Effects of celecoxib on prostanoid biosynthesis and circulating angiogenesis proteins in familial adenomatous polyposis

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Running title: ***Celecoxib and angiogenesis biomarkers in FAP***

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Abstract

Vascular cyclooxygenase(COX)-2-dependent prostacyclin(PGI_2) may affect angiogenesis by preventing endothelial activation and platelet release of angiogenic factors present in platelet α-granules. Thus, a profound inhibition of COX-2-dependent PGI_2 might be associated with changes in circulating markers of angiogenesis. We aimed to address this issue by performing a clinical study with celecoxib in familial adenomatous polyposis(FAP). In 9 FAP patients and healthy controls, pairmatched for gender and age, we compared systemic biosynthesis of PGI_2, thromboxane(TX)A_2 and prostaglandin(PG)E_2(assessing urinary enzymatic metabolites, PGI-M, TX-M and PGE-M, respectively). The impact of celecoxib (400mg/BID for 7 days), on prostanoid biosynthesis and 14 circulating biomarkers of angiogenesis was evaluated in FAP. Intestinal tumorigenesis was associated with enhanced urinary TX-M levels, unaffected by celecoxib; thus suggesting the involvement of a COX-1-dependent pathway, presumably from platelets. This was supported by the finding that in co-cultures of human colon adenocarcinoma cell line(HT-29) and platelets, enhancedTXA_2 generation was almost completely inhibited by pre-treatment of platelets with aspirin, a preferential inhibitor of COX-1. In FAP, celecoxib profoundly suppressed PGE_2 and PGI_2 biosynthesis that was associated with a significant increase in circulating levels of most pro-angiogenesis proteins but also the anti-angiogenic TIMP-2. Urinary PGI-M, but not PGE-M, was negatively correlated with circulating levels of FGF-2 and angiogenin. In conclusion, inhibition of tumor COX-2-dependent PGE_2 by celecoxib may reduce tumor progression. However, the coincident depression of vascular PGI_2, in a context of enhanced TXA_2 biosynthesis, may modulate the attendant angiogenesis, contributing to variability in the chemopreventive efficacy of COX-2 inhibitors, such as celecoxib.

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Introduction

There is increasing appreciation of the role of platelets in tumor growth and metastatic dissemination(Gay and Felding-Habermann,2011). Platelet activation can lead to the release of growth and angiogenesis factors present in α-granules into the tumor microenvironment(Italiano et al., 2008). Moreover, platelets and the factors which they release can upregulate cyclooxygenase(COX)-2, considered an early event of cell transformation(Patrono et al.,2001). In colorectal cancer, COX-2 expression is induced early in stromal cells, and subsequently at high levels in epithelial cells(Prescott, 2000), where it correlates with advanced tumor invasion and poor clinical outcomes(Sheehan et al., 1999).

Prostaglandin(PG)E_2 is a key prostanoid in tumorigenesis generated through the activity of coordinate expression of COX-2 and mPGES-1(microsomal PGE_2 synthase-1, an enzyme downstream of COX-2)(Wang and Dubois,2010). PGE_2 exerts its autocrine/paracrine effects on target cells by coupling to four subtypes of G-protein-coupled receptors classified as EP1, EP2, EP3, and EP4(E-series prostanoid receptors). Recently, we have shown that EP2 stimulation causes transactivation of the epidermal growth factor receptor signaling pathway to promote tumor cell proliferation and invasion(Donnini et al.,2007).

The possible contribution of other prostanoids to colon cancer development is less clear. Thromboxane(TX)A_2 and prostacyclin(PGI_2) play important roles in cardiovascular(CV) homeostasis (Grosser et al.,2006). In particular, TXA_2, a major product of platelet COX-1, promotes platelet aggregation and vasoconstriction, whereas PGI_2, a major product of endothelial COX-2, inhibits platelet aggregation and promotes vasodilatation. Interestingly, it has been shown that enhanced TXA_2 and PGI_2 generation by the introduction of the downstream TXA_2 synthase(TXAS) and PGI_2 synthase(PGIS), respectively, into murine colon cancer cells modified tumor growth *in vivo* via differential effects on tumor angiogenesis(Pradono et al.,2002). Tumors derived from cells expressing

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TXAS grew faster and exhibited more abundant vasculature whereas tumors from PGIS-expressing cells resulted in opposite effects(Pradono et al.,2002; De Bock et al.,2011).

Aspirin, even at low-doses(such as 75mg daily, recommended for the prevention against heart disease, which preferentially inhibits platelet COX-1)(Charman et al.,1993), reduces the incidence and mortality of colorectal cancer(Rothwell et al.,2011). This is consistent with the hypothesis that the antiplatelet effect of aspirin is central to its antitumor efficacy(Patrono et al.,2001). Enhanced systemic biosynthesis of TXA_2 is mainly from platelet COX-1 and is suppressed by low-dose aspirin in colorectal cancer(Sciulli et al.,2005). Based on this, a critical question is centered on the ability of PGI_2 to exert an antitumor effect, and if so, whether this occurs through direct inhibition of platelet activation(Grosser et al.,2006).

The selective COX-2 inhibitor celecoxib was approved by FDA for the treatment of familial adenomatous polyposis(FAP)[400mg/BID, which is a 4-fold higher dose than that recommended for analgesia]. This decision was based on the results of the clinical study showing that in patients with FAP, six months of treatment with celecoxib 400mg/BID, but not celecoxib 100mg/BID, reduced the number of colorectal polyps by roughly one third. However, marked variability in the response to celecoxib was noted, both at 100mg and at 400mg/BID(Steinbach et al.,2000). Thus, it is of clinical relevance to identify potential mechanistic contributors to this variability in response. Importantly, development of biomarkers predictive of response will allow one to avoid exposure of patients unlikely to benefit from chemoprevention to the CV hazard from this drug. Drugs, such as celecoxib, suppress vascular PGI_2 generated by COX-2 in endothelial cells, thus leaving unconstrained all mediators that stimulate platelets, elevate blood pressure, and accelerate atherogenesis, including TXA_2(Grosser et al.,2006).

In the present study, we investigated the biosynthesis of TXA_2, PGI_2 and PGE_2 *in vivo*, by the measurement of their major urinary enzymatic metabolites, in FAP patients and healthy controls,

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pairmatched for gender and age, who were nonsmokers and without clinically detectable CV risk factors. In FAP patients, we performed an open-label study with a clinically relevant dose of celecoxib (400mg/BID for 7 days) to verify the COX-isozyme involved in TXA_2 biosynthesis in patients with intestinal neoplasia. In order to exclude the possible contribution of clinically undetectable CV disease to enhanced TXA_2 biosynthesis detected in FAP, we assessed urinary levels of a major enzymatic metabolite of TX in a mouse model of colon tumorigenesis, i.e. $Apc^{Min/+}$mice(Moser et al.,1995). The hypothesis that tumor cells and their released products or microvesicles are the trigger of platelet activation and enhanced TXA_2 biosynthesis was verified by performing co-culture studies of human HT-29 colon cancer cells and platelets. Finally, we verified the hypothesis that in FAP, a profound inhibition of COX-2-dependent PGI_2 by celecoxib would be associated with complex changes in circulating markers of angiogenesis possibly because PGI_2 may constrain platelet release of angiogenesis factors present in platelet α-granules(Menter et al.,1987).

Methods

Clinical Study in FAP patients, design and assessments

Nine patients with FAP, recruited from the national hereditary colorectal tumor registry, National Cancer Institute(Milan, Italy), and 9 healthy controls, matched for gender and age, nonsmokers and without clinically detectable CV risk factors(Table 1), were enrolled to participate in the study, after providing informed consent. In the 9 patients with FAP, we performed an open-label study with celecoxib (Pfizer)(400mg/BID for 7 consecutive days) that was previously approved by the institutional ethical committee. FAP patients had not had a complete colorectal resection and had 5 or more polyps, 2mm or more in diameter, that could be assessed endoscopically. Exclusion criteria included a history of colectomy within the previous 12 months; use of non steroidal anti-inflammatory drugs(NSAIDs) or aspirin, a minimum of one or two times a week within 3 months of enrollment; abnormal results of

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serum laboratory tests(complete blood count and liver-function and renal-function tests); a history of myocardial infarction, stroke, coronary-artery bypass graft, invasive coronary revascularization, or new-onset angina within the previous 6 months or electrocardiogram evidence of recent silent myocardial ischaemia; intolerance or allergy to NSAIDs; pregnant women.

Celecoxib compliance was monitored by means of pill count and review of diaries completed by patients and the assessment of celecoxib plasma levels (Schönberger et al., 2002). Before and after treatment, blood and urine samples were collected for the assessment of different molecular and biochemical analyses. Heparinized blood samples were collected before dosing and on the 7^{th} day, 4 hr after the last dose of celecoxib, to assess the levels of 14 circulating angiogenesis proteins using an angiogenesis antibody array kit(US Biomax, Inc., Rockville, MD, USA; the list of proteins analyzed is reported in Supplementary material and methods and Supplementary Table S1). Plasma cotinine levels were determined using an enzyme immunoassay kit(Cozart Biosciences, Oxford, UK). The presence of mutations in the *APC* gene, the earliest detectable molecular abnormality in colorectal cancer (Powell et al., 1992) and in the MutY human homologue(*MYH*) gene, associated with a recessive form of polyposis (Sieber et al., 2003), was assessed as previously described (Gismondi et al., 2004). Overnight urine samples (from 8pm to 8am) were collected before treatment and on the 8^{th} day after the last dose of the drug to evaluate the urinary excretion of 11-dehydro-TXB_2(TX-M), a major enzymatic metabolite of TXA_2, by a validated radioimmunoassay(RIA) technique (Ciabattoni et al., 1987) and 2,3-dinor-6-keto-$PGF_{1\alpha}$(PGI-M), a major enzymatic metabolite of PGI_2, by reversed phase-HPLC-RIA technique[(validated by comparison with ultra performance liquid chromatography tandem mass spectrometry(UPLC/MS/MS)(Song et al., 2007)(Supplementary Figure S1 and Supplementary material and methods)]. PGI-M and TX-M are indexes of PGI_2 and TXB_2 generation *in vivo* (FitzGerald et al., 1983). Moreover, in the same urine collections we assessed the levels of 11alpha-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid(PGE-M) by UPLC/MS/MS (Song et al., 2007), an index of

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PGE_2 generation *in vivo* (Murphey et al., 2004) which has been used as a biomarker for risk assessment of colorectal cancer (Csiki et al., 2005). Metabolite levels were corrected for urinary creatinine assessed by UPLC/MS/MS.

Inhibition of human whole blood COX-1 and COX-2 by celecoxib in vitro

It was assessed using previously published whole blood assays (Patrignani et al., 1994; Patrono et al., 1980) and a brief description is reported in the Supplementary material and methods.

Studies in $Apc^{Min/+}$ mice

In vivo prostanoid generation was accomplished using $Apc^{Min/+}$mice, an established model of FAP (Moser et al., 1995). Five female $Apc^{Min/+}$mice and their wild-type C57BL/6J littermates at 11 weeks of age, were used for each group. Urine samples(100 μl/mouse) were manually collected and immediately frozen. Systemic production of PGE_2, TXA_2 and PGI_2 was evaluated by UPLC/MS/MS quantification of their major urinary metabolites (Song et al., 2007): PGE-M, 2,3-dinor-thromboxaneB_2(dinor-TX-M) and PGI-M, respectively. All animals were maintained in a pathogen-free animal facility and experiments were approved by the Institutional Animal Use and Care Committee.

Co-culture experiments with a human colon adenocarcinoma cell line(HT-29) and isolated human platelets

HT-29 cells were cultured in McCoy's 5A medium(Invitrogen, Milan, Italy) containing 10% fetal bovine serum(FBS), 1% penicillin/streptomycin and L-glutamine 2mM. For every experiment, $1x10^6$ cells were seeded in 6-multiwell containing 2 ml of McCoy 5A supplemented with FBS 0.5% and 10 μg/ml of Polimyxin B sulphate(Sigma-Aldrich, Milan, Italy) for 20 hr, alone or co-cultured with platelets. Human platelets were freshly isolated from leukocyte concentrates obtained from Stadtische Kliniken Hochst(Frankfurt, Germany), as previously reported (Albert et al., 2002)(briefly described in Supplementary material and methods). HT-29 cells($1x10^6$cells) were cultured alone or with washed human platelets($1x10^8$cells) for 20hr, at 37°C in a humidified mixture of 5% CO_2 in air. In the culture

medium, PGE_2 and TXB_2 were measured by validated RIAs (Patrignani et al., 1994; Patrono et al., 1980), while in cell lysates, COX-1, COX-2, mPGES-1 and TXAS were assessed by Western blot (Di Francesco et al., 2009)(Supplementary material and methods). Platelets, obtained by preincubating platelet rich plasma(PRP) with aspirin 300μM for 30min at room temperature before washing, were used as indicated. The effects of the highly selective COX-2 inhibitor rofecoxib(0.3μM,Witega Laboratorien, Berlin,Germany) on prostanoid biosynthesis by HT-29 cells cultured alone or with platelets for 20hr were evaluated.

Statistical analyses

Values were reported as mean±SD and median(range), as appropriate. A *P* value<0.05 was assumed to be significant. The data were compared by parametric tests(Student's *t*-test or ANOVA) or nonparametric tests when they did not pass the Kolmogorov-Smirnov normality test. The Spearman rank correlation coefficient(r_s) was calculated to quantify the statistical dependence between two variables. Linear multiple regression analysis of $\log_{10}$ transformed data was performed to test the relationship between PGI-M, PGE-M, TX-M and circulating angiogenesis proteins. Multicollinearity of biomarkers was verified by assessing the individual r^2 and VIF(Variance Inflation Factor). Variables with r^2 values greater than 0.75(so VIF was greater than 4.0) were excluded for multicollinearity. Comparisons of urinary and plasma biomarker levels between baseline and celecoxib in FAP patients were assessed by the Wilcoxon matched pairs test. All analyses were performed using GraphPad, InStat(San Diego, CA, USA). In the clinical pharmacology study, the primary hypothesis was that celecoxib would cause 60% reduction of urinary PGI-M. Assuming an intersubject coefficient of variation of 22% for urinary excretion of PGI-M (McAdam et al., 2005), 6 volunteers would allow detecting at least 46% change in its measurement between pre- and post-drug with a power of 90% based on two-tailed tests with *P*-values less than the type I error rate of 0.05. Thus, we choose a sample-size of 9 individuals. Concentration-response curves of celecoxib were fitted(using PRISM,

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GraphPad) and IC_{50}(drug concentration required for obtaining 50% of inhibition) values were calculated.

Results

Biosynthesis of prostanoids in vivo in FAP patients at baseline

The baseline characteristics of FAP patients and healthy controls, pairmatched for gender and age, are reported in Table 1. All individuals were nonsmokers without significant CV risk factors. Despite all FAP patients claiming to be nonsmokers, 1 out of the 9 exhibited plasma cotinine levels (59.4 ng/ml) compatible with a moderate smoking habit (Binnie et al. 2004). Eight patients were carriers of the *APC(*adenomatous polyposis coli) mutation and one was carrier of the *MYH* mutation.

In FAP, urinary levels of TX-M, at baseline, were 1.19±0.84, 0.93(0.10-2.90)ng/mg creatinine[mean±SD, median(range)], and resulted significantly(p<0.01) higher than the values detected in healthy controls[i.e., 0.20±0.10, 0.20(0.09-0.40)ng/mg creatinine](Table 1). Baseline urinary PGI-M levels were not significantly different in FAP and healthy controls(Table 1). In FAP, PGE-M baseline levels were significantly(p<0.05) higher than in healthy controls [22.30±16.20, 21.52(2.82-55.40) and 10.30±5.02, 11.22(4-16)ng/mg creatinine, respectively;Table 1]. They were similar to the values found in patients with recurrent non-small cell lung cancers(27.2±213.5ng/mg creatinine) (Csiki et al., 2005).

Biosynthesis of prostanoids in vivo in $Apc^{Min/+}$mice

In order to exclude the possible contribution of clinically undetectable CV disease to enhanced TXA_2 biosynthesis *in vivo* in FAP, we assessed urinary levels of a major enzymatic metabolite of TX in a mouse model of colon tumorigenesis, i.e. $Apc^{Min/+}$mice (Moser et al., 1995). In $Apc^{Min/+}$ and wild-type C57BL/6J mice, we assessed also urinary levels of PGI-M and PGE-M.

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In *Apc*$^{Min/+}$ mice, a significant increase of urinary dinor-TX-M vs wild-type mice was detected [mean±SD: 84.3±26 vs 48.2±23ng/mg creatinine, respectively, $p<0.05$, Figure 1a]. Average PGI-M was higher in *Apc*$^{Min/+}$ than in wild-type mice, but the differences were not statistically significant (Figure 1b). Urinary PGE-M was increased in *Apc*$^{Min/+}$ vs wild-type mice [5.3±1 vs 2.5±1.3ng/mg creatinine, respectively, $p<0.01$, Figure 1c]. These results confirm the data in FAP showing that enhanced *in vivo* generation of TXA_2 and PGE_2 is associated with multiple intestinal neoplasia.

Effects of celecoxib on the biosynthesis of prostanoids in vivo in FAP patients

Celecoxib, administered for 7 consecutive days did not significantly affect urinary TX-M in FAP patients(Figure 2a). In contrast, the drug caused profound and significant($p<0.01$) inhibition of PGI_2 biosynthesis by 58±9%(Figure 2b) and PGE_2 by 48±31%(Figure 2c).

The finding that celecoxib did not affect TX biosynthesis *in vivo* suggests a COX-1-dependent pathway for enhanced TX generation, presumably from platelets.

Prostanoid biosynthesis by co-culture of human adenocarcinoma cell line HT-29 and human platelets

To address whether epithelial tumorigenesis triggers platelet activation and enhances TXA_2 biosynthesis, we performed an *in vitro* study by co-culturing HT-29 cells with platelets for 20hr. As shown in Figure 3a, very low concentrations of TXB_2 were generated by HT-29 cells(mean±SD: 0.02±0.02ng/ml). Unstimulated platelets cultured alone for 20hr released 8±3 ng/ml of TXB_2. When unstimulated platelets were co-cultured with HT-29 cells for 20 hr, TXB_2 generation was significantly($p<0.01$) enhanced (57±32ng/ml). This finding suggests that colon cancer cells triggered platelet activation. TXB_2 generation was profoundly reduced by pre-treatment of platelets with aspirin under these experimental conditions, which is consistent with the enhanced TXB_2 biosynthesis in co-cultures of the 2 cell types deriving mainly from platelet COX-1 activity(Figure 3a). This notion was further supported by the finding that, under the same experimental conditions, a selective inhibitor of

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COX-2 activity, rofecoxib, did not affect TXB_2 levels, either in platelets co-cultured with HT-29 cells or in platelets cultured alone(Figure 3b). By contrast, rofecoxib significantly inhibited PGE_2 generation as detected in the culture medium of treated HT-29 cells(Figure 3c), suggesting that colon cancer cells generated PGE_2 principally *via* the COX-2 pathway. These differences in prostanoid generation can be attributed to the relative expression levels of COX-1, COX-2, TXAS, and mPGES-1 observed in HT-29 cells and platelets(Figure 3d). Altogether these results suggest that intestinal neoplasia is associated with COX-2/mPGES-1-dependent PGE_2 generation and that enhanced platelet COX-1-dependent TX generation may be triggered by tumor cell constituents, their released products and/or microvesicles.

Effects of circulating celecoxib concentrations on the activity of monocyte COX-2 and platelet COX-1 in human whole blood in vitro

We measured celecoxib levels in plasma of FAP patients 4hr after the last morning dose and we assessed the degree of inhibition on whole blood COX-1 and COX-2 activities *in vitro* produced by these concentrations. These experiments allowed us to verify whether circulating concentrations of celecoxib detected in FAP patients after dosing with 400mg BID were sufficient to suppress completely COX-2 activity *in vivo* as it has been suggested that the chemopreventive effects of celecoxib at high concentrations may occur through COX-2-independent pathways (Schiffman et al., 2009). At 4hr after the last dose of celecoxib, plasma concentrations ranged from 955 to 3566 ng/ml(1871±946.8 ng/ml,mean±SD). In Supplementary Figure S2, the sigmoidal dose-response curves of celecoxib for inhibition of whole blood COX-1 and COX-2 *in vitro* were shown. Celecoxib inhibited LPS-induced monocyte COX-2 and platelet COX-1 activities in a concentration-dependent fashion, with IC_{50} values of 128(72-228) and 3444(2116-5604) ng/ml[mean(95% Confidence Intervals,CI)], respectively. Individual plasma celecoxib concentrations detected in FAP patients were associated with inhibition by 90-95% of monocyte COX-2 activity *in vitro* and by 25-55% of platelet COX-1, *in vitro*(Supplementary Figure S2). These results indicate that circulating concentrations of celecoxib

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were appropriate to inhibit almost completely monocyte COX-2 activity and that they only modestly affected platelet COX-1 activity.

Effects of celecoxib on circulating angiogenesis biomarkers in FAP patients

We assessed the effects of celecoxib treatment for one week on circulating levels of 14 angiogenesis proteins[heparin-binding epidermal growth factor(HB-EGF), tissue inhibitor of metalloproteinases(TIMP)-1, TIMP-2, interferon-inducible protein(IP)-10, platelet derived growth factor(PDGF)-BB, keratinocyte growth factor(KGF), angiogenin, angiopoietin-1, angiopoietin-2, vascular endothelial growth factor(VEGF)-A, VEGF-D, soluble intercellular adhesion molecule(sICAM)-1, fibroblast growth factor(FGF)-2, hepatocyte growth factor(HGF)](Figure 4, 5 and Supplementary Table S1). Celecoxib caused a significant increase in the proangiogenic markers FGF-2, VEGF-D, VEGF-A, angiogenin(Figure 4a-d), and angiopoietin-2(Figure 5a). However, PDGF-BB was significantly reduced by celecoxib treatment(Figure 5b). TIMP-2, an inhibitor of angiogenesis through mechanisms involving mainly the inhibition of matrix metalloproteinases(MMP) activity (Stetler-Stevenson and Seo, 2005), was significantly(p<0.01) increased(Figure 5c), while sICAM-1, which is believed to play a role in tumor cell resistance to cell-mediated cytotoxicity (Fiore et al., 2002), was significantly(p<0.01) inhibited by celecoxib(Figure 5d).

Relationships among urinary biomarkers of prostanoid biosynthesis in vivo and circulating angiogenesis proteins in FAP patients

A statistically significant inverse Spearman's rank correlation was detected between the urinary PGI-M, assessed at baseline and after dosing with celecoxib, and the 2 growth factors HGF(r_s=-0.48, p<0.05) and FGF-2(r_s=-0.47, p<0.05)(Supplementary Figure S3). We did not find any correlation amongst urinary PGE-M or TX-M and circulating angiogenic proteins.

Celecoxib caused an increase in 6 circulating angiogenesis proteins (both pro- and anti-angiogenic)(Figure 4,5 and Supplementary Table S1). Thus, we tested the relationship among PGI-M,

TX-M and PGE-M and these circulating proteins(Supplementary Table S2). In linear multiple regression analysis of $\log_{10}$ transformed data, among the X variables which resulted independent of each other, the only one that was significantly related to TX-M was PGI-M(β:0.80; SEM:0.27; *P*=0.02), while PGI-M was positively related to TX-M(β:0.50; SEM:0.17; *P*=0.01), and inversely related to angiogenin (β:-0.44; SEM:0.16; *P*=0.02), and FGF-2(β:-0.24; SEM:0.10; *P*=0.03), in a statistically significant fashion. PGE-M was significantly correlated with TX-M (β:0.77; SEM:0.20; *P*=0.003), but not with any of the angiogenesis markers.

Discussion

Several lines of evidence support the role of COX-2-dependent PGE_2 in colon tumorigenesis (Wang and Dubois, 2010). Thus, in FAP, the administration of the selective COX-2 inhibitor celecoxib(400 mg/BID) was associated with a significant reduction of the number of colorectal polyps by roughly one third (Steinbach et al., 2000). However, marked variability in the response to celecoxib was noted (Steinbach et al., 2000). We hypothesized that inhibition of vascular COX-2-dependent PGI_2 may contribute to the variable response to celecoxib in this setting. In fact, PGI_2 may control angiogenesis by preventing endothelial activation and platelet release of angiogenic factors present in α-granules (Menter et al., 1987). Thus, we performed the present study to investigate the biosynthesis of PGI_2 and TXA_2, two key mediators of CV homeostasis (Grosser et al., 2006), and PGE_2, a well known mediator of inflammation and tumorigenesis (Wang and Dubois, 2010) in intestinal neoplasia. Importantly, we aimed to explore the impact of selective inhibition of COX-2 by celecoxib on circulating biomarkers of angiogenesis *in vivo* in FAP and to correlate them with the biosynthesis of PGI_2, TXA_2 and PGE_2 *in vivo*.

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We found for the first time that intestinal tumorigenesis is associated with enhanced TXA_2 biosynthesis *in vivo* that was not inhibited by the administration of the selective COX-2 inhibitor celecoxib. This finding suggests a COX-1-dependent pathway for enhanced TX generation, presumably from platelets. In order to exclude the possible contribution of clinically undetectable CV disease, to enhanced TXA_2 biosynthesis *in vivo*, in FAP, we assessed urinary levels of a major enzymatic metabolite of TX in a mouse model of colon tumorigenesis, i.e. $Apc^{Min/+}$ mice (Moser et al., 1995). Interestingly, we detected increased generation of TXA_2 *in vivo* as compared to wild-type mice. To address the hypothesis that epithelial tumorigenesis is associated with platelet activation, we performed an *in vitro* study using co-cultures of human colon adenocarcinoma cell line(HT-29 cells) and isolated human platelets. It is quite interesting that HT-29 cells triggered platelet TXA_2 generation which was almost completely inhibited by pre-treatment of platelets with aspirin. As previously reported there is multiplicity of molecular mechanisms that can be utilized by cancer cells to activate platelets and to enhance TXA_2 generation (Jurasz et al., 2004). Altogether our results show that intestinal tumorigenesis is associated with enhanced TXA_2 generation through the COX-1-pathway. TXA_2 has been reported to be involved in angiogenesis and development of tumor metastasis (Honn, 1983). Thus, pharmacological inhibition of TXAS has been shown significantly to inhibit tumor cell growth, invasion, metastasis and angiogenesis in a range of experimental models (Honn, 1983). Moreover, in a recent study, aspirin reduced the incidence and mortality of colorectal cancer, at doses of at least 75mg daily (Rothwell et al., 2011), recommended for the prevention against heart disease (Patrono et al., 2005), consistent with inhibition of platelet TXA_2 being central aspirin's efficacy in cancer prevention (Patrono et al., 2001).

It has been shown that tumor cell-derived products may cause endothelial dysfunction and increase vascular permeability (Padua et al., 2008). This phenomenon may facilitate the interaction of platelets with tumor constituents which are capable of inducing platelet activation (Pacienza et al., 2008). In this scenario, vascular PGI_2 may play an important role by curbing platelet activation and the release of α-

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granules, which segregate angiogenesis-regulatory proteins (Menter et al., 1987). This hypothesis was confirmed by our results showing that the inhibition of COX-2-dependent PGI_2, by celecoxib, was associated with complex changes in circulating markers of angiogenesis with enhanced levels of growth and angiogenesis factors(Figure 4,5). Interestingly, in multiple regression analysis, circulating levels of angiogenin, an inducer of angiogenesis present in platelets and released in response to agonist stimulation (Coppinger et al., 2007), were inversely related to urinary PGI-M. There was also an inverse Spearman's correlation between urinary excretion of PGI-M and plasma levels of two mediators of angiogenesis and tumor progression, i.e., FGF-2 (Hanahan and Folkman, 1996) and HGF (Jiang et al., 1999)(Supplementary Figure S3). However, we found a coincident increase of the plasma levels of a MMP inhibitor TIMP-2 (Stetler-Stevenson and Seo, 2005) and, interestingly, its concentrations inversely correlated with circulating levels of sICAM(r_s=-0.86, p<0.01), considered a hallmark of tumor cell evasion of immune surveillance (Fiore et al., 2002).

A COX-2/mPGES-1 pathway has been implicated in PGE_2 biosynthesis by colon cancer cells (Yoshimatsu et al., 2001). This has been confirmed here by our observation of enhanced systemic biosynthesis of PGE_2 in FAP patients which was profoundly reduced by celecoxib. Interestingly, we did not find any correlation between urinary PGE-M and circulating angiogenic factors, suggesting the origin of these circulating proteins to be outside the tumor proper. However, in multiple linear regression analysis, we found that PGE-M was significantly correlated with TX-M(Supplementary Table S2) which may suggest that enhanced TXA_2 generation *in vivo* contributed to COX-2-dependent PGE_2 generation in colon tumorigenesis.

In addition to COX-2-dependent prostanoids, emerging data suggest that also leukotrienes(LTs), generated from arachidonic acid(AA) through the activity of 5-lipoxygenase(5-LO), can have a role in carcinogenesis (Wang and Dubois, 2010). Both COX-2 and 5-LO use AA as the substrate for eicosanoid biosynthesis, thus, free AA accumulation, as a consequence of COX-2 inhibition by

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celecoxib, might lead to the increase of LT biosynthesis. Indeed, Duffield-Lillico et al.(2009) have recently shown that urinary levels of LTE_4(the end product of the cysteinyl LT metabolism) (Wang and Dubois, 2010)is increased in celecoxib-treated smokers with elevated COX-2 activity manifested by high baseline PGE-M levels. Whether this phenomenon occurs in FAP patients treated with celecoxib was not assessed in the present study but it deserves to be investigated in a specific study.

In summary, we show that COX-1-dependent TXA_2 is enhanced in colon tumorigenesis. In this setting, vascular COX-2-dependent PGI_2 may play a protective role by restraining the release of growth and angiogenesis factors from platelets and the generation of angiogenesis mediators from different cell types(Supplementary Figure S4). The administration of a selective COX-2 inhibitor, such as celecoxib, caused a profound inhibition of COX-2-dependent PGI_2 thus leaving enhanced TXA_2 generation unconstrained. This may explain the complex changes in circulating markers of angiogenesis with enhanced levels of both pro- and anti-angiogenesis factors. Despite inhibition of COX-2-dependent-PGE_2 may reduce tumor progression (Steinbach et al., 2000), the coincident effects on vascular PGI_2 may have undesirable effects, predisposing to thrombosis (Grosser et al., 2006) and modulating angiogenesis. These contrasting effects may contribute to the marked variability in the reduction of the number of colorectal polyps detected in patients with FAP by chronic treatment with celecoxib.

It is noteworthy that recently Pfizer has voluntarily withdrawn celecoxib's indication for reduction of colorectal polyps in patients with FAP. The sponsor was unable to provide further efficacy data, as a result of slow enrolment in an ongoing clinical trial(European Medicine Agency, 2011).

Authorship Contributions:

Participated in research design: Bertario, Dixon, Steinhilber, Patrignani

Conducted experiments: Dovizio, Tacconelli, Ricciotti, Bruno, Anzellotti, Di Francesco, Sala, Signoroni, Dixon

Contributed new reagents or analytic tools: Ricciotti, Lawson

Performed data analysis: Dovizio, Tacconelli, Bruno, Anzellotti, Di Francesco, Bertario, FitzGerald, Patrignani

Contributed to the writing of the manuscript: Dovizio, Tacconelli, Ricciotti

Wrote the manuscript: FitzGerald, Patrignani

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Footnotes

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Authors declare no conflict of interest.

MD, ST and ER contributed equally

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Legends for Figures

Figure 1. Biosynthesis of prostanoids *in vivo* in Apc$^{Min/+}$mice vs wild-type. Urinary excretion of dinor-TX-M(Panel **a**), PGI-M(Panel **b**) and PGE-M(Panel **c**) in *Apc*$^{Min/+}$mice and wild-type(WT) mice. Data were expressed as the mean±SEM. Five mice were used for each group. Metabolite levels were corrected for urinary creatinine and expressed as ng/mg creatinine. *p<0.05; **p<0.01 vs wild-type mice using Student's t-test.

Figure 2. Biosynthesis of prostanoids in FAP patients before and after celecoxib treatment (400mg/BID, for 7 consecutive days). Systemic production of TXA_2, PGI_2, and PGE_2 was assessed by measuring their enzymatic urinary metabolites, ie TX-M, PGI-M and PGE-M (Panels **a**, **b** and **c**, respectively) in samples collected before treatment(from 8pm to 8am) and on the 8th day after the last dose of the drug. Data were presented as box and whiskers, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and whiskers represent the highest and lowest values(n=9). Metabolite levels were corrected for urinary creatinine and expressed as ng/mg creatinine. *p<0.05; **p<0.01 vs baseline using Wilcoxon matched pairs test.

Figure 3. Prostanoid biosynthesis and protein expression in human adenocarcinoma cell line HT-29, human platelets and HT-29 co-cultured with platelets. Panel **a** shows TXB_2 generation by HT-29 alone(HT), HT-29 co-cultured with platelets(HT+PLT) and platelets alone(PLT) for 20hr. In some experiments, the effects of platelets pre-treated with aspirin 300μM was studied. Mean±SEM of 5-10 different experiments are shown. **p<0.01 vs HT-29 alone and platelets alone; $^{§}p$<0.01 vs untreated platelets; #p<0.01 vs HT-29 co-cultured with untreated platelets(HT+PLT). Repeated measures ANOVA was used for statistical analysis.

Panel **b** shows the effect of rofecoxib(0.3μM) on TXB_2 generation by HT-29 alone, platelets alone and HT-29 co-cultured with platelets for 20hr. Means of 2 different experiments performed in triplicate are

shown. Panel **c** shows the effect of rofecoxib(0.3μM) on PGE_2 generation by HT-29 alone. **p*<0.05 vs DMSO(vehicle). Student's *t*-test was used for statistical analysis.

In panel **d**, COX-1, COX-2, TXAS and mPGES-1 protein levels were assessed by Western blot.

Figure 4. Effect of the administration of celecoxib on circulating angiogenesis biomarkers (FGF-2, VEGF-D, VEGF-A and angiogenin) in FAP patients. Heparinized blood samples were collected before dosing and on the 7th day 4 hr after the last morning dose of celecoxib to assess the levels of circulating angiogenesis proteins.Data were presented as box and whiskers(n=9). **p*<0.05; ***p*<0.01 vs baseline using Wilcoxon matched pairs test.

Figure 5. Effect of the administration of celecoxib on circulating angiogenesis biomarkers (Angiopoietin-2, PDGF-BB, TIMP-2, sICAM-1) in FAP patients. Heparinized blood samples were collected before dosing and on the 7th day 4hr after the last morning dose of celecoxib to assess the levels of circulating angiogenesis proteins. Data were presented as box and whiskers(n=9). **p*<0.05; ***p*<0.01 vs baseline using Wilcoxon matched pairs test.

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Table 1. Baseline characteristics of FAP patients and healthy controls

Variable	FAP patients (n=9)	Controls (n=9)
Age (mean+SD), y[§]	44±11	45±10
Female, %	5, 55	5, 55
CRP, mg/l, mean±SD,	10±19,	4±2,
Median(range)[†]	3(1.10-61.60)	3.30(1.40-9.50)
Hypertension, %	0	0
LDL-cholesterol, mg/dl, mean±SD,	105±26,	106±27,
median(range)[†]	97(65-144)	103(60-147)
Cotinine, ng/ml, mean±SD,	7.20±19.60,	Not determined
median(range)	0.62(0.40-59.40)	
TX-M, ng/mg creatinine, mean±SD,	1.19±0.84,	0.20±0.10,
median(range)[†]	0.93(0.10-2.90)**	0.20(0.09-0.40)
PGI-M, ng/mg creatinine, mean±SD,	0.12±0.09,	0.12±0.05,
median(range)[†]	0.09(0.03-0.33)	0.10(0.05-0.20)
PGE-M, ng/mg creatinine, mean±SD,	22.30±16.20,	10.30±5,
median(range)[†]	21.52(2.82-55.40)*	11.22(4-16)
APC mutation, %	8, 88.9	Not determined
MYH mutation, %	1, 11.1	Not determined

Arterial hypertension, defined as current systolic/diastolic blood pressure ≥140/90 mm Hg(referring to the 1999 World Health Organization criteria for the diagnosis of hypertension).[§]Data were compared by Student's *t*-test; [†]data were compared by Mann-Whitney test; *$p<0.05$ vs healthy controls; **$p<0.01$ vs healthy controls.

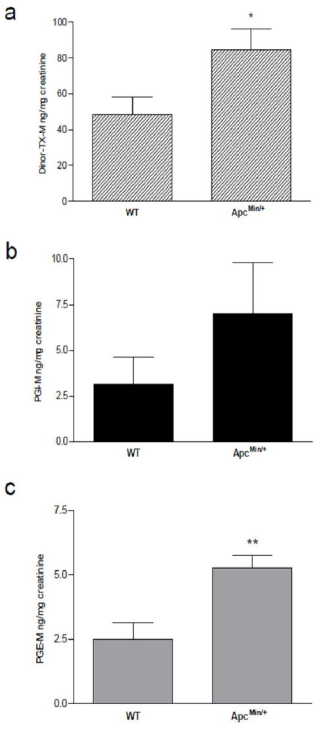

a
Dinor-TX-M ng/mg creatinine
100
80
60
40
20
0
*
WT
Apc Min/+
b
PGI-M ng/mg creatinine
10.0
7.5
5.0
2.5
0.0
WT
Apc Min/+
c
PGE-M ng/mg creatinine
7.5
5.0
2.5
0.0
**
WT
Apc Min/+

Figure 1

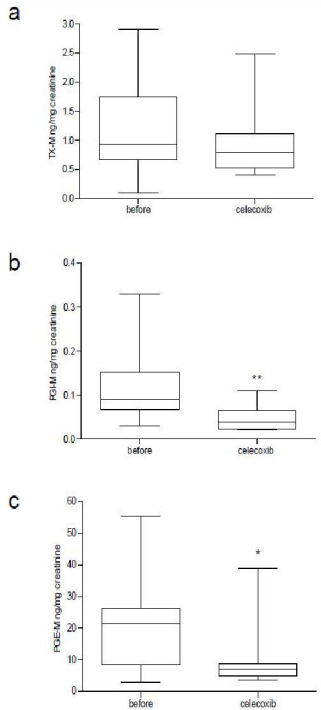
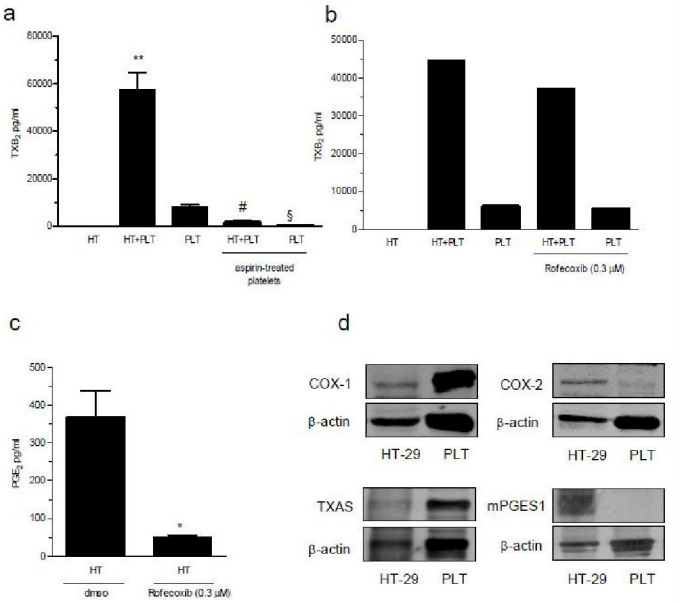

a
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TXA-M:ng/mg creatinine
before
celecoxib
b
0.4
0.3
0.2
0.1
0.0
PGIM:ng/mg creatinine
**
before
celecoxib
c
60
50
40
30
20
10
0
PGEM:ng/mg creatinine
*
before
celecoxib

Figure 2


a
60000
40000
20000
0
TXB2 pg/ml
**
#
§
HT
HT+PLT
PLT
HT+PLT
PLT
aspirin-treated platelets
b
50000
40000
30000
20000
10000
0
TXB2 pg/ml
HT
HT+PLT
PLT
HT+PLT
PLT
Rofecoxib (0.3 μM)
c
500
400
300
200
100
0
PGE2 pg/ml
*
HT
HT
dmso
Rofecoxib (0.3 μM)
d
COX-1
β-actin
HT-29
PLT
COX-2
β-actin
HT-29
PLT
TXAS
β-actin
HT-29
PLT
mPGES1
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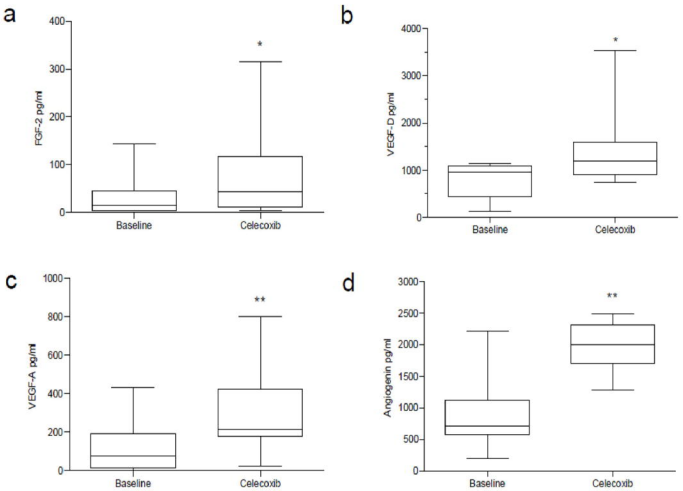
HT-29
PLT

Figure 3


a
FGF-2 pg/ml
400
300
200
100
0
*
Baseline
Celecoxib
b
VEGF-D pg/ml
4000
3000
2000
1000
0
*
Baseline
Celecoxib
c
VEGF-A pg/ml
1000
800
600
400
200
0
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Baseline
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d
Angiogenin pg/ml
3000
2500
2000
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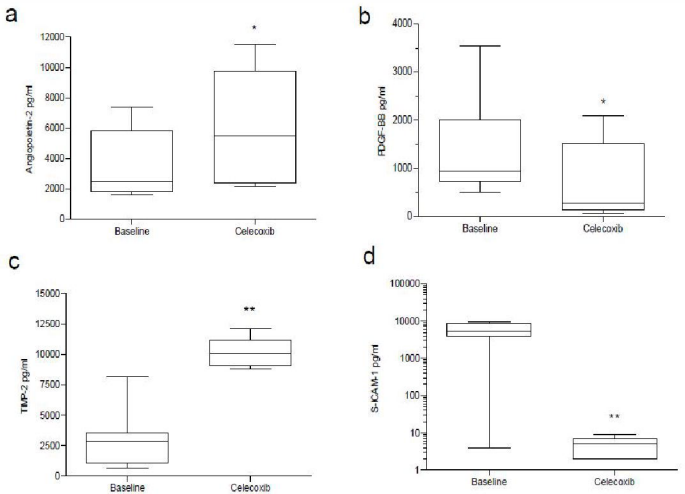
Baseline
Celecoxib

Figure 4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Angiopoietin-2 pg/ml
12000
10000
8000
6000
4000
2000
0
*
Baseline
Celecoxib
b
RDGF-BB pg/ml
4000
3000
2000
1000
0
*
Baseline
Celecoxib
c
TIMP-2 pg/ml
15000
12500
10000
7500
5000
2500
0
**
Baseline
Celecoxib
d
S-ICAM-1 pg/ml
100000
10000
1000
100
10
1
**
Baseline
Celecoxib

Figure 5

Effects of celecoxib on prostanoid biosynthesis and circulating angiogenesis proteins in familial adenomatous polyposis

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Supplementary Material and Methods

Human Angiogenesis Protein Array

The levels of human angiogenesis markers heparin-binding epidermal growth factor(HB-EGF), tissue inhibitor of metalloproteinases(TIMP)-1 and -2, hepatocyte growth factor(HGF), vascular endothelial growth factor(VEGF)A and D, interferon-inducible protein(IP)-10, platelet derived growth factor(PDGF)-BB, keratinocyte growth factor(KGF), soluble intercellular adhesion molecule s(ICAM)-1, fibroblast growth factor(FGF)-2, angiogenin, and angiopoietin-1 and -2, were assessed in plasma of FAP patients before dosing and on the 7^{th} day, 4 hr after the last morning dose of celecoxib, using an antibody protein array kit(US Biomax, Inc.) according to the vendors protocol.

Assessment of 2,3-dinor-6-keto-PGF$_{1\alpha}$ (PGI-M) by HPLC-RIA

The method consists of i) urine extraction (by using Sep-Pack C_{18} cartridges, Waters Associates, Milan, Italy), ii) separation of PGI-M from 6-keto-PGF$_{1\alpha}$ by reversed-phase(RP)-HPLC and iii) quantification of PGI-M concentrations in HPLC fractions, corresponding to the retention time (RT) of PGI-M, by a sensitive RIA. The RIA used a commercially available polyclonal antibody (Assay Designs Inc, Ann Arbor, MI, USA) developed against PGI-M, but showing high cross-reactivity with 6-ketoPGF$_{1\alpha}$ (89%). Since the anti-PGI-M antibody showed a very low cross-reactivity with TXB_2 (<0.01%), we developed a RP-HPLC method where PGI-M co-eluted with TXB_2. Thus, recovery evaluation of endogenous PGI-M was performed by assessing [^{3}H]-TXB_2 content (previously added to urine aliquots), in the same HPLC fractions that were analyzed for PGI-M by RIA.

Briefly, 3000 cpm of [^{3}H]-TXB_2 (specific activity, 150mCi/mmol; GE Healthcare Life Science, Milan, Italy) was added to 10 ml aliquots of urine samples for recovery evaluation. After adjustment of urine pH to 4.0-4.5 with formic acid, prostanoids were extracted by using Sep-Pack C_{18} cartridges and eluted with ethyl acetate and then dried with a Speed-Vac (Speed Vac Plus, Savant Instruments Inc, Farmindgale, NY, USA). All solvents were from Carlo Erba Reagents (Milan, Italy). After dryness, prostanoids were resuspended with 0.1ml water and 0.1 ml methanol, and injected into an HPLC system.

The HPLC system consisted of a Beckman model HPLC pump (Beckman, Fullerton, CA, USA), a Beckman 126 programmable solvent delivery module and a Beckman 168 programmable ultraviolet detector, with a Beckman System GOLD 32 Karat Software. A reversed-phase column was used, i.e. Novapak C18 (3.9x150mm) (Waters Associates, Milan, Italy). The mobile phase consisted of A, water/acetic acid [100:0.1 (v/v)] and B, acetonitrile/acetic acid [100:0.1(v/v)] as follows: 79% and 21% respectively, at a flow rate of 1 ml/min for 18 minutes. Then, the mobile phase was changed into A 50% and B 50%, at a flow rate of 1.5ml/min for the following 7 minutes. The absorbance was assessed at 195 nm. In these conditions, the retention times (RTs) of authentic 6-keto-PGF$_{1\alpha}$, TXB_2 and PGI-M (Cayman Chemical, Ann Arbor, MI, USA) were 14.5, 20.3 and 20.5 min, respectively. One-minute- HPLC fractions were collected and dried with the Speed-Vac and then resuspended in 0.5ml of phosphate buffer (0.02M, pH7.4). HPLC fractions eluted from 18 to 22 minutes were mixed, evaluated for [^{3}H]-TXB_2 content (to calculate the recovery that was 50%) and then stored at –80°C until the measurement of PGI-M by RIA. The assay used 2500cpm of [^{3}H]-6-keto-PGF$_{1\alpha}$ (specific activity, 185mCi/mmol; GE Healthcare Life Science, Milan, Italy) and an anti-PGI-M polyclonal antibody (at the final dilution of 1:100000) in a final volume of 1.5 ml of phosphate buffer (0.02M, pH 7.4) and was incubated for 18-24 h at 4°C. Separation of the antibody-bound from free [^{3}H]-6-keto-PGF$_{1\alpha}$ was carried out by rapidly adding 0.05ml of bovine serum albumin and 0.1 ml of a charcoal suspension (100 mg/ml) followed by centrifugation at 3000 rpm/10min at 4°C. The detection limit of the RIA was 2 pg/ml. We assessed the intra-assay and inter-assay coefficients of variation (CV) by evaluating PGI-M concentrations in urine samples (collected from 3 healthy volunteers treated with Naproxen 500 mg - to suppress endogenous PGI_2

generation - after providing their informed consent and approval by the institutional Ethical Committee) spiked with known concentrations of PGI-M: Low Quality Control (QC) (200 pg/ml added), Mid QC (400 pg/ml added) and High QC (800 pg/ml added). The intra-assay CVs (n=6) were 9, 6, and 9%, for low QC, mid QC and high QC, respectively. The inter-assay CVs (n=3) were 13, 7 and 11%, for low QC, mid QC and high QC, respectively .

Inhibition of human whole blood COX-1 and COX-2 by celecoxib in vitro

Approval from Ethics Committee of "G. d'Annunzio" University, Chieti, Italy, was obtained for peripheral whole blood collection from 3 healthy male volunteers(age range: 25-29 years) and informed consent was obtained from each subject. The same healthy volunteers were studied on different occasions. Celecoxib(1.9-19000mg/ml, kindly provided by Searle, USA) was dissolved in DMSO, and 2µl aliquots of the solutions were pipetted directly into test tubes to give final concentrations of 3.8-38000ng/ml in 1ml of whole blood. The effect of celecoxib on inducible monocyte PGE_2 synthesis was assessed by incubating the drug with heparinized peripheral venous blood samples, in the presence of lipopolysaccharide (LPS, 10µg/ml) for 24 hr at 37 °C, as previously described (Patrignani et al., 1994). The contribution of platelet COX-1 activity was suppressed by pretreating the subjects with 300mg of aspirin 48 hr before sampling. The effect of celecoxib on platelet COX-1 activity was assessed by incubating the drug with 1ml of whole blood samples (drawn from the same donors in aspirin-free periods) and then they were allowed to clot for 1 hr at 37°C (Patrono et al., 1980). Plasma PGE_2 and serum TXB_2 were measured by validated RIA (Patrignani et al., 1994; Patrono et al., 1980).

Culture of human colon adenocarcinoma cell line (HT-29)

HT-29 cell line was obtained from European Collection of Cell Cultures(ECACC, Salisbury, UK) and the quality control and authentication procedures were performed by ECACC. Cells were routinely tested for mycoplasma contamination by the authors using a PCR approach. HT-29 cells were cultured in McCoy's 5A medium (Invitrogen, Milan, Italy) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and L-glutamine 2mM. For every experiment, $1x10^6$ cells were seeded in 6-multiwell containing 2 ml of McCoy 5A supplemented with fetal bovine serum (FBS) 0.5% and 10 µg/ml of Polimyxin B sulphate(Sigma-Aldrich, Milan, Italy) for 20 hr, alone or co-cultured with platelets.

Preparation of washed human platelets

Human platelets were freshly isolated from leukocyte concentrates obtained from Stadtische Kliniken Hochst (Frankfurt, Germany) as previously described (Albert et al., 2002). In brief, venous blood was collected from healthy adult donors and leukocyte concentrates were prepared by centrifugation (4000xg, 20 min, 20°C). Leukocyte concentrate was sedimented in 5% dextran solution(Sigma-Aldrich) and the supernatant was stratified in lymphocytes separation medium (PAA) by centrifugation (800xg, 10min at room temperature). After centrifugation, platelet-rich plasma (PRP) was obtained. PRP was then mixed with PBS, pH 5.9 (3:2, v/v), centrifuged (2000xg, 15 min, room temperature), and pelleted platelets were resuspended in PBS, pH 5.9/0.9% NaCl (1:1, v/v), washed by centrifugation (2000xg, 10 min, room temperature), and finally re-suspended in McCoy 5A medium containing 0.5% FBS and 10 µg/ml of Polymixyn B sulphate.

Western blot analyses

As previously described (Di Francesco et al., 2009), cells were lysed in Triton 1% with 1mM of PMSF and 50µg of total proteins were loaded onto 4-9% Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Separated proteins were transferred to nitrocellulose membranes (GE Healthcare, Milan, Italy) and incubated with anti-COX-2 (1:1000, mouse monoclonal, Cayman Chemical, USA), anti-COX-1 (1:1000, rabbit polyclonal, Cayman

Chemical), anti-mPGES-1 (1:500, rabbit polyclonal, Cayman Chemical), anti-TXAS (1:1000, rabbit polyclonal, Cayman Chemical) and anti-β-actin (1:2000, goat polyclonal, Santa Cruz, USA) overnight at 4°C. Then, the membranes were washed in TBS-Tween-20 and incubated with the secondary antibodies. Detection of bands was performed using a LI-COR Odyssey two-color Western detection system (LI-COR Biosciences, Biosciences, Lincoln, NE, USA), according to the instructions of the manufacturer.

Supplementary Table S1. Circulating angiogenesis proteins detected before and after dosing with celecoxib, in 9 FAP patients

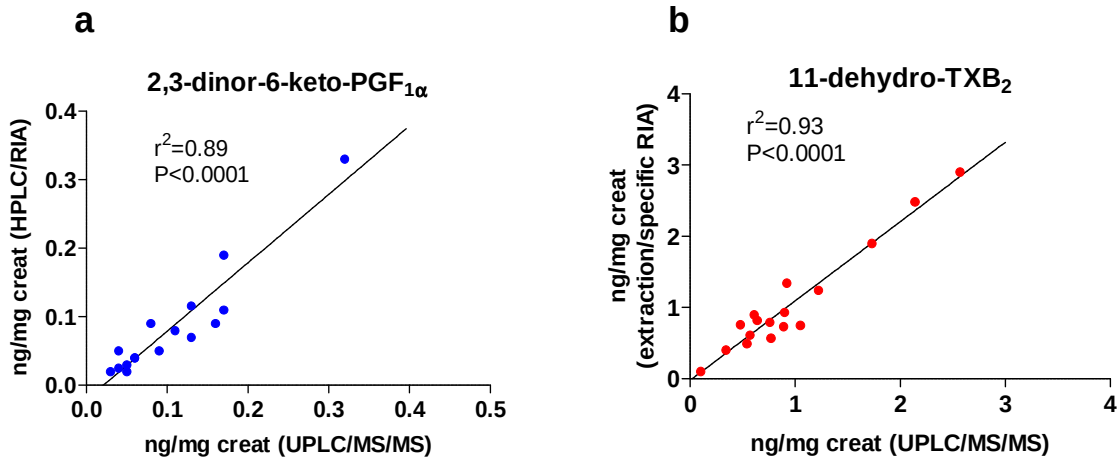
	Baseline	Post-treatment
HB-EGF	624±379, 666(106-1033)	1248±1807, 387(121-5304)
TIMP-1	4494±1107, 4525(2424-6001)	3675±1121, 3645(2053-5892)
TIMP-2	2986±2255, 2892(667-8203)	10144±1227, 10021(8821-12126)**
HGF	555±356, 489(91-1232)	659±303.5, 648(51-1099)
Angiopoietin-1	1012±899, 983(112-2906)	1084±608.5, 1375(279-1811)
Angiopoietin-2	3515±2197, 2480(1589-7385)	5799±3727, 5479(2168-11516)*
Angiogenin	890±583, 715(196-2216)	1996±381, 1993(1287-2487)**
IP-10	670±225.5, 538(437-1032)	887±483, 808(264-1490)
PDGF-BB	1380±981, 944(511-3550)	703±834, 271(70-2103)*
KGF	241±126, 224(65-484)	271±188, 322(28-572)
VEGF-A	123±137, 76(1-432)	298±227, 216(23-803)**
VEGF-D	757±377, 956(122-1145)	1429±848, 1188(739-3539)*
sICAM-1	5642±3043, 5394(4-9614)	4.9±2.6, 5(2-9)**
FGF-2	32±46, 14(4-143)	77±104, 44(4-315)*

Data were reported as pg/ml of mean±SD, median(range).
*P<0.05, **P<0.01 vs baseline; Wilcoxon matched pairs test.

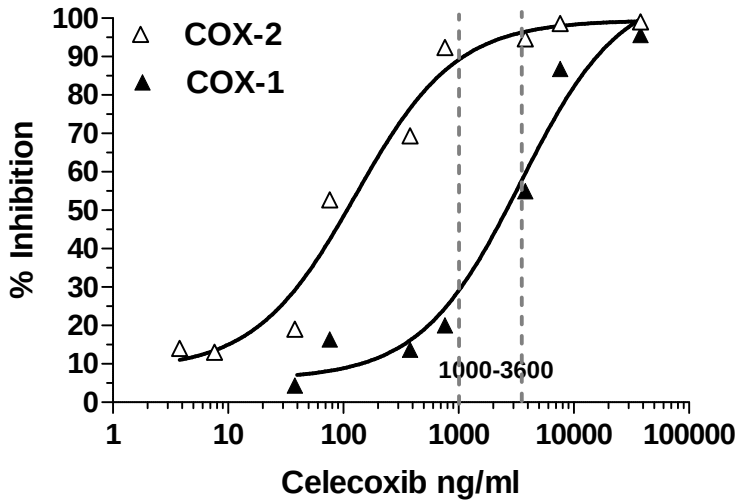
Supplementary Table S2. Linear multiple regression analysis among urinary biomarkers of prostanoid biosynthesis in vivo and circulating angiogenesis proteins in FAP patients.

Multiple Linear Regression Analysis to predict TX-M			
Variable	**β***	**SEM****	***P* value**
PGI-M	0.80	0.27	0.02
Angiopoietin-2	0.25	0.31	0.44
Angiogenin	0.31	0.24	0.23
FGF-2	0.03	0.16	0.83
Multiple Linear Regression Analysis to predict PGI-M			
Variable	**β***	**SEM****	***P* value**
TX-M	0.50	0.17	0.01
Angiopoietin-2	0.26	0.24	0.29
Angiogenin	-0.44	0.16	0.02
FGF-2	-0.24	0.10	0.03
Multiple Linear Regression Analysis to predict PGE-M			
Variable	**β***	**SEM****	***P* value**
TX-M	0.77	0.20	0.003
PGI-M	0.50	0.26	0.07
Angiopoietin-2	-0.47	0.23	0.066
Angiogenin	-0.20	0.19	0.32
FGF-2	0.13	0.12	0.29

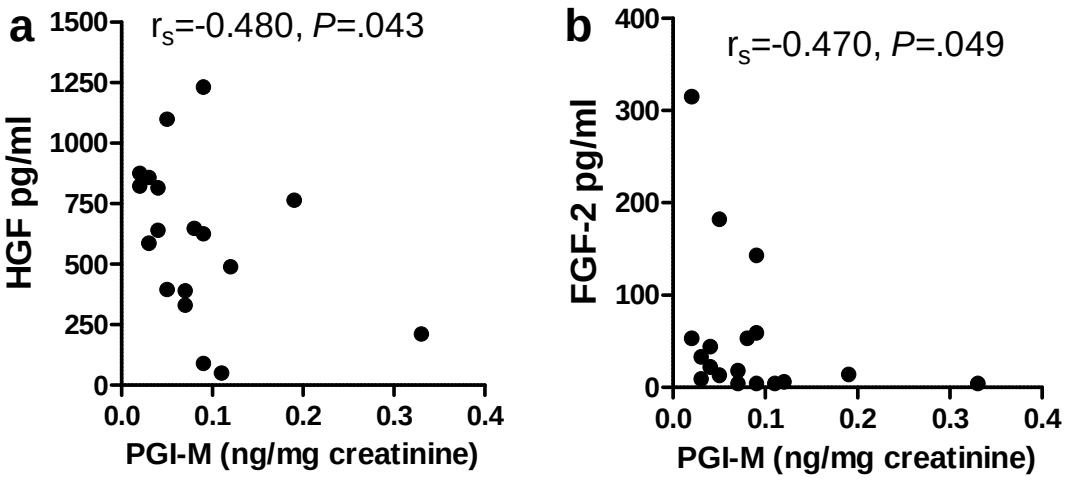
*β: standardized coefficient; **SEM: mean standard error



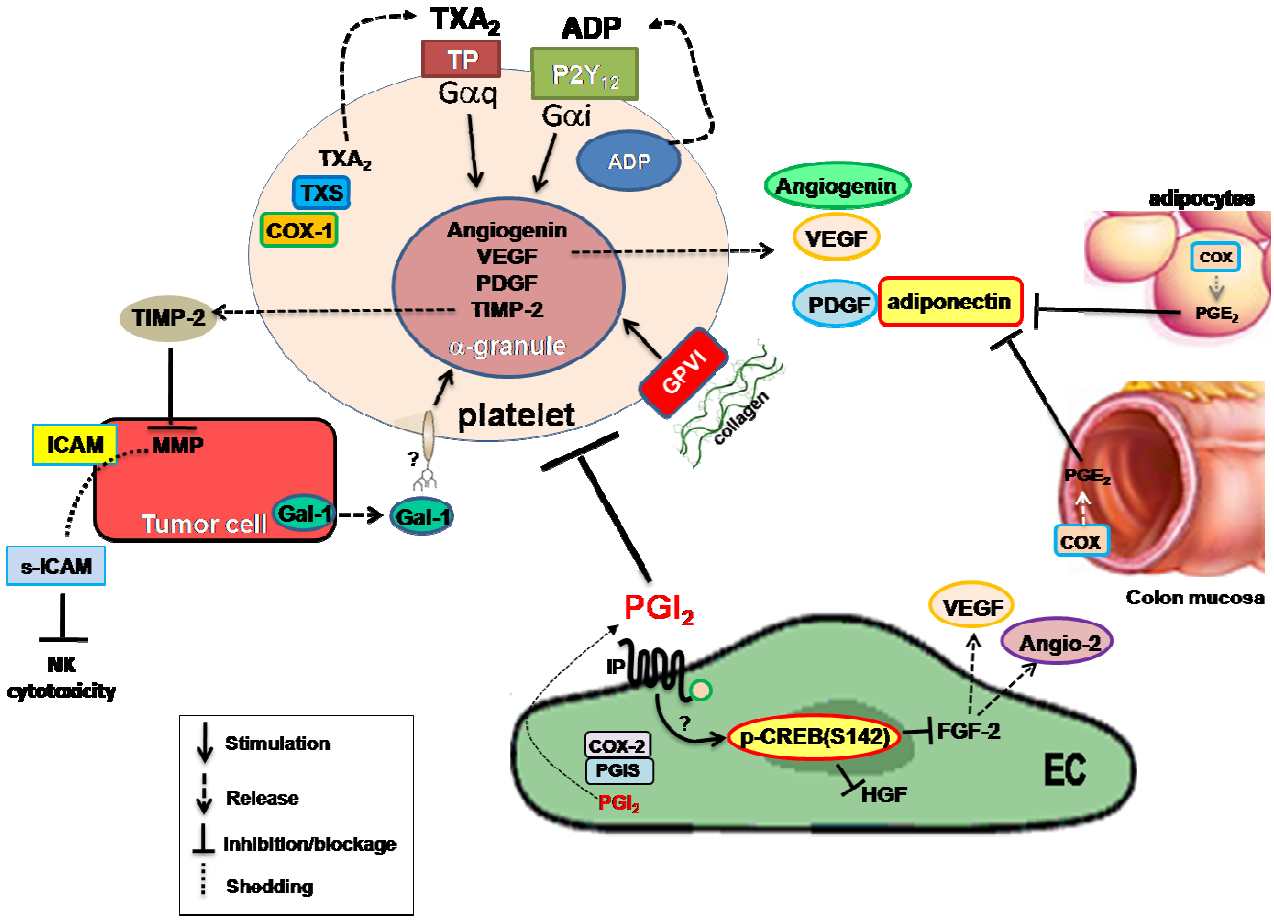
Supplementary Figure S1. Comparison of measurements of major urinary enzymatic metabolites of PGI_2 and TXB_2, i.e. 2,3-dinor-6-keto-$PGF_{1\alpha}$ (PGI-M) (a) and 11-dehydro-TXB_2, (TX-M) (b), respectively, by specific radioimmunoassay(RIAs) and ultra performance liquid chromatography tandem mass spectrometric (UPLC/MS/MS). Urinary samples (n=18) collected from FAP patients before and after dosing with celecoxib were assessed for the levels of PGI-M and TX-M using specific RIAs [PGI-M analysis is described in Supplementary Methods, while TX-M analysis was performed by a previously described and validated technique (Ciabattoni et al., 1987)] or UPLC/MS/MS (Song et al., 2007). The least-squares line and coefficient of determination r^2 were calculated by linear regression analysis using PRISM (GraphPad, San Diego, California).



Supplementary Figure S2. Effects of celecoxib on platelet COX-1 and monocyte COX-2 activities in human whole blood. Concentration-response curves for inhibition of COX-1 (▲) and COX-2 (Δ) by celecoxib *in vitro* were plotted. Celecoxib caused a concentration-dependent inhibition of COX-1 and COX-2 *in vitro* with IC_{50} values of 3444(2116-5604) and 128(72-228)ng/ml (mean, 95%CI), respectively. The range of plasma concentrations detected at 4 hr after dosing with celecoxib in FAP patients was reported.



Supplementary Figure S3. Statistical dependence between urinary levels of PGI-M and angiogenesis proteins in FAP patients. The Spearman's rank correlation coefficient(r_s) was calculated to quantify the statistical dependence between urinary levels of PGI-M and angiogenesis proteins in FAP patients. A statistically significant inverse Spearman's rank correlation was found between the urinary levels of PGI-M, assessed at baseline and after dosing with celecoxib, and HGF and FGF-2 (Panel a and b, respectively).



Supplementary Figure S4. Possible mechanisms involved in the regulation of the generation and release of angiogenesis markers by PGI_2 from endothelial cells(EC) and platelets.
In tumorigenesis, endothelial dysfunction and increased vascular permeability occur (Padua et al., 2008). This can facilitate the interaction of platelets with tumor components, such as galectin(GAL)-1, belonging to the galectin family of endogenous lectins(Pacienza et al., 2008) and/or extracellular matrix and basement membrane proteins, such as collagen. Activated platelets generate TXA_2 which participates to the release of ADP from dense granules. In platelets, the activation of TXA_2 receptor(TP), ADP receptor($P2Y_{12}$), collagen receptor(GPVI) and the binding of GAL-1 to surface components, lead to the release of α-granule content, rich in angiogenic proteins (both pro- and anti-angiogenic factors) (Italiano et al., 2008; Coppinger et al., 2007). Endothelial PGI_2, by interacting with PGI_2 receptor(IP), restrains platelet activation and α-granule release (Menter et al., 1987). Moreover, in endothelial cells, PGI_2 can restrain HGF and FGF-2 expression by interfering with the binding of CREB(cAMP responsive element binding protein) and phospho-CREB to the CRE of their promoters (Kothapalli et al., 2003). This might occur through CREB phosphorylation at Ser142 which has been shown to block the formation of CREB-CBP(CREB binding protein) complexes and the activation of target genes (Sun et al. 1994). In the presence of the inhibition of endothelial COX-2-dependent PGI_2, enhanced FGF-2 can participate in increased circulation levels of VEGF-A, VEGF-D and Angio(angiopoietin)-2. In fact, it has been reported that expression of both VEGF and Angio-2 is induced by FGF-2 (Seghezzi et al., 1998; Fujii and Kuwano, 2010). Angio-2 concentrations have been shown to be a marker of a poor prognosis in different types of cancers, ie breast cancer, non-small cell lung cancer and colorectal cancer (Shim et al., 2007). Different mechanisms may explain these findings: i) up-regulation of Angio-2 causes the exposure of the capillary endothelium to other angiogenic growth factors, including VEGF, that promote new vessel growth (Lobov et al., 2002); ii) enhanced levels of

Angio-2 may chemoattract pro-angiogenic Tie-2 expressing monocyte/macrophages and stimulate them to express tumor promoting factors (Lewis et al., 2007).
In the presence of vascular COX-2-dependent PGI_2 inhibition, platelet activation participates to the increase of circulating levels of pro-angiogenic factors, such as VEGF, PDGF and angiogenin (Folkman et al., 2007), but also anti-angiogenic factors, such as TIMP-2. The release of TIMP-2 may lead to the inhibition of MMP(matrix metallo-proteinase) activity in tumor cells thus preventing ICAM-1 shedding from tumor cells (Fiore et al., 2002). This effect is important to restrain tumor cell evasion of immune surveillance. In fact, ICAM-1-mediated cell-cell adhesion is essential for various immunologic functions, such as natural killer(NK) cell-mediated cytotoxicity (Fiore et al., 2002).
PDGF-BB, a major PDGF-isoform stored in α-granules of platelets, governs cancer cell proliferation and survival and plays a role in angiogenesis (Heldin and Westermark, 1990). In a tumor-bearing mouse, it has been shown that platelets may uptake PDGF and a sustained and persistent elevation of PDGF levels in platelets is detected (Klement et al. 2009). The decrease of circulating levels of PDGF-BB observed after PGI_2 inhibition by celecoxib detected in FAP patients, in the present study, suggests that this prostanoid influences the uptake/release machinery operating in platelets which control α-granule content. Alternatively, it can be hypothesized that depression of circulating levels of PDGF-BB may be dependent on the increase of circulating levels of adiponectin, which is able to bind PDGF-BB. In fact, adiponectin, abundantly present in human plasma, can bind directly to PDGF-BB and regulates postreceptor signal in vascular smooth muscle cells (Arita et al., 2002). Interestingly, it has been found an inverse association between total adiponectin and colorectal adenoma (Wei et al., 2005).

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