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The Role Of Oxidative Stress In The Pathogenesis Of Epithelial Ovarian Cancer

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THE ROLE OF OXIDATIVE STRESS IN THE PATHOGENESIS OF EPITHELIAL OVARIAN CANCER

by

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DEDICATION

This dissertation is dedicated to my loving husband Michael, and our son Ethan who light up my life and inspire me to do my best every single day. I would also like to dedicate this work to my parents who supported my passion for science from an early age and pushed me to pursue my dreams, to my sister and best friend, Erika, and to my brother Stephen, for their love and unequivocal support throughout. Also, to my mother, father, and brother-in-law who are overwhelmingly supportive and loving.

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LIST OF ABBREVIATIONS

ATCC:	American Type Culture Collection
AGTC:	Applied Genomics Technology Center
Ac-DEVD-pNA:	Acetyl-DEVD-p-nitroaniline
CA-125:	Cancer antigen-125
CHTN:	Cooperative Human Tissue Network
DPI:	Diphenyleneiodonium
EOC:	Epithelial ovarian cancer
FBS:	Fetal bovine serum
γ-GCS	γ-glutamylcysteine synthetase
GSH:	Glutathione
GSSG:	Glutathione disulfide
GSR:	Glutathione reductase
GST:	Glutathione s-transferase
GWAS:	Genome-wide association studies
HE4:	Human epididymis protein
H_2O_2:	Hydrogen peroxide
HOCl:	Hypochlorous acid
HIF-1α:	Hypoxia inducible factor
HGSOC:	High grade serous ovarian carcinoma
HOSEpiC:	Human ovarian surface epithelial cells
iNOS:	Inducible nitric oxide synthase
KCIGR:	Karmanos Cancer Institute's Genetic Registry
MPO:	Myeloperoxidase

NAD(P)H:	Nicotinamide adenine dinucleotide phosphate
NO_3^-:	Nitrate
NO_2^-:	Nitrite
NO:	Nitric oxide
NOS:	Nitric oxide synthase
N_2O_3:	Dinitrogen trioxide
NOX:	NAD(P)H oxidase
$ONOO^-$:	Peroxynitrite
pNA:	p-nitroaniline
PPV:	Positive predictive value
RNS:	Reactive nitrogen species
ROC:	Receiver operating characteristic
ROS:	Reactive oxygen species
SNP:	Single nucleotide polymorphism
SNO:	S-nitrosothiol
$O_2^{\bullet-}$:	Superoxide
SOD:	Superoxide dismutase
TVU:	Transvaginal ultrasound
XO:	Xanthine oxidase

CHAPTER I: INTRODUCTION AND BACKGROUND

Ovarian cancer

Ovarian cancer is the fifth leading cause of cancer death in women, the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy [1]. It is projected that in 2013, approximately 22,240 women will receive a new diagnosis of ovarian cancer and 14,230 will die from ovarian cancer [2]. Ovarian cancer can be divided into three broad subgroups, epithelial, stromal, and germ cell tumors, each with different etiologies and clinical behavior, with epithelial ovarian cancer (EOC) amounting to more than 85% of all cases [3]. Sex cord stromal tumors of the ovary account for about 5% to 8% while neoplasms of germ cell origin account for about 3% of ovarian malignancies [4]. In general, ovarian cancers originate in the epithelium of inclusion cysts, which are derived from the surface epithelium [5]. While most epithelial tumors, including a subset of low grade adenocarcinomas of the ovary (endometrioid and mucinous) follow the adenoma-carcinoma sequence, high grade serous ovarian carcinoma (HGSOC) often exhibits a unique pattern of progression in which no tumor can be found on the surface of the ovary during the early stages [5]. In contrast, in most HGSOCs, as the tumor grows, the intact ovarian outer surface is disrupted and the cancer extends to the pelvic region [6]. Early stage ovarian cancer is typically asymptomatic which results in most cases presenting with late stage (III or IV) disease [7]. Unfortunately, the five-year survival rate of patients with advanced disease is approximately 30%, with even lower survival among African-American women [7,8]. Risk factors include family history, BRCA1/2 mutations, nulliparity, obesity, advanced age, race, and pregnancy after the age of 35 [7]. On the other hand, a decreased risk of ovarian cancer occurs with the use of oral contraceptives, pregnancy and childbirth, hysterectomy and oophorectomy [7]. Currently used screening and diagnostic methods for ovarian cancer include pelvic examination and CA-125 antigen as a

tumor marker and transvaginal ultrasound (TVU), although the sensitivity and specificity of these methods are insufficient for screening the general population [9].

The deficiency in diagnostic tools, including the lack of serum markers for the detection of early neoplastic changes in epithelial cells has led to this disease being diagnosed most often in later stages resulting in poor prognosis. Recommended treatment of ovarian cancer includes a combination of surgery and a chemotherapeutics, platinums and taxanes, yet relapse is common due to the acquisition of chemotherapy resistance resulting in disease recurrence in 70% of patients who underwent treatment within 18 months [8,10]. While large-scale gene expression analyses have been done to identify differentially expressed genes in ovarian carcinoma for diagnosis of early-stage ovarian cancer, as well as the use of such markers as targets for improved therapy and treatment, to date these have not yielded reproducible prognostic indicators for identification and clinical outcomes [11-19].

Sources of oxidative stress

Oxidative stress has been implicated in the pathogenesis of several malignancies including bladder, brain, breast, cervical, gastric, liver, lung, melanoma, multiple myeloma, leukemia, lymphoma, oral, ovarian, pancreatic, prostate, and sarcoma cancers [20,21]. Moreover, there is evidence that ovarian cancer patients also have decreased levels of circulating antioxidants [21]. The imbalance between the production and elimination of free radicals and reactive metabolites leads to a state of oxidative stress and subsequent damage of important biomolecules and cells, with potential impact on the whole organism [22]. Reactive oxygen species (ROS) include superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\cdot}OH^{\bullet}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypohalous acids, and are free radical molecules that are highly destructive to cellular functions [23,24]. Various enzyme systems produce ROS, including the mitochondrial electron transport chain, cytochrome P450, lipoxygenase, cyclooxygenase, the

nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase complex, xanthine oxidase (XO), and peroxisomes (Figure 1) [25,26].

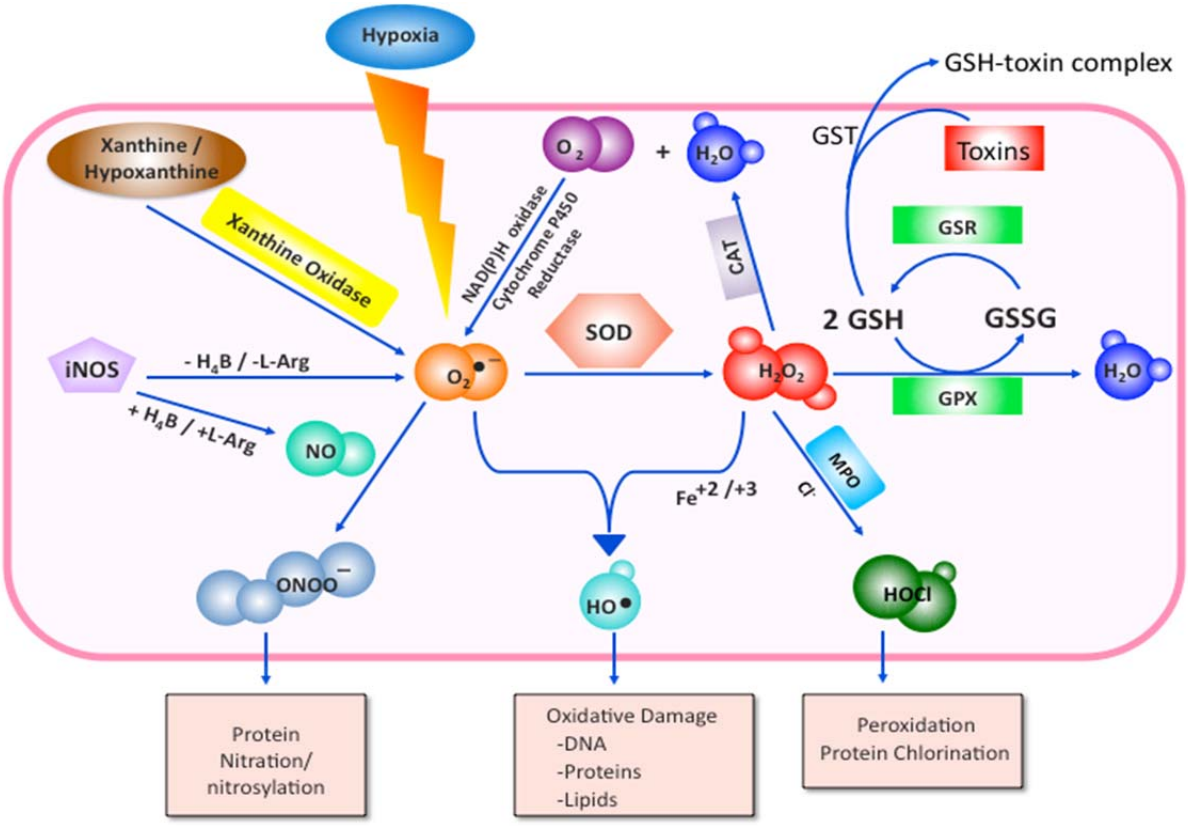

Figure 1: Summary of the mechanisms that will be examined in this study.

Mitochondrial O_2 metabolism is the dominant source of $O_2^{\bullet -}$ that results from incomplete coupling of electrons and H^+ with O_2 in the electron transport chain. Under normal conditions, ROS are maintained within narrow parameters by scavenging systems, as would be expected where those enzymes are involved in cell signaling, while ROS formed after exposure to oxidative stress can activate signaling molecules [25-33]. The redox balance is maintained by various enzyme systems that neutralize toxic reactive oxygen and nitrogen species. Superoxide dismutases (SOD) catalyze the conversion of $O_2^{\bullet -}$ to hydrogen peroxide (H_2O_2), which then can be converted to water by catalase (CAT) or glutathione peroxidase (GPX) coupled with

glutathione reductase (GSR) (Figure 1) [34]. Other important scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which utilizes glutathione (GSH) as a substrate (Figure 1). Additionally, glutathione S-transferases (GST) are involved in detoxification of varieties of environmental carcinogens and xenobiotics by catalyzing their conjugation to GSH, and subsequent removal from the cell [35]. Glutathione plays a central role in maintaining redox homeostasis, and the GSH-to-oxidized-GSH ratio provides an estimate of cellular redox buffering capacity [25,36].

Cancer cells are known to be under intrinsic oxidative stress resulting in increased DNA mutations or DNA damage, genome instability, and cellular proliferation [22,34,37-39]. The persistent generation of cellular ROS is a consequence of many factors including exposure to carcinogens, infection, inflammation, environmental toxicants, nutrients, and mitochondrial respiration [40-43]. Nitration of tyrosine residues to 3-nitrotyrosine, a hallmark of inflammation as well as a footprint of many types of oxidative injury, may play a role in the pathogenesis of human ovarian cancers [44]. Specifically, an increased expression of pro-oxidant enzymes such as inducible nitric oxide synthase (iNOS), myeloperoxidase (MPO), NAD(P)H oxidase, as well as an increase in NO as indicated by increased nitrate/nitrite has been shown in epithelial ovarian cancer (EOC) tissues and cells [45-47]. Epithelial ovarian cancer (EOC) cells have been shown to have significantly increased levels of nitric oxide (NO), which correlated with increased expression in]iNOS [46]. Also, EOC cells manifested lower apoptosis, which was markedly induced by inhibiting iNOS by L-NAME, indicating a strong link between apoptosis and NO/iNOS pathways in these cells [46]. Myeloperoxidase utilizes NO, produced by iNOS, as a one-electron substrate generating NO^+, a labile nitrosating species that is rapidly hydrolyzed forming nitrite (NO_2^-) as an end product [48-51]. The ability of MPO to generate NO^+, from NO, led us to believe that not only does MPO play a role in S-nitrosylation of caspase-3, thus

inhibiting caspases-3 activity, which is indicative of lower apoptosis in EOC cells, but also highlights a possible cross-talk between iNOS and MPO [45]. Myeloperoxidase, an abundant hemoprotein previously believed to be present solely in neutrophils and monocytes, plays an essential role in immune surveillance and host defense mechanisms, can contribute to 3-nitrotyrosine formations *in vivo* and directly modulate inflammatory responses via regulation of NO bioavailability during inflammation [44,52].

Several pro-oxidants have been demonstrated to be increased in cancer. Specifically, increased iNOS expression is elevated in breast, colorectal, brain, esophagus, lung, prostate, bladder, ovarian and pancreatic cancers [53,54]. NAD(P)H oxidase family members have also been shown to increase in association with ROS production and tumorigenicity in several cancers including colon, prostate, melanoma, breast and ovarian [55]. Myeloperoxidase has been reported to be elevated in gynecologic malignancies but has been suggested to be a result of infiltrating neutrophils and macrophages [56]. High levels of HIF-1 expression are also observed in many human cancers, and are correlated with tumorigenesis [55]. On the other hand, elevated serum GST-pi levels were observed in patients with various gastrointestinal malignancies as well as in breast, esophageal, pancreatic, hepatocellular, and biliary tract cancers [57,58]. Several other antioxidant enzymes have been indicated to be differentially expressed in cancer. Glutathione peroxidase expression is reduced in prostate, bladder and in estrogen-negative breast cancer cell lines, activity is reduced in cancerous kidney tissues but increased in breast cancer tissues [57,59,60]. Glutathione reductase levels have also been reported to be elevated in lung cancer although differentially expressed in breast and kidney cancerous tissues [61-63]. Additionally, CAT was decreased in breast, bladder, and lung cancer while increased in brain cancer [64-67]. Superoxide dismutase is highly expressed in lung, colorectal, gastric and breast cancer, while decreased SOD activity and expression have also been reported in colorectal

carcinomas and pancreatic cancer cells [28,66-70]. Collectively, this differential expression of antioxidants demonstrates the unique and complex microenvironment of cancer.

Another major source of $O_2^{\bullet-}$ in several cell types is NAD(P)H oxidase, which has been reported to be elevated in ovarian cancer [47,71-76]. NAD(P)H oxidase consists of seven isoforms: five NAD(P)H oxidases (NOX), NOXs1–5, and two NOX homologues, DUOX1 and DUOX2. NAD(P)H oxidases are differentially activated by different binding molecules, including $p22^{phox}$, $p40^{phox}$, $p47^{phox}$/NOXO1, $p67^{phox}$/NOXA1 and Rac [77]. Activation mechanisms and tissue distribution of the different members of the family are markedly different. Activation of NAD(P)H oxidase involves the translocation of regulatory elements from the cytoplasm to combine with catalytic subunits in the membrane [78]. Increased NAD(P)H oxidase activity contributes to a large number or pathologies, such as cardiovascular diseases and neurodegeneration [71]. It has been reported that mutations in specific regions of NAD(P)H oxidase subunits contribute to the enhancement of the enzyme activity with subsequent increase in $O_2^{\bullet-}$ production, contributing to enhanced levels of oxidative stress [79,80]. Specifically, an increase in NAD(P)H oxidase activity has been associated with a specific single nucleotide polymorphism (SNP) in the *CYBA* gene (rs4673), encoding the $p22^{phox}$ subunit of NAD(P)H oxidase, resulting in a CAC/TAC replacement at position 242 located on chromosome 16q24, leading to a nonconservative substitution of histidine-72 with a tyrosine [81]. This SNP is also associated with an increased risk for other diseases where oxidative stress plays a critical role in their pathophysiology, including cardiovascular disease, asthma, and diabetic nephropathy [81-83].

There is compelling evidence to support the role of oxidative stress in the pathogenesis of ovarian cancer, yet the mechanisms responsible for the dissemination of EOC are not fully understood. *The objective of this work* is to identify mechanisms of oxidative stress and their

role in the pathogenesis of ovarian cancer. The central hypothesis of this study is that ovarian cancer is controlled by mechanisms that emanate from an altered redox balance.

CHAPTER II: GENERAL METHODS

(This chapter contains previously published material. See Appendix [45,47])

Cell Lines, Media, and Cell Culture Conditions

Human epithelial ovarian cancer (EOC) cell lines, MDAH-2774 (CRL-10303™) and SKOV-3 (HTB-77™), were obtained from American Type Culture Collection (ATCC, Manassas, VA). The MDAH-2774 cell line was developed from cells in the ascitic fluid from a patient with adenocarcenoma in 1972 and forms tumors in nude mice [84]. SKOV-3 cells were developed from ascitic fluid of a 64-year-old Caucasian female. Cell lines were cultured in 75cm^2 cell culture flasks (Corning Incorporated, Corning, NY) with McCoy's 5A medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in 5% CO_2. Culture medium was replaced every two days.

The human primary ovarian surface epithelial (HOSEpiC) cells were obtained from ScienCell Research Laboratories (Carlsbad, CA). Cells were isolated from human ovarian tissue and were cultured with Ovarian Epithelial Cell Medium (OEpiCM, ScienCell), consisting of 500 ml of basal medium, 5 ml of Ovarian Epithelial Cell Growth Supplement (OEpiCGS, SicenCell) and 5 ml of penicillin/streptomycin solution (P/S, ScienCell). These cells were cryopreserved at either primary or passage one and have been utilized in other studies [85-87]. Cells were cultured according to the manufacturer's instructions.

RNA Isolation

Total RNA was extracted from cells using an RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the protocol provided by the manufacturer.

Reverse Transcription

A 20 µL cDNA reaction volume utilizing 1 µg RNA was prepared using the QuantiTect

Reverse Transcription Kit (Qiagen), as described by the manufacturer's protocol.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR kit (QIAGEN) and a Cepheid 1.2f Detection System (Cepheid, Sunnyvale, CA). Each 25-µL reaction included 12.5 µL of 2 x QuantiTect SYBR Green RT-PCR master mix, cDNA template, and 0.2 µM each of target-specific primer were selected with the aid of the software program, Beacon Designer (Premier Biosoft, Palo Alto, CA). Human oligonucleotide primers that amplify variable portions of the protein coding regions are listed in Table 1. MPO and iNOS were normalized utilizing the housekeeping gene, β-actin while all other genes listed in Table 1 were quantified using a specific standard for each gene which allows for absolute quantification of that gene in number of copies, which can then be expressed per µg of RNA. These standards have known concentrations and lengths that allowed for construction of a standard curve using a tenfold dilution series. To evaluate the validity of using β-actin as an internal standard and changes in the amounts of β-actin, mRNA was tested as an external standard. Subsequently, the normalized values of the mRNA were divided by those in controls.

Table 1. Real-time RT-PCR primer sequences and cycling conditions.

Locus	Sense (5'-3')	Antisense (3'-5')	Amplicon Length (bp)	Initial denaturation (°C / seconds)	Annealing (°C / seconds)
iNOS	GTTCTCAAGGCACAGGTCTC	GCAGGTCACTTATGTCACTTATC	127	94 / 300	56 / 60
MPO	GCT GAA TGT GTT GTC CAA GTC	TGA TGG TGC GGT ATT TGT CC	86	94 / 300	60 / 60
β-actin	AAGCAGGAGTATGACGAGTCCG	GCCTTCATACATCTCAAGTTGG	559	94 / 300	58 / 60
HIF-1α	AGCCGAGGAAGAACTATGAAC	ACTGAGGTTGGTTACTGTTGG	100	95 / 900	60 / 30
$p22^{phox}$	GTACTTTGGTGCTTACTC	GGAGCCCTTTTTCCTCTT	82	95 / 1000	54 / 30
SOD3	GCCTCCATTTGTACCGAAAC	AGGGTCTGGGTGGAAAGG	78	95 / 850	60 / 30
GSR	TCACCAAGTCCCATATAGAAATC	TGTGGCGATCAGGATGTG	116	95 / 900	59 / 30
GPX1	CAGTTCGGACATCAGGAG	ATTCACCTCGCACTTCTC	117	95 / 1400	53 / 30
GST-p1	TACCAGTCCAATACCATC	GTAGATGAGGGAGATGTA	138	95 / 1200	56 / 30
CAT	GAAGATTCTCCTGTGCTA	TAATCCAATCATCCGTCAA	174	95 / 1800	55 / 30

The conditions for the three-step polymerase chain reaction protocol were as follows: an initial cycle at as described in Table 1, followed by 35 cycles of denaturation at 95°C for 15s, annealing as described in Table 1, and extension at 72°C for 30s. Finally, a melting curve analysis was performed to demonstrate the specificity of the PCR product as a single peak. A control, which contained all the reaction components except for the template, was included in all experiments.

Immunofluorescent Staining for iNOS and MPO

Cells in Culture: EOC cells were grown on a Lab-Tek Chamber slide (Sigma Chemicals, St. Louis, MO) overnight at 37 °C. The cells were washed briefly with phosphate buffer saline solution (PBS) and fixed with 3% paraformaldehyde for 30 minutes followed by washing with PBS three times. Cells were blocked with 1% bovine serum albumin (BSA).

Tissue sections: Normal ovarian and ovarian cancer tissue sections were heated to 65 C for 2 hours followed by deparaffinization in 2 changes of xylenes (Sigma, St. Louis, MO), 5 minutes each. Tissues were rehydrated in 2 changes of 100% ethanol for 3 minutes each followed by 2 changes of 95% ethanol for 1 minute each and 2 changes of 80% ethanol for 1 minute each. Slides were rinsed in distilled water and incubated in 3% H_2O_2 for 20 minutes at room temperature to block endogenous peroxidase activity followed by washing with 0.05% Tween 20-PBS 3 times, 5 minutes each. Slides underwent antigen retrieval by being incubated in sodium citrate buffer at 95 C for 20 minutes and then were allowed to come to room temperature followed by washing with 0.05% Tween 20-PBS 3 times, 5 minutes each. Tissues sections were blocked with 10% BSA.

Slides and chambers were incubated with the FITC-conjugated iNOS and Texas Red-conjugated MPO antibodies (mouse anti-iNOS monoclonal antibody; BD Bioscience, rabbit anti-myeloperoxidase polyclonal antibody; Abcam, Cambridge, MA) diluted at 1:100 ratio for 1 hour

at room temperature. The cover slips were mounted on the slide with a drop of mounting medium containing DAPI (Invitrogen), sealed with nail polish and stored in dark at 4 °C. Slides were examined with the Axiovert 25 inverted microscope (Zeiss, Thornwood, NY) using DAPI (blue), Texas red (red) and FITC (green), fluorescent filters with excitation and emission wavelengths of 365 and 445, 470 and 525, and 596 and 613 nm respectively. Images were taken using the Axiovision software (Zeiss) and a microscope-mounted camera.

Immunohistochemical Staining for iNOS, MPO and NAD(P)H Oxidase

Tissue sections: For iNOS and MPO, benign ovarian tissues specimens (n=20), obtained from cases who underwent salpingo-oophorectomy for benign uterine pathology, and invasive epithelial ovarian cancer cases (n=20) retrieved from archival materials from the Detroit Medical Center/ Karmanos Cancer Center pathology department were utilized in this study. Case distribution by FIGO stage was as follows: 10 were stage I, and 10 were advanced stage disease (II-IV) at diagnosis. Dr. Rouba Ali-Fehmi in the Wayne State University Pathology Department, Detroit, MI, reviewed H&E stained slides from each case for validation/confirmation of diagnosis and histology. The histological diagnoses were as follows: 10 high grade serous carcinomas; 5 low grade serous carcinomas, 4 grade 1 endometrioid and one grade 1 mucinous carcinomas. The mean age of the 20 patients was 68 (range 38 to 89 years).

For NAD(P)H oxidase, twenty benign ovarian tissues specimens, obtained from patients who underwent total abdominal hysterectomy-bilateral salpingo-oophorectomy for leiomyomas, and 20 invasive EOC cases (two sections per case) were retrieved from archival materials from the Detroit Medical Center/Karmanos Cancer Center pathology department (Institutional review board number 072206MP2E). Histological diagnoses were as follows: 10 high grade and five low grade serous carcinomas, four grade 1 endometrioid and one grade 1 mucinous carcinomas. The mean age of the 20 patients was 56 (range 35-70).

After deparaffinizing and hydrating with phosphate-buffered saline (PBS, pH 7.4), sections were pretreated with 3% H_2O_2 for 10 minutes to remove endogenous peroxidases followed by incubation with goat serum for 10 minutes. Primary antibodies for MPO (Dako, Denmark, A0398) and iNOS (Santa Cruz, Santa Cruz, CA, sc7271) dilution (1:100) or a primary antibody for NAD(P)H oxidase (HPA015475, Sigma Aldrich) dilution (1:20) was applied to each section followed by washing and incubation with the biotinylated secondary antibody for 10 minutes at room temperature. Detection was performed with AEC and counterstaining was done with Mayer's hematoxylin followed by mounting.

The expression of MPO, iNOS, or NAD(P)H oxidase was assessed based on the presence of cytoplasmic staining. A gynecologic pathologist reviewed all slides, benign (2-5 slides) and malignant (5-15 slides). The most positive area in every tumor or benign cases was evaluated and only the percentage was evaluated because most of the cases had the same intensity. The scoring was assigned based on the percentage of positive epithelial cells: a zero score assigned for cases with no cytoplasmic staining in any cells; score 1 with <5% of cell staining positive; score 2 with 6-30% and score 3 with >30% of cells staining positive. For statistical analysis, cases with score 0 or 1 were considered as being negative and cases with score 2 or 3 as positive.

Measurement of Protein Concentration

Total protein concentration of cell lysates was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, Illinois) per the manufacturer's protocol. Cell lysates were prepared utilizing cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with Protease Arrest (G-Biosciences, St. Louis, MO).

CHAPTER III: CHARACTERIZATION OF THE OXIDATIVE STRESS PROFILE IN EPITHELIAL OVARIAN CANCER

(This chapter contains previously published material. See Appendix [45,47])

Approach

The objective of this chapter is to characterize the oxidative stress profile in EOC. Expression of iNOS, MPO and NAD(P)H oxidase, was determined using immunohistochemistry in EOC cell lines, SKOV-3 and MDAH-2774, normal human ovarian surface epithelial cell line, HOSEpiC, as well as in EOC tissues and their normal counterparts.

The activity levels for CAT, SOD, GPX and GSR were measured in, MDAH-2774, SKOV-3, and HOSEpiC cells utilizing ELISA assays. Cells were seeded (2.5×10^6) in 150 mm dishes and allowed to rest for 24 hrs followed by media replacement and cell collection 24 hrs later. Cells were collected for RNA and protein extraction as well as media collected for measurement of nitrate/nitrite and total GSH levels. Following RNA extraction, reverse transcription of cDNA was performed which was then utilized in real-time RT-PCR for determination of CAT, GSR, GPX1, and GST-p1 mRNA levels.

Data were analyzed with unpaired Student's t-tests comparing EOC to HOSEpiC cells. A of $p<0.05$ was considered statistically significant for all analyses.

Introduction

The imbalance between the production and elimination of free radicals and reactive metabolites leads to a state of oxidative stress and subsequent damage of important biomolecules and cells, with potential impact on the whole organism [22]. Oxidative stress has been implicated in the pathogenesis of several malignancies, including ovarian cancer [20,21]. Moreover, there is evidence that ovarian cancer patients also have decreased levels of circulating antioxidants such as vitamins C and E, in comparison to healthy controls [21]. Although there

are many players involved in the maintenance of the redox balance, we will focus on the following for this study.

Epithelial ovarian cancer cells have been reported to have significantly increased levels of NO, which correlated with increased expression of iNOS [46]. Additionally, MPO can utilize NO, produced by iNOS, as a one-electron substrate generating NO^+, a labile nitrosating species that is rapidly hydrolyzed forming nitrite (NO_2^-) as an end product [48-51]. The ability of MPO to generate NO^+ led us to believe that MPO may play a role in S-nitrosylation of caspase-3, subsequently lowering the activity of caspase-3, ultimately reducing apoptosis in EOC cells.

In addition to the ROS produced by the mitochondria, nicotinamide adenine dinucleotide phosphate (NAD(P)H)-oxidase, a flavoenzyme family member, generates a significant amount of endogenous ROS through the reduction of O_2 to $O_2^{\bullet-}$, H_2O_2, and other ROS [71,75,88,89]. It has been reported that the NAD(P)H oxidase NOX1 subunit was positively expressed in several cancers, including ovarian cancer tissues [90].

Superoxide dismutase is a major $O_2^{\bullet-}$ scavenger that converts $O_2^{\bullet-}$ to H_2O_2, which is further eliminated by both CAT and peroxidases [91,92]. Human extracellular Cu/Zn SOD (SOD3), is a unique SOD family member found in the extracellular matrix of tissues and is ideally situated to prevent cell and tissue damage, initiated by extracellularily produced ROS [93]. The loss of endogenous SOD3 activity can exacerbate oxidative stress and pathologic damage as it is a critical endogenous antioxidant enzyme involved in carcinogenesis, cancer proliferation and metastasis [94]. Catalase as well as GPX is involved in elimination of H_2O_2. Glutathione peroxidases detoxify peroxides with GSH acting as an electron donor in the reduction reaction, producing the oxidized form, GSSG, which then can be reduced by GSR, reforming GSH [95]. Reduced GSH is considered to be one of the most important scavengers of ROS, and its ratio with GSSG may be used as a marker of oxidative stress [95]. Moreover, GSR

is constitutively active and can be induced by oxidative stress and thus free GSH typically exists in the reduced form [95].

Therefore, the goal of this chapter is to examine select pro-oxidants and antioxidants in order to establish an oxidative stress profile of EOC, which will contribute to delineating the underlying mechanisms of ovarian cancer.

Methods

Cell culture of EOC cell lines, MDAH-2774 and SKOV-3, and normal ovarian surface epithelial cells, HOSEpiC, are described in *General Methods.* For SOD, CAT, GSR, GSH, and GPX assays, cells (2.5×10^6) were seeded in a 150 mm dish and allowed to rest for 24 hrs followed by media replacement and cell collection 24 hrs later. Media was collected for nitrate/nitrite and total GSH measurements. Immunohistochemistry for MPO, iNOS, and NAD(P)H oxidase is also described in *General Methods*.

Measurement of Nitrate/Nitrite

The nitrate/nitrite colorimetric assay (Cayman Chemical, Ann Arbor, MI) was used to measure the levels of stable NO by-products, nitrate (NO_2^-) and nitrite (NO_3^-), as an indication of NO production. Due to the fact that the proportion of NO_2^- and NO_3^- is variable and cannot be predicted with certainty, the sum of both NO by-products is a more accurate indicator of NO production. The assay was performed utilizing cell culture media (60 µl) according to the manufacturer's protocol. Absorbance was detected at 540 nm and a standard curve for nitrite was utilized to determine total NO_2^- and NO_3^-.

Measurement of CAT Activity

The Catalase Assay Kit (Cayman Chemical) utilizes the peroxidatic function of CAT for determination of enzyme activity and is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The assay was performed according to the

manufacturer's protocol using 2 μg total protein. A description of protein extraction and concentration determination is in *General Methods*. Formaldehyde produced is measured spectrophotometrically with Purpald as the chromogen, which is detected at 540 nm.

Measurement of SOD Activity

The Superoxide Dismutase Assay Kit (Cayman Chemical) detects SOD activity by measuring the dismutation of $O_2^{\bullet -}$ generated by xanthine oxidase (XO) and hypoxanthine. The standard curve generated using this enzyme provides a means to accurately quantify the activity of all three types of SOD (Cu/Zn-, Mn-, and Fe-SOD). The assay was performed according to the manufacturer's protocol using 0.25 μg total protein. A description of protein extraction and concentration determination is in *General Methods*. Total SOD was detected at 460 nm. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of $O_2^{\bullet -}$.

Measurement of GSR Activity

The Glutathione Reductase Assay Kit (Cayman Chemical) measures GSR activity by determining the rate of NAD(P)H oxidation which s accompanied by a decrease in absorbance at 340 nm. The assay was performed according to the manufacturer's protocol using 7 μg total protein. A description of protein extraction and concentration determination is in *General Methods*. Absorbance was read once a minute for 8 minutes and slope was utilized to calculate GPX activity. Since GSR is present at rate limiting concentrations, the rate of decrease in the absorbance at 340 nm is directly proportional to the GSR activity in the sample.

Measurement of GPX Activity

The Glutathione Peroxidase Assay Kit (Cayman Chemical) measures GPX activity indirectly by a coupled reaction with GSR [96]. Oxidized glutathione (GSSG), produced from the reduction of an organic hydroperoxide by GPX, is recycled to its reduced state by GSR and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at

340 nm. The assay was performed according to the manufacturer's protocol using 10-15 μg total protein, and was normalized per μg protein. A description of protein extraction and concentration determination is in *General Methods*. Absorbance was read once a minute for 8 minutes and slope was utilized to calculate GPX activity. The rate of decrease in the absorbance 340 is directly proportional to the GPX activity in the sample [97].

Statistical Analysis

An unpaired Student's t-test was utilized to analyze real-time RT-PCR data comparing EOC to HOSEpiC cells. A of $p<0.05$ was considered statistically significant for all analyses.

Results

MPO expression in EOC cells and tissues

Normal ovarian and EOC tissues were dually stained with antibodies targeting iNOS (green) and MPO (red). There is an increase in both iNOS and MPO fluorescence staining in ovarian cancer tissues as compared to normal ovarian tissues (Figure 2A). Co-localization of iNOS and MPO is indicated by yellow fluorescence (Figure 2A). Additionally, iNOS and MPO expression was upregulated in 70% and 65% of ovarian cancer tissue sections tested by immunohistochemistry, respectively (Figure 2B). There was no detectable expression for either MPO or iNOS in any of the normal ovarian epithelial tissue. The immunoreactivity that was observed in normal ovarian tissue sections was localized to blood vessels.

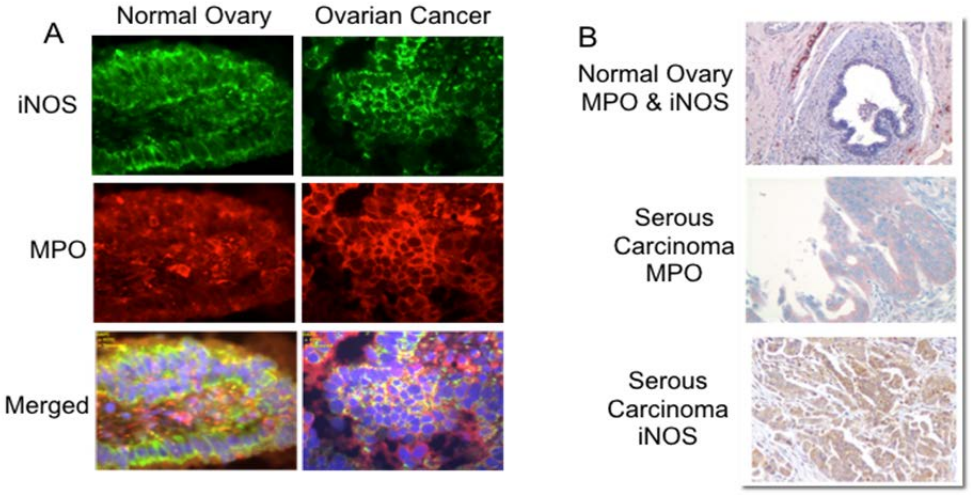

Figure 2 Immnunohistochemical staining of iNOS and MPO in normal and EOC tissues. A and B) Normal ovarian epithelium showed no staining for either iNOS or MPO. B) Strong cytoplasmic staining for iNOS and MPO is shown in a case of high grade serous carcinoma. A) Co-localization of iNOS and MPO was observed in ovarian cancer. Representative images of experiment performed in triplicate.

Co-localization of MPO and iNOS to the same cells in EOC cells.

The EOC cell lines, SKOV-3 and MDAH2774, were dually stained with antibodies targeting iNOS (green) and MPO (red). Immunoreactivity showed co-localization of iNOS and MPO (yellow) in both ovarian cancer cell lines (Figure 3).

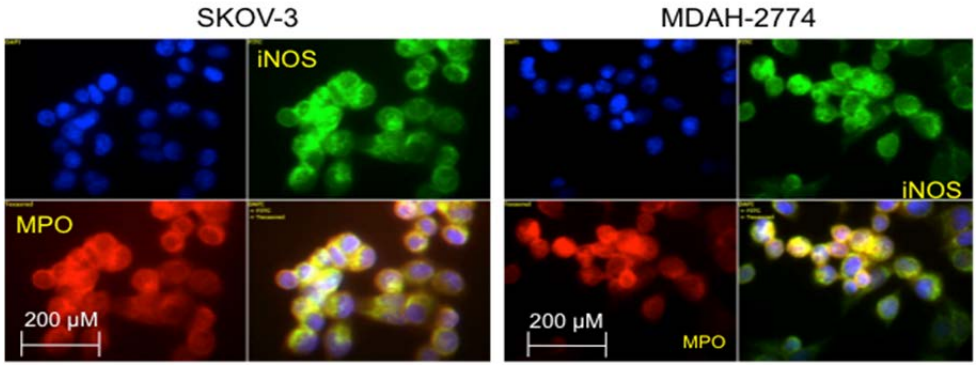

Figure 3 Co-localization of MPO and iNOS to the same cells in EOC cells. MPO and iNOS staining in MDAH-2774 and SKOV-3 EOC cells. Cells were dually stained with antibody against MPO (red), iNOS (green), and nuclei (blue) (100 x). Co-localization of MPO and iNOS is shown in yellow. Experiments were performed in triplicate.

EOC tissues expressed higher levels of NAD(P)H oxidase

NAD(P)H oxidase expression was upregulated in 65% of EOC tissue sections, tested by immunohistochemistry as compared to 20% detectable expression for NAD(P)H oxidase in normal ovarian epithelial tissue (surface epithelial inclusion cysts) (Figure 4).

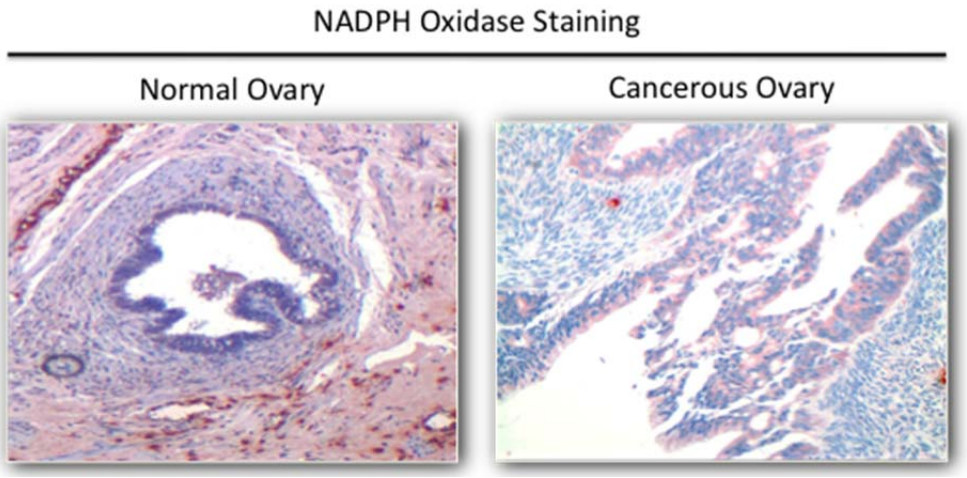

Figure 4: Immunohistochemical staining of NAD(P)H oxidase in normal ovarian and EOC tissue sections. Normal ovarian and EOC tissue sections were stained with a primary antibody for NAD(P)H oxidase, followed by biotinylated secondary antibody. Detection was performed with AEC and counterstaining was done with Mayer's hematoxylin followed by mounting and imaging (20x). The expression of NAD(P)H oxidase was assessed based on the presence of cytoplasmic staining.

EOC cells have an increase in nitrate/nitrite levels as compared to normal ovarian surface epithelial cells

Nitrate/nitrite levels significantly increased in MDAH-2774 (13.2 $\pm$ 0.5 μM, $p<0.002$) and in SKOV-3 (8.2 $\pm$ 0.0 μM, $p<0.002$) EOC cells as compared to HOSEpiC (3.9 $\pm$ 0.2 μM) cells (Figure 5).

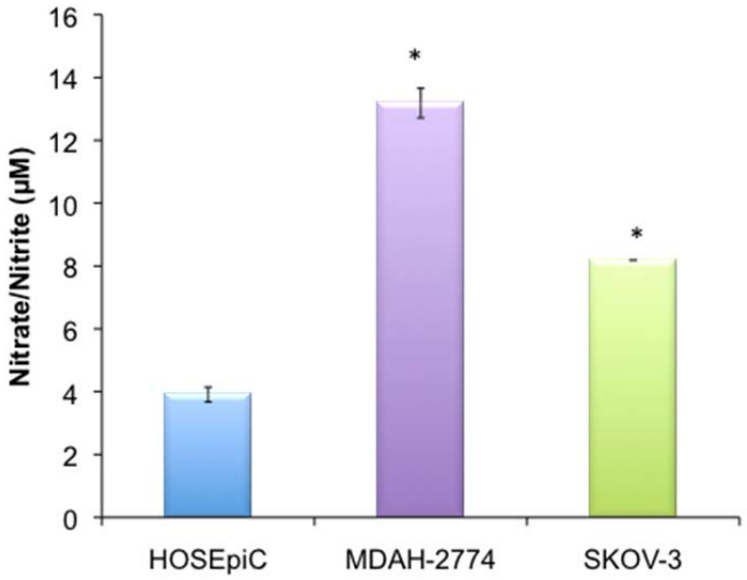

Figure 5: Nitrate/nitrite levels in EOC as compared to HOSEpiC cells. The Griess assay was performed in 24-hour media collected from normal ovarian surface epithelial (HOSEpiC) and EOC cells. *p<0.002 as compared to HOSEpiC cells. Error bars represent standard deviation of the mean.

EOC cells have an increase in CAT levels as compared to normal ovarian surface epithelial cells

There was a significant increase in CAT mRNA levels in MDAH-2774 (17.4 $\pm$ 0.1 pg/μg RNA, p<0.007) and SKOV-3 (20.6 $\pm$ 3.8 pg/μg RNA, p<0.02) EOC cells as compared to HOSEpiC (9.45 $\pm$ 1.6 pg/μg RNA), (Figure 6A). Similarly, there was an increase in CAT activity in MDAH-2774 (10.3 $\pm$ 0.06 nmol/min/ml, p<0.04) and SKOV-3 (10.1 $\pm$ 0.01 nmol/min/ml, p<0.04) EOC cells as compared to HOSEpiC (7.5 $\pm$ 0.73 nmol/min/ml) cells (Figure 6B).

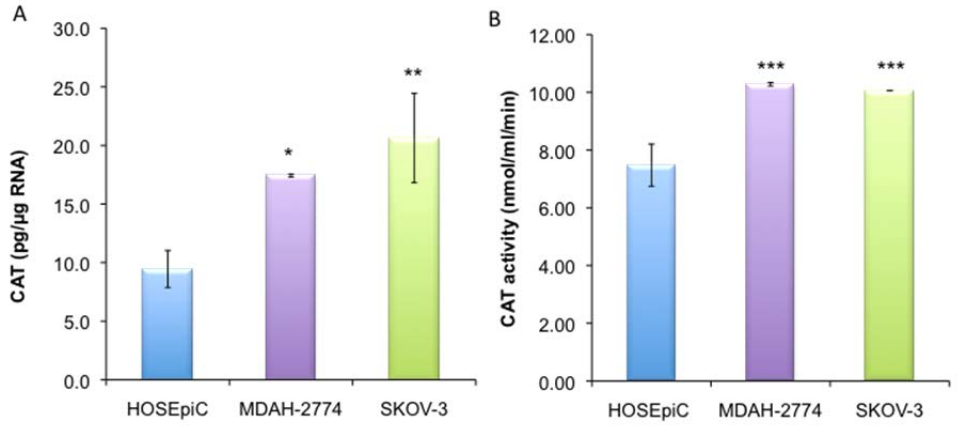

Figure 6: CAT mRNA and activity levels in EOC as compared to HOSEpiC cells. A) Real-time RT-PCR was utilized to measure CAT mRNA levels in RNA isolated from EOC cell lines MDAH-2774 and SKOV-3 as well as HOSEpiC cells. B) Catalase activity was detected in protein obtained from EOC cell lines MDAH-2774 and SKOV-3 as well as HOSEpiC cells. *p<0.007, **p<0.02, and ***p<0.04 as compared to HOSEpiC cells. Error bars represent standard deviation of the mean.

EOC cells have an increase in SOD activity levels as compared to normal ovarian surface epithelial cells

There was an increase in SOD activity levels in MDAH-2774 (to 11.1 $\pm$ 0.1 U/ml, p<0.0005) and in SKOV-3 (to 10.4 $\pm$ 0.9 U/ml, p<0.04), respectively, as compared to HOSEpiC

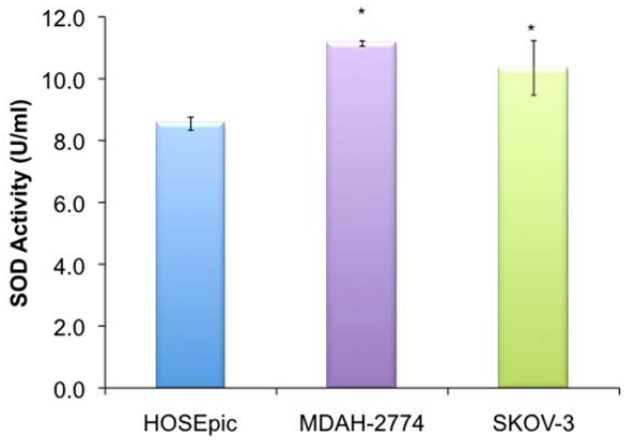

Figure 7: SOD activity levels in EOC as compared to HOSEpiC cells. SOD activity was detected in protein obtained from EOC cell lines MDAH-2774 and SKOV-3 as well as HOSEpiC cells. *p<0.005 and **p<0.04 as compared to HOSEpiC cells. Error bars represent standard deviation of the mean.

(8.6 ± 0.2 U/ml) cells (Figure 7).

EOC cells have an increase in GSR mRNA levels and activity as compared to normal ovarian surface epithelial cells

There was an increase in GSR mRNA levels in MDAH-2774 (to 27.7 ± 1.2 pg/µg RNA, $p<0.008$) and SKOV-3 (to 30.1 ± 0.6 pg/µg RNA, $p<0.008$), as compared to HOSEpiC (16.0 ± 0.8 pg/µg RNA (Figure 8A). Similarly, there was an increase in GSR activity in MDAH-2774 (to 8.5 ± 0.07 nmol/min/ml, $p<0.001$) and SKOV-3 (to 10.6 ± 0.1 nmol/min/ml, $p<0.001$) as compared to HOSEpiC (2.8 ± 0.04 nmol/min/ml) (Figure 8B).

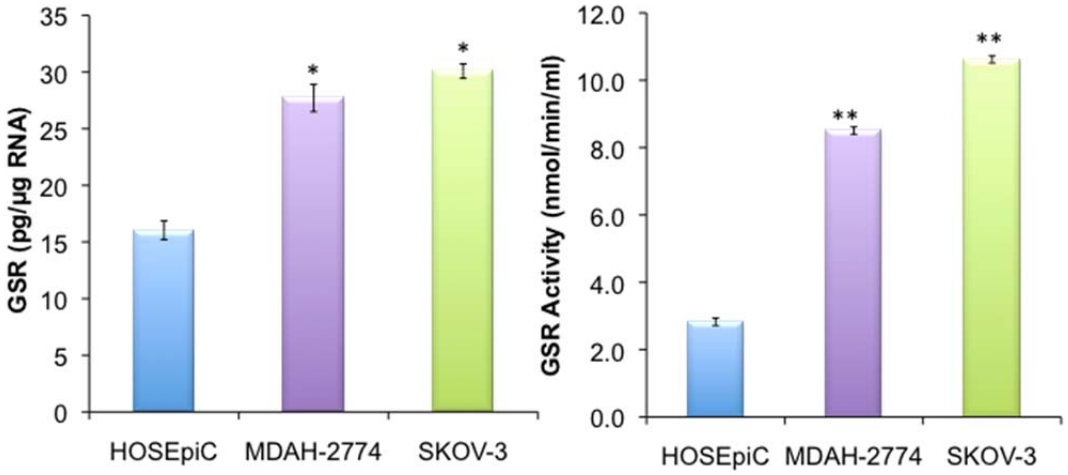

Figure 8: GSR mRNA and activity levels in EOC as compared to HOSEpiC cells. A) Real-time RT-PCR was utilized to measure GSR mRNA levels in RNA isolated from EOC cell lines MDAH-2774 and SKOV-3 as well as HOSEpiC cells. B) GSR activity was detected in protein obtained from EOC cell lines MDAH-2774 and SKOV-3 as well as HOSEpiC cells. *$p<0.008$ and **$p<0.001$ as compared to HOSEpiC cells. Error bars represent standard deviation of the mean.

EOC cells have a decrease in GPX1 levels and activity as compared to normal ovarian surface epithelial cells

There was a decrease in GPX1 mRNA levels in MDAH-2774 (to 0.75 ± 0.2 pg/µg RNA, $p<0.002$) and SKOV-3 (to 0.63 ± 0.08 pg/µg RNA, $p<0.001$), respectively, as compared to

HOSEpiC (5.50 ± 0.7 pg/µg RNA) (Figure 9A). Likewise, there was a decrease in GPX1 activity in MDAH-2774 (to 180.8 ± 4.5 nmol/min/ml/µg protein, p<0.002) and SKOV-3 (to 203.5 ± 32.1 nmol/min/ml/µg protein, p<0.002), respectively, as compared to HOSEpiC (3378.5 ± 241.3 nmol/min/ml/µg protein) (Figure 9B).

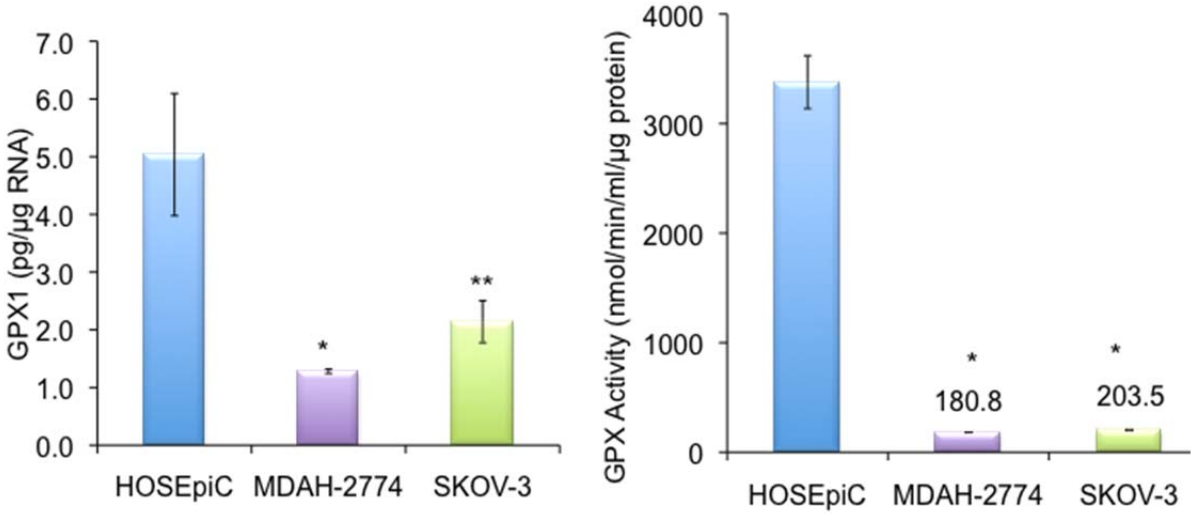

Figure 9: GPX mRNA and activity levels in EOC as compared to HOSEpiC cells. A) Real-time RT-PCR was utilized to measure GPX1 mRNA levels in RNA isolated from EOC cell lines MDAH-2774 and SKOV-3 as well as HOSEpiC cells. B) GPX activity was detected in protein obtained from EOC cell lines MDAH-2774 and SKOV-3 as well as HOSEpiC cells. *p<0.002 and **p<0.001 as compared to HOSEpiC cells. Error bars represent standard deviation of the mean.

EOC cells have an increase in GSTp1 mRNA levels as compared to normal ovarian surface epithelial cells

There was an increase in GSTp1 mRNA levels in MDAH-2774 (to 61.9 ± 2.3 pg/µg RNA, p<0.004) and in SKOV-3 (to 73.0 ± 1.1 pg/µg RNA, p<0.002), respectively, as compared to HOSEpiC (21.1 ± 2.3 pg/µg RNA) (Figure 10).

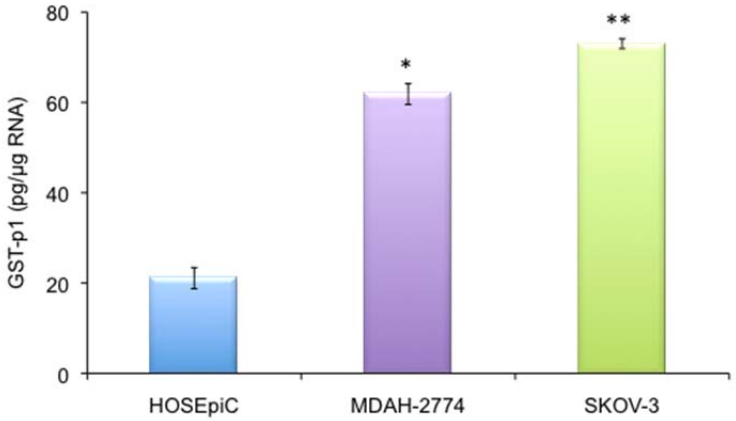

Figure 10: GSTp1 mRNA levels in EOC as compared to HOSEpiC cells. Real-time RT-PCR was utilized to measure GST-p1 mRNA levels in RNA isolated from EOC cell lines MDAH-2774 and SKOV-3 as well as HOSEpiC cells. *p<0.004 and **p<0.002 as compared to HOSEpiC cells. Error bars represent standard deviation of the mean.

Discussion

Our results clearly indicate that EOC manifests a severe pro-oxidant state, which ultimately may be responsible for the persistence and maintenance of the oncogenic phenotype. Indeed we have shown that there is an increase in key pro-oxidant enzymes iNOS, MPO, and NAD(P)H oxidase, an increase in nitrate/nitrite, as well as an increase in key antioxidant enzymes CAT, SOD, GSR, and GST-p1 in ovarian cancer (Figure 11). We also found the antioxidant enzyme GPX to be decreased in ovarian cancer (Figure 11).

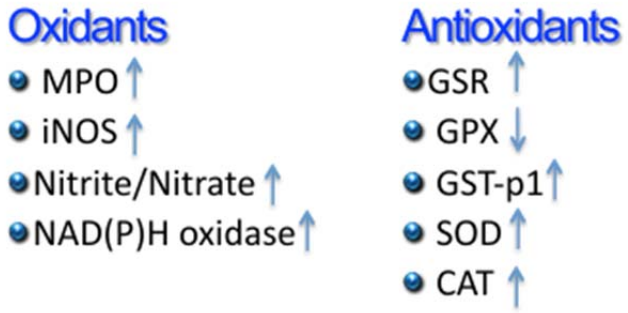

Figure 11: Summary of oxidant and antioxidant markers in epithelial ovarian cancer as compared to normal ovarian tissues and normal ovarian surface epithelial cells. MPO, iNOS, and NAD(P)H oxidase were found to be elevated in ovarian cancer tissues as compared to normal ovarian tissues. GSR, GST-p1, SOD, and CAT were elevated, while GPX1 was lower, in normal ovarian surface epithelial cells as compared to epithelial ovarian cancer cells.

Inducible nitric oxide synthase was previously found to be elevated in ovarian cancer tissues and cells [46,98]. Here, we observed an increase in NO_2^-/NO_3^- levels in MDAH-2774 and SKOV-3 EOC cells as compared to HOSEpiC cells, which is representative of increased NO production from iNOS. Nitric oxide plays an important role in cellular regulatory mechanisms, such as vasodilatation, inhibition of platelet aggregation, and modification of proteins [99-101]. Nitric oxide exists in different redox states: NO•, NO^+, and NO^- [48]. Furthermore, $O_2•^-$ reacts with NO to produce $ONOO^-$ [102]. It is well known that high concentrations of NO are proapoptotic and cytotoxic for different cells [103,104]. In contrast, low concentrations of NO have been shown to be protective against apoptosis [105-107]. In addition to the known cGMP-dependent effects, NO modifies proteins containing a cysteine residue via S-nitrosylation [101]. S-nitrosylation is a post-translational modification involving the attachment of NO to cysteine residues or transition metals [108]. Important S-nitrosylation targets are the caspase proteins and have been linked to a decrease in apoptosis.

Myeloperoxidase was expressed in ovarian cancer tissues with little or no expression in normal ovarian tissues. In addition, we observed MPO not only to be present but also to be co-localized with iNOS expression in both SKOV-3 and MDAH-2774 EOC cell lines. The presence of MPO in ovarian cancer cells is significant as MPO is believed to be present only in azurophilic granules of polymorphonuclear neutrophils and macrophages, and is released into the extracellular fluid in response to inflammation. Therefore, the presence of MPO in EOC cells supports the fact that MPO is indeed present in the actual tissues of EOC and not a result of infiltrating immune cells. The role of MPO in carcinogenesis has been implicated in both the activation of procarcinogens to genotoxic intermediates and the potentiation of xenobiotic carcinogenicity [109,110]. The co-localization of MPO and iNOS has also been demonstrated by immunohistochemical studies in cytokine-treated human neutrophils and primary granules of

activated leukocytes [111]. Furthermore, plasma levels and tissue expression of MPO in gynecologic malignancies, including ovarian cancer, were previously evaluated and it was found that gynecologic cancer patients had higher MPO as compared to control subjects [56].

Our immunohistochemical results also showed that NAD(P)H oxidase is over-expressed in EOC tissues as compared to normal ovarian tissues. Further support for this increase in ROS is demonstrated by a cross-talk between mitochondria and the $O_2^{\bullet -}$ generating NAD(P)H oxidase in ovarian tumors [90]. The mitochondria controls NAD(P)H oxidase redox signaling, therefore loss of this control contributes to tumorigenesis [90]. Another study found not only an increase in the NOX4 subunit of NAD(P)H oxidase, but that high levels of ROS production was a result of upregulated NOX4 in ovarian cancer cells as compared to immortalized ovarian surface epithelial cells [112].

Glutathione reductase is part of the GSH antioxidant system and catalyzes the reduction of GSSG to GSH in the presence of NADPH, maintaining the high GSH/GSSG ratio in the cell [61]. Glutathione, an important antioxidant, is involved in regulating mutagenic mechanisms, DNA synthesis, growth, and multidrug and radiation resistance, especially in cancer [113,114]. Due to it's antioxidant capacity, elevated GSH levels increase resistance to oxidative stress and has been observed in many types of cancer [115]. Another study examined GSH levels in tissues and found an increase in GSH in ovarian cancer as compared to benign and normal ovarian tissues [116]. Glutathione is synthesized from its constituent amino acids, glycine, cysteine and glutamic acid by the sequential action of γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase, with γ-GCS being rate limiting and can also be regenerated from it's oxidized from, GSSG, by GSR [117]. Synthesis of GSH occurs in the cytoplasm and GSH is imported into the mitochondria where it is involved in the elimination of oxidants including H_2O_2, $HO^{\bullet}$, the cytotoxic Fenton reaction product, and the cytotoxic dinitrogen dioxide (N_2O_3) and $ONOO^-$,

both which are products formed by the reaction of NO with O_2 and $O_2^{\bullet -}$, respectively [117]. In this study, GSR mRNA and activity levels were found to be elevated in EOC cells as compared to HOSEpiC cells. Inhibition of GSR has been shown to cause both reduction of intracellular GSH and accumulation of GSSG resulting in increased cellular oxidative stress [118]. GSH depletion in mitochondria has also been associated with dysfunction and loss of cell viability in response to oxidative events [113].

Another important protection against oxidative stress are the glutathione S-transferases (GST) which are involved in detoxification of varieties of environmental carcinogens by catalyzing their conjugation to GSH, and subsequent removal from the cell [35]. There are three main classes, alpha, mu and pi, which are ubiquitous throughout the tissues in the body with the pi class being most predominant in human tumors [119]. The expression of GST-pi has even been shown to be elevated in ovarian cancer as compared to normal ovarian tissue as well as being associated with resistance to chemotherapeutic drugs in ovarian cancer [119-121]. It was found that indeed, pi class (GST-p1) mRNA expression was elevated in EOC cells as compared to normal human epithelial ovarian cells. This is significant, as GST-p1 is believed to play an important protective role in tumor cell pathogenesis and survival and may play a role in the development of ovarian cancer.

One of the principal antioxidant enzymes for the elimination of H_2O_2 is GPX, which has a great affinity for H_2O_2 than CAT and thus competes with CAT for H_2O_2 as a substrate, resulting in the production of GSSG [122]. The distribution of GPX in the body and within the cells depends on the isoform, with the most prevalent form being GPX1, a cytosolic enzyme located in most tissues [123]. In this study, we found a significant decrease in both GPX1 mRNA and GPX activity levels in EOC as compared to HOSEpiC cells. The GSH redox cycle is a major source of protection against mild oxidative stress, whereas CAT becomes increasingly important

in protection against severe oxidative stress [122].

Interestingly, CAT mRNA and activity levels were elevated in EOC cells as compared to HOSEpiC cells. The conversion of $O_2^{\bullet -}$ to H_2O_2 is catalyzed by SOD, and is then converted to H_2O by CAT [34]. A recent study has demonstrated that inhibition of SOD, and subsequent accumulation of $O_2^{\bullet -}$, resulted in an increase in CAT protein levels in EOC cells as compared to normal ovarian epithelial cells [34]. We also found an increase in SOD activity in EOC cells as compared to HOSEpiC cells. An increase in SOD activity suggests an increase in production of H_2O_2, which would in turn upregulate enzymes that eliminate excess H_2O_2, such as CAT. Additionally, elevated rates of H_2O_2 generation have been detected in several human cancer cell lines, including the ovarian cancer cell line SKOV-3, again supporting the idea that SOD would be upregulated to eliminate excessive H_2O_2 [73].

Collectively, this study has demonstrated that there are elevated pro-oxidants iNOS, MPO, and NAD(P)H oxidase as well as elevated levels of antioxidants CAT, GSR, and GST-p1, and decreased levels of GPX, all which suggests ovarian cancer favors a pro-oxidant state. These key enzymes represent potential targets for future therapeutic interventions for ovarian cancer.

CHAPTER IV: MODULATION OF REDOX SIGNALING PROMOTES APOPTOSIS IN EPITHELIAL OVARIAN CANCER CELLS

(This chapter contains previously published material. See Appendix [45,47])

Approach

A distinct oxidative stress profile was described in Chapter III. In this Chapter, we sought to modulate key pro-oxidant enzymes that were upregulated in ovarian cancer, iNOS, MPO, and NAD(P)H oxidase, as compared to normal ovarian tissues and determine the effects on other key markers of oxidative stress and apoptosis.

We also observed iNOS and MPO to be co-localized in the same EOC cell, thus, to investigate this relationship we silenced iNOS or MPO utilizing specific siRNA for iNOS or MPO in EOC cell lines, MDAH-2774 and SKOV-3. Cells ($2 \text{ X } 10^6$) seeded in 60 mm x 15mm cell culture dishes and incubated with siRNA to silence either iNOS or MPO for 24 hours. Media was collected for measurement of nitrate/nitrite, and indicator of NO production from iNOS, levels following siRNA silencing of iNOS or MPO. Cells were collected for RNA and protein extraction. Following RNA extraction, reverse transcription of cDNA was performed which was then utilized in real-time RT-PCR for determination of iNOS and MPO mRNA levels following silencing of iNOS or MPO. Moreover, S-nitrosylation of caspase-3, a key control of caspase-3 activity, was determined utilizing immunoprecipitation/western blot following silencing of iNOS or MPO.

Since NAD(P)H oxidase was found to be elevated in ovarian cancer tissues, we sought to modulated NAD(P)H oxidase through its inhibition with diphenyleneiodonium in cells (5×10^6) seeded in 100 cm^2 culture dishes and treated for 24 hours. Cells were collected for RNA and protein extraction. Following RNA extraction, reverse transcription of cDNA was performed which was then utilized in real-time RT-PCR for determination of the NAD(P)H oxidase subunit

$p22^{phox}$, HIF-1α, and SOD3 mRNA levels. Expression of the $p22^{phox}$ subunit of NAD(P)H oxidase was evaluated to confirm inhibition NAD(P)H oxidase. Both HIF-1α and SOD3 protein levels were determined utilizing ELISA and immunoprecipitation/western blot, respectively. Additionally, caspase-3 activity and TUNEL staining, indicators of apoptosis, were determined for each experiment.

For the siRNA study, data were analyzed using SPSS 15.0 for Windows. A mixed model repeated measures ANOVA was used with treatment as the within factor and cell type as the between factor. Paired comparisons with a Bonferroni correction were used to compare pairs of treatments. Significant interactions between treatment and cell type were analyzed with independent sample t-tests by cell type on each treatment.

For the inhibition of NAD(P)H oxidase study, data were analyzed using SPSS 19.0 for Windows. Additionally, data were analyzed using one-way ANOVA (analysis of variance) with Student Neuman-Kuels post-hoc comparisons. $P<0.05$ was considered statistically significant for all analyses.

Introduction

In Chapter III we determined that indeed ovarian cancer exhibit a distinct oxidative stress profile favoring a pro-oxidant state. We observed an overexpression of iNOS, MPO, and NAD(P)H oxidase in ovarian cancer tissues as compared to controls. Additionally, iNOS and MPO were found to be co-localized in the same EOC cell, suggesting the existence of a cross-talk relationship. Therefore, we sought to further investigate the role of these key pro-oxidant enzymes following their modulation and the subsequent effect on additional key markers of oxidative stress as well as apoptosis.

Malignant cells are resistant to apoptosis through a mechanism that may involve alterations in their redox balance. Epithelial ovarian cancer cells have been shown to have

significantly increased levels of NO, which correlated with increased expression in iNOS [46]. Also, EOC cells manifested lower apoptosis, which was markedly induced by inhibiting iNOS by L-NAME, indicating a strong link between apoptosis and NO/iNOS pathways in these cells [46]. Myeloperoxidase utilizes NO, produced by iNOS, as a one-electron substrate generating NO^+, a labile nitrosating species that is rapidly hydrolyzed forming nitrite NO_2^- as an end product [48-51]. The ability of MPO to generate NO^+, from NO, led us to believe that not only does MPO play a role in S-nitrosylation of caspase-3 in EOC cells, but also highlights a possible cross-talk between iNOS and MPO.

Caspase-3 is known to play a critical role in controlling apoptosis, by participating in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell [107,124-126]. Caspase-3 was found to be S-nitrosylated on the catalytic-site cysteine in unstimulated human lymphocyte cell lines and denitrosylated upon activation of the Fas apoptotic pathway [127]. Decreased caspase-3 S-nitrosylation was associated with an increase in intracellular caspase activity. Caspase-3 S-nitrosylation/denitrosylation is known to serve as an on/off switch regulating caspase activity during apoptosis in endothelial cells, lymphocytes and trophoblasts [128-131]. The mechanisms underlying S-nitrosothiol (SNO) formation *in vivo* are not well understood.

Cancer cells are also known to be under intrinsic oxidative stress and manifest significantly increased levels of ROS, and thus express higher levels of SOD, which has been reported to stabilize the HIF-1α protein, enabling HIF-1α to dimerize, forming an active transcription factor [73,132-134]. Since rapidly growing tumors become hypoxic, questions are raised as to what promotes an increase in SOD, and why HIF-1α is stabilized and not degraded, with the increase in ROS under the hypoxic environment in cancer cells [135,136]. Restoration of the ROS balance in cancer cells may provide a potential therapeutic intervention to selectively

eliminate cancer cells via apoptosis. Inhibition of NAD(P)H oxidase and other pro-oxidant enzymes has been reported to significantly induce apoptosis of cancer cells [137,138]. Several agents have been utilized to test this hypothesis, however, few have been tested in ovarian cancer [139-141].

In this study, we sought to further investigate the role of oxidative stress in the pathogenesis of ovarian cancer through the modulation of several key pro-oxidants, iNOS, MPO, or NAD(P)H oxidase utilizing siRNA technology and direct inhibition of these enzymes. Identification of targets to specifically reduce pro-oxidants and/or induce apoptosis in EOC cells may provide a potential therapeutic target for selective elimination of cancer cells.

Methods

Cell culture of EOC cell lines, MDAH-2774 and SKOV-3 is described in *General Methods.* For siRNA experiments, cells (2×10^6) were plated in 60 mm x 15mm cell culture dishes. For inhibition of NAD(P)H oxidase experiments, cells were plated (5×10^6) in 100 cm^2 culture dishes and incubated for 24 hours following treatment with 10 μM DPI (Sigma-Aldrich, Saint Louis, MO) for 0, 0.5, 1, 3, 6, 12, and 24 hours. The dose of DPI was selected based on previous studies [139-141]. Cells were harvested at each time point. All experiments were performed in triplicate. Real-time RT-PCR methods are described in *General Methods.*

siRNA Design, Synthesis, and Transfection for iNOS and MPO

SiRNAs were designed after determination of target sequences by aligning iNOS and MPO sequences to an Ambion web-based algorithm. The 21-nucleotide duplex siRNA molecules with 3-dTdT overhangs were re-suspended in nuclease-free water according to the instructions of the manufacturer (Ambion, Austin, TX). To ensure stringent controls, both a 2A-based mutated control siRNA with 2 nucleotide mismatches (siRNA-2Amut) and a scrambled control sequence (siRNA-SCR) obtained from Ambion (Silencer Negative Control No. 1 siRNA,

Ambion) were used.

Cells were grown to a confluence of 30% to 40% in 12-well plates (BD Bioscience, Franklin Lakes, NJ) and transfected with the use of 3 µL NeoFX reagent (Ambion), 2 µl of 20 µmol siRNA, and OptiMEM medium (Invitrogen) up to a final volume of 100 µl. Neo FX reagent and siRNA were incubated at room temperature for 10 minutes and then applied onto 1.0 X 10^5 cells per well. Transfection mixtures were incubated with cells for 24 hours before washing cells with medium and incubated for an additional 24 hours. Experiments were performed in triplicate for each of the two cell lines.

Detection of S-nitrosylation of Caspase-3 Following siRNA Knockdown of iNOS or MPO

Ovarian cancer cell lysates from the different treatments were immunoprecipitated with anti-caspase-3 polyclonal antibody conjugated with protein A/G plus agarose beads. Immunoprecipitated caspase-3 zymogen was released by boiling the beads at 95 °C for 5 minutes. Biotinylated proteins were separated by SDS-PAGE and detected using nitrosylation detection reagent I (HRP), according to the manufacturer's protocol.

Measurement of Caspase-3 Activity

Chemicon's Caspase-3 Colorimetric Activity Assay Kit (Chemicon, Temecula, CA) was used. The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. The free pNA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-3 activity. Cells (2 x 10^6) were harvested and lysed in 300 µl of cell lysis buffer included with the kit, and concentrations were equalized for each sample set. Subsequently, 150 µg of cell lysate was combined with substrate reaction buffer containing 30 µg of caspase-3 substrate, acetyl-DEVD-p-nitroaniline (Ac-DEVD-pNA). This mixture was

incubated for 1h at 37°C, and then absorbance was measured with a plate reader (Ultramark, BIO-RAD, Hercules, CA). Background reading from cell buffers and substrate were subtracted from the readings of samples before calculating increase in caspase-3 activity.

Measurement of Apoptosis by TUNEL Assay

DNA fragmentation was assessed by the *in situ* Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique per the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) manufacturer's protocol and as we have previously described [142,143]. Positive controls were performed by treating cells with DNase I (1 mg/ml) in TdT buffer for 10 minutes at room temperature before incubation with a biotinylated nucleotide. Briefly, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C, and subjected to permeabilization for 5 min at room temperature with 0.2% Triton X-100. Next, cells were labeled with the TUNEL reaction mixture for 60 min at 37 °C. The nuclei of these cells were also stained with 4′,6′-diamino-2-phenylindole (DAPI). Fluorescein-labeled DNA, an indication of DNA fragmentation, was analyzed by using an Axiovert immunofluorescent microscope (Carl Zeiss Microimaging, Thornwood, NY) and recorded with a microscope-mounted camera (Carl Zeiss) using DAPI (blue) and FITC (green), fluorescent filters with excitation and emission wavelengths of 365 and 445, 470 and 525 nm respectively.

Measurement of SOD3 Protein Levels in EOC Cells Following Inhibition of NA(P)DH Oxidase

Immunoprecipitation (IP)/Western blot was utilized as previously described with the following changes [144]. Cells were lysed with lysis buffer and cleared by centrifugation (10 minutes at 1,000*g*, 4 °C). Protein concentration of cell lysates was measured with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) per the manufacturer's protocol. The same concentration of protein was utilized for each sample. Precleared cell lysates were incubated with anti-SOD3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at

4 °C, followed by precipitation with 20 µl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and incubated at 4 °C overnight. Adherent proteins were eluted with 1 x protein loading buffer for 5 minutes at 80 °C and analyzed by a Western blot detection kit (Visualizer, Millipore, Temecula, CA). SOD3 bands were scanned and analyzed by NIH Image J 3.0 (.U. S. National Institutes of Health, Bethesda, Maryland).

Measurement of HIF-1α Levels in EOC Cells Following Inhibition of NA(P)DH Oxidase

Protein concentration of cell lysates was measured as described in *General Methods*. Cell lysates were prepared from the various treatments of the EOC cell lines, SKOV-3 and MDAH-2774. HIF-1α was measured with an enzyme-linked immunosorbent assay kit (HIF-1α ELISA, R&D Systems, Minneapolis, MN), per the manufacturer's protocol.

Statistical Analysis

For siRNA studies, data were analyzed using SPSS 15.0 for Windows. A mixed model repeated measures ANOVA was used with treatment as the within factor and cell type as the between factor. Paired comparisons with a Bonferroni correction were used to compare pairs of treatments. Significant interactions between treatment and cell type were analyzed with independent sample t-tests by cell type on each treatment.

For inhibition of NAD(P)H oxidase studies, data were analyzed using SPSS 19.0 for Windows. Additionally, data were analyzed using one-way ANOVA (analysis of variance) with Student Neuman-Kuels post-hoc comparisons.

Results

Cross-talk between MPO and iNOS gene expression in EOC cells

To determine the relationship between iNOS and MPO gene expression in EOC cells, we utilized the siRNA technology to silence iNOS gene expression and examined MPO expression, and vice-versa. Our results clearly indicate that silencing iNOS gene expression resulted in a 47

and 36% reduction in MPO gene expression in MDAH-2774 and SKOV-3, respectively (Figure 12A). Similarly, silencing MPO gene expression also resulted in a 43 and 42% reduction in iNOS gene expression in MDAH-2774 and SKOV-3, respectively (Figure 12B). There was no difference in MPO and iNOS mRNA levels between control and scrambled nonspecific siRNA (data not shown). *p<0.05 as compared to control. Error bars represent standard deviation of the mean.

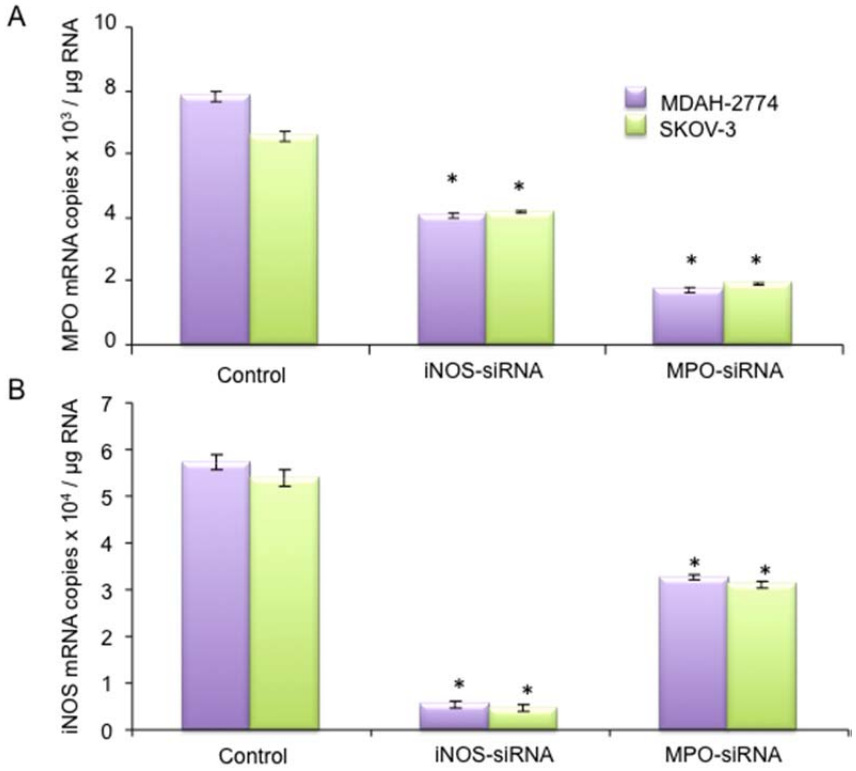

Figure 12: Real-time RT-PCR for MPO and iNOS in EOC Cells Following Silencing of Either iNOS or MPO. Total RNA isolated from EOC cells, MDAH-2774 and SKOV-3, before and after silencing MPO and iNOS gene expression using siRNA specific probes were analyzed utilizing real-time RT-PCR. There was no significant difference between controls and nonspecific scrambled siRNA (Data not shown). *p<0.05 as compared to control. Experiments were performed in triplicate.

Silencing iNOS and MPO gene expression decreased nitrate/nitrite levels.

The Griess assay was performed in 24-hour media collected from after silencing iNOS or MPO gene expression utilizing specific siRNA (Figure 13). *p<0.05 as compared to their

controls. Error bars represent standard deviation of the mean.

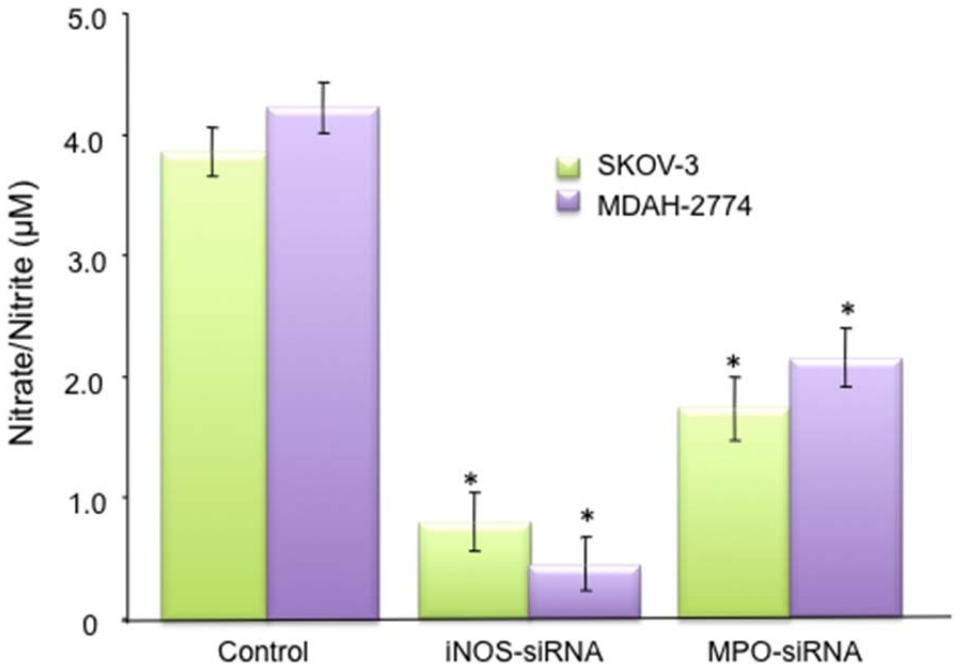

Figure 13: Nitrate/nitrite levels in EOC Cells Following Silencing of Either iNOS or MPO. Levels of nitrate/nitrite were determined in media before and after silencing MPO and iNOS gene expression using siRNA specific probes. *p<0.05 as compared to controls. Error bars represent standard deviation of the mean.

Silencing iNOS and MPO gene expression decreased S-nitrosylation of caspase-3 in EOC cells.

Since the activity of caspase-3 depends on the level of its S-nitrosylation, we investigated the level of S-nitrosylation of caspase-3 in the two cell lines before and after silencing iNOS or MPO gene expression utilizing specific siRNA. The levels of S-nitrosylation of caspase-3 were markedly lower in response to silencing either iNOS or MPO, but to a greater extent when silencing MPO (Figure 14). There was no difference in the intensity between control and scrambled nonspecific siRNA (data not shown). *p<0.05 as compared to their control. Error bars represent standard deviation of the mean.

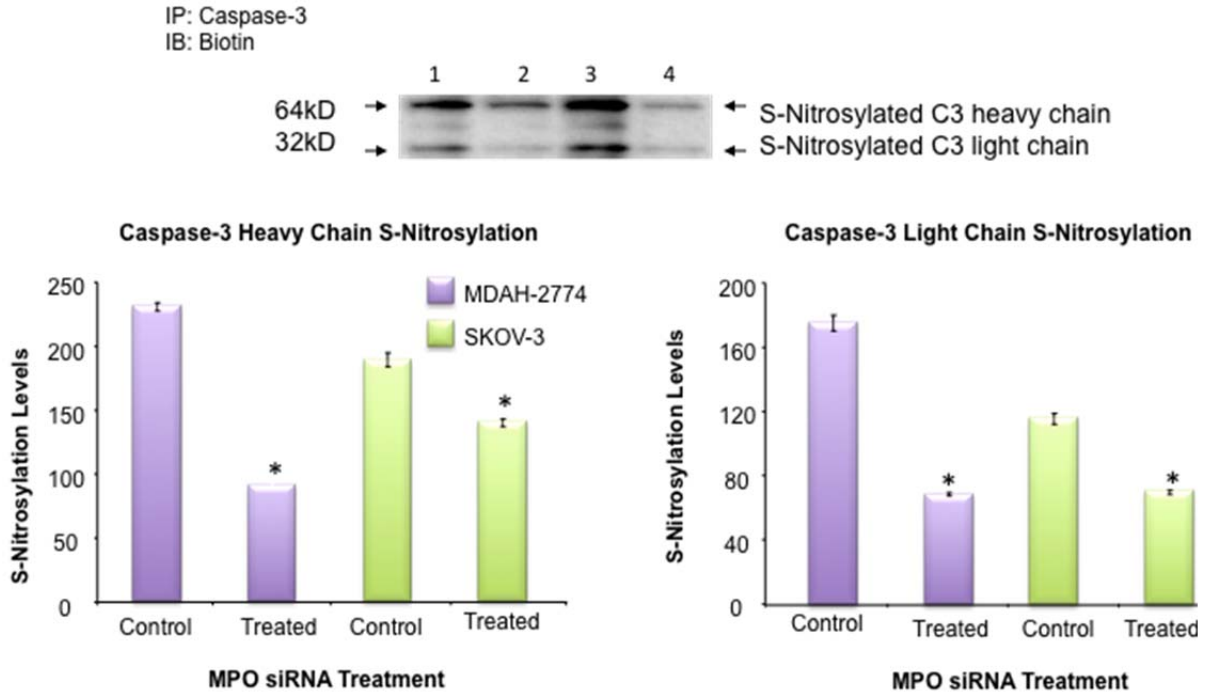

Figure 14. S-Nitrosylation of Caspase-3 in EOC Cells Following Silencing of Either iNOS or MPO. S-nitrosylation of caspase-3 in EOC cells, SKOV-3 before (1) and after silencing MPO gene expression with specific siRNA (2) and MDAH-2774 before (3) and after silencing iNOS gene expression with specific siRNA probes (4). There was no significant difference between controls and nonspecific scrambled siRNA (Data not shown). Experiments were performed in triplicate. *$p<0.05$ as compared to control.

Silencing iNOS or MPO gene expression increased caspase-3 activity and apoptosis in EOC cells.

We have previously reported that EOC cell lines SKOV-3 and MDAH-2774 manifested a marked decrease in their rate of apoptosis and significantly higher rate of proliferation [46,145]. The cause for lower apoptosis is not yet known. Caspase-3 activity increased by 161 and 418% in SKOV-3 and 156 and 446% in MDAH-2774 when silencing iNOS or MPO gene expression utilizing specific siRNA for iNOS or MPO, respectively (Figure 15A). TUNEL assay showed that these treatments were associated with increased apoptosis in both cell lines (Figure 15B). *$p<0.05$ as compared to control. Error bars represent standard deviation of the mean.

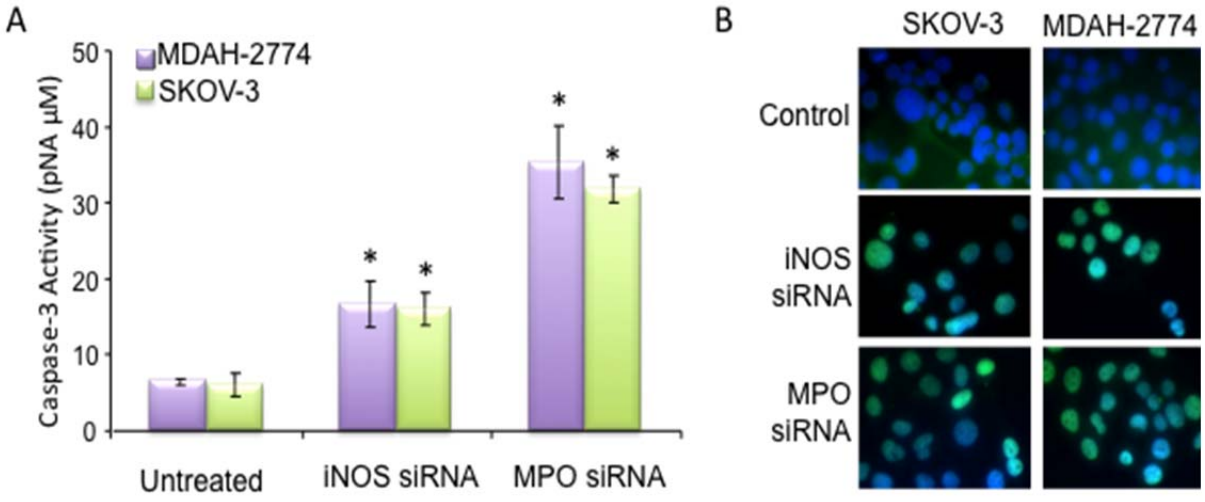

Figure 15: Measurement of Apoptosis Following Silencing of Either iNOS or MPO. A) Caspase-3 Activity was measured in EOC cells, MDAH-2774 and SKOV-3, before and after silencing iNOS or MPO gene expression utilizing siRNA specific probes. A caspase-3 colorimetric activity assay kit was utilized as described in methods. There was no significant difference between controls and nonspecific scrambled siRNA (Data not shown). As can been seen from this figure, caspase-3 activity was significantly increased when silencing MPO or iNOS. Experiments were performed in triplicate. *$p<0.05$ as compared to control. B) The amount of DNA fragmentation (apoptosis) was assessed by TUNEL assay for EOC cells, MDAH-2774 and SKOV-3, before and after silencing iNOS or MPO gene expression utilizing siRNA specific probes. Nuclei were stained with DAPI (blue) and apoptotic cells were visualized with fluorescein-12-dUTP (green). There was no significant difference between controls and nonspecific scrambled siRNA (Data not shown). Experiments were performed in triplicate.

DPI treated EOC cells had reduced levels of NAD(P)H oxidase mRNA.

Real-time RT-PCR was utilized to determine the mRNA level of the NAD(P)H oxidase $p22^{phox}$ subunit, a representative O_2 sensing subunit of NAD(P)H oxidase, in EOC cells treated with and without DPI for 0.5 hours. NAD(P)H oxidase was significantly decreased by 51.6% in SKOV-3 cells, and by 40.1% in MDAH-2774 cells (*$p<0.05$ as compared to control, Figure 16).

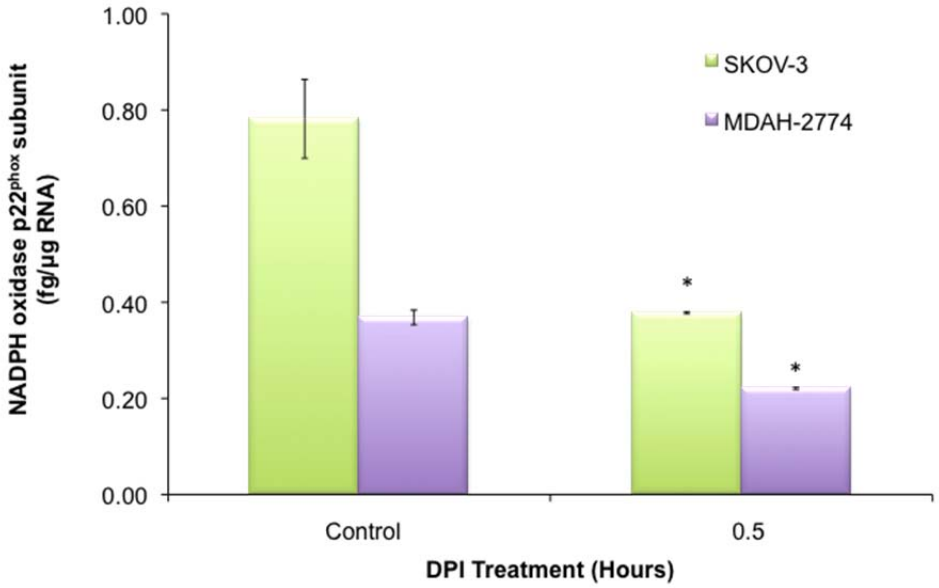

Figure 16: Real-time RT-PCR for NAD(P)H oxidase in EOC Cells Following Inhibition of NAD(P)H Oxidase. Expression of the NAD(P)H oxidase subunit $p22^{phox}$ mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM, 0.5 hrs) was measured using real-time RT-PCR. Results are representative of the mean of three independent experiments. (*$p<0.05$ as compared to their control).

DPI treated EOC cells exhibited increased caspase-3 activity and apoptosis.

Caspase-3 activity significantly increased, in a time dependent fashion, in SKOV-3 cells, from 7.62 to 20.9, 20.3, 22.0, 24.5, 39.4, and 54.3 μM and in MDAH-2774 cells from 6.61 to 19.0, 20.4, 23.1, 25.6, 39.3, and 53.1 μM at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively (*$p<0.05$ as compared to control, Figure 17A).

These results were confirmed by TUNEL staining, an indicator of the degree of DNA fragmentation, which is representative of apoptosis. Nuclei were stained with DAPI (blue) and apoptotic cells were visualized (60x) with fluorescein-12-dUTP (green). There was a significant increase in TUNEL staining (green) as compared to controls, in both EOC cell lines (Figure 17B).

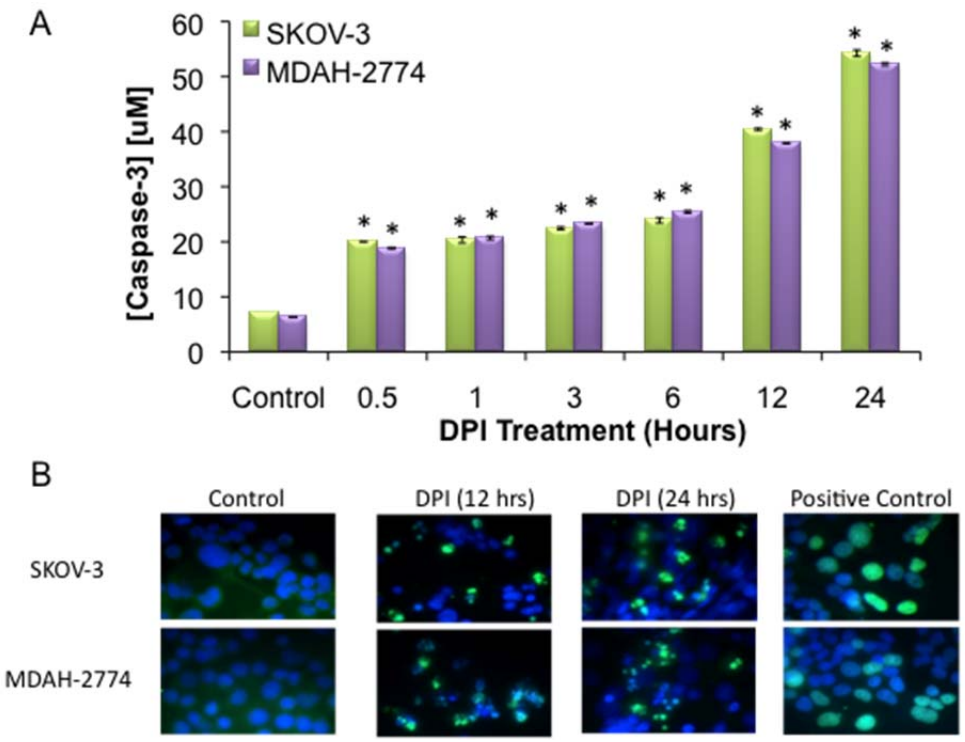

Figure 17: (A) Caspase-3 Activity and (B) Apoptosis in EOC Cells Following Inhibition of NAD(P)H Oxidase. (A) Caspase-3 activity was measured in cell lysates from SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM) at various time points. (B) The amount of DNA fragmentation (apoptosis) was assessed by TUNEL assay in MDAH-2774 and SKOV-3, before and after DPI treatment (10 μM, 12 and 24 hrs) as compared to control cells. Nuclei were stained with DAPI (blue) and apoptotic cells were visualized (60x) with fluorescein-12-dUTP (green). Results are representative of the mean of three independent experiments. (*$p<0.05$ as compared to their control).

DPI treated EOC cells had reduced levels of HIF-1α.

DPI treatment significantly reduced HIF-1α mRNA levels, in SKOV-3 cells, from 428.2 to 368.2, 318.6, 268.8, 261.3, and 206.8 pg/μg RNA at the 0.5, 3, 6, 12, and 24 hour time points, and in MDAH-2774 cells from 421.3 to 356.9, 356.1, 327.4, 260.3, 240.0 and 223.1 pg/μg RNA, at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively (*$p<0.05$ as compared to control, Figure 18A). There was no statistically significant change in HIF-1α mRNA levels at the 1 hour time point in SKOV-3 cells.

HIF-1α protein levels, in SKOV-3 cells, were significantly reduced from 1699 to 828.0,

377.2, 311.0, 291.1, 224.6, and 44.27 pg/ml total protein and in MDAH-2774 cells from 1872 to 658.1, 332.4, 257.5, 111.8, 106.1, and 22.96 pg/ml total protein at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively ($p<0.05$ as compared to control, Figure 18B).

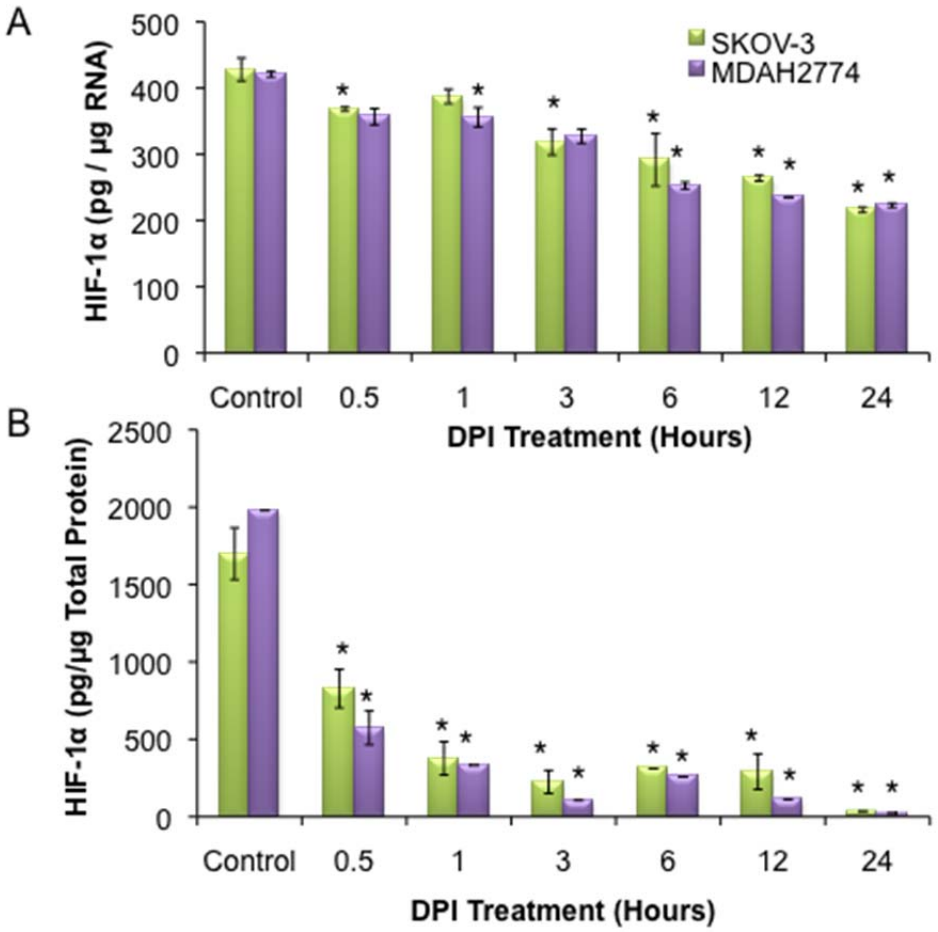

Figure 18: HIF-1α levels in EOC Cells Following Inhibition of NAD(P)H Oxidase. (A) HIF-1α mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 µM), at various time points, were measured using real-time RT-PCR. (B) ELISA was performed at different time points for cell lysates from SKOV-3 and MDAH-2774 before and after DPI treatment (10 µM) at various time points. Results are representative of the mean of three independent experiments. Results are representative of the mean of three independent experiments. (*$p<0.05$ as compared to their control).

DPI treated EOC cells had reduced levels of SOD3

DPI treatment significantly reduced SOD3 mRNA levels, in a time dependent fashion, from 31.1 to 19.5, 24.6, 15.7, 9.23, 4.63, and 3.41 fg/µg RNA at the 0.5, 1, 3, 6, 12, and 24 hour

time points, respectively, in SOKV-3 cells (*$p<0.05$ as compared to control, Figure 19A). Similarly, SOD3 mRNA levels were significantly reduced from 26.1 to 19.69, 16.0, 11.7, 8.63, and 6.68 fg/μg RNA at the 0.5, 3, 6, 12, and 24 hour time points, respectively, in MDAH-2774 cells ($p<0.05$ as compared to control, Figure 19A). There was no significant change in SOD3 mRNA levels at the 1 hour time point in MDAH-2774 cells.

Cell lysates from SKOV-3 and MDAH2774 before and after DPI treatment were precipitated with anti-SOD3 antibody and fractionated with SDS-PAGE. Membrane was probed with anti-SOD3 antibody. B: Immunoprecipitation of SOD3 from SKOV-3 (Lane 1-7), and MDAH2774 (Lane 8-14), exposed to SOD3. C: IP/Western blot results were scanned and analyzed by NIH image J 3.0. SOD3 protein levels, in SOKV-3 cells, were reduced from 251.6 to 223.2, 215.7, 181.6, and 149.0 at the 3, 6, 12, and 24 hour time points, respectively (*$p<0.05$ as compared to control, Figure 19C) and in MDAH-2774 cells from 254.4 to 237.9, 230.4, 208.5, 188.4, and 170.1 at the 1, 3, 6, 12, and 24 hour time points, respectively (*$p<0.05$ as compared to control, Figure 19C). There was no significant change in SOD3 protein levels at the 0.5 hour time point in both SKOV-3 and MDAH-2774 cells and at the 1 hour time point in SKOV-3 cells. Results were expressed as relative levels as compared to their control.

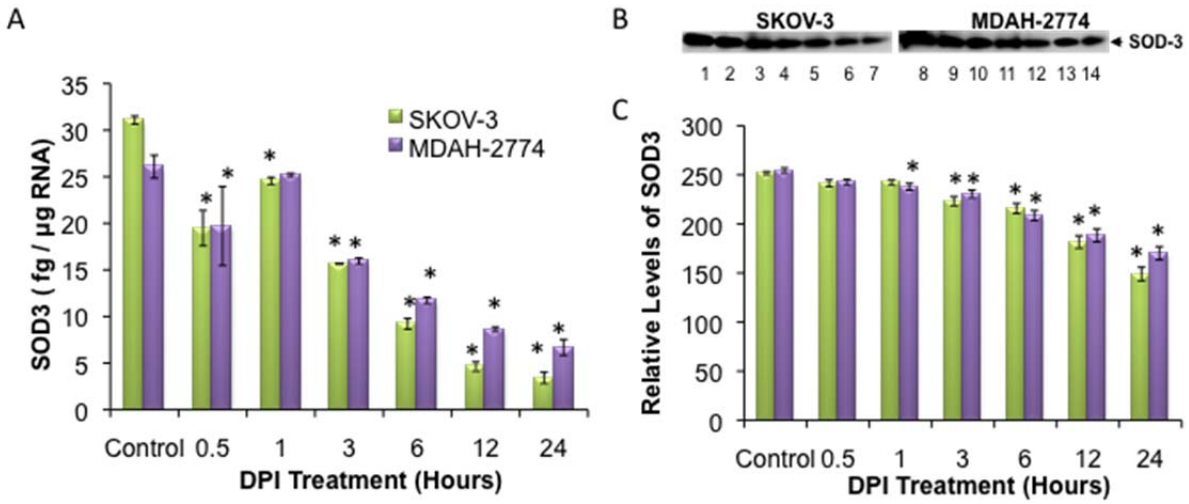

Figure 19: SOD3 levels in EOC Cells Following Inhibition of NAD(P)H Oxidase. (A) SOD3 mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 µM), at various time points, were measured using real-time RT-PCR. (B) IP/Western blot was utilized to detect SOD3 protein levels in EOC cell lines, SKOV-3 and MDAH-2774 before and after DPI treatment. Lysates were precipitated with anti-SOD3 antibody and fractionated with SDS-PAGE. Membrane was probed with anti-SOD3 antibody. Immunoprecipitation of SOD3 exposed to SOD3 antibody are as follows: Lanes 1 & 8; control, Lanes 2 & 9; 0.5 hr, Lanes 3 & 10; 1 hr, Lanes 4 & 11; 3 hr, Lanes 5 & 12; 6 hr, Lanes 6 & 13; 12 hr, and Lanes 7 & 14; 24 hr. (C): IP/Western blot results were scanned and analyzed by NIH image J 3.0. Results are representative of the mean of three independent experiments. (*$p<0.05$ as compared to their control).

Discussion

Our results clearly show that modulation of pro-oxidant enzymes iNOS, MPO, or NAD(P)H oxidase resulted in increased apoptosis as evident by an increase in caspase-3 activity and TUNEL staining in EOC cells. Furthermore, silencing of iNOS or MPO resulted in a decrease in nitrate/nitrite, and decrease in S-nitrosylation of caspase-3, suggesting a positive regulation of apoptosis through S-nitrosylation of caspase-3. On the other hand, inhibition of NAD(P)H oxidase decreased both SOD and HIF-1α expression in EOC cells, which is indicative of a decrease in $O_2^{\bullet -}$ production. Collectively, these findings further support a role for these key enzymes in the pathogenesis of ovarian cancer through regulation of apoptosis and are summarized in Figure 20.

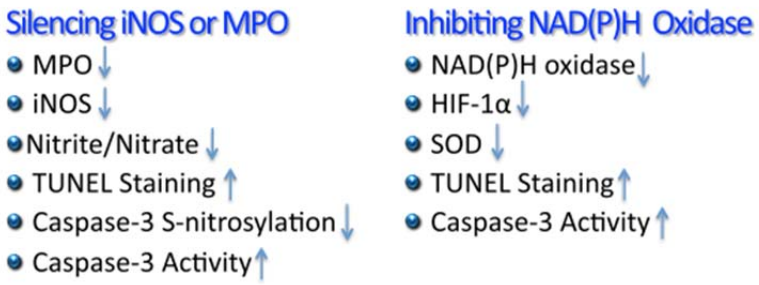

Figure 20: Summary of the effect of modulating oxidative stress in EOC cell lines, SKOV-3 and MDAH-2774. Silencing MPO or iNOS, and inhibition of NAD(P)H oxidase led to an overall increase in apoptosis in EOC cells.

Molecular alterations that lead to apoptosis can be inhibited by S-nitrosylation of apoptotic proteins such as caspases. Thus, S-nitrosylation conveys a key influence of NO on apoptosis signaling and may act as a key regulator for apoptosis in cancer cells. It is known that the effects of NO on apoptosis are not only stimulatory but also inhibitory as shown in this study. These paradoxical effects of NO on apoptosis seem to be influenced by a number of factors. It has been suggested that biological conditions, such as the redox state, concentration, exposure time and the combination with O_2, $O_2^{\bullet -}$ and other molecules, determine the net effects of NO on apoptosis [146]. Also, NO is implicated in both apoptotic and necrotic cell death depending on the NO chemistry and the cellular biological redox state [146]. We have previously demonstrated that the EOC cell lines, SKOV-3 and MDAH-2774, manifest lower apoptosis and have significantly higher levels of NO due to the presence of high levels of iNOS [46,145]. Additionally, it has been shown that MPO can consume NO in the presence of the co-substrate H_2O_2 [49]. Based on these reports we hypothesized that MPO uses the existing cellular NO to produce NO^+, which is the main source of protein S-nitrosylation, specifically caspase-3 in EOC cell lines.

Since resistance to apoptosis is a hallmark of tumor growth, identifying mechanisms of this resistance such as S-nitrosylation may be a key in cancer progression. S-nitrosylation is

reversible and seemingly specific post-translational modification that regulates the activity of several signaling proteins [147]. S-nitrosylation of the catalytic site cysteine in caspases serves as an on/off switch regulating caspase activity during apoptosis in endothelial cells, lymphocytes, and trophoblasts [129-131]. Several mechanism of denitrosylation have now been demonstrated and include activation of the Fas apoptotic pathway or through the thioredoxin or *S*-nitrosoglutathione pathways [127,148]. Thus, targeting MPO may be a potential therapeutic intervention to reverse the resistance to apoptosis in epithelial ovarian cancer cells.

In this study we also sought to determine the effects of inhibiting the generation of ROS by DPI, a well-characterized, potent inhibitor of flavoenzymes including NAD(P)H oxidase, on apoptosis of EOC cells, and whether these effects are associated with SOD3 and HIF-1α expression [149,150]. Diphenyleneiodonium has been used to inhibit ROS production mediated by NAD(P)H oxidase in various cell types [134,150,151]. We have demonstrated EOC cells to have elevated NAD(P)H oxidase, which was subsequently reduced by DPI, a NAD(P)H oxidase inhibitor, as indicated by a decrease in the NAD(P)H oxidase $p22^{phox}$ subunit. These findings are supported by the fact that increased NAD(P)H oxidase levels promote the tumorigenic potential of NIH3T3 mouse fibroblasts as well as the DU-145 prostate epithelial cells [149].

Inhibition of NAD(P)H oxidase has been reported to significantly limit the conversion of molecular O_2 to $O_2^{\bullet-}$, H_2O_2 and other ROS [137,138]. Growing evidence suggests that cancer cells exhibit increased intrinsic ROS stress, due in part to oncogenic stimulation, increased metabolic activity, and mitochondrial malfunction [152,153]. Further support for this increase in ROS is demonstrated by a cross-talk between mitochondria and the $O_2^{\bullet-}$ generating NAD(P)H oxidase in ovarian tumors [90]. The mitochondria controls NAD(P)H oxidase redox signaling, therefore loss of this control contributes to tumorigenesis [90]. In agreement with a previous study, we have shown that inhibition of NAD(P)H oxidase-dependent ROS generation with DPI

induced apoptosis in EOC cells, suggesting that the ROS produced by NAD(P)H oxidase, at least in part, exert an anti-apoptotic function [154]. This anti-apoptotic mechanism involves induced inhibition of phosphorylation of AKT and subsequent suppression of AKT-mediated phosphorylation of ASK1 on Ser-83 [154-156]. Furthermore, the anticancer drug paclitaxel-induced apoptosis of ovarian cancer cells is mediated by negative regulation of AKT–ASK1 phosphorylation signaling whereas AKT activation by H_2O_2 confers protection against apoptosis [154-156].

In addition, we have shown that DPI treatment significantly reduced SOD3 and HIF-1α mRNA levels as early as 30 minutes after treatment, with significant further reduction over the following 24 hours in EOC cells. A parallel reduction in protein levels, although not with equivalent magnitude, was also observed for both SOD3 and HIF-1α as determined by IP/Western blot and ELISA. This may be a consequence of post-translation modifications, which may result in increased stability and/or lower degradation of the proteins. These findings demonstrated that inhibition of NAD(P)H oxidase attenuates the expression of both SOD3 and HIF-1α, at the mRNA and protein levels. Moreover, our results showed that the inhibition of NAD(P)H oxidase significantly induced apoptosis of EOC cells, as assessed by both caspase-3 activity and TUNEL assays. Therefore, there appears to be a strong association between the inhibition of NAD(P)H oxidase and the subsequent reduction in SOD3 and HIF-1α levels and increase in apoptosis of EOC cells.

The correlation between HIF-1α and cellular apoptosis has previously been demonstrated in lung and hepatoma cancer cells [138,157]. Apoptosis can regulate HIF-1α through the modulation of the ratio of pro-apoptotic Bcl-2 and anti-apoptotic Bcl-2 family proteins [158]. Anti-apoptotic Bcl-2 and Bcl-xL levels have been shown to increased and pro-apoptotic BAX and BAK levels were decreased with the over-expression of HIF-1α [158]. Also, it has been

reported that inhibition of HIF-1α by rapamycin resulted in an increase in apoptosis of cancer cells, and decrease in the expression of apoptosis inhibitor Bcl-2 in ovarian cancer xenografts and that rapamycin enhanced cell death through the inhibition of cell survival signals in a number of cell lines [159]. Rapamycin has been demonstrated to decrease HIF-1α through interference with the mechanism that promotes the stabilization of this protein under hypoxic conditions [160].

Most of the generated $O_2^{\bullet -}$ undergoes a nonenzymatic or SOD-catalyzed reaction, generating H_2O_2 as an end product [161-163]. Hydrogen peroxide is freely diffusible through biological membranes, and its overproduction is extremely destructive to cells and tissues, yet it is physiologically important among ROS given its relative long half-life in the intracellular space, and that it is the precursor of the more toxic hydroxyl radicals [162-164]. It has been reported that increased SOD3 expression in ovarian cancer is a cellular response to intrinsic ROS stress [70]. However, the role of SOD3 in tumorigenesis is somewhat controversial. It has been recently demonstrated, in mice, that subcutaneous inoculation of the SOD3 gene significantly suppressed lung cancer metastasis and that over-expression of SOD3 resulted in *in vivo* inhibition of growth of B16-F1 melanoma tumors [165,166]. In contrast, inhibition of SOD has been shown to selectively induce apoptosis of leukemia and ovarian cancer cells, confirming our findings from the present study [34].

Under hypoxic conditions, high expression levels of SOD3 are reported to significantly induce the expression of HIF-1α in tumors, and thus demonstrate a relationship between the SOD3 and HIF-1α pathways [159]. The mechanism by which SOD3 upregulates HIF-1α is not well understood, but there is substantial evidence to suggest that this mechanism is modulated, in part, by the steady-state level of $O_2^{\bullet -}$ and the stabilization of HIF-1α [167]. Therefore, reduction of $O_2^{\bullet -}$ levels via inhibition of NAD(P)H oxidase may result in lowering SOD3 levels, leading to

the destabilization of HIF-1α, subsequently increasing apoptosis.

Collectively, based on previously discussed published reports and the results from this study, we conclude that lowering oxidative stress, either through inhibition of iNOS, MPO or NAD(P)H oxidase induces apoptosis in ovarian cancer cells and may serve as potential targets for future ovarian cancer therapy.

CHAPTER V: MYELOPEROXIDASE AND FREE IRON LEVELS: POTENTIAL BIOMARKERS FOR EARLY DETECTION AND PROGNOSIS OF OVARIAN CANCER

(This chapter contains previously published material. See Appendix [168])

Approach

Routine screening for ovarian cancer in the general population is not recommended because traditional screening methods lack sensitivity and specificity. We are the first to report an increase in MPO expression in epithelial ovarian cancer as compared to normal ovarian tissues and cells. This expression of MPO was surprising, as it is believed to be expressed solely in cells of hemopoietic origin. The elucidation of the function of MPO in EOC is the basis for the chapter. Recently it has been shown *in vitro* that HOCl, produced from H_2O_2 by MPO in the presence of chloride, can destroy MPO and serve as a source of free iron [169]. This study sought to identify whether a relationship exists between serum MPO and free iron with stage of ovarian cancer and their potential roles as biomarkers for the early detection of ovarian cancer. Both serum and tissue samples were collected from women with stages I through IV ovarian cancer, benign gynecologic conditions, inflammation (endometriosis), and healthy controls. Myeloperoxidase ELISA and VITROS Fe Slide assays were used to measure serum and tissue MPO and free iron levels, respectively. The VITROS Fe Slide Assay was performed in collaboration with the Chemistry Department at Wayne State University, Detroit, MI.

Data were analyzed using SPSS 19.0 for windows (SPSS for Windows, Chicago, IL). Data were analyzed using one-way ANOVA (analysis of variance) with Student Neuman-Kuels post-hoc comparisons. $P < 0.05$ were considered statistically significant for all analyses.

Introduction

The underlying pathophysiology of epithelial ovarian cancer is not clearly understood [1].

Because early-stage ovarian cancer presents with nonspecific symptoms, diagnosis is often not made until after the malignancy has spread beyond the ovaries [170]. The development of sensitive and specific methods for early detection has been a priority as a means for improving the diagnosis and treatment of this disease.

The role of MPO, an enzyme stored in the azurophilic granules of polymorphonuclear neutrophils and macrophages, and is released into the extracellular fluid in response to inflammation in carcinogenesis, has been implicated in both the activation of procarcinogens to genotoxic intermediates and the potentiation of xenobiotic carcinogenicity [109,110]. Clinical studies have documented the association between inflammation and certain cancers for decades [171]. There is convincing evidence that inflammation is a contributing factor in ovarian cancer development, but the role of complement-induced inflammation in tumor initiation or progression remains poorly investigated [172,173]. Stimulated inflammatory cells are capable of inducing genotoxic effects, such as DNA strand breaks, sister chromatid exchanges and mutations, and promotion of neoplastic transformation in nearby cells [174]. Myeloperoxidase utilizes H_2O_2, the dismutation product $O_2^{\bullet-}$, to generate HOCl, a cytotoxic and antimicrobicidal oxidant.

We have recently reported that MPO is expressed in EOC cells and tissues with limited or no expression in normal ovarian tissues [45]. Studies from our laboratory clearly indicated that MPO is responsible for the S-nitrosylation of caspase-3, which inhibited its activity [45]. Silencing MPO gene expression by the utilization of MPO specific siRNA induced apoptosis in EOC cells through a mechanism that decreases MPO-induced S-nitrosylation of caspase-3 [147]. Additionally, we have compelling evidence that leads us to believe that MPO may serve as a source of free iron under oxidative stress, where both NO and $O_2^{\bullet-}$ are elevated [175]. Recently we have shown that, hypochlorous acid (HOCl), the final product of MPO, displays the potential

capacity to mediate hemoprotein heme destruction and subsequent iron release, providing a potential link between elevated MPO and free iron [176-178].

The free iron generated by hemeoprotein destruction not only contributes to elevation of serum iron levels, but may also induce oxidative stress, which can promote lipid peroxidation, DNA strand breaks, and modification or degradation of biomolecules [179-181]. Iron reacts with H_2O_2 and catalyzes the generation of highly reactive hydroxyl radicals, which in turn further increases free iron concentrations by the Fenton and Haber–Weiss reaction [182]. Several studies from our laboratories have provided a mechanistic link between oxidative stress, MPO, higher levels of HOCl and higher free iron that could explain the observed accumulation of free iron in epithelial ovarian cancers tissues [45,175-178].

The search for non-invasive, cost-effective ovarian cancer biomarker tests has been ongoing for many years. Immunizations of mice with ovarian cancer cells has led to hybridoma validation by ELISA, while flow cytometry analysis permitted the discovery of cancer antigen (CA)-125 and mesothelin [5]. Furthermore, the screening of an array of 21,500 unknown ovarian cDNAs hybridized with labeled first-strand cDNA from ten ovarian tumors and six normal tissues led to the discovery of human epididymis protein 4 (HE4) [16]. Most interestingly, HE4 is overexpressed in 93% of serous and 100% of endometrioid EOCs, and in 50% of clear cell carcinomas, but not in mucinous ovarian carcinomas [183]. Although it is not tissue-specific, a number of independent microarray studies identified HE4 as one of the most useful biomarkers for ovarian cancer [13,16,184,185]. In addition to expression on the cellular level, secreted HE4 was detected in high levels in the serum of ovarian cancer patients [186]. Additionally, combining CA-125 and HE4 is a more accurate predictor of malignancy than either alone [187-189].

In this study, we sought to identify a positive correlation between serum MPO and free

iron with stage of ovarian cancer as well as tissue MPO and free iron with stage of ovarian cancer and assess their potential roles as biomarkers for the early detection of ovarian cancer.

Methods

Patient Population

A description of the demographics of the study groups are listed in Tables 2 and 3.

Table 2. Descriptive statistics for age including Mean, Median, and Standard deviation, n=30.

			Statistic	Std. Error
Age at Dx/Entry Into Study:	Mean		52.47	1.947
	95% Confidence Interval for Mean	Lower Bound	48.48	
		Upper Bound	56.45	
	5% Trimmed Mean		52.43	
	Median		53.00	
	Variance		113.706	
	Std. Deviation		10.663	
	Minimum		24	
	Maximum		78	
	Range		54	
	Interquartile Range		10	
	Skewness		-.030	.427
	Kurtosis		1.832	.833

Table 3. Comparative study of the cases and control based on cancer status.

			Status		p-value < 0.05 Sig. (2-sided)
			No Cancer	Ovarian Cancer	
Race	Black	Count	5	1	0.131
		% within Race:	83.3%	16.7%	
		% within Status:	33.3%	6.7%	
	Other	Count	0	1	
		% within Race:	0%	100%	
		% within Status:	0%	100%	
	White	Count	10	13	
		% within Race:	43.5%	56.5%	
		% within Status:	66.7%	86.7%	
Age	LE median (53)	Count	10	6	0.272
		% within Age:	62.5%	37.5%	
		% within Status:	66.7%	40.0%	
	GT median (53)	Count	5	9	
		% within Age:	35.7%	64.3%	
		% within Status:	33.3%	60.0%	
Region / Source	CHTN	Count	6	0	.000
		% within Region/Source:	100%	0%	
		% within Status:	40.0%	0%	
	GOG	Count	0	11	
		% within Region/Source:	0%	100%	
		% within Status:	0%	73.3%	
	KCI	Count	8	2	
		% within Region/Source:	80.0%	20.0%	
		% within Status:	53.3%	13.3%	
	Mayo Clinic	Count	1	2	
		% within Region/Source:	33.3%	66.7%	
		% within Status:	6.7%	13.3%	
Total			15	15	
			50%	50%	
			100%	100%	

Pearson Chi-Square was used for statistical test, $p<0.05$ was considered statistically significant. There was no statistically difference between the two groups based on age and race. However, the region or source where the specimen was obtained demonstrated a statistically significant difference that can be attributed to the regional and centers characteristics.

Ovarian Cancer Cases

Serum (N=15) and tissues (N=27) were collected from patients presenting to the gynecologic oncology division of Karmanos Cancer Institute with suspected EOC were invited to participate in a prior study. They underwent informed consent (Wayne State University Human Subject Committee protocol number 027201MP2E) and agreed to provide a blood sample prior to treatment (chemotherapy or surgery). Cases include early through late-stage diagnoses as well as a variety of histologies. Stage I is designated as early-stage, as compared to remaining stages II through IV (II-IV).

Benign Controls

Serum (N=14) and tissues (N=14) from patients with benign gynecologic conditions was procured through the Cooperative Human Tissue Network (CHTN). These include women diagnosed with ovarian cysts, endometriosis (inflammation), or uterine fibroids.

Healthy Controls

Healthy control sera (n=8) were procured from women recruited through a local community organization. These women presented as healthy, with no history of cancer. Basic information such as age, race, and evidence of benign gynecologic conditions, if any, was obtained at the time of informed consent. The age and racial makeup of this group overlaps with patients with ovarian cancer and benign conditions but is not explicitly matched.

Additionally, we utilized matched tissue samples from the same patient derived serum samples to assess the correlation between MPO and iron in these tissues. In this way we assessed if there was an expected discrimination in the tissues and to what extent that discrimination diluted in the serum.

Sample Collection and Processing

A single 7 cc vial of blood was obtained during normal phlebotomy and the serum was isolated after clotting. Serum was collected into a red top or SST blood tube. The serum clot was allowed to form over 1-2 hours and was pelleted in a desktop centrifuge at 2400 rpm for 10 min. The serum was removed by pipetting and ~1 ml aliquots were stored at –80 °C. Samples were labeled with a coded identifier.

Detection of Myeloperoxidase

Myeloperoxidase was detected with the Myeloperoxidase Enzyme Immunometric Assay Kit (Enzo Life Sciences, Farmingdale, NY). This is a well-established assay in our laboratory and was performed according to the manufacturer's protocol. This kit is for the quantitative

determination of MPO in biological fluids or tissues. The kit uses a monoclonal antibody to MPO immobilized on a microtiter plate to bind the MPO in the standards or sample. A native MPO Standard was provided in the kit. Rabbit polyclonal antibody to MPO was added and binds to the MPO captured on the plate followed by the addition of goat anti-rabbit IgG conjugated to horseradish peroxidase, which binds to the polyclonal MPO antibody. The enzyme reaction is stopped generating a red color detected at 450 nm. The measured optical density is directly proportional to the concentration of MPO in either standards or samples. The sensitivity of the assay, defined as the concentration of human MPO was determined to be 0.019 ng/mL. All experiments were performed in triplicate.

Detection of Free Iron

The VITROS Fe Slide method (VITROS 750, Johnson & Johnson, Rochester, USA) was used for detecting serum and tissue iron levels as described by the manufacturer's protocol. Briefly, 10 μL of sample is applied to a VITROS Fe DT Slide, which then is loaded into a VITROS 950 chemistry system (Ortho Clinical Diagnostics, Inc) located at the University Health Building. The iron in the sample was removed from transferrin at acidic pH and migrates to a reducing layer where ascorbic acid reduces it to the ferrous form. The ferrous iron then is bound to a dye, producing color that is detected (600 nm) as a rate of change in reflection density. While most females have serum iron levels between 37 and 170 μg/dL, this assay has a reportable range of 10 to 500 μg/dL, and is therefore is suitable for detection in both tissues and sera. All experiments were performed in triplicate.

Statistical Analysis

Data were analyzed using SPSS 19.0 for windows (SPSS for Windows, Chicago, IL). Data were analyzed using one-way ANOVA (analysis of variance) with Student Neuman-Kuels post-hoc comparisons. $P < 0.05$ was considered statistically significant for all analyses.

Results

Ovarian cancer stages II-IV manifested higher levels of tissue MPO.

All stages of ovarian cancer had significantly higher levels of MPO as compared to benign and inflammatory groups (Figure 19, $p< 0.05$). Also, ovarian cancer stages II-IV had a significantly higher level of MPO as compared to early-stage ovarian cancer (Figure 21, $p<0.05$). The benign group is not significantly different from the inflammatory group. At the time of running this experiment, no healthy control tissue samples were available.

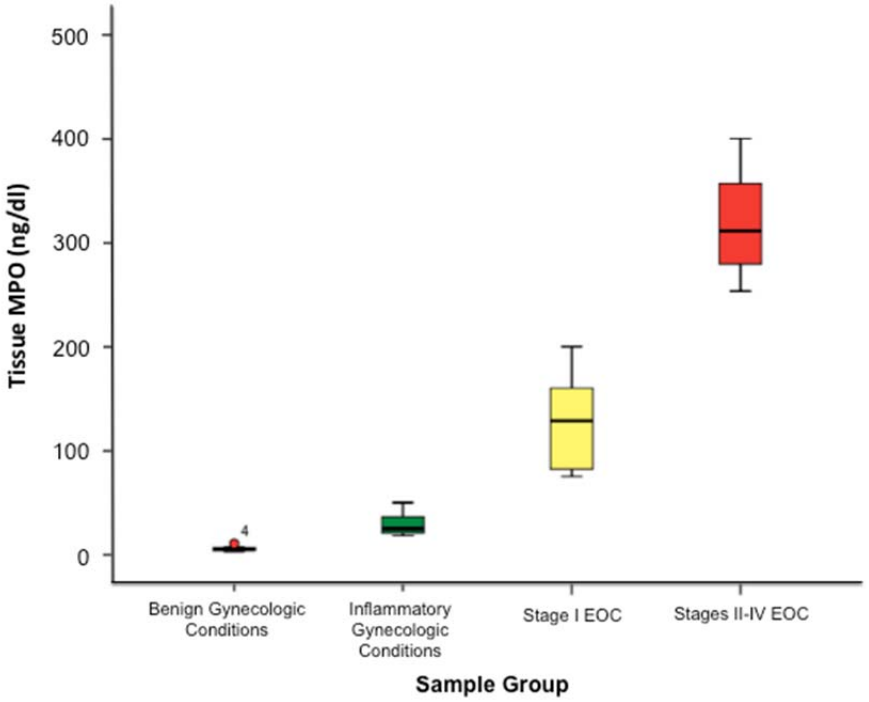

Figure 21: Tissue MPO: Tissue MPO was detected with the Myeloperoxidase Enzyme Immunometric Assay Kit, as described in methods. Early-stage ovarian cancer and ovarian cancer stages II-IV are significantly different than all other groups and each other ($p< 0.05$). The benign group is not significantly different from the inflammatory group. At the time of running this experiment, no healthy control tissue samples were available. In benign gynecologic conditions, the outlier represents a value more than three interquartile ranges above the 75^{th} percentile value.

Ovarian cancer stages II-IV manifested higher levels of serum MPO.

Ovarian cancer stages II-IV had significantly higher levels of MPO as compared to early-

stage ovarian cancer, control, benign, and inflammatory groups ($p<0.05$, Figure 22). Early-stage ovarian cancer and inflammatory gynecologic conditions are significantly different from the control and benign groups, while not significantly different from one another ($p<0.05$, Figure 22). Also, control and benign groups were not significantly different.

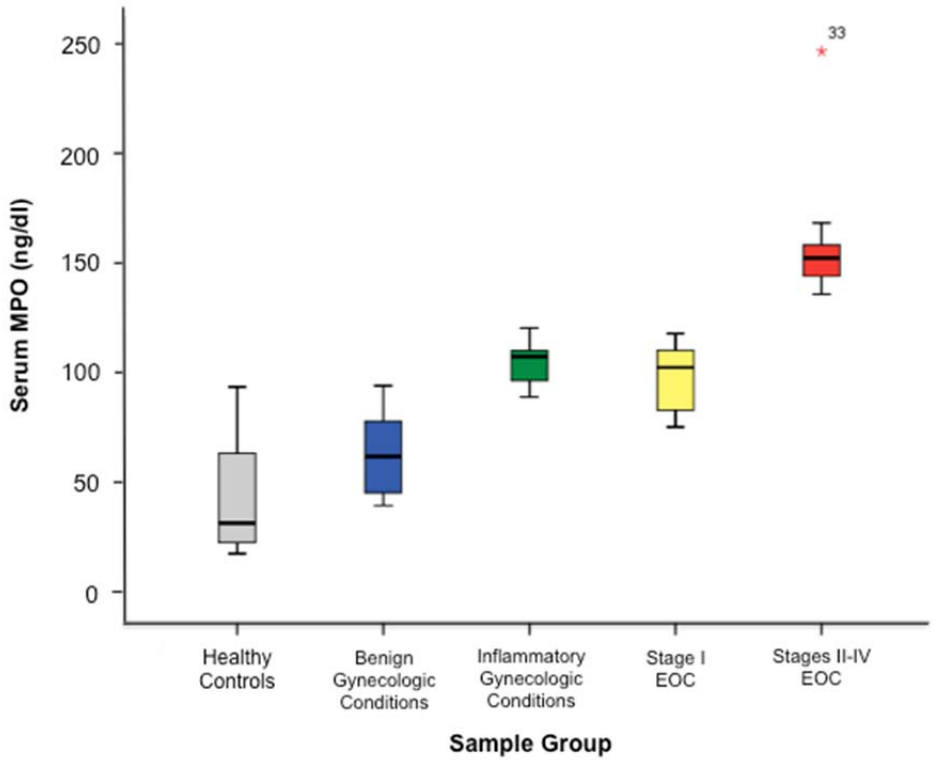

Figure 22: Serum MPO: Serum and tissue MPO was detected with the Myeloperoxidase Enzyme Immunometric Assay Kit, as described in methods. Ovarian cancer stages II-IV are significantly different than all other groups ($p<0.05$). Early-stage ovarian cancer and inflammatory gynecologic conditions are different from control and benign groups, while not one another ($p<0.05$). Control and benign groups are not statistically different. In stages II-IV, the outlier represents a value more than three interquartile ranges above the 75th percentile value.

Ovarian cancer stages II-IV manifested higher levels of tissue iron.

All stages of ovarian cancer had significantly higher levels of tissue iron than benign and inflammatory groups ($p<0.05$, Figure 23). In addition, ovarian cancer stages II-IV had higher levels of iron as compared to early-stage ovarian cancer ($p<0.05$, Figure 23). The benign group is not significantly different from the inflammatory group. At the time of running this experiment,

no healthy control tissue samples were available.

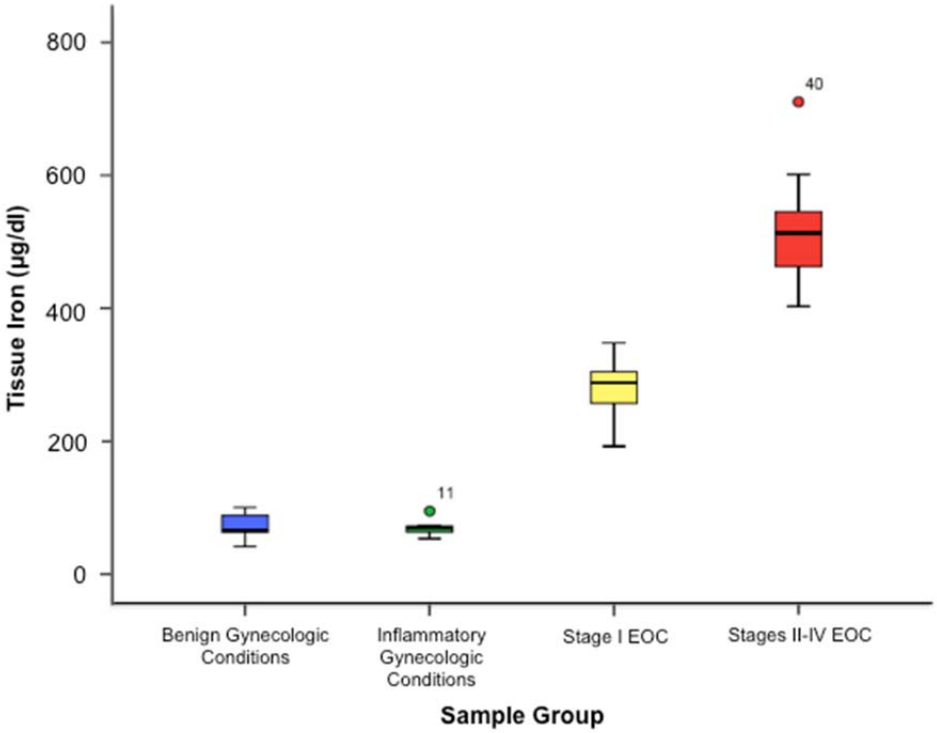

Figure 23: Tissue iron: Serum and tissue iron were detected using the VITROS Fe Slide method as described in methods. Ovarian cancer stages II-IV and early-stage ovarian cancer are significantly different than all other groups as well as each other ($p<0.05$). The benign group is not significantly different from the inflammatory group. At the time of running this experiment, no healthy control tissue samples were available. In inflammatory gynecologic conditions and stages II-IV, the outliers represent a value more than three interquartile ranges above the 75th percentile value.

Ovarian cancer stages II-IV manifested higher levels of serum iron.

All stages of ovarian cancer had significantly higher levels of serum iron as compared to control, benign, and inflammatory groups ($p< 0.05$, Figure 24). In addition, ovarian cancer stages II-IV had higher serum iron levels as compared to early-stage ovarian cancer ($p< 0.05$, Figure 24). There are no significant differences between control, benign and inflammatory groups.

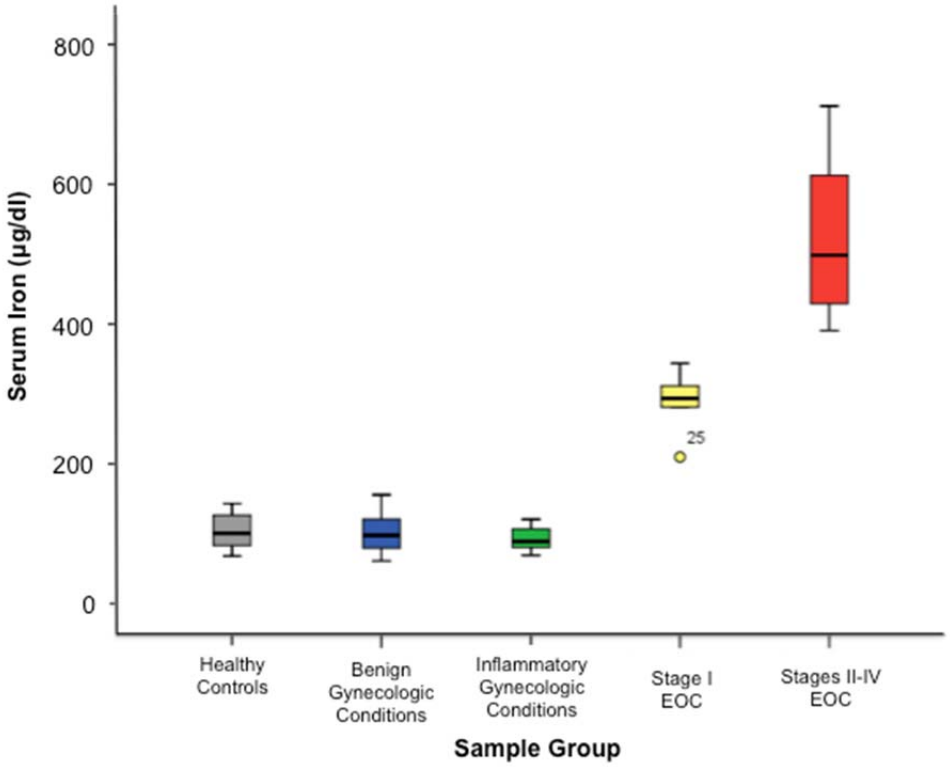

Figure 24: Serum iron: Serum and tissue iron were detected using the VITROS Fe Slide method as described in methods. Ovarian cancer stages II-IV and early-stage ovarian cancer are significantly different than other groups as well as each other ($p<0.05$). There are no significant differences among the control, benign and inflammatory groups. In stage I, the outlier represents a value more than three interquartile ranges above the 75th percentile value.

Discussion

The findings from this study, indicate a role for the combination of serum MPO and free iron as biomarkers for early detection and prognosis of ovarian cancer. Multi-marker panels have the potential for high positive predictive values (PPVs), but careful validation with appropriate sample cohorts is mandatory and complex algorithms may be difficult to implement for routine clinical use [5]. Panels of biomarkers have been extensively investigated to improve sensitivity and specificity and have included some of the most promising reported markers such as CA72-4, M-CSF, OVX1, LPA, Prostacin, Osteopontin, Inhibin and Kallikrein [190-192]. While these and other potential screening paradigms with comparable sensitivities and

specificities have been previously reported, these assays often require specialized equipment not routinely utilized in the clinical immunoassay laboratory, or rely on complex computational algorithms to generate adequate assay performance [193]. With an ovarian cancer prevalence of only 1 in 2500 among postmenopausal women in the U.S., an effective screening strategy for the general population needs to attain a sensitivity of 75% and specificity about 99.6% to attain a minimally acceptable potential PPV of 10% for the detection of all stages of ovarian cancer [193]. No single biomarker reported to date has met these thresholds.

The observation of the involvement of MPO in oxidative stress and inflammation has been a leading factor in the study of MPO as a possible marker of plaque instability and a useful clinical tool in the evaluation of patients with coronary heart disease [194]. Recent genetic studies implicated MPO in the development of lung cancer by demonstrating a striking correlation between the relative risk for development of the disease and the incidence of functionally distinct MPO polymorphisms [195].

We now have substantial evidence to believe that the presence of MPO may play a role in maintaining the oncogenic phenotype of EOC cells [45]. In support of this notion, polymorphisms at position –463 (G to A) in the promoter region of the MPO gene have been identified, with the A variant allele related to decreased transcriptional activity and decreased MPO expression, which may be associated with reduced risk of human cancers [196]. Myeloperoxidase requires heme as a cofactor, which consists of an iron atom contained in the center of a large heterocyclic organic ring [197]. Thus, the MPO G allele might produce more ROS when iron intake is high. Previous studies have investigated the combined roles of MPO and iron levels on cancer development, as well as assessed the impact of MPO genetic polymorphisms on the development of cirrhosis with hereditary hemochromatosis [41,198]. They found that the MPO GG genotype was more common in patients with cirrhosis than in

those without, modifying the clinical penetrance of hepatic iron overload with respect to hepatic fibrosis in hereditary hemochromatosis.

Myeloperoxidase levels reported for various inflammatory disorders are coincidentally lower than those levels found in all stages of ovarian cancer. A previous study reported normal serum MPO and iron levels as 62 ± 11 ng/ml and 96 ± 9 μg/dl, respectively [199]. However, there was a significant increase in serum MPO and iron levels to 95 ± 20 ng/ml, and 159 ± 20 μg/dl, respectively, in asthmatic individuals [199]. Although there was an increase in this reported serum iron, these levels still fell within the normal range (50 to 170 μg/dl) [200,201]. Other studies have showed that an elevated MPO level, reaching up to 350 ng/ml, in serum plasma, was indicative of a higher risk for cardiovascular events in patients hospitalized for chest pain [202,203]. Our data showed an overlap between serum MPO levels in early-stage ovarian cancer with inflammation. However, serum iron levels were significantly higher in early-stage ovarian cancer as compared to inflammation. Thus, there is a potential for a false positive with MPO alone in patients with cardiovascular, inflammation, and/or asthmatic disorders.

Utilizing serum iron levels alone as a biomarker is also not sufficient for early detection of ovarian cancer due to many uncontrolled variables, i.e. dietary intake, supplements, effects of other iron-generating enzymes or factors, and more importantly they are not as specific as MPO levels. Specifically, in iron deficiency anemic patients, their free iron levels may become a confounding factor in its utilization for early detection of ovarian cancer. Thus, anemia should be ruled out to eliminate any overlap that would lead to misdiagnosis. The incorporation of iron deficiency anemic patients in a logistic regression model will help determine its overlap with early-stage ovarian cancer. Additionally, currently available clinical studies focused on either biochemical or more recently, genetic markers of iron overload have reported conflicting results regarding the use of iron levels alone for diagnosis [204-207]. For these reasons, we expect that

the combination of serum MPO and iron levels to yield a higher power of specificity and sensitivity that should distinguish women with early-stage ovarian cancer from other disorders, specifically inflammation. Additionally, combining serum MPO and iron levels with the best currently existing biomarkers through the creation of a logistic regression model may increase the overall predictive values. Collectively, our data strongly supports a role for serum MPO and free iron in the pathophysiology of ovarian cancer, which thereby qualifies them to serve as biomarkers for early detection and prognosis of ovarian cancer.

To validate MPO and free iron as biomarkers for early detection of ovarian cancer, we are currently planning a follow up study on a larger population with the principal endpoint of establishing the best cutoff point using the receiver operating characteristic (ROC) method [208-210]. Also, we will generate sensitivity, specificity, and positive and negative predictive values for serum MPO and free iron alone and in combination. These results will be compared to the existing CA-125 biomarker. As a secondary endpoint, we plan to performed survival analysis using the Kaplan-Meier method with MPO and free serum levels as factors.

CHAPTER VI: A NAD(P)H OXIDASE SINGLE NUCLEOTIDE POLYMORPHISM IS ASSOCIATED WITH AN INCREASED RISK OF OVARIAN CANCER

Approach

In Chapter III, we observed an increase in NAD(P)H oxidase expression in ovarian cancer as compared to normal ovarian tissues, which serves as the rationale for this chapter. Additionally, in Chapter IV, we observed that inhibition of NAD(P)H oxidase lead to an increase in apoptosis in EOC cells, thus indicating a role for this enzyme in the pathogenesis of ovarian cancer, and serves as the rationale for this chapter. Single nucleotide polymorphisms are associated with increased risk of several cancers. We evaluated the association of a SNP (rs4673) in the *CYBA* gene, encoding the $p22^{phox}$ subunit of NAD(P)H oxidase, substituting allele C with T at position 242 located on chromosome 16q24, in high-risk individuals with or without ovarian cancer, with or without a deleterious *BRCA1/2* mutation. This NAD(P)H oxidase subunit serves as an oxygen detector and confers the activity of the enzyme.

To achieve this study, 139 individuals were recruited, in collaboration with the Karmanos Cancer Institute, Detroit, MI, based on personal and family history of cancer. Blood samples were collected and underwent DNA extraction followed by TaqMan® SNP Genotype analysis for rs4673 utilizing the QuantStudio 12K Flex Real-Time PCR System in collaboration with the Applied Genomics Technology Center (AGTC, Wayne State University, Detroit, MI). Data were analyzed using SPSS (IBM, Armonk, New York) for Mac V.21. Pearson Chi-square analyses were performed 1) to compare the SNP mutation frequencies in the present sample to that in the general population; 2) to examine the associations among *BRCA1/2* status, SNP status, and cancer status. Binary logistic regression analyses were performed to study the response of SNP status to the changes in age at enrollment, race, *BRCA1/2* status, and cancer status; using the likelihood ratio forward stepwise methods.

Introduction

While the chances of long-term patient survival are significantly increased when ovarian cancer is detected at its early stage, to date, there is no reliable method available for early detection of ovarian cancer [2]. In fact, the absence of definitive symptoms causes the survival following diagnosis and treatment of ovarian cancer to fall from a 90% 5-year survival rate in cases when detected at its early stage, to less the than 30% chance when the disease is detected at a late stage [2,211,212].

Epidemiologic studies have clearly established the role of family history as an important risk factor for both breast and ovarian cancers [213]. It is well established that germline mutations in *BRCA1* or *BRCA2* result in a predisposition to ovarian cancer, however at a rate of only 20-40%, suggesting the presence of other, unidentified mutations in other predisposition genes [214-217]. Additional genetic variations including SNPs, many of which have been identified in recent genome-wide association studies (GWAS), have been hypothesized to act as low to moderate penetrant alleles which contribute to breast and ovarian cancer risk, as well as other diseases, [216,218,219]. Single-nucleotide polymorphisms arise because of point mutations that are selectively maintained in populations that are distributed throughout the human genome at an estimated overall frequency of at least one in every 1000 base pair [220]. Non-synonymous SNPs substitute encoded amino acids in proteins, and are more likely to alter the structure, function, and interaction of the protein [216,221]. Therefore SNPs are good candidates as disease-modifiers and have been associated with an altered cancer risk.

It has been reported that mutations in specific regions of NAD(P)H oxidase subunits contribute to the enhancement of the enzyme activity with subsequent increase in $O_2^{\bullet -}$ production, contributing to enhanced levels of oxidative stress [79,80]. Specifically, an increase in NAD(P)H oxidase activity has been associated with a specific SNP in the *CYBA* gene (rs4673),

encoding the $p22^{phox}$ subunit of NAPDH oxidase, resulting in a CAC/TAC replacement at position 242 located on chromosome 16q24, leading to a nonconservative substitution of histidine-72 with a tyrosine [81]. This SNP is also associated with an increased risk for other diseases where oxidative stress plays a critical role in their pathophysiology, including cardiovascular disease, asthma, and diabetic nephropathy [81-83].

Oxidative stress has been implicated in the pathogenesis of several malignancies, including ovarian cancer [20,47]. Specifically, we have recently demonstrated an increased expression of pro-oxidant enzymes such as iNOS, MPO, NAD(P)H oxidase, as well as an increase in NO as indicated by increased nitrate/nitrite in EOC tissues and cells [46,47]. In this study, we report that the NAD(P)H oxidase *242C>T* SNP is significantly associated with not only ovarian cancer patients, but also with high-risk individuals. A combination analysis of this SNP with the currently used *BRCA1/2* mutation risk factor resulted in an even stronger association. These findings suggest a possible future role of this SNP as a biomarker to identify those at an increased risk for development of ovarian cancer.

Methods

Patient Population

A total of 139 individuals were recruited through the Karmanos Cancer Institute's Genetic Registry (KCIGR), Detroit, MI. Participants in the genetics registry were recruited through the Karmanos Cancer Institute's cancer genetic counseling service and included individuals determined to be eligible for *BRCA1* and *BRCA2* germline testing based on their personal and family history of cancer and were identified as having ovarian cancer or were "high-risk" based on this family history [211]. Research activities were conducted with the approval of Wayne State University Institutional Review Board. DNA samples were utilized to determine the frequency of the polymorphism in the *CYBA* gene that results in a CAC/TAC

replacement (rs4673). High-risk non-cancer and ovarian cancer samples used for this study were collected from participants recruited between 1999 and 2012. At the time of enrollment in the genetics registry, individual and family cancer history was obtained including history of ovarian cancer and other cancers [211]. Genetic testing for *BRCA1/2* was conducted by Myriad Genetics Laboratories (Salt Lake City, Utah) [211]. Individuals with unknown *BRCA1/2* mutation status were excluded from of *BRCA1/2* analysis. A description of the patient demographics is in Table 2.

Purification of DNA for SNP Sequencing

DNA, from blood samples, was isolated by the Applied Genomics Technology Center (AGTC, Detroit, MI). DNA was extracted with QIAamp DNA mini kit (Qiagen) [211].

TaqMan SNP Genotyping Assay for $p22^{phox}$ *242 C>T*

The TaqMan® SNP Genotyping Assay Set (Applied Biosystems, Carlsbad, CA) for rs4673 (NCBI dbSNP genome build 37, MAF source 1000 genomes) was used to genotype the *242C>T* SNP in the *CYBA* gene, which encodes the $p22^{phox}$ subunit of NAD(P)H oxidase, located on chromosome 16q24. The AGTC also performed this assay. Analysis was done utilizing the QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems).

Statistical Analysis

Data were analyzed using SPSS (IBM, Armonk, New York) for Mac V.21. Pearson Chi-square analyses were performed 1) to compare the SNP mutation frequencies in the present sample to that in the general population; 2) to examine the associations among *BRCA1/2* status, SNP status, and cancer status. Binary logistic regression analyses were performed to study the response of SNP status to the changes in age at enrollment, race, *BRCA1/2* status, and cancer status; using the likelihood ratio forward stepwise methods.

Results

The study population included 139 women with an age range of 18-90, and a median age

of 50 years. The racial distribution of the population included 92.8% White, 5.0% Black, and 2.2% other/unknown. Of the participants in the study, 35.3% had a diagnosis of ovarian cancer. The genotype frequency for the NAD(P)H oxidase SNP in individuals with ovarian cancer was as follows: CC= 30.6%, CT= 49.0%, and TT=20.4% (Figure 23). The frequency for the SNP (CT + TT) was 69.4% as compared to a minor allele frequency (MAF) of 30.3% reported for the general population (NCBI) (Figure 23, Chi Squared= 74.8, $p<0.0001$). Genotype frequency for the NAD(P)H oxidase SNP in individuals who were high-risk non-cancer was as follows: CC= 38.9%, CT= 42.2%, and TT=18.9% (Figure 25). The frequency for the SNP (CT + TT) was 61.1% as compared to 30.3% in the general population, although not quite significant (NCBI) (Figure 25, Chi Squared=3.1, $p<0.08$). There was a slight increase in the frequency of the minor allele in individuals with ovarian cancer (69.4%) as compared to high-risk non-cancer and control individuals (61.0%) (Figure 25, $p>0.05$ with d=21%).

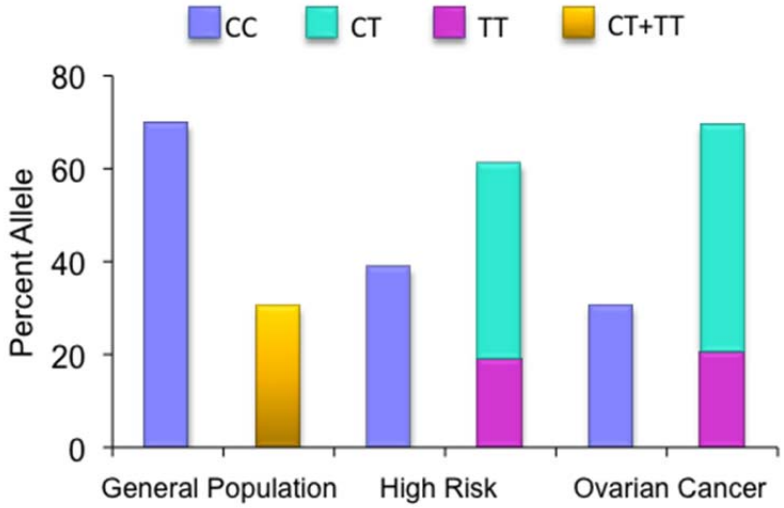

Figure 25: Frequency of NAD(P)H oxidase SNP in EOC. Frequency of the alleles at position 242 of the CYBA gene located on chromosome 16q24 present in the general population, controls, high-risk non-cancerous individuals and individuals with ovarian cancer. This SNP (rs4673, NCBI dbSNP genome build 37, MAF source 1000 genomes) in the CYBA gene, encoding the $p22^{phox}$ subunit of NAD(P)H oxidase, results in a CAC/TAC replacement.

BRCA1/2 status was available for 92.1% of the individuals (n=116) of which 11.2% with ovarian cancer were *BRCA1/2* positive as compared to 17.2% of the high-risk non-cancer individuals. For individuals with *BRCA1/2* mutations, there was an increase in the minor allele frequency in individuals with ovarian cancer as compared to high-risk non-cancer individuals (69.2 vs. 45.0%; $p>0.05$ with d=26%); for individuals without *BRCA1/2* mutations, there was also a small increase (71.0 vs. 65.4%; $p>0.05$ with d=7%).

Logistic regression analyses revealed that age at enrollment was a significant predictor of SNP status ($p=0.027$). With a one year increase in age at enrollment, the odds of an individual developing a mutated SNP increase by 3.4% after controlling for race, *BRCA1/2* status, and cancer status.

Discussion

In this study we have shown that the NAD(P)H oxidase *242C<T* SNP was not only significantly associated with patients with ovarian cancer, but also with those individuals who are considered high-risk. Patients utilized for this study were at an elevated risk of harboring a *BRCA1*/2 mutation based on personal and family history of cancer [211]. During the study period, 31.2% patients presented with or developed the disease. Our results clearly show 69.4% of those with ovarian cancer have the CT or TT alleles as compared to a MAF of 30.3% in the general population, indicating a strong association of this SNP with ovarian cancer. Yet, 61.1% of high-risk non-cancer individuals also have the CT or TT alleles, further suggesting the association of this SNP with an increased risk of development of ovarian cancer, which is again significantly higher than the reported 30.3% MAF in the general population (NCBI).

Although there are several SNPs reported for the NAD(P)H oxidase enzyme, the current study focused on the *242C>T* SNP in the *CYBA* gene (which encodes the $p22^{phox}$ subunit of NAD(P)H oxidase) located on chromosome 16q24 because of the previously reported

association between this SNP and several other diseases such as hypertension, cerebrovascular disease, diabetes and nephropathy as well as being associated with reduced respiratory burst in isolated human neutrophils [81,222-224]. This SNP has been evaluated in a limited number of cancers including lung cancer and leukemia and was found not to be associated with an increased risk [225,226]. The *A-930G* polymorphism (rs9932581) located in the promoter region is associated with higher promoter activity, hypertension, as well as an increase in exposure to oxidative stress [81,227,228]. The *A640G* polymorphism (rs1049255) is a silent variant located in the 3' untranslated region of *CYBA* and has been suggested to be associated with cardiovascular arterial disease [81,228-230]. Future studies examining the prevalence of the above mentioned SNPs is worthwhile conducting as the results of this study suggest a role for NAD(P)H oxidase in the pathogenesis of ovarian cancer.

It is well understood that the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are also strongly associated with an increased risk of ovarian cancer [231]. Of the individuals in our study who have ovarian cancer and the *BRCA1/2* mutation (10%), 69.2% had the CT or TT alleles as compared to 48.0% of high-risk non-cancer individuals, which was not statistically significant. Low statistical power due to small sample size in the present study may have limited the significance of some of the statistical comparisons. While the total sample size (n=139 overall and n=130 for *BRCA1/2* status) might appear sufficient, the cell size for individual Chi-square tests were small. For example, for individuals with *BRCA1/2* mutations, the sample size for normal and mutated SNP groups were 17 and 21, respectively, resulting in a low power of 26%. A sample size of 62 and 75, respectively, would be needed to achieve statistical power at the recommended 80% level [232].

It is known that NAD(P)H oxidase regulates several redox intracellular signaling pathways through the generation of ROS molecules. NAD(P)H oxidase utilizes NAD(P)H as an

electron donor to generate $O_2^{\bullet-}$ from molecular oxygen. NAD(P)H oxidase consists of seven isoforms: five NOXs, NOXs1–5, and two NOX homologues, DUOX1 and DUOX2 [233]. NOX enzymes are differentially activated by different binding molecules, including $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ /NOXO1, $p67^{phox}$ /NOXA1 and Rac [77]. NOX enzymes are essential for the control of many cell functions including, differentiation, proliferation, and cell death, as well as signal transduction [234]. The generation of ROS molecules, specifically $O_2^{\bullet-}$, is achieved by activation of certain redox-sensitive transcription factors, inhibition of protein tyrosine phosphatases, and modulation of the functions of some ion channels [71,235]. NAD(P)H oxidase/ROS-mediated signaling might therefore serve as an alert signal, which stimulates the cells either to adapt to the stress or to undergo apoptosis [235]. Indeed we have previously shown that inhibition of NAD(P)H oxidase significantly induced apoptosis in EOC cells [47]. Additionally, inhibition of NAD(P)H oxidase not only resulted in a reduction of its own expression, but also a significantly decreased SOD3 and HIF-1α levels in EOC cells [47]. Thus, a polymorphism favoring a pro-oxidant intercellular milieu may prove beneficial to the persistence of the cancer cell, and targeting NAD(P)H oxidase may serve as a strategy for increasing apoptosis.

Growing bodies of evidence from clinical studies support the concept that increases in NAD(P)H oxidase activity plays a central role in the etiology of various diseases [79,90,236]. Recent studies have demonstrated that NOX4 is expressed in several tumor types, including ovarian cancer [237]. NAD(P)H oxidases can be activated by mechanical forces, hormones, and cytokines [79,238]. More importantly, NAD(P)H gene polymorphisms have the ability to stimulate the activity of NAD(P)H oxidases, as well as increase the expression of its subunits [79,238,239]. Specifically, NOX2 is considered to be directly involved in the generation of $O_2^{\bullet-}$, as well as $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ [71]. Other studies have shown that polymorphisms in the

promoter region of the $p22^{phox}$ gene contribute to the upregulation of $p22^{phox}$ in spontaneously hypertensive animals [79,80]. Indeed, treatment of animals with antioxidants or inhibitors of NAD(P)H oxidase results in significantly lower ROS levels, and is associated with decreased blood pressure [239,240].

Recent GWAS have successfully identified and confirmed six SNPs that appear to influence the risk of EOC [212]. The confirmed susceptibility SNPs are rs3814113 (located at 9p22, near BNC2), rs2072590 (located at 2q31, which contains a family of HOX genes), rs2665390 (located at 3q25, intronic to TIPARP), rs10088218 (located at 8q24, 700 kb downstream of MYC), rs8170 (located at 19p13, near MERIT40), and rs9303542 (located at 17q21, intronic to SKAP1) [212]. In this study we report another SNP that is strongly associated with ovarian cancer, rs4673. This SNP is particularly important because the resulting pro-oxidant milieu is significant in the pathophysiology of several diseases, including ovarian cancer.

The findings of this study are of great importance because they can be utilized as a stepping-stone towards the development of an early screening test for identifying individuals who are at high-risk for the development of ovarian cancer.

CHAPTER VII: SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

The overall goal of these studies was to investigate the role of oxidative stress in EOC and understand the underlying mechanisms of oxidative stress in this disease. To achieve this goal, we have successfully shown that EOC favors a pro-oxidant state, which is responsible for the maintenance of the oncogenotype. We have identified key players in the redox pathway in EOC whose modulation results in increased apoptosis in EOC cells. Additionally, we have identified MPO and free iron as biomarkers for the early detection of ovarian cancer. Furthermore, we have shown that a SNP in NAD(P)H oxidase serves as a screening tool for the identification of individuals at high risk for ovarian cancer.

Collectively, this work provides the molecular basis for future clinical trials testing the potential for MPO and free iron as biomarkers for early detection of ovarian cancer. Similar studies are needed for the evaluation of NAD(P)H oxidase as a screening tool for the general population. Due to the low prevalence of ovarian cancer in U.S. women, an ovarian cancer diagnostic or screening test must have a minimum specificity of 99.6% before it can be used routinely in the general population of postmenopausal women [170,241]. Such a test may offset potential morbidity and mortality, which can be associated with complications of surgery for patients who have false-positive ovarian cancer screening tests [170,241]. An ovarian cancer screening test should also have high sensitivity and a suitable PPV [170,242]. Routine screening for ovarian cancer in the general population is not recommended because traditional screening methods are not sensitive or specific enough [170,243]. Therefore, the development of sensitive and specific methods for early detection has been a priority as a means for improving the diagnosis and treatment of this disease. Additional studies focusing on modulation of the key enzymes identified in this study are needed to assess their potential as therapeutic targets.

APPENDIX

From: Carry Koolbergen <C.Koolbergen@iospress.nl>
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To: "nfletche@med.wayne.edu" <nfletche@med.wayne.edu>
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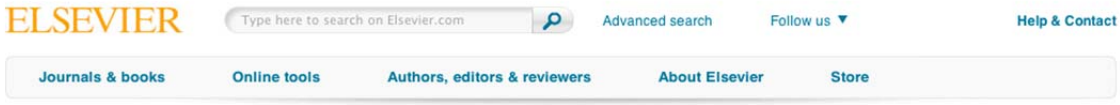

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ABSTRACT

THE ROLE OF OXIDATIVE STRESS IN THE PATHOGENESIS OF EPITHELIAL OVARIAN CANCER

by

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Major: Physiology

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Ovarian cancer is the leading cause of death from gynecologic malignancies yet the underlying pathophysiology is not clearly established. The disease is usually diagnosed in the advanced stage and carries a poor prognosis. The 5-year survival rate is greater than 75% if diagnosis of the cancer occurs at an early stage; however, this rate drops to 20% when the tumor has spread beyond its origin. Thus, a method for early detection is critically needed, which can help prolong, or even save lives. Currently, an effective screening test for ovarian cancer is lacking. Many tests have been evaluated but have been found to be lacking in sensitivity, specificity, or both. This becomes even more difficult as ovarian cancer is so rare in the general population, with an estimated prevalence of 0.04%. To date, 96% is the highest sensitivity recorded for the early detection of ovarian cancer. However, because of the low prevalence of ovarian cancer, a sensitivity and specificity of 96% would only detect 1 case of cancer per 100 oophorectomies done for positive screens due to the high false positive rate.

Clinical and epidemiological investigations have provided evidence supporting the role of reactive oxygen species (ROS) in the prognosis and metastasis of cancer due to exogenous factors that lead to chronic inflammation. In addition, cancer cells are frequently under

persistent oxidative stress, which participates in cancer progression as well as in the selection of resistant cells that are unable to be eliminated by apoptosis. A recent novel finding from our laboratory has shown that myeloperoxidase (MPO) is expressed in ovarian cancer cell lines and tissues, with minimal or no expression detected in normal ovarian tissues. Myeloperoxidase is an abundant heme protein, which plays a central role in the formation of reactive oxidant and free radical species. Additionally, under conditions of oxidative stress, MPO serves as a source of free iron.

Another source of ROS is the superoxide producing nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, which we observed to be upregulated in ovarian cancer as compared to normal ovarian tissues. It has been reported that mutations in specific regions of NAD(P)H oxidase subunits contribute to the enhancement of the enzyme activity with subsequent increase in superoxide ($O_2^{\bullet -}$) production, contributing to enhanced levels of oxidative stress.

Therefore, there is compelling evidence to support the role of oxidative stress in the pathogenesis of ovarian cancer, yet the mechanisms responsible for the dissemination of EOC are not fully understood. *The objective of this work* is to identify mechanisms of oxidative stress in the pathogenesis of ovarian cancer. The central hypothesis of this study is that ovarian cancer is controlled by mechanisms that emanate from an altered redox balance. To test this hypothesis, the following specific aims have been investigated:

Specific Aim I: Determine the oxidative stress profile in epithelial ovarian cancer and determine the effect of the modulation of specific key players of oxidative stress on the persistence of the oncogenic phenotype. To determine the oxidative stress profile, the expression of the following markers will be evaluated: NAD(P)H oxidase, nitrate/nitrite, glutathione, glutathione reductase, glutathione peroxidase, glutathione s-transferase, inducible

nitric oxide synthase, myeloperoxidase, catalase, and superoxide dismutase in epithelial ovarian cancer cells and tissues as compared to their normal counterparts. These markers were selected based on extensive preliminary and published results, utilizing a cell culture model and ovarian cancer tissues. Modulation of key players of oxidative stress will contribute to delineation of the mechanisms of development of ovarian cancer. To achieve this, a combination of knockdown or inhibition of specific pro-oxidants utilizing siRNA or direct inhibition will be performed followed by the assessment of the expression other pro-oxidants as well as the effect on apoptosis.

Specific Aim II: To assess the effectiveness of MPO and free iron levels in detection of early stage ovarian cancer. The mechanism by which MPO functions under oxidative stress is not well defined. MPO has been indicated to serve as a source of free iron under oxidative stress. The hypothesis of this aim is that serum MPO and free iron levels can be utilized to distinguish patients with stage I ovarian cancer from those with normal ovaries or benign gynecologic conditions as well as from late state ovarian cancer. MPO and free iron levels will be measured in the sera and tissues of women at the time of ovarian cancer diagnosis as compared to healthy women or those with benign gynecologic disorders respectively. The biological significance of the relationship between serum MPO and free iron, in conjunction with the poor prognosis of ovarian cancer has the potential for future clinical applications. .

Specific Aim III: To assess whether a SNP in NAD(P)H oxidase is associated with increased risk of development of ovarian cancer. Single nucleotide polymorphisms (SNPs) are associated with increased risk of several cancers. We evaluated the association of a SNP (rs4673) in the CYBA gene, encoding the $p22^{phox}$ subunit of NAD(P)H oxidase, substituting allele C with T at position 242 located on chromosome 16q24, in high-risk individuals with or without ovarian cancer, with or without a deleterious BRCA1/2 mutation. This SNP is known to

be associated an increased activity of the enzyme, resulting in an increase in superoxide production. TaqMan® SNP Genotype analysis and the QuantStudio 12K Flex Real-Time PCR System were utilized to determine the frequency of this SNP in individuals with ovarian cancer as compared to noncancerous individuals.

AUTOBIOGRAPHICAL STATEMENT

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PUBLICTIONS (on select related original work over the last 4 years)

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