EICOSANOX

SIXTH FRAMEWORK PROGRAMME

Project no **005033**

Project acronym:
EICOSANOX

Project full title:
Eicosanoids and Nitric Oxide:
Mediators of Cardiovascular, Cerebral & Neoplastic Diseases

Instrument: **Integrated Project**
Thematic Priority: **Life sciences, genomics and biotechnology for health**

Publishable Final Activity Report EICOSANOX

Period covered:	**January 1, 2005 - December 31, 2009**
Date of preparation:	**April 3, 2010**
Amended:	**May 3, 2010**
Start date of project:	**January 1, 2005**
Duration:	**5 years**
Project director:	**Jesper Z. Haeggström**
Project coordinator organisation:	**Karolinska Institutet**

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1. Publishable final activity report

The publishable final activity report of the activities and results generated within the Eicosanox project over its full duration, 2005-2009.

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1.3. Project logotype and Website
1.4. Words from the Director
1.5. General overview of the research field
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1.9. List of Eicosanox top-ranked publications
1.10. List of the 25 most cited articles

1.1. Project objectives

In the work plan, the 15 work packages are structured in five clusters (A-E) each containing 2-3 workpackages. The titles of these illustrate the general project objectives:

A. MOLECULAR STUDIES OF THE EICOSANOID AND NO CASCADES
WP1 Biochemical and molecular studies of COX, PG synthases, and NO
(In order to reinforce the interactions amongst investigators engaged in NO research, WP1 was restructured after the first year and the tasks divided between WP2, WP5, and WP6.)
WP2 Biochemical and molecular studies of COX, LOX isoenzymes, LTA4H, and LTC4S:s
WP3 Gene regulation of enzymes in the eicosanoid cascade

B. ROLE OF EICOSANOIDS AND NO IN IMMUNOLOGY AND INFLAMMATION
WP4 Role of eicosanoids in inflammatory and immune responses
WP5 Role of NO in inflammatory and immune responses

C. DISEASES OF THE CARDIOVASCULAR AND CENTRAL NERVOUS SYSTEMS
WP6 COX and PG synthases in cardiovascular diseases
WP7 LOX products as mediators of cardiovascular diseases
WP8 NOS, a friend or foe in the cardiovascular system
WP9 COX, PG synthases and NO in diseases of the central nervous system

D. CANCER AND ANGIOGENESIS
WP10 COX, PG synthases, and NO in oncogenesis
WP11 LOX isoenzymes in tumour development
WP12 COX, PG synthases, and NOS in angiogenesis

E. INFRASTRUCTURE AND COMMUNICATION
WP13 Core facilities
WP14 Training, dissemination, implementation, and technology transfer
WP15 Management

1.2. Contractors involved

In EICOSANOX, we have brought together 15 top European laboratories within Eicosanoid and NO research, one world leading Canadian group and two SMEs in a large multidisciplinary and highly competitive Consortium. The partners (contractors involved) are described below in tabular format.

Partner role	Particip. no.	Partner name	Partner short name	Country	Date enter project	Date exit project
CO	1	Karolinska Institutet (4 labs)	KI	Sweden	1	60
CR	2	University of Frankfurt (3 labs)	UFrank	Germany	1	60
CR	3	University of Milano	UNIMI	Italy	1	60
CR	5	G. D'Annunzio University	UDAnn	Italy	1	60
CR	6	University College London	UCL	U.K.	1	60
CR	7	The William Harvey Institute	QMUL	U.K.	1	60
CR	8	University College Dublin	NUID/UCD	Ireland	1	60
CR	9	NicOx S.A.	NicOx	France	1	60
CR	10	Univ. Autonoma de Madrid (2 labs)	UAM	Spain	1	60
CR	11	Biolipox AB	Biolipox	Sweden	1	48
CR	12	Queens University	QueensU	Canada	1	60
CR	13	Humboldt University	Charité	Germany	1	60
CR	14	Orexo AB	Orexo	Sweden	49	60

CO = coordinator
CR= contractor

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1.3. Project logo:

Project website: http://www.eicosanox.org/

1.4. Words from the Director

The Eicosanoids and nitric oxide (NO) are signalling molecules in many physiological and pathological processes, including severe endemic diseases, e.g. atherosclerosis, myocardial infarction, thrombosis, dementia and cancer. In addition, these inflammatory mediators are involved in many other disorders, e.g., diseases of the respiratory system (asthma), autoimmune disorders, and sequelae after severe trauma. Together, these diseases account for the vast majority of morbidity and mortality in Europe with a major socio-economic impact.

EICOSANOX is an international research consortium focused on "*Eicosanoids and Nitric Oxide*" in cardio-vascular, cerebral and neoplastic diseases. Over 5 years, 16 European research groups and 2 SMEs from 7 countries have jointly worked on an ambitious program aiming at increasing the knowledge about these autacoids with the goal to develop novel therapeutic strategies and medical treatments.

The objectives of EICOSANOX include (1) elucidating molecular properties of proteins in the eicosanoid and NO cascades; (2) delineating the role of these mediators in immunology and inflammation, and specifically in (3) cardiovascular and nervous diseases as well as (4) cancer and angiogenesis. In each of these areas, we have made significant advances, some of which can best be described as *groundbreaking with deep consequences for our understanding of the signalling systems as well as for the clinical use of drugs interfering with eicosanoids and NO.*

Due to the wealth of excellent results, only a few can be briefly commented here. Thus, for LOX enzymes, novel stimulatory factors, including CLP and glycerides, were identified. *A particular high-light was the high-resolution crystal structure of the integral membrane protein human LTC4 synthase, a remarkable scientific achievement which has opened novel avenues for drug design*. Furthermore, we have characterized gene expression profiles and demonstrated *increased mRNA levels of 5-LO pathway proteins in human atherosclerotic lesions that were found to correlate with plaque instability and LTA4 hydrolase was identified as a potential drug target.* Genes and proteins have been characterized, including the *identification of gpr17* as a novel leukotriene receptor and potential drug target. A *novel system for production of NO from nitrate and nitrite* has been elucidated and its surprising *physiological effects on the cardiovascular system* have been uncovered. Also, angiotensin II has been shown to impair endothelial function by *Tyr-phosphorylation of eNOS*. The regulation of COX-2 expression in macrophages has been described in detail and our understanding of this enzyme in tumor development has increased significantly. Cardiovascular side effects of various coxibs have been assessed and, importantly, it was found that *use traditional NSAIDs is also associated with these cardiovascular risks*, perhaps with the exception of naproxen. These clinical studies have changed our view on NSAID side effects with direct *implications for the clinical use of these important drugs*. In addition, *promising biomarkers* for predicting the relative risk of myocardial infarction associated with different NSAIDs have been identified. For our SME partners, I would like to specifically mention Biolipox's striking commercial success with mPGES-1 inhibitors, the identification of eoxins, and development and characterization of novel NO-releasing NSAIDs.

Because of these achievements, members of the consortium have regularly appeared in various European public media, filed 15 patents and created 3 spin-off companies.

Clearly, our project has increased the knowledge about the mechanisms by which eicosanoids and NO trigger and maintain physiological and pathophysiological processes, in both health and disease. In addition, we have identified new drug targets, improved existing therapies and developed novel drugs and therapeutic strategies. Hence, it seems more than likely that the long-term objectives of EICOSANOX, i.e., to significantly improve the health, and quality of life of the citizens of Europe, will be accomplished.

I dare to say that EICOSANOX has been a great success with a scientific progress that has reached far beyond our expectations. Thus, we have produced an impressive amount of high-quality science that has reached the absolute top of international prestigious journals, including, *New Engl J Med*, *Nature*, *Mol Cell, Lancet*, *Nat Chem Biol, Nat Immunol, TiBS, J Clin Invest*, *J Exp Med*, *Circulation, J Am Coll Cardiol, Genes Dev, Blood, EMBO J, and Proc Natl Acad Sci USA*. It is indeed very satisfying to see that our multi-disciplinary network has published across all fields spanning from cell, molecular and structure biology, over immunology, pharmacology, chemical biology and clinical medicine. Ten outstanding contributions have already been cited >1 133 times!

I am proud and happy over the accomplishments of EICOSANOX. It has been a true pleasure to lead a consortium composed of such motivated, enthusiastic and competent partners. As pointed out by one of our external reviewers, EICOSANOX has become "*a European point of reference*" within the field and will continue for many years to come.

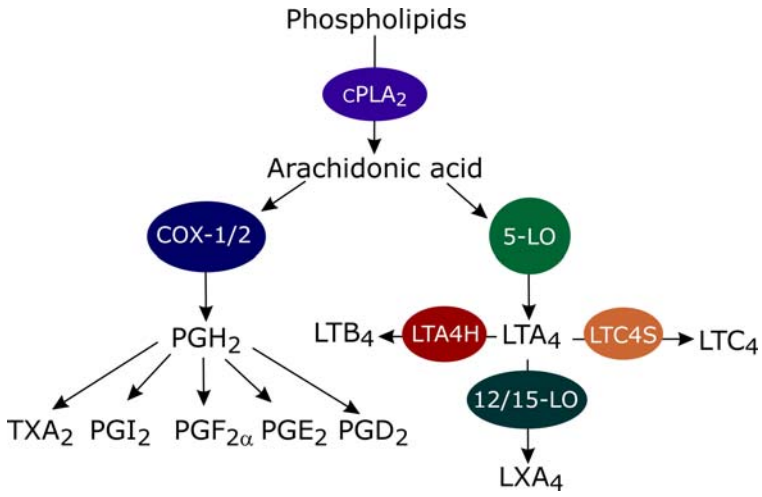
I wish all members of EICOSANOX continued success with our joint projects and look forward to future collaborations.

Jesper Z. Haeggström, Director

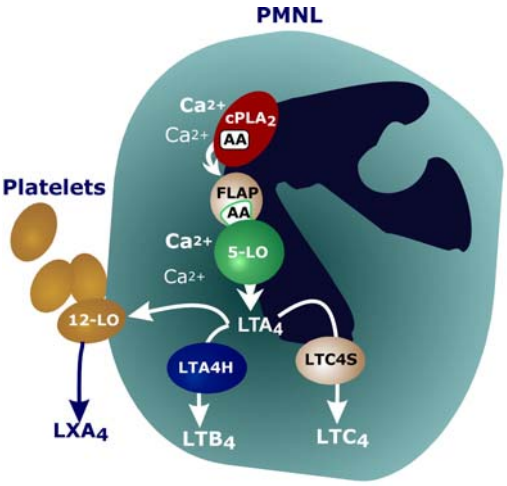
1.5. General overview of the research field

The Eicosanoids comprise a large family of potent lipid mediators derived from arachidonic acid, which include the well-known prostaglandins (PGs), thromboxanes (TX), leukotrienes (LT) and lipoxins (LX). Nitric oxide (NO) is an inorganic chemical mediator, derived from the oxidative metabolism of L-arginine. The Eicosanoids and NO are important signalling molecules in an array of physiological and pathophysiological processes, these autacoids function e.g. in the maintenance of normal cardiovascular homeostasis, kidney function, neuronal signalling, as well as cellular growth and differentiation. Under pathological conditions, these substances are central for the development of inflammatory and allergic reactions, particularly in the cardiovascular and respiratory systems, septic shock, neurodegeneration, and neoplasias. Hence, Eicosanoids and NO are regarded as critical mediators in several severe diseases such as atherosclerosis, myocardial infarction, asthma, degenerative diseases of the nervous system, and cancer. The Eicosanoids and NO are generated along three major biosynthetic pathways named after the corresponding key enzymes, i.e. the cyclooxygenase (COX), the lipoxygenase (LOX), and nitric oxide synthase (NOS) pathways. Enzymes and receptors along these pathways are very attractive drug targets and indeed, several compounds have been developed over the past decade that have successfully found their way to clinical treatment of patients.

The COX pathway. In the COX pathway, arachidonic acid is converted by COX-1 or COX-2 into prostaglandin (PG) H_2, which is then further metabolised by downstream synthases into TXA_2, PGI_2 (prostacyclin), PGE_2, PGD_2 and $PGF_{2\alpha}$. Each of these prostaglandins binds to a specific subset of G-protein coupled surface receptors. A major breakthrough was the discovery of a second enzyme, COX-2, which is induced by growth factors, cytokines, and other pro-inflammatory agents. Crystal structures of COX soon became available allowing structure-based drug design and the development of a new generation of "safe" NSAIDs that spare the gastric mucosa with less tissue damage and bleeding. Subsequently, almost all of the Coxibs were withdrawn from the market due to increased risk for thrombosis, but these compounds still have the advantage of reduced GI-bleeding as compared to conventional NSAIDs. It is now discussed whether the increased risk for cardiovascular events connected with COX-2 inhibitors, is also valid for conventional NSAIDs.



Arachidonic acid metabolism



Biosynthesis of leukotrienes and lipoxins

The LOX pathway. In the LOX pathway, arachidonic acid is oxygenated by 5-, 12- or 15-LOX into hydroperoxy- and hydroxy fatty acids, LTs, and LXs. 5-LOX transforms arachidonic acid into the unstable intermediate LTA_4, which is then further metabolised by either LTA_4 hydrolase (LTA4H) or LTC_4 synthase (LTC4S), into LTB_4 or LTC_4, respectively. The crystal structure of rabbit 15-LOX paved the way for molecular characterisation of mechanisms for LOX Ca^{2+} binding, membrane association, and translocation. Furthermore, phosphorylation of 5-LOX by MAP kinases has been demonstrated and several proteins that interact with 5-LOX have been identified, coactosin-like protein (CLP) and "Dicer", a key enzyme in the siRNA pathways of gene silencing. The crystal structure of LTA4H has been resolved, allowing a molecular analysis of the mechanisms of catalysis and structure-based inhibitor design. Four LT receptors have been cloned, two of which signal Cys-LT responses (CysLT1 & CysLT2), and two for LTB_4, (BLT1 & BLT2).

The NOS pathway. NO is a free-radical gas synthesised by endothelial, neuronal or inducible nitric oxide synthase (eNOS, nNOS, iNOS), each with a distinct primary structure, regulation, cellular and subcellular location. nNOS and eNOS are constitutively expressed and intracellular Ca^{2+} transients regulate the activity. These enzymes produce small quantities of NO over a short period of time, which is of central importance for regulation of blood flow, platelet function and neuronal signalling.

Like COX-2, iNOS is induced by LPS, cytokines and other pro-inflammatory mediators. iNOS can produce copious amounts of NO for a sustained period of time. This may lead to cytotoxic effects, which may be detrimental to host cells, or beneficial as an unspecific first line of defence against microorganisms. Defects in the NO pathway may contribute to the development of several pathophysiological conditions, including hypertension, atherosclerosis, cardiac failure, pulmonary hypertension, stroke, impotence, gastrointestinal ulcers, asthma and other CNS and systemic disorders. This makes the NO pathway a very attractive target for development of new therapeutic agents.

1.6. Work performed and end results

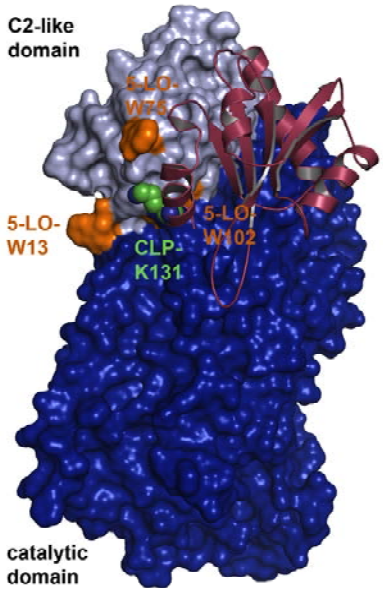
The progress of each WP is described separately.

WP 1- **Biochemical and molecular studies of COX, PG synthases, and NO**

As PGE_2 is involved in pain and fever, inhibitors of the enzyme mPGES-1 may become future anti-inflammatory drugs. New chemical entities were developed and screened for inhibitory activity on the human mPGES-1 enzyme. Lead molecules have been identified and further optimized regarding drugability aspects. A Research Collaboration and Licensing Agreement between the EICOSANOX SME Biolipox (Orexo) and Boehringer Ingelheim was signed to further discover and develop human mPGES-1 inhibitors. In fact, this was largest deal in Europe, within the biotech industry, closed in Europe that year. Certainly, it was an unusual accomplishment for the relatively small biotech company Biolipox.

WP 2-Biochemical and molecular studies of cyclooxygenases COX), lipoxygenases (LOX), LTA_4 hydrolase (LTA4H), and LTC_4 synthases (LTC4S)

12/15-LOX is the only mammalian LOX for which the structure is known, and studies of this LOX can often be extrapolated to i.e. 5-LOX. The 12/15-LOX is important for lipid peroxidation and has long been discussed in relation to chronic inflammatory diseases, such as atherosclerosis. The work performed has given additional knowledge regarding several aspects on LOX function. The principles for how certain amino acid residues determine the specificity of oxygen insertion were extended, for 12/15-LOX and other mammalian lipoxygenases (the triade concept). Regulation of the enzyme activity of lipoxygenases is complex. An arachidonic acid analogue was found to bind the catalytic domain of 15-LOX, but also to the N-terminal ß-sandwich domain. This sheds light on a long suspected regulatory second fatty acid binding site, in lipoxygenases. Also, the mode of entry of oxygen, to the active site of 12/15-LOX was elucidated. Lipoxygenases are flexible enzymes and studies were performed regarding interdomain movement, and the conformational flexibility of the entire protein. Phospholipid Hydroperoxide Glutathione Peroxidase-4 regulates cellular redox tone, and thus the activity of COX and LOX. The monomeric structure was found to consist of four α-helices and seven β-strands, with the catalytic triad localized on a flat impression on the protein surface. GPx-4 can reduce hydroperoxides in the fatty acids esterified in membrane phospholipids. Since 12/15-LOX can oxygenate esterified fatty acids, membrane integrity may depend on a balance between GPx-4 and 12/15-LOX.



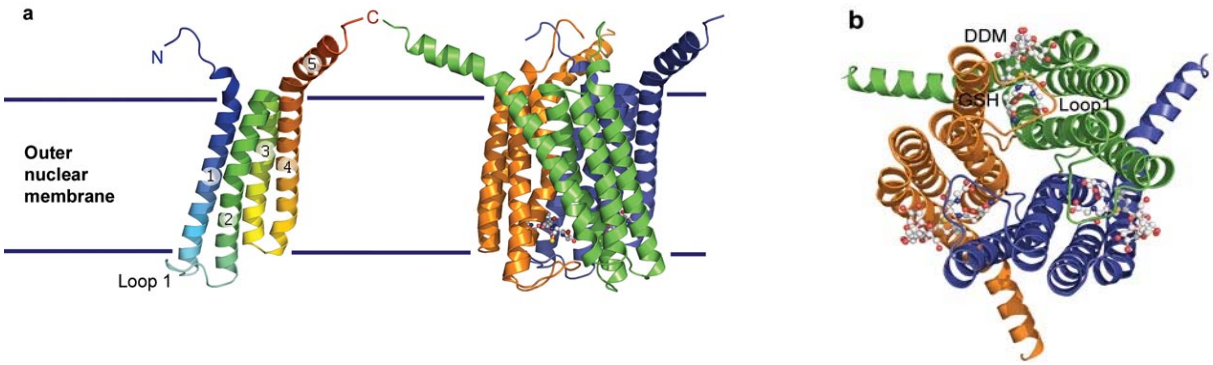
Model of 5-LOX

5-LOX catalyzes two steps in biosynthesis of leukotrienes (LTs). First, oxygenation of arachidonic acid leading to 5-HPETE, followed by dehydration to LTA_4. LTs are established lipid mediators of inflammation with effects in normal host defence. LTs are also drug targets in inflammatory diseases such as asthma. The activity of 5-LOX is regulated by factors (i.e. Ca^{2+}) binding to the C2-like ß-sandwich domain. A direct effect of glycerides on the activity of

5-LOX was described, OAG could activate 5-LOX *in vitro*. Mutagenesis indicated that also OAG directly stimulates 5-LOX by acting at the C2-like domain. Also, as found before for Ca^{2+}, OAG renders 5-LOX activity resistant against inhibition by GPx activity. Coactosin-like protein (CLP) binds to 5-LOX, and effects of CLP on 5-LOX activity were elucidated. CLP can serve as a scaffold for 5-LOX activity, upregulating the formation of leukotrienes. It was also found that CLP stabilizes 5-LOX preventing non-turnover inactivation *in vitro*. Also the binding of CLP to 5-LOX was mediated by residues in the C2-like domain. These findings establish CLP as a chaperoning scaffold factor, influencing both the stability and the activity of 5-LOX. Dicer is an RNaseIII involved in miRNA biosynthesis.
Binding of 5-LOX to the Dicer C-terminus was described. Interestingly, this resulted in a modified miRNA precursor processing activity of Dicer.

LTA4H is the enzyme which converts LTA_4 to the chemotactic LTB_4. An intriguing property of this enzyme is that it also has a peptidase activity. Extended knowledge about structure and reaction mechanisms allows for drug design, and many studies have contributed to this becoming a real possibility for LTA4H. As one piece in the puzzle, structure-function studies were performed for LTA4H from *S. cerevisiae*. By sequential mutations, selected from the homology model, functional properties were introduced into the yeast enzyme, which mimicked those of mammalian LTA4H. In another report, properties of the transition state in the peptidase reaction was described. The data provide detailed insights to the active site chemistry of M1 aminopeptidases and will aid in the development of enzyme inhibitors.

LTC4S is the enzyme which converts LTA_4 to LTC_4. This ”cys-LT” is further converted to LTD_4 and LTE_4, all three cys-LTs together make up ”slow reacting substance of anaphylaxis”, which leads to the congested airways typical for an asthma attack. As a general inflammatory mediator, LTC_4 also leads to increased vascular permeability, causing the swelling of inflamed tissue. Thus, knowledge about this enzyme is of high interest, to understand normal inflammation, and for drug development. The crystal structure was determined for human LTC4S, in its apo and GSH-complexed forms. The structure is a homotrimer, where each monomer is composed of four transmembrane segments. The active site enforces a horseshoe-shaped conformation on GSH, and effectively positions the thiol group for activation by a nearby arginine at the membrane-enzyme interface. Membrane proteins are notoriously difficult to crystallize and this represents the second high resolution crystal structure of a human integral membrane protein ever described. Accordingly, this is a remarkable scientific achievement. The structure gives important novel insights to the catalytic mechanism of the enzyme, as well as other members of the MAPEG family and is of considerable interest for drug-design.



Crystal structure of LTC4S Molina et al. *Nature*. 2007, 448:613-6

Structures, biosynthesis, and biological activities of eoxins, a group of proinflammatory arachidonic acid metabolites formed via 15-LOX, were published. EXs induced increased permeability of endothelial cell monolayer *in vitro*, indicating that EXs can modulate and enhance vascular permeability, a hallmark of inflammation.

WP 3-Gene regulation of enzymes in the eicosanoid cascade

Four partners (UFrank:a, KI:a, Charité and UAM:a) have been working in this WP and studied the regulation of gene expression under physiological and pathophysiological conditions to get insights into the functions of eicosanoid forming enzymes in inflammation, neurological disorders and cancer.

Regulation of the expression of eicosanoid forming enzymes is a key mechanism of the body to adjust the formation of eicosanoids to the physiological needs. Thus, deregulation of enzyme expression is often associated with pathophysiological processes and studies on enzyme expression provide new insights into mechanisms of physiological processes and the molecular basis of disease.

Partner 10a (UAM:a) studied the regulation of COX-2 and PG synthases (mPGES-1, PGDS) expression in macrophages and T-lymphocytes. In macrophages, UAM:a has analyzed the cooperation between LPS and G-protein cell receptors/cAMP signalling pathways in the induction of COX-2 and mPGES-1 transcriptional induction. The results point to an essential role of transcription factors NFκB and Egr-1 activation upon LPS treatment in the induction of both mPGES-1 and COX-2. UAM:a also analyzed the role of the cAMP signalling pathway in the induction of these enzymes, showing that increased cAMP production and hence PKA activation also plays an important role in the transcriptional induction of mPGES-1 and COX-2 in macrophages. These results support a positive feedback mechanism by which PGE_2 binding to EP2 or EP4 receptors further increases COX-2 and mPGES-1 expression upon macrophage activation.

In T lymphocytes, the inhibition of induced expression of COX-2 by glucocorticoids (dexamethasone) was investigated, showing that this inhibition occurs upon binding of dexamethasone to the glucocorticoid receptor (GR) even in the absence of GR binding to DNA. The data show that inhibition of COX-2 induction by dexamethasone in T cells involves a transcriptional interference between the GR and activation of transcription factor as AP-1 and NFAT.

In another set of experiments UAM:a has investigated the signals mediating COX-2 transcriptional up-regulation in colon carcinoma cell lines (Caco-2, SW620, HT-29) which contributes to cancer development. They could demonstrate for the first time that inhibitors of calcineurin phosphatase and hence of NFAT activation such as cyclosporin A or tacrolimus (FK506) are able to down-modulate the induction of COX-2 expression by various signals. Regarding the induction of COX-2 by IL-1β in colon carcinoma cells UAM:a has described the involvement of both transcriptional (NFκB–mediated) and post-transcriptional (p38MAPK –dependent stabilization of COX-2 mRNA) mechanisms.

Partner 2a (UFrank:a) investigated the regulation of 5-lipoxygenase (5-LOX) expression. They found that, surprisingly, induction of 5-LOX expression by $1,25(OH)_2D_3$/TGFβ is not mediated by response elements in the promoter but by VDREs and Smad REs located within the 5-LOX gene. Thus, it was obvious that other sequences outside of the 5-LOX promoter seem to be responsible that are located within the distal part of the 5-LOX gene. Chromatin immunoprecipitation revealed that both RXR and VDR are present at these sites and that addition of $1,25(OH)_2D_3$ and TGFβ induce elongation markers and recruitment of the elongation form of the RNA polymerase II to the distal part of the 5-LOX. The data suggest that TGFβ and $1,25(OH)_2D_3$ induce 5-LOX expression during myeloid cell maturation by the induction of transcript elongation which represents a new mechanism of gene regulation by nuclear receptors.

In previous studies UFrank:a observed that 5-LOX promoter activity is strongly downregulated by DNA methylation. It was found that the methyl-DNA binding proteins MBD1, MBD2 and MeCP2 are involved in repression of the 5-LOX promoter and that methylation of distinct Sp1-binding sites prevent binding of the transcription factor Sp1. Thus, these studies provide the basis for the understanding of the regulation of 5-LOX promoter activity by DNA methylation.

Partner 13 (Charité) studied the impact of expression silencing of eNOS, COX-1, COX-2 and various lipoxygenase and GPx isoforms on murine embryogenesis. It was found that 12/15-LOX is expressed at moderate levels (2-5 copies of 12/15-LOX mRNA per 1000 copies of GAPDH mRNA) at early developmental stages (E6.5-7.5). 12R-LOX and 5-LOX mRNA were found at low levels. COX-1 mRNA was found in small amounts at midgestation (E 10.5-15.5) but no COX-1 transcripts were detected at later stages whereas COX-2 was completely absent during the entire developmental period except from a narrow time window at midgestation when small amounts of COX-2 mRNA were found. In contrast, eNOS is expressed at relatively high levels during the entire developmental period but silencing of eNOS did not affect embryonic development.

Expression regulation of phospholipid hydroperoxide glutathione peroxidase (GPx4) in sperm was investigated. GPx4 is an intracellular antioxidant, which is high-level expressed in spermatozoa and defects in GPx4 expression regulation have been implicated in male infertility. The obtained data indicate that oligoasthenozoospermia is associated with impaired levels of spermatozoic GPx4 expression of some but not all infertile men (25 %).
In another project, partner 13 investigated the effects of IL-4 on 12/15-LOX expression in IL-4 overexpressing animals and in mice infused with IL-4. The data revealed that 15-LOX is upregulated in mice overexpressing IL-4. In patients suffering from allergic diseases there was no significant correlation between increased IL-4 and 15-LOX levels. The results suggest that allergic patients have increased circulating IL-4 levels but the increase was not high enough to induce monocytic 12/15-LOX expression.

Partner 1a (KI:a) cloned and characterized the promoter of the LTA_4 hydrolase (LTA4H) gene. The LTA4H catalyzes the conversion of LTA_4 to LTB_4 which is a mediator of inflammation and cancer. Thus, knowledge on the regulation of this enzyme might provide insights into the molecular mechanisms of inflammatory processes. So far KI:a has studied the transcriptional activity of the core promoter of LTA4H promoter, consisting on the 700 bp closest to the reported transcription initiation site which shows high transcriptional activity.

Deletions smaller than 200 bp have only low activity which suggests that the region between 200 and 700 bp comprises the LTA4H core promoter.

WP 4-Role of eicosanoids in inflammatory and immune responses

Inflammation is an essential component of maintaining the body's defence against injury however; the inflammatory response is subverted and persists in numerous chronic diseases. Persistent inflammation may result in irreversible scarring, fibrosis and eventual organ failure. Lipid mediators such as those generated by LOX, COX and NOS play critical roles in regulating inflammatory responses. Much of the research activity in work package 4 has focused on the balance between the activities of mediators that drive inflammation and those that actively promote its resolution. These investigations have lead to the identification of novel targets, biomarkers and potential mimetics that may be exploited for therapeutic benefit. Charité and UCD have expanded on the roles of LXA_4 as mediators that promote the resolution of inflammation in several diseases including arthritis. Given these discoveries several novel lipoxin analogues have been synthesized and their anti-inflammatory, pro-resolution activities investigated by KI:a and at UCD. These studies reveal that the synthetic LXs represent a group of experimental therapeutics. Intriguingly, cross desensitization of several of the receptors responding to various eicosanoids suggests an integrated network of signals that regulate the amplitude and duration of inflammatory and counter-inflammatory responses. Investigators at UDAnn have studied platelet activation in chronic renal failure in human subjects. Their data indicate increased lipid peroxidation and thromboxane-dependent platelet activation in chronic renal failure patients compared to age and sex matched controls. Investigating multiple parameters the investigators concluded that that an increase in platelet activation occurs with progressive deterioration in renal function.

Counter-regulatory roles for sphingosine kinases in TGF-β induced fibrosis have been shown. TGF-β –induced upregulation of sphingosine kinases counteracts its fibrotic drive suggesting that sphingosine 1-phosphate, a product of sphingosine kinase, or analogues of sphingosine 1-phosphate may be protective. This hypothesis was addressed by investigators at UFrank who showed that administration of the sphingosine 1-phosphate analogue FTY720 (fingolimod) clearly reduced lesion sites, improved neurological symptoms and attenuated neutrophil infiltration in a model of cerebral ischaemia. Investigators at UNIMI have characterised the pharmacology of various analogues of Lumaricoxib with a view to identifying molecules that have maximally potency as TP antagonists but diminished the inhibitory activity of COX and therefore increased cardiovascular safety profiles.

The roles of $PGF_2\alpha$ and cyPGs on T cell activation have been addressed [UAM]: $PGF_{2\alpha}$ elicits responses to upregulate activity of AP-1 and NFAT, conversely the cyclopentenone PGs repress inflammatory responses through inhibition of AP-1, NFκB and NFAT activity. The importance of EP2 and EP4 receptors in T cell migration to lymph nodes has also been demonstrated.

Using thrombin activated human platelets novel phospholipid-esterified eicosanoids have been detected [Charité]. *In vivo* generation of esterified eicosanoids has been shown in models of inflammation at distinct phases in the inflammatory and resolution responses. Investigations of the bioactions of such lipids suggest novel paradigms for lipid regulation of inflammatory responses.

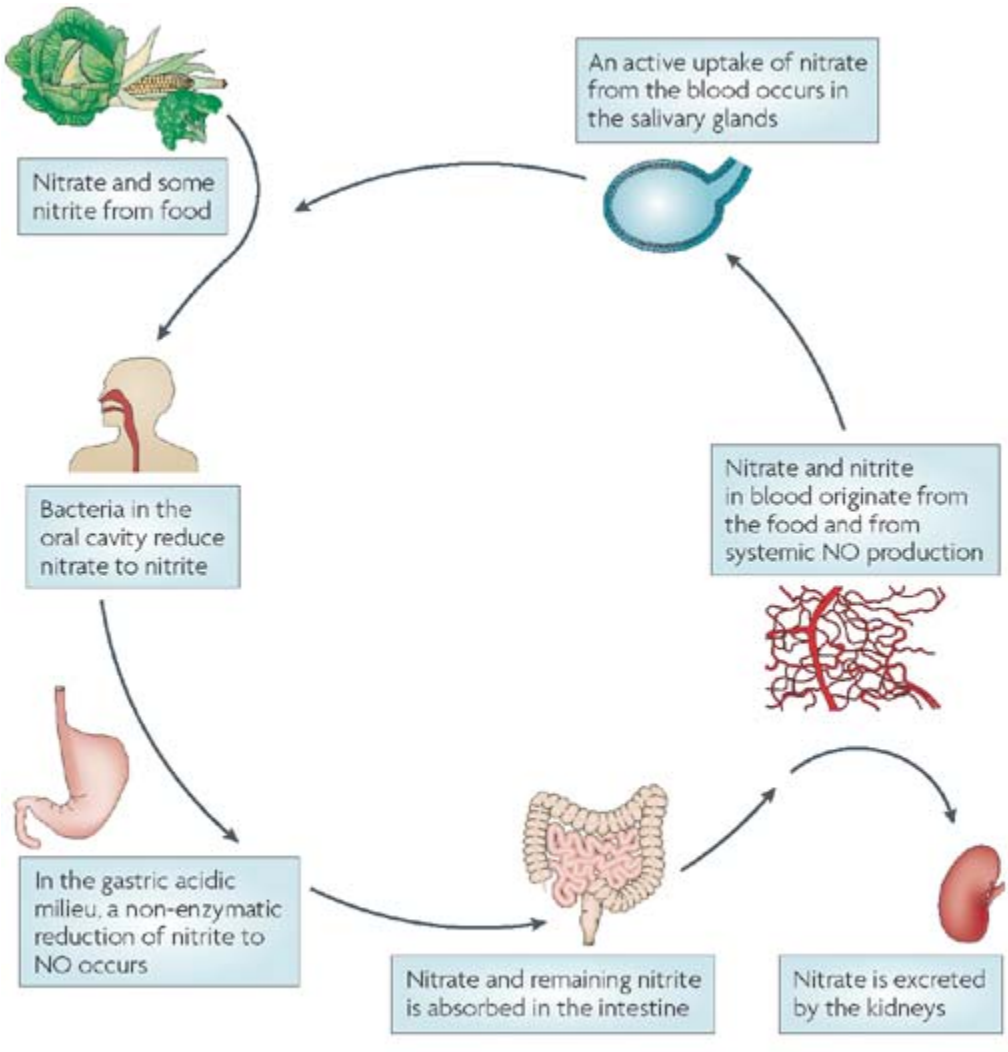
WP 5-Role of NO in inflammatory and immune responses

This WP concerned the roles of NO in inflammation and immunity. More specifically we have been studying regulation of inducible NO synthase (iNOS). iNOS regulation takes place primarily at the level of transcription. UAMa has analyzed the transcription factors involved in this regulation in macrophages, using Molecular Biology approaches as well as genetically deficient mice. We demonstrated that IFN-γ induces iNOS expression by a mechanism that involved endogenously produced TNF-α and nuclear factor-{kappa}B activation (NF-κB). IFN-γ induces iNOS and TNF transcription in mouse macrophages. iNOS expression by IFN-γ is absent in macrophages from TNF knockout mice. The TNF produced in response to IFN-γ is required for iNOS induction by activating p65/relA NF-κB member binding to the iNOS promoter. Two regions in the TNF promoter seem to be responsible for the IFN-γ response: where IRF-1 and IRF-8 bind. Those 2 factors are required not only for TNF but also for iNOS transcription factors. In summary, IFN-γ treatment induces TNF expression at transcriptional level requiring the coordinate action of IRF-1 and IRF-8. Besides, this TNF in an autocrine manner strongly potentiate iNOS transcription by inducing NF-κB activation
On the other hand, IL-4 inhibits IFNγ-induced iNOS production in macrophages by a dual mechanism. On the one hand, IL-4 downregulates TNF production by macrophages, which acts in an autocrine manner to induce iNOS transcription, on the other hand, IL-4 also affects the stability of iNOS mRNA.

We have also started to characterize a human model of iNOS deficiency. The enzyme iNOS is constitutively present and active in the nasal airways of healthy subjects. This activity can easily be monitored by sampling air from the nasal cavity and measuring NO gas content. In patients with Primary Ciliary Dyskinesia (PCD) nasal NO is virually absent. In fact a nasal NO test is now being used in the clinic as a diagnostic tool. Interestingly, these patients are very susceptible to airway infections and we have suggested the lack of NO as a possible mechanism for this. We have now examined nasal biopsies from PCD patients and somewhat surprisingly found that they do express mRNA as well as iNOS protein. So likely they have a defect in iNOS activity rather than expression.

Another major task in this WP has been the further characterization of a recently-described alternative pathway for NO generation in mammals. We have found that the supposedly inert inorganic anions nitrate and nitrite can be converted *in vivo* to form NO and other bioactive nitrogen oxides. Inorganic nitrate (NO_3^-) undergoes a serial reduction in vivo to first form nitrite (NO_2^-) and then NO. Interestingly, while the classical NO synthase pathway becomes dysfunctional during hypoxia, the nitrate-nitrite pathway is instead greatly accelerated. We have found that the first step (nitrate to nitrite) is mainly catalysed by commensal bacteria in the oral cavity and to a lesser extent by mammalian enzymes. Nitrate from endogenous or exogenous sources (our everyday diet) is actively taken up by the salivary glands and excreted in saliva. In the mouth commensal bacteria reduce nitrate to nitrite. The nitrite is then swallowed and partly reduced to NO non-enzymatically in the acidic gastric lumen. A large portion of nitrite is absorbed intact and later reduced to bioactive NO via enzymatic pathways in the blood and tissues. Generation of NO in the stomach helps to increase gastric mucosal blood flow and to stimulate mucus generation. These effects are associated with strong gastroprotection in a rat model of NSAID-induced gastric ulcer. In addition, NO is bactericidal and helps to kill potential GI pathogens entering via the oral route. Nitrite reduction to NO in tissues and blood seems to be involved in control of cardiovascular function. We could show that ingestion of nitrate leads to acute increases in circulating nitrite

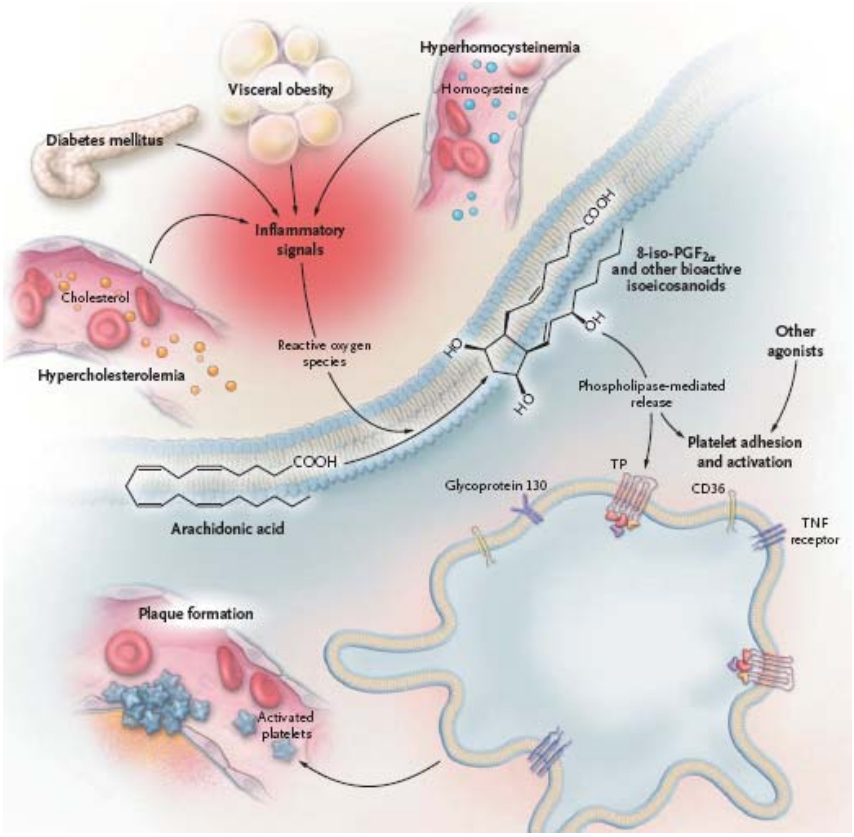
which are associated with a reduction in blood pressure in humans. This effect is highly suggestive of NO formation. Numerous groups have shown potent cytoprotective effects of nitrate /nitrite in animal models of ischemia-reperfusion injury (e.g myocardial infarction, stroke). In a recent study we found that ingestion of nitrate decreased whole body oxygen consumption in humans during exercise. This means that at an identical workload, a subject utilizes less oxygen if he has ingested nitrate prior to the test. The mechanism behind this remarkable effect is still under investigation but we suspect interaction of nitrite/NO at the mitochondrial level. In summary, an alternative pathway for NO generation in mammals has been explored. In this pathway, the simple inorganic anion nitrate- abundant in vegetables- is serially reduced to form nitrite and then bioactive NO. We believe that this pathway can serve as a backup system to ensure NO generation also in situations when the classical NO synthase pathway is malfunctioning. An intriguing nutritional aspect is the fact that nitrate is abundant in vegetables - a food group associated with reduced risk for several major disorders most notably cardiovascular disease.



WP 6-COX and PG synthases in cardiovascular diseases

Research carried out within WP6 at four different institutions (UDAnn, KI, UCL and QueensU) has greatly contributed to the advancement of our understanding of the role played by eicosanoids in atherothrombosis and of the clinical consequences of their inhibition.

A series of *in vitro* and *in vivo* studies carried out by partner UDAnn have characterized the mechanisms underlying the untoward cardiovascular effects of COX-2 inhibitors and identified the extent of COX-2 inhibition (but not COX-2 selectivity) as a major determinant of the variable cardiovascular hazard associated with nonsteroidal antiinflammatory drugs (NSAIDs). Moreover, the same Investigators have described a novel biomarker predicting the relative risk of myocardial infarction associated with different NSAIDs. Furthermore, considerable progress has been made in the mechanistic understanding of so called aspirin "resistance". Clinical studies in patients with essential thrombocythemia, type 2 diabetes mellitus and stable coronary heart disease have characterized enhanced platelet turnover and accelerated renewal of COX-1 during the 24-hour dosing interval as an important mechanism underlying less than complete inhibition of platelet COX-1 by a conventional once daily regimen of low-dose aspirin therapy. These studies have provided a rationale for exploring the efficacy and safety of a shorter dosing interval of aspirin administration in clinical settings characterized by a high cardiovascular risk and limited efficacy of low-dose aspirin (eg, diabetes mellitus).



Isoprostane formation as a biochemical link between low-grade inflammation and platelet activation in human metabolic disorders. Davì and Patrono, N Engl J Med 2007, 357;24, 2482-2494.

Work carried out at KI has demonstrated abundant expression of LTA4H in human atherosclerotic lesions and its correlation with carotid plaque instability. These studies have identified LTA4H as a potential target for pharmacological intervention in the prevention and treatment of atherothrombosis.

Work carried out at the UCL has led to the development of refined assays for the determination of platelet reactivity. Use of these assays has clarified the relationship between the antithrombotic effects of aspirin and $P2Y_{12}$ antagonists, such as clopidogrel and prasugrel, and the TXA_2-dependent pathway of platelet activation. These novel findings may have clinical implications for the optimal modality of dual antiplatelet therapy.

Overall, the major achievements obtained in WP6 have led to the **identification of novel drug targets**; the development of **novel biomarkers** to predict aspects of drug efficacy and safety; and the proposal of novel dosing regimens of existing drugs for the treatment and prevention of atherothrombosis. Leadership in the field is reflected by **two invited reviews published in** the prestigious **New England Journal of Medicine**, on low-dose aspirin as an antithrombotic agent (Patrono et al, NEJM 2005;353:2373-83) and on platelet activation and atherothrombosis (Davì & Patrono, NEJM 2007;357:2482-94). Ongoing collaboration with major pharmaceutical companies (eg, Bayer-Schering Pharma, Eli Lilly and AstraZeneca) as well as new research projects initiated within the Innovative Medicines Initiative (SUMMIT Consortium) are currently testing novel hypotheses generated within the EICOSANOX project.

WP 7-LOX products as mediators of cardiovascular diseases

The major achievements during the project period are related to the successful contributions from the laboratories of Karolinska Institutet (KI), University of Milano (UNIMI), Charité, and University of Frankfurt (UFrank). An ambitious work program within the EICOSANOX project has provided a great opportunity to develop our understanding of leukotrienes in cardiovascular disease, and created some important spin-off programs along the project.

In Karolinska Institute, there has been a great collaboration between participant A (KI:a) and participant C (KI:c), allowing the possibility to pursue an interdisciplinary approach to the role of leukotrienes in different aspects of cardiovascular disease. This has involved the generation of large clinical databases of gene expression profiles in tissue from patients with cardiovascular disease, and gene expression databases from animals with cardiovascular disease, which has allowed for the probing of specific expression profiles related to the leukotriene cascade. Additionally, the role of leukotrienes and their signalling has been evaluated in specific models of cardiovascular disease with great success, providing some novel and important answers. A series of *in vitro* and *in vivo* studies carried out by the partners have set the basis for a major leap in our understanding of the basic biological mechanisms of leukotriene action and the associated *in vivo* effects in cardiovascular disease. KI:c and KI:a made collaborative efforts to investigate the gene expression patterns and the clinical correlates of the FLAP/5-LOX and 12/15-LOX system in patients with atherosclerosis. Other projects related to the role of BLT receptors in mediating restenosis in vascular injury and the role of 5-LOX in contributing to adipose tissue inflammation in mouse models of metabolic disturbance. In addition, work performed in rat and mouse models of acute and chronic ischemic heart disease, point to cys-LTs as mediators of myocardial ischemia and suggests that anti-leukotrienes such as montelukast may represent a novel

therapeutic opportunity for treatment of some aspects of ischemic heart disease. Finally, investigations in abdominal aortic aneurysms demonstrated that leukotrienes may contribute to aneurysm remodelling.

UNIMI made successful efforts in studying the role for orphan leukotriene receptors in the response to brain ischemia in an experimental rat-model of stroke and brain ischemia. They identified a novel receptor subtype responsible for leukotriene signaling in experimental stroke and further more worked upon the development of novel pharmacological tools to block detrimental leukotriene signaling *in vivo*. To this end, UNIMI developed a new $CysLT_2$ antagonist (AP100984) which was tested for potency and selectivity in a brain model of ischemia. In addition, by using a complicated set of experiments in animal systems with targeted deletions of selected leukotriene genes and formation of LOX metabolites by circulating cells *in vivo*, workers at UNIMI were able to provide unequivocal evidence of efficient transcellular biosynthesis of cys-LTs.

Charité have been performing detailed investigations in mouse models of atherosclerosis and have contributed significantly to the generation of the gene expression database in mouse models. Charité investigated the role of 12/15-LOX in foam cell generation and developed a mathematical model of foam cell generation and concluded that mechanisms of foam cell formation are rather diverse and strongly depend on the experimental model systems, and that it is impossible to work out a general mathematical scheme, which is generally applicable. Furthermore, it was concluded that deletion of 12/15-LOX gene (in 12/15-LOX knockout mice) did not significantly alter systemic lipid accumulation and insulin responsiveness of the animals. As it turns out, the 12/15-LOX system may have role in inflammation or anti-inflammation but does not seem to be major primary target in metabolic disease.

Overall the work of this work-package has performed extremely well during the project period, and the work resulted in major publications in scientific journals and generated important new knowledge as a platform for directing future research and for further exploration of the possible therapeutic options of manipulating leukotriene system activity. Ongoing collaborations and projects are currently testing novel hypotheses generated within the EICOSANOX projects.

WP 8-NOS, a friend or foe in the cardiovascular system

The endothelial cell layer is one cell thick and is the innermost layer of the blood vessel; a position which means that it acts as a physical barrier between the circulating blood and the vascular wall. Despite its fragile appearance, the vascular endothelium generates a series of substances that can regulate the contractile level of the underlying muscle, control gene expression and the ability of blood borne cells to dock onto its surface. Needless to say this also means that the endothelium is exposed to a wide variety of stimuli and insults and vascular disease is usually preceded by endothelial cells damage and a change in its function. It follows that the maintenance of endothelial function throughout life and its restoration following the onset of vascular disease are attractive therapeutic goals.

Although the regulation of endothelial cell function and its repair are complex procedures, all of these processes are affected to a significant extent by nitric oxide (NO). Before EICOSANOX began there were few tools that could be used to measure NO levels *in vivo*, there was confusion regarding the post-translational regulation of eNOS activity by phosphorylation in endothelial cells and no clear information about the ability of circulating

cells such as platelets to generate their own NO or indeed how the targeting of these cells by NO donors could affect platelet function. In WP8 we have focused on determining the mechanisms that regulate NO production, and identified signalling mechanisms that inhibit (e.g. tyrosine phosphorylation, production of oxygen-derived free radicals) and stimulate NO output. We have also identified novel effects of NO within the endothelium (e.g. its ability to activate the AMP-activated protein kinase) and come up with a new list of genes that are sensitive to NO in the vascular wall and in other cell types. We have also made a number of observations relating to cell metabolism which might be relevant - not only for the understanding of the response to inhibition of mitochondrial respiration - but also for the understanding of the way in which glycolysis links to cell proliferation in general. Much of this work has been possible because of new methods made available by the consortium to measure nitrite, nitrate and S-nitrosothiol in plasma and tissues as well as to study the interactions of NO with the cytochrome c oxidase at stable oxygen concentrations.
NO is a potent anti–clotting agent and platelets from patients with cardiovascular disease and/or diabetes tend to clot more easily than normal. The consortium has identified novel mechanisms underlying the disturbance of platelet function in diabetes (i.e. the modification of a Ca^{2+} pump, which in turn activates a Ca^{2+}-sensitive protease that cleaves a specific subset of platelet proteins to alter their function) and identified an effective therapy (peroxisome proliferator–activated receptor-γ agonists) to repair the defect and restore platelet-sensitivity to NO in diabetic individuals. Also promising is the development of NO-releasing non-steroidal anti-inflammatory drugs (NSAIDs) and these substances have beneficial actions in platelets as in an *in vitro* model of cerebrovascular ischemia. We have also shown that a new anti-atherothrombotic agent (2NTX-99) which is both an NO donor (promotes vasorelaxation and inhibits platelets) and inhibitor of TXA_2 synthesis (the latter promotes vascular constriction and platelet activation), can control the ischemic events taking place at the level of the pulmonary vascular bed when human platelets are activated. As thrombo-embolism and a reduction of NO production are characteristic of vascular disease, such compounds have a high potential for therapeutic benefit.

All in all WP8 has produced a number of basic science studies investigating the regulation of NO production and NO-dependent gene expression that have been complemented by translational research studies in animal models and in humans. All of these studies have helped us to understand why NO production does down in disease and attempts to supplement cardiovascular system with NO-generating and/or eNOS activating compounds certainly look promising.

WP 9 -Role of eicosanoid metabolism in brain and brain development

Three partners (UNIMI, NicOx, Charité) have been working in this work package on the role of eicosanoid metabolism in neuronal dysfunction, cerebral diseases and brain development.

Partner 3 (UNIMI) focused on the pathogenesis of cerebral inflammation. To explore the involved pathomechanisms they first explored the eicosanoid metabolism of peripheral human blood cells under resting and activation conditions and found that inflammatory stimuli increase the cellular concentration of free arachidonic acid by directly inhibiting the lysophospholipid:acyl-CoA acyltransferase activity of peripheral neutrophils. These findings indicated a novel mechanism for increasing the cellular concentration of free arachidonic acid, which is the rate-limiting step for the formation of inflammatory eicosanoids. The data clearly suggests a critical regulatory role of arachidonate reacylation, which limits leukotriene

biosynthesis in concert with 5-LOX and $cPLA_2$ activation (Librizzi L, et al. Neuroscience 137, 1211-1219, 2006). To translate these findings into the pathogenesis of cerebral inflammation UNIMI developed model of isolated, intact guinea-pig brain with complete anatomical and functional integrity of the vascular bed and applied this model to explore the impact of an altered eicosanoid metabolism of inflammatory blood cells on cerebral inflammation. It was found that in vitro co-perfusion of pro-inflammatory agents and differentially pre-activated mononuclear cells induced endothelial expression of selectins and intracellular adhesion molecule-1, which moderately increased blood-brain barrier permeability. These data suggest that co-activation of mononuclear cells and cerebral vascular endothelium is essential for cell adhesion in early cerebral inflammation (Zarini S, et al. J Biol Chem. 281,10134-10240, 2006).

Partner 8 (NicOx) developed several series of NO-liberating aspirin derivatives (Ricciotti E, et al. J Immunol. In press, 2010 and Ronchetti D, et al. Br J Pharmacol. 158(2):569-79, 2009) which may be useful as anti-inflammatory drugs because of their dual functionality. As aspirin derivatives they function as COX-inhibitors, but they also serve as nitric oxide sources and thus may induce vasodilation. Partner 8 assessed the ability of these compounds to impact eicosanoid metabolism and local or systemic inflammation. One of the most intensively characterized compounds is NCX 4040, which inhibited COX-2 expression and generation of pro-inflammatory cytokines such as IL-1, IL-10, IL-18 and TNFα. Compared with aspirin NCX 4040 caused concentration-dependent accumulation of IkB in its phosphorylated form. This effect was not reversed by inhibition of guanylyl cyclase excluding the contribution of NO-dependent cGMP generation. In summary it was concluded that NCX 4040 constitutes an inhibitor of IkB-degradation by the proteasome implicating intracellular proteolysis in the inflammatory reaction. These data identify an additional inflammatory pathway and thus these findings may be important for further development of anti-inflammatory and/or chemopreventive drugs.

Partner 12 (Charité) explored the role of the EICOSANOX-relevant enzymes including the endogenous lipoxygenase/cyclooxygenase inhibitor GPx4 in neuronal function and brain development. i) They profiled expression kinetics of COX, LOX and NOS-isoforms in cerebral embryogenesis and published these data in an invited review paper (Borchert, A., et al. J. Biol. Chem. 281, 19655–19664, 2006).

ii) They explored the impact of knockdown of GPx4 expression on embryonic brain development (Savaskan, N.E., et al. *Free Radic. Biol. Med. 43,* 191-201, 2007) and found that translation of GPx4 mRNA is controlled by the regulatory translation factor Grsf1, which binds to a defined sequence in the 5'-untranslated mRNA region and recruits inactive monomeric GPx4 mRNA into translationally active polysomes. Functional knockdown of Grsf1 and GPx4 expression during mouse embryogenesis induced developmental retardations and impairment in proper brain segmentation. These data indicate that both Grsf1 and GPx4 are essential for proper brain development and that translational regulation of gene expression play a major role.

iii) They characterized the role of GPx4 in neuroinjury (Ufer, C., et al. Genes & Dev. 22, 1838-1850, 2008) and observed that following selective brain injury expression of GPx4 was upregulated in reactive astrocytes and selective knockdown of GPx4 by siRNA induced depletion of phosphatidylinositol-(4,5)-bisphosphate in the neuronal plasma membrane, which leads to neuronal apoptosis. It was concluded that astrocytic upregulation of GPx4 expression may be considered part of a protective response counteracting neuronal damage.

iv) Since GPx4 appears to be part of the protective response following neuronal injury we synthesized low molecular weight GPx4 mimics (seleno-organic compounds), which might exhibit similar properties. The structural properties (including crystal structure) of these compounds were comprehensively characterized and their catalytic mechanism as GPx-mimics was explored (Yu, S.C., et al. Chem. Eur. J. 14, 7066-7071, 2008 and Yu, S.C., et al. Org. Biomol. Chem., in press 2010). The synthetic procedure was optimized and *in vitro* pharmacology of these compounds (isoform-specific LOX- COX-inhibition) was tested. The compounds are now available for more detailed *in vivo* investigations.

WP 10-COX, PG synthases, and NO in oncogenesis

Several reports have highlighted the importance of inducible cyclooxygenase (COX-2), of microsomial prostaglandin synthase-1 (m-PGES-1) and its product PGE_2 for the progression of a variety of tumors and colon cancer. PGE_2 induces tumour progression by increasing tumour growth and invasiveness, angiogenesis and growth factor production, but the molecular mechanisms by which PGE_2 controls tumour progression are not completely understood. Four partners (UAM.a, UDAnn, Charité and NicOx) have worked in this WP.

UAM:a investigated the role of COX-2 and PG synthases in the tumoral phenotype in colon carcinoma cell lines. UAM detected for the first time the presence and activation of the nuclear factor of activated T cells (NFAT) in human colon carcinoma cells. Gastrin releasing peptide (GRP) stimulates the expression of COX-2 mRNA and protein in human colon adenocarcinoma cells through a Ca^{2+}/calcineurin (Cn)-NFAT linked pathway, resulting in enhanced invasive capacity of carcinoma cells, an effect which was inhibited by a COX-2-specific inhibitor. These findings provide the first evidence for the involvement of the Ca^{2+}/Cn/NFAT pathway in BBS-mediated induction of genes involved in colon carcinoma invasiveness such as COX-2. They went further to show that the genetic manipulation of elements of the Ca^{2+}/Cn/NFAT/COX-2/mPGES-1 pathway has profound impact in the tumoral phenotype of colon carcinoma cell. COX-2 over-expression in colon carcinoma cells, leads to an increase in migration, invasiveness, higher tumour growth and a higher tumour vascularization and more interestingly, they confer those cells a higher metastatic capacity in vivo, and specifically to the lung. COX-2 overexpression produces an increase of PGE_2 and VEGF secretion by those cells. Cn overexpression also produces a similar phenotype whereas NFAT dominant negative overexpression blocks" stimulation of migration "*in vitro*" and the tumour promoter-induced tumourogenicity. In contrast NFAT dominant negative overexpression blocks migration. Their results indicate that COX-2, mPGES1, and Calcineurin alters a great number of common genes, inducing an increase in genes related to Angiogenesis migration and motility, immune surveillance, proliferation, cellular differentiation and invasiveness and a decrease of those of apoptosis.

Charité analyzed the expression of 15-LOX-1, 15-LOX-2, COX-1, and COX-2 in clinical specimens of human ovarian carcinoma. They found a significantly higher expression of 15-LOX-2 than in normal ovary but for COX-2 there is a trend of higher expression, although not significant. In a limited pilot study, they also investigated whether the steady state concentration of COX-1 and COX-2 mRNA in the peripheral blood might constitute a predictive parameter for ovarian cancer, which, however, did not hold true.

A group in UDAnn has investigated the cross talk between PGE_2 and nitric oxide (NO) systems in tumor growth and tumor angiogenesis. They provide evidence that iNOS activity stringently controls PGE_2-induced ERK1/2 activation, and tumor growth and invasiveness

through a PKA and Src-dependent transactivation of epidermal growth factor receptor (EGFR). The recognition that iNOS/GC signalling plays a key role in squamous cell carcinoma progression has been confirmed by *in vivo* experiments. They also investigated the role of mPGES-1-derived-PGE_2 on EGFR transactivation and tumor angiogenesis, observing: i) endogenous PGE_2 controls the EGFR activity in epidermoid tumor cell line A431; ii) mPGES-1, colocalize with iNOS in tumor cells; iii) expression of mPGES-1 is crucial for iNOS expression. In addition, they also observed that PGE_2 promotes tumour angiogenesis in human colon cancer by activation of hypoxia inducible factor-1α (HIF-1α) in normoxic condition, and in turn, VEGF production. In carcinoma cell line HT29 IL-1β induces mPGES-1 expression and mPGES-1-derived PGE_2 induces HIF-1α and VEGF. Moreover, hydroxytyrosol, a derivative of olive oil, modulates tumor angiogenesis in colon cancer, inhibiting HIF-1α/VEGF expression in HT29.

Another group in UDAnn investigated the role of COX-2 pathway in mammary glands of BALB NeuT mice, a transgenic mammary tumor model. Enzymes belonging to the cyclooxygenase pathway are over-expressed in the mammary glands from the first stages of the spontaneous tumoral development. Unexpectedly indomethacin a COX unselective inhibitor delay tumour appearance and reduce tumour growth in BALB NeuT mice. Moreover indomethacin exerts a strong inhibitory action on the *in vivo* growth and the neoangiogenic activity of transplantable tumors.

These results suggest that indomethacin does not directly inhibit breast tumour cell survival but elicits its antitumor action on host derived factors, namely angiogenic molecules. Since they also suggest that immune cells and tumour associated macrophages (TAM) have a key role in inflammatory circuits that promote tumour growth and progression, UDAnn compared the cytokine expression profile of TAMs isolated from a murine transplantable breast cancer tumor (TSA) treated or not with indomethacin. A general down regulation of cytokine production in TAMs particularly evident for the classical pro-inflammatory cytokines and chemokines was observed. Indomethacin partially reverses this polarized setting.

UDAnn and NicOx have evaluated the effect of non-steroidal inflammatory drugs and NO donors in tumor progression. Several NO-NSAIDs were evaluated as novel tools for inhibition of cancer cell growth both *in vivo* and *in vitro*. Of the various strategies used by tumors to counteract immune attacks, myeloid suppressor cells (MSCs) are particularly efficient resulting in systemic T lymphocyte dysfunction. NCX-4016 (an acetylsalicylic acid (aspirin) derivative containing a nitric oxide (NO)-releasing moiety) was able to inhibit MSCs and enhanced the preventive and therapeutic effectiveness of the antitumor immunity elicited by DNA vaccine restoring T lymphocyte function.

UDAnn evaluated the role of COXs in the regulation of anti-tumour immune reactivity elicited by DNA vaccination. For this UDAnn has evaluated the effects of indomethacin administration on the antitumoral immune response elicited by anti HER-2/neu DNA vaccination in a transplantable tumor model. Indomethacin did not inhibit or ameliorate the therapeutic efficacy of DNA vaccine on the occurrence and growth of mammary tumors. The analysis of the evoked immune response failed to show differences in the amount of specific antibodies and in the cellular cytotoxic activity. Moreover, IFN-γ is a key cytokine both in the immune response elicited by DNA vaccine and in the antitumor effect of indomethacin treatment since these effects were totally lost in IFN-γ KO mice.

Finally another group of UDAnn studied the regulation of COX-2 and mPGES expression by cyclopentenone PGs (cyPG) in chemoresistant melanomas and in Kaposi Sarcoma (KS) virus-

associated Primary Effusion Lymphoma (PEL), presenting constitutively active NF-κB. High levels of COX-2 were detected in melanoma cells. Unexpectedly, cyPG did not prevent, but actually increased TNFα-induced COX-2 expression under conditions where the IKK/NF-κB signaling was impaired. cyPG-induced COX-2 up-regulation was associated with activation of the JNK/AP-1 pathway. The evidence obtained argues against a prominent role of NF-κB in COX-2 expression in melanoma cells. They also found that cyPG induced COX-2, but not mPGES, expression in cervical carcinoma HeLa cells. UDAnn investigated the molecular mechanism involved in this process and found that it is independent of NF-κB, IKK and HSF1 activity whereas it depends on JNK-1. Gene expression profile analysis of 15d-PGJ2-sensitive genes identified AIRAP as a novel human canonical heat shock gene. Moreover, UDAnn found that heat stress rapidly inhibits constitutive NF-κB activity in chemoresistant lymphomas and multiple myeloma, leading to down-regulation of the antiapoptotic protein IAP-2 and massive apoptosis, and that COX-2 is regulated by heat.

WP 11-LOX and Cancer

Three partners (UFrank:a, KIa, Charité) have been working on the potential role of eicosanoids and EICOSANOX-related enzymes in the pathogenesis of cancer development and metastasis.

In a collaborative project KI:a and UFrank:a explored expression of 5-LOX in B-cell lymphoma and found that the steady state concentration of 5-LOX protein decreases during cell proliferation. Since the efficiency of 5-LOX gene expression was hardly altered proteolytic degradation of the enzyme was explored in more detail. In fact, reduction of the full length 5-LOX was accompanied by the appearance of a 62 kDa degradation product and inhibitors of caspase-6 and -8 prevented 5-LOX cleavage suggesting the involvement of these proteolytic enzymes. Isolated human 5-LOX was cleaved by recombinant casp-6 *in vitro* and a degradation product of similar size was observed. Based on site-directed mutagenesis studies, 5-LOX is cleaved by caspase-6 after Asp170, which is located on the surface of the enzyme (Werz, O., et al. Proc Natl Acad Sci U S A 102, 13164-13169, 2005).

In additional studies, KI:a explored the potential role of eicosanoids in the pathogenesis of two different types of tumors. i) In clinical specimens of primary human neuroblostoma and in all tested neuroblastoma cell lines partner KI:a observed high expression of 5-LOX, 5-LOX-activating protein, LTA4H, LTC4S, and leukotriene receptors. In contrast, no expression was found in nonmalignant adrenal medulla where neuroblastomas typically arise. Inhibitors of leukotriene biosynthesis, leukotriene receptor antagonists and siRNA mediated expression silencing inhibited neuroblastoma cell growth by induction of G1-cell cycle arrest and apoptosis. These findings provided new insights into the pathobiology of neuroblastoma and suggest that the use of leukotriene inhibitors as a novel therapeutic strategy for neuroblastoma (Sveinbjörnsson, B., et al. FASEB J., 22(10):3525-3536, 2008).
ii) The human prostate cancer cell line DU145 expressed high amounts of microsomal prostaglandin E-synthase-1 (mPGES-1) and the enzyme was detected at higher levels in human prostate cancer when compared with benign prostate hyperplasia. Expression knockdown of mPGES-1 conferred decreased clonogenic capacity and slower growth of xenograft tumors in nude mice, which may be related to an increased apoptosis. These results suggest that mPGES-1 might constitute an alternative therapeutic target for special types of prostate cancer (Hanaka, H., et al. Proc Natl Acad Sci U S A, 106(44):18757-62, 2009).

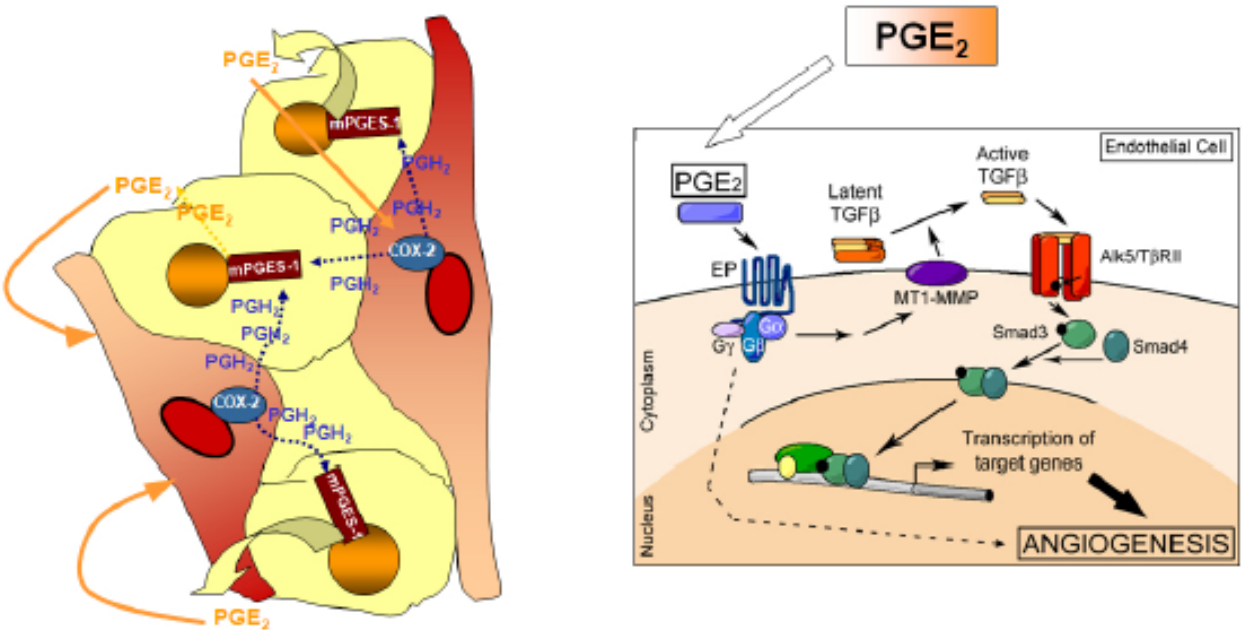
The role of 12/15-LOX in tumor pathogenesis is controversial and has been related to neoangiogenesis, which is important for growth and metastasis of solid tumors. i) In a collaborative study Charité explored the impact of 12/15-LOX overexpression on tumor growth and metastasis in an *in vivo* mouse model. Using transgenic mice that overexpress 12/15-LOX in vascular endothelial cells it was observed that 12/15-LOX overexpression inhibited tumor development and metastasis employing two different tumor models. When mammary gland and Lewis lung carcinoma cells were injected in transgenic and control mice the transgenic animals developed significantly less tumors and metastasis was strongly impaired. This inhibition was concomitant with a higher number of apoptotic cells in the primary tumors and in metastases and neoangiogenesis appeared to be disturbed (Harats, D., et al. Cancer Lett. 229(1):127-34, 2005. These findings target 12/15-LOX as a new candidate in the treatment of carcinogenesis. ii) Ovarian cancer is a major cause of lethality from gynecological malignancies. Eicosanoid-related enzymes have been implicated in the pathogenesis of various types of cancer, but little is known about the relevance of lipoxygenase isoforms in ovarian cancer. To explore the potential role of LOX- and COX-isoforms in ovarian carcinoma Charité quantified the expression of these enzymes in normal and malignant human ovarian tissue and found a highly significant increase (20-fold) in the expression of 15-LOX-2 in malignant specimens. In ovarian carcinoma metastases expression of the enzyme was also augmented. In contrast, for 12/15-LOX and COX-2 we did not observe differential expression, but there was a statistic trend for increased expression ($p=0.1$) of COX-1 and 5-LOX. These data indicate that expression of 15-LOX-2 is upregulated during ovarian carcinogenesis and that the enzyme may constitute a suitable candidate as a tumor marker (Roffeis, J., et al. Eur. J. Canc. Prev. 16, 568-575, 2007).

WP 12-COX, PG synthases, and NOS in angiogenesis

The partners within WP12 are The William Harvey Research Institute, Queen Mary University of London, U.K. (QMUL), Univ. Autonoma de Madrid, Spain (UAM:b), NicOx S.A., France (NicOx) and G. D'Annunzio University, Italy (UDAnn). The theme of WP12 was to research the roles and involvements in angiogenesis of the COX enzymes, prostanoid synthases, and the nitric oxide pathway.

The experimental achievements of WP12 can be divided into a number of areas. First of all, our studies have led us to identify a novel signalling pathway linking two effectors involved in tumour growth and angiogenesis (PGE_2 and TGFβ). Secondly, our work has revealed a novel mechanism of transcellular metabolism between endothelial and tumour cell types, which provides an alternative pathway for PGE_2 production. Given the role of PGE_2 as a mediator of the pro-angiogenic effects of COX-2, transcellular metabolism of endothelial-derived PGH_2 is a potential target for treatment of pathological angiogenesis. These two findings reveal potential targets for the treatment of angiogenesis-related disorders, such as neovascularisation-dependent retinopathies. The third part of our efforts has been devoted to the study of the protein calcineurin (CN), a unique phosphatase that transmits calcium signals from the cytosol to the nucleus and so regulates gene expression in T cells, neurons, and muscle cells. CN is the common target of the widely used immunosuppressive drugs cyclosporin A (CsA) and FK506, and our analyses have provided insights into the mechanisms by which immunosuppressants inhibit CN. This is an important area of research as the severe side effects of immunosuppressants (such as neurotoxicity, diabetes, nephrotoxicity, and hypertension) can limit their clinical use for chronic inflammatory conditions and other diseases. Importantly, however, the side effects of these drugs are at least

partly independent of CN because these drugs affect other signalling pathways. Therefore, identifying selective CN inhibitors that avoid these secondary effects is of high interest. Our studies have indentified that immunophilin-independent immunosuppressive drugs could be developed that would avoid some of the side effects associated with FK506 and CsA. Fourthly, and further to our examinations of the activities of clinically relevant drugs, we explored the pathways through which ACE inhibitors could produce their beneficial effects in cardiovascular disease. Our studies showed that ACE inhibitors have antioxidant properties that can lead to upregulation of eNOS, FGF-2 and TERT mRNA. The combination of these factors favours endothelial cell survival and prolongs endothelial lifespan, and so helps to restore endothelial cell functions after vascular damage. These effects could explain the beneficial effects of ACE inhibitors in different cardiovascular diseases associated with endothelial injury and aging. Fifthly, we looked at the effects of PGE_2 upon angiogenesis, and found that it synergizes with FGF-2 and so activates the FGF-2 pathway. This *in vitro* observation was confirmed *in vivo*, in the rabbit cornea and Matrigel plug assay, in which a strong synergism between PGE_2 and FGF-2 promoted a robust angiogenesis. Finally, in other studies developing from *in vitro* experiments to *in vivo* models, we demonstrated that activation of PPARβ/δ receptors increases *in vitro* human endothelial cell proliferation and morphogenesis and endothelial cell outgrowth from murine aortic vessels through influences on VEGF. Further to this we demonstrated that activation of PPARβ/δ promotes angiogenesis in a murine matrigel plug assay *in vivo*. These studies demonstrated that PPARβ/δ is a novel regulator of endothelial cell proliferation and angiogenesis through VEGF. This provides potential new approaches to inhibit angiogenesis, e.g. for cancer therapy, and also indicates that the use of PPARβ/δ ligands to treat dyslipidemia may need to be carefully monitored in patients susceptible to angiogenic disorders.



Novel transcellular metabolism of PGE_2 between the endothelial and tumor compartments (Salvado *et al.*, 2009)

Signaling pathway linking PGE_2 and TGFβ, two effectors involved in tumor growth and angiogenesis (Alfranca *et al.*, 2008). This work reveals potential targets for the treatment of angiogenesis-related disorders.

Overall WP12 has produced a **wide range of basic science studies** that have shed light upon the **processes regulating endothelial cell growth and angiogenesis**, and have indicated a number of **novel therapeutic pathways** that could be developed. In addition, WP12 has produced genuine collaborative research between European partners, in particular the application of angiogenesis models developed by UAM:b.

WP 13-Core facilities

This WP is devoted to Core facilities, in order to allow all groups to have access to the required state-of-the art technologies and methods.
We initially focused on the preparation of a centralized "library of methods" so that all groups could be made aware and kept up-to-date on specialized and technically demanding methods developed by individual groups. This was particularly relevant for "*in vivo*" animal or "*in vitro*" organ models for which there are basically no standardized guidelines and may represent a crucial, final step in order to acquire information of patho-physiological relevance. The groups were asked to respect a common protocol and we decided to adopt as a template the format that has been used for many years in the series "Methods in Enzymology". Texts were collected, revised and available to all groups on-line. Furthermore, we created an inventory of reagents, cell lines, methods and core facilities available at the partners' home institutions. A questionnaire was prepared and distributed to all EICOSANOX partners. Significant work was devoted to prevent redundancies and create a common, uniform "platform" of presentation of data that could be easily understood; in addition, considerable effort has been devoted to create a "centralized library" to facilitate seeking of information or advice from consortium members with expertise in particular areas. The "library" is available to all members of EICOSANOX by connecting to "www.eicosanox.org" and is constantly kept up-to-date.

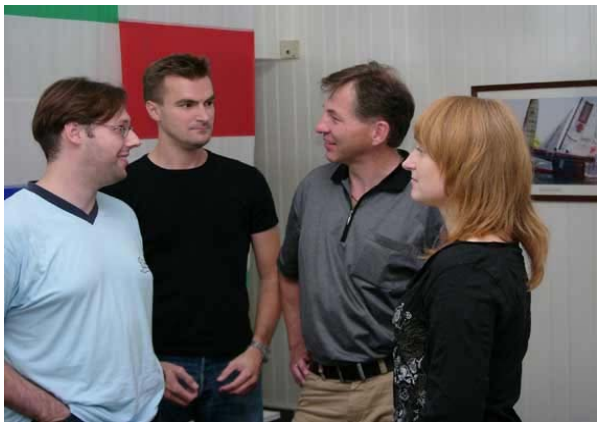
WP 14-Training, dissemination, implementation and technology transfer

Six partners (UFrank:a, NUID/UCD, Biolipox/Orexo, NicOx, KI:a and UAM:a) have been working in this WP and planned and executed training and educational programmes within the consortium, handled exchange programmes at the post graduate and postdoctoral level and established effective mechanisms for dissemination, implementation and technology transfer.

During the duration of the project, intensive training activities have been established at the consortium level. Educational and training activities at different partner institutions were announced at the EICOSANOX homepage. The consortium has successfully conducted postgraduate courses that cover scientific, technical and management skills. Summer and winter schools for graduate students have been held at various locations that covered experimental and clinical aspects of the arachidonic acid, the sphingolipid and the nitric oxide pathways. The activities included oral presentations and discussions among all participants as well as social activities. Many young investigators participated at the annual EICOSANOX meetings where the latest scientific achievements of the consortium were presented.

Education at the doctoral and postdoctoral level that covered, among other subjects, the COX and LOX pathways, CYP-mediated arachidonic acid metabolism (EETs), nitric oxide pathway, sphingolipid metabolism, proteomics and lipidomics and structural biology of the

eicosanoid-related enzymes and receptors. The activities were coordinated by UFrank:a and KI:a and PhD students from many partners participated at the courses and seminars. Courses and seminars with special emphasis on EICOSANOX related subjects were offered by UCD, KI:a, UAM, UFrank:a and UCL. Information on the programmes were gathered at the EICOSANOX website and regularly updated. An integrated education programme for postgraduate students called "Roles of Eicosanoids in Biology and Medicine" has been established and continued at KI and UFrank:a. In total, 22 theses were published during the life-time of the project (see list below).



- An integrated education programme for postgraduate students called "Roles of Eicosanoids in Biology and Medicine" is arranged by UFrank and KI.
- Meetings (2/year) open for PhD students and post docs.

List of doctoral theses

1. **Antibacterial effects of nitrite in urine** by S Carlsson, Karolinska Institutet, Stockholm, April 2005.
2. **Nitric oxide as a surrogate marker of bowel inflammation** by C. Reinders, Karolinska Institutet, Stockholm, Nov 2005.
3. **The nitrite ion. Its role in vasoregulation and host defenses** by H. Björne, Karolinska Institutet, Stockholm, Dec 2005.
4. **Catalytic mechanisms and evolution of leukotriene A_4 hydrolase** by Fredrik Otto Tholander, Karolinska Institutet, Stockholm, December 2006.
5. **Isolated and perfused brain: a method to study the cerebrovascular system** by Antonio Di Gennaro, University of Milan, November 2006.
6. **Effect of HIV-tat protein in the activation of T lymphocytes** by Alicia Mª Hidalgo Estévez, Universidad Autónoma de Madrid. February 2006.
7. **Modulación de la Actividad Transcripcional de NFAT1 por las MAP Quinasas p38 y JNK** by María Inmaculada Ortega Pérez. Universidad Autónoma de Madrid, June 2006.
8. **Investigations on the Regulation of 5-Lipoxygenase Gene Expression by DNA Methylation and Histone Deacetylation/ Acetylation** by Nicole Schnur, University of Frankfurt, Nov 2006.
9. **Leukotrienes and Leukotriene receptors: Potential roles in cardiovascular diseases** by Hong Qiu, Karolinska Institutet, Stockholm, January 2007.
10. **Studies on molecular properties and functional regulation of terminal leukotriene C_4 synthases and cystinyl-leukotriene receptor signalling in human endothelium** by Oliver Schröder, Karolinska Institutet, Stockholm, March 2007.
11. **Mechanisms of regulation of COX-2 and mPGES-1 expression in macrophages: Involvement in activation, migration and atherosclerosis** by Manuel D. Díaz Muñoz. Universidad Autónoma de Madrid, February 2008.
12. **L-arginine metabolism in experimental Chagas disease; role of arginase I and iNOS in heart tissue** by Henar Cuervo Grajal, Universidad Autónoma de Madrid. April 2008.
13. **Regulation of prostanoid biosynthesis by human endothelial cells in angiogenesis: role of COX-2 in the intracellular and transcellular synthesis of prostanoids** by **Mª Dolores Salvado**. European Thesis awarded with honours (Sobresaliente *Cum Laude*) Universidad Autónoma de Madrid, October 2008.
14. **Regulation of the matricellular protein SMOC-1 by cytokines and nitric oxide in rat mesangial cells** by Ellen Dreieicher, University of Frankfurt, Sept 2009.
15. **The role of sphingosine kinases 1 and 2 in cell growth and apoptosis in renal mesangial cells** by Lotte P. Hofmann, University of Frankfurt, November 2009.
16. **The effect of vascular cells on platelets and an assessment of platelet activity in clinical settings** by Nicola Truss, London, May 2009.

17. **Investigation of the relationship between the activation of the enzyme cyclooxygenase and the production of thromboxane A_2, platelet aggregation and thrombosis; an in vitro, ex vivo and in vivo approach** by Paul Armstrong, London, August 2009.
18. **Investigation of the potential effect of the anti-inflammatory and pro-resolution mediators LXA_4 and Ac2-26 on glial cells** by Yann Decker, Dublin, 2009
19. **Cardiopulmonary pharmacology of NTX-99: a novel antiatherothrombotic agent** by Luca Marcassoli, University of Milan, 2009.
20. **Studies on the activation of cytosolic phospholipase A_2 and 15-lipoxygenase-1 in hematopoietic cells** by Erik Andersson, Karolinska Institutet, Stockholm, April 2009.
21. **Biosynthesis and biological role of leukotrienes in human lymphocytes** by Yilmaz Mahshid, Karolinska Institutet, Stockholm, April 2009.
22. **Studies on Coactosin-like protein: Interaction with 5-liopxygenase** by Marija Rakonjac, Karolinska Institutet, Stockholm, November 2009.

Exchange programmes at the postgraduate and postdoctoral levels were offered (UFrank:a, KI:a, UAM, UDAnn, NUID/UCD) and there was a frequent exchange of scientists at the postgraduate, postdoc and senior level throughout the duration of the project.

To provide project information to other scientists and the public the PO has also maintained and updated an external website http://www.eicosanox.org/. Here the broad public can receive information about the project and its progress. Since its inception, the Eicosanox website has been visited >15,700 times. The PO has also produced 8 issues of the Eicosanox newsletter, as well as a poster and brochure. This material is available at the public web site and has been distributed to all Eicosanox research groups.

Effective means for implementation and technology transfer were established by using protected internet facilities and administrative and technology transfer services at the participating institutions. Biolipox/Orexo has discussed and evaluated discoveries and projects proposals with the Partners. During these discussions Biolipox/Orexo has provided counselling and expertise on IP and entrepreneurship. The EICOSANOX partners fostered the conversion of scientific results into intellectual property for commercial development. Their business development offices promoted this through establishing collaborative projects with industrial partners. The participating scientists were encouraged to contact the business development managers for discussions on research that may in future lead to an invention or business idea. The agencies arranged patents for inventions, negotiate licensing agreements, Materials Transfer Agreements (MTAs) and Confidentiality Agreements (CDAs). The partners have successfully patented a number of exploitable results (see separate list).

WP15-Management

A project office (PO) is required to provide efficient management and administration of the project and consists of Jesper Z. Haeggström as the Director, Anders Wetterholm and Olof Rådmark as the project managers (PM), Craig Wheelock/Alan Sabirsh as Communication Manager, and Anneli Svarén/Galina Stahmer/Maria Wejdmark as project administrators (PA). The Director, PMs and PA work in close collaboration and have provided a robust management component throughout the duration of the project.

To establish efficient communication between the partners in the Eicosanox consortium, a project intranet system has been maintained. This system, called Karolinska Project

Management (KPM), works as a 'virtual conference room' and allows project members to exchange files, work with a common calendar, have an archive for files on-line and from any Internet-connected computer. One of the 'virtual rooms' is open to all members of the consortium and here all manuscripts generated within the Eicosanox project are uploaded before submission for publication. From this area, members can download the project "handbooks", research protocols, lists of research tools, addresses, telephone numbers etc.

A web-based communication software, which may be used by all members of Eicosanox has been maintained on a server at the PO. Up to 10 persons, in one or several virtual rooms may meet simultaneously from any computer equipped with a web camera and headset. The web-based meeting room has allowed the **Work Management Group** to "meet" regularly every second week and has efficiently saved both time and travel expenses.

Project meetings: The kick-off meeting took place in Stockholm at the start of the project. The meeting provided a very nice opportunity for everybody to get to know each other and each other's work. Informal evening activities, laid the ground for an open and friendly communication between the partners. To promote fruitful collaborations within the project, the Consortium has gathered at least once per year. During these annual meetings, scientific progress of the project was discussed as well as general issues.

From left to right, Angela Monopoli (NicOx), Jesper Z. Haeggström (KI), Charlotte Edenius (Biolipox/Orexo), Carlo Patrono (UDAnn), Tim Warner (QMUL), Dieter Steinhilber (UFrank), Hartmut Kühn (Charité) and Manuel Fresno (UAM).

EICOSANOX – MEETINGS ACROSS EUROPE

- EICOSANOX Annual Meeting 2009- December, Stockholm - SWEDEN
- EICOSANOX Annual Meeting 2008 - October, Siena - ITALY
- EICOSANOX Annual Meeting 2007 - September, Aigen/Ennstal - AUSTRIA
- EICOSANOX Annual Meeting 2006 - October , Universidad Autonoma de Madrid - Madrid – SPAIN
- Cluster B meeting – October 2006, Universidad Autonoma de Madrid - Madrid – SPAIN
- Cluster A meeting - August 2006, Stockholm – SWEDEN
- Cluster C meeting - July 2006, - G.D'Annunzio University - Chieti – ITALY
- EICOSANOX Annual Meeting 2005 - October/November, Stockholm - SWEDEN
- EICOSANOX Kick Off Meeting 2005- March, Stockholm - SWEDEN

1.7. Dissemination and use

During the five years of the Eicosanox project, **333 articles** were published. The **majority** were published in journals with **impact factors >5** and no less than **34** articles were in journals **with impact factor >10**, e.g. **N Engl J Med, Nature, J Clin Invest, Lancet, Blood, Mol Cell** etc. The Eicosanox publications have so far (February 2010) been **cited >4 000** times.

Members of the consortium have presented their data at **>100 international conferences** and also commented on their work in the media. In total **22 doctoral theses** were generated in the duration of the project, **15 patents were filed and 3 spin-off companies created**.

To disseminate information to other scientists and to the public we have created a **website (>15700 visitors)**, which has been updated in a regular manner (http://www.eicosanox.org/).

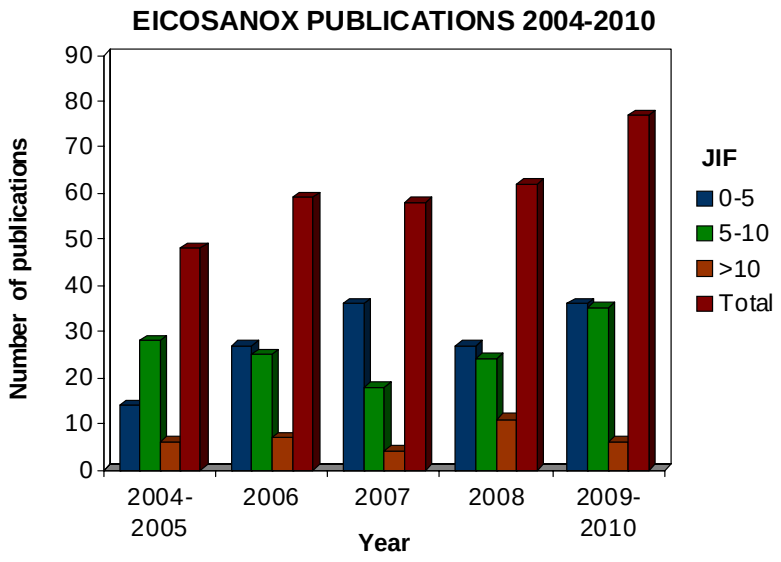
A Google search of "Eicosanox" generates >10 000 hits on the internet.

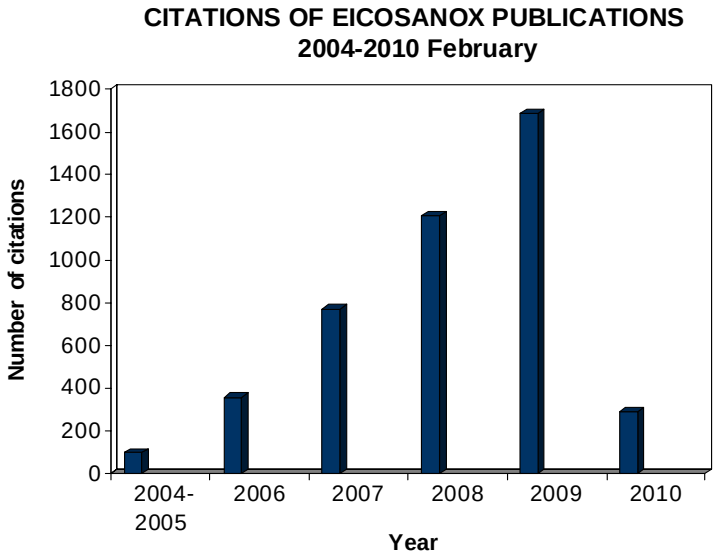
1.8. Bibliometric analysis

A bibliometric analysis was carried out for the articles published until February 2010 (Table 1). The diagrams below show the distribution of articles with respect to journal impact factors (JIF) and number of citations. On the following pages the Eicosanox top-ranked publications (JIF >10) and the 25 most cited Eicosanox publications are listed.

Year	Publ	Citations	Σ JIF	Average JIF	Cf	Σ Cf	Top5%	Share Top5%
2004-2005	66	1696	353,0	5,6	1,4	89,6	4	6%
2006	54	1281	374,8	6,9	1,9	99,6	5	9%
2007	58	804	353,0	6,1	1,5	86,9	6	10%
2008	56	425	373,5	6,7	1,6	92,3	5	9%
2009-2010	60	76	362,7	6,0	**	**	**	**
2004-2008	294	4282	1454,0	6,2	1,6	458,0	22	8,5%

Table 1. Cf, item oriented field normalized citation score; **, too recent to be stable.





1.9. List of Eicosanox top-ranked publications (JIF>10)

The NEW ENGLAND JOURNAL of MEDICINE

JIF 52,59

N Engl J Med. 2006 Dec 28;355(26):2792 -3.

Effects of dietary nitrate on blood pressure in healthy volunteers

Larsen FJ, Ekblom B, Sahlin K, Lundberg JO , Weitzberg E.

1. Bjorne H H, Petersson J, Phillipson M, Weitzberg E, Holm L, Lundberg JO. Nitrite in saliva increases gastric mucosal blood flow and mucus thickness. **J Clin Invest**. 2004, 113(1):106-14. Erratum in: J Clin Invest. 2004, ;113(3):490.
2. Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, Cannon RO 3rd, Kelm M, Wink DA, Espey MG, Oldfield EH, Pluta RM, Freeman BA, Lancaster JR Jr, Feelisch M, Lundberg JO. The emerging biology of the nitrite anion. **Nat Chem Biol**. 2005 Nov;1(6):308-14.
3. Patrono C, García Rodríguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. **N Engl J Med**. 2005, 353:2373-83.
4. Bäck M, Bu D-X, Bränström R, Sheikine Y, Yan Z-Q, and Hansson G K. Leukotriene B4 signaling through NF-{kappa}-B-dependent BLT1 receptors on vascular smooth muscle cells in atherosclerosis and intimal hyperplasia. **Proc Natl Acad Sci U S A**. 2005, 102: 17501-506.
5. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, Melani C, Guiducci C, Colombo MP, Iezzi M, Musiani P, Zanovello P, Bronte V. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. **Proc Natl Acad Sci U S A.** 2005, 102:4185-90.

6. Werz O, Tretiakova I, Michel A, Ulke-Lemee A, Hornig M, Franke L, Schneider G, Samuelsson B, Radmark O, Steinhilber D. Caspase-mediated degradation of human 5-lipoxygenase in B lymphocytic cells. **Proc Natl Acad Sci U S A**. 2005, 102(37):13164-9.

7. Chiodoni C, Iezzi M, Guiducci C, Sangaletti S, Alessandrini I, Ratti C, Tiboni F, Musiani P, Granger DN, Colombo MP. Triggering CD40 on endothelial cells contributes to tumor growth. **J Exp Med**. 2006, 203(11):2441-50.

8. Folco G, Murphy RC. Eicosanoid transcellular biosynthesis: from cell-cell interactions to in vivo tissue responses. **Pharmacol Rev**. 2006, 58(3):375-88.

9. Mitchell JA, Warner TD. COX isoforms in the cardiovascular system: understanding the activities of non-steroidal anti-inflammatory drugs. **Nat Rev Drug Discov**. 2006, 5(1):75-86.

10. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of dietary nitrate on blood pressure in healthy volunteers. **N Engl J Med**. 2006, 355(26):2792-3.

11. Ciana P, Fumagalli M, Trincavelli ML, Verderio C, Rosa P, Lecca D, Ferrario S, Parravicini C, Capra V, Gelosa P, Guerrini U, Belcredito S, Cimino M, Sironi L, Tremoli E, Rovati GE, Martini C, Abbracchio MP. The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. **EMBO J.** 2006, 25:4615-27.

12. Patrignani P, Di Febbo C, Tacconelli S, Moretta V, Baccante G, Sciulli MG, Ricciotti E, Capone ML, Antonucci I, Guglielmi MD, Stuppia L, Porreca E. Reduced thromboxane biosynthesis in carriers of toll-like receptor 4 polymorphisms in vivo. **Blood.** 2006, 107(9):3572-4.

13. Kuhn H, O'donnell VB. Inflammation and immune regulation by 12/15-lipoxygenases. **Prog Lipid Res**. 2006, 45(4):334-56.

14. Davì G, Patrono C. Platelet activation and atherothrombosis. **N Engl J Med**. 2007 Dec 13;357(24):2482-94.

15. Radmark O, Werz O, Steinhilber D, Samuelsson B. 5-Lipoxygenase: regulation of expression and enzyme activity. **Trends Biochem Sci**. 2007, 32(7):332-41.

16. Molina DM, Wetterholm A, Kohl A, McCarthy AA, Niegowski D, Ohlson E, Hammarberg T, Eshaghi S, Haeggstrom JZ, Nordlund P. Structural basis for synthesis of inflammatory mediators by human leukotriene C(4) synthase. **Nature**. 2007, 448(7153):613-6.

17. Moraes LA, Swales KE, Wray JA, Damazo A, Gibbins JM, Warner TD, Bishop-Bailey D. Nongenomic signalling of the retinoid X receptor through binding and inhibiting Gq in human platelets. **Blood**. 2007, 109(9):3741-4.

18. García Rodríguez LA, Tacconelli S, Patrignani P. Role of dose potency in the prediction of risk of myocardial infarction associated with nonsteroidal anti-inflammatory drugs in the general population. **J Am Coll Cardiol.** 2008 Nov 11;52(20):1628-36.

19. Ufer C, Wang CC, Fähling M, Schiebel H, Thiele BJ, Billett EE, Kuhn H, Borchert A. Translational regulation of glutathione peroxidase 4 expression through guanine-rich sequence-binding factor 1 is essential for embryonic brain development. **Genes Dev**. 2008 Jul 1;22(13):1838-50.

20. Santilli F, Romano M, Recchiuti A, Dragani A, Falco A, Lessiani G, Fioritoni F, Lattanzio S, Mattoscio D, De Cristofaro R, Rocca B, Davi G. Circulating endothelial progenitor cells and residual in vivo thromboxane biosynthesis in low-dose aspirin-treated polycythemia vera patients. **Blood**. 2008 Aug 15;112(4):1085-90.

21. Jansson EA, Huang L, Malkey R, Govoni M, Nihlén C, Olsson A, Stensdotter M, Petersson J, Holm L, Weitzberg E, Lundberg JO. A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. **Nat Chem Biol.** 2008 Jul;4(7):411-7.

22. Alfranca A, Lopez-Oliva JM, Genis L, Lopez-Maderuelo D, Mirones I, Salvado D, Quesada AJ, Arroyo AG, Redondo JM. PGE2 induces angiogenesis via the MT1-MMP-mediated activation of the TGF{beta}/Alk5 signalling pathway. **Blood**. 2008 Aug 15;112(4):1120-8.

23. Molina DM, Eshaghi S, Nordlund P. Catalysis within the lipid bilayer-structure and mechanism of the MAPEG family of integral membrane proteins. **Curr Opin Struct Biol**. 2008 18(4):442-9.

24. Morton J, Coles B, Wright K, Gallimore A, Morrow JD, Terry ES, Anning PB, Morgan BP, Dioszeghy V, Kuhn H, Chaitidis P, Hobbs AJ, Jones SA, O'Donnell VB. Circulating neutrophils maintain physiological blood pressure by suppressing bacteria and IFN{gamma}-dependent iNOS expression in the vasculature of healthy mice. **Blood.** 2008 May 15;111(10):5187-94.

25. Warner TD, Mitchell JA. COX-2 selectivity alone does not define the cardiovascular risks associated with non-steroidal anti-inflammatory drugs. **Lancet.** 2008 Jan 19;371(9608):270-3.

26. Qiu H, Stråat K, Rahbar A, Wan M, Söderberg-Nauclér C, Haeggström JZ. Human CMV infection induces 5-lipoxygenase expression and leukotriene B4 production in vascular smooth muscle cells. **J Exp Med**. 2008 Jan 21;205(1):19-24.

27. Randriamboavonjy V, Pistrosch F, Bölck B, Schwinger RH, Dixit M, Badenhoop K, Cohen RA, Busse R, Fleming I. Platelet Sarcoplasmic Endoplasmic Reticulum Ca2+-ATPase and {micro}-Calpain Activity Are Altered in Type 2 Diabetes Mellitus and Restored by Rosiglitazone. **Circulation**. 2008, 117(1):52-60.

28. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. **Nat Rev Drug Discov**. 2008 Feb;7(2):156-67.

29. Patrono C, Rocca B. The future of antiplatelet therapy in cardiovascular disease. **Annu Rev Med.** 2010;61:49-61.

30. Dragani A, Pascale S, Recchiuti A, Mattoscio D, Lattanzio S, Petrucci G, Mucci L, Ferrante E, Habib A, Ranelletti FO, Ciabattoni G, Davi G, Patrono C, Rocca B. The contribution of cyclooxygenase-1 and -2 to persistent thromboxane biosynthesis in aspirin-treated essential thrombocythemia: implications for antiplatelet therapy. **Blood**. 2009 Nov 3, in press.

31. Shaw MH, Reimer T, Sánchez-Valdepeñas C, Warner N, Kim YG, Fresno M, Nuñez G. T cell-intrinsic role of Nod2 in promoting type 1 immunity to Toxoplasma gondii. **Nat Immunol**. 2009 Dec;10(12):1267-74.

32. Loot AE, Schreiber JG, Fisslthaler B, Fleming I. Angiotensin II impairs endothelial function via tyrosine phosphorylation of the endothelial nitric oxide synthase. **J Exp Med**. 2009 Nov 23. in press.

33. Rodríguez A, Roy J, Martínez-Martínez S, López-Maderuelo MD, Niño-Moreno P, Ortí L, Pantoja-Uceda D, Pineda-Lucena A, Cyert MS, Redondo JM. A conserved docking surface on calcineurin mediates interaction with substrates and immunosuppressants. **Mol Cell.** 2009 Mar 13;33(5):616-26.

34. Santilli F, Rocca B, De Cristofaro R, Lattanzio S, Pietrangelo L, Habib A, Pettinella C, Recchiuti A, Ferrante E, Ciabattoni G, Davì G, Patrono C. Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays: implications for aspirin "resistance". **J Am Coll Cardiol.** 2009 Feb 24;53(8):667-77.

1.10. List of the 25 most cited articles 2004-2010

BMJ helping doctors make better decisions 306 Citations

BMJ 2006;332:1302-1308 (3 June), 2006

Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials

Kearney, PM, Baigent, C, Godwin, J, Halls, H, Emberson, JR, Patrono, C.

	All articles (307) cited 4 411 times	Total	Average Citations / Year
1.	Kearney, PM; Baigent, C; Godwin, J; et al. Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials BRITISH MEDICAL JOURNAL, 332 (7553): 1302-1305 JUN 3 2006	306	61.20
2.	Patrono, C; Rodriguez, LAG; Landolfi, R; et al. Drug therapy - Low-dose aspirin for the prevention of atherothrombosis NEW ENGLAND JOURNAL OF MEDICINE, 353 (22): 2373-2383 DEC 1 2005	203	33.83
3.	Gladwin, MT; Schechter, AN; Kim-Shapiro, DB; et al. The emerging biology of the nitrite anion NATURE CHEMICAL BIOLOGY, 1 (6): 308-314 NOV 2005	167	27.83
4.	Lundberg, JO; Weitzberg, E; Gladwin, MT The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics NATURE REVIEWS DRUG DISCOVERY, 7 (2): 156-167 FEB 2008	110	36.67
5.	Davi, G; Patrono, C Mechanisms of disease: Platelet activation and atherothrombosis NEW ENGLAND JOURNAL OF MEDICINE, 357 (24): 2482-2494 DEC 13 2007	110	27.50
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