Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Systemic-to-Tumour Copper Dissociation in Colorectal Cancer: AQP1 Water Permeability Gates Copper Delivery to Cytochrome c Oxidase

Anastasia De Luca

anastasia.deluca@uniroma2.it

University of Rome Tor Vergata https://orcid.org/0000-0002-6532-989X

Gabriella Vicano

University of Rome Tor Vergata

Chiara Carrino

University of Rome "Tor Vergata"

Rosanna Squitti

Ospedale Isola Tiberina—Gemelli Isola

Focaccio Antonio

University of Rome "Tor Vergata"

Elvia Valentini

University of Rome "Tor Vergata"

Ivan Monteleone

University of Tor Vergata https://orcid.org/0000-0002-3212-832X

Federica Allegrini

University of Rome "Tor Vergata"

Gianluca Rizzo

Ospedale Isola Tiberina—Gemelli Isola

Federica Federica Marzi

Ospedale Isola Tiberina—Gemelli Isola

Stefania Mariani

Ospedale Isola Tiberina—Gemelli Isola

Gioia Mastromoro

Ospedale Isola Tiberina—Gemelli Isola

Mauro Cozzolino

Institute of Translational Pharmacology, CNR https://orcid.org/0000-0003-0342-8961

Katia Aquilano

University of Rome Tor Vergata https://orcid.org/0000-0002-5905-9870

Mauro Rongioletti

Ospedale Isola Tiberina—Gemelli Isola

Vincenzo Tondolo

Ospedale Isola Tiberina-Gemelli Isola

Gerardo Pepe

Tor Verrgata University of Rome https://orcid.org/0000-0001-5308-2765

Article

Keywords: copper metabolism, colorectal cancer, copper transporter, KRAS, AQP1, AP1S1

Posted Date: November 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11564644/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: There is no conflict of interest

Abstract

Copper is both an essential enzymatic cofactor and, when mishandled, a trigger of copper-dependent cell death, and its availability is increasingly recognised as a determinant of cancer progression. Colorectal cancers (CRC) harboring activating *KRAS* mutations show enhanced copper dependency, but how these tumours acquire copper and transfer it to mitochondria, where it is required for cytochrome c oxidase (CcO) assembly and sets the threshold for cuproptosis, remains undefined. Here we show that colorectal tumours are copper-poor within a copper-rich systemic environment, and we identify aquaporin-1 (AQP1), alongside adaptor protein complex 1 subunit sigma 1 (AP1S1), as a non-canonical determinant of copper delivery to mitochondria. We combined TCGA and GTEx transcriptomics with an independent cohort of 104 CRC patients and 104 age- and sex-matched controls, including 78 paired tumour and adjacent-mucosa specimens assessed for circulating copper biomarkers, tissue copper content and copper-related proteins. CRC showed increased circulating total and exchangeable copper but reduced tumor copper content; these systemic changes were more pronounced in *KRAS*-mutant disease and increased with stage. In *KRAS*-mutant tumours, CD44 and AP1S1 were higher and AQP1 lower. Caco-2 and HCT-116 cells, which differ in wider genetic background including the *KRAS* status, were exposed to copper supplementation, copper chelation or AQP1 inhibition. Blocking AQP1 water permeability with AqB013, but not blocking its central cation pore with AqB011, reduced CcO abundance in both models and elicited a compensatory rise in CTR1 in HCT-116 cells, phenocopying copper chelation, whereas molecular dynamics excluded direct Cu(I) or Cu(II) permeation through the AQP1 pore. An exploratory screen of copper-gene combinations identified *AQP1–SLC31A2* as nominally associated with poorer outcome in TCGA-COAD. These findings define a systemic-to-tumour copper dissociation in CRC and place AQP1 in the route that could indirectly favor copper supply to mitochondria, identifying a water-flux-dependent step that copper-targeted strategies, including cuproptosis induction, may exploit.

Introduction

Copper is an essential micronutrient whose biological effect has expanded significantly over the past decade. It is now well-established that copper plays a crucial role in driving cancer progression and cancer cell dissemination. Cancer cell survival can be compromised by inducing "cuproptosis", a form of copper-dependent cell death [1], or tumor aggressiveness can be mitigated by inhibiting "cuproplasia", the copper-dependent mechanisms sustaining cancer cells proliferation [2, 3].

The therapeutic potential of targeting copper homeostasis to limit cancer progression has been explored in several clinical trials [4–8]. Our previous studies and others [9–11] have highlighted a strong association between copper dysregulation and the progression of breast cancer and colorectal cancer (CRC), including changes in the epithelial-to-mesenchymal transition (EMT). Our previous pilot study identified lower copper in CRC tissue together with increased circulating copper [11], although systematic reviews have reported heterogeneous and partly inconclusive results [12, 13]. Key players of copper distribution include the high-affinity copper importer CTR1, encoded by *SLC31A1*, whereas CTR2,

encoded by *SLC31A2*, participates in intracellular copper mobilization and regulates CTR1 processing [14]. Increased CTR1 expression has been reported in several malignant contexts [15–17] and may also influence cisplatin uptake [15, 18]. Cancer cells may also use alternative routes. Activated macrophages have been shown to import copper through CD44 [19], whereas in *KRAS*-mutant CRC cells macropinocytosis has been proposed as an additional route of copper acquisition [20].

In this context, Aquaporin 1 (AQP1) and AP1S1 are plausible components of such an adaptive network since both proteins have recently been included in a pan-cancer cuproplasia-related gene signature [21]. AQP1 conducts water through its monomeric pores [22] and has been reported, under specific experimental conditions, to mediate cGMP-gated monovalent cation conductance [23]; it also contributes to cancer-cell migration [24, 25]. AP1S1 is a subunit of the AP-1 adaptor complex which mediates protein sorting between the trans-Golgi network and endosomal compartments [26].

Here, we integrated public transcriptomic datasets, a matched clinical cohort and paired CRC tissues to characterize systemic and tissue copper dysregulation and to examine CTR1, CTR2, CD44, AQP1 and AP1S1 according to *KRAS* status. We further tested whether copper supplementation, chelation and pharmacological AQP1 inhibition alter copper-dependent protein readouts. Because both the assembly of cytochrome c oxidase (CcO) and the induction of cuproptosis depend on the delivery of copper to mitochondria [1], we used CcO abundance as a functional readout of mitochondrial copper availability. Finally, as an exploratory analysis, we screened combinations of copper-transport genes for association with clinical outcomes in TCGA-COAD.

Materials and Methods

Transcriptomic datasets and feature selection

Transcript-level expression data from TCGA, TARGET and GTEx were obtained from UCSC Xena [27] using the uniformly processed Toil pipeline [28] (dataset TcgaTargetGtex_rsem_isoform_tpm; version 2016-09-02). Expression values were $\log_2(\text{TPM} + 0.001)$-transformed. Colorectal, lung and pancreatic adenocarcinoma samples, respectively COAD, LUAD and PAAD, with available *KRAS* data were analyzed. An L1-penalized logistic regression (LASSO) was used to screen a 131-gene copper-related panel and a 19-gene transporter panel. Detailed preprocessing and sample numbers are reported in Supplementary Methods and Table S1.

Clinical cohort and ethics

We enrolled 104 CRC patients and 104 age- and sex-matched controls at Isola Tiberina–Gemelli Isola Hospital (October 2023–September 2024); paired tumor and adjacent mucosa were available from 78 patients. Eligibility criteria, exclusion criteria and matching are detailed in Supplementary Methods. Peripheral venous blood was collected for the measurement of copper, ceruloplasmin and exchangeable copper (ExchCu).

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee (CET Lazio, Area 3) of the Fondazione Policlinico Universitario A. Gemelli IRCCS – Università Cattolica del Sacro Cuore (protocol no. 6091/2023; approval date 26 October 2023). All participants provided written informed consent.

Copper biomarkers and tissue analyses

Total serum copper was measured colorimetrically, with approximately 30% of samples reassessed by GF-AAS [29, 30]. Exchangeable copper (ExchCu) was determined by ultrafiltration and GF-AAS. Ceruloplasmin concentration and activity, specific activity and the Cu/Cp ratio were assessed by established procedures [29–31]. Copper in tumor and adjacent mucosa was quantified by GF-AAS after tissue mineralization. Instrumental settings and quality controls are provided in Supplementary Methods.

Proteins and transcripts analyses

Tissue homogenates were prepared in ice-cold PBS with protease and phosphatase inhibitors. Protein concentrations were measured by Lowry assay, followed by SDS–PAGE, immunoblotting and ImageJ quantification; antibody specifications are provided in Table S3 and Supplementary Methods [11]. Total RNA was extracted from tissues using TRIzol, reverse transcribed and analyzed by SYBR-green RT-qPCR. ACTB was used as the internal reference, with expression calculated by the $2^{-\Delta Ct}$ method. Primer sequences are listed in Table S4.

Cell lines and treatments

Caco-2 (*KRAS* wild-type, *APC*-, *TP53*- and *SMAD4*-mutant, *PIK3CA* wild-type, microsatellite stable) and HCT-116 cells (*KRAS*^{G13D}- and P*IK3CA*^{H1047R}-mutant, *APC* and *TP53* wild-type, microsatellite unstable and mismatch repair-deficient) [32] were obtained from ATCC (Manassas, VA, USA), cultured in RPMI-1640 and McCoy's 5A, respectively, and supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO2. After dose-response assessment, cells were exposed to $CuCl_2$ (50 µM) or TRIEN (200 µM), at concentrations associated with at least 80% viability. AQP1 inhibitors AqB011 and AqB013 were tested at 50, 150 and 300 µM, alone or together with TRIEN, as described in Supplementary Methods [33–35].

Immunofluorescence and Western blot of Caco-2 and HCT-116 cells

AQP1 and AP1S1 were assessed by immunofluorescence in fixed and permeabilized cells using ApoTome microscopy and ImageJ analysis. Western blotting measured CCS, CcO and CTR1. Primary antibodies, incubation conditions and replicate information are detailed in Supplementary Methods and S3.

Prognostic analyses

Mean $\log_2$-transformed expression scores for two-, three- and four-gene combinations of *AQP1*, *CD44*, *SLC31A1*, *SLC31A2* and *AP1S1* were assessed by L2-penalized Cox regression (coefficient 0.01), stratified by *KRAS* status, across DFI, PFI, overall and disease-specific survival. Hazard ratios and nominal p-values are reported; the screening was exploratory.

Statistical analysis

Continuous variables were summarized as mean ± SD and median (IQR). Normality was assessed by the Shapiro–Wilk test. Serum measurements were compared between patients and controls with two-sided Mann–Whitney U tests. Tumour and matched adjacent mucosa were compared with the two-sided paired Wilcoxon signed-rank test in the 78 patients for whom both specimens were available; for the immunoblot analyses, in which a matched non-tumour specimen was not available for every tumour, normally distributed data were compared with Welch's unpaired t-test and non-normally distributed data with the Mann–Whitney U test. Trends across ordered groups were assessed by Spearman correlation. Cell experiments were analyzed by one-way ANOVA followed by Dunnett's post hoc correction, with n indicating independent biological replicates. Subgroup analyses by *KRAS* status and T category, the correlation matrices and the prognostic gene-combination screening were exploratory: nominal p values are reported together with Benjamini–Hochberg false discovery rates, and no confirmatory claim is made. Analyses were performed in GraphPad Prism (version 10.1.1); $p < 0.05$ was considered significant.

Results

SLC31A1 is upregulated and SLC31A2 downregulated in KRAS-driven adenocarcinomas

Among the TCGA cohorts, pancreatic (PAAD), colorectal (COAD) and lung adenocarcinoma (LUAD) show a high prevalence of *KRAS* mutations. OncoPrint analysis performed using the PANDA webserver [36] confirmed differences in mutation frequency and co-mutational landscapes (Fig. 1A–C). We compared the transcript expression levels of *SLC31A1* and *SLC31A2* between tumor (TCGA) and non-tumoral (TCGA and GTEx) tissues. *SLC31A1* mRNA was consistently upregulated in tumor tissues in all three cohorts (Fig. 1D). Conversely, *SLC31A2* was significantly downregulated in COAD and LUAD, while being upregulated in PAAD (Fig. 1F). This pattern is compatible with changes in the copper-import machinery but does not directly quantify copper uptake.

CTR1 increases and CTR2 decreases in colorectal tumours relative to matched mucosa

The final study population consisted of 104 patients with CRC and 104 age- and sex-matched controls (Table S2). Mean age was 70.8 ± 11.1 and 71.5 ± 11.4 years, respectively ($p = 0.681$), with identical sex distributions (68 males and 36 females per group). Protein analysis of CRC tissues (Tumor) and

matched adjacent normal mucosa (Adj. muc.) demonstrated increased CTR1 and decreased CTR2 levels in tumor samples (Fig. 1E and G, respectively), in agreement with TCGA-derived data, whereas no significant differences were observed at the mRNA level. Given recent evidence implicating CD44 in copper transport in activated monocyte-derived macrophages [19], we further assessed its expression. TCGA data indicated that CD44 mRNA is upregulated in COAD and PAAD and downregulated in LUAD (Fig. 1H). However, in our CRC cohort, CD44 protein levels were significantly reduced in tumor tissues compared to matched normal mucosa (Fig. 1I). Taken together, these findings are compatible with CTR1 being the principal canonical copper importer in CRC, but they also indicate that the CTR1/CTR2/CD44 axis alone does not account for the copper depletion of tumour tissue described below.

Colorectal tumours are copper-poor despite a copper-rich circulation, and the dissociation widens with stage

Copper concentration, normalized to total protein content, was significantly lower in tumor tissue than in adjacent mucosa (0.097 ± 0.049 vs 0.134 ± 0.082 μmol/mg protein; paired Wilcoxon test, $p = 2.25 \times 10^{-6}$) (Fig. 1J). In contrast, total serum copper was increased in CRC patients (16.44 ± 3.34 μmol/L) compared with controls (13.34 ± 2.69 μmol/L; $p < 0.0001$, Table S5), as was immunoreactive ceruloplasmin (30.18 ± 6.00 vs 27.18 ± 4.41 mg/dL; $p < 0.001$, Table S5). The Cu/Cp ratio was elevated (7.13 ± 1.03 vs 6.55 ± 0.93; $p < 0.001$, Table S5), ExchCu was also increased (1.50 ± 0.81 vs 1.04 ± 0.67 μmol/L; $p < 0.001$) (Table S5, Fig. 1J–L), whereas ceruloplasmin enzymatic activity was unchanged ($p = 0.7525$, Table S5). Specific activity was lower in patients (3.75 ± 0.71 vs 4.01 ± 0.64 iU/mg; $p = 0.0009$, Table S5). Stratification by sex supported the main findings. Increased serum copper, ExchCu and Cu/Cp ratio were observed in both men and women with CRC (Table S6). To examine copper homeostasis in relation to disease stage, subjects were ordered as controls, early-stage (I–II) and advanced-stage (III–IV) CRC. A positive trend across these groups was observed for serum copper (Spearman $\rho = 0.391$, $p = 1.25 \times 10^{-8}$), ExchCu ($\rho = 0.332$, $p = 7.55 \times 10^{-7}$) and Cu/Cp ($\rho = 0.336$, $p = 1.78 \times 10^{-6}$) (Table S7).

Exchangeable copper and tumour CD44 are higher in KRAS-mutant CRC

Patients with available *KRAS* and serum data included 15 *KRAS*-mutant and 11 *KRAS*-wild-type cases, thus, given the small subgroup sizes we conducted an exploratory analysis. Total serum copper, ceruloplasmin and the Cu/Cp ratio did not differ significantly, although with a tendency toward an increase in *KRAS*-mutant CRC, whereas ExchCu was significantly higher in the *KRAS*-mutant group (Fig. 2A–D). Accordingly, the levels of tissue transporters were analyzed in the available paired samples and considered together with the available TCGA datasets. In TCGA cohorts, *SLC31A1* did not differ consistently by *KRAS* status, *SLC31A2* was lower in *KRAS*-mutant COAD and LUAD, and CD44 showed tumor-type-specific differences (Fig. 2E, H and K). In our CRC cohort, no significant differences were detected in CTR1 or CTR2 protein abundance between *KRAS*-mutant and wild-type tumors (Fig. 2F, I), neither in early nor advanced T stages (Fig. 2G, J). Notably, CD44 protein was significantly increased in *KRAS*-mutant tumors (Fig. 2L), particularly in advanced T categories (Fig. 2M).

Unbiased feature selection identifies AQP1 and AP1S1 as KRAS-associated components of the copper network

To identify potential non-canonical modulators of copper uptake in CRC, we applied LASSO to a panel including 131-gene involved in copper biology and to a restricted 19-gene transporter panel. In the latter, AQP1 (β = −0.213) and AP1S1 (β = +0.314) were selected, whereas *SLC31A1* and *SLC31A2* were not retained (Table S8). Both genes were also selected in the *KRAS*-mutant COAD subset with similar coefficient directionality (Table S8B).

TCGA cohorts showed that *AQP1* expression was reduced in COAD and LUAD compared with non-tumoral tissues (Fig. S1A), independently of the available metastatic-status annotations (Fig. S1B), and correlated positively with *SLC31A2* (r = 0.51; Fig. S1C). Additionally, it showed a weak positive correlation with *SLC31A1* (r = 0.19; Fig. S1C) and a negative one with *CD44* (r = − 0.11; Fig. S1C). Lower *AQP1* was associated with longer overall survival (Fig. S1D), highlighting an association between reduced *AQP1* in tumors and better clinical outcome. In our clinical cohort, *AQP1* mRNA did not differ significantly between tumor and adjacent mucosa (Fig. 3A), whereas TCGA suggested downregulation in *KRAS*-mutant COAD (Fig. 3B), also observed in our small CRC subset (Fig. 3C). *AQP1* remained reduced across stages in TCGA (Fig. 3D), but this evidence was not confirmed in our subsets (Fig. 3E). By contrast, *AP1S1* expression was increased in TCGA tumors (Fig. S1E) but not in our cohort (Fig. 3F). Within TCGA, *AP1S1* was enriched in *KRAS*-mutant COAD (Fig. 3G–I), with possibly similar directional patterns in our small clinical subset (Fig. 3H, J).

AP1S1 abundance tracks CTR1 in KRAS-mutant tumours

In the overall CRC cohort, CTR1 and CTR2 protein abundance was strongly correlated (r = 0.837), whereas CTR2 showed a weaker correlation with CD44 (r = 0.389) (Fig. S2, upper panel). AP1S1 was weakly inversely correlated with serum copper and ceruloplasmin (r = − 0.262 and r = − 0.252). Interestingly, in the KRAS-mutant subgroup we found a strong AP1S1–CTR1 correlation (r = 0.693; Fig. S2, lower panel), although this exploratory estimate was based on approximately 15 cases and multiple pairwise tests. The observed correlation among the serum copper parameters demonstrated the expected biological consistency of our findings supporting the reliability of the analytical measurements.

AQP1 and AP1S1 respond dynamically to copper supplementation and chelation

Caco-2 (KRAS wild-type, APC-, TP53- and SMAD4-mutant, PIK3CA wild-type, microsatellite stable) and HCT-116 cells ($KRAS^{G13D}$- and $PIK3CA^{H1047R}$-mutant, APC and TP53 wild-type, microsatellite unstable and mismatch repair-deficient) [32] cells were exposed to $CuCl_2$ or TRIEN (Fig. S3). The IC_{50} values determined in HCT-116 cells were > 1000 µM for TRIEN and 347.7 µM for $CuCl_2$, whilst in Caco-2 cells IC_{50} were 628 µM for TRIEN and 99.8 µM for $CuCl_2$ (Fig. S3A). Concentrations maintaining at least 80% viability were selected, comprising 200 µM TRIEN and 50 µM $CuCl_2$. Consistent with the established copper responsiveness of CCS and mitochondrial cytochrome c oxidase (CcO), recognized as sensitive

readouts of changes in the intracellular copper content [37–39], TRIEN reduced CcO in both models and increased CCS in HCT-116 cells at 48 h, whereas copper supplementation reduced CCS in Caco-2 (Fig. S3B).

Immunofluorescence revealed distinct temporal responses of AQP1 and AP1S1 to copper perturbation (complete immunofluorescence panels and fluorescence quantifications in Figs. S4 and S5). In Caco-2 cells, copper supplementation increased AQP1 (Fig. S4A left panel, C) and AP1S1 (Fig. S5A left panel, C) fluorescence intensity at 48–72 h, whereas TRIEN reduced both signals at 72 h. HCT-116 cells showed different temporal responses. $CuCl_2$ rapidly increased AP1S1 from 24 h (Fig. S5B right panel and D), with limited effects on AQP1 (Fig. S4B right panel and D), whereas TRIEN produced biphasic changes in both proteins (Figs. S4B, D and S5B, D). In particular, AQP1 showed an initial increase, followed by a decrease and a subsequent rise at 72 h, while AP1S1 displayed a reduction at 48 h followed by a pronounced increase at 72 h. Thus, AQP1 and AP1S1 were copper-responsive in both CRC cell models.

Blocking AQP1 water permeability, but not its cation pore, depletes cytochrome c oxidase

We treated Caco-2 and HCT-116 cells with AqB011, which inhibits the AQP1 central cationic pore [33], or AqB013, which restricts water permeability [40]. Cells incubation with the specific copper chelator TRIEN was used as positive control of intracellular copper content impairment. TRIEN reduced CcO in both cell lines, whereas AqB011 did not change CcO or CTR1 significantly (Fig. 4A, B). By contrast, the inhibition of water flux by AqB013 reduced CcO in Caco-2 at 50 and 150 µM and in HCT-116 in a dose-dependent manner (Fig. 4C, D). The combination of AqB013 and TRIEN suggested a non-additive CcO response in Caco-2 and a possible additive response in HCT-116 (Fig. 4C, D). Moreover, AqB013 reduced CTR1 abundance at the highest concentration in Caco-2 (Fig. 4C), whereas CTR1 increased as CcO decreased in HCT-116 cells (Fig. 4D), a pattern compatible with a compensation mechanism probably acting to restore intracellular copper levels. However, molecular modeling predicted that neither Cu^{+} nor Cu^{2+} can permeate through AQP1 because of the electrostatic barrier at the pore constriction (Fig. S6). The positively charged arginine residues lining this region create an environment unfavorable for copper-ion transport, suggesting that any relationship between AQP1 activity and copper-dependent readouts is likely to be indirect. Because AqB013 reproduced the loss of CcO caused by copper chelation, we refer to this as a copper-delivery phenotype; its formal attribution to AQP1 requires genetic loss of function, as discussed below.

An exploratory AQP1–SLC31A2 score is nominally associated with outcome in TCGA-COAD

Using the TCGA-COAD cohort stratified by *KRAS* status, we explored all two-, three- and four-gene combinations from *AQP1*, *CD44*, *SLC31A1*, *SLC31A2* and *AP1S1* by Cox regression across four clinical endpoints (Table S9). Of all the combinations tested, only the *AQP1–SLC31A2* score reached nominal significance in both subgroups. Higher scores were associated with shorter disease-free interval in *KRAS*-mutant tumors (HR = 1.02, p = 0.034), and with shorter progression-free interval (HR = 1.01, p = 0.017) and disease-specific survival (HR = 1.01, p = 0.013) in *KRAS* wild-type tumors.

Discussion

The principal finding of this study is the identification of a dissociation between systemic copper biomarkers and tissue copper content in CRC, accompanied by changes in proteins involved in copper acquisition and intracellular handling. By integrating public transcriptomic datasets, circulating biomarkers, patient-derived tissues and *in vitro* experiments, our data suggest that copper homeostasis in CRC cannot be fully explained by the canonical CTR1/CTR2 system. In our age- and sex-matched cohort, CRC patients displayed increased serum copper, immunoreactive ceruloplasmin and ExchCu, whereas copper content was lower in tumor tissue than in adjacent mucosa. These findings extend our previous pilot observations [11]. Importantly, published meta-analyses have reported heterogeneous or inconclusive associations between copper levels and CRC [12, 13], indicating that this pattern should not be generalized without further validation. CTR1 abundance increased in tumors, whereas CTR2 and CD44 were reduced overall. After *KRAS* stratification, however, CD44 was higher in *KRAS*-mutant than in wild-type tumors. CD44 has also been linked to CRC stemness and cellular plasticity [41–44]. In view of the small subset, this observation suggests a potential link with copper acquisition in CRC cells [19, 20, 41].

A further innovative aspect is the identification of *AQP1* and *AP1S1* by exploratory LASSO feature selection. Their selection in the restricted transporter panel, while *SLC31A1* and *SLC31A2* were not retained, suggests that the two genes capture information not explained by canonical copper-transport genes alone. *AQP1* was reduced in CRC transcriptomic cohorts, particularly in the *KRAS*-mutant setting. Higher AQP1 has recently been associated with CRC progression and cell migration in an independent study [45]. AP1S1 correlated with CTR1 more strongly in the small *KRAS*-mutant subset, but neither correlation nor feature selection establishes direct transporter function.

In vitro, AQP1 and AP1S1 responded dynamically to copper supplementation and TRIEN, with distinct patterns in Caco-2 and HCT-116. The non-isogenic background prevents assigning these differences to *KRAS* alone. Nevertheless, our findings suggest that the broader genetic background, particularly oncogenic mutations driving aberrant cell proliferation such as *KRAS*, may critically influence cellular copper demand and, consequently, the regulation of copper transporters and other mechanisms driving copper homeostasis. Recent studies in isogenic *KRAS*-mutant CRC models likewise indicate mutation-specific alterations in copper trafficking [46], supporting this hypothesis. AqB013, an inhibitor of AQP1 water permeability, reduced CcO abundance in both cell models, whereas AqB011, targeting the central ion pore, did not. The AqB013–TRIEN combination suggested non-additive effects in Caco-2 and additive effects in HCT-116, further indicating context-dependent responses. CTR1 abundance showed divergent responses and may reflect adaptive regulation. Modeling predicted an unfavorable electrostatic environment for Cu^{+} and Cu^{2+} permeation through AQP1. Thus, we propose that changes in water flux, membrane organization or intracellular trafficking may indirectly affect copper-dependent pathways. Altered water permeability could increase cytosolic macromolecular crowding [47], altering the protein-collision kinetics necessary for copper transfer from CTR1 to its molecular chaperones (CCS, ATOX1, and Cox17) [48, 49]. Additionally, disrupted water flux could induce fluctuations in cytosolic pH

influencing the protonation state of cysteine ligands involved in Cu^{+} coordination [50–52] thereby stalling copper targeting to mitochondria and thus preventing the correct enzymatic assembly of CcO. The cells try to compensate for this drastic deficit of intracellular copper content by upregulating CTR1. However, although CTR1 may physically be located at the cell surface, changes in the cell membrane environment (linked to the lack of AQP1 osmosis) and in the pH render it functionally inactive or inefficient (Fig. 5). A possible role of macromolecular crowding and pH-sensitive copper coordination remains a hypothesis to be experimentally tested.

Finally, the exploratory *AQP1–SLC31A2* score showed nominal associations with adverse outcomes differing by *KRAS* status and emerging as a potential translational link between canonical and non-canonical components of the copper-trafficking network. In *KRAS*-mutant CRC, notoriously more aggressive and where tumor recurrence heavily relies on transition metal pathways [20], the signature shows a strong and direct association with a shortened Disease-Free Interval (DFI). Conversely, in the slower and biologically distinct environment typical of *KRAS* wild-type tumors, the gene signature does not impact DFI but correlates positively with a reduction in the Progression-Free Interval (PFI), possibly linking copper acquisition to chronological disease progression. However, these results were obtained after testing several gene combinations and require validation in independent cohorts before the combination can be regarded as a prognostic signature. Larger, independent cohorts and multiplicity-adjusted analyses are required.

This study has limitations that bound its conclusions. The *KRAS*-stratified analyses rest on small subsets (15 *KRAS*-mutant and 11 wild-type patients with serum data; 11 and 9 with paired tissue) and are exploratory, and the gene-combination screening tested multiple models, so the surviving associations are nominal. Caco-2 and HCT-116 are not isogenic, and the different responses therefore cannot be attributed to *KRAS* alone. The AQP1 phenotype rests on pharmacological inhibition and has not yet been confirmed by genetic loss of function with re-expression of a water-dead pore mutant. Finally, whether the copper-delivery defect produced by AQP1 blockade shifts the threshold for copper-induced cell death remains to be tested; this is the subject of ongoing work.

Conclusion

Collectively, these findings establish that colorectal tumours are copper-poor within a copper-rich systemic environment, a dissociation that widens with disease stage and is accentuated in KRAS-mutant disease, and they extend our previous pilot observation [11]. They further place AQP1 within the machinery that supplies copper to mitochondria, acting indirectly rather than as a copper transporter. Because mitochondrial copper governs both cytochrome c oxidase assembly and the threshold for cuproptosis, a step that is dispensable for copper entry yet limiting for mitochondrial copper delivery defines a potential vulnerability: tumours that rely on AQP1 to sustain mitochondrial copper may be selectively sensitised by copper-directed agents. Testing this will require genetic loss of function in isogenic *KRAS* models, direct measurement of copper flux, assays of copper-induced cell death and independent clinical validation.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee (CET Lazio, Area 3) of the Fondazione Policlinico Universitario A. Gemelli IRCCS - Università Cattolica del Sacro Cuore (protocol no. 6091/2023; approval date 26 October 2023). All participants provided written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The TCGA, TARGET, and GTEx datasets analyzed in this study are publicly available through the UCSC Xena platform. The data generated during the clinical and experimental experiments of the study are available from the corresponding authors on reasonable request, subject to institutional ethics approval and applicable privacy restrictions. Supplementary tables and figures supporting the findings are provided as Supplementary Material. Uncropped images of all immunoblots shown in the main and supplementary figures, are provided as a Source Data file.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was carried out as part of the activities of the National Center for Gene Therapy and Drugs Based on RNA Technology, funded in the framework of the National Recovery and Resilience Plan (NRRP), Mission 4 “Education and Research”, Component 2 “From Research to Business”, Investment 1.4 “Strengthening research structures for supporting the creation of National Centres, national R&D leaders on some Key Enabling Technologies”, funded by the European Union - NextGenerationEU, Project CN00000041, CUP B93D21010860004, Spoke 7 “National Center for Gene Therapy and Drugs based on RNA Technology” to G.P. and C.C. This work was also supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC) under IG 2024– ID. 30926 projects to K.A.

Authors' contributions

ADL, RS and GP: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Resources, Supervision; GV, CC, AF, EV, FA, GR, FM, SM, GM, MCAR Investigation, Visualization, Validation; GV and CC Methodology, Visualization, Writing – original draft; MC Resources; KA Investigation, Writing – review & editing; IM and VT Resources, Supervision, Writing – review & editing. All authors read and approved the final manuscript.

References

1. Tsvetkov P, Coy S, Petrova B, Dreishpoon M, Verma A, Abdusamad M, et al. Copper induces cell death by targeting lipoylated TCA cycle proteins. Science. 2022;375:1254–61. doi:10.1126/science.abf0529.
2. Ge EJ, Bush AI, Casini A, Cobine PA, Cross JR, DeNicola GM, et al. Connecting copper and cancer: from transition metal signalling to metalloplasia. Nat Rev Cancer. 2022;22:102–113. doi:10.1038/s41568-021-00417-2.
3. Tang D, Kroemer G, Kang R. Targeting cuproplasia and cuproptosis in cancer. Nat Rev Clin Oncol. 2024;21:370–88. doi:10.1038/s41571-024-00876-0.
4. Schneider BJ, Lee JSJ, Hayman JA, Chang AC, Orringer MB, Pickens A, et al. Pre-operative chemoradiation followed by post-operative adjuvant therapy with tetrathiomolybdate, a novel copper chelator, for patients with resectable esophageal cancer. Invest New Drugs. 2013;31:435–42. doi:10.1007/s10637-012-9864-0.
5. Redman BG, Esper P, Pan Q, Dunn RL, Hussain HK, Chenevert T, et al. Phase II trial of tetrathiomolybdate in patients with advanced kidney cancer. Clin Cancer Res. 2003;9:1666–72.
6. Vahdat LT, Krop IE, Goldstein LJ, Jones LW, Winer EP, Stearns V, et al. Influencing the tumor microenvironment: a phase II study of copper depletion using tetrathiomolybdate in patients with breast cancer at high risk for recurrence and in preclinical models of lung metastases. Clin Cancer Res. 2017;23:666–76. doi:10.1158/1078-0432.CCR-16-1326.
7. Gartner EM, Griffith KA, Pan Q, Brewer GJ, Henja GF, Merajver SD, et al. A pilot trial of the anti-angiogenic copper-lowering agent tetrathiomolybdate in combination with irinotecan, 5-fluorouracil, and leucovorin for metastatic colorectal cancer. Invest New Drugs. 2009;27:159–65. doi:10.1007/s10637-008-9165-9.
8. Pass HI, Brewer GJ, Dick R, Carbone M, Merajver SD. A phase II trial of tetrathiomolybdate after surgery for malignant mesothelioma: final results. Ann Thorac Surg. 2008;86:383–90. doi:10.1016/j.athoracsur.2008.03.016.
9. Vitaliti A, Roccatani I, Iorio E, Perta N, Gismondi A, Chirico M, et al. AKT-driven epithelial-mesenchymal transition is affected by copper bioavailability in HER2-negative breast cancer cells via a LOXL2-independent mechanism. Cell Oncol (Dordr). 2023;46:93–115. doi:10.1007/s13402-022-00738-w.
10. Poursani EM, Mercatelli D, Raninga P, Bell JL, Saletta F, Kohane FV, et al. Copper chelation suppresses epithelial-mesenchymal transition by inhibition of canonical and non-canonical TGF-beta signaling pathways in cancer. Cell Biosci. 2023;13:132. doi:10.1186/s13578-023-01083-7.
11. Squitti R, Tondolo V, Pal A, Rizzo G, Samanta A, Hoque M, et al. Copper dysmetabolism is connected to epithelial-mesenchymal transition: a pilot study in colorectal cancer patients. Biol Trace Elem Res. 2025;203:3604–17. doi:10.1007/s12011-024-04440-w.

12. Squitti R, Pal A, Dhar A, Shamim MA, Ventriglia M, Simonelli I, et al. Serum copper status of patients with colorectal cancer: a systematic review and meta-analysis. J Trace Elem Med Biol. 2024;82:127370. doi:10.1016/j.jtemb.2023.127370.
13. Muñoz-Bravo C, Marín-Burdallo I, González-Herrera L, González-Palacios Torres C, Lozano-Lorca M, Jiménez-Moleón JJ, et al. Copper in colorectal cancer patients: a systematic review and meta-analysis. Carcinogenesis. 2025;46:bgaf001. doi:10.1093/carcin/bgaf001.
14. Ohrvik H, Nose Y, Wood LK, Kim BE, Gleber SC, Ralle M, et al. Ctr2 regulates biogenesis of a cleaved form of mammalian Ctr1 metal transporter. Proc Natl Acad Sci U S A. 2013;110:E4279-88. doi:10.1073/pnas.1311749110.
15. Wang X, Lou Q, Fan T, Zhang Q, Yang X, Liu H, et al. Copper transporter Ctr1 contributes to enhancement of the sensitivity of cisplatin in esophageal squamous cell carcinoma. Transl Oncol. 2023;29:101626. doi:10.1016/j.tranon.2023.101626.
16. Shanbhag VC, Gudekar N, Yasir M, Conrad K, Anakpeba-Dinguyella S, Sutar P, et al. Tumor CTR1 and serum copper dynamics reveal a coordinated copper axis linked to high-grade triple-negative breast cancer biology. medRxiv. 2025. doi:10.64898/2025.12.18.25342516.
17. Xia Y, Liu L, Bai Q, Long Q, Wang J, Xi W, et al. Prognostic value of copper transporter 1 expression in patients with clear cell renal cell carcinoma. Oncol Lett. 2017;14:5791–800. doi:10.3892/ol.2017.6942.
18. Ishida S, Lee J, Thiele DJ, Herskowitz I. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. Proc Natl Acad Sci U S A. 2002;99:14298–303. doi:10.1073/pnas.162491399.
19. Solier S, Müller S, Cañeque T, Versini A, Mansart A, Sindikubwabo F, et al. A druggable copper-signalling pathway that drives inflammation. Nature. 2023;617:386–94. doi:10.1038/s41586-023-06017-4.
20. Aubert L, Nandagopal N, Steinhart Z, Lavoie G, Nourreddine S, Berman J, et al. Copper bioavailability is a KRAS-specific vulnerability in colorectal cancer. Nat Commun. 2020;11:3701. doi:10.1038/s41467-020-17549-y.
21. Pham VVH, Jue TR, Bell JL, Luciani F, Michniewicz F, Cirillo G, et al. A novel network-based method identifies a cuproplasia-related pan-cancer gene signature to predict patient outcome. Hum Genet. 2024;143:1145–62. doi:10.1007/s00439-024-02673-2.
22. Kozono D, Yasui M, King LS, Agre P. Aquaporin water channels: atomic structure molecular dynamics meet clinical medicine. J Clin Invest. 2002;109:1395–9.
23. Yu J, Yool AJ, Schulten K, Tajkhorshid E. Mechanism of gating and ion conductivity of a possible tetrameric pore in aquaporin-1. Structure. 2006;14:1411–23. doi:10.1016/j.str.2006.07.008.
24. Saadoun S, Papadopoulos MC, Hara-Chikuma M, Verkman AS. Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption. Nature. 2005;434:786–92. doi:10.1038/nature03460.

25. Monzani E, Bazzotti R, Perego C, La Porta CAM. AQP1 is not only a water channel: it contributes to cell migration through Lin7/beta-catenin. PLoS One. 2009;4:e6167. doi:10.1371/journal.pone.0006167.
26. Ghosh P, Kornfeld S. AP-1 binding to sorting signals and release from clathrin-coated vesicles is regulated by phosphorylation. J Cell Biol. 2003;160:699–708. doi:10.1083/jcb.200211080.
27. Goldman MJ, Craft B, Hastie M, Repecka K, McDade F, Kamath A, et al. Visualizing and interpreting cancer genomics data via the Xena platform. Nat Biotechnol. 2020;38:675–8. doi:10.1038/s41587-020-0546-8.
28. Vivian J, Rao AA, Nothaft FA, Ketchum C, Armstrong J, Novak A, et al. Toil enables reproducible, open source, big biomedical data analyses. Nat Biotechnol. 2017;35:314–6. doi:10.1038/nbt.3772.
29. Squitti R, Pal A, De Luca A, Rizzo G, Rongioletti M, Tondolo V. Exchangeable copper excess and zinc deficiency in the serum of patients with colorectal cancer. Biol Trace Elem Res. 2025;203:3548–58. doi:10.1007/s12011-024-04431-x.
30. Squitti R, Pal A, Ivanova ID, Marianetti M, Rongioletti M. CLSI validation of exchangeable copper determination in serum by ICP-MS: a focus on Alzheimer's disease and Wilson disease. Biomolecules. 2025;15:788. doi:10.3390/biom15060788.
31. Twomey PJ, Viljoen A, House IM, Reynolds TM, Wierzbicki AS. Copper:caeruloplasmin ratio. J Clin Pathol. 2007;60:441–2. doi:10.1136/jcp.2006.041756.
32. Hollek V, Böhning F, Florez Vargas C, Sieber A, Morkel M, Blüthgen N. Pooled single-cell screen in colorectal cancer defines transcriptional modules linked to oncogenes. Mol Syst Biol. 2026;22:435–460. doi:10.1038/s44320-025-00186-2.
33. Kourghi M, Pei JV, De Ieso ML, Flynn G, Yool AJ. Bumetanide derivatives AqB007 and AqB011 selectively block the aquaporin-1 ion channel conductance and slow cancer cell migration. Mol Pharmacol. 2016;89:133–40. doi:10.1124/mol.115.101618.
34. De Ieso ML, Pei JV, Nourmohammadi S, Smith E, Chow PH, Kourghi M, et al. Combined pharmacological administration of AQP1 ion channel blocker AqB011 and water channel blocker Bacopaside II amplifies inhibition of colon cancer cell migration. Sci Rep. 2019;9:12635. doi:10.1038/s41598-019-49045-9.
35. Dorward HS, Du A, Bruhn MA, Wrin J, Pei JV, Evdokiou A, et al. Pharmacological blockade of aquaporin-1 water channel by AqB013 restricts migration and invasiveness of colon cancer cells and prevents endothelial tube formation in vitro. J Exp Clin Cancer Res. 2016;35:36. doi:10.1186/s13046-016-0310-6.
36. Pepe G, Notturno Granieri C, Appierdo R, Ausiello G, Helmer-Citterich M, Gherardini PF. PANDA: PAN Cancer Data Analysis Web Tool. J Mol Biol. 2025;437:169158. doi:10.1016/j.jmb.2025.169158.
37. Bertinato J, L'Abbe MR. Copper modulates the degradation of copper chaperone for Cu,Zn superoxide dismutase by the 26 S proteasome. J Biol Chem. 2003;278:35071–8. doi:10.1074/jbc.M302242200.

38. Jaksch M, Paret C, Stucka R, Horn N, Müller-Höcker J, Horvath R, et al. Cytochrome c oxidase deficiency due to mutations in SCO2, encoding a mitochondrial copper-binding protein, is rescued by copper in human myoblasts. Hum Mol Genet. 2001;10:3025–35. doi:10.1093/hmg/10.26.3025.
39. Leary SC, Sasarman F, Nishimura T, Shoubridge EA. Human SCO2 is required for the synthesis of CO II and as a thiol-disulphide oxidoreductase for SCO1. Hum Mol Genet. 2009;18:2230–40. doi:10.1093/hmg/ddp158.
40. Migliati E, Meurice N, DuBois P, Fang JS, Somasekharan S, Beckett E, et al. Inhibition of aquaporin-1 and aquaporin-4 water permeability by a derivative of the loop diuretic bumetanide acting at an internal pore-occluding binding site. Mol Pharmacol. 2009;76:105–12. doi:10.1124/mol.108.053744.
41. Du L, Wang H, He L, Zhang J, Ni B, Wang X, et al. CD44 is of functional importance for colorectal cancer stem cells. Clin Cancer Res. 2008;14:6751–60. doi:10.1158/1078-0432.CCR-08-1034.
42. Paulis YW, Huijbers EJM, van der Schaft DWJ, Soetekouw PMMB, Pauwels P, Tjan-Heijnen VCG, et al. CD44 enhances tumor aggressiveness by promoting tumor cell plasticity. Oncotarget. 2015;6:19634–46. doi:10.18632/oncotarget.3839.
43. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A. 2007;104:10158–63. doi:10.1073/pnas.0703478104.
44. Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, et al. CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. Cell Stem Cell. 2014;14:342–56. doi:10.1016/j.stem.2014.01.009.
45. Xie WW, Xu L, Li Q, Zhang DQ, Zhou YB. Aquaporin 1 promotes proliferation and migration of tumor by up-regulating claudin-1 expression in colon cancer. J Pathol Transl Med. 2026;60:307–318. doi:10.4132/jptm.2026.01.01.
46. Üveges E, Gaál A, Szakács G, Bakos É, Streli C, Kregsamer P, et al. Subtype-specific alterations in copper trafficking associated with KRAS mutations in isogenic colorectal cancer cell lines. Biol Trace Elem Res. 2026;204:7038–7053. doi:10.1007/s12011-026-05171-w.
47. Ellis RJ. Macromolecular crowding: obvious but underappreciated. Trends Biochem Sci. 2001;26:597–604. doi:10.1016/S0968-0004(01)01938-7.
48. Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science. 1999;284:805–8. doi:10.1126/science.284.5415.805.
49. Kahra D, Kovermann M, Wittung-Stafshede P. The C-terminus of human copper importer Ctr1 acts as a binding site and transfers copper to Atox1. Biophys J. 2016;110:95–102. doi:10.1016/j.bpj.2015.11.016.
50. Badarau A, Dennison C. Copper trafficking mechanism of CXXC-containing domains: insight from the pH-dependence of their Cu(I) affinities. J Am Chem Soc. 2011;133:2983–8. doi:10.1021/ja1091547.

51. Davis AV, O'Halloran TV. A place for thioether chemistry in cellular copper ion recognition and trafficking. Nat Chem Biol. 2008;4:148–51. doi:10.1038/nchembio0308-148.
52. Rubino JT, Franz KJ. Coordination chemistry of copper proteins: how nature handles a toxic cargo for essential function. J Inorg Biochem. 2012;107:129–43. doi:10.1016/j.jinorgbio.2011.11.024.
53. Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed. Hillsdale, NJ: Lawrence Erlbaum Associates; 1988.
54. Jung K. Book review: Tietz textbook of clinical chemistry and molecular diagnostics, fourth edition. Clin Chem. 2006;52:1214. doi:10.1373/clinchem.2005.062638.
55. Siotto M, Pasqualetti P, Marano M, Squitti R. Automation of o-dianisidine assay for ceruloplasmin activity analyses: usefulness of investigation in Wilson's disease and in hepatic encephalopathy. J Neural Transm (Vienna). 2014;121:1281–6. doi:10.1007/s00702-014-1196-0.
56. Schosinsky KH, Lehmann HP, Beeler MF. Measurement of ceruloplasmin from its oxidase activity in serum by use of o-dianisidine dihydrochloride. Clin Chem. 1974;20:1556–63. doi:10.1093/clinchem/20.12.1556.