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Gene Expression Profiles of NO- and HNO-donor Treated Breast Cancer Cells: Insights into Tumor Response and Resistance Pathways

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Abstract

Nitric oxide (NO) synthase 2 (NOS2), a major inflammatory protein, modulates disease progression via NO in a number of pathologies, including cancer. The role of NOS2-derived NO is not only flux-dependent, which is higher in mouse vs. human cells, but also varies based on spatial and temporal distribution both within tumor cells and in the tumor microenvironment. NO donors have been utilized to mimic NO flux conditions and to investigate the effects of varied NO concentrations. As a wide range of effects mediated by NO and other nitrogen oxides such as nitroxyl (HNO) have been elucidated, multiple NO- and HNO-releasing compounds have been developed as potential therapeutics, including as tumor modulators. One of the challenges is to determine differences in biomarker expression from extracellular vs. intracellular generation of NO or HNO. Taking advantage of new NO and HNO releasing agents, we have characterized the gene expression profile of estrogen receptor-negative human breast cancer (MDA-MB-231) cells following exposure to aspirin, the NO donor DEA/NO, the HNO donor IPA/NO and their intracellularly-activated prodrug conjugates DEA/NO-aspirin and IPA/NO-aspirin. Comparison of the gene expression profiles demonstrated that several genes were uniquely expressed with respect to NO or HNO, such as *miR-21*, *HSP70*, cystathionine γ-lyase and *IL24*. These findings provide insight into targets and pathways that could be therapeutically exploited by the redox related species NO and HNO.

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Keywords

nitric oxide; nitroxyl; nitric oxide synthase; breast cancer; cell signaling; cancer biology

NOS2 in Disease

Nitric oxide (NO) synthase 2 (NOS2; aka inducible NOS, iNOS) is an inflammatory protein in humans and mice. NOS2 modulates disease initiation and progression in pathologies including asthma, neurological disorders and cancer [1–6]. While the specific activities of human and mouse NOS2 are nearly identical, the promoter regions differ dramatically, leading to significantly higher levels of NO generated by mouse vs. human cells [7–10]. These observations implicate differences in the physiological roles of NOS2-derived NO in the two species. For example, elevated NOS2-derived NO generated by murine immune cells acts as an immunotoxin [10,11]. In contrast, human NOS2-derived NO is generated at a much lower flux and mediates pro-survival signaling and cancer progression [7,12]. The flux-dependent role of NO suggests that care must be taken when extrapolating data from murine models to humans. Nonetheless, understanding of the chemical biology of NO has relied heavily on analysis in both humans and rodents.

In the 1980s, Hibbs and coworkers implicated a role for NO in the tumoricidal effects of activated murine macrophages [13,14]. Mechanistically, Stuehr and Nathan later reported the induction of apoptosis in leukemic cells by NOS2-derived NO [15]. Generation of NOS2-derived NO by cytokine stimulated rodent vascular cells also imparted cytotoxic effects in a human erythroleukemia cell co-culture model, through the involvement of nonheme iron-nitrosyl complex formation and inhibition of mitochondrial complexes I and II [16]. Similarly, Xie and coworkers demonstrated anti-tumor activity of transfected murine NOS2 in a variety of murine cancer cell lines [17]. This tumoricidal activity involved high NO fluxes, leading to nitrosation of amines to form carcinogenic nitrosamines in murine macrophages [18].

Tannenbaum and coworkers first reported elevated nitrite/nitrate levels in the urine of a subset of healthy volunteers who were immune stimulated due to a temporary infection, which suggested the involvement of NO during human response to disease and inflammation [19]. Subsequent studies demonstrated increased nitrite and nitrate in human hepatocytes, which lead to the cloning of human NOS2 [20–22]. Moncada and coworkers went on to assess NOS2 activity in human tumors and cancer cells [23–25]. They observed that while human tumor cells with high NOS2 expression grew slowly in culture, tumor xenografts of these cells exhibited more aggressive tumor characteristics, suggesting an oncogenic role of human NOS2 in disease progression. Ambs and Harris also showed that elevated NOS2 expression in human colon adenomas correlated with elevated tumor progression and angiogenesis [26–28]. They went on to show NOS2 regulation by p53 [29]; while high NO fluxes induced p53 stabilization, p53 inhibited NOS2 expression, indicating p53 regulation of NOS2 via negative feedback [30,31]. Thus, mutations in p53 can lead to chronic NOS2 expression in the tumor epithelium.

Jeannin and coworkers explored the role of NOS2 expression in tumor progression using two murine syngeneic models [32]. Two clonal populations were isolated from EMT-6 breast cancer cells; one clone (EMT-6J) expressed constitutive NOS2 and secreted high levels of NO while the other clone (EMT-6H) did not. Cytokine stimulation (IL-1β + TNF-α) induced NOS2 expression and nitrite/nitrate production in both clones with EMT-6J cells producing 15-fold higher NO than EMT-6H cells. *In vivo* studies demonstrated increased survival of EMT-6J tumor-bearing BALB/c mice when compared to the EMT-6H model, suggesting that high fluxes of NO abate tumor progression. Interestingly, when the same study was repeated in wild type and NOS2 knockout animals, tumor-bearing NOS2 knockout mice survived longer, independent of the cell clone xenograft. While elevated tumor NOS2 (15-fold greater) mediated cytotoxic and cytostatic effects on tumor growth, the authors also suggested that FAS-mediated cell death, and the involvement of CTL and NK cells may be important in the anti-tumor effects of high-flux NO. On a similar note, other reports have shown that NO donors can promote FAS and TRAIL apoptotic pathways through inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [33–37]. Thus, the impact of NO on cellular and disease processes is temporally-, spatially-, and concentration-dependent.

In contrast, our *in vitro* model indicated that NOS2 induction in human breast cancer cells produced an NO flux of <300 nM [7]. This level of NO promoted cell migration and drug resistance, which was abated by the NOS2 inhibitor aminoguanidine [7]. Importantly, aminoguanidine also dramatically decreased growth rates of MB-231 tumor xenografts and brain metastases [7]. Moreover, while the same level of NO activated NFκB, NO fluxes exceeding this level has inhibitory effects, as demonstrated by a bell shaped NO dose response. These results suggest that NO can inhibit pro-survival signaling through nitrosative mechanisms [38]. Similarly, reactive nitrogen species (RNS) associated with high NO flux mediated anti-tumor activity by inhibiting both EGFR and NFκB signaling [39]. Importantly, examination of the gene expression profile of high NOS2 expressing breast tumors suggests that this level of NO induces tumor biomarker expression that leads to increased metastasis and poor outcome [7,12]. Thus, when compared to human NOS2, fully activated murine NOS2 produces a higher localized flux of NO with vastly different effects.

Elevated tumor NOS2 expression in human tumors now correlates with increased aggressiveness in brain, lung, pancreas, breast and colon cancers [5,12,40–42] (Figure 1). Recent reports have shown that NOS2 expression in tumor versus leukocytes is a key determining factor that dictates tumor progression and immunosuppression. Elevated levels of NO in leukocytes increase cellular toxicity, which either directly or indirectly orchestrates the tumor-host immune response [43–45]. While there are contradicting reports with respect to the role of NOS2 in tumor progression, for a diagnostic point of view, NOS2 is not a single prognostic marker but rather should be employed in conjunction with other tumor biomarkers to yield detailed information about cancer phenotype. Such information may lead to improved treatment regimens and disease outcomes. Clinical studies demonstrating localization of NOS2 in the tumor cell vs. stroma or leukocyte have shown that elevated NOS2 expression in the tumor epithelium correlates with reduced disease-specific survival.

Thus, clinical studies implicate a key role of the spatial dependence of NO in modulating tumor behavior and tumor-host immune response.

Mimics of NO-flux Profile *In Vivo*

To understand the mechanisms of NO in cellular function and the dependence on NO concentration, various donors have been used to mimic NO flux conditions at the cellular level [46]. Toward this end, NONOates, due to their controlled release profiles with little influence on extraneous conditions, have emerged as indispensable tools to assess or mimic the effects of intracellular NOS-derived NO [47]. Using different NONOates at varying concentrations, our laboratory and others have identified NO flux-dependent signaling profiles that mediate different biological mechanisms. Brune and coworkers identified that HIF-1α and p53 levels are increased by different NO concentrations, thus demonstrating the NO flux-dependence of cell signaling [48–50]. Thomas *et al*. showed that low NO levels (< 50 nM steady state NO) increased cGMP-dependent mechanisms, while higher levels (500–800 nM steady state NO) led to increased HIF-1α and p53 [51,52]. Importantly, this work demonstrated a pro-tumorigenic level of NO at lower concentrations, while higher NO fluxes induced stress responses involving p53. Similar responses have been observed in a variety of cells including endothelial cells [53]. Taken together, NO-induced signaling occurs at concentrations ranging from high picomolar to low micromolar. This five-order of magnitude concentration range provides insight into the versatility of NO signaling while emphasizing the importance of concentration, spatial and temporal constraints, In particular, chromic exposure to elevated NO fluxes profoundly affects cancer phenotype [54,55].

Nitric Oxide as a Cancer Therapeutic

A wide range of NO-releasing compounds have been developed as potential therapeutic agents to exploit the diverse biological roles of NO. These donors range from organic nitrates such as glyceryl trinitrate or derivatized NSAIDs to *S*-nitrosothiols and NONOates [47,56,57]. Macromolecular NO-releasing scaffolds are also promising due to their ability to store and deliver larger NO payloads in a more controlled and effective manner compared to low molecular weight donors [58,59]. In addition to NO donors, there are numerous NOS inhibitors and scavengers that can be used for modulation of NO flux [60,61]. Strategically combining these donors and inhibitors may provide unique tools for exploring potential therapeutic applications of NO [62]. Also, drug resistance and the toxicity of therapeutics are both clinical hurdles. In the face of these obstacles, NO modulation has emerged as a powerful adjuvant for the hypersensitization of tumors to more traditional chemo- and radiation therapies. Furthermore, emerging evidence indicates that NO donors have the potential to function independently in the clinical management of cancer [63].

Nitric oxide donors of the NONOate class are routinely used as reliable sources of NO in laboratory settings [64,65]. Such donors have been instrumental in exploration of the concentration and temporal profiles of NO with respect to tumor response. However, using these compounds *in vivo* to treat solid tumors is problematic due to systemic hypotension, which has limited the amount of NO that can be delivered to the tumor. However, clinical application of sub-vasoactive levels of glyceryl trinitrate has shown some success in limiting

recurrence of prostate and lung cancers [66–68]. In addition, a new generation of prodrugs have emerged based on the strategy of activation at the lesion site to abate the adverse cardiovascular effects of systemically administered drugs that modulate NO flux [69].

Another area of research involves development of hybrid compounds of functional groups with potential therapeutic value conjugated with NO donors (e.g., ester nitrates, furoxans, benzofuroxans, NONOates, *S*-nitrosothiols, metal nitrosyl complexes) designed to release NO while maintaining the native drug activity. This approach has proved useful in targeting cardiovascular, inflammatory, bacterial, fungal, viral, parasitic, and ocular diseases as well as cancer [70]. Potent and selective NOS inhibitors are also being designed, often through an enzyme structure based process. However, the high homology shared by the NOS isoforms, is a challenge in this pursuit.

Several reports have suggested that NOS may generate nitroxyl (HNO) [71–74] in addition to NO, in a condition-dependent manner. In cardiovascular models, HNO donors were shown to elicit unique effects when compared to NO [75–77], thus suggesting related but distinct targeting mechanisms [78,79]. Donors of HNO has also been shown to have anticancer activity [80], in part due to modification of a critical thiol of the glycolytic enzyme GAPDH [80]. As with NO, several classes of HNO donor have been developed, including NONOates [81,82].

We recently demonstrated that the NONOate-based prodrugs DEA/NO-aspirin and IPA/NO-aspirin, which donate NO and HNO, respectively, were activated by esterase cleavage within the cell [83]. These prodrugs thus facilitate intracellular delivery of these redox modulators compared to the parent NONOates. Interestingly, the intracellular release of HNO by IPA/NO-aspirin and, to a lesser extent, NO by DEA/NO-aspirin inhibited proliferation of A549 non-small cell lung cancer cells. In contrast, the same prodrugs were not appreciable toxic toward primary human endothelial cells (HUVECs). This observation suggests that IPA/NO-aspirin and DEA/NO-aspirin induce cancer-specific anti-proliferative pathways. The prodrugs also were protective against the gastrointestinal toxicity associated with aspirin in rats.

Chemical Induction of NO and HNO Inside and Outside of the Cell

Our recent work has demonstrated a key role of NOS2 in estrogen receptor negative (ER(-)) breast cancer disease progression [7]. Here, we employed MB-231 ER(-) breast cancer cells to examine the presence of NOS2 responsive gene set. We also were sought to explore the effects of intracellular and extracellular delivery of HNO and NO. MB-231 cells were exposed to 50 μM aspirin, DEA/NO, IPA/NO, DEA/NO-aspirin, or IPA/NO-aspirin or to 10 mM NaOH (vehicle control) for 24 h. DEA/NO and IPA/NO are short-lived donors (half-lives of ∼5 min under physiological conditions [84,85]). Thus, NO/HNO levels will rise quickly to a maximum and then decay [86]. The aspirin derivatives are much more stable to spontaneous hydrolysis, with half-lives of 7.5 and ∼36 h [87]. In the presence of cellular esterases, hydrolysis is accelerated, such that complete dissociation of IPA/NO aspirin occurs in ∼150 min. DEA/NO-aspirin is somewhat longer-lived in cells. Slower donation will lead to lower, but more steady fluxes of HNO or NO.

Following treatment, RNAs were harvested with TRIzol, followed by reverse transcription and *in vitro* transcription to generate sufficient aRNA fragments, and then hybridized to an Affymetrix GeneChip Human Gene ST Array for transcriptome analysis. High throughput gene expression data were exported to BRB ArrayTools, Partek Genomic Analysis Suite for data mining and Ingenuity Pathway Analysis for pathway analysis.

For a global view of the RNA fingerprinting data, multivariate analysis was adopted. Non-supervised hierarchical clustering analysis using different combinations of linkage methods and distance measuring algorithms confirmed that the gene expression fingerprinting for different treatment groups is not only consistent but also unique (Figure 2). In order to identify unique gene subsets that can differentiate each treatment group, class comparison analysis was adopted to identify subsets of genes that are statistically significant and uniquely expressed among the treatment groups (Tables 1–6). Genes identified in the comparison analyses are components of various pathways, which as described below.

Nitrogen Oxide-Modulated Genes

microRNA 21 (*miRNA21*)

Micro RNAs (miRNA) are short non-coding RNAs that are involved in post-translational regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNA. *miR21* (aka oncomiR21) expression has been reported in various types of cancers including breast, colorectal and brain and is believed to be oncogenic [88–91]. In this regard, *miR21* negatively regulates the tumor suppressor PTEN [92]. Moreover, Harris and coworkers identified enhanced *miR21* and PI3K signaling in association with elevated NOS2 expression and KRAS activation in lung cancer cells [93]. *miR21* can be detected in human serum and has predicted poor therapeutic outcome in cancer patients [94–99].

When compared to control cells, IPA/NO caused roughly a three-fold increase in *miR21*, while IPA/NO-aspirin only modestly increased *miR21*. This may indicate a localization effect of HNO delivery. Interestingly, while *miR21* was induced by both DEA/NO-aspirin and IPA/NO-aspirin, our earlier studies have demonstrated cytotoxic activity of these prodrugs [100]. These contrasting observations may implicate *miR21* as a potential candidate for modulating drug resistance by these prodrugs.

Cystathionase (cystathionine γ-lyase; CTH)

Cystathionase catalyzes the last step in the trans-sulfuration pathway from methionine to cysteine and also can generate the endogenous signaling molecule hydrogen sulfide (H_2S). Pupo *et al*. reported that H_2S is pro-angiogenic and promotes migration in a tumor environment [101,102]. CTH also acts as a cysteine-protein sulfhydrase by mediating sulfhydration of target proteins. The sulfhydration reaction regulates target proteins including the NFκB subunit RelA, PTPN1 and GAPDH by converting -SH groups on specific cysteine residues to -SSH.

Our study demonstrated significant decreased *CTH* expression in the IPA/NO-aspirin treatment group when compared to control, which may in part explain the anti-angiogenic and anti-metastatic properties of HNO-releasing drugs. Fukuto and colleagues have

demonstrated HNO-mediated inhibition of GAPDH activity in human breast cancer cells and tumor xenografts, as well as reduced blood vessel density and growth of tumor xenografts [80]. The authors suggested that the anti-tumor mechanisms of HNO may stem from the inhibition of GAPDH to impede glycolysis and reduce HIF-1α levels and tumor angiogenesis. Importantly, this work demonstrated that the anti-cancer mechanisms of HNO are different from those of current clinically available anti-cancer therapeutics. Collectively, these observations suggest that HNO drugs, including IPA/NO-aspirin, may improve therapeutic tumor response.

Heat Shock 70 kDa Protein 1A/1B (HSPA1A/1B)

Heat shock 70 kDa proteins 1A and 1B are members of the heat shock protein 70 family. In conjunction with other heat shock proteins, these proteins stabilize existing protein aggregates and mediate the folding of newly translated proteins in the cytosol and in organelles. Both HSPA1A and HSPA1B are expressed in a cell-type-specific manner, and certain human tissues constitutively express varying levels of HSPA1 and HSPA2 proteins in a highly differentiated way [103]. Various diseases demonstrate elevation of HSP70 family proteins, such as human breast cancer, murine triple negative mammary cancer, metastatic breast cancer stem cells, hyperthermia, and adult T-cell leukemia [104–108]. Since silencing the HSP1A1 is cytotoxic to transformed but not normal cells, various HSP70 inhibitors are in development in the hope of triggering cell cycle arrest in cancer cells [109]. Unexpectedly, elevated HSP70 was found to be beneficial to therapy as reported in Nakatsu's breast cancer study, as increased HSPA1A enhanced sensitivity to anti-cancer drugs [110].

We found that HSP70 family proteins are significantly up-regulated in the IPA/NO-aspirin group and slightly up-regulated in the DEA/NO-aspirin and DEA/NO groups. To our surprise, IPA/NO itself down-regulates both HSPA1A and HSPA1B expression. This finding is in accordance with Nakatsu's work and strongly suggests that overexpressed HSP70 proteins triggers different downstream pathways than the commonly reported anti-apoptotic pathway.

Thioredoxin Interacting Protein (TXNIP)

Thioredoxin interacting protein mediates oxidative stress by inhibiting thioredoxin activity or by limiting its bioavailability [111]. Increased reactive oxygen species (ROS) production increases cancer progression in some tumors, including pancreatic cancer, while ROS inhibits disease progression in others such as hepatocellular carcinoma [112]. In general, TXNIP is thought to be a tumor suppressor. TXNIP functions as a transcriptional repressor, possibly by acting as a bridge molecule between transcription factors and corepressor complexes, and its over-expression induces G0/G1 cell cycle arrest [113]. In addition, TXNIP is required for the maturation of natural killer cells. It inhibits the proteasomal degradation of DDIT4 and thereby contributes to the inhibition of the mammalian target of rapamycin complex 1 (mTORC1). Elevated TXNIP has been associated with poor histological grade in phyllodes tumors [114].

Our analysis demonstrated significantly reduced *TXNIP* expression levels in only the IPA/NO-aspirin and DEA/NO-aspirin treatment groups, suggesting that IPA/NO-aspirin or DEA/NO-aspirin combined with mTOR inhibitors may further improve the cytotoxic responses of these prodrugs.

DNA-Damage-Inducible Transcript 3 (DDIT3)

DNA-damage-inducible-transcript 3 is a multifunctional transcription factor in endoplasmic reticulum (ER) stress response. It plays an essential role in the response to a wide variety of cellular stress and induces cell cycle arrest and apoptosis in response to ER stress [115]. DDIT3 positively regulates transcription of *TRIB3, IL6, IL8, IL23, TNFRSF10B/DR5, PPP1R15A/GADD34, BBC3/PUMA, BCL2L11/BIM* and *ERO1L* and negatively regulates expression of *BCL2* and *MYOD1*, ATF4-dependent transcriptional activation of asparagine synthetase (ASNS), CEBPA-dependent transcriptional activation of hepcidin (HAMP) and CEBPB-mediated expression of peroxisome proliferator-activated receptor gamma (PPARG) [115]. DDIT3 plays a regulatory role in the inflammatory response through the induction of caspase-11 (CASP4/CASP11), which induces the activation of caspase-1 (CASP1) [115]. Both caspases increase activation of pro-IL1β to mature IL-1β, which mediates inflammatory response. NO-induced apoptosis has been reported in pancreatic β cells, p53-deficient microglial cells and RAW 264.7 cells [116–118], which are triggered by DDIT3 through ER stress. Also, DDIT3 inhibits the canonical WNT signaling pathway by binding to TCF7L2/TCF4, impairing its DNA-binding properties and repressing its transcriptional activity.

Our study demonstrated decreased *DDIT3* by IPA/NO-aspirin. This may indicate that IPA/NO-aspirin and WNT inhibitors may be attractive drug combinations.

Nitric Oxide Driven Pathways

Using the Canonical Pathway Analysis module in the Ingenuity Pathway Analysis portal reveals the most significantly affected cellular pathways to be: (1) endothelin-1 (ET1) signaling, (2) NO signaling in the cardiovascular system, (3) relaxin signaling, (4) G-protein coupled receptor signaling and (5) HER2 signaling in breast cancer. Given that ET1 and NO signaling are known to play key roles in the cardiovascular system, it is not surprising to see that these two pathways are among the highest-scoring in our analysis.

(1&2) Endothelin-1

ET1 is a 21-amino acid vasoconstrictor peptide, which is able to induce cardiac hypertrophy. ET1 synthesis includes NO and its intracellular effectors cGMP, prostacyclin, atrial natriuretic peptides, and steroid hormones [119]. ET1 is considered a stress-responsive regulator working in a paracrine and autocrine fashion in a variety of organs, with both beneficial and detrimental roles in mammals [120]. In addition to its potent cardiovascular actions, ET1 causes contraction of nonvascular smooth muscle; stimulation of the release of neuropeptides, pituitary hormones, and atrial natriuretic peptide; biosynthesis of aldosterone; modulation of neurotransmitter release; and increase of bone resorption [121,122]. Furthermore, ET1 has mitogenic properties, causing proliferation and hypertrophy of

vascular smooth muscle, cardiac myocytes, mesangium, bronchial smooth muscle, and fibroblasts. ET1 also induces expression of several proto-oncogenes, including c-Fos, c-Jun, and c-Myc [123–125]. These actions are of potential significance in chronic congestive heart failure, renal disease, hypertension, cerebral vasospasm, and pulmonary hypertension, all of which are conditions commonly associated with increased expression of ET1 [126].

(3) Relaxin

Relaxin is a polypeptide hormone that is secreted by the corpus luteum into the circulation during the menstrual cycle and throughout pregnancy [127]. Binding of relaxin to its receptor also activates a tyrosine kinase pathway that inhibits the activity of a phosphodiesterase (PDE) that degrades cAMP. The consequent rise in cAMP levels activates protein kinase A (PKA), which leads to activation of transcription factors like the cAMP response-element binding protein and NFκB. PKA phosphorylates and inactivates I-kappa-B-α, the inhibitor subunit of the transcription factor NFκB, thus allowing NFκB to translocate into the nucleus and bind the NOS promoter, resulting in expression of NOS2. Relaxin can act on several of its targets by increasing the expression and/or activity of NOS isoenzymes, thereby promoting generation of NO. NOS3 can be activated by direct stimulation of the β and γ subunits of the g-proteins. This is accomplished by means of stimulation of PI3-kinase, followed by Akt/PKB, which in turn activates NOS3 by phosphorylation at Ser-1179 [128]. By stimulation of distinct signal transduction pathways, relaxin can have pleiotropic downstream effects, many of which may have useful clinical applications [129]. Relaxin elicits a vasodilatory response in several target organs. This response is mediated by the stimulation of intrinsic NO generation. In the uterus NO production via NOS is up-regulated during pregnancy by relaxin. NO induces uterine quiescence, which is deemed necessary for the maintenance of pregnancy. Relaxin increases intracellular cGMP levels in a concentration-related fashion. This effect of relaxin is likely a consequence of stimulation of NO production by this hormone. In fact, NO binds to the heme iron of soluble guanylate cyclase and thereby activates the synthesis of cGMP [130,131]. In turn, increased production of cGMP plays an important role in vasorelaxation. Moreover, relaxin induces changes in cell shape and the actin cytoskeleton that are consistent with cell relaxation [128].

(4) G-protein Coupled Receptors (GPCRs)

G-protein-coupled receptors are activated by a wide variety of external stimuli. Upon receptor activation, the G-protein exchanges GDP for GTP, causing dissociation of the GTP-bound α and β/γ subunits and triggering diverse signaling cascades. Receptors coupled to different heterotrimeric G-protein subtypes can utilize different scaffolds to activate the small G-protein/MAPK cascade, employing at least three different classes of tyrosine kinases. Src family kinases are recruited following activation of PI3Kγ by the β/γ subunits. They are also recruited by receptor internalization, cross-activation of receptor Tyr kinases, or by signaling through an integrin scaffold involving Pyk2 and/or FAK. GPCRs can also employ PLCβ to mediate activation of PKC and CaMKII, which can have either stimulatory or inhibitory consequences for the downstream MAPK pathway [132–134].

(5) HER2 signaling in breast cancer

Dysregulation of HER-mediated signaling pathways results in the growth and spread of cancer cells [135]. Inappropriate signaling may occur as a result of receptor overexpression or dysregulation of receptor activation, which may lead to increased or uncontrolled cell proliferation, decreased apoptosis, enhanced cancer cell motility, and angiogenesis [136–138].

Future for Diagnosis

Although the molecular and genetic determinants of most sporadic breast cancers remain unclear, significant advances in the understanding of events that contribute to breast cancer formation have been made. Deactivation mutations in tumor suppressor genes, such as p53, BRCA1, BRCA2, PTEN, or ATM, epigenetic functional inactivation of other tumor suppressor genes such as SYK and NES1, or activation of proto-oncogenes, such as HER2/Neu, can all play important roles in breast carcinogenesis [139]. Breast cancer is a clinically heterogeneous disease, and there is evidence that the varied clinical courses of patients with histologically similar tumors are due to molecular differences among cancers. Therefore, detailed molecular analysis of the cancer could yield important diagnostic and prognostic information as well as aid in the design of treatment regimens. Recent advances in molecular analytical techniques have led to a rapid expansion of new diagnostics designed to personalize breast cancer care [140]. However, approximately one-quarter of patients with lymph node-negative disease and one half of patients with lymph node-positive tumors will ultimately develop distant recurrent breast cancer. Standard treatment of metastatic breast cancer generally includes systemic treatment and surgery or radiation as needed and when indicated for palliation of localized symptomatic metastases [141]. The immediate challenge is to learn how to utilize the molecular characteristics of an individual and their tumor to improve tumor detection rate, enhance treatment effectiveness, reduce metastasis, and ultimately to prevent the development of breast cancer recurrence.

To better predict the clinical outcome of malignant disease, researchers have tried to use NOS2 to predict prognosis in various diseases. Toward this end, Huang has shown that NOS2 expression was higher in lymph node metastasis and recurrent groups in supraglottic squamous cell carcinoma [142]. Later, Hara and Qiu reported that elevated NOS2 expression predicted poor survival in human astrocytic glioma and immunoglobulin A nephropathy (IgAN) [143,144]. In addition, Li reported that increased NOS2 and nitrotyrosine (NT) predicted poor survival of gastric adenocarcinoma patients [145]. Also Pinlaor reported that the co-activation and co-localization of HIF1A and NOS2 was associated with poor survival of intrahepatic cholangiocarcinoma [146]. As mentioned earlier, Glynn has shown that NOS2 expression predicted poor survival of ER- breast cancer patients [12,147]. Zhang and Wang added that NOS2 expression also correlated with poor prognosis in gastric cancer patients and salivary gland adenoid cystic carcinoma [95,148]. Evidence supports the strategic combination of NOS2 with other relevant biomarkers, which could greatly improve the prognostic accuracy of various malignancies.

Besides genetically engineered mice, xenograft transplantation is another common tool adopted for cancer research. Xenograft transplantation via intravenous, intraperitoneal,

subcutaneous or orthotopic injection is a well-defined approach for breast cancer metastasis research and has been utilized for years. Since metastasis is a multi-step process, and cancer interact differently with its surrounding microenvironment is a crucial step, spontaneous metastasis from primary tumor provides an improved model to mimic human breast cancer [149]. Interestingly, tumor cells targeting different target organs have distinct gene expression profiles, and this *in vivo* selection experiment allows efficient identification of target genes and specific drugs to treat metastatic breast cancer patients. In our murine model, xenograft transplantation (231-GFP) under the mammary fat pad leads to spontaneous brain metastasis and some lung metastasis, thus providing an excellent orthotopic model for breast cancer metastasis research. Moreover, our NO/HNO prodrugs provided a class of compound that may be used together with other therapeutic agents (chemotherapy, radiation, etc.) to enhance tumor killing and reduce metastasis.

Conclusion

In this study, we identified distinct molecular signatures from NO- and HNO-donor treated breast cancer cells. Given that other cancer cells may be activated/modulated by these molecular changes, we suggest that other cell types may show similar cellular responses in the presence of NO- and HNO-donors. We are in the process of testing this hypothesis in lung and pancreatic cancer cells. However, due to the heterogeneity and sporadic nature of cancer, we may not expect that other cell types will show the exact molecular signatures as breast cancer cells. Nonetheless, such comparisons across cell types would assist in discovery and identification of key gene sets that governing the molecular response to NO and HNO.

In order to accurately assess the effects of NO production, it is important to have a basic understanding of the spatial and temporal distributions of NO at the cellular level. The latest developments in NONOate derivatives and animal models are expected to facilitate deciphering of the mechanisms by which NO modulates cellular responses under different stress conditions. Our group identified uniquely expressed genes such as *mir21*, *CTH*, *HSP70*, *TXNIP* and *DDIT3*, and key pathways such as ET-1 and relaxin signaling, in response to NO. These findings enable the fine mapping of the cellular responses modulated by NO. Strikingly, we demonstrated that the HNO releasing donor, IPA/NO, is able to suppress *HSP70* gene expression which functions like a HSP70 inhibitor while the IPA/NO derivative IPA/NO-aspirin can elevate *HSP70* gene expression to a much higher level. This could trigger an alternate cellular pathway that also leads to cancer cell death. Taken together, our findings provide insight in evaluating and improving the cytotoxicity of the NONOate derivatives especially IPA/NO-aspirin in cancer therapy.

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Abbreviations

231-GFP	MDA-MB-231 cells stably transfected with green fluorescent protein
AKT	V-Akt murine thymoma viral oncogene homolog 1
aRNA	amplified RNA
ASNA	asparagine synthetase
ATM	ataxia telangiectasia mutated
BBC3	BCL2 binding component 3
BCL2	B-cell CLL/lymphoma 2
BCL2L11	BCL2-like 11 (apoptosis facilitator)
BIM	BCL2-interacting mediator
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
CaMKII	calcium/calmodulin-dependent protein kinase II inhibitor 1
cAMP	cyclic adenosine monophosphate
CASP1	caspase 1, apoptosis-related cysteine peptidase
CASP11	SR-related CTD-associated factor 1 (SCAF1)
CASP4	caspase 4, apoptosis-related cysteine peptidase
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
c-Fos	FBJ murine osteosarcoma viral oncogene homolog
c-Jun	jun proto-oncogene
c-Myc	v-myc avian myelocytomatosis viral oncogene homolog
cGMP	cyclic guanosine monophosphate
CTC	circulating tumor cells
CTH	cystathionase (cystathionine-gamma-lyase)
CTL	cytotoxic T cell
DDIT3/4	DNA-damage-inducible transcript ¾
DEA/NO	diethylaminamine NONOate
DEA/NO-aspirin	DEA/NO-aspirin conjugate
DR5	death receptor 5
EGFR	pidermal growth factor receptor
ER	estrogen receptor

ERO1L	endoplasmic oxidoreductin-1-like protein \ (*S. cerevisiae*); ET1,endothelin-1
FAK	protein tyrosine kinase 2
FAS	Fas cell surface death receptor
GADD34	growth arrest and DNA damage-inducible protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	cyclic guanosine diphosphate
GPCR	G-protein-coupled receptor
GTP	cyclic guanosine triphosphate
HAMP	hepcidin antimicrobial peptide
HER2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
HIF1 α	hypoxia inducible factor 1 α
HNO	nitroxyl
H_2S	hydrogen sulfide
HSPA1A/1B	heat shock 70 kDa protein 1A/1B
HSP70	heat shock protein 70
IgAN	immunoglobulin A nephropathy
IL1β	interleukin 1 beta
IL23	interleukin 23
IL24	interleukin 24
IL6	interleukin 6
IL8	interleukin 8
IPA/NO	isopropylamine NONOate
IPA/NO-aspirin	IPA/NO-aspirin conjugate
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	mitogen-activated protein kinase
MB-231	MDA-MB-231 cells
mir21	microRNA 21
MMTV	mouse mammary tumor virus
mTOR	mechanistic target of rapamycin (serine/threonine kinase)
MYOD1	myogenic differentiation 1
NES1	kallikrein-related peptidase 10 (KLK10)

Neu	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
NSAID	on-steroidal anti-inflammatory drug
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NK	natural killer
NO	nitric oxide
NOS	nitric oxide synthase
NOS2	nitric oxide synthase 2 (inducible NOS)
NOS3	nitric oxide synthase 3 (endothelial NOS)
NT	nitrotyrosine
PDE	phosphodiesterase
PI3K	phosphoinositide-3-kinase
PLCβ	phospholipase C, beta
PKA	protein kinase A
PKB	protein kinase B
PKC	proetin kinase C
PPARG	peroxisome proliferator-activated receptor gamma
PPP1R15 A	protein phosphatase 1, regulatory subunit 15A
PTEN	phosphatase and tensin homolog
PTPN1	protein tyrosine phosphatase, non-receptor type 1
PUMA	P53 upregulated modulator of apoptosis
Pyk2	PITPNM family member 3 (PITPNM3)
RelA	v-rel avian reticuloendotheliosis viral oncogene homolog A
RNS	reactive nitrogen species
ROS	reactive oxygen species
Src	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SYK	spleen tyrosine kinase
TCF4	transcription factor 4
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)
TNFa	tumor necrosis factor alpha
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b
TRAIL	TNF-related apoptosis-inducing ligand
TRIB3	tribbles pseudokinase 3

TXNIP	thioredoxin interacting protein
WAP	whey acidic protein
WNT	wingless-type MMTV integration site family

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Highlights

- The biological effects of NO and HNO are governed by their cellular concentrations, plus spatial and temporal distribution both within tumor cells and in the tumor microenvironment.
- Various NO- and HNO-releasing compounds have been developed as potential therapeutics, including as tumor modulators.
- Whole genome gene expression profiling analysis indicates NO and HNO are driven different molecular pathways.

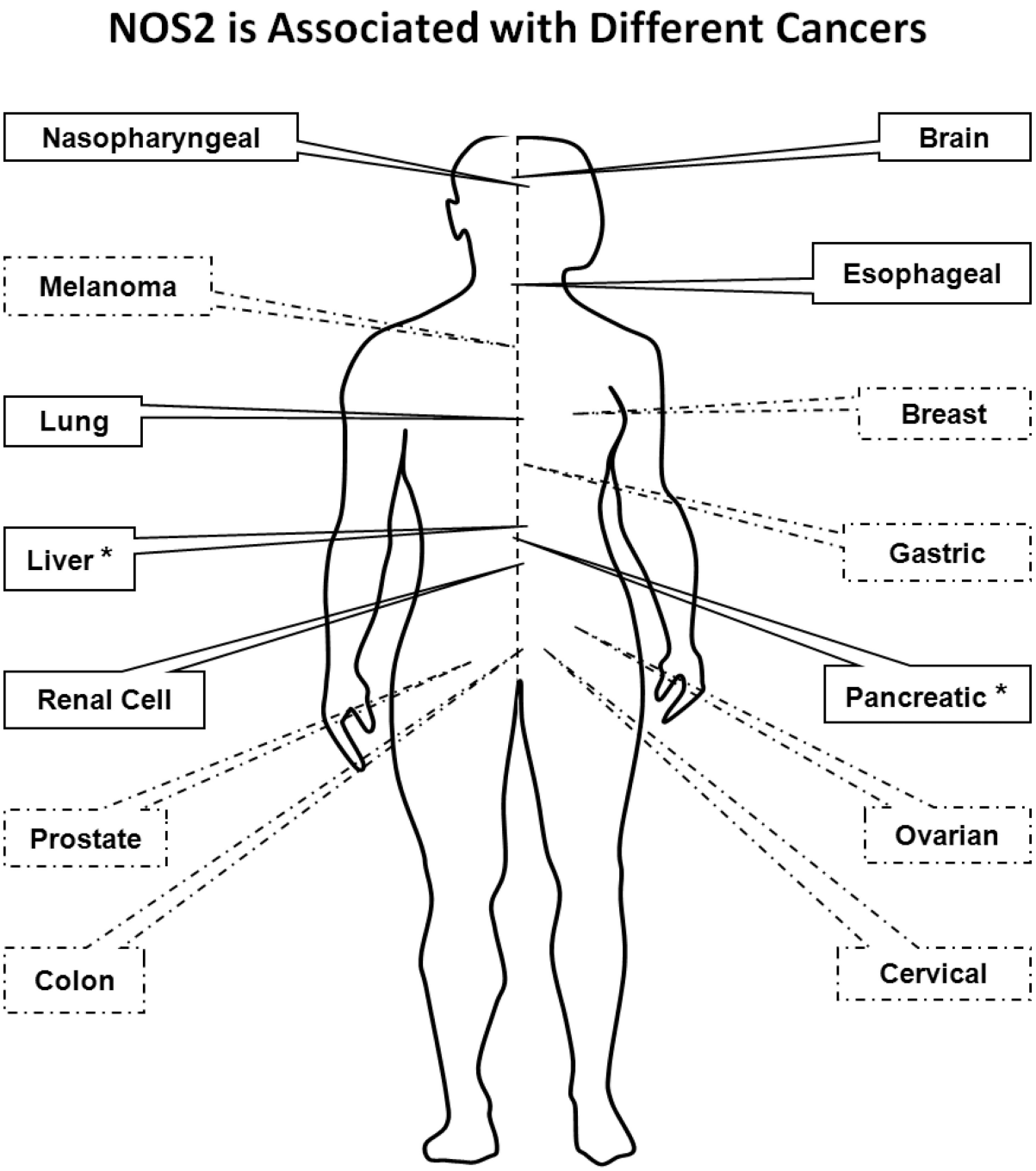
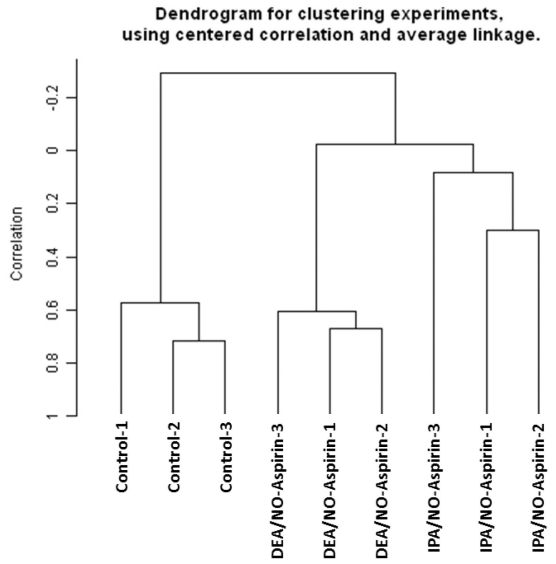


Figure 1.

NOS2 is associated with different cancers. Dashed lines: predict poor prognosis. Solid lines: weak to not predictive or not studied. Asterisk: some studies indicate that NOS2 is associated with poor prognosis.

A



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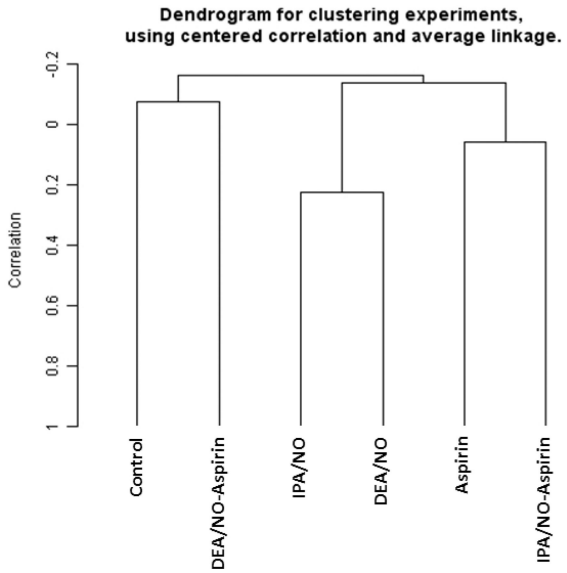

Figure 2.

Hierarchical Clustering Analysis. Non-supervised learning hierarchical clustering analysis computed with different combinations of single, complete and average linkage methods and Euclidean, centered correlation and uncentered correlation distance measurement algorithms. Figure 2A showed the clear separation of control, IPA/NO-aspirin and DEA/NO-aspirin groups. Figure 2B showed the clustering patterns when all six treated groups were analyzed together.

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Table 1

Class Comparison Analysis: Differentially expressed genes in control vs IPA/NO vs DEA/NO

Parametric p-value	FDR	Control	IPA/NO aspirin	DEA/NO aspirin	Symbol	Name
5.00E-07	0.00044	158.29	182.26	357.41	MIR21	microRNA 21
1.20E-06	0.00044	134.74	374.85	168.1	HSPA1A	heat shock 70kDa protein 1A
1.60E-06	0.00044	178.03	440.57	229.01	HSPA1B	heat shock 70kDa protein 1B
2.60E-06	0.000557	691.18	307.05	532.37	CTH	cystathionase (cystathionine gamma-lyase)
5.40E-06	0.000946	550.46	259.01	346.26	GTPBP2	GTP binding protein 2
6.70E-06	0.00108	69.32	37.01	52.14	SYT1	synaptotagmin I
7.70E-06	0.00114	276.85	470.1	427.43	MASTL	microtubule associated serine/threonine kinase-like
9.00E-06	0.00122	129.41	71.47	103.75	ZNF643	zinc finger protein 643
1.31E-05	0.00146	47.71	80.12	76.08	KATNAL1	katanin p60 subunit A-like 1
1.85E-05	0.00178	113.37	183.65	130.16	ARRB1	arrestin, beta 1
2.53E-05	0.00212	60.59	125.97	70.11	MIR27B	microRNA 27b
3.31E-05	0.00251	27.11	48.35	26.25	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
3.39E-05	0.00251	43	81.96	62.54	CSNK1G1	casein kinase 1, gamma 1
3.73E-05	0.00255	141.68	255.44	216.93	CDCA3	cell division cycle associated 3
3.84E-05	0.00255	310.44	136.8	270.54	DDIT3	DNA-damage-inducible transcript 3
4.50E-05	0.00289	1031.9	1113.08	1691.28	MMP1	matrix metallopeptidase 1 (interstitial collagenase)
4.79E-05	0.00298	26.13	37.57	49.8	C5orf54	chromosome 5 open reading frame 54
5.81E-05	0.00334	79.86	77.24	132.14	MIR100	microRNA 100
5.89E-05	0.00334	488.23	274.58	356.01	UFM1	ubiquitin-fold modifier 1
6.35E-05	0.0034	1770.85	826.61	894.51	TXNIP	thioredoxin interacting protein
6.84E-05	0.0034	289.23	156.84	189.52	MXD1	MAX dimerization protein 1
7.23E-05	0.0034	54	111.12	75.49	TGFBR3	transforming growth factor, beta receptor III
8.39E-05	0.00385	145.67	216.93	105.31	SNORD14E	small nucleolar RNA, C/D box 14E
8.80E-05	0.00394	97.67	135.92	148.06	FAM36A	family with sequence similarity 36, member A

Parametric p-value	FDR	Control	IPA/NO aspirin	DEA/NO aspirin	Symbol	Name
0.0001013	0.00438	16.26	41.79	25.53	KLRC4-KLRK1	KLRC4-KLRK1 readthrough
0.0001033	0.00438	186.29	219.91	320.13	CPA4	carboxypeptidase A4
0.0001045	0.00438	819.14	396.85	530.51	SLFN5	schlafen family member 5
0.0001127	0.00456	44.67	70.34	49.72	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
0.0001186	0.00466	151.53	241.16	184.47	SOS1	son of sevenless homolog 1 (Drosophila)
0.0001275	0.00491	125.65	56.4	94.63	SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)
0.0001711	0.00611	37.19	56.42	33.29	KLRC4	killer cell lectin-like receptor subfamily C, member 4
0.0001782	0.00624	32.9	55.11	35.37	CYP1A1	cytochrome P450, family 1, subfamily A,
						polypeptide 1
0.0002038	0.00687	95.02	163.46	105.71	TFAP2C	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)
0.0002069	0.00687	145.58	217.73	265.69	HIST1H2AK	histone cluster 1, H2ak
0.0002325	0.00747	30.48	42	53.64	MIR15A	microRNA 15a
0.0002495	0.00788	1318.22	731.84	1096.74	ASNS	asparagine synthetase (glutamine-hydrolyzing)
0.0002608	0.00811	55.23	70.89	63.14	TAS2R31	taste receptor, type 2, member 31
0.0002742	0.00826	321.72	184.71	240.24	TRIML2	tripartite motif family-like 2
0.0003194	0.00947	40.67	19.71	27.67	OLAH	oleoyl-ACP hydrolase
0.0003389	0.0096	143.33	262.36	199.65	FAM102B	family with sequence similarity 102, member B
0.0003919	0.0103	110.28	73.16	72.47	ANKRD11	ankyrin repeat domain 11
0.0004377	0.011	191.39	349.72	290.22	GMNN	geminin, DNA replication inhibitor
0.0004424	0.011	722.45	438.41	647.09	CASP4	caspase 4, apoptosis-related cysteine peptidase
0.0004676	0.011	470.21	848.73	689.63	HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)
0.0004948	0.0111	123.33	272.9	202.96	FAM111B	family with sequence similarity 111, member B
0.0005848	0.0122	80.38	103.67	96.64	ZNF699	zinc finger protein 699
0.0007473	0.0147	83.91	164	155.71	CENPI	centromere protein I
0.0007983	0.0151	442.05	561.76	586.57	CDK1	cyclin-dependent kinase 1
0.0008052	0.0151	41.62	17.11	27.66	C12orf39	chromosome 12 open reading frame 39

Parametric p-value	FDR	Control	IPA/NO aspirin	DEA/NO aspirin	Symbol	Name
0.0008272	0.0152	276.03	131.2	174.89	GDF15	growth differentiation factor 15
0.0009894	0.0177	129.32	183.21	159.07	PLK4	polo-like kinase 4
0.0010473	0.0182	190.18	247.79	233.37	GPR125	G protein-coupled receptor 125
0.0010698	0.0184	101.54	51.49	55.33	UFM1	ubiquitin-fold modifier 1
0.0011657	0.0197	36.87	36.84	26.34	SNORA2B	small nucleolar RNA, H/ACA box 2B
0.0011932	0.02	25.76	18.03	16.58	TNFSF18	tumor necrosis factor (ligand) superfamily, member 18
0.0013126	0.0213	78.71	152.05	121.04	ORC1	origin recognition complex, subunit 1
0.0013441	0.0216	32.29	50.95	43.56	DSEL	dermatan sulfate epimerase-like
0.0013774	0.0219	81.09	110.77	97.89	SERTAD4	SERTA domain containing 4
0.0014926	0.0233	161.4	206.74	119.33	SNORD12C	small nucleolar RNA, C/D box 12C
0.0015019	0.0233	184.74	180.78	124.2	EIF4A2	eukaryotic translation initiation factor 4A2
0.001705	0.0246	35.9	54.09	43.2	CDRT1	CMT1A duplicated region transcript 1
0.0018118	0.0255	140.04	220.86	208.61	MMS22L	MMS22-like, DNA repair protein
0.0018598	0.026	362.63	592.15	516.16	SKP2	S-phase kinase-associated protein 2 (p45)
0.0019382	0.0267	33.46	57.16	41.69	PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)
0.0022295	0.0296	148.85	208.38	179.76	TANC2	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2
0.002407	0.0309	122.27	209.4	183.63	ESCO2	establishment of cohesion 1 homolog 2 (S. cerevisiae)
0.0024782	0.0316	299.78	478.11	464.06	WDHD1	WD repeat and HMG-box DNA binding protein 1
0.0026491	0.0333	81.77	114.13	75.33	S100A4	S100 calcium binding protein A4
0.0027266	0.0338	53.48	91.61	72.04	SLC38A4	solute carrier family 38, member 4
0.002739	0.0338	62.92	40.49	47.37	SLC5A4	solute carrier family 5 (low affinity glucose cotransporter), member 4
0.0028887	0.0349	834.01	538.55	516.07	SNORD15B	small nucleolar RNA, C/D box 15B
0.0030393	0.0353	66.68	88.7	91.01	LRRCC1	leucine rich repeat and coiled-coil domain containing 1
0.0034645	0.0384	69.46	107.45	91.53	BMP4	bone morphogenetic protein 4
0.0034884	0.0384	919.74	1180.48	863.52	SNORD30	small nucleolar RNA, C/D box 30
0.0039179	0.0412	241.05	341.9	337.09	HSPB11	heat shock protein family B (small), member 11

Parametric p-value	FDR	Control	IPA/NO aspirin	DEA/NO aspirin	Symbol	Name
0.0039544	0.0412	121.73	189.61	186.52	CCNE2	cyclin E2
0.003966	0.0412	100.5	161.62	144.65	HIST1H3A	histone cluster 1, H3a
0.0045144	0.044	127.21	82.32	100.69	U2AF1L4	U2 small nuclear RNA auxiliary factor 1-like 4
0.0045306	0.044	89.42	133.28	131.31	NMU	neuromedin U
0.0047269	0.0451	79.93	37.91	57.23	IL24	interleukin 24
0.00486	0.0456	76.16	97.2	108.87	HSD17B7P2	hydroxysteroid (17-beta) dehydrogenase 7 pseudogene 2
0.0053066	0.0478	42.77	27.46	30.84	RAB7A	RAB7A, member RAS oncogene family
0.0055138	0.0488	34.57	49.41	39.87	OVOS	ovostatin
0.0059611	0.052	83.56	108.25	85.13	SNORD4B	small nucleolar RNA, C/D box 4B
0.0061285	0.0527	52.16	32.28	28.13	NOL7	nucleolar protein 7, 27kDa
0.0068846	0.0562	69.47	97.5	98.3	FANCB	Fanconi anemia, complementation group B
0.0070186	0.0568	23.73	22.28	26.93	MC3R	melanocortin 3 receptor
0.0071233	0.0574	301.44	496.32	409.56	CLSPN	claspin
0.0081236	0.0616	195.84	213.56	150.45	SNORA62	small nucleolar RNA, H/ACA box 62
0.0088207	0.0646	73.42	109.89	61.49	FABP4	fatty acid binding protein 4, adipocyte
0.0092606	0.0661	49.63	34.77	31.84	LLPH	LLP homolog, long-term synaptic facilitation (Aplysia)
0.0097317	0.0679	118.67	147.13	149.61	SCAI	suppressor of cancer cell invasion
0.0098834	0.0685	32.32	50.91	45.37	GPR137C	G protein-coupled receptor 137C

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Table 2

Class Comparison Analysis: Differentially expressed genes in aspirin vs Control vs DEA/NO vs DEA/NO- aspirin vs IPA/NO vs IPA/NO-aspirin

Parametric p-value	FDR	Aspirin	Control	DEA/NO	DEA/NO aspirin	IPA/NO	IPA/NO aspirin	Symbol	Name
< 1e-07	< 1e-07	157.71	134.74	180.66	168.1	138.36	374.85	HSPA1A	heat shock 70kDa protein 1A
1.00E-07	3.85E-05	217.58	178.76	253.16	228.54	196.13	441.1	HSPA1B	heat shock 70kDa protein 1B
2.00E-07	6.42E-05	129.96	158.29	212.72	357.41	336.45	182.26	MIR21	microRNA 21
3.70E-06	0.000732	2063.12	1770.85	1458.8	894.51	1869.4	826.61	TXNIP	thioredoxin interacting protein
3.80E-06	0.000732	520.53	550.46	407.47	346.26	479.93	259.01	GTPBP2	GTP binding protein 2
4.50E-06	0.000788	648.79	691.18	598.54	532.37	628.32	307.05	CTH	cystathionase (cystathionine gamma-lyase)
6.18E-05	0.00791	817.15	819.14	695	530.51	772.75	396.85	SLFN5	schlafen family member 5
7.29E-05	0.00791	34.78	28.38	58.9	22.48	27.46	32.25	FLJ16171	FLJ16171 protein
7.36E-05	0.00791	1230.98	1318.22	1113.71	1096.74	1284.57	731.84	ASNS	asparagine synthetase (glutamine-hydrolyzing)
7.59E-05	0.00791	123.22	113.37	113.62	130.16	132.96	183.65	ARRB1	arrestin, beta 1
9.90E-05	0.00954	55.15	54	71.05	75.49	69.29	111.12	TGFBR3	transforming growth factor, beta receptor III
0.0001065	0.00977	344.03	310.44	239.07	270.54	401.34	136.8	DDIT3	DNA-damage-inducible transcript 3
0.000115	0.0101	60.95	69.32	64.71	52.14	63.82	37.01	SYT1	synaptotagmin I
0.0001325	0.0109	109.96	125.65	116.2	94.63	103.04	56.4	SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)
0.0001818	0.014	275.67	289.23	173.28	189.52	241.75	156.84	MXD1	MAX dimerization protein 1
0.000231	0.0167	174.29	186.29	176.02	320.13	172.25	219.91	CPA4	carboxypeptidase A4

Parametric p-value	FDR	Aspirin	Control	DEA/NO	DEA/NO aspirin	IPA/NO	IPA/NO aspirin	Symbol	Name
0.0002426	0.0167	30.09	27.11	32.82	26.25	25.56	48.35	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
0.0003971	0.0225	116.22	145.67	199.52	105.31	103.5	216.93	SNORD14E	small nucleolar RNA, C/D box 14E
0.0004856	0.026	113.64	97.67	192.34	148.06	165.15	135.92	FAM36A	family with sequence similarity 36, member A
0.0005102	0.0262	46.31	44.67	50.76	49.72	42.76	70.34	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
0.0006235	0.0308	28.1	37.96	22.55	30.14	28.77	28.42	CXCL2	chemokine (C-X-C motif) ligand 2
0.0007044	0.0335	343.39	321.72	293.69	240.24	288.81	184.71	TRIML2	tripartite motif family-like 2
0.0007155	0.0335	163.87	184.74	198.14	124.2	103.51	180.78	EIF4A2	eukaryotic translation initiation factor 4A2
0.0007603	0.0335	242.43	276.03	160.58	174.89	269.25	131.2	GDF15	growth differentiation factor 15
0.0007652	0.0335	46.17	43	67.92	62.54	49.22	81.96	CSNK1G1	casein kinase 1, gamma 1
0.0009788	0.0355	471.51	488.23	502.42	356.01	449.83	274.58	UFM1	ubiquitin-fold modifier 1
0.0009939	0.0355	79.45	89.42	95.62	131.31	83.43	133.28	NMU	neuromedin U
0.001165	0.0401	101.31	95.02	114.88	105.71	110.36	163.46	TFAP2C	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)
0.0016524	0.0506	662.91	722.45	664.69	647.09	696.38	438.41	CASP4	caspase 4, apoptosis-related cysteine peptidase
0.0018161	0.0538	149.69	141.68	216.36	216.93	156.4	255.44	CDCA3	cell division cycle associated 3
0.0019214	0.0553	137.44	123.33	219.24	202.96	130.18	272.9	FAM111B	family with sequence similarity 111, member B
0.0019549	0.0554	98.22	83.91	121.46	155.71	95.21	164	CENPI	centromere protein I
0.0021586	0.0562	29.63	26.13	32.67	49.8	39.36	37.57	C5orf54	chromosome 5 open

Parametric p-value	FDR	Aspirin	Control	DEA/NO	DEA/NO aspirin	IPA/NO	IPA/NO aspirin	Symbol	Name
									reading frame 54
0.0022755	0.0585	53.28	47.71	62.76	76.08	49.37	80.12	KATNAL1	katanin p60 subunit A-like 1
0.0026234	0.0647	32.01	30.48	56.94	53.64	50.44	42	MIR15A	microRNA 15a
0.0026532	0.0647	37.97	41.62	37.8	27.66	38.07	17.11	C12orf39	chromosome 12 open reading frame 39
0.002932	0.0689	87.1	79.86	121.93	132.14	150.85	77.24	MIR100	microRNA 100
0.0033175	0.0726	211.91	191.39	272.01	290.22	212.27	349.72	GMNN	geminin, DNA replication inhibitor
0.0033797	0.0732	151.81	151.53	145.21	184.47	157.86	241.16	SOS1	son of sevenless homolog 1 (Drosophila)
0.0034924	0.0748	98.82	78.71	148.21	121.04	86.69	152.05	ORC1	origin recognition complex, subunit 1
0.0035543	0.0753	87.19	79.93	88.71	57.23	78.89	37.91	IL24	interleukin 24
0.0036557	0.0757	133.48	129.41	135.11	103.75	114.46	71.47	ZNF643	zinc finger protein 643
0.0040681	0.08	19.66	28.53	13.67	19.35	23.01	19.85	RNF185	ring finger protein 185
0.0042597	0.0822	34.63	37.19	35.04	33.29	48.72	56.42	KLRC4	killer cell lectin-like receptor subfamily C, member 4
0.0046958	0.0892	190.64	145.58	233.96	265.69	261.36	217.73	HIST1H2AK	histone cluster 1, H2ak
0.0050098	0.0919	145.49	143.33	192.08	199.65	143.97	262.36	FAM102B	family with sequence similarity 102, member B
0.0055921	0.0971	33.08	32.32	39.31	45.37	32.02	50.91	GPR137C	G protein-coupled receptor 137C
0.0063822	0.109	87.31	60.59	112.04	70.11	85.08	125.97	MIR27B	microRNA 27b
0.0068906	0.115	320.62	276.85	414.25	427.43	314.83	470.1	MASTL	microtubule associated serine/threonine kinase-like
0.0069605	0.115	26.56	16.26	25.57	25.53	38.79	41.79	KLRC4-KLRK1	KLRC4-KLRK1 readthrough
0.0070361	0.115	1166.34	1031.9	1010.08	1691.28	1032.54	1113.08	MMP1	matrix metallopeptidase 1 (interstitial collagenase)
0.0073541	0.116	120.93	127.21	85.92	100.69	121.26	82.32	U2AF1L4	U2 small nuclear RNA

Parametric p-value	FDR	Aspirin	Control	DEA/NO	DEA/NO aspirin	IPA/NO	IPA/NO aspirin	Symbol	Name
									auxiliary factor 1-like 4
0.0091362	0.131	416.41	362.63	588.42	516.16	370.17	592.15	SKP2	S-phase kinase-associated protein 2 (p45)
0.0093547	0.133	71.86	76.16	123.41	108.87	101.19	97.2	HSD17B7P2	hydroxysteroid (17-beta) dehydrogenase 7 pseudogene 2
0.0098301	0.134	38.32	32.29	38.33	43.56	31.37	50.95	DSEL	dermatan sulfate epimerase-like
0.009931	0.134	67.62	62.92	55.15	47.37	60.1	40.49	SLC5A4	solute carrier family 5 (low affinity glucose cotransporter), member 4

Table 3

Class Comparison Analysis: Differentially expressed genes in IPA/NO vs IPA/NO-aspirin

Parametric p-value	FDR	IPA/NO	IPA/NO aspirin	Symbol	Name
0.0004702	0.0745	401.34	136.8	DDIT3	DNA-damage-inducible transcript 3
0.0006614	0.0745	336.45	182.26	MIR21	microRNA 21
0.0028398	0.145	103.5	216.93	SNORD14E	small nucleolar RNA, C/D box 14E
0.0034745	0.145	38.07	17.11	C12orf39	chromosome 12 open reading frame 39
0.0036564	0.145	1869.4	826.61	TXNIP	thioredoxin interacting protein
0.0098684	0.262	269.25	131.2	GDF15	growth differentiation factor 15

Table 4

Class Comparison Analysis: Differentially expressed genes in DEA/NO vs DEA/NO-aspirin

Parametric p-value	FDR	DEA/NO	DEA/NO aspirin	Symbol	Name
6.19E-05	0.0253	192.41	357.21	MIR21	microRNA 21
0.0023481	0.118	1319.53	894	TXNIP	thioredoxin interacting protein
0.0028045	0.118	52.17	55.3	UFM1	ubiquitin-fold modifier 1
0.0083349	0.234	53.27	22.46	FLJ16171	FLJ16171 protein
0.0099746	0.265	22.21	28.11	NOL7	nucleolar protein 7, 27kDa

Table 5

Class Comparison Analysis: Differentially expressed genes in aspirin vs IPA/NO-aspirin

Parametric p-value	FDR	Aspirin	IPA/NO-aspirin	Symbol	Name
1.73E-05	0.00392	344.03	136.8	DDIT3	DNA-damage-inducible transcript 3
0.0001249	0.0158	37.97	17.11	C12orf39	chromosome 12 open reading frame 39
0.0002115	0.0178	2063.12	826.61	TXNIP	thioredoxin interacting protein
0.0003061	0.0221	87.19	37.91	IL24	interleukin 24
0.0072281	0.121	52.92	109.89	FABP4	fatty acid binding protein 4, adipocyte

Table 6

Class Comparison Analysis: Differentially expressed genes in aspirin vs DEA/NO-aspirin

Parametric p-value	FDR	Aspirin	DEA/NO aspirin	Symbol	Name
0.0003854	0.195	129.96	357.21	MIR21	microRNA 21
0.0039924	0.403	2063.12	894	TXNIP	thioredoxin interacting protein