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*Article*

# Effects of Molecular Iodine/Chemotherapy in the immune component of breast cancer tumoral microenvironment

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**Abstract:** Molecular iodine ($I_2$) induces apoptotic, antiangiogenic, and antiproliferative effects in breast cancer cells. Little is known about its effects on the tumor immune microenvironment. We studied the effect of oral (5 mg/day) $I_2$ supplementation alone ($I_2$) or together with conventional chemotherapy (Cht+$I_2$) on the immune component of breast cancer tumors from a previously published pilot study conducted in Mexico. RNA-seq, $I_2$ and Cht+$I_2$ samples showed significant increases in expression of Th1 and Th17 pathways. Tumor immune composition determined by deconvolution analysis revealed significant increases in M0 macrophages and B lymphocytes in both $I_2$ groups. Real-time RT-PCR showed that $I_2$ tumors overexpress T-BET ($p = 0.019$) and interferon-gamma (IFN$\gamma$; $p = 0.020$) and silence tumor growth factor-beta (TGF$\beta$; $p = 0.049$); whereas in Cht+$I_2$ tumors, GATA3 is silenced ($p = 0.014$). Preliminary methylation analysis shows that $I_2$ activates IFN$\gamma$ gene promoter (by increasing its unmethylated form) and silences TGF$\beta$ in Cht+$I_2$. In conclusion, our data showed that $I_2$ supplements induce the activation of the immune response and that when combined with Cht, the Th1 pathways are stimulated. The molecular mechanisms involved in these responses are being analyzed, but preliminary data suggest that methylation/demethylation mechanisms could also participate.

**Keywords:** molecular iodine; immune response; breast cancer.

## 1. Introduction

The immune component of the tumor microenvironment is considered one of the key players in prognosis and response to treatment [1]. In recent decades, the intratumoral presence of immune cell phenotypes has been associated with the prognosis of the disease; thus, cytotoxic lymphocytes (Th1 and CD8+), M1 macrophages and their effector molecules are considered favorable prognostic indicators [2], while immunomodulatory lymphocytes (Th2, Treg) and macrophages (TAM-M2) are found in worse prognosis scenarios [3]. At the molecular level, cytokines IL-1, IL-6, and tumor growth factor-beta (TGF$\beta$) are associated with tumor progression, whereas IL-12 and interferon-gamma (IFN$\gamma$) can inhibit cancer proliferation and/or metastasis [4]. Immune cells can switch these secretion patterns from one lineage toward another under certain circumstances, exhibiting phenotypic plasticity [5]. This functional switch –or trans-differentiation– depends on epigenetic processes [6]. Methylation/demethylation of DNA is an epigenetic mechanism concerning the transfer or removal of a methyl group onto the C5 position of the cytosine. Methylation regulates gene expression by recruiting proteins associated with gene repression or inhibiting the binding of transcription factors to DNA [7]. Conversely, active demethylation allows gene activation [6]. In the antitumor immune response, demethylation of the IFN$\gamma$ locus activates the transition from naïve to memory CD8+ T cells, promoting increased IFN$\gamma$ secretion [8]. Some dietary compounds can modify cancer

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progression, and over the past decade, numerous micronutrients have demonstrated activity as epigenetic modulators [9]. Molecular iodine ($I_2$) exerts antineoplastic effects on different cancer models [10,11], whereas in its non-oxidized form, like iodide (I-) or thyroid hormones (T4), it is not able to achieve these effects [12]. In cancer cells, $I_2$ could act as a "mitocan" agent (acronym for mitochondria and cancer) by depleting thiol reserves or disturbing the mitochondrial membrane potential (Mmp), thereby inducing apoptotic pathways [13]. Additionally, this chemical form of iodine is an effective antioxidant, even tenfold more effective than ascorbic acid [14]. Moreover, $I_2$ exhibited indirect antitumor activity by generating 6-iodolactone (6-IL) through the iodination of arachidonic acid. This iodolipid is an active ligand of peroxisomal-activated receptor type gamma (PPARγ), inducing re-differentiation by inhibiting stem signaling and triggering apoptosis [15]. In addition, $I_2$ supplementation exerts effects on the immune system, acting as a direct genetic modifier [16] or as an attractor, increasing the amount of CD8+ lymphocytes within the tumor [17]. We previously demonstrated in a breast cancer pilot study that $I_2$ supplementation exerted adjuvant effects when combined with conventional chemotherapy, reducing the residual tumor size and increasing disease-free survival [17]. The RNA-seq analysis showed that $I_2$-treated tumors exhibited significant activation of Th1, NK, and CD8 cytotoxicity pathways [17]. In the present study and using the same transcriptomic bank, we analyzed $I_2$ and the chemotherapy treatment (Cht) in the immune scenario. We describe the epigenetic patterns of immune effectors at methylation and demethylation level.

## 2. Materials and Methods

*Mammary tumors*

Tumors were collected as part of a pilot study registered at Clinicaltrial.gov (NCT03688958). Briefly, two pilot study groups were established based on the stage of cancer diagnosed: Early (stage II) and Advanced (stage III) breast cancer groups. Thirty patients were randomly assigned (double-blind) to receive either molecular iodine ($I_2$; 5 mg/day) or a placebo (vegetable colored water) for 7–35 days (as determined by the preoperative oncologist's protocol). In the Advanced group, 30 patients were randomly (double-blind) divided into the $I_2$ or placebo groups, and both groups received 4-6 cycles of neoadjuvant chemotherapy (Cht; 5-fluorouracil/epirubicin/cyclophosphamide or taxotere/epirubicin). Daily, after breakfast, $I_2$ or placebo was diluted in drinking water. During the surgical procedure, the tumor sample was kept in dry ice to avoid degradation and stored at -80ºC until further analysis.

*RNA-Seq and Transcriptomic Analysis*

Detailed constructions and all specific data analyses, including pathway and upstream regulator prediction, as well as all other analyses involving the transcriptomic data, can be found in protocols.io [18]. Briefly, total RNA was extracted with Qiazol and RNeasy (both from Qiagen). Two different pools of four individual tumor samples were used. As a normal control, we used a pool of two normal mammary gland samples from aesthetic surgeries (volume reduction). Poly-A enriched mRNA was used to construct stranded mRNA-Seq libraries following the manufacturer's instructions (KAPA Biosystems). Sequencing was carried out at Duke University Genome Sequencing Shared Resource Center (Durham, NC). The libraries were sequenced on an Illumina HiSeq 2500 platform, in which 101 bases were determined in pair-end mode. Data were assessed for quality and trimmed with FastQC and Trimmomatic, respectively. Reads were mapped to the human genome (GRCh38), and expression levels were determined by htseq-count. Differential expression analysis was performed using Fisher's exact and Benjamini-Hochberg (FDR) tests. Genes that were altered at least 2-fold or less than 0.5-fold with an FDR value equal to or lower than 0.05 were considered biological and statistically significant. The complete annotated sequences from the RNA-sequencing are available at the European Nucleotides Archives website (https://www.ebi.ac.uk/ena/erp110028).

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*Gene Set Enrichment Analysis*

Gene set enrichment analysis (GSEA) was performed with Webgestalt and GSEA with the following parameters: Organism of Interest: hsapiens; Method of Interest: GSEA; Functional Database: pathway, Kegg; Select Gene ID Type: genesymbol. Annotation of genes with immunological function was done with the Gene Ontology Consortium (wiki.geneontology.org/index.php/Immunology). The 1325 most relevant immune genes were selected.

*Th1 and Th2 differentiation genes.*

Genes known to be involved in $CD4^+$ T cell differentiation towards Th1 or Th2 cells were obtained from public datasets (KEGG hsa04658, R&D systems Pathways) and were analyzed in our differential expression gene sets.

*Deconvolution analysis*

Deconvolution studies were performed with CIBERSORT [19], which accurately quantifies the relative levels of different types of immune cells within a complex mixture of gene expression, and we used GED-IT to predict the cell type composition of tissue samples. We also used ICTD to deconvolute and identify immune cells [20].

*Real time RT-PCR*

Gene expression was quantified with the real-time quantitative polymerase chain reaction (qPCR) method previously described [21]. Total RNA was obtained according to the protocol described by the manufacturer (TRIzol reagent, Life Technologies, Inc.). Messenger RNA (2 mg) was reverse transcribed using oligo-deoxythymidine primers. Each PCR was done using a specific pair of oligonucleotides detailed in Table S1. A Rotor-Gene 3000 apparatus (Corbett Research, Mortlake, NSW, Australia) was employed to perform qPCR with a marker for DNA amplification (SYBR Green, Fermentas, Burlington, ON, Canada). Relative gene expression was calculated using a standard curve and normalized to β-actin as a housekeeping gene. Table S1 shows the oligos used for these amplifications.

*Immunohistochemistry*

The tumor tissues were cut into sections of 4 μm and treated with 3-aminopropyl-triethoxysilane for subsequent staining with hematoxylin and DBA or with specific antibodies for T-BET and IFNγ. Quantification of lymphocytes or positive-stain cells was performed using ImageJ software (Version 1.41, NIH) from three different sections of each tumor at 40x and 63x. We analyzed three tumors per experimental group.

*Methylation-specific PCR*

Tumor DNA extraction and purification (Quick-DNA Miniprep Plus Kit, Zymo and DNA Clean & Concentrator-25, Zymo) was performed following the manufacturer's instructions. Subsequently, the DNA was subjected to sodium bisulfite transformation (EZ-96 DNA Methylation MagPrep, Zimo). Promoter regions with CpG islands (FASTA and Methprime) were identified, and differential oligos for IFNγ and TGFβ were generated for these M&U regions (Table S2 and S3). Amplification was performed with both oligos together with the housekeeping gene MLH-1 in endpoint PCR. Subsequently, a nested q-PCR was performed using 4 μL of the product of the first amplification

## 3. Results

***Supplementation with $I_2$ increases the immune pathways associated with an antitumor response***

We first evaluated the expression level of genes involved in the immune response in the early and advanced tumors as compared with the normal tissue controls. Regardless of the tumor stage, $I_2$

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supplementation activates the Th1, Th17 antitumor differentiation pathways, receptor T cells, NK cytotoxicity, B cell receptor and antigen processing/presentation. Genetic overexpression was found to be twofold higher in advanced tumors than in early-stage tumors.

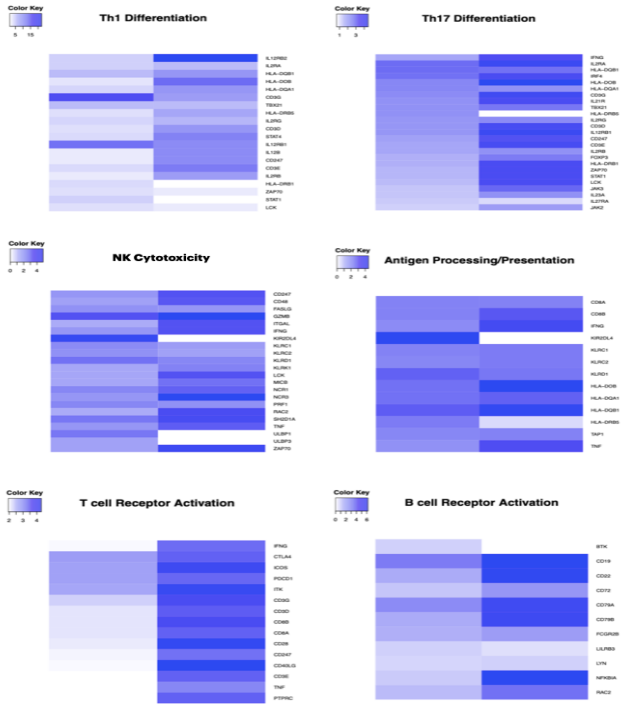


Figure 1. Immune pathways activated by iodine supplementation. The expression of genes in the $I_2$ group correspond to early-stage tumors and those of the Cht + $I_2$ group correspond to the advanced-stage tumors. A color scale specific for each pathway is depicted. The overexpressed genes for each pathway are shown in the right axis of each heatmap.

***$I_2$ increases the intratumoral ratio of antigen-presenting macrophages/dendritic cells in early-stage tumors and B lymphocytes in the advanced stage***

To identify the immune cell composition of the infiltrate, we conducted a deconvolution analysis with the CIBERSORT and ICTD algorithms. With both methods, we observed that the treatments ($I_2$ and Cht) are accompanied by an increase in the levels of M2 macrophages compared to the normal breast control group (Figure 2A-B). When analyzing the effect of the $I_2$ supplement on the proportions of intratumoral immune cells, different results were found depending on the software applied. The CIBERSORT analysis (Figure 2A) showed an increase in the relative number of macrophages M0, while ICTD interpreted this increase as dendritic cells (Figure 2B). In the case of advanced-stage tumors, supplementation with $I_2$ increased the fraction of B cells, pointing to an activation of the tumoral response in the presence of both treatments (Cht+$I_2$).

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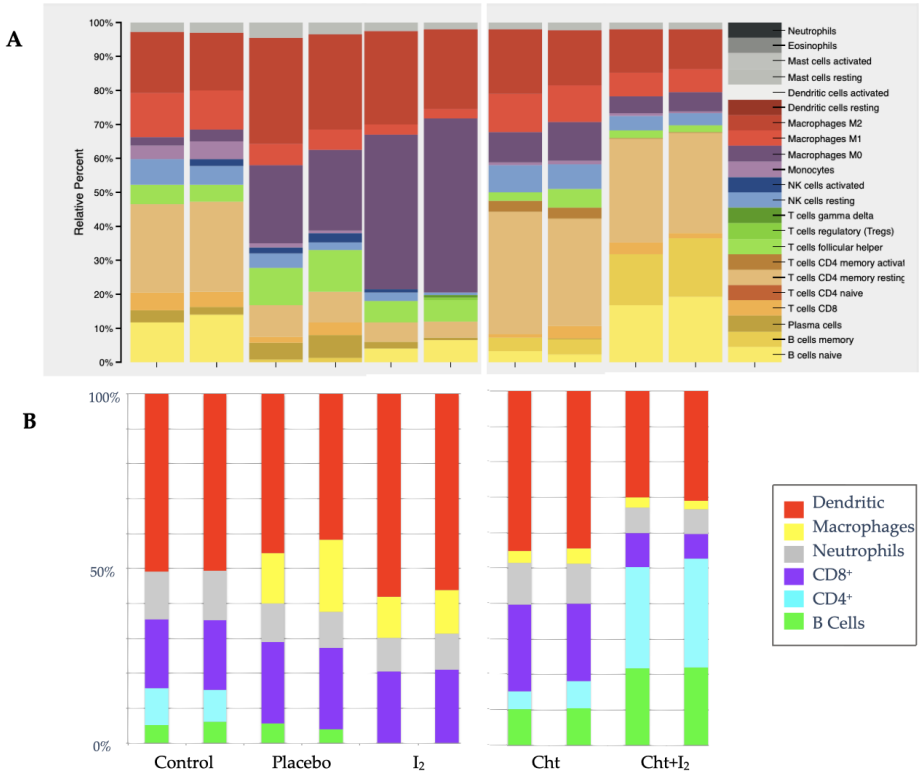


Figure 2. Deconvolution analysis of the relative composition of immune cells in normal tissue (control) and tumors. **A.** Deconvolution performed with CIBERSORT. **B.** Deconvolution obtained with ICTD. In both panels, the left bars correspond to early-stage tumors and the right bars to those in advanced stages. The composition of the immune infiltrate is color-coded and presented on the right side of each panel.

***I2 activates Th1 differentiation in the early stages of the disease, while in advanced stages it suppresses Th2 differentiation***

To corroborate the results obtained with the RNA-seq experiments, individual tumor samples were used to analyze markers of the cytotoxic IL12RB1, T-BET, IFNγ, and oncogenic GATA3 and TGFβ inducers. I2 supplementation is accompanied by a significant increase in the expression of T-BET and IFNγ, and by the repression of TGFβ in early-stage tumors (Figure 3). In advanced-stage tumors, I2 generates a decrease in the Th2 polarization marker GATA3. These data indicate that the presence of iodine at any stage induces an oncogenic polarization through Th1. The induction of T-BET and IFNγ (early stage) was also detected at the protein level in tumor tissues of patients supplemented with I2 in the early stage (Figure 4).

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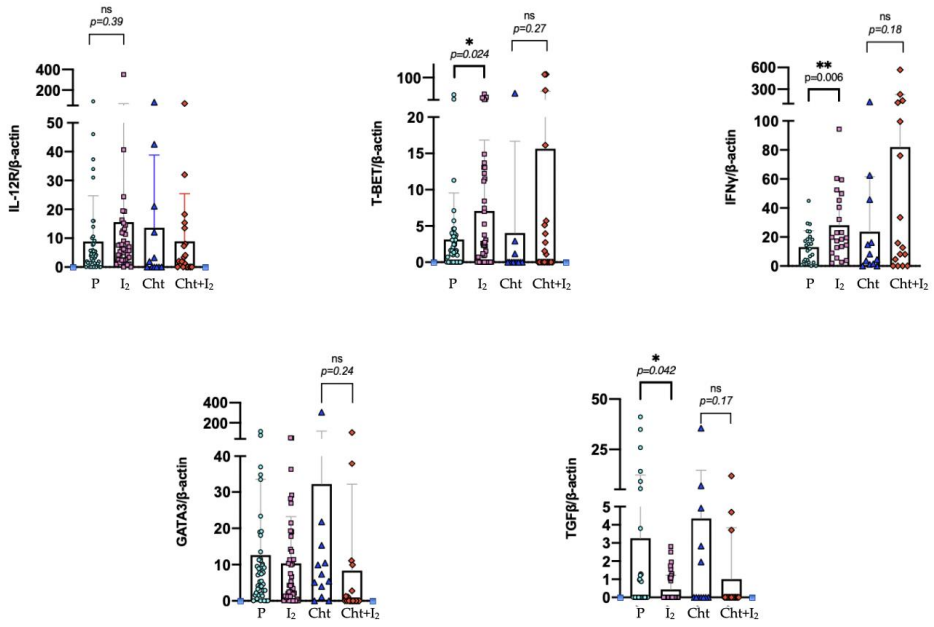


Figure 3. Gene expression of tumor suppressor cytotoxic and oncogenic inducers in individual samples. Expression was measured at the mRNA level by RT-qPCR. Data represent mean +/- SD of three independent experiments from three individual samples. Significant values correspond to a Student's t-test between $I_2$ and its respective control group (* p< 0.05). P: Placebo; $I_2$: Iodine; Cht: Chemotherapy and Cht+$I_2$: Chemotherapy and Iodine.

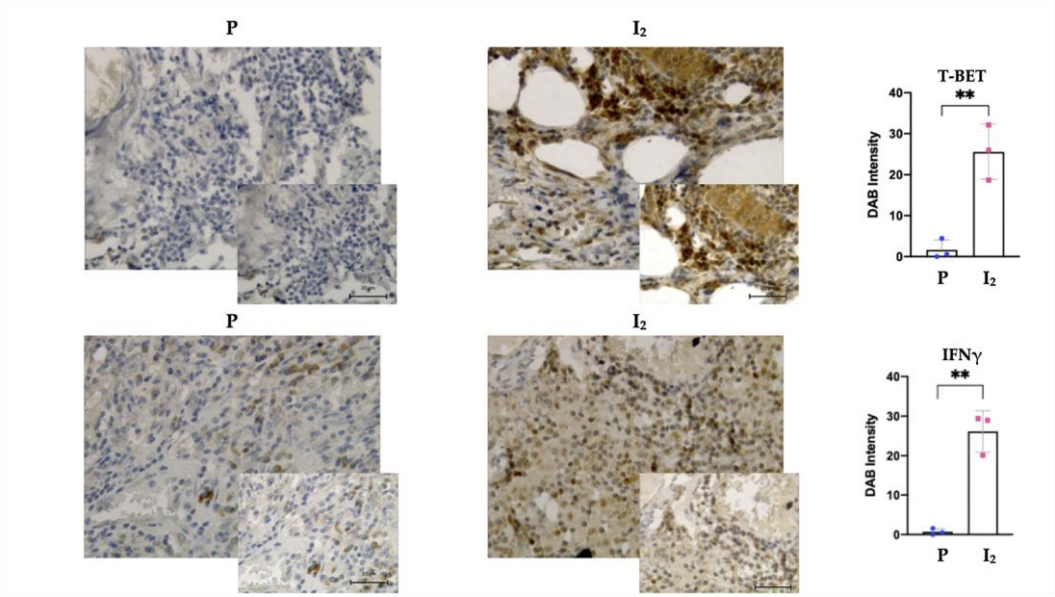


Figure 4. Protein expression of T-BET and IFNγ in individual tumor tissue samples. Data represent mean +/- SD of three independent immunochemistry experiments from three individual samples. Student's t-test ** P<0.05.

### *$I_2$ modifies the epigenetic landscape by demethylating antitumor gene promoters and maintaining methylated oncogenic genes*

To further investigate the molecular mechanisms of $I_2$ in the tumors, we evaluated the methylation status of IFNγ and TGFβ gene promoters with specific primers for the unmethylated (active) and methylated (inactive) states. Figure 5A shows that in early-stage tumors (placebo and $I_2$), there were no significant differences between unmethylated or methylated forms. In contrast, in the advanced-

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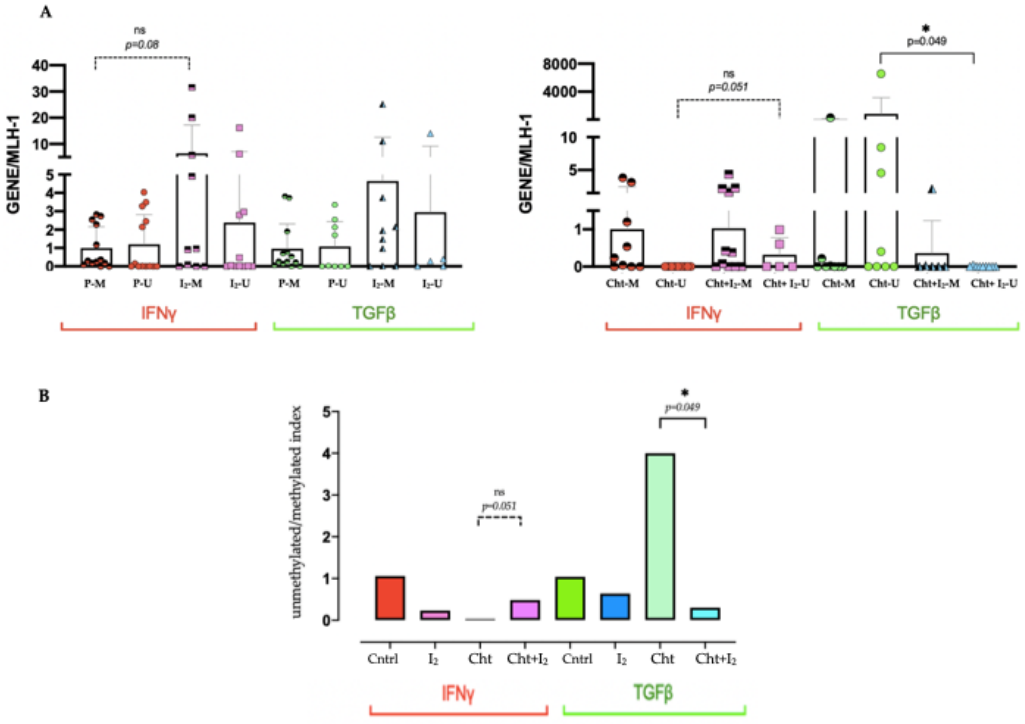
stage tumors, the presence of chemotherapy is accompanied by the absence of active IFNγ (unmethylated), and a significant number of active forms of TGFβ (unmethylated). In these conditions the presence of $I_2$ (Cht + $I_2$) showed changes through the highest levels of active IFNγ (p>0.051) and a total suppression of TGFβ (undetectable amount of unmethylated form; <0.049). These patterns become more evident when we analyzed the unmethylated / methylated index (Figure 5B), demonstrating that in tumors that were supplemented with both components (Cht + $I_2$), $I_2$ redirected the




activation of the Th1 antitumor pathway through epigenetic mechanisms.

Figure 5. Methylation pattern of IFNγ and TGFβ gene promoters. A. Amplification of the promoters (qPCR) of Unmethylated (U) or Methylated (M) forms in individual samples. The quantification was normalized by the expression of the housekeeping gene MLH-1. Left panel stage II and right panel stage III B. Unmethylated/Methylated index of each gene. Cntrl, control; $I_2$, iodine; Cht, chemotherapy; Cht+$I_2$, Chemotherapy plus iodine. Student's t-test *P<0.05

## 4. Discussion

Avoiding immune destruction and tumor-promoting inflammation in the tumor microenvironment are hallmarks of cancer initiation, and the immune component plays a key role in progression and metastasis [27]. Recognition of the critical importance of the microenvironmental component has resulted in a shift in therapeutic strategies, placing greater emphasis on treatments that include its modulation. While CAR-T cells and CTLA-4 and PD-1 blocking therapies are currently the most effective ways to reactivate the antitumor immune system, other components, some of natural origin, can reactivate the antitumor immune system and improve conventional therapies [28].

Molecular iodine is a micronutrient that shows antineoplastic properties in preclinical and clinical studies of breast cancer [29 ,30,10]. The mechanisms of action include direct antioxidant actions such as scavenging ROS and modulating mitochondrial functionality, as well as indirect actions activating PPARγ receptors, triggering apoptosis and cell redifferentiation [13, 31, 32, 33, 17]. In a previous analysis of this protocol, it was demonstrated that the $I_2$ supplement plus chemotherapy generated

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the best antitumor response (smaller tumor size and cancellation of chemoresistance) and increased the disease-free survival from 63 to 92% in five years in patients who received the $I_2$ supplement before and after surgery [17]. Transcriptomic analysis showed that $I_2$ promoted the antitumor response (Th1), increasing the presence and cytotoxic activity of intratumoral NK and CD8 + cells. In the present work, the specific analysis of the immunological profile showed that $I_2$ generally activates both the anti-oncogenic and oncogenic immune pathways (Th1, Th17), and that the presence of chemotherapy enhances the antitumor effect of $I_2$, as the response scale in these tumors (Cht + $I_2$) was more than double.

Deconvolution analysis showed that $I_2$ increases the amount of M0 (or dendritic cells) and B lymphocytes, corroborating the preponderance of the antitumor response. The two subtypes of augmented B cells were naïve B cells and memory B cells. Activated naïve B cells have been shown to promote Th1 polarization [34], while memory B cells can mount a rapid antibody response effectively controlling tumor growth [35]. Lymphocytes and macrophages are highly plastic cells that can change their phenotype in response to their microenvironment [36, 37]. Increased IFNγ synthesis has been associated with a better prognosis both by inhibition of Th2 and M2 oncogenic immune polarization [38] and by decreased angiogenic capacity [39]. Our results not only show an increase in the mRNA expression and protein content of IFNγ, but also the upstream activation of the Th1 pathway via expression of T-BET, which is the main regulator of IFNγ. T-BET (encoded by *TBX21*) is an immune cell-specific member of the T-box family of transcription factors. It is expressed in a variety of immune cells, including dendritic cells, NK, $CD4^+$ and $CD8^+$, B cells, and a subtype of Tregs. T-$BET^+$ cells function as antitumor lymphocytes by enhancing the production of cytokines such as IFNγ [40]. Previous studies have shown that the presence of intratumoral T-$BET^+$ lymphoid cells correlate with a good prognosis in all breast cancers [41]. We discovered that the Cht+$I_2$ combination not only promotes Th1 expression patterns in advanced-stage tumors, but also induces the silencing of key Th2 players such as GATA3. This transcription factor plays a critical role in the development of T cells in the thymus. Moreover, GATA3 controls the differentiation of naïve CD4 T cells and induces remodeling of the chromatin loci of Th2 cytokines and is an active repressor of IFNγ expression [42]. The mechanisms by which $I_2$ induces this transdifferentiation effect in the tumor microenvironment have received scant attention. However, it is well described that immune modulation components are regulated by epigenetic mechanisms, where natural factors derived from the diet could take part [6,43]. In fact, in cancer progression many of the changes in expression patterns are regulated at the epigenetic level by methylation/demethylation in gene promoters [44]. Recently, ascorbic acid has received great attention since this micronutrient participates as a cofactor of TET enzymes (ten eleven translocations) involved in histone and DNA demethylation and, therefore, in the epigenetic regulation of gene expression [45]. TET proteins convert 5-methylcytosine (5mC) to 5-hydroxy-methylcytosine (5hmC), 5-formylcytosine (5fC), and finally to 5-carboxytosine (5caC). Then, 5fC and 5caC are replaced by cytosine by base cleavage repair machinery [46]. Ascorbic acid increases TET-dependent 5hmC production and induces cytosine demethylation in mammals [47]. Furthermore, in a lymphoma mouse model, the intratumoral epigenome revealed a global increase of 5hmC after ascorbic acid treatment in the presence of PD1, suggesting a direct effect of ascorbic acid on CD8+ T cells and their cytotoxic function [48]. Interestingly, $I_2$ exerts antioxidant effects in the same way as ascorbic acid does, by producing electrons, and in ferric reactions that measure its capacity, $I_2$ is 10 times more potent than ascorbic acid [14]. To the best of our knowledge there are currently no studies examining the role of $I_2$ in the functionality of TETs. In conclusion, the preliminary findings from this study indicate that $I_2$, when used in conjunction with conventional chemotherapy, induces immune activation and redirects the response to the Th1 pathway through methylation and demethylation mechanisms.

**Supplementary Materials:** Table S1, Table S2, Table S3

**Author Contributions:** Conceptualization, C.A. and O.C-M.; methodology O.C-M, A.M-R., F.V-P. , E.D. and M.R-D.; software, O.C-M. and A.M-R; validation, C.A. and B.A; formal analysis O.C-M. and E.D.; investigation O.C-M.; resources, C.A and O.C-M.; data curation, O.C-M, C.A and A.M-R.; writing—original draft preparation, O.C-M.; writing—review and editing, O.C-M., C.A and B.A.; visualization, C.A., F.V-P. and A.M-R.; supervision, C.A., F.V-P. and A.M-R.; project administration, C.A.; funding acquisition, C.A and O.C-M. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** In the previous manuscript [17] these statements were described.

**Data Availability Statement:** The complete annotated sequences from the RNA-sequencing are available at the European Nucleotides Archives website (https://www.ebi.ac.uk/ena/erp110028).

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**Conflicts of Interest:** The authors declare no conflict of interest.

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