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Full-length Article

Perioperative inhibition of β-adrenergic and COX2 signaling in a clinical trial in breast cancer patients improves tumor Ki-67 expression, serum cytokine levels, and PBMCs transcriptome

Rita Haldar[a], Lee Shaashua[a], Hagar Lavon[a], Yasmin A. Lyons[b], Oded Zmora[c], Eran Sharon[d], Yehudit Birnbaum[d], Tanir Allweis[e], Anil K. Sood[b], Iris Barshack[f], Steve Cole[g], Shamgar Ben-Eliyahu[a,*]

[a] Sagol School of Neuroscience and School of Psychological Sciences, Tel Aviv University, Israel
[b] Department of Gynecologic Oncology and Reproductive Medicine, Division of Surgery, M.D. Anderson Cancer Center at University of Texas, Huston, TX, USA
[c] Department of Surgery and Transplantation, Sheba Medical Center, Ramat Gan, Israel
[d] Department of Surgery, Rabin Medical Center, Beilinson Hospital, Petach-Tikva, Israel
[e] Department of Surgery, Kaplan Medical Center, Rehovot, Israel
[f] Department of Pathology, Sheba Medical Center, Ramat Gan, Israel
[g] Department of Medicine, Department of Psychiatry and Biobehavioral Sciences, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

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ABSTRACT

Catecholamines and prostaglandins are secreted abundantly during the perioperative period in response to stress and surgery, and were shown by translational studies to promote tumor metastasis. Here, in a phase-II biomarker clinical trial in breast cancer patients (n = 38), we tested the combined perioperative use of the β-blocker, propranolol, and the COX2-inhibitor, etodolac, scheduled for 11 consecutive perioperative days, starting 5 days before surgery. Blood samples were taken before treatment (T1), on the mornings before and after surgery (T2& T3), and after treatment cessation (T4). Drugs were well tolerated. Results based on a-priori hypotheses indicated that already before surgery (T2), serum levels of pro-inflammatory IL-6, CRP, and IFNγ, and anti-inflammatory, cortisol and IL-10, increased. At T2 and/or T3, drug treatment reduced serum levels of the above pro-inflammatory cytokines and of TRAIL, as well as activity of multiple inflammation-related transcription factors (including NFκB, STAT3, ISRE), but not serum levels of cortisol, IL-10, IL-18, IL-8, VEGF and TNFα. In the excised tumor, treatment reduced the expression of the proliferation marker Ki-67, and positively affected its transcription factors SP1 and AhR. Exploratory analyses of transcriptome modulation in PBMCs revealed treatment-induced improvement at T2/T3 in several transcription factors that in primary tumors indicate poor prognosis (CUX1, THRa, EVI1, RORa, PBX1, and T3R), angiogenesis (YY1), EMT (GATA1 and deltaEF1/ZEB1), proliferation (GATA2), and glucocorticoids response (GRE), while increasing the activity of the oncogenes c-MYB and N-MYC. Overall, the drug treatment may benefit breast cancer patients through reducing systemic inflammation and pro-metastatic/pro-growth biomarkers in the excised tumor and PBMCs.

1. Introduction

Among women in the western world, breast cancer (BC) is the most prevalent malignant disease and the second leading cause of cancer-related mortality (Siegel et al., 2016). Advances in early detection and therapeutic interventions have improved survival rates, reaching up to 95% when a primary tumor (PT) is detected at an early stage. However, BC-related mortality rises up to ~73%, depending on tumor and patients' characteristics at the time of surgery, with metastatic disease accounting for the great majority of cancer related deaths (Siegel et al., 2016).

Surgical excision of the PT is crucial for cancer treatment. However, surgery may facilitate the progression of pre-existing micrometastases and/or the formation of new metastases, through numerous pro-metastatic and immune-suppressive processes (Horowitz et al., 2015; Neeman et al., 2012). Starting days before surgery (Bartal et al., 2010) and continuing throughout the perioperative period, patients experience distress and anxiety (Lutgendorf et al., 2010; Moyer and Salovey,

* Corresponding author at: Sagol School of Neuroscience and School of Psychological Sciences, Tel Aviv University, Tel Aviv 69978, Israel.
E-mail address: Shamgar@post.tau.ac.il (S. Ben-Eliyahu).

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1996), which lead to the activation of the sympathetic nervous system (SNS) and the hypothalamus-pituitaryadrenal (HPA) axis, and the consequent release of catecholamines (CAs) and glucocorticoids. These and other stress responses were reported to suppress cytotoxicity of T-lymphocytes and NK-cells (Andersen et al., 1998; Ben-Eliyahu et al., 2000), trigger pro-inflammatory processes (Steptoe et al., 2007; Sugama and Conti, 2008), and directly affect the malignant tissue and its microenvironment. It was also suggested that stress causes a shift toward a Th2 cytokine dominance (Greenfeld et al., 2007; Kim and Maes, 2003; Yang and Glaser, 2002), although this phenomenon seems more complex (Greenfeld et al., 2007). Together, these adverse effects of stress and of surgery, which occur simultaneously during the short perioperative period, were shown in translational studies to promote metastatic growth (Horowitz et al., 2015; Neeman et al., 2012).

Importantly, numerous studies indicated that excess secretion of CAs and prostaglandins (PGs; specifically PGE2) mediates many of the pro-metastatic effects of stress and surgery, while glucocorticoids seem to have a lesser role (Cole et al., 2015; Horowitz et al., 2015; Rosenne et al., 2014). Inflammation is considered a hallmark of cancer progression (Grivennikov et al., 2010; Trinchieri, 2012), and sympathetic activation has recently been shown to promote several pro-metastatic processes at the tumor and at the host levels. Both CAs and PGs are abundant during the perioperative period, and are released both by the malignant tissue (Wojtowicz-Praga, 1997) and the host, as a result of psychological and surgery-induced stress responses and tissue damage (Buvanendran et al., 2006; Traynor and Hall, 1981).

One significant mechanism throughout which CAs and PGs were suggested to promote tumor metastasis is through the modulation of the cytokine network, specifically through (i) local and systemic up-regulation of pro- and anti-inflammatory soluble factors (e.g. IL-6, CRP, TNFα and IL-10) (Baumann and Gauldie, 1994; Elenkov et al., 2005; Elenkov et al., 2000; Hinson et al., 1996), (ii) increased levels of pro-angiogenic cytokines (e.g., IL-8, VEGF) (Cole and Sood, 2012; Singh et al., 2006), and (iii) an alleged suppression of Th1 cytokine production (e.g. IFNγ) (Calcagni and Elenkov, 2006; Elenkov et al., 2000; Pockaj et al., 2004), causing a shift toward Th2 cytokine balance (Elenkov, 2008; Kalinski, 2012) and potential suppression of Th1-dependent anti-metastatic immunity (Yang and Glaser, 2002). For example, *in vitro* and *in vivo* manipulation of PGs or CAs levels restored tumor-related Th1/Th2 cytokine balance by elevating IL-12 and reducing IL-10 levels (Elenkov et al., 1996; Stolina et al., 2000); and *in vitro* adrenergic stimulation induced a β-adrenergic-dependent secretion of IL-8 by monocytes (Kavelaars et al., 1997).

Importantly, pre-clinical animal studies indicated that β-adrenergic blockade, alone or together with PGs synthesis inhibition, can reduce the immune-suppressive and metastasis-promoting effects of stress and surgery in several tumor models (Ben-Eliyahu et al., 2000; Bozinovski et al., 2016; Lee et al., 2009; Melamed et al., 2005; Roche-Nagle et al., 2004; Shakhar and Ben-Eliyahu, 1998; Sloan et al., 2010; Sood et al., 2006; Yakar et al., 2003). Specifically, the combined drug treatment counteracted the effects of stress and surgery, improving organ tumor clearance and overall long-term survival rates following PT excision (Benish et al., 2008; Glasner et al., 2010; Inbar et al., 2011). In certain cases, only the combination of the two drugs was effective (Benish et al., 2008; Glasner et al., 2010; Sorski et al., 2016), presumably because surgery simultaneously increases levels of both ligand families, and thus blockade of only one factor (either CAs or PGs) would be insufficient. A recent study also demonstrated the interplay between CAs and PGs in breast and colon cancer samples, where COX2 blockade prevented the expression of immunosuppressive factors (e.g. IL-10 & indoleamine 2,3-dioxygenase (IDO)) induced by epinephrine (Muthuswamy et al., 2017). Therefore, in the clinical study presented herein, and in an attempt to maximize our ability to demonstrate beneficial effects, only the combination of the two drugs was used.

The current randomized two-arm placebo-controlled phase-II biomarker clinical trial tested the combined perioperative use of propranolol and etodolac in BC patients. In a recent report, we provided results from this trial based on tumor whole genome mRNA analysis, indicating that the treatment positively affected pro-metastatic and pro-inflammatory biomarkers in the excised tumor tissue (Shaashua et al., 2017). Here, we report immunological results from four blood samples taken perioperatively (twice before and twice after surgery, see Fig. 2) and histological analysis of the excised tumor tissue. We aimed to test the hypotheses that drug treatment would reduce stress and immune-related inflammatory responses, identify potential mediating molecular mechanisms, and study indices of anti-metastatic immunity and molecular biomarkers of long-term cancer outcomes. To this end, we assessed (i) transcription control pathways and whole genome mRNA profile in peripheral blood mononuclear cells (PBMCs), (ii) serum levels of several cytokines and soluble factors, and (iii) markers of proliferation and cancer progression in the tumor, using immunohistochemistry (IHC) staining and mRNA profiling.

2. Materials and methods

2.1. Patients and inclusion/exclusion criteria

Thirty-eight women (age 33–70, M = 55.3, SD = 8.71) newly diagnosed with BC without known metastatic disease were recruited in three medical centers in Israel (Sheba, Rabin, and Kaplan), approximately three weeks following BC diagnosis. Groups did not differ significantly in demographic or clinical characteristics (See Table1). Exclusion criteria included (i) contraindications for any of the drugs, such as diabetes, asthma, cardiovascular diseases, and low blood pressure, (ii) chronic use of any β-blocker or COX inhibitor, and (iii) chronic autoimmune disease. The protocol (ClinicalTrials.gov Identifier: NCT00502684) was approved by the institutional review boards of all participating institutions, and written informed consent was obtained from all patients upon recruitment to the study. Two patients, one from each group, reported physical discomfort within the first 2 days of treatment (before hospitalization, see Section 3.1) and exited the study at their request without further medical examination. Thus, unless otherwise noted, the results reported are from 18 patients from each group (see CONSORT Fig. 1).

2.2. Study design and drug treatment

A multicenter double-blind placebo-controlled randomized biomarker trial was conducted, employing two equal arms of drug- and placebo-treatment (Fig. 2). Patients' randomization was stratified by age in each medical center (below or above 50). Drugs or placebo were administered for 11 consecutive days, starting five days before surgery (Fig. 2). Both drugs and the placebo were consumed orally (standard pills), and were manufactured by “Super pharm professional“. Oral BID Etodolac (400 mg) was administered throughout the treatment period. Propranolol was administered orally: (i) 20 mg of immediate release BID during the five days before surgery; (ii) 80 mg of extended release on the morning of surgery and on the evening and morning following surgery; and (iii) 20 mg of immediate release BID thereafter during five postoperative days. Identical schedule and capsules were used for placebo and medication.

2.3. Blood samples collection and preparation

Four blood samples were taken between 7 and 11 AM and were transferred at room temperature to our laboratory for processing that started exactly 2 h after blood draw. The first (T1) was taken before the initiation of medications; the second and third on the mornings before

Table1
Baseline demographic and clinical characteristics of patients.

	Control Group n = 18		Treatment Group n = 18		p
Age Mean (MIN, MAX)	55.2 (33, 70)		55.3 (41, 70)		0.97
BMI Mean (MIN, MAX)	25.7 (20.3, 32.0)		26.3 (19.4, 36.5)		0.73
Weight Mean (MIN, MAX)	68.1 (52, 86)		69.5 (50, 103)		0.75
Smoking	NO	15	NO	9	0.06
	YES (< 5 cigarette per day)	1	YES (< 5 cigarette per day)	2	
	YES (> 5 cigarette per day)	1	YES (> 5 cigarette per day)	6	
	NA	1	NA	1	
T Staging	Tis	2	Tis	0	0.33
	T1	9	T1	13	
	T2	5	T2	3	
	T3	0	T3	0	
	NA	2	NA	2	
Histological Grade	HG1	5	HG1	3	0.74
	HG2	6	HG2	10	
	HG2/3	1	HG2/3	1	
	HG3	2	HG3	1	
	DCIS/LCIS	4	DCIS/LCIS	3	
Surgical Resection	Lumpectomy	13	Lumpectomy	15	0.56
	Mastectomy	2	Mastectomy	2	
	Other[a,b,c]	3	Other[d]	1	
Metastatic Spread	No	18	No	16	0.34
	NA	0	NA	1	
			Axillary metastasis	1	
ER Status	Negative	2	Negative	1	0.83
	Positive	16	Positive	17	
PR Status	Negative	6	Negative	5	0.93
	Positive	12	Positive	13	
HER2/neu status	Negative	9	Negative	8	0.76
	NA	4	NA	3	
	Positive	5	Positive	7	
Tumor Max. Diameter	1.6 cm		1 cm		0.11
Carcinoma	Invasive	13	Invasive	14	0.6
	Non-invasive	3	Non-invasive	1	
	NA	2	NA	2	

[a] Mastectomy + Immediate reconstruction with silicone.
[b] Lumpectomy (double-Lt&Rt).
[c] Lumpectomy (+Intraoperative radiation).
[d] Mastectomy with axillary sentinel lymph node excision.

and after surgery (T2 and T3, respectively), at least 1 h after the morning medication; and the fourth (T4) at least 2 days after treatment cessation (median of 16 days post-medication, average of 16.91 days Fig. 2). One tube of 10 ml without preservative/anticoagulant was centrifuged for 30 min at 1700*g* for serum collection after being allowed to clot for 2 h. A second tube was used for harvesting PBMCs from whole blood (BD Vactainer8ml CPT mononuclear cell preparation tube containing sodium heparin; BD bioscience; San Jose, CA, USA) according to manufacturer's instructions. PBMCs suspension was washed twice in PBS (15 min at 335*g* for both washes) and re-suspended in 350 µl RLT buffer (TMO, Waltham, MA, USA), in preparation for mRNA extraction. Serum samples and PBMCs in RLT were frozen at −80 °C for future analyses that were conducted simultaneously on all samples from all patients.

2.4. Serum assessment of soluble factors

Serum cytokines levels were measured using enzyme linked immunosorbent assay (ELISA) according to manufacturer instruction. Cortisol (KGE008), high-sensitivity IL-6 (HS600B), high-sensitivity IL-8 (HS800), high-sensitivity TNFα (HSTA00D), TRAIL (DY375), IL-18 (DY318), VEGF (DY293B) and CRP (DY1707) ELISA kits were purchased from R&D systems (Minneapolis, MN, USA); high-sensitivity IFNγ (BMS228HS), high-sensitivity IL-10 (BMS215HS), high-sensitivity IL-4 (BMS225HS), high-sensitivity IL-12p70 (BMS238HS) and IL-15 (BMS2106) ELISA kits were purchased from eBioscience (San Diego, CA, USA). For elaborated information regarding low limit of detection and baseline levels of the cytokines tested see Table2. All 4 samples of each patient were assayed in duplicates within the same ELISA plate, and the intra-assay coefficient of variance (CV%) was 1–4%.

2.5. IHC staining of tumor tissue for proliferation and pro-metastatic markers.

3.5 µm sections were cut from representative formalin fixed paraffin embedded (FFPE) blocks of breast tumors, sent to Dr. Sood's laboratory at M.D. Anderson Cancer Center (University of Texas, Huston, Texas, USA), and IHC stained for Ki-67, IL-8, MMP2, MMP9 (Abcam, Cambridge, UK), and uPA/PAI1 (Acris antibodies, San Diego, California, USA), as elaborately described in (Dalton et al., 2017). We exposed control samples to secondary antibody alone, and they did not show any nonspecific staining. To quantify expression levels, a researcher naïve to group assignment examined five random 0.159 mm^2 fields at 40× magnification for each tumor and counted Ki-67 positive cells within those fields. We then calculated, for each tumor, the median of Ki-67 expression in each tumor.

2.6. Transcriptome and bioinformatic analyses

PBMCs were extracted from 8 ml of blood. The number of PBMCs used in the whole genome analyses is approximately $8*10^6$ (assuming a typical density of 1 million cells/ml), but varies slightly as a function of cell density across study subjects. Total RNA was extracted from PBMCs, tested for suitable mass (PicoGreen RNA, Thermo-Fisher) and integrity (RNA integrity number, as derived from capillary electrophoresis by Agilent TapeStation) and 200 ng of RNA were subjected to genome-wide transcriptional profiling, using Illumina Human HT-12 v4 Expression BeadChips and the manufacturer's standard protocol for cRNA synthesis (Illumina Inc., San Diego, CA, USA) with hybridized arrays scanned on an Illumina BeadLab instrument, as described in detail elsewhere (Bolstad et al., 2003). Gene expression values were quantile-normalized, $\log_2$-transformed, and subjected to general linear model analyses, identifying genes showing ≥1.20-fold difference on average expression between the drug and placebo groups, after-controlling for study site, tumor stage, age, smoking history, and BMI. Functional characteristics of the differentially expressed genes were identified by GOstat (http://gostat.wehi.edu.au/cgi-bin/goStat2.pl) Gene Ontology analysis (Beissbarth and Speed, 2004). Statistical testing of bioinformatics results was based on standard errors derived from bootstrap re-sampling of linear model residual vectors over all genes assayed.

To identify transcription control pathways that may contribute to the observed differences in gene expression, we used the Transcription Element Listening System (TELiS, http://www.telis.ucla.edu/) bioinformatic analysis of transcription factor binding motifs (TFBM) in gene promoters (Cole et al., 2005), using TRANSFAC (http://www.generegulation.com/pub/databases.html) position-specific weight matrices (Wingender et al., 1996), as previously described (Cole et al., 2007, 2010). A-priori hypotheses regarding activity of BC-relevant transcription control pathways and cytokine-related pathways were tested; Inflammatory transcription factors (TFs) (NFκB/cRel and AP-1), Interferon-related TFs (IRFs, ISRE), cytokine related factors (STAT1,

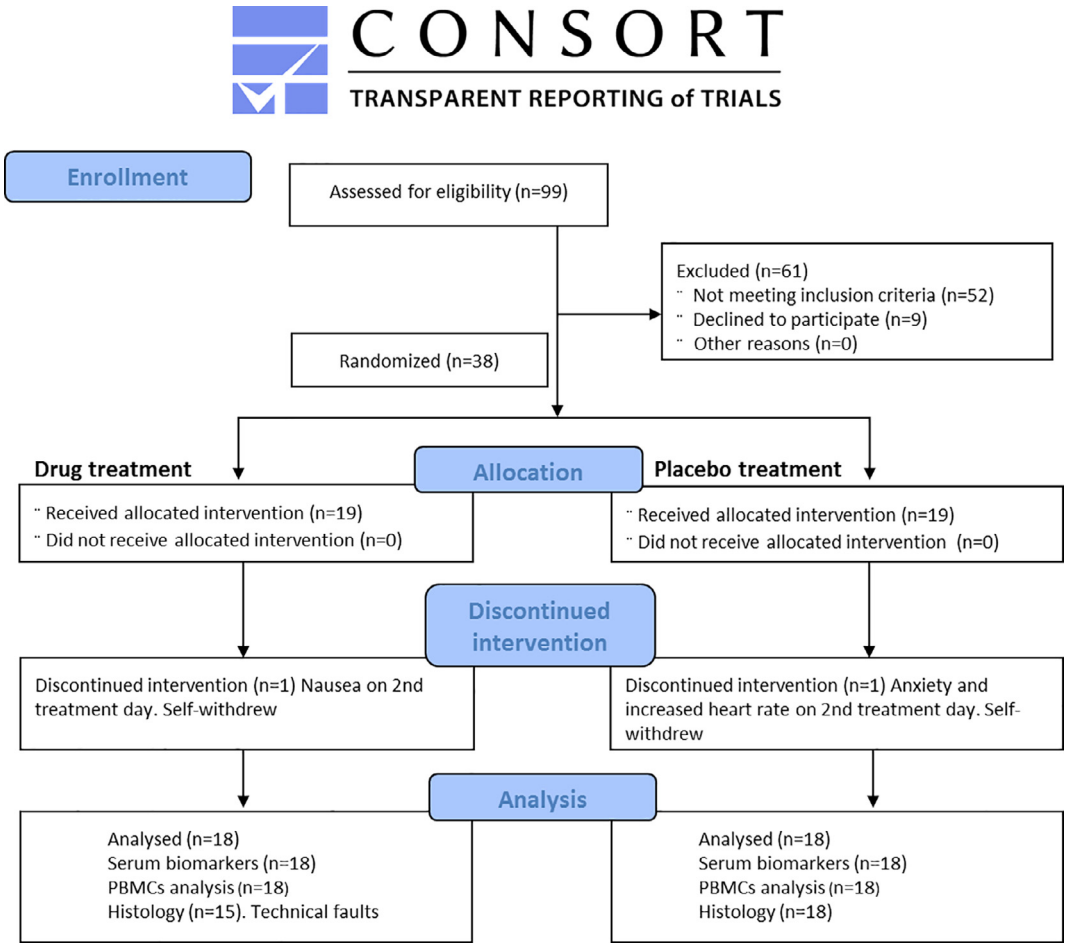

Fig. 1. CONSORT diagram of clinical trial enrollment and treatment.

STAT3, OCT1, SRF, EGR1, E4BP4, SP1), Th2-cell differentiation (GFI1, GATA3), and the neuroendocrine response factors (CREB, glucocorticoid response element [GRE])) were evaluated. A-priori hypotheses regarding TFs related to Ki-67 activity (AhR and SP1) were also studied in PBMCs and in tumor tissue. Additionally, TFs that showed group differences, but were not included in our a-priori hypotheses, were scanned to identify potential cancer-related TFs with clinical significance, to be addressed as exploratory outcomes (detailed in Section 3.9). To ensure that results were not confounded by individual differences in the prevalence of specific leukocyte subtypes within the PBMCs

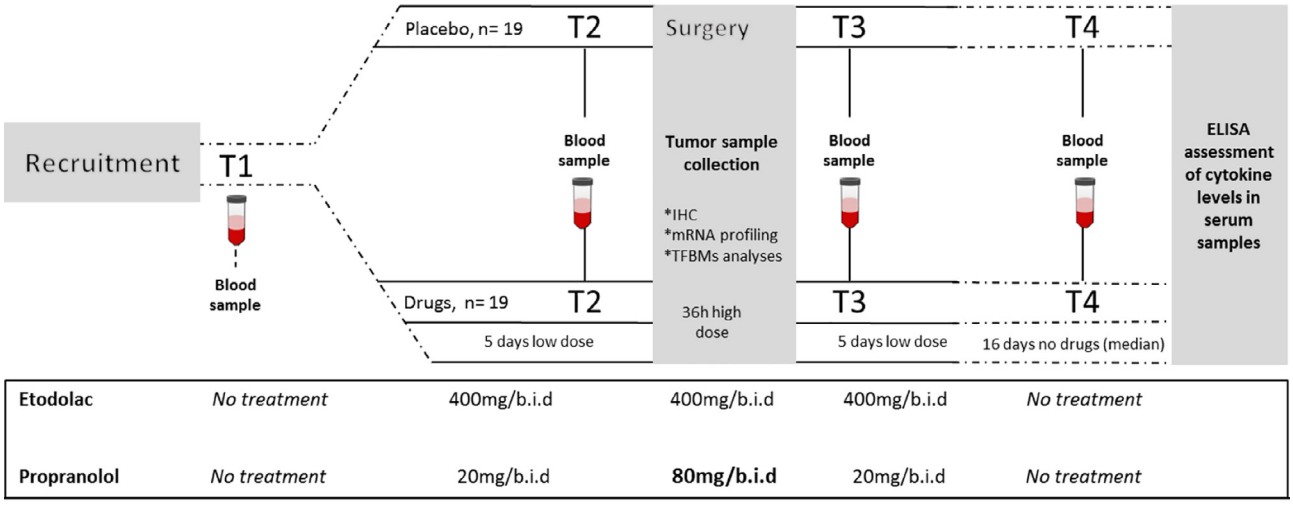

Fig. 2. Schematic presentation of the design and time schedule of the study.

Table 2
Serum cytokine baseline levels and their detection levels in the assays conducted.

	Baseline serum levels (at T1)	Low limit of detection
IL-6 Mean (MIN, MAX)	1.55 pg/ml (0.61, 2.75)	0.16 pg/ml
CRP Mean (MIN, MAX)	3030.8 ng/ml (80.3, 21,063.6)	15.6 pg/ml
IFNγ Mean (MIN, MAX)	0.25 pg/ml (0.03, 2.33)	0.03 pg/ml
TRAIL Mean (MIN, MAX)	11.22 pg/ml (2, 30.46)	2 pg/ml
IL-10 Mean (MIN, MAX)	0.9 pg/ml (0.28, 2.56)	0.2 pg/ml
Cortisol Mean (MIN, MAX)	53.21 ng/ml (19.7, 126.03)	0.16 ng/ml
IL-18 Mean (MIN, MAX)	273.46 pg/ml (146.23, 583.4)	5.86 pg/ml
IL-8 Mean (MIN, MAX)	17.14 pg/ml (7.74, 44.7)	1 pg/ml
TNFα Mean (MIN, MAX)	1.38 pg/ml (0.66, 2.57)	0.25 pg/ml
VEGF Mean (MIN, MAX)	73.8 pg/ml (9.23,238.9)	7.81 pg/ml
IL-4 Mean (MIN, MAX)	Undetectable	0.031 pg/ml
IL-12p70 Mean (MIN, MAX)	Undetectable	0.16 pg/ml
IL-15 Mean (MIN, MAX)	Undetectable	3.9 pg/ml

pool (Cole, 2010) , analyses of differential gene expression controlled for the prevalence of transcripts marking T-lymphocyte subsets (*CD3*, *CD4*, *CD8A*), B-lymphocytes (*CD19*), natural killer cells (CD56/*NCAM1*, CD16/*FCGR3A*), and monocytes (*CD14*) (Cole et al., 2007).

2.7. Statistical analysis

Unless otherwise indicated, analyses were conducted based on a-priori hypotheses derived from previous animal and human studies. Our hypothesis was that drug treatment will reduce levels of peripheral pro-inflammatory and pro-metastatic indices (specified above), and pro-liferative or pro-metastatic indices in the excised tumor tissue. For serum cytokine analyses, data was $\log_2$ transformed to stabilize variance, and then transformed within subject to % of average of T1&T4 (no-treatment time-points) in order to assess the impact of drug treatment while minimizing individual differences. Patients with unusual baseline levels (more than 4 SD from the mean) in a specific cytokine were excluded from the analysis of that cytokine, but not from other analyses. Repeated measures analysis of variance (ANOVA) was used to assess group differences (drug treatment), time (T1–T4), and interaction, and Fisher's PLSD post-hoc comparisons were performed to analyze group differences at specific time-points. In addition, a planned contrast between drug and placebo groups during treatment (T2&T3) vs off treatment (T1&T4) was used (i.e., Drugs [(T2 + T3) − (T1 + T4)] – Placebo [(T2 + T3) − (T1 + T4)]). Associations between cytokines were examined based on Pearson correlations and a-priori hypotheses derived from previous studies. For mRNA and TFs analyses, data was $\log_2$ transformed, and then the planned contrast "Drugs [(T2 + T3) − (T1 + T4)] – Placebo [(T2 + T3) − (T1 + T4)]" was used to compare the impact of drug treatment (drug vs placebo) on the difference between T2&T3 and T1&T4 within each group. When conducting TFs analyses, another two planned contrasts were used to focus only on T2 (i.e., Drugs [(T2) − (T1 + T4)] – Placebo [(T2)-(T1 + T4)]) or only on T3 (i.e., Drugs [(T3) − (T1 + T4)] – Placebo [(T3) − (T1 + T4)]) to separately study pre- or post-operative measures, respectively. Thee effect size of TFs (i.e. Fold difference TFBMs distribution (Log_2 Drugs/Placebo contrast)) was calculated by $\log_2$ of groups mean ratio. Each group mean score is based on the contrast described above (e.g., T2T3-T1T4), and the standard errors derived from bootstrap re-sampling of linear model residual vectors over all genes assayed. For histological analyses, within each patient, five regions of interest were chosen for quantifying expression levels as detailed in Section 2.5, and then the median was taken for further analysis to assess the impact of the drug treatment on these markers. T-test was used to assess group differences in expression levels.

3. Results

3.1. Drug treatment: adherence and adverse effects

Overall, no serious adverse drug-related events were evident during drug treatment, and for up to 30 days following surgery. One placebo-treated patient reported feeling anxious and showed increased blood pressure and heart rate, and one drug-treated patient reported feeling nausea. Both patients reported these minor adverse conditions within the first two days of placebo/drug treatment, and self-withdrew, without further examination. The other 36 patients did not report any physical discomfort, consume at least 95% of the medications (based on self-report and/or reports from medical staff during hospitalization), and provided all four blood samples (excluding one patient in the placebo group that did not provide the T4 sample).

3.2. Undetectable serum levels, and an overview of differentially expressed genes and TFBMs

Serum levels of IL-4, IL-12p70, and IL-15 were below detection levels in all subjects (high-sensitivity ELISA kit – see Table 2), and are not reported. Serum levels of VEGF did not differ significantly between groups or time-points, and are shown in Table 2. Approximately 370 transcripts in PBMCs (~170 up-regulated, ~200 down-regulated) showed ≥20% difference from drug- vs placebo-treated patients in T1 or T4 (non-treated time-points), whereas approximately 600 transcripts (~320 up-regulated, ~280 down-regulated) showed such differences at T2 or T3. Based on these later ~600 differentially expressed mRNAs, promoter-based bioinformatics analyses identified 42 TFBMs (out of 192 assessed) that are possibly involved in the regulation of these genes ($p < .05$) (as a function of drug treatment, T2T3 contrast, see Supplementary S2). Similarly, when separately studying T2 or T3, the respective contrasts indicated 38 and 61 significant TFBMs (see Supplementary S2). However, analyses are presented only regarding the 16 a-priori hypothesized TFs (specified in Sections 3.3–3.6 and 3.10), and 12 TFs that were not a-priori hypothesized, but were reported in previous studies to predict cancer clinical outcomes when over-expressed in the PT (N-MYC, C-MYB, PBX1, CUX1, THRa, EVI1, RORa, and T3R), or were shown to promote pro-metastatic characteristics such as angiogenesis (YY1), tumor-proliferation (GATA2) and epithelial-to-mesenchymal-transition (EMT; GATA1 and deltaEF1/ZEB1 TFs). These 12 TFs are discussed in Sections 3.9 and 4.7, and were addressed by an exploratory approach.

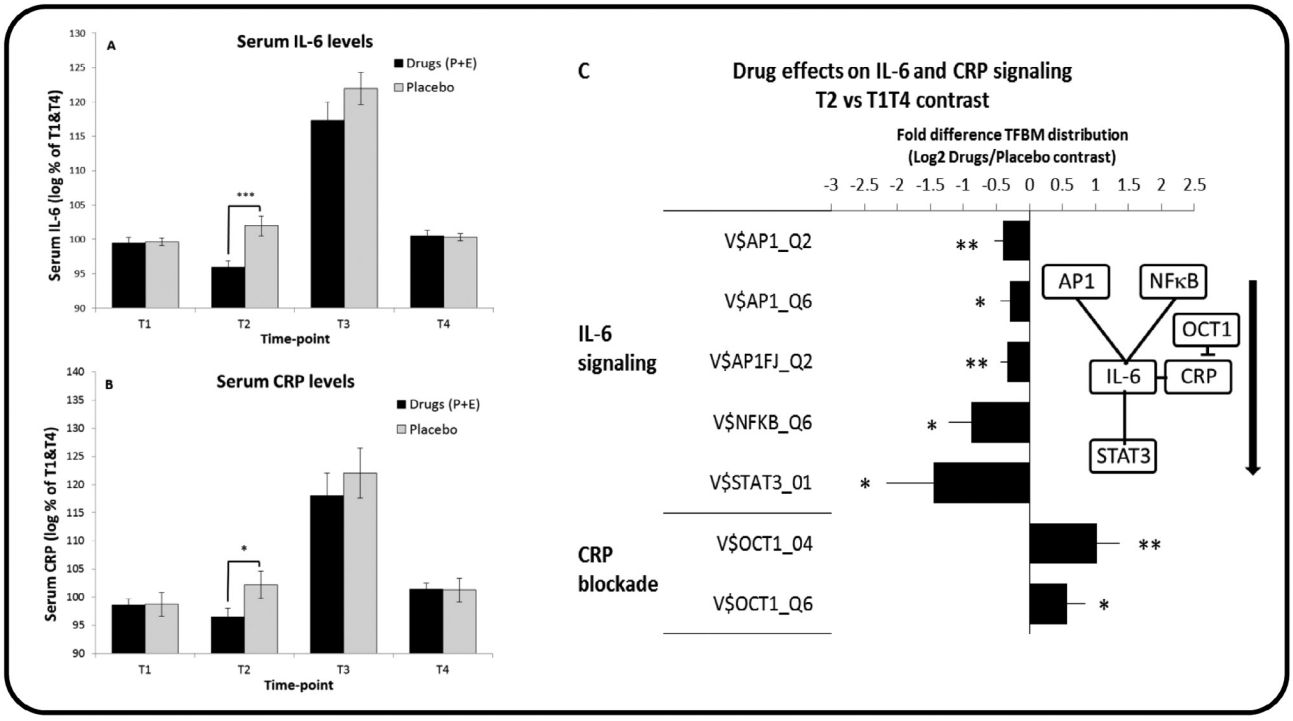

Fig. 3. Effect of drug treatment on serum levels of (A) IL-6 and (B) CRP, and (C) their relevant TFs at T2 vs. T1T4 contrast. Treatment-induced effects are consistent between serum levels and PBMCs molecular pathways of IL-6 and CRP. Data presented as mean ± SEM. Group differences at a specific time point are indicated by *($p < .05$), **($p < .01$) and ***($p < .001$).

3.3. Drug treatment reduced serum levels of IL-6 and CRP, as well as related intracellular regulatory pathways and mRNAs

Three patients from the placebo group had high baseline (before treatment) levels of IL-6 (6–32 pg/ml, compared to a mean of 1.55 pg/ml ± 0.11 SEM), but not of other cytokines, and were excluded from the IL-6 serum analysis. As we recently reported in these patients (Shaashua et al., 2017), IL-6 and CRP serum levels increased from T1 to T2 in the placebo group, but drug treatment caused opposite effects, yielding significant drug effects at T2 (IL-6, $p = .0009$; CRP, $p = .034$, Fig. 3A and B). In the morning after surgery (T3), IL-6 and CRP levels markedly increased relatively to their pre-surgical peaks (T2) in both groups ($p < .001$) (Shaashua et al., 2017). A planned contrast of drug- vs placebo-treated groups during treatment (T2 and T3) vs off treatment (T1 and T4) showed a significant reduction in IL-6 for the drug-treated group ($p = .011$), and a marginally significant reduction in CRP ($p = .059$). Here we analyze PBMCs molecular processes related to the above effects. Corresponding results with respect to IL-6 at the intracellular level were evident at T2&T3, where the drug treatment caused a 1.59-fold increase in the mRNA of the NFκB inhibitor, *NFKBIA*. Additionally, activity of the IL-6-inducing TFs, NFκB and AP-1 (Samuel et al., 2008), as well as an IL-6 downstream signaling factor, STAT3 (Heinrich et al., 2003), were down-regulated by the drug treatment at T2 vs T1T4 contrast (V$NFkB_Q6, $p = .011$; V$AP1_Q2, $p = .003$; V$AP1_Q6, $p = .037$; V$AP1FJ_Q2, $p = .002$; and V$STAT3_01, $p = .044$, Fig. 3C); and CREB, which some studies have implicated as inhibiting NF-kB activity (Wen et al., 2010), was elevated by the drug treatment ($p < .04$, Fig. 5C). Similarly, at T2T3 vs T1T4 contrast, NFκB activity was also reduced by the drug treatment (V$NFKAPPAB50_01, $p = .011$). With respect to CRP mRNA levels in PBMCs, no group difference was evident, as would be expected given that CRP is mainly produced by the liver (Pepys and Hirschfield, 2003), rather than in PBMCs. Nevertheless, and corresponding to serum levels, a TF that blocks CRP induction, OCT1, was up-regulated by the drug treatment at T2&T3 (V$OCT1_04, $p = .223$; V$OCT1_Q6, $p = .034$, Fig. 3C). IL-6 is known to enhance CRP expression (Steensberg et al., 2003), therefore we tested and found a significant correlation across all 4 time-points (Supplementary Fig. S1A, $R = .645$, $p < .0001$), and most profoundly within T3 ($R = .342$, $p = .0591$) as previously reported in BC patients (Ravishankaran and Karunanithi, 2011; Zhang and Adachi, 1999). These correlations were conducted across groups, and very similar results were evident within each group separately.

3.4. Perioperative increase in serum levels of IFNγ and TRAIL and in related intracellular regulatory pathways and mRNAs: Effects that are blocked by the drug treatment

Only 11 drug-treated and 12 placebo-treated patients had detectible serum levels of IFNγ in all four time points. In the placebo group, serum IFNγ levels significantly increased from T1 to T2 ($p = .044$), and remained high at T3, whereas drug treatment completely abrogated this elevation at T2 and at T3 ($p = .035$, $p = .013$ respectively, Fig. 4A). Corresponding outcomes were observed at the mRNA levels analyzing all 36 patients, as *STAT1*, a key IFNγ downstream signaling pathway (Bach et al., 1997), was down-regulated by the drug-treatment in PBMCs and in the tumor, causing a 26% and 29% reductions in expression levels, respectively. Additionally, numerous transcripts involved in downstream impacts of type I and type II interferons (i.e., *IFIT1*, *IFIT2*, *IFITM2*, *IFIT3*, *IFITM3*, *IFIT5*, *IFI6*, *IFI27*, *IFI30*, *IFI 35*, *IFI44*, *IFI44L*, *IFIH1*, *IRF7*, *IRF9*, *ISG15*, *OAS2*, *OAS3*, *OASL*, *MX1*, *MX2*, *PKR/EIF2AK2*) were significantly reduced by the drug treatment in PBMCs, and Gene Ontology analyses confirmed that the anti-viral

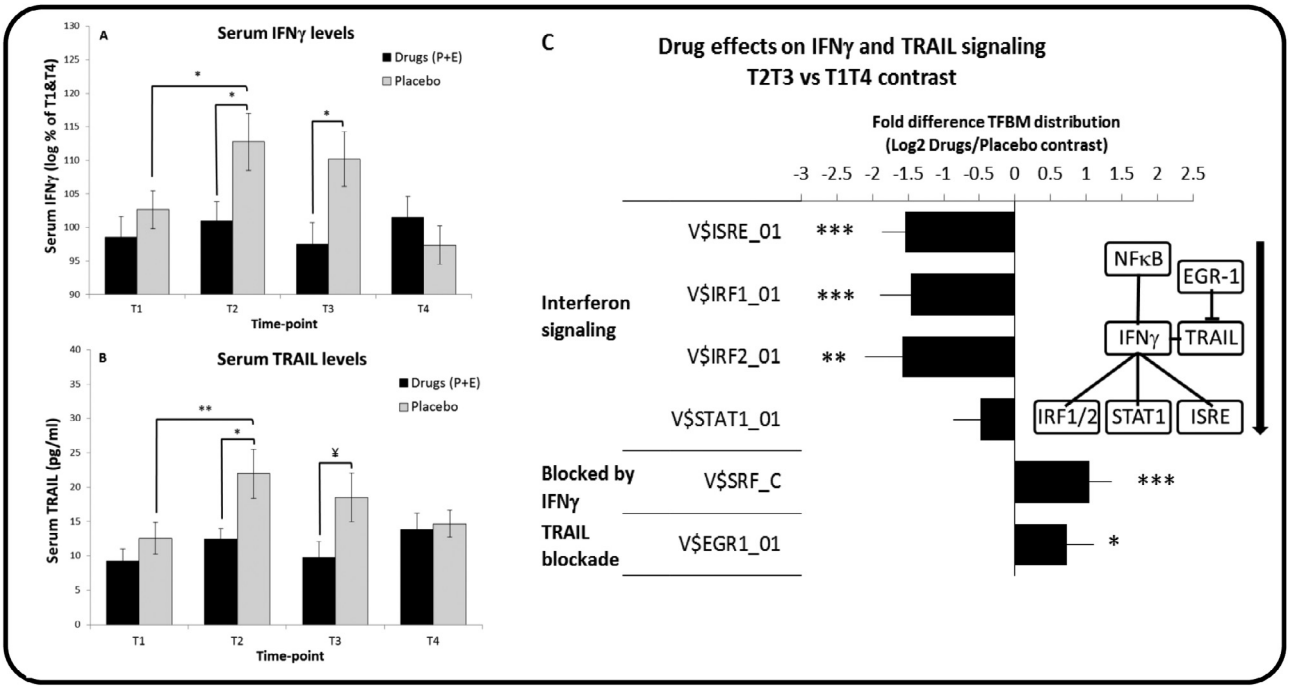

Fig.4. Effect of drug treatment on serum levels of (A) IFNγ, (B) TRAIL, and (C) their relevant TFs at T2T3 vs T1T4 contrast. Treatment-induced effects are consistent between serum levels and molecular pathways of IFNγ and TRAIL. Data presented as mean ± SEM. Group differences at a specific time point are indicated by ¥($p < .1$), *($p < .05$), **($p < .01$) and ***($p < .001$).

response family was reduced (GO:0009615; a-priori testing $p = .00014$ based on our planned assessment of IFNγ signaling; Benjamini correction for multiple comparisons $p = .0624$, if one would have assumed no a-priori focusing on IFNγ signaling). As noted above regarding IL-6 intracellular signaling, and further corroborating these findings, the NFκB inhibitors mRNA (*NFKBIA*), which reduce IFNγ production, was elevated by drug treatments, and NFκB transcriptional activity (known to induce IFNγ production) was reduced by the drug treatment. Additionally, the activity of downstream type I interferon response factors was reduced perioperatively by the drug treatment (V$ISRE_01, $p < .0001$; V$IRF1_01, $p = .0008$; V$IRF2_01, $p = .003$, Fig. 4C), and SRF, a TF that is negatively regulated by IFNγ (Shi and Rockey, 2010), was up-regulated (V$SRF_C, $p = .0009$, Fig. 4C). Overall, these outcomes correspond well with serum IFNγ levels, and are consistent across upstream and downstream mRNA and TFs, indicating a blockade of the inflammatory and interferon-related response in the treated group at T2 and T3.

Similar results were evident regarding TRAIL, which is also known to be induced by IFNγ signaling. Serum TRAIL levels were detectible only in 11 drug-treated and 16 placebo-treated patients, with marked within-patient fluctuations. Unlike other cytokines, the transformations of $\log_2$ or % of T1&T4 increased the within-group variability (mainly due to patients with low baseline levels), and thus these transformations were not used. Nevertheless, when analyzing absolute serum TRAIL levels ("raw data"), a significant increase from T1 to T2 was evident in the placebo group ($p = .0095$), which remained high at T3, whereas drug treatment abrogated these T2 and T3 elevation (T2, $p = .045$; T3 marginally significant, $p = .075$, Fig. 4B). The imbalance of 11 drug vs 16 placebo patients with detectable levels, is in the same direction of these findings, suggesting that the reduction in TRAIL levels by the drug treatment is an underestimation of the above effects. Corresponding results were evident regarding TRAIL intracellular indices, where all 36 patients provided data. Drug treatment significantly reduced TRAIL mRNA levels, causing a 45% difference (after $\log_2$ transformation) between the drug- and placebo-treated groups, and elevated the levels of EGR1 (V$EGR1_01, $p = .048$, Fig. 4C), a TF that negatively regulates TRAIL expression in monocytes (Secchiero et al., 2013) and in activated NK cells (Balzarolo et al., 2013).

3.5. Drug treatment did not consistently affect anti-inflammatory markers of cortisol and IL-10

As we recently reported in these patients, the combined use of propranolol and etodolac did not affect serum levels of cortisol or IL-10 at any time point. Both factors increased from T1 to T2 (IL-10, $p < .0001$; cortisol marginally significant, $p = .055$), and IL-10 levels further increased at T3 ($p < .0001$), before both returned to baseline levels at T4 (Shaashua et al., 2017) (Fig. 5A and B). Herein, we found that drug treatment significantly reduced IL-10 mRNA levels in PBMCs (at T2T3) and in the tumor, causing a 22% and 39% difference between the drug- and placebo-treated groups, in contrast to the increase evident in both groups in serum levels. TFs involved in IL-10 induction (Larsson et al., 2009; Motomura et al., 2011; Wen et al., 2010), showed inconsistent pattern in PBMCs, either elevated by the drug treatment (V$CREB_01, $p = .027$; V$CREB_02, $p = .0006$; V$CREB_Q4, $p = .036$; V$CREBP1_Q2, $p = .023$; V$CREBP1CJUN_01, $p = .025$; V$SP1_01, $p = .003$; V$SP1_Q6, $p = .0003$) or reduced (V$E4BP4_01, $p = .026$, Fig. 5C). Systemic cortisol levels are ascribed mostly to adrenal gland secretion (Tsigos and Chrousos, 2002), therefore time and group differences in mRNA were not expected, nor evident in PBMCs. However, glucocorticoid gene regulatory activity was significantly reduced by the drug treatment (V$GRE_C, $p = .002$; Fig. 5C), suggesting a potential lower impact of cortisol in the drug treated group.

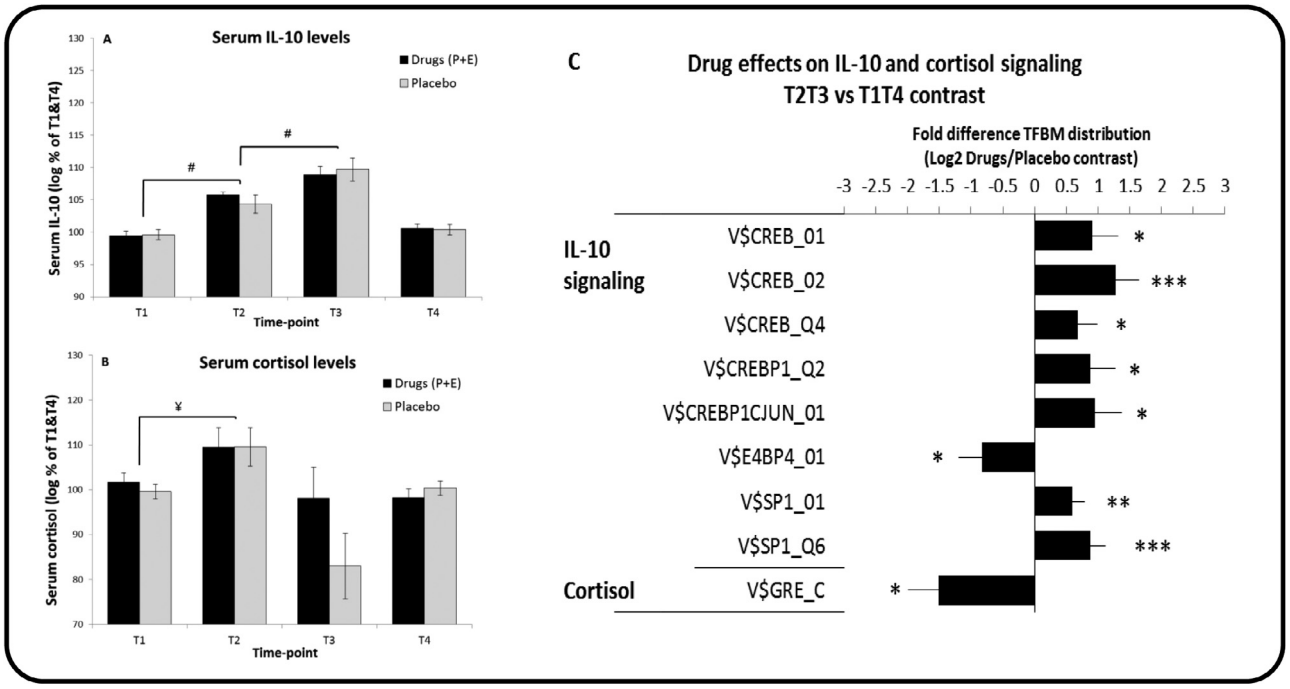

Fig. 5. Circulation levels of (A) IL-10, (B) cortisol, and (C) their relevant TFs. Drug treatment did not consistently affected IL-10 and cortisol serum levels and molecular pathways. Data presented as mean ± SEM. Group differences at a specific time point are indicated by ¥($p < .1$), *($p < .05$), **($p < .01$) and ***($p < .001$). Group differences between time-points across groups are indicated by #($p < .001$).

3.6. Pre-operative stress increased IFNγ/IL-10 serum ratio, while drug treatment reduced it by affecting Th1 cytokine secretion

Previous studies have suggested a reduction in Th1 cytokines and a shift towards Th2 polarization in response to acute mental stress, as reflected in a reduction in the IFNγ/IL-10 ratio (Marshall et al., 1998). However, in the current study, pre-operative stress increased IFNγ/IL-10 ratio at T2 compared to T1 in the placebo group, while drug treatment prevented this elevation, yielding a significantly lower Th1/Th2 ratio in the drug treated group at T2 ($p = .008$). In both groups, surgery reduced IFNγ/IL-10 ratios relatively to their pre-surgical T2 levels to a similar degree, with drug treated group remaining at a significant lower ratio at T3 ($p = .033$), before returning back to equal ratios at T4, indicating a reduction in Th1 cytokine by the drug treatment at T2 and T3. Although in the TFBMs analysis we did not assess TFs that are associated with Th1 cell-differentiation (such as T-bet and STAT4 (Zhang et al., 2014)), we did assess the transcriptional activity of two inducers of Th2 cell-differentiation (from naïve CD4+ T cells), GATA3 and GFI1 (Shinnakasu et al., 2008; Yagi et al., 2011). Surprisingly, we found that drug treatment reduced GATA3 at T2&T3 and alone at T3 (compared to T1&T4 contrasts, Fig. 6B, $p < 0001$ for both), and similarly reduced GFI1 at T2 vs T1T4 contrast (Fig. 6B, $p = .002$). This suggests that the alterations evident in serum IL-10 levels are independent of Th2 cell-differentiation in PBMCs.

3.7. Perioperative reductions in pro-inflammatory IL-18, IL-8, and TNFα serum levels

One patient from the drug-treated group had a high baseline (before treatment) level of IL-8 (100 pg/ml, compared to a mean of 17.14 pg/

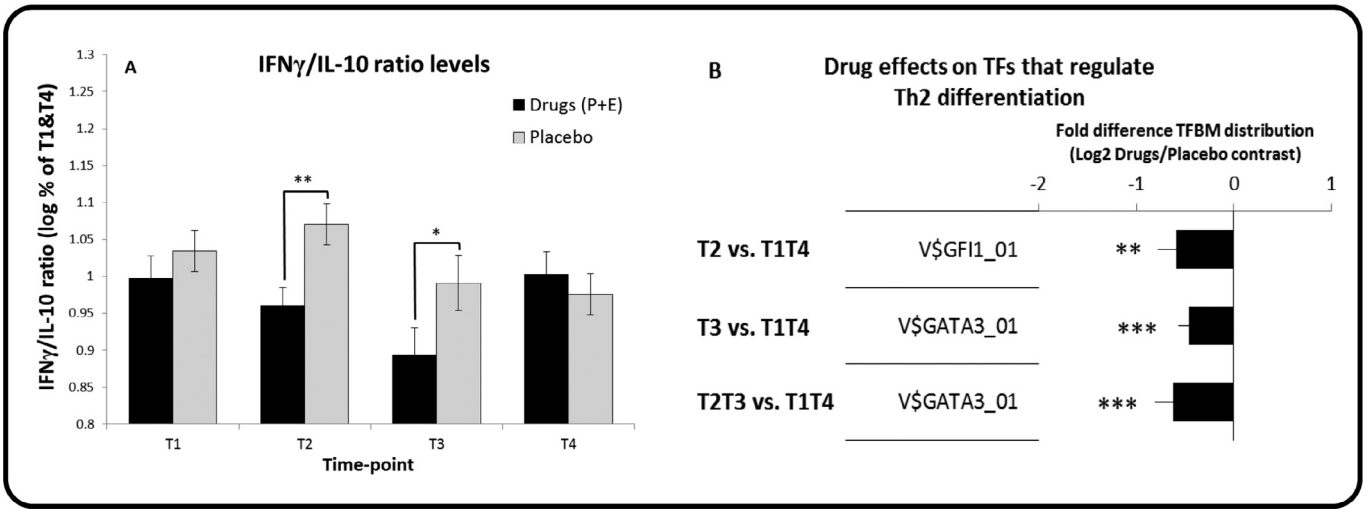

Fig. 6. Th1/Th2 polarization as indicated by (A) the ratio between IFNγ and IL-10 serum levels, and by (B) drug-induced reductions in TFs involved in Th2-cell differentiation. Data presented as mean ± SEM. Group differences at a specific time point are indicated by *($p < .05$), **($p < .01$) and ***($p < .001$).

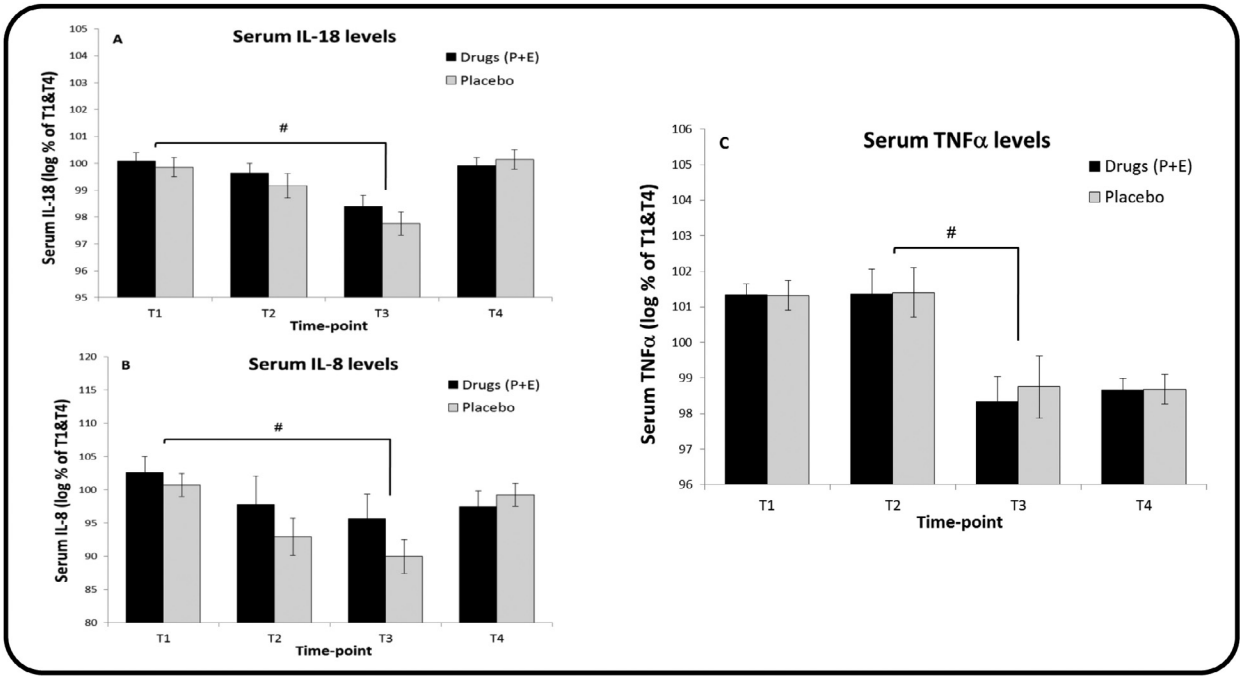

Fig. 7. Circulation levels of (A) IL-18, (B) IL-8, and (C) TNFα. Data presented as mean ± SEM. Group differences between time-points across groups are indicated by #(p < .001).

ml ± 1.49 SEM), but not of other cytokines, and was excluded from the IL-8 serum analysis. Drug treatment did not affect serum IL-18 or IL-8 levels at any time-point. In both groups, serum levels gradually decreased from T1 to T2 to T3 (T1 to T3, p < .001 for both), and returned to baseline level at T4 (Fig. 7A and B). As previous studies indicated that IL-18 induces IL-8 secretion from PBMCs (Puren et al., 1998), we tested and found a significant correlation between these two cytokines across all four time-points (Supplementary Fig. S1B, R = .288. p = .0004), and most profoundly within T2 (R = .519, p = .001). Similarly, mRNA levels of IL-18 were not affected by drug treatment. However, incongruent results were evident in IL-8 mRNA level, where drug treatment caused a significant 2.7-fold increase at T2&T3, which was not reflected in serum protein level. Although correspondences between these three levels of protein regulation and production are expected, post-transcriptional and post-translational modifications, and other regulatory mechanisms, are known and often lead to dissociations between these levels (Gunawardana and Niranjan, 2013; Vogel and Marcotte, 2012). Circulating TNFα serum levels were stable between T1 and T2 in both groups, significantly decreased following tumor excision (T3) (p < .0001), and remained low at T4 (Fig. 7C), consistent with elimination of tumor-induced TNFα secretion. Serum TNFα levels did not correlate with tumor size or histological grade. As mentioned above, NFκB, one of TNFα inductors (Jongeneel, 1995), was differentially affected by the drug treatment at the transcriptional level, as well as at the mRNA level of NFκB inhibitor (*NFKBIA*), effects that were not reflected in serum levels of TNFα.

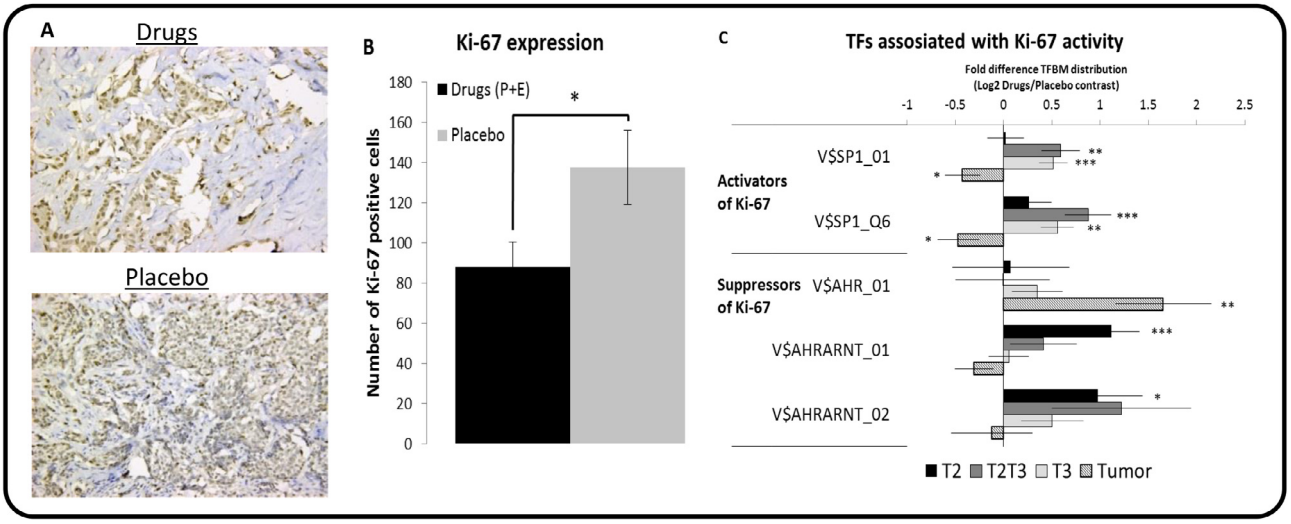

Fig. 8. Drug effects on the proliferation marker Ki-67 indicated by (A) representative pictures of drug- and placebo-treated tissues, (B) number of Ki-67 positive cells, and (C) effects on TFs associated with Ki-67 activity in PBMCs and in excised tumors. Data presented as mean ± SEM. Group differences at a specific time point are indicated by *(p < .05), **(p < .01) and ***(p < .001).

3.8. Drug treatment reduced the expression of the proliferation marker Ki-67 without affecting IL-8, MMP2, MMP9, and uPA/PAI1

In the current study, we stained for the proliferation and pro-metastatic markers Ki-67, IL-8, MMP2, MMP9, and uPA/PAI1. IHC staining of the excised tumor included all patients from the placebo group (n = 18), and, due to technical faults, only 15 patients from the drug-treated group. Drug treatment significantly reduced the expression of Ki-67 in the excised tumor tissue (p = .04, Fig. 8A and B), without affecting the other markers (IL-8, p = .65; MMP2, p = .26; MMP9, p = .76; and uPA/PAI1, p = .13; not shown). Corresponding results are evident in the activity of TFs associated with Ki-67 expression and activation. In the drug-treated group, the transcriptional activity of the Ki-67-activatin TF SP1 was downregulated in the PT (V$SP1_01, p = .018; V$SP1_Q6, p = .027), but was upregulated in PBMCs at T2T3 vs T1T4 contrast (V$SP1_01, p = .003; V$SP1_Q6, p = .0003) and at T3 alone (V$SP1_01, p = .0005; V$SP1_Q6, p = .0011). Additionally, the Ki-67-inhibiting TF AhR was up-regulated by the drug treatment in the tumor tissue (V$AHR_01, p = .0011), as well as in PBMCs at T2 (V$AHRARNT_01, p = .0002; and V$AHRARNT_02, p = .04; Fig. 8C).

3.9. Exploratory study in patients PBMCs: Biomarkers that in tumor tissue have clinical significance

Herein, we conducted an exploratory analysis of TFBMs that showed nominally significant group differences in PBMCs (T2, T2T3, or T3, compared to non-treatment time-points; T1T4), and focused only on TFs that are relevant for cancer progression, but were not included in our a-priori hypotheses (12 TFs, Fig. 9). These TFs, when over-expressed in PTs, were reported to (i) predict cancer clinical outcomes (CUX1 (Sansregret et al., 2011), EVI1 (Patel et al., 2011), THRa (Heublein et al., 2015), N-MYC (Liu et al., 2016; Mizukami et al., 1995), C-MYB (Li et al., 2016), PBX1 (Magnani et al., 2015), and RORa (Du and Xu, 2012)), or to (ii) promote pro-metastatic processes such as angiogenesis (YY1) (de Nigris et al., 2010), proliferation (GATA2) (Wang et al., 2012), EMT (deltaEF1/ZEB1 and GATA1) (Aigner et al., 2007; Hurt et al., 2008; Li et al., 2015), and stimulation of hypoxia-induced genes (p300) (Arany et al., 1996).

As we did not find any reports on the association between the activity of these TFs in PBMCs to prognosis, herein we evaluated our results based on the literature reporting correlations between TFs activity in the PTs and prognosis, to suggest potential prognostic value of our biomarkers (Fig. 9). Specifically, in patients PBMCs, both CUX1 (V$CDPCR1_01, V$CLOX_01) and EVI1 TFs were down-regulated by the drug treatment at T2T3 (V$CDPCR1_01, p < .0001; V$CLOX_01, p = .019; V$EVI1_01, p = .019) and in T3 alone (V$CDPCR1_01, p = .011; V$CLOX_01, p = .019; V$EVI1_01, p = .03; V$EVI1_03, p = .017; V$EVI1_04, p = .017), and THRa was down-regulated only at T3 (V$T3R_01, p = .002; Fig. 9). The transcriptional activity of the oncogenes c-MYB and N-MYC was up-regulated by the drug treatment at T3 (p = .046, and p = .001 respectively), and the activity of N-MYC was also up-regulated at T2 (p = .037; Fig. 9).

In addition, the transcriptional activity of EMT-inducing factors deltaEF1/ZEB1 and GATA1, as well as of the proliferation-promoting GATA2, was down-regulated by the treatment perioperatively (T2T3) (V$DELTAEF1_01, marginally significant p = .082; V$GATA1_01, p = .002; V$GATA1_02, p = .015; V$GATA1_04, p = .01; and V$GATA2_01, p = .002), and post surgically (T3) (V$DELTAEF1_01, p = .015; V$GATA1_01, p = .0001; V$GATA1_02, p = .003; V$GATA1_03, p = .011; V$GATA1_04, p = .0007; and V$GATA2_01, p < .0001; Fig. 9). Similarly, the pro-angiogenic TF YY1, was significantly reduced herein by the drug treatment in all 3 contrasts tested (p < .015 for all). The effects of the drug treatment on PBX1 and p300 were inconsistent, as the activity of V$PBX1_01 TFBM was up-regulated by the drug treatment at T2T3 (p = .007), and of p300 at T2 (V$P300_01, p = .048), while the activity of V$PBX1_02 and V$P300_01 TFBMs was reduced at T3 (p = .014, p = .039 respectively; Fig. 9). Last, the transcriptional activity of the tumor-suppressor RORa2 was up-regulated by the drug treatment at T2 (V$RORA2_01, p = .001; V$AHRARNT_01, p = .0002; and V$AHRARNT_02, p = .04; Fig. 9).

3.10. Expected correlation between pro- and anti-inflammatory cytokines

Several correlations between cytokines were expected based on the literature. Given that IL-10 was reported to increase in response to elevated IL-6 levels to restrict the pro-inflammatory response (Steensberg et al., 2003), we tested and found a significant overall correlation between IL and 6 and IL-10 (Supplementary Fig. S1C, R = .595, p < .0001), especially at T3 (R = .346, p = .0305). For the same reason, a correlation between IL-6 and cortisol levels was expected (Steensberg et al., 2003), and evident at T2 (Supplementary Fig. S1D, R = .393, p = .0229), but not at other time-points. Another significant overall correlation between pro- and anti-inflammatory cytokines was evident between IL-18 and IL-10, but contrary to the previous correlations, was negative (Supplementary Fig. S1E, R = -.261, p = .0015). TNFα has been shown to be a prominent pro-inflammatory cytokine that promotes IL-6 secretion (Akira et al., 1990). However, the correlation between these cytokines was negative herein, although weak (Supplementary Fig. S1F, R = −.191, p = .0282), with no significant correlation at any of the time-points separately. Additionally, IL-18 was identified as a potent inducer of IFNγ secretion (Nakanishi et al., 2001), therefore a significant correlation was expected, but not evident (R = −.138, p = .186). Lastly, although IL-6 and IFNγ show similar pattern of serum levels and drug effects along the four time-points studied, a mutually exclusive pattern of secretion between absolute levels of IL-6 and IFNγ was evident, where at high levels of IL-6, IFNγ levels remain low, and vice versa (Fig. 10A). A similar, but less profound pattern was evident between CRP and IFNγ (Fig. 10B).

4. Discussion

4.1. Safety and overall impact of the drug treatment

In this study, one placebo- and one drug-treated patient self-withdrew before surgery (day 2 of treatment), with no clear relation to drug treatment. Thus, drug treatment does not seem to have adverse effects, as was also found in a similar study in colorectal cancer patients (n = 34) (Haldar et al., 2017). See elaborate discussion of safety considerations in Shaashua et al. (2017).

The findings indicate potential benefits of the drug treatment in BC patients. Even before surgery, pro-inflammatory markers, including serum IL-6, CRP, IFNγ, and TRAIL, measured at the protein level and at the intracellular PBMCs molecular levels (mRNA and TFs), increased from T1 to T2 in the placebo group, whereas drug treatment attenuated these increases, without affecting serum anti-inflammatory factors (i.e. serum IL-10 and cortisol). Although psychological measures of stress were not recorded, cortisol levels increased from T1 to T2, in accordance with current literature which reports a peak in distress during the few days before surgery (e.g. Culver et al., 2002). Thus, we hypothesize that neuroendocrine stress-inflammatory responses, which were blocked by the drug treatment, underlie the rise evident in these inflammatory indices in the placebo group. In the excised tumors, drug treatment reduced the expression levels of the proliferation marker Ki-67, with corresponding results at the tumor TFs level. As inflammation is a hallmark of cancer progression, and as high serum IL-6 and CRP levels, and tumor Ki-67 expression, are negative prognostic factors, these findings suggest a clinically relevant beneficial impact for the drug treatment, which should be tested in large-scale clinical studies.

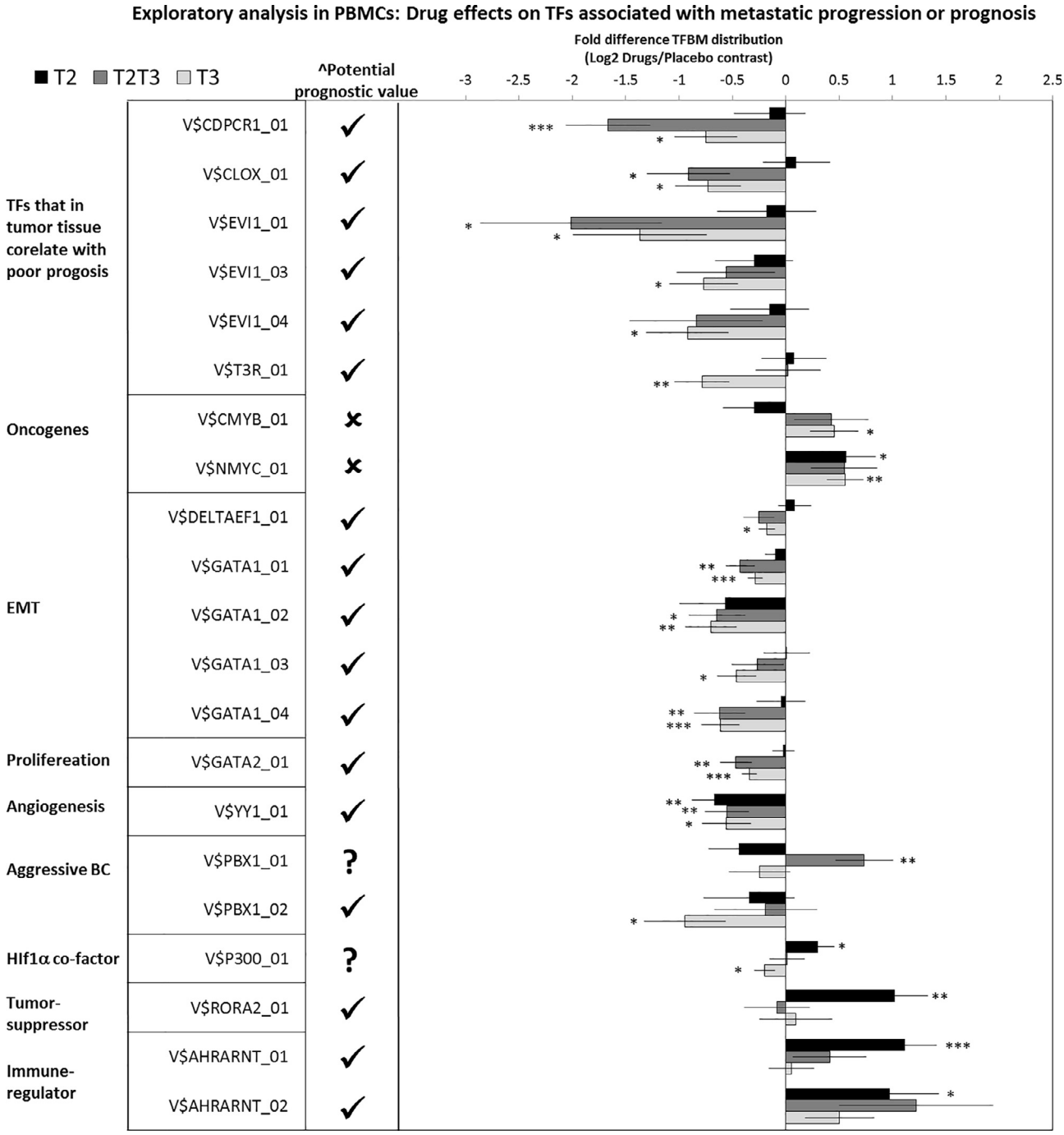

Fig. 9. Exploratory analysis in PBMCs of drug effects on TFs associated with metastatic progression or with prognosis. Data presented as Fold difference TFBM distribution ± SEM, comparing T2, T2T3, or T3 (treated time-points) to T1T4 (non-treatment time-points). ^The potential prognostic value of the drug treatment on specific TFBMs is based on previous reports regarding the expression of the TF in the primary tumor. Group differences at a specific time-point are indicated by *($p < .05$), **($p < .01$) and ***($p < .001$).

4.2. Pro-inflammatory indices and their perioperative reduction by the drug treatment

The relation between inflammation and cancer is well established (reviewed in Grivennikov et al., 2010; Trinchieri, 2012). Specifically, elevated pro-inflammatory markers, including IL-6 and CRP, were associated with reduced survival in several types of cancers, including BC (Albuquerque et al., 1995; Esquivel-Velazquez et al., 2015; Pierce et al., 2009; Zhang and Adachi, 1999), and their pre-surgical serum levels predict tumor progression and metastatic involvement (Ravishankaran and Karunanithi, 2011). Herein, serum IL-6 and CRP significantly increased in the placebo group prior to surgery, while drug treatment abolished these increases, as well as their upstream and downstream transcriptional activity in PBMCs (e.g., AP1, STAT3). The observed blockade is consistent with the expected impact of the drugs, as serum IL-6 levels were reported to be increased by stress through adrenergic mechanisms (Steptoe et al., 2007; Takaki et al., 1994), and β-adrenergic and PGE2 blockade *in vivo* or *in vitro* reduced IL-6 mRNA and protein levels (Hinson et al., 1996; Nilsson et al., 2007). As IL-6 is a prominent inducer of CRP, a reduction in IL-6 levels may have also induced the reduction in CRP levels, as indeed suggested herein by the within group/time-point correlation between these indices.

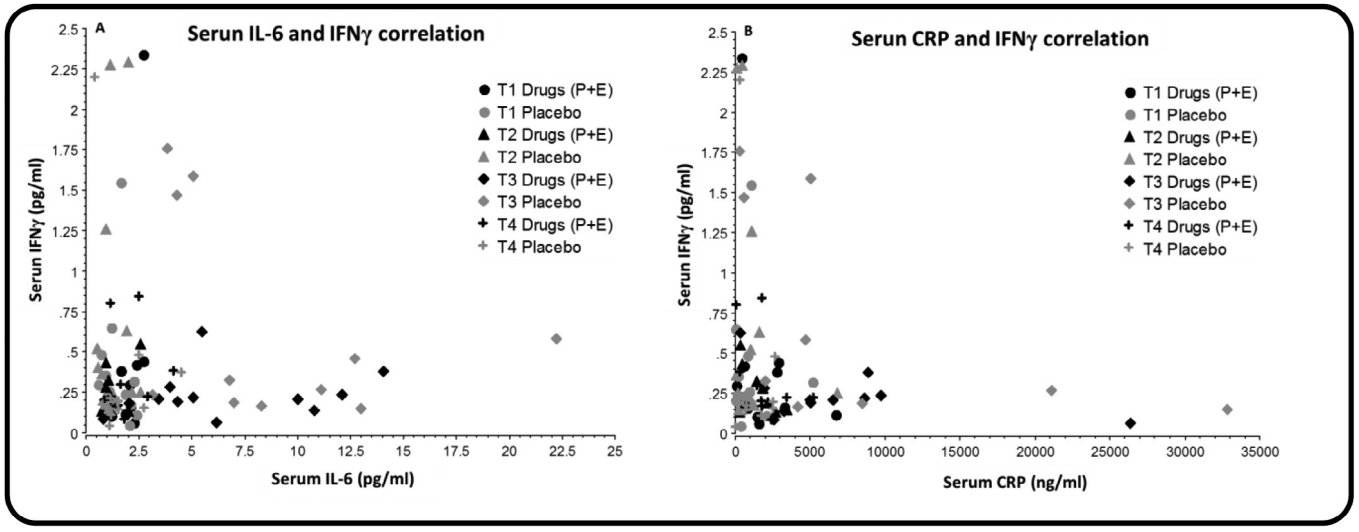

Fig. 10. A mutually exclusive pattern of secretion between levels of (A) IL-6 and IFNγ, and (B) CRP and IFNγ.

In contrast to the common belief that Th1 cytokines are suppressed by stress (Kim and Maes, 2003; Yang and Glaser, 2002) or surgery (Menger and Vollmar, 2004), leading to a shift towards Th2 immunity (Diehl and Rincon, 2002), we herein observed an increase in serum IFNγ levels from T1 to both T2 and T3 in the placebo group. Drug treatment completely abrogated this increase and reduced mRNA levels involved in downstream impacts of type I and type II interferons in PBMCs (e.g., as indicated by interferon response genes such as *IFIT1*, *IFIT2*, *IFI30*), as well as their upstream and downstream transcription pathways (NFκB and IRF respectively). Two previous studies also alluded to similar effects of psychological stress on IFNγ levels (Greenfeld et al., 2007; Xiang et al., 2012). Importantly, the evidences for reduction in Th1 cytokines are based on *in vitro* LPS-induced production (Naldini et al., 1995, 1999), rather than on actual serum levels, which are often below detection levels. Indeed, in the same patients that herein showed increased perioperative serum IFNγ levels and in a previous study (Greenfeld et al., 2007), we too found a perioperative reductions in LPS-induced IFNγ and IL-12 production that were blocked by the drug treatment (Shaashua et al., 2017). Perhaps this inverse pattern of outcomes reflects an *in vitro* exhaustion (or reduced sensitivity) of PBMCs due to their previous *in vivo* intense production of Th1 cytokines. Further discussion of such methodological cavities has been presented recently (Gotlieb et al., 2015).

Interferons have been shown to induce TRAIL production from NK- and T-cells (Smyth et al., 2003). Therefore, similar time-dependent alterations and drug effects in TRAIL levels were expected and indeed evident herein. TRAIL is widely recognized as apoptosis-inducing ligand in malignant but not in normal cells, through binding the death receptors DR4/5 (Holoch and Griffith, 2009). However, despite more than two decades of numerous attempts to take advantage of this mechanism in the clinical setting, no clear benefits have yet emerged (Holoch and Griffith, 2009; Mahalingam et al., 2009). The involvement of TRAIL in an acute stress response was previously suggested (Mundt et al., 2003), and the current study supports this notion and further demonstrates TRAIL inflammatory-like pattern of responses.

4.3. Dissociation between IL-6 and IFNγ responses

When serum IFNγ levels increased in a particular patient, serum IL-6 levels remained low, and vice versa, although across patients (by average) these two cytokines had similar response patterns to stress, surgery, and drug treatment. A similar phenomenon has been reported regarding the downstream signaling pathways of these two cytokines – STAT1 and STAT3, respectively (Bluyssen et al., 2010; Diehl et al., 2000). Because both cytokines are induced by NFκB, which is elevated during acute psychological stress (Kuebler et al., 2015) and is blocked herein by the drug treatment, our findings suggest a yet unknown mechanism of mutual inhibition between IFNγ and IL-6. As STAT1 and STAT3 play opposite roles in tumorigenesis, proliferation, apoptotic death, inflammatory, and anti-tumor immune responses (Avalle et al., 2012), it would be interesting to understand why the stress-inflammatory response of a specific patient would take one pathway but not the other, as well as the potential clinical implications of such responses on long-term cancer outcomes.

4.4. Anti-inflammatory markers

Interestingly, in the current study, IL-10 levels increased gradually from T1 to T2 and to T3, and returned to baseline levels at T4 in both groups. Thus, contrary to previous reports that stress cause a shift toward Th2 cytokine dominance (Marshall et al., 1998), herein stress increased both Th1 and Th2 cytokines (i.e., IFNγ & IL-10), while drug treatment blocked only the Th1 cytokine response (IFNγ), causing a reduction in IFNγ/IL-10 ratio. Expression of β-adrenergic receptors on different subtypes of T-cells may underlie these outcomes. Although differential expression of β2-adrenoceptors in human Th1- vs Th2-cells remains unclear due to methodological problems (Sanders, 2012), murine NK- and Th1-cells (major sources of IFNγ), but not Th2-cells, express β2-adrenoceptors (Ramer-Quinn et al., 1997), offering a possible mechanism for the observed blockade of serum IFNγ levels (through Th1), but not of IL-10 levels (Th2). More recently, β-adrenergic-dependent NFκB activation was shown to increase mRNA levels of pro-inflammatory cytokines, but not of IL-10 (Kuebler et al., 2015), suggesting the involvement of non-adrenergic regulatory mechanisms in increasing IL-10 levels herein. Noteworthy, the elevation in IL-10 levels does not seem to stem from Th2-cell-differentiation, as the Th2-differentiation-inducing GATA3 and GFI1 transcriptional activity was actually reduced by the drug treatment.

4.5. Inconsistencies with current literature

Some of our results are incongruent with prevalent assertions regarding the categorization of pro- and anti-inflammatory ligands and their signaling pathways. First, IL-18 has been suggested as an amplifier of the inflammatory cascade (Gracie et al., 2003), and as an inducer of Th1 cell response and IFNγ secretion in an AP-1 and NFκB dependent manner (Nakanishi et al., 2001). However, a significant correlation between IL-18 and IFNγ was not evident herein, as one would expect

based on this literature. Second, IL-8 was identified as a pro-inflammatory and pro-metastatic cytokine, which is also induced by NFκB, and was previously reported to be increased in the serum of BC patients and to predict lower survival rates (Benoy et al., 2004). Based on the above, one would expect both IL-8 and IL-18 to increase at T2 and T3, effects that should be antagonized by the drug treatment. However, none of these predictions occurred, and levels of both cytokines decreased from T1 to T3 without any drug effects, unlike the other pro-inflammatory ligands IL-6, CRP, IFNγ and TRAIL. Last, the prominent pro-inflammatory cytokine, TNFα, should have presumably been increased systemically following IL-1β signaling (Hietbrink et al., 2006), and induce an increase in circulating IL-6 levels (Akira et al., 1990) in an NFκB dependent manner. However, herein TNFα serum levels did not change pre-operatively (from T1 to T2), decreased in both group following tumor removal (T3), and remained low thereafter (T4), suggesting that the relatively low systemic TNFα levels evident herein were actually secreted by the excised tumor or its microenvironment.

Based on the above, the common assertions regarding the categorization and clustering of pro- and anti-inflammatory cytokines, their alleged inner-family coherence, and mutual inter-family inhibition, should be re-considered. Most outcomes in the current study are indicated by both intracellular indices and by protein levels tested in duplicate using high-sensitivity ELISA kits. We hypothesize that when cytokine levels are generally low, as evident herein and in healthy subjects (Benoy et al., 2002; Benoy et al., 2004; Eissa et al., 2005), different regulatory mechanisms may be dominant, in contrast to more severe circumstances that are characterized by at least 10 fold higher levels (Benoy et al., 2002; Bisgin et al., 2012).

4.6. Reduced Ki-67expression and associated TFs

Ki-67 is a protein expressed only in proliferating cells (Scholzen and Gerdes, 2000), and is used as a diagnostic tool in several types of cancers (Yang et al., 2017), having a predictive and prognostic value (Yerushalmi et al., 2010). Importantly, in breast tumors, high Ki-67 levels was associated with (i) cancer progression (Scholzen and Gerdes, 2000; Yang et al., 2017), (ii) higher risk for central nervous system (CNS) metastases (Ishihara et al., 2013), and (iii) reduced disease-free survival (DFS) and overall survival (OS) rates (Kontzoglou et al., 2013). Herein, a short pre-operative five-day drug treatment resulted in lower expression levels of Ki-67 in the excised tumor, indicating a treatment-induced arrest or regression in malignant proliferation. Corresponding results are evident at the molecular level, as AhR, a TF that is inversely correlated with Ki-67 labeling index (Saito et al., 2014), was significantly increased in the PT and in patients PBMCs (pre-surgically at T2). Additionally, the transcriptional activity of SP1, a TF that up-regulates Ki-67 transcription (Pei et al., 2012; Tian et al., 2011), was reduced in the PT, as would also be expected. Together, and mainly based on tumor indices, these outcomes suggest a favorable impact for the drug treatment.

4.7. Exploratory analysis: Activity of cancer-related TFs in PBMCs

To the best of our knowledge, no clinical associations between the expression or activity of TFs in PBMCs and long-term cancer outcome in BC patients is known. Given positive impacts of the drug treatment on a variety of established markers of cancer progression, herein and in our previous report (Shaashua et al., 2017), we conducted an exploratory analysis, screening for cancer-related TFs in PBMCs. Given that the drug treatment can affect both the PT and PBMCs, directly or through their local milieu, alterations in PBMCs transcriptional activity may serve as proxy for the efficacy of the drug treatment or as future predictive or prognostic indices. To this end, we focused on TFs in PBMCs that showed nominally significant effects of the drug treatment and, when expressed or active in the PT, are known to have prognostic value or to indicate pro-metastatic characteristics (a total of 12 different TFs – see Fig. 9 for all TFBMs).

Specifically, gene expression profiles in BC tissue associated the transcriptional activity of CUX1 (Sansregret et al., 2011), THRa (Heublein et al., 2015), N-MYC (Mizukami et al., 1995), EVI1 (Patel et al., 2011), c-MYB (Li et al., 2016), and PBX1 (Magnani et al., 2015) with more aggressive cancer characteristics and/or poor long-term outcomes. Herein, in PBMCs, the activity of the above TFs was positively affected (reduced) by the drug treatment at T2, T2T3, or T3, except the oncogenes N-MYC and c-MYB, which were up-regulated in PBMCs. Additionally, the transcriptional activity of the BC tumor-suppressor RORa (Du and Xu, 2012) was up-regulated by the drug treatment at T2. Similarly, drug treatment reduced the activity of TFs that were shown to promote several pro-metastatic characteristics, including (i) EMT (deltaEF1/ZEB1 (Aigner et al., 2007; Hurt et al., 2008) and GATA1 (Li et al., 2015)), (ii) promotion of angiogenesis (YY1) (de Nigris et al., 2010), and (iii) proliferation (GATA2) (Wang et al., 2012). Drug treatment also increased the activity of AhR, a TF that was previously shown to inhibit invasion and migration BC cells (Hall et al., 2010; Saito et al., 2014), and suppress Ki-67 expression. The HIF1a co-factor p300 showed inconsistent effects, both herein and in the literature (Semenza, 2007; Goodman and Smolik, 2000; Suganuma et al., 2002). Overall, these results seem mostly favorable, as 9 TFs were positively affected vs 2 that were negatively affected. This exploratory analysis may serve as a future basis for studies correlating transcriptional activity in PBMCs to long-term cancer outcomes, hopefully identifying novel predictive and/or prognostic markers. However, it is important to note that these exploratory analyses were not corrected for multiple testing (as would be typical in an exploratory/discovery-based analysis of multiple candidate biomarkers), and the present results should thus be interpreted as a provisional hypothesis-generating results that requires verification in future studies.

4.8. Limitations and conclusions

Overall, BC patients may benefit from the drug treatment employed herein through a reduction in their systemic inflammatory status, presumably in an NFκB dependent manner and independently of anti-inflammatory responses. A variety of TFs that were implicated in cancer progression, or reported to have prognostic value in PTs, were also improved by the drug treatment, albeit studied herein in PBMCs. However, one may question whether the reduction in the systemic inflammatory status and in tumor Ki-67 expression, induced by a short-term drug treatment, may improve long-term cancer outcomes. Although herein we did not directly studied metastases, we believe that some of these changes may persist beyond the treatment period, and those that are transitory in nature (e.g., stress/surgery-induced elevated IL-6 levels) may nevertheless have long-lasting impacts. Indeed, ample evidence suggests the non-proportional high impact of the short perioperative period on long-term cancer outcomes in cancer patients (Horowitz et al., 2015). In animal studies, even a single-day perioperative treatment with the same drug combination improved long-term survival after PT excision. In our other reports regarding the BC patients studied herein (Shaashua et al., 2017), and in a similar clinical study in colorectal cancer patients (Haldar et al., 2017), transcriptome profiling of the excised tumor indicated that drug treatment altered pro-metastatic molecular indices (e.g. tumor EMT), and immune and inflammatory indices, in manners that predict improved long-term outcomes. Last, as we report herein, drug treatment reduced the expression of Ki-67 in the excised PT, a known and established marker for survival in BC patients (Kontzoglou et al., 2013). Overall, the findings here and in our previous reports in BC and colorectal cancer patients (Haldar et al., 2017; Shaashua et al., 2017), indicate positive alterations in established markers of disease progression and survival. Whether such short-term changes will indeed lead to improved long-term cancer outcomes needs to be tested in larger clinical trials, which are now justified.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbi.2018.05.014.

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