Metabolomic, microbiome and transcriptomic study of crofelemer or loperamide in rat model of afatinib-induced intestine epithelial damage and diarrhea

Zheng Wang[1], Qiongya Guo[1], Wenting Li[1], Lin Fu[1], Xiaofang Li[1], Shanshan Hu[1], and Xiuling Li[1]

[1]Henan Provincial People's Hospital

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Abstract

Diarrhea is the most prevalent side effect of afatinib as an EGFR tyrosine kinase inhibitor. The current study is to investigate the potential protective mechanisms of crofelemer or loperamide in an animal model of afatinib-induced diarrhea. Rats were randomized as the control, afatinib (50 mg/kg), afatinib plus loperamide (50 mg/kg) or afatinib plus crofelemer (50 mg/kg) group. Rats received drugs or saline through oral gavage daily for consecutive 7 days. Diarrhea, weight, serum biomarkers, gut histology or ultrastructure and multiomic changes were analyzed daily or at day 8. Afatinib induced significant diarrhea, weight loss, elevated serum levels of endotoxin, IL-6, IL-1β and TNF-α in rats. Mucosal damage was most prominent in distal ileum, showing edema, inflammatory infiltration, epithelial villus atrophy or fusion and loss of tight junction. Both loperamide and crofelemer conferred protection against afatinib-induced diarrhea and gut damage. Transcriptomic enrichment analysis showed that PPAR and IL-17 signaling pathway are among the top modified pathways in the ileum and colon of the afatinib group, respectively. Metabolomic profiling identified 318 differently abundant metabolites when comparing the afatinib and the control groups, with the most prominent enriched metabolic pathways being metabolism of xenobiotics by cytochrome P450, retinol metabolism and lysine degradation. Afatinib significantly decreased microbial diversity, which is not fully restored by administration of crofelemer or loperamide. Correlation analysis showed that cecal microbiota were significantly correlated with metabolite profiles. Loperamide and crofelemer attenuate afatinib-induced diarrhea and intestinal damage in rats, possibly through regulating microbiota-metabolic axis.

Metabolomic, microbiome and transcriptomic study of crofelemer or loperamide in rat model of afatinib-induced intestine epithelial damage and diarrhea

Running title: Multiomic study of TKI-induced diarrhea

Qiongya Guo [1,+], Wenting Li [2,+], Lin Fu [1], Xiaofang Li [1], Shanshan Hu[1], Zheng Wang [3], Xiuling Li[1,*]

[1]Department of Digestive Diseases, the People's Hospital of Zhengzhou University, Zhengzhou, China

[2]Department of Tropical Medicine, the Second Affiliated Hosptial of Hainan Medical College, Haikou, China

[3]Department of Respiratory and Critical Care Medicine, the People's Hospital of Zhengzhou University, Zhengzhou, China

[+]First authorship: These authors share first authorship

*** Correspondence:** Xiulin Li email@729822969@qq.com

Abstract

Diarrhea is the most prevalent side effect of afatinib as an EGFR tyrosine kinase inhibitor. The current study is to investigate the potential protective mechanisms of crofelemer or loperamide in an animal model of afatinib-induced diarrhea. Rats were randomized as the control, afatinib (50 mg/kg), afatinib plus loperamide (50 mg/kg) or afatinib plus crofelemer (50 mg/kg) group. Rats received drugs or saline through oral gavage daily for consecutive 7 days. Diarrhea, weight, serum biomarkers, gut histology or ultrastructure and multiomic changes were analyzed daily or at day 8. Afatinib induced significant diarrhea, weight loss, elevated serum levels of endotoxin, IL-6, IL-1β and TNF-α in rats. Mucosal damage was most prominent in distal ileum, showing edema, inflammatory infiltration, epithelial villus atrophy or fusion and loss of tight junction. Both loperamide and crofelemer conferred protection against afatinib-induced diarrhea and gut damage. Transcriptomic enrichment analysis showed that PPAR and IL-17 signaling pathway are among the top modified pathways in the ileum and colon of the afatinib group, respectively. Metabolomic profiling identified 318 differently abundant metabolites when comparing the afatinib and the control groups, with the most prominent enriched metabolic pathways being metabolism of xenobiotics by cytochrome P450, retinol metabolism and lysine degradation. Afatinib significantly decreased microbial diversity, which is not fully restored by administration of crofelemer or loperamide. Correlation analysis showed that cecal microbiota were significantly correlated with metabolite profiles. Loperamide and crofelemer attenuate afatinib-induced diarrhea and intestinal damage in rats, possibly through regulating microbiota-metabolic axis.

Significance Statement: Diarrhea may influence patients' compliance and antitumor efficacy of tyrosine kinase inhibitors (TKIs). It's still unknown the exact mechanisms and optimal therapeutic strategy of TKI-induced diarrhea. In this manuscript, metabolomic, transcriptomic and microbiome changes were investigated in a model of afatinib-induced diarrhea. Diarrhea and epithelial damage are associated more with changes of metabolite and microbiome profiles than with that of transcriptomics. Key regulators are identified by multiomic integration analyses as candidates for mechanism and drug target development. The current research shows the underlying protective mechanisms of antidiarrheal agents in TKI-induced diarrhea.

Keywords: tyrosine kinase inhibitors, afatinib, diarrhea, intestine, lung cancer, transcriptomics, metabolomics, microbiome

Article type: original research

Introduction

Currently, lung cancer is responsible for highest occurrence and most death among all hematological and solid organ malignancies (Siegel et al., 2024). Molecular targeted therapy opens a new era of long-standing survival for lung cancer, and has become one of the fundamental treatment regime. In non-small cell lung cancer (NSCLC), epidermal growth factor receptor (EGFR) mutations are most prevalent among all driver gene alterations, accounting for 50-60% of all in lung adenocarcinoma (Harada et al., 2023; Hendriks et al., 2023). EGFR tyrosine kinase inhibitors (EGFR-TKIs) non-selectively targets EGFR by competing with the intracellular ATP binding site, thereof inhibiting subsequent oncogenic signaling transduction and tumor development (Hendriks et al., 2023). EGFR mutated NSCLC patients receiving EGFR-TKIs are able to survive over 3 years on average. The usage of EGFR-TKIs in lung cancer has further been expanded from single use to adjuvant or neoadjuvant therapy, with or without combination with chemotherapy, immunotherapy or antiangiogenic agents. As a second-generation EGFR-TKI, afatinib has been widely accepted in the treatment of NSCLC. Several large randomized controlled trials and real world studies support the use of afatinib in unresectable late stage or metastatic lung cancer. Except for NSCLC patients harboring common or rare EGFR mutations, afatinib has also shown potential efficacy in treating those with NRG1 fusions (Lu et al., 2021; Jiang et al., 2023; Laskin et al., 2020).

Diarrhea has been a common adverse effect in multiple clinical trials for afatinib. According to the LUX-Lung 3 trial in EGFR-mutated NSCLC, the prevalence of diarrhea in the afatinib and the control arm were

95.2% and 15.3% ([?] grade 3: 14.4% vs 0), respectively (Sequist et al., 2013). These data are consistent with subsequent trials, cohorts or real world data, which show a occurrence rate of 70% or more (Barron et al., 2016). A network meta-analysis shows that afatinib is associated with higher risk of diarrhea as compared with other EGFR-TKIs (Zhao et al., 2021). Diarrhea with afatinib tend to occur more frequently in the first month of treatment, with a reduction in frequency thereafter (Shyam Sunder et al., 2023). Current diarrhea management guidelines advise closely surveillance, prophylactic or therapeutic use of loperamide, otherwise dose reduction (eg. from 40 mg/d to 30 mg/d for afatinib). Data are inconsistent regarding the impact of dose reduction in treatment efficacy (Wang et al., 2021). Some studies advocate that reduced dose dose not impair the treatment efficacy of afatinib in EGFR-mutated NSCLC, while others raise concern that response rate and progression-free survival (PFS) decrease with dose reduction (Kim et al., 2019; Wei et al., 2019; Tan et al., 2018; Wang et al., 2018).

Despite several hypotheses proposed and tested, the nature and exact mechanism of EGFR-TKI-associated diarrhea are not fully understood. These hypotheses include secretory mechanisms controlling chloride and water transportation, epithelial leakage, drug efflux dysregulation and accumulation, endoplasmic reticulum stress-induced barrier dysfunction, mucosal inflammation, abnormal metabolism or gut microbiota abnormalities (Secombe et al., 2021; Lysyy et al., 2019; Duan et al., 2019; Kim et al., 2020; Rugo et al., 2019; Secombe et al., 2019; Cárdenas-Fernández et al., 2023; Tao et al., 2022; Van Sebille et al., 2017; Hashimoto et al., 2023). Crofelemer is an anti-secretory anti-diarrheal agent that has been approved for treating secretory diarrhea (Cottreau et al., 2012). Preclinical experiment results are inconsistent regarding crofelemer in TKI-induced diarrhea, with some showing promising protective effect, while others showing little or detrimental effects (Van Sebille et al., 2018). Individual cases support its use in TKI-induced diarrhea (Greene et al., 2021). However, limited effect of crofelemer was shown than was previously anticipated in recently published trials regarding neratinib-induced diarrhea in breast cancer patients (Pohlmann et al., 2022; Jacob et al., 2023; Guy et al., 2024). For sure, elucidation of the diarrhea mechanism may contribute to prophylaxis and management of TKI-associated diarrhea by maximizing the therapeutic effect and reducing toxicity without need of dose interruption.

In the current study, afatinib would be orally administered in rats to develop the diarrhea model. Changes in histology, ultrastructure, genetic transcription will be analyzed in jejunum and colon tissue samples. Distal colon contents are also collected for microbiome and metabolomic analysis. Loperamide or crofelemer are used as antidiarrheal agents in separate experimental groups to analyze their impact on TKI-induced diarrhea and potential protective mechanisms.

Materials and methods

2.1. Chemicals

Afatinib (439081-18-2) and loperamide (53179-11-6) was purchased from Aladdin Biotechnology (Shanghai, China). Crofelemer (4852-22-6) was purchased from Macklin Inc. (Shanghai, China).

2.2. Animals and ethics

Seven-week old Sprague-Dawley rats were purchased and raised at the Laboratory Animal Center of Zhengzhou University. The environment was maintained with a temperature of 21-23°C and relative humidity of 45-55%, with a 12 h light/ dark cycle. Food and water were given ad libitum. Rats were allowed to accommodate to living conditions for 7 days prior to grouping. This study was approved by the Ethics Committee of the Henan Provincial People's Hospital (approval number: 2018-53) and animal experimental policy of Zhengzhou University (protocol code 2022-023).

2.3. Experimental design

Rats were randomized to four groups, the control, afatinib, crofelemer or loperamide group. Chemicals were dissolved in 2 ml of PBS solution. Rats received daily chemical [or phosphate buffered saline (PBS) solution]

exposure for consecutive 7 days through a gavage tube. Doses were 60, 1 and 250, mg/kg/d, for afatinib, loperamide or crofelemer, respectively. Doses of each rat were adjusted daily according to newest measured body weight.

All rats were anesthetized with intraperitoneal injection of sodium pentobarbital and sacrificed at day 8. The whole intestinal duct were resected, removed and then flushed with sterile PBS and weighed. The cecal content samples were collected freshly for microbiome and metabolomic analysis (n=8 in each group).

Survival and body weight was documented daily. Diarrhea severity was evaluated daily by at least two observers according to a grading system as follows: grade 0, no diarrhea; grade 1, mild (soft unformed stools); grade 2, moderate (perianal staining and loose stools); grade 3, severe (watery stools and staining over legs and abdomen).

2.4. Histological and ultrastructure evaluation

Fresh samples of duodenum, jejunum, ileum and colon were collected and stored, or were fixed in 10% formalin for further preparation of paraffin wax block and sections. Hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) staining were performed following standardized protocols. An experienced pathologist evaluated the pathological alterations. Criteria were villus fusion, villus atrophy, disruption of brush border and surface enterocytes, crypt losses/architectural disruption, disruption of crypt cells, infiltration of polymorphonuclear cells and lymphocytes, dilation of lymphatics and capillaries and edema. The latter six criteria were examined in the colon. Each criterion was scored as present = 1 or absent = 0.

Utrastructure of the intestine was evaluated under transmission electron microscopy (TEM). Freshly isolated ileum and colon tissue samples were fixed in glutaraldehyde, re-fixed in osmic acid, then dehydrated and embedded, stained using lead citrate. Ultrathin sections (approximately 50 nm in thickness) were prepared and observed using a JEM-2100F transmitting electron microcopy (Hitachi, Hitachi, Japan).

2.5. Serum cytokine and entotoxin assay

Whole blood samples were collected through cardiac puncture. Serum samples were prepared by centrifugation of blood samples by 3000 rpm for 5 min, and stored at -80 until analyzed. Serum levels of IL-6, IL-1β and TNF-α were measured using enzyme linked immunosorbent assay (ELISA) kits (Neobioscience Biotechnique Co. Ltd., Shenzhen, China) following the instructions. Serum levels of endotoxin were detected using PyroGene Endotoxin Detection Assay kit (Lonza, Switzerland) with an accuracy of 0.005 EU/mL.

2.6. Microbial diversity analysis

Microbial DNA was extracted using the HiPure Stool DNA Kits (Magen, Guangzhou, China) according to manufacturer's protocols. The full length16S rDNA target region of the ribosomal RNA gene were amplified by PCR. Amplicons were extracted from 2% agarose gels, purified and quantified using commercially available kits according to the manufacturer's instructions. Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina platform according to the standard protocols. The V3-V4 region of the bacterial 16S rRNA gene was amplified using using primers 341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT. The usearch R package implements a complete pipeline to turn paired-end fastq files from the sequencer into merged, denoised, chimera-free, inferred sample sequences. The representative OTU sequences were classified into organisms by a naive Bayesian model using RDP classifier based on SILVA database, with the confidence threshold value of 0.8.

The raw data were subjected to a series of preprocessing steps, including merging, filtering, dereplication, denoising, and chimera removal, using the DADA2 R package (version 1.14). Following these procedures, the resulting clean tags were utilized to output the ASVs. The representative ASV sequences were classified into bacterial taxonomy using the RDP classifier (version 2.2) with reference to the SILVA database. After ASV annotation, the abundance statistics of each taxonomy were visualized using Krona (version 2.6). Alpha indices, including Chao1, Shannon, and Simpson, were calculated using the QIIME software (version 1.9.1), and the difference in these indices between the NC and 0.8 g/kg Pro groups was assessed by the Wilcoxon

rank test. Principal coordinates analysis (PCoA) based on weighted Unifrac distances was plotted in R project, and the Anosim test was conducted using the Vegan package (version 2.5.3).

2.7. Transcriptomic analysis

Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The mRNA was enriched by Oligo(dT) beads and fragmented, then reverse transcribed into cDNA with random primers. Second-strand cDNA was synthesized, purified, end repaired, poly(A) added, amplified and sequenced. Reads obtained from the sequencing machines were included filtered by fastp (version 0.18.0), and only high quality reads are allowed for further analysis.

Transcriptome de novo assembly was carried out with Trinity short reads assembling program. Basic annotation of unigenes were performed using BLASTx program with an E-value threshold of 1×10^{-5} to NCBI non-redundant protein (Nr) database, the Swiss-Prot protein database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the COG/KOG database. For each transcription region, a FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variations, using RSEM software. RNAs differential expression analysis was performed by DESeq2 software between two different groups (and by edgeR between two samples). The genes with the parameter of false discovery rate (FDR) below 0.05 and absolute fold change [?]2 were considered differentially expressed genes (DEGs). Functional and pathway enrichment analyses were performed with Gene Ontology (GO) or KEGG tools with a P value threshold of 0.05 (corrected through FDR correction). Protein-Protein interaction network was identified using String v10, while the interactions of hub gene within the network were visualized using Cytoscape software.

2.8. Non-targeted metabolomic analysis

The cecal content samples were thawed and subjected to LC-MS/MS analysis using an UHPLC coupled to a quadrupole time-of-flight. Raw data were converted to MzXML files for subsequent multivariate analysis. Quality control was based on principle component analysis (PCA) as well as positive ion mode (POS) and negative ion mode (NEG) analysis. PCA and OPLS-DA analysis were performed to identify metabolic discrepancies between each two groups. A variable importance in projection (VIP) score of [?] 1.0 and $P < 0.05$ (t test) was applied to identify the metabolites that best distinguished between two groups. KEGG annotations and enrichment analysis of differential expressed metabolites was further performed taking FDR [?] 0.05 as a threshold.. Heatmap and network was used to show the correlation between microbiome, transcriptomic and metabolomic data within colon content samples of each group.

2.9. Statistical analysis

Data were analyzed through Graphpad Prism software version 8.3.0 (GraphPad Software, CA, USA), R software version 4.0.5 (https://www.r-project.org/) or SPSS package version 26.0 (IBM Inc., NY, USA). Normally distributed or proportional data were expressed as mean +- standard deviation (SD), and statistical differences were determined by Student's t-test, $\chi 2$ test or one-way ANOVA, followed by post-hoc SNK test. In case of non-normally distributed data, Kruskal-Wallis rank sum test or Freidman's test was used for independent data or repeated measures respectively. P values less than 0.05 were considered statistically significant.

Results

Diarrhea and weight loss

Diarrhea was induced by afatinib, and attenuated by use of loperamide or crofelemer. The incidence and score of diarrhea increased gradually during the experiments in the afatinib, loperamide and crofelemer group. Whereas the rats of the control group had weight gain, rats of the afatinib group exhibited weight

loss, while loperamide or crofelemer attenuated weight loss induced by afatinib. There was 2 and 1 death in the afatinib and the crofelemer group, respectively.

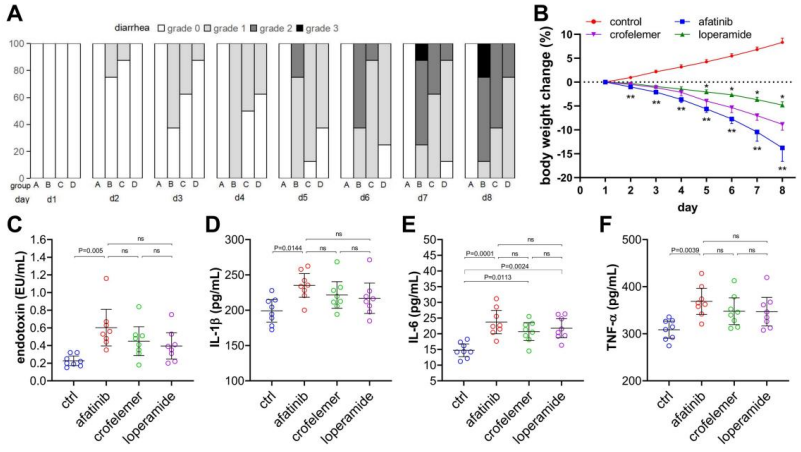

FIGURE 1 . Crofelemer or loperamide attenuates afatinib-induced diarrhea, weight loss and systemic inflammation. (A) Percent of diarrhea at each day. Groups A-D represent the control, afatinib, crofelemer and loperamide group, respectively. (B) Change of body weight relative to day 1. *: $P<0.05$ compared with the crofelemer group. **: $P<0.05$ compared with the other three groups. (C) Serum levels of endotoxin. (D) Serum levels of IL-1β. (E) Serum levels of IL-6. (F) Serum levels of TNF-α.

Histopathology and ultrastructure changes

Rats treated with afatinib showed villi loss, mucosal damage and inflammatory cell infiltration in the ileum and colon. Epithelial mucosal damage and villi loss was also shown under TEM. With the addition of crofelemer or loperamide, such histopathological changes minimally decreased, as no statistically significant differences in histopathological scoring among the four groups both in the ileum and in the colon.

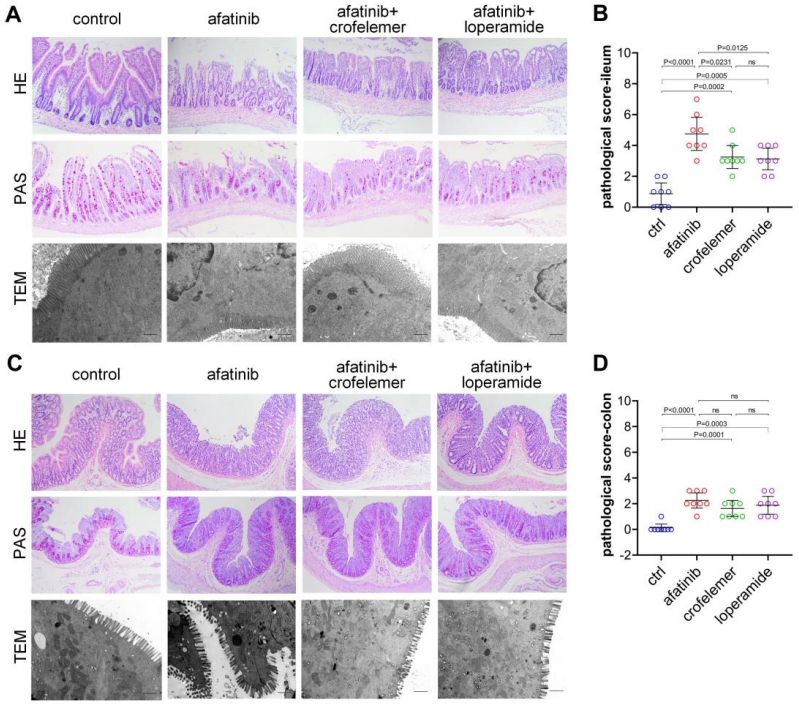

FIGURE 2 . Crofelemer or loperamide attenuates afatinib-induced gut epithelial injury, vilum loss and

pathological score. (A) pathology of the ileum. (B) pathological score of the ileum. (C) pathology of the colon. (D) pathological score of the colon. H&E and Masson' trichrome images are shown as ×100 magnified. The scale bars represent 1 μm in the transmission electron microscopy (TEM) images.

Transcriptomic analysis of the colon

DEGs in each group of colon tissue were assessed (n=5 each). Although hundreds of DEGs are also identified when comparing the control group and the other three groups, the number of DEGs between the afatinib group, the loperamide and afatinib group are significant less. When comparing the afatinib to the control group, there are 2751 DEGs (2471 up- and 280 down-regulated). Defense response, response to external stimulus and immune response are top three processed enriched in the afatinib group. KEGG enrichment identified IL-17 signaling pathway, complement and coagulation cascades, viral protein interaction with cytokine and cytokine receptor, cytokine-cytokine receptor interaction and TNF signaling pathway as most prominently enriched pathways in the afatinib group. KEGG-enriched metabolic pathways in the afatinib group include amino acids (arginine and proline), lipid (steroid hormone biosynthesis, biosynthesis of unsaturated fatty acids, linoleic acid, arachidonic acid, alpha-linolenic acid), cofactors and vitamins (retinol), xenobiotics biodegradation and metabolism (drug metabolism - other enzymes).

To explore the candidate core genes that may be regulated by crofelemer or loperamide, the DEG profiles were compared between groups A vs B and groups B vs C, or groups A vs B and groups B vs D, respectively. Thirteen genes are shown to be candidate core effector genes of crofelemer, including Egr1, Foxp3, Pim1, Stat4, Ccl4, AC112568.1, Nfkbie, Irf7, Gfi1, Nfkb1, Tap2, RT1-S3. Afatinib-associated four core genes include Oas1i, Fbl, Hspa5 and Polr1b.

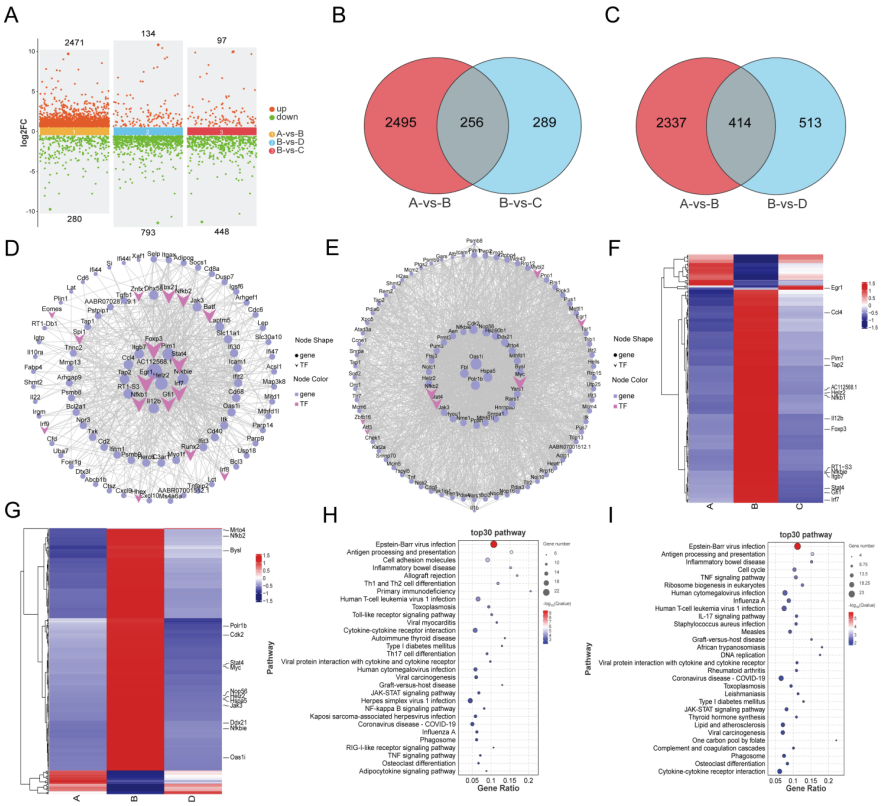

FIGURE 3 . Effects of afatinib on colon transcriptomics. (A) Scatter plot of differently expressed genes

(DEGs) in colon samples. (B) Differential gene intersection analysis of groups A vs B and groups B vs C. (C) Differential gene intersection analysis of groups A vs B and groups B vs D. (D) The PPI analysis of the 256 differential genes in Fig B. (E) The PPI analysis of the 414 differential genes in Fig C. (F-G) Heatmap distribution of the core differential genes. (H-I) KEGG enrichment analysis for differential genes.

Metabolomic analysis

Differently expressed metabolites of each group (n=8) were accessed through untargeted metabolomics. Compared with the control group, 453, 116 and 156 differentially expressed metabolites (DEMs) were identified in the afatinib, crofelemer or loperamide groups, respectively. The administration of crofelemer or loperamide resulted in 33 and 73 DEMs when comparing with the afatinib group. The most prominent enriched metabolic pathways were metabolism of xenobiotics by cytochrome P450, retinol metabolism and lysine degradation when comparing the control and the afatinib groups. When comparing the afatinib and the crofelemer group, the most prominent enriched metabolic pathways were ascorbate and aldarate metabolism, ovarian steroidogenesis and chemical carcinogenesis - DNA adducts. When comparing the afatinib and the loperamide group, the most prominent enriched metabolic pathways were vitamin B6 metabolism, microbial metabolism in diverse environments, and ABC transporters. The crofelemer and the loperamide groups differ in circadian entrainment, pathways in cancer, endocrine resistance, axon regeneration, breast cancer, synaptic vesicle cycle, prostate cancer, butanoate metabolism, gap junction, estrogen signaling pathway and GnRH secretion.

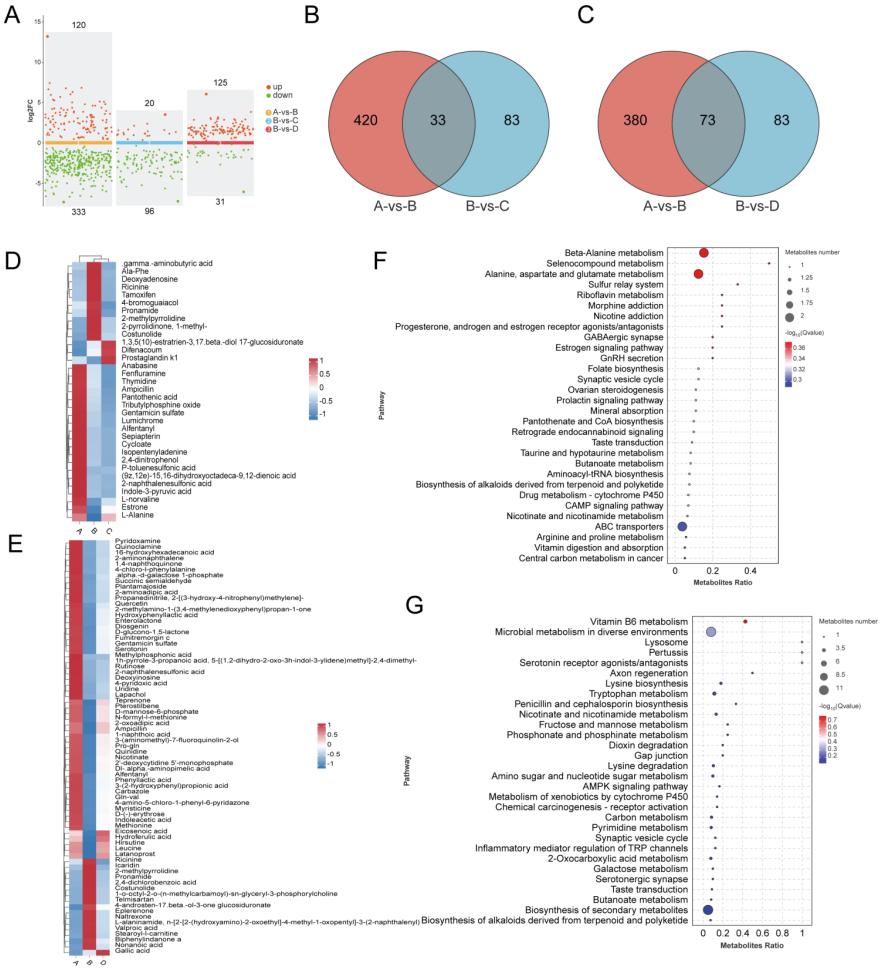

FIGURE 4 . Effects of afatinib on metabolites. (A) Scatter plot of differently expressed metabolites (DEMs) in cecal samples. (B,C) Venn diagrams of DEG intersection analysis. (D,E) Heatmap of DEMs of different groups. (F) KEGG enrichment of metabolomic pathways in groups A, B and C. (G) KEGG enrichment of metabolomic pathways in groups A, B and D.

Gut microbiome changes

Afatinib and antidiarrheal agents-induced cecal microbial changes were assessed and compared. Measured by Shannon's diversity index, the microbial diversity was decreased in other three groups compared to the control group (P <0.0001 each). However, there was no difference in microbial diversity when comparing between the afatinib, crofelemer or loperamide groups (all P >0.05). Principal coordinate analysis (PCoA) showed that these four groups clustered differently, which was confirmed with pairwise anosim and adonis comparisons (P =0.0001). Compared with the genera of the control group, the afatinib group was most abundant with*Escherichia shigella* and Rombuotsia, and lower abundant with allobaculum, lactobacillus and so on. As compared with the control group, the afatinib, crofelemer or loperamide groups had significantly higher relative abundance of Enterobacteriaceae (P =0.00087) and Erysipelotrichaceae (P =0.00017), and lower abundance of Lactobacillaceae families (P =0.03225). At the species level, the control group has significantly less abundance of *Escherichia shigella* as compared with the other three groups (all$P$ <0.011). The crofelemer and loperamide groups were different most in the abundance of Lactobacillaceae and Enterobacteriaceae families (both P <0.05), as well as*Lactobacillus murinus* and *Lactobacillus johnsonii* species (both P <0.05).

The afatinib group was more active than those of the control group in Xenobiotics biodegradation and metabolism (P =0.02542), as well as membrane transport (P =0.00748). Biological pathways estimated to be significantly altered by microbiome are shown in Figure 3 and further multiomic analyses.

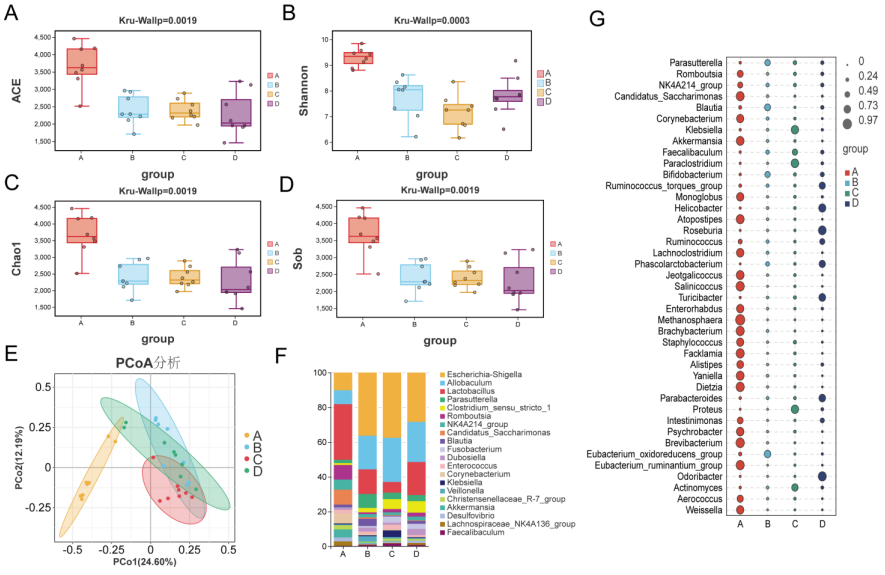

FIGURE 5 . Gut microbiota composition profiles in each group. (A) α-diversity shown by the ACE score (B) α-diversity shown by the Shannon score. (C) α-diversity shown by the Chao1 score. (D) α-diversity shown by the Sob score. (E) β-diversity shown by the PCoA analysis. (F) Stacked relative abundances at the genus level. (G) Differences of relative abundance of representative genus among each group.

Multiomic correlation analyses

3.6.1 Transcriptome and metabolite association analysis

The correlation of relative expression abundance of core different expressed genes (DEGs) and metabolites (DEMs) was analyzed. As shown in Figure 6, the core DEG-DEM correlation network maps were illustrated basing on groups A, B and C, or groups A, B and D, which shows possible crofelemer or loperamide regulating network respectively. As for the crofelemer regulatory network, the abundance of Helz2 gene is positively correlated with 4-bromoguaiacol, deoxyadenosine, pronamide, ricinine, costunolide, etc. The abundance of 4-bromoguaiacol is positively correlated with abundance of Foxp3, Pim1, Stat4, Ccl4, AC112568.1, Nfkbie, Irf7, Gfi1, Nfkb1, Tap2 and RT1-S3, while L-Alanine is negatively correlated with these genes. As for the loperamide regulatory network, the abundance of Stat4 is positively correlated with ricinine, pronamide, hirsutine, eplerenone, etc. Crofelemer and loperamide share common DEG-DEM correlations, such as Helz2/Nfkbie/Oas1i with pronamide/ricinine.

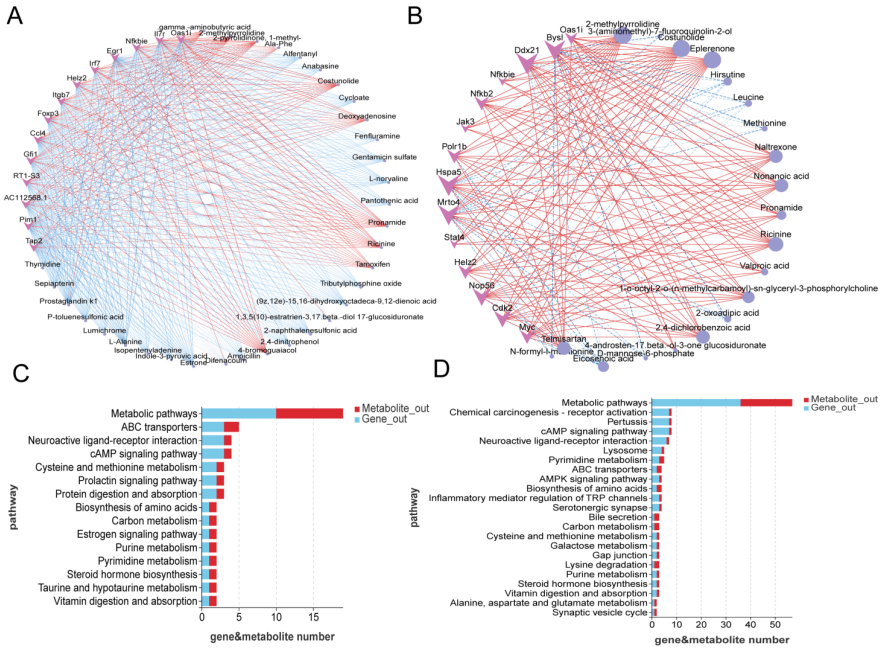

FIGURE 6 . Correlation analysis of different expressed genes (DEGs) and metabolites (DEMs). (A) Correlation network of groups A, B and C. (B) Correlation network of groups A, B and D. (C) Common transcriptomic and metabolic pathways in groups A, B and C. (D) Common transcriptomic and metabolic pathways in groups A, B and D.

3.6.2 Metabolome and microbiome association analysis

We analyzed the correlations between DEMs and different genera of different groups. As is shown in Figure 7, the abundance of *E. shigella* was positively correlated with that of ricinine, nonanoic acid, costunolide, pronamide, etc. The abundance of *Lactobacillus*was negatively correlated with that of ricinine, nonanoic acid, costunolide, pronamide, etc.

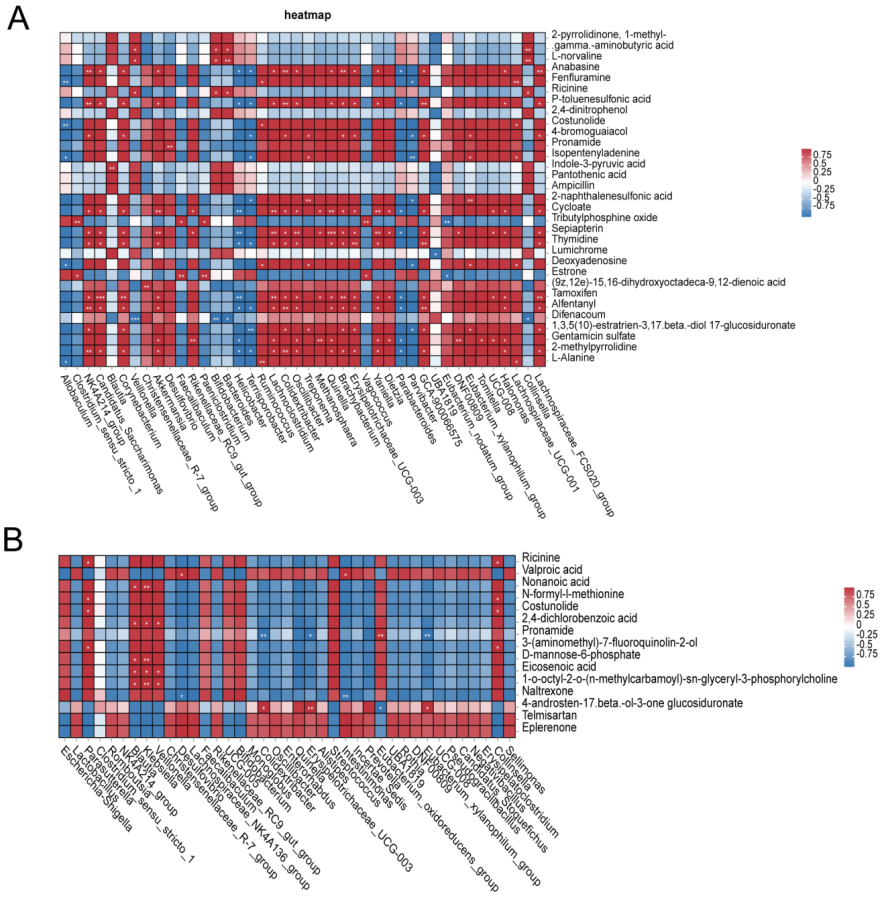

FIGURE 7 . Correlation analysis of different expressed metabolites (DEMs) and microbiome between the control and the afatinib groups. (A) Correlation of DEMs and microbiome that are regulted by crofelemer. (B) Correlation of DEMs and microbiome that are regulted by loperamide.

3.7 Transcriptomics of the ileum and correlation with the colon transcriptomics

In the ileum, we identified 448 DEGs (108 up- and 340 down-regulated) in the afatinib group compared to the control group. GO enrichment pointed out top 30 significantly different functional GOs between healthy and diarrhea rats. Lipid metabolic process, muscle contraction and blood circulation are top three processed enriched in the afatinib group. KEGG enrichment identified PPAR signaling pathway, hypertrophic cardiomyopathy, vascular smooth muscle contraction, dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy as most prominently enriched pathways in the afatinib group. Similarly, 674 (290 up- and 384 down-regulated) and 1703 (839 up- and 864 down-regulated) DEGs are identified respectively between the crofelemer or loperamide group and the control group. When comparing the crofelemer and afatinib group, the loperamide and afatinib group, or the crofelemer and group, 1295 (789 up- and 506 down-regulated), 1872 (1464 up- and 408 down-regulated) and 1159 (778 up- and 381 down-regulated) DEGs are identified, respectively. KEGG-enriched metabolic pathways in the afatinib group include amino acids (tryptophan), lipid (alpha-linolenic acid, Ether lipid, arachidonic acid, Biosynthesis of unsaturated fatty acids, Linoleic acid), cofactors and vitamins (retinol, nicotinate and nicotinamide), other amino acids (Taurine and hypotaurine), xenobiotics biodegradation and metabolism (Drug metabolism - cytochrome P450, drug metabolism - other enzymes).

Hub genes of the ileum and the colon were compared. As shown in Figure 8-D, 13 genes were identified as common hub genes in crofelemer-treated ileum and colon. Similarly, Figure 8-E shows 13 common hub genes

in loperamide-treated ileum and colon. The expression of Cdo1, Adipoq, Fabp4, cfd, Usp18, Oaslk, Trarg1 and Thrsp was regulated in response to both crofelemer and loperamide in the ileum and in the colon.

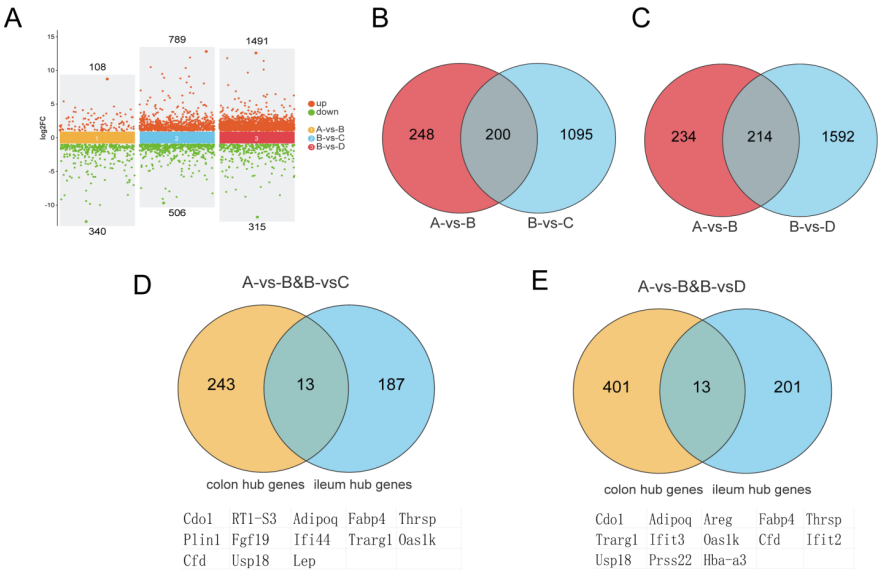

FIGURE 8. Transcriptomics of the ileum and correlation with the colon transcriptomics. **(A)** Differently expressed genes (DEGs) in the ileum of each group. **(B)** Venn diagram shows crofelemer-regulated ileum DEGs. **(C)** Venn diagram shows loperamide-regulated ileum DEGs. **(D)** Crofelemer-regulated hub genes both in the ileum and in the colon. **(E)**Loperamide-regulated hub genes both in the ileum and in the colon. Eight of these hub genes are identical to those of crofelermer-regulated common hub genes.

DiscussionIn the current study, we investigated the pathological and multiomic changes in afatinib-treated rat intestine. Similar to previous studies using afatinib or other TKIs, afatinib induces diarrhea and mucosal damage here. Multiomic data showed altered gut transcriptomic, microbiota and metabolic phenotype as well as functioning. Crofelemer or loperamide protects from afatinib-induced diarrhea. Such protective effects are associated with discrete but at least some similar changes in transcriptomic, microbiota and metabolic profiles of gut tissue and/or content. Previous studies using afatinib to induce diarrhea with a dose of 16-50 mg/kg and a duration of 6-14 days (Zhang et al., 2023). Consistent with previous studies, afatinib as an EGFR-TKI induces diarrhea, mucosal damage, villi loss and inflammatory infiltration in both the ileum and the colon. The pathological alterations are most prominent in the ileum than those in the colon. These results support the inflammatory hypothesis in TKI-induced diarrhea, as EGFR inhibition attenuates the growth, proliferation and repair process of the epithelial mucosal layer (Cárdenas-Fernández et al., 2023). Some studies also observe relatively more severe pathology in the ileum than in the colon (Secombe et al., 2021; Duan et al., 2019; Van Sebille et al., 2017; Rasmussen et al., 2010; Secombe et al., 2022). In other studies using TKIs to induce diarrhea however, pathological damage is more severe in the colon (Bowen et al., 2012, Bowen et al., 2014, Mayo et al., 2020). Similarly, in contrast to previous studies that showed significant decrease in the average body weight, in our study and another one, the body weight decreases after afatinib exposure. These inconsistent results may attribute to drug type, dose and duration of exposure, site difference in the absorption, efflux mechanisms and concentration of the drugs, mucosal or systemic inflammation status, or in the protective signaling pathways. A previous study shows that afatinib-protein conjugation is most strongly and extensively stained in the ileum, followed by the colon, which shows specifically stain with the epithelial cells and Auerbach's plexus (Yamamoto et al., 2019). In contrast, only slight staining is observed in the duodenum and jejunum. The afatinib concentration in the intestine corresponds well to these sites of EGFR expression. These results are in line with those of a previous study, which also shown that EGFR/ERBB1 is expressed at a relatively higher level in the ileum compared to the rest of

the gastrointestinal tract (Van Sebille et al., 2017). Moreover, it's suggested that spacial differences in microenvironment and metabolome between the ileum and the colon may also contribute to such discrepancy (Secombe et al., 2022). In the current study, both loperamide and crofelemer attenuate afatinib-induced diarrhea in rats. Loperamide is suggested by several guidelines as one of the major treatments in TKI-induced diarrhea. Crofelemer as an anti-secretary agent has been approved for secretary diarrhea, and is supposed to be promising in managing anticancer drugs-induced diarrhea. Preliminary studies disclosed that dacomitinib (another second-generation EGFR-TKI) -induced diarrhea in rats is associated with hyperpermeability and ileal histological damage (Van Sebille et al., 2017). A further study showed that crofelemer attenuated dacomitinib-induced chloride hypersecretion in T84 cells in vitro, but worsens dacomitinib-induced diarrhea without attenuation of barrier dysfunction and ileal damage in vivo (Van Sebille et al., 2018). A latest study showed that crofelemer prophylaxis reduced the incidence or severity of neratinib-associated diarrhea in female beagle dogs (Guy et al., 2024). Recent exploratory clinical trials have investigated the efficacy of crofelemer in TKI, chemotherapy or targeted treatment-associated diarrhea. Although limited in enrollment and power, crofelemer did show preventive and treatment potentials, especially for watery or refractory diarrhea (Greene et al., 2021; Pohlmann et al., 2022; Jacob et al., 2023). In addition, antibiotics, antidiabetic, anti-inflammatory or natural products have been tested in preclinical studies, and may provide novel treatment regimens for TKI-induced diarrhea (Cárdenas-Fernández et al., 2023). Next, biochemistry and multiomic analyses were performed to investigate the potential mechanism of afatinib-induced diarrhea and protective mechanism of loperamide and crofelemer. Afatinib induces statistically significant elevation of serum levels of endotoxin, IL-6, IL-1β or TNF-α. Among these indices, only IL-6 release is inhibited with the administration of loperamide or crofelemer. In addition, transcriptomic data show that afatinib induces expression of IL-6, IL-1β or TNF-α mRNA only in the colon other than in the ileum tissue. Loperamide, but not crofelemer, attenuates afatinib-induced expression of IL-1βmRNA and TNF-αmRNA. These results imply that crofelemer exerts less antiinflammatory activities than loperamide both in the gut and in the circulation. However, previous studies reveal that antitumor drug-induced diarrhea could be attenuated by inhibition of the proinflammatory and provocation of antiinflammatory signals (Khan et al., 2018). In addition, our model does show the intestinal and systemic inflammatory nature, which allow for the potential effects of antiinflammatory agents. Differentially expressed genes and pathway enrichment was assessed both in the colon and in the ileum of each groups. The colon and the ileum share part of similar DEGs and pathways in response to afatinib, specifically proinflammatory pathways. This may partly attribute to elevation of levels of the systemic inflammatory biomarkers. These results are supported by previous studies that colitis are associated with activation of inflammatory pathways. However, similarly to the discrepancies in the extent of pathological injury, the numbers of DEGs induced by afatinib are less of the colon than of the ileum. Enriched pathways of the ileum and the colon are also different in response to afatinib. Previous studies suggest inhibition of EGFR signaling may suppress TKI-induced diarrhea (Secombe et al., 2019). But we did not identify aberrant EGFR signaling in the ileum or in the colon, although the relative expression of EGFR was slightly increased in the colon by use of afatinib. Rather, Cdo1, Adipoq, Fabp4, cfd, Usp18, Oaslk, Trarg1 and Thrsp are genes that are commonly regulated by both crofelemer and loperamide. FABP4 and Adipoq are fatty acid regulating genes that are suggested to take a role in intestinal disorders and/or diarrhea (Mosińska et al., 2020; Mosińska et al., 2020). Such genes may regulate the process of TKI-induced diarrhea and are worthy of further investigations. In this study, cecal microbiome were analyzed and compared in each groups. Compared with that in the control group, alpha diversity significantly decreased while proportions of Proteobacteria (phylum) and *Escherichia shigella* (species) increased in all the three other groups. Simultaneously, rats of the other three groups exhibited significantly lower proportions of Romboutsia ilealis, Actinobacteriota, Patescibacteria and Verrucomicrobiota. Glycosylphosphatidylinositol (GPI)-anchor biosynthesis, Pathogenic Escherichia coli infection, steroid biosynthesis are top enriched pathogenic pathways in the other three groups as compared with the control group. The Proteobacteria consists of many Gram negative bacteria that might be opportunistic pathogenic. This corresponds to an increase in *E. shigella* abundance, and further an enrichment of pathogenic *E. shigella* infection pathway, cecal metabolomic dysregulation and elevated serum endotoxin and cytokine level. On the contrary, *Lactobacillus* as a well recongnized intestinal probiotics is shown to reverse the metabolomic dysregulation in the current

study. Previous pre-clinical studies have shown increases in abundance of gram-negative bacteria in anti-cancer drugs-induced diarrhea. These gram-negative species produce endotoxin/lipopolysaccharide (LPS) to initiate the key inflammatory mediators. Blautia has been suggested to be abundantly reside in the intestine of neratinib-induced diarrhea rat model (Secombe et al., 2022). A retrospective clinical study by Chung et al showed that the use of first-generation EGFR-TKI increases the risk of*Clostridioides difficile* infection and diarrhea (Chung et al., 2022). Moreover, both loperamide and crofelemer group showed a significant increase in the abundance of Parasutterella as compared with that of the afatinib group. Recent studies reveal the association of Parasutterella abundance with the activation of fatty acid biosynthesis and body weight gain (Henneke et al., 2022). In this scenario, microbiome mechanisms and microbiome-regulating strategies might play an important role in cancer treatment-associated digestive adverse effects (Maddern et al., 2023). It remains controversy in TKI-induced diarrhea whether diarrhea changes microbiome originally, or microbiome changes induce diarrhea. Animal and clinical evidences are accumulating to show that microbiome changes happen prior to the onset of diarrhea (Secombe et al., 2022). Our data show that group-group cecal microbial differences are smaller between the loperamide and crofelemer group than these two groups with the control or the afatinib group. These data together may indicate that loperamide and crofelemer modifies gut microbial constituents, presuming that the animals have similar microbiota background prior to the initiation of afatinib. However, there are also studies that show the impact of gut microbial background on TKI-induced diarrhea, as pre-treatment gut microbial composition may also determine or predict diarrhea severity (Conti et al., 2022; D'Amico et al., 2022). Afatinib-induced cecal metabolomic changes are further analyzed in each groups. Again, group-group cecal metabolic differences are smaller between the loperamide and crofelemer group than these two groups with the control or the afatinib group. Compared to the control group, three metabolomic pathways are different in the afatinib group, including metabolism of xenobiotics by cytochrome P450, retinol metabolism and lysine degradation. Previous studies have revealed that dietary retinol or vitamine A or lysine is responsible for maintenance of gut mucosa health and prevention of diarrhea (Li et al., 2022; Cao et al., 2022; Wang et al., 2022). The altered metabolic pathways and their components may serve as potential biomarkers and therapeutic targets. Further Pearson correlation analysis shows that the abundance of some microorganisms is associated with that of DEM and metabolic pathways. The abundance of E. shigella was negatively correlated with that of xanthine, valine, thymine, uracil, etc., and positively correlated with that of taurine and reduced L-glutathine, etc. These results are in line with previous studies, which show that bacteria abundance are associated with metabolites that may contribute to the drug-induced inflammation and diarrhea, inflammatory bowel disease, obesity, diabetes and fatty liver disease.

ConclusionsAfatinib-induced diarrhea in rats is associated with more severe mucosal damage in ileum than in colon. Crofelemer or loperamide protects from afatinib-induced mucosal damage, diarrhea and weight loss. Such protective effects are associated with changes in gut transcriptomic, microbiota and metabolic pathways of gut tissue and/or content. It's suggested to be feasible to eliminate the malregulated metabolomic and microbial profiles and supply with beneficial microorganisms for treating TKI-induced diarrhea. Further studies are needed to verify the clinical utility of these antidiarrheal agents and related mechanisms.

Data Availability Statement

The original data presented in the study are publicly available. These data can be downloaded at: https://ngdc.cncb.ac.cn/.

Author Contributions

Conceptualization, G.Q. and L.X.; methodology, G.Q. and F.L.; software, L.W., F.L. and H.S.; validation, L.X.; formal analysis, G.Q. and W.Z.; investigation, G.Q. and L.X.; resources, W.Z.; data curation, G.Q.; writing—original draft preparation, G.Q.; writing—review and editing, L.X.; visualization, F.L.; supervision,

L.X.; project administration, L.X.; funding acquisition, L.W., W.Z. and L.X. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

The following supporting information can be downloaded at: ...

References

Barron, F., de la Torre-Vallejo, M., Luna-Palencia, R. L., Cardona, A. F., & Arrieta, O. (2016). The safety of afatinib for the treatment of non-small cell lung cancer. *Expert Opin Drug Saf.* 15(11), 1563–1572. https://doi.org/10.1080/14740338.2016.1236910

Bowen, J. M., Mayo, B. J., Plews, E., Bateman, E., Stringer, A. M., Boyle, F. M., Finnie, J. W., & Keefe, D. M. (2012). Development of a rat model of oral small molecule receptor tyrosine kinase inhibitor-induced diarrhea. *Cancer Biol Ther.* 13(13), 1269–1275. https://doi.org/10.4161/cbt.21783

Bowen, J. M., Mayo, B. J., Plews, E., Bateman, E., Wignall, A., Stringer, A. M., Boyle, F. M., & Keefe, D. M. (2014). Determining the mechanisms of lapatinib-induced diarrhoea using a rat model. *Cancer Chemother Pharmacol.* 74(3), 617–627. https://doi.org/10.1007/s00280-014-2519-4

Cao, G., Yang, S., Wang, H., Zhang, R., Wu, Y., Liu, J., Qiu, K., Dong, Y., & Yue, M. (2023). Effects of *Bacillus licheniformis* on the growth performance, antioxidant capacity, ileal morphology, intestinal short chain fatty acids, and colonic microflora in piglets challenged with lipopolysaccharide. *Animals* . 13(13), 2172. https://doi.org/10.3390/ani13132172

Cárdenas-Fernández, D., Soberanis Pina, P., Turcott, J. G., Chávez-Tapia, N., Conde-Flores, E., Cardona, A. F., & Arrieta, O. (2023). Management of diarrhea induced by EGFR-TKIs in advanced lung adenocarcinoma. *Ther Adv Med Oncol* . 15, 17588359231192396. https://doi.org/10.1177/17588359231192396

Chung, Y. S., Lin, Y. C., Hung, M. S., Ho, M. C., & Fang, Y. H. (2022). Clinical impact of epidermal growth factor receptor tyrosine kinase inhibitor associated *Clostridioides difficile* infection among patients with lung cancer. *Onco Targets Ther.* 15, 1563–1571. https://doi.org/10.2147/OTT.S386807

Conti, G., D'Amico, F., Fabbrini, M., Brigidi, P., Barone, M., & Turroni, S. (2022). Pharmacomicrobiomics in anticancer therapies: Why the gut microbiota should be pointed out. *Genes* . 14(1), 55. https://doi.org/10.3390/genes14010055

Cottreau, J., Tucker, A., Crutchley, R., & Garey, K. W. (2012). Crofelemer for the treatment of secretory diarrhea. *Expert Rev Gastroenterol Hepatol.* 6(1), 17–23. https://doi.org/10.1586/egh.11.87

D'Amico, F., Barone, M., Tavella, T., Rampelli, S., Brigidi, P., & Turroni, S. (2022). Host microbiomes in tumor precision medicine: How far are we?. *Curr Med Chem* . 29(18), 3202–3230. https://doi.org/10.2174/0929867329666220105121754

Duan, T., Cil, O., Thiagarajah, J. R., & Verkman, A. S. (2019). Intestinal epithelial potassium channels and CFTR chloride channels activated in ErbB tyrosine kinase inhibitor diarrhea. *JCI Insight.* 4(4), e126444. https://doi.org/10.1172/jci.insight.126444

Greene, C., Barlesi, B., Tarroza-David, S., & Friedlander, T. (2021). Improved control of tyrosine kinase inhibitor-induced diarrhea with a novel chloride channel modulator: A case report. *Oncol Ther.*9(1), 247–253. https://doi.org/10.1007/s40487-021-00147-3

Guy, M., Teixeira, A., Shrier, A., Meschter, C., Bolognese, J., & Chaturvedi, P. (2024). Effects of orally administered crofelemer on the incidence and severity of neratinib-induced diarrhea in female dogs.*PLoS One.* 19(1), e0282769. https://doi.org/10.1371/journal.pone.0282769

Harada, G., Yang, S. R., Cocco, E., & Drilon, A. (2023). Rare molecular subtypes of lung cancer. *Nat Rev Clin Oncol.* 20(4), 229–249. https://doi.org/10.1038/s41571-023-00733-6

Hashimoto, Y., Maeda, K., Shimomura, O., Miyazaki, Y., Hashimoto, S., Moriyama, A., Oda, T., & Kusuhara, H. (2023). Evaluation of the risk of diarrhea induced by epidermal growth factor receptor tyrosine kinase inhibitors with cultured intestinal stem cells originated from crypts in humans and monkeys. *Toxicol In Vitro.* 93, 105691. https://doi.org/10.1016/j.tiv.2023.105691

Hendriks, L. E., Kerr, K. M., Menis, J., Mok, T. S., Nestle, U., Passaro, A., Peters, S., Planchard, D., Smit, E. F., Solomon, B. J., Veronesi, G., Reck, M., & ESMO Guidelines Committee. (2023). Oncogene-addicted metastatic non-small-cell lung cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol.* 34(4), 339–357. https://doi.org/10.1016/j.annonc.2022.12.009

Henneke, L., Schlicht, K., Andreani, N. A., Hollstein, T., Demetrowitsch, T., Knappe, C., Hartmann, K., Jensen-Kroll, J., Rohmann, N., Pohlschneider, D., Geisler, C., Schulte, D. M., Settgast, U., Türk, K., Zimmermann, J., Kaleta, C., Baines, J. F., Shearer, J., Shah, S., Shen-Tu, G., ... Laudes, M. (2022). A dietary carbohydrate - gut Parasutterella - human fatty acid biosynthesis metabolic axis in obesity and type 2 diabetes. *Gut Microbes.* 14(1), 2057778. https://doi.org/10.1080/19490976.2022.2057778

Jacob, S., Johnson, M., Roque, B., Quintal, L., Rugo, H. S., Melisko, M., & Chien, A. J. (2023). Crofelemer for the management of neratinib-associated diarrhea in patients with HER2+ early-stage breast cancer. *Clin Breast Cancer.* 23(7), 721–728. https://doi.org/10.1016/j.clbc.2023.06.014

Jiang, Y., Fang, X., Xiang, Y., Fang, T., Liu, J., & Lu, K. (2023). Afatinib for the treatment of NSCLC with uncommon EGFR mutations: A narrative review. *Curr Oncol.* 30(6), 5337–5349. https://doi.org/10.3390/curroncol30060405

Khan, S., Wardill, H. R., & Bowen, J. M. (2018). Role of toll-like receptor 4 (TLR4)-mediated interleukin-6 (IL-6) production in chemotherapy-induced mucositis. *Cancer Chemother Pharmacol.*82(1), 31–37. https://doi.org/10.1007/s00280-018-3605-9

Kim, Y., Lee, S. H., Ahn, J. S., Ahn, M. J., Park, K., & Sun, J. M. (2019). Efficacy and safety of afatinib for EGFR-mutant non-small cell lung cancer, compared with gefitinib or erlotinib. *Cancer Res Treat.* 51(2), 502–509. https://doi.org/10.4143/crt.2018.117

Kim, Y., Quach, A., Das, S., & Barrett, K. E. (2020). Potentiation of calcium-activated chloride secretion and barrier dysfunction may underlie EGF receptor tyrosine kinase inhibitor-induced diarrhea.*Physiol Rep.* 8(13), e14490. https://doi.org/10.14814/phy2.14490

Laskin, J., Liu, S. V., Tolba, K., Heining, C., Schlenk, R. F., Cheema, P., Cadranel, J., Jones, M. R., Drilon, A., Cseh, A., Gyorffy, S., Solca, F., & Duruisseaux, M. (2020). NRG1 fusion-driven tumors: biology,

detection, and the therapeutic role of afatinib and other ErbB-targeting agents. *Ann Oncol.* 31(12), 1693–1703. https://doi.org/10.1016/j.annonc.2020.08.2335

Li, M., Huang, Y., Jin, H., Yuan, D., Huang, K., Wang, J., Tan, B., & Yin, Y. (2022). Vitamin A ameliorated irinotecan-induced diarrhea in a piglet model involving enteric glia modulation and immune cells infiltration. *Nutrients.* 14(23), 5120. https://doi.org/10.3390/nu14235120

Lu, S., Shih, J. Y., Jang, T. W., Liam, C. K., & Yu, Y. (2021). Afatinib as first-line treatment in Asian patients with EGFR mutation-positive NSCLC: A narrative review of real-world evidence.*Adv Ther.* 38(5), 2038–2053. https://doi.org/10.1007/s12325-021-01696-9

Lysyy, T., Lalani, A. S., Olek, E. A., Diala, I., & Geibel, J. P. (2019). The calcium-sensing receptor: A novel target for treatment and prophylaxis of neratinib-induced diarrhea. *Pharmacol Res Perspect.* 7(5), e00521. https://doi.org/10.1002/prp2.521

Maddern, A. S., Coller, J. K., Bowen, J. M., & Gibson, R. J. (2023). The association between the gut microbiome and development and progression of cancer treatment adverse effects. *Cancers (Basel).*15(17), 4301. https://doi.org/10.3390/cancers15174301

Mayo, B. J., Secombe, K. R., Wignall, A. D., Bateman, E., Thorpe, D., Pietra, C., Keefe, D. M., & Bowen, J. M. (2020). The GLP-2 analogue elsiglutide reduces diarrhoea caused by the tyrosine kinase inhibitor lapatinib in rats. *Cancer Chemother Pharmacol.* 85(4), 793–803. https://doi.org/10.1007/s00280-020-04040-0

Mosińska, P., Jacenik, D., Sałaga, M., Wasilewski, A., Cygankiewicz, A., Sibaev, A., Mokrowiecka, A., Małecka-Panas, E., Pintelon, I., Storr, M., Timmermans, J. P., Krajewska, W. M., Fichna, J. (2018). FABP4 blocker attenuates colonic hypomotility and modulates white adipose tissue-derived hormone levels in mouse models mimicking constipation-predominant IBS. *Neurogastroenterol Motil.* 30(5), e13272. *https://doi.org/10.1111/nmo.13272*

Mosińska, P., Szczepaniak, A., Wojciechowicz, T., Skrzypski, M., Nowak, K., Fichna, J. (2020). Chain length of dietary fatty acids determines gastrointestinal motility and visceromotor function in mice in a fatty acid binding protein 4-dependent manner. *Eur J Nutr* . 59(6), 2481-2496. *https://doi.org/10.1007/s00394-019-02094-2*

Pohlmann, P. R., Graham, D., Wu, T., Ottaviano, Y., Mohebtash, M., Kurian, S., McNamara, D., Lynce, F., Warren, R., Dilawari, A., Rao, S., Mainor, C., Swanson, N., Tan, M., Isaacs, C., & Swain, S. M. (2022). HALT-D: a randomized open-label phase II study of crofelemer for the prevention of chemotherapy-induced diarrhea in patients with HER2-positive breast cancer receiving trastuzumab, pertuzumab, and a taxane. *Breast Cancer Res Treat.* 196(3), 571–581. https://doi.org/10.1007/s10549-022-06743-9

Rasmussen, A. R., Viby, N. E., Hare, K. J., Hartmann, B., Thim, L., Holst, J. J., & Poulsen, S. S. (2010). The intestinotrophic peptide, GLP-2, counteracts the gastrointestinal atrophy in mice induced by the epidermal growth factor receptor inhibitor, erlotinib, and cisplatin.*Dig Dis Sci.* 55(10), 2785–2796. https://doi.org/10.1007/s10620-009-1104-x

Rugo, H. S., Di Palma, J. A., Tripathy, D., Bryce, R., Moran, S., Olek, E., & Bosserman, L. (2019). The characterization, management, and future considerations for ErbB-family TKI-associated diarrhea.*Breast Cancer Res Treat.* 175(1), 5–15. https://doi.org/10.1007/s10549-018-05102-x

Secombe, K. R., Ball, I. A., Shirren, J., Wignall, A. D., Finnie, J., Keefe, D., Avogadri-Connors, F., Olek, E., Martin, D., Moran, S., & Bowen, J. M. (2019). Targeting neratinib-induced diarrhea with budesonide and colesevelam in a rat model. *Cancer Chemother Pharmacol.* 83(3), 531–543. https://doi.org/10.1007/s00280-018-3756-8

Secombe, K. R., Ball, I. A., Shirren, J., Wignall, A. D., Keefe, D. M., & Bowen, J. M. (2021). Pathophysiology of neratinib-induced diarrhea in male and female rats: microbial alterations a potential determinant.*Breast Cancer.* 28(1), 99–109. https://doi.org/10.1007/s12282-020-01133-9

Secombe, K. R., Ball, I. A., Wignall, A. D., Bateman, E., Keefe, D. M., & Bowen, J. M. (2022). Antibiotic treatment targeting gram negative bacteria prevents neratinib-induced diarrhea in rats. *Neoplasia.*30, 100806. https://doi.org/10.1016/j.neo.2022.100806

Sequist, L. V., Yang, J. C., Yamamoto, N., O'Byrne, K., Hirsh, V., Mok, T., Geater, S. L., Orlov, S., Tsai, C. M., Boyer, M., Su, W. C., Bennouna, J., Kato, T., Gorbunova, V., Lee, K. H., Shah, R., Massey, D., Zazulina, V., Shahidi, M., & Schuler, M. (2013). Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. *J Clin Oncol.* 31(27), 3327–3334. https://doi.org/10.1200/JCO.2012.44.2806

Shyam Sunder, S., Sharma, U. C., & Pokharel, S. (2023). Adverse effects of tyrosine kinase inhibitors in cancer therapy: pathophysiology, mechanisms and clinical management. *Signal Transduct Target Ther.*8(1), 262. https://doi.org/10.1038/s41392-023-01469-6

Siegel, R. L., Giaquinto, A. N., & Jemal, A. (2024). Cancer statistics, 2024. *CA Cancer J Clin.* 74(1), 12–49. https://doi.org/10.3322/caac.21820

Tan, W. L., Ng, Q. S., Lim, C., Tan, E. H., Toh, C. K., Ang, M. K., Kanesvaran, R., Jain, A., Tan, D. S. W., & Lim, D. W. (2018). Influence of afatinib dose on outcomes of advanced EGFR-mutant NSCLC patients with brain metastases. *BMC Cancer.* 18(1), 1198. https://doi.org/10.1186/s12885-018-5110-2

Tao, G., Dagher, F., & Ghose, R. (2022). Neratinib causes non-recoverable gut injury and reduces intestinal cytochrome P450 3A enzyme in mice. *Toxicol Res.* 11(1), 184–194. https://doi.org/10.1093/toxres/tfab111

Van Sebille, Y. Z. A., Gibson, R. J., Wardill, H. R., Secombe, K. R., Ball, I. A., Keefe, D. M. K., Finnie, J. W., & Bowen, J. M. (2017). Dacomitinib-induced diarrhoea is associated with altered gastrointestinal permeability and disruption in ileal histology in rats.*Int J Cancer* . 140(12), 2820–2829. https://doi.org/10.1002/ijc.30699

Van Sebille, Y. Z. A., Gibson, R. J., Wardill, H. R., Ball, I. A., Keefe, D. M. K., & Bowen, J. M. (2018). Dacomitinib-induced diarrhea: Targeting chloride secretion with crofelemer. *Int J Cancer.*142(2), 369–380. https://doi.org/10.1002/ijc.31048

Wang, J., Fan, H., Xia, S., Shao, J., Tang, T., Chen, L., Bai, X., Sun, W., Jia, X., Chen, S., & Lai, S. (2022). Microbiome, transcriptome, and metabolomic analyses revealed the mechanism of immune response to diarrhea in rabbits fed antibiotic-free diets. *Front Microbiol* . 13, 888984. https://doi.org/10.3389/fmicb.2022.888984

Wang, L. Y., Cui, J. J., Guo, A. X., & Yin, J. Y. (2018). Clinical efficacy and safety of afatinib in the treatment of non-small-cell lung cancer in Chinese patients. *Onco Targets Ther.* 11, 529–538. https://doi.org/10.2147/OTT.S136579

Wang, Z., Du, X., Chen, K., Li, S., Yu, Z., Wu, Z., Yang, L., Chen, D., & Liu, W. (2021). Impact of dose reduction of afatinib used in patients with non-small cell lung cancer: A systematic review and meta-analysis.*Front Pharmacol.* 12, 781084. https://doi.org/10.3389/fphar.2021.781084

Wei, Y. F., Lim, C. K., Tsai, M. S., Huang, M. S., & Chen, K. Y. (2019). Intracranial responses to afatinib at different doses in patients with EGFR-mutated non-small-cell lung carcinoma and brain metastases. *Clin Lung Cancer.* 20(3), e274–e283. https://doi.org/10.1016/j.cllc.2019.02.009

Yamamoto, Y., Saita, T., Yamamoto, Y., Sogawa, R., Kimura, S., Narisawa, Y., Kimura, S., & Shin, M. (2019). Immunohistochemical localization of afatinib in male rat intestines and skin after its oral administration.*Acta Histochem.* 121(8), 151439. https://doi.org/10.1016/j.acthis.2019.09.001

Zhang, L., Hu, A., Wang, Y., Yang, Y., Liu, Y., Xu, L., Wang, L., & Cheng, Z. (2023). Medication adjustment of afatinib and combination therapy with sitagliptin for alleviating afatinib-induced diarrhea in rats. *Neoplasia* . 43, 100922. https://doi.org/10.1016/j.neo.2023.100922

Zhao, Y., Cheng, B., Chen, Z., Li, J., Liang, H., Chen, Y., Zhu, F., Li, C., Xu, K., Xiong, S., Lu, W., Chen, Z., Zhong, R., Zhao, S., Xie, Z., Liu, J., Liang, W., & He, J. (2021). Toxicity profile of epidermal growth factor receptor tyrosine kinase inhibitors for patients with lung cancer: A systematic review and network meta-analysis. *Crit Rev Oncol Hematol.* 160, 103305. https://doi.org/10.1016/j.critrevonc.2021.103305

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