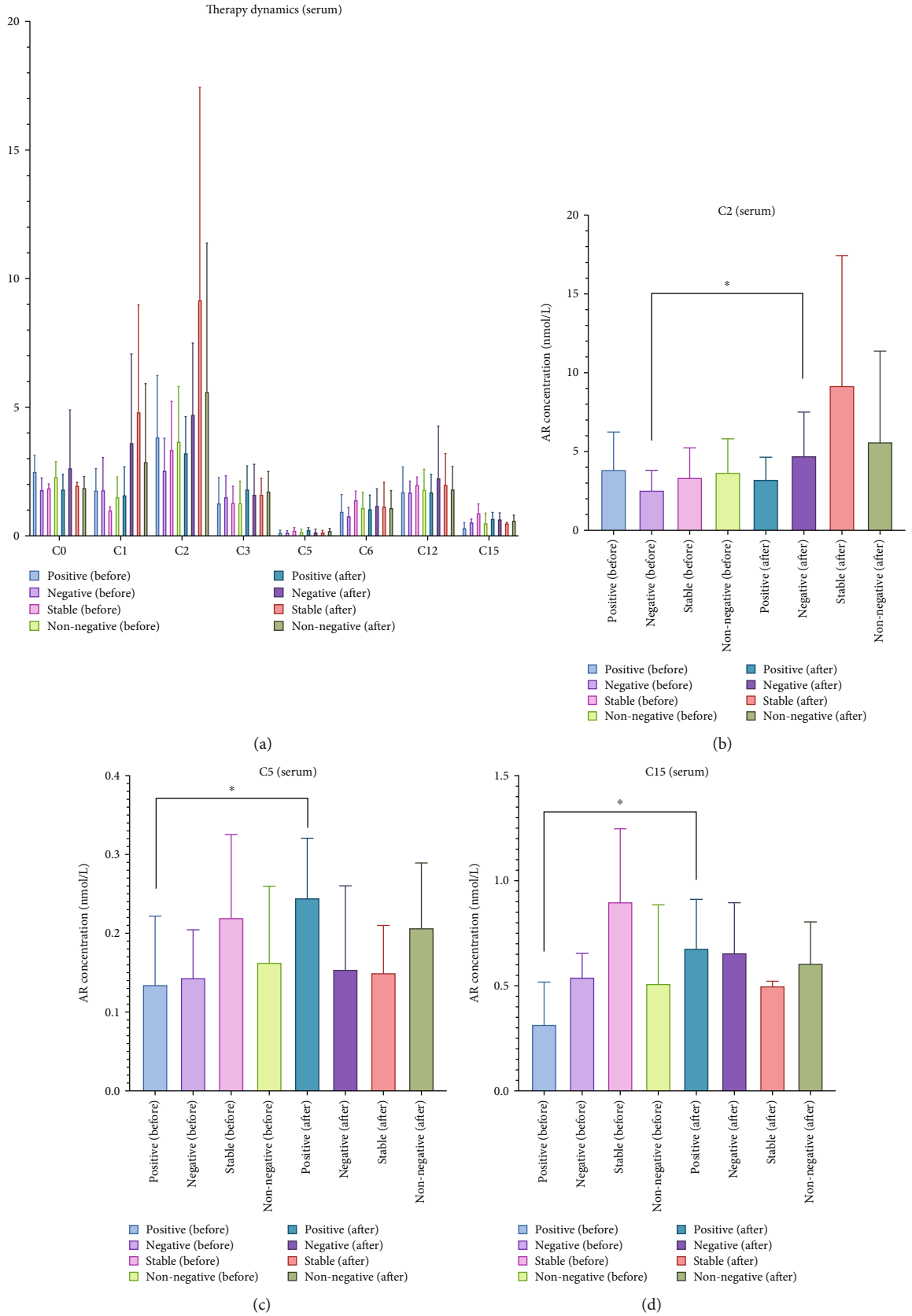



Figure 4: AR dynamics in blood serum before and after anti-PD/PD L1 therapy observed in four groups of lung cancer patients—with positive, stable, non-negative (positive + stable), and negative dynamics. (b) Dynamics of C2 (ethylresorcinol), (c) C5 (pentylresorcinol), and (d) C15 (pentadecylresorcinol) amounts in different groups of patients according to paired Wilcoxon test ($^*p < 0.05$).

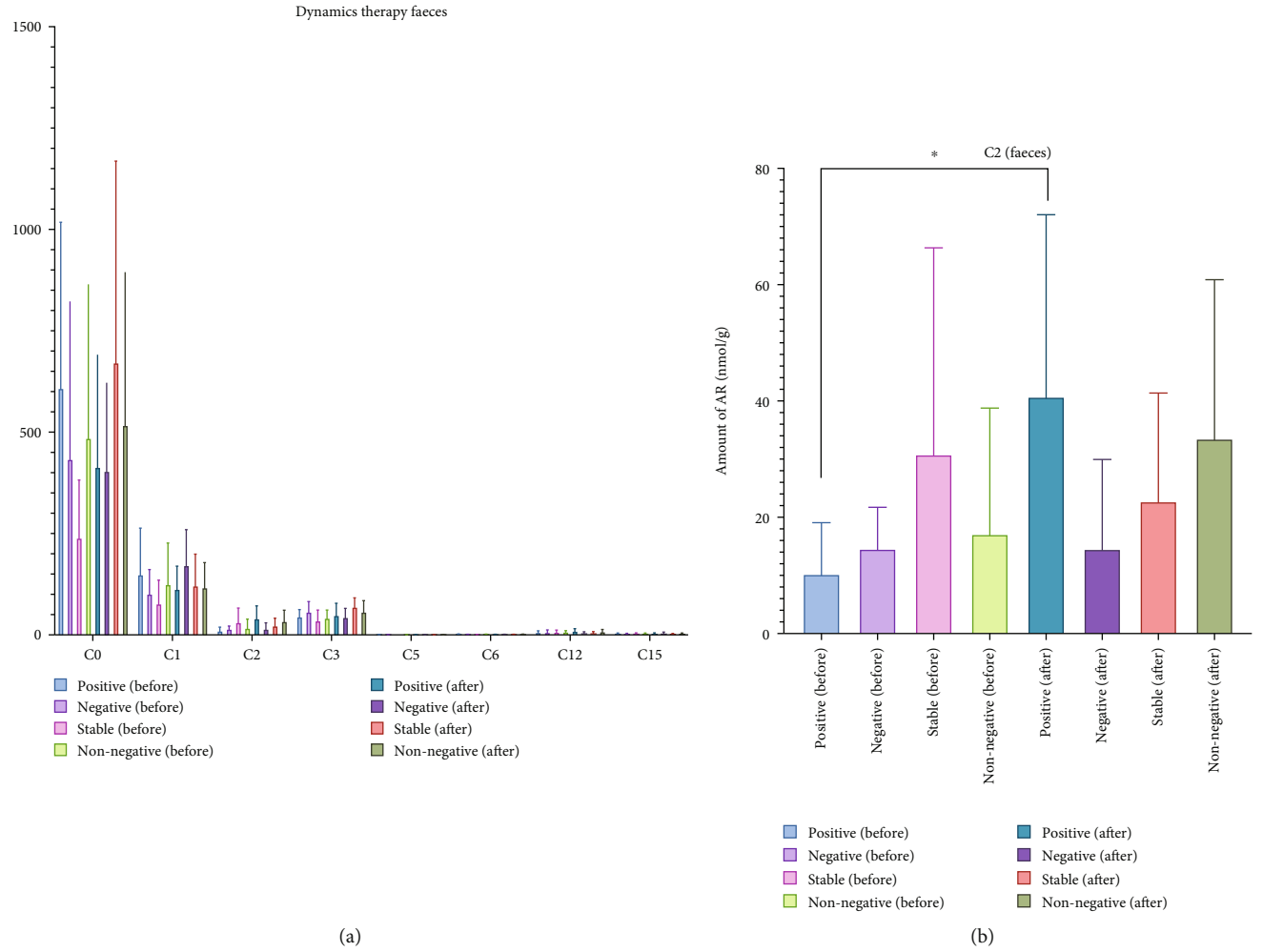



Figure 5: AR dynamics in faeces before and after anti-PD 1/PD L1 therapy observed in four groups of patients with lung cancer, with positive, stable, non-negative (positive + stable), and negative dynamics: (a) a common representation of AR dynamics; (b) dynamics of C2 (ethylresorcinol) amounts in different groups of patients according to paired Wilcoxon test ($^*p < 0.05$).

Considering that the animals were on the same diet and consumed almost the same amount of food and that mouse cells are unable to synthesise any AR molecules, a significant increase in the content of C3, C12, and C15 ($p < 0.05$) in colon contents in 14 days after the transplantation indicates a high probability of synthesis of these ARs by representatives of the human gut microbiota transplanted into gnotobiotic mice.

*3.4. A Correlation Analysis of AR Content in Blood Serum and Faeces with Immune Cell Content in Blood before and after ICI Therapy.* To further investigate whether ARs have any immunoregulatory potential, we conducted a correlation analysis of AR content in blood serum and faeces with immune cell content in blood obtained from lung cancer patients before and after ICI therapy. The results of correlation analysis for groups of patients with different post-therapeutic dynamics (positive, non-negative, and negative dynamics) are presented in Figure 7.

Before ICI therapy, we observed many correlations of ARs in both blood and faeces with granulocytes and monocytes, including TLR-carrying cells, and there was also a correlation with "exhausted" B cells, which could characterise the antimicrobial response and indicate a possible connection of ARs to the gut microbiota in patients with lung cancer. After ICI treatment, the number of statistically significant correlations between AR and immune cells decreased, and only the medium-strength correlation ($r \leq 0.6$, $p < 0.05$) of CD8 + cells with long-radical resorcinols (C6, C12) and that of monocytes with C2 molecules remained, while prior to treatment, the treatment C12 was inversely correlated with the amount of CD8 + PD-1, and the concentration of C0 was inversely correlated with CD8+ memory cells.

In blood serum, we found a greater number of correlations in the group of patients with negative dynamics before ICI therapy than in the groups with non-negative and positive (12, 8, and 3, respectively). At the same time, there were no correlations involving CD4 + PD-1 or CD8 + PD-1 cells in patients with negative dynamics in contrast to patients with non-negative dynamics. For different ARs, we observed positive and negative correlations with NKT cells and granulocytes in the group with negative dynamics, whereas there were no such correlations in the group with non-negative dynamics.

On the contrary, the number of correlations of ARs in faeces with immune cells in blood serum before ICI therapy

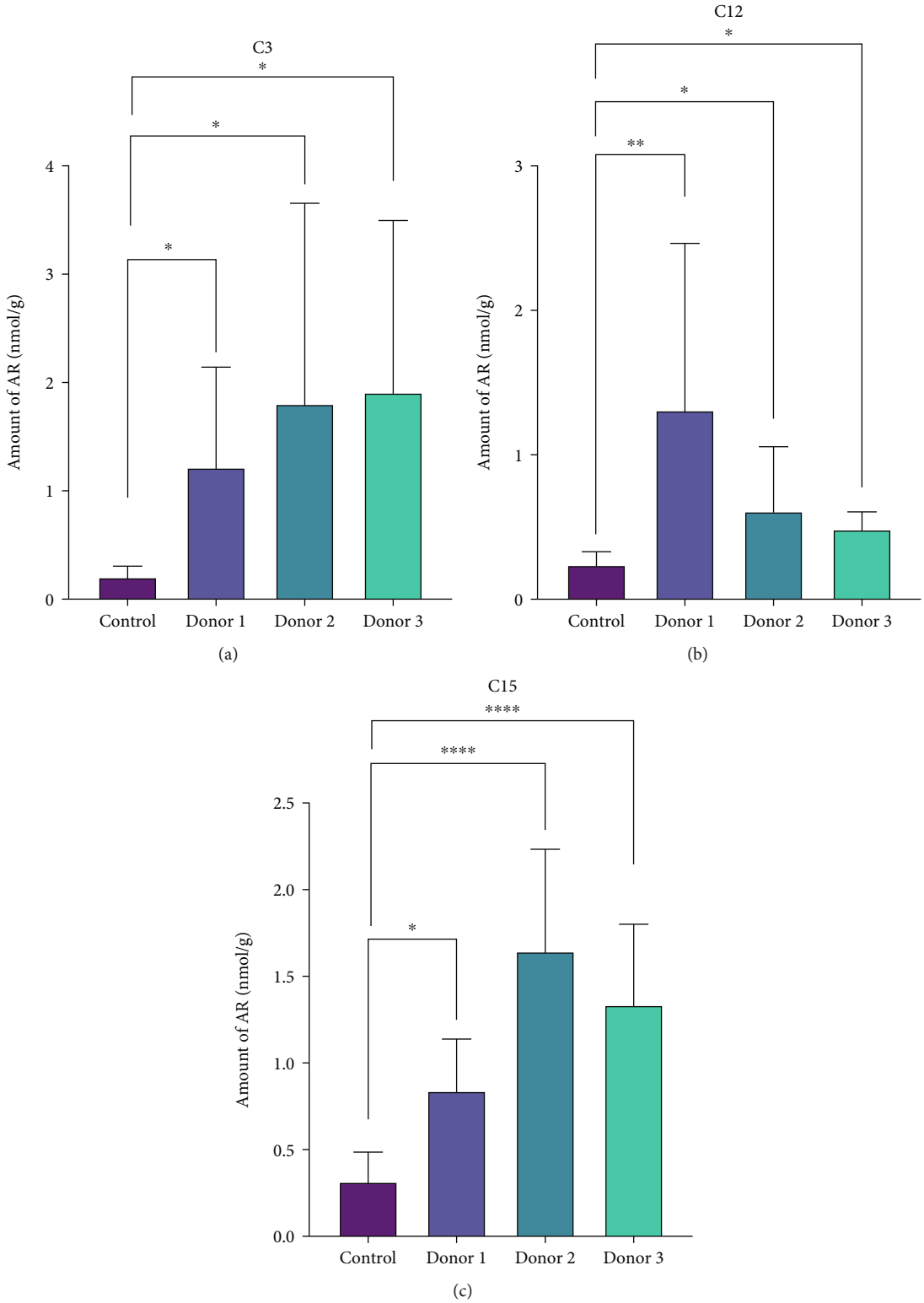
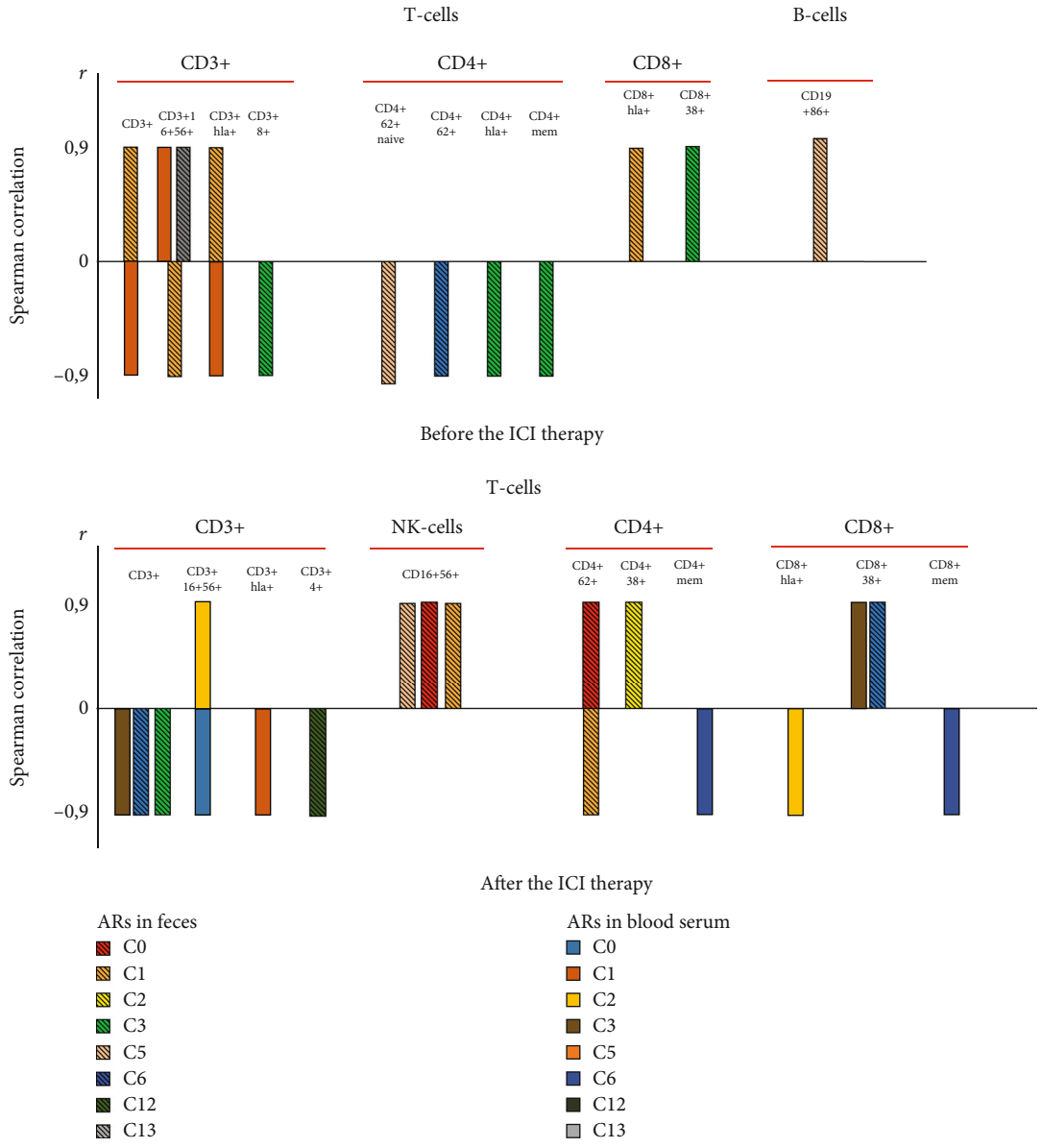


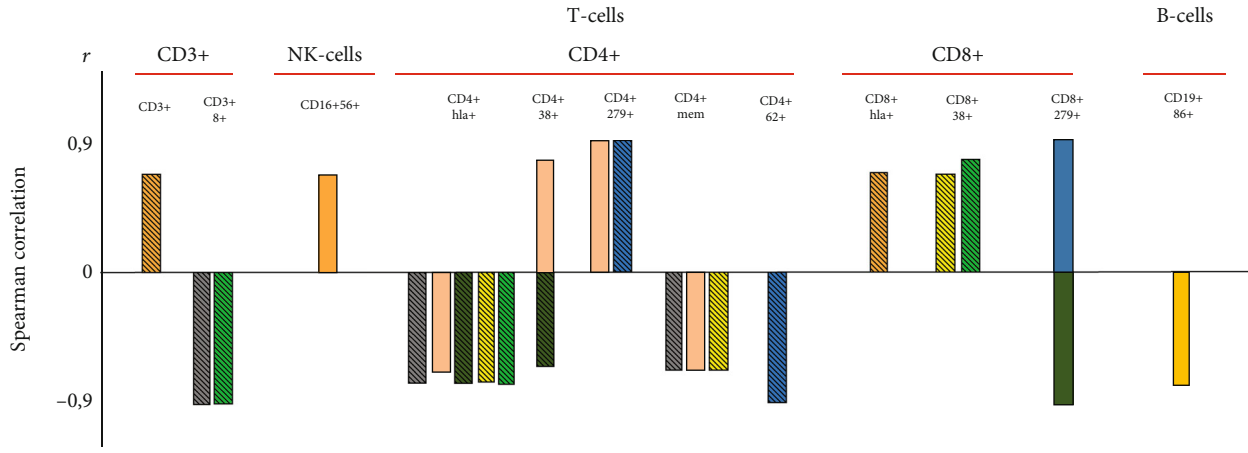

Figure 6: Amounts of (a) propylresorcinol (C3), (b) dodecylresorcinol (C12), and (c) pentadecylresorcinol (C15) in mouse colon contents (nmol/g) after microbiota transplantation from lung cancer donors (donor 1-donor 3) compared to a control group (control). Comparison of AR amounts was performed using the one-way Welch's ANOVA test followed by the $t$ test ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$).

was less in the negative dynamic group than in the negative and positive groups (7, 15, and 12, respectively). Additionally, we noted a strong positive correlation of C6 with CD4 + PD-1+ cells in the group with non-negative dynamics as in blood serum, and there were no such correlations in the group with negative dynamics as well. In the groups with

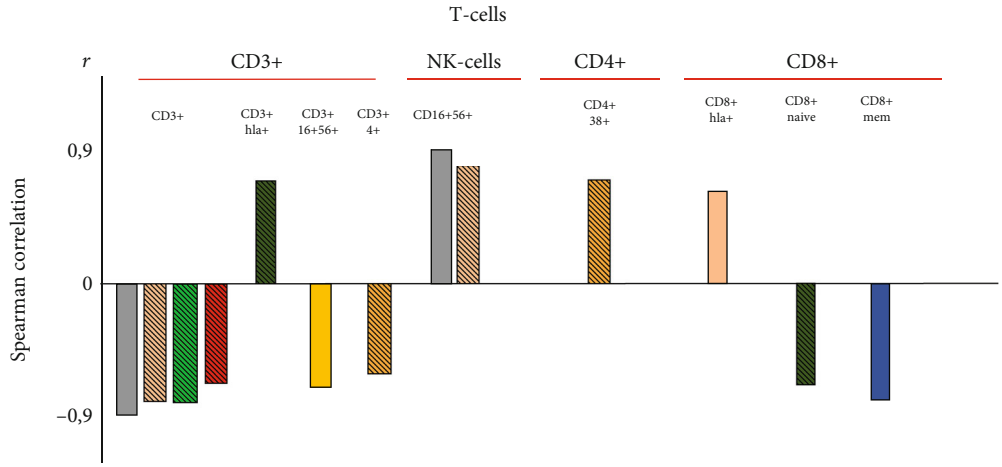



(a) Positive dynamics

Figure 7: Continued.




Before the ICI therapy



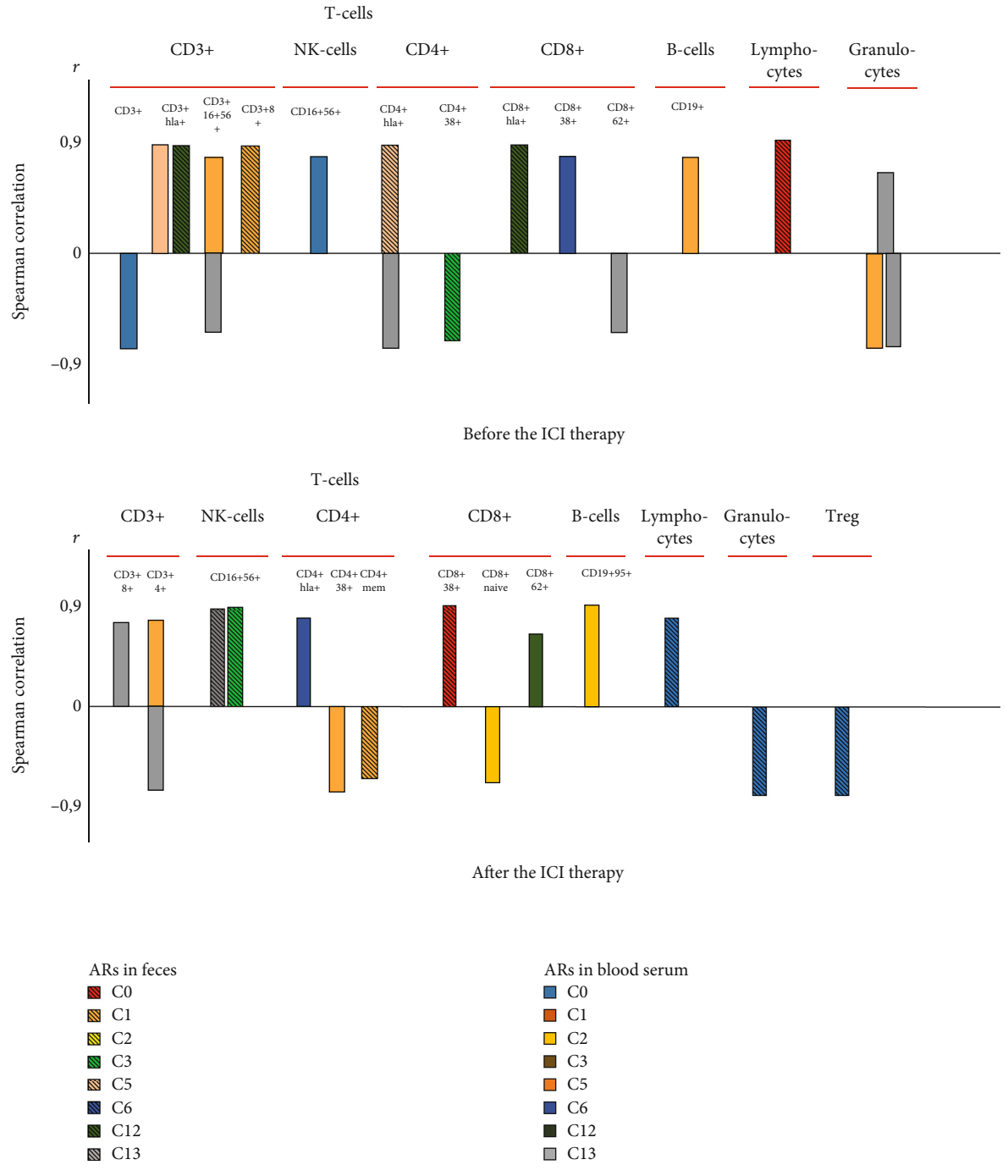

After the ICI therapy

ARs in feces
- C0
- C1
- C2
- C3
- C5
- C6
- C12
- C13

ARs in blood serum
- C0
- C1
- C2
- C3
- C5
- C6
- C12
- C13

(b) Non-negative dynamics

Figure 7: Continued.




(c) Negative dynamics

Figure 7: Correlations between AR content in serum and faeces and cell-mediated immunity parameters in patients with lung cancer before and after ICI therapy with different dynamics.

positive dynamics, activated CD8+ cells correlated with C1 and C3. Additionally, we observed strong positive correlations of several ARs with NK cells in this group in contrast to the groups with non-negative and negative dynamics.

After ICI therapy, we observed fewer correlations for all serum groups of patients compared to faeces; correlations of AR with CD4+ and CD8 + T cells were also fewer or absent. Furthermore, in nonresponsive patients, there were no correlations of AR with NK and NKT cells in contrast to the responsive group and there were no common correlations with therapy-responsive groups. In the faeces, there were many more correlations of ARs with T cells and/or NK cells. We observed many common correlations in therapy-responsive groups (such as negative correlations of ARs with CD3+ cells and positive correlations with CD4+ and CD16+) in contrast to the nonresponsive group in

which correlations significantly differed from the other groups (see Figure 7). Taken together, the data suggest the predominant impact on the functioning of immune cells of ARs containing in the stool, which appear to have microbiotic origin and depend on the composition of the microbiota.

## 4. Discussion

In recent decades, the interaction between the gut microbiota and host cells has been intensively studied. The existence of a correlation of alterations in the intestinal microbial community with various disorders, such as obesity [46], inflammatory bowel disease (IBD) [47], diabetes [48], cardiovascular [49], and liver disease [50], was shown. Furthermore, bacteria have been shown to affect the oncogenesis process [51]. The main mechanisms through which gut bacteria can influence host cell malignization are based primarily on the effects of specific toxins or virulence factors, as well as bacterial metabolites: short-chain fatty acids (SCFA), polyamines, secondary bile acids, products of polyphenols, and tryptophan (Trp) catabolism [52]. Alterations in the composition of the normal intestinal microbiome (dysbiosis) can alter the concentrations of metabolites, which in turn have pronounced effects on cellular and immune signaling and cell division. One of the most striking examples of the influence of bacterial metabolites on the host organism is the effect of Trp catabolism products. The bacterial metabolism of Trp is complex and requires the cooperation of different bacterial species [53] as well as some opportunistic pathogenic bacterial species, including *Escherichia coli*, *Proteus vulgaris*, *Paracolobactrum coliforme*, *Achromobacter liquefaciens*, and *Bacteroides* spp. which are also capable of producing indole [54].

The main Trp derivatives are indole, indolic acid (IA), indican, skatole, indole-3-acetate (I3A), tryptamine, indole-3-acetaldehyde (IAld), indole-3-lactic acid (ILA), indole-3-propionic acid (IPA), indole-3-acrylic acid (IAcrA), and tryptophol [53]. Alterations in the composition of the intestinal microbiome change the concentration of Trp and its metabolites in serum, which evidences the influence of the microbiome on Trp metabolism. According to current data, Trp metabolism plays an essential role in the suppression of anticancer immune responses and the increase in malignant properties of cancer cells, which ultimately leads to tumour progression [55]. Gut bacteria produce Trp metabolites and affect the performance of the IDO enzyme, which is active in many tumour tissues. Trp-like molecules inhibiting IDO are considered promising anticancer agents.

On the other hand, there is evidence that the gut microbiota can affect cancer treatment efficiency, as well as the development of resistance to therapy. In particular, microbiome composition was shown to affect the efficiency of immunotherapy targeting checkpoint pathways CTLA-4 (cytotoxic T lymphocyte-associated protein 4) and PD1 (programmed cell death 1)/PD-L1 (programmed death ligand 1) [56]. In the study by Tian et al., it was shown that correction of the microbiome composition by *Clostridium butyricum* reduced chemotherapy-associated side effects, such as diarrhoea, in patients with lung cancer, although the mechanism is not yet clear [57].

Thus, the alterations in Trp metabolite concentrations observed in faeces samples of different groups of lung cancer patients before and after immunotherapy reflect the alterations in gut bacteria metabolic activity. We were able to demonstrate that many of these alterations were correlated with changes in AR concentrations in both serum and faeces, indicating the involvement of AR molecules in the microbiota and host metabolism. We suppose that ARs may enter the human body not only as diet components but also as metabolites of the gut microbiota and take on the role of regulators of the microbiota composition and its metabolic activity. Our experiments concerning FMT from lung cancer donors to germ-free mice confirmed the high probability of synthesis of ARs by the human gut microbiota. The existence of a large number of strong positive and negative correlations between ARs in faeces and blood serum and different types of immune cells points to AR involvement in the host's immune response and their influence on the response to ICI therapy. Before ICI therapy, we observed many correlations of ARs in both blood and faeces with granulocytes and monocytes, including TLR-carrying cells, and there was also a correlation with "exhausted" B cells, which could characterise the antimicrobial response and indicate a possible connection of ARs to the gut microbiota in patients with lung cancer. These observations are consistent with data that altered microbiota composition or loss of barrier integrity frequently seen in cancer patients [58] leads to intestinal exposure to various pathogen-associated molecular patterns (PAMP), proinflammatory cytokines, apoptotic fragments, and toxins that break through the intestinal epithelial cell barrier. The translocation of bacteria and their components triggers the intestinal immune system through the activation of Toll-like receptors (TLRs), resulting in potentially harmful effector T cell responses. The secretion of IL-1 and IL-6 by intestinal epithelial lymphocytes increases the Th1 and Th17 response by dendritic cells and macrophages, leading to higher levels of commensal-specific IgG [59]. Furthermore, negative dynamics is likely to take place when the number of correlations of cell-mediated immune parameters with the level of AR is high in blood serum and low in faeces. We can assume that in nonresponsive lung cancer patients, the microbiota composition is changed in a way that makes the mucosal barrier more leaky, thus allowing ARs to flow to the blood. On the other hand, ARs apparently realise their effects on ICI outcomes primarily by influencing the gut microbiome and the composition of immune cells. In addition, the data obtained allow us to emphasise the assumption that the participation of PD-1 expressing PD-1 in correlations with AR may serve as a prognostic factor for the prediction of the clinical effect of ICI therapy.

The regulatory function of ARs can be carried out both directly due to entering host cells and acting through specific receptors or by influencing protein function or indirectly acting as quorum-sensing molecules that regulate gut microbiome composition and bacterial metabolism, affecting the production of other metabolically active molecules.

We suppose that ARs due to their similarity to cannabinoids could act through CB1 or CB2 receptors binding on T cells, thus influencing the activation state of T cells and the expression of PD1/PD-L1. In addition, polyphenolic xenobiotics are known to activate AhRs (aryl hydrocarbon receptors). The AhR pathway is known to be a regulator of PD1 expression in CD8+ T cells [60]. Thus, another presumptive mechanism of AR action is probably realised through activation of the AhR pathway in immune cells. However, to our knowledge, there is no exact understanding of AR mechanisms of action.

Given that according to our study, several ARs were strongly positively correlated with CD4+ and CD8 + T cells expressing PD1 (CD279 + T cells) only in that responded to treatment before therapy (these correlations disappeared after the therapy), it can be assumed that the content of AR influences the expression of PD1 in T cells, thus defining the response to ICI therapy.

## 5. Conclusions

In this study, we analysed 200 blood serum and 200 stool samples from healthy donors and lung cancer patients and managed to reveal several trends during AR profiling. AR concentrations in both faeces and serum differ dramatically between healthy and lung cancer donors. The statistically significant difference in AR concentrations ($p < 0.05$) between lung cancer and healthy donors for C0, C1, C2, and C3 alkylresorcinols in faeces samples was observed. Furthermore, AR concentrations differed in the subgroups of patients depending on their response to immunotherapy. For some ARs, we observed a correlation with the faecal Trp metabolite content. As a result of the experiments on FMT, we demonstrated a significant increase in AR concentrations in faeces, which indicates their microbial origin. Furthermore, we revealed the existence of some strong positive and negative correlations both in faeces and serum for several ARs with immune cells, and these interrelationships were quite different between the therapy-responsive and nonresponsive groups. Thus, AR content may influence the response to ICI therapy in patients with lung cancer. However, AR profile may serve as an instrument for predicting immune therapy success. All these data confirm the assumption that there exists a "tumorogenic microbiome," formed by altered gut microbiota in cancer patients. Although it is not yet known what species of microorganisms are involved in AR synthesis in the human gut, it is obvious that ARs, along with tryptophan degradation metabolites, are regulatory molecules that determine the functioning of antitumor immunity.

However, our study has some limitations. In accordance with the experiment's design, we fed mice a standard chow diet for 14 days following the transplantation of faecal microbiota, and we examined the gut microbiota and faecal compounds obtained straight from the colon of each of the ten animals in each group. All sets of mice must, however, have a time-course representation that shows the steady rise in ARs. More studies demonstrating the direct synthesis of ARs by gut microbes are required. Additionally, it should be highlighted that the patient groups for lung cancer that received ICI therapy were relatively small, and the appearance of the reprediction error must be taken into account. Therefore, we believe that the observed results point to the necessity of larger sample sizes and more research.

## Data Availability

The supplementary tables consisting raw data and statistics data used to support the findings of this study are available from the corresponding author upon request.

## Ethical Approval

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Council at the National Medical Research Centre for Oncology (protocol code № 44, date of approval 20.12.2019). The animal study protocol was approved by the Ethics Council at the National Medical Research Centre for Oncology (protocol code № 44, date of approval 20.12.2019).

## Consent

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

## Disclosure

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## Conflicts of Interest

The authors declare no conflict of interest.

## Authors' Contributions

A.V.S. and E.Y.Z. were responsible for the conceptualization. E. Y. Z, S.A.A., A.A.K., and P.A.M. were responsible for the methodology. A.A.Z. was responsible for the validation. A.A.Z., A.V.S., and O.I.M. were responsible for the formal analysis. A.B.S., S.V.T., A.S.G., and A.V.G. were responsible for the investigation. V.V.M., S.M.Y., and S.A.R. were responsible for the resources. A.Y.M. and I.A.N. were responsible for the data curation. A.A.Z. and A.V.S. wrote the original draft preparation. E.Y.Z. and A.B.S. wrote, reviewed, and edited the manuscript. A.A.Z. was responsible for the visualization. A.V.S. and S.A.R were responsible for the supervision. O.I.K. and I.A.N. were responsible for the project administration. V.V.M., S.M.Y., and S.A.R. were responsible for the funding acquisition. All authors have read and agreed to the published version of the manuscript.

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## Supplementary Materials

Supplementary Table 1: Spearman's correlations of indole metabolites in stool samples from lung cancer patients with amounts of different alkylresorcinols in blood and faeces depending on the response to therapy. Statistically significant correlations are indicated in red boxes. Supplementary Table 2: plasma levels of alkylresorcinols in different groups of lung cancer patients based on the response to therapy. *(Supplementary Materials)*

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