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CLASSIFICATION OF COLORECTAL CANCER PATIENTS BASED ON SERUM MICRONUTRIENTS: AN EXPLORATORY INVESTIGATION

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ABSTRACT

Background: Colorectal cancer (CRC) is a growing global health challenge with a multifactorial etiology encompassing genetic susceptibility, nutrition, and inflammation in the bowel.

Objective: To examine micronutrient status in CRC patients undergoing CRC resection.

Design: We performed a case-control study including 13 consecutive CRC patients and 10 healthy controls (CTRL) comparing the serum levels of 29 micronutrients, namely Copper, Zinc, Selenium, Chromium, Manganese, Carnitine, Choline, Inositol, Methylmalonic acid (MMA), Vitamin (Vit) B1, Vit B2, Vit B3, Vit B5, Vit B6, Vit C, Vit A, Vit D3, Vit E, Vit K1, Vit K2 and the amino acids Serine, Valine, Leucine, Isoleucine, Asparagine, Glutamine, Arginine, Citrulline and Cysteine.

Results: After considering the effect of age and sex, copper, arginine, and cysteine were increased, while zinc, selenium, chromium, Vit B1, Vit K1, and Vit A were decreased in CRC patients in comparison with CTRL. Zinc levels perfectly predicted the diagnosis of CRC, and were associated with lymph nodes (pN), of the pTNM staging. Copper levels in serum were strongly associated with the pathological pTNM staging of CRC.

Conclusion: Though this is a preliminary study that needs confirmation with a larger longitudinal cohort, our results show that serum micronutrients are linked to tumor growth, likely caused by increased demand from tumor tissue associated with an aberrant cell proliferation and changes in the antioxidant function.

Keywords: Colorectal cancer; pTNM; micronutrients Copper; Zinc; Cysteine, Selenium; Vitamin B1.

INTRODUCTION

Colorectal cancer (CRC) poses a significant global health challenge, ranking among the most prevalent cancers worldwide, accounting for about 10% of all neoplastic diagnosis, and contributing substantially to cancer-related morbidity and mortality (1). CRC arises from the abnormal proliferation of glandular epithelial cells, typically originating from dysplastic adenomatous polyps and progressing through stages marked by genetic alterations (2,3). Risk factors include age, genetic pathogenic variants, inflammatory bowel disease, and modifiable factors such as diet, smoking, alcohol, physical inactivity, and obesity (4).

Diet and nutrition are fundamental determinants of human health, deeply influencing various physiological processes and disease states. Among all the possible diseases impacted by dietary factors, researchers and clinicians focused their efforts on the analysis of the relationship between nutrition and cancer onset and progression. Of note, about 20–25% of cancer cases worldwide may be connected to patients' nutrition and diet (5). The complex interactions between dietary factors and cancer risk highlight the importance of a comprehensive understanding of the nutrient function in cancer onset (6) and progression and also in the therapeutic management of cancer. CRC stands as a prominent example of the complex relationship between nutrition and cancer (7). Indeed, dietary patterns and individual nutrients may synergistically interact, contributing to CRC risk modulation. Dietary patterns could exert multifaceted effects on CRC risk modulating various interconnected mechanisms including immune responsiveness and inflammation, along with indirect effects stemming from over-nutrition and obesity—both recognized as established risk factors for CRC (8).

Additionally, epidemiological evidence suggests that dietary patterns rich in certain nutrients, such as fibre, antioxidants, and specific vitamins and minerals, may confer protective effects against CRC development (9). Conversely, diets high in processed meats, saturated fats, and refined carbohydrates have been linked to an increased risk of CRC (10). The exploration of the association between nutrients and CRC extends beyond epidemiological observations including molecular mechanisms, preclinical models, and clinical studies demonstrating the alteration of several pathways underlying cancerogenesis, such as inflammation, oxidative stress, insulin resistance, and alterations in gut microbiota composition (11).

The purpose of the present case-control study was to investigate the levels of an extended panel of micronutrients and amino acids (29 in total) between healthy controls (CTRL) and CRC patients to elucidate the nutritional phenotype underlying CRC.

SUBJECTS AND METHODS

Subjects

The study protocol was approved by the local ethics committee and subjects provided written informed consent (protocol approval number 6091/2023). Patients entered in the study were

consecutively recruited at the Digestive and Colo-rectal Surgery Unit of Isola Tiberina Hospital Gemelli Isola. CTRL were blood donors consecutively enrolled at the Ematos-Fidas Unit of the same hospital. Patients' inclusion criteria were age>18 years and endoscopic and histological diagnosis of colorectal cancer (National Comprehensive Cancer Network - NCCN - Guidelines Colon Cancer 2023). The recruitment period for both patients and CTRL lasted from November 2023 to January 2024. After diagnosis, all CRC patients entered a diagnostic pathway to establish the clinical staging and the resectability of the tumor.

Clinical staging was established by abdominal-thoracic computed tomography (CT) scan and pelvic magnetic resonance imaging (MRI) in patients affected by colorectal cancer. Patients with resectable non-metastatic colon cancer underwent radical surgery. Patients with metastatic colon cancer were evaluated by a multidisciplinary team to establish the need for primary surgery or primary chemotherapy according to the most recent CRC guidelines. For each patient who underwent oncological radical colorectal resection, a pTNM tumor staging was established (12,13). Patients with a pTNM stage 4 underwent RAS/protein mitogen-activated protein kinases (MAPK) pathway genotyping to select patients who can benefit from specific drugs (e.g. monoclonal antibodies against the Epidermal Growth Factor Receptor in metastatic CRC patients without mutations); briefly: DNA was extracted from formalin-fixed paraffin-embedded samples and the mutations were detected by real-time PCR. The kit (EasyPGX) is composed of assays for the detection of *KRAS* (Kristen rat sarcoma, KRAS PROTOONCOGENE, GTPase; MIM *190070) (G12R/S/C, G12V/D/A, G13D, A59x, Q61x, K117x, A146x), *NRAS* (NRAS PROTOONCOGENE, GTPase; MIM *164790) (G12x-G13x, A59x-Q61H, Q61K, Q61R, Q61L, K117x, A146x) and *BRAF* (B-RAF PROTOONCOGENE, SERINE/THREONINE KINASE) (V66E/Ec) targeted mutations. The detection is achieved through fluorescent probes labelled with the target variants and the endogenous control gene, to exclude a false negative result. The percentage of tumor cells in the sample must be at least 70%. The test does not detect variants expressed below 5%.
Both patients and controls with conditions known to affect copper metabolism (e.g. diabetes mellitus, recent history of heart or respiratory failure, chronic liver, or renal failure a recent history of alcohol abuse or dementia) were excluded.

Fasting blood samples were collected at the Ospedale Isola Tiberina – Gemelli Isola, Rome, Italy in the morning after an overnight fast the day of the CRC surgery. Serum was separated by centrifugation at 3000 rpm, 10 min, and 4°C and then divided into 1 mL aliquots and rapidly stored at -80°C. The subjects' samples were shipped to Vibrant America Clinical Lab (3521 Leonard Ct, Santa Clara, CA 95054, US) in dry ice, for blinded biochemical analysis. The aliquots were thawed just before the assay.

Analytical methods

All participants underwent assays of 29 micronutrients, namely copper, zinc, selenium, chromium, manganese, Carnitine, Choline, Inositol, Methylmalonic Acid (MMA), Vit B1, Vit B2, Vit B3, Vit B5, Vit B6, Vit C, Vit A, Vit D3, Vit E, Vit K1, Vit K2, Serine, valine, Leucine, Isoleucine, Asparagine, Glutamine, Arginine, Citrulline, and Cysteine.

Protocol for Analysis of trace metals in serum – (Copper, Zinc, Selenium, Chromium, Manganese):

The analysis of trace metals (copper, zinc, selenium, chromium, manganese) in serum was done using the Inductively coupled plasma mass spectrometry (ICP-MS) methodology using the Agilent 8900 QQQ instrument. For the analysis of serum samples, the QC and calibrator samples were removed from the 4°C refrigerator and allowed to warm up to room temperature. For the quality control, the analytes used include Copper (Cu) and Zinc (Zn) from Sigma-Aldrich (Catalog # 94459 and 68961, respectively), Selenium (Se) and Chromium (Cr) from PerkinElmer (Catalog # N9300182 and N9300173, respectively), and Manganese (Mn) from Sigma-Aldrich (Catalog # 42071). Subsequently, all samples were thoroughly vortexed. For ICP-MS analysis, the basic diluent was prepared by combining 5 mL of tetramethylammonium hydroxide (TMAH), 20 mL of methanol, and 100 mg of ammonium pyrrolidine dithiocarbamate (APDC) in a total volume of 1 L using 18 MΩ water. This solution contained 0.5% TMAH, 0.05% Triton-X, 2% methanol, and 0.01% APDC. Additionally, the internal standard solution was prepared by adding 50 µL of 1000 µg/mL gallium (PerkinElmer) to 1 L using 18 MΩ water, resulting in a concentration of 50 µg/L gallium in the final solution. Each Agilent test tube received 3.925 mL of ICP-MS basic diluent, to which 75 µL of serum was added. For calibrators, 40 µL was added to 4 mL of ICP-MS basic diluent. Mixing of each tube was performed using a pipette. Finally, the samples were analyzed on ICP-MS.

Protocol for Analysis of Metabolism and Energy Production Group- (Carnitine, Choline, Inositol, Methylmalonic acid (MMA)):

The analysis of serum water-soluble vitamins, carnitine, choline, inositol, and methylmalonic acid (MMA) was conducted using the LC/MS (Xevo-TQ-XS) mass spectrometer (WBA0127). A 2 mL 96-well plate was utilized, with 50 µL volumes of calibrators (prepared in water), quality controls (Sigma), and serum samples added to individual wells. To each well, a volume of 320 µL of 2:1::6% meta-phosphoric acid: methanol was added and followed by thorough mixing (5-6 times). The plate underwent centrifugation at 3700 Xg for 5 minutes with low deceleration to facilitate separation. Following centrifugation, 120 µL of the supernatant from each well was carefully transferred to a final 2 mL 96-well plate. This final plate was then positioned on the LC/MS instrument for analysis.

Protocol for Analysis of Serum Levels of Water-Soluble Vit B1, Vit B2, Vit B3, Vit B5, Vit B6, Vit C:

The analysis of serum water-soluble was conducted using LC/MS (Xevo TQ-XS) in a 2 mL 96-well plate, where 50 µL of serum samples, quality controls (Sigma), and calibrators (prepared in water) were added. To each test solution, 320 µL of a mixture containing 6% meta-phosphoric acid and methanol in a 2:1 ratio was added, and the samples were mixed adequately. For LC/MS analysis, 110 µL of supernatant was transferred to a new 2 mL 96-well plate and subjected to analysis.

Protocol for Analysis of Serum Levels of Fat-Soluble Vit A, Vit D3, Vit E, Vit K1 and Vit K2:

The fat-soluble vitamins in the serum were analyzed using LCMS (Xevo-TQ-XS) mass spectrometer. For this, an appropriate volume of serum samples, calibrators (made in commercial serum, Golden West Biologicals, Catalog # MSG 3000), and quality controls (150 µL for vitamins A (Cerilliant, Catalog # V-011), D3 (Cerilliant, Catalog # V-025), and E (Cerilliant, Catalog # V-020); 50 µL for vitamins K1 (Cerilliant, Catalog # V-030) and K2 (Cerilliant, Catalog # V-044)) was added to a 2 mL 96-well microtiter plate. Each sample was diluted to a final volume of 100 µL by adding 80 µL of deionized water. Further, each well was

supplemented with 720 µL of a 1:1 mixture of isopropyl alcohol and ethyl alcohol. Internal standards were added to each sample: For vitamins A, D3, and E: 1 µg/mL of vitamin A-d4 retinyl palmitate and vitamin E phenyl- $^{13}C_6$; and for vitamins K1 and K2: 1 ng/mL of vitamin K1-$^{13}C_6$ and vitamin $^{13}C_6$ (MK-7). The plates were sealed and centrifuged at 6000 rpm for 5 minutes. After centrifugation, 400 µL of supernatant was transferred to a fresh 2 mL 96-well plate followed by the addition of 400 µL of a mixture of IPA and deionized water (at a ratio of 5:3), and the contents were gently mixed. Elution was performed using a solid-phase extraction system with an OASIS Prime HLB plate containing 10 mg of sorbent per well, in a Waters vacuum manifold equipped with a waste collection plate. The sorbent was conditioned by eluting 100 µL of 25% acetonitrile at 2.5" Hg to remove interfering substances. Subsequently, 750 µL of the reaction mixture was added to the wells of the OASIS Prime HLB elution plate and passed through the sorbent at a 5" Hg vacuum. The analytes were eluted with 60 µL of acetonitrile at a 2.5" Hg vacuum, and this step was repeated twice. The eluted analytes were then analyzed for the estimation of vitamins A, D3, E, K1, and K2.

Protocol for Analysis of amino acids in serum:
The analysis of nine amino acids (Serine, Valine, Leucine, Isoleucine, Asparagine, Glutamine, Arginine, Citrulline, and Cysteine) in serum was conducted using LC/MS (Xevo-TQ-XS) mass spectrometry. In a 2 mL 96-well microtiter plate, 20 µL of serum samples, calibrators, and quality controls were aliquoted. Materials used include Serine, Valine, Leucine, Isoleucine, Asparagine, Glutamine, Arginine, Citrulline, and Cystine from Sigma, and calibrator and QC commercial serums from Golden West Diagnostics (Catalog # MSG3000 and SP1010). Subsequently, 20 µL of internal standard solution (Chromsystems, Catalog #75146) was added to each well. 600 µL of assay solution (85:15: :acetonitrile: water, 0.2% formic acid, 10 mM ammonium formate) was added to all wells followed by centrifugation at 3700 Xg for 5 minutes with low deceleration. After centrifugation, 100 µL of supernatant from the centrifuged plate was carefully transferred to a final 2 mL 96-well plate for analysis.

The workflow depicted in Figure 1 provides an overview of the key stages involved in the study, encompassing study design, approval, experiment and sample collection, sample storage and shipment, and LCMS analysis. This graphical representation serves as a visual guide to the sequential progression of activities conducted throughout the course of the investigation.

Statistical analysis
CRC patients and CTRL subjects were described in terms of main demographic and biological characteristics and statistically compared with $\chi 2$ test, test U di Mann Whytney or Kruskal-Wallis based on type or variable distribution (continuous or categorical and Gaussian or non-Gaussian). Age and sex were taken into consideration as covariates when appropriate. Comparison among continuous biological variables were carried out by multivariate ANOVA model (MANOVA) - in order to take into account correlation among biological variables of the same category measured for the whole study sample and UNIANOVA that provides regression analysis and analysis of variance for a dependent variable based on one or more factors and/or variables including sex and age; more specifically by five MANOVA and one UNIANOVA study taking into account the effect of sex and age have been carried out accounting for the six different biological variable categories namely: i) trace metals, which included copper, zinc, selenium, chromium, and manganese; ii) metabolism and energy production group, which included carnitine, choline, inositol and MMA; iii) vitamin of the B group, which included Vit

B1, Vit B2, Vit B3, Vit B5, and Vit B6; iv) antioxidant and calcium homeostasis, which included Vit C, Vit A, Vit D3, and Vit E; v) vitamin of the K group, which included Vit K1 and Vit K2, was specifically assessed using UNIANOVA; vi) amino acid group which included serine, valine, leucine isoleucine, asparagine, glutamine arginine, citrulline, and cysteine. Variables distribution normality was assessed by the Kolmogorov-Smirnov test ($p>0.05$). The goodness of fit of MANOVA models was evaluated by the analysis of residuals (14). In case the biological variable was not normally distributed, a Kruskal Wallis test was performed (as indicated in the footnote of the tables). Multiple logistic regression and the forward likelihood-ratio method was applied to identify which biological variables better discriminated CRC patients from CTRL. Finally, correlation analyses between biological variables and tumor classification (partial correlation correcting for age) were performed. A *p*-value less than 0.05 was considered significant in all statistical analyses, except when a correction for multiple comparisons was appropriate. In this case, the Bonferroni's method was used. We used the SPSS 16.0 for Windows statistical software package (SPSS Inc., Chicago), and version 9.3.1 of the GraphPad Prism program for graphs.

RESULTS

During the trial period of our case-control study, 13 consecutive CRC patients were recruited. Serum levels of oligo-elements and vitamins obtained from CRC patients were compared to those of 10 CTRL.

CRC patient clinical characterization

The CRC and CTRL groups did not differ in sex but differed in age distribution (Table 1). Among the biological variables under study, while zinc (rho = - 0.52, $p<0.01$) and selenium (rho = - 0.45, $p=0.030$), chromium (rho = - 0.51, $p=0.013$) and Vit A (rho = - 0.473, $p=0.023$) were negatively associated, isoleucine (rho = 0.439, $p = 0.036$), arginine (rho = 0.452, $p = 0.030$) and cysteine (rho = 0.791, $p < 0.001$) were positively associated with increasing age.

In Table 1, it is reported the TNM classification based on assessing the tumor, regional lymph nodes, and distant metastasis (13) of the patients participating in the current case-control study; only 23% of the patients had a low level of CRC, corresponding to 1 as per the TNM classification.

2.1. CRC patients and CTRL comparison for oligo-elements panel

Oligo-elements were analysed in the 13 CRC patients and 10 CTRL. Various types of ANOVA analyses considering the effect of sex and age were carried out for each of the biological variable groups under study (see Methods section). While copper and manganese were higher in CRC patients (the latter not reaching the statistical threshold), zinc, chromium, and selenium were lower in CRC than in the CTRL group (Table 2; Figure 2).

Among the cases analyzed, the Real-Time PCR study led to the identification of the KRAS gene variant G12X in 2 out of 13 CRC specimens, the NRAS gene variant G13D in 1 out of 13, and the BRAF gene variant V600E in 1 out of 13 CRC specimens; cumulative data of serum levels of the essential metals under study in the patients with somatic mutations of the RAS/MAPK pathway has been reported in Table 2.

None of the biomarkers of metabolism and energy production showed a significant difference between CRC patients and CTRL group (Table 3).

Among the Vitamin B group, only the Vit B1 resulted lower in CRC patients than in the CTRL group (Table 4).

Additionally, from the UNIANOVA analysis, we found a significant reduction of vitamin K1 levels in CRC patients in comparison to the CTRL group, while vitamin K2 did not show any significant difference (Table 4).
Among vitamins exerting an antioxidant function included in the study, only Vit A significantly decreased in CRC patients in comparison with the CTRL group (Table 5). Finally, among the amino acids considered, arginine and cysteine significantly differed between CRC and CTRL groups, being both higher in CRC patients than in CTRL. Additionally, we observed a trend in decreasing of glutamine levels in CRC patients ($p = 0.05$, Table 6).

Association of the biological variables under study with the CRC diagnosis

Zinc levels perfectly predicted the diagnosis of CRC, considering the sharp separation between the serum zinc values in CRC patients, who exhibit markedly lower levels, and those in CTRL individuals, precluding the application of a regression model; the association between decreased zinc levels and the diagnosis of CRC was clear: the lower the zinc values, the higher was the probability of being affected by CRC (rho= -0.859, $p<0.0001$). A univariable binary logistic regression model was applied to evaluate the effect of the other biochemical variables under study on the probability of having CRC. Age and serum concentration of copper and arginine increased the risk of having CRC. The strongest effect observed was for copper since an increase of 1 µmol/L of Cu in serum could account for about 50% of the risk of having CRC. Conversely, selenium, chromium, Vit B1 and Vit A appeared to reduce the risk for CRC. A multivariable analysis was performed that included the biochemical variables with a p value < 0.050 at the univariable analysis. The model revealed an effect of Vit A on the probability of having CRC with a decrease of the risk of 17% (95% CI 0.692-0.990; p=0.039) for each µg/dL unit increase in Vitamin A when keeping constant all the other independent biological variables under study (Table 7).

Association between clinical indices of CRC and the biological variables under study

After correcting for the age effect (partial correlation corrected for age), serum copper correlated with indices of the size of the tumor (cT, $r = 0.627$, $p = 0.029$), number of the lymph nodes (cN $r = 0.69$, $p = 0.013$) and stage of the tumor (cStage, $r = 0.706$, $p = 0.010$) as revealed during the radiological diagnosis; the worse the diagnosis the higher the copper. After CRC surgery, values of the indices of the pathological TNM staging (pTNM), namely pT, pN, pM evaluated at the histopathological examination were analyzed in association with the biological variables under study, correcting for the age effect. The analysis revealed that copper in serum was associated with the pTNM staging ($r = 0.588$, $p = 0.044$), driven by the size of the tumor (pT, $r = 0.743$, $p = 0.006$). None of the other biological variables under study were associated with the clinical features of the patients.

DISCUSSION

The best result of the current case-control study is that copper levels in serum were strongly associated with the pathological pTNM staging of CRC. According to the guidelines of the Union for International Cancer Control (UICC) (12,13), pathological pTNM staging is the most robust prognostic factor in CRC. It builds the backbone of post-operative clinical decision-

making. More specifically, the TNM classification includes the following clinical parameters: pT indicating the extent of local invasion, pN indicating the involvement of regional lymph nodes by the tumor, and pM reporting the presence of distant metastases. Based on this 4-tiered staging system (UICC I–IV), CRC patients generally receive stage-adapted treatment. Thus, the pathological pTNM staging is one of the clinically most important parameters generated by pathologists and it is the foundation of post-operative clinical decision-making and patient management (15). In this case-control study, we demonstrated that copper serum levels were associated mainly with the extent of local tumor invasion (pT). Noteworthy, current results are coherent with the evidence showing that patients with different types of cancer (breast, prostate, lung and larynx) with a copper level in the highest quartile, have a 1.91 increase in the hazard ratio of mortality (16). Additionally, we further confirm previous studies indicating that serum copper is increased in CRC, even though data are non-univocal in literature (17). While copper is an indispensable cofactor in the body, it has been recognized as a double-edged sword as its overabundance can be detrimental to cells leading to cell death. Dysregulated intracellular copper bioavailability is known to induce oxidative stress via the Fenton-like chemistry, by contributing to the formation of the highly reactive species, hydroxyl radical (HO•) (18,19). Copper toxicity has been attributed to its activity as a catalyst for oxidative damage to tissues through redox cycling between Cu(I) and Cu (II), particularly in the presence of H_2O_2 (19,20). Moreover, copper can activate the Mitogen-activated protein kinase 1/2 (MEK1/2) which can upregulate the expression of genes associated with oxidative stress. Specifically, copper aids in the activation of MEK/ERK pathway by binding to allosteric sites, whose dysregulation is closely related to cancer initiation and progression. In addition to this pathway, copper was demonstrated to induce the PI3K/AKT signaling pathway by binding 3-phosphoinositide-dependent protein kinase 1. This action results in the activation of its downstream AKT kinase contributing to tumorigenesis (21).

Another result is that, zinc serum concentration is significantly decreased in CRC patients and perfectly predicted the diagnosis of CRC, as observed by the striking lower values for zinc serum observed in CRC patients compared to CTRL. The withdrawal of zinc from general circulation observed in the current study could be likely associated with an increased demand for zinc by i) the liver, relative to inflammation processes triggered by cancer or damaged tissues, ii) by the cancer tissues which require the metal to grow and proliferate, and iii) by the immune system, including the lymph nodes, where zinc regulates maturation of lymphocytes, reacting to the cancer condition(22). Overall, our data are in accordance with the literature showing that dysregulation of zinc homeostasis is crucial for various forms of cancer, including CRC.

Beyond its role in the activation of antioxidant enzymes, such as glutathione peroxidase, catalase (23), and the Cu, Zn SOD, and its role in inhibiting the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, engaged in mitochondria reactive oxygen species (ROS) production (24), zinc coordinates a domain of the transcriptional factors zinc finger proteins which, by binding to DNA, regulate the gene expression of a multitude of proteins and enzymes belonging to different cell pathways. Of note, through several cell signaling mechanisms such as those including RAS, Wnt, Nuclear factor-κB (NF-κB), and protein kinase 3 (PK3), zinc finger proteins control cell differentiation and cell proliferation, as well as DNA repair mechanisms whose alterations, could lead to carcinogenesis, favoring also cell migration and invasion. These proteins are also required for epigenetic modifications that can promote epithelial-mesenchymal transition. It is also noteworthy that in the subgroup of patients who

underwent the target analysis for the search for mutations in *KRAS, NRAS,* and *BRAF*, the values of serum copper levels were high, close to the upper value of the normal reference range. This finding is in agreement with previous data showing a copper dependence of KRASG12X and BRAFV600E CRC to sustain their abnormal proliferation and thus their energetic metabolism (25,26). In this regard, specific copper chelators could be particularly useful as chemotherapeutic adjuvants targeting specific signaling pathways constitutively activated in mutated KRAS, NRAS, and BRAF. Finally, zinc finger proteins play a role in cell cycle arrest and in promoting apoptosis by regulating mitochondrial metabolism and protein degradation triggered by ubiquitin and the proteasome system (recently reviewed in (27)). Besides, our results are in agreement with a meta-analysis on zinc intake in CRC showing that dietary zinc intake was negatively related to and increased risk of CRC, reducing by 80% the relative risk for CRC (28).

Interestingly other oligo-elements including vitamins (Vit B1, Vit K2, and Vit A), amino acids (arginine and cysteine), and selenium and chromium, which have a significant influence on the immune system function, were abnormal in CRC patients in comparison with CTRL. Concerning vitamins, in our study we observed that Vitamin B1 (thiamine) levels were lower in CRC patients compared to CTRL. Cancer cells exhibit an altered metabolism due to its unique requirements including a preference for aerobic glycolysis more than mitochondrial oxidation of pyruvate. This provides anabolic support and a selective growth advantage for cancer cells. As per the latest literature, these metabolic changes may be due, in part, to decreased mitochondrial function which arises from the Pyruvate dehydrogenase (PDH) complex inhibition (29). The same also holds for CRC (30). It is well established that PDH complex deficiency, a mitochondrial disorder of carbohydrate oxidation, mostly affects the brain and leads to decreased ATP production, energy deficit and decreased glycolysis (31,32). Interestingly, the human PDH complex catalyzes the thiamine-dependent decarboxylation of pyruvate. Thiamine treatment is very effective for some patients with PDH complex deficiency (33–35), suggesting a potential therapeutic strategy for combating CRC. Protein lipoylation, consists of a highly conserved lysine post-translational modification, and it is known to occur on only four enzymes, one of these is Dihydrolipoamide S-Acetyltransferase (DLAT), an essential component of the PDH complex. The lipoylation of this protein is known to be required for enzymatic function (35). Very recently, it has been shown that copper directly binds and induces the oligomerization of lipoylated DLAT proteins leading to a recently discovered pathway of cell death termed as cuproptosis (36). Taken together, the role of copper and Vit B1 in CRC seems pivotal and needs to be further elucidated. Low levels of thiamine in the serum have been linked to an increased risk of cancer, including CRC, suggesting a potential protective role for adequate thiamine levels. Genetic research has elucidated the involvement of various genes and factors in CRC development, including those related to thiamine transport such as SLC19A1 and SLC19A3. Additionally, factors like transcription factor p53, transketolase, NOX, and PARP have been implicated in CRC pathogenesis, potentially influencing cellular processes relevant to CRC. Furthermore, non-genomic mechanisms such as alterations in protein expression, inflammation, oxidative stress, and cell metabolism are also implicated in the association between thiamine levels and CRC. Thiamine may modulate these pathways, thereby influencing CRC development directly or indirectly (37).

In CRC patients, there is a notable decrease in serum Vitamin A (retinol) levels compared to the control group. This decline in retinol has been consistently observed in various studies and has been linked to poorer survival rates among CRC patients (38–40). However, evidence regarding

specific survival outcomes and dose-response relationships has been limited due to the small number of studies and patients included (41). Retinol is suggested to affect signalling cascades, notably via pathways such as the β-catenin pathway, which are pivotal in promoting cell proliferation and invasion within CRC. Additionally, it is implicated in the promotion of tumor cell differentiation, a process that potentially hinders the progression of malignancy. Furthermore, retinol exhibits the capacity to induce apoptosis in tumor cells, thereby contributing to the suppression of CRC development (42). In murine models, studies have revealed that alterations in retinoic acid (RA) metabolism significantly contribute to inflammation and tumorigenesis within the colorectal milieu. However, this deleterious effect can be mitigated by the restoration of RA concentrations, indicating a crucial role for RA homeostasis in modulating the inflammatory microenvironment and tumorigenic processes (43). Inhibition of critical pathways by retinol suggests that dietary vitamin A supplementation or retinoid chemotherapy, either alone or in combination with other medications, may hold promise for preventing the progression and metastasis of CRC (42).

Vitamin K is increasingly being reported to be associated with cancer due to its newly discovered functions including activation of the steroid and xenobiotic receptor and regulation of apoptosis, autophagy and oxidative stress (44). *In vitro* studies have shown that Vitamin K1 exerted anti-cancer activities in colon cancer cell lines (45,46). The same is also corroborated in animal studies (47).

Concerning amino acids, serum arginine levels were higher in CRC individuals as compared to CTRL, indicating a potential dysregulation in arginine metabolism. This dysregulation may contribute to the pathogenesis of CRC by facilitating tumor cell proliferation and survival. Previous studies have demonstrated that arginine is not only a critical nutrient for tumor growth but also plays a dual role in modulating the tumor microenvironment (48,49). Research by Paz et al. (50) highlighted the significance of arginine metabolism in CRC progression, showing that elevated levels of arginine promote tumor growth and metastasis through various mechanisms, including the synthesis of polyamines and nitric oxide. Furthermore, the overexpression of argininosuccinate synthetase 1 (ASS1) in CRC cells reinforces their reliance on arginine for proliferation and survival (51).

Moreover, the dysregulated arginine metabolism observed in CRC presents a therapeutic opportunity. While arginine deprivation therapy has shown promise in cancers with deficient expression of ASS1, such as liver cancer and melanoma, its efficacy in CRC may be limited due to the tumor's ability to upregulate arginine synthesis pathways (52,53). Instead, strategies aimed at increasing arginine levels or modulating its metabolism could offer a novel therapeutic approach for CRC patients. Importantly, preclinical studies using CRC mouse models have demonstrated the potential synergy between elevated arginine levels and immune checkpoint inhibitors such as PD-1/PD-L1 inhibitors (54). These findings suggest that enhancing arginine levels in combination with immunotherapy could improve treatment outcomes for CRC patients by enhancing the anti-tumor immune response and inhibiting tumor growth.

In CRC, cysteine levels have been found to be higher compared to CTRL. Cysteine can promote cancer cell proliferation and growth by activating a cellular pathway known as the Mammalian target of rapamycin complex1 (mTORC1). mTORC1 is a key regulator of cell growth and metabolism, and its activation is associated with increased cancer cell proliferation. Cysteine activates mTORC1, leading to enhanced cancer cell growth and proliferation, primarily through

the GCN2-ATF4-SESN2 axis. This signalling pathway, activated by amino acid availability, involves GCN2 phosphorylation of eIF2α, inducing ATF4 activation and subsequent SESN2 expression. The intracellular levels of essential amino acids modulate this pathway; when cysteine levels are high, they inhibit GCN2 phosphorylation, reducing ATF4 levels and resulting in decreased SESN2 expression. As SESN2 acts as a negative regulator of mTORC1, its downregulation leads to mTORC1 activation, promoting cancer cell growth (15). Additionally, cysteine can also prompt the acquisition of chemoresistance in CRC cells, making them less responsive to chemotherapy drugs like oxaliplatin and irinotecan (15). Indeed, cysteine availability is the major determinant for the synthesis of the tripeptide glutathione (GSH), a key antioxidant molecule. By increasing GSH levels, cysteine helps to eliminate ROS generated by chemotherapy drugs like, oxaliplatin and irinotecan, resulting in the reduction of their cytotoxic effects and thus protecting cancer cells from apoptosis, leading eventually to chemoresistance (15). Besides the antioxidant role associated with GSH and with a reversible post-translational modification, cysteine can be a substrate for the production of hydrogen sulphide (H_2S), which feeds the mitochondrial electron transfer chain. H_2S also mediates per-sulphidation of ATPase and glycolytic enzymes, thus facilitating cellular bioenergetics. Another role of cysteine in cancer can be as a carbon source for epigenetic regulation (55).

Also, selenium is required for the antioxidant systems of cells. It is important to note that glutathione peroxidase is another major player in the defence against oxidative stress and contains one atom of selenium located in the active site of the enzyme (56,57). Genetic alterations in the genes coding for glutathione peroxidase, especially under conditions of low selenium levels can severely lead to the increased risk of oxidative stress. This highlights the intricately crucial role of selenium in conferring protection against oxidative stress (58). Interestingly, we observed a significant decrease in selenium in CRC patients compared to the CTRL. This observation is in line with various studies that have indicated that CRC patients had lower levels of selenium than healthy controls (59). Among the various selenoproteins, Selenof (the 15 kDa selenoprotein) is widely expressed in high levels in the liver, prostate, kidney, testis, and brain. Human and animal CRC studies have indicated that the downregulation of Selenof correlated with CRC. Selenof regulates oxidative protein folding, signaling in the cellular misfolded protein response. It may also function as a redox quality control for immunoglobulins. Additionally, Selenof has been suggested to be involved in cell replication, invasion and metastasis, as well as a potential regulation of interferon (IFN)-γ-mediated signaling pathways. A study showed that Selenof-KO mice has increased levels of inflammation in the form of elevated serum interferon (IFN)-γ expression. Moreover, these mice had altered intestinal barrier integrity which can contribute to the pathogenesis of CRC. These findings reinforce the inverse relationship between dietary selenium levels and the risk of CRC, highlighting the functional role of selenoproteins in CRC (60).

Another important observation in this study was that cysteine levels in the CTRL group fell below the normal reference range, indicating a potential deviation from expected physiological levels. This observation raises concerns regarding a possible imbalance or deficiency in cysteine levels within the CTRL group. Thus, further investigation into the underlying factors contributing to this decrease is imperative to gain a deeper understanding of its implications in CRC development and progression. While the current study demonstrated a relationship between nutrient levels and CRC, future studies can aim to elucidate the association of nutrients like copper with Tumor, Node, and pTNM staging system. This may help understand whether underlying biological variables can provide information on the onset and progression of CRC. These investigations have the potential to have diagnostic, prognostic, and therapeutic implications in CRC.

The current study has several limitations regarding primarily the size of the sample that limits the interpretation of case-control study, thus the results shown must be taken with caution and need to be confirmed in bigger populations and longitudinal cohorts. Notwithstanding this weakness, the main strength of the current study is the wide range of the micronutrients analyzed that have been studied in association with the CRC pTNM staging.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by R.S., L.D.V., G.R., V.T., H.K. The first draft of the manuscript was written by R.S., H.K., J.B., and all authors commented on previous versions of the manuscript. Revising the manuscript critically for important intellectual content: A.P., A.D.L., V.T., G. R., M.R. All authors read and approved the final manuscript.

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COMPETING INTERESTS

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

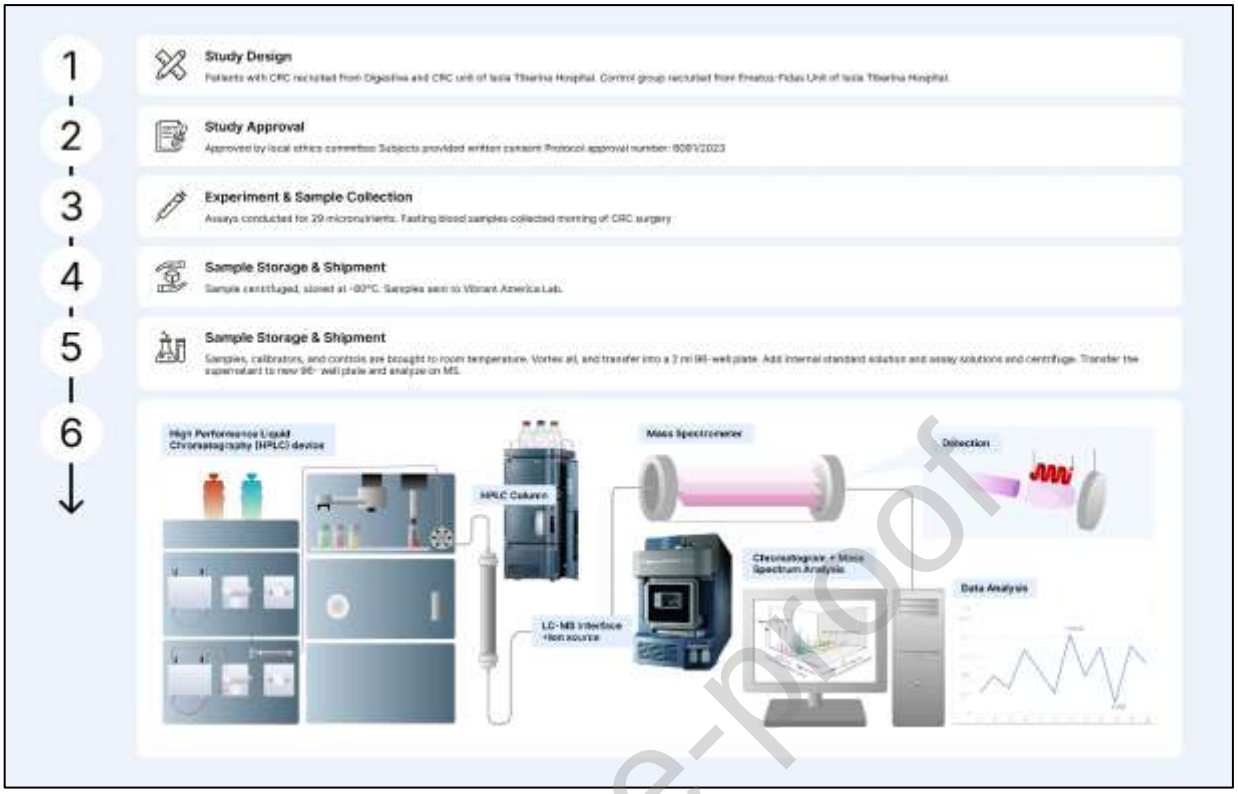

Figure 1. Study Workflow Overview

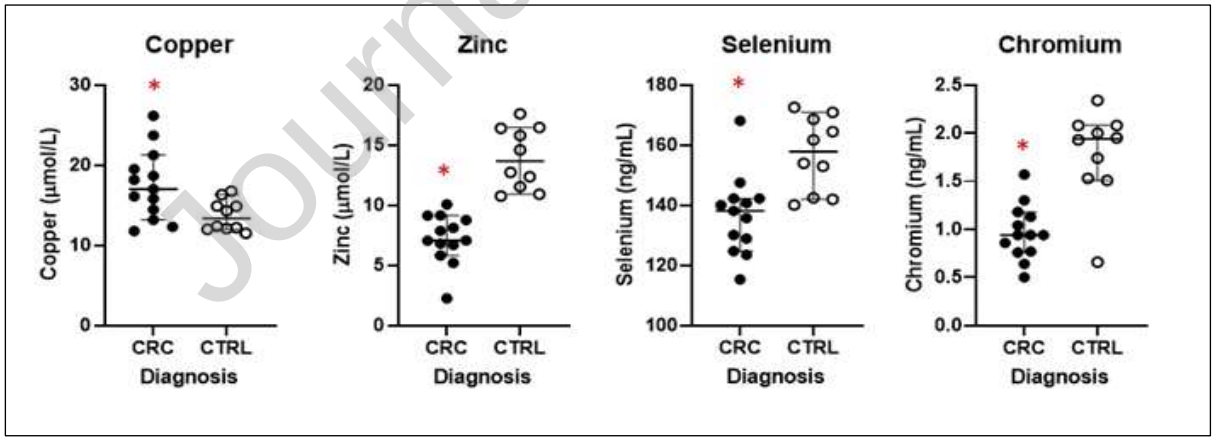

Figure 2. Comparison of the serum levels of trace metals between CRC and CTRL individuals. Data are median and 95%CI of copper (Cu), zinc (Zn), Selenium (Se), and Chromium (Cr) (*$p < 0.05$). Raw data are shown, not adjusted for subjects' differences in sex and age.

Table 1. Demographical characteristics of the study subjects.

	Heathy controls (CTRL) N= 10	Colorectal cancer (CRC) N= 13	p value
Men, n (%)	8 (80%)	6 (46.2%)	0.111[a]
Women, n (%)	2 (20%)	7 (53.8%)	
Age (± SD)	48.5 ± 9.53	68.9±14.22	0.001[b]
Patients' characteristics of the study subjects			
Patients with classification TNM 1, n (%)		3 (23%)	
Patients with classification TNM 2, n (%)		4 (31%)	
Patients with classification TNM 3, n (%)		4 (31%)	
Patients with classification TNM 4, n (%)		2 (15%)	

SD, standard deviation; a: $\chi 2$ test; b: test U di Mann Whytney

Table 2. Comparison between Colorectal cancer (CRC) and healthy controls (CTRL) subjects for trace metals [Mean, Standard deviation (SD)]; cumulative data of the serum levels of the essential metals in the patients with somatic mutations of the RAS/MAPK pathway were also reported.

	CRC patients N= 13	CTRL subjects N= 10	Test (degrees of freedom)	P value (CRC vs. CTRL)	Normal Reference range values	CRC patients with RAS/MAPK mutation N= 4
Copper, µmol/L	17.5 ± 4.4	13.7 ± 1.9	$F_{(1,22)}$=13.13	**= 0.030**	11-24.4	22.7 ± 2.9
Zinc, µmol/L	**7.2 ± 2.1**	13.9 ± 2.5	-	**= 0.002[a]**	12-24	7.3 ± 2.0
Selenium, ng/mL	136.7 ± 13.1	157 ± 12.5	-	**= 0.002[a]**	109.8-218.4	136.5 ± 5.1
Chromium, ng/mL	1.0 ± 0.3	1.8 ± 0.5	$F_{(1,22)}$=6.665	**= 0.018**	0.7-28.0	1.1 ± 0.3
Manganese, ng/mL	1.3 ± 0.2	1.1 ± 0.4	$F_{(1,22)}$=3.928	=0.062	0.3-2.0	1.3 ± 0.3

A multivariate ANOVA (MANOVA) model including zinc, chromium, and manganese was run to assess the effect of diagnosis, controlling for sex and age; [a]Kruskal Wallis test

Table 3. Comparison between CRC and CTRL subjects for metabolism and energy production group variables (mean, SD)

	CRC patients N= 13	CTRL subjects N= 10	Test (degrees of freedom)	P value	Normal Reference range values
Carnitine, nmol/mL	38.3 ± 12.9	35.8 ± 7.0	$F_{(1,22)}$=1.346	= 0.26	11.6-43.4
Choline, nmol/mL	31.0 ± 19.9	19.2 ± 3.9	-	= 0.137[a]	6.8-31.0
Inositol, nmol/L	41.6 ± 19	57.3 ± 27.1	$F_{(1,22)}$=0.255	= 0.62	20.5-60.7
MMA, nmol/L	0.3 ± 0.1	0.4 ± 0.3	-	= 0.901[a]	0.10-0.50

A MANOVA model including Carnitine, Inositol was run to assess the effect of diagnosis controlling for sex and age; [a]Kruskal Wallis test

Table 4. Comparison between CRC and CTRL subjects for vitamins group (mean, SD)

	CRC Patients N=13	CTRL subjects N=10	Test (degrees of freedom)	P value	Normal Reference range values
Vitamin B					
Vit B1, nmol/L	7.8 ± 2.5	12.5 ±2.8	$F_{(1,22)}$=6.628	**=0.019**	1.4-71.3
Vit B2, µg/L	10.78 ± 8.5	10.5 ± 6.4	-	=1[a]	5.6-126.1
Vit B3, ng/mL	21.4 ± 14.5	25.6 ± 8.1	$F_{(1,22)}$=1.260	=0.276	2.6-36.1
Vit B5, µg/L	36.42 ± 11.2	33.26 ± 5.8	$F_{(1,22)}$=2.335	=0.143	22.7-429.2
Vit B6, ng/mL	11.4 ± 7.9	29.6 ± 12.5	$F_{(1,22)}$=1.2	=0.295	2.8-76.2
Vitamin K					
Vit K1, ng/mL	0.56 ± 0.46	1.39 ± 1.0948	-	**0.030[a]**	0.10-8.10
Vit K2, ng/mL	0.86 ± 0.7	0.84 ± 0.7	$F_{(1,22)}$=0.06	0.942	0.10-5.19

A MANOVA model including vitamins, B1, B3, B5, and B6, along with a UNIANOVA model for vitamin K2, was run to assess the effect of diagnosis while controlling for sex and age; [a]Kruskal Wallis test

Table 5. Comparison between CRC and CTRL subjects for vitamins of antioxidant and calcium homeostasis (mean, SD)

	CRC Patients N=13	CTRL subjects N=10	Test (degrees of freedom)	P value	Normal Reference range values
Vit C, mg/dL	0.11 ± 0.001	0.11 ± 0.01	$F_{(1,22)}$=1.008	0.328	0.6-2
Vit A, µg/dL	30.1 ± 12.27	49.2 ± 8.95	$F_{(1,22)}$=4.636	**0.044**	40.8-154.5
Vit D3, µg/mL	0.3 ± 0.1	0.4 ± 0.2	-	0.352[a]	0.2-0.4
Vit E, mg/L	9.3 ± 2.6	9.6 ± 2.0	$F_{(1,22)}$=1.442	0.245	7.4-30.6

A MANOVA model including Vit C, Vit A, and Vit E was run to assess the effect of diagnosis controlling for sex and age; [a]Kruskal Wallis test

Table 6. Comparison between CRC and CTRL subjects for amino acids under study (mean, SD)

	CRC Patients N=13	CTRL subjects N=10	Test (degrees of freedom)	P value	Normal Reference range values
Serine, nmol/mL	213.3±24.7	189.5±23.3	$F_{(1,22)}$=2.041	0.169	94.2-246.8
Valine, nmol/mL	348.6±90.1	362.3±32.0	$F_{(1,22)}$=0.797	0.383	155.9-368.0
Leucine, nmol/mL	174.5±37.8	172.6±15.5	$F_{(1,22)}$=0.683	0.419	101.2-249.3
Isoleucine, nmol/mL	64.4±18.6	53.6±6.1	-	0.094[a]	25.5-158.9
Asparagine, nmol/mL	55.6±11.4	62.7±13.8	-	0.154[a]	39.2-89.8
Glutamine, nmol/mL	536.3±103.9	607.2±59.8	-	0.055[a]	393.5-699.3
Arginine, nmol/mL	189.4±38.7	153.6±17.6	-	**0.006[a]**	81.6-249.0
Citrulline, nmol/mL	37.6±19.9	37.3±4.6	-	0.215[a]	18.7-47.5
Cysteine, nmol/mL	11.6±11.1	**1.7±0.6**	-	**<0.001[a]**	3.4-37.0

A MANOVA model including serine, valine, and leucine was run to assess the effect of diagnosis controlling for sex and age; [a]Kruskal Wallis test

Table 7. Results of uni- and a multivariable binary logistic model developed to evaluate the effect of molecular variables on the probability of having CRC

	Univariable analysis			Multivariable analysis*		
	OR	**95% CI**	**p value**	**OR**	**95% CI**	**p value**
Age, years	1.193	1.022-1.391	**0.025**	-	-	-
Copper, µmol/L	1.491	1.009-2.203	**0.045**	-	-	-
Selenium, ng/mL	0.887	0.806-0.977	**0.015**	-	-	-
Chromium, ng/mL	0.01	0.00-0.287	**0.007**	-	-	-
Vit B1, nmol/L	0.466	0.246-0.884	**0.019**	-	-	-
Vit A, µg/dL	0.86	0.768-0.963	**0.009**	0.828	0.692-0.990	**0.039**
Arginine	1.057	1.003-1.113	**0.037**	-	-	-