American Institute of Integrative Oncology
RESEARCH & EDUCATION

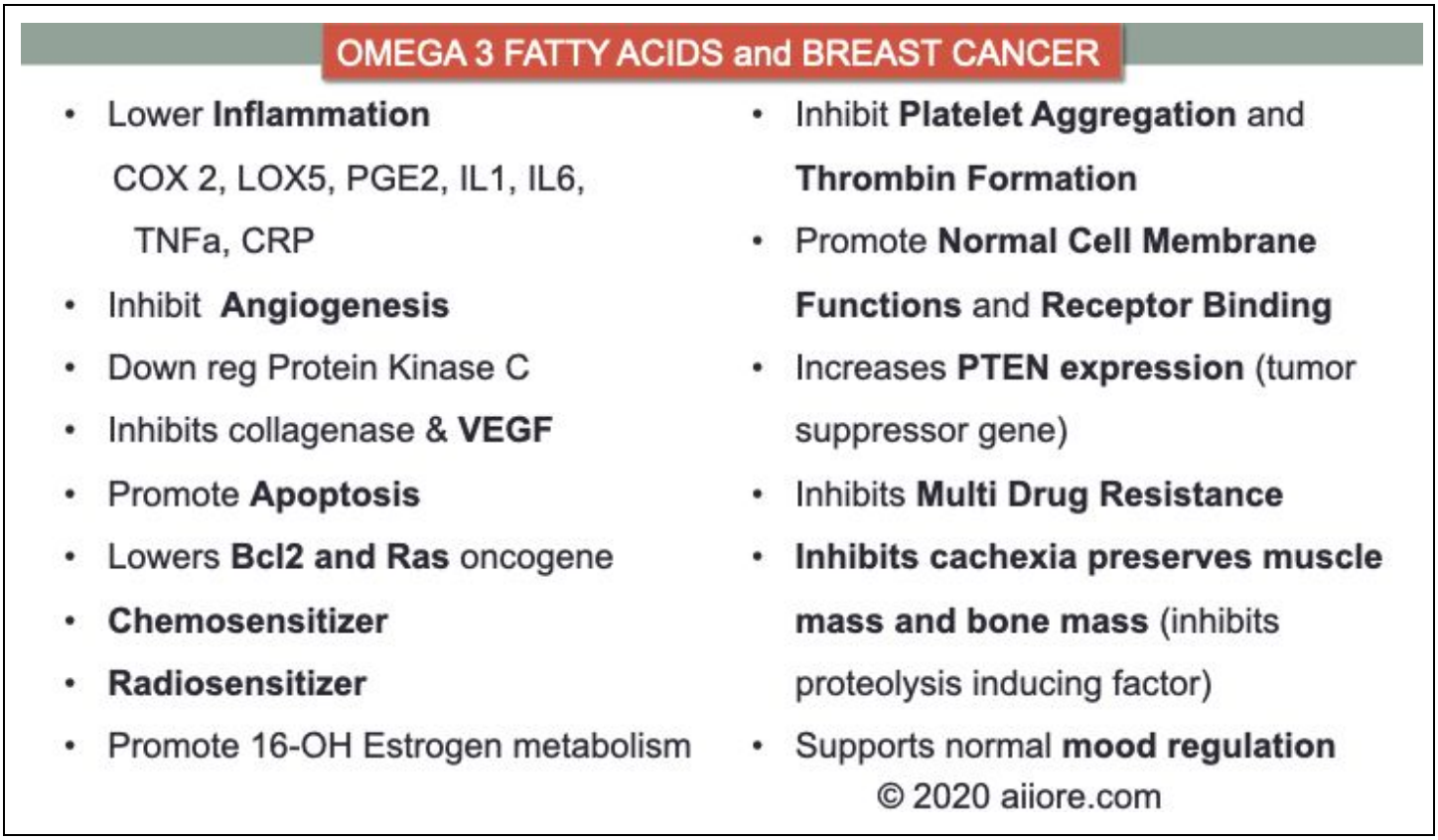
Foundations of Integrative Oncology
COURSE

GRAND ROUNDS CALL
With Dr. Nalini Chilkov
January 15th, 2020
Second Wednesday of Every Month
5:30 PM Pacific / 6:30 PM Mountain / 7:30 PM Central / 8:30 PM Eastern

Agenda

- **Clinical Pearl**
  - Omega 3 Fatty Acids and Breast Cancer
- **Case Study**
  - 64yo M Colon Cancer - Metastasis to Liver Stage 4
- **Clinical Question:**
  - Tips on how to handle peripheral neuropathy?
  - What are the particular lifestyle/diet recommendations for women with BRCA1 or BRCA2?
  - Advice/precautions around treating patients who have recently completed immunotherapy (PD-1 inhibitor)?
  - What tests do you recommend having done on the biopsy sample at the time of a recurrence other than the usual pathology, DNA testing, Chemotherapy sensitivity testing?
  -
- **Research Highlights:**
  - The Role of the Estrogen Pathway in the Tumor Microenvironment
  - Circulating Tumor Cells Predict Survival in Early Average-to-High Risk Breast Cancer Patients

Clinical Pearl: Omega 3 Fatty Acids and Breast Cancer
See the PDF of Slide Presentation and summary slide below - **Link to SLIDES -** **https://aiiore-members-only.s3-us-west-1.amazonaws.com/Grand+Rounds/2020+01+15+Clinical+Pearl+Slides+-+Omega+3+Fatty+Acids+%26+Breast+Cancer.pdf**



Case Study: 64yo M Colon Cancer - Metastasis to Liver Stage 4

Submitted by: Judy Pruzinsky L.Acs

Link to CASE STUDY - https://aiiore-members-only.s3-us-west-1.amazonaws.com/Grand+Rounds/2020-01-15+64yo+M+Colon+Cancer+-+Metastasis+to+Liver+Stage+4.pdf

Questions & Answers

Isabel Galiano: What are your tips on how to handle peripheral neuropathy?

Dr. Chilkov:

Primary Interventions: Reduce oxidative stress and promote neuronal repair

- Remove sources of high oxidative stress (smoking, sun exposure, inflammation).Iron if pro-oxidative
- Use Fe free supplements and avoid red meat.
    - Acetyl L Carnitine 2 g daily,
    - L Glutamine 3 g daily,
    - R Lipoic Acid 2 g daily,
    - O3 FA 4 g daily.
- Increase polyphenols and flavonoids in the diet.
- Promote healthy fat digestion if needed (Pancreatic enzymes and Bile supplements)
- Support healthy microbiome with prebiotics and probiotics.
- Broad Spectrum methylated B Complex daily.

- Acupuncture.

Questions & Answers

Isabel Galiano:

1. **Do you have any particular lifestyle/diet recommendations for women with BRCA1 or BRCA2?**
2. **Any difference depending on, if it is BRCA1 or BRCA2?**

I am seeing more clients that have been diagnosed with BRCA1 or BRCA2 but DO NOT have active cancers.

Dr. Chilkov:

Niacinamide is a natural PARP inhibitor: 1g bid (Douglas Lab)

- All BRCA mutations involve poor DNA repair.
- These patients should avoid all medical exams that involve radiation whenever possible (due to DNA damage)
- BRCA1 cancers are VERY AGGRESSIVE and generally tx resistant.
- BRCA2 is less aggressive but equally hard to treat..
- I do encourage all BRCA+ patients to plan to have children without delay if that is important to them.
- BRCA mutations are found in many cancers: Breast, Ovarian, Pancreatic Colorectal, Melanoma.
- Men with BRCA mutations have 80x risk of prostate and breast cancers along with the above cancers.
- Women without BRCA mutations have 12% risk of BrCA. Women WITH BRCA mutations have a 72% risk of BrCA Children with BRCA2 have higher risk of Non Hodgkins lymphoma

Interventions-Guidelines

- **Reduce all sources of oxidative stress and toxic exposures.**
- **Screen patients for SNPs** that involve Glutathione, SOD and inflammatory cytokines, p53 mutations and a larger gene panel to see the larger picture of DNA Repair *ATM, CDH1, CHEK2, MRE11A, MSH6, NBN, PALB2, PMS2, PTEN, RAD50, RAD51C, SEC23B,* or *TP53* mutations and tumor suppressor and tumor promoter genes, individually or as part of a larger gene panel that includes *BRCA1* and *BRCA2*. Include referral to genetic counselor.
- Make sure body burden of toxic metals and environmental chemicals is assessed and treated and monitored.
- Follow OUTSMART CANCER DIET Diet must be anti inflammatory and high in polyphenols, flavonoids, catechins.
- Organic plant based diet is essential to provide phytochemicals and phytophenols
- Avoid alcohol.
- Learn to manage stress and improve parasympathetic and vagal tone.
- Regular Exercise and Sleep habits must be cultivated.
- The decision to have mastectomies and ovariectomies is very personal.(mastectomies reduce risk of BrCA by 97%)
- Start and continue a program to manage the tumor microenvironment.
- Teach the patient about the Cancer Terrain, Low Glycemic diet and intermittent fasting.
- Teach patients about links between obesity, insulin and cancer risk.
- Teach patients about FIR saunas as a method of detox.
- Teach patients how to do self breast exams monthly and have breast screening every 6 months. (Ultrasound, MRI),
- Ovarian exam and ultrasound every 6 months.
- Baseline Colonoscopy.

- Pancreatic ultrasound every 6 months.
- Some women are recommended to reduce risk of estrogen positive breast cancers with SERMs and Aromatase inhibitors.
- Discuss this with patients and teach them about natural SERMs and AI (see previous GRC Clinical Pearl lecture on this topic).
- Teach patients about environmental exposures to xenoestrogens (plastics, BPA, multiple chemicals in body care and cosmetics products, animal fats and proteins)
- Never smoke. Stop smoking.
- Include Nutriceutical and Botanical Protocol to manage cancer terrain
- Patient info on Breast Cancer Genetics on Breastcancer.org

Questions & Answers

Kiran Sangha:

- **Would you have any advice/precautions around treating patients who have recently completed immunotherapy (PD-1 inhibitor)?**
- **For example, do you refrain from using anything that could potentially stimulate immunity? if so, for how long?.**
- **I'm always very cautious as I have been taught not to use anything that could stimulate immunity, including melatonin/mushrooms/ probiotics for up to 6 months after they have completed treatment, however, I would appreciate your opinion on this.**

Dr. Chilkov:

HyperInflammation and the development of autoimmune syndromes are the primary adverse effects.

- Follow the same guidelines you would use with a patient with AutoImmune Disease.
- Do not do anything that would stimulate immunity (probiotics and melatonin are OK and not contraindicated).
- However do avoid astragalus, echinacea, all medicinal mushrooms.
- Do include Curcumin, Boswellia, Omega 3 FA to modulate inflammation.
- High doses of Glycyrrhiza glabra (Chinese Raw Licorice Root Gan Cao) 2-4 g/day has a mild steroid like antiinflammatory effect)
- Implement an anti-inflammatory diet.
- Screen for hypothyroid, IBS and colitis, arthritis.
- Acupuncture can modulate states of hyperinflammation
- (See prior GRC on Immunotherapies)

Questions & Answers

Aniko Lengyel:

- **What tests do you recommend having done on the biopsy sample at the time of a recurrence other than the usual pathology, DNA testing, Chemotherapy sensitivity testing?**

Dr. Chilkov:

This depends on the amount of tissue available

Always inquire what the oncologist is planning to order in terms of tumor cell analysis

Precision Medicine a constantly evolving field

- Precision Medicine is a critical component in controlling cancer
- Interrogate a blood-based cancer or solid tumor by as many means necessary to reveal biomarkers that expose that disease's susceptibilities to standard or novel therapies.
- Identify active therapeutic agents

There are MANY MANY Labs that now do extensive tumor cell analysis in an effort to individualize treatment decisions. Some institutions do extensie testing and analysis and some do very little and just follow standard of care guidelines without any effort to individualize care..

Weisenthal Cancer Lab http://www.weisenthalcancer.com/index.html and
Nagourney Cancer Institute labs https://www.nagourneycancerinstitute.com/about
Require fresh not frozen specimen for chemosensitivity testing (also test biological targeted therapy agents)

Consultative Proteomics at University of Texas, Houston. Advance Proteomic analysis. Proteomics allows identification of expressed genes.Report includes both nutriceuticals, phytochemicals and pharmaceuticals
https://med.uth.edu/pathology/clinical-services/consultative-proteomics/

Foundation One https://www.foundationmedicine.com/genomic-testing
Genetic, Genomic and receptor analysis of tumor cells and links to available trials, Liquid Biopsy

Caris Life Sciences
Genomic and receptor analysis of tumor cells and links to available trials**.** Exosomes, Liquid Biopsies
https://www.carislifesciences.com/
For patient education https://www.mycancer.com/

Also consider Liquid Biopsy options (Circulating Tumor Cells, cell free tumor DNA, Cancer Stem Cells if tissue sample is not an option)
Guardant 360 https://www.foundationmedicine.com/genomic-testing
Biocept https://biocept.com/
Neogenomics innovative diagnostic, prognostic and predictive testing.
and Some labs can test CTCs for receptors and gene expression if tissue is not available.

- There is usually a tissue bank from prior surgeries and biopsies.
- A needle biopsy produces a very small amount of tissue and may not be sufficient for extensive testing.
- Surgical samples collect and store more frozen block tissue that can be used for current or future analysis.
- If a tissue sample is collected and then the patient undergoes treatment, the tissue sample no longer represents the post tx cells which have been transformed by the treatment.

Any patient with advanced stage cancer and/or living with cancer as a chronic illness has a heterogenous population of tumor cells.

The characteristics of metastatic lesions are typically not identical to the primary tumor. If a patient has both primary tumor and metastatic lesions, these are typically not identical tumor cells.. Always know the site of the biopsy and whether it is primary or secondary tumor cells.

Resource:

2019 San Antonio Breast Cancer Symposium [SABCS] Slide Sets (Summaries of Lectures and Presentations)
https://www.clinicaloptions.com/oncology/conference-coverage/breast-cancer-dec-2019/breast-cancer/capsule-summary-slidesets

Estrogen Decreases Breast Cancer Incidence in Postmenopausal Women, Estrogen Plus Progestin Has Opposite Effect

Jason Harris

"Use of estrogen alone and use of estrogen plus progestin have opposite effects on breast cancers," he said. "[Estrogen] alone after use for 7.2 years, now with 19.2 years follow-up, resulted in a 23% reduction in breast cancer use, which was statistically significant. The [estrogen plus progestin] use ended up increasing breast cancer by 29%."

Chlebowski added that the reduced risk for incidence associated with estrogen continued after the study period (HR, 0.83; 95% CI, 0.57-1.20). Similarly, the increased risk continued with estrogen/progestin (HR, 1.30; 95% CI, 0.99-1.70). In both cases, the effect continued over decades.

"Women who are considering estrogen alone should know that it's safer and there may be a breast cancer benefit associated with its use,"

https://www.onclive.com/conference-coverage/sabcs-2019/estrogen-decreases-breast-cancer-incidence-in-postmenopausal-women-estrogen-plus-progestin-has-opposite-affect

Resource: Book + Interview Recommendation

Estrogen Matters: Why Taking Hormones in Menopause Can Improve Women's Well-Being and Lengthen Their Lives – Without Raising the Risk of Breast Cancer.

Avrum Bluming MD is an oncologist whose practice has been 60% devoted to breast cancer. Carol Tavris Ph.D. is a social psychologist,feminist, and skeptic who writes the column "The Gadfly" for Skeptic magazine and whose many books include the classic Mistakes Were Made (But Not by Me).They were alarmed by the many misunderstandings about menopausal hormone replacement and they collaborated to set the record straight with an extensively referenced new book, https://estrogenmatters.com/

Interview with Authors: Peter Attia Podcast https://peterattiamd.com/caroltavris-avrumbluming/
#42 – Avrum Bluming, M.D. and Carol Tavris, Ph.D.: Controversial topic affecting all women—the role of hormone replacement therapy through menopause and beyond—**the compelling case for long-term HRT and dispelling the myth that it causes breast cancer.**

Research:

The Role of the Estrogen Pathway in the Tumor Microenvironment **PMID: 29463044 Int J Mol Sci. 2018 Feb; 19(2): 611.Published online 2018 Feb 19. doi: 10.3390/ijms19020611 Natalie J Rothenberger,1 Ashwin Somasundaram,1,2 and Laura P. Stabile3,4,***

ABSTRACT:

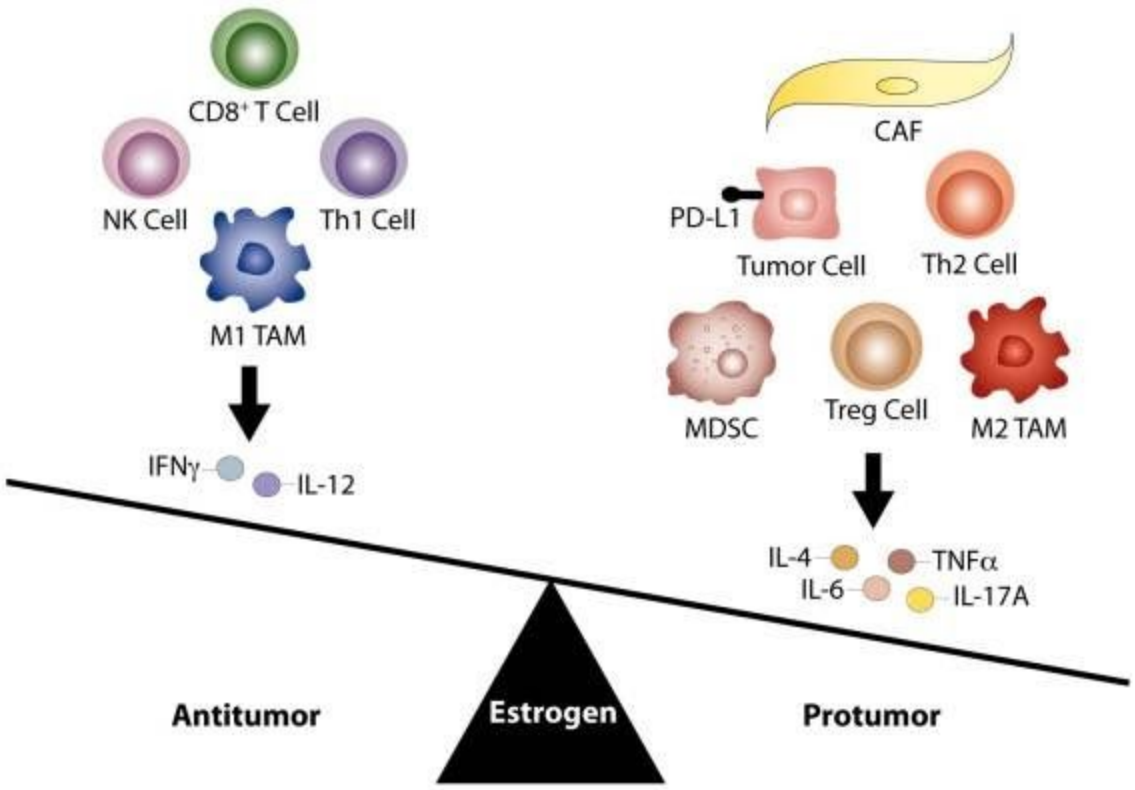
Estrogen receptors are broadly expressed in many cell types involved in the innate and adaptive immune responses, and differentially regulate the production of cytokines. While both genomic and non-genomic tumor cell promoting mechanisms of estrogen signaling are well characterized in multiple carcinomas including breast, ovarian, and lung, **recent investigations have identified a potential immune regulatory role of estrogens in the tumor microenvironment.**Tumor immune tolerance is a well-established mediator of oncogenesis, with increasing evidence indicating the importance of the immune response in tumor progression. Immune-based therapies such as antibodies that block checkpoint signals have emerged as exciting therapeutic approaches for cancer treatment, offering durable remissions and prolonged survival. However, only a subset of patients demonstrate clinical response to these agents, prompting efforts to elucidate additional immunosuppressive mechanisms within the tumor microenvironment.**Evidence drawn from multiple cancer types, including carcinomas traditionally classified as non-immunogenic, implicate estrogen as a potential mediator of immunosuppression through modulation of protumor responses independent of direct activity on tumor cells. Herein, we review the interplay between estrogen and the tumor microenvironment and the clinical implications of endocrine therapy as a novel treatment strategy within immuno-oncology.**

Conclusions:

The E2 pathway is an identified promoter of tumorigenesis in several cancers, largely for its direct genomic and non-genomic effects on tumor cells. However, evidence of ER and aromatase expression on stromal and immune cells within the TME indicates that additional mechanisms exist by which estrogens enhance malignant progression.

Evidence thus far suggests that E2 facilitates a primarily tumor-promoting and immunosuppressive TME in multiple tumor types.

The data summarized here points to the E2 pathway as a regulator of tumor immune responses, suggesting that clinical benefit may be derived from combining estrogen blocking agents with immune checkpoint inhibitors.



Research:

Circulating Tumor Cells Predict Survival in Early Average-to-High Risk Breast Cancer Patients

J Natl Cancer Inst. 2014 May; 106(5): dju066. Published online 2014 May 15. doi: 10.1093/jnci/dju06
PMID: 24832787 Brigitte Rack, et al https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4112925/

Presence of CTCs at time of diagnosis in early Breast Cancer predicted decreased progression-free survival and OS

- Prognostic relevance of CTCs after chemotherapy could be especially valuable for individualized treatment approaches to allow for the identification of patients with tumor cells evading standard chemotherapy.

Conclusions

- The SUCCESS study is the first trial to provide strong evidence for the prognostic **relevance of CTCs in early breast cancer before and after adjuvant chemotherapy** in a large patient cohort.
-
- Our data offer support for the **clinical potential of CTCs to assess the individual risk of patients at the time of primary diagnosis**
- **and may be used for treatment tailoring in the absence of other strong quantitative markers.**

- Future applications for CTCs will include the early assessment of treatment efficacy
- Phenotyping **of cells to individualize treatment strategies.**
- **CTCs may considerably contribute to the personalization of breast cancer treatment**
- **These results suggest the independent prognostic relevance of CTCs both before and after adjuvant chemotherapy in a large prospective trial of patients with primary breast cancer.**

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7. Pizato, Kiffer, Luzete, Assumpção, Correa, Melo, … Magalhães. (2019). **Omega 3-DHA and Delta-Tocotrienol Modulate Lipid Droplet Biogenesis and Lipophagy in Breast Cancer Cells: the Impact in Cancer Aggressiveness.** *Nutrients*, *11*(6), 1199. doi: 10.3390/nu11061199

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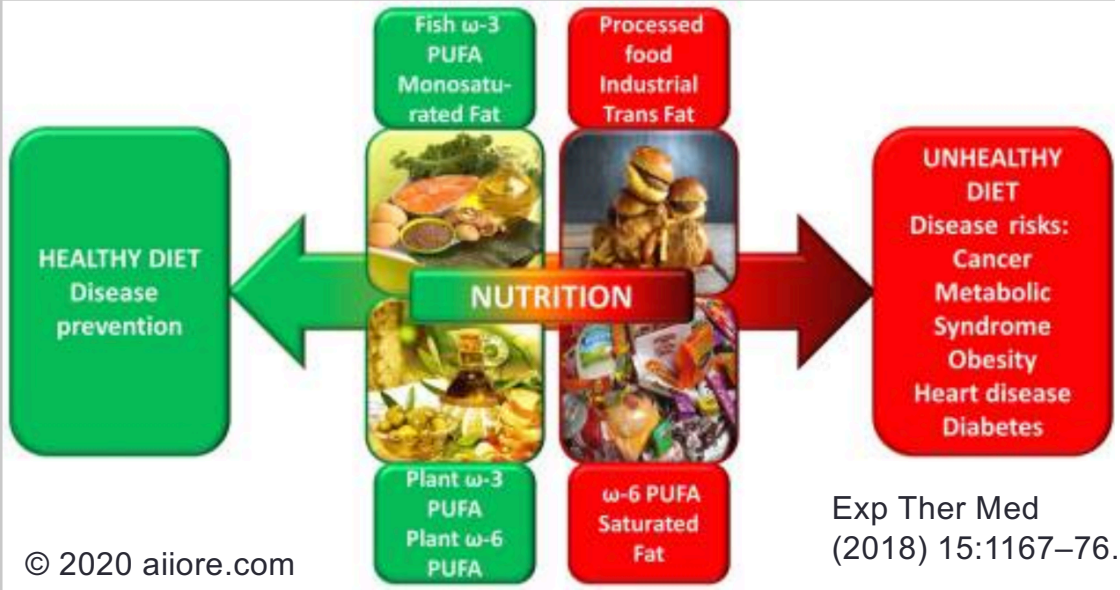
Clinical Pearl

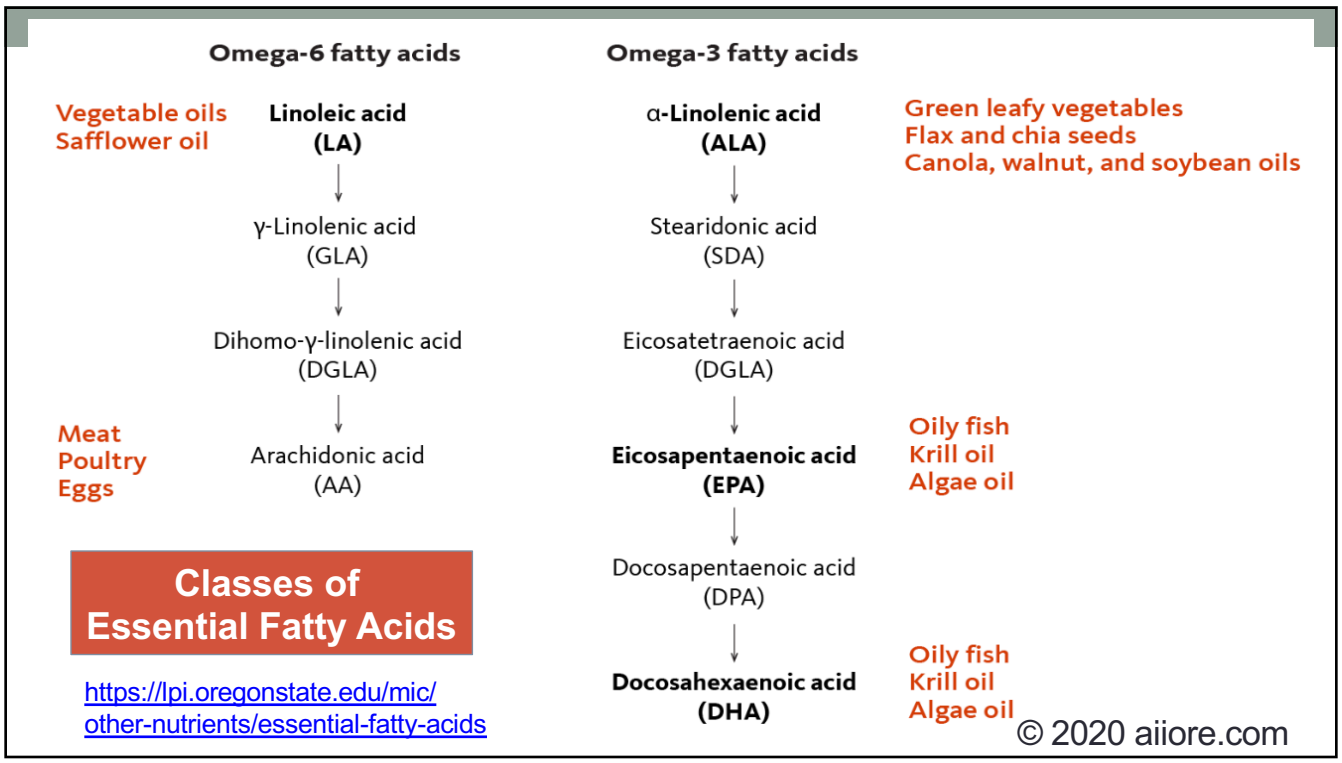
OMEGA 3 FATTY ACIDS & BREAST CANCER

Dr. Nalini Chilkov, L.Ac., OMD, Founder
American Institute of Integrative Oncology Research and Education

Impact of dietary PUFAs in disease prevention or risk.



Exp Ther Med (2018) 15:1167–76.

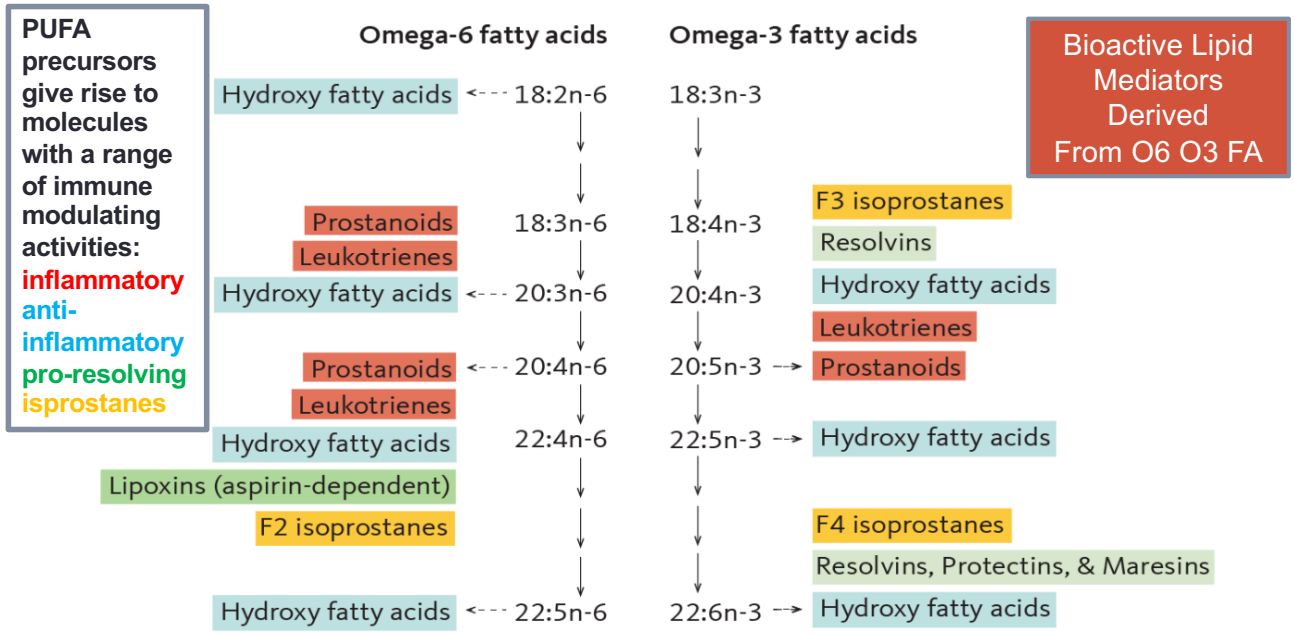


Implications of dietary ω-3 and ω-6 polyunsaturated fatty acids in breast cancer

Dietary fatty acids, have been recognized as influential factors in the **activation of carcinogenic events or disease progression,** and have been associated with a **direct connection to breast cancer prevention**. PUFAs differentially inhibit mammary tumor development by inflicting modifications to the morphology of cell membranes, and influencing signaling pathways, gene expression and apoptosis.

Exp Ther Med. 2018 Feb; 15(2): 1167–1176. Oana Zanoaga et al

PUFA precursors give rise to molecules with a range of immune modulating activities:
inflammatory
anti-inflammatory
pro-resolving
isprostanes



 https://lpi.oregonstate.edu/mic/other-nutrients/essential-fatty-acids

The human body is unable to synthesize long-chain polyunsaturated fatty acids (PUFAs) **Omega 3 DHA**, docosahexaenoic, and **EPA**, Eicosapentaenoic acid and **Omega 6 Arachidonic Acid at a reasonable rate** and therefore, **supplementation is required through dietary sources**

A diet high in polyunsaturated fatty acids, especially omega 3s, have been shown to be negatively associated with cancer development

Azrad M, Turgeon C, Demark-Wahnefried W. **Current evidence linking polyunsaturated Fatty acids with cancer risk and progression**. Front Oncol. (2013) 3:224.

Bartsch H, Nair J, Owen RW. **Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers.** Carcinogenesis. (1999) 20:2209–18.

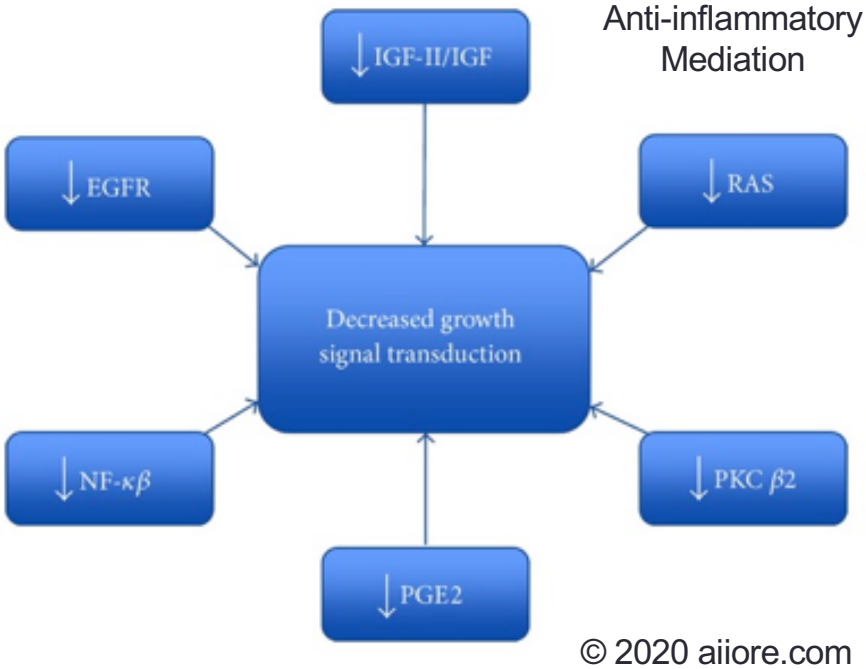
Zanoaga O, Jurj A, Raduly L, Cojocneanu-Petric R, Fuentes-Mattei E, Wu O, et al. **Implications of dietary omega-3 and omega-6 polyunsaturated fatty acids in breast cancer.** Exp Ther Med. **(2018)** 15:1167–76. 10.3892/etm.2017.5515

OMEGA 3 FATTY ACIDS and BREAST CANCER

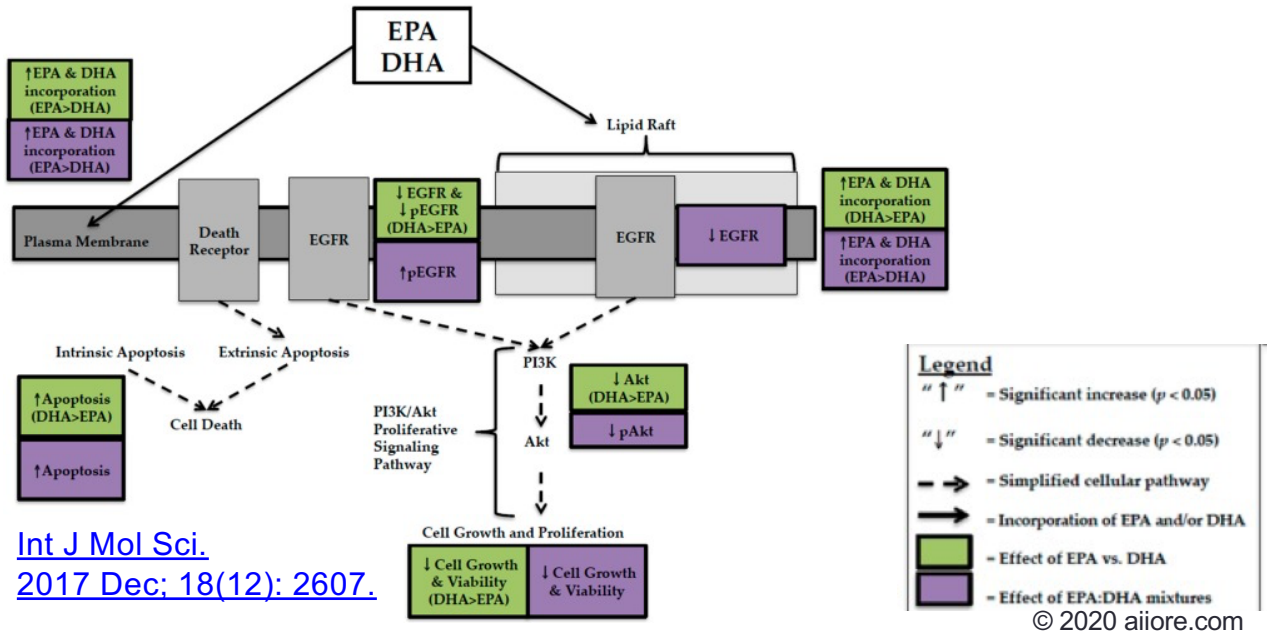
- Lower **Inflammation**
  COX 2, LOX5, PGE2, IL1, IL6, TNFa, CRP
- Inhibit **Angiogenesis**
- Down reg Protein Kinase C
- Inhibits collagenase & **VEGF**
- Promote **Apoptosis**
- Lowers **Bcl2 and Ras** oncogene
- **Chemosensitizer**
- **Radiosensitizer**
- Promote 16-OH Estrogen metabolism
- Inhibit **Platelet Aggregation** and **Thrombin Formation**
- Promote **Normal Cell Membrane Functions** and **Receptor Binding**
- Increases **PTEN expression** (tumor suppressor gene)
- Inhibits **Multi Drug Resistance**
- **Inhibits cachexia preserves muscle mass and bone mass** (inhibits proteolysis inducing factor)
- Supports normal **mood regulation**

Multi-modal putative mechanisms of action of DHA and EPA on growth signal transduction.

From:
J Lipids. 2013; 2013: 261247.
Pub online 2013 May 16.
doi: 10.1155/2013/261247



Determination of the Relative Efficacy of Eicosapentaenoic Acid and Docosahexaenoic Acid for Anti-Cancer Effects in Human Breast Cancer Models



Int J Mol Sci. 2017 Dec; 18(12): 2607.

Principle mechanisms of ω-3 polyunsaturated fatty acids in breast cancer.
Exp Ther Med. 2018 Feb; 15(2): 1167–1176.

Mechanism	Key target/gene	(Refs.)
Changes of cell membrane properties	Bcl-2; procaspase-8	(18,37)
Modulation of intracellular signaling pathways	FAK, NF-κB, MAPK, COX-2	(33,82)
Regulation of gene expression	EGFR, Her-2, Erk 1/2, AKT PTEN, Bcl-2, PDCD4, NF-κB	(70,110–112)
Antimetastatic and antiangiogenic activity	EZH2, VEGF, E-cadherin	(36,103)
Regulation of miR expression	miR-21, miR-26a/b, miR19b, miR146b, miR183	(34,42,110)

Principle mechanisms related to pro-carcinogenic effects of ω-6 polyunsaturated fatty acids in breast cancer.
Exp Ther Med. 2018 Feb; 15(2): 1167–1176.

Mechanism	Key/target gene	(Refs.)
Lipid peroxidation, DNA adducts	Redox-cycling of 4-hydroxyestradiol	(21,26,37)
Regulation of gene expression	p21WAF1/CIP1, MAPK, TGF-β, TLR	(21,42)
Antimetastatic and antiangiogenic activity	VEGF, FGF, HIF-α, E-cadherin	(21,41,122)
Regulation of miR expression	MiR19b, miR146b, miR1835p, let-7a,	(42,109)
	miR-23b, miR-27a/b, miR-21, let-7	

Nutr J. 2017; 16: 71. Pub online 2017 Oct 23. doi: 10.1186/s12937-017-0295-9
Elemárcia Martins da Silva Paixão, et al

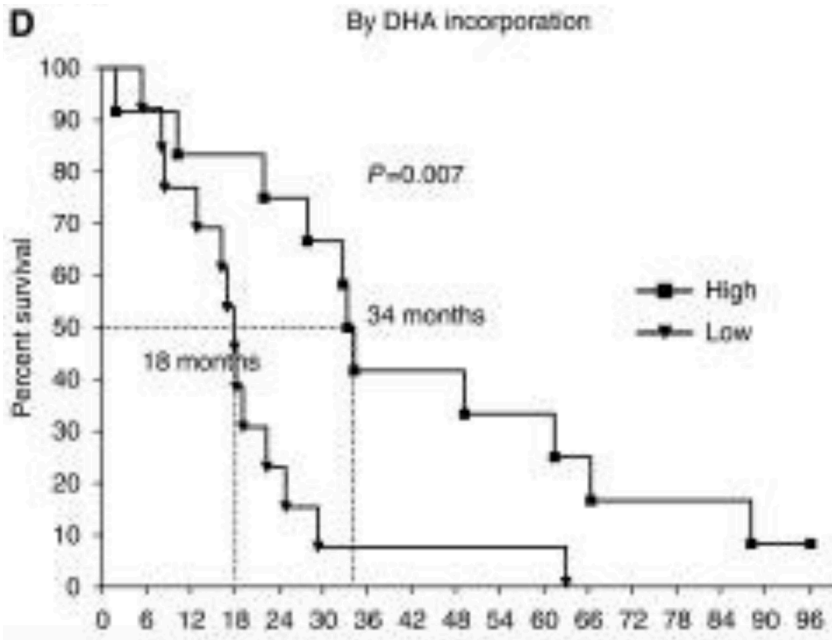
The effects of EPA and DHA enriched fish oil on nutritional and immunological markers of treatment naïve breast cancer patients: a randomized double-blind controlled trial

Metastatic Breast CA patients received 1.8 g/day of oral DHA
40% had three or more metastatic sites. Followed for 8 years

- No DHA-related adverse events were observed
- DHA, can turn malignant mammary tumors from resistant to sensitive to chemo- or radiation therapy
- DHA had a chemo-sensitizing effect on metastases and not on non-tumour tissues,
- **DHA has potential to substantially increase survival in metastatic breast cancer patients treated with chemotherapy.**
- **OS was significantly greater in the High-DHA group with a median survival time of 34 months *vs* 18 months in the Low-DHA group**

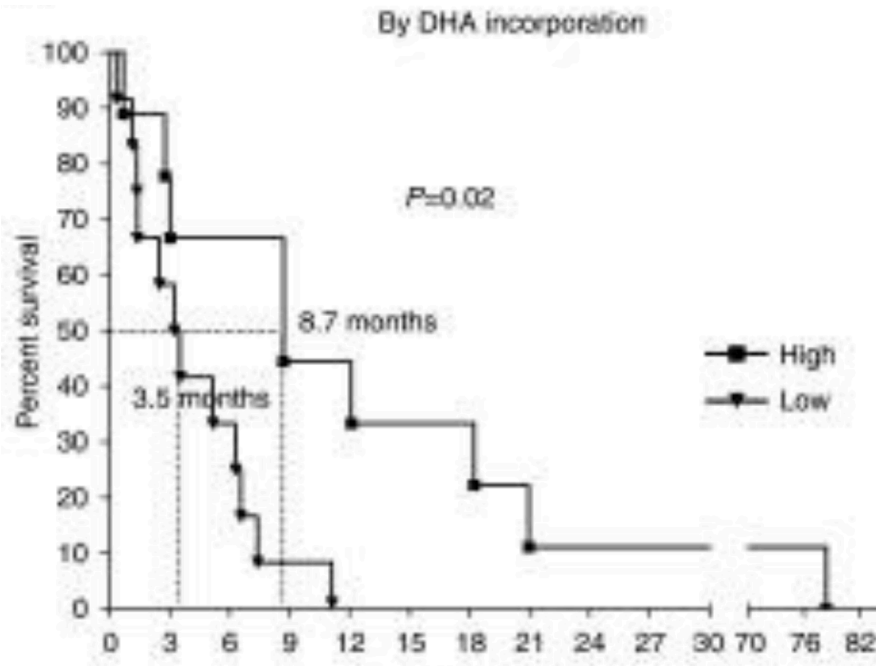
Overall Survival

High DHA
vs
Low DHA



Time To Progression

High DHA
vs
Low DHA



Altered Lipid Tumor Environment and Its Potential Effects on NKT Cell Function in Tumor Immunity

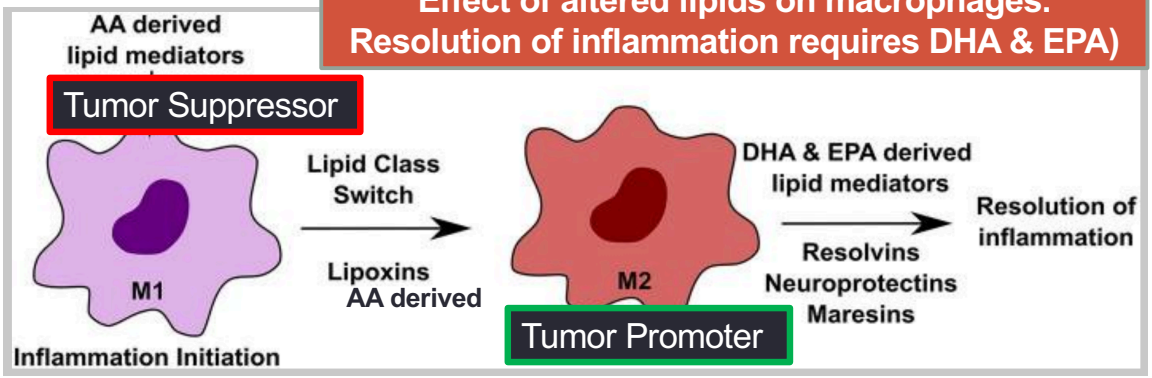
Shweta Tiwary et al Front Immunol. 2019; 10: 2187.10.3389/fimmu.2019.02187

NKT Type I Tumor Promoter M1 Macrophage
NKT Type II: Tumor Suppressor M2 Macrophage

Altered lipid composition effects tumor growth and anti-tumor immunity

Lipid changes can modulate NKT cell dependent immune functions

Effect of altered lipids on macrophages. Resolution of inflammation requires DHA & EPA)

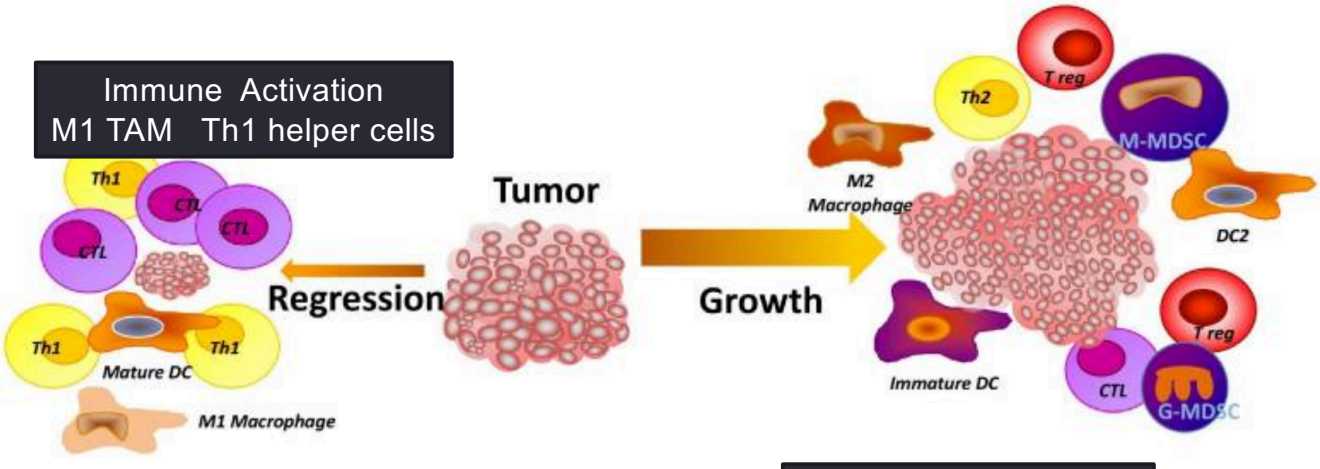


Leukocytes (TAM M1 or M2) that infiltrate a tumor can regulate growth rate, progression, angiogenesis and metastasis.

Tumor regression: Immune activation tumor infiltration by **dendritic cells (DCs), cytotoxic T cells (CTL), Th-1 helper cells** & **M1 Tumor Associated Macrophages**

Tumor growth: immunosuppression and **neoangiogenesis,** infiltration by **myeloid-derived suppressor cells**, (MDSCs), **immature DCs**, pDCs, **Th2, T reg cells & M2 Tumor Associated Macrophages**

Immune regulation and anti-cancer activity by lipid inflammatory mediators



Int Immunopharmacol. 2018 Dec; 65: 580–592.

Br J Cancer. 2009 Dec 15;101(12):1978-85. Bougnoux P et al

Improving outcome of chemotherapy of metastatic breast cancer by DHA PHASE II TRIAL

Tumour cells can be made more sensitive to chemotherapy than non-tumour cell when membrane lipids are enriched with DHA

DHA during chemotherapy was devoid of adverse side effects and can improve the outcome of chemotherapy when highly incorporated.

DHA has a potential to specifically chemo-sensitize tumors.

Asian Pac J Cancer Prev. 2019; 20(3): 911–916. Darwito Darwito et al

Effects of Omega-3 Supplementation on *Ki-67* and *VEGF* Expression Levels and Clinical Outcomes of Locally Advanced Breast Cancer Patients Treated with Neoadjuvant CAF Chemotherapy: A Randomized Controlled Trial Report

- Decreased Ki-67 expression was observed in the intervention group
- Decreased VEGF expression was seen in the intervention group
- Disease-free survival was significantly longer in the intervention group
- Overall survival in the intervention group was significantly longer

Omega-3 fatty acid supplementation improved overall survival and progression-free survival of locally advanced breast cancer treated with CAF neoadjuvant chemotherapy and mastectomy.

TRIAL

BMJ Open. 2019 Sep 17;9(9):e030502.

Comparing docosahexaenoic acid (DHA) concomitant with neoadjuvant chemotherapy versus neoadjuvant chemotherapy alone in the treatment of breast cancer (DHA WIN): protocol of a double-blind, phase II, randomised controlled trial. Newell M et al

Omega Quant Blood Test Omega 3 Index

>**THE OMEGA 3 INDEX** is defined as the amount of EPA plus DHA in red blood cell membranes expressed as the percent of total red blood cell membrane fatty acids

>**O3 FA levels of 8-12% are associated with better overall health**

>Finger Stick at home collection ($49-$99)
>Red Blood Cell Membrane (not Plasma)
https://omegaquant.com/

Bill Harris Ph.D.
>World expert on Omega 3 Fatty Acids
>Listen to Peter Attia Podcast
https://peterattiamd.com/billharris/

- Omega-3 Index
- Omega-6:Omega-3 Ratio
- AA/EPA Ratio
- Trans Fat Index
- Full Fatty Acid Profile
- Personalized dietary recommendations
- Fatty Acid Research Report
- EPA+DHA content of commonly consumed seafood
- Trans fat content of commonly consumed food

SUMMARY 2-6g EPA-DHA daily

- **Lower Inflammation**
  COX 2, LOX5, PGE2, IL1, IL6, TNFa, CRP
- Inhibit **Angiogenesis**
- **Down reg Protein Kinase C**
- **Inhibits collagenase** & **VEGF**
- Promote **Apoptosis**
- Lowers **Bcl2 and Ras** oncogene
- **Chemosensitizer**
- **Radiosensitizer**
- Promote 16-OH Estrogen metabolism
- **Inhibit Platelet Aggregation** and **Thrombin Formation**
- Promote **Normal Cell Membrane Functions** and **Receptor Binding**
- Increases **PTEN expression** (tumor suppressor gene)
- Inhibits **Multi Drug Resistance**
- **Inhibits cachexia preserves muscle mass and bone mass** (inhibits proteolysis inducing factor)
- Supports normal **mood regulation**

Case Study: 64yo M Colon Cancer - Metastasis to Liver Stage 4
Submitted by: Judy Pruzinsky L.Ac
Date Submitted: 11/05/2019

Overview:
Primary Diagnosis:

- 64-year old male.
- **In Feb 2017 was diagnosed with adenocarcinoma in the sigmoid colon.**
- **Sept 2019 metastasis to the liver, Stage 4. Disease Progression**
- Left upper lobe nodule: decreased to 5 mm from 10 mm prior 07/2019 exam.
- 7 mm hypodense splenic lesion, not significantly changed from the prior exam on 02/2017.
- Left vocal cord paresis, suggest ENT referral to exclude any mucosal lesion.
- **High Blood Pressure 150/90** - Zestoretic for b.p. which gets it down to a normal range

Update 01/2020

- **Latest blood test from 12/23/19 - See below**
- He was dismissed from chemo the last couple of weeks because his neutrophils were too low. We are thinking they will be high enough to start back this week.
- Doc wants him to continue for two not three more months at a 10% decrease in dosing.

Recent Lab Test:

- 7/1/19 - WBC 3.6 / RBC 4.18 / Hemoglobin 12.9 / CEA 4.3
- **9/6/19 - CEA 22.4 and glucose low 62? Neutrophil: Lymphocyte now 5.5 / WBC 6.4 / RBC 4.82 / Hemoglobin 14.8 all now normal range.**

Past treatment:

- Partial colectomy 12/27/18 3 months chemotherapy (FOLFOX) Feb-April 2019.

Current Treatment:

- Currently beginning 10/14/19 chemotherapy (FOLFIRI + Avastin) for 3 months duration.
- Every other week: first 1/2 day bolus, next two days infusion at home.

CORE QUESTION:

- **What are the best therapies in addition to conventional (chemotherapy)? (see Case Study document)**
- **He is considering Issels Immuno-Oncology and Hope4Care Treatment Centers.**

He is interested in knowing what your opinion of either treatment center.- see attached

Original Case Study 2017 Notes - Submitted by Judy Pruzinsky, L.Ac.

Brief Summary:
62 yo male - 2/3/17 Colonoscopy Dx - 3cm sigmoid cancerous tumor, stage unknown
CAT scan - no metastasis in the torso
PET scan - to be arranged soon
Labs:

- Low Vit D 24
- High Cholesterol, total 231
- HEAVY METALS ARE BLOOD LEVELS
- High Cadmium
- High Cesium
- V. High Lead
- V. High Mercury

Hx of hypertension and high blood pressure (150/90)
Family Hx - Sister died at age 37 metastasized breast cancer, Sister with breast lumpectomies at age 58
Oncology recommending surgery (removal of 15 inches) right away.
The patient wishes to delay surgery to try other treatments/options.

Current Diet:

- Alkaline foods
- No raw fruits or vegetables
- No sugars except what is in coconut water.
- Added sauerkraut and other fermentations, goat bone broth
- ghee as the only dairy

Current Supplements:

- green tea, antioxidants, curcumin with piperine—c. 1900 mg.
- multi-vitamins, potassium—200 mg.
- Probiophage—15 billion
- vitamin D—10,000 mg.
- salmon oil—4000 mg.

Dr. Chilkov Recommendations: Considerations

- Risk of GI Obstruction? Treatable cancer? Likelihood of progression?
- Most likely surgical resection will give the patient the most protection (only 62 yo) from recurrence, progression, and metastasis over a lifetime. If he wishes to do an AGGRESSIVE integrative approach, then he can exert control over tumor and tumor microenvironment.
- Protein repletion is important: 60 grams per day - for immunity and for the preservation of muscle mass.
- Changing diet, Tai Chi and nutritional supplements alone will not eradicate cancer. Must have a more comprehensive plan, include all factors in the tumor microenvironment and a plan to support tumor control. He can certainly consult with an ND and explore IVC, IV Artesunate, IV Curcumin, IV Mistletoe

- Adjuvant chemotherapy is often a starting place to reduce the size of the tumor so that a smaller surgery can be performed, and so a second oncology opinion may be warranted.

Dr. Chilkov Recommendations:

Cyto Toxic

- **High Dose IV Vitamin C**
- **Hyperthermia + Mistletoe Therapy concurrently**

- **Natura Health Products Phyto Cyto 60 drops 3x/day**
- **Clinical Synergy Artemax (artemisinin) 2 caps 3x/day every other week**
- **Clinical Synergy Pure Honokiol 1 am 1 pm 2 bedtime**
- **High dose melatonin 80mg per day 20 mg B L D bedtime (Vital Nutrients and Pure Encapsulations make 20 mg caps)**
- **Clinical Synergy Pectasol C Professional 7.5 gram 2x/day 30 min away from food, supplements, nutrients, herbs**

Oral Supplements-Cancer Terrain

- **Increase Dose Omega 3 Fatty Acids 2 grams 2x/day Triglyceride form**
- **Euromedica BosPro (Boswellia) 500mg 2/2x/day (2 g daily)**
- **DFH Curcumevail 2/2x/day (4 g daily)**
- **Clinical Synergy Mushroom Immune Max 2 scoops daily**

Health Concerns Marrow Plus 3/3x/day

Custom Tonic
Tumor Control inflammation Control Immune Support
2 teaspoons daily
shake well Dilute in Ginger Tea or water
take with food in stomach

250 ml	500ml	
20	40	Astragalus and Ganoderma Formula
30	60	Pinellia and Magnolia Formula
25	50	Scutellaria Baicalensis Huang qin
20	40	Oldenlandia Bai Hua She She Cao
25	50	Milk Thistle Silibium marianum
25	50	Polygonatum Yu Zhu
20	40	Red Ginseng Extract Panax ginseng Hong Ren Shen
12.5	25	Taxus brevifolia tips
12.5	25	Catharanthus
15	30	Camptotheca
10	20	Camelia_Green Tea Cha Ye

15	**30**	**Feverfew Tanacetum parthenium**
10	**20**	**Ginger root extract dried Gan Jiang**
10	**20**	**Tangerine exract Chen Pi**

- **Hope4Cancer clinic in Mexico (Antonio Jimenez MD, Director, Dr. V=Veronique Desaulniers, ND) not recommended**
- **Issels Immuno-oncology**
- **Hospice Care in the US can be excellent (Zen Hospice in San Francisco)**

Clinics outside the US

- **Sanoviv.com Baja California, Mexico (protocols developed by Paul Andersen ND)**
- **Chemothermia.com Dr. Abdul Slocum MD Istanbul, Turkey**
- **Ralf Kleef MD, Vienna Austria http://www.dr-kleef.at/en/contact**
- **International Immunology Foundation, Dr. M Ridgon Lentz MD Germany https://www.int-imm-foundation.com/en/home.html**

2019 CTRC-AACR San Antonio Breast Cancer Symposium*

DOWNLOAD ALL

December 10-14, 2019; San Antonio, Texas

Review slide sets and analyses of key data from the 2019 Breast Cancer meeting.

Breast Cancer Capsule Summary Slide Sets

HER2CLIMB: Phase III Study of Tucatinib Plus Trastuzumab and Capecitabine in Previously Treated HER2-Positive Metastatic Breast Cancer
VIEW SLIDESET
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SOPHIA: Second Interim OS Analysis of Margetuximab + CT vs Trastuzumab + CT for HER2+ MBC After Previous HER2 Therapy
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DESTINY-Breast01: Phase II Study of Trastuzumab Deruxtecan (DS-8201a) in HER2+ Advanced Breast Cancer Previously Treated With T-DM1
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APHINITY: Interim OS Analysis of Adjuvant CT Plus Trastuzumab With vs Without Pertuzumab for Patients With HER2+ EBC
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Phase II ATEMPT: Analysis of Adjuvant Trastuzumab Emtansine (T-DM1) vs Paclitaxel Plus Trastuzumab in Patients With Stage I HER2-Positive Breast Cancer
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PEARL: Palbociclib + Endocrine Therapy vs Capecitabine in Postmenopausal Women With HR+/HER- MBC and Previous AI Therapy
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KEYNOTE-522 Study of Neoadjuvant Pembrolizumab vs Placebo in Combination With Chemotherapy for Early-Stage TNBC: Subgroup Analysis of pCR
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NeoTRIPaPDL1 Michelangelo: Neoadjuvant Chemotherapy ± Atezolizumab in Early, High-Risk and Locally Advanced TNBC

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plasmaMATCH (CRUK/15/010): Evaluation of Circulating Tumor DNA Testing to Direct Targeted Therapies in Patients with Advanced Breast Cancer

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NSABP B-42 10-Yr Follow-up: Extended Adjuvant Letrozole After Previous Adjuvant AI Therapy in Postmenopausal Women With HR+ Breast Cancer

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Oral Paclitaxel With P-Glycoprotein Pump Inhibitor Encequidar vs Intravenous Paclitaxel in Metastatic Breast Cancer

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INFORM: Randomized Phase II Study of Neoadjuvant Cisplatin vs AC in Newly Diagnosed Breast Cancer With Germline *BRCA* Mutations

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Phase I Study to Assess the Effect of Trastuzumab Deruxtecan on QTc Interval and Pharmacokinetics in HER2-Expressing Metastatic or Unresectable Breast Cancer

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plasmaMATCH Cohort B: Neratinib ± Fulvestrant for Patients With *HER2* Mutation–Positive Advanced Breast Cancer as Identified by ctDNA Analysis

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Impact of Neratinib on CNS Metastases in Patients With HER2-Positive Metastatic Breast Cancer: Analysis of Data From Phase II/III Trials

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CONTROL: Phase II Trial of Antidiarrheal Prophylaxis or Neratinib Dose Escalation for Neratinib-Associated Diarrhea in Patients With HER2+ Early BC

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Estrogen Decreases Breast Cancer Incidence in Postmenopausal Women, Estrogen Plus Progestin Has Opposite Effect

Jason Harris

Rowan T. Chlebowski, MD, PhD

Updated findings from Women's Health Initiative (WHI) studies involving more than 27,000 patients demonstrated that estrogen alone as menopausal hormone therapy (MHT) decreased breast cancer incidence in postmenopausal women. In contrast, estrogen plus progestin was associated with increased incidence and death.[1]

In both cases, the result continued after discontinuation said Rowan T. Chlebowski, MD, PhD, chief of the Division of Medical Oncology and Hematology at Harbor-UCLA Medical Center. He presented the data during a press briefing at the San Antonio Breast Cancer Symposium.

"Use of estrogen alone and use of estrogen plus progestin have opposite effects on breast cancers," he said. "[Estrogen] alone after use for 7.2 years, now with 19.2 years follow-up, resulted in a 23% reduction in breast cancer use, which was statistically significant. The [estrogen plus progestin] use ended up increasing breast cancer by 29%."

Postmenopausal women aged 50 to 79 years with no prior breast cancer were recruited into 1 of 2 randomized clinical trials at 40 U.S. centers from 1993 to 1998. Those with an intact uterus received estrogen plus progestin (n = 8506) or placebo (n = 8102) for a median of 5.6 years. Women who had undergone hysterectomy received estrogen alone (n = 5310) or placebo (n = 5429) for a median of 7.2 years.

Investigators observed 231 breast cancers in women assigned to estrogen alone compared with 289 for those assigned to placebo (HR, 0.77; 95% CI, 0.65-0.92; $P = .005$). Estrogen alone patients also had a reduced risk for disease-specific death (HR, 0.56; 95% CI, 0.34-0.92; $P = .02$) and for deaths after breast cancer (HR, 0.75; 95% CI, 0.56-1.01; $P = .06$).

There were 572 breast cancers diagnosed among women among women assigned to the combination compared with 431 for those assigned to placebo (HR, 1.29; 95% CI, 1.14-1.47; $P < .0001$). These patients also had an increased risk for disease-specific death (HR, 1.45; 95% CI; $P = .06$) and deaths after breast cancer (HR, 1.29; 95% CI, 1.02-1.63; $P = .03$).

Chlebowski added that the reduced risk for incidence associated with estrogen continued after the study period (HR, 0.83; 95% CI, 0.57-1.20). Similarly, the increased risk continued with estrogen/progestin (HR, 1.30; 95%

CI, 0.99-1.70). In both cases, the effect continued over decades.

“The use of the drug was only for 5.6 years, but you can see that the increased [risk] is continuing for up to 20 years,” he said. “A woman takes estrogen plus progestin for 5 year and she is exposed to a 20 year increased breast cancer risk. [The risk] doesn't seem to be leveling off, so one can speculate it will be a lifetime risk for short-term use.”

These data directly contradict findings published this year by the Collaborative Group on Hormonal Factors in Breast Cancer and the Million Women Study.

The Collaborative Group conducted a retrospective analysis of 58 trials involving 108,647 postmenopausal women developed breast cancer at mean age of 65 years. Half the patients had used MHT. Mean MHT duration was 10 years in current users and 7 years in past users.

Among current users, there was a clear risk for breast cancer 5 to 14 years after treatment for estrogen/progestin (RR, 2.08; 95% CI, 2.02-2.15) and estrogen alone (RR, 1.33; 95% CI, 1.28-1.37).[2]

The Million Women Study included 907,162 postmenopausal women who were breast cancer-free at recruitment. Among them, about a third were current users of MHT, one-sixth were past users, and half were never-users.

Women who were on either estrogen alone or estrogen/progestin preparations at recruitment had significant excess breast cancer mortality risks (P <.0001). While there was no increased mortality found for patients who used MHT for about 5 years, those on therapy for roughly 8 years had a significant excess breast cancer mortality over the next 20 years (HR, 1.24; 95% CI, 1.12-1.38; P = .0005).[3]

Chlebowski said patients and physicians considering hormone therapy would have to weigh the data, but based on these findings from his group, estrogen and estrogen/progestin clearly do not have the same effect.

“Women who are considering estrogen alone should know that it's safer and there may be a breast cancer benefit associated with its use,” he said. “Women considering estrogen plus progestin have a little bit more difficult dilemma because they have to be willing to accept the maybe 20 year, and maybe lifetime, increased breast cancer risk.”

References

1. Chlebowski RT, Anderson GL, Aragaki AK, et al. Long-term follow-up shows estrogen alone and estrogen plus progestin have opposite effects on breast cancer incidence in postmenopausal women. Presented at:

the 2019 San Antonio Breast Cancer Symposium; December 10-14, 2019; San Antonio, Tx. Abstract GS5-00.

2. Collaborative Group on Hormonal Factors in Breast Cancer. Type and timing of menopausal hormone therapy and breast cancer risk: individual participant meta-analysis of the worldwide epidemiological evidence. *Lancet*. 2019;394(10204):1159-1168. doi: 10.1016/S0140-6736(19)31709-X.
3. Beral V, Peto R, Pirie K2, Reeves G, et al. Menopausal hormone therapy and 20-year breast cancer mortality. *Lancet*. 2019;394(10204):1139. doi: 10.1016/S0140-6736(19)32033-1.

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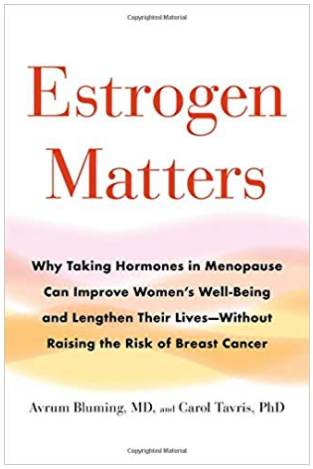

BOOK: Estrogen Matters. https://estrogenmatters.com/
Amazon US Link

About the Authors

AVRUM BLUMING, MD

Avrum Bluming received his MD from the Columbia College of Physicians and Surgeons. He spent four years as a senior investigator for the National Cancer Institute and for two of those years was director of the Lymphoma Treatment Center in Kampala, Uganda. He organized the first study of lumpectomy for the treatment of breast cancer in Southern California in 1978, and for more than two decades he has been studying the benefits and risks of hormone replacement therapy administered to women with a history of breast cancer. Dr. Bluming has served as a clinical professor of medicine at USC and has been an invited speaker at the Royal College of Physicians in London and the Pasteur Institute in Paris. He was elected to mastership in the American College of Physicians, an honor accorded to only five hundred of the over one hundred thousand board-certified internists in this country.

CAROL TAVRIS, PhD

Carol Tavris received her PhD in social psychology from the University of Michigan. Her books include *Mistakes Were Made (But Not by Me),* with Elliot Aronson; *Anger: The Misunderstood Emotion*, and *The Mismeasure of Woman.* She has written articles, op-eds, and book reviews on topics in psychological science for a wide array of publications — including the *Los Angeles Times,* the *New York Times Book Review,* the *Wall Street Journal,* and the *TLS* — and a column, "The Gadfly," for Skeptic magazine. She is a fellow of the Association for Psychological Science and has received numerous awards for her efforts to promote gender equality, science, and skepticism.

PETER ATTIA PODCAST #42 – ESTROGEN MATTERS

https://peterattiamd.com/caroltavris-avrumbluming/

Avrum Bluming, M.D. and Carol Tavris, Ph.D.: Controversial topic affecting all women—the role of hormone replacement therapy through menopause and beyond—the compelling case for long-term HRT and dispelling the myth that it causes breast cancer

In this episode, Avrum Bluming, hematologist, medical oncologist, and emeritus clinical professor at USC, and Carol Tavris, social psychologist and author of *Mistakes Were Made (But Not By Me)*, discuss their collaboration on their recent book, *Estrogen Matters*. Their book takes on the very polarizing and confusing topic of hormone replacement therapy for women suffering with symptoms of menopause. In many ways, the story and history of HRT is in striking parallel to the bad science that led up to the dietary guidelines being set forth in 1980. Carol and Avrum make a compelling case that most women benefit greatly from being on postmenopausal hormone replacement therapy, and can do so without increasing their risk of breast cancer. We also cover the history of HRT, the impact of the Women's Health Initiative, and take a deep dive into each of the clinical conditions for which HRT should be considered, such as cardiovascular disease and neurodegenerative disease, and osteoporosis, to name a few

International Journal of
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Review

The Role of the Estrogen Pathway in the Tumor Microenvironment

Natalie J Rothenberger [1], Ashwin Somasundaram [1,2] and Laura P. Stabile [3,4,*]

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Abstract: Estrogen receptors are broadly expressed in many cell types involved in the innate and adaptive immune responses, and differentially regulate the production of cytokines. While both genomic and non-genomic tumor cell promoting mechanisms of estrogen signaling are well characterized in multiple carcinomas including breast, ovarian, and lung, recent investigations have identified a potential immune regulatory role of estrogens in the tumor microenvironment. Tumor immune tolerance is a well-established mediator of oncogenesis, with increasing evidence indicating the importance of the immune response in tumor progression. Immune-based therapies such as antibodies that block checkpoint signals have emerged as exciting therapeutic approaches for cancer treatment, offering durable remissions and prolonged survival. However, only a subset of patients demonstrate clinical response to these agents, prompting efforts to elucidate additional immunosuppressive mechanisms within the tumor microenvironment. Evidence drawn from multiple cancer types, including carcinomas traditionally classified as non-immunogenic, implicate estrogen as a potential mediator of immunosuppression through modulation of protumor responses independent of direct activity on tumor cells. Herein, we review the interplay between estrogen and the tumor microenvironment and the clinical implications of endocrine therapy as a novel treatment strategy within immuno-oncology.

Keywords: estrogen; cancer; tumor microenvironment; immunotherapy; immunosuppression

1. Introduction

Estrogens are pleiotropic steroids that play a regulatory role in a myriad of physiological processes from reproduction to lipid metabolism [1]. Biosynthetically converted from precursor androgens by the enzyme aromatase (CYP19A1), estrogens exert both genomic and non-genomic biological effects mediated by interactions with one of two cognate receptors, estrogen receptor α (ERα) or estrogen receptor β (ERβ). Albeit encoded by separate genes, both ER isoforms exhibit similar functional and structural organization [1]. Displaying high sequence homology within the DNA and ligand binding domains, both receptors interact similarly with endogenous estrogens, mainly 17β-estradiol (E2) [2,3]. In addition to mediating biological mechanisms involved in homeostasis, E2 also plays a role in the development and malignant progression of multiple cancers. The oncogenic role of estrogens is well characterized in both classical and nonclassical hormone-sensitive carcinomas including breast, prostate, endometrial, ovarian, colon, and lung [4]. ERs are located in both the nucleus and the cytoplasm of tumor cells enabling tumor-promoting transcriptional regulation of genes involved in cell survival and proliferation [5,6], and non-genomic crosstalk with growth factor pathways, including epidermal growth factor (EGF), insulin growth factor (IGF), and fibroblast growth factor

(FGF) [7–9]. Due to these tumorigenic mechanisms, therapies that interfere with E2 signaling, such as selective estrogen receptor modulators or degraders (SERMs or SERDs) and aromatase inhibitors (AIs), have been developed and clinically implemented for the treatment of ER-positive breast cancer. While agents that target the estrogen pathway have been seminal in reducing breast cancer mortality over the past three decades [10], most studies in breast cancer and other cancer types have focused strictly on tumoral ER expression and signaling.

Along with tumor cells, non-cancerous cells comprising the tumor microenvironment (TME) are now recognized as critical mediators of tumor progression. Mounting evidence suggests that in addition to intracellular mechanisms such as mutational load and neoantigen presentation, interplay between cancer cells, stromal cells, immune cells, and extracellular molecules within the TME profoundly influence anti-tumor immunity and immunotherapeutic response [11–14]. The notion that enhancing tumor immunogenicity and inhibiting immunosuppressive mediators can functionally suppress progression of malignant tumors has led to the development of promising immunotherapeutic strategies. However, the clinical utility of current immunotherapies remains limited due to marginal response rates and acquired resistance mechanisms [15–17]. Therefore, greater elucidation of targetable cellular machinery involved in tumor immune evasion is necessary to improve the clinical benefit of immunotherapies.

The numerous biological effects of the E2 pathway are facilitated by distinct ER isoform expression found not only on tumor cells, but also on most immune cell types [18–21]. The impact of E2 in autoimmune pathogenesis remains heavily investigated, with reports of paradoxical and disease-dependent effects. The influence of E2 in autoimmunity is potentially concentration-dependent, and immune cell-specific. Several reviews detail E2-mediated immune responses, including transcriptional regulation of immune mediating genes possessing ERE sequences and regulation of lymphopoiesis and immune cell differentiation [22–25]. Given the prevalence of E2 modulation in both innate and adaptive immune responses, along with its evident role in tumor progression, there exist several implications for immunomodulatory effects of E2 within the TME. Herein, we will discuss findings within current literature evaluating the protumoral impact of E2 on the TME and the implications of targeting the E2 pathway in cancer to promote an anti-tumor immune response.

2. Estrogen Receptor and Aromatase Expression in Tumor Cells: Correlations with Clinical Outcome

Tumoral ER expression is reported in nearly 30 different types of cancer, predominately in hormone-sensitive tumors such as breast, ovarian, endometrial, and prostate [26,27]. Studies comparing clinicopathological characteristics with ER protein expression (typically evaluated by immunohistochemistry (IHC)) in tumor tissue show differential relation to disease prognosis based on cellular localization and cancer type. In breast cancer, while predominately expressed in the nucleus, ERα protein expression in either the nucleus and/or cytoplasm correlates with features of advanced disease, including larger tumor size and lymph node metastasis [28]. However, ERα-positive breast cancer patients exhibit improved overall survival (OS) compared to ERα-negative patients, likely owing to the clinical benefit of adjuvant endocrine therapies for ERα-positive patients [18,29]. The clinical relevance of ERβ expression in breast cancer remains controversial largely due to challenges associated with ERβ splice variants and post-translational modifications, as well as the lack of a clinically standardized ERβ antibody [19,30,31]. As an integral enzyme in estrogen production, intratumoral aromatase has also been evaluated in breast cancer. While one study reported an association between aromatase activity and poor prognosis, others have failed to correlate aromatase activity or protein expression with clinical outcomes, suggesting that paracrine sources of estrogen may be of greater significance in hormone-dependent breast cancers [32–35]. In contrast to breast cancer, non-small cell lung cancer (NSCLC) ERα protein expression is more commonly expressed in the cytoplasm and is a negative prognostic marker [36,37]. Similarly, elevated cytoplasmic ERβ protein expression in NSCLC is associated with poorer OS [38], potentially indicative of the predominance of non-genomic

mechanisms in NSCLC. Alternatively, nuclear ERβ expression in NSCLC correlates favorably with OS in some studies and negatively in others (reviewed in [39]). Tumoral aromatase protein expression and activity is also reported in NSCLC, with elevated expression identified as a predictor of poorer survival in women with early stage disease [40]. In advanced ovarian cancer tumors, while aromatase activity and ERβ mRNA expression do not correlate with any clinical outcomes [41,42], a recent meta-analysis revealed ERα protein expression was associated with improved OS [43]. Finally, while clinical correlations with aromatase have yet to be evaluated, both ERα and ERβ expression are associated with improved OS in endometrial cancer [44]. These clinical correlations, combined with mounting preclinical studies, indicate an intricate and pervasive protumoral role for hormonal signaling in multiple cancers, providing rationale for further investigation of ER expression and oncogenic cellular modulation.

3. Estrogen Receptor and Aromatase Expression and Estrogen-Mediated Effects in the Tumor Microenvironment

In addition to neoplastic cells, ERs and aromatase are also expressed on stromal and immune cells within the TME (Table 1). Numerous studies over the past decade have demonstrated that interactions between tumor cells and surrounding recruited stromal cells are integral in disrupting homeostasis and potentiating tumorigenesis (reviewed in [14,45]). Albeit highly heterogeneous within and across tumor types, regularly observed cellular components of the TME include: cancer associated fibroblasts (CAFs), tumor associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs), immune T and B cells, natural killer (NK) cells, and endothelial cells [14]. ER and aromatase expression in TME stromal and immune cells suggest a potential immunomodulatory role of ER signaling in cancer biology as detailed by cell type below.

Table 1. Estrogen receptor (ER) and aromatase expression in stromal and immune cells in the tumor microenvironment.

TME Cell Type	Cancer Type	Human Expression	Murine Expression	Method of Evaluation	Reference
Stromal	Breast	Aromatase	ERα	PCR, IHC	[46,47]
	Melanoma		ERα	IHC	[47]
	Lung		ERα	IHC	[47]
	Endometrial	Aromatase		IHC	[48]
CAF	Breast	ERα		PCR	[49]
	Prostate	ERα, ERβ		IHC	[50,51]
	Endometrial	ERα, ERβ		PCR	[52]
	Ovarian	ERα		IHC	[53]
TAM	Ovarian	ERα, ERβ		IF, IHC	[54]
	Breast	Aromatase		IHC, PCR	[55]
	Lung	Aromatase	Aromatase	IHC	[56,57]
MDSC	Ovarian	ERα	ERα	PCR, Western	[53]

Studies were identified by PubMed searches using keywords: ERα, ERβ, aromatase, stromal, CAF, TAM, MDSC, expression, cancer. CAF: cancer associated fibroblast; TAM: tumor associated macrophage; MDSC: myeloid derived suppressor cell; IHC: immunohistochemistry; PCR: polymerase chain reaction; IF: immunofluorescence; Western: western blotting analysis.

3.1. Stromal Cells

It has become increasingly evident that tumor progression is reliant not only on tumor cells present in malignant tissue, but also the distinctive stromal cells recruited to the TME that signal among the tumor cells and each other. An in vivo murine model evaluating tumor cell-independent mechanisms of ER signaling within the TME has identified ERα expression and modulation in stromal cell types. In ovariectomized syngeneic mice transplanted with ER-negative melanoma, breast, or lung cancer cells,

E2 treatment significantly enhanced tumor growth of each cell type compared to untreated controls via interactions with stromal ERα [47]. Further, E2-stimulated tumor growth was increased when evaluated in immunocompromised mice, suggesting this effect may be more reliant on the innate immune response [47]. In addition to tumor growth, E2 also enhanced angiogenesis by increasing blood vessel density 2.1-fold in E2-treated mice compared to controls, an effect reliant on host ERα expression [47]. Peritumoral aromatase expression is also reported in endometrial cancer stromal cells, correlating with advanced disease and poor OS [48,58]. Aromatase is also observed in breast cancer stromal adipocytes of obese postmenopausal women, and several studies have identified mechanistic associations between obesity, inflammation, elevated aromatase, and breast cancer development [46,59,60].

3.2. Cancer Associated Fibroblasts

CAFs are among the most prevalent stromal cell type within the TME and act as a paracrine source of chemokines and soluble growth factors that activate signaling pathways involved in tumor cell survival, invasion, and metastasis [61]. A study using nuclear receptor arrays to compare gene expression profiles between normal human breast adipose fibroblasts and primary CAFs from malignant human breast tissue, observed ERα expression in fibroblasts from primary breast cancer tissue [49]. Despite similar levels of ERα expression observed in both cancerous and normal fibroblasts, the E2 responsive gene, liver receptor homolog-1 (*LRH-1*) was upregulated in CAFs compared to normal fibroblasts [49]. *LRH-1* is also an estrogen response gene and a direct transcriptional regulator of the aromatase encoding gene *CYP19A1* [62–64]. Aromatase is found to be co-expressed in breast cancers with LRH-1, suggesting a paracrine mechanism of E2 synthesis and ER-mediated oncogenesis in the breast cancer TME [65]. Endometrial CAFs also express both ERs and can promote tumor cell proliferation when co-cultured with human endometrial tumor cells [52]. Endometrial CAFs induce in vitro tumor cell proliferation in part through activation of the phosphatidylinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling networks, which are well-known ER-mediated pathways in breast and lung cancer [52,66–68].

ERα is also expressed in prostate CAFs, however, clinical implications remain unclear with some reports identifying CAF ERα and ERβ expression as a marker of clinically advanced disease [50], while other reports suggest ERα expressing CAFs provide a protective effect against tumor cell invasion and macrophage infiltration [69,70]. In the latter studies, stromal ERα reduced both murine and human prostate cancer cell invasion using an in vitro co-culture system, and reduced lymph node metastasis of orthotopically implanted human prostate cancer cells in mice [70]. Mechanistically, ERα-positive CAFs abated migratory behavior of adjacent prostate tumor cells through reduced expression of C–C motif chemokine ligand 5 (CCL5) and IL-6 chemokines, both of which have identified roles in tumor immune recruitment, inflammation, and activation of growth factor signaling [71,72].

3.3. Tumor Associated Macrophages

Macrophages critically regulate innate immune responses under normal physiological conditions; however, several studies have shown that TAMs can promote tumor cell proliferation, an inflammatory microenvironment, and metastasis [73,74]. Macrophage immune responses are tissue-specific and dependent on polarization by different cytokines within the local microenvironment [75]. Fully polarized M1 macrophages produce proinflammatory cytokines including IFNγ, interleukin 12 (IL-12), and TNFα, that contribute to tumor rejection and antigen presentation [75]. Alternatively, macrophages exhibiting an M2 phenotype produce type-2 cytokines including interleukins 4,5,6, and 10 [75], all of which are identified promoters of tumor progression through enhanced tumor cell growth and immune evasion [76]. Infiltrating TAMs observed in malignant tumors display an M2 phenotype, representing another potential protumoral therapeutic target within the TME. TAM infiltration is observed in a wide-range of cancer types and correlates with poor prognosis [77]. For example, TAM infiltration is an independent poor prognostic predictor for ovarian

cancer, with higher infiltration observed in cancerous specimens compared to benign lesions, and density-dependent associations with five-year survival rates [78].

Co-localized expression of both ERα and ERβ is reported in human high grade serous ovarian cancer (HGSOC) TAMs, and premenopausal patients show elevated TAM infiltration compared to postmenopausal women, with highest overall TAM density observed in ERα-positive tumors [54]. Conversely, while TAM infiltration has been associated with poor prognosis in both hormone receptor positive and negative breast cancers, TAM enrichment and proliferation is more commonly observed in hormone receptor negative breast tumors [79,80]. However, M1 versus M2 polarization was not evaluated in these studies. Furthermore, a separate IHC analysis of breast cancer specimens revealed aromatase expression in TAMs, enabling local E2 production within the TME and enhanced ER-positive breast tumor cell proliferation [55]. Aromatase is also expressed in TAMs from NSCLC patient tumors [56], and both aromatase and ERβ are observed in infiltrating macrophages of preneoplasias in tobacco carcinogen-induced murine lung tumors [57].

While a paucity of data exists regarding ER expression in TAMs of several cancer types, there is evidence that E2 can induce M2 polarization and tumor infiltration. Using a polyomavirus middle T (PyMT) ER-positive breast cancer murine model, E2 increased tumoral M2 TAM infiltration, while untreated controls alternatively exhibited M1 TAM infiltration [81]. Furthermore, E2 enhanced M2 macrophage secretion of vascular endothelial growth factor (VEGF), an identified mediator of M2 macrophage recruitment [81,82]. E2 has been shown to also upregulate VEGF expression and pulmonary macrophage content in the lungs of mice exposed to a tobacco carcinogen [83]. Evaluation of E2-mediated tumor growth in a HGSOC murine model showed that E2 not only enhanced the growth of ER-negative xenografts, but also increased M2 TAM infiltration compared to untreated ovariectomized mice [54]. In addition to reports of E2-mediated TAM infiltration, a tissue microarray of patient samples coupled with in vitro analysis revealed endometrial M2 TAMs mediate ER activation through epigenetic upregulation of ERα by secreted interleukin-17A (IL-17A), increasing E2-driven malignant endometrial cell proliferation [84]. Taken together, these studies suggest a potential positive feedback mechanism between the estrogen pathway and M2 TAM infiltration in certain cancers. Targeting this interaction may therefore provide therapeutic benefit as recently demonstrated in a lung cancer xenograft model using the phytoestrogen SERM resveratrol [85]. The study showed resveratrol treatment significantly suppressed tumor growth by inhibiting M2 polarization of TAMs and decreasing activation of signal transducer and activator of transcription 3 (STAT3) signaling [85].

3.4. Myeloid Derived Supressor Cells

MDSCs are another myeloid cell present in the TME known to disrupt immune surveillance and promote tumor development [86]. ERα expression was also recently identified by IHC and confirmed by PCR and immunoblotting in MDSCs isolated from the tumor, bone marrow, and peripheral blood of human ovarian cancer patients [53]. Using an E2-insensitive syngeneic ovarian cancer model, ovariectomized mice exhibited improved survival compared to non-ovariectomized mice following tumor challenge, while E2 supplementation accelerated tumor progression and reversed the protective effect found in estrogen-depleted mice [53]. Notably, this effect was only observed in immunocompetent mice with no survival benefit of ovariectomy observed in tumor-bearing T-cell deficient immunocompromised mice, suggesting the antitumor effects of E2 deficiency is reliant on functional adaptive immunity [53]. E2-treated mice also exhibited significantly fewer helper and cytotoxic T cells, but also exhibited significantly elevated recruitment of MDSCs in both the spleen and tumor beds [53]. Specifically, the immunosuppressive activity of granulocytic MDSCs was increased in this model. ER-dependence of MDSC expansion was demonstrated using the ERα antagonist methylpiperidino pyrazole (MPP) to inhibit MDSC proliferation in vitro [53]. In the peritoneal cavity of ovarian tumor-bearing mice, E2 treatment increased activation of STAT3 signaling, a regulator of myeloid differentiation and development [87], through transcriptional upregulation of JAK2 and SRC

activity [53]. Similar findings were also observed in syngeneic lung and breast cancer murine models and the E2-stimulated tumor growth was abrogated by MDSC depletion using anti-Gr1 antibodies [53].

3.5. Tumor Infiltrating Lymphocytes (TIL)

Lymphocyte composition of the TME vastly differs based on cancer type and immune infiltrates exhibit opposing properties promoting tumor progression and antitumor immunity depending on the primary tumor [88]. For example, $CD4^+$ T cell polarization has been identified as a mediator of tumor immune surveillance. T helper 1 (Th1) T cell responses are associated with tumor suppression and upregulation of IFNγ and IL-12, while T helper 2 (Th2) responses are reliant of IL-4 production and exhibit protumor activity [89,90]. Interestingly, several murine and human studies report elevated E2 induces increased Th2 responses and upregulate IL-4 production [22,25]. A recent study utilizing an in silico machine learning based approach, identified increased immune infiltrate including Th1 T cells, B cells, and cytotoxic T lymphocytes (CTLs) in ER-negative breast tumors relative to ER-positive breast tumors [91]. This study observed an inverse correlation between ER activity and immune infiltration of each of these cells in breast cancer tissues, confirming previous reports that increased TIL, specifically $CD8^+$ T cells, in ER-negative tumors significantly correlates with improved OS [91,92]. Furthermore, a post-hoc analysis of gene expression in ER-positive breast cancer patients showed that treatment with the AI letrozole increased the infiltration of B cell and T helper lymphocyte subsets at early and late time points following treatment initiation [91].

3.5.1. Cytotoxic T Cells and Natural Killer Cells

Granule-mediated exocytosis is one pathway by which CTLs and NK cells initiate apoptosis to eliminate pathogenic and tumor cells [93]. Serine proteases such as granzyme B are deposited into the target cells to initiate caspase-dependent apoptosis [94]. Jiang et.al. cultured ERα expressing human liver carcinoma cells with E2 and showed E2 treatment upregulated expression of the granzyme B inhibitor, proteinase inhibitor-9 (PI-9), and protected the cells against NK and CTL-induced apoptosis in DNA fragmentation assays [95]. E2-induced PI-9 expression was also observed in ERα-positive MCF7 breast cancer cells, again protecting cells against NK elimination, while PI-9 knockdown blocked E2's protective effect against NK granule-mediated apoptosis [96]. These studies suggest that E2 enhances immunosuppression through inhibition of NK and CTL-mediated tumor cell elimination.

3.5.2. Regulatory T Cells

T cell activation and effector differentiation is an essential part of the adaptive immune response. FoxP3 expressing Tregs are integral in coordinating suppression of anti-tumor immune responses, secreting immunosuppressive cytokines and inhibiting responder T cell expansion [97]. Physiological doses of E2 administered to immunocompetent ovariectomized female mice have been shown to enhance $CD4^+CD25^+$ Treg expansion and upregulate Foxp3 expression in multiple tissues [98]. Furthermore, fluorescence-activated cell sorting (FACs) assays revealed ERα expressing $CD4^+CD25^-$ cells incubated with E2 acquire CD25 expression [98]. E2 transformed $CD4^+CD25^+$ T cells exhibited an immunosuppressive Treg phenotype, significantly inhibiting T cell proliferation in an in vitro mixed lymphocyte reaction [98]. Additional studies have reported E2-stimulated Foxp3 expression in murine Tregs, which is of importance considering that Foxp3 is essential for Treg functionality, and tumoral aggregation of $FoxP3^+$ Tregs in patients is a predictor of poor prognosis in multiple cancers [99–101]. For example, in early-stage NSCLC patients, nuclear ERα expression was found independently associated with increased risk of recurrence and $FoxP3^+$ lymphocyte infiltrate [102]. Further, a recent meta-analysis reported $FoxP3^+$ Treg infiltration significantly correlated with poorer OS in ER-positive breast cancer patients, but improved survival rates in ER-negative patients [103]. In addition, evaluation of ERα-positive breast tumors from patients treated with letrozole showed a significant reduction of $FoxP3^+$ Tregs post-treatment [104].

Moreover, Tregs isolated from mice treated with E2 displayed enhanced suppression and increased intracellular expression of the immune checkpoint protein programmed death-1 (PD-1), while ERα and ERβ knockout reduced Treg suppression and PD-1 expression [105]. Of note, E2 treatment also stimulates in vitro expression of the PD-1 ligand (PD-L1) on ERα-positive endometrial and breast cancer cells through activation of PI3K signaling [106]. Interactions between PD-L1 expressing tumor cells and PD-1 positive T cells induces cytotoxic T cell exhaustion, resulting in tumor immune evasion [107]. Evidence that E2 upregulates both PD-L1 and PD-1, suggests E2 signaling may critically influence the PD-1/PD-L1 pathway.

3.6. Inflammatory Cytokines and Eicosanoids

Chronic inflammation is widely recognized as an ancillary mechanism promoting tumor progression. The TME releases cytokines that activate protumoral pathways mediating proliferation, immune evasion, and metastasis [108]. IL-6, a proinflammatory cytokine, has been shown to enhance ERα-positive breast cancer cell growth and invasion [109]. Local TAFs in breast cancers act as a paracrine source of the elevated IL-6, driving STAT3 activation and ERα-positive tumor cell proliferation both in vitro and in vivo [110]. TNFα, another ubiquitous TME cytokine, regulates expression of genes associated with metastatic phenotypes in ERα-positive breast cancer cells [111]. TNFα has also been shown to upregulate aromatase expression in cultured human adipose stromal cells [112]. Transcriptional linear correlations between aromatase and the cytokines TNFα and IL-6 have been reported in patient breast cancer tissue, but not in adjacent non-cancerous tissue [113]. A similar correlation has also been seen between aromatase and the eicosanoid cyclooxygenase-2 (COX-2) [113]. COX-2 is responsible for the synthesis of inflammatory promoting eicosanoids such as prostaglandin E2 (PGE2) [114]. It is well established that PGE2 promotes upregulated transcription of aromatase through elevated cyclic adenosine monophosphate (cAMP) in breast tumors [115]. Despite conflicting reports, some epidemiological studies show that regular use of COX-2 inhibiting nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of developing ERα-positive breast cancers, but not ERα-negative cancers [116].

Significant correlations between ERα, TNFα, and NF-κB protein expression have also been reported in breast cancer tissues [117]. NF-κB signaling is well recognized for its role in tumor initiation and inflammation [118]. Constitutive activation of NF-κB is observed in several cancers, and is associated with the cytokines IL-6 and TNFα [118]. Increased DNA binding of NF-κB and activator protein-1 (AP-1) has been observed in SERM-resistant, ERα-positive breast cancer cell line models and patient specimens [119,120]. Furthermore, E2 exposure in a murine model evaluating tobacco-induced lung cancer enhanced pulmonary inflammation through increased activation of NF-κB signaling and expression of VEGF and IL-17A [83]. Alternatively, targeting E2 and inflammatory pathways with combined AI and NSAID treatment maximally prevented carcinogen-induced lung tumor development in mice, significantly reducing STAT3 and MAPK signaling, circulating IL-6, and IL-17A expression [83]. Taken together, these reports indicate potential interactions between the E2 pathway and regulators of tumor-promoting inflammation, representing another beneficial target of E2 inhibition.

4. Clinical Implications of Targeting the Estrogen Pathway in the Tumor Microenvironment

Immunotherapy is a powerful therapeutic strategy for cancer; however, the immunosuppressive TME poses major obstacles for this approach. Currently, immune checkpoint inhibitors of cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and PD-1/PD-L1 are among the most clinically evaluated immune therapies [121]. These agentshave remarkably advanced cancer treatment, significantly improving response rates and survival compared with standard-of-care chemotherapies [122–125]. However, typical response rates to these therapies remain limited to only around 20–35% of patients, with variable responses depending on stage, tumor type, and PD-L1 staining positivity [126]. Furthermore, while some patients have durable responses, mechanisms of acquired and adaptive

resistance are becoming apparent, with 25 to 33% of melanoma patients exhibiting delayed relapse on these therapies [15,16].

Recent efforts to identify molecular events underlying immune evasion and failed therapeutic response report that damaged DNA repair mechanisms, increased non-synonymous somatic mutational load, and neoantigen presentation correlate with tumor immunogenicity and improved clinical outcomes [12,13,127]. Alternatively, mechanisms facilitating immune evasion involve damage to antigen presenting capacity and recurrence of non-antigenic mutations poorly presented by MHC class 1 molecules [128,129]. While these findings provide a greater understanding of tumor immunoediting and potential biomarkers predictive of response, novel therapeutic combinations are still needed to improve the efficacy of current immunotherapeutic agents. The identification of E2 modulation of the tumor immune phenotype justifies investigation of endocrine agents to reverse tumor immune tolerance. As depicted in Figure 1, E2 signaling can modulate the immune TME through enhanced protumoral responses. Therefore, anti-estrogen therapy has the potential to not only reverse an immunosuppressive TME, but also to augment response in E2-sensitive tumors.

Recently, a high-throughput screening assay in lung cancer cells identified the anti-estrogen fulvestrant as the top compound that increased tumor sensitivity to immune-mediated lysis [130]. Fulvestrant is an ideal candidate to combine with anti-PD-1/PD-L1 agents, due to its proven safety profile and non-overlapping toxicities. These new findings of E2 action on immune cells could create a paradigm shift towards utilizing anti-estrogen therapy to target the immunosuppressive TME, thereby increasing the efficacy and duration of response of current immunotherapies [131].

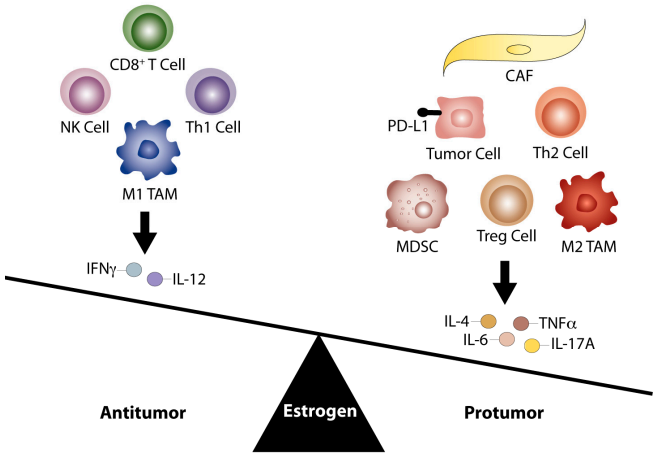

Figure 1. The E2 pathway promotes a protumor TME. The E2 pathway contributes to aberrant regulation of antitumor immunity, enhancing a greater number of protumoral responses within the TME. Current literature suggests E2 may facilitate an immunosuppressive TME by shifting the balance in favor of Th2 responses, production of tumor-promoting cytokines (IL-6, IL-4, TNFα, and IL-17A), and M2 TAM infiltration compared to Th1 responses, associated Th1 cytokines (IL-12 and IFNγ), and M1 TAM infiltration. E2 may further promote tumor immune evasion through proliferation of Treg and MDSC populations, increased tumor cell PD-L1 expression, and inhibition of $CD8^+$ T cell and NK cell induced apoptosis. CAFs may additionally support a protumor environment by supplying paracrine sources of E2 and IL-6. Therefore, targeted inhibition of the E2 pathway may act as a novel strategy to enhance the effects of immunotherapies and reverse this immune imbalance within the TME.

5. Conclusions and Perspective

The E2 pathway is an identified promoter of tumorigenesis in several cancers, largely for its direct genomic and non-genomic effects on tumor cells. However, evidence of ER and aromatase expression on stromal and immune cells within the TME indicates that additional mechanisms exist by which estrogens enhance malignant progression. It is becoming increasingly evident that cells comprising

the TME can impact tumor immunity, either beneficially through enhanced antitumoral immune responses, or detrimentally through increased protumoral responses. Evidence thus far suggests that E2 facilitates a primarily tumor-promoting and immunosuppressive TME in multiple tumor types. While checkpoint blockade immunotherapies have exhibited significant clinical success for the treatment of certain cancers, partial response rates and acquired resistance to these therapies necessitate the development of strategies to boost immunotherapeutic responses. The data summarized here points to the E2 pathway as a regulator of tumor immune responses, suggesting that clinical benefit may be derived from combining estrogen blocking agents with immune checkpoint inhibitors. Prior to clinical analysis of this combination, a more comprehensive characterization of E2-related proteins in the TME of various tumor types is necessary. There is also a need for standardized methods and CLIA-approved assays for the detection of ERβ and aromatase expression. Future studies evaluating response to current immunotherapies based on sex-differences, patient demographics including menopausal status, and obesity are warranted, given the pervasive involvement of the E2 pathway in tumor immunity.

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Abbreviations

ERβ	Estrogen receptor β
ERα	Estrogen receptor α
ERE	Estrogen response element
E2	17β-Estradiol
DC	Dendritic cell
Treg	Regulatory T cell
SERM	Selective estrogen receptor modulator
SERD	Selective estrogen receptor degrader
IHC	Immunohistochemistry
IF	Immunofluorescence
Th2	T helper 2
Th1	T helper 1
IL-4	Interleukin-4
IFNγ	Interferon Gamma
IL-6	Interleukin-6
TNFα	Tumor necrosis factor alpha
TME	Tumor microenvironment
EGF	Epidermal growth factor
IGF	Insulin growth factor
FGF	Fibroblast growth factor
OS	Overall survival
NSCLC	Non-small cell lung cancer
CAF	Cancer associated fibroblast
TAM	Tumor associated macrophage
MDSC	Myeloid derived suppressor cell
MPP	Methylpiperidino pyrazole
NK	Natural killer
LRH-1	Liver receptor homolog-1
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositide 3-kinase

MAPK	Mitogen-activated protein kinase
CCL5	C–C motif chemokine ligand 5
IL-12	Interleukin-12
VEGF	Vascular endothelial growth factor
HGSOC	High grade serous ovarian cancer
IL-17A	Interleukin-17A
STAT3	Signal transducer and activator of transcription 3
TIL	Tumor infiltrating lymphocyte
AI	Aromatase inhibitor
CTL	Cytotoxic T lymphocyte
PI-9	Proteinase inhibitor-9
PD-1	Programmed death-1
PD-L1	PD-1 ligand
COX-2	Cyclooxygenase-2
PGE2	Prostaglandin E2
NSAID	Nonsteroidal anti-inflammatory drug
AP-1	Activator protein-1
CTLA4	Cytotoxic T-lymphocyte–associated antigen 4

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ARTICLE

Circulating Tumor Cells Predict Survival in Early Average-to-High Risk Breast Cancer Patients

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Background Circulating tumor cells (CTCs) have been shown to predict reduced survival outcomes in metastatic breast cancer.

Methods CTCs were analyzed in 2026 patients with early breast cancer before adjuvant chemotherapy and in 1492 patients after chemotherapy using the CellSearch System. After immuno-magnetic enrichment for cells expressing the epithelial-cell adhesion molecule, CTCs were defined as nucleated cells expressing cytokeratin and lacking CD45. The patients were followed for a median of 35 months (range = 0–54). Kaplan–Meier analyses and the log-rank test were used for survival analyses. All statistical tests were two-sided.

Results Before chemotherapy, CTCs were detected in 21.5% of patients (n = 435 of 2026), with 19.6% (n = 136 of 692) of node-negative and 22.4% (n = 299 of 1334) of node-positive patients showing CTCs ($P < .001$). No association was found with tumor size, grading, or hormone receptor status. After chemotherapy, 22.1% of patients (n = 330 of 1493) were CTC positive. The presence of CTCs was associated with poor disease-free survival (DFS; $P < .0001$), distant DFS ($P < .001$), breast cancer-specific survival ($P = .008$), and overall survival (OS; $P = .0002$). CTCs were confirmed as independent prognostic markers in multivariable analysis for DFS (hazard ratio [HR] = 2.11; 95% confidence interval [CI] = 1.49 to 2.99; $P < .0001$) and OS (HR = 2.18; 95% CI = 1.32 to 3.59; $P = .002$). The prognosis was worst in patients with at least five CTCs per 30 mL blood (DFS: HR = 4.51, 95% CI = 2.59 to 7.86; OS: HR = 3.60, 95% CI = 1.56 to 8.45). The presence of persisting CTCs after chemotherapy showed a negative influence on DFS (HR = 1.12; 95% CI = 1.02 to 1.25; $P = .02$) and on OS (HR = 1.16; 95% CI = 0.99 to 1.37; $P = .06$)

Conclusions These results suggest the independent prognostic relevance of CTCs both before and after adjuvant chemotherapy in a large prospective trial of patients with primary breast cancer.

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The prognostic relevance of disseminated tumor cells (DTCs) in the bone marrow of patients with early breast cancer has been confirmed with the highest level of evidence. A pooled analysis of 4703 patients reported poor outcomes in patients with DTCs before the initiation of primary therapy (1), and 726 patients with persistent DTCs during recurrence-free follow-up showed an increased risk for distant relapse and a shortened overall survival (OS) (2). Based on these results, it was hypothesized that DTCs may underlie subsequent metastatic spread (3).

Increasing evidence suggests that circulating tumor cells (CTCs) in the peripheral blood are associated with reduced progression-free survival and OS in metastatic disease (4–8). Whereas the detection of CTCs before the start of a new treatment has been associated with poor prognosis, the enumeration of CTCs shortly after the initiation of therapy provides additional information regarding treatment response (4,7).

Although conclusive data for the prognostic relevance of CTCs are available for metastatic disease, only a few prospective trials in smaller patient cohorts have been performed for early breast cancer that suggest the prognostic relevance for CTC detection (9–16). In the SUCCESS (Simultaneous Study of Gemcitabine-Docetaxel Combination adjuvant treatment, as well as Extended Bisphosphonate and Surveillance-Trial) trial (EUDRA-CT No. 2005-000490-21), CTCs were statistically significantly associated with node-positive disease. The presence of CTCs both before the start of systemic adjuvant treatment and after completion of chemotherapy was associated with deteriorated survival. Prognostic relevance independent of lymph node metastases was confirmed in multivariable analysis.

Methods

Patients

Eligible patients were defined as women with breast cancer (stages pT1–T4, pN0–N3, M0) who agreed to participate in the phase III SUCCESS study. SUCCESS was a prospective, randomized adjuvant study comparing three cycles of fluorouracil-epirubicin-cyclophosphamide (FEC; 500/100/500 mg/m^2) followed by 3 cycles of docetaxel (100 mg/m^2) every 3 weeks vs three cycles of FEC followed by 3 cycles of gemcitabine (1000 mg/m^2 d1,8)-docetaxel (75 mg/m^2) every 3 weeks. After the completion of chemotherapy, the patients were further randomized to receive either 2 or 5 years of zoledronate. Hormone receptor–positive women received adequate endocrine treatment. The research questions associated with CTC analysis, the blood sampling time points, and the methodology were prospectively designed, and the prognostic value of the CTCs was defined as a scientific objective of the study protocol. The study was approved by 37 German ethical boards (lead ethical board: Ludwig-Maximilians-University Munich) and conducted in accordance with the Declaration of Helsinki.

Blood samples for CTC enumeration were collected from 2090 consecutive patients after complete resection of the primary tumor and before adjuvant chemotherapy after written informed consent was obtained. Sixty-four patients were excluded because of test failure or a time interval of more than 96 hours between the blood collection and sample preparation. A follow-up evaluation after chemotherapy and before the start of endocrine or bisphosphonate treatment was available for a subgroup of 1492 patients (Supplementary Figure 1, available online).

The primary surgery consisted of either breast conservation (n = 1414 of 2012; 70.3%) or mastectomy (n = 598 of 2012; 29.7%) leading to R0 resection in all case patients. Sentinel node dissection was performed in all cN0 patients (sentinel node dissection as the only axillary intervention; n = 692 of 2026; 34.2%) followed by complete axillary node dissection in case patients with positive sentinel nodes. The cN1 patients primarily received axillary node dissection (n = 1334 of 2026; 65.8%). Radiotherapy was performed according to national guidelines (17–19) and was used in all case patients that received breast-conserving treatment.

Preparation of Blood Samples and Detection of CTCs

CTCs were analyzed using the CellSearch System (Veridex, Raritan, NJ). Peripheral blood was drawn into three CellSave tubes (30 mL), shipped at room temperature to the central laboratory at the University of Munich, and analyzed within 96 hours of collection.

The samples were centrifuged for 10 minutes at 800 × *g*. The plasma was removed, and a dilution buffer was added. This mixture was overlaid on 6 mL of Histopaque (Sigma, Steinheim, Germany) and centrifuged for 10 minutes at 400 × *g*. Subsequently, 7.5 mL of this sample containing the buffy coat was processed on the CellTracks AutoPrep system using the CellSearch Epithelial Cell Kit (Veridex). After immuno-magnetic enrichment with an anti-Epcam antibody, the cells were labeled with fluorescent anticytokeratin (CK8,18,19–phycoerythrin) and anti-CD45 antibodies (CD45–allophycocyan), and 4,6-diamidino-2-phenylindoledihydrochloride was used to detect the intact cells.

The identification and enumeration of CTCs were performed using the CellTracks Analyzer II. CTCs were defined as nucleated cells lacking CD45 and expressing cytokeratin. All positive samples were reviewed by two independent investigators. The samples with at least one CTC per 30 mL of blood were regarded as CTC positive.

The blood from 84 individuals with no clinical evidence of malignant disease was processed blinded and used as a negative control. Four of these negative controls (4.9%) included cells that fit the definition of epithelial cells and could be interpreted as CTCs (one control had one epithelial cell, two controls had two, and one control had three epithelial cells).

Follow-up and Patient Evaluation

The median follow-up was 35 months (range = 0–54 months). The patients were followed at the study sites at 3-month intervals for the first 3 years and every 6 months thereafter. Follow-up included clinical examination (each visit), mammography (every 6 months), and symptom-driven examinations if necessary. All data were obtained from the electronic case record forms of the SUCCESS study. The quality of the data was ensured by electronic data management, including automated plausibility checks and regular monitoring visits to the study site by an independent clinical research organization (Alcedis, Gießen, Germany).

Statistical Analyses

The endpoints were defined according to the STEEP criteria, with disease-free survival (DFS) as the primary endpoint (20). The product-limit method according to Kaplan–Meier was used to estimate survival (21). The survival estimates in different groups were compared using the log-rank test. The Cox proportional hazards regression model was used for the analyses taking into account all variables simultaneously (22). The assumption of proportional hazards was checked by plotting the log(-log(S(t)) against time on study. In both endpoints, OS and DFS, the lines were parallel and no influence of time could be seen.

The χ^2 and Cochran–Armitage tests for trends in cases of more than two categories were used to analyze and compare frequencies for categorical variables. Continuous variables were compared using a *t* test. *P* less than .05 was considered significant in two-sided tests. No adjustment of the error probability for multiple testing was performed. SAS software,version 8.02 (SAS Institute, Cary, NC) was used.

Results

Prevalence of CTCs in Early Breast Cancer

Patient characteristics of 2026 patients with primary breast cancer are shown in Table 1. CTCs were detected in 21.5% of the patients (n = 435 of 2026) after the complete resection of the primary tumor and before the start of systemic treatment (median = 1.0 cell; range = 0–827 per 30 mL of blood). The patients with lymph node metastases were statistically significantly more often CTC-positive than node-negative patients. The frequency of CTC positive patients was 19.6% (n = 136 of 692) in the N0 group and 22.4% (n = 299 of 1334) in the N1 to N3 group (*P* < .001), whereas the presence of any CTC was not statistically significantly associated with other clinico-pathological characteristics or local and systemic

Table 1. Patient characteristics at baseline for circulating tumor cell count before chemotherapy (n = 2026)*

Characteristic	CTC ≥ 1† No. (%)	CTC = 0† No. (%)	*P*	CTC ≥ 5† No. (%)	CTC = 0–4† No. (%)	*P*
No. of patients	435 (21.5)	1591 (78.5)		63 (3.1)	1963 (96.9)	
Age in years (mean ± SD)	53.8 ± 10.3	53.2 ± 10.5	.26‡	55.03 + 9.87	53.30 + 10.52	.19‡
Tumor size¶						
pT1a	1 (0.2)	16 (1.0)	.19§	0 (0)	17 (0.8)	.31§
pT1b	19 (4.4)	86 (5.4)		3 (4.8)	102 (5.2)	
pT1c	139 (32.0)	561 (35.3)		20(31.8)	680 (34.6)	
pT2–4	268 (61.6)	906 (56.9)		40 (63.5)	1134 (57.8)	
pTx	7 (1.6)	22 (1.4)		0 (0)	29 (1.5)	
Lymph node metastases¶						
Absent (pN0)/ pNX	136 (31.3)	556 (35.0)	<.001§	15 (23.8)	659 (33.6)	<.001§
1–3 axillary (pN1)	178 (40.9)	747 (47.0)		23 (36.5)	921 (46.9)	
4–9 axillary (pN2)	72 (16.5)	208 (13.0)		16 (25.4)	257 (13.1)	
≥10 axillary (pN3)	49 (11.3)	80 (5.0)		9 (14.3)	126 (6.4)	
Grading#						
G1	14 (3.2)	85 (5.3)	.19‡	1 (1.6)	98 (5.0)	.12‡
G2	206 (47.4)	740 (46.5)		37 (58.7)	909 (46.3)	
G3	212 (48.7)	753 (47.3)		25 (39.7)	940 (47.9)	
Gx	3 (0.7)	13 (0.8)		0 (0)	16 (0.8)	
Hormone receptor status						
Negative	128 (29.4)	450 (28.3)	.64‖	13 (20.6)	565 (28.8)	.16‖
Positive	307 (70.6)	1141 (71.7)		50 (79.4)	1398 (71.2)	
Her2-neu status						
Undefined	10 (2.3)	41 (2.6)	.54‖	3 (4.8)	48 (2.4)	.95‖
Negative	322 (74.0)	1152 (72.4)		45 (71.4)	1429 (72.8)	
Positive	103 (23.7)	398 (25.0)		15 (23.8)	486 (24.8)	
Histological type						
Undefined	12 (.8)	2 (0.5)	.15§	0 (0)	14 (0.7)	.13§
Ductal	344 (79.1)	1285 (80.8)		45 (71.4)	1584 (80.7)	
Lobular	62 (14.3)	176 (11.1)		12 (19.0)	226 (11.5)	
Mixed ductal-lobular	27 (6.2)	118 (7.4)		6 (9.5)	139 (7.1)	
Menopausal status						
Premenopausal	169 (38.9)	672 (42.2)	.20‖	17 (27.0)	824 (42.0)	.02‖
Postmenopausal	266 (61.1)	919 (57.8)		46 (73.0)	1139 (58.0)	
Primary operation						
Breast conserving	295 (67.8)	1119 (70.3)	.27‖	45 (71.4)	1369 (69.7)	.84‖
Mastectomy	138 (31.7)	460 (28.9)		18 (28.6)	580 (29.5)	
Radiotherapy						
Performed	341 (78.4)	1211 (76.1)	.11‖	46 (73.0)	1506 (76.7)	.68‖
Not performed	94 (21.6)	380 (23.9)		17 (27.0)	457 (23.3)	
Systemic therapy						
Chemotherapy–FEC-D	205 (47.1)	820 (51.5)	.10‖	26 (41.3)	999 (50.9)	.13‖
Chemotherapy–FEC-DG	230 (52.9)	771 (48.5)		37 (58.7)	964 (49.1)	
Endocrine treatment	266 (61.2)	967 (60.7)	.88‖	32 (50.8)	990 (50.4)	.78‖
Trastuzumab	83 (19.4)	329 (21.2)	.41‖	9 (14.3)	229 (11.7)	.52‖

* CTC = circulating tumor cell; FEC-D = fluorouracil-epirubicin-cyclophosphamide (500/100/500 mg/m^2, FEC) followed by docetaxel (100 mg/mg^2); FEC-DG = fluorouracil-epirubicin-cyclophosphamide (500/100/500 mg/m^2, FEC) followed by gemcitabine (1,000 mg/m^2 d1,8)-docetaxel (75 mg/m^2); SD = standard deviation.

† Per 30 mL of blood.

‡ Two-sided *t* test.

§ Two-sided Cochran–Armitage test for trend.

‖ Two-sided χ^2 test.

¶ Tumor-node-metastasis (TNM) was classified according to the revised American Joint Committee on Cancer TNM classification (23).

Histopathological grading of the primary tumors was performed according to Elston–Ellis (24).

treatment. High CTC numbers of five or more were more frequent in postmenopausal patients (P = .02) (Table 1).

CTC analysis after completion of adjuvant chemotherapy was performed in a subgroup of 1492 patients. At this time point, CTCs (median = 1 cell; range = 0–124 cells per 30 mL of blood) were detected in 22.1% of the patients (n = 330 of 1493).There was no difference in CTC counts before and after chemotherapy (Supplementary Table 1, available online).

Prognostic Relevance of CTCs for DFS

One hundred fourteen patients (6%) relapsed, including 16 patients with locoregional disease and 98 patients with distant metastases. CTCs were detected in three patients (19%) with locoregional relapse and in 35 patients (30%) with distant metastases.

The disease-free probability at 36 months was 88.1% for patients with CTCs and 93.7% for patients without CTCs. The presence of CTCs was statistically significantly predictive of

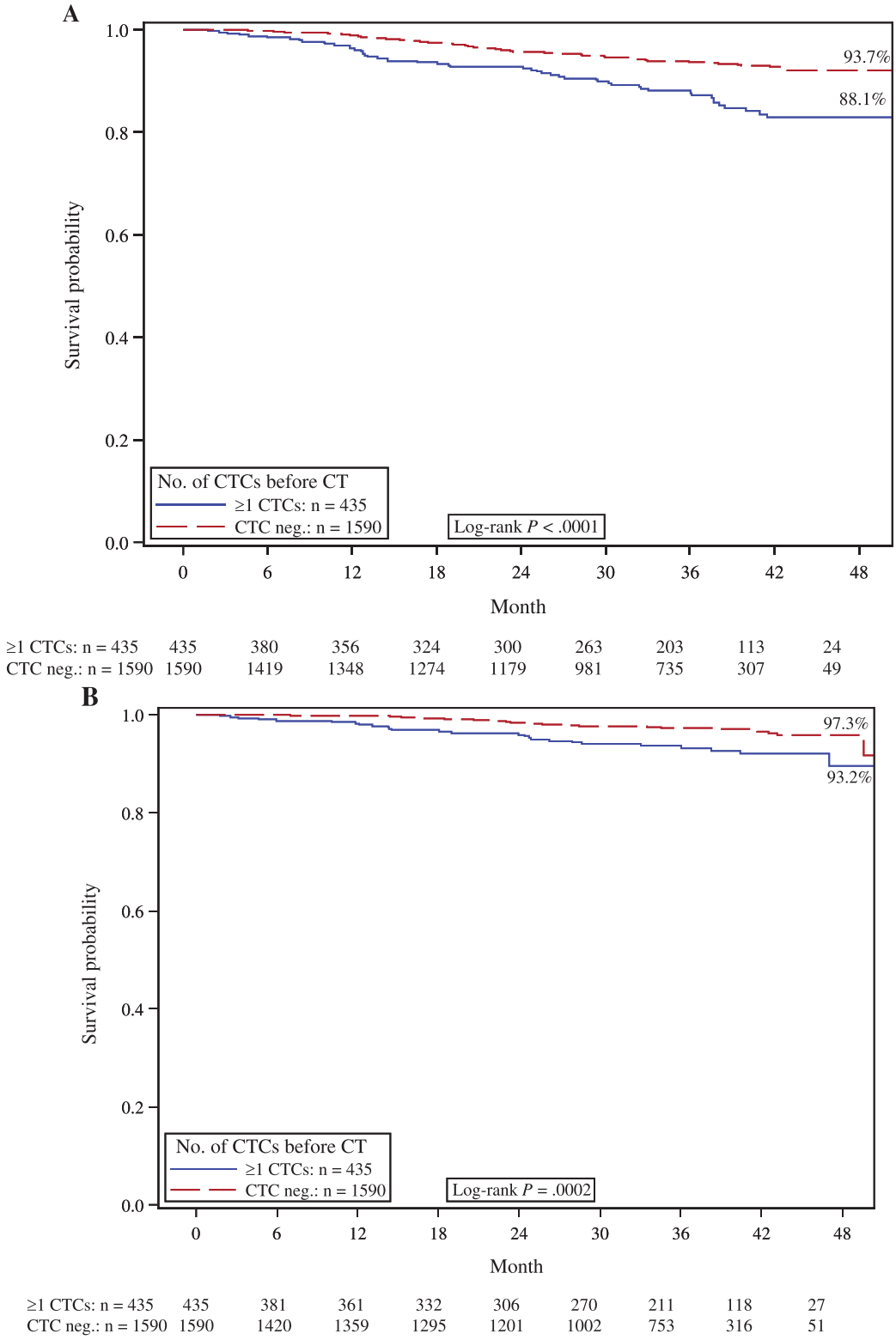


Figure 1. Kaplan–Meier analysis according to the presence or absence (neg.) of peripheral blood circulating tumor cells (CTCs) before chemotherapy (CT). **A**) Disease-free survival. **B**) Overall survival. **C**) Distant disease-free survival. **D**) Breast cancer–specific survival. Two-sided log-rank test.

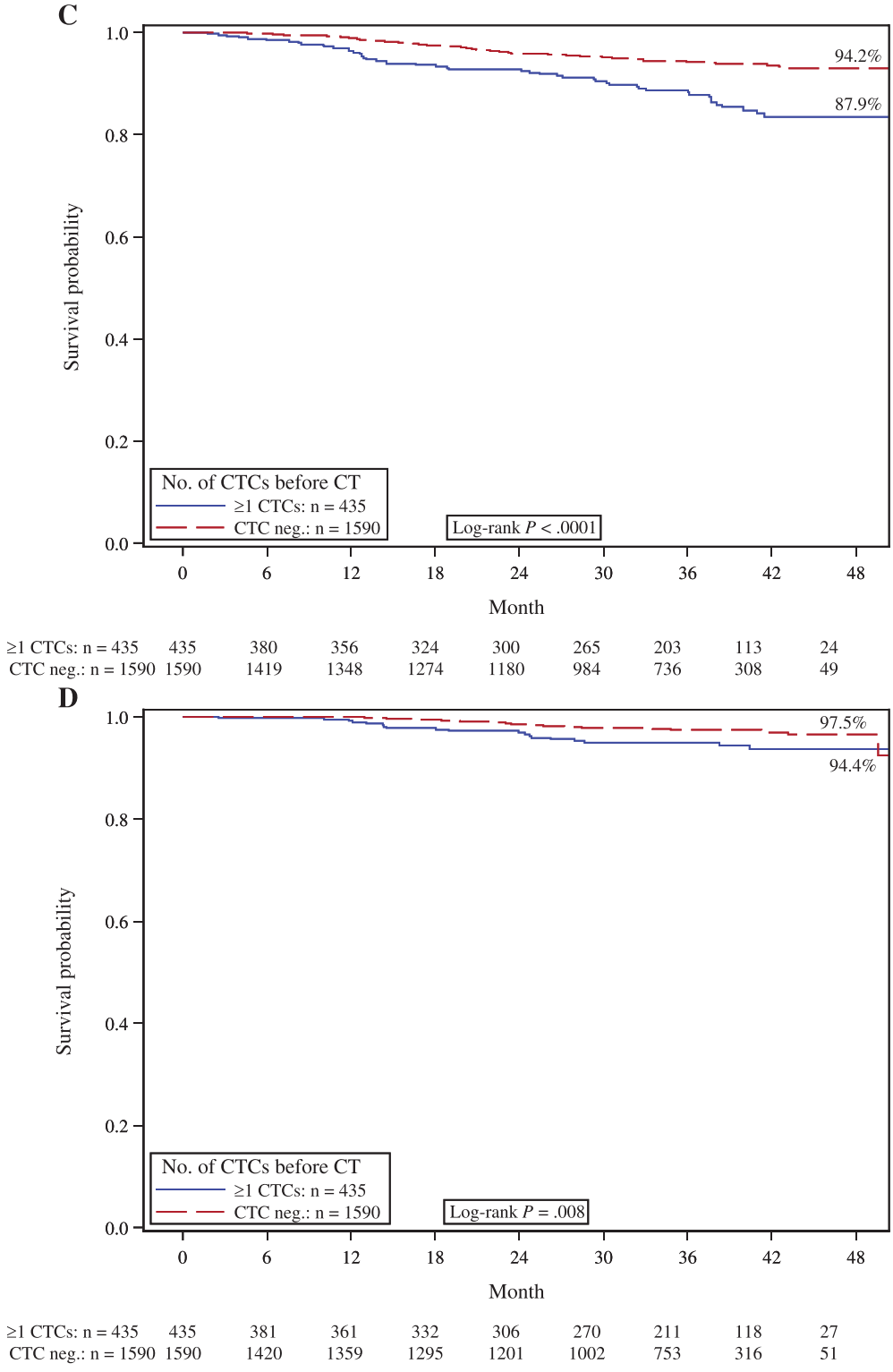


Figure 1. Continued

reduced DFS (log-rank test, $P < .0001$) (Figure 1A). The distant DFS at 36 months was 87.9% for CTC-positive patients and 94.2% for CTC-negative patients (log-rank test, $P < .001$).

In the multivariable proportional hazards model, the presence of one or more CTCs was confirmed to be an independent prognostic factor for reduced DFS (hazard ratio [HR] = 2.11; 95% confidence interval [CI] = 1.49 to 2.99; $P < .0001$) in addition to negative hormone receptor status, lymph node involvement, unfavorable grading, and tumor size greater than 2 cm (Table 2).

In a subgroup analysis, the patients were stratified according to lymph node status. The presence of CTCs was associated with reduced DFS in all node-positive subgroups (ie, in patients with 1–3 [log-rank test, $P = .008$), 4–9 [log-rank test, $P < .0001$), and ≥10 involved lymph nodes [log-rank test, $P = .001$]), whereas no statistically significant difference was observed for DFS in node-negative patients (log-rank test, $P = .23$) (Supplementary Figure 2A, available online).

Prognostic Relevance of CTCs for Survival

Sixty-six patients died during follow-up, including 54 who died of breast cancer, and 12 patients who succumbed to other causes. The CTC positivity rate was 40.9% (n = 27 of 66) for the patients who died compared with 20.8% (n = 408 of 2026) for the patients who survived. The overall death rate and the breast cancer death rate were both statistically significantly higher in patients with CTCs. A total of 4.6% of the CTC-positive patients died of breast cancer compared with 2.2% of the CTC-negative patients. The Kaplan–Meier estimate for 36-month survival was 93.2% for CTC-positive patients and 97.3% for CTC-negative patients. The presence of CTCs was associated with reduced breast cancer–specific survival (log-rank test, $P = .008$) and OS (log-rank test, $P = .0002$) (Figure 1, D and B, respectively). In the multivariable analysis, CTC detection remained a statistically significant prognostic predictor of poor survival (HR = 2.18; 95% CI = 1.32 to 3.59; $P = .002$) (Table 3).

Analysis of Different CTC Cutoff Values

An exploratory proportional hazard analysis was performed using several CTC levels as cutoffs to evaluate the influence of the cutoff on the hazard ratios of OS and DFS adjusted for standard risk factors and treatment. The patients were grouped and compared according to three different CTC cutoff values (0 vs ≥1; 0–1 vs ≥2; 0–4 vs ≥5 CTCs in 30 mL of blood). DFS and OS were statistically significantly reduced in the group with the higher CTC levels for all three cutoff values (Table 4).

Patients with five or more CTCs were at highest risk for recurrence. At 36 months, 28.1% of patients presented with recurrent disease and 14.3% had died, compared with 7.1% and 3.4% of patients with less than five CTCs, respectively (log-rank test, $P < .0001$ and $P = .005$) (Figure 2). The results indicated that patient outcome was associated with the absolute number of CTCs because the hazard ratios consistently increased with increasing cutoff values. The risk of recurrence or death more than doubled when a cutoff value of five or more CTCs was used (DFS: HR = 4.51, 95% CI = 2.59 to 7.86; OS: HR = 3.60, 95% CI = 1.56 to 8.45) compared with a cutoff value of one or more CTCs (DFS: HR = 2.11; OS: HR = 2.18) (Table 4). To investigate the relationship between outcome and number of CTCs, the hazard ratio of the number of CTCs present compared with no CTCs was calculated, adjusted for the standard risk factors and treatment. For all clinical endpoints, patient prognosis deteriorated continuously with increasing CTC numbers (Figure 3).

Table 2. Univariate and multivariable proportional hazards model for disease-free survival for circulating tumor cell count before chemotherapy (n = 2026)*

	Univariate analysis		Multivariable analysis	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
CTCs in blood, negative vs positive	2.257 (1.595 to 3.195)	<.0001	2.107 (1.487 to 2.986)	<.0001
Hormone receptor status, positive vs negative	2.187 (1.559 to 3.066)	<.0001	1.972 (1.363 to 2.854)	.0003
Lymph node involvement, N0 vs N1–3	1.780 (1.187 to 2.670)	.005	2.942 (1.922 to 4.505)	<.0001
Grading, G1 vs G2–3	3.109 (2.124 to 4.551)	<.0001	3.254 (2.146 to 4.935)	<.0001
Tumor size, T1 vs T2–4	2.205 (1.496 to 3.251)	<.0001	2.082 (1.405 to 3.083)	.0003
Menopausal status, pre vs post	1.221 (0.864 to 1.725)	.26	1.018 (0.717 to 1.445)	.92
Histology, lobular/mixed vs ductal	1.308 (0.822 to 2.083)	.26	0.931 (0.575 to 1.508)	.77

* Cox proportional hazards models. All statistical tests were two-sided. CI = confidence interval; CTC = circulating tumor cell; HR = hazard ratio.

Table 3. Univariate and multivariable proportional hazards model for overall survival for circulating tumor cell count before chemotherapy (n = 2026)*

	Univariate Analysis		Multivariable Analysis	
Variable	**HR (95% CI)**	***P***	**HR 95% CI**	***P***
CTCs in blood, negative vs positive	2.447 (1.491 to 4.015)	.0004	2.177 (1.320 to 3.588)	.002
Hormone receptor status, positive vs negative	3.414 (2.098 to 5.556)	<.0001	2.997 (1.763 to 5.095)	<.0001
Lymph node involvement, N0 vs N1–3	2.465 (1.290 to 4.709)	.006	4.254 (2.182 to 8.293)	<.0001
Grading, G1 vs G2–3	4.097 (2.271 to 7.392)	<.0001	3.549 (1.864 to 6.760)	.0001
Tumor size, T1 vs T2–4	2.969 (1.618 to 5.446)	.0004	2.665 (1.441 to 4.930)	.002
Menopausal status, pre vs post	1.990 (1.157 to 3.421)	.013	1.518 (0.876 to 2.629)	.14
Histology, lobular/mixed vs ductal	2.020 (0.923 to 4.423)	.08	1.262 (0.559 to 2.850)	.58

* Cox proportional hazards models. All statistical tests were two-sided. CI = confidence interval; CTC = circulating tumor cell; HR = hazard ratio.

Table 4. Multivariable proportional hazards model for disease-free survival and overall survival for different circulating tumor cell cutoff values*

Variable	HRs (95% CI) adjusted for treatment		
	0 vs ≥1 CTC per 30 mL blood	0–1 vs ≥2 CTC per 30 mL blood	0–4 vs ≥5 CTC per 30 mL blood
DFS			
CTCs in blood, negative vs positive	2.11† (1.487 to 2.986)	3.19† (2.141 to 4.763)	4.51† (2.586 to 7.864)
Hormone receptor status, positive vs negative	1.97† (1.36 to 2.85)	1.98† (1.366 to 2.861)	1.98† (1.365 to 2.869)
Lymph node involvement, N0 vs N1–3	2.94† (1.92 to 4.51)	2.77† (1.807 to 4.241)	2.84† (1.859 to 4.349)
Grading, G1 vs G2–3	3.25† (2.15 to 4.94)	3.39† (2.236 to 5.145)	3.32† (2.186 to 5.026)
Tumor size, T1 vs T2–4	2.08† (1.41 to 3.08)	2.13† (1.440 to 3.159)	2.19† (1.485 to 3.246)
Menopausal status, pre vs post	1.02 (0.88 to 2.63)	1.00 (0.705 to 1.423)	0.99 (0.699 to 1.410)
Histology, lobular/mixed vs ductal	0.93 (0.58 to 1.51)	0.91 (0.559 to 1.466)	0.94 (0.579 to 1.516)
OS			
CTCs in blood, negative vs positive	2.18† (1.32 to 3.59)	2.57† (1.416 to 4.659)	3.60† (1.564 to 8.445)
Hormone receptor status, positive vs negative	3.0† (1.76 to 5.10)	3.04† (1.786 to 5.163)	3.05† (1.790 to 5.190)
Lymph node involvement, N0 vs N1–3	4.25† (2.18 to 8.29)	4.07† (2.085 to 7.947)	4.19† (2.149 to 8.161)
Grading, G1 vs G2–3	3.55† (1.86 to 6.76)	3.65† (1.920 to 6.954)	3.66† (1.924 to 6.977)
Tumor size, T1 vs T2–4	2.67† (1.44 to 4.93)	2.74† (1.479 to 5.058)	2.85† (1.548 to 5.255)
Menopausal status, pre vs post	1.52 (0.88 to 2.63)	1.49 (0.856 to 2.580)	1.49 (0.859 to 2.583)
Histology, lobular/mixed vs ductal	1.26 (0.56 to 2.85)	1.23 (0.546 to 2.779)	1.25 (0.556 to 2.823)

* CI = confidence interval; CTC = circulating tumor cell; DFS = disease free survival; HR = hazard ratio; OS = overall survival. Cox proportional hazards models. All statistical tests were two-sided.

† Statistically significant.

CTC Detection in Different Breast Cancer Subtypes

Breast cancer is a heterogeneous disease and classified into molecular subtypes, which we analyzed with regard to the presence or absence of CTCs. We grouped the primary tumors according to their immunohistochemical phenotype. Luminal cancers were defined as estrogen receptor and/or progesterone receptor positive (n = 1155; 57.0%), basal-like tumors were defined as estrogen, progesterone, and HER2 negative (n = 347; 17.1%), and HER2-like tumors were defined as HER2 positive (n = 501; 24.7%). Following this classification, no association of CTC positivity with luminal, basal-like, or HER2-like tumors (χ^2 test, all $P \geq .5$) was found. In the largest subgroup of luminal patients, the presence of CTCs was associated with a reduced DFS (HR = 1.24; 95% CI = 1.16 to 1.33; $P < .001$) and OS (HR = 1.28; 95% CI = 1.16 to 1.44; $P < .001$).

Relevance of CTCs Persisting After Adjuvant Chemotherapy

A total of 85.7% of CTC-positive patients were free of recurrence at 36 months compared with 91.1% of CTC-negative patients. After chemotherapy, 22.1% of patients (n = 330 of 1493) were CTC positive. The presence of persisting CTCs after chemotherapy showed a negative influence on DFS (HR = 1.124; 95% CI = 1.02 to 1.25; $P = .02$) and on OS (HR = 1.162; 95% CI = 0.99 to 1.37; $P = .06$).

Four patient groups were formed according to their CTC status both before and after chemotherapy: persistently positive patients, persistently negative patients, patients with positive prechemotherapy CTC status changing to negative, and patients with negative prechemotherapy CTC status changing to positive. The Kaplan–Meier estimate for 36-month OS was 92.8% for persistently CTC-positive patients and 97.6% for persistently CTC-negative patients. For DFS, the estimates were 85.9% for persistently CTC-positive patients and 93.9% for persistently CTC-negative patients. The presence of CTCs both before and after chemotherapy compared with all other subgroups was associated with a statistically significantly reduced DFS (log-rank test, $P = .005$) (Figure 4) and a trend toward a reduced OS (log-rank test, $P = .10$).

Discussion

For the first time, we could show CTCs to be a prognostic marker for reduced DFS, distant DFS, breast cancer–specific survival, and OS before the start of systemic treatment and for DFS after completion of adjuvant chemotherapy in the setting of a large, multicenter, prospective, randomized trial. Prognostic relevance independent of other prognostic markers was confirmed in multivariable analysis both for DFS and OS. The strength of this prognostic effect increased with higher CTC levels.

The prevalence of at least one CTC per 30 mL of blood was 21.5%, which is within the CTC positivity range found by other investigators (9,14,15). In smaller cohorts, CTCs were reported in 18% to 30% of patients with early breast cancer (9,12,14,15,25) and more frequently in patients with metastatic disease, with a prevalence of 70% (4,26). Lucci et al. recently published data on 302 breast cancer patients at the time of surgery: CTCs were detected in 24% of patients, and their presence predicted decreased progression-free survival and OS (15). Our trial confirmed these data in a much larger patient cohort, extending the data to patients after completion of chemotherapy. Based on the evaluation at sequential time points, we provided the prevalence, course, and prognostic relevance of CTCs before and after adjuvant chemotherapy within the same patients and could confirm our results in multivariable analysis. Because of the large number of patients, subgroup analyses taking into account the different CTC levels and biological breast cancer subtypes were performed. All patients were average-risk to high-risk early breast cancer patients for whom chemotherapy is

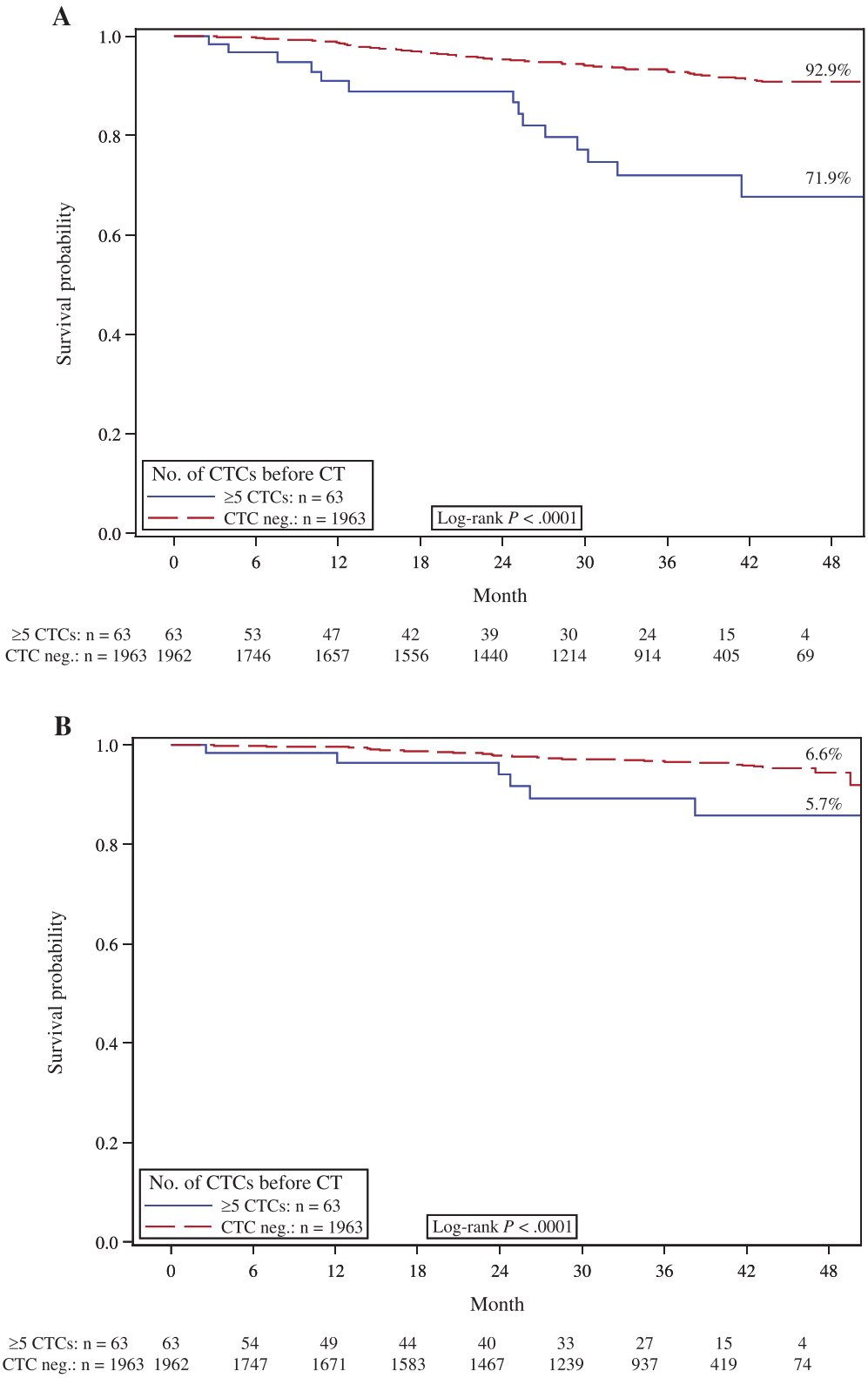


Figure 2. Kaplan–Meier analysis according to the presence or absence of five or more peripheral blood circulating tumor cells (CTCs) before chemotherapy (CT). **A**) Disease-free survival. **B**) Overall survival. Two-sided log-rank test.

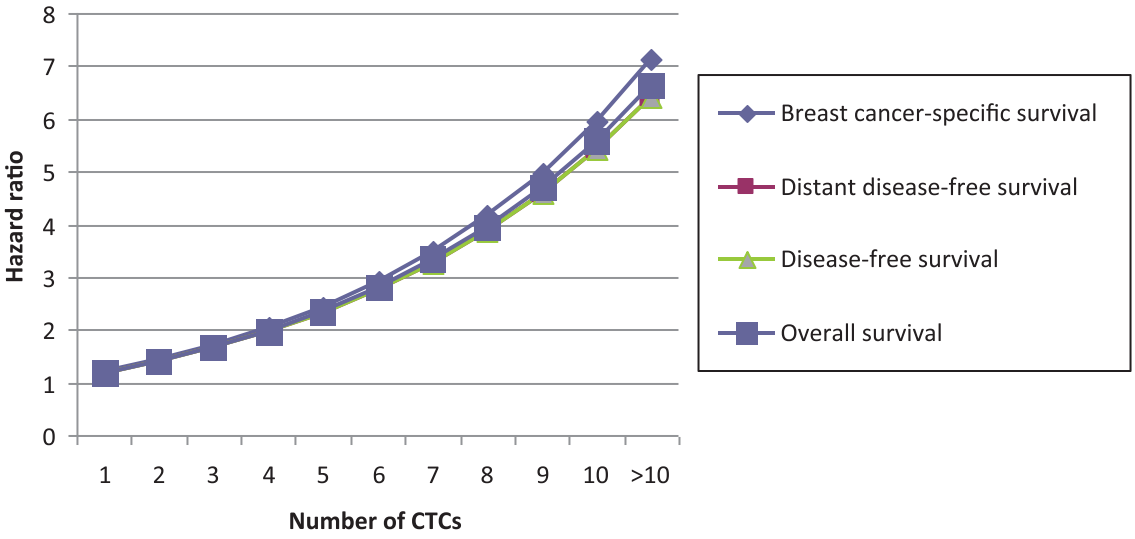
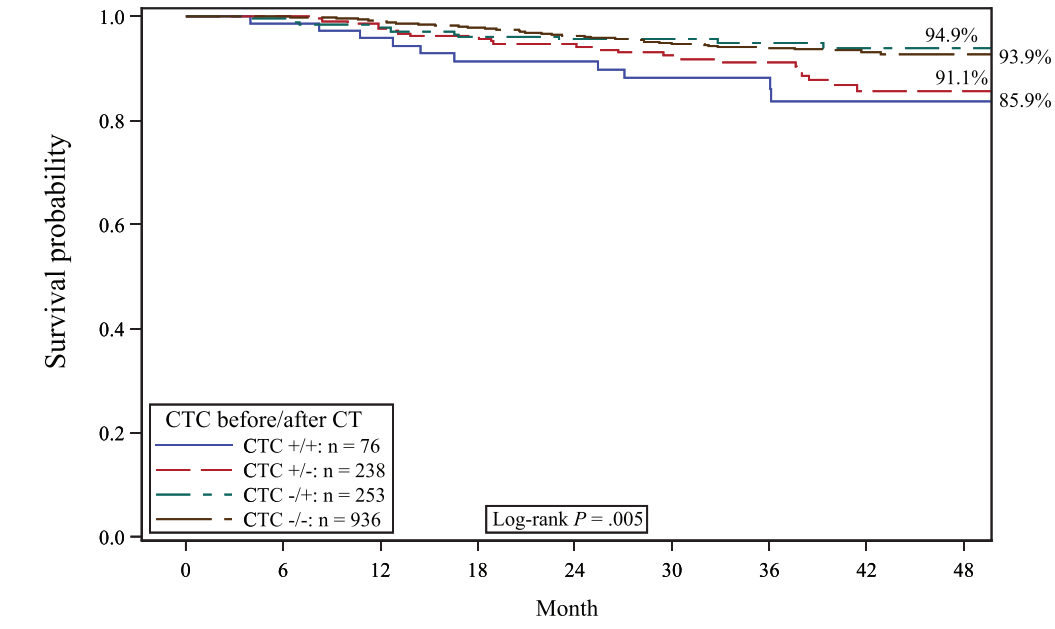


Figure 3. The correlation of hazard ratios with increasing numbers of circulating tumor cells (CTCs) per 30 mL of blood according to survival endpoints.



CTC +/+: n = 76	76	72	67	60	59	51	40	22	8
CTC +/-: n = 238	238	218	207	190	173	153	124	72	14
CTC -/+: n = 253	253	234	220	209	195	164	127	59	9
CTC -/-: n = 936	936	874	839	796	738	620	487	210	38

Figure 4. Kaplan–Meier analysis for disease-free survival according to the presence (+) or absence (–) of peripheral blood circulating tumor cells (CTCs) before and after chemotherapy (CT). Two-sided log-rank test.

recommended. Therefore, the observation that the presence of CTCs at primary diagnosis is associated with worse prognosis is likely to remain of limited impact for the modification of treatment algorithms in this group of patients. In contrast, the prognostic relevance of CTCs after chemotherapy could be especially valuable for individualized treatment approaches to allow for the identification of patients with tumor cells evading standard chemotherapy.

Although basal-like tumors are commonly treated with chemotherapy, decisions regarding adjuvant chemotherapy are much more difficult in the luminal subgroup. Despite recent advances in technology, such as the Oncotype DX or gene arrays, the benefit of a treatment with considerable side effects still remains unclear in the individual patient, leading to a general overtreatment in many cases. Because we observed an increased risk of recurrence,

especially in the subgroup of luminal patients, the detection of CTCs can help select patients at risk by providing tumor biological information beyond the available diagnostic tests. Furthermore, because axillary operation will increasingly be confined to sentinel node biopsy, CTCs could be a helpful tool for selection of high-risk patients who might benefit from a more aggressive dose-dense chemotherapy regimen (27,28).

The limitations of this study include the short median follow-up of 35 months. This short follow-up in the context of a very good prognosis results in small absolute differences in the rate of recurrence and death. Despite this limited number of events in our data, as well as in the study published by Lucci et al., both trials consistently demonstrate a clear prognostic relevance of CTCs in early breast cancer. In addition, the number of cells detected by the CellSearch system is relatively low and limited to cells with expression of Epcam and cytokeratin 8/18/19. In contrast, basal-like tumors with low Epcam expression have been shown to contain a high frequency of stem cells (29–31) and are associated with very poor prognosis (32). CTCs with decreased epithelial marker expression as a result of the epithelial–mesenchymal transition could be missed by the CellSearch methodology (33). Epcam-independent detection approaches could increase the capacity to detect CTCs with stem cell phenotype. Nevertheless, the CellSearch system has shown highly reproducible and automated detection of CTCs in interlab validation trials (34,35).

Although the presence of persisting CTCs after chemotherapy was associated with worse outcome, survival of patients without CTCs before chemotherapy was the same irrespective of CTC status after chemotherapy. This might be explained by various effects of chemotherapy on CTCs. Tumor cell mobilization by chemotherapy or bone marrow stimulating agents such as granulocyte colony stimulating factor is a known phenomenon (36), whereas adjuvant chemotherapy reduces the number of proliferating CTCs (37,38). These differential effects could influence the metastatic potential of CTCs. The development of new techniques for CTC phenotyping could help to identify tumor cells responsible for subsequent metastatic disease.

Modern breast cancer treatment is tailored to the individual tumor characteristics (19,39). Changes in the tumor phenotype from the primary tumor to that of distant metastasis are a known phenomenon and may lead to treatment changes in up to 20% of patients (40,41). Given the chromosomal abnormalities and the overexpression of HER2 and stem cell markers in CTCs (9,24,42–44), improved phenotyping could help to identify treatment-relevant targets and resistance mechanisms (45). Clinical intervention trials are currently being performed to evaluate the predictive role of CTCs to tailor the treatment in primary and metastatic disease (SWOG S0500, TREAT CTC, and DETECT III) (46).

In conclusion, the SUCCESS study is the first trial to provide strong evidence for the prognostic relevance of CTCs in early breast cancer before and after adjuvant chemotherapy in a large patient cohort. Our data offer support for the clinical potential of CTCs to assess the individual risk of patients at the time of primary diagnosis and may be used for treatment tailoring in the absence of other strong quantitative markers. Future applications for CTCs will include the early assessment of treatment efficacy as well as the phenotyping of cells to individualize treatment strategies. Thus, in addition to established parameters, the use of CTCs may considerably contribute to the personalization of breast cancer treatment (36).

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