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ORIGINAL RESEARCH

The effect of non-oncology drugs on clinical and genomic risk in early luminal breast cancer

B. Waissengrin[1,2†], T. Zahavi[3†], M. Salmon-Divon[3], A. Goldberg[3], I. Wolf[1,2], T. Rubinek[1,2], T. Winkler[4], O. Farkash[1], A. Grinshpun[5], A. Zubkov[6], M. Khatib[7], S. S. Shachar[1,2], N. Keren[1,2], I. Carmi-Levy[8], U. Ben-David[2] & A. Sonnenblick[1,2*]

[1]The Oncology Division, Tel Aviv Sourasky Medical Center, Tel Aviv; [2]Sackler School of Medicine, Tel Aviv University, Tel Aviv; [3]Department of Molecular Biology, Adelson School of Medicine, Ariel University, Ariel; [4]Department of Human Molecular Genetics & Biochemistry, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; [5]Breast Oncology Center, Department of Medical Oncology, Dana Farber Cancer Institute, Boston, USA; [6]Pathology Department, Pathology Institute, Tel Aviv Medical Center, Tel Aviv; [7]Division of General Surgery, Tel Aviv Medical Center, Tel Aviv; [8]Aummune Ltd, Tel Aviv, Israel

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Background: An effect of non-oncology medications on cancer outcome has been proposed. In this study, we aimed to systematically examine the impact of commonly prescribed non-oncology drugs on clinical risk and on the genomic risk [based on the Oncotype DX recurrence score (RS)] in early breast cancer (BC).

Experimental design: We collected data on clinical risk (stage and grade), genomic risk (Oncotype DX RS), and on non-oncology medications administered to 1423 patients with estrogen receptor-positive human epidermal growth factor receptor 2-negative BC during the month of their surgery. The influence of various medications on clinical and genomic risks was evaluated by statistical analysis.

Results: Out of the multiple drugs we examined, levothyroxine was significantly associated with a high Oncotype DX RS (mean 24.78; $P < 0.0001$) and metformin with a low Oncotype DX RS (mean 14.87; $P < 0.01$) compared with patients not receiving other non-oncology drugs (mean 18.7). By contrast, there were no differences in the clinical risk between patients receiving metformin, levothyroxine, or no other non-oncology drugs. Notably, there was no association between the consumption of levothyroxine and metformin and proliferation marker (Ki67) levels, but both drugs were significantly associated with progesterone-related features, suggesting that they influence genomic risk through estrogen-dependent signaling.

Conclusions: The results of this study indicate a significant impact of metformin and levothyroxine on clinical decisions in luminal BC, with potential impact on the clinical course of these patients.

Key words: breast cancer, estrogen receptor, levothyroxine, metformin, Oncotype DX, genomic risk, clinical risk

INTRODUCTION

Adjuvant chemotherapy treatment decisions for women with estrogen receptor (ER)-positive human epidermal growth factor receptor 2 (HER2)-negative early-stage breast cancer (BC) are guided by the tumor's genomic signatures and the specific patient's clinical risks. The clinical risk considers the tumor's TNM (tumor—node—metastasis) staging and grade, as well as the patient's age and medical and family history. The genomic risk involves molecular tests for measuring gene expression linked to the risk of disease recurrence. Among the commercially available assays,[1,2] Oncotype DX, which is based on a 21-gene assay with a scoring range from 0 to 100, has demonstrated prognostic and predictive value in quantifying BC recurrence in both premenopausal and postmenopausal patients with local[3] and locally advanced[4] BC. Its endorsement was founded on a number of prospective[3,4] and retrospective[5-9] clinical trials.

Various associations between non-oncology medications and their anticancer potential have been proposed, as well as the risk for cancer development and cancer outcomes.[10,11] It has recently been shown that many non-oncology drugs can affect the growth and survival of cancer cell lines.[12] While meta-analyses and real-world data supported these positive outcomes[13-15] in various cancer types and medications, strong molecular evidence for this concept is lacking.

To date, there are no clear-cut clinical guidelines for recommending the prophylactic administration of non-oncology medications with the aim of lowering the risk of cancer or improving outcomes. Neither are there any

*Correspondence to: Dr Amir Sonnenblik, MD, Tel Aviv Sourasky Medical Center, 6 Waizman Street, Tel Aviv 64239, Israel; Tel: +972-3-6972446
E-mail: amirson@tlvmc.gov.il (A. Sonnenblick).

†These authors contributed equally.

published clear-cut restrictions nor recommendations for avoidance of other medications that might increase the risk of worse prognosis.

In this study, we aimed to systematically examine the impact of non-oncology drugs on the clinical risk and on the genomic risk [based on Oncotype DX recurrence score (RS)] in early BC and their impact on clinical decisions.

MATERIALS AND METHODS

Setting and patients

This hospital-based, retrospective cohort study included data from the Tel Aviv Sourasky Medical Center, Tel Aviv, Israel. The study received ethics committee approval from the institutional review board (TLV18-0426). Eligible participants were patients diagnosed with stage I-III invasive BC, with ER- and/or progesterone receptor (PR)-positive and HER2-negative tumors for whom molecular genomic test scores were available (study group). Their data on clinical and pathological parameters, including age, tumor size, lymph node involvement, histology, grade, Ki67, genomic score, as well concomitant non-oncology medications (such as statins, aspirin, antihypertensive medication, insulin, levothyroxine, metformin, proton pump inhibitors, benzodiazepines, vitamin D, and calcium supplement) taken during the month prior to undergoing surgery, were collected. The control group comprised patients with BC who had not received any known non-oncology medicines.

Statistical analysis

Distributions of the genomic risk, the clinical risk, and the probability of adjuvant chemotherapy were compared with a two-tailed *t*-test between the control group and the groups of patients who had received various non-oncology medications. A multivariate linear or logistic regression analysis was carried out to test for a correlation between the genomic risk or the clinical risk and the clinical features, respectively. The genomic risk was determined by the Oncotype DX RS. According to the TAILORx study,[3] an RS $\geq$26 was considered high risk. The clinical risk assessment was based on an algorithm used in the MINDACT trial (see Appendix Table S13 in Cardoso et al[1]). The Oncotype DX assay uses the following expression unit criteria for PR expression: PR negative $<$5.5 and PR positive $\geq$5.5.[16] The probability for an adjuvant chemotherapy recommendation was calculated based on the genomic and clinical risk according to the model suggested by the phase III TAILORx results[3] and the subsequent analysis by Sparano et al.[17] The data were analyzed with a chi-square test by the Prism statistical program, and $P \leq 0.05$ was considered significant.

In addition, we wished to evaluate the impact of metformin and levothyroxine on the genomic scores of other genomic tests (PAM50 and MammaPrint) by analyzing 170 and 271 patients' results, respectively. We compared the frequency of patients presenting high or low genomic risk in MammaPrint between control (no known drugs) patients and metformin or levothyroxine groups and distribution and median expression levels of the risk of recurrence as determined by the PAM50 test results for control (no known drugs) patients and metformin or levothyroxine groups. An unpaired Student's *t*-test was used to test the significance for normally distributed data for all results. The *P* values are indicated.

Gene expression analysis

RNA-seq data from a primary BC clinical study that measured gene expression before and after 2 weeks of treatment with metformin were analyzed.[18] The dataset included the fold change of normalized expression levels for 36 patients. The average fold change for all 36 patients was calculated for each gene, and the genes were ranked from highest to lowest average fold change. A preranked gene set enrichment analysis was carried out on the ranked genes[19] using the KEGG (Kyoto Encyclopedia of Genes and Genomes) signature collection from the Molecular Signature Database (MSigDB).[20] The connectivity map L1000-LINCS database of perturbational experiments (https://clue.io/cmap) was also queried.[21] The response to metformin was queried against the Touchstone transcriptional signatures and sorted based on the connectivity score of the MCF-7 BC cell line to the 171 perturbational classes.

Reagents and cell lines

MCF-7 cells were purchased from American Type Culture Collection (ATCC) and were routinely maintained in Dulbecco's modified Eagle medium or phenol red-free Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum [FBS; Gibco heat inactivated], or charcoal—dextran-stripped FBS (Biological Industries, Sartorius group) and 1% L-glutamine, at 5% CO_2. Cells were authenticated using STR analysis and confirmed to be mycoplasma free using the MycoBlue Mycoplasma Detector Kit (Vazyme).

In vitro cancer cell line quantitative reverse transcription PCR analysis

For analysis of PR expression, in response to 50-μM metformin treatment, for 24 h, MCF-7 cells were seeded in six-well plates containing phenol red-free media supplemented with 10% charcoal-stripped FBS. Twenty four hours later, cells were treated with or without 10 nM 17β-estradiol (E2) and 50 μM metformin (both obtained from Sigma). Twenty-four hours later, total RNA was extracted using the HP RNA isolation kit (Roche) and RNA concentration measured using NanoDrop (Thermo Scientific); 1 μg RNA was taken for reverse transcription using the qScript cDNA Synthesis Kit (Quanta Biosciences). Messenger RNA (mRNA) levels were determined using quantitative reverse transcription PCR (qRT-PCR). Primers were designed using Primer Express (Applied Biosystems) and synthesized by Integrated DNA Technologies (listed in the next section). Equal loading was determined using GAPDH-specific primers. Amplification reactions were performed with PerfeCTa SYBR Green FastMix ROX (Quantabio) in triplicate using StepOnePlus (Applied Biosystems).

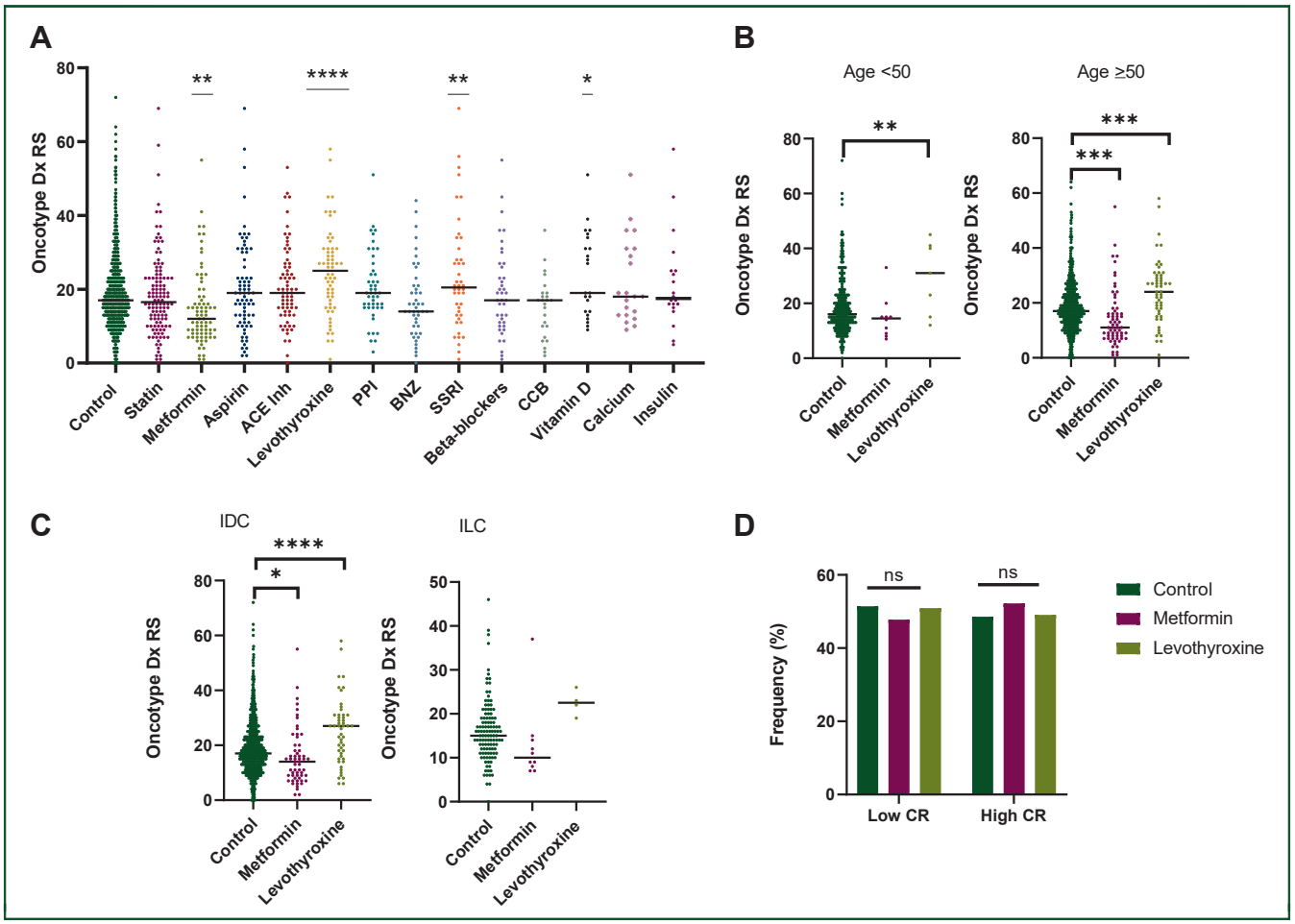

Figure 1. Non-oncology drugs are associated with different Oncotype DX recurrence scores (RSs). (A—C) Distribution and median expression levels of Oncotype DX RS. An unpaired Student's *t*-test was used to test the significance for normally distributed data. The *P* values are indicated. (A) Metformin, levothyroxine, vitamin D, and selective serotonin reuptake inhibitor (SSRI) are associated with different median Oncotype DX RS compared with control (no known drugs) patients. (B) Differences in Oncotype DX RS for control (no known drugs) patients and metformin or levothyroxine groups according to *patient age*. (C) Differences in Oncotype DX RS between control (no known drugs) patients and metformin or levothyroxine groups according to *histological subtypes* of the tumors. *<0.05, **<0.01, ***<0.001, ****<0.0001. (D) Comparison of the frequency of patients presenting high or low *clinical risk (CR)* between control (no known drugs) patients and metformin or levothyroxine groups.

BNZ, benzodiazepines; CCB, calcium channel blockers; IDC, invasive duct carcinoma; ILC, invasive lobular carcinoma; Inh, inhibitor; ns, not significant; PPI, proton pump inhibitors.

qRT-PCR primers used were GAPDH (forward: AGGGCCCTGACAACTCTTTT; reverse: TTACTCCTTGGAGGCCATGT) and PR (forward: CGC GCT CTA CCC TGC ACT C and reverse: TGA ATC CGG CCT CAG GTA GTT).

RESULTS

Non-oncology drugs are associated with different Oncotype DX RS

Our cohort included 1423 patients with ER-positive HER2-negative early BC. The mean age was 57 years. Most patients had no lymph node involvement (1008, 71%), and exhibited infiltrating ductal carcinoma histology (1158, 81%) and low-intermediate-grade tumors (1103, 79.6%). The mean Oncotype DX score was 18.95 (range 0-72). Assessment of the effect of multiple non-oncology drugs on the distribution of the Oncotype DX RS identified several drugs that were associated with different Oncotype DX scores in comparison to one control group that comprised patients with no known drugs (Figure 1A) and the other control group that comprised patients among whom the drug was not tested (supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2022.100648). Filtering for non-oncology drugs with at least 50 samples and significant *P* values in comparison to both of those control groups identified levothyroxine and metformin as being associated with higher and lower mean Oncotype DX RS, respectively (Table 1 and Figure 1A). Levothyroxine was significantly associated with a high RS (mean 24.78; $P < 0.0001$), and metformin was associated with a relatively low Oncotype DX RS (mean 14.87; $P < 0.01$) in comparison to patients who were not receiving medications (mean 18.7; Supplementary Figure S2A,B, available at https://doi.org/10.1016/j.esmoop.2022.100648), and this effect persisted irrespective of age or histology type, although metformin seems to affect Oncotype RS more in postmenopausal patients (Figure 1B and C). By contrast, the clinical risk (assessed by tumor size and grade and nodal status as in the

Table 1. Pathologic and clinical characteristics of the study and control groups

Characteristics	Control (no known drugs) ($N = 1018$), *n* (%)	Metformin ($N = 77$), *n* (%)	Levothyroxine ($N = 60$), *n* (%)
Age (years)			
<50	262 (26)	12 (16)	7 (12)
≥50	756 (74)	65 (84)	53 (88)
Histology			
IDC	828 (82)	58 (75)	51 (85)
ILC	116 (11)	10 (13)	4 (7)
Other	74 (8)	9 (12)	5 (8)
Tumor size (cm)			
≤2	754 (74)	53 (69)	53 (88)
>2	233 (23)	24 (31)	7 (12)
NA	31 (3)		
Involved nodes			
Negative	702 (69)	52 (68)	42 (70)
Mic	210 (21)	14 (18)	11 (18)
Positive	68 (6)	7 (9)	4 (7)
NA	38 (4)	4 (5)	3 (5)
Grade			
G1 or G2	657 (64)	47 (61)	29 (48)
G3	191 (19)	14 (18)	25 (42)
Gx	170 (17)	16 (21)	6 (10)
Ki67			
≤15%	444 (44)	43 (56)	27 (25)
>15%	206 (20)	23 (30)	21 (35)
NA	368 (36)	11 (14)	12 (20)
BMI (kg/m^2)			
≤25	224 (22)	18 (23)	12 (20)
>25	122 (12)	21 (27)	14 (23)
NA	672 (66)	38 (50)	30 (50)
Oncotype DX RS, mean (range)	18.7 (0-72)	14.87 (1-55)	24.78 (1-58)

BMI, body mass index; IDC, invasive duct carcinoma; ILC, invasive lobular carcinoma; NA, not available; RS, recurrence score.

MINDACT study)[1] was not different between patients treated with levothyroxine or metformin and the control group (Figure 1D). A multivariate regression analysis was carried out to assess the relative contribution of levothyroxine or metformin to the Oncotype DX RS after controlling for covariates, including tumor size, lymph node involvement, patient age at diagnosis, nuclear grade, and ER/PR expression. Both levothyroxine ($\beta = 3.81$, $P < 0.01$) and metformin ($\beta = -3.7$, $P < 0.01$) were found to independently influence the Oncotype DX RS (Table 2).

The genomic influence of levothyroxine and metformin through estrogen-dependent modules

To better understand the underlying molecular basis through which levothyroxine and metformin impact the Oncotype DX score, we sought to determine whether the main modules that generate the genomic score, namely, the proliferation level and the hormone expression, are affected by the drug use. There was no detectable difference in the levels of the classic proliferation marker Ki67 between patients who did and those who did not use the drugs (Figure 2A). By contrast, the PR and ER expression levels were different between patients who received levothyroxine or metformin and those who did not (Figure 2B). The two drugs showed an opposite effect: metformin was associated with a higher PR expression mainly on cells without estrogen exposure (Figure 2C), whereas levothyroxine was associated with a lower PR expression. This difference was observed at the mRNA level (qRT-PCR) for both drugs (Figure 2B), and at the protein level (immunohistochemistry) for levothyroxine (Figure 2C). ER mRNA, but not protein, expression levels were significantly different between patients who received metformin compared with controls (Figure 2B and C). No differences were observed in HER2 expression between the different groups (Figure 2D).

We used additional clinical datasets to investigate the association between metformin and hormonal signaling. A gene set enrichment analysis[22] of RNA-seq data from a primary BC clinical study before and 2 weeks after treatment with metformin[18] confirmed the association between metformin treatment and the expression of gene sets associated with steroid hormone biosynthesis (normalized enrichment score: 1.72; $P < 0.001$; q-value $= 0.019$; Figure 2E and Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100648). We then queried the Broad Institute Connectivity Map, which is a genome-scale library of cellular signatures that catalogs transcriptional responses to chemical perturbations.[21] Specific to the BC cell line (MCF-7), metformin was strongly connected to PR-modulating drugs (#1 and #11 top connections: Figure 2F and Supplementary Table S2 available at https://doi.org/10.1016/j.esmoop.2022.100648). Consistent with these data and in support of our clinical data, we

Table 2. Univariate and multivariate linear regression analyses between Oncotype DX scores and the different pathologic and clinical features of age, tumor size, grade, hormone receptor expression, lymph node status, as well as administration of the non-oncology medications metformin or levothyroxine

Variable	Univariable estimate (95% CI)	*P* value	Multivariate estimate (95% CI)	*P* value
Age	−0.03 (−0.09 to 0.02)	0.27		
Tumor size	0.99 (0.13 to 1.86)	0.02		
Grade	4.57 (3.43 to 5.70)	<0.001	3.78 (2.67 to 4.89)	<0.001
ER (IHC)	−1.37 (−2.73 to −0.01)	0.048	−0.34 (−1.6 to 0.92)	0.6
PR (IHC)	−2.87 (−3.39 to −2.35)	<0.001	−2.7 (−3.26 to −2.2)	<0.001
HER2 (IHC)	0.59 (−0.29 to 1.48)	0.18		
Nmic	−0.48 (−1.39 to −0.43)	0.3		
Metformin	−4.19 (−6.74 to −1.64)	0.0013	−3.72 (−6.24 to −1.21)	0.004
Levothyroxine	7.07 (4.2 to 10)	<0.001	3.8 (1 to 6.63)	0.008

CI, confidence interval; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; PR, progesterone receptor.

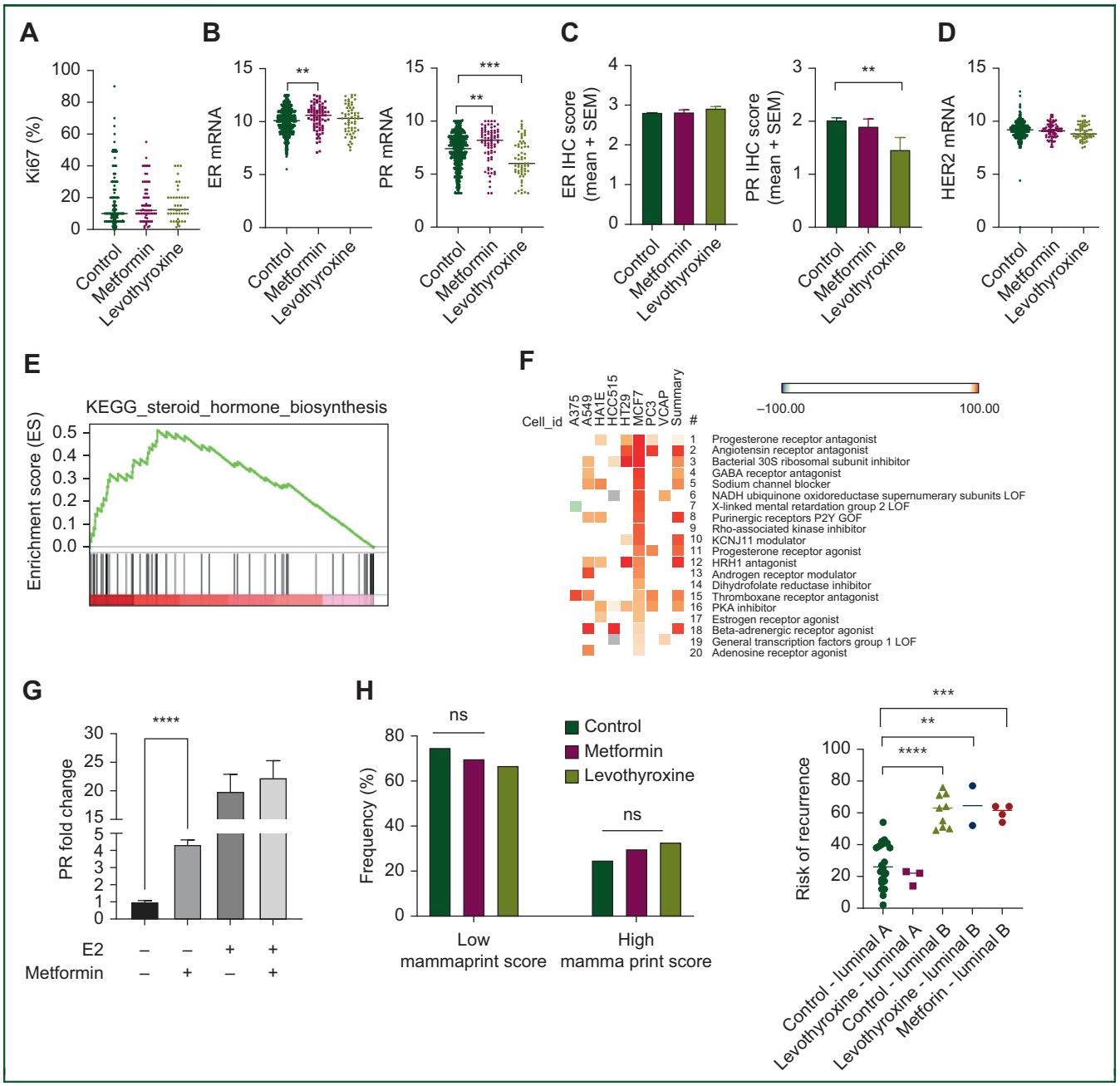

Figure 2. Levothyroxine and metformin influence genomic risk through estrogen-dependent modules. (A) Distribution and median expression levels of Ki67 (%) for control (no known drugs) patients and metformin or levothyroxine groups. (B–D) Differences in hormone receptor expression between control (no known drugs) patients and metformin or levothyroxine groups. (B) ER and PR mRNA expression. (C) ER and PR protein expression (immunohistochemistry). (D) HER2 mRNA expression. (E) A GSEA enrichment plot of the gene set 'Kegg_Steroid_hormone_biosynthesis'. The preranked analysis was performed with fold-change-normalized gene expression data from 36 patients 2 weeks before and after metformin treatment. The fold change refers to normalized expression after treatment/normalized expression before treatment. (F) A connectivity map plot of the 20 perturbational classes most associated with the response of MCF-7 to metformin. (G) PR fold change as a result of metformin treatments and E2 presence. Histogram depicts the mean fold change ($n = 3$) relative to the mean of vehicle control ± SD. Unpaired two-tailed *t*-test was used to detect statistically significant differences between metformin treatment for the −E2 and +E2 groups. In the absence of E2, metformin treatment shows significant increase in PR expression ($P < 0.0001$), whereas in the presence of E2 no significant results were observed ($P = 0.589$). (H) Left: comparison between the frequency of patients presenting high or low genomic risk in MammaPrint between control (no known drugs) patients and metformin or levothyroxine groups. Right: distribution and median expression levels of the risk of recurrence as determined by the PAM50 test results for control (no known drugs) patients and metformin or levothyroxine groups. An unpaired Student's *t*-test was used to test the significance for normally distributed data for all results. The *P* values are indicated. **<0.01, ***<0.001, ****<0.0001.

ER, estrogen receptor; GSEA, gene set enrichment analysis; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; mRNA, messenger RNA; PR, progesterone receptor; SD, standard deviation.

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demonstrated that metformin treatment in MCF-7 cells led to elevated PR levels in estrogen-deprived conditions (Figure 2G). These results support the notion that metformin impacts the Oncotype DX RS through its effect on hormonal-dependent signaling.

To support our observation that the impact of levothyroxine and metformin on the Oncotype DX RS is through hormonal-dependent genes, we evaluated the impact of both drugs on the genomic scores of other genomic tests (PAM50 and Mamma Print), which are considered to be less influenced by hormonal-related features,[23] and, as expected, we found that these genomic scores were less influenced by the two drugs (Figure 2H). In agreement with these results, the effect of metformin and levothyroxine on the distribution of Oncotype DX RS was significant only in patients who were PR positive but not in those who were PR negative (Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2022.100648).

Recommendation to receive adjuvant chemotherapy based on levothyroxine and metformin treatment

The current guidelines to recommend adjuvant chemotherapy according to clinical risk and genomic risk (Oncotype DX) in early luminal BC were suggested by Sparano et al.[3] based on the TAILORx trial results and they have been adopted worldwide. Implementation of this model was done on our cohort of patients. We found that the probability of an adjuvant chemotherapy recommendation among women above the age of 50 years was significantly higher in patients treated with levothyroxine compared with patients treated with metformin (49% versus 14.5%; *P* 0.0001, respectively).

DISCUSSION

ER-positive HER2-negative BC is the most common BC worldwide.[24] To date, the treatment decision making in the early stages is greatly influenced by molecular tests that determine the risk of recurrence and the benefit of chemotherapy. The results of our study demonstrated that specific commonly prescribed medications, namely, levothyroxine and metformin, influence the molecular score and thereby affect the likelihood of recommending adjuvant chemotherapy.

Hypothyroidism is a common disease, especially among elderly women, and it is treated with thyroid hormone supplementations, one of which is levothyroxine. The latter was found in preclinical data to be a growth factor for various cancers[25] by bearing an influence on cancer cell proliferation[26,27] and on cancer cell defense pathways, for example, anti-apoptosis and proangiogenesis.[28,29] Initial clinical data of induced hypothyroxinemia are now emerging for patients with solid tumors.[30,31] Our current results showed that levothyroxine has an elevating effect on the Oncotype DX RS. Goldvaser et al.[32] had attempted to associate extended levothyroxine usage with the Oncotype DX RS. In contrast to our findings, those authors reported an association between extended levothyroxine usage and a lower Oncotype DX RS, suggesting that levothyroxine *per se* is not a risk factor. Future research is required to reconcile these findings and to identify potential confounding factors (e.g. euthyroidism, duration of treatment) that may affect the genomic risk of patients treated with levothyroxine. Metformin, a widespread medication for type 2 diabetes mellitus, was shown to play a key role in tumorigenesis. Numerous preclinical data and retrospective studies have suggested that metformin may also reduce the incidence of BC and improve cancer prognosis.[33-38] The clinical data, however, are inconclusive,[39-41] and a recent prospective study failed to demonstrate benefit from adjuvant metformin.[42] We found an association between metformin use and a relatively low Oncotype DX RS, with no change in the clinical risk, in comparison with patients who did not receive metformin. Our results are supported by those of others who showed that patients treated with metformin had a significantly lower Oncotype DX score.[32] Our data suggest that the effect of metformin on the Oncotype RS is mediated through its effect on ER and PR signaling.

Importantly, we did not find any difference in clinical risk between the patients who received the two studied medications and those that did not. The Ki67 levels were not altered by either of them, suggesting that there had been no impact of either drug on proliferation. However, we did demonstrate an impact of progesterone-related features, suggesting that the two drugs influence the genomic risk through their effect on hormone-dependent signaling. This observation may explain why other molecular tests, which are less influenced by the hormonal pathway (e.g. MammaPrint and Prosigna) did not reflect the same differences noted in the Oncotype DX RS. Interestingly, metformin promoted PR expression via inhibition of a mammalian target of rapamycin in endometrial cancer,[43] providing a possible mechanism by which metformin affects PR levels and the Oncotype DX RS.

This work shares the same limitation with others that have a retrospective design. Another drawback is the inability to test the direct impact of the drugs on therapeutic outcome due to the inherent biases and confounders, especially the fact that treatment decisions are influenced by the Oncotype DX RS, which, in turn, is affected by the non-oncology drugs received by the patients. This complicates the efforts to move from correlation to causation.

In conclusion, our observation of an association between levothyroxine and metformin and the Oncotype DX RS needs to be further validated and interrogated functionally. Nevertheless, we believe that it is reasonable to consider whether or not the patients are prescribed levothyroxine or metformin when selecting the genomic test and/or when interpreting the results of the test for patients with early luminal BC for which the physician considers genomic testing.

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DISCLOSURE

BW is on the speakers bureau for MSD, BMS, Merck, AstraZeneca, Roche; reports a consulting or advisory role in MSD; travel, accommodations, and expenses covered by MSD and Sanofi. IW is on the speakers bureau and reports a consulting or advisory role in MSD, BMS, Takeda, AstraZeneca, Roche, Novartis, and Pfizer. SSS reports a consulting or advisory role in Pfizer, Novartis, Roche, Medison, MSD, AstraZeneca, Eli Lilly, ProGenetics, and Gilead; and travel, accommodations, expenses covered by Pfizer. AS is on the speakers bureau for Teva, Roche, Pfizer, Novartis, and Medison; reports a consulting or advisory role in Eli Lilly, Pfizer, Novartis, Roche, Gilead, MSD, and Roche; travel, accommodations, expenses covered by Neopharm, Celgene, Medison, and MSD. TZ, MSD, AG, TR, TW, OF, AG, AZ, MK, NK, ICL, UBD have no interests to declare.

DATA SHARING

The datasets generated during this study are available from the corresponding author on reasonable request.

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