

Multi-omics analysis reveals coordinated lipid and iron metabolism alterations associated with pathological complete response to neoadjuvant immunochemotherapy in LA OPSCC

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Abstract

Background: Neoadjuvant immunochemotherapy (nCIT) is used for locally advanced oropharyngeal and hypopharyngeal squamous cell carcinoma (LA OPSCC), but the biological basis of pathological complete response (PCR) remains unclear. Metabolic reprogramming beyond immune activation may play a critical role.

Methods: Serum metabolic profiles (iron, ferritin, lipids) from 116 LA OPSCC patients receiving nCIT were analyzed, and quantitative MRI assessed treatment-induced damage. Multi-omics sequencing was performed on pre-/post-treatment samples from 23 patients (10 PCR, 13 non-PCR). Immunofluorescence validated selected markers.

Results: Post-treatment serum iron and triglycerides were significantly elevated in PCR patients ($p=0.0252$ and $p=0.013$) only in primary tumors, not lymph nodes. MRI showed increased T1 and T2* values selectively in primary tumors of PCR patients ($p=0.017$, $p=0.015$), correlating with serum markers. Metabolomics and transcriptomics identified significant lipid metabolism enzyme regulation. Targeted lipidomics revealed upregulation of plasmalogen PE, phosphatidylserine, and phosphatidylcholine in PCR tumors. Proteomics showed SELENOI (involved in PE-P synthesis) upregulated in PCR patients, with strong positive correlation with TFRC ($\rho=0.951$, $p<0.0001$). Immunofluorescence confirmed elevated SELENOI, TFRC, 4-HNE, and ferritin in PCR tumors, along with increased malondialdehyde and lipid ROS, consistent with ferroptosis.

Conclusions: Integrated multi-omics reveals that PCR following nCIT is associated with a coordinated lipid remodeling signature involving SELENOI-associated PE-P enrichment and TFRC-associated iron availability, suggesting a dual priming state for ferroptosis. These findings identify candidate biomarkers for further validation.

Background

Local advanced head and neck squamous cell carcinoma (HNSCC) remains a significant global health concern, with locally advanced oropharyngeal and hypopharyngeal squamous cell carcinoma (LA OPSCC) contributing notably to both the incidence and mortality rates. Oropharyngeal cancer, primarily associated with HPV infection, accounts for approximately 389,846 new cases and 177,757 deaths annually[1]. In contrast, hypopharyngeal cancer, often linked to tobacco and alcohol use, has a relatively lower incidence but poorer prognosis, with a 5-year survival rate of approximately 30–35%. Despite advancements in treatment, these cancers remain challenging owing to late-stage diagnosis and high recurrence rates.

The NCCN guidelines have demonstrated the efficacy of Neoadjuvant chemoimmunotherapy (nCIT) in treating locally advanced oropharyngeal and hypopharyngeal cancers [2]. The combination of platinum-based chemotherapy (e.g., cisplatin or carboplatin) and PD-1/PD-L1 inhibitors (e.g., nivolumab or pembrolizumab) has shown significant clinical benefits in various cancers, including lung, esophageal,

and oral cancers[3]. The mechanism of action involves platinum-based chemotherapy that promotes immunogenic cell death (ICD), which releases tumor antigens and activates the immune system, making tumor cells more susceptible to PD-1/PD-L1 blockade. In clinical trials, this combination has shown improved overall survival (OS) and progression-free survival (PFS) in patients with tumors expressing PD-L1[4]. Consequently, predictive biomarkers, such as PD-L1 expression, are primarily confined to characterization of the tumor immune microenvironment. However, this paradigm fails to fully explain the spectrum of clinical responses, particularly the presence of responders among PD-L1 negative patients, suggesting the involvement of alternative pivotal mechanisms independent of canonical immune pathways.

Beyond its immunomodulatory effects, the efficacy of nCIT is profoundly influenced by the metabolic landscape of the tumor microenvironment (TME). The metabolic interplay between nCIT and tumor biology, particularly iron and lipid metabolism, presents a compelling yet underexplored axis. Chemotherapeutic agents such as those in the taxane-platinum (TP) regimen are known to induce significant metabolic rewiring. They promote oxidative stress, leading to pervasive lipid peroxidation and concurrent disruption of iron homeostasis by elevating the labile iron pool (LIP). These conditions collectively establish a metabolic context ripe for ferroptosis, an iron-dependent form of regulated cell death driven by the accumulation of lipid peroxides [5,6]. Complementarily, immune checkpoint inhibitors contribute to this landscape by reprogramming lipid metabolism within the TME. They enhance the metabolic fitness of T-cells, which is crucial for their anti-tumor effector functions, while simultaneously disrupting the lipid adaptive pathways that tumor cells use to evade immune surveillance. This duality suggests that strategic manipulation of the iron-lipid axis could convert a potential resistance mechanism into a powerful adjunct to nCIT.

However, the current paradigm for nCIT efficacy remains predominantly centered on immune activation and PD-L1 as a biomarker, largely overlooking the contribution of coordinated metabolic reprogramming. It is unknown whether a distinct metabolic signature defines therapeutic response in tumor cells. Building upon this conceptual framework, our study employed an integrated multi-omics approach to test the hypothesis that an iron-centric lipid metabolic shift is a fundamental, yet unrecognized, mechanism driving the effective nCIT response in OPSCC tumor cells.

Methods

1. Clinical sample collection and analysis

1.1 Patient Cohort

This study is a retrospective analysis of 116 patients who received neoadjuvant therapy with taxane-platinum chemotherapy combined with immune checkpoint inhibitors as part of their standard-of-care treatment in a short-run hospital from Jan,2024-Dec to,2025(**Table 1**). All patients were treated according to the institutional guidelines, and no study-specific interventions were performed. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review

Board (IRB) of Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University. Given the retrospective nature of the study, the requirement for informed consent was waived by the IRB.

Including of that, 23 patients with immediately frozen specimens were selected for the sequencing analysis (**Table 2**). All patients underwent surgical resection following nCIT. There was no delay in surgical resection owing to immune-related adverse events. All patients underwent baseline and post-nCIT pathological evaluations to assess treatment response. Prior to therapy initiation, comprehensive staging imaging and pathological confirmation of diagnosis were performed for all patients.

1.2 Treatment Protocol

The standard institutional management for LA OPSCC involves a multimodal approach centered on neoadjuvant systemic therapy. The treatment protocol, established through a multidisciplinary consensus, consisted of TP chemotherapy combined with ICIs (pembrolizumab, tislelizumab, or nivolumab). Following the completion of neoadjuvant therapy, all patients underwent definitive surgical resection. The pathological response was determined from the surgical specimens and classified as a pathological complete response (PCR) or non-complete response (non-PCR). All treatment plans and response assessments were reviewed and finalized by the Institutional Head and Neck Cancer Multidisciplinary Tumor Board. The pTR grading system was defined as follows: pTR-0 (Minimal Response), <10% of the tumor area; pTR-1 (Partial Response), 10% to 49% of the tumor area; pTR-2 (Major/Complete Response), ≥50% of the tumor area. In this study, PCR was defined as pTR-2, that is, a tumor regression of ≥50% following treatment.

1.3 Evaluation and Data Collection

Patients who received nCIT were evaluated at regular intervals throughout the treatment course. The nCIT regimen was administered for 3-week cycles. Treatment response was assessed after the completion of neoadjuvant therapy and prior to definitive surgery based on clinical examination, restaging imaging, and pathological evaluation of resected specimens. Baseline patient and tumor characteristics, treatment details, pathological responses, and oncologic outcomes were prospectively collected using predefined case report forms.

2. Serological Biomarker Analysis

Fasting venous blood samples were collected from patients before and after nCIT. Indicators of lipid metabolism, including free fatty acids (FFA), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), and total cholesterol (TC), were quantified using enzymatic colorimetric methods on a clinical biochemical analyzer. Concurrently, iron metabolism was assessed by measuring serum iron (Fe) levels via atomic absorption spectrometry and serum ferritin concentrations using an enzyme-linked immunosorbent assay (ELISA).

3. Imaging Evaluation (MRI)

3.1 Image Acquisition

All patients underwent pre-treatment (within one week prior to nCIT) and post- nCIT (within one week prior to definitive surgery) magnetic resonance imaging (MRI) examinations of the head and neck using a 3.0T clinical MRI scanner (e.g., Siemens Skyra, Philips Ingenia, or GE Discovery). The standardized imaging protocol included: **Anatomical Sequences:** Axial T2-weighted fast spin-echo sequence with fat suppression; Axial T1-weighted spin-echo sequence before and after intravenous administration of a gadolinium-based contrast agent.

3.2 Image Analysis and Tumor Delineation

Primary tumors and metastatic CLNs were identified and delineated on the pre- and post-treatment MRI scans by two experienced head and neck radiologists in consensus, who were blinded to the pathological response outcomes. Volumes of interest (VOIs) were manually contoured slice-by-slice on the axial T2-weighted images. For each patient, the following regions were segmented: **Primary Tumor:** The entire visible tumor volume in the oropharynx or hypopharynx. **Metastatic CLNs:** The largest and most representative metastatic node, or a composite volume of multiple nodes in cases of confluent disease.

These VOIs were then co-registered and propagated to the quantitative T1 and T2* maps using the scanner's proprietary software or a dedicated image analysis platform (e.g., 3D Slicer). Rigid registration was applied to correct for minor patient motion between sequences.

3.3 Quantitative Parameter Extraction

For each VOI (primary tumor and CLN), the mean native T1 and T2* relaxation values (in milliseconds) were extracted from the respective parametric maps on both pre- and post-treatment scans. The relative change (Δ) in each parameter was calculated as: $\Delta\text{Parameter} = (\text{Post-treatment value} - \text{Pre-treatment value}) / \text{Pre-treatment value}$. Patients were subsequently grouped based on the pathological assessment of the surgical specimen into PCR and non-PCR groups for statistical comparison.

4. Transcriptomic and metabolic sequencing

4.1 Sample Collection and Preparation

Matched biopsy (pre-treatment) and surgical resection (post-treatment) specimens from the 23-patient were immediately processed following collection. Each tissue sample was divided into three aliquots: snap-frozen in liquid nitrogen for RNA and metabolite extraction, fixed in formalin for pathological assessment (IHC), and embedded in OCT compound for immunofluorescence (IF) analysis. Only samples with a tumor cell content greater than 60%, as confirmed by a dedicated pathologist on a hematoxylin and eosin (H&E)-stained section, were used for sequencing. Paired pre- and post-treatment surgical specimens were analyzed. The primary analysis focused on the longitudinal change between

these two time points. In PCR patients, post-treatment specimens contain tumor bed tissue, and the detected signals reflect treatment-related tissue remodeling and immune activity.

4.2 RNA Sequencing and Transcriptomic Analysis

RNA Extraction and Library Preparation: Total RNA was extracted from snap-frozen tissues using a commercial kit (Qiagen RNeasy Mini Kit), according to the manufacturer's instructions. RNA integrity was verified using an Agilent 2100 Bioanalyzer, and only samples with an RNA Integrity Number (RIN) > 7.0. Strand-specific RNA-seq libraries were prepared using a standardized kit (Illumina TruSeq Stranded mRNA Kit) and sequenced on an Illumina NovaSeq 6000 platform to generate 150 bp paired-end reads.

4.3 Metabolomic Profiling and Analysis

Metabolite Extraction: Metabolites were extracted from ~20 mg of snap-frozen tissue using a methanol:acetonitrile:water (2:2:1, v/v) extraction solvent. The extracts were vortexed, incubated at -20°C, and centrifuged to remove proteins. The supernatant was collected and dried under vacuum. The dried metabolites were reconstituted in an appropriate solvent for mass spectrometry analysis.

LC-MS Analysis: Metabolite separation was achieved using a reversed-phase UHPLC system (Thermo Scientific Vanquish) with a C18 column (e.g., ACQUITY UPLC BEH C18). The mobile phase consisted of water and acetonitrile, both with 0.1% formic acid, using gradient elution, and metabolite profiling was performed using a high-resolution mass spectrometer (Thermo Q-Exactive HF-X) operating in both positive and negative electrospray ionization (ESI) modes. Data were acquired in full scan and data-dependent MS/MS (dd-MS²) modes.

4.4 Mass Spectrometry-Based Proteomics

Tissue samples were homogenized in lysis buffer (8 M Urea and 100 mM Tris-HCl, pH 8.0) supplemented with protease and phosphatase inhibitors. Proteins were reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAA), and digested with trypsin (enzyme-to-protein ratio 1:50) overnight at 37°C. The resulting peptides were desalted using C18 solid-phase extraction (SPE) cartridges and dried under a vacuum.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using a Q-Exactive HF-X mass spectrometer coupled to an EASY-nLC 1200 system (Thermo Fisher Scientific). The peptides were separated on a reverse-phase C18 column using a gradient of acetonitrile in 0.1% formic acid. The mass spectrometer was operated in data-dependent acquisition (DDA) mode, with a full MS scan (350-1500 m/z), followed by high-energy collisional dissociation (HCD) of the top 20 most intense ions.

Raw data were processed using the MaxQuant software against the human UniProt database. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation and N-

terminal acetylation were set as variable modifications. Label-free quantification (LFQ) was performed and proteins were identified with a false discovery rate (FDR) of <1%.

5. Immunohistochemistry (IHC) and Immunofluorescence (IF)

The pathological tumor response (pTR) was quantitatively assessed using post-treatment surgical or biopsy specimens based on the proportion of the tumor area occupied by treatment-induced histological features, including tumor necrosis, keratin debris, and multinucleated giant cell/histiocytic infiltrate.

Results

1. Serum metabolite profile reveals evaluated triglycerides and iron in primary tumor

Firstly, 116 patients receiving nCIT was retrospectively analyzed for serum metabolite iron and fatty profile (Free Fatty Acids, HDL, LDL, Triglycerides, Total Cholesterol, Serum Iron, Ferritin) in primary tumor and CLN. Mann-Whitney U tests revealed no pre-nCIT biomarkers significantly differed between groups, but some significant associations between primary tumor response and specific post-nCIT blood biomarkers (**Table 3**). In post-nCIT, serum iron and triglyceride levels are significantly higher in the PCR group compared to the non-PCR group ($p=0.0252$; $p = 0.013$) (**Figure 1A, B**).

However, no statistically significant differences were found for any blood biomarkers or iron markers—either pre- or post-treatment—between the PCR and non-PCR groups in CLN (**Table 4**). To investigate the interrelationships between iron metabolism markers (Fe, Ferritin) and lipid metabolism markers (free fatty acids, HDL, LDL, total cholesterol, triglycerides), correlation analyses were performed. The results are summarized in **Figure 1C**. Although weak, serum iron showed positive correlations with free fatty acids ($p = 0.0467$) and total cholesterol ($p = 0.0466$), but no significant correlations with other lipid parameters (HDL, LDL, triglycerides). Ferritin exhibited no significant correlation with any lipid parameter (all $p > 0.05$). These findings indicate that at the systemic level, iron markers (Fe, ferritin) are largely independent of lipid metabolism.

To investigate the distribution of this parameter across different primary tumor and lymph node response statuses, patients were divided into four groups: Group A (PCR+LPCR), Group B (PCR+non-LPCR), Group C (non-PCR+LPCR), and Group D (non-PCR+non-LPCR). Pairwise comparisons were performed using the Mann-Whitney U test (**Supplementary Table 1-2**). Statistical differences were observed between Group A and Group C ($p = 0.038$), Group A and Group D ($p = 0.021$), and Group B and Group C ($p = 0.045$). In contrast, no significant differences were found between Group A and Group B ($p = 0.842$) or between Group C and Group D ($p = 0.891$). The comparison between Group B and Group D showed borderline significance ($p = 0.067$). These findings indicate that the differences in this parameter are primarily driven by primary tumor response status (PCR vs non-PCR) rather than lymph node response status (LPCR vs non-LPCR). Thus, this parameter effectively discriminates primary tumor responders from non-responders.

These findings suggest that patients achieving PCR may exhibit a distinct metabolic profile characterized by elevated triglycerides and iron. However, the correlations between serum iron markers (Fe, Ferritin) and lipid parameters are generally weak. These findings suggest that iron and lipid metabolism are relatively independent at the serum level.

2. Quantitative MRI Captures Primary Tumor-Specific Tissue Damage

To investigate whether it is a tumor-localized phenomenon, quantitative MRI parameters, particularly T1 and T2* mapping, are sensitive to microstructural and metabolic alterations within the tumor microenvironment. T1 relaxation time is primarily influenced by tissue water content, cellularity, and the presence of edema or necrosis, while T2* relaxation is highly susceptible to local magnetic field inhomogeneities caused by paramagnetic substances, such as deoxyhemoglobin and iron deposition[7-9].

To substantiate the occurrence of heightened redox reactions in patients achieving PCR, we utilized contrast-enhanced neck MRI to visualize differential treatment-induced responses in primary tumors and metastatic cervical lymph nodes in 77 patients (**Figure 1D**). Marked morphological changes and signal abnormalities, including volume reduction, structural obliteration, necrosis, and decreased contrast uptake, were observed on imaging. These primary tumors and lymph nodes were surgically resected, and post-treatment responses were determined based on pathological evaluation. Regions of interest encompassing the primary tumors and cervical lymph nodes were delineated to calculate relative signal intensities. Quantitative MRI analysis revealed that in primary tumors, patients achieving PCR exhibited significantly higher T1 and T2* values compared to those with non-PCR. Specifically, both T1 and T2* values were significantly elevated in the PCR group relative to the non-PCR group ($p = 0.017$ and $p = 0.015$, respectively) (**Figure 1E**). In contrast, T1 and T2* values involving lymph node MRI parameters were not statistically different. The increase in T2* likely reflects pronounced treatment-related tissue injury and inflammation, while the prolonged T1 relaxation time supports the presence of tissue edema, disruption of membrane integrity, and ongoing inflammatory processes. Collectively, these changes were captured by MRI as markedly elevated T1 and T2* signals.

To explore whether the observed serum profiles could be associated with underlying tissue-level changes, we performed correlation analyses between quantitative MRI parameters and the serum biomarkers (Fe²⁺, ferritin, and triglycerides) in patients with available paired data (**Table 5**). Stratified analysis revealed that these correlations were predominantly driven by the PCR group, in which post-treatment primary tumor MRI T2* relaxation values correlated with Fe²⁺ ($R = 0.27$, $p = 0.0491$), while MRI T1 relaxation values correlated with triglycerides (T1, $R = 0.29$, $p = 0.039$), whereas no significant correlations were observed in the non-PCR group (**Figure 1F, G, Supplementary Table 3**). Correlations involving lymph node MRI parameters were not statistically different (**Supplementary Table 4**). Nevertheless, the p-values for T2* with Fe²⁺ ($p = 0.0491$) and T1 with triglycerides ($p = 0.039$) are modest, their consistency with the hypothesized biological pathway—linking redox activity to imaging changes—supports their interpretative value. These findings indicate that MRI-detected tissue damage in

responding primary tumors not lymph nodes is closely linked to systemic metabolic changes—particularly iron release and lipid mobilization.

3. Integrated multi-Omics analysis

Given the observed discordance in serum metabolite profile and quantitative MRI parameters between primary tumors and metastatic lymph nodes following nCIT, we next sought to investigate the underlying molecular basis for these differential treatment responses. To dissect the molecular mechanisms driving these differential responses and to identify potential biomarkers that distinguish PCR from non-PCR tumors, we performed multi-sequencing of pre-nCIT biopsy and post-nCIT surgical specimens from 23 LA OPSCC patients. Ten patients achieved PCR, while 13 patients did not.

3.1 Transcriptomics

We began identifying genes with significantly altered expression levels ($p < 0.05$) between PCR (N=10) and non-PCR (N=13). Principal component analysis (PCA) shows significantly altered gene profiles over the course of nCIT and over the PCR and non-PCR groups (**Figure 2A**). Using the Gene Ontology (GO) database, all significantly altered genes were categorized according to their major biological processes. These genes were consolidated into three core biological process categories: immune response, metabolism, oxidative processes, and cell proliferation (**Figure 2B**). Based on GO pathway enrichment analysis, the genes associated with achieving PCR were strongly linked to fundamental cellular processes involving mitochondrial function, ion transport (especially sodium and iron), and lipid metabolism (**Figure 2C**). This suggests that nCIT is closely tied to the metabolic and transport activities of cancer cells, potentially influencing cell death mechanisms, such as ferroptosis. Enriched Terms in Transmembrane Transporter Activity included GO:0034220 | ion transmembrane transport, GO:0022857 | transmembrane transporter activity, GO:0035725 | sodium ion transmembrane transport, and GO:0015670 | carbon dioxide transport. Enriched Terms in Iron Ion Response and Lipid Binding, including GO:0010039 | response to iron ions and GO:0008289 | lipid binding. These findings highlight the importance of the mitochondrial and ion transport pathways in mediating PCR. KEGG pathway enrichment analysis revealed significant enrichment in the PCR group, which remodeled the lipid metabolic landscape, particularly glycerophospholipid metabolism, thereby altering the membrane fluidity (**Figure 2D**). The activation of transmembrane transporters potentially influences intracellular drug concentrations and antigen presentation efficiency. The GSEA results revealed a significant enrichment in the "negative regulation of lipid catabolic process" ($p = 0.0019$), characterized by a pronounced negative enrichment score (**Figure 2E**).

This finding indicates that lipid catabolic processes are markedly suppressed in patients who respond effectively to nCIT. These results suggest that targeting iron transport and lipid peroxidation could synergize with nCIT to improve the response rates in LA OPSCC. Those results lead us to conduct an in-depth analysis, specifically focusing on the "iron and lipid metabolism-related gene cluster. The analysis revealed ten intersecting KEGG pathways (**Figure 2F**), including oxaloacetate and dicarboxylate metabolism, chemical carcinogenesis, reactive oxygen species, ferroptosis, glycerophospholipid

metabolism, and oxidative phosphorylation. Analysis of the genes within these intersecting pathways identified several genes involved in iron ion transport, including FTH1, TFRC, STEAP3, ATP6V0D2, CYCS, and SLC5A6 etc.. Further investigation of these proteins was conducted to elucidate the mechanism by which nCIT promoted iron-dependent lipid peroxidation in LA OPSCC.

3.2 Metabolomics

Twelve patients with high-quality tissue specimens (6 PCR vs. 6 non-PCR) were selected for metabolomics analysis. PCA score plots derived from unsupervised clustering demonstrated clear separation between the two groups, with PCR and non-PCR samples clustering independently, indicating distinct pretreatment metabolic landscapes between responders and non-responders (**Figure 3A**). The PCA1 and PCA2 explained 74.41% and 72.41% of the total variance, respectively, indicating a clear separation between PCR and non-PCR groups. The PCR cohort possessed 1,166 distinct differential metabolites compared to the non-PCR cohort. A total of 1,166 distinct differential metabolites were identified between the PCR and non-PCR cohorts. The pie chart shows the distribution of these metabolites across superclasses: Lipids and lipid-like molecules accounted for the largest proportion (36.28%), followed by Organoheterocyclic compounds (20.22%), Organic acids and derivatives (13.45%), Benzoic acids and derivatives (13.45%), Organic oxygen compounds (5.69%), and Nucleosides, nucleotides, and analogues (4.15%). This highlights that lipid-related metabolic alterations are the predominant feature distinguishing responders from non-responders (**Figure 3B**). Lipid-related metabolic molecules are significantly upregulated in PCR group (**Figure 3C**). A bar chart further illustrating the proportion of each metabolite superclass, with lipids and lipid-like molecules being the most abundant category.

Heatmap showed 18 metabolites that significantly differed between PCR and non-PCR groups. These metabolites reflect alterations in phospholipid, fatty acid, and energy metabolism (**Figure 3D**): patients with PCR exhibit elevated polyunsaturated phosphatidylcholines (PUFA-PCs), particularly phosphatidylcholine (PC) (18:1/22:6), enhancing membrane fluidity and facilitating immune synapse formation[10,11]. Increased lysophosphatidylcholine (LysoPC (22:6/0:0)) suggests enhanced phospholipase A2 activity, generating lipid mediators that promote T cell recruitment and activation[12]. The redox cofactor NAD and its ratio reflect the altered cellular redox state in the responders. Elevated antioxidant flavanones in non-responders suggest compensatory mechanisms against therapy-induced oxidative stress[13]. Enrichment of PUFA-containing phospholipids in patients with PCR creates a permissive environment for lipid peroxidation and subsequent ferroptosis. Shifts in dihydroxyacetone phosphate levels indicate glycolytic flux alterations and glycerolipid synthesis adaptations. Accumulation of adipic acid, a fatty acid ω -oxidation product, suggests mitochondrial metabolic rewiring in treatment-responsive tumors[14].

A hierarchical or circular plot showed, the PCR cohort revealed that lipid metabolism and membrane remodeling constituted the most prominent modules, a finding consistent with our prior analyses (**Figure 3E, Suppl. seq analysis data**). The key lipid species, PC (18:1/22:6), LysoPC (22:6/0:0), and DG

(2:0/PGJ2/0:0), exhibited strong correlations, indicating coordinated metabolic reprogramming critical for treatment efficacy [15]. The robust association between PC and LysoPC signifies the treatment-induced vigorous remodeling of membrane phospholipids. Concurrently, the correlation of the second messenger DG with the PC/LysoPC axis forms a complete signaling cascade, linking structural changes in the plasma membrane to intracellular signal transduction. Collectively, this coordinated lipid module establishes a foundational metabolic state that simultaneously primes the tumor microenvironment for robust immune activation and sensitizes cancer cells to ferroptosis, driven by the peroxidation of PUFA-rich phospholipids such as PC (18:1/22:6).

A chord diagram linking differential metabolites to their respective chemical subclasses (**Figure 3F**), including glycerophospholipids, fatty acids, flavonoids, cinchona alkaloids, pyridines, benzothiophenes, sterols, and carboxylic acids. Glycerophospholipids and fatty acids are among the most frequently represented subclasses, which indicates a significant shift in their profile in the PCR cohort strongly indicated active membrane remodeling.

The ROC curve indicated that these metabolites (e.g., LysoPC (22:6) linked to lipid peroxidation) serve as robust biomarkers, indicating that PCR treatment induces significant oxidative stress and metabolic dysregulation (**Figure 3G**), with an AUC closer to 1 indicating a better classifier. The GSEA also results revealed a significant enrichment in the "Glycerolipids metabolism" term (Normalized Enrichment Score [ES] = 0.9186, $p = 0.0018$, FDR = 0.0986), characterized by a pronounced negative enrichment score (**Figure 3H**). Therefore, this provides a mechanistic explanation that glycerophospholipid remodeling underlies the successful eradication of tumors in patients approaching PCR.

3.3 Targeted Lipidomics

To directly quantify therapy-induced alterations in oxidized lipids and polyunsaturated fatty acid (PUFA)-containing phospholipids—we performed targeted oxylipidomics analysis using LC-MS/MS on paired pre- and post-treatment tumor tissues from PCR ($n=10$) and non-PCR ($n=13$) patients. PCR tumors exhibit significant accumulation of peroxidation-susceptible PUFA-phospholipids. As shown, a total of 28 lipid species were differentially expressed between PCR and non-PCR groups (**Figure 4A**). Notably, multiple phosphatidylserine (PS), phosphatidylglycerol (PG), and PC species are upregulated in PCR patients. Membrane contact site (MCS) ratio showed key 12 lipids were significantly upregulated ($\text{Log}_2\text{FC} > 1.5$, $p < 0.05$) in the PCR group, while 16 lipids ($\text{Log}_2\text{FC} < -1.7$, $p < 0.05$) were downregulated (**Figure 4B**). Key upregulated lipids in PCR tumors include: PC (14:0_18:2) and PC (18:2_16:1): Phosphatidylcholine species containing linoleic acid (18:2, a PUFA). These represent the most abundant membrane phospholipids and serve as primary substrates for lipid peroxidation. Their accumulation creates a membrane environment "primed" for ferroptosis execution. PE-AA-OOH and PE-AdA-OOH (phosphatidylethanolamine hydroperoxides): These oxidized PE species represent the direct molecular executors of ferroptosis. Phosphatidylethanolamine (PE) is the preferred phospholipid class for ferroptotic lipid peroxidation due to its high content of arachidonic acid (AA, 20:4) and adrenic acid (AdA, 22:4). Their significant accumulation in PCR tumors indicates the ongoing ferroptosis execution *in situ*.

PS (16:0_22:6): Phosphatidylserine containing docosahexaenoic acid (DHA, 22:6), one of the most oxidation-sensitive PUFAs in cellular membranes. DHA-containing phospholipids are considered "super-substrates" for ferroptotic lipid peroxidation due to their high bis-allylic hydrogen content. The PCR group exhibited significant downregulation of LPE (20:0) and LPI (18:1), both lysophospholipid species. Reduced lysophospholipid levels may reflect altered membrane phospholipid catabolism or remodeling under lipid peroxidation stress, potentially representing a compensatory response to extensive oxidative membrane damage.

Cells regulate lipid chain length to maintain membrane fluidity homeostasis (homeoviscous adaptation) [16]. Highly aggressive tumors often upregulate the synthesis of monounsaturated fatty acids (MUFAs) and long-chain fatty acids (LCFAs) to preserve membrane integrity while resisting external stressors such as chemotherapy and immune attack[17]. Through glycerophospholipid remodeling, particularly a shift toward enrichment in long-chain polyunsaturated fatty acids (LC-PUFAs), the tumor cell membrane becomes highly susceptible to chemotherapy-induced oxidative stress, thereby facilitating tumor eradication.

Analysis of lipid chain length and unsaturation revealed distinct remodeling patterns of triglycerides (TGs), phosphatidylcholines (PCs), and plasmalogen-phosphatidylethanolamines (PE-Ps), phosphatidylserine (PS), Lysophospholipids (LPC, LPE, LPS, LPI) (**Figure 4C**). In the PCR group, TG abundance decreased progressively with increasing carbon chain length from C41 to C49 (from 1000 to 200 arbitrary units), suggesting selective consumption or reduced synthesis of very-long-chain triglycerides in responding tumors. This pattern is consistent with a shift away from energy storage (lipid droplet formation) toward membrane-destructive pathways.

The PCR group exhibited consistently higher levels of PC across most detected chain lengths (C28–C40) compared to the non-PCR group, with the most pronounced differences observed at C30 (10.7-fold), C34 (1.9-fold), and C36 (1.6-fold). Even more striking, PS levels were dramatically increased in the PCR group, particularly at C36 (340 vs. 60 in non-PCR, 5.7-fold) and C38 (120 vs. 40, 3-fold). Confirming and extending our previous observations, PE-P levels were uniformly and substantially higher in the PCR group across all chain lengths (C32–C40), with the largest differences at C36 (100 vs. 30, 3.3-fold) and C38 (60 vs. 10, 6-fold). PE-P possesses dual functionality: antioxidant defense (via its vinyl-ether bond) and promotion of non-lamellar membrane structures. Its elevation serves both as a compensatory response to treatment-induced oxidative stress and as a direct contributor to increased mitochondrial membrane permeability. Importantly, PE-P species containing polyunsaturated fatty acid (PUFA) chains are preferred substrates for lipid peroxidation. Their marked accumulation in PCR tumors renders cells exquisitely sensitive to ferroptosis, a regulated cell death pathway driven by iron-dependent phospholipid peroxidation.

Lysophospholipids (LPC, LPE, LPS, LPI) show class-specific patterns. LPC (C15–C18) and LPE (C18, C20) were both elevated in the PCR group (e.g., LPE C20: 82 vs. 21 in non-PCR, 3.9-fold), indicating accelerated membrane remodeling and turnover, as well as potential immunomodulatory effects via

G-protein-coupled receptor signaling. In contrast, LPS C18 was uniquely higher in the non-PCR group (21.0 vs. 14.0 in PCR, 0.67-fold). PI levels were modestly elevated in the PCR group at C36–C40, suggesting enhanced phosphoinositide signaling (IP₃/DAG pathway) in responders.

This lipidomic signature renders tumor cells susceptible to ferroptosis—via PE-P as a lipid peroxidation substrate—and enhances apoptotic clearance via PS externalization. Integrating lipidomics, metabolomics, and transcriptomics data reveals that pathological complete response to neoadjuvant immunochemotherapy is driven by a coordinated reprogramming of tumor lipid metabolism (**Figure 4D**).

3.3 Integration of Proteomics with Existing Multi-Omics Data

Having established a distinct lipidomic and metabolomic signature associated with PCR, we next performed proteomic profiling to identify the upstream regulatory proteins and downstream effectors governing this metabolic reprogramming.

Among significant 1445 quantifiable proteins (**Suppl. seq analysis data**), Reactome pathway enrichment analysis revealed that differentially expressed proteins were most highly enriched in iron and lipid metabolism-related pathways (**Figure 5B**). Pathways related to lipid metabolism (e.g., *Metabolism of lipids*, *Fatty acid metabolism*, *Phospholipid metabolism*), peroxisomal function, and iron uptake (*Iron uptake and transport*) are prominently enriched in the PCR group.

We then analyzed the relative RNA and protein level correlation (**Suppl. seq analysis data**). 15 showed significant differential abundance between the two groups (fold change > 1.5 or < 0.67, $p < 0.05$) (**Figure 5C**). Specifically, the PCR group exhibited significantly elevated protein and RNA levels of **GPAT4**, **SELENOI**, **CDS2**, **DEGS1**, and **LPCAT1** (all ↑, **Figure 5B**). Among these, **SELENOI** (ethanolamine phosphotransferase 1, also known as EPT1) is of particular mechanistic importance. SELENOI catalyzes the final step of PE and PE-P synthesis via the Kennedy pathway. Its 5.3-fold upregulation in responders provides a direct enzymatic explanation for our lipidomic finding of markedly elevated PE-P species (particularly C36 and C38, 3- to 6-fold higher than non-PCR). Given that PUFA-containing PE-P species are preferred substrates for iron-dependent lipid peroxidation, SELENOI-driven PE-P accumulation directly primes responder tumor cells for ferroptosis. Concurrently, the upregulation of **LPCAT1** (lysophosphatidylcholine acyltransferase 1) facilitates the incorporation of PUFAs into phosphatidylcholine (PC), the most abundant membrane phospholipid. This not only explains the elevated PC levels observed in our lipidomics but also increases the overall membrane PUFA content, thereby expanding the substrate pool for lipoxygenase-mediated lipid peroxidation. The coordinated upregulation of **GPAT4**, **CDS2**, and **DEGS1** further supports a global reprogramming of glycerolipid and sphingolipid metabolism toward membrane remodeling and turnover.

Beyond lipid metabolism, we observed increased protein levels of pro-apoptotic factor **BAD** and NF-κB pathway components **TRAF2** and **IRAK1** in the PCR group. This suggests that, in addition to ferroptosis, apoptotic signaling and inflammatory responses may concurrently be activated in responders. Conversely, the mixed regulation of lipid signaling proteins **SGPP1** and **PLPP2**, alongside oxidative stress

modulators **PAFAH1B2** (↓) and **NCF2** (↑), may reflect fine-tuning of pro-survival and pro-death signals within the tumor microenvironment.

Collectively, these proteomic findings bridge our transcriptomic observations and lipidomic phenotypes, establishing a causal chain from enzymatic upregulation (SELENOI, LPCAT1) to lipid substrate accumulation (PE-P, PC-PUFA) to execution of cell death (ferroptosis and apoptosis), ultimately culminating in pathological complete response. Notably, 40% (6/15) of differentially abundant proteins exhibited discordant directionality between mRNA and protein levels, suggesting that post-transcriptional regulation plays a significant role in shaping the responder lipidome. The hypothesized protein-level changes corresponding to key lipidomic findings are summarized in **Figure 5D**.

To explore the functional relationship between the core enzyme driving PE-P synthesis and key regulators of iron metabolism and ferroptosis, we performed spearman correlation analysis using protein expression data. Strikingly, SELENOI showed a strong positive correlation with TFRC (transferrin receptor 1, $\rho = 0.951$, $p < 0.0001$) (**Figure 5E**). SELENOI also exhibited strong positive correlations with ELOVL7 ($\rho = 0.881$, $p = 0.00019$), a fatty acid elongase involved in PUFA synthesis, and with DEGS1 ($\rho = 0.874$, $p = 0.00031$), a sphingolipid desaturase that modulates membrane fluidity. Additionally, SELENOI correlated positively with iron transport-related proteins, including SLC25A28 (mitochondrial iron transporter, $\rho = 0.832$, $p = 0.0014$) and HSCB (iron-sulfur cluster assembly, $\rho = 0.748$, $p = 0.0074$) (**Suppl. seq analysis data**). In contrast, SELENOI showed no significant correlation with GPX4 ($\rho = 0.301$, $p = 0.342$), the central ferroptosis defense enzyme, nor with FTH1 ($\rho = -0.329$, $p = 0.297$), the iron storage protein.

The strongest positive correlation between SELENOI and TFRC provides a mechanistic link between phospholipid remodeling and iron metabolism in responders (**Figure 5F**). Protein expression of SELENOI and TFRC also are significantly evaluated in PCR group compared with non-PCR group. TFRC is the rate-limiting gateway for cellular iron import and a well-established pro-ferroptotic factor. Its coordinate upregulation with SELENOI suggests that responder tumors simultaneously increase both the **substrate** (PUFA-PE) and the **cofactor** (iron) required for lethal lipid peroxidation. This dual priming mechanism—increasing both the fuel and the catalyst—may explain the particularly high ferroptosis vulnerability of PCR tumors. These findings suggest that SELENOI promotes ferroptosis susceptibility primarily through enhancing substrate availability of PE-P synthesis and iron uptake.

2. Tumor tissue molecular markers confirm ferroptosis execution

Immunofluorescence analysis revealed additional insights into the dynamics of iron and lipid metabolism. The intense 4-HNE signal colocalized with the cytoplasmic region, indicating widespread lipid peroxidation, a hallmark of ferroptosis, was significantly increased in the PCR group. Additionally, intense 4-HNE signals co-localized with the cytoplasmic region where the FTH1 signal was diminished (**Figure 6A**), SELENOI and TFRC exhibited synchronized distinct subcellular localization in this group (**Figure 6B, C**), providing spatial insight into the cooperative mechanism driving ferroptosis in responders. TFRC predominantly localized to the plasma membrane and perinuclear vesicles, reflecting

its canonical role in iron uptake and recycling. Notably, as it previously reported, the convergence of SELENOI-derived lipid substrates and TFRC-supplied iron likely occurs at the interface of the ER and mitochondria, the primary sites of lipid peroxidation[18]. The strong positive correlation between their expression levels, coupled with their distinct yet complementary spatial distribution, suggests a functional compartmentalization: SELENOI remodels the membrane environment to generate peroxidation-prone phospholipids, while TFRC delivers the essential iron cofactor.

This spatial coordination amplifies local oxidative stress, lowering the threshold for ferroptotic cell death in PCR patients. Furthermore, strengthening our findings, we detected a significant increase in the level of malondialdehyde (MDA), another well-established end-product of lipid peroxidation, in the PCR group compared to that in the non-PCR group (**Figure 5D**). In coincidence with MDA, Phen Green stain and C11-BODIPY stain showed increased iron and lipid ROS (**Figure 5E, F**). The mechanistic connection between SELENOI upregulation and TFRC co-upregulation remains to be elucidated; whether SELENOI directly regulates TFRC expression or whether both are co-regulated by a common upstream transcription factor (e.g., HIF-1 α , NRF2, or IRP/IRE system) warrants further investigation.

Discussion

In this study, we employed an integrated multi-omics approach to investigate the molecular determinants of pathological complete response to neoadjuvant chemoimmunotherapy in locally advanced oropharyngeal and hypopharyngeal squamous cell carcinoma. Our findings converge on a coherent and novel model: effective nCIT response is driven by an iron-centric, lipid metabolic reprogramming.

The convergence of our transcriptomic, metabolomic, proteomic, targeted lipidomic and immunohistochemical described the metabolomic landscape of PCR tumors—characterized by accumulation of peroxidation-susceptible PUFA-containing phospholipids (particularly PE species) and elevated lysophosphatidylcholines—creates a membrane environment ripe for oxidative demolition [19-23].

Multi-omics analysis was performed in a step-by-step manner. Firstly, we found the primary tumor-specific metabolic associations. Elevated post-treatment serum iron and triglycerides, as well as MRI-detected T1/T2* prolongation, are associated with PCR in primary tumors but not in metastatic lymph nodes. This suggests that iron-lipid metabolic coupling may be a localized, site-specific event. Second, we found the lipid remodeling is a prominent feature of the PCR-associated multi-omics signature. Metabolomics and lipidomics consistently show that lipids and lipid-like molecules constitute the largest class of differential metabolites (36.28%). PCR tumors exhibit marked upregulation of PE-P (C36/C38, up to 6-fold), PS (C36, 5.7-fold), and PC, alongside reduced long-chain triglycerides and LPS C18. Proteomics identified SELENOI (an enzyme in PE-P synthesis) as upregulated 5.3-fold in PCR patients. Spearman correlation revealed a strong positive correlation between SELENOI and TFRC ($\rho=0.951$), suggesting a coordinated relationship that may link phospholipid remodeling and iron

metabolism in responders. Third, we found the ferroptosis-associated markers are elevated in PCR tumors: Immunofluorescence showed increased 4-HNE, ferritin, SELENOI, and TFRC, along with elevated MDA and lipid ROS, consistent with the occurrence of ferroptosis-like lipid peroxidation in PCR tumors. In final, multi-omics integration (transcriptomics, metabolomics, lipidomics, proteomics) of nCIT response in LA OPSCC reveals coordinated changes in lipid and iron metabolism that converge on a ferroptosis-associated phenotype. This finding represents, to our knowledge, the first clinical evidence linking specific phospholipid remodeling and iron metabolism to therapeutic response in LA OPSCC.

Importantly, within hypothesis-generating our study indicated several clinical Implications. The first, candidate predictive biomarkers: SELENOI and TFRC protein expression (assessable by immunohistochemistry) emerged from multi-omics analysis as candidate biomarkers associated with PCR. Their potential to predict nCIT response requires prospective validation; The second, MRI as a non-invasive monitoring tool: Quantitative T1 and T2* mapping detected treatment-induced changes selectively in primary tumors. Further studies are needed to validate MRI as an early response biomarker for nCIT; The third, Site-specific metabolic heterogeneity: The discordant findings between primary tumors and lymph nodes suggest that metabolic vulnerabilities may differ by anatomical site, which could inform future biopsy strategies or combination treatment. The fourth, hypothesis for future mechanistic studies: The correlated upregulation of SELENOI and TFRC, along with elevated lipid peroxidation markers, generates the hypothesis that a dual-priming mechanism for ferroptosis may contribute to nCIT response. Functional experiments (e.g., SELENOI overexpression/knockdown, iron chelation, ferroptosis inhibition) are required to test causality.

Several important limitations warrant consideration. First, the primary aim of this exploratory study was to characterize biological differences between responders and non-responders; therefore, patients were not randomized to different immune checkpoint inhibitors (pembrolizumab, tislelizumab, or nivolumab). The small subgroup sizes precluded meaningful comparisons of PCR rates across different ICI agents. Second, analyses based on endpoint samples inherently face challenges in causal inference. The molecular features observed in non-PCR samples may represent the state of surviving tumor cell clusters with acquired resistant phenotypes that emerged under therapeutic pressure, rather than the initial causes of resistance. We cannot entirely exclude the possibility that signals detected in non-PCR samples predominantly originate from resistant cellular subclones that withstood immune attack. Third, our immunohistochemical and immunofluorescence analyses were performed on post-nCIT surgical specimens, which reflect the molecular state of residual tissue comprising mixed cell populations (tumor cells, immune cells, and stromal components). While we observed clear associations between the identified markers and treatment response, multiplex immunofluorescence staining will be required in future studies to precisely localize these proteins to specific cell compartments (e.g., tumor vs. immune cells) and to confirm that the observed signals are not solely consequences of altered tissue composition. Fourth, due to limited availability of pre-treatment biopsy samples, most could not be matched with post-operative samples for rigorous within-patient paired analysis. Consequently, our primary findings should be interpreted as robust correlative biomarkers strongly associated with treatment outcome, rather than definitive causal drivers. To prospectively validate the predictive value of

these pathway features and decipher their causal roles, future studies must systematically collect pre-treatment paired biopsy samples within large prospective cohorts. Finally, in PCR patients, post-treatment surgical specimens contain no viable tumor cells; thus, any positive IF signal must originate from ADH (Atypical dysplasia hypertension) within the tumor microenvironment (tumor bed with treatment-related changes, inflammatory infiltrate, and stromal components). This biologically meaningful signal reflects the cellular composition and activity of the post-treatment tissue bed rather than residual malignancy.

In summary, this integrated multi-omics and immunofluorescence study provides a detailed molecular landscape of pathological complete response to neoadjuvant immunochemotherapy in LA OPSCC. The data reveal that PCR is associated with a coordinated lipid remodeling signature involving SELENOI-associated PE-P enrichment and TFRC-associated iron availability, together with elevated ferroptosis-related markers. These findings generate the hypothesis that a dual-priming ferroptosis mechanism may contribute to treatment response, and they identify SELENOI and TFRC as candidate biomarkers for further investigation. Functional validation studies are now needed to establish causality and explore therapeutic targeting of this pathway.

Conclusion

In this study, we performed integrated serum metabolomics, quantitative MRI, and multi-omics sequencing (transcriptomics, proteomics, metabolomics, and lipidomics) to dissect the molecular basis of discordant treatment responses between primary tumors and metastatic cervical lymph nodes in LA OPSCC patients receiving nCIT. Our multi-omics analyses converged on a coherent mechanistic model: coordinated reprogramming of phospholipid metabolism—centered on SELENOI (EPT1)-driven plasmalogen-PE (PE-P) synthesis—establishes a ferroptosis-prone state in responder tumors, culminating in complete tumor eradication.

Declarations

Ethics approval and consent to participate

Patient consent for publication:

Written informed consent for publication was obtained from all patients included in this study in accordance with the guidelines of the journal and ethical standards. The patients were informed about the nature of the study, including the potential publication of anonymized clinical data.

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Tables

Table 1. All Patient Characteristics and nCIT Overview (n=116)

Variable	Total Cohort (n=116)
Age (years), median [IQR]	58 [49-66]
Primary Tumor Response[†]PTR[‡], n (%)	
PCR	75
Non-PCR	41
CLN Response, n (%)	
LPCR	61
Non-LPCR	55
Tumor/CLN Response	
PCR / PCR	47
PCR / non-PCR	28
non-PCR / PCR	14
non-PCR / non-PCR	27
nCIT Regimen	
TP + K	40
TP + T	32
TP + O	44

Table 2 Clinical and Pathological Characteristics of patient for the sequencing analysis (n=23)

Characteristic	Category	Nummer	Percentage (%)
Sex	Male	22	95.7%
	Female	1	4.3%
Age (years)	≤ 64 (median age)	12	52.2%
	> 64 (median age)	11	47.8%
Tobacco	yes	23	100%
	no	0	0
Alcohol	yes	18	78.3%
	no	5	21.7%
Tumor Location	Oropharynx	12	52.2%
	Hypopharynx	11	47.8%
p16 Status	Positive (P)	13	56.5%
	Negative (N)	5	21.7%
	Not Reported	5	21.7%
Pathological Response (primary tumor)	PCR	10	43.5%
	non-PCR	13	56.5%
CLN	LPCR	15	66.2%
	Non-LPCR	8	34.8%
Treatment Regimen	TP + K	9	39.1%
	TP + T	8	34.8%
	TP + O	6	26.1%
CPS Score (n=12)	CPS < 10	3	25.0%
	CPS ≥ 10	9	75.0%
	Not Reported	11	/
T Stage	T1	1	4.3%
	T2	3	13.0%
	T3	8	34.8%
	T4	11	47.8%
N Stage	N1	6	26.1%

	N2	12	52.2%
	N3	5	21.7%

Abbreviations:

K: Pembrolizumab (Keytruda); **O:** Nivolumab (Opdivo); **T:** Tislelizumab; **TP:** Paclitaxel + Platinum; **TPF:** Docetaxel + Platinum + 5-FU; (L)**PCR:** (Lymph) Pathological Complete Response; **mPR:** Major Pathological Response ; **CLN** : cervical lymph nodes

Table 3. Blood Biomarkers by Primary Tumor Response (PCR vs. Non-PCR) from Cohort 2

Biomarker	Timepoint	PCR Median (N=75)	Non-PCR Median (N=41)	p-value
Free Fatty Acids (μmol/L)	Pre-nCIT	455.0	455.5	0.832
	Post-nCIT	367.0	384.5	0.257
HDL (mmol/L)	Pre-nCIT	1.14	1.11	0.386
	Post-nCIT	1.16	1.07	0.113
LDL (mmol/L)	Pre-nCIT	2.96	2.97	0.966
	Post-nCIT	3.13	2.96	0.069
Triglycerides (mmol/L)	Pre-nCIT	1.42	1.33	0.281
	Post-nCIT	1.58	1.35	0.013
Total Cholesterol (mmol/L)	Pre-nCIT	4.96	4.89	0.474
	Post-nCIT	5.17	4.89	0.054
Serum fe ²⁺ (μmol/L)	Pre-nCIT	8.08	7.87	0.197
	Post-nCIT	7.59	7.16	0.025
Serum ferritin (ng/mL)	Pre-nCIT	314.0	312.0	0.938
	Post-nCIT	507.5	492.5	0.987

*Note: $p < 0.05$

Table 4. Blood Biomarkers by CLN Response (LPCR vs. Non-LPCR) from Cohort 2

Biomarker	Timepoint	LPCR	Non-LPCR	U Statistic	p-value
		N = 52	N =64		
		Median	Median		
Free Fatty Acids mmol/L	Pre-nCIT	462.5	455.0	1298.5	0.756
	Post-nCIT	367.0	376.0	1267.0	0.589
HDL mmol/L	Pre-nCIT	1.16	1.11	1203.5	0.284
	Post-nCIT	1.16	1.07	1156.0	0.156
LDL mmol/L	Pre-nCIT	2.94	3.03	1356.5	0.968
	Post-nCIT	3.13	2.96	1123.5	0.089
Triglycerides mmol/L	Pre-nCIT	1.42	1.33	1245.5	0.423
	Post-nCIT	1.55	1.35	1189.0	0.218
Total Cholesterol mmol/L	Pre-nCIT	4.96	4.89	1289.5	0.674
	Post-nCIT	5.17	4.89	1167.5	0.134
Serum Iron μmol/L	Pre-nCIT	8.08	7.88	156.5	0.345
	Post-nCIT	7.59	7.35	142.0	0.178
Ferritin ng/mL	Pre-nCIT	314.0	314.5	645.5	0.892
	Post-nCIT	522.5	497.0	612.5	0.587

- Iron metabolism: serum iron, serum Ferritin
- Lipid profile: Free Fatty Acids (FFA), High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL), Triglycerides (TG), Total Cholesterol (TC)
Pathological response was assessed post-operatively and categorized as:
- Primary Tumor: PCR vs. non-PCR
- Clinical Lymph Node (CLN): LPCR vs. non-LPCR

Table 5. Correlation Matrix: Available MRI Primary Tumor Values vs Serum Biomarkers from PCR group from Cohort 2 (N = 77)

Time point	Serum Marker	MRI Parameter	r (Spearman)	P value
Pre-nCIT	Fe ²⁺	Primary tumor T1	-0.220	0.478
		Lymph node T1	-0.086	0.581
		Primary tumor T2*	-0.102	0.531
		Lymph node T2*	0.014	0.928
	Ferritin	Primary tumor T1	-0.171	0.179
		Lymph node T1	0.015	0.924
		Primary tumor T2*	-0.011	0.946
		Lymph node T2*	0.079	0.619
	Triglycerides	Primary tumor T1	0.29	0.039*
		Lymph node T1	0.295	0.055
		Primary tumor T2*	0.101	0.529
		Lymph node T2*	-0.158	0.317
Post-nCIT	Fe ²⁺	Primary tumor T1	-0.060	0.707
		Lymph node T1	0.080	0.613
		Primary tumor T2*	0.27	0.0491*
		Lymph node T2*	-0.041	0.794
	Ferritin	Primary tumor T1	0.301	0.049
		Lymph node T1	0.177	0.262
		Primary tumor T2*	-0.129	0.408
		Lymph node T2*	-0.028	0.860
	Triglycerides	Primary tumor T1	0.115	0.463
		Lymph node T1	0.164	0.300
		Primary tumor T2*	-0.054	0.731
		Lymph node T2*	-0.082	0.607

Figures

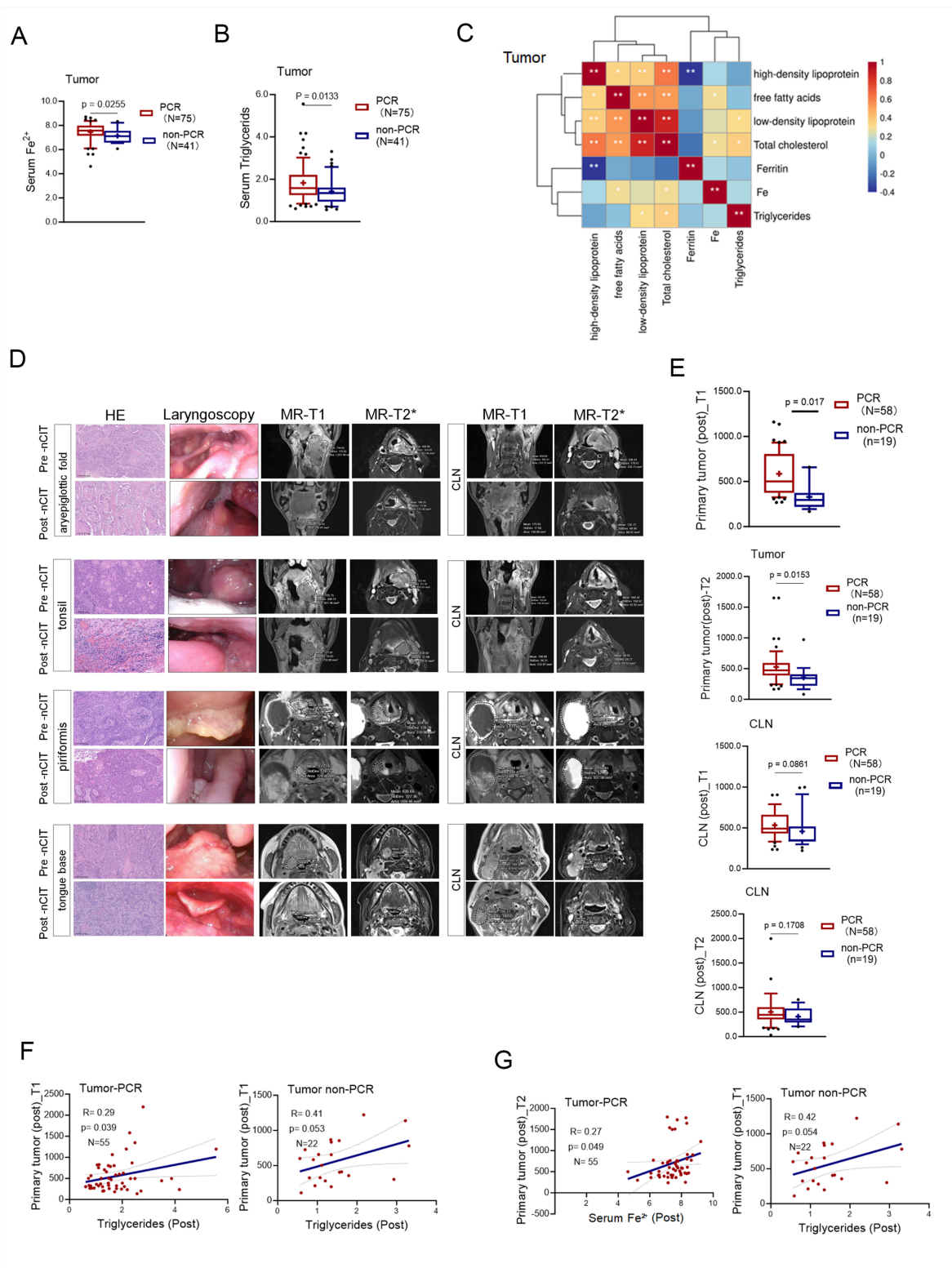


Figure 1

Serum metabolic profiling, quantitative MRI, and correlation analyses reveal primary tumor-specific iron-lipid metabolic coupling associated with PCR response following nCIT

A-B. Post-treatment serum iron (A) and triglyceride (B) levels in patients with and without pathological complete response (PCR) in primary tumors. Data are presented as mean $\pm$ SD (PCR n=75, non-PCR

n=41).

C. Correlation matrix of serum biomarkers in patients receiving nCIT (n=116). The heatmap displays pairwise correlation p-values for seven serum metabolites/ biomarkers: iron (Fe), ferritin, free fatty acids (FFA), high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (TC), and triglycerides (TG). Each cell represents the p-value from Spearman correlation analysis. The color gradient indicates significance levels: red shades denote $p < 0.05$ (statistically significant), while blue/white shades denote $p \geq 0.05$ (non-significant).

D. T1 and T2-weighted MRI images of three patients with head and neck squamous cell carcinoma before (top) and after (bottom) two cycles of nCIT. From top to bottom:

Primary lesions were located at the left aryepiglottic fold, with metastatic lymph nodes in right cervical levels II-IV. After treatment, the primary lesions showed PCR, while the metastatic cervical lymph nodes achieved PCR.

Primary lesions were located at the left tongue base, with metastatic lymph nodes in right cervical levels II-IV. After treatment, the primary lesions showed non-PCR, while the metastatic cervical lymph nodes achieved PCR.

The primary lesion was located in the right pyriform sinus, with metastatic lymph nodes in right cervical levels II-IV. After treatment, both the primary lesion and cervical lymph nodes showed non-PCR.
The primary lesion was located at the right tongue base, with metastatic lymph nodes in right cervical levels I-III. After treatment, the primary lesions achieved PCR, while the metastatic cervical lymph nodes showed non-PCR.

E. Quantitative MRI parameter analysis for patients achieving PCR versus non-PCR in primary tumors and cervical lymph nodes. Box plots show differences in signal values on T1 mapping and T2* mapping sequences between the groups.

F. Correlation analysis between triglycerides and serum iron levels in patients with primary tumors achieving PCR or non-PCR.

G. A significant positive correlation was exclusively observed in the PCR group (n = 55).

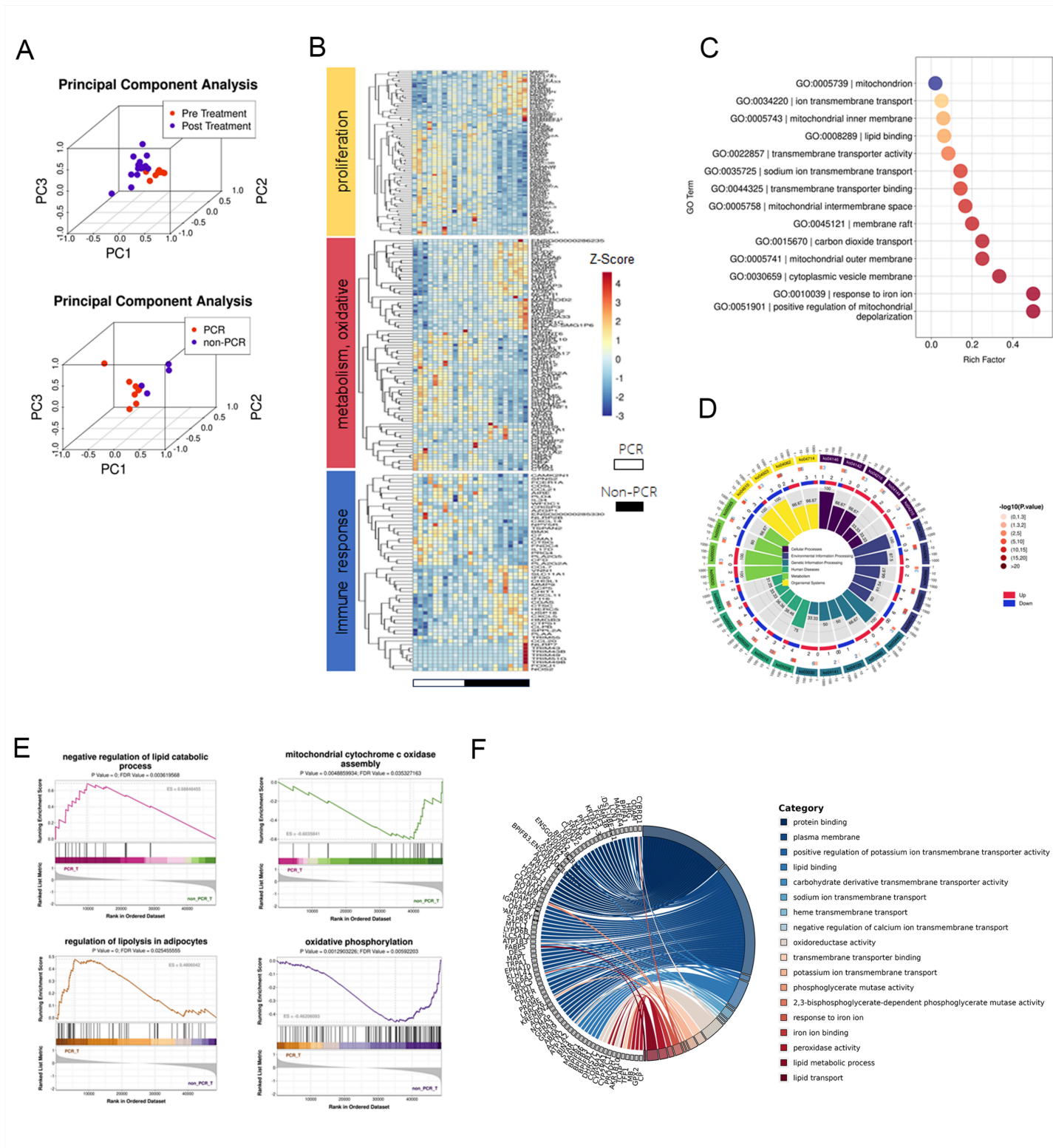


Figure 2

Transcriptomic profiling reveals global gene expression remodeling and enrichment of iron and lipid metabolism-related pathways in patients with PCR response following nCIT

A. Principal component analysis (PCA) shows significantly altered gene profiles over the course of nCIT (above), over the PCR and non-PCR groups (down). Each dot represents the averaged value from all

tumor.

B. Heatmap of differentially expressed genes across response groups. The color gradient from blue (low expression) to red (high expression) represents Z-scores, visually demonstrating transcriptomic remodeling associated with the treatment response.

C-E. Functional enrichment analysis of genes associated with PCR vs. non-PCR. Results from (C) Gene Ontology (GO) terms, (D) KEGG pathways, and (E) Gene Set Enrichment Analysis (GSEA) collectively revealed the key biological processes underlying PCR status.

F. The Enrichment Chord Plot shows a significant integrated result of KEGG pathways and corresponding genes.

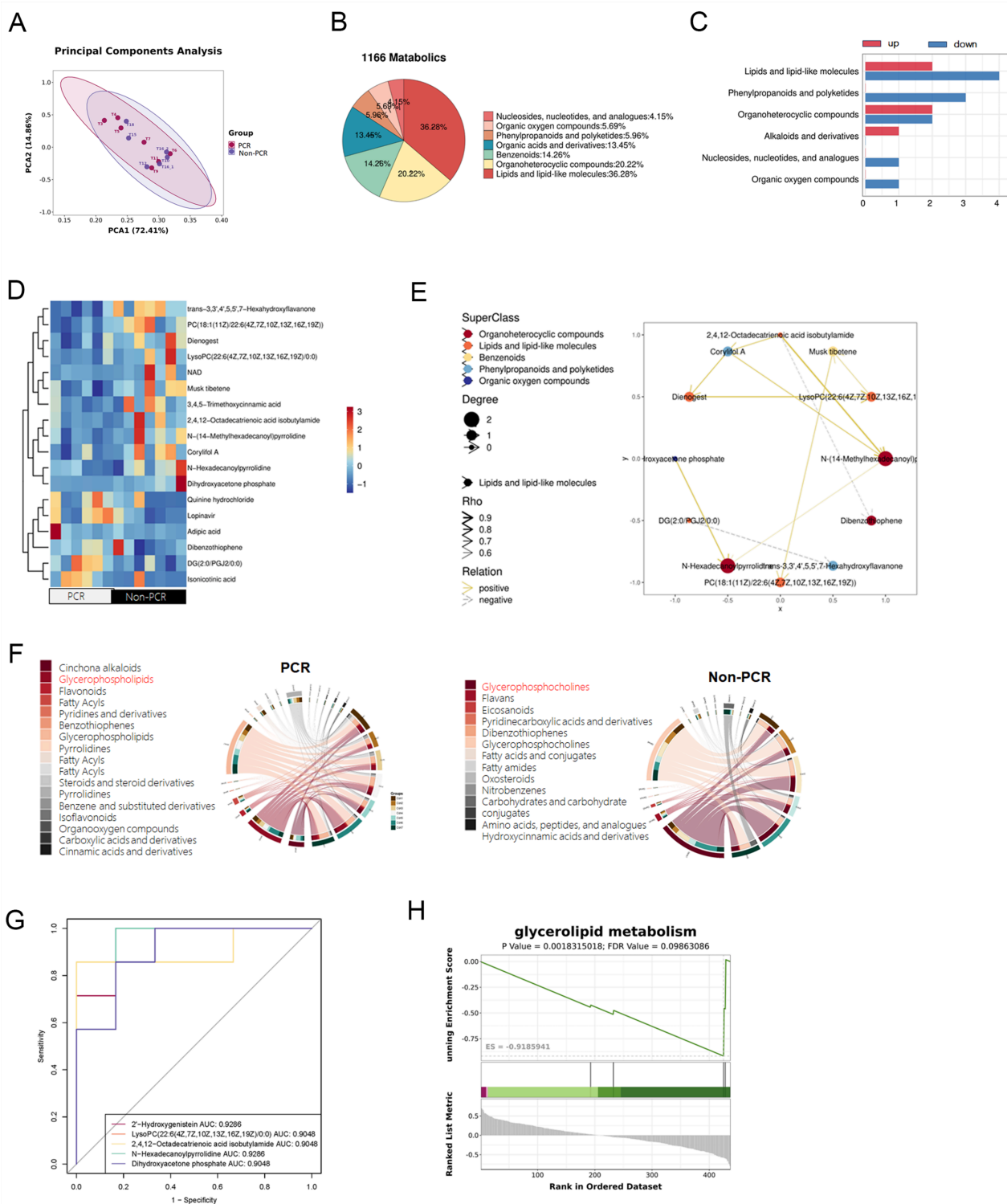


Figure 3

Metabolomic profiling reveals lipid-related metabolic alterations as a key feature of patients achieving PCR following nCIT.

A. PCA score plots showing global metabolomic profile changes over the course of nCIT (upper panel) and between PCR (red) and non-PCR (blue) groups (lower panel). Each dot represents an individual

tissue sample.

B. Pie chart shows the categorical distribution of a total of 1,166 distinct differential metabolites between the PCR and non-PCR groups. The pie chart shows the distribution of these metabolites across superclasses.

C. A bar chart (or additional pie chart) further illustrating the proportion of each metabolite superclass.

D. Heatmap of 18 differential metabolites between PCR and non-PCR groups. The color gradient from blue (low expression) to red (high expression) represents Z-scores, visually demonstrating significant metabolic disparities between the two groups.

E. Correlation network diagram of differential metabolites within the PCR group. Nodes represent individual metabolites, and edges between nodes indicate significant positive correlations (Pearson correlation; $r > 0.6$ and $p < 0.05$). A higher node degree (number of connections) suggests greater potential influence within the metabolic network.

F. Circos plot visualizing global differences in metabolite abundance between PCR (**left**) and non-PCR (**right**) groups. The links between the inner and outer rings intuitively reflect the changing patterns of metabolites across the two groups.

G. ROC Curves for Discriminating PCR vs. non-PCR: X-axis (1-Specificity): Represents the False Positive Rate. A lower value indicates better specificity. Y-axis (Sensitivity): Represents the True Positive Rate. A higher value indicates better sensitivity. Diagonal Line (Reference): Represents random guessing with no diagnostic value (AUC=0.5). All curves are well above this line, indicating excellent diagnostic performance. AUC (Area Under the Curve): Measures the overall diagnostic accuracy. An AUC closer to 1 indicates a better classifier.

H. Results from Gene Set Enrichment Analysis (GSEA) collectively reveal key biological processes- Glycerolipids metabolism underlying PCR status. The running enrichment score curve (green line) and the ranked list of metabolites (black vertical lines) are shown.

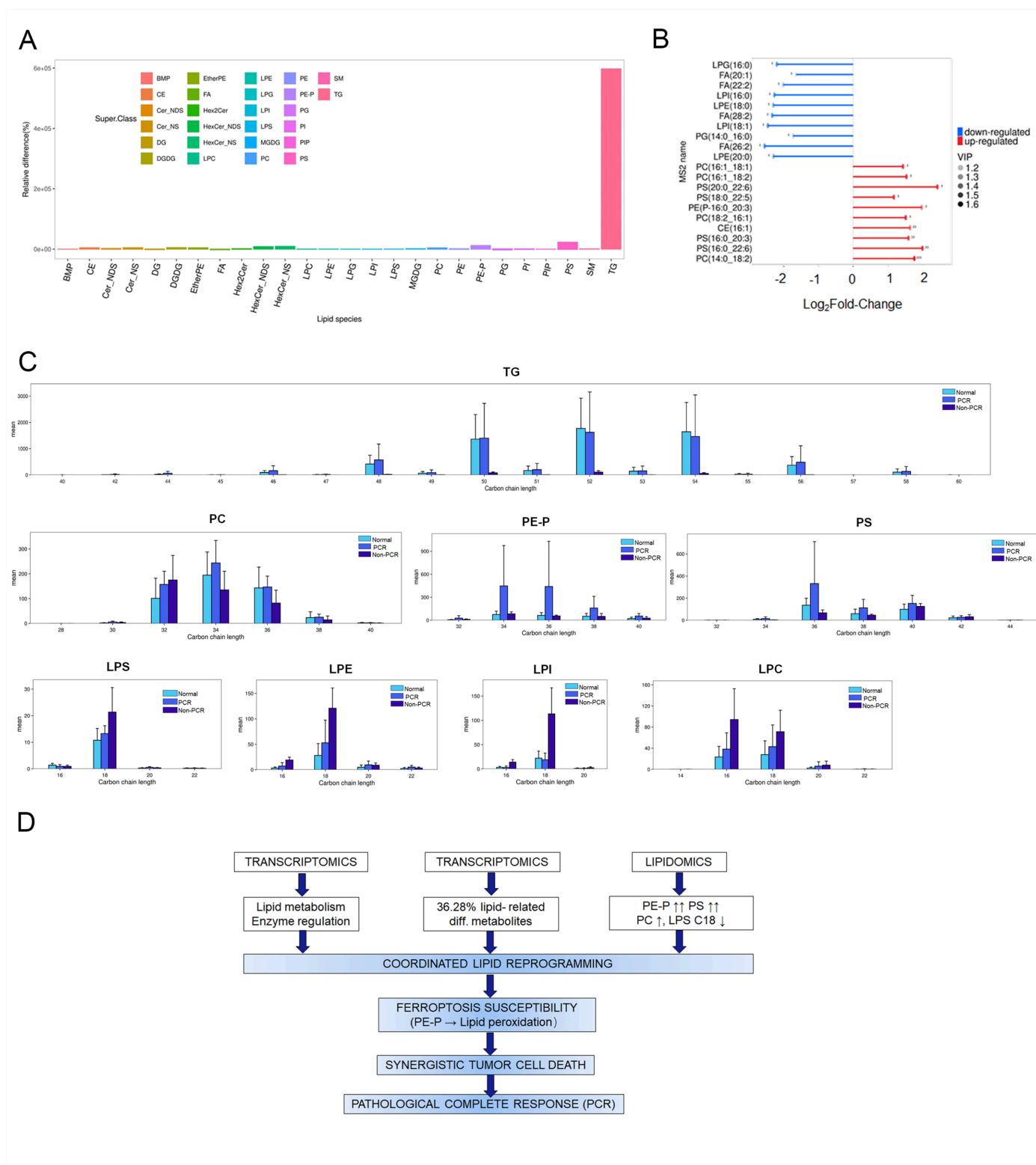


Figure 4

Integrated lipidomics analyses identify coordinated lipid remodeling as a hallmark of PCR

A. Relative differences of individual lipid species are shown as a bar plot (or heatmap). Lipid species are categorized by superclass (BMP, CE, Cer_NDS, DG, LPC, etc.) and specific lipid species are listed on the y-axis. The LogFold-Change color scale (blue: downregulated in PCR, red: upregulated in PCR) indicates

the magnitude of change. Notably, multiple phosphatidylserine (PS), phosphatidylglycerol (PG), and PC species are upregulated in PCR patients.

B. Membrane contact site (MCS) ratio represent the enrichment of lipids at membrane contact sites (e.g., ER-mitochondria junctions).

C. Carbon chain length distribution of major lipid classes. The profiles of TG, PC, PE-P, PS, and LPC across carbon chain lengths (C40 to C50) are shown.

D. Proposed mechanistic model. Coordinated lipid reprogramming—driven by transcriptomic and lipidomic alterations—enhances ferroptosis susceptibility (PE-P serving as a substrate for lipid peroxidation), leading to synergistic tumor cell death and ultimately PCR. This model summarizes the central finding of Integrated multi-omics regulatory network.

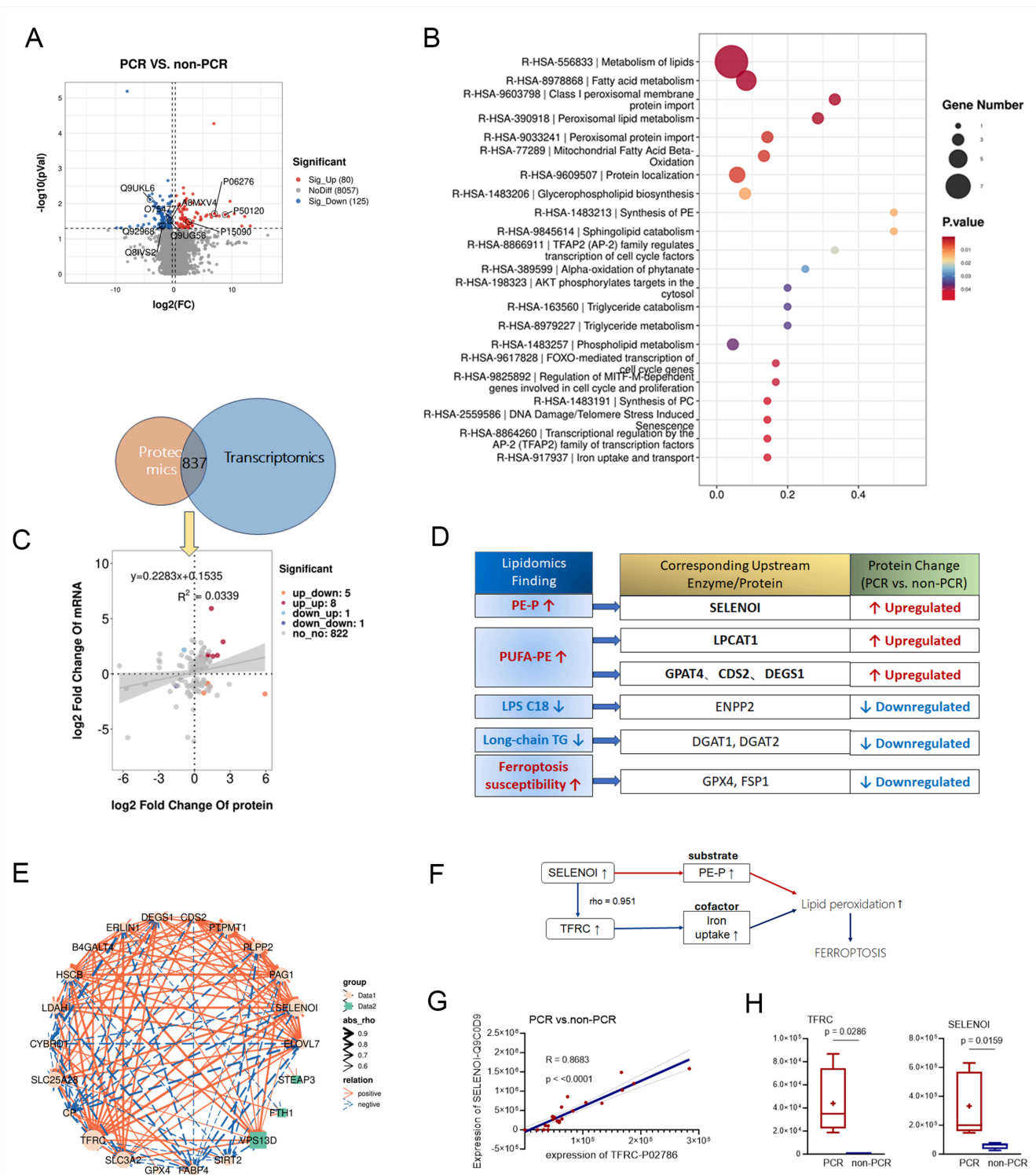


Figure 5

Proteomic profiling and pathway enrichment reveal lipid metabolism and SELENOI-centered networks as key features of PCR

A. Volcano plot displaying the overall distribution of differentially expressed proteins. Proteins with statistically significant up-regulation (red) and down-regulation (blue) in the PCR group are highlighted

against non-significant proteins (gray).

B. Reactome pathway enrichment analysis. Enriched pathways among the differentially expressed proteins. Bars represent the adjusted p-value ($-\log_{10}(\text{p-value})$).

C. The number of proteins quantified by proteomics (837) and the number of transcripts analyzed by transcriptomics. The overlapping region indicates proteins with corresponding mRNA data, enabling integrative analysis. The axis shows the correlation between mRNA expression and protein, which divided into Up-Up, Up-down, Down-down, Down-Up. The grey area represents the mRNA expression and protein with no significant correlations.

D. Mechanistic table linking lipidomic phenotypes to upstream enzymes. For each key lipidomic finding (e.g., PE-P $\uparrow$, PS $\uparrow$, LPS C18 $\downarrow$, long-chain TG $\downarrow$, ferroptosis susceptibility $\uparrow$), the corresponding upstream enzyme/protein and its expected direction of change in PCR vs. non-PCR are listed. Upregulated enzymes (SELENOI, LPCAT1, GPAT4, CDS2, DEGS1) are shown in red, downregulated enzymes (ENPP2, DGAT1, DGAT2, GPX4, FSP1) in blue.

E. A circular heatmap displaying correlation coefficients (ρ) between SELENOI and selected proteins involved in phospholipid/sphingolipid metabolism (DEGS1, CDS2, ERLIN1, PTPMT1, B4GALT4, PLPP2, HSCB, PAG1). Positive correlations (red) indicate co-expression patterns that support a coordinated functional module centered on SELENOI.

F. Proposed mechanistic model. SELENOI and TFRC co-upregulation establishes a dual-priming mechanism for ferroptosis. The strongest positive correlation between SELENOI and TFRC ($\rho = 0.951$, $p < 0.0001$) provides a mechanistic link between phospholipid remodeling and iron metabolism in responders.

G. Correlation analysis between expression of SELENOI and TFRC levels of primary tumors.

H. Box plots (median with interquartile range) of TFRC and SELENOI in PCR versus non-PCR group. The p-values were calculated using two-tailed Student's t-test.

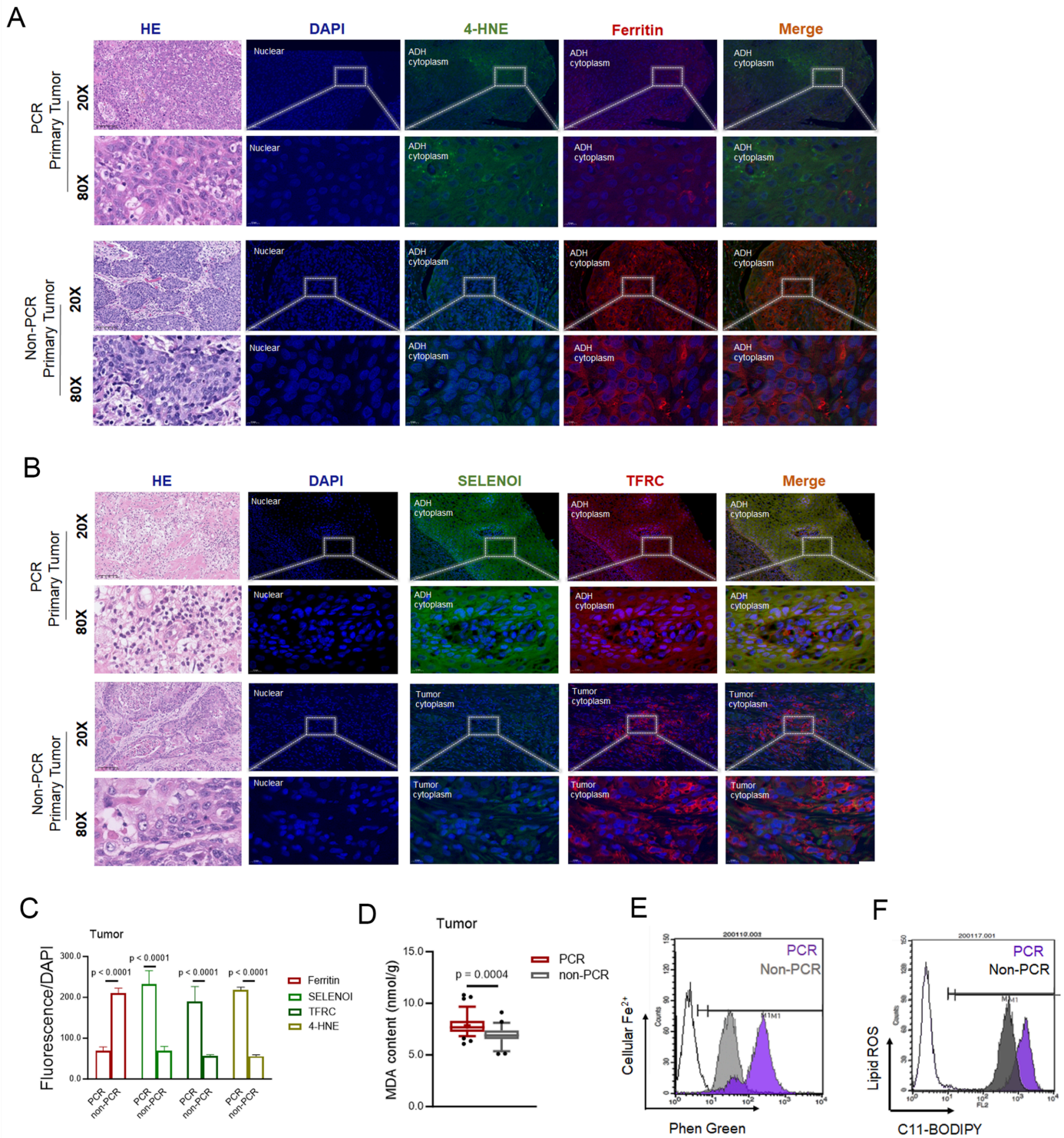


Figure 6

Immunofluorescence and functional assays validate SELENOI/TFRC upregulation and elevated ferroptosis markers in PCR tumors

A-B. Representative HE stains and immunofluorescence images showing the expression and localization of 4-HNE and Ferritin (A), TFRC and SELENOI (B) in primary tumors. Nuclei were stained with DAPI (blue).

The merged channels show protein co-localization.

C. Quantitative analysis of 4-HNE and Ferritin, TFRC and SELENOI immunofluorescence signal intensity in primary tumors. Data are presented as mean $\pm$ SD (PCR n=29, non-PCR n=28).

D. Bar graph (mean $\pm$ SEM) quantifying MDA concentrations in tumor tissues from the PCR and non-PCR groups. Data are presented as mean $\pm$ SD (PCR n=29, non-PCR n=28).

E-F. Bar graphs showing results of labile iron pool (Phen Green probe) (E) and lipid ROS (C11-BODIPY) (F) measurements.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [suppldata.docx](#)
- [seqananlysisdata.rar](#)
- [seqananlysisdata.xlsx](#)