Am J Cancer Res 2021;11(9):4070-4091
www.ajcr.us /ISSN:2156-6976/ajcr0135493

Review Article

Subclinical doxorubicin-induced cardiotoxicity update: role of neutrophils and endothelium

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Received May 20, 2021; Accepted July 23, 2021; Epub September 15, 2021; Published September 30, 2021

Abstract: Doxorubicin (DOX) is a highly effective chemotherapy agent that often causes cardiotoxicity. Despite a number of extensive studies, the risk for DOX cardiotoxicity remains unpredictable. The majority of the studies on DOX-induced cardiotoxicity have been focused on the effects on cardiomyocytes that lead to contractile dysfunction. The roles of systemic inflammation, endothelial injury and neutrophil recruitment, all induced by the DOX, are increasingly recognized as the mechanisms that trigger the development and progression of DOX-induced cardiomyopathy. This review explores recent data regarding the possible mechanisms and biomarkers of early subclinical DOX-associated cardiotoxicity.

Keywords: Doxorubicin, cardiotoxicity, mechanisms, neutrophils, coronary vasculature, biomarkers

Introduction

Anthracyclines, such as doxorubicin (DOX) are very potent chemotherapeutic drugs that have significantly improved cancer survival [1]. DOX and other anthracyclines (i.e. epirubicin, daunorubicin, idarubicin) are used for treatment of many cancers of the breast, endometrial and gastric tissues, childhood solid tumors, soft tissue sarcomas, and pediatric leukemia, as well as post-heart and bone marrow transplantation [2]. Although many anti-neoplastic therapies are cardiotoxic, anthracycline-induced cardiomyopathy and heart failure (HF) are the most thoroughly studied [3]. Cardiotoxicity of anthracyclines may not be detected until several years after the treatment and may significantly impact a patient's survival and quality of life independently of the oncological prognosis [4]. Survivors of childhood cancer treated with DOX are at the greatest risk of cardiovascular morbidity and mortality. About 60% of pediatric cancer patients are treated with DOX-based chemotherapy [5] and 10% of these patients develop cardiomyopathy up to 15 years after the end of chemotherapy [6]. The majority of the studies on DOX-induced cardiotoxicity are focused on the effects on cardiomyocytes that lead to contractile dysfunction. The role of the systemic inflammation associated with neutrophil recruitment and vascular endothelial injury has been recently recognized as a mechanism that triggers the development and progression of DOX-induced cardiomyopathy. This review discusses the available data regarding the possible mechanisms and biomarkers of the early subclinical DOX-associated cardiotoxicity.

Clinical aspects

Clinically recognized DOX-induced cardiotoxicity can occur at any point during and after treatment with anthracyclines. Acute/subacute cardiovascular complications can arise from the initiation of therapy to several weeks after treatment termination [7] and manifest themselves as chest pain, palpitation, dysplasia, and/or tachycardia arrhythmias, as well as a decline in the left ventricle ejection fraction (LVEF) from more than 10% to 50% [8]. Electrocardiograms reveal nonspecific ST-T changes, left axis deviation, and decreased amplitude of QRS complexes [9]. The mechanism of the acute cardiotoxicity is not quite clear, but

may be associated with DOX-induced myocardial edema or inflammatory response, which is reversible and can be controlled with appropriate treatment [10]. DOX-induced cardiotoxicity may become clinically evident after one-year chemotherapy completion (late-onset chronic cardiotoxicity), which is characterized by dilated cardiomyopathy, including dilation of ventricles, in some cases of atria, reduced LVEF and contractile function, diastolic dysfunction, and mural thrombi in some patients [11]. DOX-induced pathological alterations indicative of restrictive cardiomyopathy following DOX chemotherapy have been reported in survivors of childhood cancer (i.e. reduced LV mass and diffuse interstitial fibrosis) [12] and in experimental animals (i.e. decreased end-diastolic LV volume, increased posterior wall thickness and a ratio between the E-wave and A wave velocities of the mitral valve >2) [13].

Monitoring

Several different methods exist for the assessment of chemotherapy-induced cardiotoxicity, including electrocardiography, echocardiography, biopsy, scintigraphy, serum analysis, and some genomic markers [14]. Each of these has certain limitations, such as low sensitivity, high invasiveness, elevated costs, and/or relatively late detection of heart dysfunction. The assessment of a decreased LVEF decrease radionuclide scintigraphy or angiocardiography or 12-lead electrocardiogram have been the most common non-invasive methods in clinical practice to monitor cardiotoxicity. However, LVEF is considered a fairly late manifestation of cancer chemotherapy-associated cardiotoxicity, reflecting the presence of irreversible myocardial damage and is usually insensitive to early stages of subclinical injury [15]. Endomyocardial biopsy, traditionally considered as the "gold standard" test for the evaluation of DOX cardiomyopathy is invasive and does not correlate with the subsequent risk of congestive HF [16]. Traditional blood-based cardiac biomarkers, such as cardiac troponins and B-type natriuretic peptide (BNP), have been suggested in the diagnostics of HF, but several studies failed to detect any correlation between their blood results and DOX-induced cardiotoxicity [17]. High-sensitivity troponin assays have been shown to have better accuracy in the diagnostics of acute coronary syndrome and cancer patients treated with anthracyclines [18], but they have failed to predict DOX cardiotoxicity in breast cancer patients in a recently clinical study [19].

Risk factors

A major risk for DOX-induced cardiotoxicity is the total dose of the drug administered. DOX-induced cardiotoxicity is usually cumulative dose-dependent, which begins with the first dose. There has not been established a "safe" dose of DOX which does not result in cardiotoxicity [20]. Clinical HF incidences of 3%, 7%, 18% and 40% have been shown with cumulative doses of 400, 550, 600 or >650 mg/m^2 respectively, therefore doses below 450 mg/m^2 are recommended [21]. The risk of DOX-induced cardiotoxicity increases with other added chemotherapy drugs, such as trastuzumab or cyclophosphamide, or with added chest or mediastinal radiation [22]. Other suspected risk factors are the age at diagnosis [23], follow-up time, and female sex [24]. Prepubescent girls are at higher risk of DOX-induced cardiotoxicity in comparison with boys [25], as are women older than 65 [26]. It has been suggested that women with higher circulating estrogen are more resistant than an age-matched man to DOX-induced cardiomyopathy [27]. The increased risk of cardiomyopathy induced by DOX in the young and older patients has also been attributed in part to the immature liver function in young children and declining liver activity among older adults, both of which slow DOX clearance and prolong exposure to circulating DOX [28]. Because the liver is a major site of DOX clearance, any alteration in DOX metabolism caused by liver disease or concurrent medications would be expected to result in elevated levels of DOX and increased exposure to toxic concentrations of the drug [29]. Additionally, hypertension, diabetes, dyslipidemia, obesity also increase the risk of DOX-induced cardiotoxicity [30].

Susceptibility to DOX cardiotoxicity is largely individual with some patients developing cardiomyopathy at doses of 200-400 mg/m^2, while others tolerating well >1000 mg/m^2 [31], suggesting the presence of a genetic predisposition. Several recent studies have addressed the existence of gene variants predisposing to DOX-induced cardiotoxicity. Candidate SNPs or gene panels have previously been associated

Table 1. Candidate SNPs associated with sensitivity to DOX-induced cardiotoxicity

Mechanistic pathways	Genes
DOX metabolism and transport	SLC28A3, ABCC1, ABCC2, UGT1A6, SULT2B1, [141]
Oxidative stress	ABCC5, NOS3, [142], HAS3 [143], HFE [144]
DNA damage	RARG [145]
Immunity and inflammation	TNF-alpha [146], C6orf10 [147], MICA, NFKBIL1, LTA [148], NOTCH4 [149], HLA-C [150]

with the DOX metabolism and transport, oxidative stress, and DNA damage [32], some of which are shown in **Table 1**. The overall reproducibility of the published studies has been limited, due to small cohorts, failure to assess population ancestry, and lack of replication, including our study on breast cancer patients with or without DOX-induced cardiotoxicity [33]. We have identified 15 SNPs in nine genes in the human leukocyte antigen (HLA) region (NFKBIL1, TNF-alpha, ATP6V1G2, MSH5, MICA, LTA, BAT1, NOTCH4), and three SNPs in the psoriasis susceptibility region of HLA-C as potential candidates for association with DOX-cardiotoxicity in breast cancer patients (**Table 1**). The function of most of these molecules remains insufficiently characterized, although evidence suggests a role in immune and inflammatory responses [34]. All of the candidate genes in our study are located on chromosomes 6p32 and 6p33. Six of the candidate SNPs in the BAT1-NFKBIL1-LTA region in our dataset have previously been reported in association with myocardial infarction [35] and autoimmune disorders [36]. SNPs within TNF-α, NOTCH4, and C6orf10 have been associated with coronary artery disease, and myocardial infarction, while SNPs within MSH5, MICA, and HLA-C have been reported in autoimmune inflammatory disorders (**Table 1**). The telomeric class III region of HLA bordering the class I region is particularly gene-dense containing at least 10 genes in addition to TNF alpha within an 82 kb interval, including BAT1, ATP6V1G2, NFkBIL1, LTA, TNF, LTB, LST1, NCR3, AIF-1, BAT3 and BAT2 [37]. Our findings are consistent with reports showing the presence of susceptibility loci within the HLA-gene region for coronary artery disease (CAD) [38] and inflammatory/autoimmune disorders [39]. Autoimmune features and rheumatic manifestations have been reported in cancer patients after chemotherapy [40] including rheumatism, and systemic lupus erythematosus (SLE) in those with breast cancer [41].

Cellular and molecular mechanisms and biomarkers oxidative stress

The *in vitro* metabolism of DOX in cardiac and liver microsomal membranes has been described in several earlier studies. The initial reduction of DOX to a semiquinone free radical is catalyzed by NADPH-dependent cytochrome P450 reductase and reconversion to the quinone, a process involving one-electron reduction, which leads to the persistent production of superoxide anion radical and secondary ROS (e.g., $O_2\cdot$, H_2O_2, OH·) [42, 43]. H_2O_2 and $O_2\cdot$- may also generate highly reactive and toxic hydroxyl radicals (OH) during the iron-catalyzed Haber-Weiss reaction, resulting in an iron cycling between Fe^{3+} and Fe^{2+}, thus altering iron homeostasis [44]. The increased production of ROS induced by DOX leads to excessive oxidative stress, strongly linked to cell damage involving reduced protein synthesis and redox modifications of proteins, lipids, and DNA [45].

DOX accumulates primarily in the mitochondria [46], which accounts for DOX cardio-selective toxicity, combined with a less active antioxidant network in the heart compared with other tissues such as the liver [47]. DOX, being a cationic drug, binds to the negatively charged phospholipid cardiolipin located on the inner mitochondrial membrane, leading to disruption of the activity of complexes I-IV of the electron transport chain, peroxidation of lipids, oxidative damage of proteins and mitochondrial DNA, loss of ATP levels and mitochondrial permeability transition 3 one integrity [48]. The Keap1-Nrf2 pathway is the major regulator of cytoprotective responses to oxidative and xenobiotic stress by activating antioxidants and anti-electrophiles [49]. The key signaling protein within the pathway is the transcription factor Nrf2, which under the regulation of Keap-1 can protect the cells and tissues from oxidative stress by increasing the expression of several downstream cytoprotective genes, including antioxidants and phase II and phase III detoxification

enzymes [50]. It has been demonstrated that deficiency of Nrf2 amplified DOX-induced cardiotoxicity and cardiac dysfunction [51]. We have examined the effect of DOX on the early (48 hours post-injection) gene expression of rat hearts and found that at this time-point there was significant downregulation of NRF2 gene (NFE2L2), cardiolipin gene (CRLS1), along with a widespread reduction in the expression of multiple genes encoding for proteins of complexes I-IV, and ATP synthase [52]. We observed significant downregulation of mitochondrial oxidative phosphorylation (OXPHOS) complexes I-IV and ATP synthase, including, 25 transcripts coding for NADH dehydrogenase in complex I, all four transcripts coding for succinate dehydrogenases in complex II, four transcripts of ubiquinone-cytochrome c reductase in complex III, 13 genes coding for several subunits of cytochrome c oxidase in complex IV, and 8 ATP synthases from complex V. Downregulation of OXPHOS complexes and the associated increased superoxide production and 4-HNE protein adducts tend to predispose to hypertension, coronary artery disease and HF [53].

Along with the increased oxidative stress, DOX also reduces the antioxidant defense of the cells, for example, reducing SOD, and catalase content or activity, thus contributing to enhance and prolong mitochondrial damage [54]. Various antioxidant supplements have shown some protection when combined with DOX, including vitamins C and E [55], glutathione and metallothionein [56], and glutamine [57]. Chandran et al. [58] demonstrated that co-administration of MitoQ, a triphenylphosphonium-conjugated analog of coenzyme Q, to rats treated with DOX resulted in improved LV function. Because MitoQ is a mitochondria-targeted antioxidant, enrichment of mitochondrial membranes with the active antioxidant is beneficial against DOX toxicity.

Calcium homeostasis dysregulation

Intracellular ionized Ca concentration ($[Ca^{2+}]i$) regulates cardiomyocytes' contractility through excitation-contraction coupling, a process that links the electric excitation of the sarcolemma surface membrane (action potential) to the mechanical contraction. During the cardiac action potential, Ca^{2+} enters the cell through the L-type calcium channel, which triggers additional Ca^{2+} release from the sarcoplasmic reticulum (SR). The elevated $[Ca^{2+}]i$ concentration allows Ca^{2+} to bind to the myofilament protein troponin C (Tn-C), which then switches on the contractile machinery [59]. For relaxation to occur $[Ca^{2+}]i$ must decline and allow Ca^{2+} to dissociate from troponin, which involves SR Ca^{2+}-ATPase (SERCA2a), $Na+/Ca^{2+}$-exchanger (NCX), and plasmalemmal Ca^{2+}-ATPase (PMCA) [60]. The elevated $[Ca^{2+}]I$ levels resulting in mitochondrial Ca^{2+} overload, combined with unregulated ROS production, causes opening of the mitochondrial permeability transition pore (MPTP), causing permeabilization of the mitochondrial inner membrane to molecules of less than 1.5 kDa in molecular weight [61]. MPTP opening results in inner membrane potential reduction and collapse, respiratory chain uncoupling, halt of mitochondrial ATP synthesis, and eventually, mitochondrial swelling, rupture, and cell death, as reported in DOX cardiotoxicity in the human heart [62].

DOX binds and activates sarcoplasmic reticulum (SR) ryanodine receptors (RYRs) to increase cytosolic ionized Ca^{2+}, while at the same time downregulating calcium transport ATPase (SERCA), which pumps Ca^{2+} back to SR, leading to an abnormal cytoplasmic Ca^{2+} and increased generation of ROS [63, 64]. It has been shown that DOX directly affects RYR2 activity by rapid reversible activation of the channel, followed later by irreversible inhibition [65]. We have tested the effects of RYR antagonist dantrolene (DNT) on DOX-induced cardiotoxicity in a rat model of breast cancer [66]. We found that DNT improved DOX-induced alterations in the echocardiographic and histopathological parameters, without affecting the anti-tumor efficacy of DOX. Rats treated with DNT lost less body weight, had higher blood GSH levels and lower troponin I level than DOX-treated rats. These data indicate that DNT can provide protection against DOX cardiotoxicity without reducing its antitumor activity.

Topoisomerases

Topoisomerases (Tops) catalyze the relaxation of DNA supercoils and unknotting of DNA helices and strands [67]. Top2 enzymes introduce double-strand breaks in the DNA molecule, passes another unbroken DNA helix through it, and then re-ligates the cut strands. DOX binds to both DNA and Top2, forming the DOX-Top-DNA complex, which inhibits Top2 activity,

resulting in DNA double-strand breaks, activating DNA damage response, and apoptosis [68]. Top2 isoforms, Top2α and Top2β, are differentially regulated during the cell cycle in normal and neoplastic tissues, as Top2α is overexpressed in highly proliferative tumor cells and undetectable in quiescent cardiomyocytes [69], while Top2β is overexpressed in terminally differentiated quiescent cells, such as cardiomyocytes [70, 71]. Top2β is a mechanism thought to be responsible for DOX-induced cardiotoxicity through enhancement of oxidative stress and impairment of mitochondrial biogenesis [72]. Cardiomyocyte-specific deletion of Top2β protected the cardiomyocytes from DOX-induced DNA double-strand breaks and mice from the development of DOX-induced HF [73]. Currently, dexrazoxane, a Top2 poison, an iron chelator, and free radical scavenger, is the only approved drug for the treatment of DOX-induced cardiotoxicity in clinical settings [74].

Heat shock proteins

DOX-induced oxidative stress and ATP depletion induce high expression of heat shock proteins (HSPs), which regulate the activity of multiple signaling intermediates involved in the execution of apoptotic signaling pathways [75]. For example, HSP10 and HSP60 overexpression led to an increase in the post-translational modification of Bcl-2 proteins induced by DOX [129], HSP20 reduced DOX-associated oxidative stress, and cardiotoxicity via interacting with AKT phosphorylation [76]. A significant 3,9-fold upregulation of the HSP90 gene (HSPCB) was detected in the rat hearts at 48 hours post-DOX administration [85], a finding that correlated with the 16-fold HSP90 induction by cardiac ischemia [77], due to ROS accumulation [78] and reduction of ATP concentration [79].

Cellular senescence

Many chemotherapeutic drugs, such as anthracyclines, cyclophosphamide, cisplatin, mitoxantrone, and gamma irradiation are known to alter cellular states and induce senescence in cancer cells and the tumor microenvironment [80]. Cellular senescence is a potent tumor-suppressive mechanism that arrests the growth of cells at risk for malignant transformation [81]. Senescence-associated secretory phenotype (SASP) is characterized by arrested cell growth, resistance to apoptosis, high metabolism and secretion of proinflammatory cytokines (e.g. IL-6, IL-1α-6, -8, 10), growth factors (e.g. IGF/IGFBP, FGF, TGF-β, IFN-γ), proteases (e.g. MMP1, MMP-3). It has been demonstrated that cell cycle inhibitors p16 and p21 are overexpressed by senescent cells, making them the most well-established senescence markers [82]. The contribution of senescent cells to coronary heart diseases [83] and atherosclerosis [84] have been shown in several studies. Studies in cancer showed that therapy-induced senescence can stimulate immunosurveillance to eliminate tumor cells, but it can also be a source of chronic inflammation and drug resistance [85]. For example, treatment of breast cancer patients with DOX and alkylating agents induced cellular senescence in a p16INK4a-dependent, telomere-independent fashion [86]. Demaria et al. [87] showed that DOX-induced senescence could persist and contributed to local and systemic inflammation in mice, and elimination of the senescent cells reduced several short- and long-term effects of the drugs, including bone marrow suppression, cardiac dysfunction, cancer recurrence, and physical activity and strength.

Inflammation, endothelial dysfunction, and hypercoagulability

Several inflammatory markers may be able to predict future cardiovascular events [88]. For example, IL-6 is associated with an increased risk of myocardial infarction and cardiovascular death [89]. Elevated pro-inflammatory cytokines, such as TNF, IL-6, monocyte chemotactic protein 1 (MCP-1), and Interferon-γ (INF-γ) were found in the serum of mice after administration of DOX [90]. Wang et al. [91] demonstrated that DOX-induced upregulation of the proinflammatory Toll-like receptor TLR4 in macrophages was associated with DOX-triggered leakage of endotoxin into the circulation of rats. Elevated levels of CRP were associated with decreased LVEF in patients with varying cardiovascular diseases ranging from myocardial infarction to HF [92]. We used multiplex assays for chemokines to examine plasma samples collected before and after the first cycle of DOX-based chemotherapy of breast cancer patients [19]. The results showed that the initial DOX dose-induced chemokine “immune/inflammatory signature” includ-

ing CCL23, CCL27 and MIF was able to predict the abnormal decrease of LVEF after chemotherapy. Previous studies showed the association of CCL23 and MIF with coronary atherosclerosis, myocardial infarction, and CCL27 was reported in autoimmune diseases (**Table 2**), but no report correlated these chemokines with cardiotoxicity.

DOX-induced inflammation is associated with endothelial dysfunction, which is a complex process involving ROS production, pro-inflammatory cytokines secretion and inactivation of NO production, resulting in disruption of vascular contractility [93]. Dysfunctional endothelial cells (ECs) secrete TNF-α, IL-1β, IL-6, IL-8, which along with granulocyte colony-stimulating factor (G-CSF) promote mobilization of neutrophils from the bone marrow and their recruitment to the vascular endothelium [94]. Neutrophils adhere to the endothelium and release neutrophil granular peptides and proteins, such as myeloperoxidase (MPO), elastase, matrix metalloproteases (MMPs) disrupting inner endothelial junction. Further damage can be induced by the production of neutrophil extracellular traps (NETs), which enhance inflammation and endothelial permeability. Neutrophils activate platelets, which bind to the extracellular matrix (ECM) beneath the endothelial layer and create platelet plug to maintain hemostasis within an injured vessel [95]. The interactions between ECs, neutrophils, and platelets are influenced by several factors released from ECs, such as thrombomodulin (TM), von Willebrandt factor (vWF), P-selectin, which modulate platelet activity, coagulation, and vascular contractility, all of which contribute to the thrombotic formation, myocardial infarction, coronary artery disease and ischemic stroke [93]. It is known that cancer chemotherapy increases the risk of cancer-related thrombosis, which is a major risk factor for cardiovascular diseases [96]. We have found, that circulating biomarkers of inflammation, hypercoagulability and endothelial injury before or after the initial infusion of DOX-based chemotherapy were able to predict the risk of early subclinical DOX-induced cardiotoxicity in breast cancer patients [97]. Patients with an abnormal decline of LVEF had significantly elevated levels of MPO and TM both at baseline, and after the first dose of DOX-based chemotherapy relative to patients with normal LVEF. The first dose of DOX also induced higher circulating levels of thrombin-anti-thrombin complex (TAT) complex, C-reactive protein (CRP), markers of NETs, vWF, and P-selectin in patients with cardiotoxicity in comparison with patients without. These findings indicate that the risk of DOX-induced cardiotoxicity in breast cancer is associated with endothelial dysfunction, inflammation, and prothrombotic state before and after the first dose of chemotherapy. Furthermore, the increased circulating levels of MPO and TM before and after the first DOX infusion in cancer patients with DOX-induced low LVEF suggest their potential to be used as predictive biomarkers and the need for future validation studies. In addition, the circulating levels of TAT, NETs, CRP, and vWF might also be potentially predictive for the risk of DOX-induced cardiotoxicity after the first chemotherapy dose.

Cardiac microvascular ECs, being the most abundant cell type in adult myocardium are in direct contact with the adjacent cardiomyocytes and fibroblasts, and actively secret many proteins, which can modulate cardiac contractility and remodeling [98, 99]. DOX-induced endothelial damage has been associated with the development of severe chronic vascular diseases, such as the atherosclerosis [100]. A prospective study of 7289 childhood survivors showed that 10% developed coronary artery disease 10 years after diagnosis [101]. A recent review by Luu et al. [102] indicates that the initial endothelial damage could be asymptomatic with a long delay between the end of DOX treatment and the onset of vascular disorder, but with time, the declining health of the endothelium progressively renders ECs more vulnerable to chronic inflammatory stressors. The ability of DOX to affect cardiac microvascular endothelial cell permeability *in vivo* has been reported in rat studies where cardiac permeability changes correlated with decreased LV function [103]. Wilkinson et al. [104] showed that DOX could increase cardiac microvascular endothelial cell permeability, which could potentially lead to cardiomyopathy. It has been shown that DOX reduces the expression of phosphorylated eNOS (Ser1177), which leads to a decreased bioavailability of NO and endothelial dysfunction [105]. Urschel et al. [106] demonstrated that DOX administration enhanced proinflammatory TNF-α signaling by activating NF-κB, which led to endothelial dysfunction characterized by increased permeability, enhanced oxidative stress, followed by

Table 2. Candidate circulating biomarkers for prediction of DOX-induced cardiotoxicity

Biomarker	Function
CCL23	Elevated in coronary atherosclerosis [151] Promising biomarker of injury in patients with ischemic stroke through upregulation of TNF-α [152] Elevated in autoimmune diseases (psoriasis and eczema) [153]
CCL27	Associated with diminished kidney transplant and autoimmune disease (psoriasis and eczema) [154]
MIF	Elevated in myocardial ischemia/reperfusion injury [155] Upregulated during the progression of atherosclerosis [156] Elevated in myocardial infarction and inflammatory diseases [157]
PGLYRP1 (Tag7, PGRP1)	Increased gene expression and protein levels in patients with myocardial infarction [158] Present in atherosclerotic plaques [159] Circulating levels were independently associated with coronary and peripheral atherosclerosis [160, 161] Elevated circulating levels predicted poor outcome in patients with HF [162] Marker of coronary artery disease and HF [163]
CAMP (hCAP-18/LL-37) (Cramp in mouse)	Associated with acute HF rodents [164] Presence in human atherosclerotic plaques [165, 166] Associated with platelet activation and induction of thrombosis [167] Produced in atherosclerotic lesions, where it may function as an immune modulator [168]
MMP9	Involved in plaque destabilization resulting in acute coronary syndrome and stroke [169, 170] Elevated circulating levels predicted poor outcome in acute coronary syndrome [171] Role in remodeling atherosclerotic plaques in myocardial infarction [172] Higher levels associated with acute coronary syndrome [173-176]
MMP8	Circulating levels correlate with acute coronary syndrome [177] Baseline serum is a significant predictor of LV remodeling and cardiovascular outcome after myocardial infarction [178] Serum levels associated with subclinical atherosclerosis [179]
MPO	Circulating levels correlate with acute coronary syndrome [180, 181] Marker of ischemic heart disease and acute coronary syndrome [182] Associated with a risk of coronary artery disease [183] Serum levels predict acute coronary syndrome [184] Circulating levels appeared as a strong independent marker of coronary artery disease [185] Potential biomarker of the risk for a subsequent cardiac dysfunction in cancer patients [186]
DEFA 1-4, human neutrophil peptidases (HNPs)	Exert pro-atherosclerotic properties by promoting monocyte adhesion, platelet activation, and foam cell formation [187] Significantly increased in acute coronary syndrome and sepsis [187]
CEACAM8 (CD66b) (Ly6G in mice)	Upregulation in granulocytes polymorphonuclear leukocytes was a risk factor for atherothrombosis [188] Mice treated with anti-LY6G to deplete neutrophils were protected against CD8+ T-cell-dependent myocarditis [189]
TM	Facilitates the thrombin-mediated activation of protein C and plays role in coagulation, fibrinolysis and inflammation [190] Marker of generalized endothelial injury [191] Elevated circulating levels in intravascular coagulation and venous thrombosis [192] Circulating levels predict the risk of coronary heart disease [193, 194]
OLR1 (LOX-1)	Marker of atherosclerosis and vasculopathy [195] Marker of coronary artery disease, stroke, and acute aortic dissection [196] Correlate with acute coronary syndrome [197] Elevated in hypertensive rats [198] Upregulated during myocardial ischemia-reperfusion [199] Expressed in atherosclerotic lesions [200]
Markers of NETs	Elevated circulating levels independently associated with severe coronary atherosclerosis and a prothrombotic state [201] Presence in human coronary and ischemic stroke thrombi [202] Biomarker of STEMI myocardial infarction [203, 204] Biomarker of acute ischemic stroke, coronary and peripheral artery disease [205] Markers of acute myocardial infarction [206] Activate ECs and platelets, resulting in endothelial dysfunction, proinflammatory immune response, and thrombotic lesions [207, 208] Presence in atherosclerotic lesions [209] Increase with age [210]
TAT complex	Diagnosis of hypercoagulability [211] Diagnosis and assessment of treatment-induced intravascular coagulation, deep vein thrombosis, and pulmonary thromboembolism [212, 213] Elevated circulating levels associated with coronary artery disease [214] Increased plasma levels associated with atrial fibrillation [215]

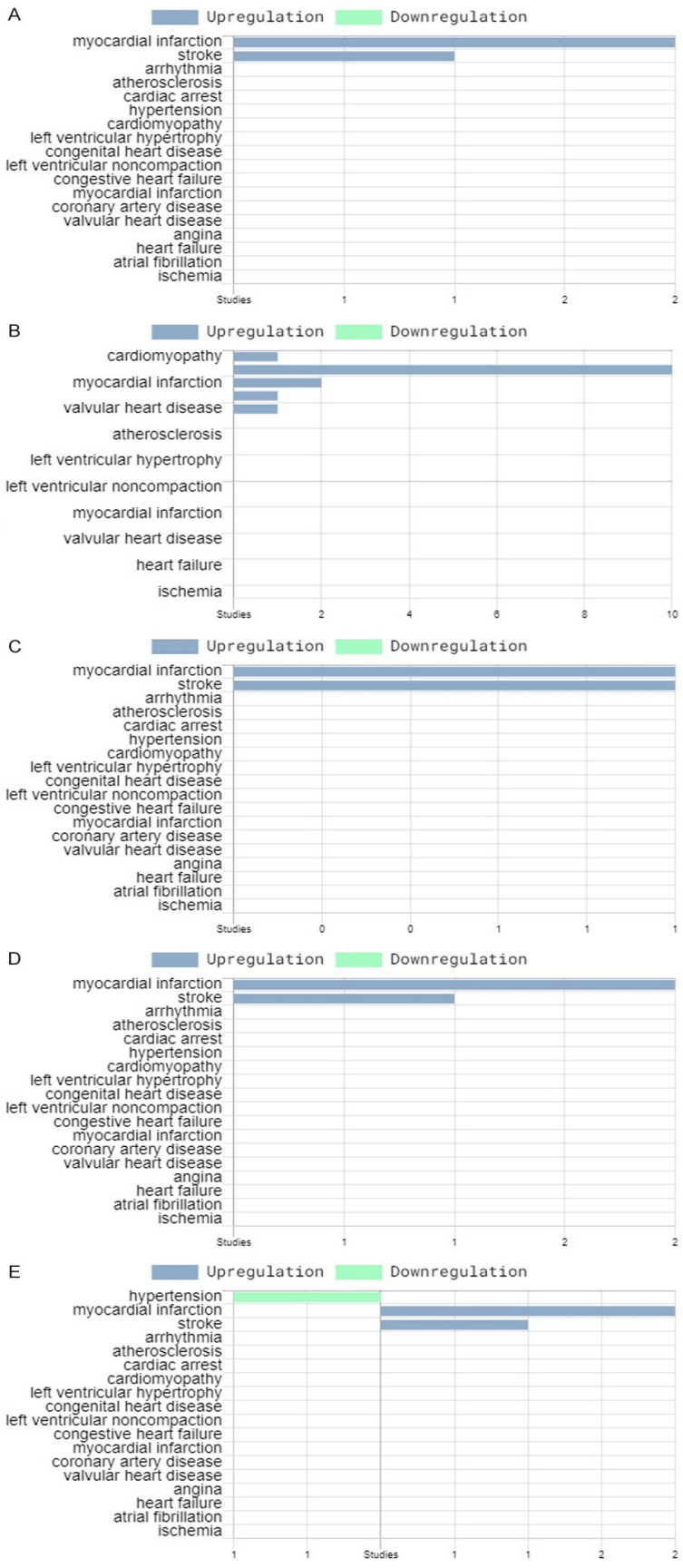
CRP	A well-known marker of inflammation [216] and endothelial dysfunction [217, 218] Marker of coronary artery disease [219] Serum levels correlate with severity of coronary artery lesions [220] Associated with atherothrombosis [221] Predict cardiovascular disease [222] Marker of early vascular aging [223]
vWF	Elevated plasma levels associated with myocardial infarction [224], coronary artery disease [225] and ischemic stroke [226]
P-selectin	Promotes acute myocardial infarction [227] Associated with atherothrombosis [228]
hsa-mir-1	Downregulated in cardiac hypertrophy [229]
Has-mir-16-5p	Aggravates myocardial infarction injury by targeting IRS1 [230]
hsa-mir133a	Downregulated in cardiac hypertrophy [231]
hsa-miR-92	Upregulated in dilated cardiomyopathy [232]
hsa-mir-34a	Upregulation in myocardial infarction [233]
Has-mir-15a	Upregulated in myocardial ischemia [234]
hsa-mir-30	Downregulated in dilated cardiomyopathy [235]

adhesion of leukocytes to the activated endothelium. Clayton et al. [107] showed that DOX-induced aortic stiffness through TNFα-mediated endothelial inflammation, which was associated with adverse structural changes, including collagen deposition (fibrosis), elastin fragmentation, and formation of AGEs (advanced glycation end-products). AGEs, also known as glycotoxins are highly oxidant compounds with pathogenic significance in many degenerative diseases, such as atherosclerosis, Alzheimer's disease, diabetes, and chronic kidney disease [108, 109]. Chow et al. [110] assessed endothelial-dependent vasodilatation in anthracycline-treated patients and impairment of endothelial-dependent arterial vasodilatation, which was sustained for months to years, suggesting its important role in the progression of coronary disease. These findings correlate with several clinical studies showing that chemotherapy with DOX induces damage to the coronary microcirculation, which might contribute to the adverse cardiovascular outcomes in cancer survivors, including those who did not develop symptomatic cardiotoxicity [111].

Gene expression of circulating blood cells

The effect of DOX on gene expression of easily obtainable tissue such as the blood is an opportunity to identify non-invasive biomarkers for the prediction and identification of DOX-induced cardiotoxicity. McCaffrey et al. [112] demonstrated that breast cancer patients with low LVEF after DOX chemotherapy had significantly altered transcripts in comparison with patients with normal LVEF, including genes associated with apoptosis, immunity, detoxification, and drug transport, in comparison with patients who maintained normal LVEF. Two of the decreased genes were suggested as potential biomarkers of DOX-induced cardiotoxicity, T Cell Leukemia/Lymphoma protein 1A (*TCL1A*), major pro-survival factor for cardiomyocytes, and *ABCB1*, which codes for the multidrug resistance protein 1 (*MDR1*), an efflux pump for DOX, potentially leading to higher cardiac levels of the drug. Doroshow et al. [113] showed that patients exposed to continuous DOX infusions have increased DNA-base oxidation within their blood cells compared to pre-treatment.

We have found a high similarity between the gene expression profiles of peripheral blood cells (PBCs) and cardiac tissue in rats with DOX-induced cardiotoxicity [114]. Of the ~4,000 differentially regulated genes in each heart tissue and PBCs of rats, at 48 hours after DOX administration, 2400 genes were similarly differentially regulated. Therefore, in the subsequent clinical study, we have examined the potential of PBMC transcriptome profile after the first dose of DOX chemotherapy to predict DOX-induced cardiomyopathy in breast cancer patients [115]. The results showed that significantly altered transcripts coding for proteins of neutrophils, macrophages, and monocytes were able to predict the risk for DOX-induced cardiotoxicity. The top upregulated DOX-induced transcripts associated with abnormal LVEF decline include neutrophilic anti-microbial proteins such as alpha-defensins (*DEFA1-4*); cathelicidin (*CAMP*); MPO; peptidoglycan recognition protein1 (*PGLYRP1*); matrix metalloproteases (*MMP*) 8 and 9); carcinoembryonic anti-



gen-related cell adhesion molecule 8 (*CEACAM8*, known as *CD66b*), glycosyl-phosphatidylinositol glycoprotein (*CD177*). These findings are in agreement with the reported role of neutrophils in cardiovascular diseases by Frangogiannis et al. [116]. Some of the neutrophil transcripts (*CAMP*, *MMP9*, *PGLYRP1*, *MMP8*) in our clinical study were similarly dysregulated in the rat blood cells in our previous study [52]. We have recently found significant elevation of the plasma proteins coded by *CAMP*, *MMP9*, *PGLYRP1*, *CEACAM8*, *ELANE*, and *MPO*, suggesting that these molecules carry the potential for an early prediction of the risk for LVEF decrease. Another significantly upregulated transcript in the group of patients with low LVEF in our study is also *OLR1* (oxidized low-density lipoprotein receptor 1), which codes for lectin-type oxidized LDL receptor 1 (LOX-1) protein. LOX-1 is suggested as a marker of atherosclerosis and induces vascular endothelial cell activation, and dysfunction, resulting in pro-inflammatory responses, pro-oxidative conditions, and apoptosis [117]. Recent studies showed that OLR1 was highly expressed in polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) cells of cancer patients [118] and in low-density granulocytes of patients with lupus who are at high risk of cardiovascular diseases [119], but no data are available on its role in DOX-induced cardiotoxicity. Neutrophil granular proteins are known to be involved in the inflammation and progression of cardiovascular diseases including atherosclerosis, thrombosis, and acute coronary syn-

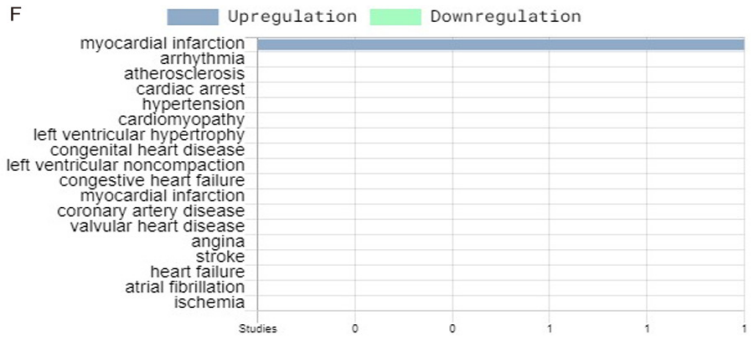


Figure 1. Analysis of six of the top upregulated genes in the blood of breast cancer patients with subclinical DOX-induced cardiotoxicity [115] using HeartBioportal database: (A) MPO; (B) MMP9; (C) CEACAM8; (D) CAMP; (E) PGLYRP1; (F) ELANE. The elevated expression of the six genes after the initial DOX dose in patients with abnormally decline LVEF correlate with upregulation in cardiovascular diseases.

drome [120, 121]. The elevation of neutrophil granular proteins, including *MPO, MMP9, CAMP, PGLYRP1, MMP8, CEACAM8* and *DEFA1* has been predictive of atherosclerosis, coronary artery disease, and myocardial infarction (**Table 2**). We have analyzed the published transcriptional data related to the gene expression of the top upregulated transcripts for cardiovascular diseases through the genetics database for cardiovascular diseases HeartBioPortal (https://www.heartbioportal.com/) [122]. The analysis showed that elevation of several of these genes, including *DEFA4, CAMP, PGLYRP1, MMP9, MPO, CEACAM9, ELANE, OLR1* was associated with myocardial infarction (**Figure 1**). This finding confirms our hypothesis that the early subclinical toxicity of chemotherapy with DOX might in part, be mediated by neutrophil granular proteins through enhancing cardiovascular inflammation, coronary artery disorder, and atherosclerosis, which predispose to myocardial infarction (**Figure 2**). Therefore, peripheral blood transcriptome and proteome biomarkers, such as *MPO, CAMP, PGLYRP1, MMP8, MMP9, CEACAM8, DEFA1-4, OLR-1, ARG-1, ELANE* could provide early indications about DOX-induced cardiomyopathy.

Circulating microRNAs

The involvement of miRNAs in cardiovascular biology and pathology, and their potential as biomarkers has been demonstrated in several studies on cardiovascular diseases [123, 124]. In a clinical study, we have examined the potential of circulating miRNAs to predict the risk of DOX-induced cardiotoxicity in breast cancer patients [125]. We have identified 32 differentially regulated microRNAs (DEmiRs) in patients with an abnormal decline of LVEF >10% in comparison with patients who maintained normal LVEF. Several of the DEmiRs in patients with an abnormal decline of LVEF EF have been reported previously in patients with cardiovascular diseases, including downregulation miR-1 and miR-133 in cardiac hypertrophy, upregulation of miR-92 in dilated hypertrophy, upregulation of miR-34a in myocardial infarction, downregulation of miR-15b in myocardial infarction and downregulation of miR-30 in dilated cardiomyopathy (**Table 2**). Elevated miR-23b suppressed IL-17-, TNF-α- and IL-1β-induced NF-κB activation, which correlated with its downregulation in inflammatory disorders, such as SLE and rheumatoid arthritis [126]. Several of DemiRs have been associated with inflammatory and autoimmune diseases such as elevated miR-16, miR-486, miR 92, miR-532, miR-140 [127-129]. None of these miRNAs have been reported as potential candidate biomarkers for the prediction of DOX-induced cardiotoxicity. The analysis of the targets of DEmiRs in the group of patients with low LVEF showed their association with several of the differentially regulated mRNA in the same patients.

DNA methylation of peripheral blood cells

In our recent study, we have determined the whole-genome DNA methylation of blood cells from breast cancer patients treated with DOX-based chemotherapy [130]. The results showed that 379 differentially methylated CpGs at baseline and 136 CpGs after the first chemotherapy dose significantly correlated with LVEF status. Pathway enrichment analysis using GO, Reactome, KEGG showed that the most significantly positively enriched pathway was “RNA splicing” (included *RBM17, DHX9, EIF4A3, CACS3, DHX15, HNRNPH1, HNRNPR, HNRNPU, SF1, SNRPN*) and the most significantly negatively enriched pathway was IFN-γ signaling (included *IRF6, HLA-DRB1, TRIM14, HLA-A, and*

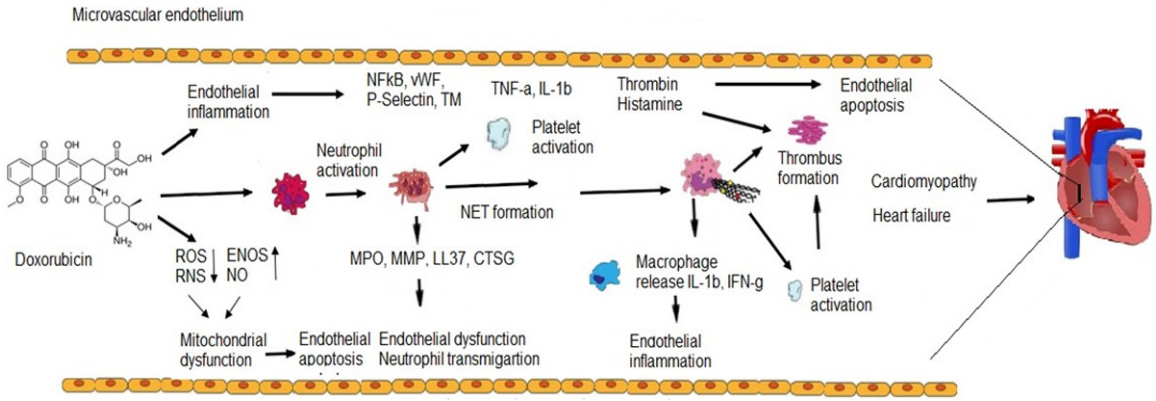


Figure 2. DOX-induced cardiomyocytes damage is preceded by vascular endothelial injury associated with neutrophil degranulation and NET formation. DOX induces ROS generation, inactivation of NO production, endothelial dysfunction and recruitment of neutrophils. Pro-inflammatory cytokines secreted by monocytes, lymphocytes and ECs activate neutrophil degranulation. Neutrophil granule proteins participate in the disruption of EC structure, leading to endothelial inflammation, neutrophil transmigration and apoptosis. NETs provide scaffold for platelet activation and deposition, thus promoting thrombosis formation.

HLA-F). Overexpression of *DHX15* and *EIF4A3* splicing factors have been implicated in the contractile function of cardiomyocytes [131, 132] and in cancer [133]. IRF6, a member of the IFN family of transcription factors is one of the significantly hypomethylated genes before the start of chemotherapy that predicted the risk of DOX-induced cardiotoxicity in our study. These findings correlate with previous reports showing that IRF6 has a protective role in the response to endotoxic shock [134] which is one of the suggested mechanisms of DOX-induced inflammation and multiorgan toxicity [135]. Downregulation of IRF6 has been demonstrated in several cancers, including breast cancer [136]. Further analysis with IPA software showed upregulation of estrogen receptor (ER) signaling, ErbB signaling, and Thrombin signaling. ErbB receptor tyrosine kinases, epidermal growth factor receptor (EGFR), and ErbB2 (neu, HER2) are often overexpressed, amplified, or mutated in many forms of cancer, including breast cancer, making them important therapeutic targets and predictors of the therapeutic response to DOX [137]. At the same time, *ERB2* overexpression in the heart leads to hypertrophy [138]. It is well known that ER signaling plays an important role in breast cancer progression and the majority of human breast cancers start as estrogen-dependent [139]. The activation of the coagulation cascade in which thrombin plays a key role is closely related to inflammation, development of cardiovascular diseases, and HF prognosis [140]. Accordingly, our previous study demonstrated that elevated markers of inflammation, hypercoagulability, and endothelial function (i.e. thrombomodulin, myeloperoxidase, thrombin-anti-thrombin complex) before and after the first dose of DOX chemotherapy were able to predict the early subclinical DOX-induced cardiotoxicity in patients with breast cancer [97].

Conclusions

DOX is a powerful chemotherapy agent that has improved substantially the cure rate and survivorship of patients with various types of cancer. Unfortunately, the cardiotoxicity of DOX remains an important health concern. Despite the years of substantial research, there is still insufficient understanding of the mechanisms governing cardiac toxicity and no efficient treatment or means for early prediction. DOX-induced cardiotoxicity begins asymptomatically with the initial dose and develops into asymptomatic cardiac dysfunction, and subsequent HF. Our studies have demonstrated that the initial dose of chemotherapy with DOX in cancer patients induced neutrophil activation that could be detrimental to the vascular integrity, as the release of the harmful cargo of their granules could compromise vascular integrity or induce a prothrombotic state. We propose that neutrophil degranulation and NETosis during the early stages of vascular inflammation are key mechanisms of DOX-associated cardiovascular toxicity. Neutrophil granular proteins and NETs could

potentially act as predictive biomarkers, as well as novel therapeutic targets for DOX-induced cardiotoxicity.

Acknowledgements

This work was supported in part with grants from Susan G Komen for the Cure (grant# BCTR78206), Arkansas Breast Cancer Research Program and the University of Arkansas for Medical Sciences (1UL1RR029884), NIH/NIA Claude Pepper Center (P30AG028718), and funds from the Laura F. Hutchins, M.D. Distinguished Chair for Hematology and Oncology.

Disclosure of conflict of interest

None.

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