Effects of Inflammation and Non-Steroidal Anti-Inflammatory Drugs on Renin-Angiotensin System and Cytochrome P450 Metabolites of Arachidonic Acid

by

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Abstract

Patients suffering from inflammatory disorders are at higher risk of cardiovascular (CV) morbidity and mortality compared to general population. Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to treat pain and inflammation associated with inflammatory disorders such as rheumatoid arthritis (RA). However, since the withdrawal of rofecoxib from the market, due to reports of life threatening CV incidents in its users, other NSAIDs are also suspected for such risks. Combined effects of inflammation and NSAIDs use on CV/renal risks are not well understood. However, many hypotheses have been suggested to explain the mechanisms behind these risks, yet none is conclusive and further pharmacological and pharmacokinetic explanations are needed to be explored.

The renin angiotensin system (RAS) and cytochrome P450 (CYP) metabolites of arachidonic acid (ArA) are two major systems responsible for maintaining CV homeostasis in the body. Both systems contain distinct components in their pathways which physiologically oppose each other enabling a naturel balance. RAS component includes angiotensin converting enzymes (ACE, ACE2), physiologically active peptides (Ang-II, Ang-(1-7)) and angiotensin receptors (AT1R, AT2R Mas). The ACE/Ang-II/AT1R are group into what is called as cardiotoxic axis and ACE2/Ang-(1-7)/Mas are group into what is called as cardioprotective axis. Similarly, CYP pathway consists of CYP-enzymes and their arachidonic acid (ArA) metabolites (i.e., 20-hydroxyeicosatetraenoic acid; 20-HETE and epoxyeicosatrienoic acids; EETs, respectively). They are grouped into hydroxylase pathway (i.e., CYP-hydroxylases and 20-HETE) which is cardiotoxic in nature and epoxygenase pathway (i.e., CYP-epoxygenases and EETs) which is cardioprotective in nature. Constitutively, a balance exists between these components. However, under inflammatory conditions as well as in CV diseases this balance is altered indicating cardiotoxicity.

We hypothesized that NSAIDs induced CV/renal risks involve their effects on RAS and ArA systems in the heart and kidney tissues. Also the extent of tissue exposure to NSAIDs may govern such risks. To test these hypotheses we first performed a systematic review of randomized control trials and observational studies. Looking for published evidence that NSAIDs differ in their extent of CV/renal

risks. Based on these findings we choose four NSAIDs (rofecoxib, celecoxib, meloxicam, and flurbiprofen) to be dosed in Sprague Dawley rats, to investigate their extent of tissue accumulation and how it affects RAS and ArA pathways in adjuvant arthritis (AA) rat model of inflammation.

Our results suggest that rofecoxib have highest while meloxicam and celecoxib have least potential of causing CV toxicity in population. Rofecoxib and flurbiprofen were also found to have higher tissue accumulation compared to meloxicam which minimally distributes in heart and kidney tissues. Celecoxib was an exception, which have high tissues accumulation yet possess less CV risks. Our results in AA rats showed that inflammation results in lower ACE2/Ang-(1-7)/Mas the cardioprotective axis, over ACE/Ang-II/ AT1R cardiotoxic axis. NSAIDs restored the constitutive balance, perhaps due to their anti-inflammatory properties. However, difference exists in terms of NSAIDS effects on ArA metabolism. Rofecoxib and flurbiprofen when dosed in inflamed rats, further increased 20-HETE/EETs cardiotoxic/cardioprotective metabolites concentration in the plasma and heart of AA rats. But meloxicam and celecoxib were devoid of these effects. These findings also correspond to higher tissue accumulation and higher CV risk reported for rofecoxib and flurbiprofen compared to meloxicam.

We concluded that inflammation has detrimental effects on both RAS and ArA metabolism. NSAIDs effects on RAS are anti-inflammatory and beneficial in nature, however, some NSAIDs alter ArA metabolism resulting in higher concentration of cardiotoxic metabolites in the body. Moreover, NSAIDs with higher tissue distribution (e.g., rofecoxib, flurbiprofen) are more likely to interfere with ArA metabolism and may pose higher CV risks. While, NSAIDs which minimally distributes (e.g., meloxicam) into heart and kidney tissues poses significantly safer cadiorenal profiles. We also found that plasma and heart profiles of ArA metabolites of are very similar to each other, thus plasma ArA metabolites can serve as surrogate biomarkers of NSAIDs induced cardiotoxicity.

Preface

This thesis is an original work by Muhammad Waheed Asghar, completed under supervision of Prof. Dr. Fakhreddin Jamali at the University of Alberta. The animal studies performed on Sprague Dawley rats, received research ethics approval from the University of Alberta Research Ethics Board, Project Name " Pharmacokinetics and Pharmacodynamics of anti-rheumatic and cardiovascular drugs", No AUP00000267, 11/2/2015 .

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In chapter 6 and 7 the data on effect of inflammation on renin angiotensin system and arachidonic acid metabolites was a collaborated project with Dr. Ali Aghazadeh-Habashi, under supervision of Prof. Dr. Fakhreddin Jamali being the primary author. Muhammad Waheed Asghar is also a co-author of the publication coming out of this shared project "Association of the renin-angiotensin system components and arachidonic acid metabolites under inflammatory condition in the rat with adjuvant arthritis".

DEDICATION

This work is nicely dedicated to my family and parents

Mr. Asghar Ali Rohail and Mrs. Zahida Asghar

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AA	Adjuvant arthritis
Ang-(1-7)	Angiotensin 1-7 peptide
Ang-II	Angiotensin-II peptide
AUC	Area under the concentration curve
AT1R	Angiotensin-II Type 1 receptor
AT2R	Angiotensin-II Type 2 receptor
ACE	Angiotensin converting enzyme
ArA	Arachidonic acid
CV	Cardiovascular
COX	Cyclooxygenase enzyme
CLASS	Celecoxib Long-term Arthritis Safety Study
CYP	Cytochrome P450 enzyme
DHET	Dihydroxyeicosatrienoic acid
EET	Epoxyeicosatrienoic acids
ELISA	Enzyme-linked immunosorbent assay
HETE	Hydroxyeicosatetraenoic acids
GI	Gastro intestinal
HPLC	High performance liquid chromatography
IC50	half maximal inhibitory concentration
ICD-10	International Statistical Classification of Diseases Version-10
Mas	Mas receptor
MI	Myocardial infarction
NSAIDs	Non-steroidal anti-inflammatory drugs
NF-κB	Nuclear factor-kappa-B
nM	Nano moles
mM	Milli moles
mg	Milligram
OA	Osteoarthritis
OR	Odds ratio
OR’	Combined odds ratio
PK	Pharmacokinetics
PD	Pharmacodynamics
pKa	Ionization constant
RA	Rheumatoid arthritis
RR	Relative risk ratio
RAS	Renin angiotensin system
RCT	Randomized controlled trials
sEH	Soluble epoxide hydrolase
TNF	Tumor necrosis factor
µM	Micro moles
µL	Micro liter
µg	Micro gram
VIGOR	VIOXX Gastrointestinal Outcomes Research Trial

Chapter 1

1. Introduction

1.1. Inflammation

Inflammation is a physiological response of the body towards harmful stimuli, damage to its cells and/or invasion by a pathogen. Inflammation is a protective mechanism that prevents the spread of infection, eliminates pathogens, disposes of dead cells and sets the stage for healing and repair. The process of inflammation involves complex physiological mechanisms, including activation of the immune system, release of chemical mediators and tissue response all collectively constitute the body's biological response towards a harmful stimuli [1].

1.2. Causes of inflammation

Inflammation can be triggered by many types of stimuli of both immunogenic or non-immunogenic nature, for example physical agents (e.g., ionizing radiation, cold, heat), chemical agents (e.g., acids, alkali, astringents, corrosive chemicals, reducing agents, and bacterial toxins), microbial infections (e.g., bacteria, and viruses), hypersensitivity reactions (e.g., allergic reactions, anaphylactic reactions, and asthma), dead cells (e.g., infarction) and autoimmunity (e.g., rheumatoid arthritis) [2, 3].

Various physical and chemical stimuli can trigger inflammation by causing necrosis and trauma in the tissues. Such damage to the body cells results in the release of inflammatory mediators which then provokes inflammatory response by the immune system to digest the dead cells and repair the damage [2, 4]. Infections caused by bacterial, viral, fungal and other microbes all result in inflammation [5]. In particular, bacterial infections trigger strong inflammatory responses due to the release of exotoxins (synthesized by bacterial cell) and endotoxins (degradation products of bacterial cell wall). These toxins are potent activators of a host's immune system, leading to a more intense inflammatory response [6].

Hypersensitivity is a form of immunological reaction characterized by excessive release of immune mediators in response to a trigger. The excess of these chemicals may result in tissue assault, tissue damage, followed by inflammation. Hypersensitivity reactions are classified as type-I (IgE-mediated), type-II (IgG or IgM mediated), type-III (immune complex mediated), and

type-IV (cell mediated) reactions based on the onset and severity of symptoms. Inflammation is a common component in all types of hypersensitivity reactions. Examples of hypersensitivity reactions can be given with allergies and asthma. [7].

Autoimmunity or autoimmune disorders are also a type of immunological disorder in which the immune system targets body's own cells and produces antibodies against it. This leads to self-destruction of body tissues, release of inflammatory mediators, followed by inflammation. Examples of such disorders are RA, Graves' disease, and myasthenia gravis. [8, 9].

1.3. Chemical mediators of inflammation

The process of inflammation is marked by the release of series of endogenous chemical mediators such as histamine, cytokines, and prostaglandins. These chemical mediators help increase the blood flow to the inflamed area, increase the permeability of local blood vessels, and assist in immune cells infiltration, accumulation of clotting factors, antibodies and leucocytes at the site of infection. These chemicals also act as messenger molecules and carry the chemotactic signals to help the immune system detect the antigen, eliminate the harmful stimuli and start the repair process.

Inflammatory mediators are either cell derived (e.g., inflamed and immune cells) or synthesized in the organs (e.g., liver) from their precursor proteins [10]. The cell-derived mediators can be synthesized at the site of inflammation (e.g., leukotrienes, prostaglandins, platelet activating factors, cytokines and neuropeptides) or pre-formed and stored in intracellular granules (e.g., histamine, and serotonin) to be release upon activation. Liver is the main site of synthesis of these mediators from precursor proteins (e.g., complement proteins, Hagman factor, bradykinin, fibrins) which are then released in the plasma to be converted to active forms upon activation [10, 11].

Regardless of the type of trigger and nature of the stimuli, almost identical inflammatory mediators are released during all types of inflammation. Several inflammatory mediators have been identified until now, but vasoactive amines (histamine and serotonin), eicosanoids (e.g., prostaglandins, leukotrienes and thromboxane), cytokines (TNFα and interleukins), chemokines, transcription factors (NF-κB), lysozymes, reactive oxygen species, complement proteins, coagulation factors and nitric oxide (NO) are most important [Table 1].

Table 1: Chemical mediators of inflammation (modified from [12])

Mediator	Type	Mediator	Source
Cell derived	Pre-formed and stored intracellularly	• Histamine • Serotonin • Lysozymes	Mast cells, basophils, platelets.
	Newly synthesized at the site of inflammation	• Prostaglandins • Leukotrienes • Platelet-activating factors • Nitric oxide (NO) • Cytokines • Substance-P	Leukocytes, mast cells, lymphocytes and nerve fibers
Plasma precursor proteins derived	Complement system	• Anaphylatoxins (C3a, C5a) • Membrane complex (C5b-9)	Circulatory cells
	Coagulation system	• Kinin system (bradykinin) • Coagulation system • Fibrins	Tissue (e.g., liver)

Histamine and serotonin (5-HT) are vasoactive amines synthesized in basophils, mast cells and platelets. Histamine is a potent vasodilator of vascular smooth muscle. It increases vascular permeability and blood flow to the inflamed area. In non-vascular smooth muscles (e.g., bronchi), histamine causes bronchoconstriction through its effects on H_1-receptors found in the lungs. Histamine also stimulates inflamed cells to secrete chemotactic agents to attract eosinophils and facilitates leucocyte infiltration at the site of inflammation. On the other hand, serotonin is a neurotransmitter that acts through central nervous system (CNS) and causes changes in mood, body temperature, appetite and sleep.

Prostaglandins are the product of metabolism of arachidonic acid (ArA) by cyclooxygenase (COX) and tissue specific synthases. Prostaglandins play important role in the body as well as they mediate the effects of inflammation. They are involved in controlling body

temperature through their actions in the central nervous system, and they are also responsible for elevated body temperature in various disease conditions. Some prostaglandins are also involved in pain sensation. Anti-inflammatory drugs such as NSAIDs act by inhibiting prostaglandin synthesis[13]. Many prostaglandins have been identified till now, but most important are PGE2, and PGI2 (prostacyclin). PGE2 is synthesized in the heart, kidney, and spleen as well as trace amounts by other tissues. PGE2 is involved in platelet aggregation, T-cell proliferation and lymphocyte migration, interleukin secretion and cAMP dependent vasodilatation. PGE2 also enhances the effects of bradykinin and histamine causing uterine contraction during the process of delivery [14]. PGI2 is mainly produced in the heart, vascular smooth muscles and endothelial cells. PGI2 inhibits platelet aggregation, decreases T-cell proliferation, lymphocyte migration, inhibits IL-1 and IL-2 secretion, but induces cAMP dependent vasodilatation [14, 15].

Thromboxane is derived from ArA by thromboxane synthase enzymes. Thromboxane is known to stimulate platelet aggregation, and play role in blood clotting. Thromboxane also cause lymphocyte proliferation, vasoconstriction and bronchospasm [14].

Leukotrienes are products of ArA metabolism by lipoxygenase enzyme. They are produced in the leucocytes, mast cells, epithelial cells and basophils. Leukotrienes induces chemotaxis, platelet aggregation, T-cell proliferation, bronchospasm, increases vascular permeability and increase secretion of cytokines and interferons [15].

Cytokines are low molecular weight (~5–20 kDa) proteins which act as signaling molecules in the body. They are excreted by various cells in the body including the immune cells, macrophages, lymphocytes and mast cells. Trace amounts of are also produced by the endothelial cells, fibroblasts and inflamed cells. Cytokines includes chemokines (IL-8), interleukin (IL-1, IL-6), tumor necrosis factor (TNF), interferons -γ (INF -γ) and growth factor. They are involved in controlling the process of maturation and proliferation of immune cells in the body [16].

Cytochrome P450 (CYP) enzymes are widely expressed in various tissues of the body. Two main categories of CYPs involved in metabolism of ArA, namely the hydroxylases, which convert ArA into 20-hydroxyeicosatetraenoic acids (20-HETE) and the epoxygenases which convert ArA into epoxyeicosatrienoic acids (EETs). 20-HETE and EETs are biologically active and are involved in regulation of vascular tone, diuresis, angiogenesis, and ischemia

preconditioning after reperfusion injury. Recently role of CYP metabolites in the process of inflammation has also been suggested [17]

Transcription factor such as nuclear factor kappa-B (NF-κB) is part of the inflammatory signaling and is largely known for its role in controlling the pro-inflammatory genes. NF-κB also plays a role in the leukocyte activation, anti-apoptotic functions and resolution of inflammation. NF-κB acts as feedback controller of inflammation that limits the magnitude and duration of inflammatory response[18].

Lysozymes are proteolytic enzymes found in the cytoplasmic granules of macrophages and leucocytes. Reactive oxygen species are diverse group of highly reactive molecules produced during the normal process of cellular metabolism in the mitochondria of inflamed cells. Lysozymes and reactive oxygen species are involved in digestion degeneration and elimination of invading microbes and dead tissue [19] .

The complement system consists of plasma proteins which help antibodies in identification of antigens. The complement proteins are traditionally considered as part of innate immunity required as body's defense. There are some reports of involvement of complement in renal diseases as well. They play a crucial role in inflammation as they supplement and assist the antibodies to trigger the immune response observed during inflammation [20].

The coagulation system acts in connection with the complement proteins to form clots around inflamed area and prevent infection from spreading. Along with the coagulation systems many other factors affecting blood clotting such as the Kallikrein Kinin system which controls blood pressure and degrades blood clots during fibrinolysis [20].

Bradykinin is a potent vasodilator peptide that increases the blood supply to an inflamed area. It is known to stimulate phospholipase enzyme and increase the ArA supply for the production of eicosanoids, prostaglandins and leukotrienes biosynthesis [20].

Nitric oxide (NO) is a soluble free radical synthesized in endothelial cells and macrophages. NO acts as cytotoxic agent to kill the invading microorganism. It also causes systemic vasodilation. However, owing to its short life all these effects remain strictly local [20].

1.4. The immune system and the inflammatory response

The immune system is an integral part of inflammation. Both the cellular and humoral components of immune systems are involved in the pathophysiology of inflammation. In some cases, inflammation results from excessive immune response e.g., hypersensitivity reactions and autoimmune diseases. The inflammatory mediators released during the process of inflammation often act as chemotactic agents to recruit the immune cells at the site of inflammation to clear out the infection and digest the dead cells. One of the differential features of inflammation is the extracellular infiltration of leucocytes, proliferation and maturation of immune cells. Once the infection is cleared, the immune system resets to its normal state, reducing the leucocyte population and letting the anti-inflammatory and healing processes take over.

The type of immune cells involved in the inflammatory process differs with the type of inflammation and nature of stimuli. In acute inflammation, predominantly neutrophils infiltrate the intracellular exudate. In chronic inflammation however, macrophages, monocytes and lymphocytes are found in the intracellular exudate. Other immune cells that participate in inflammatory process are granulocytes (polymorph neutrophils, eosinophils, and basophils), lymphocytes, monocytes, mast cells, macrophages, natural killer cells, platelets and fibroblasts. The humoral immunity also participates in the inflammatory response and synthesizes antibodies to eliminate the invading microbe [7, 21].

1.5. Types of inflammation

Inflammation can last from hours to days or years depending upon the stimuli. Based on duration inflammation is classified into two types; acute and chronic. Although the initial symptoms for both acute and chronic inflammation are the same but duration, intensity and types of immune cells involved and symptoms that resolve later are different in both forms of inflammation [Table 2] [22, 23].

Table 2: Difference between acute and chronic inflammation (modified from [12])

Feature	Acute inflammation	Chronic Inflammation
Onset	Fast, acute	Slow, delayed
Cell involved	Neutrophils	Macrophages/monocytes and lymphocytes
Tissue injury	Mild to moderate, self-limited	Severe and progressive
Signs and symptoms	Visible, mild in nature	Hidden, not prominent but subtle

1.5.1. Acute inflammation

Acute inflammation is the preliminary and transit response of the body towards a harmful stimuli. The duration of acute inflammation ranges from few hours up to days and more, depending on the stimulus. The typical symptoms of acute inflammation include redness, swelling, heat radiation, pain, edema and loss of organ function. Such symptoms emerge due to edema and swelling of the inflamed area caused by vasodilation of blood vessels, increased vascular permeability and extracellular fluid accumulation as well as release of chemical mediators. This local fluid accumulation helps to dilute the toxins, allow the antibodies to neutralize the antigens and help the lymphatic system to take up the remains of microbes and/or dead cells. The accumulation of neutrophils in extracellular fluid of inflamed tissue is differential diagnosis of acute inflammation.

1.5.2. Chronic inflammation

Chronic inflammation is a prolonged and subsequent response that follows the initial inflammatory response towards harmful stimuli. This happens when the acute inflammatory response is not sufficient to neutralize or eliminate the stimuli. Thus inflammatory process continues and develops into chronic inflammation. Chronic inflammation can be exemplified with tuberculosis, gastric ulceration, body infestation, particulate objects (e.g., tissue implants) and autoimmune reactions (e.g., rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis).

Chronic inflammation not only differs from acute inflammation in its duration the type of immune cells involved but also differs in its presenting symptoms. Whereas, the immune cells such as monocytes, lymphocytes and macrophage are involved in the process of chronic inflammation. The presenting symptoms of chronic inflammation include chronic ulceration, chronic abscesses, thickening of the vascular walls, hardening of the tissue structure and loss of organ function [24].

1.6. Pathophysiology of inflammation

The biological mechanisms behind the inflammatory response are coordinated through multiple systems and series of processes in the body, such processes can be classified into three types of responses: vascular, cellular and immune.

The initial release of inflammatory mediators results in vascular response marked by vasoconstriction of small blood vessels in the inflamed area. This is followed by vasodilation of the arterioles, increased vascular permeability and increased blood flow resulting in redness, radiation of heat, swelling and flair formation in the inflamed area [25].

The cellular response begins with the infiltration of leucocytes in the inflamed area and is evident with the progression of inflammatory symptoms and remodeling of the tissue. The cellular response is completed in four distinct steps: 1) pavement: a process of antigen recognition, pooling of blood and release of inflammatory mediators; 2) emigration: infiltration of leucocytes; 3) chemotaxis: increase in the local population of leucocytes and macrophages at the site of inflammation; and 4) phagocytosis: involving the actual process of digestion of microbes or dead cells by the immune cells [3, 12, 25].

Lastly, the immune response involves a coordinated response toward the invading microbe from T-cell-mediated cellular immunity and antibody-mediated humoral immunity. Initially, T-cells are activated to recognize the antigen, release chemotaxis mediators that in turn activate the B-cells. B-cells upon activation produce antibodies against the antigen. T-cells act as the regulators of the immune response as they control the recruitment of immune cells at the site of inflammation [26].

After the harmful stimuli are removed the tissue repair process starts. This is marked by the appearance of fibroblasts in the inflamed area. First the fibrin and collagen fibers cordon off

the inflamed area from the surrounding healthy tissue and provide a favorable inside environment for the digestion of the dead cells and further tissue repair. This stage is characterized by a loss of tenderness, resistance to stretch, and immobilization, as the tissue adapts and recovers from the removal of dead cells and harmful stimuli. This process may take months to years until the tissue achieves steady state and resumes normal function [3, 12, 25].

1.7. Animal models of inflammation

Animals models, especially murine models (mice and rats), have long been used in pharmaceutical research to understand disease processes and to develop new therapeutics for inflammation and immune disorders. Although these animal models are vital in understanding the pathophysiology of inflammation, yet there are some inevitable differences between humans and animals pathophysiology of inflammation that needs to be considered. Thus, caution should be exercised in extending animal data to humans. Animal models of RA, inflammatory bowel disease and multiple sclerosis have been successfully developed and utilized in pharmaceutical research and development [27].

1.8. Animal models of rheumatoid arthritis

Animal models of RA often share the hallmarks symptoms of RA in humans, such as swelling of joints, cellular infiltration, joint erosion, and the production of cytokines. However, difference in genetic makeup and environmental factors between humans and animals, contribute variability in these animal models [Table 3].

Another issue with animal models is that, by default, they are acute in nature and disease phenotype appearing quickly within 12-21 days after exposure to the antigen. Thus, most RA therapies developed using these animal models naturally target the acute phase pain and inflammation and are rarely effective at treating long-term conditions [27, 28]. In humans RA diagnosis is often made after the visible signs and symptoms such as morning stiffness, pain and swelling have emerged. These development of newer techniques and biomarkers for early RA diagnosis can help better define the disease and design therapies that target the disease at later stage [27].

Various murine models for RA have been developed and successfully used in research. But rats are preferred over mice for development of clinically symptomatic RA, because rats can

handle the pain, trauma and stress associated with RA better than mice. Moreover, the process involved in immune activation in rats resemble to those in humans, but not in mice. The onset, duration, metabolic products and cytokine profile of inflammatory genes in mice also differ from humans. Thus, mice are deemed inappropriate for RA models, but are recommended for immunological studies because of the ease of manipulation of their genome [29]. The rat models of RA usually categorized based on the type of induction agent (i.e., adjuvant) [Table 3].

Table 3: Animal models of rheumatoid arthritis (adopted from [30]).

A. Rat models	
• Collagen-induced arthritis • Adjuvant-induced arthritis • Pristane-induced arthritis • Formalin-induced arthritis • Kaolin- and Carrageenan-induced arthritis	
B. Mouse models	
Induced models • Collagen-induced arthritis • Pristane-induced arthritis • Proteoglycan-induced arthritis • Zymosan-induced arthritis • Serum transfer model of arthritis	Genetic models • K/BxN (T cell receptor KRN, MHC-II molecule gene expressing) • HuTNF Tg (TNF gene modulation) • IL-1RA-/- (Interleukin-1 receptor antagonist knock outs)

1.9. Adjuvant arthritis rat model

Adjuvant arthritis (AA) is a rat model of rheumatoid arthritis, where dried powder of heat-killed *Mycobacterium butyricum* is used as antigen to stimulate the immune system and mimic RA. Induction of AA involves intra-dermal injection of emulsion of antigen in oil, also called as Complete Freund's adjuvant (CFA), at the caudal aspect of the hind limb [31].

AA is known for rapid onset of disease and polyarticular response (involving multiple joints) within 10-14 days of CFA injection. Typical symptoms of AA include joint swelling, lymphocyte infiltration and bone degradation, and other symptoms similar to human RA. As the disease progresses it results into permanent joint malformation and stiff bones, but rats are

euthanized before that within 20-25 days of CFA injection. The involvement of spleen (splenomegaly), swelling of lymph nodes, macrophage activation, T-cells in the synovial fluids, increased plasma levels of interleukins, TNF-α, IFNγ, nitric oxide and C-reactive protein are also reported in AA rats [32, 33]..

Changes in biosynthesis of inflammatory mediators, particularly those synthesized by the COX enzymes are also observed in AA rat model. The end point of most studies utilizing AA is to stop joint erosion and minimize the joint pain, which is also the aim in most human clinical trials. This makes AA a preferred animal model for RA research and the development of NSAIDs-related therapies [27]. Sprague-Dawley rats are usually used in AA rat model for being cheaper and more readily available [33]. This model is not gender specific yet males are preferred as they can better tolerate the stress and pain associated with it [34].

Despite all the advantages, AA rat model has its draw backs, such as, besides causing inflammation it is painful, stressful and causes animal discomfort. It is also accompanied with reduced food intake, weight loss that can lead to mortality [35, 36]. These factors may have a significant impact on disease outcome and experimental results. Studies have suggested effective use of harm reduction strategies (clinical, non-clinical) and reducing of pain in AA rat model can minimize animal morbidity and mortality. This can be achieved by monitoring the progress of disease (e.g. arthritis index) and use of analgesic or possibly discontinue the study if animal suffering exceeds the acceptable level [36]. Ling et. al., have reported that early phase pre-AA model, 5-6 days after CFA injection, demonstrate significantly elevated levels of inflammatory mediators and is also accompanied by a significant reduction in hepatic enzymes without showing any signs of pain and arthritis. He concluded that if animals are carefully monitored for symptoms of arthritis (e.g. arthritis index <4) the unnecessary pain and distress to the animals can be avoided [37].

1.10. Effects of inflammation

With growing understanding of inflammatory processes, inflammatory mediators and involvement of body mechanisms in inflammatory response of the body, it has become imperative to investigate how inflammation will affect the function of various organs in the body.

1.10.1. Local effects of inflammation

The local effects of inflammation are characterized by an increased blood supply, increased vascular permeability of the capillaries and leaking of the blood vessels. This causes fluids to pool and leads to redness, heat radiation, edema and swelling in the inflamed area. This fluid accumulation helps dilute the toxins, help antibodies to reach the antigens and neutralize them, and helps lymphatic system to take up the dead cells debris.

1.10.2. Systemic effects of inflammation

Inflammatory response of the body involving multiple organs and systems and is not just limited to the inflamed area. The release of inflammatory mediators in the circulation during inflammation may cause various systemic effects as well. At this stage the preliminary symptoms develop into systemic signs including pyrexia, weight loss, anorexia, nausea, increased erythrocyte sedimentation rate, leukocytosis, amyloidosis and many other hematological changes. Compromised organs function such as reduced renal function, changes in hepatic metabolism, altered ischemic reconditioning and increased CV risk are also the systemic consequence of inflammation [38].

Pyrexia is the increase in body temperature caused by bioactive proteins (pyrogen) released by leucocytes in the process of digesting the invading bacteria. Pyrogens and some prostaglandins act on the thermoregulatory center in the hypothalamus, increasing the body temperature.

Weight loss is a common systemic effect of inflammation caused by the negative nitrogen balance during the process of inflammation, indicating the consumption of energy. Reactive hyperplasia of the reticuloendothelial systems and swelling or enlargement of the systemic lymph nodes is also common in acute and chronic inflammation. But enlargement of spleen is only characteristic to microbial infections (e.g. malaria and infectious mononucleosis).

Hematological changes such as increase in the erythrocyte sedimentation rate are non-specific to the release of cytokines, thromboxane and leukotrienes by immune cells which increase the thrombogenic potential of blood.

Neutrophilia, increased neutrophil population in circulation, is typically seen in pyogenic infections. Eosinophilia (increased number of eosinophils) is seen in allergic disorders,

monocytosis (increased number of monocytes) (increased number of monocytes) is seen in bacterial and viral infections (e.g., tuberculosis, typhoid), and lymphosis (increased number of lymphocytes) is seen in chronic bacterial infections. Other hematological changes include ulcers (abscess), hemolysis (due to toxins) and chronic bone marrow depression.

Longstanding chronic inflammation also involves elevated levels of serum amyloid-A protein an abnormal shredded protein fragments that can result in plaque formation and leads to systemic (reactive) amyloidosis, organ failure and death [39].

Anemia is rare, though it occurs typically in ulcerative conditions (e.g., ulcerative colitis) or due to chronic bone marrow depression. Other constitutional effects such as enlarged local and systemic lymph nodes and splenomegaly are more profound in infections (e.g., , malaria, tuberculosis).

Systemic inflammation is reported as underlying cause of heart diseases including atherosclerosis, heart failure, thrombosis, myocardial infarction and heart failure [24, 40]. Blood levels of inflammatory cytokines are often linked with the severity and risk associated with these disease [41]. However, acute or short-term inflammation may actually help prepare the body to handle the stress and damage caused by ischemic heart diseases and help in recovery processes.

1.10.3. Pharmacokinetics and pharmacodynamics effects of inflammation

Inflammation involves various biochemical changes in the body to adapt defensive capabilities, e.g., altered plasma protein profile, induction of inflammatory genes and secretion of inflammatory mediators. These biochemical changes and release of cytokines then altered genes for regulatory proteins. This phenomenon was first noticed with the discovery of C-reactive proteins in the plasma of infectious pneumococcal pneumonia patients, where C-reactive proteins was found to be upregulated in inflammation [42]. Such a gene regulation is mainly reported at the translational level, but some post-translational modifications (e.g. enzymatic or non-enzymatic modification of proteins) can also participate [43].

Inflammation is associated with production of large quantities of cytokines and other inflammatory mediators. Cytokines such as IL1 and TNF-α are known to inhibit the mRNA followed by reduced levels of physiologically significant regulatory proteins [44]. It is possible

that such proteins also have a role in regulating the pharmacokinetic (PK) and pharmacodynamics (PD) of drugs. For example, cytochrome P450 in the liver and other organs that are involved in the metabolism of a large number of endogenous as well as therapeutic compounds are reported to be altered during inflammation. This can lead to changes in disposition and metabolism of these drugs [45, 46]. Many other proteins, enzymes and transporters are also suspected of such alteration in inflammation [47, 48]. Thus, it is important to study the effect of inflammation on the PK and PD of the drugs used in patients suffering from inflammatory disorders.

CV mortality is the most common comorbidity in patients suffering from inflammatory disease like RA. Hence, most RA patients are prescribed with CV medication such as calcium channel blockers, beta blockers and angiotensin receptor blockers [38]. Clinical studies have shown that inflammation status may directly determine the failure of blood pressure therapy in high-risk patients [49]. Many research activities are now focused on the effects of inflammation on PK and PD of CV medication to understand the treatment failures of CV medication in RA patients [50].

1.10.3.1. Effect of inflammation on pharmacokinetics

Changes in drug concentrations reported in various inflammatory disorders indicate altered PK of these drugs [51]. Although the mechanisms behind such changes are not well understood, altered drug metabolism and reduced renal clearance are reported in inflammatory disorders [37]. Inflammation affects plasma protein concentration by increasing alpha glycoproteins [52] and reducing plasma albumin [53]. Basic drugs that bind to alpha glycoproteins and acidic or neutral drugs bind to albumin and lipoproteins. These changes in plasma glycoproteins [52] and albumin [53] can translate in higher unbound concentration of drugs [54]. It is important to note that only free (unbound) fraction of a drug is available to cause therapeutic by binding to specific receptors.

For example, verapamil, a calcium channel blocker, are reported to have more free fraction available in RA patients, due to reduced plasma protein binding and decreased hepatic metabolism [55]. Similar observations were made for the beta blocker propranolol, where inflammation reduced its clearance but increased the plasma concentration of propranolol.

1.10.3.2. Effect of inflammation on pharmacodynamics

Previous studies have investigated the effects of inflammation on pharmacodynamics of drug actions [56-58]s. One such study has reported a higher free fraction of verapamil, but despite the increased plasma concentration the dormotropic effects were decreased in RA patients [55]. The authors concluded that inflammatory cytokines down-regulate the calcium channel proteins, leading to a loss of the dormotropic effect despite the higher plasma concentration of verapamil [55]. This might also be due to altered binding of calcium to the ion channels [58], or changes in the receptor functioning caused by decoupling the G-protein from the intracellular protein kinase-C enzymes, making receptors dysfunctional [59, 60].

In another study, infliximab appeared to lower the inflammation and restore the normal PK and PD of verapamil which was altered by inflammation [61]. Clinically in patients with Crohn's disease (an inflammatory disorder) are reported not to respond to verapamil therapy during remission phase of disease, but the drug responses were recovered by reduction in the severity of inflammation [57]. Similarly, inflammation reduced the potency of propranolol a beta blocker despite increased plasma concentration, due to down regulation of the target protein on beta-adrenergic receptors [62]. The angiotensin II type 1 receptor (AT1R) blockers such as valsartan and losartan are reported to conserve their pharmacodynamic effects in RA patients [63, 64].

1.11. Diseases associated with inflammation

High levels of inflammatory cytokines such as interleukins (e.g., IL-1, IL-6), tumor necrosis factor (TNF-α) and C-reactive protein are identified as markers of inflammatory burden in the body. Among these, C-reactive protein is reported to be a biomarker for CV risk associated with inflammation [65]. Inflammatory markers are also elevated in depression, dementia and Alzheimer's disease [66]. Other diseases associated with inflammation includes, arthritis, asthma, chronic ulcers, tuberculosis, Crohn's disease, diabetes, hepatitis, cancer, various neurological and immune disorders.

1.12. Arthritis

Arthritis is referred as an inflammation of the joints. It is a musculoskeletal disorder used to describe various rheumatic conditions affecting joints, tissues around joints, ligaments, cartilage

and connective tissue. Arthritis is one of the most common chronic diseases in the world. In United States one-third of the population over 45 years of age has, at some point, suffered from objective joint pain, swelling, and loss of mobility due to arthritis [67].

Although arthritis is considered as disease of elderly, a significant number of the younger population (<25 years) is also affected. Arthritis is more prevalent in females compared to males [68]. Being chronic disorder arthritis has a substantial impact on the quality of life [68].

1.12.1. Types of Arthritis

There are many of types of arthritis, ranging from mild forms (e.g., tendinitis, bursitis and fibromyalgia) to sever and systemic forms (e.g., osteoarthritis; OA and rheumatoid arthritis; RA). Although the exact triggers of arthritis is not known, but the risk factors include autoimmunity, infection (bacterial, viral, microbial), genetics, environment, hormones, cigarette smoking and physical or emotional stress [69].

Arthritis is classified into many types: 1) Infectious arthritis (e.g. arthritis secondary to bacterial and viral infections); 2) degenerative arthritis (e.g., diffuse idiopathic skeletal hyperostosis): 3) rheumatic arthritis (e.g., rheumatoid arthritis, juvenile arthritis); 4) traumatic arthritis (e.g., work-related trauma to joints, radiation-induced arthritis, Raynaud's disease); 5) metabolic arthritis (e.g., gout, pseudo-gout) etc. [70]. But the most common forms of arthritis are OA, RA and gout.

1.13. Rheumatoid arthritis (RA)

RA is an autoimmune disorder of unknown origin characterized by the production of antibodies directed against the body's own synovial tissues, cartilage and bone, resulting in inflammation, pain, and swelling of the joints and surrounding tissue [71]. Although RA is disorder of joints, signs and symptoms of RA may spread to other organs as well, such as fever, loss of renal function, CV abnormalities etc.

1.13.1. Epidemiology of RA

RA is the leading causes of disability in the world with an estimated 0.1-1% prevalence in general population [72]. Although RA is considered predominantly a disease of the elderly with more than half of new RA cases occur between the ages of 40 and 70 years, younger people

especially 25-50 years old are also affected with this disease. RA is three times more prevalent in women than men [69].

In 2005, an estimated 1.3 million of United States population are reported to have RA and 22% of arthritis related deaths were reported to be CV related. RA costs a total of 128 billion USD$ to the their economy in direct and indirect cost of treatment [73].

RA affects over 0.9% of Canadians and more than 272,000 people living with RA and related disorders and an estimate 1.3% increase is expected in next 30 years as the population ages. Overall 50% of people with RA are work disabled and 0.74% of the Canada's labor force (1/136) is suffering from RA. This is expected to increase to 1/68 in the next 30 years. RA will cost Canadian economy an estimated 39 billion USD$ in direct and indirect costs over the next 30 years [74].

1.13.2. Pathophysiology of RA

In RA, immune system of the body starts making antibodies against its own cells, a phenomena referred to as autoimmunity. These antibodies then attack the synovial lining and membranes around the joints resulting in inflammation, pain and swelling of the joints. RA is characterized by thickness of the synovium, stiffness of joints, loss of alignment and loss of mobility [75].

The pathophysiology of RA's involves complex interaction between immune cells (i.e., macrophages, mast cells and natural killer cells) and humoral immunity [75]. During RA the synovial membrane is either damaged or infiltrated by the inflammatory cells releasing inflammatory cytokines. These cytokines then recruit antigen-specific macrophages and promote T- cell activation. Once T-cells are activated the T-helper cells are recruited at the site of inflammation. T-helper cells then trigger the humoral immunity by activating B-lymphocyte. In response to this B- lymphocytes secrete antibody against the synovial membrane [76]. This production of auto-antibodies, compelled with the release of cytokines in the synovial fluid creates an inflammatory environment in the joint leading to RA [77].

1.13.3. Causes and risk factors of RA

The exact causes of RA are not known, however, both genetic and the environmental factors are suspected to trigger immune system that leads to RA. Polymorphism on a specific HLA gene is reported to increase the risk of developing RA by five times as compared to the general

population [78]. This HLA gene is meant to control the immune response towards an antigen; hence, any abnormality to this gene can trigger abnormal immune response leading to RA. Other gene polymorphisms suspected to be involved in RA are DRB1, PTPN22, OLIG3/TNFAIP3, STAT4 and TRAF1/C5. However, these genes only explain a part of causes of RA [79, 80].

Other risk factor for development of RA includes, microbial infections, gender (women), oral contraceptives, hormone replacement therapy, breastfeeding, menstrual history, obesity, physical and emotional stress or trauma. Other environment related factors such as cigarette smoke, air pollution, insecticides, occupational hazards, mineral oil, silica and pollens may also play roles in the development and progression of RA [81].

1.13.4. Symptoms of RA

The primary symptoms of RA are swelling of joints, pain and redness. RA mostly affects larger joints, such as the wrists, fingers, knees, and ankles, simultaneously on both sides of the body. The disease often starts slowly, with joint stiffness and pain, which further develops into morning stiffness, warm and tender joints which become stiff over time. The morning stiffness usually lasts for an hour after patient wakes up and wears off as the joint and tendons warm up during the day. Other RA symptoms include chest pain, shortness of breath, dry itching eye with discharge, finger numbness, burning and tingling in the extremities, and sleep disturbances [75, 81].

1.13.5. Diagnosis of RA

Ideally, the diagnosis of RA should be done early in the course of the disease, i.e., within six months of the appearance of preliminary symptoms. Then an appropriate treatment should be started before greater damages emerge. However, early symptoms of RA are nonspecific and hard to diagnose including malaise, fatigue, weakness, muscle soreness and low grade fever with some weight loss. When RA is suspected, severity and type of RA should be determined and subsequently the course of proper treatment should be planed [81]. Diagnosis of RA should include the followings.

Medical history: Personal habits, recent and current symptoms such as, early morning stiffness, inflamed nodules, low-grade fever and restricted movement. This should be followed by a physical examination of the joints to see the number of joints involved, pain, swelling and

tenderness of joints, on both sides of the body.

Blood tests: If suspected, a complete blood test should be ordered, along with erythrocytes sedimentation rate and specific inflammatory markers of RA such as C-reactive proteins and rheumatic factor to confirm the diagnosis.

Imaging tests: An X-ray, ultrasound or magnetic resonance imaging of affected joints should be done for differential diagnosis and to measure the extent of erosion of the bone, narrowing of the joint cavity, and the overall extent of damage to the joint [81].

1.13.6. Complications of RA

Commonly reported complication in RA patients includes diseases affecting almost every system in the body such as;

Life-time risk of mortality: RA increases the risk of death due to underlying inflammation. A recent North American cohort study has reported twice the risk of death in RA patients compared to healthy individuals.

Cardiovascular diseases: The most common diseases found in conjunction with RA are CV diseases, particularly myocardial infarction and congestive heart failure. An estimated 40% of the deaths in RA patients are due to CV complications.

Infections: Infections are common in RA patients, including tuberculosis that is the second leading cause of death in RA patients. However, it is not clear if the underlying cause of this super infection is the dysfunction of the immune system during RA or the side effects of anti-rheumatic drugs, which are targeted to suppress the immune system.

Central nervous system diseases: A prevalence of anxiety and depression has been reported in patients suffering from RA.

Malignancies and cancer: RA patients at some point in their lives suffer from malignancies such as lymphomas, leukemia, multiple myelomas and cancer.

Carpal tunnel syndrome: RA can affect the nerves supplying to the hands, at advance stages of the disease leading to curving of fingers and hands, a condition called as carpal tunnel syndrome.

Lung diseases: Patients suffering from RA experience dramatic hemodynamic changes and accumulation of fluids exudate in the lungs that results in significant loss of surface area, leading to shortness of breath, scarring, and asthma [81].

1.13.7. Treatment of RA

There is no actual cure for RA, rather, there are therapeutic and non-therapeutic approaches available to help manage the symptoms control the disease and avoid complications. Therefore, RA requires lifelong treatment including exercise, medication and possible surgery to protect the joint. But overall goals of RA therapy remain the same to relieve symptoms, prevent joint damage, improve physical function, prevent complications, and improve quality of life. Various approaches are used to achieve these goals, as classified in the following categories.

1.13.7.1. Non-pharmacological approaches

The non-pharmacological approach adopted to treat RA includes properly designed physical activity that may include walking, swimming, cycling, light aerobics, and jogging. However, heavy exercises need to be avoided. The aim of physical activity is to protect the joints in question from losing mobility and to strengthen the muscles around them. If done correctly and regularly, physical activity can reduce pain and fatigue, strengthen the joints, improve mobility, help elevate the biochemical changes, counter depression and improve the quality of life.

According to the public health agency of Canada [82], physical activity for RA patients should include three kinds of exercise in their physical activity and exercise plans: 1) moderate flexing to reduce pain, prevent stiffness of tendons and improve mobility; 2) strengthening exercises to maintain and increase muscle tone, and protect the joint from damage; and 3) endurance exercises to strengthen the heart, circulation, metabolism to maintain the active lifestyle, control weight and reduce depression [83].

1.13.7.2. Pharmacological intervention

Drugs are often used in combination with physical activity to achieve the goals of RA therapy. They are mainly used to treat the symptoms of inflammation (swelling, pain and edema), and/or to limit the progression of disease and joint damage. Various drug options are available for the treatment of RA following are the example of most commonly used anti-rheumatic drugs.

a) Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are used in the treatment for RA for their analgesic, antipyretic and anti-inflammatory properties [84]. NSAIDs exert their pharmacological effects by inhibition of prostaglandin synthesis through inhibition of COX enzymes. NSAIDs are classified based on their COX-2 selectivity, categorising them into non-selective NSAIDs (e.g., Aspirin, ibuprofen, naproxen, ibuprofen, etc.) and selective COX-2 inhibitors (e.g., rofecoxib, celecoxib and meloxicam etc.). NSAIDs are discussed in detail in section 1.13.

b) Glucocorticoids

Glucocorticoids are steroidal compounds used to treat RA for their immuno-suppressive and anti-inflammatory properties. Glucocorticoid compounds, such as prednisolone, act by suppressing the immune system, blocking the synthesis of inflammatory mediators in RA. However, the use of glucocorticoids is only limited for extreme situations and for short-term use only. The side effects of glucocorticoids include weight gain, diabetes, thinning of the bones and infections due to immune suppression. [85].

c) Disease modifying anti-rheumatic drugs

Diseases modifying anti-rheumatic drugs (DMARDs) are immune modifying agents that slow down the progression of RA and protect the joint(s) from further damage. It is worth mentioning that while NSAIDs target the symptoms, DMARDs actually target the progression of the disease. Examples of DMARDs are methotrexate, sulfasalazine cyclophosphamide, gold salts, penicillamine and minocycline [86].

d) Biologic response modifiers or biologics

Biologics or biological response modifiers are monoclonal antibodies (MAbs) or fusion proteins targeted towards pro-inflammatory cytokines (e.g., TNF-α, IL-1), signaling proteins (e.g., cytotoxic T-lymphocyte-associated antigen-4 immunoglobulin: CTLA-4-Ig) and T-cell antigen (CD20)[87]. Examples of biologics are i.e., anti-TNF-α monoclonal antibody (MAb) (e.g., adalimumab, etanercept, and infliximab), Anti IL-1 receptor MAb (e.g., anakinra), Anti CD20 MAb (e.g., rituximab), anti CTLA-4-Ig (e.g., abatacep) and anti-IL-6 receptor MAb (e.g.,

tocilizumabi). Biologics are usually well tolerated, but because of their high cost, injectable dosage form their use is reserved for severe conditions and non-responding RA patients [87, 88].

e) Other drugs

Other drugs used for in the treatment of RA include immune-suppressing agents (e.g., , cyclophosphamide and cyclosporine) and cardiovascular medications (e.g., statins, beta adrenergic receptor blockers and anti-histaminic drugs) [69, 81].

1.14. Therapeutic strategies for the treatment of RA

Below is the layout of therapeutic approaches recommended by Canadian rheumatology association [Figure 1]. Initially, non-pharmacological methods along with DMARDs and occasional NSAIDs are used to treat primary symptoms of RA. But if results are not satisfactory, glucocorticoids and then biologics can be added to the therapy as needed. However, symptomatic treatment with NSAIDs and/or surgery is always an option.

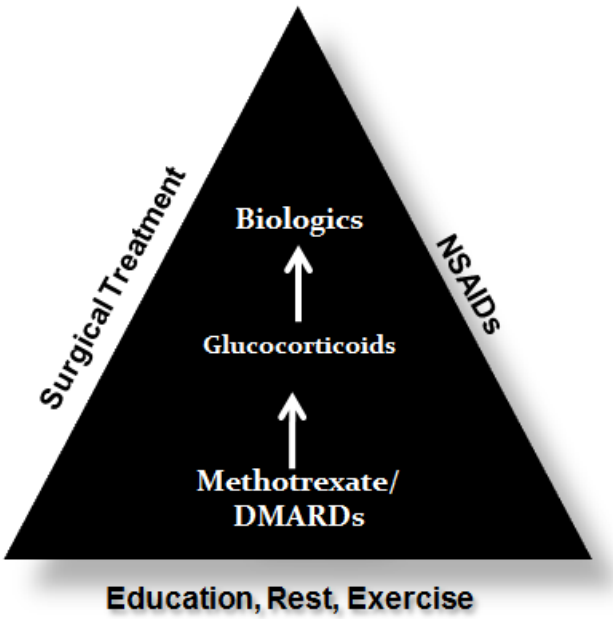


Figure 1: Canadian guidelines for the management of RA (modified from [89]).

1.15. Non-steroidal anti-inflammatory drugs (NSAIDs)

Acetylsalicylic acid was the first NSAID introduced with the name of Aspirin in 1899 [90]. Followed by the introduction of indomethacin and ibuprofen, in 1964 and 1969, respectively [91]. Since then, many compounds belonging to various chemical categories have been introduced as NSAIDs [90]. NSAIDs are cited as one of the most prescribed drugs, approximately 2.5% of all prescriptions around the world are for NSAIDs [92]. In a drug utilization survey 2013, diclofenac was declared the most commonly used NSAIDs in the world [93]. Most NSAIDs are completely absorbed after oral administration, have high protein binding and are metabolized by the liver [94, 95] and eliminated almost completely through renal and fecal routes [95, 96].

1.15.1. Classification of NSAIDs

NSAIDs includes members of various chemical categoriers. NSAIDs are also categorised based on their selectivity towards COX-2 enzyme [Table 4] and chemical structure [Table 5] [97]. [98].

1.15.2. Therapeutic use of NSAIDs

NSAIDs are used to treat the swelling, pain and inflammation associated with rheumatic diseases such as RA [99], OA [100], ankylosing spondylitis, juvenile arthritis, psoriatic arthritis, systemic lupus erythematosus, rheumatic fever, Kawasaki disease, gout, patent ductus arteriosus, dysmenorrhea, pericarditis, Reiter's disease and other musculoskeletal disorders [101]. NSAIDs are also indicated for the management of thrombosis to minimize the risk of CV incident. Recent studies have highlighted that NSAIDs can also be useful in the prevention of colon cancer [102] and Alzheimer's disease [103].

Table 4: Classification of NSAIDs based on cyclooxygenase (COX) selectivity (adopted from [104]

Highly COX-2 selective NSAIDs	Moderately COX-2 selective NSAIDs	Non Selective NSAID
Etoricoxib	Celecoxib	Ibuprofen
Lumiracoxib	Meloxicam	Flurbiprofen
Rofecoxib	Etodolac	Naproxen
Valdecoxib		Diclofenac

Table 5: Chemical classification of NSAIDs (modified from [105])

Chemical nature	Example
Salicylic acid derivatives	Acetyl salicylic acid, sulfasalazine
Propionic acid derivatives	Ibuprofen, naproxen, ketoprofen, flurbiprofen
Carboxylic acids derivatives	Etodolac
Acetic acid derivatives	Diclofenac, indomethacin, ketorolac, sulindac
Indoleacetic and Indeneacetic acids derivatives	Indomethacin, sulindac, etodolac
Enolic acid derivatives (oxicams)	Piroxicam, meloxicam
Pyrroloppyrrole derivatives	Ketorolac
Fenamic acid derivatives	Mefenamic acid, meclofenamic acid
Diaryl heterocyclic compounds	Rofecoxib, celecoxib, veldecoxib, paracoxib, etoricoxib, lumaricoxib.
Non acidic	Nabumetone

1.15.3. Mechanism of action of NSAIDs

In 1971, Vane and Piper discovered the mechanism of action of NSAIDs, that they exert their analgesic, anti-inflammatory and antipyretic effects by blocking the prostaglandin synthesis through their inhibitory effects on prostaglandin synthetase enzyme, which was later named cyclooxygenase enzyme [106]. COX enzyme exists in two isoforms COX-1 and COX-2. COX-1 isoform is constitutively expressed in the tissues and is necessary for normal functioning of the organs. COX-2 is also expressed constitutively but in low quantities, however, its expression is high during inflammation [107, 108] [Figure 2]. This observation led to the development of a newer category of NSAIDs called selective COX-2 inhibitors (COXIBs) (e.g., rofecoxib, celecoxib, meloxicam) [109]. Recently, another variant of the COX enzyme (COX-3), has also been identified but its function is not yet understood [110].

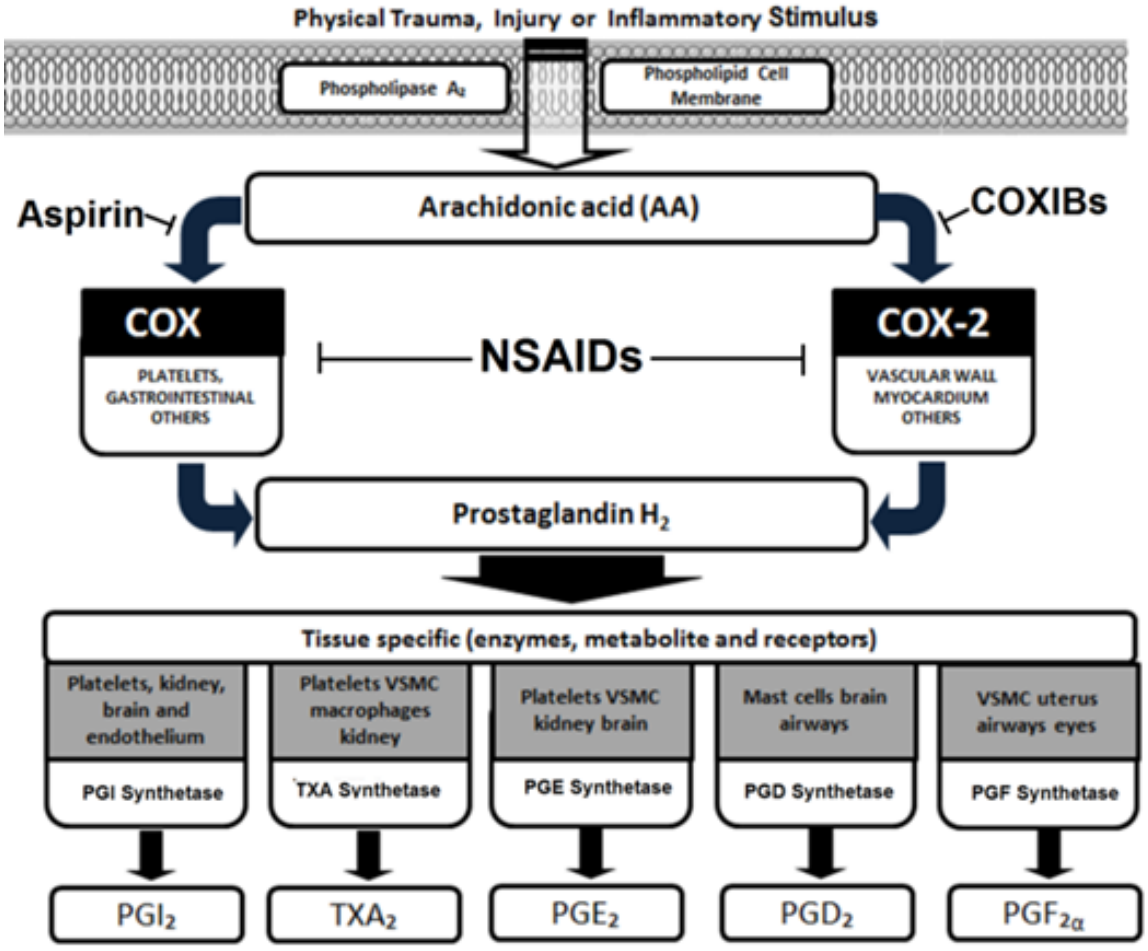


Figure 2: Cascade of prostaglandin synthesis (modified from [111]).

1.15.4. Adverse effects of NSAIDs

NSAIDs are generally well tolerated if used within therapeutic doses and for shorter duration. However, in situations including high doses, altered drug exposure they may cause gastrointestinal, CV and renal side effects [112]. NSAIDs adverse effects range from mild symptoms including dyspepsia, vomiting, and gastric discomfort to severe life-threatening situations like renal failure, congestive heart failure, myocardial infarction and sudden death [113].

1.15.4.1. Gastrointestinal side effects

Gastrointestinal adverse effects are the first to be noted in NSAIDs users and are readily noticed in the upper gastrointestinal tract with symptoms such as dyspepsia, heartburn and nausea. The effects are mild in nature and disappear with the loss of exposure [114]. Proper management of these symptoms with gastro-protective agents is sufficient to avoid these adverse effects [115].

However, less frequent but serious GI side effects occur in the lower gastrointestinal tract, which are often go unnoticed and hard to diagnose, leaving them unattended to develop into serious risks [116]. The visible symptoms of the lower gastrointestinal tract adverse effects include flatulence, stool bleeding, darkening of stools and lesions in gastrointestinal lumen [117]. All of these adverse effects can progress into more serious conditions [118]. Although all NSAIDs cause lower gastrointestinal effects to some extent, delayed dosage forms (e.g., enteric coated, sustained release) may augment such risk [119]. Concomitant use of mucosal protective agents (e.g., misoprostol) [120], H2-receptor blockers (e.g., famotidine) [121] or proton pump inhibitors (e.g., omeprazole) [122] can minimize the GI damage caused by NSAIDs. However, these stratgies are mainly aimed at upper GI side effects of NSAIDs and their effectiveness in mitigating lower GI effects is not yet established.

1.15.4.2. Renal side effects

Renal effects are the second most frequent side effects observed in NSAIDs users [123]. NSAIDs can cause renal papillary necrosis, intersitial nepehtritis, perforated renal tubules, hyperkalemia, fluid and solvent retention or renal failure [124]. These effects of NSAIDs are dose- and duration-dependent and often reversible. In rare cases, if the acute renal failure persists for a long time it may develop into a chronic and permanent condition [125].

Prostaglandins play an important role in filteration function of nephron and assists in urine formation. The prostaglandin receptors found in the medullary interstitium, collecting ducts once stimulated can cause vasodilation of capillaries thus increasing salt and water excretion increasing urine volume [126]. NSAIDs interfere with normal renal function by inhibiting the prostaglandin sythesis in the kidney, resultig in loss of electrolytes excretion and decrease urine formation. This in turn leads to hypertension and increases the chnaces of CV incident [127]. NSAIDs also interfere with the renal effects of diuretics, ACE inhibitors and angiotensin receptor blockers. Caution is advised in using NSAIDs in the patients with compromised renal function as that may lead to renal failure [128].

1.15.4.3. Cardiovascular effects

After the introduction of COX-2 selective, rofecoxib on the market and its subsequent withdrawal the focus of NSAIDs induced adverse effects was switched from gastrointestinal to CV complications [129]. Initially it was thought that CV risk is limited to the newer selective COX-2 inhibitors only, but later studies have shown that even the nonselective NSAIDs possess CV risks [110].In addition one has to account for the additive CV risk of underlying inflammatory disease in NSAIDs users [130]. There is a good chance that the CV inidencets reported in NSAIDs user might actually be caused by the underlying inflammation. Moreover, the reduced efficay of CV medication reported in inflammatory diseases may also contribute towards increased CV risks [55].

VIOXX Gastrointestinal Outcomes Research (VIGOR) trials compared the mortality rates between rofecoxib (50 mg) and naproxen (500 mg) in 8000 RA patients. It concluded that although mortality was equal in the comparators groups (0.2% each), the myocardial infarction incidents were four-fold higher in rofecoxib users compared to naproxen (0.4% vs 0.1%)[131]. However, critics of this study suggest that patients used in this trial were prevented from aspirin use. That might have caused this increase in CV incidents instead of rofecoxib alone.

Celecoxib Long-Term Arthritis Study (CLASS) compared celecoxib (400 mg, twice a day) with ibuprofen (800 mg, three times a day) and diclofenac (75 mg, twice a day) in arthritis patients. It found no difference independent of aspirin use [132]. This suggests that NSAIDs induced CV risk is independent of their COX-2 selectivity.

Mukherjee et al. suggested that the observed difference in VIGOR and CLASS trials is actually due to difference in comparators used in two trials. VIGOR compared rofecoxib with naproxen while CLASS compared celecoxib with ibuprofen and diclofenac [133]. Naproxen is known for its low CV risks; hence, the increase in risk reported for rofecoxib is due to low CV risk in naproxen users. In contrast, ibuprofen and diclofenac themselves increase the chances of CV incidents, thus when compared with celecoxib they pose CV risk similar to celecoxib, rendering the difference insignificant. Another explanation for the difference VIGOR and CLASS trials is the duration of treatment, as CLASS trial might have been, too short to observe any possible CV outcome [133].

A recent review of NSAIDs in medium and low income countries has revealed that NSAIDs including rofecoxib, etoricoxib and diclofenac have the highest CV risk compared to naproxen. Whereas, meloxicam and indomethacin demonstrated moderate CV risk and etodalac exhibited no risk when compared with naproxen. Celecoxib and ibuprofen exhibit CV risk in high doses only, not in low doses when used for shorter durations [93].

1.15.4.4. Risk of Death

Mortality is a widely used endpoint reported in clinical trials. All-cause mortality is a term used to describe death [134]. All-cause mortality associated with NSAIDs is still a controversial topic. Some reports suggest that NSAIDs increase the risk of mortality due to their potential of causing CV effects [135], yet others suggest that NSAIDs actually treat the inflammation, hence reduce chances of ACM [136]. However, one thing is certain that mortality is not uniform for all NSAIDs.

Kerr at el. have reported a high risk of mortality for non-selective NSAIDs, followed by rofecoxib, meloxicam, diclofenac and celecoxib [135]. A Danish study measuring the risk of death, MI, and stroke, concluded a high risk for rofecoxib and diclofenac in a cohort of patients 10 years and older. Ibuprofen was also observed to have a trend towards risk but not a significant one, while naproxen was completely devoid of risk [137].

Studies showing lower ACM in NSAIDs user are criticized for shorter exposure to NSAIDs (<30 days), inconsistency of exposure, smaller sample size and use of healthy volunteers without inflammation thus not representing the actual risk of death associated with NSAIDs [138]. Overall, there is no clear evidence if NSAIDs use is associated with risk of death

or it should be attributed to the underlying inflammation. Further clinical trials are needed to determine actual risk of death associated with different NSAIDs.

1.15.5. Mechanism of NSAIDs cardiotoxicity

Multiple mechanisms are likely to be involved in determining the CV toxicity of NSAIDs. Including differences in thrombogenicity, endothelial function, oxidative stress and renal effects [139]. NSAIDs are suspected to increase the chances of thrombotic events caused by imblance in COX-2–mediated prostacyclin production without inhibition of COX-1–mediated thromboxane biosynthesis. This results in higher thrombogenicity in the body that can lead to CV incident [140].

Physiochemical properties such as ionization constant (pKa), solubility and partition coefficient (log P) may contribute towards tissue distribution characteristics of NSAIDs. Animal studies have shown that those NSAIDs which distribute relatively more into the kidney tissues tend to interfere more with the renal function [141].

An ion channel hypothesis have recently been proposed, suggesting that differential CV toxicity of NSAIDs may actually be related to NSAIDs effects on potassium channels (Kv7 family) and L-type calcium channels which are found in vascular smooth muscle cells. NSAIDs like celecoxib are reported to activate the Kv7 channels, and block L-type calcium channels in VSMC resulting blood pressure and less chances of CV incident [142].

A pH-dependent ion trapping phenomenon is also suggested for some NSAIDs like meloxicam. In healthy tissue extracellular pH is higher than intracellular pH. But in inflamed tissues extracellular pH will decrease below intracellular pH. Most NSAIDs are derivatives of weak acids, a lower extracellular pH in inflamed tissues mean more non-ionic form of NSAIDs which will then penetrate more into tissues[143]. Once inside tissue, the higher intracellular pH will make NSAIDs ionized and get trapped. Meloxicam's limited tissue distribution reduces the risk of CV and renal effects compared to other NSAIDs which distributes more into the tissues [144].

The role of drug transporters in NSAIDs induced CV toxicities is yet to be explored. However, few reports have suggested that NSAIDs not only block prostaglandin synthesis but also interfere with the transport of prostaglandin across the cell membranes. PGE2 is a

prostaglandin synthesized on the surface of endoplasmic reticulum and is stored in the cytosol. At physiological pH PGE2 is impermeable to the body membranes and needs to be transported by prostaglandin transporters. Multidrug resistance protein (MRP4) is a prostaglandin efflux transporter involved in the release of PGE2. NSAIDs like indomethacin block MRP4 mediate release of PGE2. This might result in higher thrombogenic potential at narrow blood vessels of heart and brain resulting in CV event [145].

A role for oxidative stress caused by the use of NSAIDs is suggested in disrupting the integrity of cell membranes. The use of rofecoxib is associated with oxidative stress in cardiomyocytes that can lead to disruption of the cell membranes thus increasing the risk of CV incident [146].

Metabolomics profiling of murine plasma has revealed that thrombogenic metabolites of ArA are increased by rofecoxib treatment which contributes towards increasing thrombogenicity of blood resulting in higher CV risk [147].

1.15.6. Effect of duration of treatment on NSAIDs associated CV risks

Evidence from observational studies and clinical trials has suggested that the CV risk of NSAIDs increases with longer duration of use [148]. However, with short-term use (e.g., post-operative analgesic use) of NSAIDS the CV incidents are not observed [149]. The higher CV risk reported in VIGOR trial is also attributed to a long duration of use [133]. It is suggested that the elevated risk of MI [149], congestive heart failure [136] and atrial fibrillation [150] in NSAIDs users require use of NSAIDS for longer than 180 days to be significant. Overall, there is contradicting evidence over the duration of NSAIDs use and side effects thus the mechanisms involved in early and late toxicity of need to be investigated further.

1.15.7. Effect of dose on NSAIDs associated CV risks

Several meta-analysis and cohort studies of NSAIDs user have reported that the hazard of toxicity increases with an increased dose [151, 152]. Olsen *et al.,* have reported a dose dependent increase in CV related death in diclofenac and rofecoxib users (hazard ratios 1.96, 95% CI; 1.79-2.15 and 1.66, 95% CI; 1.44-1.91, respectively) [153]. Lévesque *et al.,* also reported a dose effect relationship in current users of rofecoxib and MI risk, as the risk was more pronounced at

higher doses compared to low doses (relative risk ratio 1.73, 95% CI; 1.09 to 2.76 verses 1.24, 95% CI; 1.05 to 1.46, respectively [154].

One example of dose associated toxicity is ibuprofen which at lower doses (<1200 mg /day) has aspirin like cardioprotective effects [137], at higher dose however it causes MI risk [153]. Naproxen is another example of a drug that produces a different outcome at different doses. Naproxen is neutral at higher doses but at lower doses it is cardioprotective [137]. The literature contains many examples of NSAIDs which exhibit a higher risk of MI but are well tolerated at lower doses, such as diclofenac, celecoxib, rofecoxib. All this suggests the relevance of dose in determining the adverse effects of NSAIDs.

1.16. Selection of NSAIDs for this study

Previously our group has reported that rofecoxib and celecoxib have a higher kidney-to-plasma concentration ratio as compared to meloxicam. The study also reported that meloxicam did not affect the electrolyte excretion, while celecoxib and rofecoxib did [141]. Interestingly, systematic reviews of randomized clinical trials also suggested a more favorable CV safety profile for meloxicam over celecoxib and rofecoxib [155]. These observations suggest that the tissue distribution of NSAIDs correlates with their potential to alter the CV and renal complications.

We wished to extend these finding to the heart tissue and investigate what pharmacological implications it might have on CV risks. To do so, we kept meloxicam as our main comparator, and included the following NSAIDs, each representing a different COX-2 selectivity and pharmacokinetic profile: rofecoxib is a positive control; celecoxib is a widely prescribed COXIBs; flurbiprofen represents non-selective NSAIDs and is commonly used as a painkiller.

1.17. Rofecoxib

1.17.1. Chemistry

Rofecoxib, also known as MK-0966 or [4-(4'-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone], is a methyl-sulphonyl-phenyl derivative (stilbenes) with a molecular formula of $C_{17}H_{14}O_4S$ [Figure 3]. The molecular weight of rofecoxib is 314.36 g/mol [156, 157].

1.17.2. Physicochemical properties

Rofecoxib is an off-white-to-pale-yellow amorphous powder with a melting point of 204-208° C. It is practically insoluble in water, sparingly soluble in acetone and slightly soluble in methanol, isopropyl alcohol and ethanol. It is reported that rofecoxib exists only in a single polymorphic form. The theoretical log P (partition coefficient) value is computed to be 2.14 [156], but the American Chemical Society has reported a log P of 1.635. However, rofecoxib does not have any acid and basic moieties detectable in its structure, thus it's not possible to measure its pKa (ionization coefficient) [156, 157]. However, rofecoxib is a highly lipophilic compound in nature and is known to cross the blood brain barrier [158].

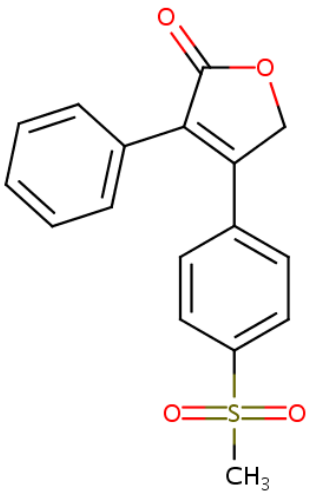


Figure 3: Chemical structure of rofecoxib

1.17.3. Pharmacodynamics

The mechanism of action for anti-inflammatory, analgesic and anti-pyretic properties of rofecoxib involves inhibiting prostaglandin synthesis enzyme also called as cyclooxygenase. Rofecoxib is reported to be 1000 times more selective in inhibiting COX-2 over COX-1. In-vitro experiments on lipopolysaccharide (LPS) induced Chinese hamster and human mononuclear cells suggests that rofecoxib has a half maximal inhibitory concentration (IC50) for COX-2 from 0.018-0.0446 µM, its IC50 for COX-1 is reported to be > 50 µM.

Rofecoxib was indicated for the treatment of pain and inflammation associated with arthritis, dysmenorrhea, acute migraines, and joint and musculoskeletal pain for adults. It was

approved for juvenile RA in children who were two years or older and weigh more than 10 kg. Rofecoxib was available in 12.5, 25 and 50 mg doses for adults and was marketed as tablet and suspension dosage forms [129, 159].

1.17.4. Pharmacokinetics

Rofecoxib follows a complex, nonlinear pharmacokinetic profile. The oral bioavailability after a single therapeutic dose is reported to be 93%, with a time to maximum plasma concentration (Tmax) of 5 h. Rofecoxib yields a maximum plasma concertation (Cmax) of approximately 207 µg/L within 3 h and a steady state plasma concentration (Css) of 321 µg/L after four days of administration of a 25 mg dose.

Food delays the Cmax of rofecoxib by 1-2 h [159]. Rofecoxib is shown to have a dual Cmax: one at 1 h and the other at 10 h due to reversible metabolites (i.e., 5-hydroxy-rofecoxib) and glucuronide conjugates which reabsorb after being excreted in the intestine through biliary excretion [156].

Rofecoxib is highly bound to plasma proteins (87%) at plasma concentrations of 0.05-25 mg/L. The human tissue distribution is not well characterized, however, in animals it is reported to have a high tissue-to-plasma ratio. The apparent volume of distribution at a steady state is reported to be 91 L for a 12.5 mg dose and 86 L for a 25 mg dose [156].

Rofecoxib is extensively metabolized by the cytochrome P450 (CYP) 1A2, 3A4, 2C9, 2C8 and cytosolic reductases enzymes in the liver. It is metabolized to two inactive metabolites (dihydro-rofecoxib 56% and trans-dihydro-rofecoxib 8.8%); that are recovered in the urine. Rofecoxib is metabolized by hepatic metabolism and less than 1 % of the administered dose is excreted unchanged. The elimination half-life of rofecoxib is 17 h at a steady state [156, 159]. The major route of excretion of rofecoxib metabolites is urine, however, Halpin *et al.,* have reported a 14 % of the single radio labeled dose recovered from the feces [160].

1.17.5. Specialty population

The area under the concentration curve (AUC) for rofecoxib is 34 % higher in elderly population (>65 years) compared to younger adults. However, no dose reduction is recommended for elderly, only a lower loading dose is required by the therapeutic guidelines [161]. Antacids containing calcium carbonate and magnesium or aluminum salts decrease the absorption of

rofecoxib [162]. The AUC is increased by 69% in moderate hepatic impaired patients as reported in only four patients; however, there is no report on severe hepatic failure patients[159].

End stage renal failure patients have shown a decrease of 18% and 9%, respectively in Cmax and AUC, because of reduced renal excretion. No data was found for rofecoxib pharmacokinetics in advanced renal disease patients. Overall, the use of rofecoxib is not recommended in renal and hepatic failure patients, however, in mild to moderate conditions lowest dose possible are recommended to be used with caution [162].

1.17.6. Drug interaction

There is no clinically relevant interaction between rofecoxib and other arthritis medication including prednisolone and methotrexate (7.5-15 mg/week). However the plasma concentration of rofecoxib is increased when administered with methotrexate, cimetidine, warfarin and rifampicin [159].

1.17.7. Adverse effects

Rofecoxib is generally well tolerated if used as therapeutic doses. In OA patients, mild symptoms of headache (5.8%), nausea (8.3%), abdominal pain (8.7%), diarrhea (6.2%), dyspepsia (2.9%) and epigastric discomfort (3.7%) are reported with 12.5 mg/day doses of rofecoxib. In RA patients, the most common adverse effects for rofecoxib include diarrhea, headache, fatigue and dizziness [159].

Gastrointestinal adverse effects: COXIBs like rofecoxib were developed with an aim to reduce the GI adverse effects of traditional NSAIDs. Initial reports have suggested that the incidents of perforation, ulceration and bleeding (PUB) are lower in rofecoxib compared to traditional NSAIDs [163]. However, recent analysis of clinical trials and data obtained through litigation have revealed no superiority [107].A meta-analysis of eight clinical trials revealed significantly fewer incidences of PUB in six weeks (p=0.004) after 12 months (p 0.01) of rofecoxib treatment in OA patients. It was also confirmed by an endoscopic examination of study subjects [164]. The VIGOR trial of RA patients fewer patients quit the trial because of GI side effects in rofecoxib group (3.5%) compared to naproxen (4.9%) [131].

Cardiovascular adverse effects: Rofecoxib a being highly selective COX-2 inhibitor blocks COX-2 and its products. This may cause thrombogenic state in the body leading to

infarction, angina pectoris, transient ischemic attacks, deep vein thrombosis, peripheral thrombosis and cerebrovascular events. Studies comparing rofecoxib with a placebo for their incidences of cardiogenic thromboembolic events resulted into 2.71 incidences per 100 patient years for rofecoxib compared to 2.57 per 100 patient years in the placebo. However there was no statistical significance [165].

Renal adverse effects: The COX isoforms and their products play important roles in the kidney. Inhibition of prostaglandins (e.g., PGE2) production may adversely affect the renal function and may result in peripheral edema, weight gain and attenuation of antihypertensive effects of diuretics, hypertension and rarely chronic heart failure.

Rofecoxib treatment is reported to reduce the renal production of PGE2 and PGF1 by 40% to 50% and result in sodium retention [166]. Schwartz *et al.,* have reported that compared to placebo rofecoxib reduces urinary sodium and potassium excretion, and creatinine clearance but increased mean systolic blood pressure in elderly patients receiving a normal-salt diet. However, rofecoxib effects were not any different from celecoxib and naproxen [167].

Rofecoxib treatment is also linked to a modest increase in the serum potassium concentration. In an animal study rofecoxib, celecoxib, diclofenac, and flurbiprofen significantly reduced excretion rate of sodium and well as potassium compared to placebo. Rofecoxib was also found to reduce the urine flow that can lead to nephrotoxicity [168]. In a case repot, by Morales *et al.,* rofecoxib treatment significantly reduced the glomerular filtration rate in the nephrons, at 12.5 and 25 mg doses compared to placebo [169].

Rofecoxib treatment may cause renal impairment in high risk patients with a history of renal and cardiac disorders, diabetes and hypertension. Therefore, it should not be prescribed to these high risk patients [156, 159].

1.18. Meloxicam

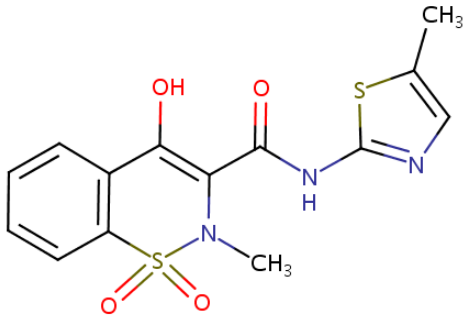
1.18.1. Chemistry

Meloxicam is a derivative of enol-carboxyamide and is member of oxicam class of NSAIDs. Chemically, meloxicam is 4-hydroxy-2, methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzo-thiazine-3-carboxamide,1,1-dioxide [Figure 4]. It has formula of $C_{14}H_{13}N_3O_4S_2$ and molecular weight of 351.4 g/mol [157, 170].

1.18.2. Physicochemical properties

Meloxicam exhibits tautomerism in its structure. At physiological pH (pH 7.4) it exists in anionic form, while, in acidic conditions it converts into cationic form. Meloxicam is a zwitterionic and exhibits two ionization points with pKa values of 1.29 and 4.35. Because of its zwitterionic nature its log P value cannot be determined experimentally, however, some studies have reported the computed log P value of 0.07 at pH 7.4 [157, 171].

A)



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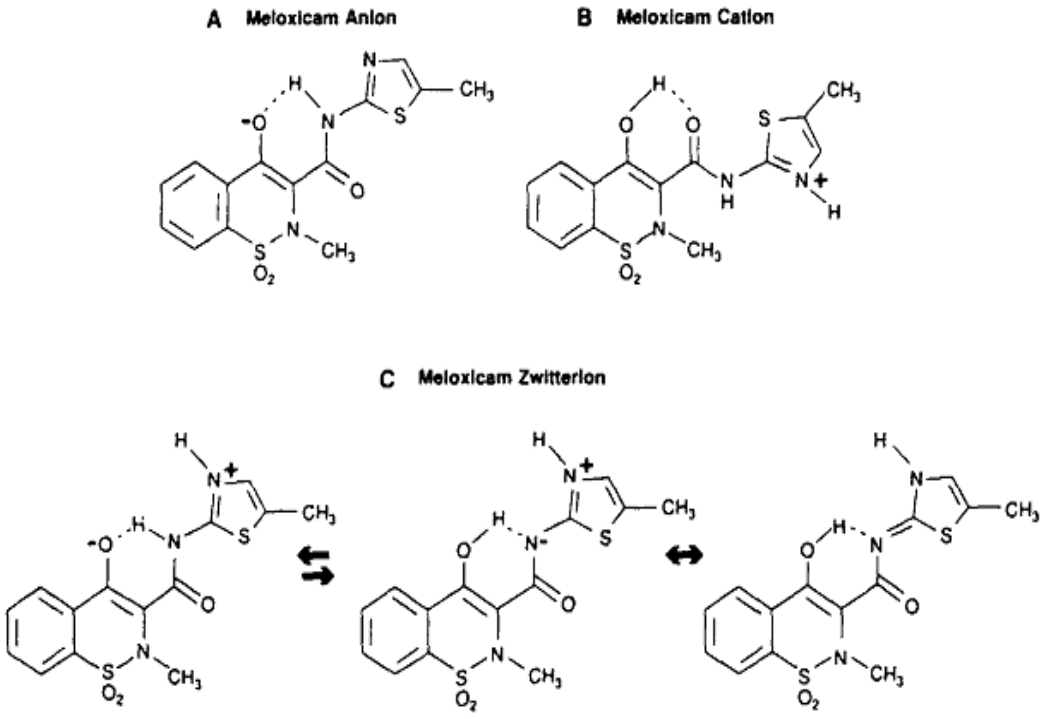

Figure 4: Chemical structure of meloxicam (A) and meloxicam ions (B) (with permission from [171]).

1.18.3. Pharmacokinetics

Meloxicam can be administered through oral, intramuscular, intravenous and rectal routes. It follows a dose-dependent linear pharmacokinetics and is almost completely absorbed after oral administration with 89% oral bioavailability. Food does not be affect bioavailability of meloxicam. Its Tmax varies from 9-11 h after the administration of a 30 mg dose orally. The steady state plasma concentration of meloxicam is achieved after 3-4 days of oral administration. Meloxicam has a long plasma half-life of 22-24 h and undergoes enterohepatic recirculation. The apparent volume of distribution for meloxicam is estimated to be 10-15 L in humans. It also binds to plasma proteins, specifically to albumin (>99%) [170]. Synovial fluid in the joints is the primary site of action for NSAIDs; meloxicam readily penetrates the synovial fluids with a mean synovial concentration of 50 ± 15% of the administered dose is reported 24 h post dose after seventh day of administration [170, 172].

Meloxicam is extensively and completely metabolized by the liver, mainly by phase-I metabolic enzymes involving CYP2C9, and to a lesser extent by CYP3A4. No phase-II metabolism is reported for meloxicam. Two inactive metabolites of meloxicam, namely the 5-hydroxy-methyl-metabolite (M7) and 5-carboxy-derivative (M5) are identified in humans. M7 formed by CYP3A4 metabolism is the major metabolites accounting for 51% of the total metabolite concentration [173].

About 60–65% of the radioactively labelled meloxicam is eliminated in the urine and 35-40% in the feces. Only 0.2% of unchanged drug is found in the urine and 1.6% in the feces [173]. Total body clearance of meloxicam varies between 0.42-0.7 L/h with an elimination half-life of 13 to 20 h [174]

1.18.4. Pharmacodynamics

Meloxicam is indicated for the symptomatic relief of OA, RA and juvenile RA. It is approved in a therapeutic dose of 7.5, 15 mg per day. Meloxicam and is available in oral and intramuscular injection dosage forms. It exhibits anti-inflammatory, analgesic, anti-pyretic properties through inhibition of cyclooxygenase enzymes. Meloxicam was introduced before COX isoforms were discovered. However, after the discovery of COX-2, meloxicam was reclassified as preferentially COX-2 inhibitor [106].

Meloxicam is 3 and 8 times more selective towards COX-2 than COX-1. It has IC50 of 2 nmol/] for COX-2 and IC50 of 33.7 nmol/L for COX-1 reported in synovial fluid obtained from osteoarthritic patients. Notably the concentration of meloxicam in the synovial fluids, as seen after 6 h after 15 mg doses in RA patients, is higher than the IC50 for COX-2. Studies comparing the potency of meloxicam have reported that meloxicam has a lower COX-2 inhibitory properties than rofecoxib, but higher than ibuprofen, naproxen and indomethacin [175].

Meloxicam exhibits substantial dose dependency in its anti-inflammatory, anti-exudative and anti-edematous properties as observed in the adjuvant arthritis rat model. The analgesic effects of meloxicam were found to be equipotent to other NSAIDs [176]. It exhibits no centrally mediated analgesia when observed for its central analgesic effect using hot-plate and tail-clamp tests. It does not impart central analgesia even at doses above the anti-inflammatory doses [176].

The antipyretic effects of meloxicam were not observed under normothermic conditions, but it does reduce the fever once fever is induced in a dose-dependent manner. Additionally, meloxicam also had uricosuria effect on rats. This effect was the same as observed with piroxicam and indomethacin [176]. NSAIDs inhibit the ability of chondrocytes to repair any damage to human cartilage through their inhibitory effects on proteoglycan synthesis. But meloxicam, owing to its selective inhibitory activity on inflammatory mediators, exhibits a slightly favorable profile. It increases the synthesis of peptidoglycans and hyaluronic acid (HA) in osteoarthritis explants, and significantly reduces the net loss of proteoglycan molecules in synovial fluids, exerting favorable effects upon the overall metabolic turnover. The exact mechanism through which meloxicam exerts these effects is still unknown; however, its ability to normalize the catabolic changes under mild-to-moderate OA is proven [177].

1.18.5. Drug interaction

Unlike other NSAIDs, meloxicam does not interact with platelets and with anticoagulating actions of warfarin [178]. Meloxicam may interact with the metabolism of other drugs which are substrate for these CYP enzymes, such as, tolbutamide, sulfaphenazole, ketokonzole and nifedipine [172, 179].

1.18.6. Specialty population

Meloxicam is highly bound to plasma proteins, only a small free fraction available for distribution into the tissues. An increase in the free fraction of meloxicam is reported in renal failure patients; thus, a lower dose is recommended for use in moderate-to-severe renal failure patients. [172]. The pharmacokinetics of meloxicam is also affected by renal and hepatic insufficiencies due to altered plasma proteins and lower hepatic metabolism [180].

1.18.7. Adverse effects

Gastrointestinal adverse effects: Meloxicam, being a preferential COX-2 inhibitor, is expected to cause minimal gastrointestinal effects. But studies have shown that meloxicam cause lesions in gastric mucosa in a dose-dependent manner. But it causes less ulceration compared to indomethacin using the rat model [176].

Renal adverse effects: In an animal model meloxicam do not affect the rate of excretion electrolytes compared to placebo [168]. The prostaglandin synthesis in the kidney was also not affected, but may interfere with the diuretic effects of furosemide [181].

Cardiovascular adverse effects: Meloxicam has no effect on platelet aggregation, nor it affect the thrombogenicity of blood [182]. Thus it can be expected that it may posse lower CV risk. In a prescription events monitoring study the CV and thromboembolic events were lower in meloxicam users compared to celecoxib and rofecoxib users [183, 184]. Meloxicam (7.5 and 15 mg doses), was reported to have less risk of CV and cerebrovascular thromboembolic events compared with diclofenac and celecoxib [185]. However, little is known about its long-term use.

1.19. Celecoxib

1.19.1. Chemistry

Celecoxib is the first NSAID introduced as selective COX-2 inhibitor (COXIBs). It's an aromatic hetero-monocyclic organic compound belonging to the group called phenyl-pyrazoles that is structurally similar to sulphonamides. Celecoxib is chemically designated as 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulphonamide. It has an empirical formula of $C_{17}H_{14}F_3N_3O_2S$ and molecular weight of 381.38 g/mol [Figure 5] [186].

1.19.2. Physicochemical properties

Celecoxib is available as a pale yellow to white fluffy and amorphous powder. Its melting point is 158° C, a log P value of 3.47 and a pKa of 11.1. Celecoxib has limited water solubility in water (3.3 mg/L) [157].

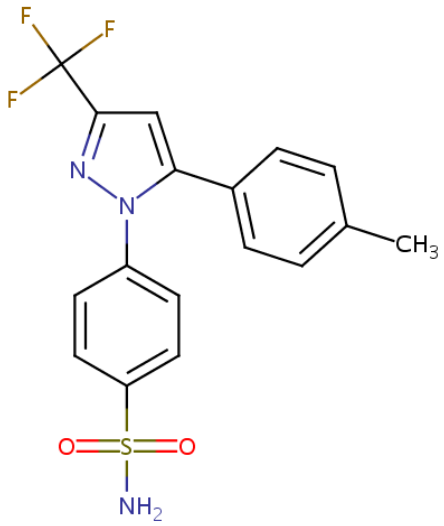

Figure 5: Chemical structure of celecoxib

1.19.3. Pharmacokinetics

Celecoxib is rapidly and completely absorbed after oral administration and peak plasma concentration is achieved within 3 h. Celecoxib follows a linear pharmacokinetic pathway in the therapeutic dose ranging from 100-600 mg [187]. Food, especially a high fat meal, alters the absorption of celecoxib, increases AUC by 11% which has no clinically relevant impact on its pharmacokinetics [188].

Celecoxib highly bound to plasma proteins (>98%), mainly albumin. It evenly distributes between the plasma and erythrocytes. In humans, the volume of distribution for celecoxib is reported to be 5.7-7.1 L/kg. This unexpectedly large volume of distribution is linked to the lipophilic nature of the compound, indicating extensive tissue distribution [189].

Celecoxib is extensively metabolized and only 2% of the parent drug is excreted unchanged. It is metabolized by CYP2C9 (>75%) and to some extent by CYP3A4 (<25%). CYP2C9 catalyze the hydroxylation of celecoxib which is followed by oxidation by cytosolic

alcohol dehydrogenases (ADH1 and ADH2) and conjugation with glucuronic acid to form inactive metabolites. The metabolites of celecoxib are identified as SC-60613, SC-62087 and the glucuronide conjugate of SC-62807. SC-62807 is the major metabolite of celecoxib that constitutes 19% and 54% of the administrated dose in urine and feces, respectively [189]. Celecoxib and its metabolites are mainly excreted in urine (27%) and feces (70%) with an elimination half-life between 11.2 and 15.6 hours, and total clearance (CL) 30 L/h [189].

1.19.4. Pharmacodynamics

Celecoxib is a potent anti-inflammatory, analgesic and anti-pyretic compound. It selectively inhibits more COX-2 enzyme over COX-1 enzyme. In vitro assays on human recombinant COX enzymes celecoxib demonstrated an IC50 value of 15 ± 1 μg/L for COX-1 and 0.04 ± 0.01 μg/L for COX-2 [189].

Celecoxib is indicated for the treatment of RA, OA, acute pain, menstrual pain, colon and rectal polyps and familial adenomatous polyposis. Other diseases that celecoxib is known to benefit includes, dysmenorrhea, familiar adenomatous polyposis and prevention of colorectal adenomas. ankylosing spondylitis and juvenile RA [190] . The approved therapeutic doses of celecoxib are 50 mg, 100 mg, 200 mg and 400 mg as capsules [189]. In systemic review, celecoxib has been found to be equipotent in relieving pain and inflammation in arthritis patients. It also have significantly favorable GI safety and tolerability compared to other NSAIDs [191].

1.19.5. Specialty population

Celecoxib pharmacokinetics is affected by age, 40% higher Cmax and 50% higher AUC were reported in patients 65 years and older compared to younger adults. These effects of age are more significant in female patients [125].

Celecoxib use is not recommended during pregnancy. Women in the late stages of pregnancy should avoid celecoxib. Celecoxib is known to cause premature closure of the ductus arteriosus in animals however, its evidence in humans is still lacking [125]. Celecoxib is also excreted in breast milk in concentration equal to plasma concentrations. But no reports on breast feeding associated toxicity with celecoxib are found in literature [189].

Genetic variations in CYP2C9 are suggested to cause significant alterations in systemic drug exposures and clearance of celecoxib. Thus dose individualization is required in susceptible individuals [189].

1.19.6. Adverse effects

Celecoxib Long-term Arthritis Safety Study (CLASS) involving 8059 arthritis patients showed that celecoxib is superior to diclofenac and ibuprofen in treating inflammation and has less GI complications [132]. In a systematic review of clinical trials, celecoxib is reported to have a lower rate of GI complications compared to the placebo group in OA patients [190].

Renal adverse effects: Celecoxib inhibits renal PGE2 synthesis, causing sodium and water retention in medullary thick ascending loop of Henle and inhibits water reabsorption in the collecting ducts similar to naproxen [192]. It also interacts with carbonic anhydrase enzyme and can block diuresis and cause hyperchloremia and metabolic acidosis. However, the therapeutic doses of celecoxib do not have any clinically relevant effects on carbonic anhydrase enzyme [193]

Cardiovascular adverse effects: Two long-term trials, CLASS and the PreSAP trial (Prevention of Spontaneous Adenomatous Polyps) have concluded that celecoxib does not increase CV risks [190]. However, Adenoma Prevention with Celecoxib (APC) trial have concluded that celecoxib dose dependently, increased the risk of mortality, myocardial infarction, thromboembolism and stroke as compared to placebo [194]. Owing to such contradicting evidence on CV safety of celecoxib, it is suggested that CV risk for celecoxib is relative and depends on patient related risk factors and the dose of celecoxib use.

1.20. Flurbiprofen

1.20.1. Chemistry

Flurbiprofen is non-selective NSAID with anti-inflammatory and analgesic properties. It's a propionic acid derivative of phenyl-alkanoic acid with a chiral center. Flurbiprofen exists as racemate of S and R enantiomers. Its chemical name is 2-(3-fluoro-4-phenylphenyl) propanoic acid. It has molecular weight of 244.26 g/mol and molecular formula of $C_{15}H_{13}FO_2$, [Figure 6]. Its melting point is 110-111° C [157].

1.20.2. Physicochemical properties

Flurbiprofen is a white-to-slightly-yellow crystalline powder which is slightly soluble in water (8 mg/L), but readily soluble in most polar solvents. It has a log P value between 3.57-3.94 and pKa of 4.50 [157, 195, 196].

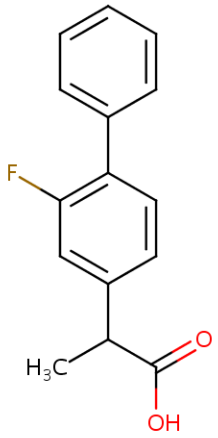

Figure 6: Chemical structure of Flurbiprofen

1.20.3. Pharmacokinetics

Flurbiprofen is readily and completely absorbed after oral administration. The peak plasma concentration 12 µg/mL is achieved in 1.5 to 3 h after a single dose of 100 mg orally. Food has no effect on its absorption, but may reduce its Cmax. Flurbiprofen is highly bound to plasma proteins (>99%), primarily albumin. The apparent volume of distribution for flurbiprofen in adults is reported to be 14 L. The synovial concentration of flurbiprofen after 6 h of oral administration of a 100 mg dose is comparable with that of plasma concentration [197].

Flurbiprofen is metabolized both by cytochrome P450 enzymes and glucuronidation pathways. The metabolites of flurbiprofen are devoid of any biological activity and 4-hydroxy-flurbiprofen is the principle metabolite formed by CYP2C9. Flurbiprofen absorption and metabolism is non-stereoselective and inter-conversion of R-and S-enantiomers is minimal. Flurbiprofen is excreted 20-25% (unchanged), 40-47% (4-hydroxy), 20-25% (3-hydroxy-4-methoxy) and 5% (3, 4-dihydroxy) in the urine [198]. The elimination half-life of R-flurbiprofen is (4.7 h) and S- flurbiprofen (5.7 h) [199].

1.20.4. Pharmacodynamics

Flurbiprofen is a non-selective NSAID and is structurally and pharmacologically similar to other NSAIDs like ibuprofen and ketoprofen. It reversibly inhibits the COX enzymes and blocks the prostaglandin synthesis. It's one of the most potent NSAID in terms of blocking the prostaglandin synthesis. The S-enantiomer is reported to possess most of the anti-inflammatory properties attributed to flurbiprofen. However, both enantiomers possess the analgesic properties [195].

Flurbiprofen is indicated for symptomatic treatment of RA, OA, ankylosing spondylitis and inflammation-induced pain associated with dysmenorrhea, bursitis, tendonitis and soft tissue trauma. It is used orally, topically and intraocular prior to eye surgery, to prevent meiosis. The usual initial adult dose of flurbiprofen is 150 to 200 mg daily in three or four divided doses. If necessary, the dosage may be increased to 300 mg daily in divided doses [197].

1.20.5. Specialty population

There are reports of higher drug exposure in elderly patients using flurbiprofen. This might be due to less plasma albumin and reduced hepatic metabolism [198]. Urine is the main route of excretion for flurbiprofen and its metabolites thus patients suffering with renal failure may require dose adjustment [199].

1.20.6. Adverse effects

Flurbiprofen is generally well tolerated in therapeutic doses, with chances of abdominal discomfort, dyspepsia and constipation and, rarely, central nervous system effects.

Gastrointestinal adverse effects: The most common adverse effects reported in flurbiprofen users are gastrointestinal related, such as abdominal discomfort, dyspepsia, nausea, vomiting, elevated liver enzymes, flatulence and constipation. Other less-frequent adverse effects include bloody diarrhea, gastric ulcer, gastric jaundice (cholestatic and non-cholestatic), hematemesis, hepatitis and stomatitis [198].

Cardiovascular adverse effects: Recent epidemiological studies have shown that patients treated with flurbiprofen might be at risk of CV complications such as CHF, MI, hypertension and other vascular disturbances. Some studies have also reported vasodilatation, hypotension, palpitation and tachycardia in flurbiprofen users [197].

Renal adverse effects: The renal effects of flurbiprofen are neither very common nor often reported. Flurbiprofen is known to cause interstitial nephritis, renal failure, dysuria, oliguria, hyperkalemia, hyperuricemia, and proteinuria [197].

Central Nervous System (CNS) adverse effects: CNS disturbances such as headache, nervousness, anxiety, insomnia, increased reflexes and tremors are common in flurbiprofen users [197].

1.21. Biomarkers of cardiovascular risk in rheumatoid arthritis

Biomarkers are biological indicators used to represent the state of the body or a body system. They can either be an indigenous compound or a physiological parameter that can be observed to differentiate between a normal physiological condition and pathological states. Biomarkers can also be used to monitor the pharmacological response of a drug or therapeutic progress of a treatment.

Biomarkers provide an early warning for risk and adverse effects and are crucial to monitor carefully in the high-risk patients. If a biomarker is to be used as a diagnostic tool, it should be sensitive and specific and have a high predictive power.

An ideal biomarker should have the following characteristics to qualify as a reliable indicator: 1) easily accessible from the body, 2) safe and easy to measure, 3) cost effective, 4) adoptable and modifiable from sample to sample, 5) consistent across individuals.

Various kinds of biomarkers have been developed, such as some indigenous compound (e.g., C-reactive protein, rheumatic factor) and physiological parameters (e.g., blood pressure, electrocardiogram). These biomarkers are either used alone or in combination with other techniques, depending upon specificity and sensitivity that is needed to monitor the disease or make a diagnosis [200].

Chapter 2

2. Thesis rationale and hypotheses

2.1. Rationale

Inflammation increases the risk of cardiovascular (CV) incidents [201-203]. The nonsteroidal anti-inflammatory drugs (NSAIDs) that are used to treat inflammation are also associated with adverse CV and renal effects [107, 113]. Meta-analysis of randomized clinical trials (RCTs) has concluded that long term use of NSAIDs, increases the risk of CV/renal incidents. However, uncertainty remains about the nature of such risks and the relative safety of various NSAIDs [139, 152, 155, 204-207].

Multiple mechanisms are likely to be involved in cause CV toxicity in NSAIDs users. Most popular belief is that NSAIDs increase the thrombogenic potential in the body, by selectively inhibiting cyclooxygenase-2 (COX-2) and its antithrombotic products (e.g. prostacyclin). In turn increase the chances of MI, thromboembolic events and renal failure [140]. However, studies have suggested that even nonselective NSAIDs (e.g., ibuprofen) pose si milar CV/renal risks [208, 209]. Overall, it can be said that CV/renal risks of NSAIDs is neither a class effect nor it can be explained with COX-2 selectivity of alone; rather there may be other pharmacological explanations that needed to be explored.

Epidemiological studies have revealed that the CV/renal risks of NSAIDs are multifaceted, and are difference in its nature on different body systems [204, 205]. For example, a meta-analysis of CV risk in celecoxib users found that the risk of cerebrovascular events was different from the risk of myocardial events [206].Likewise, for rofecoxib the risk of myocardial infarction was different from the risk of stroke [210]. This suggests that CV risk of NSAIDS is variable across different organs perhaps due to difference in exposure to the drug. Overall, there is inadequate information to address this anomaly and it obviously worth in-depth investigation.

Our group has shown that rofecoxib and celecoxib distributes more in the kidney tissues with higher kidney-to-plasma concentration ratios than that of meloxicam when dosed in rats. This observation corresponded with a reduced renal function in rofecoxib and celecoxib but not in meloxicam treated rats [141]. It was concluded that a lower tissue distribution might, at least in part, be responsible for the observed lower renal effects observed in rats dosed with

meloxicam. Meloxicam is also reported to have more favorable overall CV safety profile compared with other NSAIDs in observational studies [152, 204, 211]. This suggests that tissue exposure may have a role to play in NSAIDs induced renal adverse effects. Perhaps the NSAIDs accumulation in a tissue has pharmacological implication on local prostaglandin synthesis and organ function [192].

Renin angiotensin system (RAS) a multi-organ system involved in regulating blood pressure, electrolyte excretion, fluid balance, and overall CV homeostasis [212]. It comprises of angiotensin converting enzymes (ACE, ACE2), angiotensin peptides (Ang-II, Ang-(1-7) and angiotensin receptors (AT1R, AT2R Mas). These RAS component are grouped together as ACE/Ang-II/AT1R is called as cardiotoxic axis and ACE2/Ang-(1-7)/Mas is called as cardioprotective axis. We have previously reported in our laboratory that an important cardioprotective component of RAS, the ACE2 enzyme, is downregulated in adjuvant arthrtis (AA) rat heart [213], altering the constitutive balance between two RAS enzymes, ACE and ACE2. This down regulation of cardioprotective components of RAS during inflammatory diseases, might be contributing toward a higher CV risks [130].

Cytochrome P450 (CYP) metabolites of arachidonic acid (ArA) are involved in regulating vascular tone [214] as well as renal [215] and heart function [216]. Recent studies have speculated the role of CYP metabolites of ArA in CV/renal risks of NSAIDs[17, 217]. Two kinds of CYP enzymes are involved in ArA metabolism, the hydroxylases and epoxidases. The product of hydroxylases is a cardiotoxic metabolite 20-hydroxyeicosatetraenoic acid (20-HETE), and the product of epoxidases are group of cardioprotective metabolites called as epoxyeicosatrienoic acids (EETs). 20-HETE has a pro-inflammatory [218, 219], vasoconstrictive [220], and myogenic [221] effects in heart but in the kidney it has a dual effect. In the renal tubules, 20-HETE inhibits electrolyte absorption, whereas in the Bowman's capsule and capillaries it is vasoconstrictive. On the other hand, EETs are potent vasodilating, anti-inflammatory, anti-mutagenic metabolites [222] that confer cardio-protection [216, 223]. Once produced, these metabolites have shorter half-lives, binding with plasma proteins and/or get esterified [224]. This suggests that local eicosanoid production in a tissue is important.

In an animal study our laboratory has reported a direct relationship between renal function, and NSAIDs concentration in kidney tissue [141].It was reported that rofecoxib,

celecoxib, diclofenac, and flurbiprofen, but not meloxicam significantly reduced urinary excretion of electrolytes. This was in line with the human clinical trial data that reported that meloxicam had the least adverse renal effects compared to other NSAIDs. Warner et al., have also reported similar finding that celecoxib but not meloxicam altered renal electrolyte secretion [98]. This suggests that the extent of renal tissue exposure influences renal function; this might also be the case for other organs as well. Limited data are available on NSAIDs tissue concentration in humans. However, a clear concentration versus response is reported in animals.

The above information suggests that further investigation of NSAIDS tissue accumulation and its consequences on the heart and kidneys is warranted. In this thesis we will investigate the influence of NSAIDs treatment on tissue based RAS and CYP mediated ArA metabolism in adjuvant arthritic (AA) rat model of inflammation. NSAIDs with different COX-2 selectivity, physiochemical and pharmacokinetics properties will be used in our studies to inform us about possible, if any, relationship between NSAIDs tissue accumulation and CV/renal risks. We will also explore the possibility that ArA metabolites can be used as biomarkers to predict NSAIDs-associated CV/renal toxicity [Appendix-I].

2.2. Thesis hypotheses

- Extent of NSAIDs exposure influences their CV/renal risks, independent of their COX-2 selectivity. Also the type of comparator used in a study, underlying inflammation in the subjects and concomitant use of aspirin are significant factors in estimation of such risks.

- NSAIDs which accumulate more readily in tissues than plasma, may also have more pronounced pharmacological implications on renin angiotensin system (RAS) and arachidonic acid (ArA) metabolism by cytochrome P450 enzymes in that tissue, compared to the NSAIDs which minimally distribute in the tissues.

- NSAIDs down regulate the cardioprotective components of RAS (enzyme/peptides/receptors, respectively) i.e., ACE2/Ang-(1-7)/Mas; over cardiotoxic ones i.e., ACE/Ang-II/AT1R. The plasma ratio of angiotensin peptides (i.e. Ang-(1-7)/Ang-II) can serve as a biomarker of cardiotoxicity.

- NSAIDs disrupt the CYP-mediated metabolism of ArA, which parallels with their CV/renal risks reported in clinical trials. The plasma ratio of ArA metabolites (i.e., 20-HETE/EETs) can serve as a surrogate biomarker for NSAIDs-induced cardiotoxicity.

2.3. Thesis objectives

- A systemic review of clinical trial and observational studies will be done to test the theory that extent of NSAIDs exposure is associated with CV/renal risks categorized into myocardial, vascular, and renal categories. Further to see if such risk is affected by type of comparator, underlying inflammation and concomitant use of aspirin.

- Extent of tissue drug accumulation will be measure by dosing pharmacokinetic equivalent dose of rofecoxib, meloxicam, celecoxib and flurbiprofen in adjuvant arthritic (AA) rats and their tissue to plasma concentration ratios will be calculated in heart and kidneys.

- Effects of NSAIDs treatment on the renin angiotensin system (RAS) will be investigated in AA rat plasma, heart, and kidneys. To investigate if plasma concentration of RAS peptides can predict cardiotoxicity of NSAIDs.

- Effects of NSAIDs treatment on cytochrome P450 metabolism of arachidonic acid (ArA) and the product eicosanoid profile will be investigated in AA rat plasma, heart, and kidneys. To investigate if plasma ratio of ArA metabolites can predict cardiotoxicity of NSAIDs.

Chapter 3

3. Material and methods

3.1. Materials

High performance liquid chromatography (HPLC) grade; acetonitrile, anhydrous acetonitrile, hexane, methanol, acetone, formic acid (96%), N, N-diisopropylethlamine, butylated hydroxytoulene, 16-hydroxydecanoic acid and indomethacin (Sigma-Aldrich, Oakville, ON, Canada); water for HPLC (Caledon Laboratories Ltd., Toronto ON, Canada); HPLC grade, methanol trimethylamine, glacial acetic acid, analytical grade sulfuric acid, sodium acetate (Fisher Scientific Corp., Ottawa, ON, Canada); Polyethylene glycol MW 200 and piroxicam (Sigma-Aldrich, Oakville ON, Canada); rofecoxib powder (Yick-Vic Chemicals & Pharmaceuticals Ltd., Kowloon, Hong Kong); ketoprofen racemate powder (Sigma-Aldrich, Oakville ON, Canada); meloxicam powder (Unichem Laboratories Ltd., Bombay, India); celecoxib powder was a gift from Searle (Harbor Beach MI, USA); ibuprofen racemate powder (Upjohn, Don Mills ON, Canada) and flurbiprofen racemate (Sigma Chemical Corp., MO, USA); potassium dihydrogen tetraphosphate buffer (KH_2PO_4), ethyl acetate, chloroformate, isooctane, and isopropanol (BDH Chemicals, Edmonton AB, Canada);, L-Leucinamide for the derivatization of flurbiprofen enantiomers (Sigma Aldrich Oakville ON, Canada) and diammonium hydrogen orthophosphate buffer (Merck KGaA, Darmstadt, Germany).Standards for arachidonic acid metabolites (Cayman Chemical Company, Ann Arbor MI, USA); 2-(2,3-naphthalimino) ethyl-trifluromethanesulphonate (Molecular Probes, Eugene OR, USA); Oasis HLB 1CC (30 mg) solid-phase extraction cartridges (Waters Corporation, Milford MA, USA); tris buffer (pH 7.4), sodium orthovanadate, benzamide, sodium dodecyl sulfate SDS (0.1%), EDTA (Sigma Aldrich Co., MO, USA) and lowery protein assay kit (Bio-Rad Laboratories, Hercules CA, USA). Western blot analyses; sample reducing buffer (Thermo Fisher Scientific Inc., Waltham, MA USA), polyacrylamide gel, nitrocellulose membrane, and Tween-20 (Bio-Rad Laboratories, Hercules, CA, USA), bovine serum albumin (Fisher Scientific Company, Ottawa, ON, Canada). The primary antibodies for Western blots included 1) mouse monoclonal ACE antibody (Abcam Inc.,Toronto, ON.Canada -ab77990); 2) rabbit polyclonal ACE2 antibody (Abcam Inc.,Toronto, ON.Canada -ab87436); 3) rabbit polyclonal anti-alpha tubulin antibody (Abcam Inc.,Toronto, ON.Canada -ab4074); 4) mouse monoclonal [AC-15] to beta Actin

(Abcam Inc.,Toronto, ON.Canada -ab6276); 5) rabbit polyclonal anti-Mas receptor antibody (LifeSpan BioSciences, Inc, Seattle, WA, USA-LS-B3564); 6) Mouse monoclonal to Angiotensin II Type 1 Receptor antibody (Abcam Inc.,Toronto, ON.Canada - ab9391) and 7) rabbit monoclonal to Angiotensin II Type 2 Receptor (Abcam Inc.,Toronto, ON.Canada - ab92445).The horseradish peroxidase secondary antibodies used were: goat anti-mouse secondary antibody (#170-5047) and goat anti-rabbit secondary antibody (#170-5046) (Bio-Rad, Laboratories, Hercules, CA, USA). Immune-Star Chemiluminescence ECL Kit (#170-5070) (Bio-Rad, Laboratories (Hercules, CA, USA) and Fujifilm plates (FUJIFILM North American Corp. Mississauga, Canada) were used to capture the image. Hhydrochloric acid and p-hydroxymercury benzoate (#55540-5G) (Sigma-Aldrich, Seelze, Germany), 1, 10-phenanthroline (431788-25G), phenylmethsylsulphonyl fluoride (P7626-5G), pepstatin-A (P5318-5MG), EDTA (431788-25G) (Sigma Aldrich, Cleveland, OH, USA), and trifluoroacitic acid (Y1040) (Peninsula Lab, San Carlos, CA, USA). Commercially available ELISA kits for Ang-II and Ang-(1-7) cat. #1133 and 1330, respectively (Peninsula Lab, San Carlos, CA, USA).

3.2. Methods

3.2.1. Selection of NSAIDs

Based on our previous observation on kidney-to-plasma concentration for rofecoxib, celecoxib and meloxicam and its correlation with reduced urinary electrolyte excretion [141]. We wished to see if heart-to-plasma concentration for these NSAIDS follows the same trend and is correlated with CV risk reported in literature.

For this purpose we choose meloxicam as our main comparator, owing to its unique pharmacokinetic, tissue distribution characteristics. However, because of the large number of possible NSAID comparisons, we restricted ourselves to the following NSAIDs, each representing a different COX-2 selectivity and pharmacokinetic profile: rofecoxib is a positive control and carries the highest CV risk that we aim to explore; celecoxib is a widely prescribed selective COX-2 inhibitor reported to cause low CV risk at therapeutic doses; flurbiprofen is commonly used as an over the counter pain killer and was chosen for this study to represent non-selective NSAIDs.

3.2.2. Dose calculation

The doses we have used are rofecoxib (10 mg/kg), meloxicam (0.5 mg/kg), celecoxib (15 mg/kg), or flurbiprofen (5 mg/kg) per day. All the doses used in this study were weight-normalized pharmacokinetics rat equivalent to their average therapeutic human recommended for rheumatoid arthritis (RA) treatment in humans. We searched the literature studies reporting AUC after the administration of weight-normalized rat doses and human area under the plasma level time curve (AUC), after administration of the dose recommended for RA treatment in in human. Then we simply calculated the proportion of rat doses which would produce the AUC-rat equivalent to that of AUC in -human. It was assumed that the absorption process and protein binding is similar in both rats and humans and that AUC will serve as a reliable indicator of the drugs systemic exposure [225].

3.2.3. Animal handling

Adjuvant arthritis (AA) protocol for rats was approved by the Health Sciences Animal Care and Use Committee of University of Alberta, Edmonton, Canada. Adult male Sprague-Dawlay rats (n=18) weighing 230-250 g were purchased from Health Sciences Laboratory Animal Services. Animals were housed in the standard rat cages under ambient temperature and ventilation, with 12 h, day and night cycles and standard rat chow and free access to drinking water. After three days of acclimatization, the rats were randomly allocated to either of groups (n=3 per group) control, inflamed and inflamed treated with rofecoxib (10 mg/kg), meloxicam (0.5 mg/kg), celecoxib (15 mg/kg), or flurbiprofen (5 mg/kg).

Rats in inflamed and inflamed treated group were anesthetized with isoflurane/oxygen mixture (2/0.75%) as per the manufacturer's protocol with *Mycobacterium butyricum* (0.5 mg/mL) suspended in squalene and injected intra-muscularly in the tail base. Rats in the control group were injected with pyrogen free sterile solution of normal saline. Caution was exercised not to inject the emulsion into the veins, pulling the plunger out to see if any blood is coming, before injecting the contents of injection.

After 12 days, rats developed visible signs of inflammation associated with adjuvant arthritis. Rats in inflamed treated groups started to receive respective dose of NSAIDs suspended in polyethylene glycol (PEG-200). But the rats in control and inflamed groups received blank

PEG-200 only. All the doses were administered through an oral gavage and after adjusting for the body weight of each rat every day, for next 7 days [Appendix-II].

3.2.4. Assessment of adjuvant arthritis

The emergence of arthritis and later progress of disease was measured by monitoring the physical and visual signs and symptoms of the experimental adjuvant arthritis (AA), according to published methods [35].

After inoculation with the adjuvant, rats were observed daily for the physical sign of AA including, swelling of paws, joints and ankle, involvement of tarsal , metatarsals and front paw [226]. Paw and joint diameters were measured using micrometer caliper (Mitutoyo Canada Inc., Toronto, ON) and water displacement test was done to measure paw volume, by dipping the hind paws in measure amount of water. Daily change in the rat body weight was recorded using the animal balance.

Arthritis index (AI), a kind of disease score was calculated according to published criteria [35]. For each hind paw involved in swelling, a score between 0-4 was assigned (0=not involved, 1=single joint, 2=more than one joint, 3=several joints with moderate swelling, 4=several joints, ankles and severe swelling). For each forepaw involved in swelling, a score between 0-3 was assigned (0=not involved, 1=single joint, 2=more than one joint, 3=involvement of wrists and joints with swelling).

AI was calculated by adding all the scores from both hind paws and both forepaws together with maximum of 14. A score of >5 was considered an emergence of signs and symptoms of disease, and treatment would be started. For biochemical assessments of arthritis serum nitrates and nitrites were quantified in using Griess reagent according to a published method [227].

3.2.5. Sample collection

After 7 days of dosing and 24 h after the last dose, rats were euthanized using isoflurane/oxygen mixture (0.75/2 %) as per manufacturers protocol [228]. Blood was collected using heart puncture then heart and kidney were surgically removed and processed as described under given paragraphs.

1) For the tissue distribution study, blood was collected using heart puncture with a 18-gauge needle pushed in the ventricle. Blood was collected in glass tube containing 60-USP units of sodium heparin (BD Diagnostics, NJ, USA). After gentle mixing the tubes were centrifuged at 2,500-2,800 revolutions per min (15000-2000 × g) at 4° C, for 10 min. Then plasma was separated in Eppendorf tubes, snap frozen with liquid nitrogen and then stored at -80° C until analyzed. The heart, kidneys surgically removed and divided into three portions before washed with normal saline, snap frozen with liquid nitrogen and stored at -80° C until analyzed with high-performance liquid chromatography (HPLC).
2) For the nitrites and nitrate measurement, a portion of blood was collected in clean glass tube and was kept on at room temperature for 20 min. Serum was carefully separated using a micropipette in Eppendorf tubes and snaps frozen with liquid nitrogen to be stored at -80° C until analyzed.
3) For the RAS measurement, a portion of blood was collected in a glass tubes. 50 µL of saline solution of "protease inhibitor cocktail" was added per each 1 mL of blood. The protease inhibitor cocktail contained 1 mM of p-hydroxymercury benzoate, 30 mM of 10-phenanthroline, 1 mM of phenylmethsylsulphonyl fluoride, 1 mM of pepstatin-A enzyme and 7.5% of ethylenediaminetetraacetic acid (EDTA). No heparin was added in these samples as it interferes with the analysis. After gentle mixing tubes were centrifuged at 2,500-2,800 revolutions per min (15000-2000 × g) at 4° C, for 10 min. Then plasma was separated in Eppendorf tubes, snap frozen with liquid nitrogen and then stored at -80° C until analyzed. A portion of heart and kidney tissues was washed with above mentioned protease inhibitor cocktail and stored in labelled polypropylene containers, snap-frozen in liquid nitrogen and stored at -80° C until analyzed.
4) For the ArA metabolites measurement, a portion of blood was collected in glass tubes containing 60-USP units of sodium heparin (BD Diagnostics, NJ, USA). To every 1 mL of blood, 200 µL of saline solution containing 0.113 mM of butylated hydroxytoluene (BHT) and 10 µM of indomethacin was added to prevent chemical and enzymatic decomposition of fatty acids, respectively. After gentle mixing the tubes were centrifuged at 2,500-2,800 revolutions per min (15000-2000 × g) at 4° C, for 10 min. Then plasma was separated in Eppendorf tubes, snap frozen with liquid nitrogen and then stored at -80° C until analyzed. The heart, kidneys surgically removed and a portion of these was washed with normal saline

containing butylated hydroxytoluene (0.113 mM) and indomethacin (10 µM) and stored in labelled polypropylene containers, snap-frozen in liquid nitrogen and stored at -80° C until analyzed using HPLC fluorescent detections.

3.2.6. Western blot analysis of angiotensin converting enzymes

Western blot analysis was used to measure the protein density of angiotensin converting enzymes (ACE and ACE2) in the rat organs (heart and kidney) using a published method with some modifications. Briefly, the previously frozen organs were thawed at room temperature. Approximately 30 mg of tissue was weighed and minced in 50 mM Tris homogenization buffer (pH 7.4) also containing protease inhibitor cocktail (1/150 mL), sodium orthovandate (27.6 mg/150 mL), benzamide (15 mg/150 mL), sodium dodecyl sulphate and EDTA (45 mg/150mL). The tissue homogenate was centrifuged at 8000 revolutions per min (7000 g) for 20 min at 4° C. Then supernatant was collected in Eppendorf tubes and debris was discarded. Total protein was measured in the supernatant using the Lowry method.

From each sample, a volume containing approximately 50 µg of protein or equivalent was incubated with sample reducing buffer (Fisher Scientific, Ottawa, Canada) in a water bath at 90° C for 5 min. Samples were electrophoresed at 200 mV in 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes previously soaked and washed in washing buffer i.e., phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20. Membranes were incubated overnight at 4° C in 15 mL in a blocking solution containing 5% skimmed milk and 1.5% BSA in washing buffer.

On next day, nitrocellulose membranes were thoroughly washed with portions of 15 mL washing buffer 4 times for 10 min each on a rocking platform. Membranes were then incubated with 10 mL of phosphate-buffered saline (pH 7.4) dilution of respective of primary antibody, for 2 h at room temperature, as per manufacturer protocol. The dilutions used for this purpose were 1) mouse monoclonal ACE antibody (ab77990) at 1/100 dilution produced a 150 kDa band; 2) rabbit polyclonal ACE2 antibody (ab87436) at 1/1000 dilution produced a 92 kDa band, 3) rabbit polyclonal anti-alpha tubulin antibody (ab4074) at 1/10,000 dilution produced a 50 kDa band; 4) mouse monoclonal [AC-15] to beta Actin (ab6276) at 1/10000 produced a 42 kDA band; 5) rabbit polyclonal anti-Mas receptor antibody (LS-B3564) at 1:50000 dilution produced a 37 kDA band; 6) Mouse monoclonal to Angiotensin II Type 1 Receptor antibody (ab9391) at 1:400

dilution produced a 40 kDA band; and 7) rabbit monoclonal to Angiotensin II Type 2 Receptor (ab92445) at 1/5000 dilution produced a 41 kDA band.

Following incubation with primary antibodies, the nitrocellulose membranes were thoroughly washed with 15 mL washing buffer 4 times for 10 min each. These membranes were then incubated with the secondary antibody (anti-mouse or anti-rabbit, as required) in a dilution of 1/60:000 for 1 h at room temperature. After 1 h the secondary antibody was recovered and membranes were lightly washed with 15 mL washing buffer 4 times for 10 min each. The binding of secondary antibody was then visualized using Immune-Star Chemiluminescence ECL Kit (Bio-Rad, Laboratories (Hercules, CA, USA-cat#170-5070). Images were captures on Fujifilm plates and later scanned to quantify the optical density of bands made by target protein relative to optical density of bands loading control (tubulin or beta-actin).

3.2.7. ELISA analysis for angiotensin peptide measurement

To measure angiotensin peptides, previously stored plasma tissues (heart, kidney) samples were thawed at room temperature and homogenized with 0.045 N HCL in ethanol (10 mL/g of tissue), containing 0.90 µmol p-hydroxymercury benzoate, 131.5 µmol 1, 10-phenanthroline, 0.90 µmol PMSF, 1.75 µmol pepstatin-A, 0.032% EDTA, and 0.0043% protease-free bovine serum albumin. The homogenate was centrifuged at 2500-3000 revolutions per min (750 g) for 10 min to remove the tissue debris and supernatant was recovered in clean glass tubes. These samples were evaporated in a rotary dryer and then reconstituted with 1 mL portion of 0.003% trifluoroacetic acid. Afterwards the peptides were extracted on Oasis HLB C18 extraction cartages (Pennsylvania laboratories, San Carlos, CA, USA) according to the manufacturer's protocol.

Extracted samples were kept in a freeze-drier overnight and reconstituted again with 1 mL solution of 0.003% trifluoroacetic acid. The protein concentration was determined in these samples using the Lowry method. Ang-II and Ang-(1-7) peptide concentrations were determined using commercially available ELISA kits (Peninsula Lab, cat #1133 and 1330, respectively) according to the manufacturer's protocol.

3.2.8. HPLC analysis of arachidonic acid metabolites

The method for extraction of ArA metabolites in rat plasma and tissues was adopted from a previously published methods [229, 230]. Briefly, previously stored samples were thawed at

room temperature. Then 200 µL plasma or approximately 30 mg of tissue (heart or kidney) were weighted into a glass tube containing 200 µL methanol and 0.4 µL of 96% formic acid on ice (4° C). While plasma samples were kept on ice 0° C , the tissue sample were homogenized using a tissue homogenizer (Omni-TH Thomas Scientific, NJ, USA) at 15,000 rpm for 1 min on ice 0° C. Both plasma and tissue samples were centrifuged at 14,000 revolutions per min (10,000 g) for 10 min at 0° C and resulting supernatant was transferred to 2 mL Eppendorf tubes and diluted with 1.8 mL of 10% methanol in distilled water. Then 30 µL of solution of 16-hydroxydecanoic acid (0.01 mg/mL of ethanol) was added as internal standard to each sample.

ArA metabolites were extracted using Oasis HLB C18 extraction cartages (Pennsylvania laboratories, San Carlos, CA, USA), previously conditioned with 1 mL methanol, 1 mL acetone, 2 mL hexane, 1 mL acetone, 1 mL methanol and 2 mL water. After samples were loaded on to extraction cartridges a light vacuum was applied to facilitate the extraction. Cartridges were then washed with 3 mL water and 1 mL 10% methanol and allowed to dry under a nitrogen stream. Once dried, the ArA metabolites were eluted with 2 mL of anhydrous acetonitrile and solvent was evaporated under stream of nitrogen. Standard dilution (2.5-0.01 µg/mL for each metabolite) were prepared using standards solution of ArA metabolites (Cayman Chemical Company, Ann Arbor MI, USA), then 30 µL of solution of internal standard i.e., 16-hydroxydecanoic acid (0.01 mg/mL of ethanol) was added in each tube before drying them under nitrogen stream.

Dried samples and standards were reconstituted with 136 µL anhydrous acetonitrile in glass tubes and kept under anhydrous conditions. A 10 µL portion of freshly prepared solution of 2-(2, 3-naphthalimino) ethyl-trifluoromethane sulphonate (2 mg/mL in anhydrous acetonitrile) as derivatizing agent, and 4 µL of N, N-diiospropylethylamine (catalyst) was added to each glass tubes and incubated for 30 min in desiccator at 4º C. Afterwards incubation the contents were dried under nitrogen stream to stop the reaction. Then reconstituted with 1 mL 20% methano, and loaded onto a fresh set of preconditioned Oasis HLB C18 extraction cartages. These loaded cartridges were washed with 3 mL of water and 1 mL of 30% methanol to remove excess derivatizing mixture and dried under a nitrogen stream. Then derivatized ArA metabolites were eluted with 2 mL anhydrous acetonitrile and eluent was evaporated to dryness under nitrogen stream. The residue was reconstituted with 100 µL 90% acetonitrile in water and 10 µL of each reconstituted sample was injected on to HPLC.

A published reverse-phase HPLC method with fluorescent detection was validated for our use [229] on a Shimadzu Prominence HPLC system (Mandel Scientific, Guelph ON, Canada) equipped with a DGU-20A5 degasser, LC-20 AT dual pump, SIL-20A auto sampler, CTO-20AC column oven, CBM-20A communication module, and RF-10AXL fluorescence detector. The detector was set at excitation and emission wavelengths of 260 and 360 nm, respectively. Data were acquired and analyzed using CLASS-VP 7.4 software provided with the system. Reverse phase chromatographic separation was achieved on two C18 columns (100 × 4.6 mm, 3.0 mm I.D.) connected in series and maintained at 30° C A C18 guard column (4.0 mm L × 3.0 mm) (Phenomenex, Torrance CA, USA) was attached prior to these analysis columns. Simultaneous elution of ArA metabolites was performed using a linear gradient of 0.05% formic acid water (A) and 0.05% formic acid acetonitrile (B) in a 124 min run pumping the solvent mixture at flow rate of 0.8 mL/min. Initially mixture of 45% A and 55% B was increased to 55:65% (A: B) over next 40 min, followed by a plateau for 25 min. Then 25:75% (A:B) over next 20 min, before reaching to 5:95% (A:B) over 10 min, which was held there for next 22 min, before dropping down to initial 45:55% (A:B) and 7 min pre-equilibration period prior was allowed before the next run. Sample chromatogram of ArA metabolites and biological samples is included in appendix [Appendix-III].

3.2.9. HPLC analysis for NSAIDs

3.2.9.1. Rofecoxib Assay

The concentrations of rofecoxib rat plasma, heart and kidneys were measured using a previously published reverse-phase HPLC method with modifications [231]. Briefly, to a weighted amount of heart and kidney tissues, twice the volume of their weight, HPLC-grade water was added in glass tubes. Heart and kidney samples were homogenized using a hand-held tissue homogenizer for 1 min on ice.

Rofecoxib stock solution (1 mg/mL) was prepared by dissolving pure drug in ethyl acetate. Standard dilutions containing 0.01, 0.025, 0.05, 0.1, 0.25, 0.1, 0.2, 0.5, 1, 2 and 3 µg/mL of rofecoxib were made by spiking portions of 200 µL blank rat plasma with above mentioned stock solution. Similarly, stock solution of internal standard i.e., ketoprofen (2 mg/mL), was prepared in methanol with a few drops of 0.01 M, NaOH to help dissolve the compound before making up the final total volume.

In separate glass test tubes, 200 µL of tissue homogenate, 200 µL of rat plasma and 200 µL of standard dilution were taken. Then 100 µL of (2 mg/mL) ketoprofen solution, 200 µL 0.05 M acetate buffer (pH 4.5), and 6 mL of ethyl acetate were added to these glass tubes. Tubes were vortexed mixed for 3 min and then centrifuged at 7000 revolutions per min (2500 g) for 3 min. The organic layer was removed and evaporated in a sample concentrator (Speed-Vac, Emerson instruments, Scarborough, ON, Canada). Lastly, samples were reconstituted with 200 µL of mobile phase and 100 µL of it was injected onto the HPLC system.

The reverse-phase chromatographic HPLC system consisted of, Sil-9A model auto-sampler, diode-array detector model CR601 (set at 272 nm lambda max) and data were acquired with chromatopac integrator (Shimadzu, Nakagyo-ku, Kyoto, Japan). The mobile phase consisted of 77%: 23%: 0.1%: 0.03 %, water: acetonitrile: acetic acid and trimethylamine, respectively. The mobile phase was pumped at a flow rate of 1 mL/min using a Waters 6000-A HPLC pump (Waters Corp, Mississauga, ON, Canada). Chromatographic separated was achieved on a C18 analytical column (10 cm x 4.6 mm i.d. 5 µm particle size), (Phenomenex, Torrance, CA, USA) attached to a C8 guard-column (Waters Crop, MA, USA).

3.2.9.2. Meloxicam Assay

The concentrations of meloxicam in rat plasma, heart and kidneys were measured using a previously published reverse-phase HPLC method with modifications [232]. Briefly, to a weighted amount of heart and kidney tissues, twice the volume of their weight, HPLC-grade water was added in glass tubes. Heart and kidney samples were homogenized using a hand-held tissue homogenizer for 1 min on ice.

Stock solutions of meloxicam (5 mg/ 100 mL) and internal standard i.e., piroxicam (10 mg/ 100 mL) were prepared by separately dissolving them in methanol. Standard dilution was made by spiking 100 µL of blank rat plasma to obtain dilutions of 0.05, 0.1, 0.5, 1, 5, 10, 25 and 50 µg/mL.

100 µL of tissue homogenate, 100 µL of meloxicam treated rat plasma and 100 µL of standard dilution were taken in separate glass test tube. To each tube 50 µL of (0.1 mg/mL) piroxicam solution, 100 µL of 1M hydrochloric acid, 6 mL of chloroform were added to each tube, vortexed for 3 min and centrifuged at 7000 revolutions per min (2500 g) for 3 min. The organic layer was removed and evaporated in a sample concentrator (Speed Vac, Emerson

instruments, Scarborough, ON, Canada). Lastly, samples were reconstituted with 100 µL of mobile phase and 50 µL of it was injected to HPLC.

The HPLC system consisting of, Sil-9A model auto-sampler, diode-array detector model CR601 (set at 364 nm lambda max) and data were acquired with chromatopac integrator (Shimadzu, Nakagyo-ku, Kyoto, Japan). The mobile phase consisted of 5:4:1, v/v, 50 mM diammonium hydrogen orthophosphate buffer: methanol and acetonitrile, respectively. Mobile phase was pumped at a flow rate of 1 mL/min using a Waters-6000A HPLC pump (Waters Corp, Mississauga, ON, Canada). Chromatographic separated was achieved on a C18 analytical column (10 cm x 4.6 mm i.d. 5 µm particle size), (Phenomenex, Torrance, CA, USA) attached to a C8 guard-column (Waters Crop, MA, USA).

3.2.9.3. Celecoxib Assay

The concentrations of celecoxib in rat plasma, heart and kidneys were measured using a previously published, reverse-phase HPLC method with modifications [233]. Briefly, to a weighted amount of heart and kidney tissues, twice the volume of their weight, HPLC-grade water was added in glass tubes. Heart and kidney samples were homogenized using a hand-held tissue homogenizer for 1 min on ice.

Stock solutions of celecoxib (100 mg/mL) and ibuprofen (100 mg/mL) were prepared by separately dissolving the drugs in methanol. Two standard curves were constructed for celecoxib by spiking 100 µL of blank rat plasma with various proportions of celecoxib stock solution to make a concentration of 0.02, 0.05, 0.1, 0.25, 0.5, 1 µg/mL and 1.0, 2.5, 5.0, 25, 100 µg/mL of celecoxib.

100 µL of tissue homogenate, 100 µL of rat plasma and 100 µL of standard dilutions were taken in separate glass test tube. 100 µL ibuprofen solution (20 mg/mL), 0.2 mL of 0.6 M sulphuric acid and 5 mL of isooctane: isopropanol mixture (95:5 v/v) was added to each tube. Tubes were vortexed for 3 min and centrifuged at 7000 revolutions per min (2500 g) for 3 min. The organic layer was removed and evaporated in a sample concentrator (Speed Vac, Emerson instruments, Scarborough, Canada). Lastly, the samples were reconstituted with 200 µL of mobile phase, and 150 µL of what was injected to HPLC.

The HPLC system consisting of, Sil-9A model auto-sampler, diode-array detector model CR601 (set at 254 nm lambda max) and data were acquired with chromatopac integrator

(Shimadzu, Nakagyo-ku, Kyoto, Japan). The mobile phase consisted of 47:53:0.1:0.03 % acetonitrile: water: acetic acid and trimethylamine, respectively. The mobile phase was pumped at a flow rate of 1 mL/min using a Waters-6000A HPLC pump (Waters Corp, Mississauga, ON, Canada). Chromatographic separated was achieved on a C18 analytical column (10 cm x 4.6 mm i.d. 5 µm particle size), (Phenomenex, Torrance, CA, USA) attached to a C8 guard-column (Waters Crop, MA, USA).

3.2.9.4. Flurbiprofen Assay

The concentrations of flurbiprofen in rat plasma, heart and kidneys were measured using a previously published, reverse-phase HPLC method with modifications [234]. Briefly, to a weighted amount of heart and kidney tissues, twice the volume of their weight, HPLC-grade water was added in glass tubes. Heart and kidney samples were homogenized using a hand-held tissue homogenizer for 1 min on ice.

Flurbiprofen (10 mg/ 100 mL) and internal standard i.e., ketoprofen (10 mg/ 100 mL) stock solution were prepared in purified water. A series of dilutions were made by spiking 500 µL of blank rat plasma to make final concentrations of 0.02 - 25 µg/mL and 25 to 100 µg/mL.

500 µL of tissue homogenate, 500 µL of rat plasma and 500 µL of standard dilutions were taken in separate glass test tube. To each tube 200 µL of 0.6 M sulphuric acid was added, and after 5 min wait, 100 µL of 1M NaOH, 50 µL of ketoprofen solution (10 mg/100 mL) and 3 mL isopropanol: isooctane (5:95, v/v) were added. Tubes were vortexed for 3 min and centrifuged at 7000 revolutions per min (2500 g) for 3 min. Organic layers were removed in clean test tube and another 3 mL of HPLC-grade water was added to each tube. The mixtures were vortexed for 3 min and centrifuged at 7000 revolutions per min (2500 g) for 3 min. The organic layer was discarded and 350 µL of 0.6 M sulphuric acid solution was added to the remaining aqueous layer. After vortex for 1 min, 3 mL of chloroform was added to this aqueous layer and the mixture was again vortexed for another 3 min and centrifuged at 7000 revolutions per min (2500 g) for 3 min. The aqueous layer was then discarded and the chloroform layer was evaporated to dryness in sample concentrator (Speed Vac, Emerson instruments, Scarborough, Canada).

To the evaporated residue was added, 100 µL trimethylamine (50 mM in anhydrous acetonitrile), 50 µL of chloroformate (60 mM in anhydrous acetonitrile) and 50 µL L-

Leucinamide (1M in anhydrous acetonitrile). Tubes were incubated at room temperature for 2 min to derivatize the flurbiprofen enantiomers. Then 50 µL of HPLC water was added to stop the reaction, and 10 µL of this solution was injected to HPLC.

The HPLC system consisting of, Sil-9A model auto-sampler, diode-array detector model CR601, flurbiprofen and ketoprofen were detected at 250 and 275 nm multiple wavelengths and data were acquired with chromatopac integrator (Shimadzu, Nakagyo-ku, Kyoto, Japan). Chromatographic separation was achieved on Partisil 5 ODS-3, Octadecyl-bonded silica (10 cm, x 4.6 mm, 5 µ particle size) (Whatsman Inc. Clifton, N.J. USA) attached to C8 Novo-Pak guard-column (Waters Corp, MA, USA). The mobile phase consisted of 36:65:0.02%, v/v, acetonitrile; (0.067 M) KH_2PO_4; and trimethylamine and was pumped at a flow rate of 1 mL/min using Waters 6000-A HPLC pump (Waters Crop, MA, USA).

3.2.10. Statistical methods

The results of systematic review are presented as combined odds ratio (OR') ± 95% confidence interval (95% CI), which was calculated from individual odds ratios (OR) reported in eligible studies. We used inverse variance method and Review Manager-5® 2014 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark) to calculate these OR' ± 95% CI. The significance of difference between OR' was noted, by a universal rule for reading OR, if 95% CI is not overlapping 1.00 (OR for reference group) it's significantly different from control/placebo.

The results of drug concentration assays are presented as mean ± standard deviation (SD) of respective NSAID in rat plasma (µg/mL), heart (µg/g) and kidney (µg/g) tissues. The tissue to plasma ratios are calculated individually for each rat, for each NSAID concentration in the heart over plasma (ratio) and kidney over plasma (ratio) and then mean ± SD was calculated. No statistical test was done on tissue to plasma ratios, as we don't intend to compare them against each other.

The results of Western blot analyses are presented here as mean ± SD of ratio of optical densities (OD) of the bands obtained for target protein and loading control beta-actin or tubulin. The values optical densities were obtained by processing the Western blot images through ImageJ software (developed by multiple contributors worldwide and available through University of Wisconsin-Madison). The mean values obtained for the control group are

compared with mean of the inflamed group using two tailed Students t-test using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA). For multiple comparison between inflamed group and NSAIDs treated groups we used one way analysis of variance (ANOVA) followed by Bonferroni adjustment using GraphPad Prism® statistics software (GraphPad Software, Inc. La Jolla, CA 92037 USA). Results are considered significant at $p < 0.05$.

The results of ELISA analyses are presented as mean ± SD of concentration of respective peptides (Ang-(1-7) and Ang-II) in the rat plasma (pg/mL), heart (pg/g) and kidney (pg/g) tissues. The Ang-(1-7)/Ang-II ratios are calculated individually for each rat, for plasma, heart and kidney samples and then mean ± SD was calculated. The mean values obtained for control group are compared with mean of inflamed group using two tailed Students t-test using Microsoft Excel Microsoft Corp., Redmond, WA, USA).). For multiple comparison between inflamed group and NSAIDs treated groups we used one way analysis of variance (ANOVA) followed by Bonferroni adjustment using GraphPad Prism® statistics software 2015 (GraphPad Software, Inc. La Jolla, CA 92037 USA). Results are considered significant at $p < 0.05$.

The results of arachidonic acid (ArA) metabolites are presented as concentration mean ± SD of concentration of respective metabolites (20-HETE, EETs and DHETs) in the rat plasma (ng/mL), heart (ng/mg) and kidney (ng/mg) tissues. The 20-HETE/EETs ratios are calculated individually for each rat, for plasma, heart and kidney samples and then mean ± SD was calculated. The mean values obtained for control group are compared with mean of inflamed group using two tailed Students t-test using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). For multiple comparison between inflamed group and NSAIDs treated groups we used one way analysis of variance (ANOVA) followed by Bonferroni adjustment using GraphPad Prism® statistics software 2015 (GraphPad Software, Inc. La Jolla, CA 92037 USA). Results are considered significant at $p < 0.05$. Pearson correlation coefficient (r value) is calculated between plasma, heart or plasma and kidney metabolites concentrations using SAS statistical software (SAS Institute Inc., NC, USA). Statistical significance of correlation was considered at $p < 0.05$.

Chapter 4

4. The Effect of COX-2 Selective Meloxicam on Myocardial, Vascular and Renal Risks: A Systematic Review *

4.1. Introduction

With the withdrawal of rofecoxib from the market in 2004 due to reports of increased cardiovascular (CV) complications in its users, the CV/renal effects of NSAIDs have been placed under spotlight [129, 201]. While the mechanisms behind these effects have remained mainly unclear, but the cyclooxygenase (COX) potency [235] and selective inhibition of COX-2 over COX-1 by some NSAIDs have been discussed in literature. In 2005 the United States Food and Drug Administration (FDA) issued a warning for the users of selective COX-2 inhibitors as well as for non-selective NSAIDs [236]. Thus generalizing the side effect of NSAIDs beyond the COX-2 selectivity, as it was suggested in more recent studies as well [201, 237]. Later, it was discovered that the risk of CV event associated with NSAIDs use can be lowered with concomitant use of low-dose aspirin [238]. Consequently, the FDA issued another label warning for all NSAIDs users, excluding those patients who also use low-dose aspirin [239]. Meanwhile, many epidemiological studies have suggested that CV/renal risks of NSAIDs are not homogeneous across all NSAIDs as some exhibit higher and some lower potential of causing such incidences[204]. Overall, it can be said that the CV/renal risks of NSAIDs are neither a class effect nor can be explained with COX-2 selectivity alone; rather there may be some other explanations yet to be explored.

Previously, in experimental animals, our laboratory has reported that rofecoxib and celecoxib have much higher kidney to plasma concentration ratio than another COX-2-selective NSAID, meloxicam. This observation in line with reduced renal function as exhibited by reduced urinary sodium and potassium excretions associated with the former two, but it was not observed with meloxicam use [141]. It was concluded in that study that study that a lower tissue

* Asghar, Waheed, and Fakhreddin Jamali. "The effect of COX-2-selective meloxicam on the myocardial, vascular and renal risks: a systematic review." *Inflammopharmacology* 23.1 (2015): 1-16.

distribution might, at least in part, be responsible for the observed lower renal risk for meloxicam. Interestingly, subsequent systematic reviews and observational studies, although not focusing on meloxicam, also suggested a more favorable overall CV safety profile for the drug. Meloxicam which is now available as its generic versions, hence, is not actively marketed, may have favor-able safety profiles as compared with other NSAIDs [152, 204, 211]. Etodolac, another COX-2-selective NSAID also appears to have favorable CV/renal profile, similar to that of meloxicam [201, 211, 240].

Many studies reporting the CV/renal effects of NSAIDs lack critical details to enable meaningful conclusions. For example, some previous systematic reviews that included meloxicam in their comparisons, have reported composite CV outcomes irrespective of the difference in the nature of the reported adverse outcomes, duration of use, dose, dosage and type of comparators (placebo vs active control trials). More importantly, some have even ignored the influence of underlying inflammatory disease and concomitant use of ASA, pooling the data for combine risk estimation [152, 155, 204, 205]. It is well established that inflammatory conditions, in general, and arthritis, in particular, are associated with increased morbidity and mortality mainly due to CV complications [202]. In addition, since emphasis is usually given to the composite CV/renal risks, the nature and origin of these adverse effects (e.g., myocardial, vascular and renal) have remained mainly unknown [206, 210]. A differentiation between these outcomes is essential as not all NSAIDs carry the same potential of influencing a particular system in the body.

We hypothesized that meloxicam (1) is not associated with lethal CV risk, (2) the risks, if any, is dependent on the dose and concomitant use of aspirin, and (3) the underlying inflammation and the nature of the drug used as the comparator plays significant role in estimating CV/renal risks. Accordingly, we assessed the CV/renal risk of meloxicam after categorizing the outcomes reported in NSAIDs users into categories such as, myocardial, vascular and renal outcomes. In addition, we examined the effect of the underlying inflammatory disease, dose, concomitant use of aspirin and the type of comparator (placebo, active control).

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4.2. Methods

4.2.1. Literature Search

A web search was executed using predefined keywords to find studies published in databases consisting of MEDLINE, EMBASE, CINHAL, IPA, PASCAL, Cochrane, EBM and Google Scholar until April 2014. The FDA and selected pharmaceutical companies websites and/or any relevant literature were also searched. Reference lists from review articles were also checked for more information. Further, some authors were contacted for clarification on their reported outcomes and/or to provide risk values of individual metrics. Two reviewers independently reviewed the short-listed studies for inclusion/exclusion criteria. Any disagreements and conflicts were mutually resolved.

4.2.2. Inclusion criteria

Comparative studies such as randomized controlled trial (RCT), case–control and cohort studies reporting myocardial and/or all-cause mortality outcomes (upon >90 days exposure), and/or vascular/renal outcomes (upon any exposure) to meloxicam were included in our analysis. No language restriction was imposed on the search output. Further, to reduce the risk of bias, studies were excluded if (1) the patients were using NSAIDs in combination (except for aspirin), (2) drug switching, dose adjustment, and/or use of extra-oral route of administration had occurred, (3) patient missing prescription follow-up before the anticipated index date, and/or (4) hazard ratio was used as the measure of the risk. We abstracted the CV/renal risk estimates reported for meloxicam compared to placebo or other NSAIDs (rofecoxib, celecoxib, diclofenac, etodolac, ibuprofen, naproxen and indomethacin).

The choice of >90 days of exposure limit for myocardial or mortality outcomes was made to capture the effects of long-term meloxicam exposure. As it is repeatedly been reported that NSAIDs exposure shorter than <30 days results in insignificant or negligible risk estimates [241]. Further, many studies have reported that ≥90 days exposure is required for the emergence of NSAIDs fatal adverse effects such as mortality and myocardial infarction (MI) [242]. However, for the studies reporting renal and vascular outcome, there was no exposure limit set for the use of NSAIDs. Because it has been reported both in humans [243] as well as animals [141], that peak renal effects of NSAIDs appear for a short time and they tend to plummet after a

few days of exposure [123]. are also prompt so that they may emerge shortly after the commencement of the therapy [244].

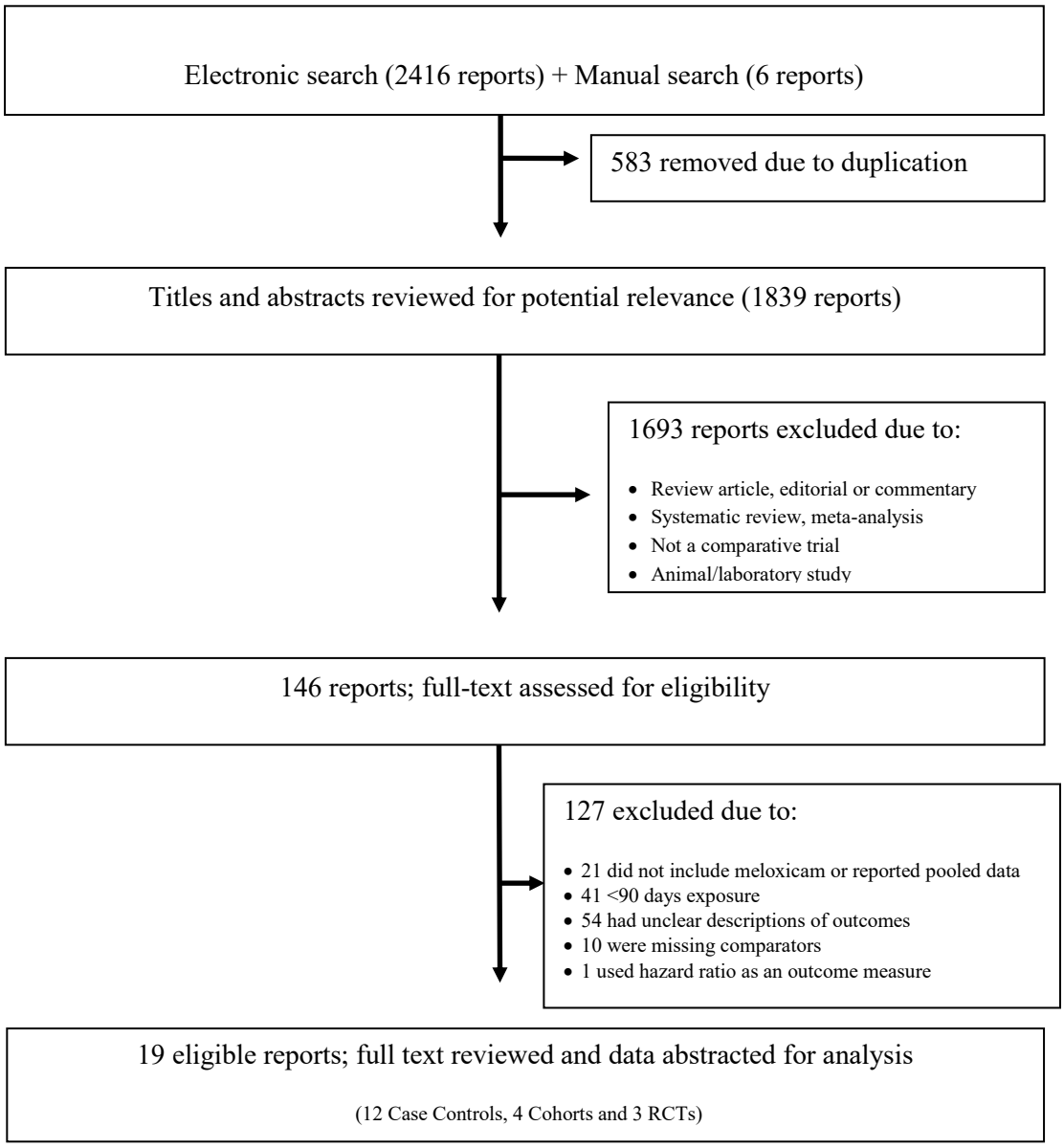


Figure 7: Flow diagram for the selection of randomized controlled trials, cohort or case-control studies reporting meloxicam use.

4.2.3. Data analysis and outcome measures

Upon reviewing, we categorized the reported outcomes in the eligible studies according to the ICD-10 classification [245]. To avoid the possibility of double counting of data, from each study, we used the World Health Organization's Adverse Reaction Terminology (WHO-ART) to prioritize the outcomes based on severity; only the data reflecting for the top outcome was used [246]. Fully adjusted relative risk (RR) and odds ratios (OR) with a 95% confidence interval (CI) were extracted for each drug and for each outcome. The combined risk estimates (OR') were calculated using inverse variance weighted method and utilizing Cochrane recommended Review Manager® software (The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) using relative risk (RR) and odds ratios (OR) with 95 % confidence interval (CI) for each CV outcome [247]. All 19 studies [136, 211, 235, 240, 243, 248-261] included in our review were adjusted for common variables such as age, sex, comorbidities and concomitant medication, other variables were also recorded here as study characteristics [Table 6].

For primary analyses, the data from various studies were pooled together to estimate the risk for myocardial, vascular and renal outcomes categories separately as well as all pooled together to calculate the overall risk estimate (i.e., composite outcome). For the secondary analysis, however, due to the scarcity of reports, we only tested the possibility of changes in the all composite risks, and analyzed the data for the effect of the dose, underlying inflammation, concomitant use of aspirin and the type of control (placebo vs other NSAID).

4.2.4. Heterogeneity

The heterogeneity in analysis caused by variation in outcomes across the studies and between the outcomes was determined using I_2 statistics, [Table 8] using published criteria (0-40%: not significant, 30-60%: moderate heterogeneity, 50-90%: substantial heterogeneity, and 75-100%: considerable heterogeneity) [262]. The methodological quality of the studies included in our analysis was tested using the Newcastle-Ottawa scale method. The case control studies scored 7-8 out of a total 9 scores. The cohort and RCTs scored 6 to 7 out of a total 10 scores, so met the minimum standards of quality [263].

4.3. Results

As depicted in [Figure 7] we initially identified 2,422 studies out of which 146 potentially relevant reports were extracted and read in full text. Of those, we excluded 21 as they did not

include meloxicam or the drug was grouped with other NSAIDs; 41 were excluded as their exposure time was not clearly defined; 54 were excluded as they did not report the outcomes of interest; 10 studies were excluded for being non-comparative trials; one study was excluded because of the use of hazard ratio only [264] that cannot be compared directly with the studies reporting OR, RR. Finally, 19 studies [136, 211, 235, 240, 243, 248-261] were found eligible and meeting all of the inclusion/exclusion criteria and had meloxicam in their comparisons. The characteristics of the 19 studies and the patients involved are listed here, [Table 6 and 7], respectively. For some observations we found only limited studies having meloxicam in their comparisons. Hence, our estimated risks for some observations may have low statistical power [Table 8].

4.3.1. Primary analysis

According to the ICD-10 classification system [245], the outcomes were defined as myocardial (codes, I20-25, I46-52), vascular (codes I60-89, I96-99, I74) and renal (codes, I10-15, N00-N29, S37, E87). OR' values were calculated for each of these categories. For the composite OR' calculation the OR for all three categories were combined.

4.3.1.1. Myocardial outcomes

Five studies [136, 235, 240, 248, 249] that reported >90 days meloxicam exposure included data on the myocardial risk. The results [Table 8] suggest that >90 days exposure to meloxicam and naproxen is not associated with any increased risk of myocardial outcomes, while rofecoxib and diclofenac do increase such risk. For other examined NSAIDs, the number of eligible reports was insufficient for conclusive results.

4.3.1.2. Vascular outcomes

We found seven studies [211, 250-255] that reported the effect of meloxicam exposure of any duration on the vascular system. Meloxicam was found to exhibit an elevated OR'. Data on rofecoxib were inconclusive as we found only one eligible study reporting vascular risk. Other NSAIDs also elevated OR' in the following order, naproxen, diclofenac, ibuprofen, indomethacin and celecoxib. We found no eligible study with report of vascular outcomes for etodolac [Table 8].

4.3.1.3. Renal outcomes

Seven studies [243, 249, 256, 257, 259-261] were found reporting meloxicam exposure of any duration and its effect on renal system [Table 8]. Meloxicam was found with no increase in renal risk OR', neither did ibuprofen. However, the use of all other NSAIDs resulted in increased risk of renal incidences. For etodolac, however, only one eligible report was found that was suggestive of no increased risk.

4.3.1.4. Composite CV/renal risk

Composite risk was calculated by combining all the risk estimates for CV/renal outcomes reported in the 19 eligible studies [136, 211, 235, 240, 243, 248-261]. Meloxicam was found to elevate the composite odds ratio [Table 8]. Likewise increased OR' values were found for other NSAIDs with the following rank order: rofecoxib> diclofenac> indomethacin> celecoxib> meloxicam> naproxen> ibuprofen. Only etodolac was not associated with elevated composite risk, however, OR' was calculated based on two studies only.

4.3.1.5. All-cause mortality

We found only one eligible study [136] assessing all-cause mortality for meloxicam. However, with that limited data meloxicam ibuprofen, naproxen and diclofenac were associated with no risk of death. For rofecoxib, celecoxib, etodolac or indomethacin, there was no eligible report that met our inclusion criteria.

Table 6: Characteristic of the studies included in the final analysis (n=19)

Reference	Type of study	Exposure definition	Outcome reported (ICD-10 disease classification)	Covariates adjusted
Garcia et. al., (2008)	Case Control	***Past Use***: Single NSAIDs use without switching. Mean exposure ended between 91-365 days before the index date. Why are some of these punctuated and some not? Be consistent.	Myocardial infarction (I21, I22)	Age, sex, race, calendar year, body mass index, prior hospitalization, smoking, pre-existing diseases and concomitant drug use [Table 7].
Singh *et al.,*(2004)	Case Control	24,196 patients from 28 trials, who had been followed for 33-180 days	Cardiovascular thromboembolic complications (I24.0)	None
van Staa et al.(2008)	Case Control	Current exposure period from the date of an NSAID prescription to the end of expected duration plus 3 months	Myocardial infarction (I21, I22)	Age, sex, race, calendar year, body mass index, smoking, alcohol use, socioeconomic status, pre-existing diseases and concomitant drug use [Table 7].
Schneider *et al.,*(2006)	Case Control	Current new use in the 30 days before the index date.	Acute renal failure (N17.9)	Age, sex, hospitalization, aged care status, pre-existing diseases and concomitant drug use [Table 7].
Mangoni *et al.,* (2010)	Case Control	NSAIDs' exposure over the 2-year period before the index date (20+ supplies of NSAID within the past 2 years)	All-cause mortality (R96, R99, I46)	Age, prior-hospitalization, aged care status, pre-existing diseases and concomitant drug use [Table 7].
Jick *et al.,*(2000)	Case Control	Patients were exposed to one of the study drugs within 90 days before the index date	Myocardial infarction (I21, I22)	NR
Chang *et al.,*(2010)	Case Control	Case period as 1 to 30 days before the index date and control period as 91 to 120 days before the index date	Stroke (I64)	Pre-existing diseases [Table 7].
Biere-Rafi *et al.,*(2011)	Case Control	Chronic use (from to 365 days), and long-term use (more than 1year)	Pulmonary embolism(I26)	None
Mangoni *et al.,* (2010)	Case Control	NSAIDs' exposure over the 2-year period before the index date (20+ supplies of NSAID within the past 2 years)	Stroke (I64)	Age, prior hospitalization, aged care status, pre-existing diseases and concomitant drug use [Table 7].
Lafrance *et al.,*(2012)	Case Control	Single NSAID exposure (date of prescription+ No of days supplied+30 days)	Risk of hyperkaliemia (E87.5)	Age, sex, race, hospitalization, pre-existing diseases and concomitant drug use [Table 7].
Huerta *et al.,*(2005)	Case Control	Most recent prescription ended within 30 days (current) before index date.	Renal failure (N1-N19)	Age, sex, race, calendar year, body mass index, prior-hospitalization, pre-existing diseases and concomitant drug use [Table 7].
Lapeyre-Mestre *et al.,*(2013)	Case Control	Any exposure	Renal complications (N10-N16) Altered renal function (N25.9)	Age, sex
Layton *et al.,*(2003)	Cohort	Prescriptions written by physician spell on first ref. in England for meloxicam (1996-1997) and celecoxib (2000) months after starting treatment	CV thromboembolic events (I24.0)	Age, sex

Table 6: Continued.

Reference	Type of study	Exposure definition	Outcome reported (ICD-10 disease classification)	Covariates adjusted
Layton *et al.,*(2003)	Cohort	Prescriptions written by GPs in England for meloxicam (1996-1997) and rofecoxib (1999).	CV thromboembolic events (I24.0)	Age, sex.
Varga *et al.,*(2013)	Cohort	All 428 patients using NSAIDs for a minimum of four consecutive days were enrolled.	CV thrombotic events (I24.0)	None
Winkelmayer *et al.,*(2008)	Cohort	Patients were followed until 45 days after the index date.	Acute kidney injury (S37.0)	Multiple comparisons
Shi *et al.,*(2004)	RCT	Randomized treatment allocation.	Renal adverse drug reaction (increase in urinary protein and edema) (N00-N29, E87)	None
Arvind *et al.,*(2004)	RCT	Randomized treatment allocation.	Renal adverse drug reaction (N00-N29, E87)	None
Hosie *et al.,*(1996)	RCT	Following a washout period of three days, patients were randomly assigned to the treatment.	CV adverse drug reaction (I46-I51)	None

Table 7: Characteristics of the patients in the studies included in final analysis

Reference	No. of Subjects		Age (years)	% Male	Concomitant drug use	Existing Diseases
	Cohort	Case/Control	Mean± SD			
Garcia *et al.,* (2008)	-	8852/ 20000	50-84	NR	Oral-anticoagulants, anti-hypertensives, anti-hyperlipidemic drugs, oral steroids, statins and aspirin.	Rheumatoid arthritis, osteoarthritis, diabetes, anemia, hypertension, hyperlipidemia, obesity, and coronary artery disease.
Singh *et al.,* (2004)	-	18 / 8667	18-65>	32.2	Non-selective NSAIDs	Gastric ulcer
van Staa *et al.,*.(2008)	-	729294 /443047	58±0.2	46	Anti-coagulants, anti-hypertensives, oral steroids, cardiac glycosides, diuretics, statins and aspirin.	Diabetes, cardiovascular diseases
Schneider *et al.,* .(2006)	-	4228 / 84540	78 ± 5.7	Case (46.1) Control (32.3)	Oral anticoagulants, corticosteroids, aspirin psychotropic drugs, thyroid drugs, nephrotoxic drugs and radio-contrast media.	Diabetes, hypertension, cardiovascular diseases, atherosclerosis, hyperlipidemia, respiratory diseases, GIT disease, peptic ulcer, renal failure, cancer, and malignancy related to kidney disease
Mangoni *et al.,* .(2010)	-	83623/ 1662099	81 ± 5	68	Antidiabetics, anticoagulant, anti-hyperlipidemic drugs, cardiovascular medication, dementia, respiratory medication, and aspirin.	Rheumatoid arthritis, diabetes, obesity, hypertension, ischemic heart disease, renal failure, respiratory diseases, and liver disease
Jick *et al.,*.(2000)	-	19/61	NR	NR	NR	NR
Hosie *et al.,* (1996)	336	--	64±12	68	Non-selective NSAIDs	osteoarthritis,
Chang *et al.,* (2010)	-	16251 / 28424	68.8	54	Anti-diabetics, anti-coagulants, insulin, anti-hyperlipidemic drugs, anti-hypertensives, cardiovascular medication,	Rheumatoid arthritis, osteoarthritis, diabetes, anemia, hypertension, hyperlipidemia, atrial fibrillation, renal failure, liver disease, ulcer,
Biere-Rafi *et al.,*.(2011)	-	4433 / 16802	60	43	Anticoagulant, antiplatelet drugs, antihypertensive, antidiabetic	Acute infection, inflammatory bowel disease, surgery, congestive heart failure and cancer
Mangoni *et al.,* (2010)	-	6624/ 132150	81 ± 5	65	Antidiabetics, anticoagulant, anti-obesity, cardiovascular medication, dementia, respiratory medication and aspirin	Rheumatoid arthritis, diabetes, obesity, hypertension, ischemic heart disease, renal failure, respiratory diseases, liver disease and dementia

Table 7 continued.

Reference	No. of Subjects		Age (years) Mean± SD	% Male	Concomitant drug use	Existing Diseases
	Cohort	Case/Control				
Layton *et al.,* (2003)	36545	--	<39-80	33	Aspirin, or other anticoagulant/antiplatelet agents (only reported for 51 cases)	Osteoarthritis, ischemic heart disease, thromboembolism (only reported for 51 cases)
Layton *et al.,* (2003)	34355	--	<39-80	33	Aspirin or other anticoagulant or antiplatelet agents (reported for 74 cases)	Osteoarthritis, ischemic heart disease, thromboembolism (Only reported for 74 cases)
Varga et al.(2013)	428	-	63.2±16.4	40	NR	Diabetes, hypertension, congestive heart failure, ischemic heart disease,, stroke, renal failure , thromboembolism
Lafrance *et al.,* (2012)	-	18326/ 355106	Case: 67.1± 12.1 Control: 66.8± 12.0	Case:(97.6) Control: (98.8)	Oral anticoagulant, antiplatelet drugs, cardiovascular medications, corticosteroids, nephrotoxic drug, dietary supplement (potassium)	OA, RA, diabetes, hypertension, cardiovascular diseases, hyperlipidemia, hyperkalemia, renal failure, respiratory diseases, cancer, peptic ulcer,, liver disease, musculoskeletal disorder, mental
Winkelmayer *et al.,* (2008)	183446	-	78.0	30	Diuretics , cardiovascular medications and radio-contrast media	Diabetes, hypertension, kidney disorder, ischemic heart disease liver disease, gout and cancer
Huerta *et al.,* (2005)	-	103/ 5000	50-84	NR	Anti-diabetics and anti-hypertensives	Diabetes, cardiovascular diseases
Shi *et al.,* (2004)	461	--	46.9 ±14.4	37 ± 13	Methotrexate, folic acid tablets, focal intra-articular injection	Rheumatoid arthritis
Arvind *et al.,* (2004)	254	-	43.4 ± 12.7	9	NR	Rheumatoid arthritis, osteoarthritis,
Lapeyre *et al.,*(2013)	-	169/ 38506	40	NR	NR	NR

NR= Data not reported. SD= standard deviation

Table 8: Increased risks (OR') of cardio-renal outcomes associated with the use of NSAIDs in the 19 studies

Comparator	RCTs & Cohort		Case Control		Increased risks OR' (95% CI)				
	Total studies	# of patient (events/total)	Total studies	# of patient (cases/controls)	Myocardial	Vascular	Renal	Composite	I2 (%)
Reference	0	0 / 0	9	73891 / 1314567	1	1	1	1	n/a
Meloxicam	9	261/123,009	10	928 / 8746	1.13 (0.98, 1.32) n= 5	1.35 (1.18, 1.55) n= 7	0.99 (0.72, 1.35) n= 7	1.14 (1.04, 1.25) n= 19	62
Rofecoxib	4	475/82,722	3	470 / 5397	1.46 (1.10, 1.93) n= 3	1.38 (0.71, 2.68) n= 1	1.51 (1.35, 1.68) n= 3	1.50 (1.36, 1.66) n= 7	61
Celecoxib	5	549/112,701	5	657 / 5680	1.33 (1.00, 1.77) n= 2	1.20 (1.01, 1.43) n= 4	1.31 (1.12, 1.52) n= 4	1.27 (1.14, 1.41) n= 10	25
Ibuprofen	2	89 /17,816	8	2634 / 23102	1.03 (0.95, 1.11) n= 2	1.50 (1.36, 1.65) n= 5	1.00 (0.93, 1.08)` n= 3	1.10 (1.05, 1.15) n= 10	89
Naproxen	2	63/15,452	12	1766 / 22267	1.10 (0.98, 1.23) n= 4	1.68 (1.44, 1.97) n= 5	1.13 (1.04, 1.22) n= 5	1.18 (1.11, 1.25) n= 14	77
Diclofenac	6	37/6,892	11	4134 / 19842	1.34 (1.24, 1.44) n= 5	1.67 (1.56, 1.78) n= 6	1.42 (1.24, 1.61) n= 6	1.47 (1.40, 1.53) n= 17	91
Etodolac	0	0 / 0	2	385 / 7536	1.32 (0.69, 2.51) n= 1	n/a n= 0	1.07 (0.95, 1.21) n= 1	1.08 (0.96, 1.21) n= 2	0
Indomethacin	2	58/6,099	4	454 / 2755	1.47 (0.90, 2.40) n= 1	1.27 (1.03, 1.56) n= 3	1.55 (1.35, 1.78) n= 2	1.46 (1.31, 1.64) n= 6	65

n= number of eligible reports used to calculate odds ratio , n/a = not applicable, reference= Placebo or non-NSAID drug

Table 9: The effect of dose, disease, aspirin use and type of comparator (placebo or other NSAIDs) on the composite CV/renal risks.

Comparator	Dose[a]		Disease		Aspirin use		Type of Comparator	
	Low	High	Adjusted	Not adjusted	Adjusted	Not adjusted	Non-user[b]	Other NSAIDs[c]
Reference	1	1	1	1	1	1	1	1
Meloxicam	1.15 (0.98, 1.35) n= 4	1.11 (0.98, 1.25) n= 6	0.94 (0.81, 1.08) n= 4	1.34 (1.18, 1.52) n= 7	1.16 (1.05, 1.27) n= 8	1.19 (0.70, 2.05) n= 6	0.99 (0.87, 1.13) n= 7	0.99 (0.61, 1.62) n= 5
Rofecoxib	1.63 (1.33, 2.00) n= 2	6.63 (4.10, 10.72) n= 2	1.37 (1.18, 1.60) n= 1	1.46 (1.26, 1.70) n= 2	1.82 (1.49, 2.22) n= 3	1.41 (1.25, 1.58) n= 2	1.52 (1.35, 1.72) n= 3	1.50 (1.27, 1.76) n= 2
Celecoxib	1.34 (1.08, 1.67) n= 2	1.86 (1.27, 2.73) n= 3	1.23 (1.03, 1.47) n= 1	1.24 (1.06, 1.45) n= 4	1.26 (1.13, 1.41) n= 5	1.52 (1.09, 2.12) n= 3	1.31 (1.14, 1.50) n= 3	1.67 (1.26, 2.21) n= 3
Ibuprofen	1.03 (0.92, 1.17) n= 2	1.72 (1.14, 2.61) n= 2	0.98 (0.92, 1.03) n= 4	1.41 (1.30, 1.54) n= 4	1.08 (1.03, 1.13) n= 7	1.34 (0.94, 1.91) n= 3	0.98 (0.93, 1.04) n= 6	1.69 (1.35, 2.11) n= 1
Naproxen	0.90 (0.88, 0.93) n= 3	1.44 (1.07, 1.93) n= 4	1.09 (1.02, 1.17) n= 4	1.35 (1.18, 1.54) n= 5	1.18 (1.11, 1.25) n= 8	1.40 (0.89, 2.20) n= 3	1.11 (1.04, 1.18) n= 7	1.30 (1.00, 1.69) n= 1
Diclofenac	1.17 (1.08, 1.26) n= 3	1.83 (1.56, 2.16) n= 5	1.21 (1.13, 1.30) n= 4	1.56 (1.47, 1.66) n= 7	1.46 (1.40, 1.53) n= 7	2.09 (1.69, 2.58) n= 4	1.29 (1.21, 1.38) n= 6	1.07 (0.75, 1.52) n= 3
Etodolac	n/a n= 0	n/a n= 0	1.07 (0.95, 1.21) n= 1	1.57 (0.76, 3.25) n= 1	1.07 (0.95, 1.21) n= 1	n/a n= 0	1.08 (0.96, 1.21) n= 2	n/a n= 0
Indomethacin	1.28 (1.14, 1.44) n= 2	1.27 (1.13, 1.43) n= 2	1.36 (1.15, 1.60) n= 1	1.54 (1.32, 1.81) n= 3	1.33 (1.17, 1.51) n= 3	0.38 (0.04, 3.32) n= 1	1.37 (1.17, 1.60) n= 2	2.15 (1.66, 2.78) n= 1

a, Meloxicam, 7.5 vs 15 mg; rofecoxib, <25 vs >25 mg; celecoxib, 200 vs 400 mg; ibuprofen <=1200 vs 1200-2400 mg; naproxen, 750 vs => 1000 mg; diclofenac, 100 vs 150-300 mg; indomethacin, 75 vs 100-200 mg. b, All case control. c, All RCTs and cohorts, 'n' indicates the number of eligible reports used to calculate OR, n/a = data not available

4.3.2. Secondary Analysis

4.3.2.1. Effect of dose

There were 7 eligible studies [211, 235, 240, 243, 249, 259, 260] reporting information on the size of the meloxicam dose and accordingly its effect on CV/renal outcomes. Meloxicam was found to increase the CV/renal risk neither at lower nor at higher doses [Table 9]. For other NSAIDs, however, in general, OR' was increased with dose elevation. For example rofecoxib showed a striking dose-OR' dependency with many fold increase in CV/renal risk at higher doses. Likewise, the risk associated with diclofenac was found to be dose dependent. For ibuprofen and naproxen the composite CV risk did not increase with the use of low but substantially rose after high doses. There was a trend toward elevated OR' of celecoxib and indomethacin, however, CI of the two dose ranges overlapped. No data on dose effect were found for etodolac in the eligible studies.

4.3.2.2. Effect of disease

There were 11 eligible studies [136, 211, 235, 240, 250, 252, 256-260] reporting the underlying inflammatory diseases and its effect on CV/renal outcomes. In general, the composite OR' values were reduced when adjustments were made for the diseases [Table 9]. Only for meloxicam and ibuprofen CV/renal risk rather diminished after adjustment. In case of etodolac the data was inconclusive due to the scarcity of reports.

4.3.2.3. Effect of aspirin use

There were 13 eligible studies reporting concomitant use of aspirin [136, 235, 240, 243, 250-256, 258, 261] and its effect on CV/renal outcomes [Table 9]. In general, the use of aspirin resulted in widening of the 95 % CI around the calculated composite CV/renal risk estimates. This observed increased variability resulted in diminished elevated composite risk of meloxicam, ibuprofen and naproxen, rendering it insignificant; however, the estimates remained higher. For other NSAIDs, our observations were inconclusive due to the scarcity of eligible reports.

4.3.2.4. Effect of type of comparison

We found 12 eligible studies [136, 235, 240, 243, 249, 252-254, 256-258, 260] providing sufficient information on the CV/renal risk of meloxicam as compared to a placebo or other NSAIDs. All reports that used a placebo as the comparator were case-control studies; hence, the

placebo groups were only the nonusers of NSAIDs [Table 9]. All of the patients included in the studies were given an NSAID as comparator (meloxicam vs diclofenac, naproxen or celecoxib) and had underlying inflammation.

The OR' values calculated for meloxicam were not elevated when tested versus either non-users or users of other NSAIDs. Whereas, the OR' values for rofecoxib and celecoxib were elevated when compared with both users and non-users of other NSAIDs. There was no risk associated with ibuprofen in the non-users, but, OR' was elevated when compared to other NSAIDs. On the other hand, OR' values for diclofenac was elevated when tested versus non-users but not when compared to other NSAIDs. Overall, the decision to make the analysis based on a comparison with placebo or another NSAID as comparator significantly influenced the calculation of CV/renal risks [Table 9].

4.4. Discussion

Many meta-analyses and systematic reviews have concluded that NSAIDs increase CV/renal risks. However, uncertainty remains about the nature of such risks as well as the relative safety of NSAIDs [139, 152, 155, 204-207].

Our analysis agrees with the limited number of studies that have looked for, and found, that NSAIDs are not only heterogeneous in their extent of CV/renal risks but also differ in the nature of their adverse effects on different body systems. For example, a systematic review and meta-analysis of CV risk in celecoxib users found that the risk of cerebrovascular thrombotic events (OR' 1.0, 95% CI; 0.51–1.84) was different from the risk of MI events (OR' 2.26, 95% CI; 1.0-5.1) in a total of 12,780 patients included in 6 RCTs [206].Likewise, for rofecoxib the risk of MI events (RR, 2.24; 95% CI 1.24–4.02) was different from the risk of stroke RR (1.02, 95% CI; 0.54–1.93) [210]. Similarly, rofecoxib is reported to adversely affect myocardial [210], vascular [253, 254] and renal systems [265], but naproxen is reported to cause no significant myocardial risk [207].

Here in our analysis the data point to meloxicam as having limited CV/renal risks associated to its use. Further analysis of data for myocardial, vascular and renal risk revealed that meloxicam is devoid of risks categorized under myocardial and renal incidences, but poses a significant risk of vascular incidences only [201, 235, 266-268] The inability of meloxicam to increase renal risks may be attributed, among other plausible mechanisms, to its limited

distribution into the kidneys as has been reported in experimental animals [141]. It is plausible that a sufficient local presence of the drug is needed to inhibit prostaglandin synthesis and cause renal complications[123]. Recently, it has been reported that meloxicam restores the doxorubicin-induced suppressed cardiac antioxidant enzyme activities in mice [269]. Such mechanisms are in line with the lack of increased myocardial risk of meloxicam.

The relatively better overall safety profile for meloxicam compared to those reported for various NSAIDs has repeatedly been cited in the literature [152, 204, 211, 248]. Nevertheless, long term use of meloxicam (>90 days) does carry some CV/renal risks [241, 242]. However, the data presented herein suggest, for the first time, that the mechanism behind the elevated risk for meloxicam use is mainly at the vascular level with no measurable effect on the myocardial and renal systems. Similarly, Kearney et al. have reported that selective COX-2 inhibitors increase the risk of major vascular events by threefold but elevate the myocardial events by only three quarters [208]. However, the authors pooled data generated following the use of all COX-2-selective NSAIDs assuming an across the class side effect profile.

It is important to mention that the vascular risks of some NSAIDs may be controlled with low dose aspirin [270] to counter the thrombogenic side effects of the treatment [238], albeit, may add other risks such as bleeding and gastrointestinal complications. On the contrary, there is no well-developed therapeutic management strategy to minimize the myocardial and renal side effects of NSAIDs. The use of misoprostol has been suggested to control the renal side effect of drugs but the strategy has not been adopted in practice [123, 271].

Aspirin is known for its cardioprotective effects. At low doses, it reduces the incidence of myocardial infarction and stroke by approximately one quarter in NSAIDs users [238, 270, 272]. This, however, appears not to be a class effect since by replacing aspirin from its receptors on platelet, some NSAIDs (e.g., ibuprofen, indomethacin and naproxen) abolish the cardioprotective properties of the drug. Such an interaction does not take place for meloxicam [273], sulindac and celecoxib [274]. The concomitant use of ASA has a negligible effect on the already small increased risk of meloxicam use [Table 9]. For rofecoxib, we found only two eligible reports but it has been reported that ASA reduces the CV complications associated with the drug [154]. The lack of class effect may explain, at least, in part our observation that the adjustment for the ASA use does not influence the CV risks in the same direction for all examined NSAIDs [Table 9].

Our analysis highlights the problem of pooling data without considering the heterogeneous nature of the outcomes and the NSAIDs used. NSAIDs appear to cause CV/renal risks of different intensity independent of their COX-2 selectivity [201]. Many investigators have studied the CV/renal risk of NSAIDs, comparing users versus non-users of the drugs [205, 207]. This approach undermines the potential effect of the disease, since the users, and not the non-users, are likely taking the drugs to treat an inflammatory condition. It is well established that inflammation increases the risk of CV incidents [201, 202]. Rheumatoid arthritis (RA) is an inflammatory disorder well-documented adverse CV [201, 202] and renal [203] effects that contribute to the chances of increased CV/renal risks. Interestingly, however, the results of a recent longitudinal suggests that the risk of major CV complications in newly diagnosed RA patients under therapy with NSAIDs, but with no history of stroke or MI, is lower than or equal to that of controls [275]. The authors explain the discrepancy between their results and those reported previously to the fact that they compared their outcomes between patients who were taking the drug and the general population. Another plausible explanation may be the inflammatory status of the patients (i.e., active disease *vs* remission due to therapy) [276]. It has been shown that the response to calcium channel blockers as a reflection of the cardiac status is influenced by the severity of the inflammatory condition [57]. Nevertheless, a differentiation between the risks caused by the underlying disease from that associated with the use of the drug is impossible unless adjustment is made to account for the contribution of each covariate. In our analysis, adjusting for underlying disease resulted in the loss of the risks for meloxicam. This suggests that the little risk observed in meloxicam users may be attributable to the underlying inflammation rather than meloxicam itself. Our limited data on ibuprofen suggest the same as well.

Some studies have suggested that CV/renal risk of NSAIDs are dose dependent [235, 277]. Indeed, Moore et al. found no CV risks associated with over-the-counter doses of ibuprofen and diclofenac [278]. Vioxx Gastrointestinal Outcome Research (VIGOR) trial reported a five times increase in CV risk of 50 mg rofecoxib versus 500 mg naproxen [131]. This lead to the withdrawal of rofecoxib from the market, owing to its higher potential to cause CV incident [129]. Later, the results of Adenomatous Poly Prevention on Vioxx (APPROVe) trial that compared 25 mg rofecoxib with placebo reported only a twofold increase [279]. This difference in the magnitude of the risk was attributed to the changes in the size of the rofecoxib dose as well as the use of the comparator, i.e., naproxen in one and placebo in the other. A

similar observation has been made for celecoxib [208]. For meloxicam, no such dose dependency was noticed in determining its risk profile, due, perhaps, to its low magnitude of CV/renal risks.

Another complicating issue is the use of an NSAID as a reference. Ideally, the choice of comparator should be made to assess the therapeutic advantage of alternative drugs. In practice, however, a comparator is used either to test the relative outcome or to favor a particular event. The outcomes of VIGOR and APPROVe trials are examples of such discrepancies [208, 280]. For example in VIGOR trial diclofenac was used to compare CV risk of rofecoxib, but diclofenac itself is associated with substantial increase in CV risks, thus when used as reference in a study can diminish the CV risk of the comparator (RR, 0.92; CI, 0.81-1.05) [280]. When in subsequent assessments diclofenac that is by itself associated with substantial increased CV risks is used as the comparator, the risk of vascular events in the NSAIDs users diminishes (RR 0.92; CI 0.81–1.05)[274].However, when naproxen was used as comparator and rather safe NSAID, the vascular risk rises (RR 1.57; CI, 1.21-2.03) [208]. Similarly, Chen *et al.,* have reported a difference in the outcome measures with different type of comparators [155]. It is therefore reasonable to criticize those systematic reviews that have made their conclusions by pooling all the risks together ignoring the effect of comparator used in different trials [152, 155, 204, 280].

For meloxicam, the composite CV/renal risk is negligible regardless of if being compared with a placebo or other NSAIDs and adjusted for the underlined disease. For other NSAIDs, however, the OR' varied depending on the type of comparator used [Table 9]. Recently, not surprisingly, the CV risk of celecoxib has been found to be equal to that of other NSAIDs in patients with RA or osteoarthritis [281]. This highlights the importance of considering the type of comparator in assessing the CV risk of NSAIDs. Table 9 also depicts how the type of the study affects the outcomes: for example, randomized controlled trials (RCT) may include patients treated with a placebo, while other observational studies may include healthy subjects (controls) or the users of other NSAIDs (cohorts) as comparators. This difference in the study type may also influence the measured increased risk.

Patients with inflammatory conditions that need anti-inflammatory treatment are warned of increased risks of cardio-renal side effects of NSAIDs. This places the patient and the caregiver in a dilemma as the decisions are limited either to avoid using the drugs to treat

inflammation or to risk the CV adverse outcomes. However, the available reports on the toxicity of NSAIDs often ignore the origin of the cardio-renal side effects, the CV effects of the underlying disease, the dose dependency of the out-comes, and the concomitant use of ASA. It is also important to note that not all NSAIDs are equally harmful, particularly if they used in low therapeutic doses [201], or with low dose ASA. In addition, the increased risk associated with the use of some NSAIDs, although statistically established, may have limited therapeutic relevance due to the low calculated risk values[201, 275]. Nevertheless, the possibility of CV side effects cannot be put aside when patients are under therapy with NSAIDs as the US FDA has recently emphasized [239, 282]. Hence, careful monitoring of long-time NSAIDs users particularly those with compromised CV function [283] and reduced renal function and elderly population is necessary. In patients with very low kidney function, topical NSAIDs may be considered for their low systemic exposure [284].

Our analysis of the available data suggests that meloxicam, a generic, therefore, cost effective NSAID, is associated with lower CV and renal risk than what has been reported for other drugs of the class. Etodolac can also be considered as a relatively safe NSAID but the limited available data prevent us from making an unequivocal claim. Both meloxicam and etodolac are COX-2 selective[201]; hence, are expected to be less harmful to the gastrointestinal tract as well. Nevertheless, patients must be advised and monitored for the possibility of gastrointestinal complications particularly those on concomitant ASA use. Indeed, relatively recent data suggest that the use of low dose ASA is associated with rare but sever intestinal side effects that may be attributed to the enteric-coated nature of the available formulations [201].

Further clinical trials on these drugs are timely. How-ever, since the original sponsors of these NSAIDs have lost their market exclusivity, they are unlikely to finance further clinical trials. Nevertheless, the public will greatly benefit from further studies that focus on the long-term use of these available generic drugs.

This study is not without shortcomings. Other than meloxicam, we were able to include only limited data on some NSAIDs because of the scarcity of eligible reports. To highlight this point, the number of studies used in our analysis for individual NSAIDs are listed in the [Tables 6]. Second, the focus of the most studies that we included in our analysis was not the CV and/or renal risks as they reported the latter as secondary outcomes. This might have added to the

heterogeneity of our analysis. Last, the studies included in our analysis were heterogeneous as some reported RR while others did odds ratio. Even though there have been published methods stating that one can use the inverse variance random effect method to combine all these outcomes [285], still it causes heterogeneity as observed in our analysis. The heterogeneity of the type of studies that we pooled to calculate the increased risk values is another point to consider. Indeed such heterogeneity may result in increased variability in the risk estimates and, consequently, mask the outcome.

4.5. Conclusion

NSAIDs are heterogeneous in causing CV/renal risks in terms of both extent and the nature of their adverse effects. Meloxicam causes limited risk and its side effects are mainly of vascular in nature, hence, clinically manageable. The usefulness of the available information on the relative CV/ renal risks of the NSAIDs may be questioned for not considering various important covariates such as the underlying disease, does dependency, ASA use and the type of comparator. Further clinical trials on the safety of meloxicam are desired to ascertain the exact nature of its CV risk

Chapter 5

5. Comparison of tissue accumulation of NSAIDs in the heart and kidneys of adjuvant arthritis rats

5.1. Introduction

The non-steroidal anti-inflammatory drugs (NSAIDs) are a diverse group of compounds used for the treatment of inflammatory disorders. NSAIDs are rapidly and completely absorbed after oral administration and are highly protein binding (>90%) with smaller volume of distribution. NSAIDs are primarily metabolized in the liver. The elimination half-lives of NSAIDs vary from 0.25 to 70 h, [107]. NSAIDs are categorized as low extraction ratio drugs and their metabolism is dependent on the availability of the plasma free fraction and intrinsic metabolizing capacity of hepatic metabolizing enzymes [286]. Because only the free fraction is available for metabolism, any condition (e.g., inflammatory conditions, drug interactions) that can alter protein binding can affect disposition of NSAIDs in the body. Some NSAIDs are excreted as conjugates through biliary excretion and are reabsorbed in the small intestine thus exhibiting double peak phenomena (e.g., rofecoxib) [287].

The category of NSAIDs includes members of various chemical classes. Although chemically different they share most of their pharmacological properties. However, differences exist in their safety and toxicity profiles. Many theories have been proposed to explain the apparent difference in CV/ renal safety of NSAIDs [107]. But none of these is conclusive.

Previously in experimental animals, it was reported that rofecoxib and celecoxib have a higher kidney-to-plasma concentration ratio compared to meloxicam. This correlates well with the reduction in urinary excretions of sodium and potassium in rofecoxib and celecoxib treatments. Which was not observed in meloxicam treated rats [141]. Interestingly, systematic reviews of randomized clinical trials also suggested a more favorable CV safety profile for meloxicam than for other NSAIDs [152, 204, 211, 241, 242, 248]. The physiochemical properties and plasma protein binding are discussed in literature as the rate limiting step for NSAIDs disposition [288]. This emphasizes the importance of the systemic tissue-drug exposure in determining the CV/renal risk of NSAIDs [289].

The relationship between NSAIDs systemic exposure and their adverse effects can also be explained by their half maximal inhibitory concentration (IC50) for COX inhibition.

Prostaglandins products of COX-2 enzyme are not only involved in development of inflammation in the inflamed areas, but are also involved in constitutively maintaining the tissue homeostasis to preserve the function of healthy tissue. Prostaglandins are important endogenous mediators of peripheral vascular and local renal as well as coronary systems. Experimental studies have demonstrated that low perfusion of renal and coronary tissues stimulate the prostaglandin release which in turn decreases vascular resistance, increases blood flow and provide ischemic protection to the tissues [290]. In the kidneys, prostaglandins are also involved in salt excretion, volume control and renin excretion. Inhibition of renal prostaglandin synthesis is reported to increase salt reabsorption, reduced urine volume and hypertension [291]. Similar implications of inhibition of prostaglandin synthesis are suggested to occur in the lungs, brain and other tissues [292]. NSAIDs exert their pharmacological effects by blocking the prostaglandin synthesis both in the inflamed area and in healthy tissue. But for therapeutic purposes it's the NSAIDs concentration at the site of action that is important. In rheumatic diseases, probably the major site of action for NSAIDs is synovium. NSAIDs can reach the synovial fluid concentrations high enough to meet the IC50 for COX-2 inhibition [293]. Although, human data on NSAIDs concentration in the tissues are lacking, however, in an animal study it was reported that even though the concentration of meloxicam in the kidney tissues is higher than rofecoxib and celecoxib [141, 289]. Yet it did not alter the renal function accordingly the electrolyte excretion in urine. This may be because of higher IC50 for COX-2 inhibition for meloxicam. In literature, the IC50 values for COX-2 inhibition by meloxicam, rofecoxib and celecoxib are reported to be 2.1, 0.84 and 0.83 mmol/L, respectively. It was concluded in that meloxicam tends to stay in the blood (low tissue over plasma concertation ratio). But it also requires a much higher concentration, is the kidney tissues to inhibit local prostaglandin synthesis and onward cause any renal adverse effects [141].

Herein, we have tested the hypothesis that NSAIDs differ in their disposition in heart-tissue which might be linked with CV risks reported for these NSAIDs. Such that NSAIDs which have high heart-tissue-plasma concertation ratio may also have high CV/renal risks than other NSAIDs which minimally accumulate in the heart tissue [209].

5.2. Objective

The objective of this study was to measure systemic drug exposure and tissue accumulation of meloxicam compared to compared to other NSAIDs including rofecoxib, celecoxib and flurbiprofen in adjuvant arthritis rats heart and kidney tissues.

5.3. Hypotheses

- NSAIDs differ in their extent of tissue distribution.
- NSAIDs with higher tissue concentration compared to their concentration in the plasma accumulate in the tissues resulting in organ dysfunction.
- Meloxicam has limited tissue-distribution compared to other NSAIDs.

5.4. Methods

Materials used in this study, selection of NSAIDs, dose calculation, animal handling, sample collection, and method of analysis are discussed in detail in section 3.1 and 3.2.

5.5. Results

Changes in the body weight gain, arthritis index and serum nitrites, 20 days post adjuvant injection were used to determine the extent of inflammation. Which were compared with baseline observations [Table 10]. For determination of NSAIDs concentration in the plasma and tissue samples, we used previously published, high-performance liquid chromatography (HPLC) methods with some modification as mentioned in the method section.

We found that rofecoxib and celecoxib concentration in rat the heart and kidneys were significantly higher than their steady state trough (Css) in the plasma [Figure 8(A), 10(A), respectively]. The tissue accumulation for these two NSAID is also evident from the high tissue-to-plasma concentration ratios. For rofecoxib, the heart-to-plasma (H/P) ratio was 3.475 ± 0.40 and the kidney-to-plasma (K/P) ratio was 2.33 ± 0.74, suggesting a greater accumulation of rofecoxib in the heart almost doubles the amount in the kidney [Figure 8(B)]. For celecoxib, the heart-to-plasma (H/P) ratio was 2.750 ± 1.26 and the kidney-to-plasma (K/P) ratio was 2.65 ± 0.85, indicating that almost the same amount of celecoxib is concentrated in both heart and kidney tissue [Figure 10(B)].

We found that flurbiprofen enantiomers exhibited a distinct tissue-specific drug disposition: the heart-tissue concentration after 24 h post-dose and after seven days of dosing

was significantly higher than its steady state (Css) trough levels in the plasma [Figure 11(A)], with a high heart-to-plasma (H/P) ratio of 16.9 ± 3.85 and 28.1 ± 7.28 for S-flurbiprofen and R-flurbiprofen, respectively. Such tissue accumulation of flurbiprofen enantiomers was not seen in the kidney tissue; rather, the 24-hour post-dose kidney-tissue concentration of flurbiprofen 7 days post-dose was lower than steady state (Css) trough level in the plasma [Figure 11(A)]. The kidney-to-plasma (K/P) ratio was 2.051 ± 0.59 and 0.514 ± 0.05 for flurbiprofen (S+) and flurbiprofen (R-), respectively Figure 11(B)]

We were able to reproduce the findings from a previous study [141], which suggested that meloxicam accumulate in the kidney tissues to much less extent compared to other NSAIDs [141]. Likewise, in the present study, the 24-hour post-dose kidney concentration of meloxicam after seven days of dosing was found lower than its steady state trough (Css) in the plasma with a kidney-to-plasma (K/P) ratio of 0.14 ± 0.4. We also observed the same trend for meloxicam in the heart, where the 24-hour post-dose heart tissue concentration of meloxicam was lower than its steady state trough (Css) in the plasma [Figure 9(a)] and the heart-to-plasma (H/P) ratio was 0.10 ± 0.04 [Figure 9(B)].

5.6. Discussion

Pharmacokinetic studies have shown that the plasma concentration of NSAIDs is not well correlated with the therapeutic response of these drugs, especially under inflammatory conditions [294]. The reason might be because plasma is not the primary site of action for NSAIDs [294]. The primary site of action for NSAIDs is not the central vascular compartment (i.e., plasma); instead it is peripheral tissue compartment. NSAIDs concentration in the tissue might give us a better estimate of NSAIDs efficacy. Similarly, NSAIDs concentration in the peripheral tissue compartment needs to be considered for dose–concentration–adverse effect relationships to measure their toxicity potential.

The human data on tissue concentrations of NSAIDs are rare, hoever, animal studies have shown a clear relationship between NSAIDs concentration in the tissues and their toxicity [143, 295]. For example in adjuvant arthritis rats a direct relationship was reported between the urinary electrolytes excretion, as a measure of renal function and the concentration of NSAIDs in kidney tissues [16]. It was found that rofecoxib, celecoxib, and diclofenac significantly reduce the urinary excretion of electrolytes, indicating renal failure, but meloxicam did not have this effect.

These effects were also independent of COX-2 selectivity, as meloxicam and celecoxib both have similar COX-2 selectivity, yet only celecoxib altered the renal electrolyte secretion [98]. These findings in animal coincide those generated from randomized clinical trials, suggesting that risk of toxicity increases with an increased in NSAIDs exposure [151, 152, 209].

Another aspect to tissue drug exposure is the potency of NSAIDs that's to say what concentration of an NSAID is required in a tissue to influence that organs function. Harirforoosh, et. Al., have reported that although the kidney-to-plasma concentration ratio for meloxicam was low but its concentration in the kidney was higher than rofecoxib and celecoxib [16]. He concluded that because the IC_{50} values for COX-2 inhibition by meloxicam is much higher than rofecoxib and celecoxib that is 2.1, 0.84 and 0.83 mmol/L, respectively. Thus, more meloxicam is required to inhibit COX-2 in the kidney tissues compared to rofecoxib and celecoxib [141]. Also, meloxicam being a zwitterion and possesses a unique physiochemical profile. Once absorbed and at physiological pH, meloxicam gets converted into an enolic (polar) form, restricting further tissue distribution and penetration through physiological membranes [171]. However, studies have shown that the synovial concentration of meloxicam is well above the IC_{50} of COX-2 in the joints, making it a favorable NSAID [296]. The exposure-dependent toxicity is not just limited to NSAIDs; other classes of drugs, such as cyclosporine-A [297, 298], gentamycin [299] and other aminoglycosides [300, 301], have also exhibited such characteristics where higher tissue accumulation correlates with higher tissue-specific toxicities. Higher tissue concentration of gentamycin in the deep tissue compartment was associated with lower creatinine clearance and nephrotoxicity [299].

Several epidemiological studies have reported that NSAIDs differ in their adverse effects on CV and renal system [204, 205]. Here we are suggesting that differences in tissue distributions of NSAIDs might, at least in part, play a role in their adverse effect profile. How the difference cardiotoxicities of NSAIDs as reported in randomized control trials [173, 302, 303]. In a review, Jamali et al. have suggested that physiochemical properties and pharmacokinetics differences amongst NSAIDs might play a role in deterring the high CV risk associated with rofecoxib [104].

5.7. Conclusion

We confirmed the selective accumulation of rofecoxib and celecoxib in the kidney, previously reported in literature. In this study we also found the same trend of NSAIDs tissue accumulation in the heart tissues. Also we found that both enantiomers of flurbiprofen also show higher accumulation in heart and kidneys tissues. We suggested that this local accumulation of NSAIDs in the cardiac and renal tissues may impact the physiological mechanism involved in tissue homeostasis and organ function. However, the data presented here are limited to animals and inflammation model, thus further investigation is required to assess whether these findings explain the adverse events seen with the chronic use of NSAIDs in humans.

Table 10: Changes in body weight, Arthritis Index and serum nitrite observed in control, inflamed rats, and inflamed rats treated with NSAIDs.

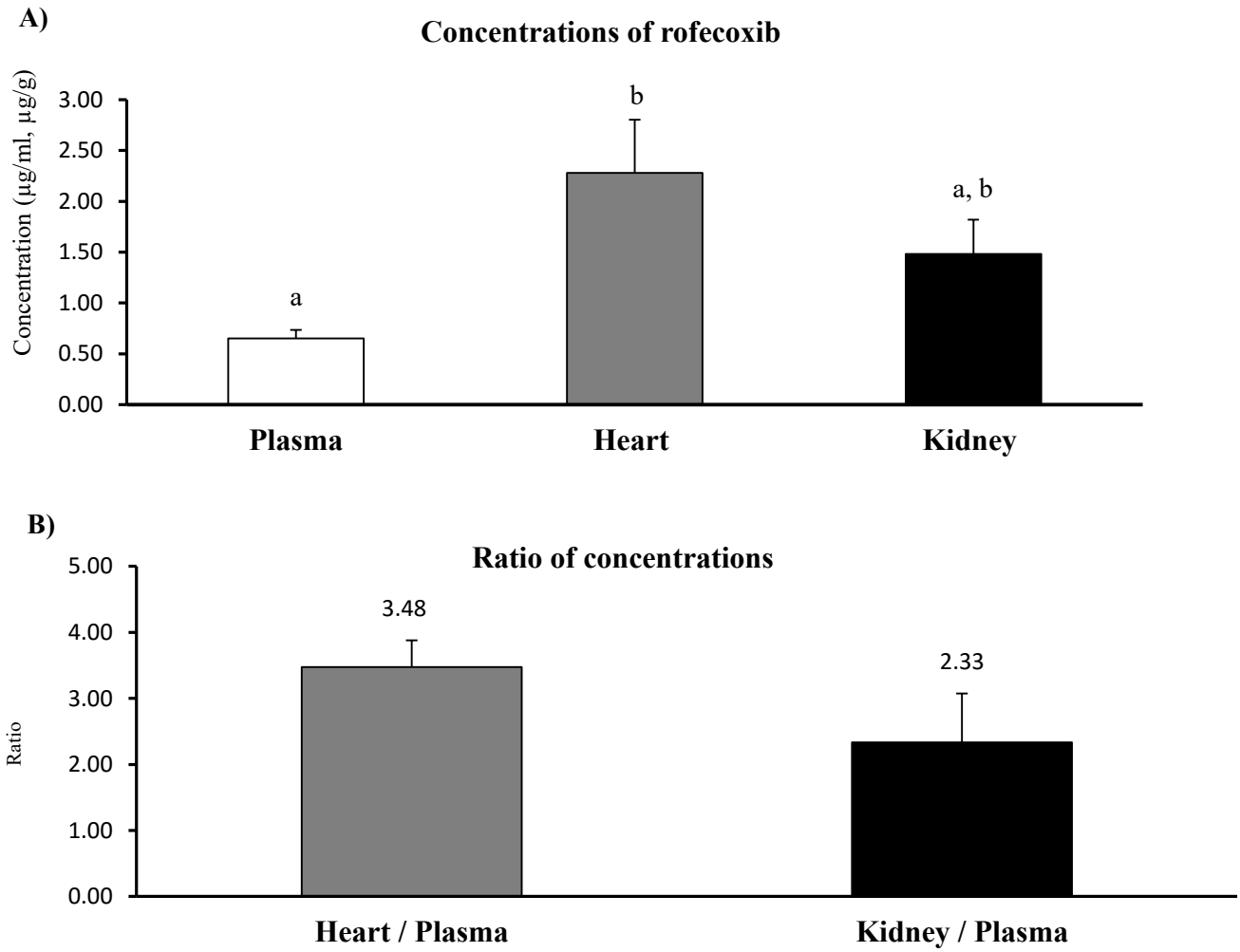
Body weight (g), n=3		
Group	**Weight gained compared to baseline, Mean (SD)**	
	Pre-Dose (g)	***Post-Dose (g)***
Control	50.1 (5.4)θ	-
Inflamed	17.8 (8.3)θ	-
Rofecoxib	11.8 (3.8)	42.68 (8.7)
Meloxicam	17.6 (3.7)	39.51 (10.2)
Celecoxib	11.8 (5.6)	39.14 (13.6)
Flurbiprofen	14.7(4.7)	33.08 (8.5)

Serum nitrite concentration (µM), n=3		
Group	**Serum nitrite (µM) Mean (SD)**	
	Pre-Dose	***Post-Dose***
Control	67.1 (16.3)θ	-
Inflamed	208.0 (24.3)θ	-
Rofecoxib	212.4 (20.7)	55.5 (10.7)
Meloxicam	211.0 (20.6)	65.8 (8.7)
Celecoxib	219.4 (21.4)	62.5 (10.5)
Flurbiprofen	222.4 (21.6)	77.5 (10.1)

Physical assessment of arthritis , n=3					
Group	**Arthritis Index Mean (SD)**		**Paw and Joint**		
			Paw Diameter (µm)	**Joint Diameter (µm)**	**Paw Volume (µL)**
	Pre-Dose	Post-Dose	Post-Dose	Post-Dose	Post-Dose
Control	0.0 (0.0)ǂ	-	-20 (38)θ	518 (120)θ	280 (83.7)θ
Inflamed	4.3 (1.2)ǂ	-	1321 (606.9)θ	2963 (563.6)θ	1500 (265)θ
Rofecoxib	5.0 (1.0)	1.3 (0.6)	115 (34.5)	495 (95.1)	250 (61.8)
Meloxicam	4.3 (1.5)	2.0 (1.0)	140 (23.0)	545 (95.7)	380 (70.7)
Celecoxib	5.0 (1.0)	2.0 (1.0)	165 (29.9)	525 (93.2)	340 (67.1)
Flurbiprofen	4.7 (1.5)	2.0 (1.0)	195 (43.7)	690 (117.6)	520 (74.2)

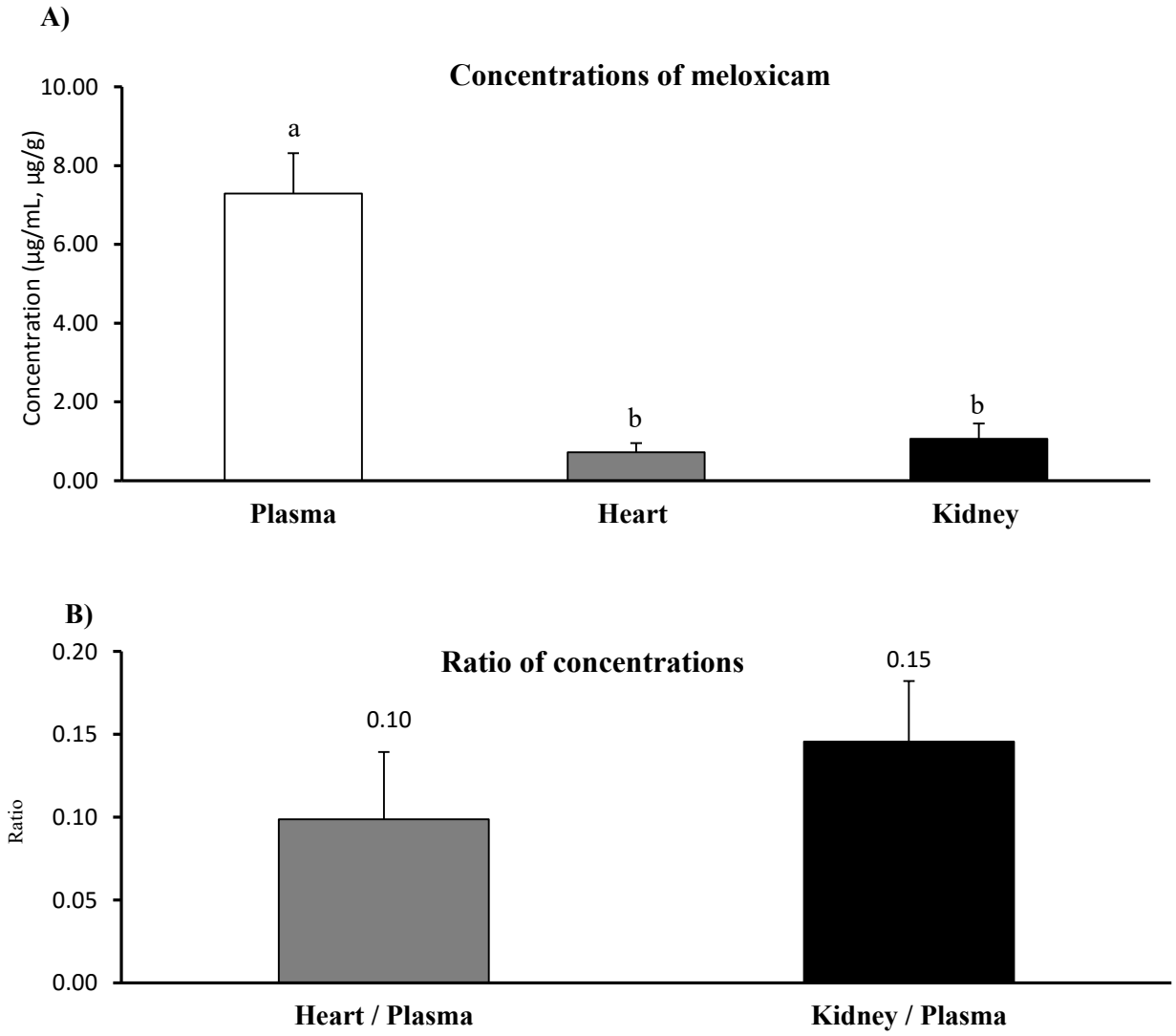
θ= Animals in these groups were not treated and were euthanized on 12^{th} -$14^{th\ a}$ day after adjuvant injection

Rats in inflamed NSAIDs treated groups were euthanized on 20^{th} 22^{nd} day after adjuvant injection



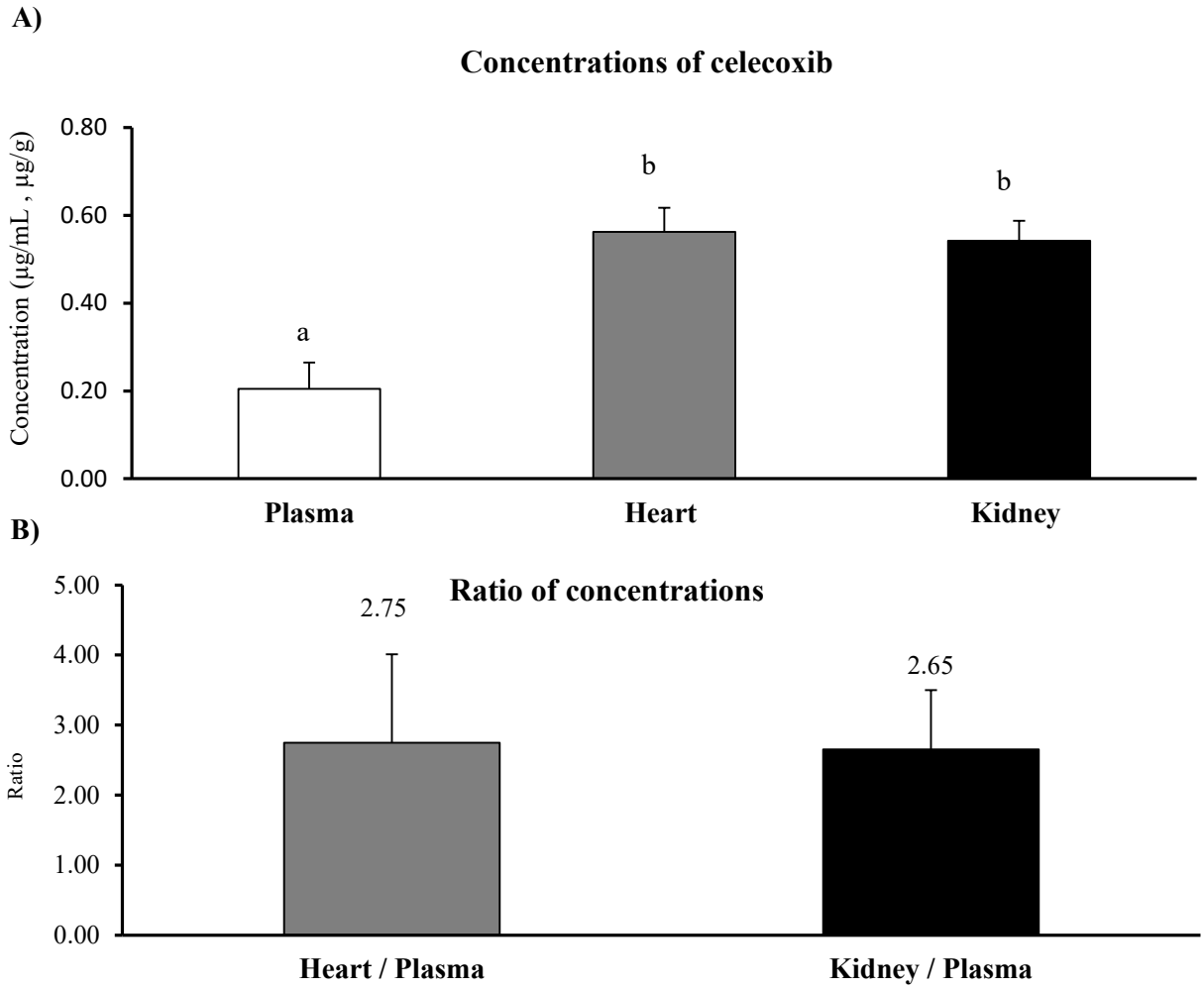
Rat #	Body weight (gm)	Plasma Conc. (µg/ml)	Heart Conc. (µg/gm)	Kidney Conc. (µg/gm)	Heart /Plasma (ratio)	Kidney /Plasma (ratio)
Rat -1	386	0.75	2.85	1.18	3.81	1.57
Rat -2	406	0.61	1.83	1.84	3.03	3.04
Rat -3	380	0.60	2.15	1.43	3.59	2.39
Mean	391	0.651	2.279	1.484	3.475	2.334
SD	13	0.085	0.522	0.336	0.402	0.737

Figure 8: A) Concentrations of rofecoxib in rat plasma, heart and kidneys (µg/mL, µg/g, and µg/g, respectively) and B) ratio of tissue-to-plasma concentrations , (a, b, c) represents significant difference b/w each other using ANOVA ($p<0.05$).



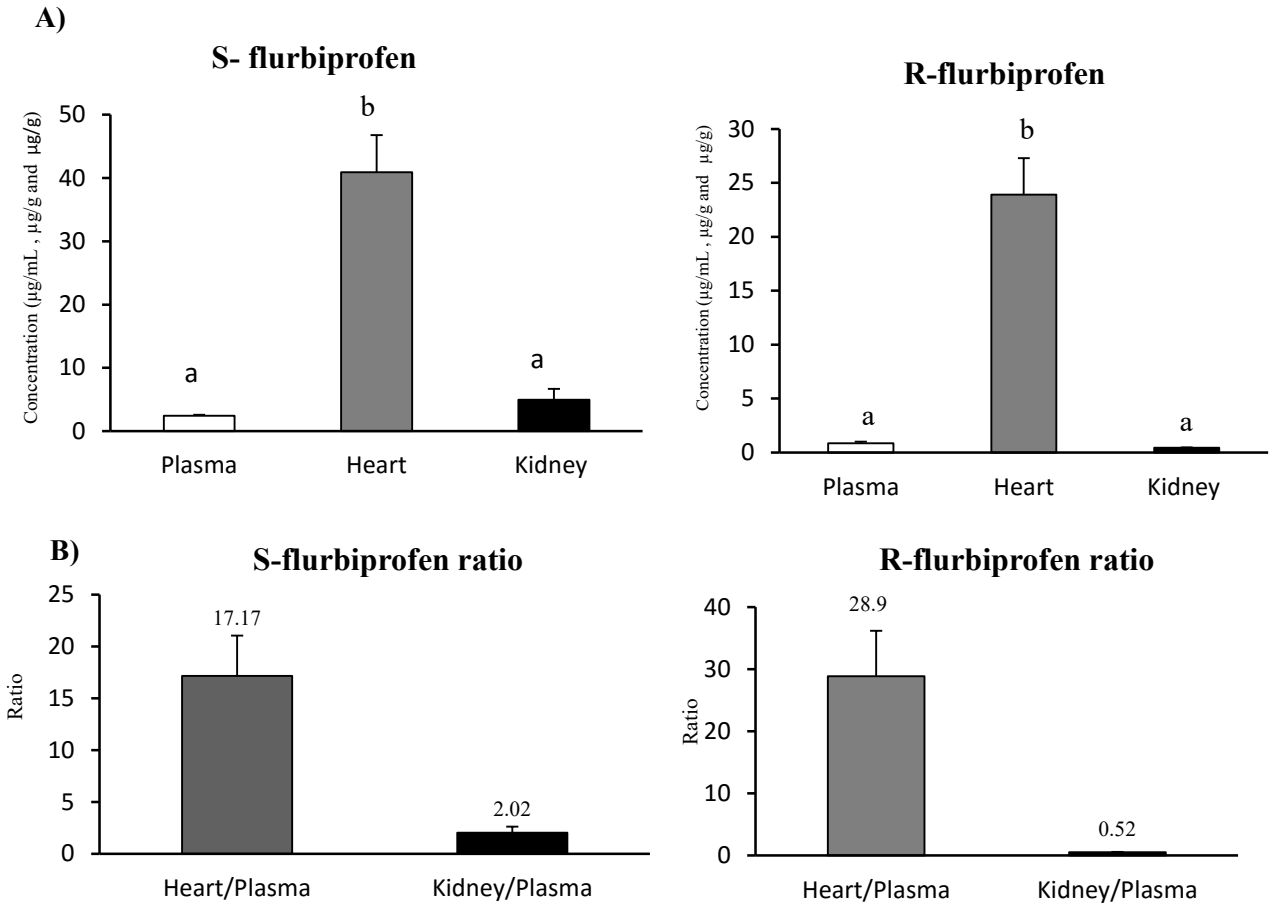
Rat #	Body weight (gm)	Plasma Conc. (µg/ml)	Heart Conc. (µg/gm)	Kidney Conc. (µg/gm)	Heart /Plasma (ratio)	Kidney /Plasma (ratio)
Rat -1	370.5	8.04	0.45	1.48	0.06	0.18
Rat -2	459.3	7.71	0.88	1.00	0.11	0.13
Rat -3	352.5	6.12	0.83	0.70	0.13	0.12
Mean	394	7.289	0.720	1.062	0.099	0.146
SD	57	1.029	0.232	0.392	0.041	0.036

Figure 9: A) Concentrations of meloxicam in rat plasma, heart and kidneys (µg/mL, µg/g, and µg/g, respectively) and B) ratio of tissue-to-plasma concentrations, (a, b, c) represents significant difference b/w each other using ANOVA ($p<0.05$).



Rat #	Body weight (gm)	Plasma Conc. (µg/ml)	Heart Conc. (µg/gm)	Kidney Conc. (µg/gm)	Heart /Plasma (ratio)	Kidney /Plasma (ratio)
Rat -1	421	0.26	0.53	0.59	2.03	2.26
Rat -2	468	0.21	0.53	0.50	2.52	2.37
Rat -3	457	0.14	0.63	0.54	4.42	3.79
Mean	449	0.205	0.562	0.542	2.750	2.651
SD	24	0.060	0.055	0.045	1.260	0.850

Figure 10: A) Concentrations of celecoxib in rat plasma, heart and kidneys (µg/mL, µg/g, and µg/g, respectively), and B) ratio of tissue-to-plasma concentrations, (a, b, c) represents significant difference b/w each other using ANOVA ($p<0.05$.



S-flurbiprofen

Rat #	Body weight (gm)	Plasma Conc. (µg/ml)	Heart Conc. (µg/gm)	Kidney Conc. (µg/gm)	Heart / Plasma (ratio)	Kidney/ Plasma (ratio)
Rat -1	399	2.20	47.64	2.99	21.61	1.36
Rat -2	390	2.50	38.45	5.64	15.35	2.25
Rat -3	419	2.52	36.74	6.20	14.56	2.46
Mean	403	2.41	40.94	4.94	16.98	2.05
SD	15	0.18	5.86	1.72	3.86	0.59

R-flurbiprofen

Rat #	Body weight (gm)	Plasma Conc. (µg/ml)	Heart Conc. (µg/gm)	Kidney Conc. (µg/gm)	Heart / Plasma (ratio)	Kidney/ Plasma (ratio)
Rat -1	399	0.79	27.75	0.40	35.08	0.51
Rat -2	390	0.74	22.60	0.42	30.68	0.57
Rat -3	419	1.03	21.43	0.49	20.86	0.47
Mean	403	0.85	23.92	0.44	28.10	0.51
SD	15	0.15	3.36	0.04	7.28	0.05

Figure 11: A) Concentrations of flurbiprofen enantiomers in rat plasma, heart and kidneys (µg/ mL, µg/ g, and µg/ g, respectively) and B) ratio of tissue-to-plasma concentrations, (a, b, c) represents significant difference b/w each other using ANOVA ($p<0.05$).

Chapter 6

6. Effect of NSAIDs on renin-angiotensin system in adjuvant arthritis rat plasma, heart and kidneys

6.1. Introduction

The multi-organ renin angiotensin system (RAS) helps to regulate blood pressure, electrolyte excretion, fluid balance, and maintains homeostasis in the cardiovascular (CV) system [212]. The RAS also plays an important role in pathophysiological conditions such as hypertension, heart failure, and myocardial infarction (MI) [304, 305]. It exerts most of its effects through its component enzymes, peptides, and receptors, which are widely expressed in vascular smooth muscle, heart, kidney, lung, liver, and other organs [Figure 12].The RAS causes its effects mostly in an endocrine manner, secreting biologically active peptides in the blood and influencing aldosterone and vasopressin hormones [306, 307]. However, owing to the differential expression of RAS components in different body organs, tissue-specific roles of the biologically active peptides and corresponding receptors are yet to be recognized as paracrine factors [308, 309].

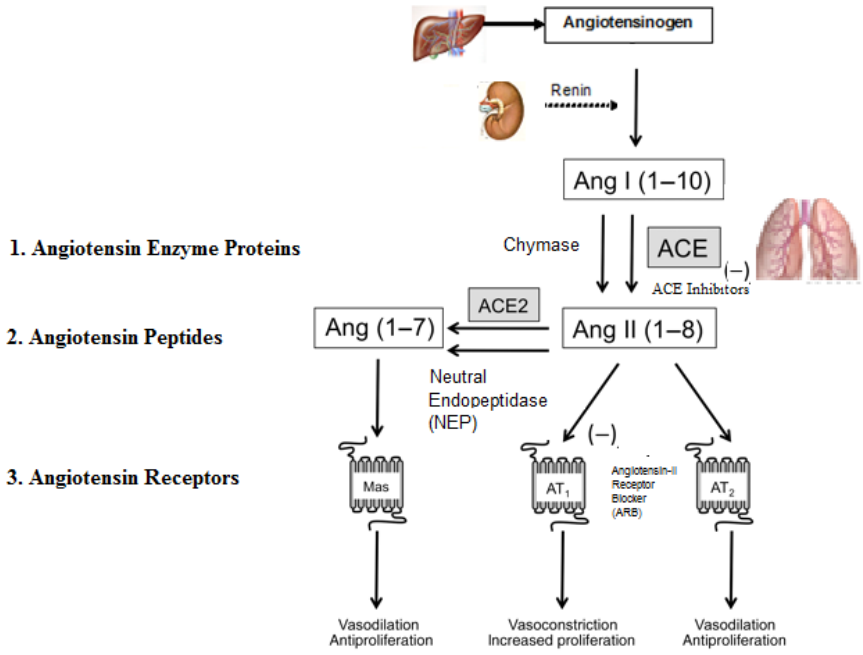


Figure 12: Renin-angiotensin system; RAS (with permission from [310]).

The classic pathway for the synthesis of RAS peptides starts with the production of renin by juxtaglomerular cells on the afferent arterioles of the kidney [311]. Although most renin is secreted by the kidney, other tissues also secrete protein in the circulation. Pro-renin can be converted into renin by trypsin activation [312]. Another component of the RAS is angiotensinogen, a protein excreted by the liver into the circulation [313]. Once in the circulation, angiotensinogen, functions as a donor of the decapeptide angiotensin-I (Ang-I), which is cleaved from the N-terminus by renin [314]. Angiotensin then takes multiple metabolic pathways to produce physiologically active angiotensin (Ang) peptides, e.g., Ang-II, Ang-III, Ang-IV, and Ang-(1-7). Many peptidases including chymase, chymotrypsin, angiotensin converting enzymes (ACE, ACE2), and amino peptidase-A, are involved in conversion of angiotensin into RAS peptides [315]. These peptidases act both in ACE-dependent and ACE-independent pathways. The major pathway is the conversion of angiotensin-I (Ang-I) into angiotensin-II (Ang-II) through angiotensin converting enzyme (ACE). ACE is widely spread across the endothelial cells of vascular tissues and lungs [309, 314, 316]. Although many other ACE-independent pathways for the production of Ang-II have been identified, the predominant concentration of Ang-II in the circulation is from the renin-ACE pathway [317, 318]. The circulatory concentration of angiotensinogen is many-fold higher than the concentrations of Ang-I and Ang-II. The rate limiting step in the production of angiotensin peptides is the renin-catalyzed production of Ang-I [319].

Independent of ACE, cathepsin and kallikreins can directly convert angiotensinogen into Ang-I or Ang-II. Also, in the pulmonary arteries, chymase is reported to participate in the production of Ang-II [320]. Studies have shown that, in humans, chymase is synthesized and stored in endothelial and mesenchymal cells where it is responsible for 80% of the Ang-II found in the circulation [321]. However, the role of chymase in the kidney is not well understood. It has been reported that in ACE knockout mice, there is no change in Ang-II content, but there was a 14-fold increase in chymase activity. That may be a balancing response to maintain the constitutive basal Ang-II level in the kidney [322]. In dogs, 20% of Ang-II was shown to be chymase dependent [323]. In humans, an increased chymase expression is reported in rejected kidney [324] and in patients with renovascular hypertension [325] and diabetes [326, 327]. This suggests that chymase has a role in renal diseases.

Among all RAS peptides, Ang-II is the most potent; it is involved in both endocrine and paracrine signaling in an organ specific manner [328]. Ang-II mediates its biological effects through its specific G protein-coupled, surface-type receptors, the Ang-II receptor type 1 (AT1R) and the Ang-II receptor type 2 (AT2R) [308, 309]. Ang-II causes vasoconstriction in vascular smooth muscle, enhances myocardial contractility, and stimulates aldosterone secretion and the release of catecholamine secretion, thus increasing sympathetic activity. Ang-II also regulates sodium transport in renal tubules in the kidney [308, 309]. The pathophysiological effects of Ang-II as a pro-inflammatory mediator, and in the cell proliferation, migration, apoptosis, differentiation, and intracellular cell signaling, suggest a role in the cell injury [319]. Several peptidases deactivate and convert Ang-II to smaller peptides such as Ang-III, Ang-IV, and Ang-(1-7). These smaller peptides also have biological activity, mostly opposite to that of Ang-II, but, their plasma levels are much lower than that of Ang-II. However, inside the tissues they might be able to counter the effects of Ang-II [315, 329].

Angiotensin receptors (ATR) are expressed in heart, kidney, gonads, pituitary gland, placenta, peripheral blood vessels, and central nervous system. The precise roles of angiotensin receptor subtypes are still under debate, but AT1Rs are believed to mediate vasoconstriction, increasing the peripheral resistance and blood pressure elevating effects of Ang-II [330-332]. AT1R expression is ubiquitous throughout peripheral blood vessels and cardiovascular and renal systems [331]. AT2Rs are predominant in the developing fetus heart but their expression is gradually reduced with age in the adult heart [333, 334]. Recently, it has been shown that AT2R expression is modulated in cardiovascular diseases (hypertension, arteriosclerosis, myocardial infarction, heart failure) where it is involved in tissue remodeling, hypertrophy, and inflammation [335].

ACE is a carboxypeptidase that cleaves two amino acids from the N-Terminus of Ang-I to make it an octapeptide, angiotensin-II (Ang-II) [309, 314, 316]. Another carboxypeptidase, ACE2, cleaves one amino acid from the C-terminus of Ang-I to make a nanopeptide, Ang-(1-9); Ang-(1-9) is not converted to Ang-II and is devoid of Ang-II activity [336-339]. It has been proposed that ACE2 is a key factor in the control of the ACE-dependent pathway for the production of Ang-II by channeling the alternate pathway of Ang-I degradation into Ang-(1-9). ACE2 is also involved in the degradation of Ang-II into a heptapeptide, Ang-(1-7), that counters the effects of Ang-II, as it possess completely opposite physiological properties [337, 339, 340].

ACE2 is abundantly expressed in kidney epithelial cells and proximal tubule cells [336, 339, 340], where it plays a significant role in maintaining a constitutive balance among Ang peptides by converting Ang-II into Ang-(1-7). Levels of Ang-II are elevated in ACE2 knockout mice [341]. Likewise, ACE2 expression in the kidneys of hypertensive [342] and diabetic rats [343] is high compared to that in normal rats. It is suggested that ACE2 is crucial in balancing the excessive Ang-II and to counter its physiological effects [344].

Previously, it was believed that only the angiotensin receptors (AT1R, AT2R) are involved in mediating the physiological effects of angiotensin peptides. Later it was found that Ang-(1-7) acts as a ligand for a Mas-related G protein-coupled receptor, simply called a Mas receptor [345]. Mas receptors are orphan heterotrimeric guanine nucleotide-binding protein-coupled receptors, found in peripheral blood vessels and in cardiovascular and renal systems, and are involved in mediating the vasodilating, antihypertensive, the antihypertrophic, antiatherosclerotic effects of Ang-(1-7) [346]. Ang-(1-7) endogenously opposes the effects of Ang-II via its effect on Mas receptors [337, 338, 340, 347]. Genetic studies have shown that Ang-(1-7) effects are lost upon deletion of Mas receptor genes in mice [346].

In the kidney, the AT1R modulates the sodium and water reabsorption by stimulating the release of aldosterone. In contrast, the AT2R enhances vasodilation through countervailing cardiac hypertrophy, vascular injury, pressure overload, and atherosclerosis. Ang-II has been implicated in various cardiovascular conditions such as hypertension, atherosclerosis, coronary artery disease, restenosis, and heart failure [333, 348]. Most Ang-II effects are believed to be mediated through the AT1R and the AT2R, but there is a growing opinion that the involvement of Ang-(1-7) and its Mas receptor play a role in the RAS [331, 332, 349]. Along with Ang-II, some other peptides are also formed by the action of amino peptidases such as Ang-III and Ang-IV, but studies have shown the most of the characteristic functions of RAS peptides, such as vasoconstriction, salt and water retention, and hypertension, are due to Ang-II through its effects on the AT1R [350]. On the other hand, Ang-(1-7) and Ang-IV affect the AT2R, to counter the protective or buffering effect [351] of Ang-II on the AT1R.

6.2. Objectives

This study explores the effects of NSAIDs treatment on the RAS components at the level of angiotensin proteins, bioactive peptides, and receptors. For this purpose we choose four NSAIDs

(i.e., rofecoxib, meloxicam, celecoxib and flurbiprofen) to study what changes they might cause in RAS components in adjuvant arthritis (AA) rat model of inflammation. The overall question asked is: Can the differential cardiovascular safety profile of different NSAIDs be explained by RAS behaviors

6.3. Hypotheses

- NSAIDs have differential effects on RAS components (enzymes, peptides, and receptors).
- Downregulate the cardioprotective axis i.e., ACE2/Ang-(1-7)/Mas in NSAIDs treatment can lead to CV/renal risks.
- The plasma ratio of angiotensin peptides (Ang-(1-7)/Ang-II) can serve as biomarkers of cardiotoxicity.

6.4. Methods

Materials used in this study, selection of NSAIDs, dose calculation, animal handling, sample collection, and method of Western blot and ELISA analysis are discussed in detail in section 3.1 and 3.2.

6.5. Results

We studied the effects of NSAIDs treatment on RAS (angiotensin enzymes, peptides and receptors). The results of Western blot measuring protein expression and ELISA analyses measuring peptide concentration in AA rat plasma, heart and kidneys are presented below.

6.5.1. Angiotensin converting enzymes

We confirm our previous finding [213] that inflammation results in significant downregulation in the density of ACE2 *(p*=0.04) as observed in inflamed rat heart. Similar observations were made in inflamed rat kidneys *(p*=0.03). However, density of ACE remained unchanged both in AA rat heart and kidneys [Figure 13] [Table 11].

In the heart, NSAIDs treatment resulted in significant upregulation of ACE2, compared with inflamed- rats (p: rofecoxib, 0.02; meloxicam, 0.03; flurbiprofen, 0.03) with the exception of celecoxib which showed the trend that was not significant, likely, due to a larger extent of variations as compared to other NSAIDs. However, the density of ACE in AA rat heart remained unchanged upon NSAIDs treatment. In the kidneys, same upregulation of ACE2 in the NSAIDs

treated rats as those made in the heart. . However, the effect reached statistical significance only following rofecoxib treatment *(p*=0.04). No change was observed in the density of ACE in AA rats and NSAIDs treated rat kidneys [Figure 14] [Table 11].

The ACE2/ACE ratio, although numerically increased by NSAIDs, the effects were not significant neither in the heart nor in the kidneys [Figure 13, 14] [Table 11].

6.5.2. Angiotensin peptides

We found that despite no change in the density of ACE, the plasma and heart concentration of Ang-II (a product of ACE) was significantly increased in AA rats as compared with controls *(p*=0.03) [Figure 15]. NSAIDs did not have significant effects on Ang-II [Figure 16] [Table 12]. However, Ang-II concentration was significantly lower following administration of meloxicam as compared with other NSAIDs. None, of the examined NSAIDs did alter Ang-II concentrations in the kidneys. In plasma and kidney, NSAIDs had no effect on Ang-(1-7), another important RAS peptide. With the exception of flurbiprofen, NSAIDs elevated the peptide concentrations in the heart. There was an insignificant trend toward increased Ang-(1-7) concentration in the heart following administration of flurbiprofen as well [Figure 15, 16] [Table 12].

6.5.3. Angiotensin receptors

In the heart, only celecoxib and flurbiprofen were able to reduce AT1R *(p*=0.03, 0.03, respectively) compared to untreated inflamed rats. In the kidneys, however, all the NSAIDs reduced the AT1R expression compared to inflamed rats [Figure 17, 19] [Table 13].

The density of Mas receptors was elevated by meloxicam in the heart, while other NSAIDs had no significant effect when compared with inflamed rats. Celecoxib treatment resulted in significantly higher Mas optical density than rofecoxib and flurbiprofen. In the kidneys, none of the NSAIDs significantly influences the Mas receptor. However, the Mas density of the rats treated with meloxicam was significantly higher than other NSAIDs. Same trend was observed in the kidney but it lacked significance [Figure 19] [Table 13].

NSAIDs had no significant effect on the optical density of AT2R in both heart and kidney with the exception of flurbiprofen which significantly increased the receptor density in the heart [Figure 18, 20] [Table 13].

6.6. Discussion

Inflammation is a systemic disease that affects almost every organ and system in the body, including RAS. The RAS is involved in maintaining the circulatory homeostatic blood pressure, electrolyte, and fluid balance in the body. RAS exerts most of its effects in an endocrine manner, secreting its bioactive peptides into circulation to cause its physiological effects. But recent studies have shown that RAS components expressed all over the body and in different organs are also involved in local function as well [309, 352]. This highlights the significance of studying the local RAS especially in the heart and kidneys, which are directly involved in maintaining the blood pressure in the body.

The physiological effects of RAS are mediated through its peptides, mainly Ang-II and Ang-(1-7). Under normal conditions ACE is responsible for the production of Ang-II. But ACE independent pathways (i.e., chymase) also plays a role, especially under disease states [353, 354]. We observed that in inflammation the density of ACE was not altered in AA rat heart and kidneys; accordingly the Ang-II levels in inflamed rat heart and kidneys were also comparable with normal healthy rats. This might be because chymase is not expressed in rat heart and does not contribute towards ACE independent Ang-II production. However, the plasma concentration of Ang-II was significantly elevated during inflammation, possibly due to the contribution of ACE independent mechanisms in other organs (e.g. liver), acting in endocrine manner.

ACE2, another major RAS enzyme, is responsible for the production of Ang-(1-7). The physiological effects of Ang-(1-7) are opposite to that of Ang-II, thus it confers protection to the body cells from Ang-II. In our Western blot, ACE2 protein gave us double bands, at the said molecular weight. We could not find any explanation in the literature. Nevertheless, we choose only to analyze the upper, denser band, consistently across all the groups. We observed a significant decrease in the density of ACE2 in the inflamed rat heart and kidneys [Table 11]. That might be a result of subcellular interactions of inflammatory cytokines with the ACE2 gene which could alter the transcription or translation of ACE2 protein. This reduced expression of ACE2 was also found to be associated with reduced Ang-(1-7) concentration in the inflamed rat heart but not in the inflamed rat kidney [Table 12]. This might be because of ACE independent neutral endopeptidase (NEP) expression during inflammation. It might have compensated for the loss of Ang-(1-7) production in inflamed rat kidney. Such compensatory mechanism is not

reported in the heart [355].Thus the Ang-(1-7) levels in the heart were observed to be lower when ACE2 density is downregulated in inflammation.

Angiotensin receptors (AT1R, AT2R, and Mas) are expressed all over the body and are responsible for binding of RAS peptides and mediating their physiological effects of that organ. Ang-II has affinity for both AT1R and AT2R, but most of Ang-II effects are mediated through AT1R [356]. AT2R is known to regulate the AT1R through a negative feedback mechanism and the AT2R also competes for Ang-II binding with no consequential physiological activity [357, 358]We observed that the density of both AT1R and AT2R receptors increased with increased Ang-II levels in inflamed rat heart and kidney. This may provide more binding sites for the hypertensive, thrombogenic, and mutagenic effects of Ang-II. The Ang-(1-7) receptor, Mas, was downregulated in the inflamed rat heart; this coupled with lower Ang-(1-7) concentration in the inflamed heart might indicate a compromised heart situation. NSAIDs treatment, with the exception of rofecoxib, brought the elevated density of angiotensin receptors (AT1R, AT2R) down which was upregulated by inflammation. In case of flurbiprofen there was no apparent role of Mas in countering the effects of AT1R. Meloxicam, on the other hand, not only lowered the density of angiotensin receptor protein but also upregulated the Ang-(1-7) specific Mas receptor, exerting a dual cardioprotective action through both angiotensin and non-angiotensin receptors in AA rat heart and kidney.

For Western blot analysis of angiotensin enzymes we used beta-actin as loading control. But for angiotensin receptor Western blot we had to switch to tubulin as loading control., as AT1R and AT2R had their molecular weight in the same range as beta actin. But in our Western blot analysis for angiotensin receptors (AT1R, AT2R, Mas) the images we produced for tubulin (loading control) got overexposed, compromising its optical density measurement. But as we have reported in thi study that NSAIDs do not differ in terms of their effects on RAS components. Thus we donot expect the angiotensin receptor to be different even if tubulin (loading control) is overexposed. We did not made any attempts to redo the experiments, however, we suggest that results for angiotensin receptor Western blots should only be seen in context of this study and not be generalized.

The most interesting finding of this study was that NSAIDs had no differential effects on RAS enzyme, peptide or receptor. NSAIDs treatment simply corrected the imbalance caused by

inflammation, perhaps by treating inflammation, independently of their COX-2 selectivity or CV/renal risks.

6.7. Conclusion

This study confirmed the previous finding that inflammation downregulates the density of ACE2 in adjuvant arthritis rat heart. We found same downregulations in ACE2 density in the inflamed rat kidneys as well. For the first time, we are reporting that inflammation creates an imbalance between cardioprotective and cardiotoxic components of renin angiotensin system. By increasing the concentration of Ang-II and upregulating its receptor AT1R at the same time lowering Ang-(1-7) concentration and downregulating its Mas receptor in inflamed rat heart and kidneys. This imbalance might explain high CV incidents in inflammatory diseases. In addition we are reporting for the first time that NSAIDs corrected the imbalance in RAS perhaps due to their anti-inflammatory properties. However, based on our results we did not see any difference in the plasma ratio of RAS-peptides (Ang-(1-7) and Ang-II) thus we cannot use them as a biomarker of CV risks.

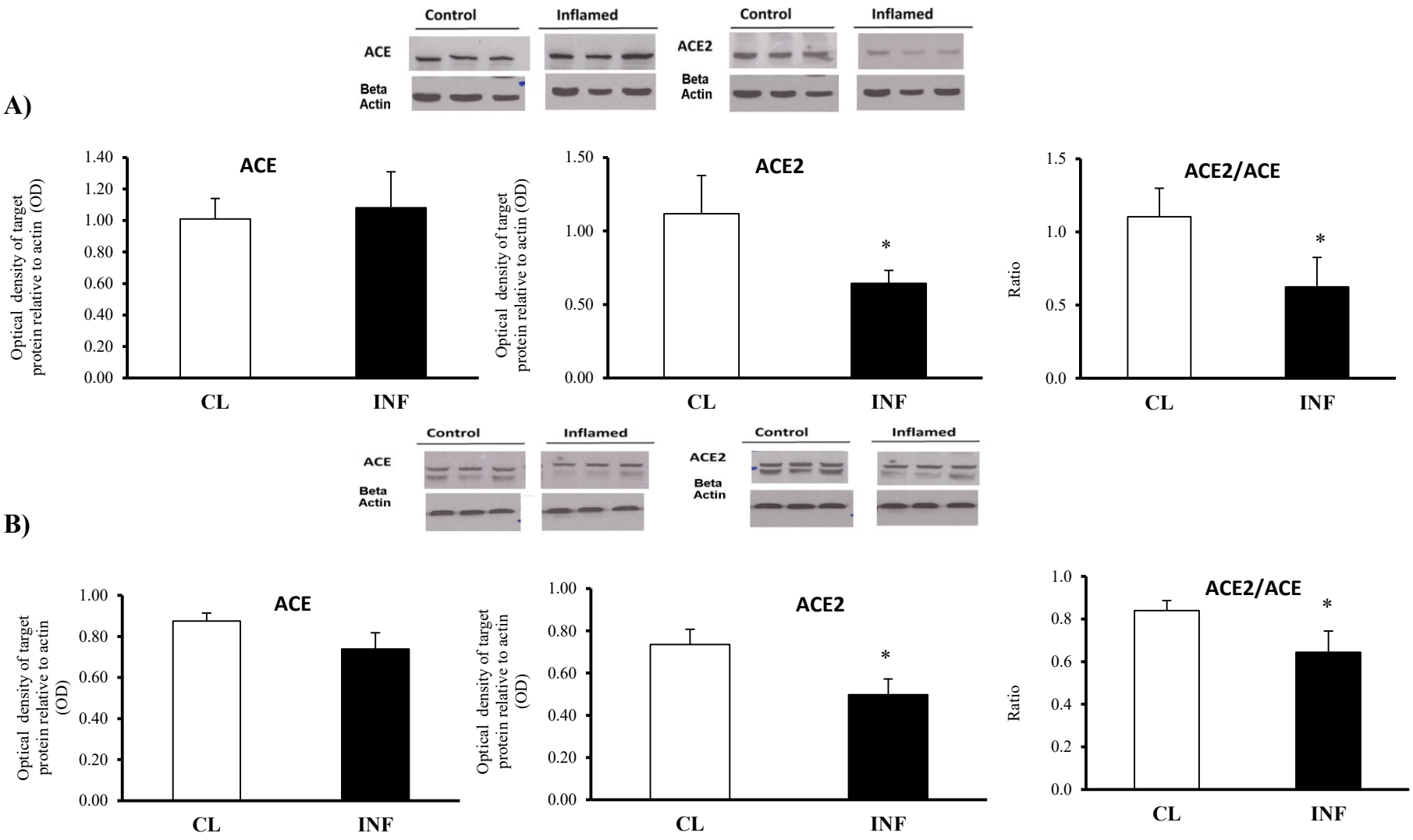


Figure 13: Densities of ACE, ACE2 presented as ratio of optical density (OD) of target protein /beta-actin in heart (A) and kidneys (B) of control and inflamed rats, n = 3 each (CL=Control, INF=Inflamed not treated) * significantly different from control rats using Student's t-test ($p < 0.05$), (Ali Aghazadeh-Habashi, W. Asghar and F. Jamali.[359]).

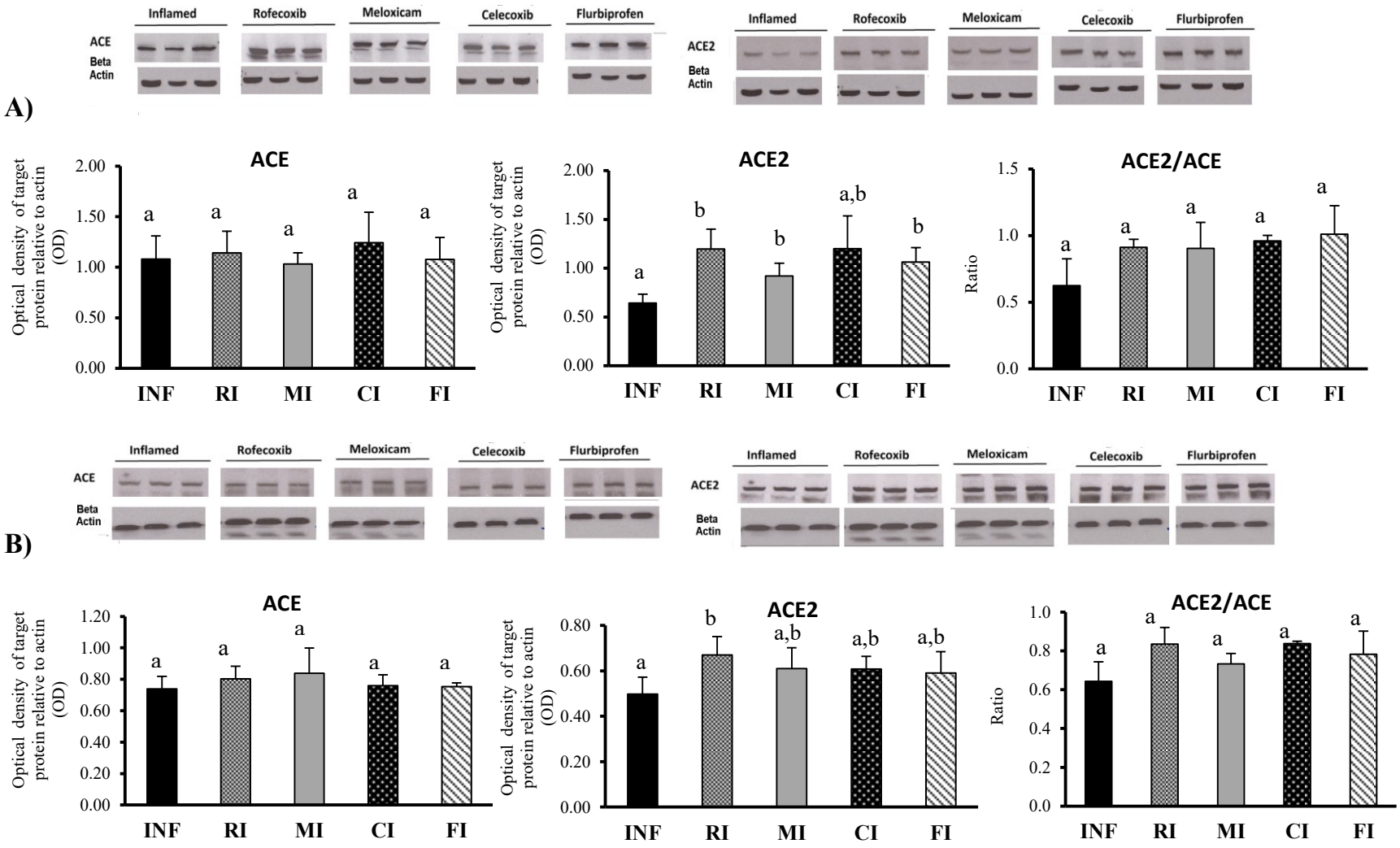


Figure 14: Densities of ACE, ACE2 presented as ratio of optical density (OD) of target protein /beta-actin in heart (A) and kidneys (B) of inflamed and NSAIDs treated rats, n=3 each (INF=Inflamed not treated, RI=Inflamed treated with rofecoxib, MI=Inflamed treated with meloxicam, CI=inflamed treated with celecoxib, FI= inflamed treated with flurbiprofen), (a, b, c) represents significant difference b/w each other using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

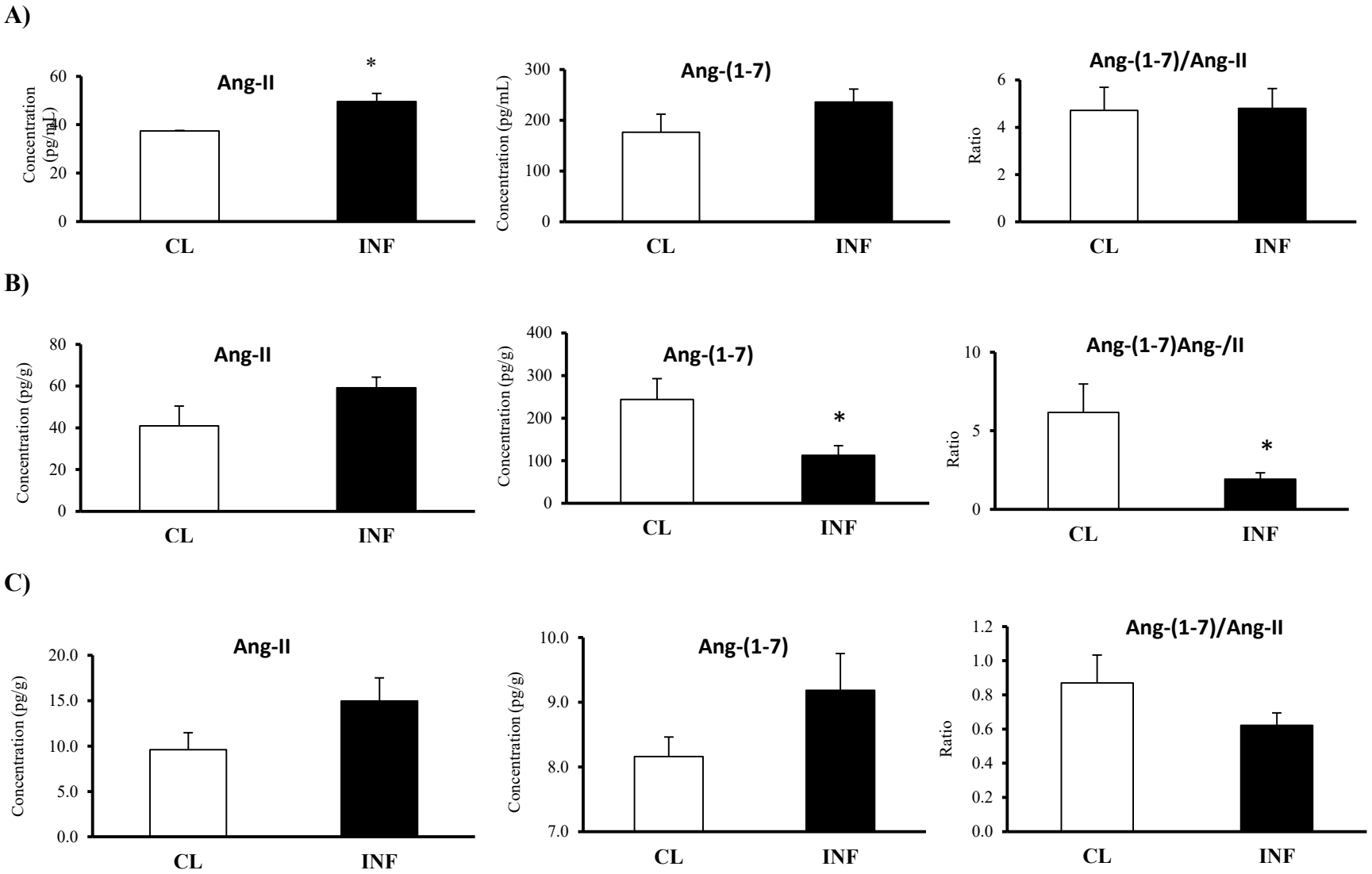


Figure 15: Angiotensin peptide concentrations (pg/mL and pg/g) in plasma (A), heart (B) and kidneys (C) of control and inflamed rats, n = 3 each (CL=Control, INF=Inflamed not treated), *significantly different from control rats using Student's t-test ($p < 0.05$), (Ali Aghazadeh-Habashi, W. Asghar and F. Jamali.[359]).

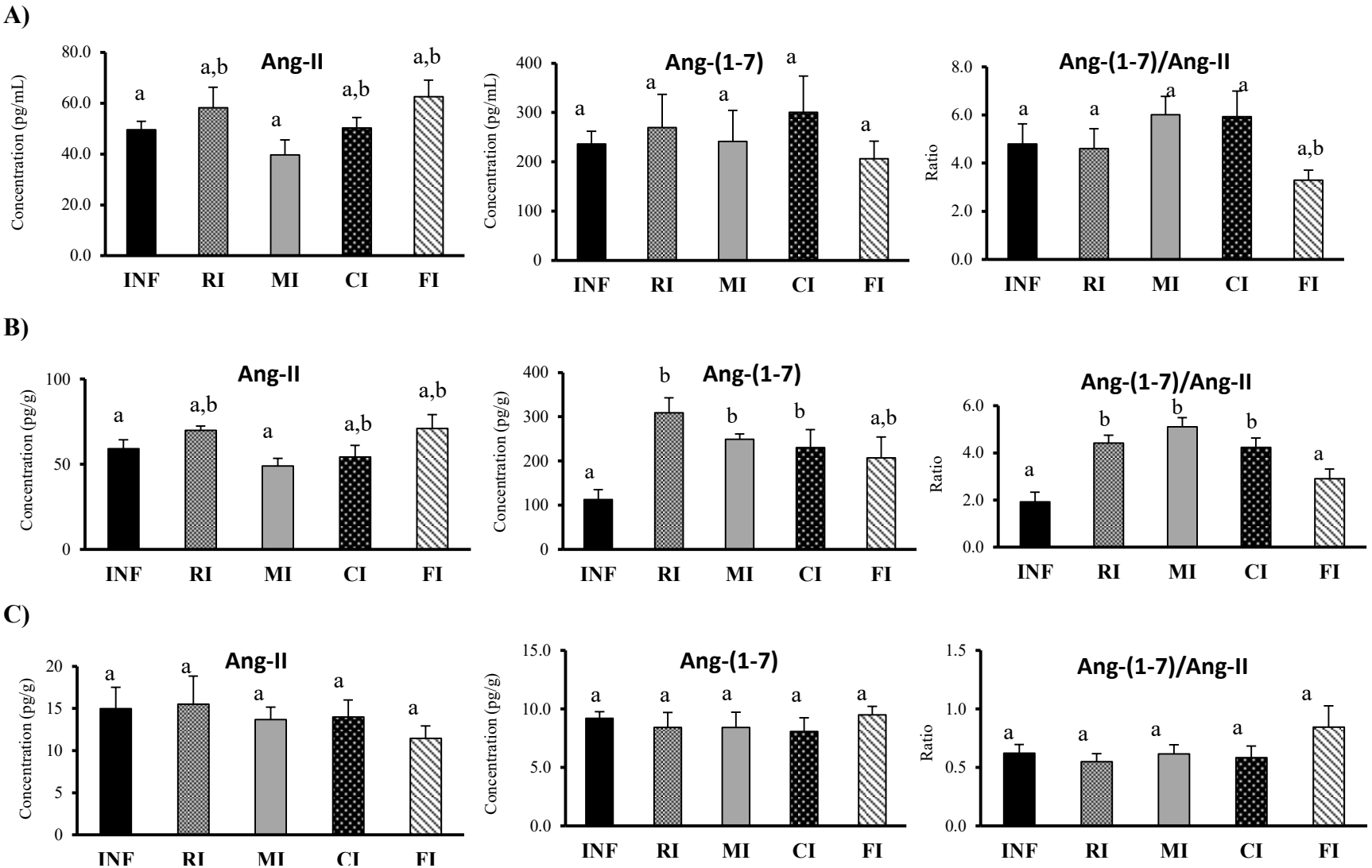


Figure 16: Angiotensin peptide concentrations (pg/mL and pg/g) in plasma (A), heart (B) and kidneys (C) of inflamed and NSAIDs treated rats, n=3 each (INF=Inflamed not treated, RI=Inflamed treated with rofecoxib, MI=Inflamed treated with meloxicam, CI=inflamed treated with celecoxib, FI= inflamed treated with flurbiprofen), (a, b, c) represents significant difference b/w each other using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

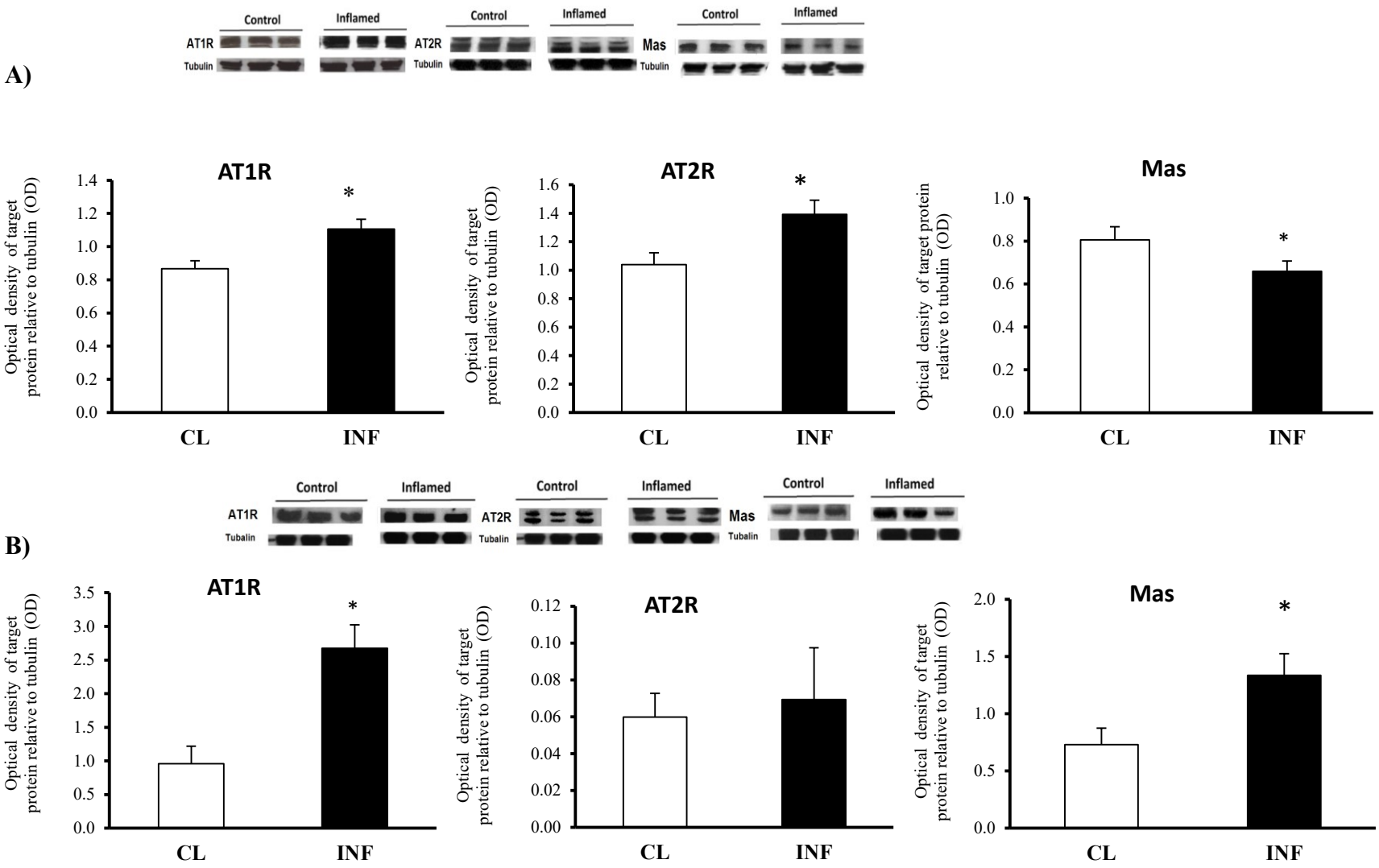


Figure 17: Densities of angiotensin receptors (AT1R, AT2R, Mas) presented as ratio of optical density (OD) of target protein/tubulin in heart (A) and kidneys (B) of control and inflamed rats, n = 3 each (CL=Control, INF=Inflamed not treated), *significantly different from control rats using Student's t-test ($p<0.05$), (Ali Aghazadeh-Habashi, W. Asghar and F. Jamali.[359]).

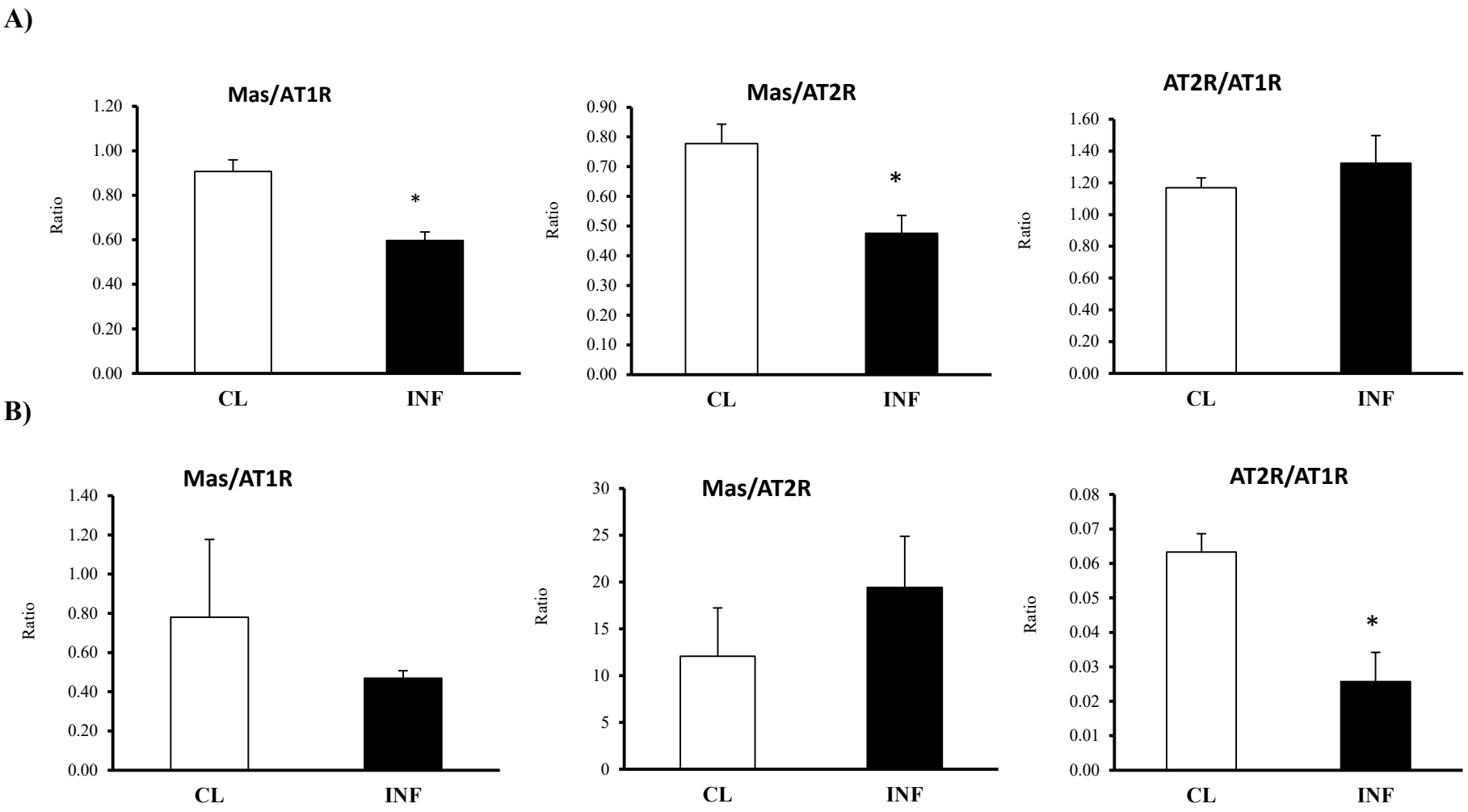


Figure 18: Ratios of densities of AT1R, AT2R) and Mas in heart (A) and kidneys (B) of control and inflamed rats, n = 3 each (CL=Control, INF=Inflamed not treated), *significantly different from control rats using Student's t-test ($p<0.05$), (Ali Aghazadeh-Habashi, W. Asghar and F. Jamali.[359]).

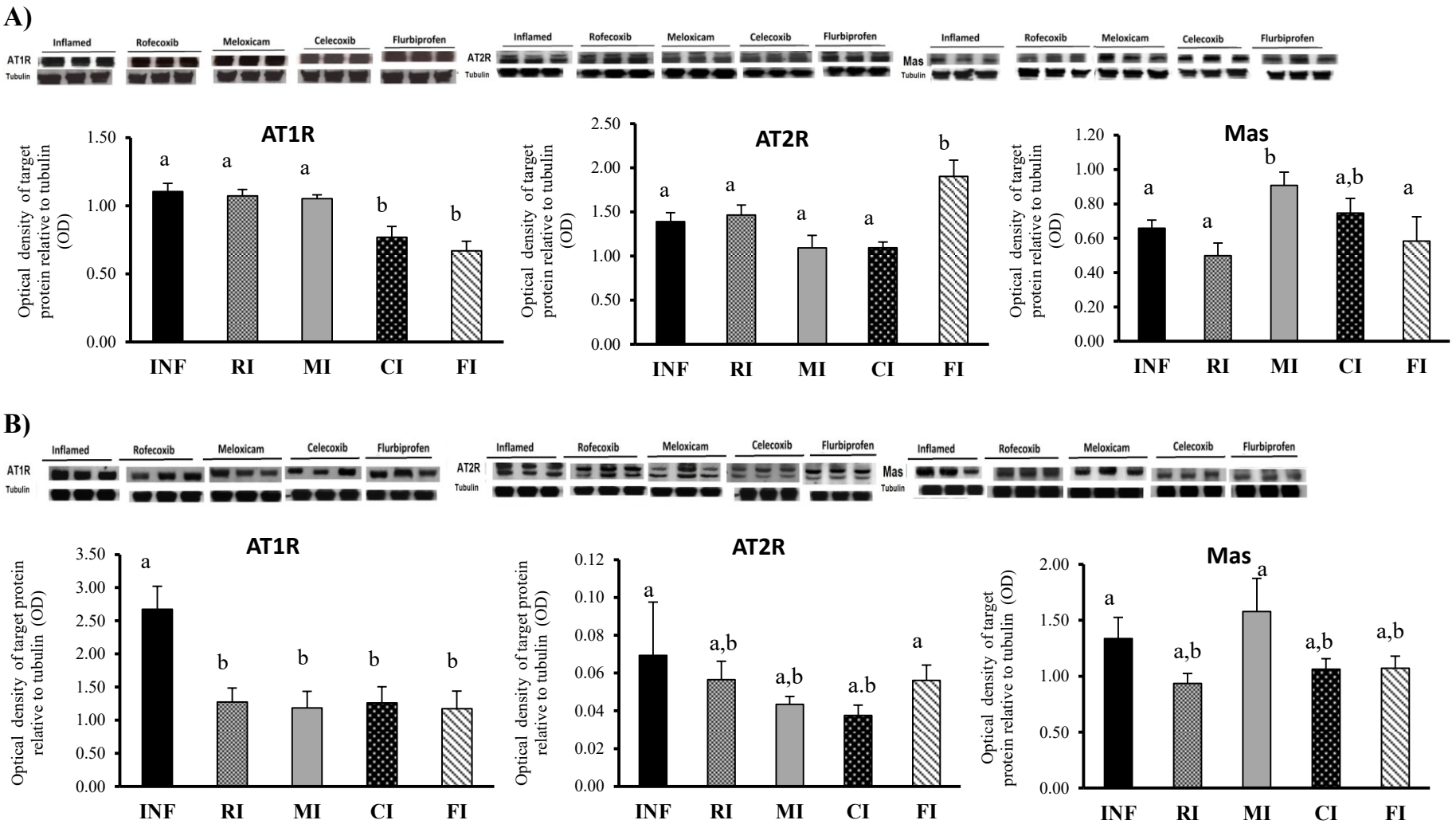


Figure 19: Densities of angiotensin receptors (AT1R, AT2R, Mas), presented as ratio of optical density (OD) of target protein/tubulin in heart (A) and kidneys (B) of inflamed and NSAIDs treated rats, n=3 each (INF=Inflamed not treated, RI=Inflamed treated with rofecoxib, MI=Inflamed treated with meloxicam, CI=inflamed treated with celecoxib, FI= inflamed treated with flurbiprofen), (a, b, c) represents significant difference b/w each other using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

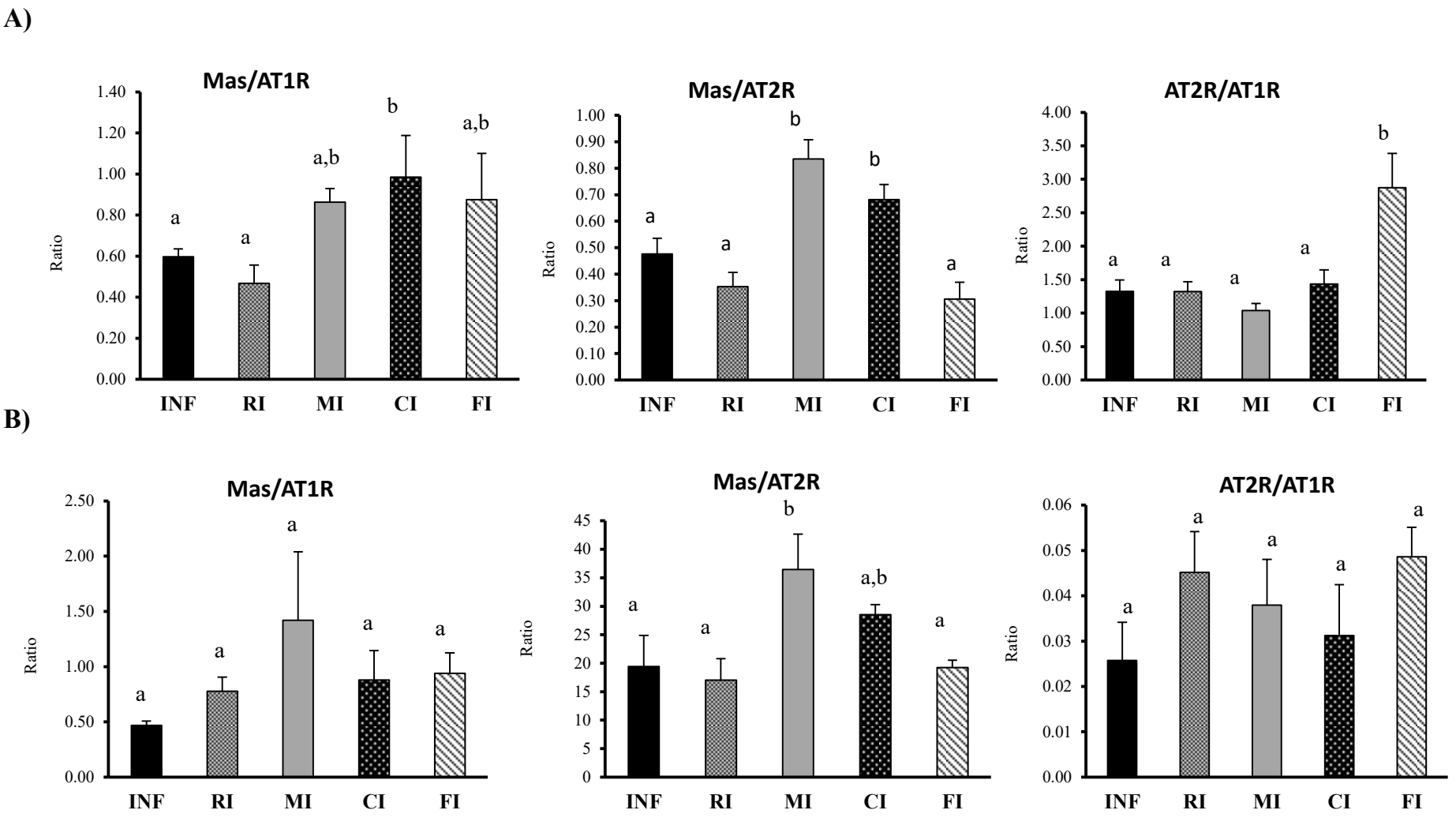


Figure 20: Ratios of densities of (AT1R, AT2R and Mas in heart (A) and kidneys (B) of inflamed and NSAIDs treated rats, n=3 each (INF=Inflamed not treated, RI=Inflamed treated with rofecoxib, MI=Inflamed treated with meloxicam, CI=inflamed treated with celecoxib, FI= inflamed treated with flurbiprofen), (a, b, c) represents significant difference b/w each other using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

Table 11: Effect of NSAIDs treatment on density of ACE, ACE2 and ACE2/ACE ratio in adjuvant arthritis rat heart and kidneys.

	ACE2	ACE	ACE2/ACE
Heart			
Control	1.12 ± 0.26	1.01 ± 0.13	1.11 ± 0.19
Inflamed	0.64 ± 0.09*	1.08 ± 0.23	0.62 ± 0.20*
Rofecoxib	1.20 ± 0.20†	1.14 ± 0.21	0.91 ± 0.06
Meloxicam	0.92 ± 0.13†	1.03 ± 0.11	0.90 ± 0.19
Celecoxib	1.20 ± 0.34	1.24 ± 0.30	0.96 ± 0.04
Flurbiprofen	1.06 ± 0.15†	1.08 ± 0.22	1.01 ± 0.21
Kidney			
Control	0.74 ± 0.07	0.87 ± 0.04	0.84 ± 0.05
Inflamed	0.50 ± 0.07*	0.74 ± 0.08	0.64 ± 0.10*
Rofecoxib	0.67 ± 0.08†	0.80 ± 0.08	0.84 ± 0.08
Meloxicam	0.61 ± 0.09	0.84 ± 0.16	0.73 ± 0.05
Celecoxib	0.61 ± 0.06	0.76 ± 0.07	0.84 ± 0.01
Flurbiprofen	0.59 ± 0.09	0.75 ± 0.02	0.78 ± 0.12

Mean ± SD of densities of target protein over beta-actin (loading control) (n = 3)

* Significantly different from control rats using Student's t-test ($p < 0.05$)

† Significantly different from inflamed using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

Table 12: Effect of NSAIDs treatment on Ang-(1-7), Ang-II concentrations (pg /mL, pg /g, and pg /g, respectively) and Ang-(1-7)/Ang-II ratio in adjuvant arthritis rat heart and kidneys.

Plasma	**Ang-(1-7)**	**Ang-II**	**Ang-(1-7)/ Ang-II**
Control	176.40 ± 35.62	37.43 ± 0.23	4.72 ± 0.98
Inflamed	236.29 ± 25.63	49.62 ± 3.29*	4.80 ± 0.84
Rofecoxib	269.42 ± 67.23	58.20 ± 8.11	4.61 ± 0.82
Meloxicam	241.31 ± 63.26	39.63 ± 5.98	6.02 ± 0.78
Celecoxib	300.37 ± 73.83	50.25 ± 4.17	5.93 ± 1.07
Flurbiprofen	206.22 ± 35.62	62.55 ± 6.51	3.29 ± 0.42
Heart			
Control	243.98 ± 48.80	40.92 ± 9.48	6.16 ±1.81
Inflamed	112.90 ± 22.21*	59.14 ± 5.20	1.92 ± 0.41*
Rofecoxib	309.18 ± 33.81†	69.94 ± 2.54	4.41 ± 0.34†
Meloxicam	248.94 ± 32.32†	48.91 ± 4.51	5.11 ± 0.39†
Celecoxib	230.47 ± 40.28†	54.33 ± 6.74	4.23 ± 0.40†
Flurbiprofen	207.23 ± 46.99	71.05 ± 8.14	2.90 ± 0.42
Kidney			
Control	8.16 ± 0.30	9.61 ± 1.88	0.87 ± 0.16
Inflamed	9.19 ± 0.57	14.99 ± 2.53	0.62 ± 0.07
Rofecoxib	8.42 ± 1.28	15.51 ± 3.33	0.55 ± 0.07
Meloxicam	8.42 ± 1.30	13.70 ± 1.47	0.62 ± 0.08
Celecoxib	8.07 ± 1.17	13.99 ± 1.03	0.58 ± 0.10
Flurbiprofen	9.49 ± 0.73	11.45 ± 1.48	0.84 ± 0.18

Mean ± SD of concerntrations (n = 3);

* Significantly different from control rats using Student's t-test ($p < 0.05$)

† Significantly different from inflamed using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

Table 13: Effect of NSAIDs treatment on density of AT1R, AT2R, Mas and their ratios in adjuvant arthritis rat heart and kidneys.

	AT1R	AT2R	Mas	Mas/AT1R	Mas/AT2R	AT2R/AT1R
Heart						
Control	0.87 ± 0.05	1.04 ± 0.08	0.81 ± 0.06	0.91 ± 0.05	0.78 ± 0.07	1.17 ± 0.06
Inflamed	1.11 ± 0.06*	1.39 ± 0.10*	0.66 ± 0.05*	0.60 ± 0.04	0.48 ± 0.06*	1.32 ± 0.17
Rofecoxib	1.07 ± 0.05	1.46 ± 0.11	0.50 ± 0.07	0.47 ± 0.09	0.35 ± 0.05	1.32 ± 0.15
Meloxicam	1.05 ± 0.03	1.09 ± 0.14	0.91 ± 0.08†	0.86 ± 0.07	0.83 ± 0.07†	1.04 ± 0.11
Celecoxib	0.77 ± 0.08†	1.09 ± 0.07	0.75 ± 0.09	0.98 ± 0.20†	0.68 ± 0.06†	1.44 ± 0.21
Flurbiprofen	0.67 ± 0.07†	1.90 ± 0.19†	0.58 ± 0.14	0.87 ± 0.23	0.31 ± 0.06	2.88 ± 0.51†
Kidney						
Control	0.96 ± 0.26	0.06 ± 0.01	0.73 ± 0.14	0.78 ± 0.40	12.08 ± 5.17	0.06 ± 0.01
Inflamed	2.68 ± 0.35*	0.07 ± 0.03	1.34 ± 0.19*	0.47 ± 0.04	19.42 ± 5.47	0.03 ± 0.01*
Rofecoxib	1.27 ± 0.21†	0.06 ± 0.01	0.94 ± 0.09	0.78 ± 0.13	17.01 ± 3.77	0.05 ± 0.01
Meloxicam	1.18 ± 0.25†	0.04 ± 0.001	1.58 ± 0.30	1.42 ± 0.62	36.45 ± 6.23†	0.04 ± 0.01
Celecoxib	1.26 ± 0.24†	0.04 ± 0.01	1.06 ± 0.09	0.88 ± 0.27	28.51 ± 1.79	0.03 ± 0.01
Flurbiprofen	1.17 ± 0.27†	0.06 ± 0.01	1.07 ± 0.11	0.94 ± 0.19	19.22 ± 1.30	0.05 ± 0.01

Mean ± SD of densities of target protein over tubulin (loading control) (n = 3), * significantly different from control rats using Student's t-test ($p < 0.05$), † significantly different from inflamed using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

Chapter 7

7. Analysis of cytochrome P450 metabolites of arachidonic acid in adjuvant arthritis rat plasma, heart and kidneys; search for a biomarker for NSAIDs cardiotoxicity

7.1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to treat the pain and swelling associated with inflammatory disorders such as rheumatoid arthritis (RA). NSAIDs exert anti-inflammatory, analgesic, and antipyretic effects mainly by blocking prostaglandin synthesis from its precursor arachidonic acid (ArA). ArA is a phospholipid component of the cell wall which is cleaved by the enzyme phospholipase and is made available for further metabolism by the enzymes cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) [360]. These enzymes convert ArA into lipid metabolites such as prostanoids (prostaglandins, prostacyclin, thromboxane), leukotriene, and eicosanoids, respectively [216]. These metabolites are biologically active compounds and play a vital role in renal, pulmonary, and vascular systems, as well as during inflammation [361, 362]. The COX and LOX pathways are well-characterized as both are targets for anti-inflammatory drugs. However, the CYP pathway received much less attention until it was discovered that the CYP metabolites of ArA have roles in regulating vascular tone [214] and renal [215] and heart functions [216].

It is well established that inflammation increases the risk of cardiovascular (CV) incidents and is involved in the pathogenesis and prognosis of CV diseases [202]. NSAIDs are used to treat the pain and inflammation associated with RA. However, long-term use of NSAIDs is found to increase the risk of CV/renal events [201]. Although the mechanisms behind such increase in CV/renal risks are not yet known, the CYP metabolites of ArA are suspected to be involved [17, 217]. CYP metabolites of ArA are also involved in homeostasis of many physiological and pathophysiological processes in the body, especially in the heart and kidney. Examples of CYPs involved in ArA metabolism are ω-hydroxylase and epoxidases which produce 20-hydroxyeicosatetraenoic acid (20-HETEs) and epoxyeicosatrienoic acids (EETs), respectively. These CYPs are widely expressed in the vasculature, heart, kidney, and liver where they produce respective eicosanoids in a tissue-specific manner [Figure 21]. A balanced production of CYP metabolites is essential for controlling the function of the body locally and systemically.

Members of the CYP4A subfamily were the first to be discovered in the ω-hydroxylation of ArA. A group of hydroxylases—CYP4A, 4B, and 4F—convert ArA into 20-

hydroxyeicosatetraenoic acids (20-HETE) [363]. These CYPs are expressed in the vasculature and renal arterioles, where they produce the low quantities of 20-HETE that are required for its uricosuric function in the kidney [364]. An over-expression of CYP4A and elevated levels of 20-HETE are reported in hypertension, congestive heart failure, myocardial infarction (MI), diabetes, and inflammatory disorders [363]. Studies have shown that a high concentration of 20-HETE results in endothelial dysfunction, diminished vasodilatory response of vascular smooth muscle cells to acetylcholine, higher oxidative stress, promotion of platelet aggregation, and high blood pressure which can result in CV incidents [365].

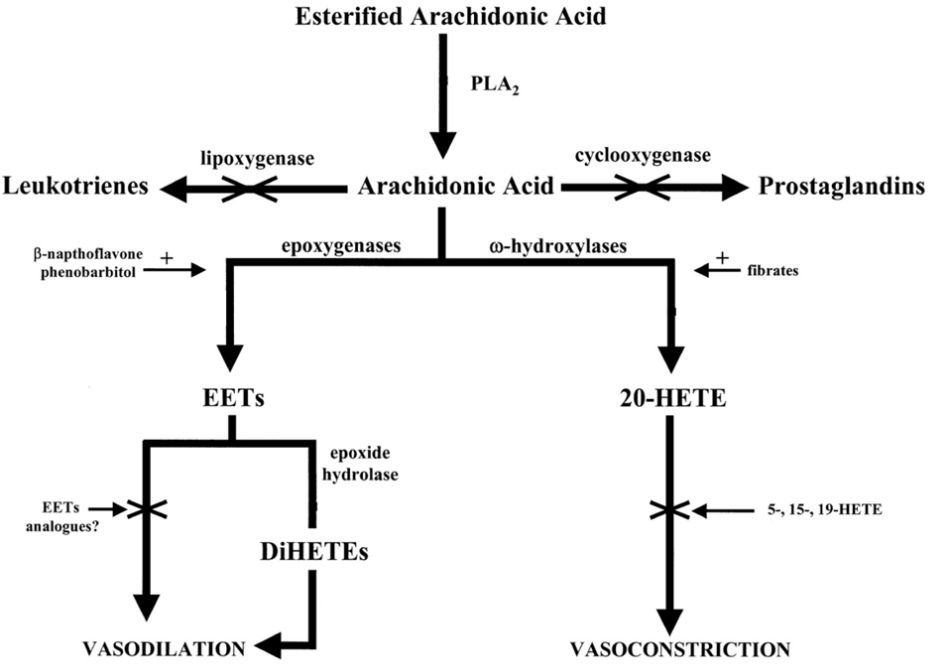


Figure 21: Pathways for the metabolism of arachidonic acid (with permission from [366]).

The biological significance of CYP metabolites was unknown until recently, when it was discovered that reducing the activity of CYP4A reduces blood pressure in spontaneously hypertensive rats [367]. This led to the discovery that 20-HETE is a potent vasoactive eicosanoid involved in maintaining vascular tone and blood pressure homeostasis [368]. 20-HETE induces vasoconstriction in blood vessels by inhibiting the conductance of calcium-activated potassium channels, resulting in the depolarization of vascular smooth muscle cells [220]. The myogenic response to 20-HETE is observed in blood vessels all over the body, specifically in arterioles that supply to skeletal muscles, and the cerebral, renal, and mesenteric system [221]. 20-HETE also carries myogenic and angiogenesis signals, which suggests roles in cell proliferation and cell

growth [369]. In the kidney, 20-HETE acts in a dual manner. In the kidney cortex, it is involved in auto regulating blood flow to the nephrons by exerting vasoconstrictive effects [370]. In the kidney medulla, 20-HETE exerts uricosuric effects by promoting the excretion of electrolytes and increasing urine volume. 20-HETE blocks the (Na+)/(K+)-ATPase, an active transporter involved in electrolyte reabsorption and found in the proximal tubules, and also inhibits (K+) channels in the ascending loop of Henle, thus limiting the availability of (K+) for the (Na^+K^+2CL) transport involved in electrolyte and water reabsorption in the collecting tubules [371].

Epoxygenases, including CYP 1A, 2B, 2C, 2D, 2E, and 2J, convert ArA into four regioisomers: 14,15-, 11,12-, 8,9-, and 5,6-EETs [216]. 14,15-EETs and 11,12-EETs are predominant regioisomers in most tissue and account for 67–80% of the total EETs produced by CYP epoxygenases in rats [372]. 14, 15-EETs accounts for 41% of EETs in rat hearts [373], and 11, 12- EETs accounts for 58% of EETs in the kidney [374]. Epoxygenases are expressed in vascular endothelium, and in coronary and renal arterioles. EETs are known for their potent vasodilator, anti-inflammatory, and anti-mutagenic effects, as they increase the open state of large conductance calcium-dependent potassium channels, hyperpolarizing vascular smooth muscle [222].

CYP2J2 is the major enzyme responsible for the extrahepatic production of EETs in humans and is widely expressed in the extrahepatic tissues such as heart [375], kidney, proximal tubules, collecting ducts [376], gastrointestinal tract [377], and lung [378]. Wu *et al.,* reported that CYP2J2 is highly expressed in the heart in humans and mice [375]. CYP2J2 produces equal amounts of 14,15-, 11,12-, 8,9-, and 5,6-EETs [375]. These EETs serve a dual purpose in the heart. First EETs inhibit the contractile activity of VSMC by blocking sodium channels [379] and L-type calcium channels, resulting in vasodilatation [380]; second, EETs enhance the recovery of ventricular repolarization through activation of K+ channels, thus limiting the infarct size and conferring protection under ischemic conditions [381]. It is speculated that EETs play a role in mitochondria by significantly improving the viability of starved cardiac cells [382]. In the cardiovascular (CV) system EETs lower blood pressure, reduce infarct size [383] and repair damaged tissue [223], and confer cardio protection [223].

CYP2C11 is the predominantly expressed epoxygenase in rat kidney. EETs produced by CYP2C11 inhibit sodium transport in the kidney up to 50%, thus reducing renal function [384]. Loss of sodium excretion is a typical side effect of nonsteroidal anti-inflammatory drugs (NSAIDs). In the pancreas, EETs regulate the secretion of insulin and glucagon, thus they might have a role in diabetes linked complications [385]. CYP2C9 is the major enzyme involved in EET production in the liver, with 14,15-EETs as the predominant product [386].

EET metabolism involves hydrolysis by soluble epoxide hydrolases (sEH) or microsomal epoxide hydroxylases (mEH) [373] which convert EETs into corresponding dihydroxyeicosatrienoic acids (DHETs) [387]. DHETs possess similar but little physiological activity compared to their respective EETs [388]. The only exception is in the coronary microvasculature where EETs and DHETs are reported to be equipotent [389]. Under disease conditions such as hypertension, sEH expression is upregulated resulting in more DHETs, as seen in rat kidney. Also, sEH inhibitors have been found to reduce blood pressure by increasing EET concentrations in spontaneously hypertensive rats [390].

Evidence suggests that EETs confer cardio-protection through anti-inflammatory, antihypertensive, anti-mutagenic properties [375]. Whereas, 20-HETE is a proinflammatory mediator with vasoconstrictive, thromgogenic and mutagenic properties [373], these can lead to increased CV risks [373, 391]. Studies have shown that a balanced production of metabolites from both epoxygenases and hydroxylases is required to maintain CV homeostasis. Studies have reported an altered eicosanoids profile in cardiac disorders such as hypertension [392], ischemia and infarction [393] and inflammation [394] indicating its involvement in CV incidents. In animals, the pharmacological inhibition or deletion of the sEH gene, which is responsible for degradation and elimination of EETs, can limit the infarct size [223, 395], improve post ischemic functional recovery [396], and provide antiarrhythmic effects, perhaps due to higher EETs concentrations [397].

ArA metabolites being lipid derived metabolites have a lipophilic nature and concentrate in tissue rather than plasma exerting their effects in an autocrine manner. Studies have shown that these metabolites when introduced systemically they bind to plasma proteins, get esterified and taken up by the cells. This is the reason that in vitro administration of eicosanoids worked, and in vivo administration of the same eicosanoids failed to produce the expected physiological effects [224]. This emphasizes the significance of local eicosanoid production to understand their organ-specific effects. Eicosanoids, once formed, are rapidly incorporated into membrane phospholipids. High concentrations (milli molar range) of EETs have been extracted from kidney, platelets, vascular smooth muscle, heart, and brain [398]. These lipid-bound eicosanoids act as a local reservoir and are released in response to vasoactive hormones and other stimuli. Bradykinins produced during inflammation are reported to stimulate the release of EETs from phospholipid stores [399]. Angiotensin (Ang)-II, is also reported to increase the release of 16-, 17-, 18-, 19-, and 20-HETEs in renal vessels in isolated perfused kidney. 20-HETE inhibitors when given in vivo did not result in inhibition of 20-HETE effects, as seen in vitro in organ infusion studies [400]. This

suggests that eicosanoid reservoirs don't deplete immediately after the administration of inhibitors. This concept might explain the delayed CV adverse effects as seen with long-term use of NSAIDs.

The prolonged use of NSAIDs, particularly rofecoxib, is suspected to be linked to higher CV incidents. Epidemiological studies have suggested a higher rate of MI and heart failure in rofecoxib users [107]. NSAIDs are known to cause reduced excretion of urinary electrolytes and to reduce urine volume; however, these effects are not universal for all NSAIDs. Some recent studies have suggested that ArA metabolites might be involved in the development of CV/renal risks reported in NSAIDs treated patients [217]. However, there was a lack of discrimination regarding which NSAIDs exhibit such effects and what mechanisms are involved, especially under a disease state. Therefore, it is important to understand how NSAIDs and inflammation affect the normal heart and kidney function in patients suffering from RA.

7.2. Objectives

The present study investigates the effect of long-term NSAIDs exposure on the CYP metabolism of ArA in the plasma, heart, and kidney of adjuvant arthritis (AA) rats. We investigate whether these eicosanoids present a potential CV/renal risk reported for different NSAIDs and if plasma concentration of any of these eicosanoids can be used as a biomarker for NSAIDs induced cardiotoxicity.

7.3. Hypotheses

- NSAIDs disrupt the CYP-mediated metabolism of ArA.
- NSAIDs differentially influence the ArA profile which parallels with their CV/renal risks reported in clinical trials.
- The plasma eicosanoid profile can serve as a reliable biomarker to predict the cardiotoxicity of NSAIDs.

7.4. Methods

Materials used in this study, selection of NSAIDs, dose calculation, animal handling, sample collection, and method of analysis are discussed in detail in section 3.1 and 3.2.

7.5. Results

We measured constitutive levels of eicosanoids in rat plasma, heart, and kidney [Tables 14, 15, and 16] and made two type of comparison 1) between inflamed AA rats and healthy control rats, 2) between inflamed AA rats and NSAIDs treated inflamed rats. In the present study, inflammation altered the ArA pathway consequently changing the balance between anti-inflammatory and pro-

inflammatory eicosanoids in AA rats. We also found very strong correlations between plasma levels of eicosanoids and levels in heart and kidney tissues in arthritic rats. These results are discussed in detail in coming sections.

7.5.1. ArA metabolites in rat plasma

We found that 20-HETE levels were significantly higher in the plasma of inflamed adjuvant arthritis (AA) rats, compared to healthy controls (p=0.04) [Figure 22] [Table 14]. In case of EETs, there was no difference in total EETs levels in the plasma between inflamed and control rats. Individually, neither 11, 12- nor 8,9-EET was changed in inflamed rats plasma, however, 14,15-EET was significantly lower in inflamed group compared to controls (p=0.03) [Figure 24][Table 15]. The DHET metabolites also showed the same trend, with no difference in total DHET, 11,12- and 8,9-DHET levels between inflamed and controls. Only exception was 14, 15-DHET which was significantly high in inflamed rat plasma (p=0.04) [Figure 22, 23] [Table 16].

NSAIDs treatment also altered the plasma profile of ArA metabolites in AA rats. Such that the plasma levels of 20-HETE in rofecoxib and flurbiprofen treated rats were found to be significantly higher compared to inflamed rats (p=0.03 and 0.02, respectively) [Figure 23] [Table 14].In case of EETs, all the NSAIDs lowered the plasma levels of total EETs compared to inflamed rats, but was only rofecoxib treated rats levels were significantly different compared to inflamed rats (p=0.03) [Table 15]. Individually, the plasma levels of 14,15- EET in meloxicam and celecoxib treated rats were rather significantly higher than inflamed rats (p=0.04, 0.03, respectively). But the plasma levels 11,12- EET were significantly lower in all NSAIDs compared to inflamed rats. Same was the trend for 8,9- EET levels in NSAIDs treated rats plasma, however, it was not statistically significant [Figure 23, 25] [Table 15].

Total DHET levels in meloxicam and celecoxib treated rats plasma was significantly lower compared to inflamed rats (p=0.03, 0.02, respectively), while it was comparable in rofecoxib and flurbiprofen treatments. Individually, 14,15-DHET levels in celecoxib and flurbiprofen treated rats plasma were significantly lower from inflamed rat, (p=0.03, 0.03, respectively), but were comparable in rofecoxib and meloxicam treatments. Other DHET were also lower in NSAIDs treated rats plasma but no statistical significance was found when compared with inflamed rat [Figure 23, 25] [Table 16].

When put together as cardiotoxic/cardioprotective ArA metabolites ratios, the 20-HETE/total-EET and 20-HETE/14,15-EET were significantly high in inflamed rats plasma compared to same in control rats. While treatment with rofecoxib and flurbiprofen resulted in even

higher 20-HETE/total-EET and 20-HETE/14,15-EET significantly compared to inflamed rats. This was not observed for meloxicam and celecoxib treatments [Figure 26, 27].

7.5.2. ArA metabolites in rat heart

The 20-HETE levels in the hearts of inflamed rats were found to be significantly higher than healthy controls *(p*=0.03) [Figure 22] [Table 14]. But the total, 14,15-, 11,12- or 8,9- EET were comparable between inflamed and control rats hearts [Figure 27, 29[Table 15]. Similarly, the total, 14,15-, 11,12- and 8,9-DHET levels were not different in inflamed hearts from that in control rats heart [Figure 22, 23][Table 16].

NSAIDs treatment resulted in lower 20-HETE levels in the AA rats heart compared to inflamed rats, but only meloxicam and celecoxib treatments brought it down significantly *(p*=0.02 and 0.02, respectively) [Figure 23][Table 14]. All NSAIDs significantly lowered the total EETs levels in heart compared to inflamed rats with the exception of their levels in meloxicam treated rats which were comparable to inflamed rats hearts. Individually, NSAIDs treatment had no effect on levels of 14,15-EET in the heart comparable to inflamed rats. For 11,12- and 8, 9- EETs their levels in heart were significantly lowered by NSAIDs treatment compared to inflamed rats [Figure 30][Table 15]. All NSAIDs lowered the total DHET levels in the heart compared to inflamed rats. Same was observed for 11,12- ,8,9-DHET in NSAIDs treated rats compared to inflamed group, but no statistical significance was found for 14,15- DHET [Figure 23, 25][Table 16].

When put together as cardiotoxic/cardioprotective ArA metabolites ratios, the 20-HETE/total-EET, 20-HETE/14,15- EET were significantly high in inflamed rats heart compared to controls. For rofecoxib and flurbiprofen treated rats the 20-HETE/total-EET ratios were significantly higher compared to inflamed rats, this was not observed for meloxicam and celecoxib treatments. The 20-HETE/14,15-EET was comparable in NSAIDs treated rats heart and inflamed rats, with exception of celecoxib, which was significantly higher compared to inflamed rats [Figure 26, 27].

7.5.3. ArA metabolites in rat kidney

In the kidneys, the 20-HETE levels were significantly lower in inflamed rats compared to healthy controls (*p*=0.02) [Figure 22] [Table 14]. Total EETs levels were comparable in the kidneys of inflamed and control rats [Figure 22][Table 15]. The DHET metabolites were also comparable, with no difference between inflamed and control rats kidney [Figure 22] [Table 16].

NSAIDs treatment increased the 20-HETE levels in kidneys compared to inflamed rats, however, no statistical significance was observed. 20-HETE levels in meloxicam treated rats kidney

were the highest among other NSAIDs [Figure 23] [Table 14]. In case of EETs, all the NSAIDs lowered the kidney levels of total EETs compared to inflamed rats. Individually, no significant difference was found between total, 11,12- and 8,9-EETs in the kidneys of NSAIDs treated rats compared with inflamed rats. However, 14, 15-EET levels in the NSAIDs treated rats kidney were significantly lower compared to inflamed rats, with exception of flurbiprofen which had no effect [Figure 23, 25][Table 15]. Similarly, total, 14,15-, 11,12-, 8,9-DHET levels were not different in the kidneys of NSAIDs treated rats compared to inflamed rats, with exception of flurbiprofen which has high 14, 15-DHET levels in the kidney [Figure 24,25][Table 16].

The 20-HETE/total-EET and 20-HETE/11,12-EET were significantly low in the kidneys of inflamed rat compared to controls. All NSAIDs showed a trend towards an increase in these ratios, but only meloxicam was able to significantly increase it compared to inflamed rats. 20-HETE/14,15-EET was significantly higher in meloxicam, rofecoxib and celecoxib treated rats kidney compared to the same in inflamed rats kidney [Figure 26, 27].

7.5.4. Association between plasma and tissue ArA metabolites

We found a trend towards positive correlation between plasma and heart 20-HETE levels, whereas a negative correlation was observed between plasma and kidney 20-HETE levels. Individually the 14,15-, 11,12- and 8,9-EET concentrations in the plasma did not correlate well with the same in the tissues, but when added together as total EETs it was positively correlated with the total EETs levels in the heart ((r = 0.795, p = 0.03). When made into 20-HETE/14,15-EET, 20-HETE/11,12-EETs and 20-HETE/T-EETs the plasma ratios correlated well with the heart ratios (r = 0.793, p = 0.03; r = 0.813, p = 0.02 and r = 0.874, p = 0.003, respectively).No such relationship was found for these ratios in the kidney [Table 17].

7.6. Discussion

One of the most important findings in our study is that systemic inflammation has tissue dependent effects on the production of 20-HETE levels in the body. 20-HETE levels were increased in plasma and heart but were reduced in the kidney. Increased 20-HETE levels in the heart may cause vasoconstriction, an increase in blood pressure, and a higher thrombotic state, all of which can lead to a CV incident [373] [373, 391]. Several studies identified 20-HETE as potent pro-inflammatory mediator and considered it as indices of inflammation, in line with our findings [218, 219].

In the kidney tissues, however, a lower level of 20-HETE, in response to inflammation, is linked with reduced renal function. Because in the kidney, 20-HETE has uricosuric effects, that is, it is involved in excretion of electrolytes and in reducing the fluid overload; thus lower 20-HETE in

the kidney reflects a loss of renal function[401, 402]. 20-HETE plays a dual role in the kidney—as a pro- and antihypertensive agent. 20-HETE exerts vasoconstrictive effects on glomerular blood vessels as part of the tubuloglomerular feedback response [17], which is one of several mechanisms that the kidney uses to regulate the glomerular blood flow and filtration rate. Studies have found that perfusion of the loop of Henle with exogenous ArA intensified the tubuloglomerular feedback response, whereas, the tubuloglomerular feedback response was blocked by inhibition of 20-HETE production through CYP inhibition [371].In the proximal tubule, 20-HETE reduces sodium transport, and in the thick ascending loop of Henle it limits the availability of K+ for transport by a Na+-K+-2Cl- transporter, which enhances natriuresis. Thus, in the proximal tubule, 20-HETE acts as an antihypertensive and tissue protective agent [403, 404]. The significantly decreased level of 20-HETE in inflamed animals could be an indication of a loss of natriuresis, which would have a pro-hypertensive effect. 20-HETE function as pressure natriuretic response in the kidney which inhibits Na+ transport in the renal tubule, when blood pressure is high, thus increasing Na+ excretion, increase urine volume and reduced blood pressure [403, 404]. In support of our results, there are several reports indicating that a deficiency of 20-HETE production in the kidney results in the development of salt-sensitive hypertension [404-406]. Williams et al. provided evidence to support the hypothesis that lower renal production of 20-HETE contributes to the elevation of Cl^- transport in the loop of henle and the development of hypertension [407].

It has been reported that 11,12- and 14,15-EETs are the main EETs produced in rabbit kidney and rat pre-glomerular microvessels [370, 408], and that only 14,15- and 11,12-EETs caused significant vasodilation in isolated perfused rabbit kidney [409] and in rat pre-glomerular microvessels of a juxtamedullary nephron preparation [410]. Consistent with these findings, Arima et al. reported that 11,12-EETs dilated rabbit pre-constricted renal artery [411]. However, in another study, 11,12-EETs, 14,15-EETs, and their hydrated products (DHETs) acted as vasoactive agents and induced relaxation in bovine adrenal artery [412]. In this study we found overall reduced concentrations of EETs, particularly 14,15-EETs and 11,12-EETs, in inflamed plasma and heart of AA rats. This indicates that in inflammatory conditions with higher plasma 20-HETE concentrations, a lower EET concentration can mediate vasoconstriction and cause hypertension. Lower 14,15-EETs and 11,12-EETs suggest a compensatory mechanism for blood pressure homeostasis in kidney [Table 15]. All NSAIDs tend to mitigate the effects of inflammation and accordingly bring 20-HETE levels down and increase EET levels to a normal level. We found that some NSAIDs affected renal and cardiac systems more than other members of the class. This may be due to differences in NSAID physiochemical characteristics.

We found significant correlations between eicosanoid levels in plasma and those in heart and kidney in AA rats, suggesting that the plasma concentration of ArA metabolites can predict the concentration of vasoactive metabolites in the heart and kidney. If this relationship is validated, the plasma profiling of ArA metabolites can be used as a surrogate biomarker for eicosanoid changes in the heart during inflammation as well as during NSAIDs treatment. In fact, our findings correlate with the epidemiological findings of NSAIDs' CV risk. Thus, measuring the plasma ratio can give us an idea of changes that are happening in the heart and can be used to gauge NSAIDs induced cardiotoxicity. A significant positive correlation was observed between 20-HETE/14,15-EET, 20-HETE/11,12-EET and 20-HETE/total-EET ratios in plasma and heart, (r = 0.793, *p* 0.03; r = 0.0.813, *p* 0.02; r = 0.874, *p* 0.003, respectively) [Table 17]. This emphasize the usefulness of these plasma ratios as an indicator of the same ratios in the heart; the above three ratios correlate positively between the plasma and the heart. Such a relationship was lacking for these ratios in kidney, as in kidney both the 20-HETE (uricosuric) and EETs (protective) have bifacial effects. Thus instead of eicosanoid ratios, we calculated the correlation between plasma and kidney concentrations of 20-HETE and EETs. We found that 20-HETE, 14,15-EETs, and 14,15-DHETs concentrations correlate negatively between plasma and kidney (r = -0.788, *p* 0.03; r = 0.751, *p* 0.05; r = 0.799, *p* 0.02, respectively) [Table 17]. ArA metabolites in plasma and heart were positively correlated, but the correlation of ArA metabolites between plasma and kidney was negative. This discrepancy could be explained by different systemic and local expressions of CYP and tissue-dependent physiological effects of ArA metabolites.

Previously in our laboratory, a direct relationship was observed between renal function and concentration of NSAIDs in rat kidney tissue. Rofecoxib, celecoxib, but not meloxicam significantly reduced the urinary excretion of electrolytes, indicating renal failure. This finding was in line with the extent of tissue distribution of these NSAIDs. Rofecoxib and celecoxib were more concentrated in the kidney, with a higher kidney to plasma ratio than meloxicam. It was concluded that the extent of tissue drug exposure has a role to play in kidney function. Exposure dependent toxicity is not limited to NSAIDs, other classes of drugs such as cyclosporine-A [297, 298], gentamycin [299], and other aminoglycosides [300, 301] have also exhibited a relationship between high tissue accumulation and high tissue specific toxicity. A high tissue concentration of gentamycin in a deep tissue compartment was associated with lower creatinine clearance and nephrotoxicity [299]. This suggests the importance of measuring the extent of tissue drug exposure relative to plasma concentration when studying the adverse effect profiles of NSAIDs [289]. In the present study we report a similar trend in heart tissue exposure to NSAIDs which might be linked to

high CV related adverse effects in rofecoxib and flurbiprofen users but not in meloxicam users [209].

Epidemiological studies have reported differences in cardiorenal toxicities of NSAIDs independent of COX-2 selectivity; recently, even nonselective NSAIDs were found to adversely affect CV and renal function as well [93, 152, 153]. Davies and Jamali have suggested that physiochemical properties and pharmacokinetic differences among NSAIDs might play a role in deterring the high CV risk associated with rofecoxib [104]. We suggest that tissue distribution of NSAIDs at least in part plays a role in determining how these NSAIDs affect the molecular mechanisms such as the ArA pathway involved in the functioning of an organ. The observed changes in ArA systems and a subsequent change in the balance between cardiotoxic and cardioprotective metabolites are well correlated with tissue to plasma ratios of NSAIDs. In the present study we found that differences exist in CYP metabolites in the ArA profile of NSAIDs treated rats. That is, the observed 20-HETE/total-EETs ratios in the plasma and heart of AA rats correspond with epidemiological studies that show that rofecoxib and flurbiprofen have higher, and celecoxib and meloxicam have lower, CV risks in the population [Figure 28] [Figure 29].

This suggest that levels of ArA metabolites in the plasma can indicate the CV/renal risks associated with NSAIDs which concentrate more in the tissue than in the plasma. Thus blood is a useful biological medium to identify changes in the ArA metabolite profile in the heart in response to NSAIDS exposure. If carefully extrapolated from animal to human, these findings can help to identify high risk patients and increased CV/renal risks during the course of NSAIDs treatment.

7.7. Conclusion

We observed that inflammation alters the CYP metabolites of arachidonic acid such that it results in higher 20-HETE but lower EETs in plasma and heart of adjuvant arthritis rats compared to controls rats. This indicates higher cardiotoxicity potential during inflammation from higher 20-HETE levels and loss of protective effects of EETs. However, the lower 20-HETE levels in the kidney suggest a loss of its uricosuric effects and loss of renal function. Together they may contribute towards higher CV/renal risks as reported in inflammatory disorders.

NSAIDs treatment in inflamed rats resulted in lowering of 20-HETE levels in plasma and heart. Moreover, rofecoxib and flurbiprofen, but not meloxicam and celecoxib, also lowered 11,12-EET and 14,15-EET concentrations in NSAIDs treated adjuvant arthritis rats. Interestingly, the effects of NSAIDS on the plasma eicosanoid profile corresponded well with NSAIDs effects on the heart metabolites profile, thus we can say that plasma is a suitable medium to depict heart eicosanoids profile. Also, the observed 20-HETE/total-EETs showing higher ratio for rofecoxib and

flurbiprofen, corresponds agreed with available epidemiology data, suggesting that rofecoxib and flurbiprofen are associated with higher risk of cardiovascular incidents, perhaps due to changes in these metabolites. We suggest that plasma concentration of arachidonic acid metabolites are good predictors of CV risk associated with NSAIDs use and it can be used to optimize NSAID treatments and identify high-risk individuals.

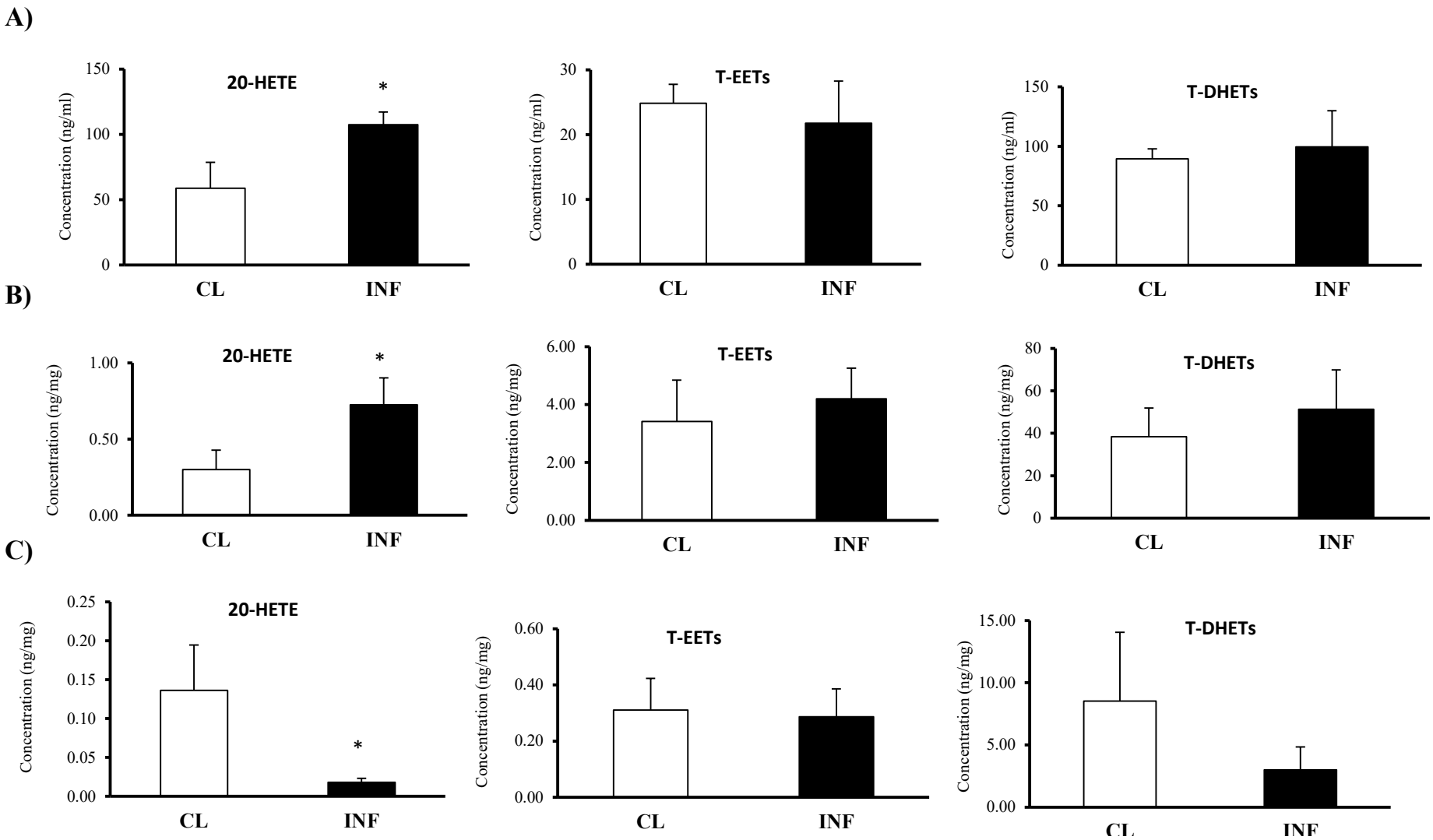


Figure 22: Concentrations of 20-HETE, total EETs, and total DHETs, in plasma (A) , heart (B) and kidneys (C) of control and inflamed rats, n = 3 each (CL=Control, INF=Inflamed not treated), *significantly different from control rats using Student's t-test ($p < 0.05$), (Ali Aghazadeh-Habashi, W. Asghar and F. Jamali.[359]).

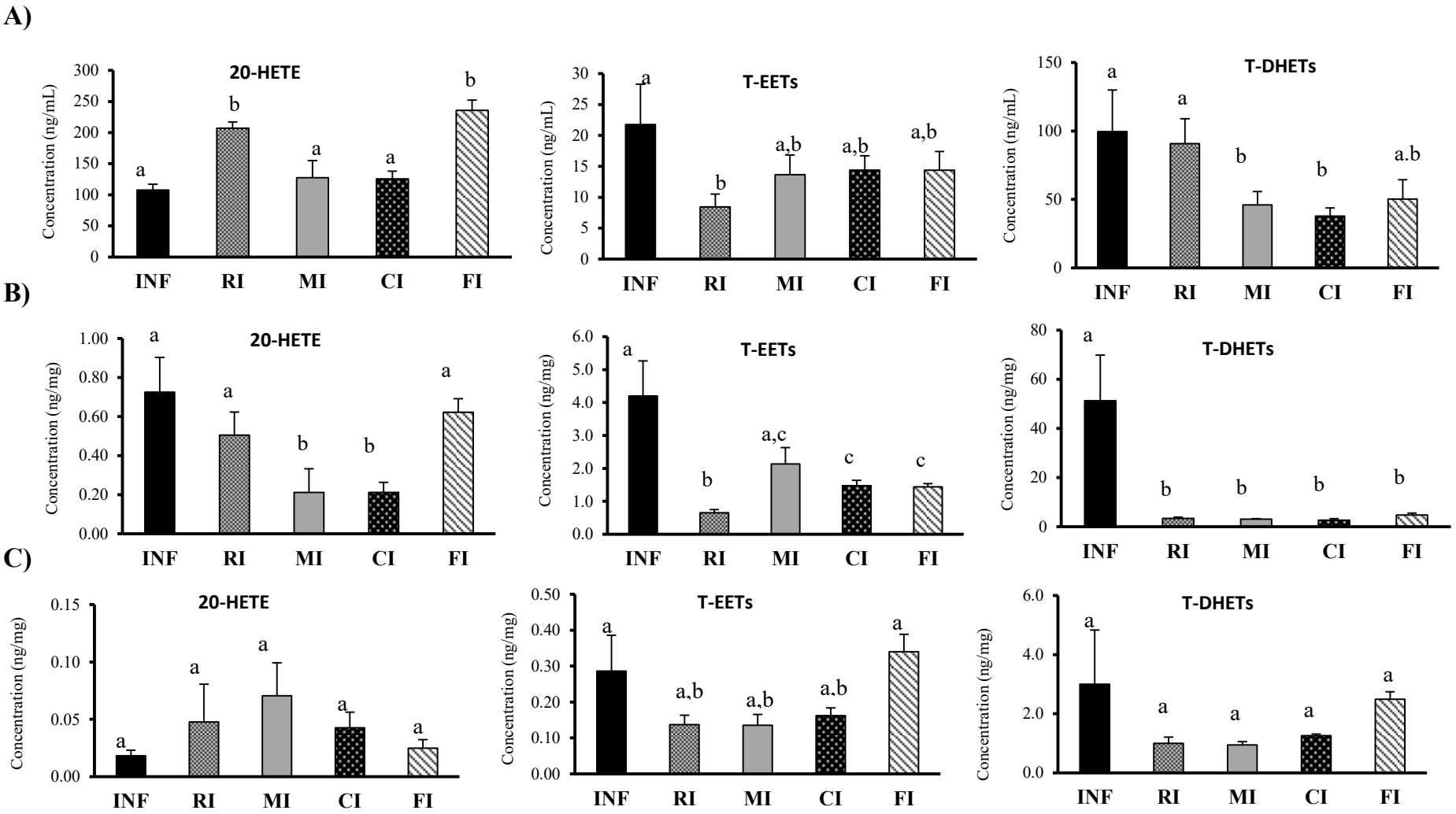


Figure 23 : Concentrations of 20-HETE, total EETs, and total DHETs in plasma (A) , heart (B) and kidneys (C) of inflamed and NSAIDs treated rats, n=3 each (INF=Inflamed not treated, RI=Inflamed treated with rofecoxib, MI=Inflamed treated with meloxicam, CI=inflamed treated with celecoxib, FI= inflamed treated with flurbiprofen)**,** (a, b, c) represents significant difference b/w each other using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

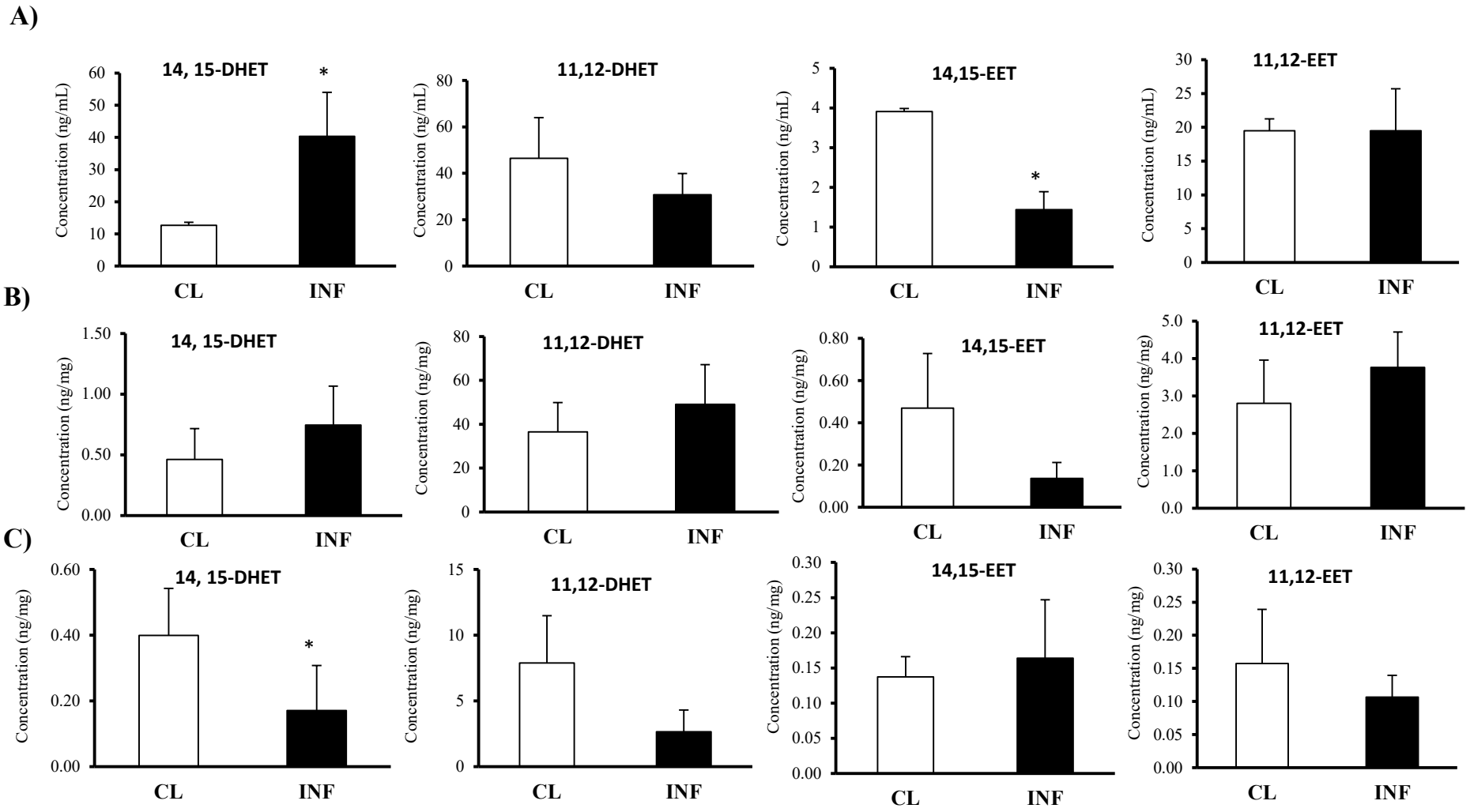


Figure 24: Concentrations of 14,15-EETs, 14,15-DHETs, 11,12-EETs, and 11,12-DHETs in plasma (A) , heart (B) and kidneys (C) of control and inflamed rats, n = 3 each (CL=Control, INF=Inflamed not treated), *significantly different from control rats using Student's t-test ($p < 0.05$), (Ali Aghazadeh-Habashi, W. Asghar and F. Jamali.[359]).

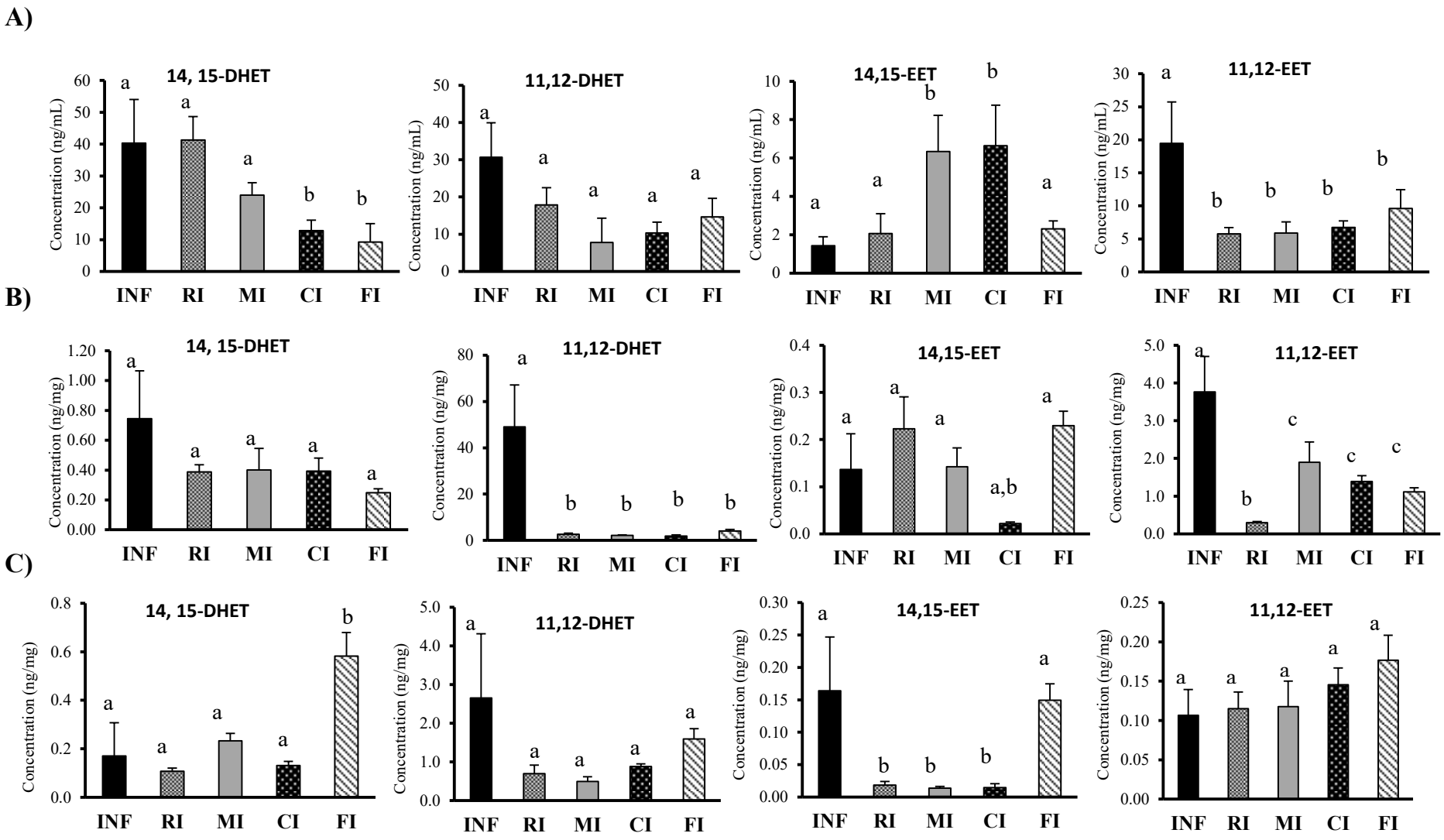


Figure 25: Concentrations of 14,15-EETs, 14,15-DHETs, 11,12-EETs, and 11,12-DHETs in plasma (A) , heart (B) and kidneys (C) of inflamed and NSAIDs treated rats, n=3 each (INF=Inflamed not treated, RI=Inflamed treated with rofecoxib, MI=Inflamed treated with meloxicam, CI=inflamed treated with celecoxib, FI= inflamed treated with flurbiprofen), (a, b, c) represents significant difference b/w each other using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

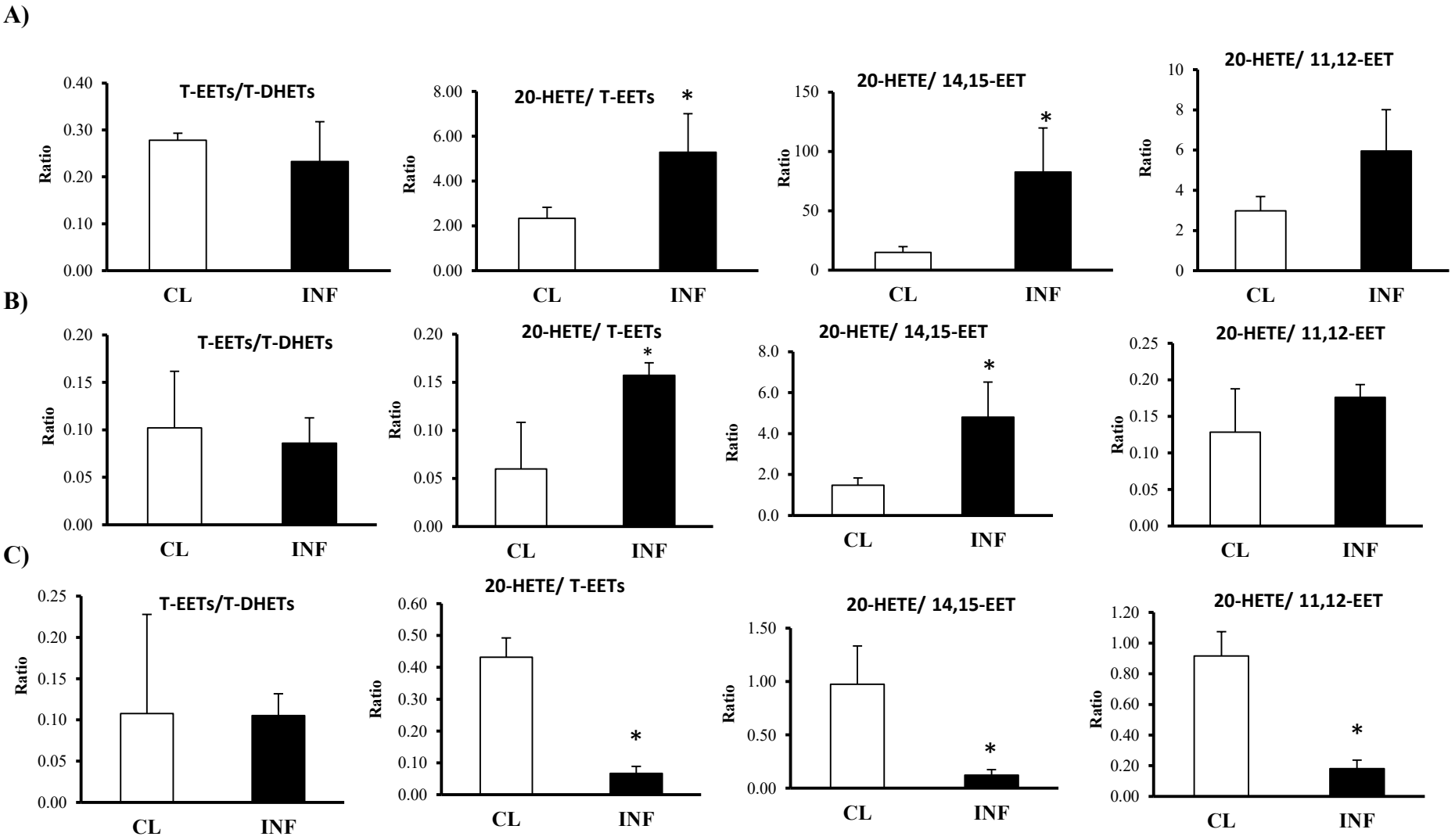


Figure 26: T-EETs/T-DHETs, 20-HETE/T-EETs, 20-HETE/14,15-EET and 20-HETE/11,12-EET ratio in plasma (A) , heart (B) and kidneys (C) of control and inflamed rats, n = 3 each (CL=Control, INF=Inflamed not treated), *significantly different from control rats using Student's t-test ($p < 0.05$), (Ali Aghazadeh-Habashi, W. Asghar and F. Jamali.[359]).

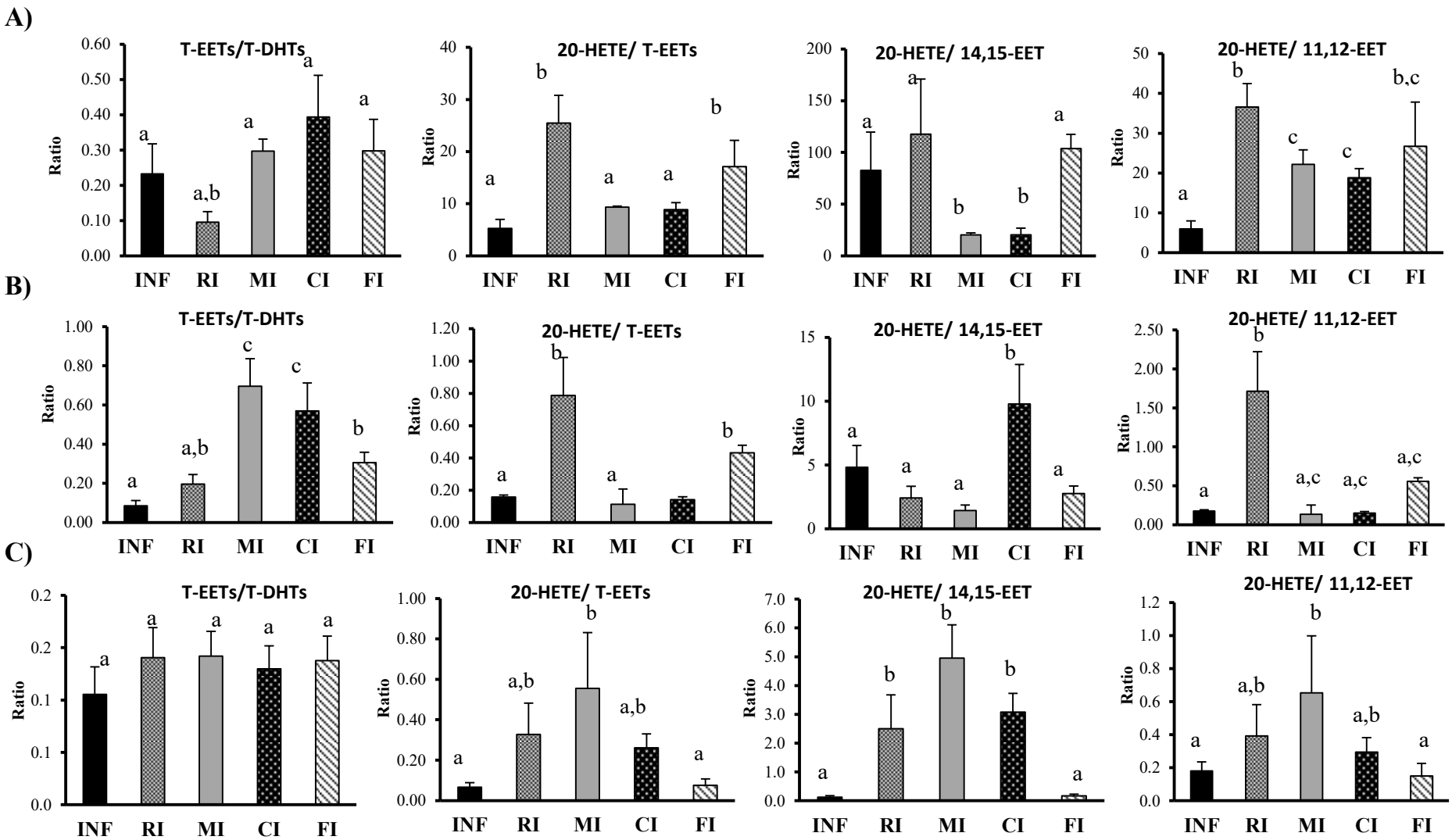


Figure 27: T-EETs/T-DHETs, 20-HETE/T-EETs, 20-HETE/14,15-EET and 20-HETE/11,12-EET ratio in plasma (A) , heart (B) and kidneys (C) of inflamed and NSAIDs treated rats, n=3 each (INF=Inflamed not treated, RI=Inflamed treated with rofecoxib, MI=Inflamed treated with meloxicam, CI=inflamed treated with celecoxib, FI= inflamed treated with flurbiprofen), (a, b, c) represents significant difference b/w each other using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

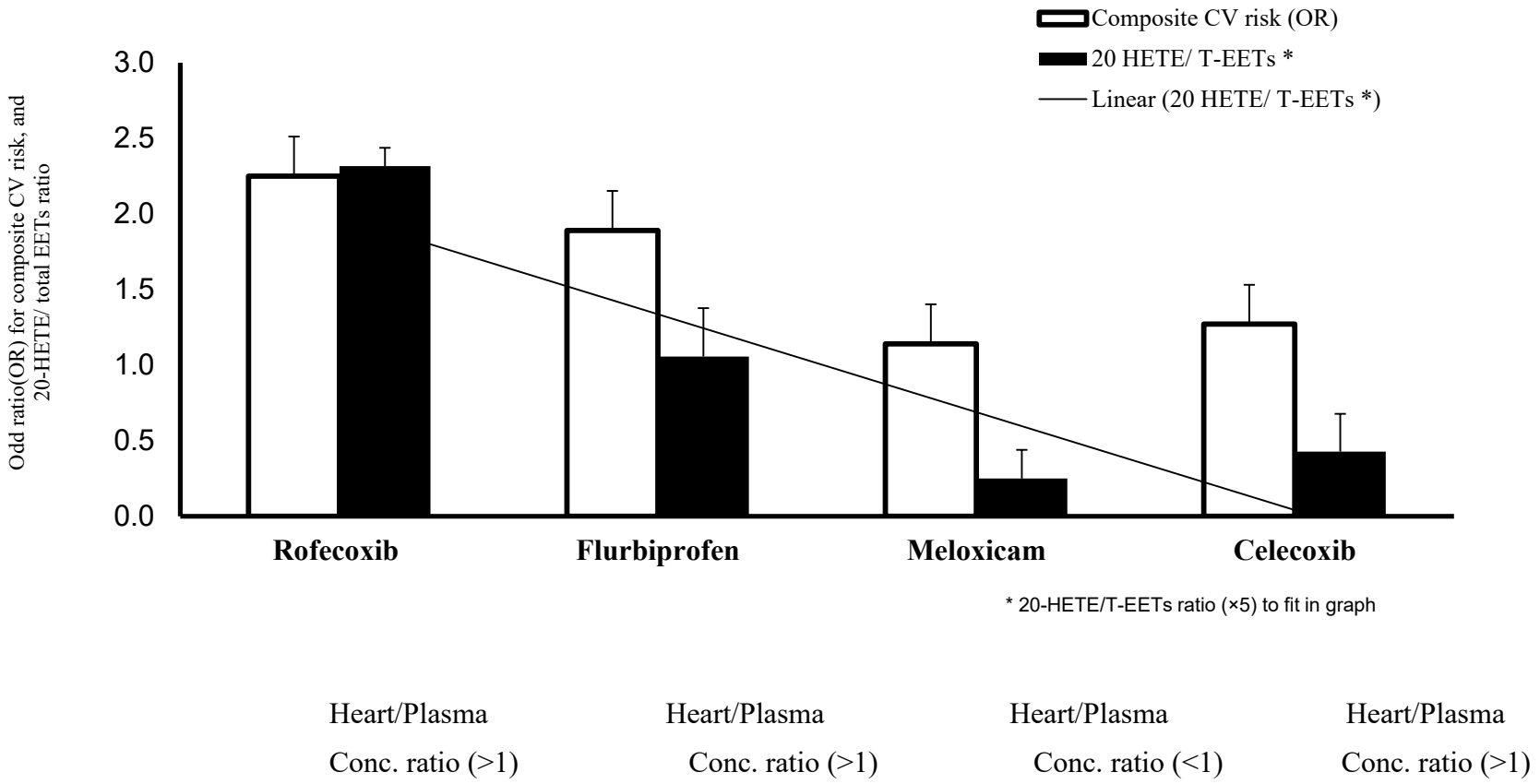


Figure 28: Association between CV/renal risks (OR) and 20-HETE/total EETs ratio in observed in adjuvant arthritis rats heart (n = 3 each), (OR) (reported by Asghar *et al.,* [209]).

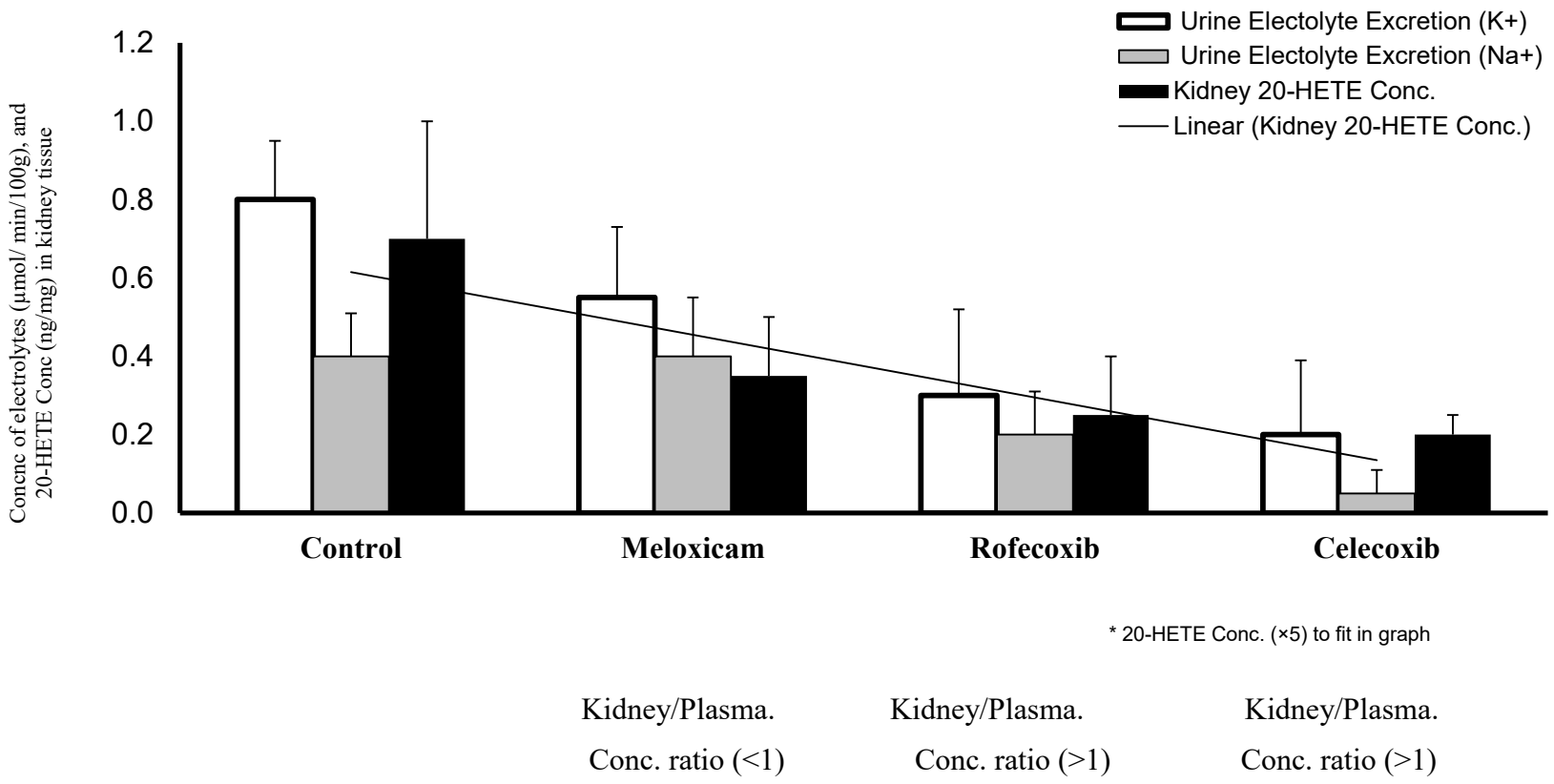


Figure 29: Association between urinary electrolytes (Na+, K+) excretion (µmol/ min/100 g) and 20-HETE concentration in the kidney tissues (ng/mg) observed in adjuvant arthritis rats (n = 3 each) Urinary electrolytes (Na+, K+) excretion is previously reported by Harirforoosh, S., A. Aghazadeh-Habashi, *et al.* [141], electrolytes excretion data was not available for flurbiprofen.

Table 14: Hydroxyeicosatetraenoic acid (HETE) concentrations in adjuvant arthritis rat plasma, heart, and kidneys.

Group	Concentration (ng/mL) in rat plasma, n=3 per group				
	20-HETE	18-HETE	15-HETE	12-HETE	Total HETEs
Control	58.90 ± 19.68	13.62 ± 1.76	9.43 ± 2.84	44.19 ± 21.18	126.13 ± 38.62
Inflamed	107.50 ± 9.59*	35.16 ± 10.36*	17.78 ± 5.86	46.58 ± 46.90	207.02 ± 54.95*
Rofecoxib	206.93 ± 9.99†	14.74 ± 3.61†	2.77 ± 0.95†	16.55 ± 2.02	240.99 ± 11.77
Meloxicam	127.37 ±27.56	6.34 ±0.73†	5.89 ±2.59†	14.73 ±4.69	154.34 ±26.62
Celecoxib	125.36 ±12.80	11.19 ±4.70†	10.99 ±0.57	25.46 ±1.03	173.00 ±14.02
Flurbiprofen	235.62 ±16.74†	16.73 ±3.23†	5.54 ±2.31†	14.65 ±1.56	272.54 ±18.33
Group	Concentration (ng/mg) in rat hearts, n=3 per group				
	20-HETE	18-HETE	15-HETE	12-HETE	Total HETEs
Control	0.30 ± 0.13	1.94 ± 1.05	0.52 ± 0.14	0.18 ± 0.09	2.87 ± 1.03
Inflamed	0.73 ± 0.18*	2.97 ± 1.00	0.78 ± 0.27	0.91 ± 0.17*	5.31 ± 1.37*
Rofecoxib	0.50 ± 0.12	0.96 ± 0.32†	0.44 ± 0.02	0.45 ± 0.04†	2.36 ± 0.42†
Meloxicam	0.18 ± 0.06†	0.95 ± 0.40†	0.31 ± 0.08†	0.22 ± 0.08†	1.70 ± 0.46†
Celecoxib	0.21 ± 0.05†	0.70 ± 0.18 †	0.43 ± 0.11	0.60 ± 0.13†	1.95 ± 0.24†
Flurbiprofen	0.62 ± 0.07	0.80 ± 0.48†	0.28 ± 0.07†	0.70 ± 0.06	2.24 ± 0.72†
Group	Concentration (ng/mg) in rat kidneys, n=3 per group				
	20-HETE	18-HETE	15-HETE	12-HETE	Total HETEs
Control	0.14 ± 0.06	0.10 ± 0.05	0.27 ± 0.10	0.30 ± 0.16	0.80 ± 0.25
Inflamed	0.02 ± 0.01*	0.02 ± 0.02	0.14 ± 0.03	0.02 ± 0.01*	0.19 ± 0.05*
Rofecoxib	0.05 ± 0.03	0.15 ± 0.01†	0.15 ± 0.03	0.07 ± 0.02	0.42 ± 0.05
Meloxicam	0.07 ± 0.03	0.07 ± 0.03	0.15 ± 0.04	0.18 ± 0.02	0.47 ± 0.10
Celecoxib	0.04 ± 0.01	0.16 ± 0.02†	0.31 ± 0.05†	0.05 ± 0.01	0.57 ± 0.06†
Flurbiprofen	0.02 ± 0.01	0.17 ± 0.05†	0.41 ± 0.06†	0.68 ± 0.05†	1.28 ± 0.15†

Mean ± standard deviation of concentration of metabolites

***** Significantly different from control rats using Student's t-test ($p < 0.05$)

† Significantly different from inflamed using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

Table 15: Epoxyeicosatrienoic acids (EETs) concentrations in adjuvant arthritis rat plasma, heart, and kidneys.

Group	Concentration (ng/mL) in rat plasma, n=3 per group			
	14,15-EET	11,12-EET	8,9-EET	Total EETs
Control	3.91 ± 0.08	19.50 ± 1.75	1.45 ± 1.10	24.86 ± 2.92
Inflamed	1.44 ± 0.45*	19.48 ± 6.24	0.86 ± 0.19	21.78 ± 6.50
Rofecoxib	2.06 ± 1.03	5.76 ± 0.94†	0.60± 0.21	8.42 ± 2.11†
Meloxicam	6.34 ± 1.88†	5.87 ± 1.70†	1.42 ± 0.40	13.64 ± 3.21
Celecoxib	6.64 ± 2.12†	6.73 ± 0.99†	1.00 ± 0.75	14.37 ± 2.34
Flurbiprofen	2.31 ± 0.42	9.60 ± 2.83†	2.45 ± 0.41	14.36 ± 3.04
Group	Concentration (ng/mg) in rat hearts, n=3 per group			
	14,15-EET	11,12-EET	8,9-EET	Total EETs
Control	0.47 ± 0.52	2.80 ± 1.16	0.14 ± 0.11	3.41 ± 1.43
Inflamed	0.14 ± 0.08	3.76 ± 0.94	0.30 ± 0.11	4.20 ± 1.06
Rofecoxib	0.22 ± 0.07	0.30 ± 0.03†	0.13 ± 0.02	0.65 ± 0.10†
Meloxicam	0.14 ± 0.04	1.90 ± 0.54†	0.10 ± 0.04†	2.14 ± 0.49
Celecoxib	0.02 ± 0.004	1.39 ± 0.15†	0.06 ± 0.02†	1.48 ± 0.16†
Flurbiprofen	0.23 ± 0.03	1.12 ± 0.10†	0.09 ± 0.01†	1.31 ± 0.10†
Group	Concentration (ng/mg) in rat kidneys, n=3 per group			
	14,15-EET	11,12-EET	8,9-EET	Total EETs
Control	0.14 ± 0.03	0.16 ± 0.08	0.02 ± 0.01	0.31 ± 0.11
Inflamed	0.16 ± 0.08	0.11 ± 0.03	0.02 ± 0.003	0.29 ± 0.10
Rofecoxib	0.02 ± 0.01†	0.12 ± 0.02	0.004 ± 0.002	0.14 ± 0.03
Meloxicam	0.01 ± 0.004†	0.12 ± 0.03	0.004 ± 0.001	0.14 ± 0.03
Celecoxib	0.01 ± 0.01†	0.15 ± 0.02	0.002 ± 0.001†	0.16 ± 0.02
Flurbiprofen	0.15 ± 0.03	0.18 ± 0.03	0.01 ± 0.002	0.34 ± 0.05

Mean ± standard deviation of concentration of metabolites

* Significantly different from control rats using Student's t-test ($p < 0.05$)

† Significantly different from inflamed using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

Table 16: Dihydroxyeicosatetraenoic acids (DHETs) concentrations in adjuvant arthritis rat plasma, heart, and kidneys.

Group	Concentration (ng/mL) in rat plasma, n=3 per group				
	14,15-DHET	11,12-DHET	8,9-DHET	5,6-DHET	Total DHETs
Control	12.71 ± 0.88	46.41 ± 17.63	19.17 ± 13.53	11.09 ± 5.26	89.38 ± 8.39
Inflamed	40.60 ± 13.68*	30.72 ± 9.22	14.66 ± 9.81	13.85 ± 3.45	99.57 ± 30.44
Rofecoxib	41.32 ± 7.31	17.88 ± 4.61	13.60 ± 4.28	17.88 ± 7.11	90.68 ± 18.19
Meloxicam	23.98 ± 3.93†	7.75 ± 6.54	6.11 ± 2.62	8.21 ± 1.06	46.06 ± 9.63†
Celecoxib	12.85 ± 3.24†	10.35 ± 2.92	7.88 ± 2.51	6.67 ± 1.12	37.74 ± 5.99†
Flurbiprofen	9.27 ± 5.73†	14.66 ± 4.96	18.55 ± 4.12	7.72 ± 5.98	50.21 ± 14.16
Group	Concentration (ng/mg) in rat heart, n=3 per group				
	14,15-DHET	11,12-DHET	8,9-DHET	5,6-DHET	Total DHETs
Control	0.46 ± 0.25	36.51 ± 13.44	1.22 ± 0.52	0.24 ± 0.08	38.44 ± 13.48
Inflamed	0.66 ± 0.31	49.06 ± 18.14	1.30 ± 0.29	0.39 ± 0.12	51.41 ± 18.50
Rofecoxib	0.39 ± 0.05	2.61 ± 0.55†	0.16 ± 0.03†	0.26 ± 0.05	3.41 ± 0.54†
Meloxicam	0.40 ± 0.15	2.21 ± 0.18†	0.22 ± 0.06†	0.23 ± 0.03	3.07 ± 0.29†
Celecoxib	0.39 ± 0.09	1.83 ± 0.59†	0.05 ± 0.01†	0.42 ± 0.05	2.70 ± 0.66†
Flurbiprofen	0.25 ± 0.03	4.02 ± 0.74†	0.33 ± 0.06†	0.19 ± 0.06	4.79 ± 0.77†
Group	Concentration (ng/mg) in rat kidney, n=3 per group				
	14,15-DHET	11,12-DHET	8,9-DHET	5,6-DHET	Total DHETs
Control	0.40 ± 0.14	7.88 ± 10.79	0.15 ± 0.16	0.10 ± 0.04	8.53 ± 11.08
Inflamed	0.17 ± 0.14*	2.65 ± 1.66	0.09 ± 0.03	0.08 ± 0.04	3.00 ± 1.84
Rofecoxib	0.11 ± 0.01	0.70 ± 0.22	0.13 ± 0.02	0.06 ± 0.02	1.00 ± 0.22
Meloxicam	0.23 ± 0.03	0.50 ± 0.12	0.17 ± 0.05	0.05 ± 0.01	0.95 ± 0.11
Celecoxib	0.13 ± 0.02	0.88 ± 0.07	0.15 ± 0.03	0.09 ± 0.01	1.26 ± 0.06
Flurbiprofen	0.58 ± 0.10†	1.59 ± 0.27	0.25 ± 0.03	0.06 ± 0.01	2.49 ± 0.25

Mean ± standard deviation of concentration of metabolites

* Significantly different from control rats using Student's t-test ($p < 0.05$)

† Significantly different from inflamed using one way ANOVA followed by the Bonferroni adjustment ($p<0.05$).

Table 17: Correlations between plasma concentrations of ArA metabolites with their levels in the heart and kidneys in adjuvant arthritis rats

Comparison (n=12)		Pearson's correlation coefficient and two tail test for significance											
		20-HETE	14, 15-EET	11,12-EET	14, 15-DHET	11,12-DHET	T- EET	T-DHET	20-HETE /14, 15-EET	20-HETE/ 11,12-EET	20-HETE/ T-EET	T-EET/ T-DHETs	20-HETE /T-DHET
Plasma Vs Heart	r	**0.767**	0.716	0.232	0.319	0.269	**0.795**	0.030	**0.793**	**0.813**	**0.874**	0.694	0.114
	p	**0.04***	0.109	0.86	0.75	0.75	**0.03***	1.0	**0.03***	**0.02***	**0.003***	0.11	0.96
Plasma Vs Kidney	r	**-0.788**	-0.537	0.331	**0.799**	0.237	0.342	0.245	-0.736	-0.054	-0.347	0.330	0.738
	p	**0.03***	0.272	0.73	**0.02***	0.86	0.72	0.85	0.06	0.99	0.71	0.73	0.06

*Significant correlations using Students t-test for correlation ($p < 0.05$).

Chapter 8

8. Summary and suggestions

8.1. General Conclusion

Our results suggest that NSAIDs are heterogeneous in causing CV/renal risks both in the extent and in the nature of their actions. Meloxicam caused limited CV/renal risk of vascular origin that is clinically manageable. We also found that the CV/renal risks associated with NSAIDs, if any, are dependent on dose, concomitant use of aspirin, and other factors such as underlying inflammation. This study confirmed that rofecoxib, celecoxib, and flurbiprofen accumulate in tissue more readily than meloxicam [141]. Such tissue accumulation of NSAIDs can influence intracellular components and homeostatic mechanisms, ultimately leading to CV/renal consequences.

Inflammation influences the RAS by downregulating the components of the cardioprotective axis (i.e., ACE2/Ang-(1-7)/Mas) over the cardiotoxic axis (i.e., ACE/Ang-II/AT1R) [359]. However, NSAIDs treatment corrected this imbalance. Our results also suggest that the Ang-(1-7)/Ang-II ratio in the plasma of inflamed rats do not correspond with cardiotoxicities reported in inflammation, thus cannot be used as a biomarker of CV/renal risks. We concluded that NSAIDs effects on RAS are anti-inflammatory in nature and do not reflect their cardiotoxicity potential.

We also studied the effect of NSAIDs on CYP metabolites of ArA. We found that the levels of cardiotoxic metabolites (i.e., 20-HETE) are increased and levels of cardioprotective metabolite (i.e., EETs) are decreased in AA rat plasma and heart. Higher 20-HETE can result in vasoconstriction of blood vessels, increased blood pressure, and increased thrombogenicity of blood. While, lower EETs means reduced cardioprotection and reduced survival of cardiac myocytes. This along with decreased levels of 20-HETE in the kidneys, and loss of its uricosuric effects may contribute towards higher CV/renal risks. Treatments with rofecoxib and flurbiprofen, but not treatments with meloxicam and celecoxib, resulted in a further increase in 20-HETE levels but lower 11,12- and 14,15-EETs concentrations in AA rat plasma and heart. Interestingly, plasma levels of eicosanoids correlated well with those in heart, suggesting that plasma concentrations of ArA metabolites can predict the concentrations of vasoactive

metabolites in the heart. The observed 20-HETE/total-EETs ratio in plasma were in agreement with the available epidemiology data, suggesting that celecoxib and meloxicam are less likely than rofecoxib and flurbiprofen to cause cardiotoxicity. Thus plasma eicosanoid ratios can serve as biomarkers of NSAIDs-induced cardiotoxicity.

We concluded that adverse effects of NSAIDs might, in part, be a result of their effects on the modulation of CYP enzymes involved in ArA metabolism. The 14,15-EETs, 11,12-EETs and 20-HETE can act as surrogate biomarkers of NSAIDs induced CV/renal risks. These biomarkers were tested in only one animal species and the findings of these studies need to be performed in other animal species and eventually in humans. We did not study the functional consequences of NSAIDs tissue distribution; rather, we relied on outcomes reported in clinical trials to measure the CV risk associated with some NSAIDs. Further studies are needed to validate these observations and to validate the suggested biomarkers to accurately estimate the CV/renal risks.

8.2. Future directions

Based on the results of this thesis the following are my suggestions for future studies to verify and extend these findings.

a) Pharmacodynamics analysis of effects of NSAIDs exposure

I recommend measuring both the gene product and the protein levels of cytochrome P450 using RT-PCR and Western blot, respectively. To demonstrate which of these proteins are affected upon exposure to NSAIDs in AA rat heart and kidneys.

b) Assessment of heart and kidney functions after NSAIDs exposure

I recommend measuring the excretion of electrolytes into urine that will indicate how renal function is affected upon exposure to NSAIDs. I also recommend investigating the effect of NSAIDs exposure on the heart function with in-vitro studies using Langendorff method followed by in-vivo studies using echocardiography.

c) Validation and assessment of clinical utility of biomarkers

I recommend further validation of the biomarkers identified in this thesis in another animal species followed by testing their clinical utility for use in human samples.

8.3. Limitations and suggestions

Despite my best efforts, the studies included here in this thesis have their limitations. One limitation was the small sample size in animal studies (6 experimental groups and 3 animals in each group). Thus any future research should address the low number of animals to overcome this shortcoming.

Lastly, in the systematic review and meta-analysis, some conclusions are made based on limited available reports. This was due to our narrow inclusion criteria which required inclusion of meloxicam in the reported studies. Any future research should either broaden the inclusion criteria, or look for a statistical model that can compare the outcomes from different studies not having a common comparator.

The housekeeping protein tubulin bands generated in the Western blot analysis of AT1R, AT2R and Mas appeared to be overexposed [Figure 19]. I therefore, suggest that these results are preliminary.

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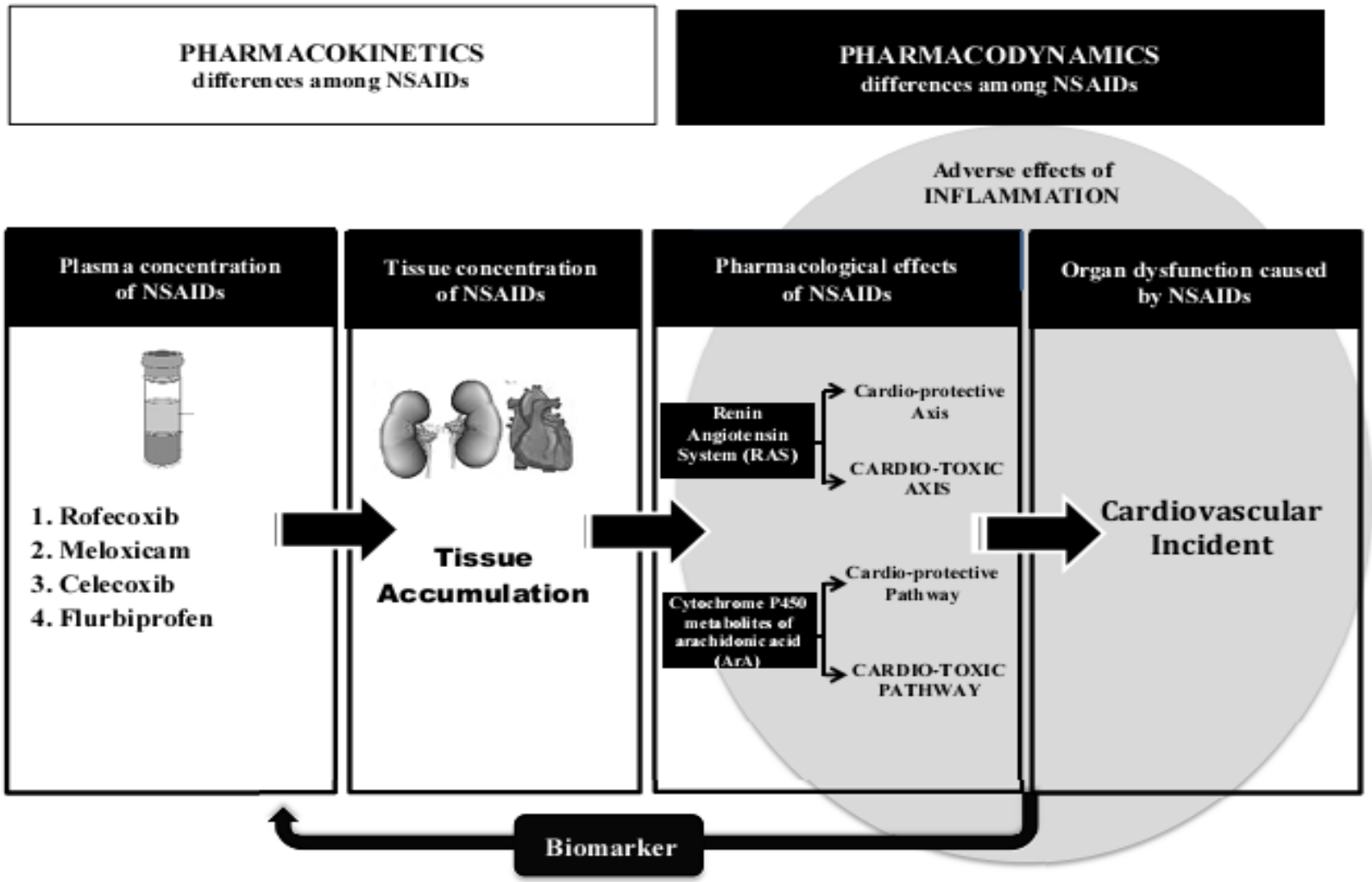
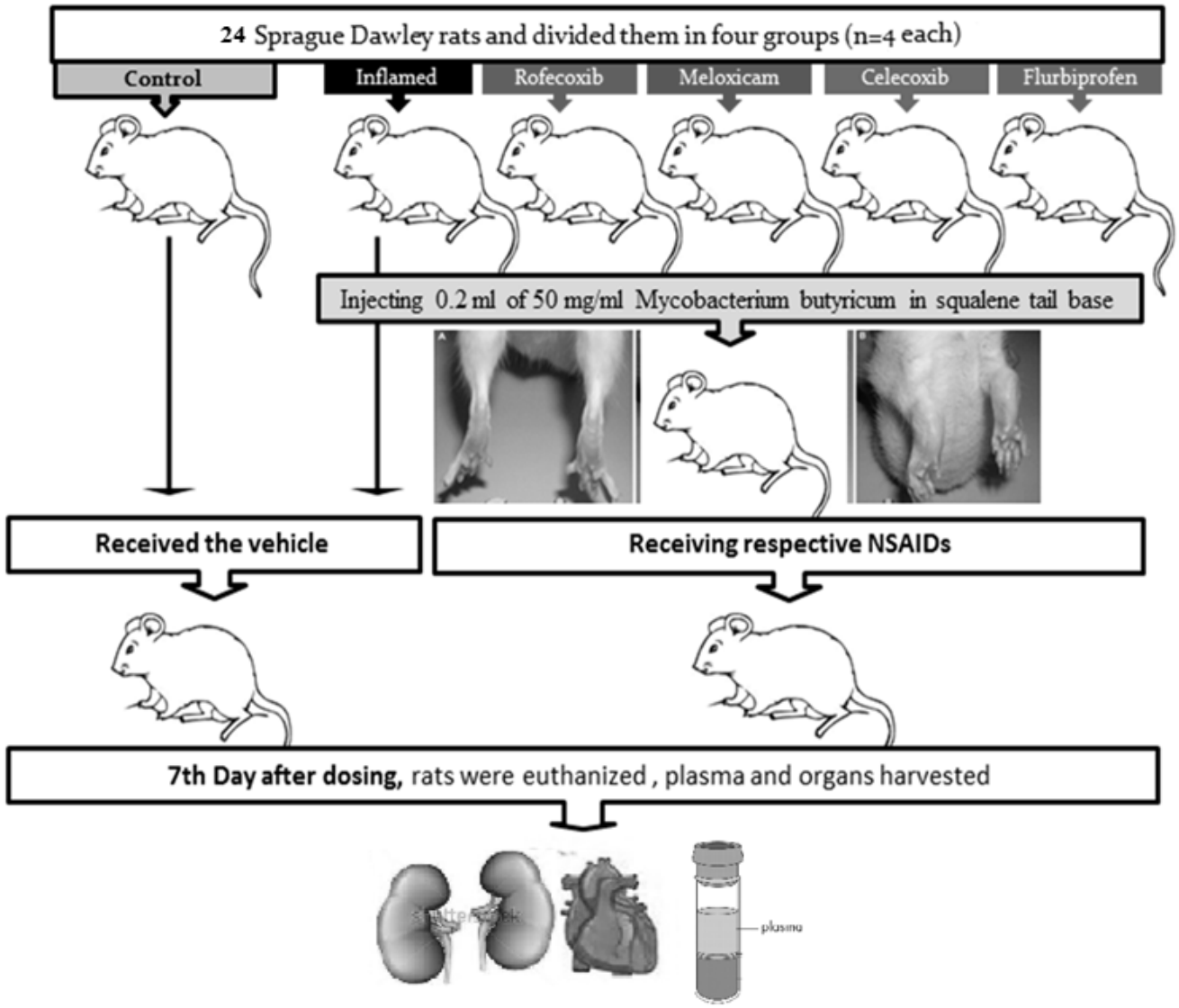
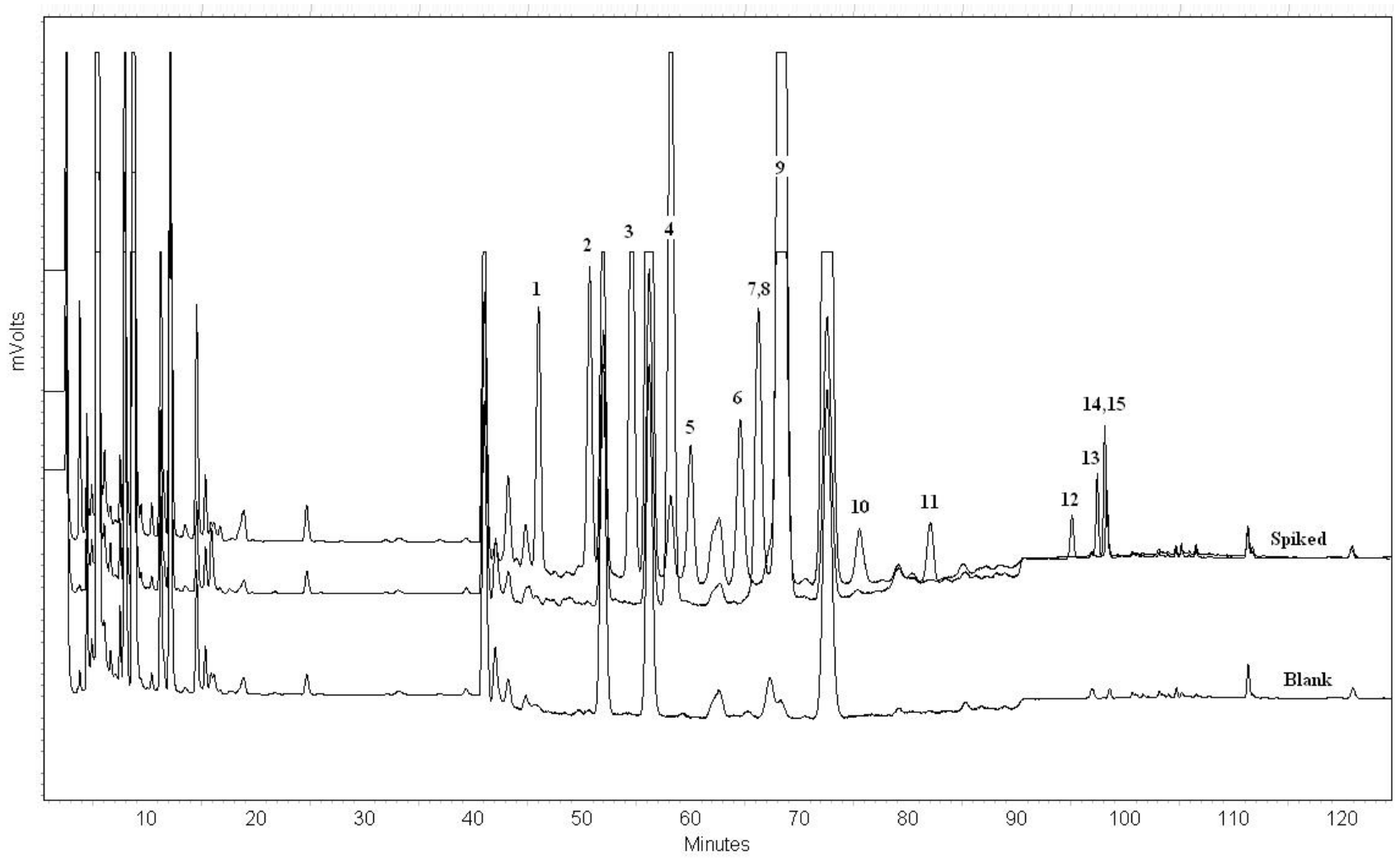
Appendixes



Appendix-I: Schematic diagram of thesis rationale and hypothesis.



Appendix-II: Flow chart of experimental method used in animal handling.



Appendix-III: Representative chromatograms of ArA metabolites in blank and normal rat plasma and spiked (10 ng) rat plasma. Labeled as (1) 14,15-DHET; (2) 11,12-DHET; (3) 8,9-DHET; (4) 5,6-DHET; (5) 20-HETE; (6) 18-HETE; (7, 8) 16-HETE, and 17-HETE together; (9) internal standard; (10) 15-HETE; (11) 12-HETE; (12) 14,15-EET; (13) 11,12-EET; (14,15) 8,9-EET; 5,6-EET together

Appendix-IV: List of publications from this thesis.

1. Asghar, Waheed, and Fakhreddin Jamali. "The effect of COX-2-selective meloxicam on the myocardial, vascular and renal risks: a systematic review." Inflammopharmacology 23.1 (2015): 1-16.
2. Ali Aghazadeh-Habashi, Asghar, Waheed, and Fakhreddin Jamali. Association of the Renin-Angiotensin System Components and Arachidonic Acid Metabolites under Inflammatory Condition in the Rat with Adjuvant Arthritis (Conference paper; Canadian Society for Pharmaceutical Sciences 2014). Journal of Pharmacy & Pharmaceutical Sciences, 2014. 17(3).