Impaired microvascular reactivity in patients treated with 5-fluorouracil chemotherapy regimens: Potential role of endothelial dysfunction

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ABSTRACT

Background: 5-fluorouracil (5-FU) is the second most common cancer chemotherapy associated with short- and long-term cardiotoxicity. Although the mechanisms mediating these toxicities are not well understood, patients often present with symptoms suggestive of microvascular dysfunction. We tested the hypotheses that patients undergoing cancer treatment with 5-FU based chemotherapy regimens would present with impaired microvascular reactivity and that these findings would be substantiated by decrements in endothelial nitric oxide synthase (eNOS) gene expression in 5-FU treated human coronary artery endothelial cells (HCAEC).
Methods: We first performed a cross-sectional analysis of 30 patients undergoing 5-FU based chemotherapy treatment for cancer (5-FU) and 32 controls (CON) matched for age, sex, body mass index, and prior health history (excluding cancer). Cutaneous microvascular reactivity was evaluated by laser Doppler flowmetry in response to endothelium-dependent (local skin heating; acetylcholine iontophoresis, ACh) and -independent (sodium nitroprusside iontophoresis, SNP) stimuli. *In vitro* experiments in HCAEC were completed to assess the effects of 5-FU on eNOS gene expression.
Results: 5-FU presented with diminished microvascular reactivity following eNOS-dependent local heating compared to CON (P = 0.001). Iontophoresis of the eNOS inhibitor L-NAME failed to alter the heating response in 5-FU (P = 0.95), despite significant reductions in CON (P = 0.03). These findings were corroborated by lower eNOS gene expression in 5-FU treated HCAEC (P < 0.01) compared to control. Peak vasodilation to ACh (P = 0.58) nor SNP (P = 0.39) were different between groups.
Conclusions: The present findings suggest diminished microvascular function along the eNOS-NO vasodilatory pathway in patients with cancer undergoing treatment with 5-FU-based chemotherapy regimens and thus, may provide insight into the underlying mechanisms of 5-FU cardiotoxicity.

1. Introduction

5-fluorouracil (5-FU) is one of the most frequently administered chemotherapeutic agents used in the treatment of cancer and is a central component of the preferred treatment regimens for gastrointestinal and head/neck malignancies (e.g., FOLFOX, FOLFIRI, FOLFIRINOX). Despite established effectiveness as an anticancer agent, the efficacy of 5-FU-containing regimens can be limited by its off-target cardiovascular side effects (i.e., cardiotoxicity), which most commonly arise as angina with or without ST-segment alterations [1–5]. The onset of symptomatic cardiotoxicity has been documented to occur in 1–20 % of patients treated with 5-FU [1,3,5–8], however, these incidence rates have been suggested to be underestimated [8,9]. Rezkalla and colleagues report

Abbreviations

5-FU	5-fluorouracil
%ΔCVC	Percent change in cutaneous vascular conductance from baseline
ACh	Acetylcholine
CVC	Cutaneous vascular conductance
eNOS	Endothelial nitric oxide synthase
HCAEC	Human coronary artery endothelial cells
L-NAME	NG-Nitroarginine methyl ester
MAP	Mean arterial pressure
SNP	Sodium nitroprusside

asymptomatic ST segment alterations suggestive of decreased myocardial perfusion in greater than 50 % of 5-FU treated patients [10]. Importantly, the development of adverse cardiovascular side effects during cancer treatment can prompt deviation from optimal chemotherapy dosing regimens [11] resulting in an increased likelihood of cancer reoccurrence and significant reductions in overall survival [12–14]. As such, there is a critical need to investigate the mechanistic underpinnings by which 5-FU alters vital aspects of cardiovascular health.

To date, the pathophysiological mechanisms driving 5-FU cardiotoxicity remain poorly understood. Among the most prominent theories is the development of coronary epicardial or microvascular spasm provoked by a direct effect of 5-FU on the coronary endothelium and/or smooth muscle [3,4,15]. However, evidence of the effect of 5-FU on the coronary circulation is limited to single-patient case reports [16,17]. This paucity of evidence is seemingly due, at least in part, to the high invasiveness required in investigating the coronary circulation. As such, others have turned to the peripheral vasculature to better understand how 5-FU may impact vascular function. While reductions in flow-mediated dilation in large vessels such as the brachial artery following 5-FU treatment have been demonstrated [18], it remains unknown whether 5-FU or 5-FU-containing regimens negatively affect the microcirculation. In our previous work we have used laser Doppler flowmetry coupled with iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) to demonstrate alterations in cutaneous microvascular reactivity in chemotherapy [19] and radiation [20] treated patients with cancer. However, to our knowledge, there are currently no studies of this nature specific to 5-FU-based regimens. Therefore, the present investigation aimed to assess potential impairments in cutaneous microvascular reactivity in patients treated for cancer with 5-FU-based chemotherapy regimens. Additionally, to further explore the mechanisms driving these impairments, we utilized a human coronary artery endothelial cell (HCAEC) culture model treated with multiple concentrations of 5-FU. We hypothesized that 5-FU treatment would diminish endothelial-dependent microvascular reactivity, as determined by responses to local skin heating and iontophoresis of ACh, and that these impairments would be corroborated by decreased eNOS gene expression in HCAEC.

2. Methods

2.1. Participants

We used a case-control study design in which 30 patients undergoing cancer treatment with 5-FU-based chemotherapy regimens (5-FU) were matched with 32 control (CON) participants. Cancer diagnoses for patients undergoing 5-FU-based treatment included colon/rectal, pancreatic, appendiceal, duodenal, stomach/esophageal, and common bile duct cancers which were confirmed by the patient's oncologist. Patients treated with the following 5-FU-based chemotherapy regimens: FOLFOX

(folinic acid, 5-FU, oxaliplatin), FOLFIRINOX (folinic acid, 5-FU, irinotecan, oxaliplatin), FOLFIRI (folinic acid, 5-FU, irinotecan), or FOLFOX → FOLFIRI, were eligible to enroll in the study (Table 1). Despite components of some of these regimens differing slightly, current chemotherapy practice generally includes the use of multiple agents depending on cancer type, tumor stage, and the patient's response to treatment. Therefore, we included patients treated with a variety of standard 5-FU-based regimens. Patients were matched with control subjects for age, sex, body mass index (BMI), and smoking status, as well as prior non-cancer related health history, including the presence of cardiovascular disease and documented history of cardiovascular disease risk factors (hyperlipidemia, high blood pressure, and metabolic disease) (Table 2). Further, we recorded any medications used to treat the aforementioned cardiovascular disease risk factors (diabetic, hypertensive, and cholesterol medications). All participants provided written informed consent approved by the institutional review boards at Kansas State University, with all procedures conforming to the Declaration of Helsinki.

All testing was conducted during a single 90–120-minute visit, with the subject in a seated recliner at the local cancer treatment facility (Stormont Vail Cotton O'Neil Cancer Center, Topeka, KS) or the investigators' dedicated laboratory space (Lafene Health Center, Kansas State University, Manhattan, KS). Data collection for all patients with cancer was arranged to begin immediately following the completion of a regularly scheduled 46-hr continuous infusion of 5-FU chemotherapy with all experiments starting within 90-minutes of completing the infusion. Matched control experiments were scheduled based on participant availability. Generally, patients with cancer received a single bolus injection of 5-FU immediately followed by a 46-hour continuous infusion of 5-FU with doses, durations, and use of additional chemotherapies varying slightly between patients depending on the individually prescribed regimen. Upon completion of therapy and removal of the 5-FU pump, patients were seated in a reclined chair and instrumented with an automated arm blood pressure cuff (HEM-907XL, OMRON Healthcare, Kyoto, Japan) for measurement of systolic and diastolic blood pressures (taken in duplicate). All blood pressure measurements were made at heart level.

2.2. Laser Doppler flowmetry

To assess cutaneous microvascular reactivity, we conducted experiments using laser Doppler flowmetry in combination with both endothelium-dependent and -independent stimuli. Due to the availability of patient time and measurement sites (two per participant), we completed one or two of the following experimental studies in each participant: 1) local skin heating at 42 °C, 2) local skin heating with iontophoresis of the nitric oxide synthase inhibitor NG-Nitroarginine methyl ester (L-NAME; see *Microvascular Reactivity to Localized heating and L-NAME iontophoresis*), 3) iontophoresis of ACh (see *Microvascular Reactivity to ACh*), and 4) iontophoresis of sodium nitroprusside (SNP) (see *Microvascular Reactivity to SNP*). Iontophoresis was selected as the mode of drug delivery to avoid the vascular consequences of needle trauma associated with inserting intradermal microdialysis fibers [21] and to minimize the patient time requirement, invasiveness, and pain. It is well established that chemotherapy-induced neuropathic pain is one of the most common and severe off-target effects of anticancer agents, with symptoms often affecting the extremities of the upper and lower limbs [22,23]. Therefore, the use of intradermal microdialysis was contraindicated in this population. For all experiments, two drug delivery electrodes (PF 383, Perimed, Järfälla, Sweden) instrumented with laser Doppler flowmeters and temperature regulators were randomly assigned and placed immediately proximal to the wrist or distal to the elbow on the ventral right forearm. The skin was pre-cleaned with commercially available alcohol wipes before probe placement, and the probes were held in place with two strips of surgical tape. A single hydrogel drug dispersive electrode (PF 384; Perimed) was fixed to the

Table 1
Individual experimental cohort cancer diagnosis and treatment information. The components of the common 5-FU based chemotherapy regimens used in treatment of patients included in this study are as follows: FOLFOX (Leucovorin, 5-fluorouracil, Oxaliplatin), FOLFIRINOX (Leucovorin, 5-fluorouracil, Irinotecan, Oxaliplatin); FOLFIRI (Leucovorin, 5-fluorouracil, Irinotecan). Some patients may have also received capecitabine (CAPE) which is an oral prodrug that is converted to 5-FU upon consumption.

	Local Heating (n = 17)		Local Heat + L-NAME (n = 7)		Ach (n = 17)		SNP (n = 6)	
Cancer Type, n	Stages I-III	Stage IV	Stages I-III	Stage IV	Stages I-III	Stage IV	Stages I-III	Stage IV
Colon/Rectum	3	3	2	2	3	5	2	1
Pancreatic	3	1	1	0	3	1	2	0
Appendiceal	0	3	0	1	0	2	0	0
Stomach/Esophageal	1	0	0	0	1	0	0	1
Duodenal	2	0	0	0	2	0	0	0
Common Bile	1	0	1	0	0	0	0	0
Chemotherapy Regimen, n	Alone	+ CAPE	Alone	+ CAPE	Alone	+ CAPE	Alone	+ CAPE
FOLFOX	6	3	2	2	7	1	3	0
FOLFIRINOX	6	0	2	0	4	1	3	0
FOLFIRI	0	0	0	0	0	1	0	0
FOLFOX + FOLFIRI	1	1	1	0	2	1	0	0
Cumulative 5-FU dose mg/m^2, median (25th-75th percentile)	12,000 (7,000 – 31,720)		9,000 (2,800——27,600)		25,045 (12,500 – 42,200)		21,000 (15,100——27,100)	
Additional Chemotherapies, name (n)	bevacizumab (n = 2), gemcitabine (n = 4), trastuzumab (n = 2), panitumumab (n = 1)		gemcitabine (n = 2), panitumumab (n = 1)		bevacizumab (n = 3), gemcitabine (n = 3), trastuzumab (n = 2), panitumumab (n = 3), mitomycin (n = 1), paclitaxel (n = 1), cetuximab (n = 1)		paclitaxel (n = 1), carboplatin (n = 1), nivolumab (n = 1)	

Table 2
Demographic information for combined study cohorts. Group means for age, BMI, and MAP were compared using independent samples t-tests. Proportion of patients in each group who were women, have been diagnosed with CVD, who have been diagnosed with common CVD risk factors, who take medications associated with CVD and/or CVD associated risk factors, and who are current smokers were compared between groups using Fisher's exact test. BMI = body mass index. CVD = cardiovascular disease. MAP = mean arterial pressure. Data presented as mean ± SD or n (%).

	Local Heating			Local Heating + L-NAME			ACh			SNP		
	CON	5-FU	P-value	CON	5-FU	P-value	CON	5-FU	P-value	CON	5-FU	P-value
n	20	17	n/a	7	7	n/a	15	17	n/a	9	6	n/a
Age, years	62.0 ± 12.9	62.9 ± 10.6	0.81	62.4 ± 8.5	61.1 ± 11.2	0.81	58.7 ± 16.5	63.5 ± 12.1	0.35	57.3 ± 9.3	58.5 ± 12.8	0.86
BMI, kg/m^2	30.0 ± 5.0	29.9 ± 4.7	0.94	27.5 ± 5.5	31.6 ± 4.7	0.16	30.6 ± 4.5	28.3 ± 4.2	0.16	26.6 ± 3.8	30.8 ± 5.4	0.10
MAP, mmHg	93 ± 9	86 ± 11	0.04*	98 ± 11	86 ± 11	0.07	90 ± 7	84 ± 10	0.08	86 ± 10	84 ± 10	0.79
No. of women, n (%)	9 (45)	7 (41)	>0.99	3 (43)	3 (43)	>0.99	7 (47)	4 (24)	0.27	4 (44)	3 (50)	>0.99
No. with ≥ 1 CVD risk factor, n (%)	12 (60)	15 (88)	0.07	5 (71)	7 (100)	0.46	8 (53)	10 (59)	>0.99	2 (22)	6 (100)	0.01*
CVD risk meds., n (%)	9 (55)	13 (76)	0.30	5 (71)	7 (100)	0.46	8 (53)	8 (47)	>0.99	2 (22)	3 (50)	0.33
Diagnosed CVD, n (%)	5 (25)	3 (18)	0.70	1 (14)	1 (14)	>0.99	4 (27)	3 (18)	0.68	1 (11)	1 (17)	>0.99
Current smoker, n (%)	0 (0)	2 (12)	0.20	0 (0)	2 (29)	0.46	0 (0)	2 (12)	0.49	0 (0)	1 (17)	0.40

skin of the same forearm at least 10 cm away from the drug delivery electrodes. The drug delivery and dispersive electrodes formed a complete circuit via connection to a USB power supply (PF 751; Perimed) that controlled current delivery intensity, duration, and interval using available software (PerIont Software; Perimed). An integrated laser Doppler flowmeter (PeriFlux 5010; Perimed) measured cutaneous red blood cell flux (RBC flux), of which was used as an index of cutaneous blood flow and recorded using data acquisition software (DI-720; DATAAQ Instruments, Akron, OH) for future offline analyses. The laser Doppler flowmeter was calibrated according to factory standards using Brownian motility standard solution (PF 1001; Perimed). Upon completion of setup and prior to any local heating or iontophoresis experiments, steady resting RBC flux values were assured and at least two minutes of baseline RBC flux was collected from each probe with the localized probe temperature set to 33 °C.

To account for potential differences and fluctuations in mean arterial pressure (MAP) between participants, all RBC flux data are presented as percent changes in cutaneous vascular conductance (CVC) from baseline. MAP was calculated as MAP = 1/3 (pulse pressure) + diastolic blood pressure. CVC was calculated as CVC = (RBC flux / MAP) x 100. Relative changes in CVC from baseline to peak (%ΔCVC) were calculated as: [(peak - baseline CVC) / baseline CVC] x 100, as described previously [19,20].

2.3. Microvascular reactivity to local heating and L-NAME iontophoresis

Local heating of the forearm skin elicits a biphasic vasodilatory response which culminates in a plateau in cutaneous blood flow that is

highly reliant on nitric oxide (NO) [24–30]. Briefly, local heating stimulates an initial axon-mediated increase in blood flow that occurs within the first few minutes of heat onset [25,31], followed by a brief nadir and successive gradual rise to a sustained endothelium-derived NO-dependent plateau [24–27]. To assess this NO-dependent response, one of the two laser Doppler probes was randomly selected for a standardized local heating protocol [24] (n = 20 CON, n = 17 5-FU). Following a short baseline period, the local probe temperature was increased from 33 °C by 1 °C every 10 s until a temperature of 42 °C was achieved. The local temperature remained clamped at 42 °C for ∼ 35-minutes until a plateau in RBC flux was reached. The highest 5-minute average of RBC flux during the plateau period was used for subsequent calculations of CVC, as described above. This protocol has previously been used as a non-invasive/pain-free method of assessing the endothelial-derived NO vasodilator pathway in participants afflicted by external stressors [32] or various disease states [24,29,33–36].

In a subset of these participants (n = 7 CON, n = 7 5-FU), an additional local heating site was included at a second laser Doppler probe in which otherwise identical procedures to those described above were preceded by iontophoresis of a 2 % L-NAME solution (Product #: N5751, Sigma-Aldrich, St. Louis, MO, USA). For these experiments, 200 μL of 2 % L-NAME dissolved in deionized water was added to the drug delivery electrode and placed at the predetermined forearm site as mentioned above. Following the collection of baseline measures, 20-minutes of L-NAME iontophoresis commenced using a 50 μA anodal current, as demonstrated by others [32,37]. Before probe placement, measurement sites were pretreated for ∼ 10-minutes with a topical anesthetic (removed via alcohol swab prior to probe placement) that included 4 % lidocaine (Aspercreme, Chattem, Chattanooga, TN) to minimize any potential discomfort caused by the current and simultaneously inhibit a current induced increase in basal RBC flux. Notably, the use of like anesthetic procedures has been shown to blunt the initial sensory neuron-related spike in RBC flux without affecting the sustained heated plateau [25]. As such, data comparing potential differences in the initial peak in RBC flux are not presented herein.

2.4. Microvascular reactivity to ACh

The cutaneous vasodilatory response to exogenous ACh is mediated by a combination of factors including endothelial-derived NO, prostanoids, and endothelial-derived hyperpolarizing factors and therefore is often used as a standard method to assess broad endothelium-dependent microvascular reactivity [38–42]. As such, to test potential endothelium-dependent differences beyond the primarily NO-mediated effects of local heating, 5-FU patients (n = 17) and controls (n = 15) completed an ACh iontophoresis protocol. As demonstrated previously by our group [19,20], a 100 μA anodal current was used to deliver 200 μL of 2 % ACh solution (Product #A6625, Sigma-Aldrich) dissolved in deionized water in 7 sequential iontophoresis pulses (i.e., doses) separated by a 60-second rest interval. Data recording persisted for at least 5-minutes into the recovery period following administration of the final iontophoresis pulse. The local probe temperature for ACh iontophoresis remained clamped at 33 °C throughout the experiment. The ACh-induced peak was considered the highest 10-second mean CVC recorded at any point during the 7 successive ACh iontophoresis pulses [19,20]. To evaluate the cumulative effect of the ACh-induced hyperemic response, area under the curve (AUC) was calculated using %ΔCVC over the course of the ACh iontophoresis period.

2.5. Microvascular reactivity to SNP

Endothelium-independent microvascular reactivity was evaluated in a separate cohort of CON (n = 9) and 5-FU (n = 6) participants via iontophoresis of SNP. Briefly, a 20 μA cathodal current was used to deliver 200 μL of a 1 % SNP solution (Product #: 71780, Honeywell, Charlotte, NC, USA) dissolved in 0.9 % saline over the course of 400 s

[43]. Following completion of SNP delivery, data recording continued for at least 5-minutes to ensure a peak vasodilatory response had been achieved. The SNP peak was considered the highest 1-minute CVC average recorded during the 400-s delivery period or in the 5-minutes following the iontophoresis protocol.

2.6. Human coronary artery endothelial cells (HCAEC)

Previously frozen primary HCAEC (PCS-100–020; ATCC, Manassas, VA, USA) were plated at 35,000 cells per well in tissue culture treated 6-well plates (3516; Corning, Glendale, AZ, USA) and maintained in 2 mL vascular cell growth medium (PCS-100–030 supplemented with PCS-100–041; ATCC) and placed in an incubator at 37 °C/5% CO2 as per manufacturer instructions. Following a 24-hour attachment period, growth medium was aspirated, and wells were randomly assigned to receive fresh growth medium, growth medium treated with 0.7 mM of 5-FU, or growth medium treated with 7 mM of 5-FU, for 24 h (n = 3 cultures per group). These concentrations were selected for their use in studies demonstrating *ex vivo* vascular reactivity in response to 5-FU [44,45] though are higher than those expected in the human circulation during 5-FU treatment [46–49]. Following the 24-hour treatment period, the growth medium was aspirated, and all wells were washed twice with sterile 1x D-PBS before cell collection via incubation with Trypsin EDTA for Primary Cells (PCS-999–003; ATCC). Before proceeding, all wells were inspected to ensure similar cell removal from each well. Cells were then centrifuged according to manufacturer standards and subsequently lysed using a 1 % DNase I lysis solution (Ref#: AM8728, Thermo Fisher Scientific) prior to storage at −80 °C for future analyses.

2.7. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Complimentary DNA (cDNA) was prepared from cell lysates using the TaqMan Fast Cells-to-Ct kit (Product #: 4399003; Life Technologies, Carlsbad, CA) according to manufacturer instructions. cDNA samples were subsequently used in two-step RT-PCR experiments using TaqMan gene expression assays (Applied Biosystems; Bedord, MA, USA) for eNOS (assay ID: Hs01574659_m1) and GAPDH (assay ID: Hs99999905_m1) on 96-well TaqMan Plates in a QuantStudio 3 RT-PCR System (Applied Biosystems). All samples were run in duplicate for genes of interest and endogenous control (GAPDH) and analyzed using the comparative threshold (ΔΔCt) method.

2.8. Statistical analysis

All data were analyzed using a commercially available software package (GraphPad Prism 9; San Diego, Ca, USA). Demographic variables (proportion of male/female, cardiovascular disease risk, etc.) were compared using Fisher's exact test. Independent-samples t-tests and Welch's *t*-test were used to determine pair differences between 5-FU and CON groups and within-group differences between L-NAME (L + SH) and standard local heating (SH) sites for normally distributed data with and without equal variance, respectively. Differences in baseline measurements for standard heating and L-NAME treated sites, gene expression in HCAEC, and potential differences in local heating or ACh-induced vasodilation between chemotherapy regimens or cancer stages were compared using one-way ANOVA, while multiple comparisons were assessed using Tukey's HSD when appropriate. A two-way repeated measures ANOVA was used to compare CVC differences following each ACh-iontophoresis delivery period between groups (group x impulse) with the Holm-Šidák method used to analyze multiple comparisons. To provide additional insight on the magnitude of differences between groups, effect sizes (ES) were calculated using Hedges' g unless standard deviations were significantly different between the groups (i.e., F-test < 0.05); in which case, Glass's delta was calculated

instead. The threshold values for ES were classified as: small $= 0.2$, moderate $= 0.5$, and large $= > 0.8$ [50]. All data are presented as mean $\pm$ standard deviation unless otherwise stated. Cumulative 5-FU doses received by each cohort are presented as median (25th-75th percentile). Alpha was set to 0.05 for all tests.

3. Results

3.1. General subject demographics

Cancer diagnosis, staging, and 5-FU chemotherapy regimen data are reported for each individual cohort in Table 1, whereas participant demographics are presented in Table 2. There were no statistical differences between individual cohorts for participant age, BMI, percentages of men and women, participants diagnosed with cardiovascular disease, use of cardiovascular-related medications, or percentage of current smokers (all P > 0.05) (Table 2). In addition, no differences were present concerning the number of participants with cardiovascular disease-related risk factors aside from the SNP cohort in which the 5-FU treated patients presented with a significantly higher percentage of participants with risk factors for CVD compared to the control group (22 % of CON vs 100 % of 5-FU; P = 0.01) (Table 2).

3.2. Local heating and L-NAME iontophoresis

The median 5-FU dose received by patients with cancer in the local heating cohort was 12,000 mg/m^2 (25th-75th percentile: 7,000 – 31,720) (Table 1). Fig. 1a illustrates a representative tracing of the %Δ CVC response to local heating in a patient treated with a 5-FU-based chemotherapy regimen and an age- and sex-matched control subject. The response to local heating expressed as %ΔCVC was significantly lower in the 5-FU-based chemotherapy group (n = 17) compared to controls (n = 20) (CON: 566 $\pm$ 305 %; 5-FU: 282 $\pm$ 172 %; P = 0.001, ES = 0.93). (Fig. 1b). Importantly, the CVC at baseline was not different between groups (CON: 6.9 $\pm$ 3.1 PU mmHg^{-1}; 5-FU: 8.8 $\pm$ 5.8 PU mmHg^{-1}; P = 0.42); however, 5-FU patients did present with significantly lower resting MAP (CON: 93 $\pm$ 9 mmHg; 5-FU: 86 $\pm$ 11 mmHg; P = 0.04) (Table 2). No differences in %ΔCVC were present between any of the 5-FU based regimens (FOLFOX (n = 9): 265 $\pm$ 162 %; FOLFIR-INOX (n = 6): 343 $\pm$ 202 %; FOLFOX + FOLFIRI (n = 2): 176 $\pm$ 117 %; P = 0.48). Similarly, when patients treated with 5-FU based regimens were grouped by cancer stage, there were no differences %ΔCVC between the groups (Stage II: 391 $\pm$ 132 %, n = 5; Stage III: 221 $\pm$ 211 %, n = 5; Stage IV: 248 $\pm$ 154 %, n = 7; P = 0.25).

Given that the work of others has demonstrated the role of NO in the vasodilator response to local skin heating [24–29], we further evaluated the local heating response in a subset of these participants following iontophoresis of the constitutive NOS inhibitor L-NAME (CON: n = 7, 5-FU: n = 7). The median 5-FU dose received by patients with cancer in the L-NAME subgroup was 9,000 mg/m^2 (25th-75th percentile: 2,800–27,600). No resting CVC differences were detected between standard heating and L-NAME testing sites within each group or between groups (CON + SH = 6.3 $\pm$ 2.3 PU mmHg^{-1}, CON + L = 6.3 $\pm$ 2.9 PU mmHg^{-1}, 5-FU + SH = 8.80 $\pm$ 5.8 PU mmHg^{-1}, 5-FU + L = 5.6 $\pm$ 2.3 PU mmHg^{-1}; main effect: P = 0.38). Further, there were no differences in resting MAP between the groups (CON: 98 $\pm$ 4 mmHg; 5-FU: 86 $\pm$ 4 mmHg; P = 0.07) (Table 2). In control subjects, the L-NAME treated site (L + SH) had a significantly lower %ΔCVC when compared to the standard local heating (SH) site (SH: 564 $\pm$ 371 %; L + SH: 175 $\pm$ 122 %; P = 0.03; ES = 1.05) (Fig. 2a, left). Interestingly, this difference was not reciprocated in the 5-FU group as the SH site was not significantly different from the L + SH site (SH: 327 $\pm$ 118 %; L + SH: 333 $\pm$ 227 %; P = 0.95; ES = 0.03) (Fig. 2a, right), suggesting that NO is not contributing significantly to the local heating-induced vasodilatory response in patients undergoing treatment with 5-FU based chemotherapy regimens. The difference between the %ΔCVC at the SH and L +
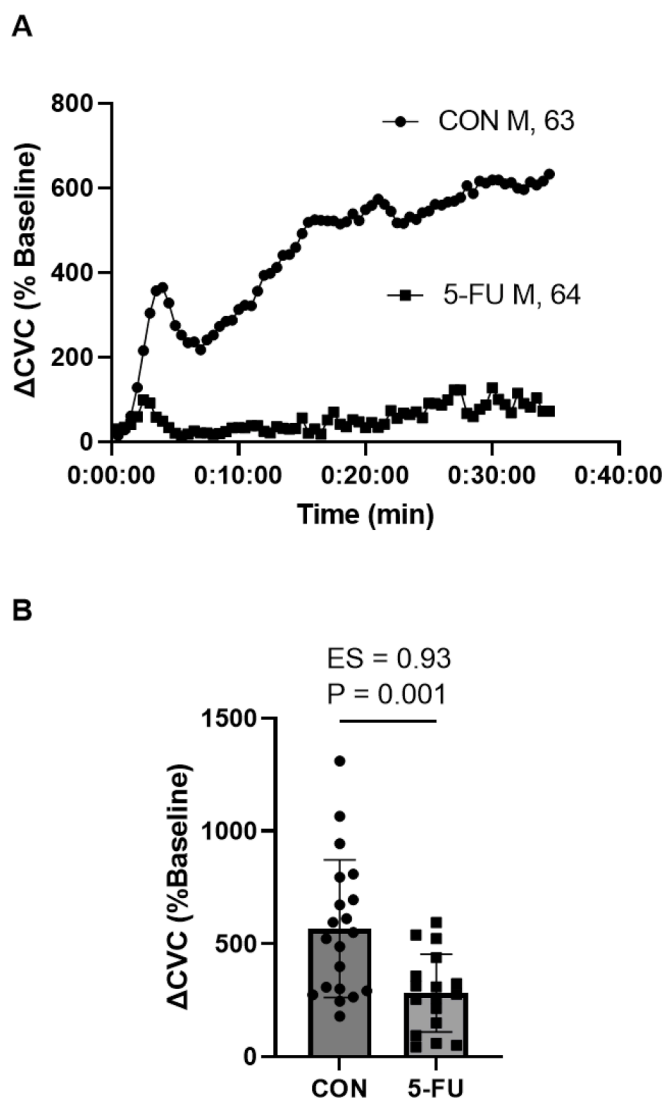
Fig. 1. Impaired cutaneous microvascular reactivity following local heating in cancer patients treated with 5-FU. A) A representative tracing of the rise in CVC from baseline during a 35-minute local heating protocol for a 5-FU treated patient (5-FU, Male 64 years old) and matched control (CON, Male, 63 years old). B) 5-FU patients (n = 17, 7 women) presented with a significantly lower %ΔCVC than control subjects (n = 20, 9 women) (CON: 566 $\pm$ 305 %; 5-FU: 282 $\pm$ 172 %; P = 0.001, ES = 0.93) following 35-minutes of local heating suggesting the presence of impairment along the eNOS-NO vasodilator pathway following 5-FU chemotherapy. Data were compared using the Welch's *t*-test and presented as mean $\pm$ SD.

SH sites was calculated and subsequently compared between the groups. The matched controls had a significantly greater difference between the SH and L + SH sites compared to the patients receiving 5-FU-based chemotherapy (CON: 389 $\pm$ 388 %; 5-FU: −6 $\pm$ 247 %; P = 0.04; ES = 1.21) (Fig. 2b).

3.3. ACh iontophoresis

The median 5-FU dose for 5-FU treated patients in the ACh cohort was 25,045 mg/m^2 (25th-75th percentile: 12,500 – 42,200) (Table 1). There were no differences detected in baseline CVC (CON: 6.5 $\pm$ 2.9 PU mmHg^{-1}; 5-FU: 11.3 $\pm$ 9.2 PU mmHg^{-1}; P = 0.22) or MAP (CON: 90 $\pm$ 4 mmHg; 5-FU: 84 $\pm$ 3 mmHg; P = 0.08) (Table 2) between the groups. Both 5-FU (n = 17) and CON (n = 15) groups experienced significant increases in %ΔCVC with successive ACh doses (P = <0.001) (Fig. 3a).
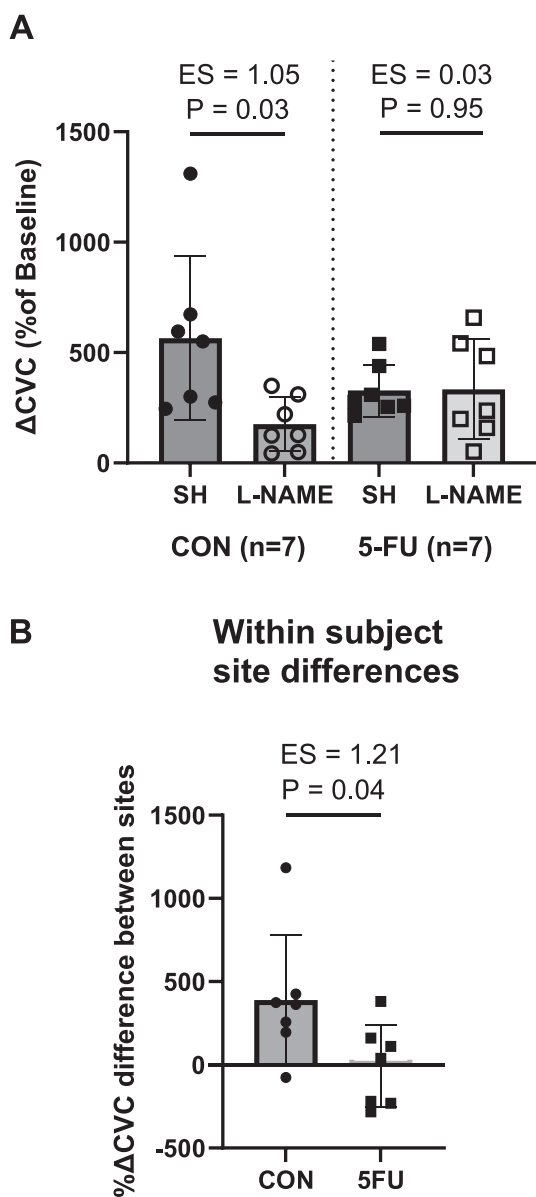
Fig. 2. L-NAME fails to reduce the local heating response in 5-FU-treated cancer patients. A) L-NAME iontophoresis significantly reduced the %ΔCVC response to local heating in control participants (n = 7, 3 women) (SH: 564 ± 371 %; L + SH: 175 ± 122 %; P = 0.03, ES = 1.05) but not in patients undergoing cancer treatment with 5-FU (n = 7, 3 women) (SH: 327 ± 118 %; L + SH: 333 ± 227 %; P = 0.95, ES = 0.03) suggesting a lesser contribution of NO to the heated plateau following treatment with 5-FU chemotherapy. B) The absolute difference in the percent change between the SH and L treated sites was significantly greater in control participants compared to 5-FU treated cancer patients (CON: 389 ± 388 %; 5-FU: −6 ± 247 %; P = 0.04, ES = 1.21). SH = standard heating; L + SH = L-NAME + standard heating. Data were compared using Welch's *t*-test (A, control cohort) and independent-samples *t*-test (A, 5-FU cohort; B) and presented as mean ± SD.

However, contrary to our hypothesis, there were no statistical differences between groups at any of the ACh iontophoresis impulse time points (P = 0.44) (Fig. 3a) nor in the peak ACh induced response from baseline (CON: 920 ± 416 %; 5-FU: 835 ± 448 %; P = 0.58) (Fig. 3b). Similarly, no differences AUC were present between groups (CON: 315,327 ± 148,879 %ΔCVC · sec; 5-FU: 266,300 ± 178,510 %ΔCVC · sec; P = 0.28) indicating no differences in the total cutaneous hyperemic response to ACh. Peak ACh response from baseline was not different between any of the 5-FU based regimens (FOLFOX: 963 ± 405 %, n = 8;
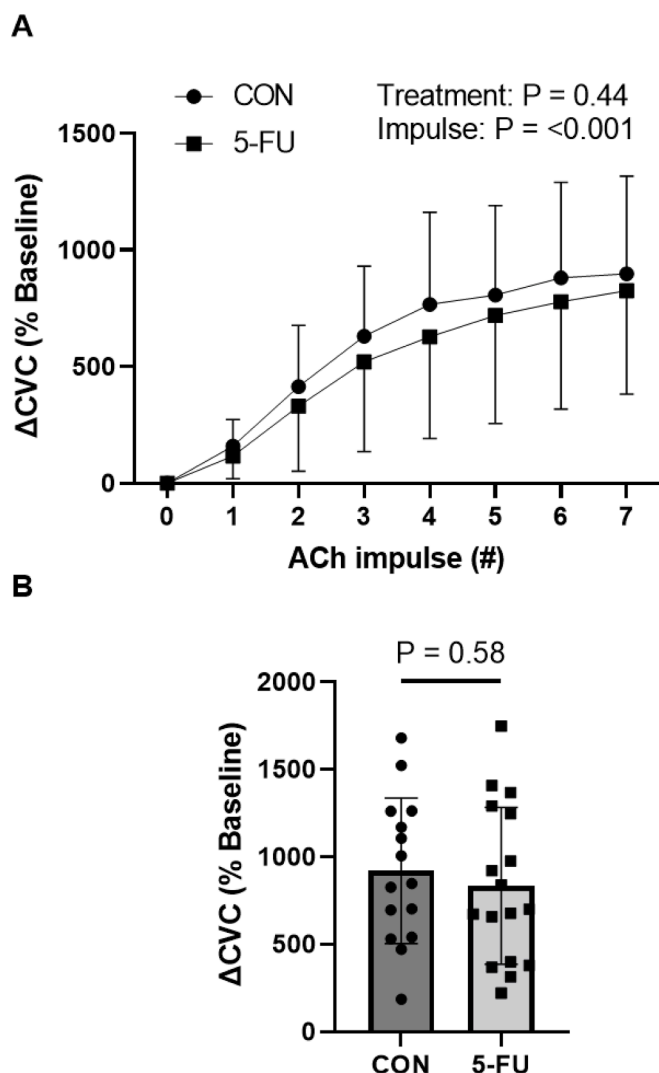

Fig. 3. Cutaneous microvascular reactivity in response to ACh iontophoresis is maintained in 5-FU-treated cancer patients. A) ACh iontophoresis resulted in a significant rise in %ΔCVC with successive ACh impulses (P = < 0.001); however, this rise was not different between patients treated with 5-FU (n = 17, 4 women) and control subjects (n = 15, 7 women) (P = 0.44). B) This finding was corroborated by the similarity in the peak response to ACh between groups (CON: 920 ± 416 %; 5-FU: 835 ± 448 %; P = 0.58), suggesting some endothelium-dependent vasodilatory pathways may remain intact following 5-FU-based chemotherapy. Data were compared using two-way repeated measures ANOVA (A) and independent-samples *t*-test (B). All data are presented as mean ± SD.

FOLFIRINOX: 844 ± 570 %, n = 5; FOLFOX + FOLFIRI: 522 ± 400 %, n = 3; FOLFIRI: 701 %, n = 1, not included in analysis; P = 0.99). Likewise, cancer stage did not impact the peak vasodilatory response to ACh (Stage II: 693 ± 585 %, n = 3; Stage III: 945 ± 498 %, n = 6; Stage IV: 805 ± 405 %, n = 8; P = 0.32). Taken together, these findings suggest that 5-FU treatment may not hinder all endothelium-dependent vasodilatory pathways.

3.4. SNP iontophoresis

The cumulative median dose of 5-FU received by patients in the SNP cohort was 21,000 mg/m^2 (25th-75th percentile: 15,100 – 27,100) (Table 1). Baseline CVC before SNP delivery was not different between CON (n = 9) and 5-FU (n = 6) groups (CON: 7.0 ± 1.6 PU mmHg^{-1}; 5-FU: 6.0 ± 2.6 PU mmHg^{-1}; P = 0.38) nor was the response to SNP

iontophoresis (CON: 188 ± 269 %; 5-FU: 245 ± 234 %; P = 0.39) suggesting no difference between the groups in the ability of the smooth muscle to respond to an NO donor.

3.5. In vitro experiments

There was a significant main effect for group on the eNOS gene expression of HCAEC (P = 0.002, ES = 0.72). Multiple comparisons revealed HCAEC incubated with both 0.7 mM (0.711 ± 0.12 a.u.; P = 0.008, ES = 0.52) and 7.0 mM (0.578 ± 0.13 a.u; P = 0.002, ES = 0.69) of 5-FU for 24-hours presented with significant decrements in eNOS gene expression when compared to HCAEC incubated in standard growth medium (1.11 ± 0.06 a.u.) alone. However, there were no differences between the high and low 5-FU concentrations (P = 0.34) (Fig. 4).
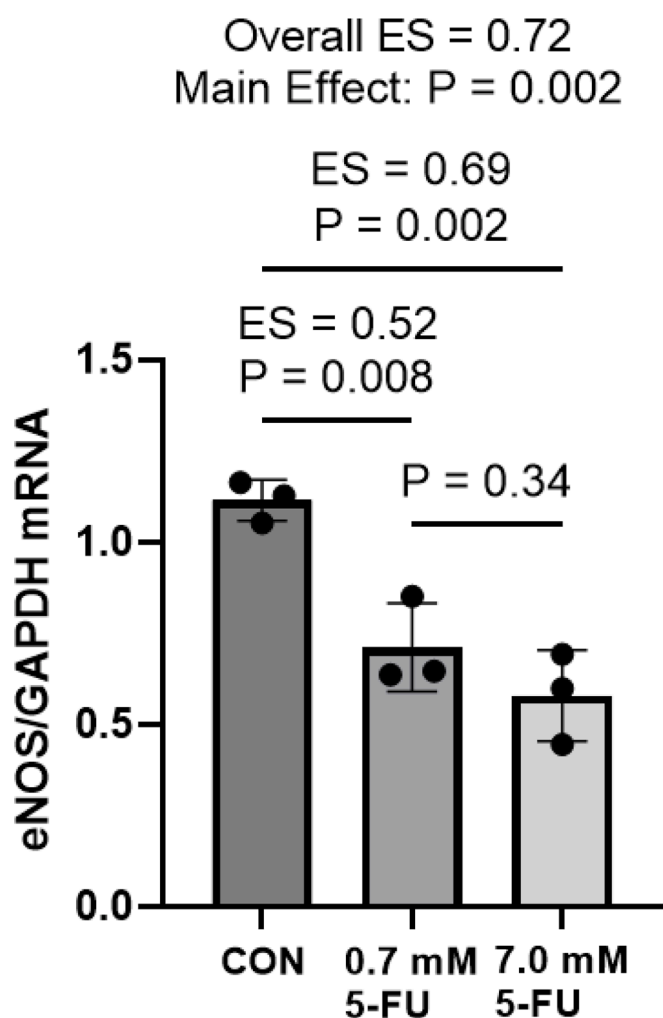

Fig. 4. eNOS gene expression is reduced in 5-FU treated HCAEC. There was a significant main effect for differences in eNOS gene expression between the groups following the 24-hr. incubation period (P = 0.002, ES = 0.72). CON cells (1.114 ± 0.06 a.u) had significantly higher levels of eNOS gene expression compared to cells treated with both 0.7 mM (0.711 ± 0.12 a.u; P = 0.008, ES = 0.52) and 7.0 mM (0.578 ± 0.13 a.u; P = 0.002, ES = 0.69) of 5-FU, while no differences were detected between the two 5-FU concentrations (P = 0.34). n = 3 cultures per group. All samples were run in duplicate. Data were analyzed using One-way ANOVA and are presented as mean ± SD. a.u. = arbitrary units. eNOS = endothelial nitric oxide synthase.

4. Discussion

4.1. Study findings

Our primary finding was that patients undergoing treatment for cancer with 5-FU-based chemotherapy regimens have significant impairments in cutaneous microvascular reactivity in response to local skin heating compared to matched controls—a process primarily facilitated by eNOS-derived NO production [24,30]. The inability of L-NAME to reduce the local heating-induced rise in %ΔCVC in 5-FU treated patients, along with the lack of %ΔCVC differences between groups following iontophoresis of SNP, support these findings and suggest potential perturbations occur along the eNOS-NO vasodilator pathway in patients treated with 5-FU based regimens. Our follow-up experiments in HCAEC culture substantiate this notion, as decrements in eNOS gene expression were evident in HCAEC treated with high and low concentrations of 5-FU compared to control. Interestingly, the vasodilatory response to iontophoresis of ACh was unaltered following 5-FU treatment. This finding implies that endothelium-dependent vasodilator pathways relying on contributions from vasodilatory substances other than NO alone [38–40] may remain intact and confirms the ability of the smooth muscle to respond to vasodilatory stimuli. Taken together, these findings offer evidence for 5-FU-induced microvascular impairment, presumably through pathways involving eNOS-derived NO, and thus may provide valuable insight into the mechanistic pathophysiology of 5-FU-related cardiotoxicity.

Dysfunction of the vascular endothelium is a well-established precursor to a vast array of cardiovascular diseases and events and is generally attributed, at least in part, to decrements in NO bioavailability (for review, see [51]). Specifically, impairments in endothelium-dependent vasodilatory function within the coronary circulation are associated with angina [52], coronary vasospasm [53], and myocardial ischemia [54]—the main cardiotoxicities often affiliated with 5-FU treatment. Alterations to both epicardial and microcirculatory vessels are believed to contribute to these maladies, with microcirculatory dysfunction considered one of the earliest signs of impending cardiovascular disarray [55]. Importantly, endothelial inadequacies in these populations are not exclusive to the coronary vasculature alone and are also apparent throughout the systemic circulation [56]. Pertinent to the present investigation, the cutaneous circulation has regularly been used to assess potential mechanistic vascular alterations in disease states such as hypertension [36], type II diabetes [57], hypercholesteremia [33], kidney disease [35], psoriasis [58], postural tachycardia syndrome [29], coronary artery disease [59], heart failure [60], and cancer [19,20,61]. Given the invasiveness in investigating the coronary circulation, this latter point is critical as evaluation of cutaneous vascular beds may deliver a first glimpse into global endothelial microcirculatory health of at-risk populations and *in vivo* justification for the more invasive and conclusive study of the coronary vasculature. It is important to note, however, that while some have demonstrated statistically significant correlations between vasodilatory responses in the cutaneous and coronary circulations [62], these correlations appear relatively modest and, to our knowledge, have only been studied in a small group of healthy participants. Thus, future work that aims to further characterize this association would be beneficial in understanding how alterations in cutaneous microvascular function reflect those occurring within the coronary vasculature.

To the best of our knowledge, the present investigation is the first attempt at deciphering how 5-FU-based chemotherapy regimens may impact microcirculatory function in patients undergoing cancer treatment. Südhoff and colleagues [18] report the occurrence of brachial artery vasoconstriction—rather than the typical vasodilation—in 50 % of patients receiving 5-FU in response to a standard flow-mediated dilation (FMD) protocol [18]. Given that endothelium-derived NO mediates up to 67 % of the vasodilatory response to FMD [63], these data imply the presence of NO-dependent alterations in conduit vessel

function immediately following 5-FU treatment. The findings herein support this notion, albeit in the microcirculation, with the vasodilatory response to local heating in 5-FU patients significantly reduced to that in matched controls. Further, given that the change in %ΔCVC following NOS inhibition with L-NAME was insignificant in 5-FU treated patients, coupled with the lack of differences demonstrated between groups in response to iontophoresis of SNP, we can postulate that NO-mediated dilation may indeed be diminished. As such, our findings suggest that 5-FU-based regimens may result in either lesser production of eNOS-derived NO or increased scavenging of NO by reactive oxygen species (ROS) [64]. Additional insight is provided by our observed decrements in eNOS gene expression in HCAEC treated with 5-FU. This finding is consistent with those of other preclinical models which have demonstrated alterations in eNOS protein content and/or phosphorylation status following 5-FU treatment [65,66]. While altered gene expression in cell culture models may not always reflect changes seen *in vivo*, we feel that our similar finding of alterations in eNOS and/or eNOS mediated pathways in both of our experimental models offer compelling support for future study of the impact of 5-FU based regimens on NO-mediated vasodilatory pathways in the coronary circulation.

Though microcirculatory responses to local heating were altered following 5-FU treatment, to our surprise, ACh-induced vasodilatory pathways were seemingly unaffected. Prior work by our group has shown apparent attenuation in ACh-induced cutaneous microvascular reactivity in cancer populations treated with heterogeneous chemotherapy regimens compared to controls [19] and between radiated and contralateral non-radiated sites in patients with breast cancer [20]. Similarly, Mourad and colleagues note a reduced cutaneous red blood cell flux (not corrected for arterial pressure) in response to iontophoresis of the ACh analog pilocarpine in metastatic colon cancer patients following 6-months of bevacizumab treatment [61]. Interestingly, these patients were also treated with the 5-FU containing regimens FOLFIRI and XELOX (5-FU prodrug capecitabine and oxaliplatin). However, the authors do not mention how the 6-months of bevacizumab treatment overlapped with the 5-FU regimens, the dose of 5-FU administered, or the time between 5-FU administration and experimental testing. Therefore, differences in cutaneous microvascular reactivity between the present work and those stated above may result from methodological differences including cancer type and severity, the cancer-treatment regimen, and the presence of underlying comorbidities. Despite these differences, the fact that ACh-induced vasodilation was maintained in 5-FU treated patients lends further support to the ability of the smooth muscle to respond to vasodilatory stimuli suggesting that the observed local heating alterations occur upstream of the smooth muscle layer.

This now begs the question: With local heating and ACh iontophoresis protocols both acting through endothelium-dependent vasodilatory pathways, how could one present with differences while the other remains similar between groups? There are several possible explanations. First, local heating and ACh-induced cutaneous vasodilation are facilitated by different endothelium-dependent pathways. The plateau phase of the local heating protocol is mediated primarily (~70 %) via endothelium-derived NO [24]. In contrast, cutaneous vasodilatory responses to exogenous ACh occur due to the combined actions of NO, prostanoid, and non-prostanoid/non-NO dependent pathways [38,40,42]. Moreover, multiple reports demonstrate minimal contributions from NO in response to exogenous ACh in the cutaneous microcirculation [39,41]. Thus, whereas reductions in NO may drastically impact the vasodilatory response to local heating, this reduction may not be equally reciprocated in response to ACh iontophoresis. Second, other chemotherapy drugs regularly included in 5-FU-based regimens have been demonstrated to alter the function of sensory neurons—which are key contributors to the initial peak in cutaneous RBC flux seen during local heating. Specifically, oxaliplatin is used alongside 5-FU in both FOLFOX and FOLFIRINOX regimens and is one of the most frequently reported causes of peripheral neuropathy in treated patients. While an alteration to the sensation of heat secondary to oxaliplatin is possible, it appears

unlikely as oxaliplatin seemingly influences cold but not heat sensitivity [67,68]. Further, inhibition of sensory neurons with EMLA cream has been demonstrated to diminish the initial axon reflex to local heat without altering the NO mediated plateau [25,69] suggesting mechanisms beyond those mediated by sensory neurons are responsible for the group differences observed in the present study.

The present data support the involvement of the eNOS-NO signaling in the pathophysiology of 5-FU-induced vascular dysfunction. Given the *in vitro* and *in vivo* evidence of impaired eNOS-NO vasodilatory pathways presented herein, coupled with preclinical works suggesting eNOS alterations demonstrated elsewhere [65,66], it seems worthy of consideration that reductions in NO bioavailability could facilitate a pathological phenotype within the vasculature of patients treated with 5-FU based regimens. Furthermore, reductions in NO bioavailability give rise to superoxide and endothelium-derived ET-1 [70], the latter of which has been measured at high levels within the blood of 5-FU treated patients [71,72] as well as within the aorta of 5-FU treated rats [65]. Thus, despite the *ex vivo* data of Mosserri et al. [44] suggesting no difference in the vasoconstrictor response between endothelium-denuded and intact aortas, these *in vivo* findings are provocative in that decrements in eNOS gene expression as well as NO-mediated vasodilation are known precursors to many of the symptoms experienced by 5-FU treated patients. It is worth noting, however, that although our *in vitro* findings suggest the *in vivo* alterations in NO dependent cutaneous microvascular reactivity may occur at the level of the endothelium, we cannot entirely discount the possibility of perturbations in the eNOS-NO vasodilator pathway happening downstream of the NO produced by eNOS, as assessment of the production of ROS were beyond the scope of the present investigation. Further, the concentrations of 5-FU used in our cell culture studies are higher than those seen in clinical practice. As such, it is possible that physiological concentrations of 5-FU may act on eNOS through post translational modification rather than via reductions in total eNOS gene or protein expression. Reduced phosphorylation of eNOS Ser-1777—a suggested eNOS activation site—has been demonstrated in rodent cardiac tissue following 5-FU treatment [65]. Likewise, Gajalakshmi and colleagues found reductions in eNOS Ser-1177 along with reduced NO production and alterations in eNOS cellular localization in epithelial cells treated with the 5-FU prodrug capecitabine suggesting that 5-FU may interfere with several steps along the eNOS-NO pathway [73]. To our knowledge, such findings have yet to be recapitulated in endothelial cells. Future research aimed at elucidating how these factors intertwine to influence coronary vascular health in patients undergoing 5-FU treatment could ultimately provide insight into the mechanisms driving both acute and long-term cardiovascular complications associated with this drug.

While this particular study—along with the field of cardio-oncology in general—has largely focused on the cardiovascular consequences of anti-cancer treatment, it is important to consider the potential contribution of the cancer itself to the development of cardiovascular pathology in this population. Our group has previously found reductions in cardiac and left ventricular mass in tumor bearing, treatment naïve rodents compared to non-cancer counterparts [74] while others have recently demonstrated reduced cardiac volumes and increased systolic strain rate in treatment naïve patients with lymphoma or breast cancer [75]. To the best of our knowledge, the effect of cancer itself on microvascular function has yet to be established. In the present study, there was no significant difference in the local heating or ACh-induced vasodilatory responses when 5-FU cohorts were split based on tumor stage, however, it is important to note that this study was not designed to specifically address this question and we were unable to control for other potential confounding variables (e.g., age, sex, etc.) amongst these groups. Given the growing literature indicating the development of cardiovascular abnormalities prior to the receipt of anti-cancer treatment, future studies aimed at elucidating how the cancer and/or its severity impacts vascular function are warranted.

4.2. Study limitations and considerations

This study provides important findings toward a better understanding of the mechanisms driving the onset of 5-FU-induced cardiotoxicity. However, it does not come without experimental considerations. Importantly, these findings should be kept in context of the model in which they were collected, as impairments in the cutaneous microcirculation may not be wholly recapitulated within the coronary microcirculation or other vascular beds. Khan and colleagues [62] show a modest, albeit statistically significant, correlation between coronary velocity reserve and iontophoresis of both ACh and SNP in healthy subjects. Nevertheless, more research is needed to fully elucidate whether functional responses in the cutaneous circulation are reflective of those in the coronary vasculature. Further, iontophoretic currents can induce non-specific vasodilatory responses that may occur in parallel with those elicited by the administered drug. While we cannot wholly negate the potential contributions of the current, we took numerous precautions to ensure the minimal effect, as detailed in our methodology and confirmed via statistical analyses. Our decision to employ iontophoresis rather than other more invasive protocols (e.g., microdialysis) was made to minimize the risk of potential discomfort experienced by patients. However, this decision requires an increased reliance on consistent resting CVC values across groups and measurement sites. Importantly, we found no statistical differences in our resting CVC values. It is important to note that microvascular function can be influenced by several diseases and individual behavioral factors. In this study, we did our best to match known risk factors between 5-FU and control cohorts, however, we were unable to do so perfectly despite the lack of statistical differences between matched groups in all but one of these categories (CVD risk factor category in the SNP studies). As such, we do not feel the small variability in these risk factors (e.g., smoking status, sex) drastically impacts our findings. Our sample population was treated with a variety of chemotherapeutic regimens containing 5-FU, along with several other medications (e.g., angiotensin-converting enzyme inhibitors, calcium channel blockers, diabetic medications, etc.) as is common in clinical practice, that we were unable to control for in the present investigation. These medications were documented by the investigators and their use (minus chemotherapeutics) was of similar proportion between groups (all P > 0.05). When sample size permitted, we compared the local heating and ACh-peak vasodilatory responses between base regimens (i.e., FOLFOX, FOLFIRINOX, FOLFOX + FOLFIRI) and found no differences between the individual regimens on endothelium-dependent vasodilation. In some scenarios, patients were also treated with additional cardiotoxic therapies (e.g., bevacizumab, gemcitabine) and while these proportions are small for each group (Table 1), it is possible that these therapies also contributed to our findings. Unfortunately, given the relatively small sample size used in this study, we were unable to assess the potential effect of these additional therapies or that of the total dose of 5-FU on microvascular endothelial function. Lastly, a small proportion of our participants in the local heating (n = 3) and ACh (n = 6) cohorts had previously been treated for cancer prior to the present diagnoses. In all instances but one (gemcitabine, local heating), these chemotherapy regimens were 5-FU based. Given that repeating the statistical analyses without these individuals in the data set does not alter the outcome of any of our statistical tests, we do not feel inclusion of these participants in the final data set impacts the interpretation of our findings.

5. Conclusion

The present study demonstrates significantly lower cutaneous microvascular reactivity in patients with cancer undergoing treatment with 5-FU chemotherapy compared to matched healthy controls. Of note, these findings suggest alterations along vasodilatory pathways reliant on endothelium-dependent eNOS-derived NO production. This conclusion is supported by our *in vitro* findings of decreased eNOS gene expression in 5-FU-treated HCAEC. This study offers mechanistic insight into the vascular toxicities associated with 5-FU chemotherapy and provides the experimental basis for future investigation into potential alterations along the eNOS-NO vasodilatory pathway within the coronary circulation and its relation to acute and long-term clinical outcomes following 5-FU chemotherapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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