AOP ID and Title:

SNAPSHOT

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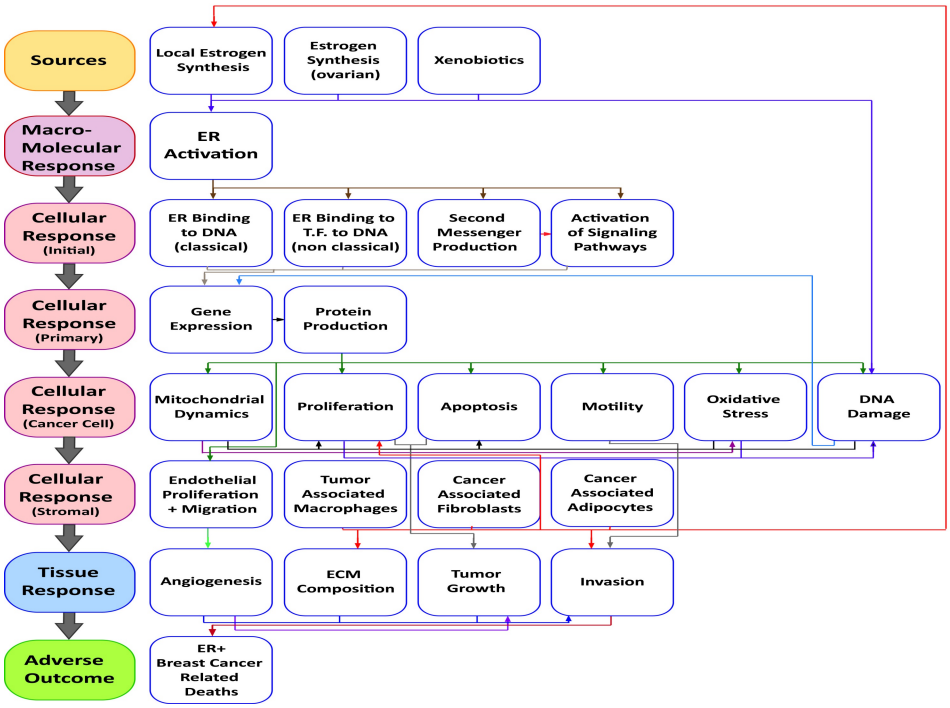
AOP 200: Estrogen receptor activation leading to breast cancer

Short Title: ER activation to breast cancer

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite			Under Development

Abstract

Endocrine disrupting chemicals (EDC), particularly estrogen receptor (ER) agonists, are thought to contribute to the incidence of breast cancer. The majority (approximately 75 percent) of breast cancer cases express the estrogen receptor. Both animal and human studies strongly support that activation of the estrogen receptor stimulates breast cancer development and progression. We created the ER-mediated breast cancer AOP to frame how ER activation (the MIE) leads to breast cancer (the AO). For more information regarding the AOP, refer to the Morgan & Johnson et al. (2015) citation.

Activation of the estrogen receptor in breast epithelial cells stimulates genomic and non-genomic changes, which alters epithelial gene expression and subsequent protein production. Consequently, breast epithelial cells experience increased proliferation, decreased apoptosis, dysfunction of mitochondrial dynamics, increased DNA damage, increased cell motility, and increased oxidative stress. These cellular changes translate to a tissue level where ductal hyperplasia and cell invasion is increased.

While breast epithelial cells are the cancer cell type in ER+ adenocarcinomas, other cell types of the microenvironment interact with the AOP. For example, endothelial cells express ER and upon ER activation, undergo gene expression and protein production changes. Consequently, endothelial cell proliferation and migration is increased, leading to increased angiogenesis, which supports the proliferation of breast cancer

epithelial cells. While estrogens do not target fibroblasts, adipocytes, or macrophages directly, they become activated as breast cancer progresses. It is not well understood if there is a direct relationship between estrogen signaling and stromal cell activation, however, activated cells stimulate cancer cell proliferation, influence chemical response, increase cell motility, and rearrange the extracellular matrix. Moreover, adipocytes contribute to the AOP through metabolism of testosterone to estrogen, and fibroblasts have been shown to regulate estrogen receptor regulated genes in epithelial cells. Therefore, due to how the breast microenvironment interacts with and stimulates the AOP, we have included activation of these cell types into our framework.

Overall, the ER-mediated breast cancer AOP is a useful framework that can identify both readouts and components of the breast microenvironment that are important in disease progression.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1181	Activation, Estrogen receptor (https://aopwiki.org/events/1181)	Activation, Estrogen receptor
2	KE	1182	Increase, Cell Proliferation (Epithelial Cells) (https://aopwiki.org/events/1182)	Increase, Cell Proliferation (Epithelial Cells)
3	KE	1183	Decreased, Apoptosis (Epithelial Cells) (https://aopwiki.org/events/1183)	Decreased, Apoptosis (Epithelial Cells)
4	KE	177	N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/events/177)	N/A, Mitochondrial dysfunction 1
5	KE	1088	Increased, Oxidative Stress (https://aopwiki.org/events/1088)	Increased, Oxidative Stress
6	KE	1187	Increased, ER binding to DNA (classical pathway) (https://aopwiki.org/events/1187)	Increased, ER binding to DNA (classical pathway)
7	KE	1188	Increased, ER binding to T.F. to DNA (non-classical pathway) (https://aopwiki.org/events/1188)	Increased, ER binding to T.F. to DNA (non-classical pathway)
8	KE	1189	Increased, Proliferation (Endothelial cells) (https://aopwiki.org/events/1189)	Increased, Proliferation (Endothelial cells)
9	KE	1190	Increased, Migration (Endothelial Cells) (https://aopwiki.org/events/1190)	Increased, Migration (Endothelial Cells)
10	KE	1191	Increased, Non-genomic signaling (https://aopwiki.org/events/1191)	Increased, Non-genomic signaling
11	KE	1192	Increased, Ductal Hyperplasia (https://aopwiki.org/events/1192)	Increased, Ductal Hyperplasia
12	KE	1194	Increase, DNA damage (https://aopwiki.org/events/1194)	Increase, DNA Damage
13	KE	1195	modulation, Extracellular Matrix Composition (https://aopwiki.org/events/1195)	modulation, Extracellular Matrix Composition
14	KE	1196	Increased, Invasion (https://aopwiki.org/events/1196)	Increased, Invasion
15	KE	1197	Activation, Fibroblasts (https://aopwiki.org/events/1197)	Activation, Fibroblasts
16	KE	1198	Activation, Macrophages (https://aopwiki.org/events/1198)	Activation, Macrophages

Sequence	Type	Event ID	Title	Short name
17	KE	1213	Increased, Angiogenesis (https://aopwiki.org/events/1213)	Increased, Angiogenesis
18	KE	1239	Altered, Gene Expression (https://aopwiki.org/events/1239)	Altered, Gene Expression
19	KE	1240	Altered, Protein Production (https://aopwiki.org/events/1240)	Altered, Protein Production
20	KE	1241	Increased, Motility (https://aopwiki.org/events/1241)	Increased, Motility
21	KE	1242	Increased, Second Messenger Production (https://aopwiki.org/events/1242)	Increased, Second Messenger Production
22	AO	1193	N/A, Breast Cancer (https://aopwiki.org/events/1193)	N/A, Breast Cancer

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation, Estrogen receptor (https://aopwiki.org/relationships/1246)	adjacent	Increased, ER binding to DNA (classical pathway)	High	High
Increase, Cell Proliferation (Epithelial Cells) (https://aopwiki.org/relationships/1247)	adjacent	Increased, Ductal Hyperplasia	High	High
Decreased, Apoptosis (Epithelial Cells) (https://aopwiki.org/relationships/1248)	adjacent	Increased, Ductal Hyperplasia	High	High
Activation, Estrogen receptor (https://aopwiki.org/relationships/1249)	adjacent	Increased, ER binding to T.F. to DNA (non-classical pathway)	High	High
Increased, ER binding to DNA (classical pathway) (https://aopwiki.org/relationships/1250)	adjacent	Increase, Cell Proliferation (Epithelial Cells)	High	High
Increased, ER binding to T.F. to DNA (non-classical pathway) (https://aopwiki.org/relationships/1251)	adjacent	Increase, Cell Proliferation (Epithelial Cells)	High	High
Increased, Ductal Hyperplasia (https://aopwiki.org/relationships/1252)	adjacent	N/A, Breast Cancer	High	High
Increased, Proliferation (Endothelial cells) (https://aopwiki.org/relationships/1266)	adjacent	Increased, Angiogenesis	High	High
Increased, Migration (Endothelial Cells) (https://aopwiki.org/relationships/1267)	adjacent	Increased, Angiogenesis	High	High
Activation, Estrogen receptor (https://aopwiki.org/relationships/1294)	adjacent	Increased, Non-genomic signaling	Moderate	High
Increased, Non-genomic signaling (https://aopwiki.org/relationships/1295)	adjacent	Increased, ER binding to T.F. to DNA (non-classical pathway)	High	High
Increased, ER binding to DNA (classical pathway) (https://aopwiki.org/relationships/1296)	adjacent	Altered, Gene Expression	High	High
Increased, ER binding to T.F. to DNA (non-classical pathway) (https://aopwiki.org/relationships/1297)	adjacent	Altered, Gene Expression	High	High
Altered, Gene Expression (https://aopwiki.org/relationships/1298)	adjacent	Altered, Protein Production	High	High

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Altered, Protein Production (https://aopwiki.org/relationships/1299)	adjacent	Increased, Oxidative Stress	High	High
Increased, Oxidative Stress (https://aopwiki.org/relationships/1300)	adjacent	Increase, DNA damage	High	High
Increase, DNA damage (https://aopwiki.org/relationships/1301)	adjacent	Altered, Gene Expression	High	High
Increased, Non-genomic signaling (https://aopwiki.org/relationships/1302)	adjacent	Altered, Gene Expression	High	High
Altered, Protein Production (https://aopwiki.org/relationships/1303)	adjacent	Increased, Proliferation (Endothelial cells)	High	High
Altered, Protein Production (https://aopwiki.org/relationships/1304)	adjacent	Decreased, Apoptosis (Epithelial Cells)	High	High
Altered, Protein Production (https://aopwiki.org/relationships/1305)	adjacent	Increased, Motility	Moderate	Moderate
Increased, Motility (https://aopwiki.org/relationships/1306)	adjacent	Increased, Invasion	Moderate	Moderate
Activation, Estrogen receptor (https://aopwiki.org/relationships/1307)	adjacent	Increased, Second Messenger Production	Moderate	Moderate
Increased, Second Messenger Production (https://aopwiki.org/relationships/1308)	adjacent	Increased, Non-genomic signaling	Moderate	Moderate

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
cat	Felis catus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9685)
dog	Canis lupus familiaris	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9615)

Sex Applicability

Sex	Evidence
Unspecific	High

Sex. While females have a higher incidence of breast cancer, estrogen-receptor mediated breast cancer can occur in males and females.

Life stages. Breast cancer affects adult women and men. Older adult women have a higher probability of having an ER+ breast cancer (vs. ER-) than younger adult women.

Taxonomic applicability. Breast cancer occurs naturally in humans, cats, and dogs. *In vivo* studies primarily study breast cancer in mice.

Weight of Evidence Summary

The weight of evidence for the KERs related to epithelial cells is mostly strong. The KERs between ER activation, motility, and invasion were labeled as a moderate weight of evidence due to discrepancies in the literature regarding whether ER activation decreases motility/invasion, vs. increases motility/invasion. ER activation leading to non-genomic signaling was labeled as moderate due to the limited evidence supporting this KER. For non-epithelial cell types, we labeled the KERs relationship as mostly weak. ER activation has direct effects on endothelial cells as they express ER and several studies have correlated ER activation with increased proliferation, migration, and angiogenesis. Macrophages, fibroblasts, and adipocytes are influenced by and stimulate breast cancer progression, however, the exact correlation between ER activation and these events is still unclear.

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Appendix 1

List of MIEs in this AOP

Event: 1181: Activation, Estrogen receptor (https://aopwiki.org/events/1181)

Short Name: Activation, Estrogen receptor

Key Event Component

Process	Object	Action
signaling	estrogen receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	MolecularInitiatingEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

List of Key Events in the AOP

Event: 1182: Increase, Cell Proliferation (Epithelial Cells) (https://aopwiki.org/events/1182)

Short Name: Increase, Cell Proliferation (Epithelial Cells)

Key Event Component

Process	Object	Action
cell proliferation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
epithelial cell

Event: 1183: Decreased, Apoptosis (Epithelial Cells) (https://aopwiki.org/events/1183)

Short Name: Decreased, Apoptosis (Epithelial Cells)

Key Event Component

Process	Object	Action
apoptotic process		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
epithelial cell

Event: 177: N/A, Mitochondrial dysfunction 1 (https://aopwiki.org/events/177)

Short Name: N/A, Mitochondrial dysfunction 1

Key Event Component

Process	Object	Action
	mitochondrion	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment. (https://aopwiki.org/aops/48)	KeyEvent
Aop:77 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony death/failure 1 (https://aopwiki.org/aops/77)	KeyEvent
Aop:78 - Nicotinic acetylcholine receptor activation contributes to abnormal role change within the worker bee caste leading to colony death failure 1 (https://aopwiki.org/aops/78)	KeyEvent
Aop:79 - Nicotinic acetylcholine receptor activation contributes to impaired hive thermoregulation and leads to colony loss/failure (https://aopwiki.org/aops/79)	KeyEvent
Aop:80 - Nicotinic acetylcholine receptor activation contributes to accumulation of damaged mitochondrial DNA and leads to colony loss/failure (https://aopwiki.org/aops/80)	KeyEvent
Aop:87 - Nicotinic acetylcholine receptor activation contributes to abnormal foraging and leads to colony loss/failure (https://aopwiki.org/aops/87)	KeyEvent
Aop:3 - Inhibition of the mitochondrial complex I of nigro-striatal neurons leads to parkinsonian motor deficits (https://aopwiki.org/aops/3)	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent
Aop:178 - Nicotinic acetylcholine receptor activation contributes to mitochondrial dysfunction and leads to colony loss/failure (https://aopwiki.org/aops/178)	KeyEvent
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Mitochondrial dysfunction is a universal event occurring in cells of any species (Farooqui and Farooqui, 2012). Many invertebrate species (drosophila, C, elegans) are considered as potential models to study mitochondrial function. New data on marine invertebrates, such as molluscs and crustaceans and non-Drosophila species, are emerging (Martinez-Cruz et al., 2012). Mitochondrial dysfunction can be measured in animal models used for toxicity testing (Winklhofer and Haass, 2010; Waerzeggers et al., 2010) as well as in humans (Winklhofer and Haass, 2010).

Key Event Description

Mitochondrial dysfunction is a consequence of inhibition of the respiratory chain leading to oxidative stress.

Mitochondria can be found in all cells and are considered the most important cellular consumers of oxygen. Furthermore, mitochondria possess numerous redox enzymes capable of transferring single electrons to oxygen, generating the superoxide (O2-). Some mitochondrial enzymes that are involved in reactive oxygen species (ROS) generation include the electron-transport chain (ETC) complexes I, II and III; pyruvate dehydrogenase (PDH) and glycerol-3-phosphate dehydrogenase (GPDH). The transfer of electrons to oxygen, generating superoxide, happens mainly when these redox carriers are charged enough with electrons and the potential energy for transfer is elevated, like in the case of high mitochondrial membrane potential. In contrast, ROS generation is decreased if there are not enough electrons and the potential energy for the transfer is not sufficient (reviewed in Lin and Beal, 2006).

Cells are also able to detoxify the generated ROS due to an extensive antioxidant defence system that includes superoxide dismutases, glutathione peroxidases, catalase, thioredoxins, and peroxiredoxins in various cell organelles (reviewed in Lin and Beal, 2006). It is worth mentioning that, as in the case of ROS generation, antioxidant defences are also closely related to the redox and energetic status of mitochondria. If mitochondria are structurally and functionally healthy, an antioxidant defence mechanism balances ROS generation, and there is not much available ROS production. However, in case of mitochondrial damage, the antioxidant defence capacity drops and ROS generation takes over. Once this happens, a vicious cycle starts and ROS can further damage mitochondria, leading to more free-radical generation and further loss of antioxidant capacity. During mitochondrial dysfunction the availability of ATP also decreases, which is considered necessary for repair mechanisms after ROS generation.

A number of proteins bound to the mitochondria or endoplasmic reticulum (ER), especially in the mitochondria-associated ER membrane (MAM), are playing an important role of communicators between these two organelles (reviewed Mei et al., 2013). ER stress induces mitochondrial dysfunction through regulation of Ca2+ signaling and ROS production (reviewed Mei et al., 2013). Prolonged ER stress leads to release of Ca2+ at the MAM and increased Ca2+ uptake into the mitochondrial matrix, which induces Ca2+-dependent mitochondrial outer membrane permeabilization and apoptosis. At the same, ROS are produced by proteins in the ER oxidoreductin 1 (ERO1) family. ER stress activates ERO1 and leads to excessive production of ROS, which, in turn, inactivates SERCA and activates inositol-1,4,5- trisphosphate receptors (IP3R) via oxidation, resulting in elevated levels of cytosolic Ca2+, increased mitochondrial uptake of Ca2+, and ultimately mitochondrial dysfunction. Just as ER stress can lead to mitochondrial dysfunction, mitochondrial dysfunction also induces ER Stress (reviewed Mei et al., 2013). For example, nitric oxide disrupts the mitochondrial respiratory chain and causes changes in mitochondrial Ca2+ flux which induce ER stress. Increased Ca2+ flux triggers loss of mitochondrial membrane potential (MMP), opening of mitochondrial permeability transition pore (MPTP), release of cytochrome c and apoptosis inducing factor (AIF), decreasing ATP synthesis and rendering the cells more vulnerable to both apoptosis and necrosis (Wang and Qin, 2010).

Summing up: Mitochondria play a pivotal role in cell survival and cell death because they are regulators of both energy metabolism and apoptotic/necrotic pathways (Fiskum, 2000; Wieloch, 2001; Friberg and Wieloch, 2002). The production of ATP via oxidative phosphorylation is a vital mitochondrial function (Kann and Kovács, 2007; Nunnari and Suomalainen, 2012). The ATP is continuously required for signalling processes (e.g. Ca2+ signalling), maintenance of ionic gradients across membranes, and biosynthetic processes (e.g. protein synthesis, heme synthesis or lipid and phospholipid metabolism) (Kang and Pervaiz, 2012), and (Green, 1998; McBride et al., 2006). Inhibition of mitochondrial respiration contributes to various cellular stress responses, such as deregulation of cellular Ca2+ homeostasis (Graier et al., 2007) and ROS production (Nunnari and Suomalainen, 2012; reviewed Mei et al., 2013).). It is well established in the existing literature that mitochondrial dysfunction may result in: (a) an increased ROS production and a decreased ATP level, (b) the loss of mitochondrial protein import and protein biosynthesis, (c) the

reduced activities of enzymes of the mitochondrial respiratory chain and the Krebs cycle, (d) the loss of the mitochondrial membrane potential, (e) the loss of mitochondrial motility, causing a failure to re-localize to the sites with increased energy demands (f) the destruction of the mitochondrial network, and (g) increased mitochondrial Ca2+ uptake, causing Ca2+ overload (reviewed in Lin and Beal, 2006; Graier et al., 2007), (h) the rupture of the mitochondrial inner and outer membranes, leading to (i) the release of mitochondrial pro-death factors, including cytochrome c (Cyt. c), apoptosis-inducing factor, or endonuclease G (Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013), which eventually leads to apoptotic, necrotic or autophagic cell death (Wang and Qin, 2010). Due to their structural and functional complexity, mitochondria present multiple targets for various compounds.

How it is Measured or Detected

Mitochondrial dysfunction can be detected using isolated mitochondria, intact cells or cells in culture as well as in vivo studies. Such assessment can be performed with a large range of methods (revised by Brand and Nicholls, 2011) for which some important examples are given. All approaches to assess mitochondrial dysfunction fall into two main categories: the first assesses the consequences of a loss-of-function, i.e. impaired functioning of the respiratory chain and processes linked to it. Some assay to assess this have been described for KE1, with the limitation that they are not specific for complex I. In the context of overall mitochondrial dysfunction, the same assays provide useful information, when performed under slightly different assay conditions (e.g. without addition of complex III and IV inhibitors). The second approach assesses a ‘non-desirable gain-of-function’, i.e. processes that are usually only present to a very small degree in healthy cells, and that are triggered in a cell, in which mitochondria fail.

I. Mitochondrial dysfunction assays assessing a loss-of function.

1. Cellular oxygen consumption.

See KE1 for details of oxygen consumption assays. The oxygen consumption parameter can be combined with other endpoints to derive more specific information on the efficacy of mitochondrial function. One approach measures the ADP-to-O ratio (the number of ADP molecules phosphorylated per oxygen atom reduced (Hinkle, 1995 and Hafner et al., 1990). The related P/O ratio is calculated from the amount of ADP added, divided by the amount of O_2 consumed while phosphorylating the added ADP (Ciapaite et al., 2005; Diepart et al., 2010; Hynes et al., 2006; James et al., 1995; von Heimburg et al., 2005).

2. Mitochondrial membrane potential (Δψm).

The mitochondrial membrane potential (Δψm) is the electric potential difference across the inner mitochondrial membrane. It requires a functioning respiratory chain in the absence of mechanisms that dissipate the proton gradient without coupling it to ATP production. The classical, and still most quantitative method uses a tetraphenylphosphonium ion (TPP+)-sensitive electrode on suspensions of isolated mitochondria. The Δψm can also be measured in live cells by fluorimetric methods. These are based on dyes which accumulate in mitochochondria because of Δψm. Frequently used are tetramethylrhodamineethylester (TMRE), tetramethylrhodaminemethyl ester (TMRM) (Petronilli et al., 1999) or 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1). Mitochondria with intact membrane potential concentrate JC-1, so that it forms red fluorescent aggregates, whereas de-energized mitochondria cannot concentrate JC-1 and the dilute dye fluoresces green (Barrientos et al., 1999). Assays using TMRE or TMRM measure only at one wavelength (red fluorescence), and depending on the assay setup, de-energized mitochondria become either less fluorescent (loss of the dye) or more fluorescent (attenuated dye quenching).

3. Enzymatic activity of the electron transport system (ETS).

Determination of ETS activity can be dene following Owens and King's assay (1975). The technique is based on a cell-free homogenate that is incubated with NADH to saturate the mitochondrial ETS and an artificial electron acceptor [I - (4 -iodophenyl) -3 - (4 -nitrophenyl) -5-phenylte trazolium chloride (INT)] to register the electron transmission rate. The oxygen consumption rate is calculated from the molar production rate of INT-formazan which is determined spectrophotometrically (Cammen et al., 1990).

4. ATP content.

For the evaluation of ATP levels, various commercially-available ATP assay kits are offered based on luciferin and luciferase activity. For isolated mitochondria various methods are available to continuously measure ATP with electrodes (Laudet 2005), with luminometric methods, or for obtaining more information on different nucleotide phosphate pools (e.g. Ciapaite et al., (2005).

II. Mitochondrial dysfunction assays assessing a gain-of function.

1. Mitochondrial permeability transition pore opening (PTP).

The opening of the PTP is associated with a permeabilization of mitochondrial membranes, so that different compounds and cellular constituents can change intracellular localization. This can be measured by assessment of the translocation of cytochrome c, adenylate kinase or AIF from mitochondria to the cytosol or nucleus. The translocation can be assessed biochemically in cell fractions, by imaging approaches in fixed cells or tissues or by life-cell imaging of GFP fusion proteins (Single 1998; Modjtahedi 2006). An alternative approach is to measure the accessibility of cobalt to the mitochondrial matrix in a calcein fluorescence quenching assay in live permeabilized cells (Petronilli et al., 1999).

2. mtDNA damage as a biomarker of mitochondrial dysfunction.

Various quantitative polymerase chain reaction (QPCR)-based assays have been developed to detect changes of DNA structure and sequence in the mitochondrial genome. mtDNA damage can be detected in blood after low-level rotenone exposure, and the damage persists even after CI activity has returned to normal. With a more sustained rotenone exposure, mtDNA damage is also detected in skeletal muscle. These data support the idea that mtDNA damage in peripheral tissues in the rotenone model may provide a biomarker of past or ongoing mitochondrial toxin exposure (Sanders et al., 2014a and 2014b).

3. Generation of ROS and resultant oxidative stress.

a. General approach. Electrons from the mitochondrial ETS may be transferred ‘erroneously’ to molecular oxygen to form superoxide anions. This type of side reaction can be strongly enhanced upon mitochondrial damage. As superoxide may form hydrogen peroxide, hydroxyl radicals or other reactive oxygen species, a large number of direct ROS assays and assays assessing the effects of ROS (indirect ROS assays) are available (Adam-Vizi, 2005; Fan and Li 2014). Direct assays are based on the chemical modification of fluorescent or luminescent reporters by ROS species. Indirect assays assess cellular metabolites, the concentration of which is changed in the presence of ROS (e.g. glutathione, malonaldehyde, isoprostanes,etc.) At the animal level the effects of oxidative stress are measured from biomarkers in the blood or urine.

b. Measurement of the cellular glutathione (GSH) status. GSH is regenerated from its oxidized form (GSSH) by the action of an NADPH dependent reductase (GSSH + NADPH + H+ à 2 GSH + NADP+). The ratio of GSH/GSSG is therefore a good indicator for the cellular NADH+/NADPH ratio (i.e. the redox potential). GSH and GSSH levels can be determined by HPLC, capillary electrophoresis, or biochemically with DTNB (Ellman’s reagent). As excess GSSG is rapidly exported from most cells to maintain a constant GSH/GSSG ratio, a reduction of total glutathione (GSH/GSSG) is often a good surrogate measure for oxidative stress.

c. Quantification of lipid peroxidation. Measurement of lipid peroxidation has historically relied on the detection of thiobarbituric acid (TBA)-reactive compounds such as malondialdehyde generated from the decomposition of cellular membrane lipid under oxidative stress (Pryor et al., 1976). This method is quite sensitive, but not highly specific. A number of commercial assay kits are available for this assay using absorbance or fluorescence detection technologies. The formation of F2-like prostanoid derivatives of arachidonic acid, termed F2-isoprostanes (IsoP) has been shown to be more specific for lipid peroxidation. A number of commercial ELISA kits have been developed for IsoPs, but interfering agents in samples requires partial purification before analysis. Alternatively, GC/MS may be used, as robust (specific) and sensitive method.

d. Detection of superoxide production. Generation of superoxide by inhibition of complex I and the methods for its detection are described by Grivennikova and Vinogradov (2014). A range of different methods is also described by BioTek (http://www.biotek.com/resources/articles/reactive-oxygen-species.html (http://www.biotek.com/resources/articles/reactive-oxygen-species.html)). The reduction of ferricytochrome c to ferrocytochrome c may be used to assess the rate of superoxide formation (McCord, 1968). Like in other superoxide assays, specificity can only be obtained by measurements in the absence and presence of superoxide dismutase. Chemiluminescent reactions have been used for their increased sensitivity. The most widely used chemiluminescent substrate is lucigenin. Coelenterazine has also been used as a chemiluminescent substrate. Hydrocyanine dyes are fluorogenic sensors for superoxide and hydroxyl radical, and they become membrane impermeable after oxidation (trapping at site of formation). The best characterized of these probes are Hydro-Cy3 and Hydro-Cy5. generation of superoxide in mitochondria can be visualized using fluorescence microscopy with MitoSOX™ Red reagent (Life Technologies). MitoSOX™ Red reagent is a cationic derivative of dihydroethidium that permeates live cells and accumulates in mitochondria.

e. Detection of hydrogen peroxide (H_2O_2) production. There are a number of fluorogenic substrates, which serve as hydrogen donors that have been used in conjunction with horseradish peroxidase (HRP) enzyme to produce intensely fluorescent products in the presence of hydrogen peroxide (Zhou et al., 1997: Ruch et al., 1983). The more commonly used substrates include diacetyldichloro-fluorescein, homovanillic acid, and Amplex® Red. In these examples, increasing amounts of H_2O_2 form increasing amounts of fluorescent product (Tarpley et al., 2004).

Summing up, mitochondrial dysfunction can be measured by: • ROS production: superoxide (O2-), and hydroxyl radicals (OH–) • Nitrosative radical formation such as ONOO– or directly by: • Loss of mitochondrial membrane potential (MMP) • Opening of mitochondrial permeability transition pores (MPTP) • ATP synthesis • Increase in mitochondrial Ca2+ • Cytochrome c release • AIF (apoptosis inducing factor) release from mitochondria • Mitochondrial Complexes enzyme activity • Measurements of mitochondrial oxygen consumption • Ultrastructure of mitochondria using electron microscope and mitochondrial fragmentation measured by labelling with DsRed-Mito expression (Knott et al, 2008) Mitochondrial dysfunction-induced oxidative stress can be measured by: • Reactive carbonyls formations (proteins oxidation) • Increased 8-oxo-dG immunoreactivity (DNA oxidation) • Lipid peroxidation (formation of malondialdehyde (MDA) and 4- hydroxynonenal (HNE) • 3-nitrotyrosine (3-NT) formation, marker of protein nitration • Translocation of Bid and Bax to mitochondria • Measurement of intracellular free calcium concentration ([Ca2+]i): Cells are loaded with 4 µM fura-2/AM). • Ratio between reduced and oxidized form of glutathione (GSH depletion) (Promega assay, TB369; Radkowsky et al., 1986) • Neuronal nitric oxide synthase (nNOS) activation that is Ca2+-dependent. All above measurements can be performed as the assays for each readout are well established in the existing literature (e.g. Bal-Price and Brown, 2000; Bal-Price et al., 2002; Fujikawa, 2015; Walker et al., 1995). See also KE Oxidative Stress, Increase (https://aopwiki.org/wiki/index.php/Event:209)

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Event: 1088: Increased, Oxidative Stress (https://aopwiki.org/events/1088)

Short Name: Increased, Oxidative Stress

Key Event Component

Process	Object	Action
oxidative stress		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:171 - Chronic cytotoxicity of the serous membrane leading to pleural/peritoneal mesotheliomas in the rat. (https://aopwiki.org/aops/171)	KeyEvent
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality (https://aopwiki.org/aops/138)	KeyEvent
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality (https://aopwiki.org/aops/177)	KeyEvent

AOP ID and Name	Event Type
Aop:186 - unknown MIE leading to renal failure and mortality (https://aopwiki.org/aops/186)	KeyEvent
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Event: 1187: Increased, ER binding to DNA (classical pathway) (https://aopwiki.org/events/1187)

Short Name: Increased, ER binding to DNA (classical pathway)

Key Event Component

Process	Object	Action
estrogen receptor binding	deoxyribonucleic acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Event: 1188: Increased, ER binding to T.F. to DNA (non-classical pathway) (https://aopwiki.org/events/1188)

Short Name: Increased, ER binding to T.F. to DNA (non-classical pathway)

Key Event Component

Process	Object	Action
estrogen receptor binding	serotransferrin	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Event: 1189: Increased, Proliferation (Endothelial cells) (https://aopwiki.org/events/1189)

Short Name: Increased, Proliferation (Endothelial cells)

Key Event Component

Process	Object	Action
endothelial cell proliferation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
endothelial cell

Event: 1190: Increased, Migration (Endothelial Cells) (https://aopwiki.org/events/1190)

Short Name: Increased, Migration (Endothelial Cells)

Key Event Component

Process	Object	Action
endothelial cell migration		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
endothelial cell

Event: 1191: Increased, Non-genomic signaling (https://aopwiki.org/events/1191)

Short Name: Increased, Non-genomic signaling

Key Event Component

Process	Object	Action
signaling		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
epithelial cell

Event: 1192: Increased, Ductal Hyperplasia (https://aopwiki.org/events/1192)

Short Name: Increased, Ductal Hyperplasia

Key Event Component

Process	Object	Action
hyperplasia		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
mammary duct

Event: 1194: Increase, DNA damage (https://aopwiki.org/events/1194)

Short Name: Increase, DNA Damage

Key Event Component

Process	Object	Action
	deoxyribonucleic acid	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent
Aop:216 - Excessive reactive oxygen species production leading to oocyte apoptosis-associated reproduction decline (https://aopwiki.org/aops/216)	KeyEvent
Aop:266 - D1 protein blockage leading to apoptosis associated growth inhibition (https://aopwiki.org/aops/266)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Event: 1195: modulation, Extracellular Matrix Composition (https://aopwiki.org/events/1195)

Short Name: modulation, Extracellular Matrix Composition

Key Event Component

Process	Object	Action
	extracellular matrix	morphological change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Event: 1196: Increased, Invasion (https://aopwiki.org/events/1196)

Short Name: Increased, Invasion

Key Event Component

Process	Object	Action
	epithelial cell	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Organ term

Organ term
mammary duct

Event: 1197: Activation, Fibroblasts (https://aopwiki.org/events/1197)

Short Name: Activation, Fibroblasts

Key Event Component

Process	Object	Action
cell activation		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
fibroblast

Event: 1198: Activation, Macrophages (https://aopwiki.org/events/1198)

Short Name: Activation, Macrophages

Key Event Component

Process	Object	Action
macrophage activation		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
macrophage

Event: 1213: Increased, Angiogenesis (https://aopwiki.org/events/1213)

Short Name: Increased, Angiogenesis

Key Event Component

Process	Object	Action
angiogenesis		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Event: 1239: Altered, Gene Expression (https://aopwiki.org/events/1239)

Short Name: Altered, Gene Expression

Key Event Component

Process	Object	Action
gene expression		abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent
Aop:275 - Histone deacetylase inhibition leads to neural tube defects (https://aopwiki.org/aops/275)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Event: 1240: Altered, Protein Production (https://aopwiki.org/events/1240)

Short Name: Altered, Protein Production

Key Event Component

Process	Object	Action
translation	protein	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Event: 1241: Increased, Motility (https://aopwiki.org/events/1241)

Short Name: Increased, Motility

Key Event Component

Process	Object	Action
cell motility		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Event: 1242: Increased, Second Messenger Production (https://aopwiki.org/events/1242)

Short Name: Increased, Second Messenger Production

Key Event Component

Process	Object	Action
second-messenger-mediated signaling		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

List of Adverse Outcomes in this AOP

Event: 1193: N/A, Breast Cancer (https://aopwiki.org/events/1193)

Short Name: N/A, Breast Cancer

Key Event Component

Process	Object	Action
	Breast Neoplasms	pathological

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1246: Activation, Estrogen receptor leads to Increased, ER binding to DNA (classical pathway) (https://aopwiki.org/relationships/1246)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1247: Increase, Cell Proliferation (Epithelial Cells) leads to Increased, Ductal Hyperplasia (https://aopwiki.org/relationships/1247)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1248: Decreased, Apoptosis (Epithelial Cells) leads to Increased, Ductal Hyperplasia (https://aopwiki.org/relationships/1248)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1249: Activation, Estrogen receptor leads to Increased, ER binding to T.F. to DNA (non-classical pathway) (https://aopwiki.org/relationships/1249)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1250: Increased, ER binding to DNA (classical pathway) leads to Increase, Cell Proliferation (Epithelial Cells) (https://aopwiki.org/relationships/1250)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1251: Increased, ER binding to T.F. to DNA (non-classical pathway) leads to Increase, Cell Proliferation (Epithelial Cells) (https://aopwiki.org/relationships/1251)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1252: Increased, Ductal Hyperplasia leads to N/A, Breast Cancer (https://aopwiki.org/relationships/1252)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1266: Increased, Proliferation (Endothelial cells) leads to Increased, Angiogenesis (https://aopwiki.org/relationships/1266)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1267: Increased, Migration (Endothelial Cells) leads to Increased, Angiogenesis (https://aopwiki.org/relationships/1267)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1294: Activation, Estrogen receptor leads to Increased, Non-genomic signaling (https://aopwiki.org/relationships/1294)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**Moderate**	**High**

Relationship: 1295: Increased, Non-genomic signaling leads to Increased, ER binding to T.F. to DNA (non-classical pathway) (https://aopwiki.org/relationships/1295)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1296: Increased, ER binding to DNA (classical pathway) leads to Altered, Gene Expression (https://aopwiki.org/relationships/1296)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1297: Increased, ER binding to T.F. to DNA (non-classical pathway) leads to Altered, Gene Expression (https://aopwiki.org/relationships/1297)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1298: Altered, Gene Expression leads to Altered, Protein Production (https://aopwiki.org/relationships/1298)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1299: Altered, Protein Production leads to Increased, Oxidative Stress (https://aopwiki.org/relationships/1299)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1300: Increased, Oxidative Stress leads to Increase, DNA Damage (https://aopwiki.org/relationships/1300)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1301: Increase, DNA Damage leads to Altered, Gene Expression (https://aopwiki.org/relationships/1301)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1302: Increased, Non-genomic signaling leads to Altered, Gene Expression (https://aopwiki.org/relationships/1302)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1303: Altered, Protein Production leads to Increased, Proliferation (Endothelial cells) (https://aopwiki.org/relationships/1303)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1304: Altered, Protein Production leads to Decreased, Apoptosis (Epithelial Cells) (https://aopwiki.org/relationships/1304)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**High**	**High**

Relationship: 1305: Altered, Protein Production leads to Increased, Motility (https://aopwiki.org/relationships/1305)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**Moderate**	**Moderate**

Relationship: 1306: Increased, Motility leads to Increased, Invasion (https://aopwiki.org/relationships/1306)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**Moderate**	**Moderate**

Relationship: 1307: Activation, Estrogen receptor leads to Increased, Second Messenger Production (https://aopwiki.org/relationships/1307)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**Moderate**	**Moderate**

Relationship: 1308: Increased, Second Messenger Production leads to Increased, Non-genomic signaling (https://aopwiki.org/relationships/1308)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	**adjacent**	**Moderate**	**Moderate**