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ORIGINAL RESEARCH

Exploring the Toxicological Effects of Acetyl Tributyl Citrate Exposure on Osteoarthritis Based on Machine Learning, Network Toxicology and Molecular Docking Analysis

Yifang Zhu[1], Lin Deng[1], Junxiang Xia[1], Jing Yang[1], Dan Zhao[1], Min Li[2]

[1]Clinical Laboratory, Sichuan Province Orthopedic Hospital, Chengdu, Sichuan, People's Republic of China; [2]Department of Rheumatoid Osteoarthropathy, Sichuan Province Orthopedic Hospital, Chengdu, Sichuan, People's Republic of China

Correspondence: Yifang Zhu, Email 53113072@qq.com

Objective: To investigate the potential toxicological effects of acetyl tributyl citrate (ATBC) on osteoarthritis (OA) and elucidate the underlying mechanisms using bioinformatics, machine learning, and network toxicology.

Methods: ATBC targets were identified from multiple databases, and OA-associated differentially expressed genes (DEGs) were sourced from GSE51588. Intersection analysis identified common targets. Functional enrichment and protein-protein interaction (PPI) network analysis were performed. Machine learning algorithms (LASSO, Random Forest and SVM) validated core targets, with ROC curves assessing diagnostic potential. Immune infiltration differences were analyzed via Cibersort. Molecular docking confirmed ATBC binding to core targets, and an adverse outcome pathway (AOP) framework was developed to elucidate ATBC's role in exacerbating OA through key genes and pathways.

Results: Intersection analysis identified 40 common targets related to both ATBC and OA. Functional enrichment analysis revealed that these targets were significantly involved in calcium signaling pathways and neuroactive ligand-receptor interactions, both of which are implicated in OA pathogenesis. The PPI network analysis identified TNF, MMP8, CXCR4, and SLC2A1 as core targets. Machine learning algorithms further validated these core targets. ROC curve analysis showed that these genes have diagnostic potential, with AUC values ranging from 0.762 to 0.970. Immune infiltration analysis using Cibersort revealed significant differences in immune cell infiltration between OA and control groups, with core targets showing distinct correlations with various immune cells. Molecular docking confirmed strong binding affinities between ATBC and the core targets, with binding energies less than −5 kcal/mol. A novel adverse outcome pathway (AOP) framework was established, suggesting that ATBC may influence the expression of TNF, CXCR4, MMP8, and SLC2A1, with the calcium signaling and neuroactive ligand-receptor interaction pathways potentially contributing to immune dysregulation and OA progression.

Conclusion: The identification of key targets (TNF, MMP8, CXCR4, and SLC2A1) and molecular docking results elucidates potential mechanisms by which ATBC exposure may exacerbate OA progression. The AOP provides evidence for joint-health risk assessment of plasticizers and offers readily measurable biomarkers for regulatory toxicology and future therapeutic development.

Keywords: osteoarthritis, acetyl tributyl citrate, network toxicology, molecular docking, machine learning, bioinformatics

Introduction

Osteoarthritis (OA) is a prevalent degenerative joint disease characterized by the progressive deterioration of articular cartilage, leading to joint pain, stiffness, and functional impairment.[1] It is a significant cause of disability among the elderly population, imposing a substantial burden on healthcare systems worldwide.[2] The pathogenesis of OA is multifactorial, involving genetic, biomechanical, and environmental factors.[3] Despite extensive research, the exact mechanisms underlying OA development and progression remain incompletely understood, and there is a lack of effective early diagnostic markers and curative treatments.

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Acetyl tributyl citrate (ATBC) is a widely used plasticizer and solvent in various industrial applications, including food packaging, cosmetics, and pharmaceuticals.[4] Its chemical structure and properties make it an effective plasticizer, enhancing the flexibility and durability of materials.[5] Although ATBC was not on the European REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) Authorisation or Restriction lists and the 2023 French RMOA (Risk Management Options Analysis) concluded that no additional measures are presently required, the same report emphasized the need for continuous monitoring. Due to its high mobility, ATBC is more likely to leach from plastic articles than most phthalates.[6] It is only loosely retained by weak secondary bonds to the polymer matrix.[4] Therefore, concerns regarding the potential toxicological effects of ATBC exposure have been raised, particularly in the context of long-term and low-dose exposure.[7–9] Animal data show that 20 mg/kg/d ATBC disturbs glucose/lipid homeostasis and provokes hepatic steatosis and cognitive deficits in type-2-diabetic mice,[8] while nanomolar concentrations suppress osteoclast proliferation and upset bone remodeling.[4] Moreover, ATBC also alters estrogen, androgen and thyroid-hormone signaling.[10]

Despite the increasing awareness of ATBC's potential health risks, the specific toxicological effects of ATBC exposure on OA remain largely unexplored. As estrogen and thyroid-hormone are chondro-protective, we hypothesize that such endocrine interference could indirectly accelerate OA progression. Traditional toxicological studies often focus on acute or high-dose exposures, which may not accurately reflect the real-world scenario of chronic low-dose exposure.[8] Moreover, the complex interplay between ATBC exposure and the pathophysiological processes of OA necessitates a comprehensive and integrative approach to elucidate the underlying mechanisms.

Recent advances in bioinformatics, machine learning, and network toxicology offer powerful tools to address these challenges. Bioinformatics enables the analysis of large-scale biological data, providing insights into the molecular mechanisms of diseases and the potential toxicological effects of chemicals.[11,12] Machine learning algorithms can identify patterns and associations in complex datasets, facilitating the prediction of adverse outcomes and the identification of key biomarkers.[13,14] Network toxicology,[15,16] which integrates systems biology and toxicology, allows for the construction of comprehensive networks to model the interactions between chemicals and biological systems, thereby revealing the potential pathways and mechanisms involved in toxicity.

This study aims to explore the toxicological impact of ATBC exposure on OA using an integrative approach combining bioinformatics, machine learning, and network toxicology. By analyzing microarray data and constructing toxicological networks, we seek to identify potential biomarkers and pathways associated with ATBC-induced OA. This research not only contributes to a deeper understanding of the toxicological mechanisms of ATBC but also provides valuable insights for the early diagnosis and prevention of OA. Furthermore, the findings may inform future regulatory policies regarding the safe use of ATBC and other similar chemicals.

Methods

Network Toxicology Analysis of Potential ATBC Targets

We obtained the chemical structure and the simplified molecular input line entry system (SMILES) notation of ATBC from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The potential targets of ATBC were identified via the ChEMBL (https://www.ebi.ac.uk/chembl/), CTD (https://ctdbase.org/), TargetNet (http://targetnet.scbdd.com/), Swiss Target Prediction databases (http://swisstargetprediction.ch/), and STITCH (http://stitch.embl.de/), with the species specified as "Homo sapiens". After integrating the targets and eliminating duplicates, the resulting list of ATBC-related targets was employed to construct a comprehensive target repository.

Identification of ATBC-Induced OA Targets

The dataset GSE51588 encompasses gene expression profiles of 40 OA samples and 10 normal samples, which were retrieved from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Subsequently, the differentially expressed genes (DEGs) between the disease and control groups of the dataset GSE51588 were calculated using the Linear Model for Microarrays (LIMMA) package in R (version 4.3.2), with the criteria for DEGs set at p-value < 0.05 and $|\log2FC| \geq 1$ (fold change). Additionally, Venn diagrams were employed to identify common potential targets

between ATBC targets and OA targets utilizing the "VennDiagram" package (version 1.7.3) in R, and the intersecting portion was regarded as a potential target for ATBC-induced OA.

Functional Analysis with Potential Targets of ATBC-Induced OA

We performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis with clusterProfiler package by R 4.3.2 and the results were visualized using ggplot2 package (version 3.4.4) to investigate the molecular pathways implicated in the potential target genes associated with ATBC-induced OA. Our analysis encompassed three GO domains (biological process, cellular component, and molecular function) as well as KEGG pathways to elucidate the biological roles and molecular mechanisms of the identified genes. Functional pathways with significant enrichment were identified based on an adjusted p-value of less than 0.05. The results were visualized in the form of bubble chart and heat map.

Constructing Protein Interaction Networks and Topological Analysis

To investigate the potential targets of ATBC-induced OA, the cross-over genes were introduced into the STRING database. The analysis was conducted with the species restricted to "Homo sapiens" and the "Minimum Required Interaction Score" set at "Medium Confidence > 0.4", which helped to focus on the active target proteins associated with the target genes. Subsequently, the data obtained from STRING was transferred to Cytoscape software (version 3.9.0), a powerful tool for network biology visualization and analysis. It enables the calculation of various parameters for each node in the network graph and the visualization of molecular connections. This process allows for the computation of topological properties of network nodes and edges, thereby generating protein-protein interaction (PPI) network graphs. Moreover, the MCOD plugin was utilized to determine the Degree values for individual targets, and the top ten targets were picked out.

Core Targets Selection by Machine Learning

In our study, we harnessed the power of three machine learning algorithms to pinpoint the core targets linked to ATBC-induced OA from top targets with MCOD plugin. These algorithms were the Least Absolute Shrinkage and Selection Operator (LASSO) regression, Random Forest (RF) and the Support Vector Machine (SVM). To optimize feature selection, we deployed the Recursive Feature Elimination (RFE) algorithm, which is designed to identify the optimal combination of variables that maximizes model performance. The core targets were determined as the genes that were identified by all the three algorithms.

For the application of LASSO, we utilized the "glmnet" package (version 4.1.7) with specific parameter settings: standardize = True, alpha = 1, family = "gaussian", and nfolds = 3. Subsequently, the R package "randomForest" (version 4.7.1.1) was employed by us to identify significant genes through the random forests method. Initially, we built a random forest model comprising 1000 trees based on the discovery cohort. The optimal number of trees was ascertained via cross-validation error. Following that, we sorted ten genes according to their importance and pinpointed the top 8 most important ones. As for SVM-RFE, we used the "mlbench" (version 2.1–6) and "caret" (version 6.0–94) packages in R 4.3.2. The predictor matrix was Z-score-normalized prior to modeling. A radial-basis SVM was trained with a nested resampling scheme: the inner loop performed 10-fold cross-validation to select the optimal combination of $C \in \{0.1, 1, 10\}$ and σ estimated by sigest() (range 0.01–0.05). Recursive feature elimination was run in the outer loop with dropping 10% of features per step, retaining the subset with maximum balanced accuracy averaged across a stratified 10-fold cross-validation repeated 3 times. Random seed 2024 was fixed for full reproducibility and final selected genes were carried forward as SVM-RFE hits. Having evaluated the three aforementioned algorithms, we proceeded to identify the common genes that emerged from the outcomes of all these algorithms. These overlapping genes were then designated as the potential core targets underlying ATBC-induced OA.

Assessing Diagnostic Accuracy of Core Targets

GSE51588 was utilized to graphically depict the expression levels of the core genes. In this context, genes exhibiting a p-value of less than 0.05 were deemed to be significantly differentially expressed. Subsequently, we generated receiver

operating characteristic (ROC) curves, leveraging the mRNA expression data derived from normal and OA samples within the GSE51588 dataset. This step was crucial for further appraising the diagnostic potential of the identified core targets. Ultimately, the area under the curve (AUC) was computed to gauge the diagnostic precision of the ROC curves.

Immune Infiltration Analysis

To assess the infiltration levels of immune cells in OA individuals within the GSE51588 cohorts, the Cibersort algorithm was employed. The Wilcoxon method was then applied to explore the differences in immune cell proportions between the normal and OA groups. Moreover, the study aimed to investigate the correlation between ATBC-induced core target genes and the infiltration of these 22 immune cells, and to further clarify the regulatory mechanisms through which ATBC-induced core target genes influence immune cells.

Molecular Docking

To delve deeper into the intermolecular interactions between ATBC and the core target proteins identified in this study, the molecular docking method was employed. This approach allowed us to predict the binding mode and affinity of ATBC. Initially, the Pubchem ID and three-dimensional chemical structure was retrieved from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/) and saved as a mol2 file. Subsequently, the RCSB PDB database (http://www.rcsb.org/) was utilized to select a high-resolution crystal structure of the protein target to serve as the molecular pair acceptor. The selected protein structure was processed using PyMOL 2.6.0 software to remove water molecules and phosphate groups, and it was saved as a PDB file. Subsequently, we employed AutoDockTools 1.5.7 software to conduct a series of preprocessing steps. Specifically, we removed water molecules, added hydrogen atoms, and truncated both the ligand and receptor. After these steps, the processed structures were saved as pdbqt files. Utilizing AutoDockTools 1.5.7 again, we performed docking simulations between the ligand and receptor. Based on the docking outcomes, we assessed the binding activity between artemisinin and the targets. The results, which exhibited negative values, suggested that the common targets of OA possess binding activity with artemisinin. Notably, lower binding energies corresponded to stronger binding activity and superior docking performance. Moving forward, the docking results were exported as PDBQT files and subsequently converted to PDB files via OpenBabelGUI. Ultimately, PyMOL software was utilized to analyze and visualize the docking results.

Construction of the Adverse Outcome Pathway

By examining phenotypes that were strongly associated with ATBC and OA, we identified potential key events (KEs) based on the analysis of target phenotypes and gene-phenotype networks. The genes associated with these phenotypes were designated as potential molecular initiating events (MIEs). Subsequently, an adverse outcome pathway (AOP) was constructed to link ATBC exposure to OA. This was achieved by defining and connecting these events considering the levels and biological relationships of the various phenotypes as reported in the literature. The flowchart of this study was showed in Figure 1.

Results

Network Toxicology and Bioinformatics Analysis of Potential ATBC-Induced OA Targets

In this study, we employed network toxicology to investigate the potential pathogenic effects of ATBC in the context of OA. First, we identified 580 targets using the ChEMBL, CTD, TargetNet, Swiss Target Prediction, and STITCH databases. Afterwards, we identified the differential expression genes correlated with OA by analysis of GSE51588. The signal intensity was normalized (Figure 2A) and Principal Component Analysis (PCA) was performed to explore the overall gene expression patterns and identify potential differences between the OA and control groups (Figure 2B). The PCA plot illustrated that the samples were clearly separated into two distinct clusters, indicating significant differences in gene expression profiles between two groups. To further investigate the DEGs between two groups, the volcano plot was generated (Figure 2C). A total of 966 DEGs were filtered out by the criteria of $|logFC| \geq 1$ and adjust P value <0.05. The

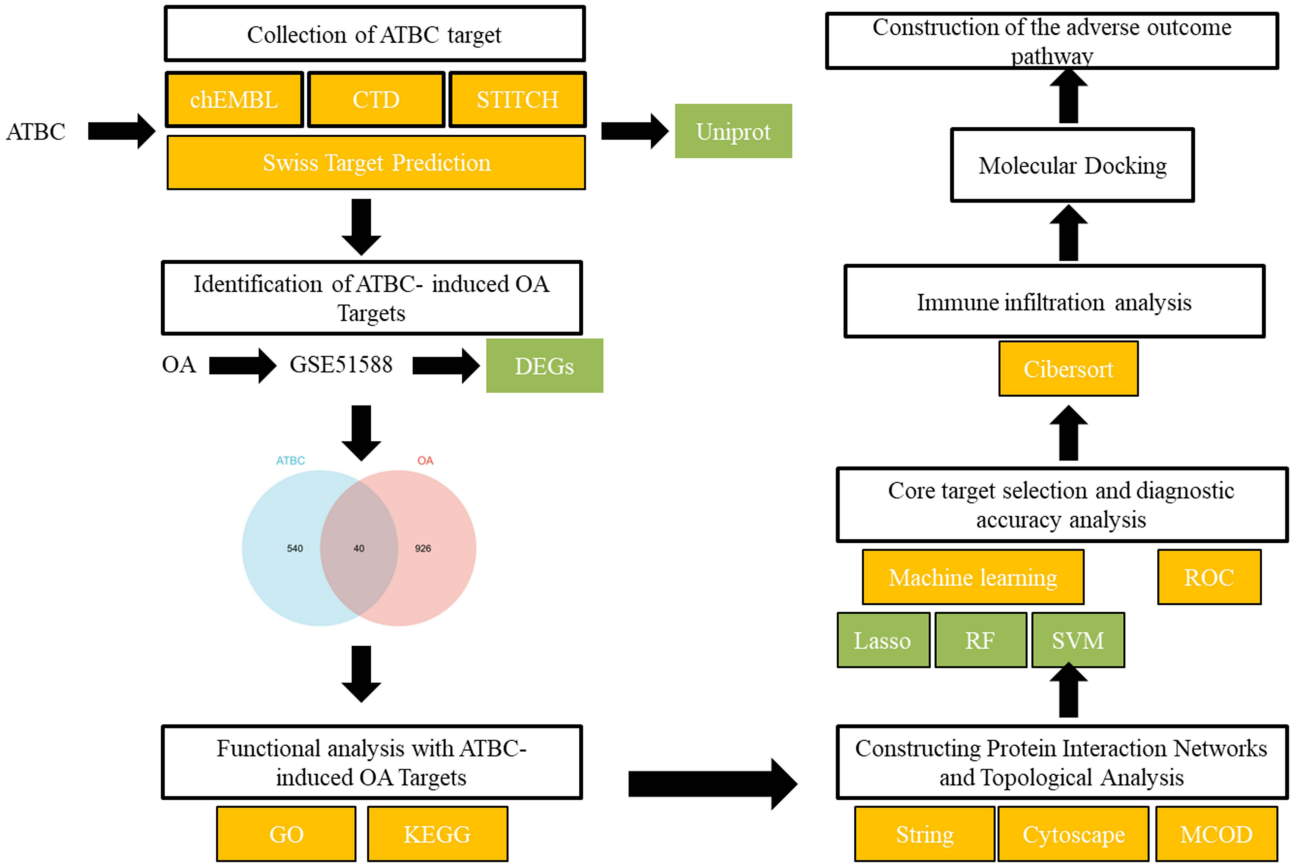

Figure 1 The flowchart of this study.

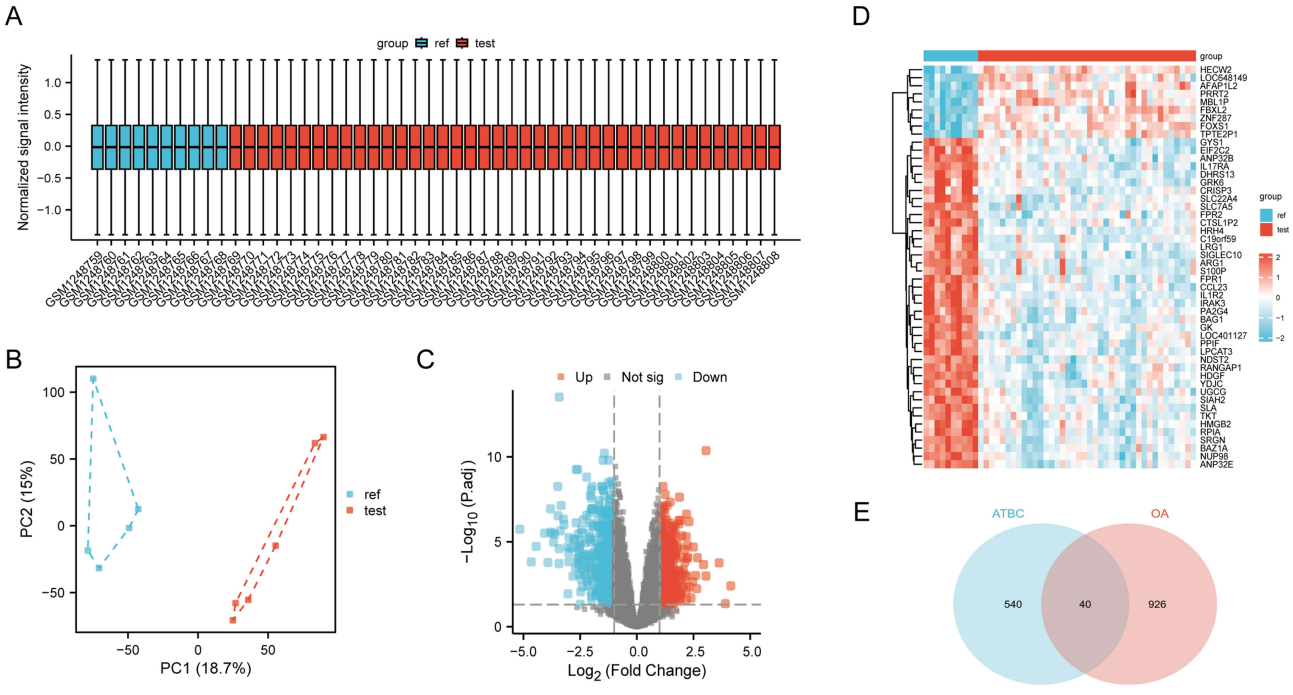

Figure 2 OA-related differential expression genes analysis and ATBC-induced OA targets screening. (**A**) Signal intensity normalization. (**B**) PCA plot of gene expression profiles. (**C**) Volcano plot of differentially expressed genes. (**D**) Heat map of top 50 differentially expressed genes. (**E**) Venn diagram displaying the overlap between ATBC-related targets and OA-related DEGs.

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top 50 DEGs were showed by heat map (Figure 2D). 40 common targets were obtained by intersecting 580 ATBC-related targets and 966 OA-related DEGs (Figure 2E).

Functional Analysis with Potential Targets of ATBC-Induced OA

GO and KEGG pathway enrichment analysis were performed to investigate the biological functions and pathways associated with the ATBC-induced OA targets. The results are summarized in Figure 3. As shown in Figure 3A, the GO enrichment analysis revealed several significantly enriched BP terms related to calcium ion regulation and cellular adhesion. Specifically, enriched terms such as regulation of blood circulation, positive regulation of cytosolic calcium ion concentration and leukocyte migration indicated that the DEGs were involved in processes related to calcium signaling and immune responses. Additionally, the CC analysis (Figure 3B) highlighted the enrichment of terms associated with synaptic membranes and vesicle lumens, suggesting that the DEGs may play roles in neurotransmission and synaptic function. The MF analysis (Figure 3C) further identified enriched terms related to neurotransmitter receptor activity and protease binding, indicating potential involvement of the DEGs in signal transduction and protein degradation pathways. The KEGG pathway analysis (Figure 3D) revealed significant enriching in the calcium signaling pathway and neuroactive ligand-receptor interaction pathways. Key genes involved in these pathways, such as ADORA3, AVPR2, XTSG and CXCR4 were identified (Figure 3E), suggesting that these observed gene expression changes may impact calcium signaling and neurotransmitter receptor interactions.

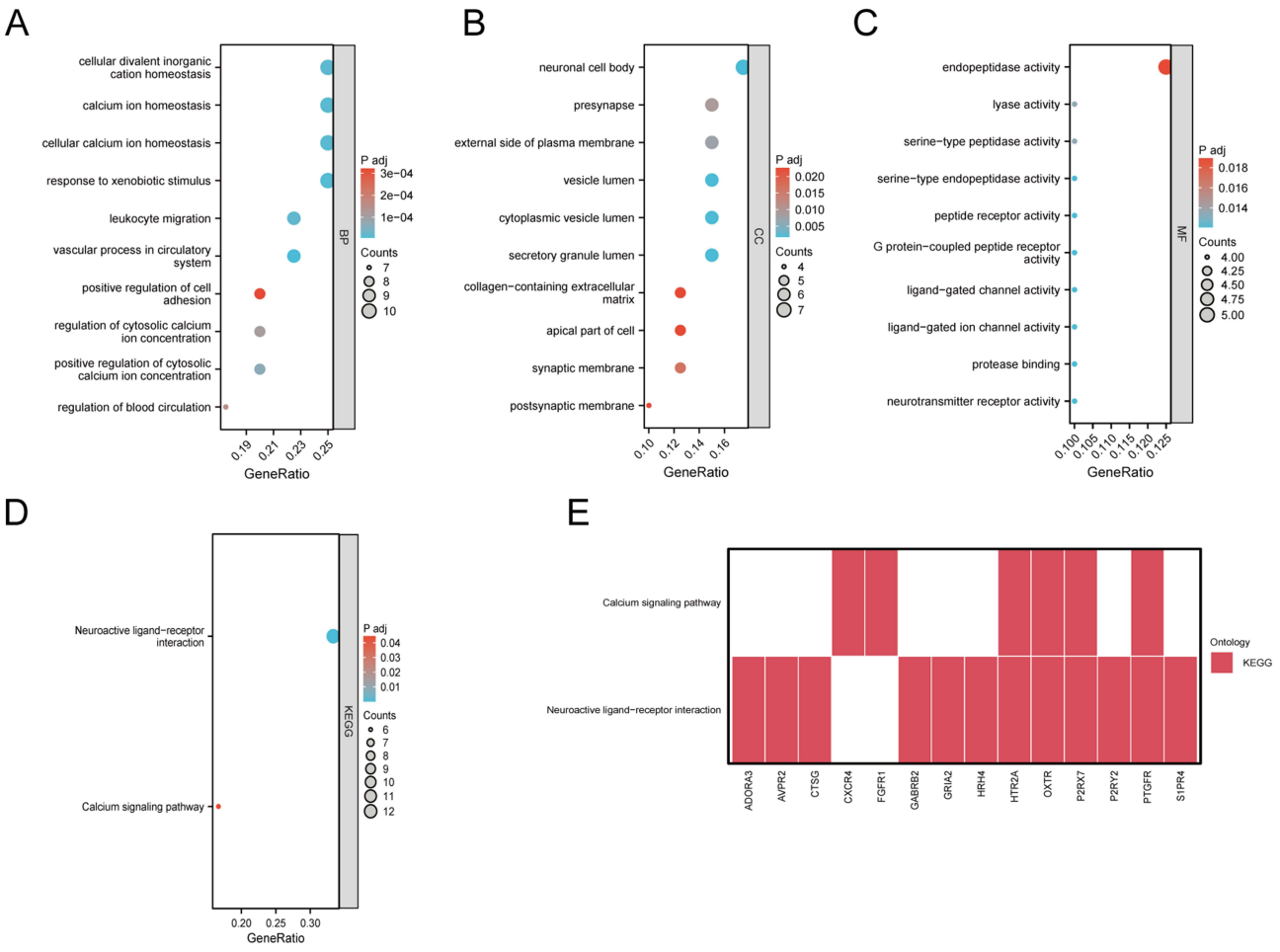

Figure 3 Enrichment analysis of ATBC-related OA targets. (**A**) bubble chart of BP (biological process) enrichment analysis. (**B**) bubble chart of CC (cellular component) enrichment analysis. (**C**) bubble chart of MF (molecular function) enrichment analysis. (**D**) bubble chart of KEGG pathway analysis. (**E**). Heat map of enrichment KEGG pathway. P adj, adjust P value.

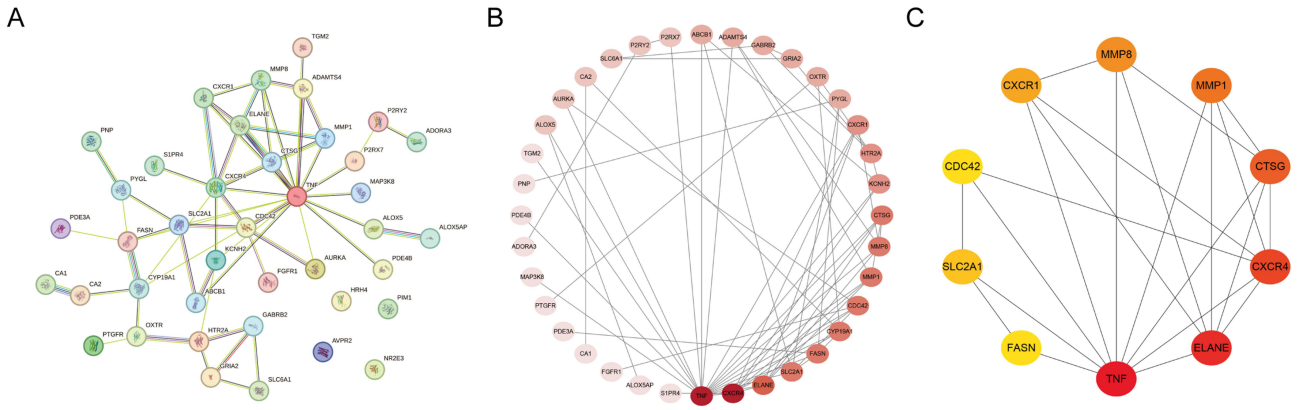

Figure 4 Construction of protein-protein interaction network. The interaction network of was constructed using the STRING database (**A**) and Cytoscape software (**B**). The top 10 ranked genes were identified using the CytoHubba plugin (**C**).

Construction of Protein Interaction Network

The 40 ATBC-related OA targets were imported into the STRING database to construct a PPI network, which comprised 40 nodes and 61 edges (Figure 4A). The network was built using Cytoscape software, and the nodes were ranked by degree (Figure 4B). The top ten targets by MCC degree, identified using the CytoHubba plug-in (Figure 4C and Table 1), were TNF, ELANE, CXCR4, CTSG, MMP1, MMP8, CXCR1, CDC42, SLC2A1, FASN. These targets were selected as the primary ATBC- induced OA targets for further analysis.

Core Targets Selection by Machine Learning

In this study, we employed three machine learning algorithms (LASSO, RF and SVM) to further identify core targets. The results of the gene selection process were resented in Figure 5, which illustrates the key genes identified by each algorithm. As shown in Figure 5A, the trajectories of variable coefficients under different regularization parameters λ. We identified 6 genes as potential core targets by LASSO algorithm and showed with deviance plot (Figure 5B). On the other hand, the feature importance plot provided a clear visualization of which features were most critical in a predictive model based on the Random Forest algorithm and then the 8 more important genes were included in the next step of analysis (Figure 5C). The SVM regression algorithm further filtered out 8 core targets (Figure 5D). A Venn diagram integrating the results from both methods identified four definitive core targets associated with ATBC-induced OA: TNF, MMP8, CXCR4 and SLC2A1 (Figure 5E).

Table 1 Topological Character of Top 10 Targets

No.	Gene Name	Uniprot ID	Protein Names	Degree	Closeness Centrality	Betweenness Centrality	Neighborhood Connectivity
1	ELANE	P08246	Neutrophil elastase	6	0.750	0.032	5.333
2	MMP8	P22894	Neutrophil collagenase	4	0.643	0.007	6.000
3	MMP1	P03956	Interstitial collagenase	4	0.643	0.000	6.500
4	CXCR1	P25024	C-X-C chemokine receptor type 1	4	0.643	0.007	6.250
5	CXCR4	P61073	C-X-C chemokine receptor type 4	6	0.750	0.072	5.167
6	TNF	P01375	Tumor necrosis factor	9	1.000	0.449	4.111
7	CTSG	P08311	Cathepsin G	5	0.692	0.016	5.800
8	SLC2A1	P11166	Solute carrier family 2, facilitated glucose transporter member 1	3	0.600	0.014	4.667
9	FASN	P49327	Fatty acid synthase	2	0.563	0.000	6.000
10	CDC42	P60953	Cell division control protein 42 homolog	3	0.600	0.014	6.000

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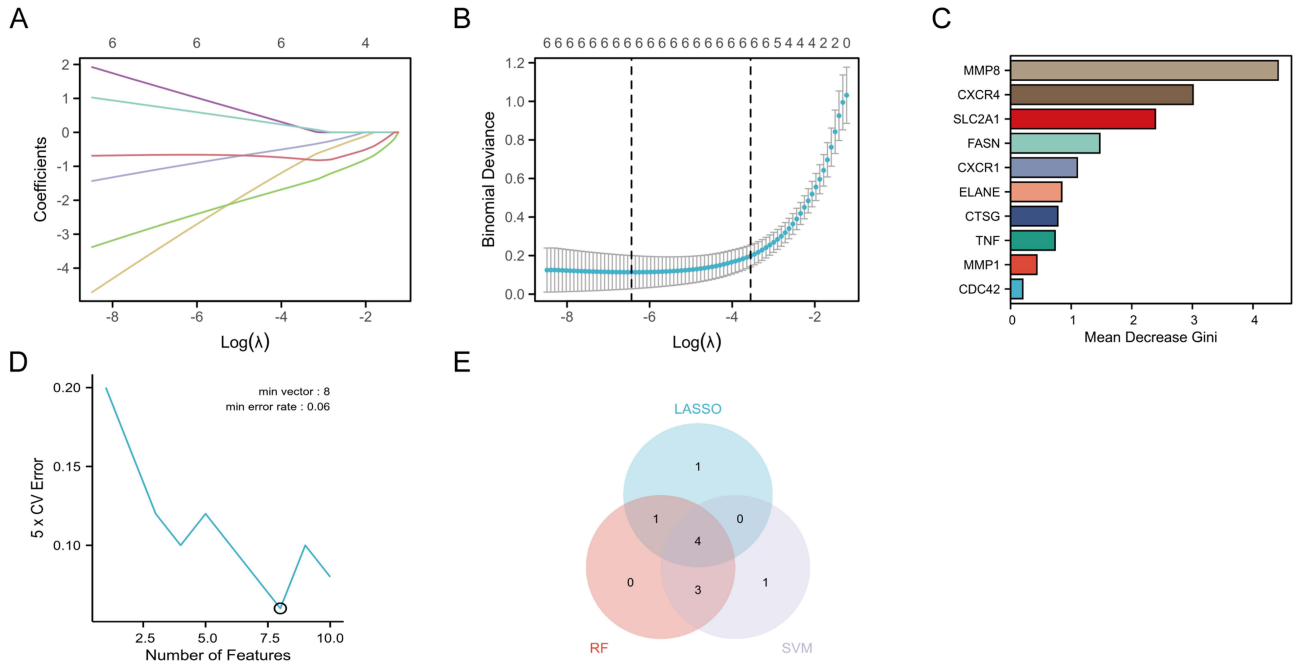

Figure 5 Identification of core targets by Machine Learning Algorithms. Core genes were identified by LASSO (**A** and **B**), RF (**C**) and SVM algorithm (**D**). Common core targets were further obtained by venn diagram (**E**).

Assessing Diagnostic Accuracy of Core Targets

We analyzed the RNA-seq dataset of osteoarthritis patients (GSE51588) from the GEO database to obtain the core gene expression levels. A significant upregulation (TNF) or downregulation (CXCR4, MMP8 and SLC2A1) was observed in the expression level of subchondral bone tissues in osteoarthritis compared to normal group (Figure 6A). We plotted receiver operating characteristic (ROC) curves and calculated the area under the curve (AUC) to assess the diagnostic performance of four core genes. The AUC values for TNF, CXCR4, MMP8 and SLC2A1 were 0.762, 0.955, 0.970 and 0.920, respectively (Figure 6B). These findings highlight the potential of these four core targets in OA pathogenesis and future diagnosis.

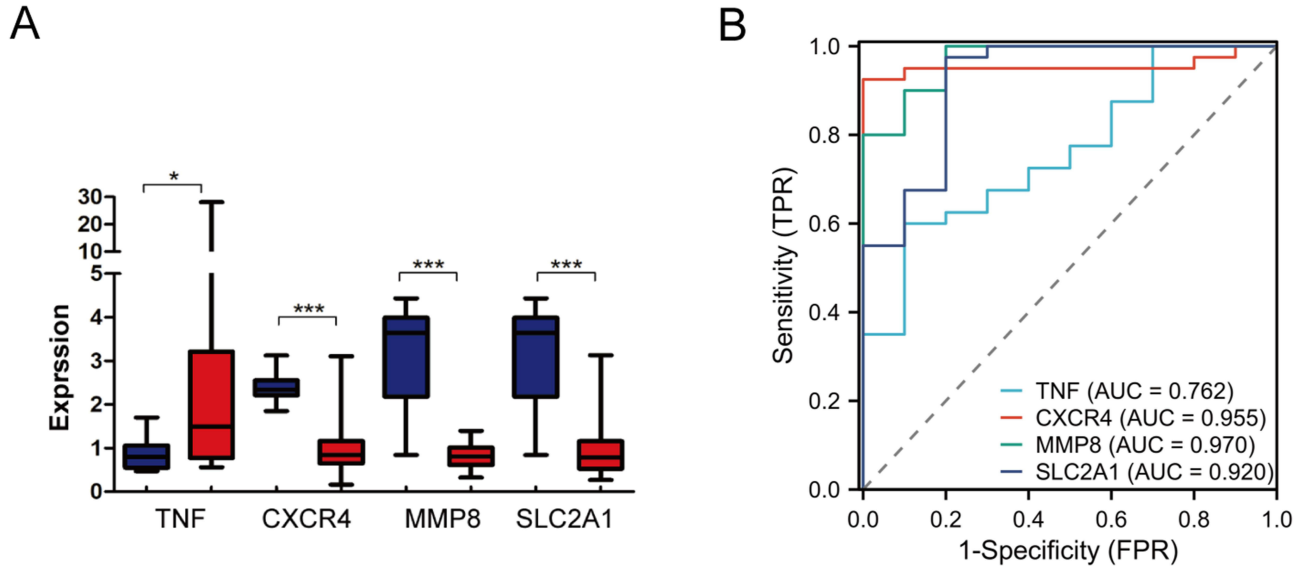

Figure 6 RNA sequence analysis of core gene expression in OA samples compared to normal control. (**A**) Expression levels of four key targets in GSE51588 dataset. *P<0.05, ***P<0.001. (**B**) Diagnostic effectiveness of the four core targets in distinguishing normal and OA samples with receiver operating characteristic (ROC) curves. **Abbreviations**: TPR, True Positive Rate; FPR, False Positive Rate.

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Immune Infiltration Analysis

To gain a comprehensive understanding of the immune landscape in OA, we conducted immune infiltration analysis to assess the composition of 21 immune cell types in both OA and control groups, as well as their correlations with four core genes. Our analysis revealed significant differences in the infiltration levels of several immune cell types between the two groups. Specifically, we observed notable variations in the infiltration of B cell memory, T cells (including CD8+ T cells, CD4+ naive T cells, resting CD4+ memory T cells, and follicular helper T cells), M2 macrophages, dendritic cells (both activated and resting), resting mast cells, eosinophils, and neutrophils (Figure 7A and B). Furthermore, we examined the specific correlations between different immune cell types (Figure 7C). This step was crucial in uncovering the complex interplay among various immune cells within the OA microenvironment.

Subsequently, we delved into the relationships between the four core targets and immune cell infiltration (Figure 7D). In terms of gene expression correlations, TNF expression levels exhibited a positive with correlation memory B cells and regulatory T cells (Tregs), while showing a negative correlation with activated CD4+ memory T cells, M0 macrophages, and neutrophils. CXCR4 expression levels, on the other hand, were positively correlated with resting CD4+ memory T cells, M0 macrophages, eosinophils, and neutrophils, but negatively correlated with naïve CD4+ T cells and M1 macrophages. MMP8 expression levels demonstrated a positive relationship with resting NK cells, resting mast cells, and eosinophils, while being negatively related to memory B cells, CD8+ T cells, Tregs, M2 macrophages, and activated dendritic cells. Lastly, SLC2A1 gene levels were positively associated with plasma cells, resting CD4+ memory T cells, and eosinophils. Conversely, they were negatively related to B memory cells, CD8+ T cells, Tregs, γδ T cells, M1 macrophages, and activated dendritic cells. These findings collectively highlight the intricate relationships between the core genes and immune cell infiltration in OA, shedding light on the underlying immune mechanisms that contribute to the disease pathology.

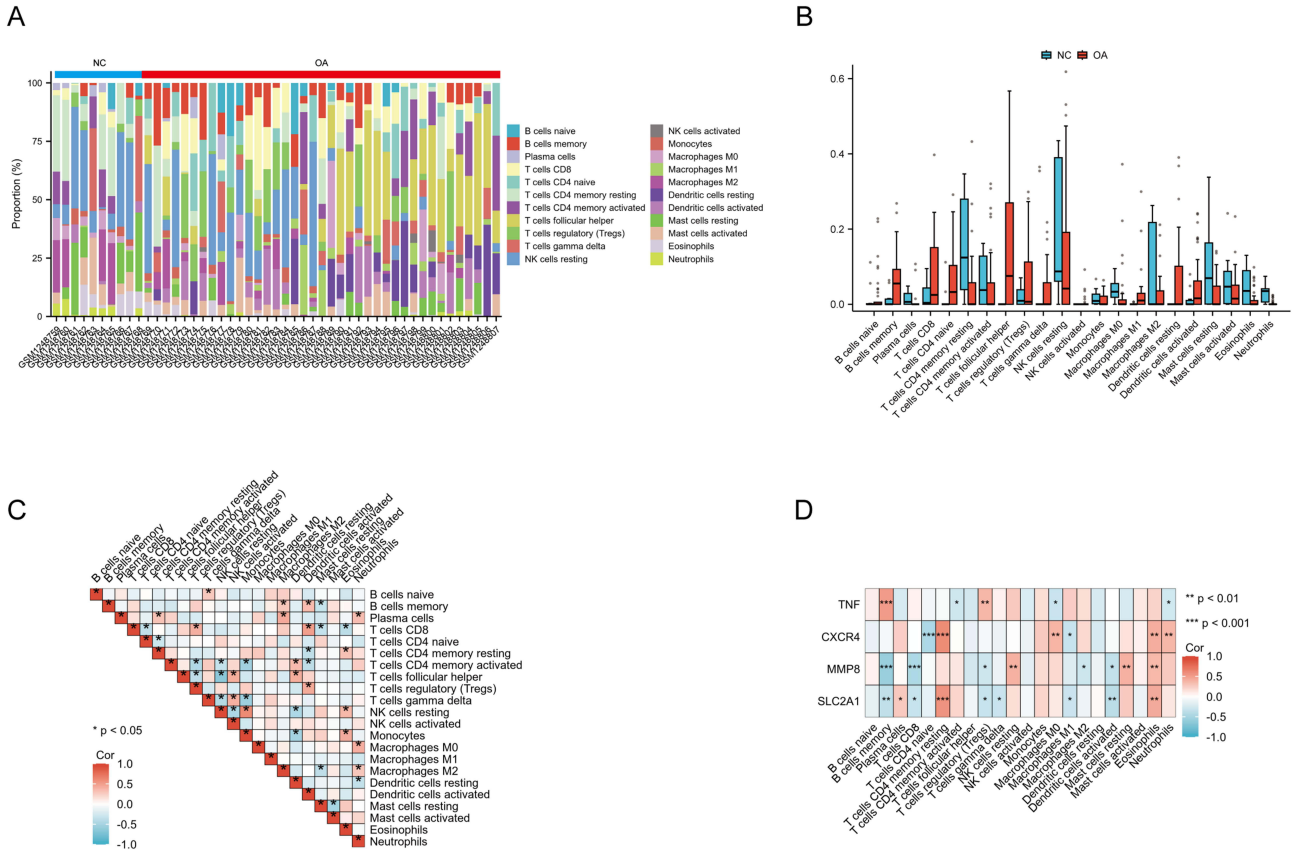

Figure 7 Immune infiltration analysis of OA and control from the GSE51588 dataset. (**A** and **B**) CIBERSORT analysis of 21 different immune cell types in OA and control groups. (**C**) Correlations between various immune cell types. (**D**) Correlations between core target genes and immune cell infiltration in OA. Cor, Correlation. *P<0.05, **P<0.01, ***P<0.001.

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Table 2 The Docking Results of ATBC with Core Targets

Molecule	Binding Energy (kcal/mol)	Interaction Type
TNF	−5.4	Hydrogen bond Hydrophobic interaction
CXCR4	−6.1	Hydrogen bond Hydrophobic interaction
MMP8	−6.3	Hydrogen bond Hydrophobic interaction
SLC2A1	−5.9	Hydrogen bond Hydrophobic interaction

Molecular Docking Verification

We conducted a stepwise molecule docking analysis between the four core targets (TNF, CXCR4, MMP8, and SLC2A1) and ATBC using AutoDock vina 1.1.2 software. The docking results showed that the binding energies of ATBC to the targets were less than −5 kcal/mol, indicating excellent binding affinity (Table 2). After completing the docking steps, PyMOL software was used to visually analyze the binding pattern between the ligand and protein. ATBC bound to TNF through hydrogen bonding and hydrophobic interactions with ILE97, CYS77, THR79 and ASN92 (Figure 8A). It also had stable interactions with CXCR4 residues THR142, ALA141, ILE1009, ARG1008, and LYS225 via hydrogen bonding and hydrophobic interactions (Figure 8B). ATBC interacted stably with MMP8 residues HIE207, HIE162, ILE159, HIE197, TYR219, VAL194, and TYR189 through hydrogen bonding and hydrophobic interactions (Figure 8C). ATBC stably interacted with SLC2A1 through intricate hydrogen bonding and robust hydrophobic interactions involving key residues LYS256, THR258, PRO401, ILE259, GLY84 and PHE81, as visualized in Figure 8D.

Development of the Adverse Outcome Pathway

Subsequently, a novel adverse outcome pathway (AOP) framework was established. Within this AOP network, the expression and activity of TNF, CXCR4, MMP8, and SLC2A1 might be influenced by ATBC. Moreover, the calcium signaling pathway neuroactive ligand-receptor interaction pathway could also be implicated, potentially resulting in immune dysregulation and the occurrence and development of OA (Figure 9). This study successfully constructed

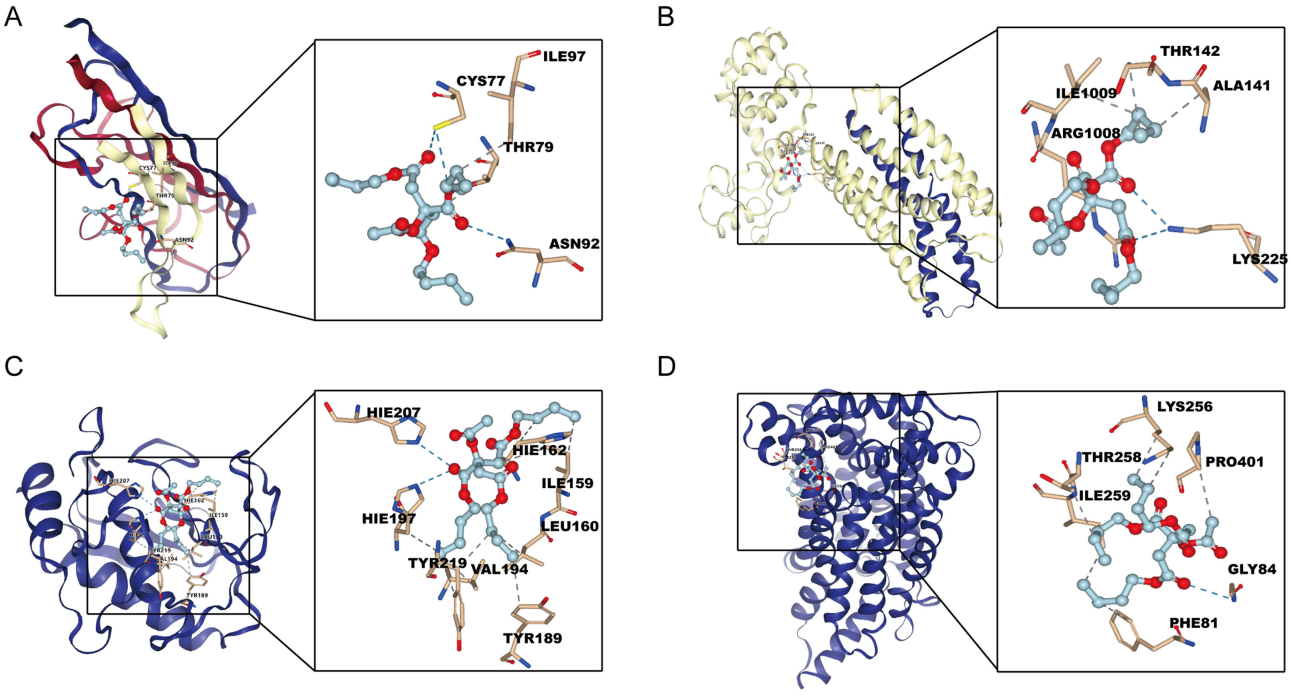

Figure 8 The molecular docking results for ATBC and four core targets, including TNF (**A**), CXCR4 (**B**), MMP8 (**C**), and SLC2A1 (**D**).

Figure 9 The Adverse Outcome Pathway (AOP) hypothesis of ATBC-induced OA.

a comprehensive AOP framework by systematically integrating the identified core genes mentioned above, thereby providing a theoretical foundation for elucidating the mechanisms by which ATBC exacerbates OA.

Discussion

The present study delves into the potential toxicological impact of ATBC exposure on OA through a multifaceted approach encompassing bioinformatics, machine learning, and network toxicology analysis. The findings offer valuable insights into the underlying mechanisms and potential diagnostic targets associated with ATBC-induced OA, contributing to the broader understanding of this disease.

The identification of 40 common targets by intersecting ATBC-related targets and OA-related differentially expressed genes (DEGs) serves as a crucial foundation for subsequent functional analysis. The GO enrichment analysis revealed significant involvement of these targets in BP of calcium ion regulation and cellular adhesion, and the enriched KEGG pathway were calcium signaling pathway and neuroactive ligand-receptor interaction pathways. These pathways are well-documented in the context of OA pathogenesis. Calcium signaling is intricately linked to the pathogenesis of OA through its involvement in inflammation, cytokine regulation, cellular senescence, mitochondrial dysfunction, and mechanical stress responses. Understanding these mechanisms provides valuable insights for developing targeted therapies to treat OA and improve patient outcomes. For instance, the increased inflammatory factor and ROS level may activate the calcium signaling pathway, which stepwise increase chondrocyte apoptosis and ultimately exacerbate OA.[17] TRPC1, a calcium channel protein, has been implicated in the early stages of OA and plays an important role in calcium signaling in chondrocytes. Sambale et al[18] found that loss of TRPC1 was linked to the early stages of OA and plays a critical role in calcium signaling in chondrocytes. The dysregulation of calcium ion homeostasis can lead to the activation of various signaling pathways that contribute to the breakdown of extracellular matrix components in articular cartilage.[19] Similarly, neuroactive ligand-receptor interactions have been associated with pain and inflammatory responses in OA.[20] Neuroactive ligand-receptor interactions are among the key pathways implicated in OA. Transcriptome analysis and network pharmacology studies have identified this pathway as significantly enriched in differentially expressed genes in OA cartilage.[21] For instance, a study on retinoic acid-induced OA highlighted the neuroactive ligand-receptor interaction pathway as one of the critical signaling pathways involved in OA pathogenesis.[22] Additionally, the active components of traditional Chinese medicine Xianlinggubao capsule, which was used for OA treatment, have been shown to act on this pathway, suggesting it may act as therapeutic target.[21] So far, studies found that the neuroactive ligand-receptor interactions played a multifaceted role in OA, contributing to pain,[23] cartilage degradation,[22] and bone remodeling.[24] Understanding these mechanisms provides valuable insights for developing targeted therapies to alleviate OA symptoms and slow disease progression. Future research should focus on elucidating how ATBC influences the specific neuroactive ligands and receptors and exploring their potential as therapeutic targets.

The construction of the PPI network and the subsequent selection of core targets using machine learning algorithms (LASSO, RF, and SVM) highlighted the most relevant genes associated with ATBC-induced OA. TNF, MMP8, CXCR4, and SLC2A1 emerged as definitive core targets, which were not only highly connected within the PPI network but also exhibited significant expression changes in OA patients. TNF, a well-known pro-inflammatory cytokine, has been extensively studied in OA due to its role in promoting inflammation and cartilage degradation.[25] The upregulation of TNF in OA tissues, as observed in our study, is

consistent with previous findings.[26] MMP8, a member of the matrix metalloproteinase family, is involved in the degradation of cartilage matrix proteins.[27] Studies have shown that MMP8 levels are significantly elevated in the synovial fluid and cartilage of patients with OA.[28,29] This increase is associated with the severity of the disease, suggesting that MMP8 plays a crucial role in the progression of OA.[30] MMP8 degraded type II collagen and other cartilage matrix proteins, leading to the loss of cartilage integrity, which is a hallmark of OA and contributing to joint instability and pain.[30,31] MMP8 can also affect bone remodeling by degrading collagen in the subchondral bone, leading to increased bone resorption and the formation of osteophytes, which are characteristic features of OA.[31] Targeting MMP8 through pharmacological inhibitors or gene therapy has shown promise in preclinical models of OA. For example, the use of specific MMP inhibitors has been shown to reduce cartilage degradation and improve joint function.[32,33] CXCR4, a chemokine receptor, plays a significant role in the pathogenesis of OA by influencing various biological processes, including chondrocyte function, inflammation, and bone remodeling. Studies have shown that CXCR4 expression was significantly elevated in the synovial fluid and cartilage of patients with OA.[34,35] CXCR4 was found expressing on chondrocytes and synovial cells, where it mediates the effects of SDF-1 (stromal cell-derived factor-1), contributing to cartilage degradation and inflammation.[34,36]

Targeting CXCR4 through pharmacological inhibitors or genetic modification has shown promise in reducing inflammation and cartilage degradation in preclinical models of OA.[37,38] Enhancing the SDF-1/CXCR4 axis may promote the migration and differentiation of MSCs, which could be a potential therapeutic strategy for OA.[39,40] SLC2A1, also known as GLUT1, is a facilitative glucose transporter that plays a crucial role in glucose uptake and metabolism. Recent studies have highlighted its involvement in the pathogenesis of OA, particularly in modulating chondrocyte metabolism and inflammatory responses.[41,42] GLUT1-mediated glucose uptake promoted glycolysis in chondrocytes, leading to the production of lactate. Elevated lactate levels can induce inflammation and contribute to cartilage degradation. SLC2A1 played a significant role in the pathogenesis of OA by modulating glucose metabolism,[41,42] inflammation,[41] and cartilage degradation.[42] Targeting SLC2A1 and its associated pathways holds promise for developing novel therapeutic strategies to mitigate OA progression and improve joint health.

The diagnostic accuracy assessment of these core targets through ROC curve analysis further validated their potential as biomarkers for OA. The AUC values obtained for TNF, CXCR4, MMP8, and SLC2A1 indicated their ability to distinguish between OA and control groups with a reasonable degree of accuracy. This research also underscores the crucial role of the core targets identified in this study in the development of OA.

This study provides in-silico evidence that ATBC aggravates OA via calcium-signaling and neuroactive ligand-receptor pathways, findings that hold translational potential. Manocchio et al[43] showed that a single intra-articular injection of carboxymethyl-chitosan (CMC) rapidly and durably improves pain and function in advanced knee OA, while down-regulating synovial TNF-α and MMP-8 within four weeks-two molecules we identified as ATBC-sensitive targets. This convergence suggests that ATBC-exposed individuals with high synovial TNF/MMP8 signatures may benefit from CMC or similar biologic formulations. Moreover, the four core targets (TNF, MMP8, CXCR4 and SLC2A1) can serve as measurable ATBC-exposure-response biomarkers for patient stratification.

The immune infiltration analysis provided a comprehensive view of the immune landscape in OA, revealing significant differences in the infiltration levels of various immune cell types between OA and control groups. The correlations between the core targets and immune cell infiltration highlighted the intricate interplay between these genes and the immune system. TNF's positive correlation with memory B cells and Treg cells, and its negative correlation with activated CD4+ memory T cells, M0 macrophages, and neutrophils, suggested its role in modulating immune cell activity. Similar findings have also been discovered in other studies. They found that TNF-α can stimulate the recruitment of various immune cells, including T cells and B cells, into the synovial fluid, which will exacerbate synovial inflammation and joint damage.[44] CXCR4, MMP8, and SLC2A1 also exhibited distinct correlation patterns with different immune cell types, indicating their involvement in shaping the immune microenvironment in OA. These findings are consistent with previous studies that have explored the immune mechanisms underlying OA.[42,45,46] The immune system plays a crucial role in OA pathogenesis, with various immune cells contributing to inflammation, cartilage degradation, and pain. The identified core targets may serve as potential therapeutic targets to modulate immune responses and alleviate OA symptoms. However, the immune infiltration results may be biased by the following confounding factors: First, the subchondral bone specimens in GSE51588 may mixed with bone marrow hematopoietic

cells, which could systematically raise the monocyte and neutrophil scores; Second, age, gender and BMI were all independently correlated with the M1 macrophage score; Third, the use of glucocorticoids or NSAIDs can inhibit the expression of neutrophil-related genes, leading to an underestimation of true infiltration. Regrettably, the information of age, gender, BMI, medication history and tissue purity is lacking in GSE51588. The above variables need to be corrected in cohorts with larger sample sizes and more complete clinical phenotypes to verify the causality of the ATBC-immune interaction In the future research.

The molecular docking verification of the binding affinity between ATBC and the core targets provides further evidence of the potential toxicological impact of ATBC exposure. The binding energies obtained for TNF, CXCR4, MMP8, and SLC2A1 indicate strong interactions with ATBC, suggesting that ATBC may directly influence the expression and activity of these genes. The detailed binding patterns analyzed using PyMOL software reveal specific interactions between ATBC and key residues of these proteins, highlighting the structural basis for their potential functional effects. These findings are novel and provide a molecular-level understanding of the impact of ATBC exposure on OA.

Finally, the development of the AOP framework integrates the identified core genes, pathways, and immune mechanisms to elucidate the potential mechanisms by which ATBC exacerbates OA. The AOP embeds these molecular events into two key events-calcium signaling pathway, neuroactive ligand–receptor imbalance, each of which is already measurable in mainstream OA models: IL-1β-stimulated chondrocyte model, synovium-cartilage co-culture neuro-invasion model and DMM mouse model. The AOP framework can serve as a valuable tool for future research to explore the underlying mechanisms and identify potential therapeutic targets for OA. Besides, from a preventive standpoint, our AOP framework identifies calcium signaling as the molecular initiating event, providing a quantifiable endpoint (serum or urinary Ca^{2+} flux) for future epidemiological studies linking plasticizer exposure to early cartilage changes. Regulatory agencies could incorporate synovial-fluid TNF/MMP8/CXCR4/SLC2A1 assays into joint-health surveillance for high-exposure occupations. However, multi-level validation, from cellular co-cultures to population biomonitoring, remains essential before the framework can inform regulatory decisions on ATBC exposure limits.

While a toxicology-based approach provides valuable insights into ATBC-induced OA, we acknowledge its limitations. Direct experimental evidence linking ATBC exposure to OA in animal models was not assessed. Validating network-based computational findings through carefully designed experiments is crucial to ensure reliable conclusions. Future research should include in vitro and in vivo validation of identified key targets and signaling pathways. Large-scale, long-term epidemiological studies are also needed to track and analyze the dynamic association between ATBC exposure and OA incidence. Such studies will provide a solid theoretical foundation for developing prevention and treatment strategies for ATBC-induced OA and other environmental health risks.

In conclusion, our study provides a comprehensive analysis of the toxicological impact of ATBC exposure on OA through an integrative approach. The identified core targets, pathways, and immune mechanisms offer valuable insights into the disease pathogenesis and potential diagnostic and therapeutic avenues. Future studies should focus on validating these findings in larger cohorts and exploring the therapeutic potential of targeting these core genes to alleviate OA symptoms and improve patient outcomes.

Conclusions

Our study utilized network toxicology and computational simulations to investigate the potential targets and molecular mechanisms of ATBC-induced OA, identifying 40 candidate targets. Machine learning algorithms further emphasized four key targets: TNF, MMP8, CXCR4 and SLC2A1, through which ATBC may exacerbate OA progression. Molecular docking confirmed stable interactions between ATBC and these core targets. Additionally, we developed an AOP framework to illustrate the toxic effects of ATBC on OA, aligning with established OA pathophysiology which has been implicated in OA progression. These results suggested that the environmental pollutant ATBC may contribute to the progression of OA, laying the groundwork for future research. However, these findings require further validation through additional pharmacological and clinical studies.

Generative AI Statement

The authors state that no generative AI was utilized in creating this manuscript.

Data Sharing Statement

The original contributions presented in the study are included in the article.

Ethical Approval

The study utilized publicly available data from the GEO database (GSE51588), which has been anonymized and do not involve any identifiable personal information. Formal ethical approval was waived by our Institutional Review Board (IRB) in accordance with Article 32 of the Chinese "Regulations on Ethical Review of Life Sciences and Medical Research Involving Human Subjects" (effective February 18, 2023).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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.

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