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# Celecoxib pre-treatment in human colorectal adenocarcinoma patients is associated with gene expression alterations suggestive of diminished cellular proliferation

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## ABSTRACT

Cancer cells treated with the cyclooxygenase-2 inhibitor celecoxib show growth inhibition and induced apoptosis. This study was conducted to determine if the same processes are relevant to celecoxib's effects on human colorectal adenocarcinomas treated *in vivo*. A cohort of 23 patients with primary colorectal adenocarcinomas was randomised to receive a 7-d course of celecoxib (400 mg b.i.d.) or no drug prior to surgical resection. Gene expression profiling was performed on resected adenocarcinomas from the cohort of patients. Using fold change (>1.5) and *p*-value (<0.05) cut-offs, 190 genes were differentially expressed between adenocarcinomas from patients receiving celecoxib and those that did not. The celecoxib pre-treated samples showed decreased expression levels in multiple genes involved in cellular lipid and glutathione metabolism; changes associated with diminished cellular proliferation. Celecoxib pre-treatment for 7 d *in vivo* is associated with alterations in colorectal adenocarcinoma gene expression which are suggestive of diminished cellular proliferation.

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## 1. Introduction

The inducible form of cyclooxygenase (COX), COX-2 is markedly up-regulated in pathological conditions such as inflammation and cancer.<sup>1</sup> For instance, a majority of colorectal adenocarcinomas display increased expression levels of the COX-2 protein.<sup>2</sup> Additionally, COX-2 expression levels have been associated with invasion depth,<sup>3</sup> liver metastasis<sup>4</sup> and poor clinical outcome.<sup>5</sup> Thus, the pharmacological inhibition of COX-2 activity has been explored as a therapeutic option

for the management of colorectal cancer. In fact, administration of celecoxib, a selective COX-2 inhibitor, for 6 months resulted in a 28% reduction in the mean number of colorectal polyps in patients with familial adenomatous polyposis.<sup>6</sup> Furthermore, clinical trials of colorectal cancer patients are currently underway investigating the addition of celecoxib to conventional chemotherapeutic regimens.<sup>7</sup>

Investigations into celecoxib's mechanism of action have shown that *in vitro* administration leads to growth arrest and induction of apoptosis in a variety of cancer cells.<sup>8–12</sup>

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Interestingly, celecoxib's anti-cancer effects appear to be independent of COX-2 inhibition, involving the inhibition of cell cycle progression, induction of apoptosis and inhibition of angiogenesis (reviewed in [13]). Investigations into celecoxib's mechanisms of action *in vivo* have also been conducted. Gene expression analysis of healthy colon tissue from patients with hereditary non-polypoid colon cancer (HNPCC) receiving celecoxib for 12 months displayed gene expression changes, suggestive of alterations in immune response, cell adhesion and transforming growth factor- $\beta$  signalling.<sup>14</sup>

However, the mechanism with which celecoxib exerts its chemotherapeutic effects in colorectal tumours *in vivo* is currently not known. We conducted a Phase 0 study to examine the effects of celecoxib pre-treatment on primary colorectal adenocarcinoma gene expression to gain insight into the mechanisms underlying celecoxib's effects *in vivo*. Our results indicate that celecoxib pre-treatment *in vivo* is associated with multiple gene expression changes consistent with alterations in cellular lipid metabolism, glutathione metabolism and cell adhesion, in addition to differential expression of select genes involved in the immune response, apoptosis and angiogenesis. These results suggest that celecoxib tips the balance away from cellular proliferation towards growth inhibition in colorectal adenocarcinoma cells.

## 2. Materials and methods

Patients undergoing surgical resection of histologically proven primary colorectal adenocarcinomas were consented for participation in the study, which was approved by the Washington University, School of Medicine Institutional Review Board (Table 1). The patients enrolled in this study were randomised to receive either 400 mg celecoxib two times per day or no COX-2 inhibitor for 7 d prior to surgical resection. This dose of celecoxib (400 mg b.i.d.) was chosen because it elicited a significant decrease in the mean number of colorectal polyps in patients with familial adenomatous polyposis.<sup>6</sup> A 7-d treatment regimen allowed the pharmacological evaluation to be performed without disrupting the surgical management of the colorectal adenocarcinomas. Patients in both groups were not allowed to take aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) before surgical resection to be eligible to participate in this study. Patients had received no prior therapy for their cancer. There were no apparent differences in either tumour cellularity or histology of the resection samples in the two groups.

Total RNA was isolated from surgically resected, histologically confirmed colorectal primary adenocarcinomas using a TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol at the Siteman Cancer Center Tissue Procurement Core (St. Louis, MO). Total RNA (5  $\mu$ g) from each sample was converted to double stranded cDNA using a dT-T7 promoter primer. The double stranded cDNA was then used as a template to synthesise biotinylated RNA, which was fragmented and hybridised to the Affymetrix HG\_U95av2 microarray chip (Santa Clara, CA) using Affymetrix's labelling and hybridisation protocol within the Siteman Cancer Center Multiplex Gene Expression Core (St. Louis, MO).

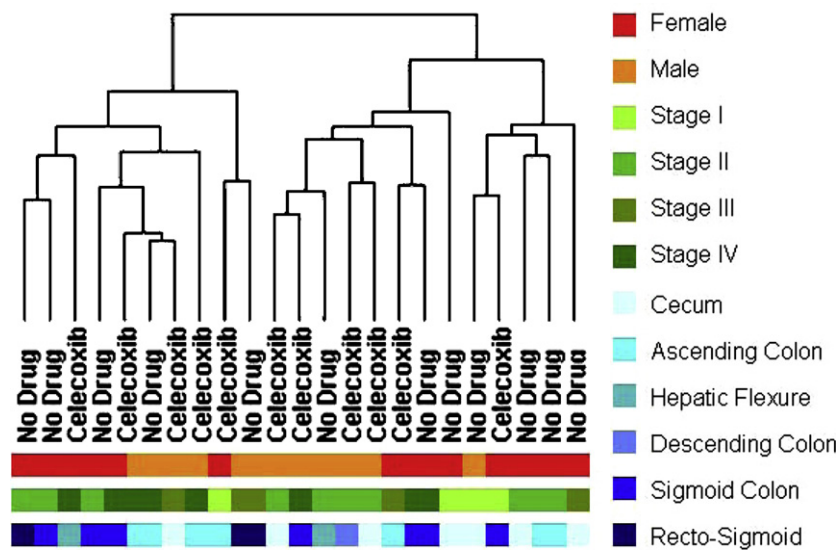
**Table 1 – Patient demographics**

	Celecoxib	No drug
Gender		
Male	6	4
Female	5	8
Tumor site		
Ascending colon	3	2
Cecum	3	4
Descending colon	1	0
Hepatic flexure	1	1
Recto-sigmoid colon	0	2
Sigmoid colon	3	3
Clinical stage		
I	2	2
II	3	6
III	2	2
IV	4	2
Pathological grade		
Poorly differentiated	2	2
Moderately differentiated	7	9
Well differentiated	2	1

The array data were imported into GeneSpring GX 7.3 (Santa Clara, CA) and normalised using GC-RMA. Genes with normalised expression standard deviations <0.04 across all samples, essentially unchanging genes, were excluded from further analysis. Unsupervised, hierarchical clustering was performed on all samples and all changing genes ( $n = 10,083$  genes) using uncentred correlation as the similarity matrix with complete linkage clustering was performed with Eisen's clustering programme.<sup>15</sup> Using a volcano plot in GeneSpring, 190 genes were identified with an absolute fold change greater than 1.5 and non-parametric  $p$ -value < 0.05 between adenocarcinomas from patients receiving celecoxib pre-treatment and adenocarcinomas from patients receiving no drug. Hierarchical clustering<sup>15</sup> was performed on all samples using the 190 differentially expressed genes identified in the volcano plot, using uncentred correlation as the similarity matrix with complete linkage clustering. The resulting heatmap and dendrogram were visualised using Java TreeView.<sup>16</sup> Gene expression enrichment analysis was performed using the analytical tools available at the database for annotation, visualization, and integrated discovery (DAVID) website: <http://david.abcc.ncifcrf.gov/>.<sup>17</sup> The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE11237.

## 3. Results

Unsupervised hierarchical clustering using all genes with differing levels of expression across all samples ( $n = 10,083$  genes) showed no obvious separations based on gender, tumour site, clinical stage or celecoxib pre-treatment status (Fig. 1). Differentially expressed genes associated with celecoxib pre-treatment were identified by comparing gene expression intensities in celecoxib-treated patients and patients receiving no drug using a volcano plot. Using a >1.5

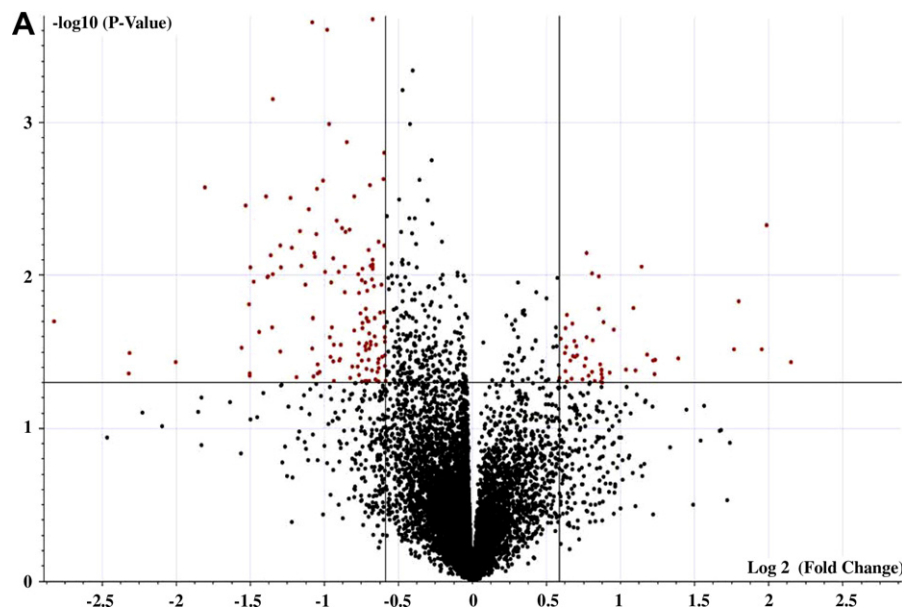


**Fig. 1 – Clustering of gene expression in colorectal adenocarcinoma patients.** Unsupervised hierarchical clustering was performed on all samples using 10,083 genes with a normalised expression standard deviations greater than 0.04. The dendrogram indicates that the samples do not cluster based solely on any of the recorded phenotypic characteristics.

absolute fold change and  $p < 0.05$  cut-offs, 35 genes displayed higher expression values and 155 genes displayed lower expression values in adenocarcinomas from patients receiving celecoxib relative to adenocarcinomas from patients receiving no drug (Fig. 2A and Supplemental Table 1). Hierarchical clustering of the adenocarcinoma samples across the 190 differentially expressed genes separated the samples into three main sub-clusters (Fig. 2B). In sub-cluster A, 8 of 9 tumour samples were from patients receiving no celecoxib

pre-treatment. In sub-cluster B, 6 of 7 tumour samples were from patients receiving celecoxib pre-treatment. Sub-cluster C contains tumour samples from 4 patients receiving celecoxib and 3 patients receiving no drug.

Gene annotation enrichment analysis was performed to examine which biological processes were altered by celecoxib pre-treatment. Celecoxib pre-treatment for 7 d was associated with the alteration of 23 genes involved in cellular lipid metabolism, four genes involved in glutathione metabolism



**Fig. 2 – Differentially expressed genes associated with celecoxib pre-treatment. (A)** Volcano plot of gene expression changes between colorectal adenocarcinomas from patients pre-treated and not pre-treated with celecoxib. **(B)** Clustering using the 190 differentially expressed genes. Red indicates increased expression; green indicates decreased expression and black indicates no difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

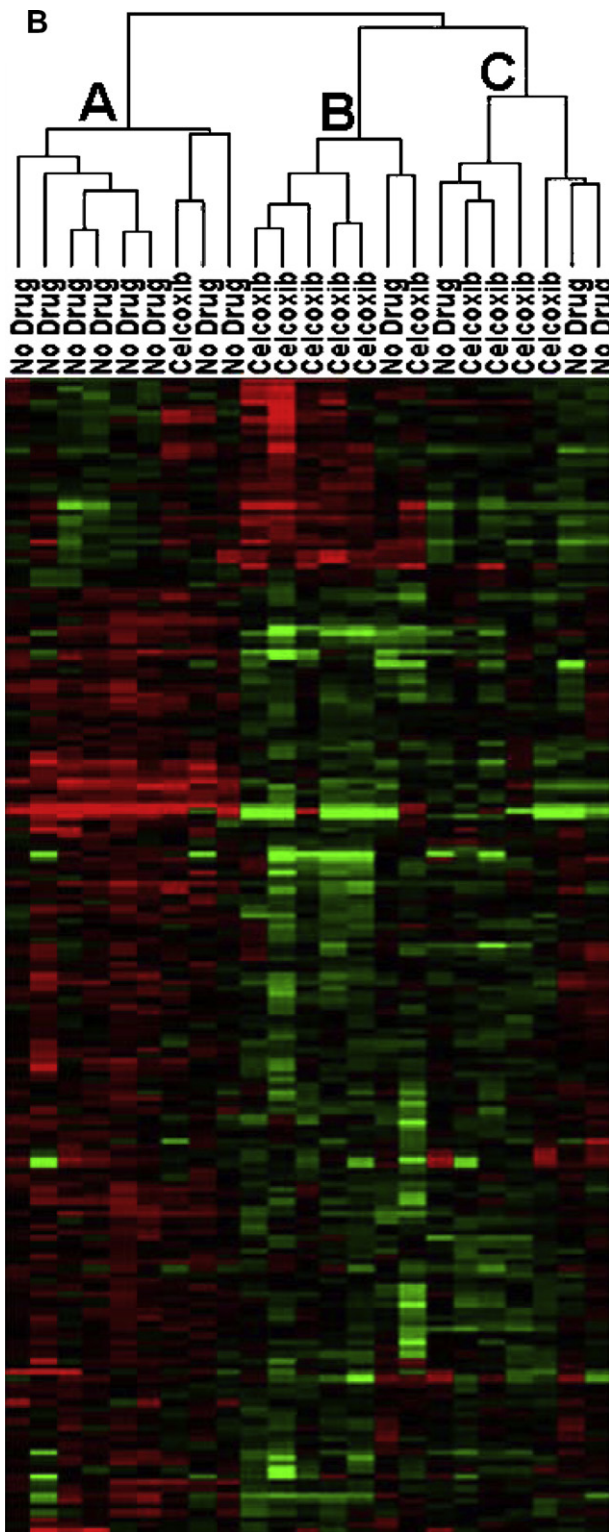


Fig. 2 (continued)

and 19 genes involved in cell adhesion (Table 2). In addition to these significantly altered biological processes, tumour samples from patients receiving celecoxib pre-treatment displayed changes in expression levels for genes associated with growth arrest (growth arrest-specific 6, 1.7-fold change), apoptosis (BCL2-associated X protein, -1.9-fold change), angi-

ogenesis (angiogenin, -2.8-fold change; kallikrein-1, -2.6-fold change) and inflammatory/immune response (interleukin-6, 4.4-fold change; S100 calcium binding protein A8, 2.4-fold change).

#### 4. Discussion

This first analysis of gene expression alterations associated with celecoxib pre-treatment in patients with primary colorectal adenocarcinoma revealed changes of putative biological relevance. Pre-treatment for only 7 d with pharmacological doses of celecoxib (400 mg b.i.d.) resulted in alterations in numerous genes involved in cellular lipid metabolism, glutathione metabolism and cell adhesion. In general, most of the genes involved in cellular lipid metabolism (>90%) were down-regulated in patients receiving celecoxib pre-treatment relative to patients receiving no drug. Importantly, several transcripts involved in fatty acid  $\beta$ -oxidation (ACAA2, ECH1, HADHSC and HSD17B4; see Table 2 for gene names) were down-regulated, suggesting that the tumours from patients receiving celecoxib pre-treatment have a diminished capacity to generate energy. Since fatty acid metabolism has been shown to be elevated in colorectal adenocarcinomas compared to normal colonic tissue,<sup>18</sup> celecoxib may exert its anti-proliferative effects, at least in part, through counteracting the increase in fatty acid metabolism associated with colorectal carcinogenesis.

Celecoxib pre-treatment was also associated with a decrease in the relative expression of transcripts involved in glutathione metabolism, including three  $\gamma$ -glutamyltransferases (GGT1, GGT2 and GGTL4) and glutathione reductase. The decreased relative expression of these transcripts suggests that tumours from patients receiving celecoxib pre-treatment have a reduced capacity to handle oxidative stress. Additionally, increased expression of GGT1 has been linked to the proliferative ability of colon cancer cells *in vitro*<sup>19</sup> by providing a growth advantage over cells devoid of GGT1 expression.<sup>20</sup> Furthermore, the proliferation of colon cell lines is associated with increased activity of glutathione reductase, whilst decreased cell proliferation is associated with decreased cellular glutathione content.<sup>21</sup> The decreased expression of glutathione metabolism related genes in this study therefore suggests a decrease in cellular glutathione content by celecoxib pre-treatment and by extension, perhaps decreasing the proliferative ability of the tumours.

In addition to altering expression of genes involved in lipid and glutathione metabolism, celecoxib pre-treatment affected the expression level of genes involved in cell adhesion. Several genes encoding for extracellular matrix proteins, including collagen, laminin and von Willebrand factor, showed increased relative expression in celecoxib pre-treated patients. These results are in agreement with previous findings on the effects of celecoxib and other NSAIDs on gene expression. For instance, colorectal cancer cells treated *in vitro* with aspirin were also associated with the up-regulation of collagens.<sup>22</sup> Alterations of genes involved in cell adhesion were also noted in healthy colons from HNPCC patients treated with celecoxib for 12 months.<sup>14</sup> Another extracellular matrix protein, tenascin C, showed a relative increase in



**Table 2 – Differentially expressed genes in three overrepresented biological processes associated with celecoxib pre-treatment**

Symbol	Gene name	Fold change
Cellular lipid metabolism: $p < 0.000002^*$		
ACAA2	Acetyl-coenzyme A acyltransferase 2	-1.86
ACADSB	Acyl-coenzyme A dehydrogenase, short/branched chain	-1.63
AGPAT2	1-Acylglycerol-3-phosphate O-acyltransferase 2	-1.65
AKR1C1/AKR1C2	Aldo-keto reductase family 1, member C	1.72
C14ORF1	Chromosome 14 open reading frame 1	-1.58
CDS1	CDP-diacylglycerol synthase 1	-2.89
CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	-2.15
DGAT1	Diacylglycerol O-acyltransferase homolog 1 (mouse)	-2.04
DHCR24	24-Dehydrocholesterol reductase	-1.55
ECH1	Enoyl coenzyme A hydratase 1, peroxisomal	-1.51
FABP1	Fatty acid binding protein 1, liver	-2.82
FDFT1	Farnesyl-diphosphate farnesyltransferase 1	-1.58
HADHSC	L-3-hydroxyacyl-coenzyme A dehydrogenase, short chain	-2.33
HMGCR	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase	-1.81
HSD17B2	Hydroxysteroid (17-beta) dehydrogenase 2	-2.11
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4	-1.66
PCCA	Propionyl coenzyme A carboxylase, alpha polypeptide	-2.60
PCCB	Propionyl coenzyme A carboxylase, beta polypeptide	-2.25
PIGF	Phosphatidylinositol glycan anchor biosynthesis, class F	-1.60
RARRES2	Retinoic acid receptor responder (tazarotene induced) 2	1.75
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	-2.54
UGT1A1–A10	UDP glucuronosyltransferase 1 family	-2.84
UGT8	UDP glycosyltransferase 8	-2.54
Glutathione metabolism: $p < 0.001^*$		
GGT1	gamma-Glutamyltransferase 1	-1.54
GGT2	gamma-Glutamyltransferase 2	-1.80
GGTL4	gamma-Glutamyltransferase-like 4	-2.12
GSR	Glutathione reductase	-1.67
Cell adhesion: $p < 0.02^*$		
CCL2	Chemokine (C-C motif) ligand 2	2.62
CIB1	Calcium and integrin binding 1 (calmyrin)	-1.64
CLDN7	Claudin 7	-1.71
COL16A1	Collagen, type XVI, alpha 1	2.34
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102 kDa	-1.68
FCGBP	Fc fragment of immunoglobulin G (IgG) binding protein	-7.09
GMDS	GDP-mannose 4,6-dehydratase	-2.07
ITGA6	Integrin, alpha 6	-1.58
LAMA4	Laminin, alpha 4	1.81
LGALS4	Lectin, galactoside-binding, soluble, 4 (galectin 4)	-2.57
LOXL2	Lysyl oxidase-like 2	1.52
MAEA	Macrophage erythroblast attacher	-1.51
MUC5AC	Mucin 5AC, oligomeric mucus/gel-forming	-2.84
PECAM1	Platelet/endothelial cell adhesion molecule (CD31 antigen)	2.21
TNC	Tenascin C (hexabrachion)	1.82
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	1.93
TSPAN1	Tetraspanin 1	-2.61
VCAM1	Vascular cell adhesion molecule 1	1.69
VWF	von Willebrand factor	1.80

Note: Fold change represents the difference in expression in celecoxib pre-treated patients versus patients receiving no drug pre-treatment.

\*  $p$ -Value represents the likelihood that the number of differentially expressed genes belonging to the biological process occurred by chance as determined by the Fischer's exact test.

expression levels following celecoxib pre-treatment. Tenascin C expression has been found to be minimal in normal colonic mucosa, but elevated expression levels are observed in colorectal adenocarcinomas.<sup>23</sup>

Other gene expression alterations observed in patients receiving celecoxib pre-treatment suggest a trend towards

growth inhibitory influences in the tumours. For instance, the inflammatory modulators, interleukin-6 and chemokine (C-X-C motif) ligand-6 (CXCL6) displayed greater relative expression levels in tumour samples from patients receiving celecoxib pre-treatment. Similar increases in inflammatory modulators, including interleukin-6, CXCL2 and CXCL3, have

been noted in a breast epithelial cell line treated with 50  $\mu$ M celecoxib for 48 h, a treatment regimen associated with growth inhibition and induced apoptosis.<sup>9</sup> Angiogenin, a potent mediator of angiogenesis, has been found to promote proliferation of gastric cancer cells<sup>24</sup> and has also been correlated with focal macrophage infiltration in colorectal cancer.<sup>25</sup> In this study, celecoxib pre-treatment was associated with decreased expression of angiogenin, again suggesting that celecoxib diminishes the proliferative ability of colorectal cancer cells.

We must note that the cut-offs for differential expression between the two treatment groups were of relatively low stringency. However, by using low stringency cut-offs for selecting differentially expressed genes, we have achieved the goal of this pilot study by identifying gene expression alterations associated with celecoxib pre-treatment, *in vivo*. It is promising that even a short course of celecoxib resulted in gene expression changes in colorectal adenocarcinomas. Previously, a 12 month regimen of celecoxib was found to induce expression changes in over 1000 genes in healthy colonic mucosa from HNPCC gene carriers.<sup>14</sup> Many of the expression changes were found in genes involved in immune response, cell adhesion, transforming growth factor- $\beta$  signaling, cell proliferation and angiogenesis. Similarly, the 7-d regimen of celecoxib employed in this study showed gene expression alterations in colorectal tumour tissue, most notably changes in genes involved in cell proliferation, cell adhesion and immune response. Interestingly, no genes involved in transforming growth factor- $\beta$  signalling were differentially expressed by the 7-d pre-treatment regimen of celecoxib, possibly indicating that these effects of celecoxib occur after prolonged exposure.

How do the results from this study inform on the usefulness of celecoxib as an option for colorectal cancer chemotherapy? This is an important issue, since the prolonged use of several selective COX-2 inhibitors, including celecoxib, has been found to increase the risk of cardiovascular side effects.<sup>26,27</sup> However, the cardiovascular risks associated with long-term use of celecoxib are rather ambiguous, as no increased cardiovascular risk was observed in Prevention of Spontaneous Adenomatous Polyps (PreSAP) trial (<http://www.fda.gov/cder/drug/infopage/celebrex/celebrex-hcp.htm>). Celecoxib appears to be safer than other selective COX-2 inhibitors, but care should be taken when given to patients already at risk for cardiovascular events.<sup>28</sup> In regards to the therapeutic potential in colorectal cancer, celecoxib given by itself at currently recommended doses (400 mg b.i.d.) appears to lessen cellular proliferation of colorectal tumours, as evidenced by the results of this study. However, the observed gene expression alterations appear to be relatively minor and it still remains to be determined if a longer course of celecoxib treatment would significantly arrest the growth of colorectal cancers. However, combination therapy of celecoxib with other chemotherapeutics does not appear to be as promising. A recent Phase II study looking at the effects of celecoxib treatment in metastatic colorectal cancer patients indicates that celecoxib adds no added benefit to combined 5-fluorouracil/oxaliplatin chemotherapy.<sup>29</sup> Additionally, previous *in vitro* findings suggest that the combined use of celecoxib with 5-fluorouracil could be counterproductive, as the growth arrest elicited by celecoxib interferes with 5-fluorouracil mediated cytotoxicity.<sup>29,30</sup> Further investigation is war-

ranted to determine if celecoxib can be employed as an adjunct to chemotherapeutic regimens that do not rely on enhanced cellular proliferation for cytotoxicity.

In conclusion, our results suggest that celecoxib reduces the proliferative capacity of colorectal adenocarcinomas. As with previous results, this small, short-term study indicates that celecoxib treatment is associated with gene expression alterations in the tumour itself that are indicative of decreased cellular lipid and glutathione metabolism, along with an increased immune response. These gene expression alterations, taken together, suggest that celecoxib pre-treatment leads to growth inhibitory influences on colorectal tumours. This study suggests possible gene expression alterations mediating the effects of celecoxib treatment on colorectal adenocarcinoma cells *in vivo*. Additionally, these results should provide the impetus for larger studies in the future, either using celecoxib treatment alone or in combination with other chemotherapeutic regimens.

### Conflict of interest statement

None declared.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ejca.2008.05.010](https://doi.org/10.1016/j.ejca.2008.05.010).

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