# Heme-Related Gene Expression Signatures of Meat Intakes in Lung Cancer Tissues

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Lung cancer causes more deaths worldwide than any other cancer. In addition to cigarette smoking, dietary factors may contribute to lung carcinogenesis. Epidemiologic studies, including the environment and genetics in lung cancer etiology (EAGLE), have reported increased consumption of red/processed meats to be associated with higher risk of lung cancer. Heme–iron toxicity may link meat intake with cancer. We investigated this hypothesis in meat-related lung carcinogenesis using whole genome expression. We measured genome-wide expression (HG-U133A) in 49 tumor and 42 non-involved fresh frozen lung tissues of 64 adenocarcinoma EAGLE patients. We studied gene expression profiles by high-versus-low meat consumption, with and without adjustment by sex, age, and smoking. Threshold for significance was a false discovery rate (FDR) $\leq$0.15. We studied whether the identified genes played a role in heme–iron related processes by means of manually curated literature search and gene ontology-based pathway analysis. We found that gene expression of 232 annotated genes in tumor tissue significantly distinguished lung adenocarcinoma cases who consumed above/below the median intake of fresh red meats (FDR = 0.12). Sixty-three (~28%) of the 232 identified genes (12 expected by chance, *P*-value $<$ 0.001) were involved in heme binding, absorption, transport, and Wnt signaling pathway (e.g., CYPs, TPO, HPX, HFE, SLCs, and WNTs). We also identified several genes involved in lipid metabolism (e.g., NCR1, TNF, and UCP3) and oxidative stress (e.g., TPO, SGK2, and MTHFR) that may be indirectly related to heme-toxicity. The study's results provide preliminary evidence that heme–iron toxicity might be one underlying mechanism linking fresh red meat intake and lung cancer. 

Key words: gene expression; heme–iron; lung cancer

## INTRODUCTION

Lung cancer is the leading cancer-related cause of death worldwide [1]. Exposure to cigarette smoking is the dominant risk factor for over 85% of all lung cancers. Nevertheless, other environmental determinants such as dietary factors may contribute to lung carcinogenesis. Of interest, within the past 5 yr, several studies, including ours [2], showed that higher intakes of fresh red meat (e.g., steak, hamburger, pork), processed meat (e.g., baloney, salami, and hot dogs), and derived meat mutagens (heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs)) were associated with increased risk of lung cancer [3–5]. The studies span across different populations—from both developed [2,3] and developing countries [5]. Developed countries tend to consume high meat intake in their diets while developing countries are quickly adapting to this type of dietary behavior. Data from GLOBOCAN 2008 showed that the majority of new lung cancer cases (55%) are now occurring in the developing countries [1]. In light of the potential role of meat as a modifiable risk factor of lung cancer and coupled with the public health implication of increasing consumption of meat worldwide, characterizing the mechanisms by which meat and related derivatives contribute to the etiology of lung cancer is both warranted and timely.

Several mechanisms may explain the relationship

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Abbreviations: PAHs, polycyclic aromatic hydrocarbons; NOCs, *N*-nitroso compounds; EAGLE, environmental and genetics in lung cancer etiology; FDR, false discovery rate; GO, gene ontology; HPX, hemopexin.

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between intake of red or processed meat and carcinogenesis, including the carcinogenic role of dietary heme–iron, meat mutagens, saturated fat, and *N*-nitroso compounds (NOCs) [6]. The vast literature of studies published on the biologic and mechanistic processes of meat-related carcinogenesis has focused on colon cancer. While the precise mechanism remains to be discerned, Bastide et al. [7] recently highlighted the role of heme–iron toxicity as the contributing factor in colorectal cancer risk. The authors asserted that heme–iron, responsible for the ruby color seen in red meat, more than dietary saturated fat and derived meat-mutagens explains the relationship. The reasons included the consistent lack of association found between saturated fat [8] and white meat (a significant contributor to HCAs derived from high cooking temperature) [9] and colorectal cancer. Conversely, dietary heme–iron, found in high concentration in red meat compared to white meat, is consistently found to be associated with colorectal cancer risk [7].

Bastide et al. [7] conducted a meta-analysis of prospective cohort studies showing an 18% increased risk of colon cancer with higher intake of dietary heme–iron. In their review of experimental studies of animals and humans, they suggested two major possible mechanisms for the effect of heme–iron: (1) heme catalyzes the endogenous formation of NOCs and (2) heme catalyzes the formation of cytotoxic and genotoxic aldehydes by lipoperoxidation.

The literature on dietary heme–iron and lung cancer risk is limited compared to that for colorectal cancer. Of two recent studies that published on the association between dietary heme–iron and lung cancer risk, one showed a significant positive association [4] while the other showed no association [10]. With respect to mechanistic understanding of heme–iron's relationship on lung cancer in humans or animals, the literature is non-existent.

We previously showed that high intakes of fresh red meat, processed meat, and meat mutagens were significantly associated with increased risk of lung cancer in the environmental and genetics in lung cancer etiology (EAGLE) study, a large population-based case–control study conducted in Italy [2]. The studied Italian population consumed a high amount of fresh red and processed meat regularly in their diets. As a result, the dietary data on meat consumption in the EAGLE study covered a wide range of intake permitting comparisons between extreme intakes. Additionally, EAGLE has gene expression data from fresh lung tumor and adjacent normal tissues of subjects with dietary meat data [11]. As a follow-up study on the positive associations between meat intake and lung cancer in EAGLE, we investigated the gene expression signatures between consumers of meat intakes in a subset of lung adenocarcinoma patients with both data on meat intake and genome-wide expression profiles. Our primary aim was to evaluate the heme-hypothesis in relation to meat-related lung carcinogenesis by specifically assessing the expression profiles of relevant genes in heme binding, absorption, and transport pathways. We hypothesized that the heme–iron expression profile in lung tissues of high consumers of fresh red meats and processed meats will differ from those of low consumers. To our knowledge, this is the first mechanistic investigation of this nature on human tissues and dietary meat consumption.

## MATERIALS AND METHODS

### Study Population, Sample Collection and Gene Expression Data

The present study is based on 64 lung adenocarcinoma participants from the EAGLE case–control study. These 64 individuals had both dietary meat information and microarray gene expression data from tumor (49 subjects) and non-involved (42 subjects) fresh-frozen lung tissue samples (Table 1) of which 27 pairs were from the same individuals (i.e., 27 patients with both tumor and non-involved

Table 1. Samples Characteristics

| | T, *n* (%) | N, *n* (%) |
|---|---|---|
| Fresh red meat | | |
| Below median[a] | 19 (38.8) | 17 (40.5) |
| Above median[a] | 30 (61.2) | 25 (59.5) |
| Processed meat | | |
| Below median[a] | 18 (36.7) | 18 (42.9) |
| Above median[a] | 31 (63.3) | 24 (57.1) |
| Smoking | | |
| Never | 13 (26.5) | 12 (28.6) |
| Former | 15 (30.6) | 15 (35.7) |
| Current | 21 (42.9) | 15 (35.7) |
| Sex | | |
| M | 29 (59.2) | 29 (69.0) |
| F | 20 (40.8) | 13 (31.0) |
| Age | | |
| 45–59 | 11 (22.4) | 9 (21.4) |
| 60–64 | 11 (22.4) | 9 (21.4) |
| 65–69 | 12 (24.5) | 13 (31.0) |
| 70–79 | 15 (30.7) | 11 (26.2) |
| Stage | | |
| IA | 5 (10.2) | 7 (16.7) |
| IB | 14 (28.6) | 14 (33.3) |
| IIA | 1 (2.1) | 1 (2.4) |
| IIB | 15 (30.6) | 10 (23.8) |
| IIIA | 8 (16.3) | 5 (11.9) |
| IIIB | 3 (6.1) | 2 (4.8) |
| IV | 3 (6.1) | 3 (7.1) |
| Paired T–N | | |
| Yes | 27 (55.1) | 27 (64.3) |
| No | 22 (44.9) | 15 (35.7) |
| Total | 49 (100.0) | 42 (100.0) |

T, tumor; N, normal.
[a]Median intake of fresh red meat (female/male: 1.2/1.5 times per week) and processed meat (female/male: 3.4/4.2 times per week), based on the sex-specific distribution of the EAGLE controls.

samples, 22 patients with only tumor samples and 15 patients with only non-involved samples, for a total of 64 patients). The power to detect 85% true positive (false discovery rate (FDR) = 15%) differentially expressed genes (fold change (FC) = 1.25 and standard deviation = 0.7) based on a two sample *t*-test was 79.5% and 74% for the tumor (49 samples) and non-involved (42 samples) analyses, respectively.

The EAGLE study has been described previously [11]. Briefly, 2100 consecutive patients with primary lung cancer were enrolled in EAGLE between 2003 and 2005 from 13 hospitals within the Lombardy region of Italy and 2120 population controls were matched to cases by age (5 yr categories), sex, and residence (five municipalities). The study was approved by the institutional review boards of the enrolling hospitals and the National Cancer Institute (NCI). All participating subjects signed an informed consent form. Lung cancer histology was ascertained by the EAGLE local pathologists and reviewed by a pathologist from the NCI.

The individuals included in this gene expression study were randomly selected from the EAGLE lung adenocarcinoma patients to include similar numbers of never, former and current smokers, and of male and female. Subjects were 45–79 yr old, had histologically confirmed primary adenocarcinoma of the lung, stages I–IV, and provided detailed smoking and dietary information (Table 1).

Lung tissue samples had been snap-frozen in liquid nitrogen within 20 min of surgical resection. A single pathologist confirmed the hospital-based diagnosis of adenocarcinoma, estimated the presence of malignant cells in each sample based on H&E-stained sections, and classified the samples as tumor or non-involved.

Gene expression was measured using the Affymetrix Chip HG-U133A. Corresponding CEL files and information conforming to the MIAME guidelines are publicly available on the GEO database (accession number = GSE10072). Quality assurance data were presented in a previous publication [12].

### Statistical Analysis

Gene expression data were processed and normalized using the robust multichip average (RMA) method [13] and log transformed. All 22 283 Affymetrix HG-U133A probe sets were used in the analysis. Average linkage hierarchical clustering of samples was based on one minus Pearson correlation.

Analyses were carried out separately in 49 tumor and in 42 non-involved tissues (of which 27 were from the same subjects) among patients from all stages of lung adenocarcinoma and from resectable stages only. Analyses excluding one patient who underwent radiotherapy before surgery were also performed. Fresh red meat and processed meat intakes were derived from a detailed self-reported food frequency questionnaire [2]. For each meat group, we assigned individuals to two categories using cutoffs based on the median meat intake of sex-specific distribution of ~2000 controls from the larger EAGLE study. The median intake (25th, 95th percentiles) of fresh red meat were 1.2 (0.5–13.1) and 1.5 (0.7, 5.0) times per week for females and males, respectively. Similarly, the median intakes of processed meats were 3.4 (1.8, 10.5) and 4.2 (2.3, 12.8) times per week.

We compared the gene expression in tissue of patients who consumed above the median of meat (fresh red meat and processed meat) intake compared to those below the median. We used an ANOVA analysis adjusted for sex, because sex and meat intake were significantly associated in the studied patients. We also verified that adjusting for age and smoking status (never, former, current cigarette smoking) did not change the results. We further investigated whether residual confounding by age, sex, and smoking might affect our findings by checking whether the expression of the identified genes was associated with these factors. To control for multiple comparisons, we computed the maximum FDR using the Benjamini–Hochberg technique [14] based on a single gene–probe *P*-value threshold of 0.001. We considered as significant signatures with an FDR $\leq$ 0.15. The above statistical analyses were conducted using the R program version 2.12.

We then conducted a manually curated literature search in order to investigate whether the identified meat-related gene expression signature was involved in any of the four meat carcinogenesis related processes hypothesized by Bastide et al. [6,7]. These four processes included (1) heme–iron, (2) oxidative stress, (3) lipids, and (4) heterocyclic amines or PAH mutagens. The PubMed, Entrez Gene, and Gene Ontology databases were searched independently by two investigators using the following keywords: gene symbol AND (1) heme–iron, heme, metal ion transport or binding; (2) *N*-nitroso, oxidative stress; (3) lipid, fatty acid; and (4) heterocyclic amine, PAH, benzo-*a*-pyrene (BaP). To estimate the statistical significance of each search result, we computed the number of genes that would be expected by chance alone and the corresponding *P*-value based on the *z*-statistic (referred to as $P\text{-value}_{\text{search}}$).

In addition, we used the GoMiner software [15] to assess whether the above mentioned or other processes were significantly enriched by genes whose expression showed differences by meat intake. The GoMiner algorithm assigns genes to gene ontology (GO) functional categories [16] and rank-orders the GO categories by computing the ratio of changed genes in the category divided by the total number of genes in the category, divided by the same ratio for the entire microarray. The significance of this ratio of proportions is evaluated using a one-sided Fisher exact test.

## RESULTS

The median intakes of fresh red meat (25th, 95th percentile range) and processed meat for the tumor samples in the present study were 2.0 (1.1, 6.5) and 5.5 (2.8, 16.6) times per week, respectively. For non-involved lung samples, the distributions of meat intakes were comparable to tumor samples for fresh red meat (2.0 times per week) and slightly lower for processed meat (5.0 times per week).

Using lung tumor samples we identified a significant gene expression signature of above the median versus below the median intake of fresh red meat (FDR = 0.12), consisting of 235 up-regulated and 34 down-regulated gene probes (Supplementary Table S1 and Supplementary Figure S1), corresponding to 232 unique annotated genes. To verify that our results were not due to alteration of gene expression because of advanced tumor stages, we conducted the same analysis restricted to resectable lesions (stages I–IIIA). The results were similar to those from all stages, thus in the present report we present results from all stage tumors. Similarly, excluding one patient who underwent radiotherapy before surgery did not alter the results. We did not observe significant gene expression differences by processed meat intake.

Analyses by fresh red or processed meat intake in non-involved lung tissues did not provide significant gene expression signatures (FDRs $> 0.15$). More precisely, only 43 gene–probes were differentially expressed ($P$-value $< 0.001$) by red meat intake (30 down-regulated and 13 up-regulated) with a corresponding FDR of 0.76. This result indicates that 76% of the 43 gene–probes identified in non-involved tissues (i.e., 33) might be due to chance. Consequently, we did not follow up on these 43 gene-probes further and only results from the analysis by red meat intake in tumor samples were followed up.

We then investigate whether the 232 genes identified in the class comparison by fresh red meat intake in tumor samples were involved in: (1) heme–iron, (2) oxidative stress, (3) lipids, and/or (4) heterocyclic amines or PAH mutagens [7]. The results of the manually curated literature search are shown in Table 2. We found: (1) 63 genes involved with heme–iron ($P$-value$_{search} < 0.001$); (2) 10 genes involved in oxidative stress ($P$-value$_{search} = 0.326$); (3) 19 genes involved in lipid metabolism ($P$-value$_{search} = 0.005$); (4) no genes involved in metabolism from mutagens such as PAH and BaP. The identified heme-related genes are involved in a range of heme-related activities, including heme-binding (e.g., HPX and NENF), absorption (e.g., HFE), biosynthesis (e.g., ALAS2), heme/iron-mediated Wnt signaling (e.g., WNTs, LEF1, PTPRT, TNF). We also identified several genes involved in oxidative stress (e.g., MTHFR, SGK2) and lipid metabolism (e.g., MYCN, RBP4, NCR1). Several of these genes were present in multiple categories: CYP4A11, CYP3A3 in all three categories; TPO, CYP2C8, and TGM3 both in heme–iron and oxidative stress functions; PRKD3, TNF, SNX3, PDEAD, PLSCR3, PRKCA, and ALAS2 both in heme–iron and lipid related processes; UCP3 both in oxidative stress and lipid metabolism. We did not observe significant associations between the expression of these genes with age, sex, and smoking, suggesting that our findings are not confounded by these factors.

Finally, using GOMiner we classified the 232 genes according to GO molecular functional categories and verified whether the four a priori processes involved in meat carcinogenesis were among the most enriched GO categories. The heme binding related function was significantly over-represented among GO categories (Fisher exact $P$-value = 0.038). Contrarily, functions related to oxidative stress, lipids, and mutagens metabolism were not significantly enriched based on a $P$-value threshold of 0.05. Of note, other GO categories of genes related to meat consumption were identified using GOMiner, including transcription factor activity ($P$-value = 0.001), SH3 domain binding ($P$-value = 0.004), and potassium channel activity ($P$-value = 0.011).

## DISCUSSION

We previously observed an increased risk of lung cancer with increasing higher intake of red meat in a large population-based case–control study in Italy [2]. In a follow-up study to characterize the mechanisms underlying meat-related lung carcinogenesis, and to test the heme–iron hypothesis [7], we examined the gene expression patterns in a subset of the same subjects with available dietary meat information and gene expression data derived from fresh frozen lung adenocarcinoma tissues. We found a significant tumor gene expression signature of red meat intake consisting of 269 differentially expressed gene–probes ($P$-value $< 0.001$, FDR = 0.12), corresponding to 232 unique annotated genes. Among these, 60 genes involved in heme–iron processes ($P$-value$_{search} < 0.001$) were identified to differentiate consumers of higher fresh red meat intake compared to low consumers in our study as hypothesized. We additionally identified genes involved in lipid and oxidative stress, which may be indirectly related to heme.

Epidemiologic prospective cohort studies showed a positive relationship between dietary heme–iron and colorectal cancer (pooled RR based on five cohort studies: 1.18; 95% CI: 1.06–1.32; $P$-heterogeneity = 0.18) [7]. Recent finding from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial, showed that total binding capacity and unsaturated iron binding capacity, indicators of reduced iron overload, was inversely associated with colorectal cancer risk [17]. Analysis of existing experimental evidence further provides biologic plausibility to the heme hypothesis. Dietary heme,

Table 2. Genes Involved in (1) Heme–Iron, (2) Oxidative Stress, and (3) Lipid Pathways Identified Through Manually Curated Literature Search

| (1) Chr. location | Symbol | Name | FC | *P*-value | Heme related function |
|---|---|---|---|---|---|
| 1p33 | CYP4A11 | Cytochrome P450, family 4A, polypeptide 11 | 1.17 | 0.0004 | Heme binding |
| 1p35.1 | TRIM62 | Tripartite motif-containing 62 | 1.13 | 0.0008 | Metal ion binding |
| 1p36.31 | THAP3 | THAP domain containing, apoptosis assoc. protein 3 | 1.17 | 0.0001 | Metal ion binding |
| 1q21.2 | OTUD7B | OTU (ovarian tumor protein) domain containing 7B | 1.15 | 0.0010 | Metal ion binding |
| 1q21.2-q22 | CADM3 | Cell adhesion molecule 3 | 1.12 | 0.0004 | Metal ion binding |
| 1q32.3 | NENF | Neuron derived neurotrophic factor | 1.17 | 0.0008 | Heme binding |
| 2p16.3 | SLC3A1 | Solute carrier family 3, member 1 | 1.13 | 0.0004 | Metal ion binding |
| 2p21 | PRKD3 | Protein kinase D3 | 0.71 | 0.0011 | Metal ion binding |
| 2p22.3 | SLC30A6 | Solute carrier family 30 (zinc transporter), member 6 | 1.17 | 0.0006 | Metal ion transport |
| 2p25 | TPO | Thyroid peroxidase | 1.24 | 0.0002 | Heme binding |
| 2q12 | SLC5A7 | Solute carrier family 5 (choline tr), member 7 | 1.15 | 0.0002 | Metal ion transport |
| 2q24.3 | SCN2A | Sodium channel, voltage-gated, type II, alpha subun | 1.29 | 0.0002 | Metal ion binding |
| 3q26.2 | PHC3 | Polyhomeotic homolog 3 (Drosophila) | 1.28 | 0.0004 | Metal ion binding |
| 3q27 | HRG | Histidine-rich glycoprotein | 1.10 | 0.0012 | Heme binding |
| 4q23-q25 | LEF1 | Lymphoid enhancer-binding factor 1 | 0.71 | 0.0009 | wnt signaling |
| 6p21.3 | HFE | Hemochromatosis | 1.14 | 0.0006 | Iron absorption |
| 6p21.3 | TNF | Tumor necrosis factor | 1.18 | 0.0002 | Induce heme oxyenase |
| 6q21 | SNX3 | Sorting nexin 3 | 0.84 | 0.0003 | Iron transport |
| 6q26 | PLG | Plasminogen | 1.17 | 0.0003 | Metal ion binding |
| 7q21.1 | CYP3A43 | Cytochrome P450, family 3A, polypeptide 43 | 1.13 | 0.0010 | Heme binding |
| 7q22.1 | AGFG2 | ArfGAP with FG repeats 2 | 1.13 | 0.0009 | Metal ion binding |
| 7q22.1 | ZNF3 | Zinc finger protein 3 | 0.62 | 0.0009 | Metal ion binding |
| 8q24 | KCNQ3 | Potassium voltage-gated channel, KQT-like, mem.3 | 1.18 | 0.0010 | Metal ion binding |
| 9q34.3 | LHX3 | LIM homobox 3 | 1.18 | 0.0006 | Metal ion binding |
| 9q34.3 | RNF208 | Ring finger protein 208 | 1.18 | 0.0010 | Metal ion binding |
| 10q11.2 | ZNF33B | Zinc finger protein 33B | 1.26 | 0.0011 | Metal ion binding |
| 10q22.3-23.2 | LDB3 | LIM domain binding 3 | 1.28 | 0.0001 | Metal ion binding |
| 10q23.33 | CYP2C8 | Cytochrome P450, family 2C, polypeptide 8 | 1.21 | 0.0003 | Heme binding |
| 10q24.1 | ZNF518A | Zinc finger protein 518A | 1.14 | 0.0009 | Metal ion binding |
| 10q24.2 | CPN1 | Carboxypeptidase N, polypeptide 1 | 1.19 | 0.0002 | Metal ion binding |
| 10q26.3 | ADAM12 | ADAM metallopeptidase domain 12 | 0.59 | 0.0007 | Metal ion binding |
| 11p13-p12 | SLC1A2 | Solute carrier family 1, member 2 | 1.17 | 0.0001 | Metal ion transport |
| 11p15.5-15.4 | HPX | Hemopexin | 1.18 | 0.0002 | Heme binding |
| 11q13.5 | WNT11 | Wingless-type MMTV integration site family, m11 | 1.22 | 0.0015 | wnt signaling |
| 12p12 | PDE4D | Phosphodiesterase 3A, cGMP-inhibited | 1.21 | 0.0004 | Metal ion binding |
| 12q13 | WNT1 | Wingless-type MMTV integration site family, m1 | 1.17 | 0.0005 | wnt signaling |
| 12q14 | GNS | Glucosamine (*N*-acetyl)-6-sulfatase | 0.63 | 0.0006 | Metal ion binding |
| 12q21.1 | CSRP2 | Cysteine and glycine-rich protein 2 | 0.60 | 0.0002 | Metal ion binding |
| 14q31 | ITPK1 | Inositol 1,3,4-triphosphate 5/6 kinase | 1.16 | 0.0008 | Metal ion binding |
| 14q31 | NRXN3 | Neurexin 3 | 1.21 | 0.0006 | Metal ion binding |
| 16p11.2 | MYST1 | MYST histone acetyltransferase 1 | 1.27 | 0.0007 | Metal ion binding |
| 16p13.13 | TNP2 | Transition protein 2 | 1.14 | 0.0001 | Metal ion binding |
| 16q24.3 | CDH15 | Cadherin 15, type 1, M-cadherin (myotubule) | 1.21 | 0.0001 | Metal ion binding |
| 17p13.1 | PLSCR3 | Phospholipid scramblase 3 | 0.83 | 0.0010 | Metal ion binding |
| 17q22-q23.2 | PRKCA | Protein kinase C, alpha | 1.17 | 0.0002 | Metal ion binding |
| 17q24.2 | ERN1 | Endoplasmic reticulum to nucleus signaling 1 | 1.15 | 0.0008 | Metal ion binding |
| 18p11.32 | CETN1 | Centrin, EF-hand protein | 1.15 | 0.0007 | Metal ion binding |
| 18q22-q23 | ZNF236 | Zinc finger protein 236 | 1.21 | <0.0001 | Metal ion binding |
| 19p13.3-.2 | MAP2K7 | Mitogen-activated protein kinase kinase 7 | 1.19 | 0.0011 | Heme induced erythroid differen. |
| 19q13.1-qter | GRIN2D | Glutamate receptor, ionotropic, *N*-methyl D-aspartate | 1.19 | 0.0013 | metal ion binding |
| 20p12 | PLCB1 | Phospholipase C, beta 1 | 1.19 | 0.0015 | metal ion binding |
| 20q11.2 | TGM3 | Transglutaminase 3 | 1.17 | 0.0009 | Heme related oxidative stress |
| 20q12 | KCNS1 | Potassium voltage-gatedchannel, delayed-rectifier, S1 | 1.27 | 0.0006 | Metal ion binding |
| 20q12-q13 | PTPRT | Protein tyrosine phosphatase, receptor type, T | 1.17 | 0.0014 | wnt signaling |

(*Continued*)

Table 2. (*Continued*)

| (1) Chr. location | Symbol | Name | FC | *P*-value | Heme related function |
|---|---|---|---|---|---|
| 20q13.12 | L3MBTL | Letal(3)malignant brain tumor-like 1 | 1.13 | 0.0009 | Metal ion binding |
| 20q13.1-.2 | PTPN1 | Protein tyrosine phosphatase, non-receptor type 1 | 1.21 | <0.0001 | Metal ion binding |
| 20q13.2 | KCNB1 | Potassium voltage-gated channel, Shab-related, m1 | 1.14 | 0.0013 | Metal ion binding |
| 21q22.1 | KCNJ6 | Potassium inwardly-rectifying channel, subfamily J6 | 1.17 | 0.0013 | Metal ion binding |
| 21q22.12 | KCNE1 | Potassium voltage-gated channel, Isk-related, m1 | 1.22 | 0.0009 | Metal ion binding |
| 22q13.1 | NPTXR | Neuronal pentraxin receptor | 1.13 | 0.0004 | Metal ion binding |
| Xp11.21 | ALAS2 | Aminolevulinate, delta-, synthase 2 | 1.22 | 0.0002 | Heme biosynthesis |
| Xq22.3 | TEX13A | Testis expressed 13A | 1.25 | 0.0012 | Metal ion binding |
| Xq23 | PAK3 | p21 protein (Cdc42/Rac)-activated kinase 3 | 1.32 | 0.0005 | Metal ion binding |

| (2) Chr. location | Symbol | Name | FC | *P*-value | Oxidation related function |
|---|---|---|---|---|---|
| 1p33 | CYP4A11 | Cytochrome P450, family 4A, polypeptide 11 | 1.17 | 0.0004 | Oxidoreductase activity |
| 1p36.3 | MTHFR | Methylenetetrahydrofolate reductase | 1.19 | <0.0001 | Oxidoreductase activity |
| 2p25 | TPO | Thyroid peroxidase | 1.24 | 0.0002 | Antioxidant activity |
| 7q21.1 | CYP3A43 | Cytochrome P450, family 3A, polypeptide 43 | 1.13 | 0.0010 | Oxidoreductase activity |
| 10q23.33 | CYP2C8 | Cytochrome P450, family 2C, polypeptide 8 | 1.21 | 0.0003 | Oxidoreductase activity |
| 11q13 | UCP3 | Uncoupling protein 3 | 1.16 | 0.0014 | Oxidative phosphorylation |
| 15q14-q15 | IVD | Isovaleryl-CoA dehydrogenase | 1.16 | 0.0013 | Oxidoreductase activity |
| 19q13.12 | GAPDHS | Glyceraldehyde-3-phosphate dehydrogenase sper | 1.17 | 0.0004 | Oxidoreductase activity |
| 20q11.2 | TGM3 | Transglutaminase 3 | 1.17 | 0.0009 | Response to oxidative stress |
| 20q13.2 | SGK2 | Serum/glucocorticoid regulated kinase 2 | 1.15 | 0.0003 | Response to oxidative stress |

| (3) Chr. location | Symbol | Name | FC | *P*-value | Lipid related function |
|---|---|---|---|---|---|
| 1p33 | CYP4A11 | Cytochrome P450, family 4A, polypeptide 11 | 1.17 | 0.0004 | Lipid metabolism |
| 2p21 | PRKD3 | Protein kinase D3 | 0.71 | 0.0011 | Lipid binding |
| 2p24.1 | MYCN | v-myc myelocytomatosis viral related oncogene | 1.12 | 0.0014 | Fatty acid |
| 6p21.3 | TNF | Tumor necrosis factor | 1.18 | 0.0002 | Lipid metabolism |
| 6q21 | SNX3 | Sorting nexin 3 | 0.84 | 0.0003 | Lipid binding |
| 7q21.1 | CYP3A43 | Cytochrome P450, family 3A, polypeptide 43 | 1.13 | 0.0010 | Lipid metabolism |
| 10q23-q24 | RBP4 | Retinol binding protein 4, plasma | 1.17 | 0.0004 | Lipid binding |
| 11p15.4 | MYOD1 | Myogenic differentiation 1 | 1.19 | 0.0004 | Fatty acid |
| 11p15.5 | PNPLA2 | Patatin-like phospholipase domain containing 2 | 1.17 | 0.0003 | Lipase |
| 11q13 | UCP3 | Uncoupling protein 3 | 1.16 | 0.0014 | Lipid-induced oxidative stress |
| 11q13.5 | NEU3 | Sialidase 3 (membrane sialidase) | 1.24 | 0.0007 | Lipid metabolism |
| 11q23.1-2 | APOC3 | Apolipoprotein C-III | 1.17 | 0.0012 | Lipid binding |
| 12p12 | PDE3A | Phosphodiesterase 3A, cGMP-inhibited | 1.21 | 0.0004 | lipId metabolism |
| 17p13.1 | PLSCR3 | Phospholipid scramblase 3 | 0.83 | 0.0010 | Lipid tranport |
| 17q22-q23.2 | PRKCA | Protein kinase C, alpha | 1.17 | 0.0002 | Lipid binding |
| 19q13.42 | NCR1 | Natural cytotoxicity triggering receptor 1 | 1.21 | 0.0004 | Suppress lipid accumulation |
| 20q11.23 | BPI | Bactericidal/permeability-increasing protein | 1.14 | 0.0015 | Lipopolysaccharide binding |
| Xp11.21 | ALAS2 | Aminolevulinate, delta-, synthase 2 | 1.22 | 0.0002 | Lipid binding |

FC, fold change; and *P*-value were obtained through ANOVA class comparison analysis, adjusted by sex, between above and below median intake of red fresh meat.

including nitrosyl heme, can promote colon carcinogenesis in rats [18,19]. Results from feeding studies of humans also suggested that heme–iron, not protein or inorganic iron, was responsible for endogenous intestinal *N*-nitrosation derived from red meat diet [20], although there are no studies based on lung epithelium. It is biologically plausible that dietary iron absorbed in the gut can be

transported to the lung and consequently affect lung carcinogenesis. While heme–iron is found in animal products and easily absorbed, most dietary iron is found in the form of non-heme. A study showed that lung non-heme–iron levels increased ~twofold in rats fed with a high iron diet [21].

Using gene expression data from lung tumor tissues, we found that, collectively, heme binding genes were significantly up-regulated in consumers of high fresh red meat intake compared to low consumers by GO and an independent manually curated search of PubMed (Table 2). These genes included hemopexin (HPX) and several cytochrome P450 genes (CYP450s). HPX has the highest binding affinity for heme and is induced in the presence of heme–iron overload [22]. The heme-HPX complex plays multifaceted roles, including modulating gene expression and heme biosynthesis [23]. CYP4A11 and CYP3A43 covalently bind to heme and metabolize a variety of xenobiotics including saturated fatty acids [24]. Since CYP450's gene expression can be induced by smoking, we verified that the analyses adjusted by smoking yielded the same results.

In addition to heme-binding genes, genes responsible for heme absorption (e.g., HFE) and biosynthesis (e.g., ALAS2) were differentially altered between high-versus-low meat consumers in the present study. HFE expression regulates iron absorption by regulating the interaction of transferrin receptor with transferrin (TF), an iron transporter [25]. We also observed two TF probes that were up-regulated; however, they did not reach multiple comparison test *p*-value (probe 214064_at, $P$-value = 0.04 and 220109_at, $P$-value = 0.06). We observed several up-regulated genes belonging to the solute carrier protein (SLC) group involved in transporting heme into the bloodstream [26], including SLC30A6, SLC5A7, SLC1A2 ($P$-values < 0.001) and SCL11A2 ($P$-value = 0.003).

Iron has been shown to increase WNT signaling [27] and the WNT signaling pathway is involved in a plethora of oncogenic processes (e.g., tumor development, progression, proliferation, and differentiation) [28]. The WNT signaling has been associated with colorectal cancer [29] and lung cancer [28]. Furthermore, dietary heme–iron has been shown to modulate genes controlling proliferation and differentiation in rats [30]. In our study, we observed significant up-regulation of five genes related to the WNT signaling pathway (WNT1, WNT11, PTPRT, WSIP1, and TNF). Recently, Hebels et al. [31] published findings from a dietary intervention study showing that increased fecal water genotoxicity, after 7 d of high red meat (300 g/d; beef products) consumption, strongly modulated expression of WNT signaling genes ($P$-value <0.01). Interestingly, the authors proposed that red meat intake-induced fecal water genotoxcity is attributable to the heme content of red meat.

Two mechanisms have been hypothesized involving heme–iron in cancer: (i) the endogenous formation of carcinogenic NOCs and (ii) the formation of cytotoxic and genotoxic aldehydes via lipoperoxidation [7]. NOCs cause the formation of reactive oxygen species and form DNA adducts. The lipophilic nature of heme can perturb lipid bilayers which consequently can promote the production of reactive oxygen species [32]. Heme oxyradicals can initiate oxidative DNA damage [33]. In the present study, we also identified genes that are involved in oxidative stress, including MTHFR, and in lipid metabolism (Table 2). MTHFR is a key regulator of folate metabolism and variants have been shown to impact genetic instability in lung cancer patients [34] and influence DNA methylation. MTHFR has been implicated in lung cancer risk in some studies [35] while a pooled study found no association [36]. Of interest, recently MTHFR variants were shown to modify DNA adducts in blood and lung tissues [37].

In addition to the aforementioned mechanisms attributed to heme–iron toxicity reviewed by Bastide et al., one possible explanation specific to the lung could be related to hemoglobin and myoglobin. A large repository of heme–iron in the human body is contained in hemoglobin, which transports oxygen from the lungs to the rest of the body, and to a lesser extent myoglobin, found in the muscle cells. Although hemoglobin is predominantly found in erythroid cells, it has been shown to be expressed in alveolar epithelial Type II cells of the lung [38]. Though preliminary, some studies have found an association between hemoglobin and myoglobin in some tumor cells [39,40], suggesting that these hemoproteins may contribute to lung carcinogenesis.

We were surprised by the lack of differential heme–iron gene expression by processed meats in both tissue types. One possible explanation is the reduced amount of heme–iron content relative to fresh red meat. Although heme–iron is present in processed meats, the content is substantially less (~50%) than in fresh red meat [41]. Processed meat has been commonly hypothesized to cause cancer via the exogenous production of NOCs in nitrates found in cured meats [42]. Therefore, heme-toxicity may not be a predominant carcinogenic mechanism relating to processed meats. We did not observe a differential gene expression by meat intake in non-involved tissues. Data from in vitro and animal models have suggested that normal and tumor cells differ in their uptake and metabolism of certain dietary-related constituents. For example, the metabolic rates of *cis*-unsaturated fatty acids (*c*-UFAs) are entirely different in tumor cells versus normal cells [43]. This contributes to the selectively tumoricidal property of *c*-UFA [44] in rats. Similarly, it is biologically plausible that there are molecular characteristics in tumor tissues which potentially cause a more

pronounced presence of meat-related components, such as heme–iron or lipids, and consequently permitted the observed differential expression of related genes while such occurrences are less detectable in normal tissues. Nevertheless, these possible explanations for the observed lack of differential gene expression are only speculative and require additional research from experimental studies.

Although specific to lung cancer, the findings of the present study extend beyond the lung. To our knowledge, it is the first study to use human cancer tissues to specifically investigate heme–iron toxicity as a biologic process underlying the relationship between tumor tissues linked to dietary data of meat intake in a population-based study, also unique is the fact that the studied population consumed a high range of meat intake frequently, which made the investigation of altered gene expression by meat intake possible. We specifically used cut-off points for the analyses based on the distribution of meat intake in the controls from the larger EAGLE study to reflect more closely the intake in the general population.

Our results could be affected by changes due to the tumor behavior. Ideally, pre-diagnosed lung tissue samples would be preferable for measuring gene expression related to meat intake, but it is not feasible. However, the similar signature found in early stages and overall stages suggests that meat-related expression changes are identifiable regardless of the tumor characteristics of the samples. Although promising, our findings are preliminary and warrant confirmation in a larger study with both dietary meat information and expression data. Another possible limitation is that, although the FFQ in the EAGLE study was targeted to obtain information on specific foods common in the Northern Italian population, it was relatively limited in scope and portion size was not asked. Frequency of intake may not reflect actual intake and the possibility of misclassification of meat intake exists; however, this would most likely be non-differential. Thereby, it would not alter the interpretation of the differential expression observed between higher-versus-lower consumers of fresh red meat. Red meat intake for this population was similar to some European countries (e.g., France, Spain, and the Netherlands) [45]. Investigations using different populations are recommended as dietary habits and genetic profiles vary by ethnic groups. Lastly, other pathways and biological processes, for example, those identified by the GOMiner classification, need to be explored in similar studies.

In conclusion, we observed gene expression signatures involved in processes relating to heme–iron, oxidative stress, and lipid metabolism to be differentially altered in lung tumor tissue between high versus low consumers of fresh red meat. The study's findings of heme-binding genes provide additional evidence that heme–iron toxicity may be an underlying mechanism linking meat intake and cancer.

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