

Vitamin C administration by intravenous infusion increases tumour ascorbate content in patients with colon cancer: a clinical intervention study

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Research

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Abstract

Background: The use of high dose ascorbate infusions in cancer patients is widespread, but without evidence of efficacy. Several mechanisms whereby ascorbate could affect tumour progression have been proposed, including (i) the localised generation of cytotoxic quantities of H₂O₂, (ii) ascorbate-dependent activation of the 2-oxoglutarate-dependent dioxygenases that control the hypoxia-inducible factors (HIFs) and that are responsible for the demethylation of DNA and histones and (iii) increased oxidative stress induced by dehydroascorbic acid. We hypothesise that the dysfunctional vasculature of solid tumours results in compromised delivery of ascorbate to poorly perfused regions of the tumour and that this ascorbate deficit acts as an additional driver of the hypoxic response via upregulation of HIFs. Using a randomised 'therapeutic window of opportunity' clinical study we aimed to determine whether ascorbate infusions affected tumour ascorbate content and tumour biology.

Methods: Patients with colon cancer were randomised to receive infusions of up to 1 g/kg ascorbate for four days before surgical resection (n=9) or to not receive infusions (n=6). Ascorbate was measured in plasma, erythrocytes, tumour and histologically normal mucosa at diagnostic colonoscopy and at surgery. Protein markers of tumour hypoxia or DNA damage were monitored in resected tissue.

Results: Plasma ascorbate reached millimolar levels following infusion and returned to micromolar levels over 24 h. Pre-infusion plasma ascorbate increased from 38 ± 10 mM to 241 ± 33 mM ($p < 0.0001$) over four days and erythrocyte ascorbate from 14 ± 15 mM to 2004 ± 814 mM ($p < 0.005$). Tumour ascorbate increased from 15 ± 6 to 28 ± 6 mg/100g tissue ($p < 0.0001$) and normal tissue from 14 ± 6 to 21 ± 4 mg/100g ($p < 0.001$). A gradient of lower ascorbate was evident towards the tumour centre in both control and infusion samples. Lower expression of hypoxia-associated proteins was seen in post-infusion tumours compared with controls. There were no significant adverse events and quality of life was unaffected by ascorbate infusion.

Conclusions: This is the first clinical study to demonstrate that tumour ascorbate levels increase following infusion, even in regions of poor diffusion, and that this could modify tumour biology.

Trial registration: ANZCTR Trail ID ACTRN12615001277538

Background

The use of high dose vitamin C (ascorbate) infusions as a complementary therapy for cancer is widespread (1), despite a lack of robust evidence for efficacy. Ascorbate is administered to cancer patients in amounts far in excess of the recommended daily intake, in the absence of any guidelines on recommended dosages. This practice was initiated when early clinical experiments in late stage cancer patients reported improved patient survival following daily intravenous infusion of around 10 g of ascorbate, compared to historical controls (2). However, randomized, placebo-controlled trials (RCT) from the Mayo Clinic failed to show significant benefit with the same amount of ascorbate given orally (3).

Differences between the studies with respect to study design and methods of ascorbate administration are now thought to be significant in explaining this discrepancy.

Pharmacokinetic and modelling data have demonstrated that tight regulation of ascorbate uptake and excretion means that plasma concentrations do not readily exceed 100 μM following oral intake (4, 5). In contrast, intravenous ascorbate administration bypasses uptake regulation in the gastrointestinal tract and results in dose-dependent increases in plasma levels, even in excess of 20 mM (4, 6–8). These levels are not maintained following infusion and ascorbate is cleared with a half-life of < 2 h (4, 6–8). That this difference in pharmacokinetics may be critical for a potential anti-cancer effect remains much discussed, particularly as case studies suggesting a clinical benefit from ascorbate infusions continue to surface (9–17).

Current research is focussed on the identification and verification of several proposed mechanisms whereby ascorbate could affect tumour progression. These include: (i) the localised generation of cytotoxic quantities of H_2O_2 as a consequence of ascorbate oxidation (15, 18, 19); (ii) ascorbate-dependent activation of the 2-oxoglutarate-dependent dioxygenases that down-regulate the hypoxia-inducible factors (HIFs) (20–22) and that are responsible for the demethylation of DNA and histones (11, 17, 23–25) and (iii) increased oxidative stress induced by dehydroascorbic acid taken up into tumour cells via the glucose transporters (26). These mechanisms all require effective delivery of ascorbate to the tumour.

We have previously measured the diffusion of ascorbate through cell layers *in vitro* and have modelled its transport through tumour tissue (27). Poor vascularisation of tumour tissue not only affects oxygen availability that drives up-regulation of the HIFs, but will also limit the distribution of ascorbate to poorly-perfused regions of the tumour (27). Pharmacokinetic data for plasma ascorbate concentrations from patients treated with high dose infusion are available (4, 6–8) but whether levels of ascorbate in tumour tissue are increased following such treatment in patients has never been reported. We have shown increase tumour ascorbate in a pre-clinical model, with associated decreased expression of HIF-dependent protein markers (28).

We hypothesise that the dysfunctional vasculature of solid tumours results in compromised delivery of ascorbate to poorly vascularised regions of the tumour and that the ascorbate deficit acts as an additional driver of the hypoxic response via HIF upregulation. In this study we aimed to determine whether supra-physiological plasma concentrations can overcome poor perfusion in solid tumours and whether increasing tumour ascorbate could affect the prevalence of hypoxia markers. Patients with colon cancer were treated with four daily ascorbate infusions immediately prior to surgical resection of their cancer. We measured ascorbate levels in plasma, red blood cells and tumour tissues and the expression of HIF-dependent proteins. As ascorbate can improve surgical wound healing (29) and quality of life for cancer patients (30), we also monitored patient quality of life and recovery following surgery.

Materials And Methods

Materials

Unless stated otherwise, all chemicals were from Sigma-Aldrich (St Louis, MO, USA).

Ethics and patient consent: Ethical approval was obtained from the New Zealand Health and Disability Ethics Committees (15/STH/145). This trial was registered on the Australian New Zealand Clinical Trials Registry (ANZCTR Trail ID ACTRN12615001277538, Universal trial no. U1111-1173-0882). Patients were recruited at Christchurch Hospital, the main tertiary hospital on the South Island of New Zealand. Patients gave informed consent separately to two parts of the study: 1) prior to diagnostic colonoscopy for additional biopsies for tumour and normal tissue; 2) after cancer diagnosis was confirmed, for randomisation to either the control arm (no infusions) or intervention arm (four consecutive days of high dose vitamin C infusions prior to surgery) of the trial. Randomisation was carried out by the study co-ordinator and the recruiting clinician and patient were blinded to this process. Patients declared ethnicity using the New Zealand census question, and an option of sample disposal with karakia (blessing) was offered.

Selection criteria: Participant recruitment and selection is shown in **Supplementary Fig. 1**. Criteria for inclusion in part 1 (additional biopsies at colonoscopy) were strong clinical suspicion of colorectal cancer and planned confirmatory biopsy. Patients were selected by the attending clinician according to clinical criteria (including anemia, blood in stool or change in bowel habit). If a tumour was found on colonoscopy, the patient was considered for part 2 of the study. Inclusion criteria were (all required): histologically confirmed colon cancer; good functional status (ECOG grade 0 or 1); age ≥ 18 years; adequate bone marrow, hepatic, renal and cardiac functions. Exclusion criteria were (one required): likely to receive neoadjuvant therapy (e.g. for rectal cancer); serious gastrointestinal disorders including active bleeding; inability to give informed consent; diabetes (high dose ascorbate can have a hypoglycaemic effect and interfere with some glucose monitoring tests); regular use of vitamin C supplements containing ≥ 1 g/day; serum creatinine > 175 $\mu\text{mol/L}$; known erythrocyte glucose-6-phosphate dehydrogenase deficiency (1); serious or uncontrolled infection; cardiac or neurological conditions; pregnant or lactating women; current calcium oxalate nephropathies with the potential to impair urinary flow (31). An adverse response to the test dose of 25 g IV ascorbate given on the first intervention day would also exclude patients from continuing on the study. Adverse events were monitored by the research nurses and clinicians, and recorded using the Common Terminology Criteria for Adverse Events terms and grading (CTCAE, version 4.0).

Trial design: The study comprised a therapeutic window of opportunity-style study design with an intervention administered between diagnostic colonoscopy and planned surgical resection. Biopsy samples of both normal mucosa and suspected tumour were taken at colonoscopy, and approximate location recorded. A fasting blood sample was also taken. Following colonoscopy, patients who met all inclusion criteria were randomised at a ratio of 3:2 into the infusion or control arm of the study. Patients in the infusion arm received a first dose of 25 g of ascorbate four days prior to their planned surgical resection, and then daily ascorbate infusions of up to 1 g/kg body weight (capped at a total daily dose of

75 g) for three days (days 2–4). Patients in the control arm did not receive ascorbate infusions, but consented to blood and tissue samples to be taken at resection. Surgery was carried out on day five. Blood samples were taken daily prior to infusion, and also immediately after infusion in three patients. Patients attended Christchurch Hospital for their planned surgery where the resected specimen was immediately transferred to Anatomical Pathology, Canterbury District Health Board, for pathological review and tissue banking.

Ascorbate infusion

Ascor L 500® (McGuff Pharmaceuticals, Inc., Santa Ana, CA), equivalent to 500 mg/mL ascorbic acid, was diluted in 250 ml sterile water for injection (25 g ascorbic acid on day 1) or in 500 ml water (up to 75 g ascorbic acid for days 2–4) immediately prior to use, and infused into the median cubital vein at 0.5–1 g/min. Infusions and monitoring were carried out by experienced research nurses with supervision by an oncologist at Christchurch Clinical Studies Trust. During infusion, the diluted ascorbate was kept at room temperature protected from light. Infusions took between one and two hours on average. Patient vital signs were recorded before, after 10 min and immediately after infusion.

Quality of life

Patients completed a validated quality of life and symptom analysis questionnaire (EORTC QLQ-C30) (32) and fatigue questionnaire (MFSI-SF) (33), prior to the first infusion and on the day of the last infusion (the day before surgery).

Patient follow-up

Patients were followed up for 30 days following surgery, with complications recorded using the Clavien-Dindo (CD) score. Length of hospital stay and readmissions were retrieved from their medical records. Recurrences, metastases and death were recorded up until 561–745 days post-surgery.

Blood samples

Peripheral blood (5 ml) was collected into K3-EDTA vacutainer tubes, immediately placed on ice and processed within 2 h. Plasma was removed following centrifugation at 1000 g for 10 min at 4°C and an aliquot mixed with an equal volume of ice-cold 0.54 M perchloric acid containing 50 µM DTPA. Precipitated protein was removed by centrifugation and supernatants were stored at –80°C. The erythrocytes were prepared for ascorbate analysis, with lysis in a five-fold volume of ice-cold milliQ water containing 500 µM DTPA and removal of haemoglobin by ultrafiltration as previously described (34).

Tissue samples

During diagnostic colonoscopy, tumour and histologically normal biopsy tissue samples were taken. Normal, uninvolved colon mucosa was sampled at a distance ≥ 10 cm from the tumour. Biopsy material was immediately put on ice and snap frozen in liquid nitrogen within 20 minutes. At cancer resection,

surgical specimens were examined by an expert pathologist, and surplus viable tumour and uninvolved normal tissue at a distance of ≥ 10 cm from the tumour were sampled. Multiple regions of the tumour (edge/periphery, mid and central) were sampled to assess tumour heterogeneity (schematic shown in **Supplementary Fig. 2**). Tissue was processed and snap frozen in liquid nitrogen within 45 min of surgical removal and stored at -80°C .

Tissue processing for ascorbate measurement

Frozen normal bowel and tumour biopsy tissue samples were weighed and homogenised in ice-cold 10 mM potassium phosphate buffer (pH 7.4) in 1.5 ml Eppendorf tubes using a plastic micro tissue homogeniser (Thermo Fisher, Auckland, New Zealand) on ice. Extraction procedures of these samples (10–20 mg) were optimised using mouse breast cancer tissue (University of Otago Animal Ethics Committee approval C01/16). Resection tissue samples (> 30 mg) were ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle on dry ice as previously described (35). The resulting powder was weighed and homogenized with 10 mM potassium phosphate buffer (pH 7.4) for ascorbate extraction. An equal volume of 0.54 M perchloric acid containing 50 μM DTPA was added to the extracts and samples stored at -80°C until analysis.

Ascorbate analysis

Ascorbate content of tissue lysates, plasma and erythrocyte samples was measured by high-performance liquid chromatography (HPLC) with electrochemical detection (Thermo Fisher Scientific, Waltham, MA, USA) and quantified against a standard curve (1.25 μM to 40 μM , prepared daily) (34). Samples were analysed with and without reduction with 10 mg/ml tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for 3 h at 4°C , which allowed quantification of both ascorbate and dehydroascorbic acid (34).

Tissue processing for protein analyses

Frozen powder from resected tissue was homogenized with 10 mM potassium phosphate buffer for vascular endothelial growth factor (VEGF) ELISA, or with RIPA buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Complete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland)] for immunoblot analysis.

Western blotting

SDS-PAGE was carried out on tissue homogenates with each sample standardized to 1.5 μg DNA per well. Proteins were separated on 4–12% Bis-Tris Plus SDS gels and transferred to 0.45 μm polyvinylidene difluoride membranes (Life Technologies, Carlsbad, USA). Membranes were incubated overnight at 4°C with primary antibodies against carbonic anhydrase IX (CA-IX) (1/200, R&D Systems, AF2188), glucose transporter 1 (GLUT1) (1/1000; Abcam, ab32551), phosphorylated histone protein γH2AX (1/2000; Abcam, ab11174) or β -actin (1/10,000, Sigma, A5316), and for 1 h at room temperature with secondary horseradish peroxidase-conjugated antibodies (1/5000, DAKO). β -Actin was used as loading control. For

GLUT1, the same positive control (20 µg protein of 1% O₂ treated T24 cell lysate) was run on each gel to normalize signals between the blots. For CA-IX, a clear cell renal cell carcinoma tissue sample (University of Otago Human Ethics committee H14/020, (36), adjusted to 1.5 µg DNA per well, was similarly run on each gel. For γH2AX, a positive control (WiDr cells from ATCC treated for 4 h with 20 mM ascorbate, 20 µg protein) was run on each gel. Protein bands were visualized using the ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, USA), and quantified with the Alliance 4.7 imaging system and the ImageJ software. (35, 36)

VEGF ELISA

VEGF protein levels were measured in tissue lysates using the human VEGF DuoSET ELISA Development Kit (R&D Systems, DY293B) according to the manufacturer's instructions.

Statistics

Data were analysed with GraphPad Prism version 8.4.2 with significance set at $p < 0.05$. Ascorbate levels in normal tissue before and after infusion were compared using paired t-tests. Unpaired t-tests and mixed effects models with ANOVA were used to analyse changes in erythrocyte levels and differences in tumour ascorbate and hypoxia marker expression. Pearson's correlations were used to determine relationships between normal and tumour tissue ascorbate levels and least squares fit was used to determine associations between plasma and tissue ascorbate levels.

Results

Patient accrual and characteristics

Patients who presented at Christchurch Hospital for a colonoscopy due to suspicion of colon cancer were approached for consent (Supplementary Fig. 1). Of 53 patients consented for additional biopsies, 15 met all inclusion criteria and consented to the second part of the study. Of the remainder, 30 did not have cancer, four had mid-low rectal cancer and likely to receive neoadjuvant therapy, three consented to biopsy only and one withdrew prior to biopsy. Of the 15 enrolled study participants, nine patients were randomised to the vitamin C infusion arm and six to the control arm. The control cohort were all men, whereas the infusion cohort contained three women and six men. The average age was similar in both cohorts and ethnicity was predominantly European.

Tumour characteristics

Clinicopathological data for the study participants is shown in Table 1. Tumour stages I-III/IV were represented in both cohorts, with variable presentation of positive nodes, vascular/perineural invasion, and the presence of infiltrating lymphocytes (Table 1).

Table 1
Patient and clinicopathological data.

Parameter		Control	Infusion
		N = 6	N = 9
Gender	Female, male	0, 6	3, 6
Age	Mean ± SD (years)	74 ± 6	72 ± 8
Pathology data:			
AJCC stage	I and II	4	4
	III and IV	2	5
Grade	Well differentiated	3	0
	Moderate diff/low grade	3	9
	Poor diff/high grade	0	0
Tumour size	Mean ± SD (mm)	46 ± 28	37 ± 11
Lymph node			
	Positive	0	4
Vascular/perineural invasion	Positive	1	6
Infiltrating lymphocytes	Positive	1	5
Follow up observations:			
Average length of hospital-stay	Mean ± SD (days)	9.3 ± 5.4	5.8 ± 2.4
Readmission within 30 days		2	1
Surgical complications	No. of patients	3	3
Clavien-Dindo	Grade I (episodes, patients)	2,2	1,1
	Grade II (episodes, patients)	7,3	2,2
	Grade III (episodes, patients)	0,0	2,1
	Grade IV/V (episodes, patients)	0,0	0,0
Metastasis		1	1
Death within 2 years		2	1

Plasma ascorbate levels and effects of infusion

Prior to ascorbate infusion, fasting plasma ascorbate concentrations were similar for both control and infusion groups. Levels ranged from 3.0–64.8 μM ascorbate, with means \pm SD of $32.5 \pm 18.2 \mu\text{M}$ for control and $37.4 \pm 21.5 \mu\text{M}$ for infusion patients (Fig. 1A). At diagnostic biopsy baseline, two patients were defined as ascorbate deficient and at risk of scurvy ($< 11 \mu\text{M}$), two were marginally deficient ($11\text{--}23 \mu\text{M}$), seven inadequate ($23\text{--}50 \mu\text{M}$) and four adequate ($> 50 \mu\text{M}$), with cut-offs as previously defined (37). Upon repeated measurement prior to surgery, or before first infusion, an intervening period of 31 days (median, range 5–90 days), average plasma levels remained low, with 7/12 patients now classified as ascorbate deficient or marginally deficient, compared with 4/12 at diagnostic presentation (Fig. 1A).

Plasma ascorbate monitoring in three patients immediately after infusion indicated that levels reached $\sim 10 \text{ mM}$ following the first infusion of 25 g ascorbate and $\geq 20 \text{ mM}$ following subsequent infusions at 1 g/kg or 75 g maximum (Fig. 1B). At 24 h post-infusion, most of the ascorbate had been cleared and plasma levels had returned to the micromolar range as expected. However, baseline plasma levels increased steadily over the four infusion days and were above the $100 \mu\text{M}$ normal saturating level in the days following infusions (Fig. 1C). These daily levels increased significantly from $38 \pm 28 \mu\text{M}$ on day one, to $139 \pm 65 \mu\text{M}$ on day two, to $207 \pm 65 \mu\text{M}$ on day three, to $241 \pm 88 \mu\text{M}$ on day four prior to the final infusion ($p < 0.001$) (Fig. 1C).

Erythrocyte ascorbate levels and effects of infusion

Mature red blood cells do not express the specific vitamin C transporters that allow active ascorbate accumulation against a concentration gradient (38). Consequently, ascorbate levels in red blood cells reflect passive accumulation or uptake of dehydroascorbate via the glucose transporters (39), and are generally similar to plasma concentrations (34). Ascorbate concentrations in erythrocytes at biopsy were $14 \pm 15 \mu\text{M}$, compared to $38 \pm 28 \mu\text{M}$ in matching plasma. Erythrocyte ascorbate increased significantly immediately post-infusion (Fig. 1D) and levels continued to increase over the four-day infusion period ($p < 0.005$). In contrast to results with plasma, erythrocyte levels were sustained at post-infusion peaks for 24 h, with the ascorbate concentration measured pre-infusion equivalent to post-infusion on the previous day (Fig. 1D). Therefore, the erythrocyte ascorbate concentration accumulated to levels significantly higher than in plasma, with average concentrations of 2 mM on days three and four compared to $\sim 0.2 \text{ mM}$ in plasma (Fig. 1E).

Tissue ascorbate levels

Tumour biopsy samples taken during colonoscopy showed similar levels of ascorbate for both normal mucosa and tumour tissues in the control and infusion cohorts (Fig. 2A,C, and Table 2). At resection, ascorbate levels in normal mucosa or in tumour tissue were unchanged in the control cohort (Fig. 2A,B and Table 2). In contrast, with ascorbate infusion over four days, both tumour and normal tissue ascorbate increased significantly (Fig. 2C,D). Ascorbate in tissue from the tumour periphery appeared higher than tissue from mid or central regions in both control and infusion samples (Fig. 2B,D; **Supplementary Fig. 2A,B**). Tumour ascorbate increased significantly post-infusion and was higher than in

tumours from the control cohort, regardless of whether the samples were derived from peripheral, middle or central locations within the tumour (Fig. 2B,D and Table 2).

Table 2

Ascorbate levels in normal mucosal bowel tissue and tumours pre- and post-ascorbate infusions.

Ascorbate content (mg/100 g tissue)			
Sample	Control Cohort	Infusion cohort	Significance
Normal mucosa:	15.4 ± 4.1 (6)	14.1 ± 5.9 (9)	0.662
Biopsy	12.1 ± 4.0 (6)	20.9 ± 3.6 (9)	0.0006 ***
Resection			
Tumour:	14.5 ± 3.6 (6)	14.8 ± 6.4 (9)	0.925
Biopsy	14.9 ± 5.9 (5)	28.1 ± 6.1 (9)	0.0014 **
Resection	13.9 ± 8.5 (6)	22.8 ± 4.4 (7)	0.033 *
Periphery	9.7 ± 3.7 (4)	23.2 ± 4.1 (4)	0.0026 **
Mid-tumour			
Central			
Sample	Normal mucosa	Tumour tissue	Significance
VEGF (pg/mg tissue)	0.823 ± 0.209 (14)	1.534 ± 0.211 (14)	0.0031**
GLUT1 (Relative protein levels)	1.176 ± 0.376 (15)	1.687 ± 0.385 (15)	0.023*
CA-IX (Relative protein levels)	0 ± 0 (14)	0.073 ± .037 (14)	0.0078**
γH2AX (Relative protein levels)	0.002 ± 0.001 (14)	0.028 ± 0.013 (14)	0.068
Results show means ± SE for samples from (n) individual patients. Significance levels are recorded for paired t-tests between data from the normal mucosa and tumour tissue from each individual patient.			
Results show means ± SD for samples from (n) individual patients. Significant differences are recorded for unpaired t-tests between data from the control and infusion cohorts, with higher levels being recorded in normal mucosa and tumour tissue (periphery, mid and central regions) in the infusion cohort.			

There was a close correlation between ascorbate levels in tumour tissue and adjacent normal tissue in all cases, at baseline and following resection (Pearson $r = 0.821$, $p = 0.0002$ and $r = 0.867$, $p < 0.0001$ for baseline and resection tissue, respectively) (Fig. 3A,C). These data are for measurements from the tumour periphery. Similar correlations were also seen between normal tissue and other parts of the tumour (mid-tumour $R^2 = 0.56$, $p = 0.003$, tumour centre $R^2 = 0.70$, $p = 0.01$).

When data from both patient cohorts is combined, a non-linear saturation curve is evident which is similar for normal and tumour tissue, by plotting plasma levels on the day of biopsy vs biopsy tissue levels in normal mucosa and tumour periphery (Fig. 3B). There was a strong linear relationship between plasma and tissue levels (tumour and normal) at plasma ascorbate concentrations below $40 \mu\text{M}$ (Fig. 3B). Above these levels, the association between plasma and tissue ascorbate followed a non-linear saturation curve (least squares fit, $R^2 = 0.64$ and 0.62 for normal and tumour, respectively (Fig. 3B). At resection, a similar linear relationship between plasma levels, taken on the day prior to surgery, and tissue levels was seen at lower plasma levels (least squares fit, $R^2 = 0.85$ and 0.69 for normal and tumour periphery, respectively, Fig. 3D). However, ascorbate infusions affected tumour and normal tissue differently at higher ascorbate concentrations. Increasing plasma ascorbate in the physiological range (up to $100 \mu\text{M}$), affected both normal mucosa and tumour tissue equally, whereas tumour levels increased more readily than normal mucosal tissue levels when concentrations exceeded $100 \mu\text{M}$ (Fig. 3B,D,E).

Expression of HIF-dependent proteins and markers of oxidative stress following infusion. We monitored the levels of VEGF, GLUT1 and CA-IX as markers of HIF activation in the surgical resection samples, as previously (22, 36) and have compared tumour and adjacent normal mucosal tissue in control and infusion patient samples. Hypoxic marker protein levels were measured by western blotting (GLUT1, CA-IX) (**Supplementary Fig. 3A**) and by ELISA (VEGF). Expression of GLUT1, CA-IX and VEGF was significantly higher in tumour tissue compared with normal mucosal tissue (**Supplementary Table 1**, Fig. 4A,B,C). When comparing post-infusion tumour tissues with control tumours, HIF-associated proteins expression appeared to be lower in tumours following infusion, with a significant difference in GLUT1 levels ($p = 0.002$) and a strong trend in CA-IX ($p = 0.051$) (Fig. 4A,B,C). To determine whether ascorbate impacted HIF transcriptional activity in the tumour tissue, we derived a HIF pathway score for each tumour sample by normalizing the relative expression values for each protein as percent expression of the highest sample and combining the GLUT1, CA-IX and VEGF scores for each sample. There was a trend for a lower HIF pathway score in the post-infusion tumour samples than in the control tumour tissue (Fig. 4D; $p = 0.057$). There also was a significant negative correlation between tumour ascorbate levels and VEGF expression ($r = -0.383$, $p = 0.023$) and the HIF Pathway score ($r = -0.430$, $p = 0.01$) (**Supplementary Fig. 4**).

Levels of the phosphorylated histone DNA repair protein γH2AX were measured as a marker of oxidative DNA damage (40). Colorectal cancer cells (WiDr) treated *in vitro* with 20 mM ascorbate for 2 h or 4 h

showed a robust γ H2AX immunoreactive band (**Supplementary Fig. 3B**), and were used as positive control for the tissue samples. Measured levels of γ H2AX were very low in tumour tissue and generally undetectable in normal mucosal tissue (**Supplementary Table 1, Supplementary Fig. 3C**). There was no difference in levels detected between the post-infusion and control cohorts ($p = 0.697$) (Fig. 4E).

Adverse events and quality of life

Ascorbate infusion was carried out at 0.5 g/min and increased to 1 g/min after 10 min if tolerated, but decreased if any discomfort was noted or any adverse symptoms occurred. Adverse events were Grade 1 according to CTCAE, version 4.0; asymptomatic or mild symptoms, and intervention was not indicated. Three of nine patients reported no discomfort or adverse events. For 5/36 infusions, infusion flow rate was reduced from 1 g/min to 0.75 g/min or 0.5 g/min due to increased blood pressure or tingling in fingers. Elevated blood pressure was noted on 9/36 infusions, which all resolved post-void (**Supplementary Fig. 5**). Three events of tingling fingers (transient) and three of light-headedness (transient) were reported. Otherwise, data on patient vital signs during infusions were unremarkable (**Supplementary Fig. 5**). Quality of life was recorded at colonoscopy and prior to resection, showing universally high functional scores, low symptom scores, and low fatigue with high vigour scores in this cohort. These levels did not change following the course of four high dose ascorbate infusions (**Supplementary Fig. 6**).

Follow-up

While the trial was not powered to determine clinical efficacy, patients were followed up for the first 30 days and then up to two years post-surgery (Table 1). Patients in the control cohort tended to have a longer hospital stay post-surgery (9.3 days vs 5.8 days, $p = 0.105$). Surgical complications were all minor (CD 1–2 complications) except for one patient in the infusion arm that suffered an anastomotic leak and subsequent enterocutaneous fistula (CD Grade III). One patient in each cohort developed metastases to the liver. Two patients in the control group died at nine months and twelve months and one in the infusion cohort at nine months (Table 1).

Discussion

Our window-style clinical intervention study, has provided detailed information on the uptake of ascorbate into tumour tissue following high dose ascorbate infusions. Whether this treatment affects tumour ascorbate content has not previously been reported and this is important when determining the potential for clinical efficacy. While often discussed (9, 41) and raised in clinical studies with cancer patients using high dose infusions (8, 13, 16, 42) there has been no information on tumour ascorbate levels or biology. Our novel data shows that ascorbate infusion over four days significantly increased both normal mucosa and tumour ascorbate levels in patients with colon cancer. This is consistent with predictions from modelling ascorbate diffusion uptake through multicellular tissue layers that suggested that variable plasma ascorbate concentrations would be reflected in tissue levels (27). Indeed we noted a linear relationship between both normal mucosa and tumour tissue ascorbate and plasma levels when

concentrations were below saturation. It appears that tumour tissue accumulated higher ascorbate concentrations than normal mucosa when plasma levels were elevated beyond saturation, as was apparent in the post-infusion samples. This suggests that there may be an advantage in supplying ascorbate at higher concentrations than can be achieved by dietary intervention alone. However, our study does not determine whether the same effect on tumour content could have been achieved with lower infusion doses of ascorbate as predicted by our modelling study (27). This would require further testing.

Plasma ascorbate concentrations were below average for most of our cohort at recruitment, and generally decreased further in the period between diagnosis and surgery. This is not surprising as increased prevalence of ascorbate insufficiency in cancer patients has also been noted by others (43, 44). Peak plasma levels achieved following infusion were similar to those reported by others (6, 7) and clearance of these high millimolar levels occurred over the following 24 h, as expected. It was of interest that the baseline plasma levels increased daily over the four day infusion period and were maintained above 100 μM . This may reflect the accumulation of ascorbate in erythrocytes, where levels were seen to increase to millimolar levels following infusion and to be maintained at these concentrations for the following 24 h. Given that red blood cells are a major compartment of whole blood, this represents a significant ascorbate pool of ascorbate that could be available via leakage from the cells to buffer plasma levels over the infusion period, resulting in continually elevated baseline plasma status. Red blood cell ascorbate levels are known to closely match plasma levels (34, 45). Here we report that erythrocyte ascorbate concentrations remain elevated following infusion, with potential effects on plasma status. This finding deserves consideration when determining an effective dosing regimen for ascorbate by infusion.

Our data suggest that achieving supraphysiological plasma ascorbate concentrations could be advantageous for the delivery of ascorbate to tumour tissue. We have proposed that ascorbate delivery will be compromised in poorly perfused areas (27) and the data from our study showing notably lower ascorbate levels in central tumour than in peripheral regions are consistent with this prediction. Elevation of plasma ascorbate by infusion could overcome the challenge of delivery to poorly vascularised core regions of solid tumours by increasing the efficacy of delivery (46). Our previous modelling data indicated that delivery to all areas of the tumour would occur when plasma ascorbate levels approached 1 mM. These levels are readily achievable via infusion (8, 13, 16, 42). Our data also indicates that the generation of an ascorbate reservoir in erythrocytes could contribute to sustained elevated plasma levels.

The tissue protein expression levels showed a similar inverse relationship between ascorbate and markers of hypoxia as we have previously observed in retrospective analyses of human endometrial, breast, renal and colorectal tumour samples (22, 35, 36, 47). A similar finding was described in thyroid cancer (48). Biopsy material was insufficient for the protein expression analysis, and only comparisons between the control and infusion cohorts were possible, but these suggest that HIF activation may be moderated by elevating tumour ascorbate. We also monitored levels of the phosphorylated histone protein γH2AX , which is elevated in tissues following DNA damage, initiated by oxidative stress such as

radiation treatment (40), and which we and others have observed in cells following *in vitro* exposure to millimolar ascorbate concentrations (49). We could not detect γ H2AX in normal mucosal tissue and only extremely low levels in tumour tissue. That there was no difference between the levels in tumours from the control or infusion cohorts suggests that ascorbate levels following infusion did not reach concentrations sufficient to induce an oxidative stress in these tissues. It has also previously been noted that oxidative damage by ascorbate is limited in a hypoxic environment (50) and this may impact on the likelihood of intra-tumour oxidative stress initiated by ascorbate. These findings require validation in a much larger cohort that was beyond the scope of the current study.

None of the patients in the infusion cohort reported significant adverse events (> Grade 1) associated with the infusion and there was little change in quality of life, supporting prior safety data for this vitamin. Our prior observational studies showed that higher tumour levels of ascorbate were associated with improved disease-free and disease-specific survival in colorectal and breast cancer (22, 35), but this intervention trial was not powered to address treatment efficacy.

In summary, our study has provided a rich dataset and robust measurements acquired for each patient, with the matched plasma, normal and tissue data both before and after an intervention with high dose ascorbate by infusion. This is the first demonstration that tumour ascorbate levels are significantly increased following high dose ascorbate infusion, which fills a fundamental gap in researching the claimed biological effects of ascorbate infusions in patients with (colon) cancer.

Conclusions

Many patients with cancer access high dose vitamin C infusions via complementary medicine providers, but evidence of efficacy is lacking. Our study of patients with colon cancer shows that ascorbate concentrations are lower in the tumour core than in the periphery. Administration of ascorbate by intravenous infusions over four days prior to surgical resection resulted in supra-physiological plasma concentrations and compartmentalisation of high ascorbate concentrations into erythrocytes. Ascorbate levels increased in all tumour regions, suggesting that increased plasma availability ensured effective accumulation throughout the tumour. Markers of tumour hypoxia were lower in the post-infusion tumours compared with the control cohort, which suggests that increasing ascorbate levels could moderate the activation of the hypoxia-inducible factors. Our data provide a rationale for further investigation into the potential advantages for the targeted use of vitamin C infusions, rather than oral supplementation, in patients with solid tumours.

List Of Abbreviations

Hypoxia-inducible factor (HIF)

Randomized controlled trial (RCT)

Common Terminology Criteria for Adverse Events terms and grading (CTCAE)

Clavien-Dindo (CD)

High-performance liquid chromatography (HPLC)

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)

Vascular endothelial growth factor (VEGF)

Carbonic anhydrase IX (CA-IX)

Glucose transporter 1 (GLUT1)

Phosphorylated histone protein (γ H2AX)

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the New Zealand Health and Disability Ethics Committees (15/STH/145), and all participants gave informed consent.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JG consented patients and performed many of the colonoscopies and operations, HRM coordinated sample collection, AC obtained ethical approval, co-ordinated the study and carried out quality of life assessments, CW and SB processed and analysed samples, JP co-ordinated and provided oversight of all ascorbate analyses, and analysed erythrocyte levels, TE and BAR were responsible for patient safety and clinical oversight, GUD and MCMV designed and managed the study, analysed the data and wrote the manuscript. All authors contributed to data analysis and edited and approved the final manuscript.

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Figures

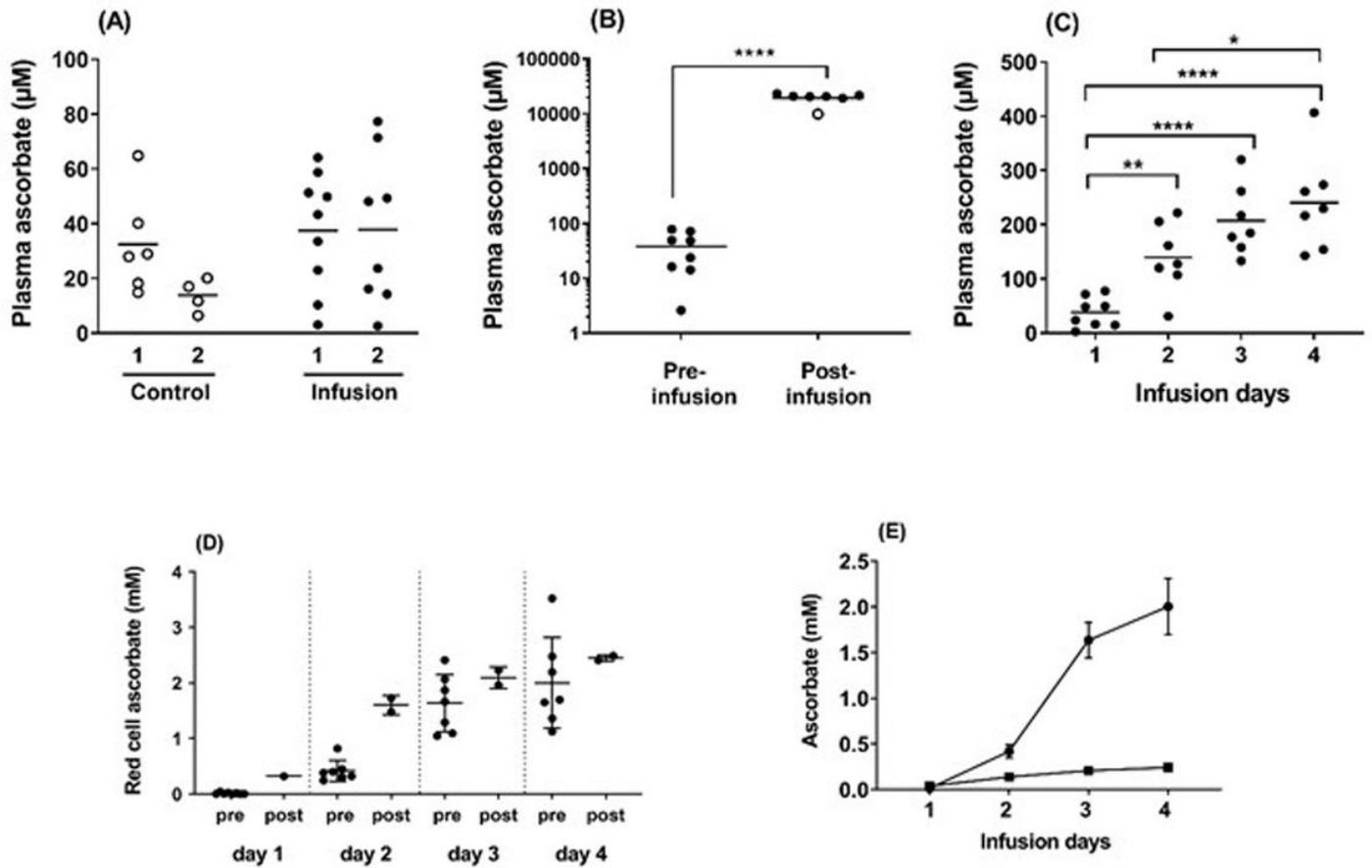


Figure 1

Plasma and red blood cell ascorbate levels in patients at baseline, immediately post-infusion and over the course of high dose ascorbate infusion. IVC was administered daily for four days and blood taken pre- and post-IVC infusion as indicated. The first infusion comprised 25 g ascorbate and subsequent infusions were at 1g/kg body weight, with a maximum of 75 g. (A) Baseline plasma ascorbate from patients in the control (□) and infusion (●) cohorts at colonoscopy (1) and at surgery (2) (control patients) or immediately prior to first infusion (Infusion patients). (B) Plasma ascorbate in samples taken prior to first infusion and immediately after infusion with high dose ascorbate. (□) sample following 25 g infusion. *** $p < 0.0001$ (unpaired t-test) (C) Plasma ascorbate immediately prior to consecutive high dose ascorbate infusions on days 1-4. Significant differences were observed between day 1 plasma ascorbate and the subsequent days ** $p < 0.005$; *** $p < 0.0001$ (unpaired t-tests). There was a significant difference between concentrations at days 1 and 4, * $p < 0.05$ and a one way ANOVA indicated a significant difference between days 1-4, $p < 0.0001$. (D) Red cell ascorbate concentrations pre- and post-infusion; means \pm SD. Unpaired Student's t test showed no significant differences between red cell ascorbate concentrations on day 2 post-infusion and pre-infusion day 3, and post-infusion day 3 and pre-infusion day 4. (E) Plasma (□) and red cell (●) ascorbate concentrations measured prior to infusion each day; means \pm SD. A mixed-effects model indicated a significant difference between days, with day 1 different to day 2, 3 and 4; $P < 0.005$ for red cells and $P < 0.05$ for plasma.

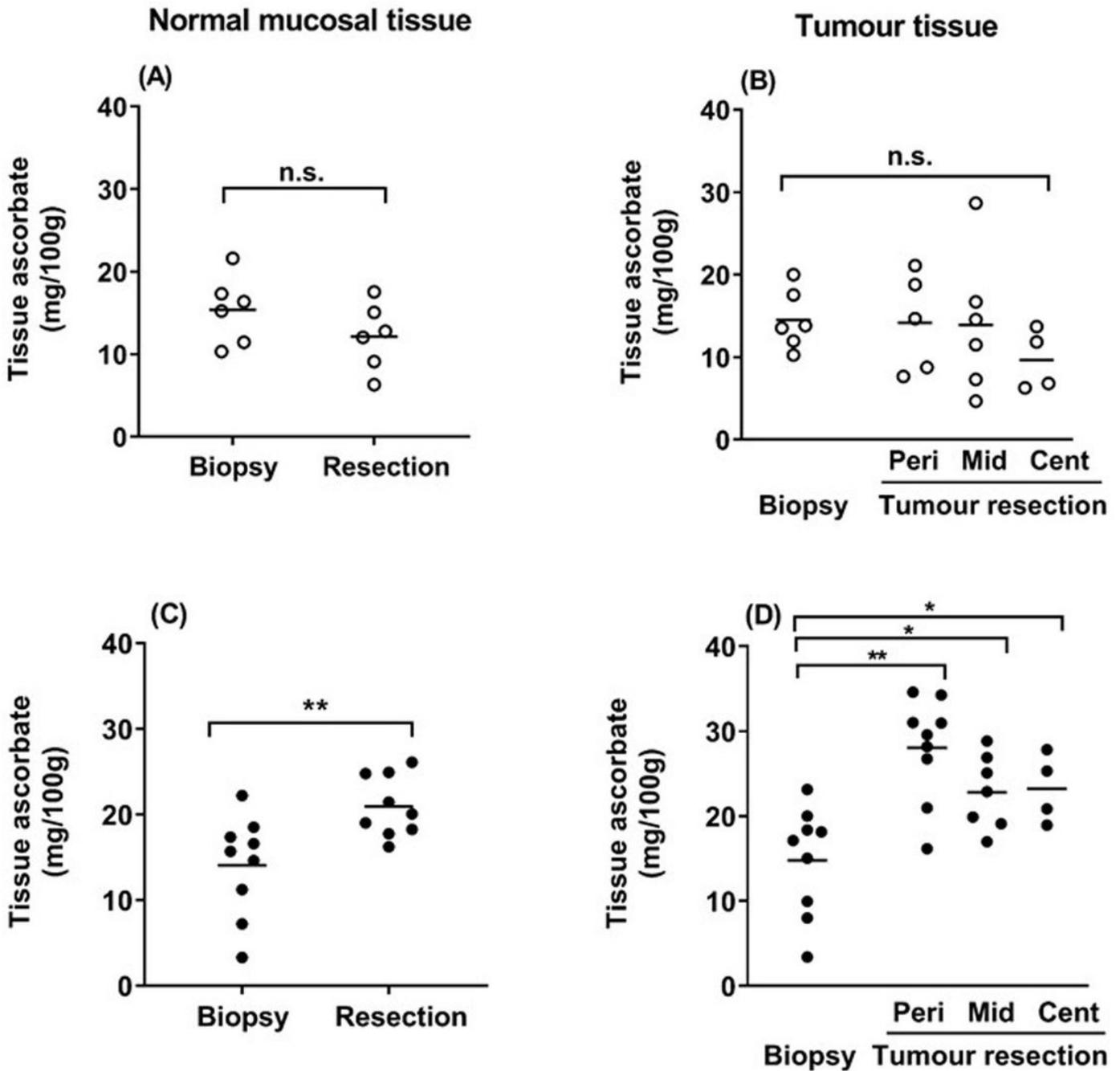


Figure 2

Ascorbate levels in patients with colorectal cancer in normal mucosa and in tumour tissue. Tissue levels in control (A,B) and infusion patients (C,D), with samples taken at diagnostic colonoscopy and after resection. Ascorbate levels were unchanged in normal mucosa (A) and in tumour tissue (B) from control patients, but were significantly increased in both normal mucosa (C) and tumour tissue (D) following infusions. Tumour lesions were sampled at multiple locations as follows: edge/periphery (Peri), mid-tumour (Mid) and central core (Cent) (B,D); not every part of the tumour was sampled in every patient.

Individual data for control ($n \leq 6$) and infusion cohorts ($n \leq 9$) with means are shown. Results were compared using paired t-tests for changes following infusion, as shown. * $p < 0.05$, ** $p < 0.01$.

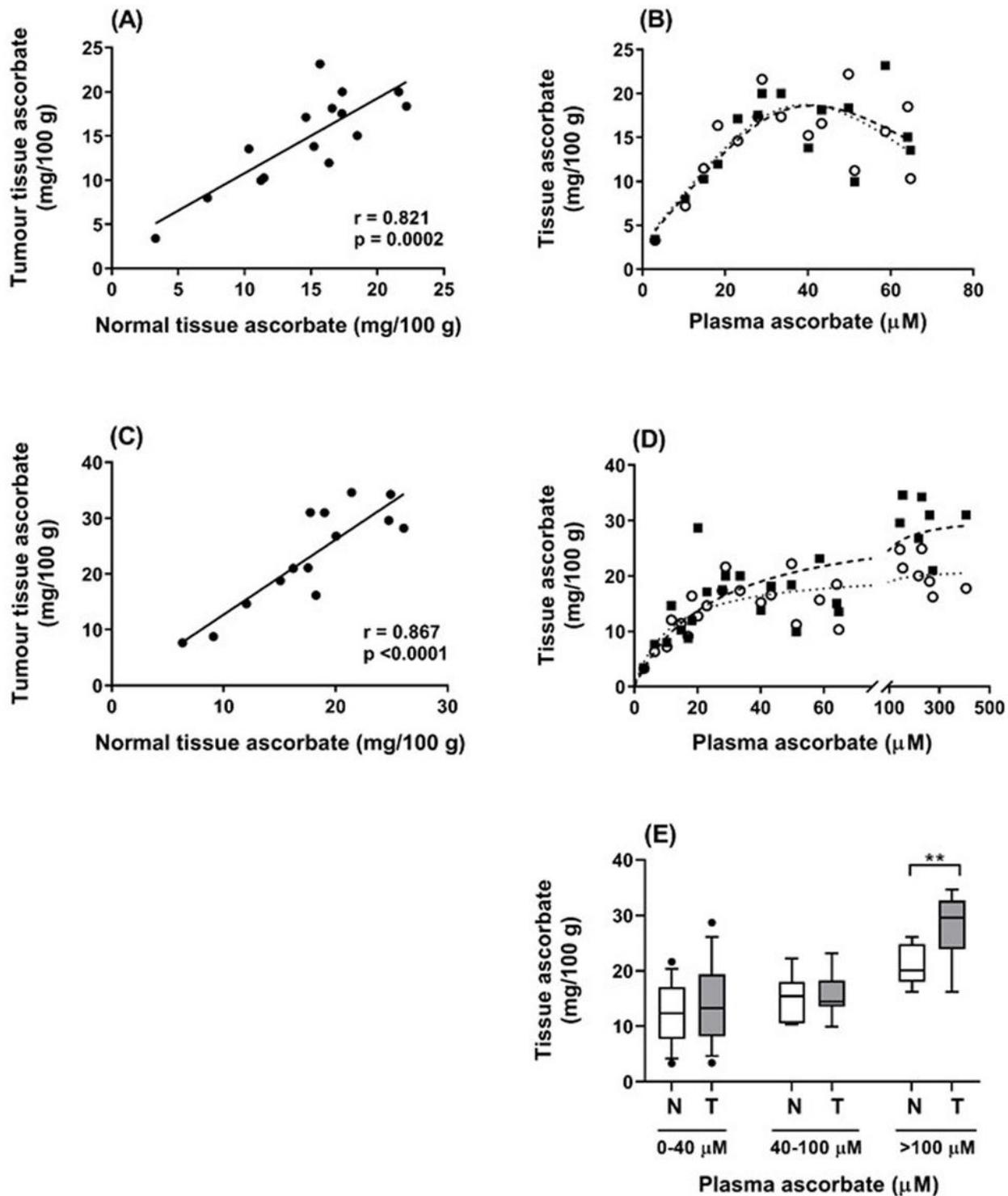


Figure 3

Associations between tumour and normal tissue ascorbate, and between tissue and plasma ascorbate levels in patients with colon cancer. (A) At biopsy, tumour and normal mucosa tissue ascorbate is closely correlated (Pearson $r=0.821$, $p=0.0002$). (B) Association between plasma ascorbate levels and normal

mucosa (□) and tumour (■) tissue in patients at biopsy (least squares fit, R² for best fit 0.64 and 0.62 for normal (.....) and tumour (—), respectively). (C) At resection, a correlation between tumour periphery and normal tissue is seen (Pearson $r=0.867$, $p<0.0001$). (D) Association between plasma levels (pre-infusion on day prior to resection) and normal mucosa (□) and tumour periphery (■), with non-linear saturation curve fit indicated (R² for best fit 0.85 and 0.69 for normal and tumour, respectively). Individual data for control (n= 6) and infusion cohorts (n= 9). (E) Comparison of normal mucosa (N) and tumour (T) tissue ascorbate levels with increasing plasma levels. There is a significant difference between mucosal and tumour tissue when plasma levels exceed the normal physiological maximum of 100 μM (unpaired t-test, $p = 0.008$).

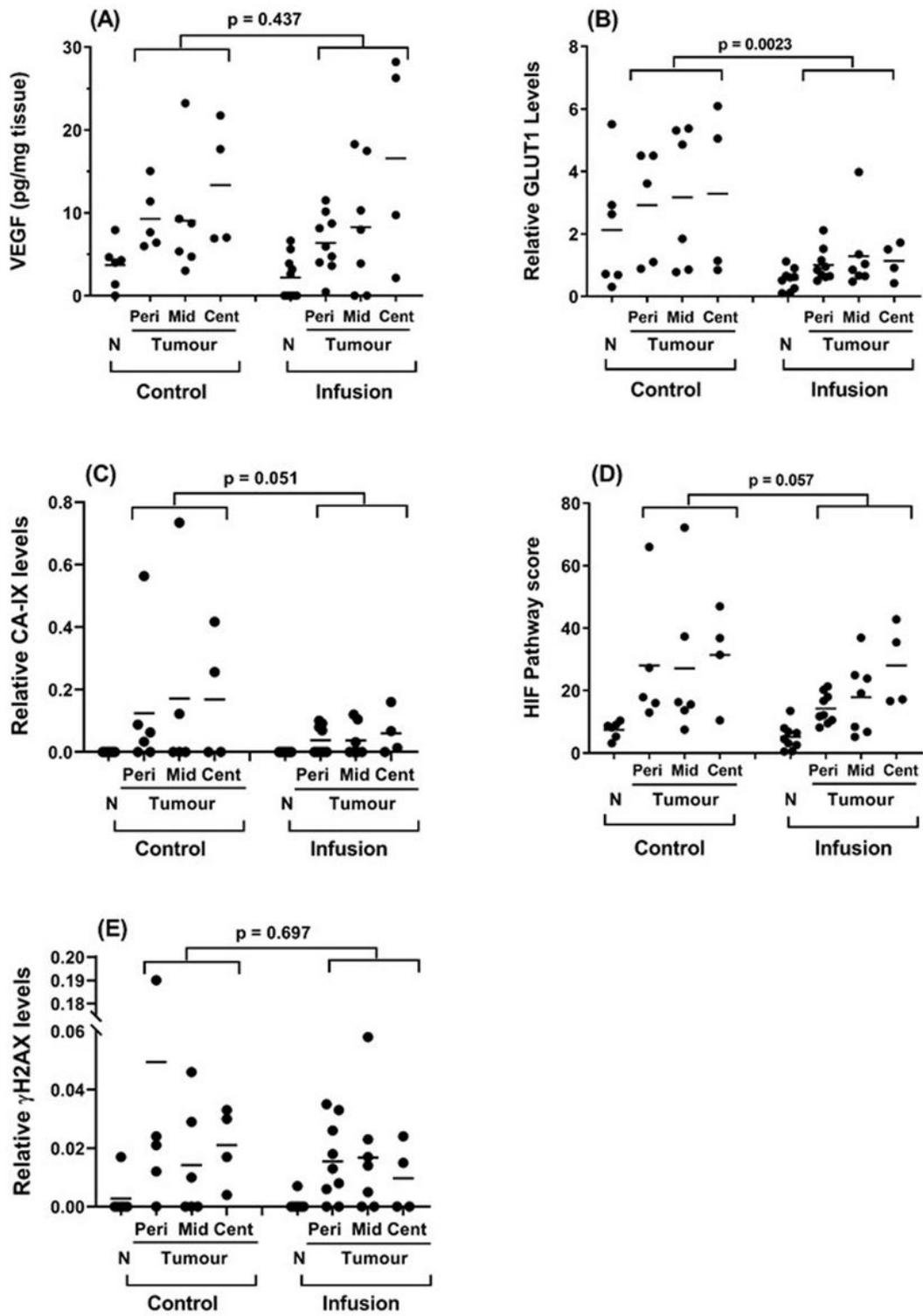


Figure 4

Protein expression associated with the hypoxic response and oxidative stress response in bowel mucosal tissue and in tumour tissue in control and infusion cohorts. Relative levels of (A) VEGF, (B) GLUT1, (C) CA-IX, were monitored in normal mucosal and tumour tissue from each individual. Expression of each protein was elevated in tumour tissue relative to control. Levels were lower in tumour tissue (all tumour regions) from the infusion cohort when compared with the levels in control tumour tissue (unpaired t-test results

shown). (D) HIF Pathway score was derived from the combined measurement of the three proteins for each tumour sample. (E) Relative levels of γ H2AX in tumour and normal mucosal tissues. Comparative statistics for combined tumour samples from peripheral, mid and central regions of the tumours are shown (unpaired t-tests). In addition, one way ANOVA with fitting for a mixed effects model (Brown-Forsythe and Welch test) indicated a significant increase in the tumour regions from periphery to central for the combined HIF Pathway score ($p=0.02$).

Supplementary Files

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