Glucosamine Anti-Inflammatory Dose-Effect Correlation and Its Influence on the Renin-Angiotensin System Components and Arachidonic Acid Metabolites under Inflammatory Condition in the Rat with Adjuvant Arthritis

by

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Abstract

Crystalline glucosamine (GlcN) sulfate salt formulation is approved as a prescription drug with anti-inflammatory effects for the management of osteoarthritis (OA) in the European Union, while it is considered as a nutraceutical in North America. The effectiveness of GlcN for ameliorating the signs and symptoms of OA in humans is still the subject of scientific debate. In animal studies using high doses, its effectiveness is undisputable; however results of clinical trials in humans are controversial.

One of the objectives of this thesis was to address the possible causes of controversy around GlcN effectiveness in humans, and to establish a minimum effective dose (MED) needed to produce minimum anti-inflammatory effective concentration in the rat adjuvant arthritis animal model. Many explanations have been offered as the root of the controversy including superiority of a crystalline sulfate salt over the hydrochloride form, industry bias, insensitive assessment metrics, and poor methodology. Herein, we rule out a difference in bioequivalence between GlcN salts and suggest additional factors; i.e., inconsistency in the GlcN content of some products used in trials, under-dosing of patients, as well as variable pharmacokinetic indices as possible reasons for the lack of GlcN efficacy observed in some studies. Clinical trials using high doses of pharmaceutical grade GlcN, or formulations with greater bioavailability should yield positive results. Our results indicate that the MED is between 40 to 80 mg/kg/day that generates a maximum plasma concentration in the range of 1.37 ± 0.24 to 5.31± 6.84 mg/L, close to the rang of concentration reported for pharmaceutical grades of GlcN in humans. GlcN efficacy is dose and concentration dependent and if the data could be extrapolated to humans, using a higher than the commonly tested 1500 mg/kg /day dosage regimen may provide more clear treatment outcomes.

Inflammation influences the body as whole and imbalances the homeostasis of different regulatory systems in particular. Renin-angiotensin system (RAS) and arachidonic acid (ArA) pathway are two main regulators of cardiovascular (CV) systems which could be affected by inflammation. Other objectives of this thesis were to investigate if experimentally induced adjuvant arthritis (AA), as an animal model for systemic inflammation, alters different components of the RAS such as angiotensin converting enzymes (ACE) expression, angiotensin (Ang) peptides systemic and local concentration, and their associated receptors expression level. We also investigated the effect of AA on ArA metabolites concentration in the plasma, heart and kidney tissues. We correlated the RAS components and ArA metabolites concentration in order to identify reliable biomarkers for prediction of CV complications in vulnerable individuals suffering from inflammatory conditions such as rheumatoid arthritis (RA). GlcN with anti-inflammatory effects is a good candidate for modulating the detrimental effect of inflammation on the RAS and ArA pathways. We studied the impact of GlcN on these two systems. GlcN was able to reestablish the disturbed balance of the RAS and ArA pathway by reinstating the ratio of cardioprotective (vasodilator, anti-proliferative and anti-hypertrophic) over cardiotoxic (vasoconstrictive, proliferative and pro-hypertrophic) components of these systems. GlcN by presenting modulatory effects on the RAS and ArA pathways could be an alternative over NSAIDs which their long term use imposes CV side effects in RA patients.

This dissertation is dedicated

to my parents and my siblings for their love, encouragement and support,

and

to my lovely wife, Marzyeh, and our wonderful son, Yashar, for their

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LIST OF ABBREVIATION AND SYMBOLS

AA	Adjuvant arthritis
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACEI	Angiotensin converting enzyme inhibitor
ACR	American College of Rheumatology
AI	Arthritis index
Ang 1-7	Angiotensin-(1-7)
Ang II	Angiotensin II
ADAM	1-Aminoadmantan
ANOVA	Analysis of variance
ArA	Arachidonic acid
ARBs	Angiotensin II receptor blockers
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
AUC	Area under plasma concentration-time curve
BP	Blood pressure
CD	Cluster of Differentiation
CL	Control
CL-GlcN	Control glucosamine treated
C_{max}	Maximum plasma concentration
CO_2	Carbon dioxide
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CS	Chondroitin sulfate
CTLA	Cytotoxic T lymphocyte associated
CV	Cardiovascular
CV%	Coefficient of variation
CYP	Cytochrome P450

DHT	Dihydroxyeicosatrienoic
DMARD	Disease modifying anti-rheumatic drugs
EC_{50}	Half maximal effective concentration
ED_{50}	Median effective dose
EET	Epoxyeicosatrienoic acid
FDA	Food and Drug Administration
FMOC-Cl	9-Fluorenylmethoxycarbonyl chloride
Fruc-6-P	Fructose-6-phosphate
GAG	Glycosaminoglycan
GFAT	Glutamine:fructose-6-phosphate amidotransferase
GAIT	Glucosamine/chondroitin Arthritis Intervention Trial
GlcN	Glucosamine
GlcN-6-p	Glucosamine-6-phosphate
GlcN-3-sulfate	Glucosamine-3-sulfate
GlcN-6-sulfate	Glucosamine-6-sulfate
GlcNAc	N-acetyl-Glucosamine
GlcN-HCl	Glucosamine hydrochloride
GlcN-S	Crystalline glucosamine sulfate
GLUT	Glucose transporters
GNPDA	Glucosamine-6-phosphate deaminase
GSK-3β	Glycogen synthase kinase-3 β
KCl	Potassium chloride
HBP	Hexosamine biosynthetic pathway
HETE	Hydroxyeicosatetraenoic
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel diseases
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
INR	International normalized ratio
IL	Interleukin

IL-1β	Interleukin-1-beta
iNOS	Inducible nitric oxide
INF	Inflamed
INF-GlcN	Inflamed glucosamine treated
IS	Internal standard
LC	Liquid chromatography
LC-FL	Liquid chromatography- fluorescence detector
LC- MS/MS	Liquid chromatography-mass spectroscopy
LD_{50}	Median effective dose
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MEC	Minimum effective concentration
MED	Minimum effect dose
mg	Milligram
MMPs	Matrix metalloproteinase
mL	Milliliter
mRNA	Messenger ribonucleic acid
MRM	Multiple reaction monitoring
MSM	Methylsulfonylmethane
MW	Molecular weight
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
ng	nanogram
NE-OTf	2-(2,3-naphthalimino) ethyl-trifluoromethanesulphonate
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
O-GlcNAc	O-linked GlcNAc

OGT	O-linked GlcNAc transferase
PEG	Polyethylene glycol
PEG2	Prostaglandin E2
PBS	phosphate buffered saline
PK	Pharmacokinetic
PD	Pharmacodynamic
pg	Picogram
PPAR	Peroxisome proliferator-activated receptor
RA	Rheumatoid arthritis
RAS	Renin-angiotensin system
SD	Standard deviation
sHE	Soluble epoxide hydrolase
SPE	Solid phase extraction
t1/2	Half life
TALH	Thick ascending loop of Henle
T-DHT	Total DHTs
T-EET	Total EETs
Tmax	Time to reach maximum concentration
TNF-α	Tumor necrosis factor-alpha
TGF	Tubulo-glomerular feedback
UDP-GlcNAc	Uridine diphosphate-N-acetyl-glucosamine
UDP-GalNAc	Uridine diphosphate-N-acetyl galactosamine
µg	Microgram
µM	Micromolar
UV	Ultraviolet
WOMAC	Western Ontario and McMaster Universities Osteoarthritis index

Chapter 1

1 Introduction

1.1 Inflammation

Inflammation is typically recognized as an important step for control of microbial assault or tissue injury and is essential for maintaining tissue homeostasis under a variety of harmful conditions. The discovery of a growing number of inflammatory mediators and unveiling their effects on target tissues is one of the most intriguing aspects of studying inflammation. Inflammation is the body's reaction to injury and danger and is composed of a communication network that basically controls and processes the homeostasis of the body. This network detects the risk and controls the damage, and healing process, which are all important elements in the maintenance of an organism's integrity. Recent studies indicated that inflammation and inflammatory response are common bases of several complex diseases, including chronic inflammatory rheumatic disorders, CV diseases, diabetes, obesity, cancer, asthma, and ageing (1).

The inflammatory process is composed of cycles of repair and remodeling after any assault or injury, and involves different inflammatory mediators. These processes, as characteristics of a highly developed complex biological system, are highly robust and reliable. Inflammation can be initiated by a number of different triggers including the exposure to microbial products, tissue injury, and metabolic stress. Contact with the trigger could happen due to a barrier malfunction or loss of normal immune tolerance. Regardless of the type of trigger, a general set of cellular pathways is initiated including activation of signaling through Toll-like receptors, nuclear factor (NF)-kB, and the formation of inflammasome, which are composed of several proteins. The inflammasome is responsible for endoplasmic reticulum stress and the release of several

inflammatory cytokines (2). The resultant inflammatory response manifests itself by redness, swelling, heat, pain, and loss of function. This response engages different interactions among many cell types, which causes the production of an enormous number of chemical mediators. The nature of the trigger and the location and length of the exposure time govern the onset and intensity of the inflammatory response, including the exact cells and mediators involved. These aspects control the initiation and maintenance of the inflammatory response. After controlling or eliminating the trigger, the course of the response changes and some mechanisms start to terminate the inflammation with the aim of limiting further damage to the host and beginning tissue repair. This process of self-regulation, which involves the engagement of feedback mechanisms, is called resolution of inflammation. During this phase, the body starts the inhibition of pro-inflammatory signaling cascades, the down- regulation of receptors for inflammatory mediators, the secretion of anti-inflammatory cytokines and the activation of regulatory cells. The resolution process is vital for proper control of inflammation in order to maintain health and homeostasis.

However, any inflammatory condition that happens due to a loss of tolerance or lack of regulatory processes has the potential to become pathologic. Inflammation may be categorized as acute or chronic. An acute form of inflammation is the early response of the body to a trigger that could be an infectious agent or tissue damage. This response is accomplished by the enhanced movement of plasma and leucocytes from the blood into the site of infection or tissue damage. A series of biochemical reactions take place to start and to propagate the inflammatory response. This involves the local vascular system, the immune system, and various cells within the injured tissue. The release of chemotactic mediators such as leukotrienes, complement factors, and platelet activating factors attracts neutrophils to the site of injury. Consequently, it is

followed by infiltration of neutrophils into the tissue after binding to endothelial surface adhesion molecules. Then, tissue macrophages and infiltrated neutrophils invade the assaulting organism. Furthermore, other biochemical agents are also released from neutrophils to add more toxic effects against the invading organism and the surrounding tissues.

This acute inflammatory response is usually self-limiting and once the trigger is contained or removed the resolution phase will be activated. Evidence suggests that the resolution process as a dynamic, harmonized program starts in the first few hours after an inflammatory response initiates. Granulocytes, after entering the injured tissue, start to switch from secretion of arachidonic acid-derived prostaglandins (PGs) and leukotrienes to lipoxins, which begins the termination sequence of inflammation. Consequently, neutrophil recruitment stops and programmed death of neutrophils by apoptosis engages. Then apoptotic neutrophils undergo phagocytosis by macrophages, leading to neutrophil clearance (3). There are specific anti-inflammatory mediators, and specific cell types, and pro-resolving lipid mediators which are involved in this active process of resolution (3,4).

Chronic inflammation happens when inflammation continues for extended periods due to failure of immune cells to remove the harmful agent or when the trigger is still persistent. It involves a continuing shift in the type of cells attracted to the site of insult and concurrent damage and repair of the tissue due to the current inflammatory process. The initially protective inflammatory response could become pathological as a result of diminished tolerance threshold and/or loss of regulatory processes such as resolution. Where this becomes severe, permanent damage to host tissues can happen. Then, the situation could turn into a chronic inflammatory disease characterized by significantly increased concentrations of inflammatory mediators at the site of tissue damage and in the systemic circulation. Depending on the level of these mediators,

this state may be considered as high grade inflammation. This situation can be seen in inflammatory conditions such as rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), atopic dermatitis, psoriasis and asthma. Low grade inflammation implies to such circumstances which apparent clinical sign and symptoms can be negligible or absent. Compared to high grade inflammation, the levels of inflammatory mediators in the systemic circulation in low grade inflammation do not reach the same level. This situation can be seen in adipose tissue as a characteristic of obesity (5).

Acute and chronic inflammations have common features. Both conditions consist of two stages: an afferent stage, in which the presence of a trigger is detected by some types of immune cells, and an efferent stage, in which an inflammatory response is generated to remove the alleged harmful invader. The generated response consists of four main episodes, regardless of the type of inflammation. The first episode is enhanced blood flow to the site of inflammation. The second episode is increased capillary vessel permeability induced by endothelial cells which allows the extravasation of larger molecules that in normal conditions are not capable of crossing the endothelium. This process delivers some soluble inflammatory mediators to the site of inflammation. The third episode is the migration of leukocyte into the surrounding tissue through the permeable capillaries, supported by chemotactic mediators released from the site of the injury (Figure 1.1). The fourth episode is the release of inflammatory mediators from leukocytes at the site of inflammation. Depending on the inflammatory cell type involved, the nature of the trigger, the anatomical site of inflammation, and the stage during the inflammatory response, these released mediators may include PGs, leukotrienes, cytokines, chemokines, or reactive oxygen species. These mediators are involved in amplification of the inflammatory process in different ways. For example, as chemoattractants they impose their effect by attracting other

mediators, while some other mediators escape the inflammatory site into the circulation, from which they can apply their systemic effects (Figure 1.1). For instance, interleukin (IL)-6, which enters into the bloodstream from an inflammatory site, can induce acute-phase protein C-reactive protein (CRP) synthesis by the liver; at the same time, tumor necrosis factor-alpha (TNF-α) imposes metabolic effects within the skeletal muscle, adipose tissue, and bone. Therefore, inflammation at one site of the body can cause inflammation induced changes at distal sites (6).

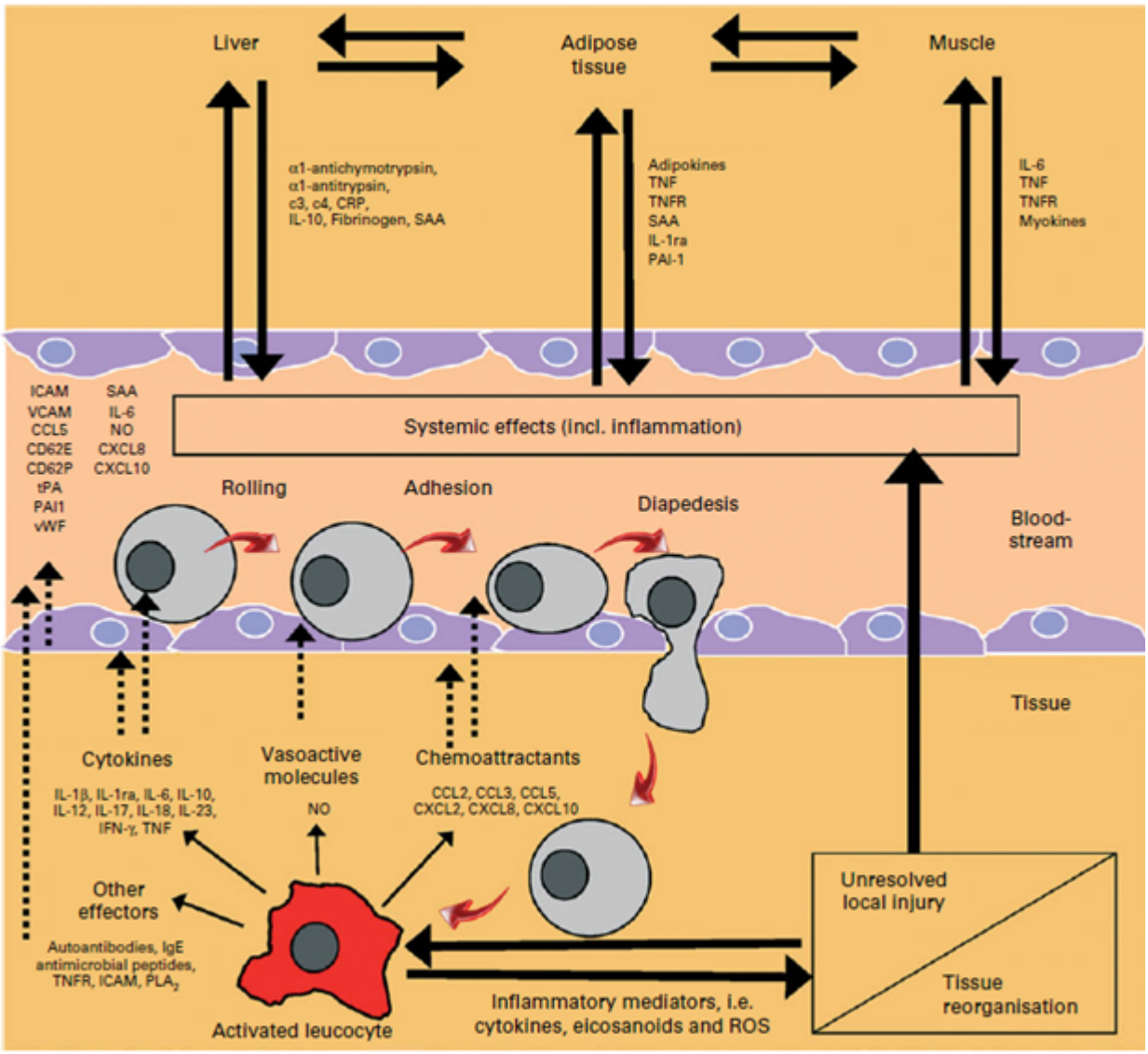

Figure 1.1. An overview of inflammation

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1.2 The effect of inflammation on pharmacokinetics and pharmacodynamics of cardiovascular drugs

It has been shown that inflammation decreases the hepatic clearance of many drugs (7-9). Inflammation also causes significant changes in plasma proteins and target receptors expression, which can translate not only to pharmacokinetic (PK) alteration but also in pharmacodynamic (PD) changes. Our laboratory is one of the pioneer labs working in the field of drug-disease interactions, mostly focused on cardiovascular (CV) drugs.

CV complications are one of the major comorbidities of RA patients, accounting for 35% to 50% of excess mortality in this population (10). Therefore, CV drugs are one of the mainstays of therapeutic approaches in RA patients. Our group was one of the first investigators to notice that despite a pronounced increased drug concentration, the response to some CV drugs was decreased. Meyo et al. (11) studied the effect of RA on the PK and PD of verapamil, a calcium channel blocker, in healthy and patients with RA. The result of this study indicated that IL-6 and NO concentrations as inflammatory biomarkers were significantly higher in patients with RA and were correlated with disease severity. Verapamil PK was altered likely due to changes in protein binding, decreased clearance, and/or altered hepatic blood flow. Interestingly, despite increased serum drug concentrations, a significant decrease in dromotropic effect was observed. The authors attributed this observation to receptor down regulation which may be caused by pro-inflammatory cytokines (11).

In Crohn's disease, another type of inflammatory condition, patients with severe disease did not respond to CV therapy with verapamil. Despite the lower drug concentration in patient with remission, the drug response increased by reduction of the severity of disease (12).

β-blocker drugs such as propranolol may have the same fate as verapamil in patients with inflammatory conditions. This hypothesis was tested by Guirguis et al. (13). Propranolol metabolizes mostly by the liver and, as with verapamil, the inflammatory conditions reduce the clearance of propranolol and hence increase its plasma concentration. Despite the elevated propranolol concentrations, similar to Mayo et al.'s observation for verapamil, the prolongation of PR interval, as drug response to β-blocker, was significantly reduced in adjuvant arthritis (AA) rats when compared with control rats. The PK alteration as a result of inflammation does not apply to all β-blockers. For instance, sotalol, another β-adrenergic antagonist contrary to the verapamil and propranolol, is cleared through the kidney and therefore, its PK parameters are not altered by the hepatic effect of inflammation; however, the AA resulted in a significant reduction on pharmacological response to sotalol (14). Therefore, it was concluded that the reduced response to propranolol in rats with AA is suggestive of altered β-adrenergic receptors function (13).

The next question was whether the intensity of the disease, which can be evaluated by measurement of inflammatory mediator levels, plays any role in this setting. Infliximab, as an anti-TNF-α, reduces pro-inflammatory mediators and reverses the reduced drug response to β-blockers in the arthritic rat (14). On the other hand, in human study, comparing infliximab treated patients with RA in remission against patients with active disease revealed that infliximab restored the altered plasma protein levels and hepatic drug metabolism activity, which consequently resulted in relatively normal verapamil PK and PD. Therefore, it was concluded that the lower level of proinflammatory mediators in the infliximab group may explain this observation (15).

Overall, these studies have indicated that reduced drug-receptor binding secondary to a down-regulation of the target proteins expression is a possible explanation for the reduced response. However, this reduction of response due to inflammation does not apply to all CV drugs. For example, Daneshtalab et al. reported that the potency of valsartan and losartan, as Ang II receptor type 1(AT1R) antagonists (ARBs), were not diminished by RA, which distinguishes this class of drugs from β-adrenergic receptor antagonists and calcium channel blockers (16,17). ARBs have anti-inflammatory effects, which might reverse the effect of inflammation on receptor expression of target proteins. Furthermore, the same phenomenon was observed for pravastatin, which also presents anti-inflammatory effects (18).

Similar to ARBs and statins, GlcN, as an anti-inflammatory agent, was able to restore the inflammation-induced alteration of verapamil pharmacokinetics (19). It had been reported that inflammation alters the constitutive balance in the renin-angiotensin system (RAS) enzymes (20). It is not known if this imbalance influences angiotensin peptide production and AT1R, AT2R, as well as Mas receptor expression. Furthermore, the effect of GlcN treatment on the expression of RAS protein and production of peptide components has not been investigated.

1.3 Effect of inflammation on renin angiotensin system

The RAS is one of the major regulators of human physiology and specifically it has a key role in renal and CV homeostasis (21). Its systemic actions include the regulation of blood pressure (BP), fluid, and electrolyte balance through harmonized effects on the heart, blood vessels, and the kidneys. At the local level, the regulation of regional blood flow and the control of tissue response to a range of stimuli are also governed by the RAS. It is composed of a number of different elements that dynamically regulate the function of the CV system. In order

to provide a simultaneous rapid and balanced response to certain stimulants, these elements must perform opposing functions.

The RAS is mostly endocrine in nature and exerts its effects on vascular smooth muscles, renal tubules, the heart, and other organs through its three sets of components such as: I) enzymes; angiotensin converting enzyme (ACE) and ACE2, II) peptide products; angiotensin (Ang) II, Ang III, Ang IV, and Ang 1-7, and III) target receptors; AT1R, Ang II type 2 receptor (AT2R), and G-coupled protein receptor of Ang 1-7 (Mas receptor). Within the RAS, angiotensinogen is synthesized by the liver and metabolized by renin into inactive peptide Ang I. Subsequently, ACE metabolizes Ang I in order to generate Ang II, which imposes its physiological actions through AT1R and AT2R.

Constitutively, the RAS consists of two opposing axes. The first axis is constituted of the ACE/Ang II/AT1R receptor, which is responsible for physiologic and pathologic effects such as inflammation, vasoconstriction, cell proliferation, and fibrosis (22). The second axis is composed of the ACE2/Ang 1-7/Mas receptor, which results in anti-inflammatory, antifibrotic, antiproliferative, and vasodilatory effects (23). The key peptidase activity of ACE2 is degradation of Ang II to Ang 1-7 by which it changes the balance within the RAS cascade from vasoconstriction and proinflammatory to vasodilatation and anti-inflammatory actions, hence functioning effectively as a negative regulator of the RAS (24). The activation of the RAS plays an important role in the physiology and pathophysiology of various renal and CV diseases. Ang II, one of the main effectors of the ACE/Ang II/ AT1R axis, regulates BP, fluid, and electrolyte homeostasis. It contributes to the inflammatory response in the vascular walls (21). Ang II enhances the formation of cardiac and vascular reactive species causing endothelial dysfunction.

It has been shown that endothelial dysfunction is reduced by RAS blockers, such as ACE inhibitors (ACEIs) and ARBs (25).

Prior to the discovery of ACE2, all of interventional approaches for targeting the RAS were focused on ACE and Ang II. However, the importance of the ACE2/Ang 1-7/Mas receptor axis in the regulation of the RAS has become evident from the results of recent studies which are extensively reviewed elsewhere (23). Several studies have demonstrated that ACE2/Ang 1-7/Mas modulate the inflammatory response. Ang 1-7 negatively regulates the proinflammatory mediator's expression and release, and modulates fibrogenic pathways (26-29). It has been shown that tissue and circulating concentrations of Ang II were increased and Ang 1-7 concentrations were reduced as a result of *Ace2* deficiency (30). In addition, Wei et al. (31) have demonstrated that *Ace* knockout mice have reduced circulating Ang II, while tissue concentrations are not significantly modified, suggesting that a substantial amount of Ang II is generated by non-ACE pathways. These results indicate that, in some sites and especially in the CV system, ACE2 may be more important than ACE in regulating the local levels of Ang II and Ang 1–7. Previously, it had been reported that inflammation alters the constitutive balance in the RAS enzymes (20). This merits further investigation if this imbalance influences angiotensin peptides production and AT1R, AT2R, and Mas receptor expression as well. Although these findings indicate that RAS is associated with the development of CV complications, the process by which the RAS impacts the pathophysiology of vasculopathies in patients with RA is not clear and requires further investigation.

1.4 Effect of inflammation on arachidonic acid pathway

The arachidonic acid (ArA) pathway is another important system in regulating body functions. ArA is a fatty acid membrane component that after cleavage by phospholipase A2 due

to various stimulants or tissue damage becomes available for oxidative metabolism by multiple enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX) or cytochrome (CYP) 450 to form an array of biologically active metabolites. CYP enzymes are expressed in many tissues including the heart, kidney, liver, lung, adrenal gland, and vasculature (32,33). The enzymes metabolize ArA to eicosanoids such as hydroxyeicosatetraenoic (HETEs) and epoxyeicosatrienoic acids (EETs). The latter is further metabolized to dihydroxyeicosatrienoic acids (DHTs) (Figure 1.2) (34). ArA has a central role as a precursor of a variety of mediators produced by either constitutive or inducible enzymes or by nonenzymatic pathways. ArA metabolite are involved in biological functions of different tissues such as the brain (35-38), heart (39-41), kidneys (33,42-44), liver (45-47), lungs (48-51), and blood vessels (52-54). These lipid-derived ArA metabolites are involved in physiological processes through the intracellular signaling and are implicated in the pathophysiology of various disease states and inflammatory conditions such as hypertension, renal disorders, atherosclerosis, stroke, diabetes, obesity, and cancer (53). Ample recent preclinical and epidemiologic data suggest that modulation of eicosanoid metabolism may be a feasible clinical therapeutic strategy for the management of different pathological disorders, in particular CV disease (33).

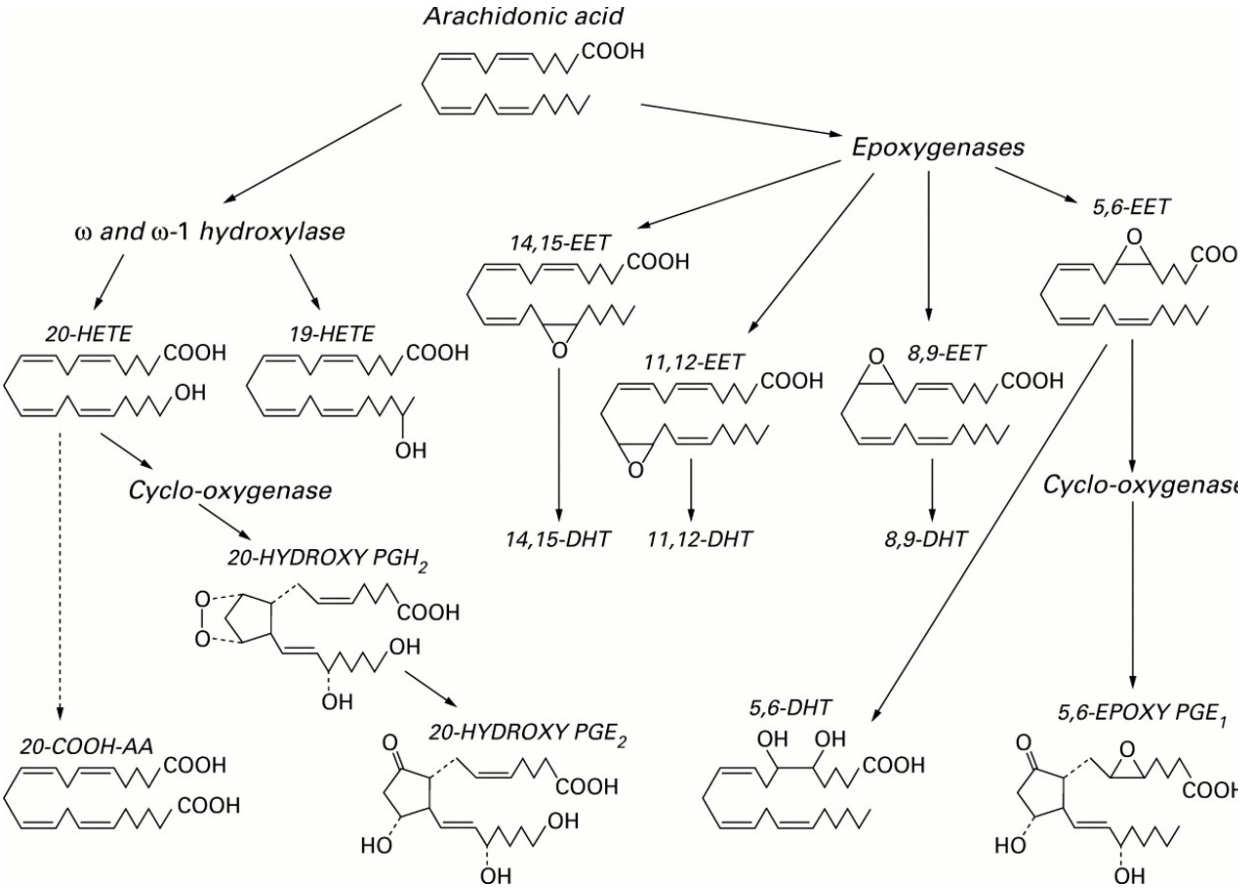

Figure 1.2. Arachidonic Acid pathway

Hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs), and dihydroxyeicosatrienoic acids (DHTs). Adopted from reference NO. (34) with permission.

ArA metabolites have important roles in the regulation and homeostasis of cardiac, renal, and pulmonary functions. Among these ArA metabolites, 20-HETE is produced in a tissue and cell-specific fashion and plays an important role in the regulation of vascular tone in these tissues. 20-HETE is a potent vasoconstrictor produced in vascular smooth muscle cells. The main subfamilies of CYP4 involved in this process are CYP4A and CYP4F in human and animal species (55). Different pathological conditions such as ischemic cerebrovascular diseases, hypertension, diabetes, kidney diseases, and cancer are linked to changes in 20-HETE production (33,43). The development of hypertension and CV diseases has linked to CYP4A enzyme activity (56,57). In the blood vessels, CYP4A enzymes are expressed in endothelial cells all over the vasculature net and their expression increases as vessel diameter decreases. This is in concert with increased concentrations of 20-HETE in narrower vessels due to higher rate of its production. This is why 20-HETE is called the eicosanoid of microcirculation (58). 20-HETE performs a dual action on the renovascular system: on one hand, it has an anti-hypertensive effect on renal tubules by inhibiting tubular ion transport and inducing diuresis. On the other hand, in the vasculature it imposes a pro-hypertensive effect by increasing vascular resistance and inhibiting endothelial-dependent vessel relaxation.

Once formed, ArA metabolites are subject to metabolism by other enzymes. For instance, the metabolism of 20-HETE happens through similar pathways as ArA, such as COX, LOX and CYP monooxygenases. COX metabolizes 20-HETE to produce 20-hydroxy PGs which have less vasoconstrictive properties than the parent compound in rat aortic rings (59,60). Platelet lipoxygenase and COX are also able to metabolize 20-HETE to inactive compounds (61). In the rat kidney, 20-hydroxy-EETs as epoxygenases derived metabolites of 20-HETE have been

demonstrated an anti-inflammatory and tissue protective effect through activation of the peroxisome proliferator-activated receptor (PPAR)-α (62).

Several studies previously identified some of these ArA metabolites as potent pro-inflammatory agents and considered them as indices of inflammation (63-68). *In vitro* studies using an acute inflammatory model of LPS in mice and rat have reported similar results (32,69).

EETs, as other important members of ArA metabolites with potent vasodilatory and anti-inflammatory effects, have been shown to induce smooth muscle hyperpolarization and relaxation through activation of different types of K^+ channels (70). Soluble epoxide hydrolase (sEH) metabolizes EETs to DHTs with less vasodilatory activity than the parent EETs (71,72).

1.5 Rheumatoid arthritis

RA is a chronic, progressive, and systemic autoimmune inflammatory condition that causes considerable pain, swelling, and stiffness in many joints and leads to joint destruction, diminished function, and disability (73). It is an unpredictable long term condition with variable episodes of disease activity. Patients with RA are at a higher risk of developing CV disease, which accounts for 35% to 50% higher mortality in this population (10). Life expectancy after a CV event is shortened by 3 to 18 years compared to patients suffering from CV diseases without RA comorbidity. Inflammation plays a major role in the pathogenesis and prognosis of CV diseases such as hypertension and acute myocardial infarction (74-77). Inflammation has been implicated in the initiation and development of atherosclerosis, a chronic inflammatory condition in the walls of arteries. Evidence suggests that common proinflammatory cytokines are involved in the development and progression of both atherosclerosis and RA (78). The immunological mechanisms involved in the pathogenesis of atherosclerosis resemble those which occur in RA development (79). In patients with RA, higher levels of circulatory inflammation mediators (78)

such as TNF-α, IL-1, IL-6, IL-17, CRP, vascular cell adhesion molecule-1, and intracellular adhesion molecule-1 have been found to be risk factors for CV events and mortality (80). It has been clearly established that systemic biomarkers of inflammation, even at considerably lower levels than those seen in patients with RA, independently predict CV events in individuals with or without existing heart diseases (81). This evidence indicates that we need to pay closer attention to patients with RA in order to reduce the CV related mortality and morbidity in this population.

1.6 Osteoarthritis

Osteoarthritis (OA), formerly known as degenerative joint disease, is a progressive joint disease that happens when a body fails to repair the injured joint tissues, resulting in a destruction of cartilage and bone. It is the most common form of arthritis (82) affecting 9.6% of men and 18.0% of women older than 60 years of age worldwide (83). Pain and morning stiffness as primary clinical symptoms of OA are caused by catabolic and anabolic process imbalances in joints, which results in articular cartilage erosion in synovial joints. There are other radiographic findings such as joint space narrowing and osteophyts. Under healthy situations, a cytokine-mediated balanced anabolic and catabolic process maintains integrity of the cartilage. However, in OA joints some catabolic enzymes such as matrix metalloproteinase and aggrecanase are over-expressed and the balance of the process shifts toward catabolism, which results in cartilage damage. Consequently, some pro-inflammatory cytokines such as IL-1β and TNF-α are released by the synovial membrane that starts a cascade of inflammatory reactions (84). Inflammation results in joints swelling and sensation of pain and stiffness. As the inflammation progresses, more catabolic enzymes become involved and more articular cartilage damage occurs.

OA as a debilitating, progressive disease and RA as a multisystem disease with underlying immune mechanism are different from each other. However, several studies have focused on possible correlation of cytokine levels in RA and OA with some common clinical pathological features (85). In fact, the development of RA and OA is associated with the alteration of plasma levels of some cytokines, such as IL-1β, IL-6 and TNF-α (85). Additionally, the lack of physical activity in individuals with OA potentially increases the risk for CV disease. The association has been reported between severity of disability and increased rate of CV disease and mortality incidence among individuals with OA (86,87).

OA can be categorized as primary or secondary based of the cause and the location of joints involved. The knee as the most prevalent site and the hand, hip, foot and spine joints are usually the ones that involved with primary OA. Alternatively, secondary OA is prevalent in the shoulder, elbow, wrist, and ankle joints, generally occurring after joint injury or infection. The severity of OA depends on the extent of the damage and the type of tissue involved. In more advanced OA, when the bone tissue is also affected, the pain and loss of mobility have a profound effect on quality of life. The financial burden of OA is high, and includes the direct cost of treatment, transportation, adaptation of home and life for the patients and their caregivers, and indirect costs such as those due to loss of income as a result of disability, which is more than functional impairment. The psychological impact of OA on quality of life such as feelings of helplessness and depression can influence individual's functioning. These substantial psychological and financial costs of OA necessitate early diagnosis and treatment.

1.7 Adjuvant arthritis animal model

In order to study the pathogenic processes of arthritis, rodent models of RA and OA are useful tools. Among these animal models, AA, as an experimental model of polyarthritis, has been

widely used for testing of antiarthritic agents (88). As mentioned above, despite the difference in the nature of RA and OA, these inflammatory conditions share some common inflammatory mediator profiles and clinical pathological features (85). The rat model of AA also shows several common features with both OA and RA and provides a reliable animal model for studying these diseases in humans. (88,89)

1.8 Anti-inflammatory therapy of RA

Available treatment options aim to control the inflammatory process in order to reduce symptoms and delay joint destruction. Considering the higher CV risk (cardiac thrombosis, myocardial infarction and stroke) in this population, early management through single or combination drug therapy strategies and involvement of multidisciplinary team could control the disease better and prevent the development of other comorbidities.

1.8.1 Steroidal anti-inflammatory agents

Glucocorticoids are a class of steroidal hormones with potent anti-inflammatory and immunosuppressive properties. They have numerous pharmacological actions and a many therapeutic indications such as inhibition of the synthesis of the proinflammatory PGs and leukotrienes, suppression of immune cells, and reduction of the production of pro-inflammatory mediators. Glucocorticoids are indicated in the management of autoimmune diseases, asthma, organ transplants, renal diseases and inflammatory conditions. However, they may cause serious side effects, and therefore their usage should be weighed against their side effects. They are indicated only for short-term use.

1.8.2 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) control inflammation and associated pain by blocking both COX-1 and COX-2 enzymes and thus preventing PGs synthesis. This action also explains the gastrointestinal adverse effect of NSAIDs, as COX-1 is responsible for maintaining the integrity of normal physiologic function in gastric mucosa and other tissues (90). The design of selective NSAIDs is justified by this concept. This class of NSAIDs selectively inhibits the COX-2 enzyme, resulting in diminished gastrointestinal adverse effects; however, their use has been associated with increased fluid retention, elevated BP, and impaired renal function (91). These adverse effects along with increased thrombotic events reported in patients receiving COX-2 inhibitors such as rofecoxib, resulted in its withdrawal from the market in 2004 (92). The other members of COX-2 inhibitors may also carry the same risk. Therefore, due to potential risk of CV events, the use of NSAIDs should be limited in patient with RA and CV diseases.

1.8.3 Disease modifying antirheumatic drugs

It is important to note that neither corticosteroids nor NSAIDs are able to arrest RA disease progression. Favorable management of RA requires rapid and sustained suppression of inflammation with disease modifying antirheumatic drugs (DMARDs). These agents are defined as medications that slow down or stop the progression of disease. Disease modification is most realistically confirmed by the ability of these agents to decrease radiographic progression of the disease. They have different mechanisms of action; however, it has been reported that the relative efficacy of methotrexate, sulfasalazine, intramuscular (*i.m.*) gold, and penicillamine is similar. Antimalarial agents such as chloroquine and hydroxychloroquine present with less

efficacy (93). The side effects of DMARDs are also a matter of concern and in some cases, such as penicillamine, due high incidence of toxicity its use is very rare.

1.8.4 Biologics

Biologic response modifiers or biologics are a group of drugs that has been developed in the last decade. As a class of medications, biologics have added major therapeutic options for the treatment of many diseases. Due to their effectiveness, safety, and better knowledge on mechanism action at their initial targets in altered immune system, the use of biological therapies is expanding as an adjunct to DMARDs for treatment of RA (94). Although these targeted therapies are often well tolerated by patients, these agents are not considered as first line medications due to the high costs, the inconvenient intravenous route of administration, and some side effects such as serious infection. The biologics are mainly targeted toward cytokines, B lymphocytes and co-stimulation molecules. Based on their mechanism of action, they are classified as novel anti-TNF alpha blockers (fully humanized or pegylated), anti-IL agents (toward IL-1, IL-6), B-cell-directed therapies (toward CD20, CD22), or co-activation signaling (CTLA4-Ig) (95). As a result of a better understanding of biologics' mode of action and improved industrial capacity for their production, the therapeutic options using these agents for treatment of autoimmune diseases are quickly expanding. Most of these agents are approved by FDA only for treatment of RA; however, because of their efficacy and safety record, they are used in off-label indications for patients with resistant autoimmune conditions. The adverse reaction profiles are similar for all biologics, with infection as the most severe side effect. By conducting screening tests before initiation of biologic therapy, some of the serious infections such tuberculosis recurrence have been virtually eradicated. Table 1.1 presents the list of some of the available biologics for RA treatment.

Table 1.1 Biologic therapy in autoimmune diseases such as RA. Adapted from reference No. (95,96)

Drug (trade name)	Mechanism of action
Infliximab (Remicade)	Chimaeric monoclonal antibody against TNF-α
Etanercept (Enbrel)	Fusion protein that mimics soluble TNF receptor
Adalimumab (Humira)	Fully humanised monoclonal antibody against TNF-α
Certolizumab (Cimzia)	PEGylated Fab fragment of fully humanised Anti-TNF-α
Golimumab (Simponi)	Fully humanised monoclonal antibody against TNF-α
Rituximab (Mabthera)	Chimaeric monoclonal antibody to CD20
Abatacept (Orenica)	Fusion protein of an immunoglobulin and extracellular domain of CTLA-4
Tocilizumab (RoActemera)	Humanised monoclonal antibody to IL-6 receptor
Anakinra (Kineret)	Recombinant IL1 receptor antagonist
Canakinumab (Ilaris)	Recombinant IL1 receptor antagonist
Rilonocept (Arclyst)	Recombinant IL1 receptor antagonist
Ofatumumab (Arzerra)	Human monoclonal antibodyto CD20 molecule
Belimumab (Benlysta)	Human monoclonal IgG1 gamma
Epratuzumab (Lymphocide)	IgG1 monoclonal antibody directed against the CD22 molecule
Sifalimumab	An anti-IFN-α monoclonal antibody

TNF, tumor necrosis factor; CTLA, cytotoxic T lymphocyte associated

1.9 Anti-inflammatory treatment options for coexisting illnesses with RA

In patients with RA the risk of accelerated atherogenesis and coronary heart disease is greater than in healthy subjects (10). The new concepts of immunological mechanisms involved in the pathogenesis of atherosclerosis resemble those which occur in RA development (79). This suggests that the systemic inflammation that characterizes RA may play an important role in the higher incidence of atherosclerosis seen in patients with RA. Risk factors for atherosclerosis should be aggressively sought and addressed in this population. In particular, smoking cessation may be fruitful, since smoking has been associated with increased severity of arthritis. Recent use of statin and ARBs as an adjunctive therapy in patients with RA may address the risk of CV disease, since these classes of drugs should decrease both inflammation and atherosclerosis.

1.9.1 Statins

Anti-inflammatory activities of statins in inflammatory conditions have been well documented by both basic research and clinical studies. In the 1980s these agents were introduced as cholesterol synthesis blockers by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase which resulted in a lower plasma level of cholesterol. In the last three decades, it has been shown that statins possess anti-inflammatory and antioxidant activities resulting in the beneficial reduction of CV risk in both humans and animal models (97). Statins have been demonstrated to have protective anti-inflammatory effects in several inflammatory conditions (98-100).

As an inflammatory disease of the joints, arthritis is associated with elevated levels of inflammatory mediators. The rat model of AA was used to evaluate the anti-inflammatory effect of atorvastatin on joint inflammation and associated oxidative stress. The results show that atorvastatin produced a dose-dependent reduction in joint inflammation and normalized the

levels of oxidative stress (101). Other studies have reported antioxidant, antinociceptive, and anti-inflammatory effects for atorvastatin and rosuvastatin, and a reduction in leukocyte activity and cytokine release effects for lovastatin in different animal models (102,103).

Statins have been shown to control both acute and chronic inflammatory processes. As mentioned above, statins demonstrate these anti-inflammatory effects by lowering pro-inflammatory mediator levels. In a human study, patients with Crohn's disease received 80 mg atorvastatin once daily for 13 weeks and then followed up for 8 weeks after the treatment. The statin effects on pro-inflammatory mediator levels such as CRP, CD14, and TNF-α were assessed. The results of this study indicated that atorvastatin therapy downregulated the expression of these mediators and reduced intensity inflammation in some of the patients with Crohn's disease (104).

1.9.2 Angiotensin enzyme inhibitors and angiotensin receptor blockers

Several studies on ACEIs and ARBs have shown that the RAS action extends far beyond BP control and electrolyte balance (105,106). Activation of AT1R by Ang II through stimulation of multiple signaling pathways, several growth factor receptors, reactive oxygen species, and other pro-inflammatory responses mimics several cytokine-like actions (107). Ang II production can be regulated by nuclear factor-κB (NF-κB) and in turn it is also capable of activating NF-κB and increasing the expression of NF-κB dependent genes (107). Ang II is also associated with the expression of pro-inflammatory cytokines, such a TNF, IL-1β and IL-6 (108).

Recent studies indicate that the balance between pro- and anti-inflammatory cytokines within the brain has a central determinant role in the control of hypertension. An Ang II-induced imbalance in cytokines status is arbitrated by glycogen synthase kinase-3 (GSK-3)β-mediated changes in downstream transcription factors in neuronal cells. GSK-3β is a proline-

directed serine-threonine kinase that was initially identified as a phosphorylating and inactivating agent of glycogen synthase. It has been shown that Ang II-induced effects could be improved by GSK-3β blockage. This suggests GSK-3β as a key therapeutic target for hypertension that is characterized by augmented pro-inflammatory cytokines and NF-κB activation (109).

The pro-inflammatory effects of Ang II and therapeutic strategies that interfere with the components of the RAS (such as ACEIs and ARBs) characterize Ang II as a reasonable therapeutic target to reduce inflammation and its related risks. This is suggested by the results of large clinical trials in patients with different inflammatory conditions, such as RA, diabetes, hypertension, renal and Crohn's diseases (110-114).

Regarding ACEIs, there is no supporting data indicating ACEIs ability in reducing plasma levels of major inflammatory markers in hypertension models. However, the results of different trials using ARBs seem to univocally confirm that there is an anti-inflammatory effect of drugs blocking AT1R receptor in different settings.

Olmesartan is one of the most effective ARBs currently available in the market. Its rapid and reliable antihypertensive effectiveness allows a high percentage of patients to reach their target BP quickly, which is associated with beneficial effects on reduction of oxidative stress, inflammation, and atherosclerotic lesion formation. In a review article, Agabiti Rosei et al. (115) reported that olmesartan is able to control inflammation in hypertensive patients, decrease oxidative stress in patients with type 2 diabetes mellitus, and restore the normal wall: lumen ratio of small resistance arteries in patients with hypertension. Furthermore, a 2-year study involving hypertensive patients with carotid atherosclerosis showed olmesartan reduced the intima-media thickness of the carotid artery and significantly reduced the volume of large atherosclerotic

plaques (116). These reports conclude that olmesartan may reduce CV risk by concurrent regulation of BP and neutralizing the proatherogenic effects of Ang II (115).

As another ARB, irbesartan has a selective PPAR-γ modulatory action; therefore, it may possess anti-inflammatory and antioxidative effects, as well as beneficial effects on glucose and lipid metabolism. In a human study Taguchi et al (117) investigated the different effects of irbesartan, by enrolling high-risk hypertensive outpatients who were suffering from at least one complication such as coronary artery disease, CV disease, or diabetes. Alterations in lipid parameters, inflammatory markers, and oxidative stress indicators were evaluated. The result has shown significant decreases in triglycerides, CRP, and oxidative stress indexes after 12 weeks of irbesartan therapy. There was also significant increase in high-density lipoprotein cholesterol level in patients treated with irbesartan. The authors conclude that irbesartan was able to decrease oxidative stress and inflammation and show beneficial effects on lipid metabolism and metabolic syndrome, indicating that it may be useful option for treatment of high-risk hypertensive patients (117).

The effect of losartan, another ARB, has been investigated in rats with AA, an animal model of human RA (118). AT2R activation by its ligands mostly counteracts the AT1R actions. Wang et al. investigated the role of AT2R in the treatment of AA in rats with losartan from day 14 to day 28 post-arthritis induction. Ang II, TNF-α levels, and AT1R and AT2R expressions were evaluated along with an arthritis index calculation and histological examination. The results of the study indicated that after losartan treatment AT1R expression was downregulated, while AT2R expression was up-regulated, positively correlating with arthritis index reduction. The conclusion was that the up-regulation of AT2R may offer an additional mechanism by which losartan applies its anti-inflammatory effects in rats with arthritis (118).

Ulcerative colitis is a chronic inflammatory bowel disease caused by distorted immune responses and the production of pro-inflammatory cytokines. Recent evidence is indicative of the involvement of the pro-inflammatory hormone Ang II in inflammatory bowel disease. Nagib et al. (119) investigated the possible role of olmesartan in the improvement of ulcerative colitis. Their findings indicate that olmesartan dose-dependently ameliorated the colonic histopathological and biochemical injuries. This suggests olmesartan as an alternative for treatment of ulcerative colitis due to its anti-inflammatory and antioxidant effects (119).

RAS has been shown to be associated with the pathology of ageing and hypertension, both of which are the main risk factors for the development of CV and renal diseases. In an animal study, Mihailavic-Stanojevic et al. (120) investigated the effect of losartan on the cardiac function of young and old spontaneously hypertensive rats. Losartan treatment significantly improved aortal blood flow and resistance in younger animals when compared with aged-matched non-treated littermates. In aged rats, losartan treatment significantly reduced BP and improved age related impairment of left ventricular weight index, vascular resistance, and glomerular filtration. It was concluded that losartan treatment restored age related systemic and regional impairment of hemodynamics and left ventricular hypertrophy in old spontaneously hypertensive rats. These results could be translated into lower CV risk in elderly patients treated with losartan due to the reduction of ventricular hypertrophy, vascular resistance and pressure overload, and preserving kidney function (120).

Candesartan as an ARB drug is associated with prevention or delaying the development of coronary heart disease. In a randomized, double-blind, placebo-controlled crossover study, placebo or candesartan were given to patients with mild to moderate hypertension during a two months period. Candesartan therapy was able to significantly lower both systolic and diastolic

BP, plasma CRP, soluble CD40 ligand, and fasting insulin levels. It was concluded that candesartan therapy significantly reduced inflammation and improved insulin sensitivity in hypertensive patients (121).

1.10 Therapeutic choices for OA treatment

Figure 1.3 presents a pyramid approach for managing patients with OA. In this approach each layer is added one to another during the course of therapy stepwise. That means therapy starts with patient education and other non-pharmacological approaches, then, if needed, acetaminophen (paracetamol in Europe) will be added as the first line drug therapy. If the results are not satisfactory, the next layer could be added on top of the previous layer and so on (82).

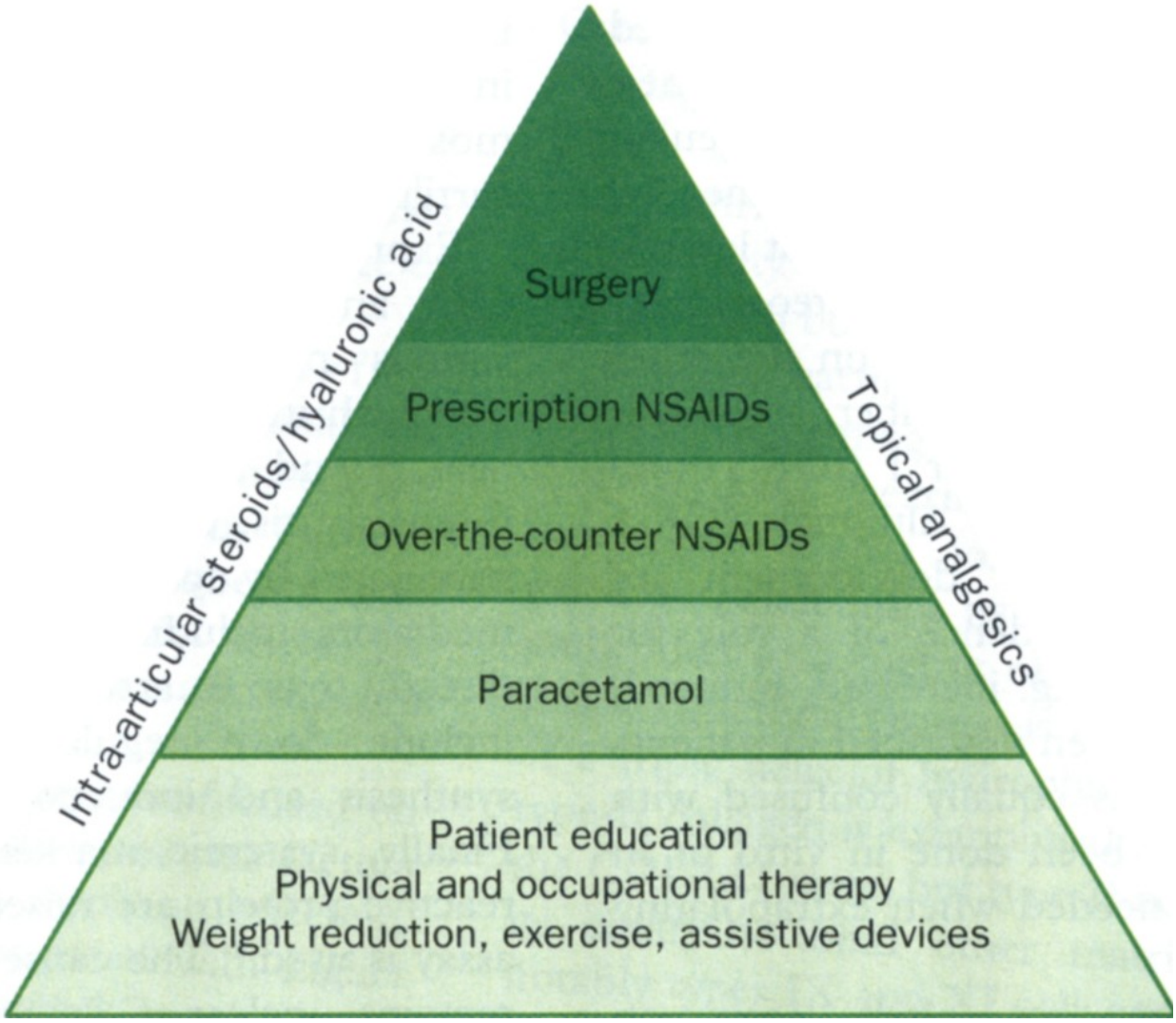

Figure 1.3. Pyramid approach to the management of osteoarthritis.

Adopted from reference No. (82) with permission

The current treatment strategy of OA is mostly based on improvement of reduced quality of life and the control of symptoms such as pain and functional limitation. There are non-pharmacological and pharmacological options.

1.10.1 Non-pharmacological approaches

Non-pharmacologic therapies, including patient education, physical therapy, weight reduction, exercise, and bracing are equally recommended for OA patients. Their excellent safety makes them important components of the treatment plan for OA patients. Traditional therapy options such as acupuncture, arthroscopic debridement, and electrical stimulation play more controversial roles in OA management and are less available. Surgery is the last-resort option for OA patient whose disease is refractory to less-invasive treatment methods.

1.10.2 Pharmacological intervention

1.10.2.1 Acetaminophen and non-steroidal anti-inflammatory drugs

Pharmacological options are better studied in OA than non-pharmacologic treatments. Acetaminophen or paracetamol is often considered first line agents for treatment OA. This agent is safer than NSAIDS, and usually recommended by most physicians before moving on to more aggressive treatment. Older studies report the efficacy of acetaminophen to be the same as NSAIDs (122); however, more recent studies report higher efficacy for NSAIDs than acetaminophen (123). Due to the inflammatory nature of established OA in most patients, NSAIDs with anti-inflammatory and analgesic effects are the most widely used class of drugs in this population. However, because of the adverse effects associated with long-term use of NSAIDS, acetaminophen is considered first-line therapy.

1.10.2.2 Narcotics

Opioid analgesics are sometimes used to treat the pain of OA; however, they are not considered as a first-line pain management option. According to recommendations of American Academy of Rheumatology, these agents should be typically reserved for patients who are intolerant of or who have failed other traditional options for management of the pain (124). These agents should be used in low-doses for a short term, especially in elderly patient while considering the incidence of their side effects such as sedation and constipation. The use of narcotic drugs is not well studied in OA, but a few studies reported efficacy and safety in carefully selected patients (125,126).

1.11 Glucosamine

GlcN alone or in combination with chondroitin sulfate (CS) have been widely used as modifying agents for OA. Although GlcN has a status of prescription drug in Europe, it is considered a food supplement in the North America, and thus it is not FDA regulated. GlcN is available in tablet, capsule, liquid and topical semisolid dosage forms, which are formulated as a hydrochloride or a sulfated salt. In some dosage forms it is often found in combination with various amounts of CS and/or methylsulfonylmethane (MSM). GlcN is a part of the structure of the polysaccharides, chitosan and chitin, which compose the exoskeletons of crustaceans and other arthropods, cell walls in fungi, and in many higher organisms. It is a major precursor in the biochemical synthesis of glycosaminoglycans that are present in connective tissues (127). In addition to its chondroprotective properties, similar to a disease-modifying antirheumatic drug, GlcN appears to inhibit or halt the underlying immune process and prevent long-term damage and even reverse the inflammatory process (128,129). GlcN has been shown pharmacological effect against the progression of RA (130), OA (131,132) in human and experimentally induced

arthritis in rats (133). Although the mechanism of action of GlcN is not fully understood, there are several *in vitro* studies suggesting a promising pharmacological effect through mechanisms such as the inhibition of proteoglycan degradation and stimulation of proteoglycan synthesis, suppression of IL-1β-induced cyclooxygenase-2 and matrix metalloproteinase-13 expression, reduction of TNF-α level, suppression of NO and PGE_2 production (134). Anti-inflammatory effect of GlcN is also associated with the inhibition of NF-κB activation by promoting the O-GlcNAc of nuclear components of transcription process (135).

CS, on the other hand, is one of the principle glycosaminoglycans in healthy cartilage. It presents beneficial effects on management of OA symptoms. It is likely, however, that the action of CS is far more complex than originally believed. It affects both anabolic and catabolic pathways in cartilage in multiple ways. It is able to maintain viscosity in joints, stimulate cartilage repair, and inhibit enzymatic degradation of the cartilage (136,137). CS shares the same mechanism as GlcN and partly mediates its effect on NF-κB nuclear translocation (135). The multicenter, double-blind, placebo- and celecoxib-controlled Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) revealed that the combination of GlcN and CS did not result in an effective improvement of OA pain when compared to a placebo. However, there was a significant improvement on pain reduction in a subgroup of patients with moderate- to severe knee OA (138).

GlcN is an amino monosaccharide found in the proteoglycan molecules that contribute to the strength, flexibility and elasticity of connective and cartilage tissues (133). GlcN is involved in the synthesis of glycosaminoglycan, proteoglycan and hyaluronic acid, and contributes to their structural backbone. It mechanism of action as asymptomatic slow acting drugs for OA is elaborated elsewhere (135). It has been widely evaluated for its efficacy in relieving the symptoms of OA and its disease-modifying potential. Similar to a disease-modifying

antirheumatic drug, GlcN appears to inhibit or halt the underlying immune process and prevent long-term damages. GlcN has shown pharmacological effect against the progression of RA (130), OA (131,132) in humans and in experimentally induced arthritis rats (133,139). Nevertheless, even though many animal studies confirmed potent anti-inflammatory actions for GlcN, human clinical trials have demonstrated controversial results ranging from strong effectiveness to marginal or negligible potency (140). Nevertheless, the popularity of GlcN in the treatment of joint diseases has resulted in over $2 billion global sale per year since 2008 (141,142).

1.11.1 Physicochemical properties

GlcN is the biochemical precursor of all nitrogen-containing sugars. It is available as different salt forms (Figure 1.4).

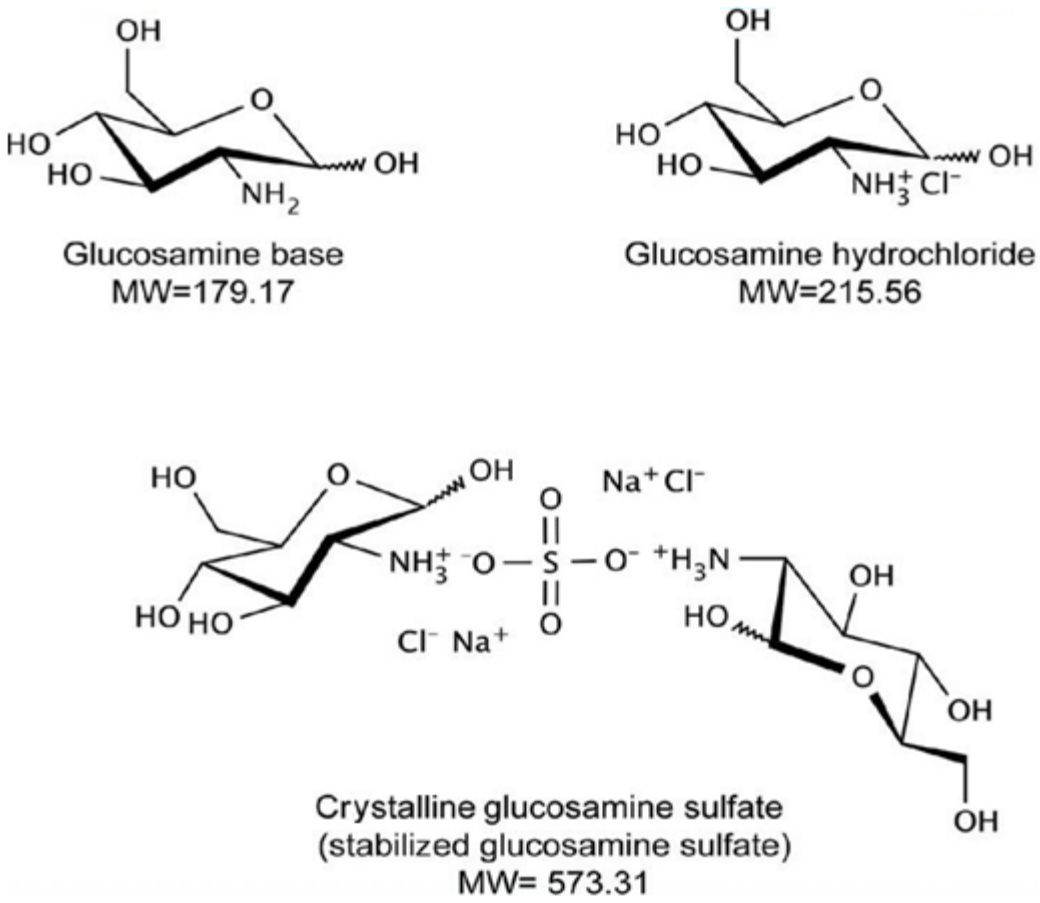

Figure 1.4. Chemical structure of GlcN and its salts.

Adopted from reference No.(143) with permission

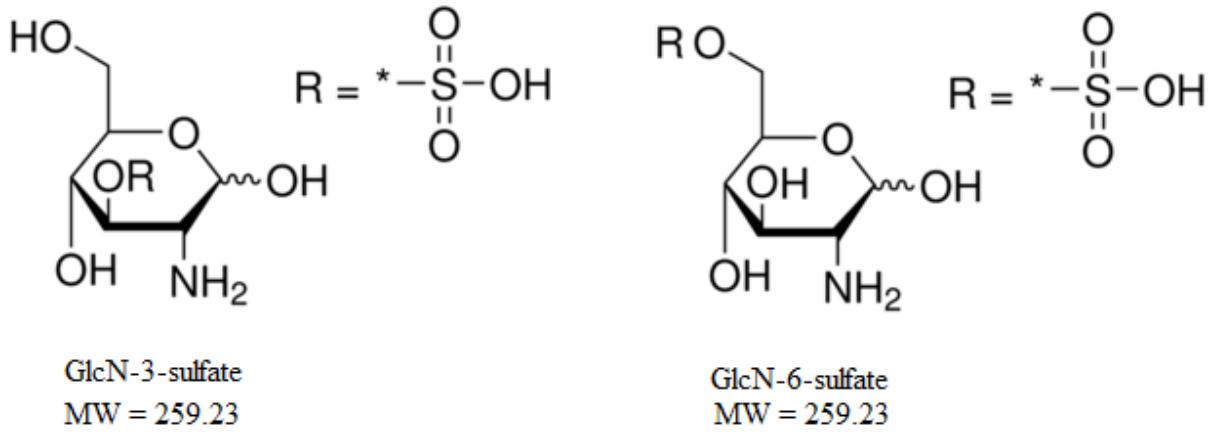

Figure 1.5. Chemical structure of covalently bound GlcN-3-sulfate and GlcN-6-sulfate.

Adopted from the Sigma-Aldrich website (www.sigmaaldrich.com)

The pharmacologically active forms of different GlcN containing chemicals are ionic HCl and sulfate salt forms, which deliver GlcN free base after dissolution in gastric acidic media. There are also covalently bound sulfated glucosamine compounds available (Figure1.5). These compounds do not present pharmacological activity (144-146).The molecular weight of GlcN as free base is 179.17, while it is 215.56, 259.23 and 456.43 for its HCl, sulfate salts and covalent bound forms, respectively. The sulfate salt of GlcN is very unstable, and therefore it usually co-crystallized with sodium chloride (NaCl) or potassium chloride (KCl) to produce stabilized crystalline GlcN sulfate with a molecular weight of 573.31 or 605.52, respectively. In this complex structure, GlcN, sulfate, sodium or potassium and chloride ions exist in a stoichiometric ratio of 2:1:2:2, respectively. GlcN is very soluble in water, sparingly soluble in methanol or ethanol, and insoluble in ether or chloroform. With a pKa of 6.91, both HCl and crystalline sulfate salts freely dissolve in gastric fluid with pH of 1.2 to form GlcN free base.

1.11.2 Pharmacological effects

GlcN has been the subject of a remarkable number of research studies for its effects on relieving the symptoms of OA and these studies have been reviewed extensively (147-168). GlcN is one of the building blocks of glycosaminogylcans (GAG) and it has been suggested that exogenous GlcN is able to enhance the production of GAG and regeneration of cartilage (169-172). More recent studies suggest that therapeutic benefits of GlcN for management of OA (84,173-175) or RA (130,176-178) are mediated through its anti-inflammatory activities. Indeed, it has been shown that GlcN sulfate was able to inhibit NF-κB nuclear translocation and IL gene expression in human osteoarthritic chondrocytes (179). GlcN has been presented as a potent transcriptional regulator of iNOS and other genes involved in the general inflammation process in microglia cells treated with LPS (180,181). Anti-inflammatory effects of GlcN have been also

reported in systemic inflammatory complications such as atherosclerosis (174,182), lung inflammation (183), brain ischemia (184), and cancer (185). Intravenous administration of GlcN during resuscitation improves recovery from trauma hemorrhage and increases the survival rate by improving cardiac function, organ perfusion, mean arterial pressure and attenuation of circulating inflammatory cytokines levels. These results may suggest a vasoactive and/or positive inotropic effect of glucosamine in hypovolemic shock (186,187).

GlcN has been studied in several inflammatory conditions in animals, such as AA in rats (19,133) and mice (177). As mentioned above, the observed effects in these studies are mainly attributed to the anti-inflammatory properties of GlcN that are achieved by lowering the serum levels TNF-α, IL-6, and IL-1-induced gene expression. GlcN inhibits the cytokine intracellular signaling cascade through inhibition of NF-κB activation (143). Additional reported mechanisms of anti-inflammatory action for GlcN include suppression of IL-1-β-induced production of IL-8, NO, and PGE2, inhibition of phosphorylation of p38 mitogen-activated protein kinase in synoviocytes (133,188), and suppression of neutrophil functions such as superoxide generation, phagocytosis, granule enzyme release and chemotaxis (189).

Several studies suggest that GlcN modifies the symptom of OA and halts the disease progression with a favorable safety profile (190,191). No symptoms of toxicity were observed after oral administration of GlcN to animals. The median lethal (LD_{50}) doses of 8.0, 6.6, 1.6g/kg GlcN sulfate were reported after oral, intraperitoneal (*i.p.*) or intravenous (*i.v.*) administration in mice, respectively (148).Similarly, animal studies strongly suggest its disease-modifying effects and anti-inflammatory properties (170,192-194). However, the compound remains, perhaps, the most misunderstood therapeutic agent in use. The controversy over GlcN effectiveness will be discussed in following chapters.

1.11.3 Pharmacokinetics

1.11.3.1 Absorption

GlcN is highly soluble in water. Due to its slightly acidic nature with a pKa value of 6.91, it presents in complete ionization in the stomach at pH 1-3, while in the pH of 6.8 in the small intestine it is ionized about 50%. This level of ionization facilitates its absorption from the small intestine (195). It has been reported that GlcN is a substrate for glucose facilitative transporters (GLUT1, 2 and 4) with higher affinity to GLUT2. However in hepatocytes, its transport through the membrane is entirely mediated by GLUT2. In the intestinal mucosa, GLUT2 present as a main transporter for mediation of sugars transport such as glucose, galactose and fructose from enterocytes to the blood stream (196). Ibrahim et al. reported the involvement of GLUT2 in the transport of GlcN through the rat intestinal wall using quercetin, as a GLUT2 inhibitor (197).

The oral bioavailability of GlcN is relatively low and has been reported as 19% (198), 12% (199) and 2.5% (200) in rats, dogs, and horses, respectively. It was 26% in humans after administration of radiolabeled GlcN sulfate (201). This low bioavailability is not due to a lack of absorption through the gastrointestinal tract, since only 11.3% of the administrated dose was recovered in feces (195). The hepatic first pass metabolism was also ruled out as the systemic availability of GlcN after the *i.p.* and *i.v.* route of administration were similar. Therefore, it has been concluded that possibly gut rather than hepatic metabolism is responsible for the observed low oral bioavailability of GlcN (198). This could be due to utilization of GlcN in intestinal epithelial cell through the hexosamine biosynthetic pathway (HBP).

1.11.3.2 Distribution

GlcN does not bind to plasma or tissue proteins in different species (202). The transport of GlcN through the membranes is mediated by GLUT2; however, it was not completely blocked by GLUT2 inhibitor (197). Therefore, its passive transport cross the membranes enables GlcN to distribute in the body fluids. The reported values for GlcN volume of distribution at a steady state are very variable. This variability is probably attributable to the differences in sensitivity of the methods of analysis and the duration of blood sample collection. The volume of distribution is reported to be 0.6 L/kg in dogs and horses (199,200), 2.1 L/kg in rats (198) and 0.07 L/kg in humans (202).

Tissue distribution of GlcN in the rat organs was studied using a radiolabeled compound. Plasma radioactivity appeared due to *i.v.* administration of radiolabel GlcN, which rapidly declined in the first 30 min, peaked at 2 h post-dose, and slowly declined, with a $t_{1/2}$ of 28 h. Tissue radioactivity was detected after 10 min in all organs with the highest activity in the liver and kidneys. This activity was detectable in all organs several days after the dose administration (195).

It has been reported (195) that the incorporation of GlcN into plasma proteins after oral administration of the radiolabeled compound followed similar PK patterns seen after parenteral doses. The plasma radioactivity appeared rapidly in15 min but in lower levels than that after *i.v.* administration. It peaked 4 h after-dosing, then declined with a $t_{1/2}$ of 18 h between the 8^{th} and 48^{th} h. Similar to the *i.v.* route, the highest radioactivity was observed in the liver and kidneys. It reached its peak level in plasma, liver and kidneys after 4 h, and in the femoral cartilage after 8 h. Radioactivity in liver and kidneys was 2-10 and 1.2-4 times higher than in plasma , respectively. In femoral cartilage, the radioactivity was higher than plasma and similar to the

liver after 72 and 120 h post-dosing. The radioactivity rapidly decreased in the intestinal content up to 1 h and then reached its highest level after 2 h, followed by a slow decline (195). This increase of radioactivity in the intestine after early decline may suggest the uptake and biotransformation of GlcN by intestinal tissues.

The distribution of GlcN to joint and synovial fluid was studied in horses. The synovial concentration of GlcN was measured after the *i.v.* and nasogastric administration of 20 mg/kg GlcN HCl. The basal synovial concentration was below the detection limit before dose administration. The concentration elevated to reach 1.5-2.5 and 0.05-0.12 μg/mL after 1 h post-*i.v.* and nasogastric dosing, respectively. There was a correlation between GlcN in serum and synovial concentrations. The GlcN serum concentration was not detectable after 6 h post-dose, while it could be detected in the synovial fluid at 12 h post dose (203). This result indicates that GlcN rapidly distributes to the synovial fluid and slowly eliminates from its site of action.

Persiani et al. reported in humans that GlcN distributed to the synovial fluid and its concentration increased more than 20 fold of basal level after two weeks of daily administration of 1500 mg crystalline GlcN sulfate. Moreover, there was a significant correlation between plasma and synovial concentrations and the synovial level at 3 h after oral dose was about 25% of the corresponding plasma concentration at that time point (204).

The distribution of GlcN may be influenced by joint inflammation as it has been shown that the maximum concentration in the inflamed joint of the horse was four times higher than healthy control animals after nasogastric administration of the same dose of GlcN. The effect of inflammation on synovial concentration did not reflect on the plasma concentration, indicating that joint inflammation only changed the GlcN distribution in the joint but did not affect its oral absorption (205).

1.11.3.3 Metabolism

GlcN is not metabolized by liver cytochrome P450; however, it is transformed and incorporated by liver into plasma glycoprotein through hexosamine pathway (201,206).There is no report of any GlcN induction or inhibition effect on the human liver cytochrome P450, particularly CYP1A2, CYP2E1, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 (207).Therefore, there is no possibility of a drug-drug interaction between GlcN and other CYP substrates. However, some cases of interaction between warfarin and GlcN resulting in increased international normalized ratio (INR), have been reported (208). The mechanism of such interaction in fully elaborated.

GlcN degrades into carbon dioxide, water and urea. After the *i.v.* administration of radiolabeled GlcN sulfate, about 16% of the dose was excreted as labeled carbon dioxide ($^{14}CO_2$) in the first 6 h, and reached 50% after 144 h post-dose. These values were 61% and 81% after 6 h and 144 h post-oral dose, respectively (209). The higher level of CO2 production after oral administration of GlcN was attributed to its metabolism by gut (210).

The HBP is a common metabolic pathway that is involved in the metabolism of both glucose and GlcN (Figure 1.6). Through the glucose transporter, glucose enters the cell and is phosphorylated and converts into fructose-6-phosphate (Fruc-6-p). Subsequently, Fruc-6-p enters into the glycolysis pathway in order to produce energy. A small portion of Fruc-6-p enters into the HBP, and converts into GlcN-6-phosphate (GlcN-6-p) by glutamine:fructose-6-phosphate amidotransferase (GFAT). In this enzymatic reaction, glutamine serves as a donor of the amino group. GlcN-6-p then rapidly acetylates and isomerizes to N-acetylglucosamine-1-phosphate to form uridine diphosphate-N-acetylglucosamine as a precursor of glycoproteins, glycolipids and proteoglycans (141,211).

Exogenous GlcN also can be transported by a glucose transporter and once in the cell, enters into HBP to be phosphorylated by a hexokinase. This phenomenon occurs only at very high plasma concentrations (i.e., 10-30 mM) that are not attainable by oral dosing of GlcN (141,212-214).

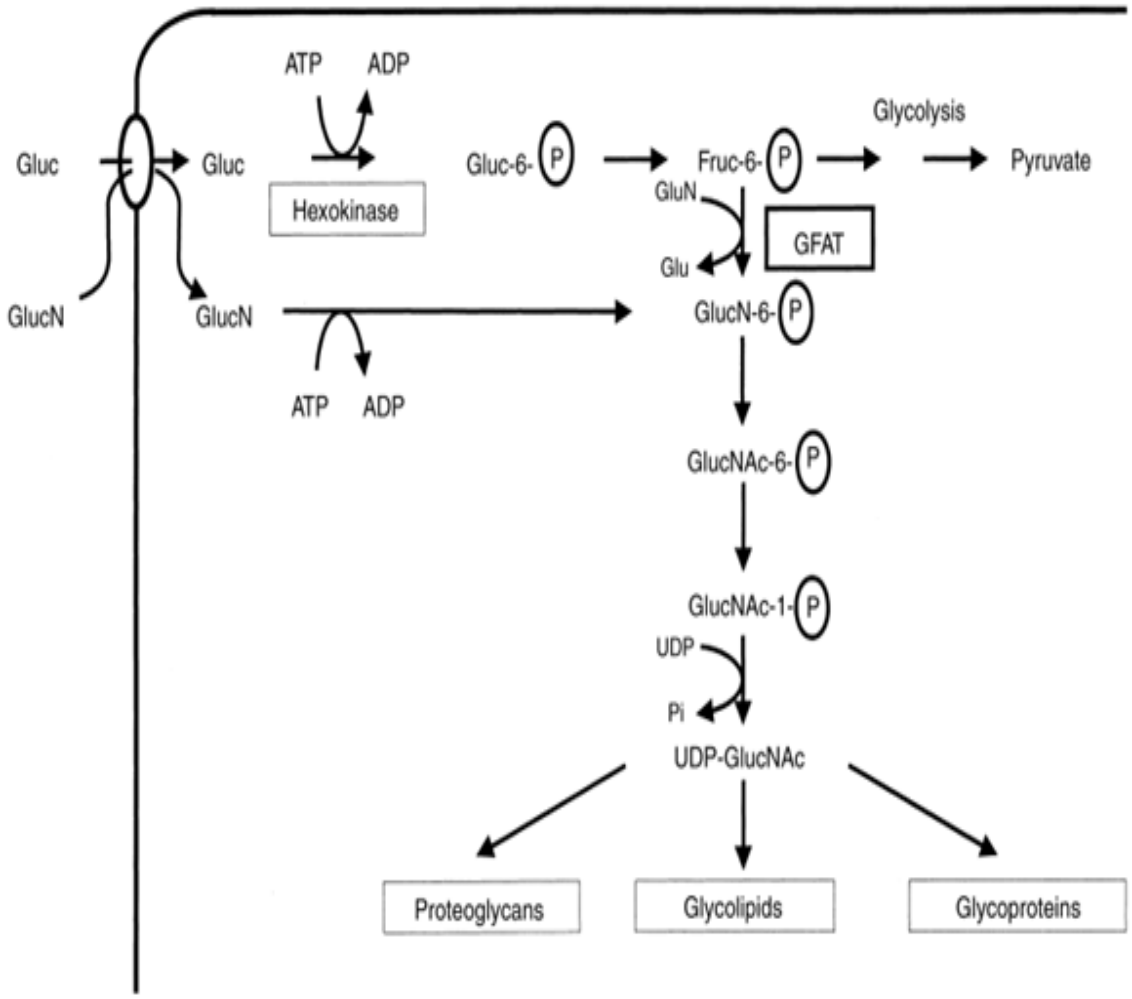

Figure 1.6. The hexosamine biosynthetic pathway.

Adopted from reference No.(211) with permission

1.11.3.4 Elimination

GlcN has a short half-life and eliminates rapidly from the plasma. It ranges from 1.5 to 4 h in different animals and humans (199,200,203,215-218). Bioequivalence studies indicate that there is no difference in the half-life of GlcN-HCl and sulfate salts (140,203). Due to lack of sensitivity of assays, reported half-lives are possibly not representing the true values. Using more sensitive LC-MS, GlcN plasma concentration remained above the baseline level even after 48 h post-oral administration and a longer half-life was reported (219). A radioimmunoassay after i.v or oral administration of ^{14}C-GlcN in healthy volunteers indicated that GlcN is rapidly incorporated into plasma protein by the liver, resulting on a longer half-life of 70 hours (201).

The percent excretion of unchanged GlcN through urine after 120 h post *i.v., i.m.* and oral doses were 28, 37 and 21% , respectively (160). Approximately 11% of the oral dose was recovered in feces after 24-72 h, while it was less than 1% for *i.v.* or *i.m.* doses (201). In a human study, the total amount excreted in the urine 13 h post-oral dose was 3.16% or 2.43 % of the absorbed portion of the GlcN dose as HCl or sulfate salts, respectively (140). Excretion by exhalation as CO_2 through the lungs accounted for about the half of the administered dose (160).

GlcN total body clearance after the *i.v.* administration is 2.61 L/h/kg in rats (198), 9.73 L/h in dogs (199) and 39.25 L/h in horses (200).

1.12 The glucosamine controversy; a pharmacokinetic issue[1]

GlcN a naturally occurring aminosugar is widely used to treat OA. Several studies suggest that GlcN modifies the symptom of OA and halts the disease progression with a favorable safety profile (190,191). Similarly, animal studies strongly suggest disease-modifying effects and anti-

[1] *A version of this section has been published. Aghazadeh-Habashi, A. & Jamali, F. J Pharm Pharm Sci.2011;14 (2):264-273.*

inflammatory properties (Table 1.2). However, the compound remains, perhaps, the most misunderstood therapeutic agent in use. While some reports suggest beneficial effects, other clinical trials and subsequent meta-analyses are inconclusive with their results ranging from strongly effective to negligible or no benefit to patient (220-228).

This review is presented in an attempt to shed new light onto the reasons for the controversy surrounding the issue of beneficial effects of GlcN. Since two thoughtful reviews on the topic have appeared since 2009 (229,230), herein, we briefly mentioned the source of controversy and focused on new issues.

1.12.1 Evidence for and against the beneficial effects of GlcN

In general, animal and *in vitro* studies have focused on the effect of GlcN on damaged joints and particularly on the site-specific beneficial effects (Table 1.2). Although results of such studies are often difficult to extrapolate to effects in humans, it appears that, indeed, GlcN does positively influence the biology of damaged issues. These studies ascribe anti-inflammatory properties to GlcN through inhibition of various pro-inflammatory mediators such as NO, COX-2, matrix metalloproteinase (MMP) but mainly in the context of OA (84,179,231-241). In addition to these studied, Hua et al (133) , have reported the inhibitory effect of GlcN on the emergence of AA in the rat (242). They have shown that daily administration of GlcN commencing on the day of adjuvant injection to induce AA, inhibits the emergence of the disease. AA is a type of severe arthritis that influences all joins and is associated with various systemic signs and symptoms. AA is often considered as a model for RA (170).

Table 1.2 Selected studies on the effectiveness of GlcN on experimental osteoarthritis (OA) and adjuvant arthritis (AA)

Animal	Salt	Dose, mg/kg	Duration, weeks	Outcome	Reference
Rabbit (OA)	HCl	27	8	Detectable, site-specific, partial disease-modifying effect	(170)
Rabbit (OA)	HCl	20 and 100	8	Dose-dependent increase of glycosaminoglycan content in contralateral knee	(193)
Rabbit (OA)	HCl	100	8	Improved subchondral bone turnover, structure, and mineralization	(194)
Rat (OA)	Sulfate	250	10	Attenuates the development of OA; reduces nociception; modulates chondrocytes metabolism;	(243)
Rabbit (OA)	Sulfate or HCl	800-1000	8	Chondroprotective effect; reduced MMP-1	(244)
Rat (OA)	HCl	1000	8	Chondroprotective by inhibiting degradation and enhancing synthesis of type II collagen	(192)
Rat (AA)	HCl	300	3	Suppress progression of AA	(133)

Despite the overwhelming evidence generated using experimental animals in favour of beneficial effects for GlcN in the treatment of arthritis, randomized human clinical trials are not conclusive as some have observed benefit for both pain and joint function (190,245) and others have seen no or negligible positive effects, (246,247). Similarly, subsequent meta-analysis and systemic reviews that included the original reports were not quite in agreement (248). To the best of our knowledge, the latest meta-analysis done by Wandel et al. that has pulled together data from both GlcN alone and various combinations of GlcN and chondroitin (222). The authors did not find any beneficial effect for the treatments. They were later criticized for the study design and criteria used (249,250).

Among the reported clinical trials a few have attracted considerable attention. They include the studies that were typically sponsored by the European producer of GlcN crystalline sulfate (GlcN-S, Rottapharm, Monza, Italy) that generally demonstrated positive results, (150,251,252). The other highly publicized trial is the independent Glucosamine/chondroitin Arthritis Intervention Trail (GAIT) (246). This study tested the effectiveness of GlcN-HCl alone and in combination with chondroitin and concluded that none of the treatments were superior to placebo in pain relief but suggested beneficial effects of the combination is a pre-specified group with severe knee pain. Previously a Cochrane Collaboration review of clinical trials conducted on GlcN had also concluded that "Pooled results from studies using a non-Rotta preparation or inadequate allocation concealment failed to show benefit in pain and WOMAC function, while those studies evaluating the RottaPharm preparation showed that GlcN was superior to placebo in the treatment of pain and functional impairment resulting from symptomatic OA" (248). WOMAC is a self-administered knee and hip OA index. To make the matter even more complicated 2 two-year follow-up reports of a cohort of patients enlisted in GAIT have detected

no significant different between the groups treated with GlcN-HCl, celecoxib or placebo (224,253). This is when other studies have suggested effectiveness for celecoxib in the treatment of OA (254). The observed lack of superiority over placebo of two years of treatment with GlcN and celecoxib is attributed to a low baseline level of pain that can render the treatment effect difficult to assess, and also as the placebo group demonstrated improvement, to the very high expectation bias on the part of 2 years of treatment (253). The authors suggest re-evaluation of the assessment factors involved in designing future OA trails. Interestingly, despite the publicity around the negative results of the GAIT study, GlcN has maintained its popularity among OA patients (142,255).

1.12.2 Potential sources of controversy

1.12.2.1 Inconsistency of commercially available products and it consequence on clinical trials outcomes

We have previously shown that, at least for the Canadian products, 13 out of 14 tested formulations contained substantially lower than label claim of the active ingredient (Table 1.3) (256). This is mainly due to the physical instability of GlcN that is overcome with crystallization in the presences of KCl. The crystals of the active ingredient, therefore, are diluted with KCl. Except for the European Union, where GlcN is regulated as a pharmaceutical, the quality control of the commercially available products of GlcN is a prerogative of the manufacturer, and hence, it may use the diluted crystals without allowance given for the co-crystallization.

A typical clinical trial report has either no mention of the validity of the label-claim of the product used or contains only a statement conveying the manufacturer's claim with no assurance indicating an actual dose-potency measurement. While the commercially available marketed

formulation used in the GAIT study (246) is reported to be tested for its active ingredient content. For the studies included in the meta-analysis of Wandel et al. (222) assurances from the manufacturers appear to be deemed sufficient as a measure of the products active ingredient content accuracy (257,258). It is, therefore, reasonable to suggest that the patients in some of the reported clinical trials may have been under-dosed.

It is important to note that the results of some of the GlcN clinical trials with negative outcomes do not totally rule out potential benefits or trend toward efficacy of the treatment in some patients (138,224,253,258). This, coupled with the possibility of under-dosing with the less than claimed 1500 mg/day regimens, highlight the need for clinical trials using higher pharmaceutical grade GlcN doses or formulations that yield greater plasma concentrations.

Table 1.3 Content of GlcN (mg/capsule or tablet) in commercially available products in Canada.

Preparation	Capsule or Tablet	Labeled Content, mg	Sulfate or HCl	GlcN, mg	GlcN, mg Expressed as Sulfate	% of Stated Amount (as base)
1	T	500	S + C	542	688	108
2	C	500	S	409	519	82
3	C	500	S	277	351	55
4	C	500	S	325	445	65
5	C	500	S	330	419	66
6	C	500	S	248	315	50
7	T	1500	S	634	804	42
8	C	500	S	233	295	41
9	C	500	S	298	378	60
10	C	500	S	231	293	46
11	C	500	S	274	348	55
12	C	500	S	238	302	48
13	C	300	S	169	214	56
14	C	500	S	262	332	52

Adopted from Ref (256).

1.12.2.2 Dose-effect relationship

Almost all of the human clinical trials are carried out with a dosage regimen of 1500/day. Interestingly, at a certain stage, investigators should have known that such a dosage regimen was going to yield concentration substantially lower than those used in *in vitro* or in animal studies (229,241,259). Also, the trend toward GlcN's beneficial effect reported by several authors {{}}(138,224,258), should have alarmed them of the possibility of under-dosing. Nevertheless, to the best of our knowledge, no attempt was made to test a higher dosage regimen even by the investigators involved in the GAIT study that used a pharmaceutical grade formulation. The physical size of GlcN products (e.g., 1.4 g for a 500 mg tablet) might have been, at least in part, a deterrent for using higher dose.

It is well-known that most reported animal data on the pharmacological properties of GlcN are generated following high doses. However, the MED, hence, MEC of GlcN in animals has also remained unknown. Consequently, the gap between plasma GlcN concentration in human and that associated with effectiveness in experimental animals is unknown. The available human pharmacokinetic data demonstrate great inter-study variations (Table 1.4). The reported peak concentration following a 1500 mg dose ranges from 0.492 to 3.36 mg/L; i.e., a 6-fold difference between the highest (GlcN crystalline sulfate) and the lowest (GlcN-HCl as used in GAIT). This brings up an important question: Is the source of the discrepancy between the outcomes of various clinical trials a difference in GlcN bioavailability from different formulations?

Table 1.4 GlcN pharmacokinetic data generated from human studies

Dose, mg	C_{max}		AUC_{0-t}	$AUC_{0-\infty}$	Reference
	mg/L	µM	mg.h/L		
1500	3.11±2.2	17.4±11.96	9.85±4.1 (8 h)[d]	10.3±4.1	(217)
500[a]	1.11±0.51	6.2±2.85	5.25±2.16 (14 h)[d]	5.31±2.16	(260)
500[a]	3.36[b]	19.0[b]	NR	19.7[b]	(261)
1500	1.60±0.42	8.95±2.37	20.22±5.02 (48 h)[d]	14.6±4.14	(219)
1500[e]	0.492±0.16	2. 75±0.9	NR	2.38±0.94	(218)
1500	0.90±0.43[c]	5.04±2.4[c]	NR	NR	(259)

a, indices were calculated for 1500 mg doses; b, geometric means no SD; all value at steady state except 'c"; d, the value in bracket indicate the time point of last measured concentration; all sulfate except 'e' NR, not reported.

Although the values listed in Table 1.4 are from different studies, hence, are not generated according to a cross-over or simultaneous parallel fashion, the fact that the human exposure to GlcN following ingestion of the HCL salt (218) is so much lower than that of the sulfate salt raises a good question that we cannot address. It is intuitively accepted that following ingestion and subsequent dissolution in the gut, both sulfate and HCl salts of GlcN are immediately ionized to GlcN, hence, the nature of the salt becomes irrelevant. If so, the differences between the two products should be at the level of formulation and not the nature of the salt.

To assess the effect of the salt nature on the bioavailability of GlcN, we present a preliminary set of data generated following a random cross-over oral gavage to the rat (male Sprague-Dawley rats, 300-360 g, n=5/group) of equal doses (equivalent to 100 mg/kg GlcN base grounded and suspended in PEG 400) of HCl (Sigma-Aldrich Canada, LTD, Oakville, ON) as used in the GAIT trial (Personal communication with J.D. Sandy) (218) or the crystalline sulfate (Dona, Lot No. PR 24080004, RottaPharm, Monza, Italy, purchased from a community pharmacy in Florence, Italy). We have previously reported the dosing and sampling methods (198,262) as well as an assay (262) used.

Based on this small animal and human study, no significant difference in bioavailability indices was observed between the two formulations (Figure 1.7, Table 1.5). This cross-over assessment indicates that the nature of the salt may does not influence the bioavailability of GlcN administered orally.

The rat data presented herein confirms a previous observation that, in horses, GlcN-HCl and sulfate are bioequivalent (203) but does not rule out the effect of formulation *per se*. We therefore, designed a brief open-label cross-over bioavailability study in healthy volunteers to directly compare GlcN-HCl as used in the GAIT study with a RottaPharm product. We used the

urinary excretion data for comparison as, despite the limited excretion of intact GlcN, it has been found to be a reliable and less variable measure of the pharmacokinetic indices of GlcN (263).

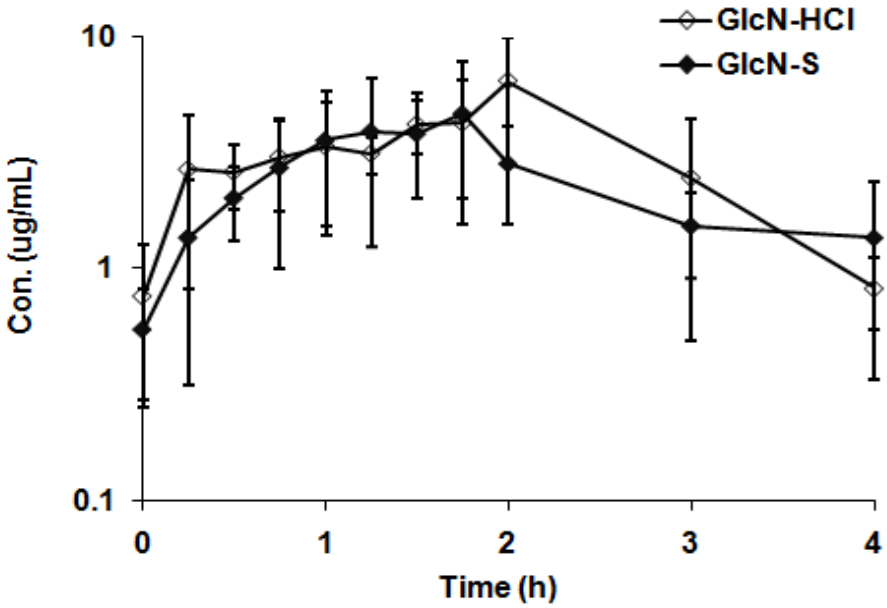

Figure 1.7. Glucosamine plasma concentration vs. time after oral administration of 100 mg/kg as HCl or sulfate salts into the rat (n= 5 /group)

Table 1.5 GlcN pharmacokinetic indices following cross-over oral administration of single 100 mg/kg doses of the compound as HCl or crystalline sulfate to 5 rats.

Parameters	GlcN-HCl	GlcN-S
t_{max}, h	1.50±0.73	1.30±0.33
C_{max}, mg/L	7.49±2.76	7.92±1.84
90% Confidence interval of geometric means, %		94.9 (80.9%-109.0)
AUC_t mg.h/.L	13.59±3.64	10.12±2.54
90% Confidence interval of geometric means, %		112.4 (100.4%-124.4)

Data are presented as mean±SD (n=5/group).

GlcN plasma concentrations were measured according to a previously described method (262). Formulations were grounded and suspended in polyethylene glycol 400 before administration. Their active ingredient content measured by HPLC (215) was 99.9% for the HCl and 95.2% for the sulfate. Concentrations in zero h samples demonstrate endogenous GlcN. Male Sprague-Dawley rats (300-360 g; n=5/group) were dosed through a gastric gavage after an overnight food deprivation but free access to water. Blood samples were collected over a 4 h post-dose period, plasma separated and stored at -20° C until analyzed for GlcN.

Four healthy volunteers (1 female and 3 males, 47±12.5 yr, 81.8 ±11.6 kg, 177±7 cm) took 1500 mg GlcN crystalline sulfate (Dona, 250 mg tablets Lot No. PR 24080004, RottaPhram, Monza, Italy) or its equivalent of GlcN-HCl (Sigma-Aldrich Canada, LTD, Oakville, ON) dispensed in capsules as used in GAIT study. They took the formulations after an overnight fast with 250 mL water in a random fashion with a 2 week washout period. GlcN was measured in total urine output for 13 h post-dose using HPLC (262). No significant differences were found between the two products in either urinary excretion rate plots (Figure 1.8) or in the total amount excreted (Table 1.6). This cross-over study suggests that the formulations used in the GAIT study and those reported for GlcN crystalline sulfate are seem to be bioequivalent and rules out the possible effect of formulation between the two products.

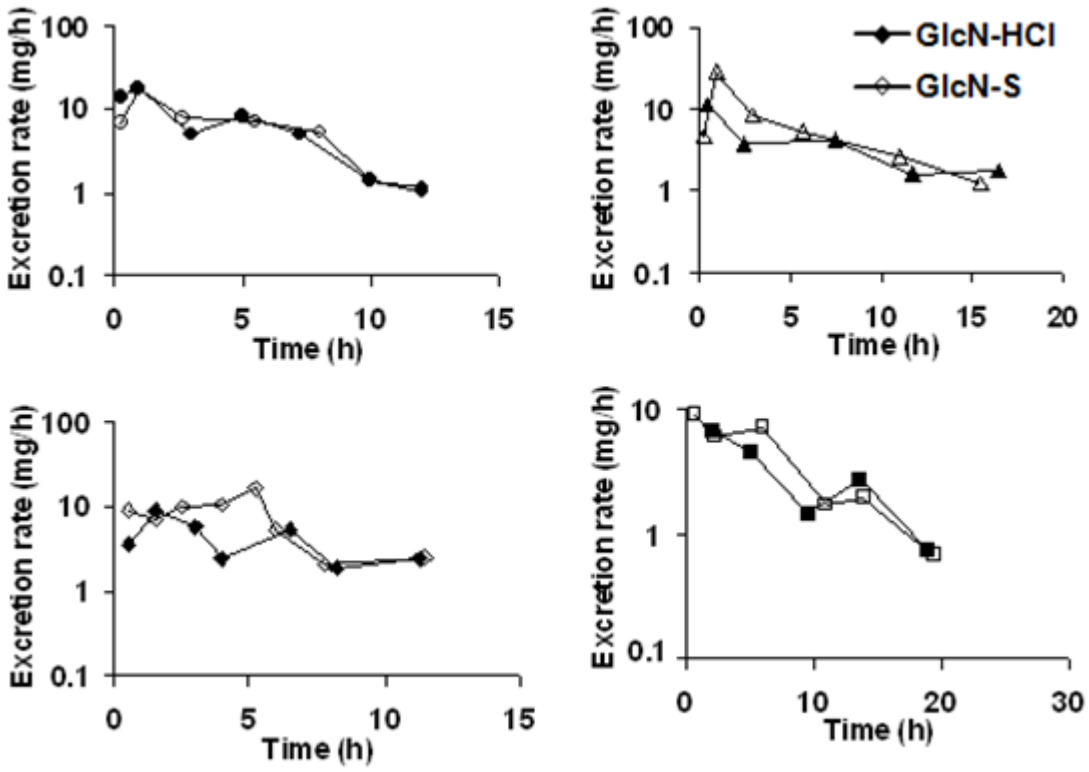

Figure 1.8. Glucosamine urinary excretion rate

GlcN urinary excretion rate vs. mid time point of urine collection period following single oral dose of 1500 mg GlcN crystalline sulfate or its equivalent HCl salt to humans. Each graph represents one individual.

Table 1.6 Individual subjects pharmacokinetic indices of GlcN in urine after an oral dose of 1500 mg GlcN crystalline sulfate or its equivalent HCl salt in human

	GlcN excreted in 13 h					
	GlcN-HCl			GlcN sulfate		
Subjects	$t_{1/2}$, h	mg	% dose[a]	$t_{1/2}$, h	mg	% dose[a]
1	1.69	37.2	3.10	2.12	38.8	3.23
2	4.71	40.92	3.41	7.37	30.3	2.53
3	5.87	36.0	3.00	8.35	25.3	2.11
4	6.54	37.7	3.14	5.17	22.2	1.85
Mean	4.70	38.0	3.16	5.75	29.2	2.43
SD	2.14	2.12	0.18	2.76	7.23	0.60
CV, %		6.0				24.7
90% Confidence interval of geometric means, %					108.7 (103.1-114.3)	

a, calculated based on 26% absolute bioavailability of radiolabeled GlcN (201)

Our data are preliminary and, need to be confirmed with a more detailed study. Nevertheless, the rat and human data on the bioequivalence of GlcN salts and formulations suggest that the HCl formulation used in the GAIT study and the commercially available RottaPharm tablets, indeed, yield equal body exposure to the compound. The discrepancy in the reported pharmacokinetic indices in general and the relatively low concentration of the formulation used in the GAIT study in particular, needs further attention.

It is known that inflammatory conditions may inhibit clearance of drugs that are efficiently undergoing hepatic metabolism (i.e., first pass effect) (8,264,265). There are two reasons to rule out this possibility for the present case: i) there is no evidence of efficient hepatic metabolism for GlcN as its low bioavailability appears to be probably due to an intestinal first-pass (198) and ii) except for one (259), all studies have been carried out in normal volunteers.

Some authors have suggested industry bias as one of the sources of the differences between the two sides of the debate (228). Such a notion, however, does not address the issue of plasma concentration differences between the two formulations. Among the sets of data reported thus far, those of Jackson et al (i.e., the HCl formulation used in the GAIT study) stands out for its substantially lower peak plasma concentration (0.49 mg/L). Since other studies with peak concentrations range of 0.9 to 3.36 mg/L are not all sponsored by a single industry source, it is reasonable to rule out the possibility of an industry bias for the reported pharmacokinetic data. Hence, the question remains as to the reason for such a low bioavailability for the formulation used in the GAIT study and the possibility of a link between the negative beneficial effects of the latter. This is particularly important since several authors have suggested a concentration dependent-effect for GlcN (229,241). For example, it has been suggested that in order to protect

cartilage from stimulated loss of aggrecan, continual presence of GlcN in plasma is required (266).

Based on *in vitro* and animal data, several investigators have pointed out the need for higher concentration in human studies to reach therapeutic levels (229,230,241,267){{}}. Repeated 300 mg/kg does of GlcN to the rat completely inhibit emergence of AA (133). Such a regimen yield peak plasma concentration of approximately 17 mg/L (19) which is much greater than those reported following 1500 mg/kg doses to humans (Table 1.4). The 300 mg/kg regimen, however, is not necessarily the minimum effect dose (Table 1.2). Indeed, we have observed similar preventive effects of GlcN following doses of 80 mg/kg (268). This regimen resulted in a peak concentration of 4.5 mg/L that is still higher that those listed in Table 1.4 but rather is an achievable level. This should prompt investigators to assess GlcN efficacy following higher doses of GlcN or formulations with improved bioavailability.

1.12.2.3 Therapeutic outcome measurements

In assessing the effectiveness of GlcN in OA, double blind placebo-controlled methods are used. The outcome measurement includes various measures such as WOMAC arthritis index, various pain scales, radiographic techniques, joint space narrowing. The sensitivity of these methods to differentiate between treatments may be questioned. Indeed, some investigators who were following GlcN effects in a sub-group of patients enrolled in the GAIT study have not even been able to differentiate between a non-controversial treatment (celecoxib) (253) and placebo so that they suggested a re-evaluation of the assessment factors involved is designing future OA trails. Interestingly, these authors attribute the observed lack of superiority over placebo of the two years treatment with GlcN and celecoxib to the possibility of a low baseline level of pain and a placebo effect due to a very high expectation bias on the part of two years of treatment

(253). If so, the same must be applied to other clinical trials as well and, indeed, it gives more credence to the issue of heterogeneity across studies (228). With such assessment difficulties in place, the possibility of detecting moderate beneficial effects on mild to moderate OA is expected to be remote. This is, perhaps, why patients ignore the results of the scientific studies and continue using GlcN. It may be that some but not all patients do benefit from the treatment.

GlcN has anti-inflammatory properties that are evident only upon administration of high doses or formulations with high bioavailability to experimental animals. The discrepancy between the reported human clinical data is probably not due the nature of the salt or formulation properties. Regardless of the formulation used, following the commonly used 1500 mg/day doses, no or marginal beneficial effects may be observed because of under-dosing which stems from low GlcN bioavailability and inconsistency in active ingredient content of commercially available products. Limited and erratic bioavailability of GlcN may also contribute to the problem. In addition, insensitive clinical outcomes and inclusion of patients with low baseline pain might have contributed to the unsatisfactory treatment outcome.

The source of the controversy in the efficacy of GlcN seems to be pharmacokinetic in nature. At this stage there is an obvious need to determine the minimum effective GlcN dose and/or concentration and conduct clinical trials following higher doses of GlcN or formulations with improved bioavailability.

1.13 Thesis rationale, hypotheses, and objectives

1.13.1 Rationale

Despite mounting number of animal studies in favour of the beneficial effects of GlcN in the treatment of arthritis, randomized human clinical trials are not conclusive as some have observed benefit for both pain and joint function and others have seen no or negligible positive

effects(220-228). The source of the discrepancy between the outcomes of various clinical trials could be a difference in GlcN bioavailability from different formulations.

The animal data regarding anti-inflammatory effect of GlcN are mostly generated using high doses and there is a disparity between human plasma GlcN concentration and that attributed to its effectiveness in animal studies. Therefore, the MED and consequently the MEC of GlcN are still unknown.

It has been shown that inflammatory conditions alter the metabolism of ArA metabolites (69) and disrupt the balance between cardioprotective (EETs) and cardiotoxic (HETEs) metabolites (32). The AA induced inflammation also changes the metabolism of ArA metabolite and disturbs their balance.

There are several analytical methods reported for determination of ArA metabolites in biological matrixes, including HPLC with UV, FL, MS, MS/MS detection. Some of these methods lack sensitivity and selectivity for simultaneous quantification of these metabolites, other utilize expensive instruments with high operational and maintenance costs. Development of a simultaneous sensitive, reliable, and cost-effective HPLC method to assay bioactive ArA metabolites in biological samples seems essential.

Inflammation has a great impact on the physiological systems in the body by altering the expression levels of regulatory proteins. The RAS, which regulates BP, fluid, and electrolyte homeostasis, can be affected by inflammation. Inflammation alters the RAS and changes the expression of its components (ACE and ACE2 enzyme expression levels, Ang peptides concentration, and their receptors; AT1R, AT2R and Mas).

The RAS and ArA pathways as two main systems involved in homeostasis of renal and CV function are in intimate interaction and their components level are correlated with each other.

Clinical trials of GlcN have confirmed several positive and disease-modifying effects in the treatment of OA (131,132). Animal studies have also suggested potent anti-inflammatory effects (134). It is expected that GlcN treatment could lead to an improvement in the signs and symptoms of arthritis by exerting its anti-inflammatory action and be able to modulate inflammation effect on the RAS and ArA pathways.

Rofecoxib, as an NSAID, caused CV and renal complications, which resulted in its withdrawal from the market (92). The importance of ArA metabolites role in CV disease and risk associated with COX-2 inhibitors is recently recognized (53,269). There is no evidence of such adverse effects after long term treatment with GlcN. Although GlcN and rofecoxib both elicit anti-inflammatory actions, it is expected that GlcN be able to restore the disturbed balance between the cardioprotective and cardiotoxic axes of the RAS and ArA pathways in comparable degrees.

1.13.2 Hypotheses

GlcN presents anti-inflammatory effect in animal model of rat with adjuvant arthritis and prevents development and progression of arthritis, if it is administered in a sufficient and higher than minimum effective dose.

Inflammation modulates ArA pathway and alters pro-inflammatory (cardiotoxic) and anti-inflammatory (cardioprotective) metabolites concentrations.

Inflammation modulates the RAS and alters its components at enzyme, peptide and receptor level.

The RAS and ArA pathways are interacting and influencing each other in healthy and disease state and their components are correlated.

GlcN as an anti-inflammatory agent is able to correct the deleterious effect of inflammation on ArA pathway by restoring the balance between cardiotoxic and cardioprotective components.

GlcN as an anti-inflammatory agent is able to correct the harmful effect of inflammation on the RAS by restoring the altered expression of enzymes, receptors, and peptides concentration and reinstating the balance between cardiotoxic and cardioprotective components.

1.13.3 Objectives

To perform a dose escalation study to determine the MEDof GlcN-HCl in the progression of inflammation.

To investigate effect of GlcN on the development of arthritis and to correlate GlcN dose and plasma concentration with the response.

To develop a sensitive HPLC-FL method for determination of ArA metabolites concentration in biological samples.

To determine the effect of AA on the plasma, heart and kidney concentration of ArA metabolites and RAS components.

To correlated plasma and tissue level of the RAS components and ArA metabolites.

To investigate the effect of GlcN treatment on the RAS components expression level and ArA metabolites concentration in biological tissues.

Chapter 2

2 General Materials and Methods

2.1 Chemicals and reagents

Glucosamine HCl, mannosamine HCl, amantadine HCl, and fluorenylmethyloxycarbonyl chloride (FMOC-Cl) were purchased from Sigma-Aldrich Canada, LTD, (Oakville, ON, Canada); high performance liquid chromatography (HPLC) grade acetonitrile and water were obtained from Caledon Laboratories Ltd, (ON, Canada).

ArA metabolites standards were purchased from Cayman Chemical Company (Ann Arbor, MI. USA). They consisted of analogues of HETEs, EETs, and DHTs. The fluorescent label 2-(2,3-naphthalimino) ethyl-trifluoromethanesulphonate (NE-OTf) was obtained from Molecular Probes (Eugene, OR, USA). HPLC grade acetonitrile, methanol, acetone, hexane, anhydrous acetonitrile, N,N-diiospropylethylamine, 16- hydroxydecanoic acid, formic acid (96%), butylated hydroxytoluene, and indomethacin were acquired from Sigma-Aldrich (Oakville, ON, Canada). HPLC grade water used in preparation of different methanol-water solutions was obtained from Caledon Laboratories Ltd, (ON, Canada). Solid phase extraction (SPE) cartridge Oasis HLB 1cc (30 mg) was purchased from Waters Corporation (Milford, MA, USA).

For RAS component analysis using western blotting sodium orthovandate, benzamide, sodium dodecyl sulfate and EDTA were obtained from Sigma Aldrich (Oakville, ON, Canada) Polyacrylamide gel (10%) nitrocellulose membrane, Tween 20 were purchased form Bio-Rad (Hercules, USA).Bovine serum albumin and reducing sample buffer were obtained from Fisher Scientific (Ottawa, ON, Canada). All antibodies for ACE, ACE2, AR1R, AT2R, Neutral endopeptidase (NEP) and tubulin proteins were supplied by Abcam (Cambridge, MA, USA). Mas receptor and chymase antibodies were obtained from LifeSpan Bioscience and Biorbyt LLC

(California, USA) respectively. The goat anti-mouse secondary antibody and Immune-Star™ Chemiluminescence ECL Kit were purchased from Bio-Rad (Hercules, USA).

For Ang peptides analysis p-hydroxymercury benzoate, 1,10-phenanthroline, phenylmethylsulphonyl fluoride, pepstatin A, EDTA were obtained from Sigma-Aldrich (Oakville, ON, Canada). Trifluoroacitic acid and Ang-II, Ang1-7 ELISA kits were supplied by Peninsula Lab LLC (San Carlos, USA).

2.2 Animals

The study protocol was approved by the Health Sciences Animal Care and Use Committee at the University of Alberta. Healthy adult male Sprague-Dawley rats (230–250 g, approximately 2 month-old) were obtained from the Health Sciences Laboratory Animal Services. Animals had free access to food and water and were housed under standard temperature, ventilation, and hygienic conditions with 12 h light and dark cycle and were allowed sufficient time to acclimatize before the experiment.

2.2.1 Glucosamine dose/concentration-effect correlation study

The rats were divided into two groups of control and inflamed (n=4/group). Animals were anaesthetized with isoflurane/oxygen and injected at the tail base with 0.2 mL of adjuvant, containing 50 mg/mL of *Mycobacterium butyricum* in squalene solution (Difco Laboratories, Detroit, MI, USA). The control group received normal saline instead of adjuvant.

2.2.1.1 Assessment of experimental adjuvant arthritis

Subsequent to induction of arthritis, changes in the joint and paw diameters were measured daily using a caliper with 25 μm sensitivity (Mitutoyo Canada Inc., Toronto, ON). The paw

volume was monitored daily using the water replacement method (8). The daily percent change in the body weight was recorded using a regular animal balance. Serum nitrite/nitrate concentrations were quantified using the Griess reaction (270) in samples collected immediately after jugular vein insertion of cannula for serial blood sampling.

The progression of AA was monitored daily by assigning an arthritis index (AI) using a macroscopic scoring system as described before (19): For each hind paw on a 0-4 scale, 0, indicated no sign; 1, involvement of single joint; 2, involvement of >1 joint and/or ankle; 3, involvement of several joints and ankle with moderate swelling; or 4, involvement of several joints and ankle with severe swelling. For each forepaw on a 0-3 scale, 0, indicated no sign; 1, involvement of single joint; 2, involvement of > 1 joint and/or wrist; or 3, involvement of wrist and joints with moderate-to-severe swelling. AI for each rat was calculated by adding the scores for each individual paw. A maximum score of 14 could, thus, be obtained. An AI score of ≥ 5 was considered as significant emergence of the disease, and in the preventive experiment, animals were euthanized when they reached this score. During the treatment regimens, the experiment continued regardless of the AI score.

2.2.1.2 Ameliorating regimen

AA was induced by an intradermal injection of adjuvant at tail base, and following emergence of AA (AI ≥ 5) they were randomly assigned into two groups (n = 3/group) of INF-placebo and INF-GlcN and received a once daily dose of water or GlcN (160 mg/kg) for 6 days, respectively.

2.2.1.3 Preventive regimens

The rats were divided into two main groups, healthy controls (Cont, n = 4) and inflamed (INF). On Day 1, after induction of arthritis, the INF groups were divided into five sub-groups (n

= 3-4/group), INF-0, INF-20, INF-40, INF-80, and INF-160. The animals were received daily doses of water, 20, 40, 80 and 160 mg/kg GlcN, respectively, through an oral gastric gavage with commencing on day 1 for up to 16 days. The healthy controls (Cont-0) were received daily doses of water instead.

To assess the reversibility of the effect of GlcN, on day 16 when animals in group INF-160 were still showing minimal or no sign of arthritis (AI, 0–1), the GlcN regimen was discontinued for 4 days to allow the signs of arthritis to emerge (AI, 3–5). Subsequently, the group was administered 300 mg/kg.day–1 GlcN for 4 days when the AI was reduced to 0–1. At that point, the group was subjected to a pharmacokinetics experiment as described under *"Pharmacokinetic Study."*

2.2.1.4 Pharmacokinetic study

The aims of this experiment were to study the effect of inflammation on GlcN pharmacokinetics and its linearity over the range of the examined doses and, additionally, to evaluate the correlation of GlcN plasma concentration with its pharmacological effect. Animal groups of Cont-0, INF-40, and INF-80 groups (on day 16) and INF-160 which were treated with 300 mg/kg GlcN for last 4 days were used for this part of study (see section 2.2.1.3). They were anaesthetized with oxygen/isoflurane and cannulated in the right jugular vein (197). After an overnight recovery, the last doses of 40, 80, or 300 mg/kg were orally administered to the AA groups. The control group received 80 mg/kg GlcN. Serial blood samples were collected and plasma separated and stored in a -20° C freezer until assayed for GlcN.

2.2.2 Effect of inflammation on ArA metabolites and RAS component

AA was introduced by injection of adjuvant as explained mentioned in section 2.2.1, in order to study the effect of GlcN on the ArA and RAS systems in the rats with arthritis. Animals were divided into two groups of control and inflamed. Control groups were further divided to subgroups of Control (CL), Control-GlcN (CL-GlcN) and inflamed group were divided to inflamed (INF), inflamed-GlcN (INF-GlcN). GlcN groups received a daily dose of 160 mg/kg/day (as GlcN-HCl solution in water) for 22 days commencing day 1. CL and INF groups received water using oral gavage technique. At the end of the experiment, blood samples were collected by cardiac puncture. Heart and kidney tissues were rapidly removed and weighted. Blood and tissue samples were divided into two portions and prepared for ArA and RAS component assays as described in the following sections.

2.2.2.1 Sample collection for ArA metabolites study

Aliquots of 200 µL of saline containing butylated hydroxytoluene (0.113 mM) and indomethacin (10 µM) were added to 1mL of blood. Plasma was separated after centrifugation at 10,000 g for 10 min at 0° C. The heart and kidney tissues were snap frozen in liquid nitrogen along with plasma samples and stored at −80° C until analyzed.

2.2.2.2 Sample collection for RAS component study

Blood

To aliquots of 1 mL blood samples in heparin free polypropylene tubes, 50 µL of cocktail solution containing 1 mM p-hydroxymercury benzoate, 30 mM 1, 10-phenanthroline, 1mM phenylmethsylsulphonyl fluoride, 1 mM peptstian A and 7.5% EDTA was added. After centrifugation for 10 min at 250 g, the plasma was separated and stored at -80° C until analyzed.

Heart and kidney tissue

The heart and kidney tissues were washed with ice cold phosphate buffered saline (PBS), immediately frozen in liquid nitrogen and stored at -80° C until analyzed.

2.3 Glucosamine assay

GlcN plasma concentrations were determined using a pre-column derivatization reversed-phase HPLC method previously reported by our laboratory (262). Briefly, 100 µL of rat plasma samples were spiked with 50 µL of 10 µg/ml mannosamine as an internal standard. Plasma proteins were precipitated by adding 200 µL acetonitrile followed by 1 min vortex-mixing and centrifugation for 3 min at 10,000 g. In a test tube, to 100 µL of the separated supernatant, 50 µL of borate buffer (0.2 M, pH 8.5) was added, followed by 50 µL of freshly prepared solution of FMOC-Cl as a derivatizing agent (8 mM, dissolved in acetonitrile). The mixture was shaken for 1 min and incubated for derivatization in a water bath at 30° C for 30 min. Subsequently, 50 µL of amantadine HCl, in order to react with excess of reagent (271), was added and samples were mixed and diluted with 1 mL acetonitrile-water (1:1). 5 µL of the final solution was then injected into the HPLC system (Shimadzu prominence, Mandel Scientific, Guelph, ON Canada). The chromatographic separation was achieved on a Phenomenex C18 (100mm X 4.6 mm, id 3 µm) reversed phase column, using 0.1% acetic acid/acetonitrile gradient mobile phase at a 1 mL/min flow rate and column oven temperature of 40° C. Detection was carried out with a fluorescence detector at excitation and an emission wavelength of 256 nm and 315 nm, respectively. The method was validated over the range 0.05-20 µg/ml with CV < 15%.

2.4 ArA metabolites assay

For ArA metabolites assay in biological samples, we developed a new HPLC-fluorescence (LC-FL) method which is explained in chapter 3 in details.

2.5 RAS components analysis

2.5.1 Angiotensin converting enzyme measurement (western blot analysis)

The level of proteins of interest i.e., ACE, ACE2, angiotensin target proteins i.e., AT1R, AT2R and mass receptor MAS was determined in the heart and kidney tissues using previously reported Western blot method (105). Animal sample collection was described at a section 2.2.2.2. Accurately weighted portion of these organs were thawed at room temperature, minced and homogenized in the given volume of 50 mM Tris buffer (pH 7.4) containing protease inhibitor cocktail (1/150 mL), sodium orthovandate (27.6 mg/150 mL), benzamide (15 mg/150 mL), sodium dodecyl sulphate (SDS) (0.1%) and EDTA (45 mg/150mL). The tissue homogenate was then subjected to centrifugation at 7000 g at 4º C for 20 min. The debris was discarded and total protein concentration was measured using Lowery method with a commercially available protein assay kit (Bio-Rad, Hercules, USA). From each sample a measured volume containing 50 µg of protein was incubated with reducing sample buffer for 5 min at 90º C. Afterwards samples were loaded onto 10% polyacrylamide gel and electrophoresis was run at 200 V until the marker reached to the lower end of plate. The proteins were then transferred to a nitrocellulose membrane previously soaked and washed in washing buffer containing 0.1% Tween 20 in phosphate buffered saline. The membrane was incubated over-night at 4º C in blocking solution containing 5% fat-free milk and 1.5% bovine serum albumin in washing buffer. After thoroughly washing with 15 mL washing buffer 10 min each four time, the membranes were incubated at room temperature for 2 hours with the following primary antibodies: ACE (anti-mouse, ab77990;

Abcam; 1:100), ACE2 (anti-rabbit, ab87436; Abcam; 1:1000), Mas receptor (anti-rabbit, LS-B3564; LifeSpan; 1:50000), AR1R (anti-rabbit, ab9391; Abcam; 1:400), AT2R (anti-goat, ab19134; Abcam; 1:800), Neutral endopeptidase (NEP) (anti-rabbit, ab126593; Abcam; 1:5000), chymase (anti-rabbit, orb4912; Biorbyt; 1:500), Tubulin (anti-rabbit, ab4074; Abcam; 1:10000). Following primary antibody treatment the membranes were thoroughly washed in 5 mL washing buffer for 10 min for four times, and then incubated for 1 hour at room temperature with respective Goat Anti-Mouse Secondary Antibody (# 170-5047; Bio-Rad) is a conjugate of horseradish peroxidase secondary antibody used in the concentration of 1:30000. Afterwards antibody solution was recovered for later use and membranes were treated with Immune-Star™ Chemiluminescence ECL Kit (#170-5070; Bio-Rad) to visualize and quantified as the density of the image on a film plate (Fujifilm, Canada). Results were presented as the ratio of densities between the protein of interest and Tubulin bands.

2.5.2 Angiotensin Peptide Measurement (ELISA Analysis)

In order to substantiate the results obtained from ACE protein measurement obtained from western blotting analysis, we also measured their respective biologically active peptide products in the plasma, heart, and kidney. The animal sample collection was described in section 2.2.2.2. Before analysis, samples were thawed and brought to room temperature. The heat and kidney tissues were homogenized with 0.045 N HCL in ethanol (10 ml/g tissue), containing 0.90 µM p-hydroxymercury benzoate, 131.5 µM 1,10-phenanthroline , 0.90 µM phenylmethylsulphonyl fluoride , 1.75 µM pepstatin A , 0.032% EDTA , and 0.0043% protease free bovine serum albumin and subjected to centrifugation at 750 g for 10min. Afterwards, debris were discarded and samples were evaporated and reconstituted again in 0.003% trifluoroacitic acid.

Peptides were extracted from plasma, heart and kidney tissue samples using an Oasis HLB extraction column according to the manufacturer's protocol (Peninsula Lab LLC USA, EIA protocol). After extraction, the samples were freeze dried overnight. The freeze dried plasma, heart, and kidney samples were used to measure Ang-II, Ang1-7 using an ELISA kit. According to the manufacturer, the cross reactivity of a polyclonal Ang-II antibody is less than 0.001% with Ang-1 and Ang 1-7. For a polyclonal Ang1-7 antibody cross reactivity was less than 0.08% for Ang-I and Ang-II. Protein concentration in a crude homogenate was determined using the Lowry method and a commercially available protein assay kit (Bio-Rad Laboratories, Hercules, CA). The ratio of Ang1-7 / Ang-II was determined individually only for both peptides.

2.6 Data analysis

The pharmacokinetic parameters were determined using the non-compartmental approach. Doses were normalized based on the rat body weight. The area under the plasma concentration-time curve (AUC_{0-t}) was calculated using the linear trapezoidal rule up to the last measured plasma concentration. The parameters were determined for each individual animal and the sample population averages were calculated. The observed peak plasma concentration (C_{max}) and the time-to-peak concentration (T_{max}) were recorded.

To calculate the median effective dose (ED_{50}) and half maximal effective concentration (EC_{50}), the best fit line through the observed data were estimated using the nonlinear regression approach (GraphPad Prism version 5.3 for windows, GraphPad Software, San Diego, California, USA). Data was confirmed using the sigmoidal dose-response (variable slope) equation passing through the origin. The response to GlcN treatment, a reduction in AI, expressed as percent relief and calculated by the assumption that the maximum effect (100% relief) was achieved when AI was equal to zero, i.e., equal to that measured for healthy rats. No effect (0% relief) was

considered as AI = 5. The ED_{50} and EC_{50} were determined using the software by extrapolation of the 50% effect at Y axis to find the corresponding dose or concentration at X axis.

2.7 Statistical analysis

The values are presented as mean ± SD. The relationship between two parameters was analyzed using the Pearson regression correlation coefficient. Statistical analysis was performed using GraphPad Prism statistical software version 5.3 for Windows (GraphPad Software, San Diego, California, USA). Statistical significance ($p < 0.05$) between observations were examined based on the number of means involved, using either the Student's *t*-test or one way ANOVA followed by the Tukey's Multiple Comparison test at $\alpha = 0.05$.

Chapter 3

3 Glucosamine Dose/Concentration-Effect Correlation in the Rat with Adjuvant Arthritis [2]

3.1 Introduction

The animal data regarding pharmacological effects of GlcN are mostly generated using high doses so that the MED and consequently MEC is still unknown (133). Therefore, there is a disparity between human plasma GlcN concentration and that attributed to effectiveness in animal studies. The inter-study variability of current human pharmacokinetic data is high (140). For example, there is over 6-fold difference on reported maximum plasma concentration between the pharmaceutical grade products available in Europe (3.36 mg/L) (217) and the formulation used in a more recent clinical trial (0.492 mg/L) (218) following the same dosage regimen. This mater raises the question that whether oral bioavailability of GlcN from different formulations is responsible for the discrepancy between the outcomes of various clinical trials, or patients are under-dosed when treated with the common 1500 mg/day regimen. Surprisingly, after many clinical trials, which reported no or marginal effects for 1500 mg/day GlcN in OA, the subsequent studies (272-276) still used the same regimen with no attempt to try a greater daily dose. To the best of our knowledge, no information is available as to the GlcN MED or concentration in animals or human. Herein, we report the GlcN dose/concentration-effect relationship in AA.

3.2 Hypothesis

GlcN presents anti-inflammatory effect in AA rat and prevents development and progression of arthritis if it is administered in a sufficient and higher than minimum effective dose.

[2] *A version of this chapter has been published Aghazadeh-Habashi A, Kohan MH, Asghar W, Jamali F. J Pharm Sci 2014;103(2):760-767*

3.3 Objectives

To perform a dose escalation study to determine the MED of GlcN-HCl in progression of inflammation.

To investigate GlcN effect on the development of arthritis and to correlate GlcN-HCl dose and plasma concentration with response.

3.4 Results

3.4.1 Ameliorating effect of GlcN

As depicted in Figure 3.1, the elevated AI, as a measure of signs and symptoms of already established arthritis, dropped rapidly and significantly after 4 days of 160 mg/kg/day GlcN dosing. In contrast, AI gradually increased to the highest level in placebo treated animals until they were sacrificed.

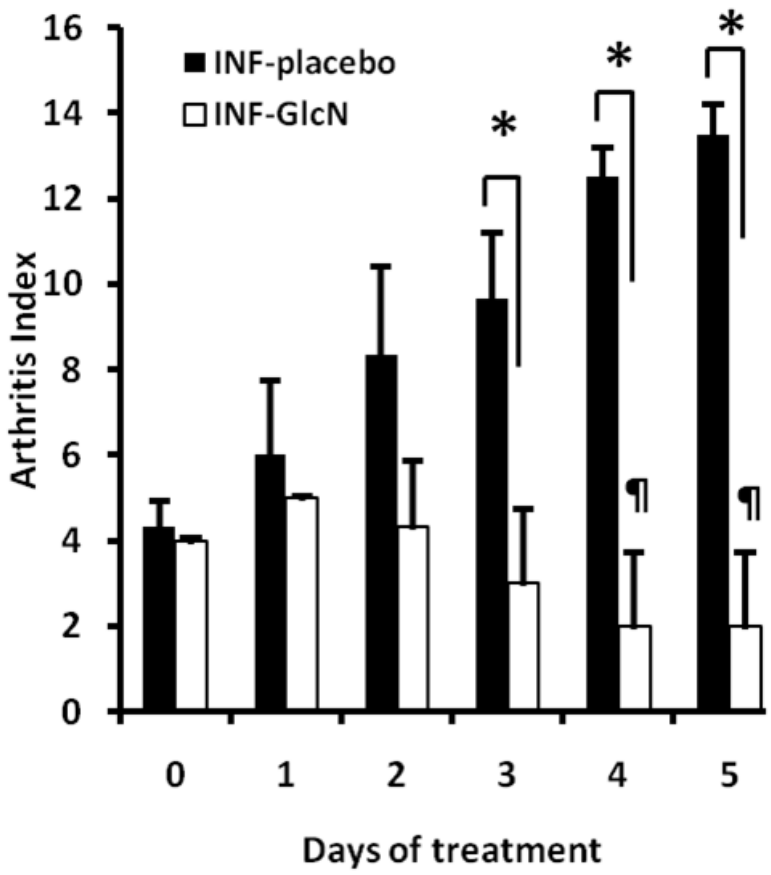

Figure 3.1. Ameliorating effect of GlcN

Ameliorating effect of daily doses of placebo or 160 mg/kg GlcN on the signs and symptoms of established AA (AI ≥ 5) induce by tail base injection of adjuvant, (n = 3/group). *significantly different between groups ($p < 0.01$). ¶ Significantly different from GlcN treatment day 0 ($p < 0.001$). Error bars are standard deviation. INF-placebo; animal with AA treated with water, INF-GlcN, animal with AA treated with GlcN-HCl

3.4.2 Preventive effect of GlcN

AA was evident 10-12 days post- adjuvant injection of the (Figure 3.2). It resulted in significant reductions in body weight gain and elevation of the serum nitrite/nitrate concentration 16 days post-adjuvant injection (Table 3.1). Both indices stayed at the healthy levels by all GlcN dosing regimens administered after the adjuvant injection. AA manifested itself by initial scaling and redness of paw sole, erythema of ankle joints followed by involvement of the metatarsal and interphalangeal joints. The symptoms spread progressively with time into other parts of hind and forepaws. Animals that were injected the adjuvant but not received GlcN showed significant emergence of AA (Figure 3.2). They were, hence, euthanized when AI was elevated (≥ 5). Those that received GlcN, on the other hand, responded to the therapy in a dose-dependent fashion (Figure 3.2).

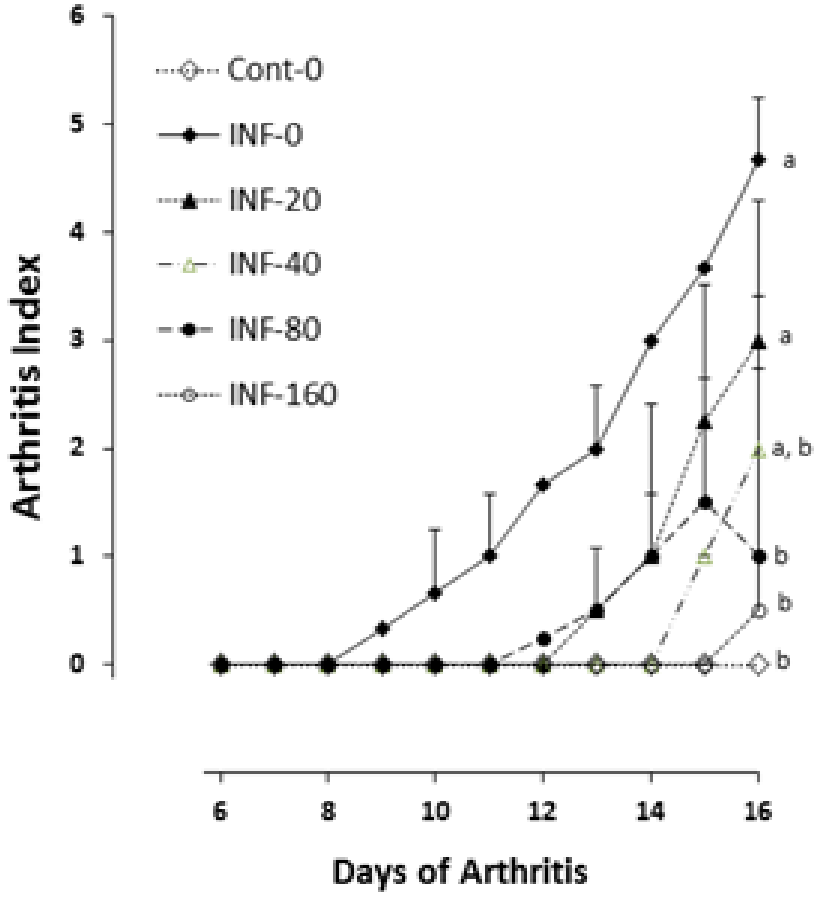

Figure 3.2. Preventive effect of GlcN

Effect of different daily doses of GlcN on emergence of arthritis induced by tail base injection of adjuvant; n = 3-4/group, Different characters indicate significant differences between groups on day 16. Error bars are standard deviation. Cont-0, INF-0, INF-20, INF-40, INF-80, INF-160 groups received, water or GlcN dose of 20, 40, 80, 160 mg/kg/day, respectively.

Table 3.1 Effect of different daily doses of GlcN on percent body weight gain and serum nitrite 16 days post adjuvant injection

Weight gain, % change from day 1			
Group	Mean	vs. INF-0,%	$p <$
Cont-0	50.1 (5.4)	32.3	0.001
INF-0	17.8 (8.3)		
INF-20	39.5 (12.0)	21.7	0.05
INF-40	47.2 (8.7)	29.4	0.01
INF-80	43.4 (14.8)	25.6	0.05
INF-160	44.6 (13.1)	26.8	0.05
Serum nitrite concentration, µM			
Cont-0	67.1 (16.3)	-141.3	0.001
INF-0	208 (24.3)	0.0	
INF-20	95.6 (14.8)	-112.8	0.01
INF-40	73.7 (15.3)	-134. 8	0.01
INF-80	58.3 (27.4)	-150.1	0.01
INF-160	69.3 (11.1)	-139.1	0.001

The data are expressed as arithmetic mean (SD) and % of mean differences vs. INF-0; (n=3-4), p <0.05 was considered significant.

Table 3.2 Changes from baseline observed following daily doses of GlcN administered for 16 days to control and rats with AA (INF)

Animal Group	GlcN Dose (mg/kg/day)	Paw Diameter (µm)	Joint Diameter (µm)	Paw Volume (µL)
Cont-0	0	-20 (38)	518 (120)	280 (83.7)
INF-0	0	1321 (606.9)¶	2963 (563.6)¶	1500 (265)¶
INF-20	20	121 (326)	686 (648)	575 (499)
INF-40	40	323 (123)	527 (206)	475 (330)
INF-80	80	165 (451)	533 (298)	450 (100)
INF-160	160	165 (243)	222 (104)	225 (275)

Data are expressed as arithmetic means (SD); ¶ Significantly different from other groups; p < 0.001, n = 3-4/group.

The preventive effect of GlcN was evident following the treatment with all doses of GlcN. In the treated animals, paw and joint diameters and paw volumes were significantly smaller than those that were not treated (Table 3.2). The effect was dose dependent albeit variable (Table 3.2). With regards to AI, however, a partial effect (reduction of AI) appears with the 20 mg/kg/day dose but was only significant after the 80 mg/kg/day dose (Figure 3.2).

Serum nitrite concentration and body weight gains (Table 3.1) were maintained at the healthy levels by all GlcN doses.

As depicted in Figure 3.3 and Table 3.3, experimental inflammation had no effect on GlcN pharmacokinetic. The relationship between the dose and AUC and C_{max} of GlcN were linear in the rats with arthritis (Figure 3.4). Similarly, the dose elevation influence neither T_{max} nor t1/2 of the drug.

The AI was significantly correlated with dose ($p < 0.01$, r = -0.647), C_{max} ($p < 0.05$, r = -0.651) and AUC ($p < 0.01$, r = -0.833) , while the AUC-AI relationship demonstrated the least degree of variability and strongest correlation (Figure 3.5).

The linear regression lines between % relief of symptoms vs. logarithm dose or concentration did not pass through the origin and presented with X axis intercepts; therefore sigmoidal model with nonlinear regression approach was used. The modeling based on the relationship between logarithm of dose or concentration and percent improvement in AI (Figure 3.6) revealed ED_{50} and EC_{50} of 33.1 mg/kg/day and 1.36 mg/L, respectively.

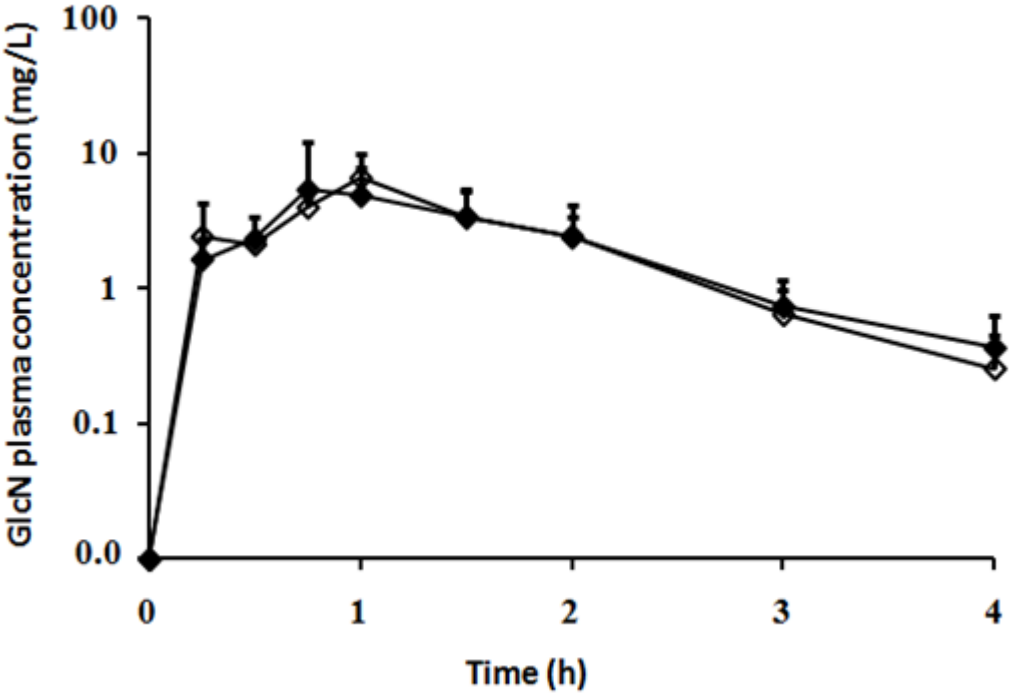

Figure 3.3 GlcN plasma concentration-time curve

GlcN concentration-time profiles after oral administration of 80 mg/kg/day to arthritic (◊, n = 3) and non-arthritic (♦, n = 4) rats; Error bars are standard deviations

Table 3.3 GlcN pharmacokinetic indices after oral administration of 80 mg/kg to arthritic (n =3) and non-arthritic rats (n = 4).

	Non-arthritic control	Arthritic	
AUC $_{0\text{-}4\ h}$ (mg.h/L)	7.47 (2.33)	8.77 (2.04)	$p > 0.05$
C_{max}(mg/L)	5.31 (6.84)	6.50 (3.51)	$p > 0.05$
t_{max} (h)[a]	1.25 (0.35)	0.92 (0.14)	$p > 0.05$
$t_{1/2}$ (h)	0.83 (0.33)	0.83 (0.61)	$p > 0.05$

Data presented as mean (SD), [a] arithmetic mean of individual t_{max}

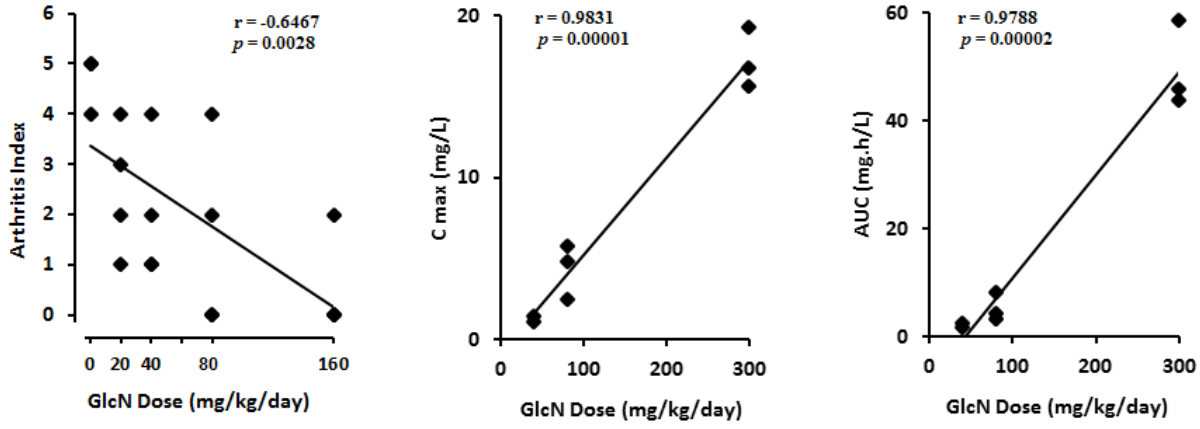

Figure 3.4 Correlation between GlcN daily dose and arthritis index, C_{max}, and AUC

Relationship between GlcN daily dose and arthritis index, C_{max} and AUC ; —, regression lines

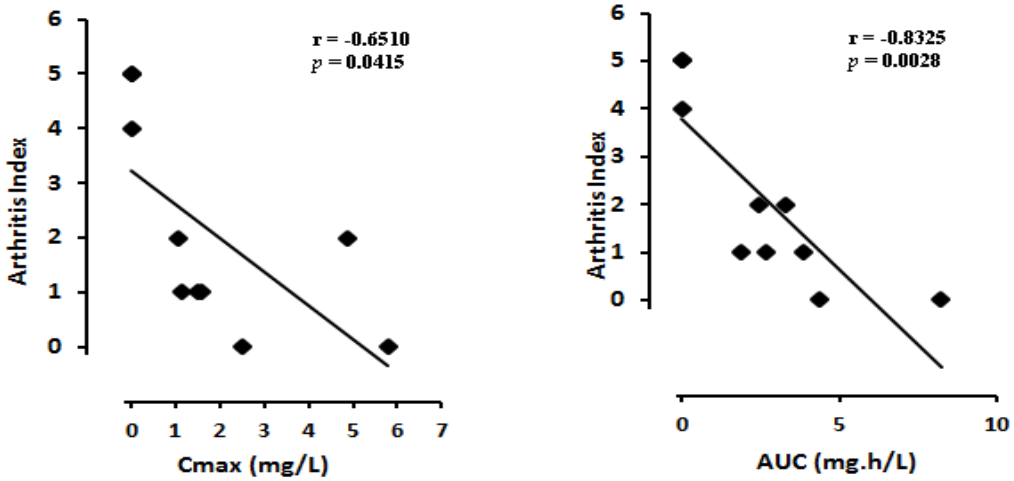

Figure 3.5. Correlation of Cmax and AUC with arthritis index

Relationship between C_{max} , and AUC and GlcN dose; —, regression lines

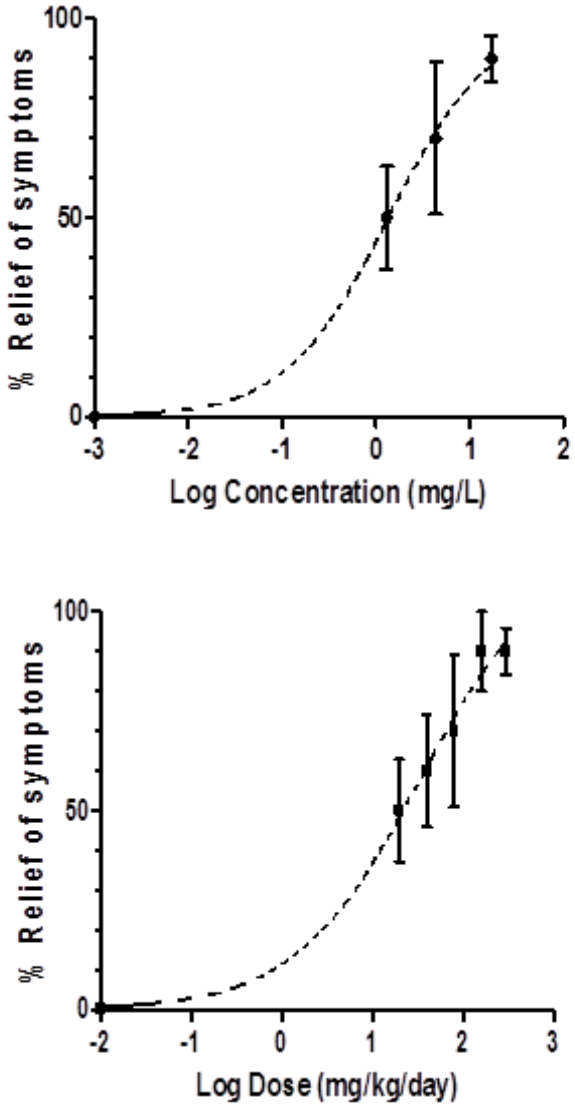

Figure 3.6. GlcN EC50 and ED50

Logarithm of mean GlcN dose or concentration vs. percent difference in arthritis index before and after treatment. The best fit line (….) through the observed data was estimated using the nonlinear regression approach (GraphPad Prism version 5.3 for windows, GraphPad Software, San Diego, California, USA). Data was confirmed using the sigmoidal dose-response (variable slope) equation passing through the origin. The response to GlcN treatment, a reduction in AI, expressed as percent relief of symptoms and calculated by the assumption that the maximum effect (100% relief) was achieved when AI was equal to zero, i.e., equal to that measured for healthy rats. No effect (0% relief) was considered as AI=5, (n= 3-4/ group)

3.5 Discussion

In the present work, a single injection of Mycobacterium butyricum resulted in 100% incidence of AA. Data from our first experiment clearly demonstrated that GlcN is effective in reducing the signs and symptoms of AA after its emergence (Figure 3.1). After the onset of AA (AI ≥5), treatment with daily doses of 160 mg/kg GlcN-HCl significantly brought down AI. When untreated, AI continually rose until the animal was euthanized. The result from this arm of the study, however, did not provide information regarding the dose-dependency of the effect. Subsequently, we planned a dose-effect study that, due to the associated ethical consideration, focused on the preventive effect of the compound. We assumed that the preventive effects of GlcN reflect the compound's ameliorating properties based on our observations made on the NSAIDs, in general (unpublished data), and ketoprofen in particular (277).

In the preventive experiment, the emergence of AA in the untreated rats was evident through significant increased AI (Figure 3.2), reduced weight gain and increased serum nitrite concentration (Table 3.1) and swelling of the joints (Table 3.2). In humans, a rise in the serum nitrite has been reported to be a reliable predictor of rheumatoid arthritis (11).

To the best of our knowledge this is the first study to identify the dose-dependency of GlcN intervention in arthritis. Hua et al have previously reported the suppressing effect of GlcN on AA after repeated daily doses of 300 and 1000 mg/kg with no sign of dose-dependency. Indeed, both doses were equally effective in suppressing arthritis, i.e., the maximum attainable effect (133). Based on our present data, the observation of Hua et al suggests that, similar to many other studies reporting animal observations (193,243), they had used doses beyond what is needed to attain the maximum effect. We observed that GlcN suppressed the elevation of AI in a dose-

dependent fashion. Doses as low as 20 mg/kg showed some positive results when individual metrics were considered (Table 3.2, Figure 3.2). However, when AI on day 16 was considered (Figure 3.2), a significant effect was not detected until 80 mg/kg/day was used. AI is a hybrid measure of the disease, hence, inherently it includes more variables than metrics such as paw diameter. The effect of the 40 mg/kg/day regimen on AI was not significantly different from the higher tested doses but its difference with the placebo did not reach significance either. This may be due to smaller number of animal used in the study or the variability in response and the type of statistical test (one-way ANOVA) used. As compared with the tests that compare only two means, a comparison among more than two means requires greater consistency in response detect significant differences. Nevertheless, these results indicated that the MED of GlcN ranges from 40 to 80 mg/kg/day. However, the 40 mg/kg/day dose generates plasma concentrations of 1.37±0.24 mg/L (observed) or 1.69 mg/L (estimated from sigmoidal regression line). These values are close to the calculated ED_{50} (33.09 mg/kg/day) and EC_{50} (1.36 mg/L) based on the applied curve fitting approach despite the potential error involved due to the lack of experimental data point at both extreme ends of the relationship (Figure 3.6). These therapeutic C_{max} values are comparable with the reported values in human (1.60±0.42 mg/L) who participated in the clinical trials and receiving 1500 mg/L/day pharmaceutical grade GlcN sulfate with potentially positive outcomes (140). However, the range observed, herein, is much higher than that the concentration of 0.49 mg/L reported for the formulation used in Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) (218). The common daily dose used in almost all of clinical trials is 1500 mg (140,221) which has not generally resulted in a constant concentration range or consistent therapeutic outcomes (140). For some of these trials, the low plasma concentration can be attributed to the reported lower than the label claim content of glucosamine regardless of the salt

used except for the pharmaceutical grade sulfate used in Europe (256). The reason for the low concentration reported for the well-publicized GAIT study is unknown as the formulation used therein was a pharmaceutical grade GlcN-HCl (personal communication) which seems to be bioequivalent to a prescription-grade GlcN sulfate product available in Europe (Dona, RottaPharm, Monza, Italy) (140). The GAIT results that suggested only marginal beneficial effects for a sub-group of patients and the lack of availability of prescription-quality preparations in the US, have influenced the negative opinions regarding the use of GlcN in OA expressed by the American College of Rheumatology (ACR) (278). Interestingly, a follow up of the same GAIT study reported no beneficial effect for celecoxib as compared with placebo either (253) suggestive of either a lack of effect of the drug in OA or the unreliability of measuring tools of OA. Surprisingly ACR did not issue a negative recommendation against celecoxib as it did for GlcN.

The closeness of the GlcN therapeutic plasma concentration in the rat and humans is interesting and agreeable with the belief expressed by several authors (229,259). The fact that GlcN is not plasma protein bound may, in part, have a role in this observation. Our data suggest that an increase in the dose will proportionally elevate GlcN plasma concentration, hence, the beneficial effects of the drug. This exposure dependency of GlcN effect observed in the rat, may provide a hint as to the reason why clinical trials of the compound are controversial; the therapeutic plasma concentration range is not reached due either to the use of products that contain less than label claim (140) or because the examined dosage regimen is marginally effective, thereby, results in great variations in response and lack of statistical significance as we have observed in the lower range of our examined doses (i.e., 40 mg/kg/day). A higher than

commonly tested 1500 mg/day GlcN dosage regimen, therefore, may results in a more clear response to the drug.

GlcN prevents the rise in serum nitrite (a stable metabolite of NO) concentration in response to the experimentally induced arthritis (Table 3.1). The role of NO in the inflammatory conditions has been extensively reported (279,280). The fact that GlcN influences the serum nitrite concentrations in AA is another evidence for the anti-inflammatory properties of the compound and maybe involves separate pathways not related to AA. Interestingly, the preventive effect of GlcN on serum nitrite concentration was significant in response to the lowest examined regimen (20 mg/kg/day) and was not dose-dependent (Table 3.1). This may suggest that the compound's anti-inflammatory properties appear even with doses that are not fully effective against AA. This may have therapeutic relevance in the treatment of inflammatory conditions that are less severe than systemic arthritis. Another indicator of AA progression is the reduced body weight gain rate (Table 3.1) that is suggested to be due to the inability of animal in the cage to reach food as a result of the inflammatory condition (281). Interestingly, GlcN at a lower dose than those demonstrating significant therapeutic benefits prevents this effect of AA in reducing the weight gain.

Although AI demonstrated a significant dependence on GlcN dose, and C_{max}, it was best correlated with the GlcN exposure or AUC as depicted in Figure 3.5. The variability observed in the effect vs. dose relationship (Figure 3.4, 3.6) may be explained by the inconsistency in the absorption process. For the C_{max}, often, precise identification of the parameter is difficult due to the limitation in blood sample collection. AUC, on the other hand, is a less variable parameter, hence, better reflects the systemic exposure of the drug. For a compound such as GlcN that has

to be distributed through the body to elicit its effect, a measure of exposure, i.e., AUC, is expected to be better correlated with the effect.

Following repeated doses of GlcN to arthritic animals, a linear relationship was observed between plasma concentration and the dose within the examined range of 20-300 mg/kg (Figure 3.4). A linear pharmacokinetic pattern has also been reported for single doses of GlcN administered to healthy rats (197). The similarity between the healthy and AA rats in the linearity of GlcN pharmacokinetics may be a reflection of the fact that the experimental disease did not influence the GlcN disposition (Figure 3.3, Table 3.3).

Inflammation reduces the metabolic clearance of drugs which are efficiently metabolized (11,282). The effect of AA on pharmacokinetics of GlcN has been reported to be insignificant after single doses (19). Since GlcN is used for chronic treatments, we compared its plasma concentration vs. time in control rats with those with AA following repeated doses. Our observation confirms that inflammation does not influence GlcN pharmacokinetics after single or repeated doses (Figure 3.3, Table 3.3).

The present study has a few limitations. Firstly, it has used a model of systemic inflammation that resembles RA while most human studies are focused on OA. Although AA is associated with severe affliction of the joints, its etiology is different from OA. Nevertheless, our data present the potential beneficial effects of GlcN on severe inflammatory conditions involving joints. Secondly, with the exception of the 160 mg/kg dose study, the therapeutic range presented herein, is based on GlcN preventing effects assuming a link between the preventing and ameliorating effects. Thirdly, the identification of the therapeutic concentrations range was made using an animal model and a limited sample size/group, hence, may not necessarily reflect that in humans. The present study, therefore, should be considered as the first step toward a better

understanding of GlcN mode of action and resolving the issues surrounding the controversy in the use of the compound as a drug.

In summary, GlcN possesses preventive and ameliorating effects on AA in a dose and concentration dependent manner. The MED is approximately between 40 and 80 mg/kg that corresponds with maximum plasma concentrations that are within the range reported for pharmaceutical-grade products used in human trial that have reported beneficial effects. Considering the observed dose-dependency of the GlcN effect and the possibility of under-dosing, mainly, due to the use of inferior products, future clinical trials should consider using pharmaceutical grade GlcN and, perhaps, higher than 1500 mg/kg/day doses.

Chapter 4

4 Simultaneous Determination of Bioactive Arachidonic acid Metabolites by Reversed-Phase HPLC Method Using Fluorescence Detection and Application to Rat and Human Plasma, and Rat Heart and Kidney Samples

4.1 Introduction

Ample recent preclinical and epidemiologic data suggest that modulation of eicosanoid metabolism may be a feasible clinical therapeutic strategy for the management of different pathological disorders including CV diseases (33). Estimation of plasma and tissue concentration of ArA metabolites is, therefore, important for understanding their role in physiological and pathophysiological processes. In addition they may play the role of biomarkers of various conditions.

Several liquid chromatography-mass spectrometry (LC-MS) as well as ultraviolet and fluorescence high performance liquid chromatography (HPLC) assays have been reported in the literature for the determination of ArA metabolites family (Table 4.1). A few of the LC-MS methods have focused only on particular members of this family (283,284), or they lack sensitivity and/or selectivity to detect the basal levels in biological matrixes (285). In order to overcome these shortfalls and improve sensitivity, some researchers have incubated AA with microsomal fraction of CYP enzymes, have stimulated endothelial cells culture or have used higher sample volume (286-288). In our hands, a previously reported LC-MS method (287) under the conditions used by others (69,289), did not produce consistent results due to the following reasons: first, it was an indirect assay using microsomal fraction and incubation with external ArA to enhance the ArA metabolites concentration, therefore, the method was not suitable for the estimation of basal concentrations; second, it demonstrated unacceptable intra

and inter-day variability and, third, it was not selective for resolution of structurally similar ArA metabolites. Theoretically, any LC-MS method for separation of compounds with equal mass relies on LC component, and if the chromatographic behaviors of the compounds of interest are not sufficiently different, the peaks of interest do not well-resolve (288). To address the latter issue, Martín-Venegas et al used an LC-MS/MS with multiple reaction monitoring (MRM) mode capable of using the combination of the parent mass and unique fragment ions (288) however, they declared that even MRM method was not specific since each eicosanoid presents several m/z fragment transitions. The advantage of LC-MS/MS in provision of high sensitivity is, however, compromised by the high instrumental, operational and maintenance cost. Alternatively, HPLC-fluorescence (LC-FL) approach has been explored. The previously reported LC-FL, however, are not without problem neither. The isocratic elution approach used in two of these assays results in less than desired peak resolutions (285,290). For instance Maier et al (285) have reported the same retention time for several metabolites. Alternatively, Yue et al (291) have reported a LC-FL method using gradient elution that resulted in improved peak resolution reported for their standard AA metabolites solutions. They, however, have not presented chromatographs of biological samples; nor have they reported the effect of matrixes on the accuracy of the assay. Using Yue et al method we found unknown peaks interfering with our peaks of interest. We, therefore, developed an improved LC-FL assay based on two previously reported methods (285,291) that provided a greater sensitivity and improved selectivity so that is suitable for a wider applicability for simultaneous analysis of AA metabolites in different biological specimens of human and rat

.

Table 4.1. Comparative list of HPLC methods reported in literature for measurement of ArA metabolites

NO	Method	LOD, pg	LOQ, pg	N[a]	Sample Type[b](D/IN)[c]	Reference
1	LC-UV, Isocratic	800-1500	NR	7	Std sol., biological samples (D)	(292)
2	LC-FL, Isocratic	500	NR	7	Std sol., urine (D)	(285)
3	LC-FL, Isocratic	500	NR	5	Std sol., platelet (D)	(290)
4	LC-FL, gradient	2-20	20-70	13	Std sol., (D)	(291)
5	LC-FL	2	NR	5	Endothelial cell (D)	(286)
6	LC-MS, gradient	1	NR	9	Std sol. (D), microsome (IN)	(287)
7	LC-MS, LC–MS/MS	NR	750	2	Tissue microsome (IN)	(293)
8	LC–MS/MS	NR	NR	4	Corona microsome (IN)	(294)
9	LC-MS/MS, MRM	0.33-32	1.4-105	15	Std sol, cell culture (D)	(288)
10	LC–MS/MS, MRM	NR	1-40	32	Plasma (D)	(295)
11	LC–MS/MS, MRM	0.2-619	1.4-768	14	Std sol., Plasma and tissue (D)	(296)
12	UHPLC–MS/MS, MRM	NR	0.57-5.6	10	Tissue (D)	(297)

[a] Number of metabolites included; [b]The type of sample chromatograph presented (Std Sol, standard solution no biological specimen); [c] Sample preparation needs (D) or does not need amplifying (IN) and measurement of assay, direct or after incubation of microsomal fraction; LOD, Limit of detection; LOQ, limit of quantification; NR, not reported.

4.2 Objective

To develop a simultaneous sensitive, reliable and cost-effective HPLC method for determination of bioactive ArA metabolites in biological samples in order to study the effect of inflammation and anti-inflammatory treatment with GlcN and rofecoxib on their concentrations

4.3 Materials and Methods

ArA metabolites standards were purchased from Cayman Chemical Company (Ann Arbor, MI. USA). They consisted of analogues of HETEs, EETs, and DHTs. The fluorescent label 2-(2,3-naphthalimino) ethyl-trifluoromethanesulphonate (NE-OTf) was obtained from Molecular Probes (Eugene, OR, USA). HPLC grade acetonitrile, methanol, acetone, hexane, anhydrous acetonitrile, N,N-diiospropylethylamine, 16- hydroxydecanoic acid, formic acid (96%), butylated hydroxytoluene, and indomethacin were acquired from Sigma-Aldrich (Oakville, ON, Canada). HPLC grade water used in preparation of different methanol-water solutions was obtained from Caledon Laboratories Ltd, (ON, Canada). Solid phase extraction (SPE) cartridge Oasis HLB 1cc (30 mg) was purchased from Waters Corporation (Milford, MA, USA).

4.3.1 Animals

The study protocol was approved by the Health Sciences Animal Care and Use Committee at the University of Alberta. Healthy adult male Sprague-Dawley (SD) rats (230–250 g, approximately 2 month-old) were obtained from the Health Sciences Laboratory Animal Services. Animals had free access to food and water and were housed under standard temperature, ventilation, and hygienic conditions with 12 h light and dark cycle and were allowed sufficient time to acclimatize before the experiment.

4.3.2 Rat and human biological sample collection

The animal study protocol was approved by the Health Sciences Animal Care and Use Committee and University of Alberta. Adult healthy male Sprague-Dawley rats (230–250 g) were obtained from the Health Sciences Laboratory Animal Services. Animals had free access to food and water and were housed under standard temperature, ventilation and hygienic conditions. They were anesthetised using isoflurane. Through cardiac puncture aliquots of 1 mL blood samples were collected and 200 μL of saline containing butylated hydroxytoluene (0.113 mM) and indomethacin (10 μM) was added to inhibit auto-oxidation and enzymatic degradation of the eicosanoids, respectively. Plasma was separated after centrifugation at 10,000 g for 10 min at 0° C. Heart and kidney organs were rapidly removed, snap frozen on liquid nitrogen along with plasma samples and stored at −80º C until analyzed. Human blood samples were collected from two healthy volunteers according to a protocol approved by the University of Alberta Human Ethics Committee (12); plasma separated and aliquot of 200 μL were analyzed for AA metabolites as described above.

4.3.3 Sample preparation

Standard ethanolic solutions were prepared by serial dilution of a working solution (5 μg/mL of each eicosanoids) to achieve concentration range of 0.01-2.5 μg/mL. 16-Hydroxydecanoic acid (30 μL of 1 mg/mL in ethanol) was used as internal standard. Aliquots of approximately 30 mg rat frozen heart or kidney tissues were accurately weighed and placed in a glass tube and homogenated in a mixture of 200 μL of methanol and 0.4 μL of 96% formic acid containing 0.113 mM butylated hydroxytoluene and indomethacin (10 μM). For the assay of the metabolites in plasma, to aliquot of 200 μL samples was added 0.4 μl of 96% formic acid. Subsequently, the samples were spiked with internal standard and separated by centrifugation at

10,000 g for 10 min at 0º C. The supernatants were diluted with 1.8 mL of 10% methanol before loading on SPE cartridges on a vacuum manifold. The SPE cartridges were previously conditioned with 1 mL methanol, 1 mL acetone, 2 mL hexane, 1 mL acetone, 1 mL methanol and 2 mL water. We modified the procedure (291) by adding an additional extraction step to remove unwanted endogenous compounds: The loaded cartridges were washed with 3 mL of water and 1 mL of 10% methanol. The retained eicosanoids in cartridges were eluted with 2 mL anhydrous acetonitrile followed by evaporation to dryness under nitrogen.

4.3.4 Fluorescent labeling

The labeling procedure [13] was also slightly modified to increase the sensitivity of the assay. The dried plasma, tissue and standard samples were reconstituted with 136 µL acetonitrile. An aliquot of 10 µL of a freshly prepared NE-OTf solution (2 mg/mL in saturated potassium fluoride solution in anhydrous acetonitrile) was added to each tube along with 4 µl of pure N, N-diiospropylethylamine as catalyst at 4º C and kept for 30 min in a desiccator for completion of the reaction. Subsequently, the reaction was terminated by drying of the samples under nitrogen. The samples, then, were reconstituted with 1 mL of 20% methanol to be loaded on a fresh set of pre-conditioned SPE cartridges. The cartridges were washed with 3 mL water followed by1 mL 30% methanol then dried under nitrogen to remove any moisture. The derivetized eicosanoids were eluted with 2 mL anhydrous acetonitrile, subsequently evaporated to dryness under nitrogen. The residue was reconstituted in 0.1 mL of 90% acetonitrile in water and 10 µL was injected into the HPLC column.

4.3.5 HPLC System

A Shimadzu Prominence HPLC system (Mandel Scientific, Guelph, ON, Canada) consisting of a DGU-20A5 degasser, a LC-20AT dual pump, a SIL-20A autosampler, a CTO-20AC column oven, a RF-10AxL fluorescence detector, and a CBM-20A communication module was used. Chromatographic separations were performed on C18 columns (2 columns of 100X 4.6 mm i.d., 3µm, connected as series) guarded with a security Guard Cartridge C18 (4.0 mm L x 3.0 mm i.d.) purchased from Phenomenex (Torrance, CA, USA). Simultaneous separation of ArA metabolites was performed using a gradient acetonitrile:water system. Mobile phases consisted of 0.05% formic acid in HPLC-grade water (A) and 0.05% of formic acid in acetonitrile (B) at an initial mixture of 45:55 A and B, respectively. Mobile phase B increased linearly from 55% to 65% over 40 min and remained plateau for 25 min. Then, in a linear gradient mode increased to 75% over 20 min and afterward to 95% over 10 min where it remained plateau for 22 min and dropped back to initial conditions (55%) with a 7 min pre-equilibration period prior to the next sample run. The run time of the sample was 124 min. The flow rate was 0.8 mL/min and the column oven temperature was set at 30º C. The detection was done at excitation and emission wavelengths of 260 and 396 nm, respectively. Data acquisition was carried out using Shimadzu Class VP 7.4 version software.

4.3.6 Validation

The method was validated for its specificity, linearity, and accuracy, precision, limits of detection (LOD), and limits of quantification (LOQ). Blank samples (derivatization reaction without any added ArA metabolites) and standard solutions (10 µL) containing either each eicosanoid alone or in combination of all metabolites of interest were injected into the HPLC to test specificity by observing the lack of interfering peaks. Initially, we constructed calibration

curves with eicosanoid concentrations of 0.0005, 0.001, 0.005, 0.01, 0.1, 0.5, 1.0, 10 and 2.5 μg/mL with the coefficient of variation (CV%) for the 0.0005 μg/mL concentration being 19.3%. However, subsequently we realized that to analyze biological samples of interest, such low concentrations were not needed. Therefore, our working calibration curves were prepared by serial dilution of ethanolic mixture of ArA metabolites standard solutions to yield standard samples containing 0.01, 0.1, 0.5, 1.0, 10 and 2.5 μg/mL of each compound. This range was broken down into two ranges of 0.01-1.0 and 0.5-2.5 μg/mL for better linearity on different analytes. The curves were constructed by plotting a metabolite/internal standard peak area ratio versus the given concentration of each metabolite. Three calibration curves were constructed the same day to determine intra-day variability. The assay was repeated on three different days to determine the inter-day variability. The accuracy was determined from % error = (mean observed concentration – added concentration) x100/ added concentration. The CV% was used to estimate the assay precision. The LOD was defined as the concentration that resulted in a higher than signal-to-noise ratio of 3:1. The LOQ was determined at the lowest concentration on the calibration curve for which the assay precision was lower than 20%.

4.3.7 Recovery and Stability

The recovery of ArA metabolites was determined by extracting known amounts of standard compounds from samples. They were then labeled with fluorescent dye and analyzed as described above. The peak responses were compared with that of standards without extraction. ArA metabolites stock solutions were kept in -20º C and protected from light all the time and all sample preparation process took place in a dimmed light exposure. Standard solutions, fluorescent labeling reagent and catalyzing solutions were freshly prepared.

The stability of ArA metabolites in biological samples during freeze-and-thaw cycles was examined by comparing their peak response after derivatization. Additionally, the stability of the underivetized ArA metabolites in the rat plasma and homogenated biological samples was investigated at 0 and 4 h storage time at room temperature (25º C). The derivatized ArA metabolites in final samples (dissolved in 90% acetonitrile in water) were stored in autosampler (at room temperature) waiting in line for maximum of 24 h to be analyzed. Their stability in sample vials during analysis was tested using standard solution of these compounds (0.1, 1.0 and 2.5 µg/mL or 1, 10, 25 ng on column). Samples were prepared and derivetized as mentioned before and analyzed at 0, 12 and 24 h after derivatization. The % accuracy and CV% were calculated.

4.3.8 Matrix effect

Matrix effect was assessed by subtracting the baseline peak areas observed for non-spiked from spiked biological samples with standard solutions, and comparing the values with those yielded by standard ethanolic solutions in the absence of matrixes. Triplicate solutions containing 0.1, 1.0 and 2.5 µg/mL (1, 10, 25 ng on column) were used.

4.3.9 Application to human and rat biological specimens

The method was used to detect AA metabolites in the plasma, heart and kidney of the rat and in the human plasma

4.4 Results

The fluorescent labeling reaction and procedure was adapted from two previous studies (285,291). The application of two-step sample clean up and using labeling reagent dissolved in saturated potassium fluoride solution in anhydrous acetonitrile, that we added to the procedure,

improved the selectivity and sensitivity of the assay as compared with those reported previously(285,291). As depicted in Figure 4.2 and Figure 4.4, peaks of interest were adequately separated, however, due to the close chemical structures and chromatographic behaviors, those representing 16- and 17-HETE were co-eluted with a retention time of 68 min. The same was true for the peaks representing 8,9- and 5,6-EET with retention time of 98 min. Each of these two pairs of co-eluting metabolites possesses the same pharmacological properties, hence, a measurement of the sum is not without value. It has been observed that 5,6-EET is highly unstable (298). This parameter is calculated as the ratio of retention time of the peak of interest over that of internal standard. The relative retention times of the metabolites ranged from 0.68 to 1.43 with CV% of <4.3% for triplicate injections. The matching retention time and the increased response proportional to increasing concentration were used to confirm the identity of the peaks. The identity and characterization of peaks representing ArA metabolites have been addressed previously (285,291).

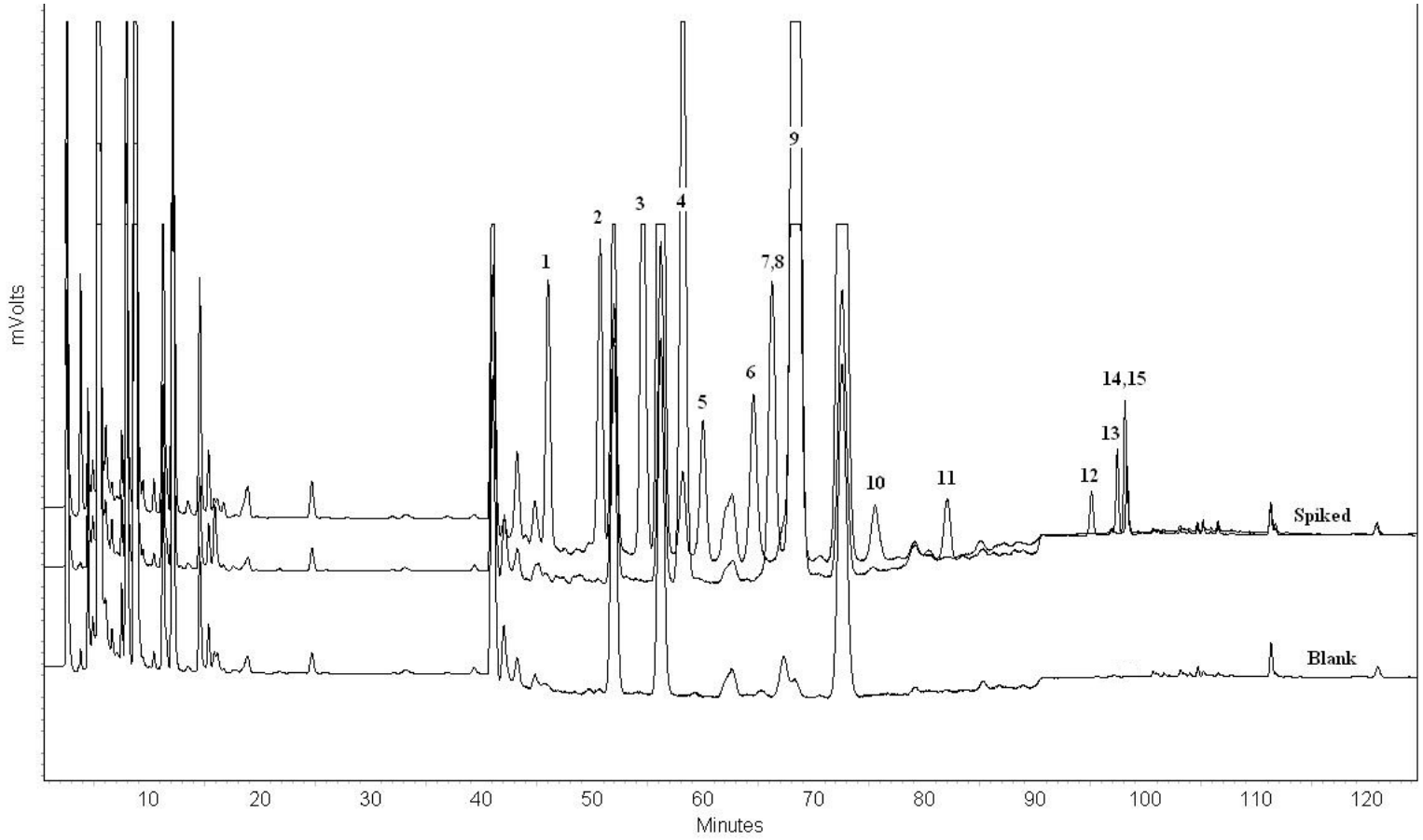

Figure 4.1. Representative chromatograms of ArA metabolites

Representative chromatograms of ArA metabolites in blank and two spiked standard solutions each containing a selected number of the compounds of interest; 1) 14,15-DHT, 2) 11,12-DHT, 3) 8,9- DHT, 4) 5,6- DHT, 5) 20-HETE, 6) 18-HETE, 7) 16-HETE, 8) 17-HETE, 9) Internal Standard, 10)15-HETE, 11)12-HETE, 12)14,15-EET, 13) 11,12-EET, 14) 8,9-EET and 15) 5,6-EET.

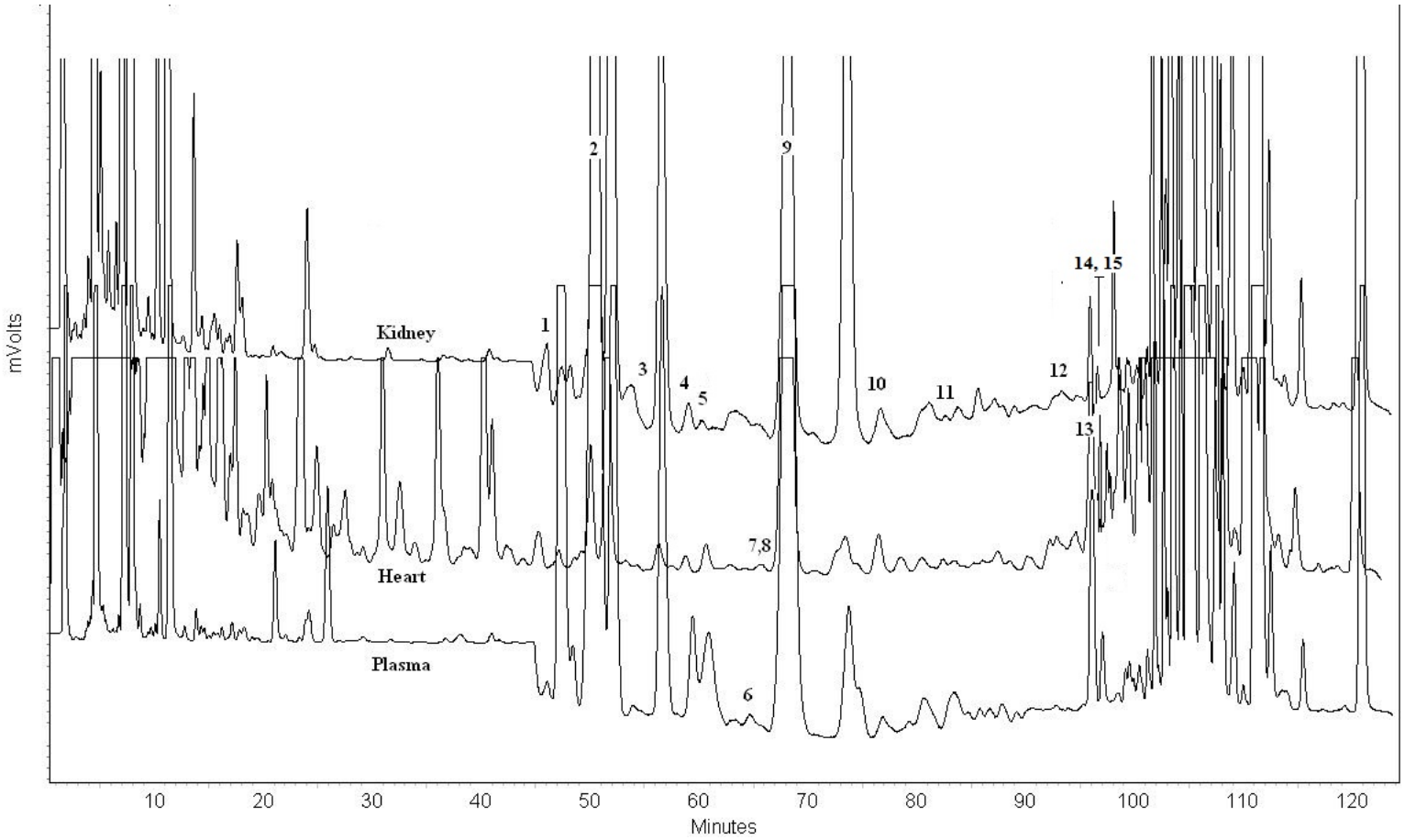

Figure 4.2. Representative chromatograms of ArA metabolites in rat plasma, heart and kidney samples

Representative chromatograms of ArA metabolites in rat plasma, heart and kidney samples the peak identifiers are same as Figure 4.1.

As has been reported by others (48,285,287,299), due to the presence of detectable basal concentrations of these metabolites, construction of standard curves using plasma or tissue matrices is impossible. However, as we have tested for the first time for the HPLC-FL approach, the differences in the detector response ranged from 1.5 to18.6% regardless of the biological samples used. This suggests that the use of non-spiked ethanolic solutions of the metabolites as standard solutions provide reliable results.

The assay was linear over the working concentration range of 0.01-2.5 μg/mL which we break it to two shorter ranges of 0.01-1 and 0.5-2.5 μg/mL (Figure 4.3) corresponding to 0.1-10 and 5-25 ng on column, respectively, based on the observed concentrations in the tested specimens. As depicted in Table 4.2, validation data were generated based on the above range. Nevertheless, based on our initial 0.0005-2.5 μg/mL range calibration curves, depending on the eicosanoid of interest, LOD was 1-20 pg and LOQ ranged from 5 to70 pg on column. We used 0.2 mL plasma but 30 mg of other tissues. Hence, the sensitivity of the assay was 0.5-7.0 ng/mL for plasma and 0.0016-0.023 ng/mg for tissues. The observed sensitivity made it possible to detect basal level of these metabolites in the human plasma and the rat plasma, heart and kidney tissues. The overall inter- and intra-day variations were less than 19.8% for the lowest concentration (0.01 μg/mL) of the working standard solutions range. The accuracy ranged from 0.0 to18.9% (Table 4.2).

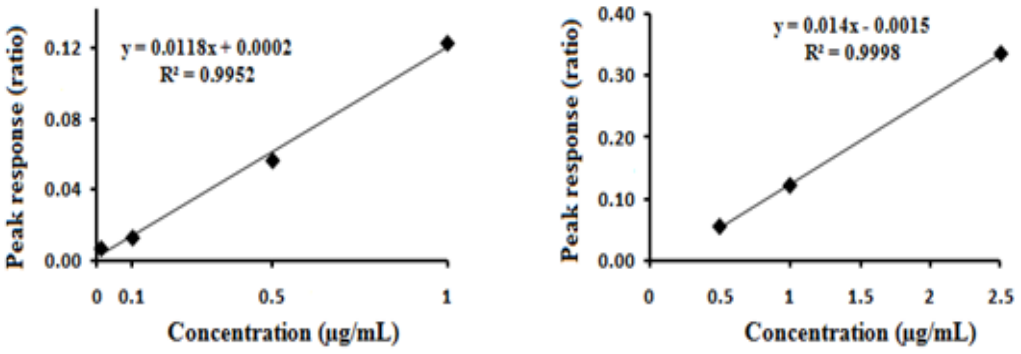

Figure 4.3. Representative calibration curves

Calibration curves for 20-HETE generated by coupling derivatization reaction and LC-FL. The concentration of standard solution ranges form 0.01-1.0 and 0.5-2.5 µg/mL.

Table 4.2 Precision (CV%) and accuracy of ArA metabolites analysis

Amount added (ng)a	0.1			1			10			25		
	Mean	CV%	%Accuracy	Mean	CV%	%Accuracy	Mean	CV%	%Accuracy	Mean	CV%	%Accuracy
Intra-day												
14,15-DHT	0.10	18.2	-2.0	1.0	14.0	-0.2	9.8	4.8	-1.9	25.3	1.9	1.2
11,12-DHT	0.11	14.2	12.7	1.0	19.0	1.2	9.0	9.9	-9.8	25.4	1.4	1.6
8,9-DHT	0.10	15.3	-2.8	1.1	15.9	5.2	9.1	10.1	-8.6	25.3	1.4	1.3
5,6-DHT	0.09	12.6	-12.2	1.0	19.6	-3.4	9.3	8.7	-6.6	25.3	1.4	1.0
20-HETE	0.09	3.0	-16.2	1.1	14.1	8.9	9.2	9.0	-8.5	25.3	0.9	1.3
18-HETE	0.09	19.3	-13.2	1.1	18.1	14.0	9.0	2.0	-10.1	25.5	10.6	2.0
16&17-HETE	0.09	15.7	-18.9	1.2	6.7	16.3	9.2	6.2	-7.7	25.3	10.6	1.2
15-HETE	0.10	18.6	4.3	1.2	12.6	15.1	9.2	2.3	-8.4	25.3	6.5	1.3
12-HETE	0.11	18.6	15.6	1.1	1.5	14.1	9.6	5.6	-3.9	25.2	4.8	0.9
14,15-EET	0.11	13.5	12.1	1.1	19.8	5.3	9.0	14.6	-10.2	25.3	1.7	1.3
11,12-EET	0.10	13.9	2.1	0.9	10.9	-6.6	9.5	5.4	-4.6	25.2	0.8	0.8
8,9 &5,6-EET	0.09	15.3	-7.8	1.0	10.8	-0.4	9.9	7.8	-1.1	24.9	0.6	-0.3
Inter-day												
14,15-DHT	0.10	18.6	-4.0	0.9	12.4	-10.7	9.6	8.0	-3.7	25.5	3.0	1.9
11,12-DHT	0.11	18.3	10.0	0.9	6.0	-12.7	9.1	9.0	-9.4	25.5	2.2	2.1
8,9-DHT	0.09	17.0	-7.8	0.9	4.6	-13.8	9.0	12.0	-10.0	25.4	1.7	1.5
5,6-DHT	0.09	15.7	-5.2	0.8	15.0	-18.6	9.1	11.9	-8.7	25.3	1.6	1.3
20-HETE	0.08	11.5	-16.9	1.0	5.0	-2.8	9.0	9.7	-9.7	25.4	2.5	1.5
18-HETE	0.09	17.8	-10.7	1.1	17.2	12.0	9.8	8.0	-1.7	25.2	9.5	1.0
16&17-HETE	0.08	19.0	-17.8	1.1	7.8	14.3	9.4	7.2	-5.8	25.5	8.8	1.9
15-HETE	0.11	14.4	8.0	0.9	10.6	-6.2	9.6	4.3	-4.2	25.3	4.5	1.4
12-HETE	0.11	19.4	9.0	1.2	3.5	14.1	9.4	7.6	-5.6	25.4	5.8	1.5
14,15-EET	0.11	18.8	13.0	1.0	16.9	-3.6	9.0	18.2	-10.2	25.3	2.6	1.4
11,12-EET	0.09	22.0	-10.4	0.9	15.0	-14.9	9.6	7.2	-4.5	25.2	1.1	0.8
8,9 &5,6-EET	0.09	11.5	-8.0	1.0	13.0	0.0	10.0	6.1	0.3	25.0	0.9	0.0

Values are mean, n=3 experiments. a, amount injected to the LC column.

The percent recovery of ArA metabolites from plasma, heart and kidney samples in the low, medium and high concentration ranged from 89.8 to108.9% (Table 4.3).The examined ArA metabolites were found to be stable during freeze-and-thaw cycles and sample preparation process (CV% < 10%). ArA metabolites were stable in room temperature for the tested period of time (4 h). The fluorescence-labeled products of ArA metabolites were stable after derivatization and during the sample analysis. The CV% for accuracy and precision of derivetized metabolites after 24 h storage time at room temperature inside the auto-sampler were less than 5 and 10%, respectively.

Table 4.3 Percent recovery of ArA metabolites analytical standards from samples after solid phase extraction

Amount added (ng)[a]	1		10		25	
Compound	Mean	CV%	Mean	CV%	Mean	CV%
14,15-DHT	99.8	14.0	98.1	4.8	101.2	1.9
11,12-DHT	101.2	19.0	90.2	9.9	101.6	1.4
8,9-DHT	105.2	15.9	91.4	10.1	101.3	1.4
5,6-DHT	96.6	19.6	93.4	8.7	101.0	1.4
20-HETE	108.9	14.1	91.5	9.0	101.3	0.9
18-HETE	103.2	18.1	89.9	12.0	102.0	10.6
16&17-HETE	108.8	6.7	92.3	6.2	101.2	10.1
15-HETE	108.2	12.6	91.6	12.3	101.3	6.5
12-HETE	106.9	10.5	96.1	5.6	100.9	4.8
14,15-EET	105.3	19.8	89.8	14.6	101.3	1.7
11,12-EET	93.4	10.9	95.4	5.4	100.8	0.8
8,9 &5,6-EET	99.6	10.8	99.0	7.8	99.7	0.6

Values are mean; n=3 experiments, [a] amount injected to the LC column.

4.4.1 Analysis of biological samples

The method was used to quantify ArA metabolites in the rat plasma, heart and kidney samples (Figure 4.2) and in human plasmas (Figure 4.4). Most of the metabolites such as 14,15-DHT, 11,12-DHT, 8,9-DHT, 5,6-DHT, 20-HETE, 18-HETE, 12-HETE, 15-HETE, 14,15-EET, 11,12-EET and 8,9-EET were quantifiable at their basal level in the rat plasma, heart and kidney and human plasma. The concentration of these ArA metabolites ranged from 1.5 to 58.9 ng/mL in the rat plasma. It ranged from 0.14 to 36.5 and 0.02 to7.9 ng/mg of tissue in the rat heart and kidney, respectively. In the plasma of the two healthy subjects the ArA metabolites concentration were 39.5-597 and 15.6-1651 ng/ml, respectively.

A comparison of the rat plasma, heart and kidney sample chromatograms revealed that the ArA metabolites concentrations were tissue dependent (Figure 4.2). There were some unidentified peaks unique to the heart samples (retention time range of 15-25 and 30-40 min) which needs to be further investigated. In addition, another unknown peak with the retention time of 47 min had much higher intensity in plasma than in other examined tissues. For the rest of the chromatogram, although the relative intensities of the peaks representing the basal level of the ArA metabolites were different, the overall profiles of the chromatograms were similar for all examined tissues. The major metabolite present at high concentration in all three biological matrixes was 11, 12-DHT.

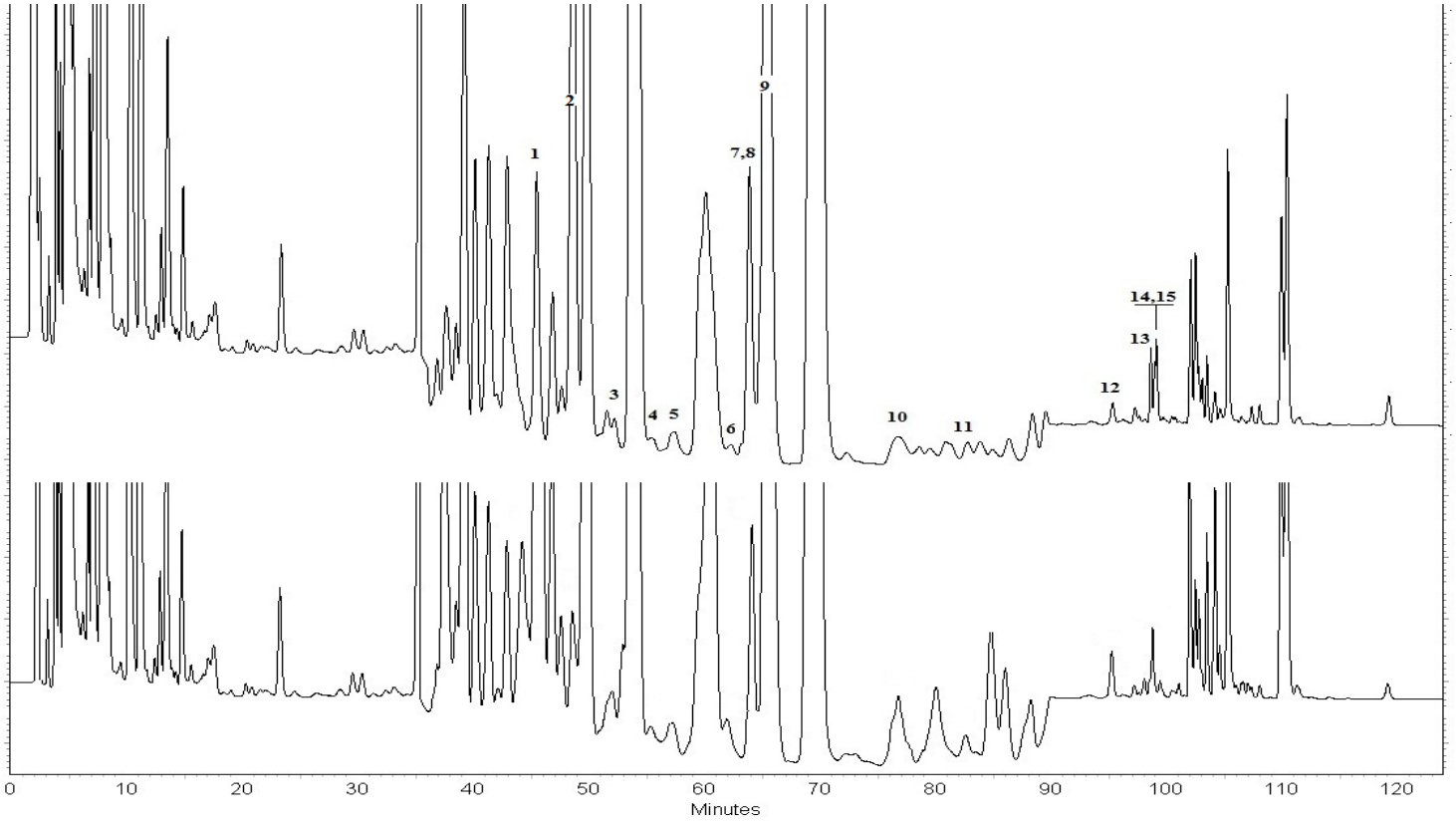

Figure 4.4. Representative chromatograms of ArA metabolites in human plasma

Representative chromatograms of ArA metabolites in human plasma samples from two individuals; the peak identifiers are same as Figure 4.1. .

4.5 Discussion

Under healthy conditions, ArA metabolites are present in plasma and tissues in low concentrations. They, however, are elevated in response to pathological stimulations. Herein, we present a facile, sensitive and selective assay suitable for real-time measurement of 10 ArA metabolites. Four other metabolites are also measurable but presented as two co-eluted pairs of peaks, each as sum of two structurally similar metabolites; i.e., 17 plus 16-HETE, and 8,9 plus 5,6-EETs. Since each pair of these co-eluted metabolites possesses the same pharmacological properties, a measurement of the sum is not without value. The co-elution of these metabolites is not clearly addressed in the previous LC-FL or LC-MS methods. For instance, Maier et al. (285) have referred to several of the HETE metabolites as "subterminal HETEs" and presented them as a cluster of peaks or just reported them as individual peaks but some with the same retention times. Yue et al. (291) included only one out of four co-eluting metabolites into their chromatogram of standard solution. Similarly, using the LC-MS methods, the peaks with equal mass are reported individually with very close retention times so that the differences in the retention times are often less than the width of the individual peaks (295). Therefore, the co-elution of these metabolites seems to be a general issue with all the reported assays including LC-MS/MS using MRM (288,297).

The LOQ of the present method ranges from 5 to 70 pg on column, depending on the metabolite. Two of the reported LC-FL methods have comparable sensitivity to the present method. They, however, have not reported any evidence of selectivity of the assay as the authors did not present chromatograms containing biological samples (Table 4.1). We achieved a better selectivity by improving the labeling reaction condition and sample clean up procedures. For a lower than presented LOQ, it appears the LC-MS/MS methods is an alternative.

The sensitivity of the present method appears to be sufficient for the measurement of the basal levels of at least twelve out of fourteen tested metabolites including the co-eluted ones. Plasma concentrations depending on the metabolite were about 3-40 fold higher in the human compared with the rat. The lowest basal concentration was observed for 8,9-EET in rat plasma (1.5 ng/mL), heart (0.14 ng/mg) and kidney (0.02 ng/mg). In human plasma also 8,9-EET demonstrated the lowest concentration (39.5 ng/mL). These values are higher than the quantification limit of the method for all of the tested ArA metabolites.

The evaluation of real time concentrations of these ArA metabolites, and more importantly the ratio of cardioprotective vs. cardiotoxic ArA metabolites, are critical for understanding their physiopathological role in the body functions. Therefore, a direct measurement of these ArA metabolites in biological matrixes is essential. Additionally, despite the reported detection limit of about 1 pg per injection (Table 4.1), in our hand, the LC-MS method of Nithipatikom et al (287) as applied by others (69,289), demonstrated insufficient sensitivity and unacceptable reproducibility for simultaneous and direct determination of low basal concentration of ArA metabolites in biological tissues.

Recently, it has been reported that the process of freeze-thawing results in substantial increased concentration of ArA metabolites in the liver tissues as compared to fresh samples (297). The authors have attributed this change to de-esterification of ArA metabolites from cell membranes. Hence, unless, the biological samples are tested quickly after collection, the generated data does not present free ArA metabolites in tissues (297). In plasma, on the other hand, there is no evidence for esterified ArA metabolites. The instability of ArA metabolites in tissues introduces procedural complications. Alternatively, one can de-esterify the esters by various means including acidification (285,288,291,295), as we have, and measure the total

concentration of ArA metabolites in the samples. Indeed, Hammond and O'Donnell (300) have demonstrated that esterified ArA metabolites mediate biological properties in their own right, even more potent than the parent compounds. In the heart and kidney tissues, it seems that our approach yields the total rather non-esterified eicosanoid, since, we used acidified freeze-thawed samples, and under repeated freeze-thawing and storage, we did not detect significance changes in the concentrations. Hence, we suggest the use of plasma ArA metabolites as biomarkers of the cardiac condition when the status of ArA metabolism in deep tissues (e.g., heart and kidney) is investigated. To support this notion, and in agreement with earlier reports (33,301-307) regarding the therapeutic relevance of total tissue eicosanoid, we plotted ArA metabolites concentrations in homogenized heart tissue (total) versus those measured in plasma and noticed strong positive correlations (See chapter 5).

The present study is an optimization of two previously reported methods (285,291). We used the same derivatization approach but modified the reaction conditions to 1) increase the reaction yield by using saturated potassium fluoride in the reaction medium and 2) to eliminate the interfering unwanted peaks by doubling the solid phase extraction procedure. In addition, the use of gradient elution further improved our selectivity as compared with that reported by Yue et al. method (291) that, in our hands, yielded crowded chromatograms when applied to biological samples. Our modifications improved the LC-FL assay in terms of both sensitivity and selectivity.

The present study has few limitations. First, the LC-FL methods do not provide information on the molecular mass of the peaks of interest as LC-MS methods do. We relied only on the relative retention time, the proportional increase in the peak size by increasing the amount injected and the published information on the peak identification (285,291). Second, we

observed a few unidentified peaks that need to be further identified. Third, the present method has a longer run time as compared with LC-MS methods.

4.6 Conclusions

The advantage of the present LC-FL over the other methods is the relatively low cost of instrumentation, maintenance and operation and application for different tissues with improved selectivity and sufficient sensitivity for simultaneous assay of ArA metabolites.

Chapter 5

5 Association of the Renin-Angiotensin System Components and Arachidonic Acid Metabolites under Inflammatory Condition in the Rat with Adjuvant Arthritis

5.1 Introduction

There are several reports in the literature addressing the involvement of the RAS components as proinflammatory or anti-inflammatory modulators in the inflammatory conditions such as hypertension, atherosclerosis, stroke and kidney diseases (308-316). Similar accusations were also reported for ArA pathway (53,64,317-319). Additionally, some studies were reported the possible interaction between one or two components of the RAS and ArA pathways in some CV or renal disease (320-323) however, their focus were usually on one system rather than both. Hence, the mechanism of interaction between these two systems and their mutual role in modulation of body functions is not fully explored. RAS and ArA pathways consist of several biologically active components with opposing physiological functions, therefore, a better knowledge about their intra-system balances and inter-system interactions seems essential to explain the regulatory mechanism of these systems in the body, especially in CV and renal systems.

The role of angiotensin peptides and ArA metabolites in the pathogenesis of RA as an inflammatory condition is largely unknown. Therefore, a direct and *in vivo* study of several opposing members of these two systems is needed to get a better picture about their involvement in inflammation, which it could help to identify some of the components of these systems as reliable biomarkers in this setting. Using rat AA model, a well established animal model representing human RA, we demonstrate that there are significant changes in the plasma, heart and kidney concentration of ArA metabolites and the RAS components under inflammatory

conditions. In addition, we found a relationship between plasma and tissue concentration of these components. The aim of the current work is to correlate ArA metabolites and RAS component level in the plasma with their concentration in the heart and kidney in healthy and inflammatory states to introduce reliable biomarkers of CV risk. If these biomarkers could be extrapolated to the human, they will provide useful hints for planning future clinical trials in order to identify vulnerable individuals with RA at risk of CV events.

5.2 Hypothesis

Inflammation modulates ArA pathway and the RAS by altering their components levels locally and systemically in the body. These two pathways are interacting and influencing each other in healthy and disease states and their components are correlated with each other

5.3 Objectives

To determine the effect of AA on the plasma, heart and kidney concentration of ArA metabolites

To determine the effect of AA on the plasma, heart and kidney level the RAS components

To correlate the plasma, heart and kidney tissue level of the RAS components and ArA metabolites with each other.

5.4 Results

5.4.1 ArA metabolites concentration in the plasma, heart and kidney

Table 5.1 represents the plasma, heart, and kidney concentration of ArA metabolites in control and rat with arthritis. The results of the present study demonstrated that the animals with arthritis showed significant ($p < 0.05$) elevation of plasma concentrations of 18- and 20-HETE when compared to healthy control animals (Figure 5.1). The 12-HETE concentration was

comparable between the two groups. The total CYP-epoxygenase-derived EETs plasma concentration was similar between control and arthritic rats. Nevertheless, a significant ($p < 0.05$) increase in 14,15-EET concentration was observed in the INF group. There was no significant difference in observed total T-DHTs concentration between rats with AA and healthy animals; however, as an exception, the 14,15-DHT concentration was significantly ($p < 0.05$) higher in the INF group. The ratio of 20-HETE over total EETs (T-EETs) as a marker of vasoconstrictive/ vasodilator metabolites ratio was significantly ($p < 0.05$) higher in the INF group (Figure 5.1). Additionally, the sEH enzyme is responsible for hydroxylation of EETs to DHTs and ratio of DHTs/EETs, can be considered as an index of sEH enzyme activity. This index, 14,15-(DHT/EET), was highly significant ($p < 0.01$) for the conversion of 14,15-EET to14,15-DHT in INF group.

The heart tissues samples from rats with arthritis presented significantly ($p < 0.05$) higher concentrations of 20-HETE and 12-HETE when compared with control rats (Table 5.1, Figure 5.2). Although the 15-HETE and 18-HETE concentrations were higher in the INF group, they were not significantly different from control animals. Although there was no significant change in the heart EET and DHT concentrations between inflamed and control groups, the ratio of DHTs/T-EETs (as an indicator of sEH enzyme activity) was significantly ($p < 0.05$) higher in inflamed animals. In addition, inflammation resulted in a significant increase in the ratios of 20-HETE/T-EETs, 20-HETE/ 14,15-EET, 20-HETE/DHTs, 20-HETE/T-EETs+DHTs, and 14,15-(DHT/EET) The same result was observed for the ratio of 12-HETE over T-EETs, T-DHTs and 11,12-EET in inflamed animals.

The overall tissue concentrations of ArA metabolites in the kidney were lower than the heart samples. Moreover, the trends of their concentration changes were contrary to that of the plasma

and heart tissues (Table 5.1, Figure 5.3). A highly significant ($p < 0.05$) decline in 12, 15, 18, and 20-HETE concentrations were noticed in the kidney samples of the INF group. However, the individual or total EET concentration was not altered by inflammation and consequently, the ratio of 20-HETE/T-EET followed the same trend as the 20-HETE concentration between the two groups. In contrast to the plasma and heart, inflammation caused insignificant reduction in the activity of sEH in the kidney and as a result, the individual and T-DHTs concentrations were lower in inflamed animals. The ratio of kidney DHTs/EETs was comparable between two groups however, a significant difference was noted for 14,15-(DHT/EET) (Table 5.1).

Table 5.1 ArA metabolites concentration and their ratio in the plasma, heart and kidney of control and arthritic rats (n=3-4/group)

Plasma ArA metabolites	Plasma (ng/mL)				Heart (ng/mg tissue)				Kidney (ng/mg tissue)			
	Control		INF		Control		INF		Control		INF	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14,15-DHT	12.71	0.88	40.35*	13.68	0.46	0.25	0.66	0.31	0.40	0.14	0.17	0.14
11,12-DHT	31.50	22.26	30.72	9.22	36.51	13.44	49.06	18.14	7.88	10.79	2.65	1.66
8,9-DHT	12.45	1.90	14.66	9.81	1.22	0.52	1.30	0.29	0.15	0.16	0.09	0.03
5,6-DHT	11.09	5.26	16.83	2.02	0.24	0.08	0.39	0.12	0.10	0.04	0.08	0.04
T-DHT	67.74	18.20	102.55	25.89	38.44	13.48	51.41	18.50	8.53	11.08	3.00	1.84
20-HETE	58.90	19.68	107.50*	9.59	0.30	0.13	0.73*	0.18	0.14	0.06	0.02*	0.00
18-HETE	13.62	1.76	35.16*	10.36	1.94	1.05	2.97	1.00	0.10	0.05	0.02*	0.02
15-HETE	9.43	2.84	17.78	5.86	0.52	0.14	0.78	0.27	0.27	0.10	0.14*	0.03
12-HETE	44.19	21.18	46.58	46.90	0.18	0.09	0.91*	0.17	0.30	0.16	0.02*	0.00
14,15-EET	3.91	0.08	1.44*	0.45	0.47	0.52	0.14	0.08	0.14	0.03	0.16	0.08
11,12-EET	19.50	1.75	19.48	6.24	3.25	0.90	3.51	0.66	0.16	0.08	0.11	0.03
8,9-EET	1.45	1.10	0.86	0.19	0.14	0.11	0.30	0.11	0.02	0.01	0.02	0.00
T-EET	24.86	2.92	21.78	6.50	3.98	1.06	3.95	1.15	0.31	0.11	0.29	0.10
DHTs+EETs	92.60	16.22	124.33	25.82	41.85	12.76	55.61	19.13	8.84	11.12	3.28	1.93
Ratio												
DHTs/EETs	2.79	0.98	5.01	2.14	8.56	2.61	13.86*	1.50	26.39	26.70	9.97	2.61
20-HETE/EETs	2.33	0.50	5.28*	1.73	0.06	0.05	0.15*	0.01	0.43	0.06	0.07*	0.02
20-HETE/DHTs	0.94	0.51	1.10	0.34	0.01	0.00	0.01*	0.00	0.05	0.06	0.01	0.00
20-HETE/EETs+DHTs	0.66	0.29	0.90	0.26	0.01	0.00	0.01*	0.00	0.04	0.05	0.01	0.00
20-HETE/14,15-EET	15.03	4.75	82.63*	37.13	1.12	0.78	5.58*	2.08	0.98	0.36	0.12*	0.05
20-HETE/11,12-EET	2.98	0.71	5.96	2.05	0.10	0.08	0.17	0.02	1.91	0.22	0.16*	0.09
20-HETE/8,9-EET	49.35	18.68	130.58*	37.90	2.93	2.34	2.26	0.79	9.99	3.34	1.21*	0.47
15-HETE/EETs	0.38	0.12	0.83	0.25	0.17	0.06	0.18	0.02	0.89	0.30	0.49	0.07
15-HETE/DHTs	0.15	0.07	0.19	0.10	0.02	0.01	0.02	0.01	0.09	0.12	0.05	0.02
15-HETE/EETs+DHTs	0.11	0.04	0.15	0.07	0.01	0.01	0.02	0.01	0.08	0.08	0.05	0.01
12-HETE/EETs	1.75	0.76	2.49	2.89	0.06	0.05	0.22*	0.02	0.92	0.21	0.06*	0.02
12-HETE/DHTs	0.73	0.46	0.41	0.33	0.01	0.00	0.02*	0.00	0.09	0.10	0.01	0.00
12-HETE/EETs+DHTs	0.51	0.29	0.34	0.30	0.00	0.00	0.02*	0.00	8.84	11.12	3.28	1.93
14,15-(DHT/ EET)	3.26	0.28	27.97**	1.64	1.93	1.56	5.41*	2.06	2.84	0.57	1.03*	0.74

Significantly different from CL group,* $p < 0.05$, **$p < 0.01$

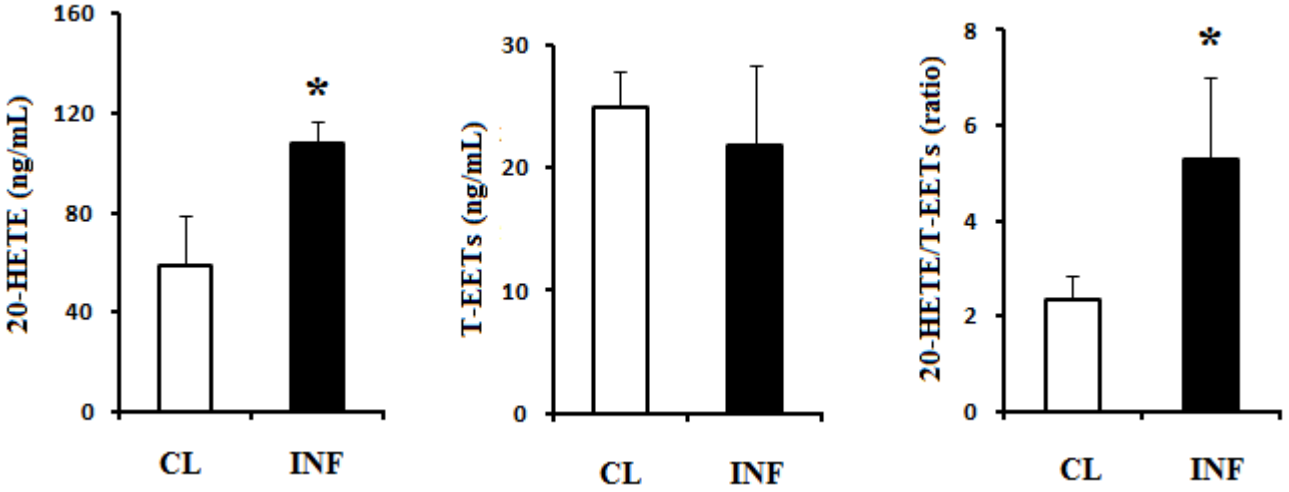

Figure 5.1. Effect of inflammation on the plasma concentration of ArA metabolites.

The plasma concentration of 20-HETE, T-EETs and their ratio in control (CL) and inflamed (INF), *statistically significant differences ($p < 0.05$) (n=3-4/group).

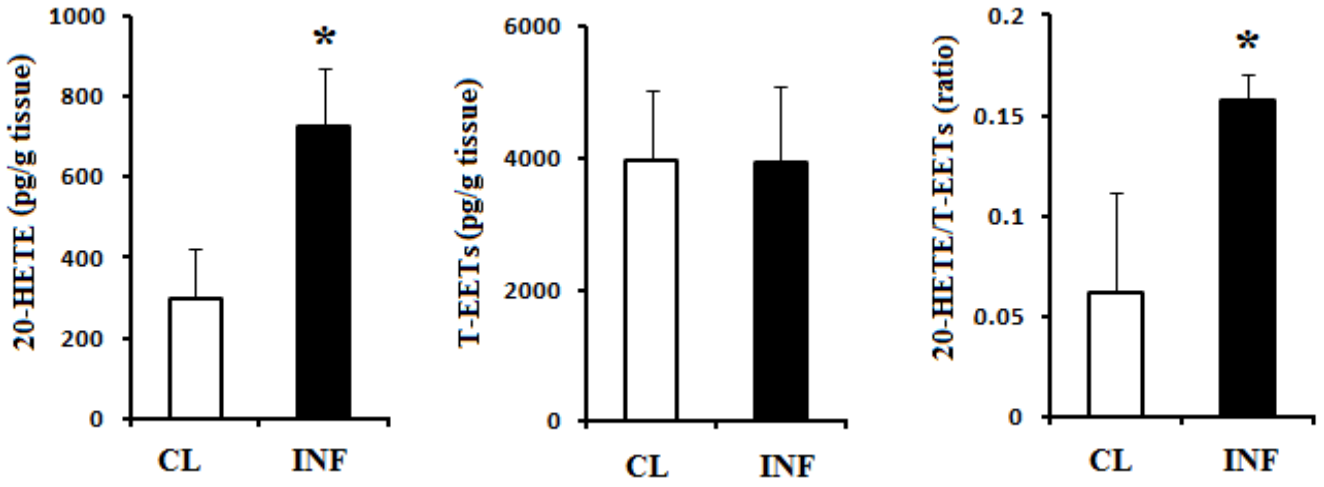

Figure 5.2. Effect of inflammation on the heart concentration of ArA metabolites.

The heart concentration of 20-HETE, T-EETs and their ratio in control (CL) and inflamed (INF), *statistically significant differences ($p < 0.05$) (n=3-4/group).

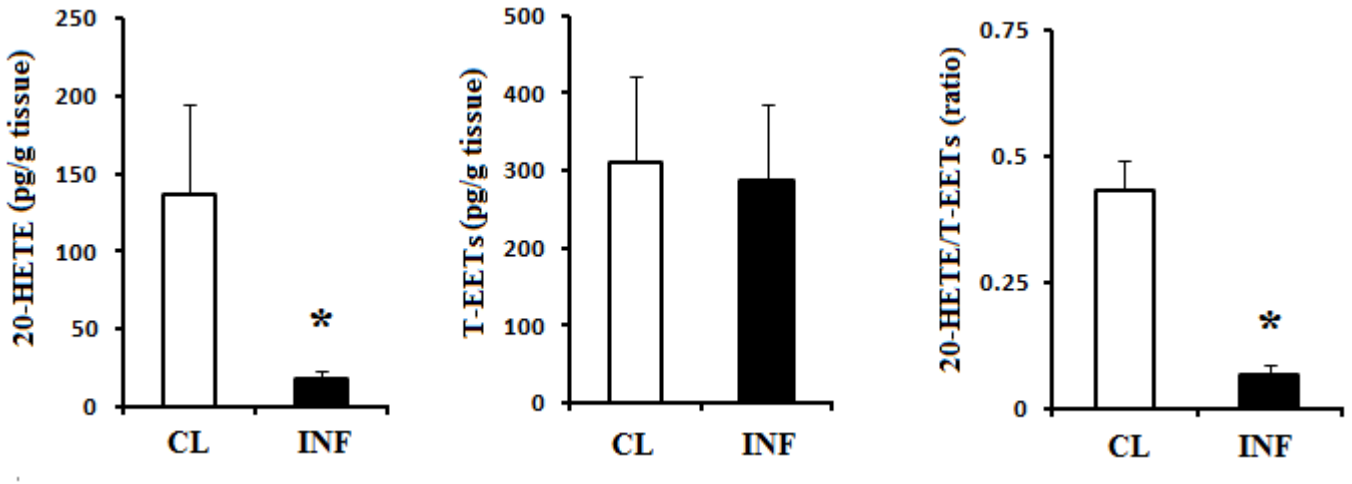

Figure 5.3. Effect of inflammation on the kidney concentration of ArA metabolites.

The kidney concentration of 20-HETE, T-EETs and their ratio in control (CL) and inflamed (INF), *statistically significant differences ($p < 0.05$) (n=3-4/group).

5.4.2 RAS component analysis

The effect of adjuvant arthritis on the heart and kidney ACE, ACE2 protein expression, and their ratio is presented in Figures 5.4 and 5.5. As previously reported by us (20), in both tissues ACE protein expression was not altered due to inflammation; however, ACE2 protein expression was significantly reduced in the INF group and consequently it influenced the ACE2/ACE ratio as well. Accordingly, angiotensin peptides, Ang II, Ang 1-7, and their ratios are also affected by inflammation (Figure 5.6 -5.8). In the plasma, the Ang II concentration was higher in the INF group; however, Ang 1-7 and Ang1-7/II were comparable between groups. In the heart, the same trend was observed for Ang II but the Ang 1-7 concentration and Ang 1-7/II were significantly lower in the INF group. Similarly, in the kidney the Ang II concentration was significantly elevated due to inflammation; however, for Ang 1-7 and the ratio of Ang 1-7/II the difference between groups failed to reach a significant level.

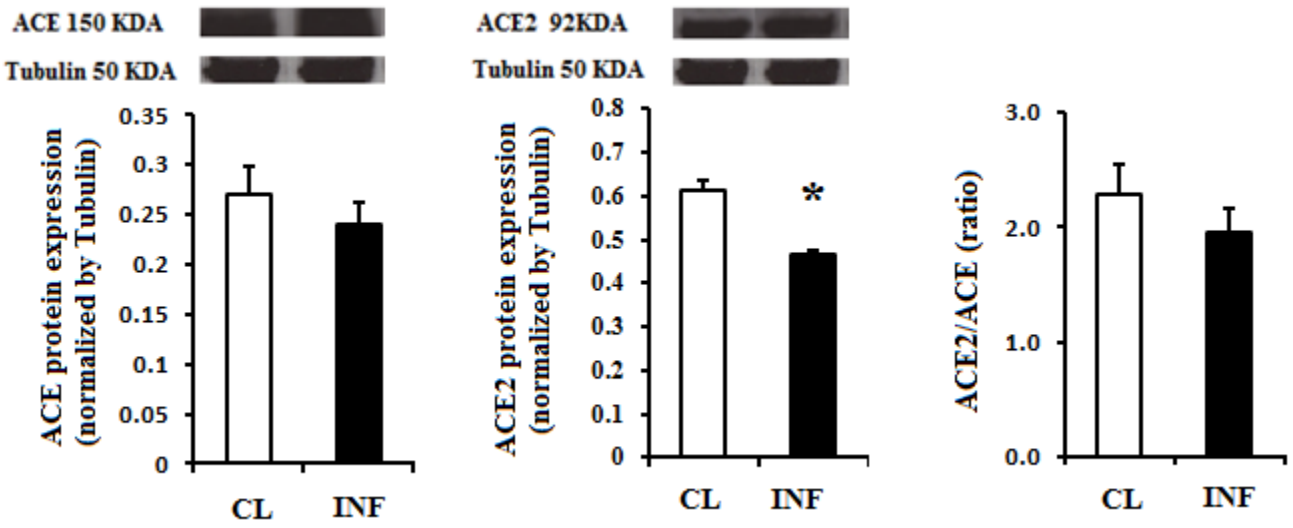

Figure 5.4. Effect of inflammation on ACE, ACE2 protein expression level and their ratio in the rat heart

The heart tissue ACE and ACE2 protein expression levels and their ratio in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

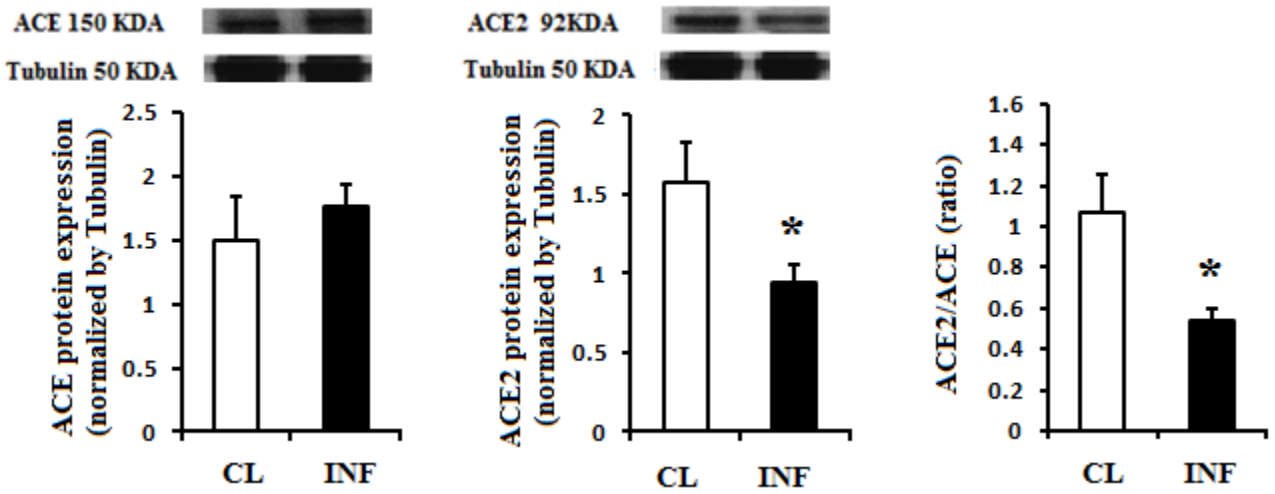

Figure 5.5. Effect of inflammation on ACE, ACE2 protein expression level and their ratio in the rat kidney

The kidney tissue ACE and ACE2 protein expression levels and their ratio in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

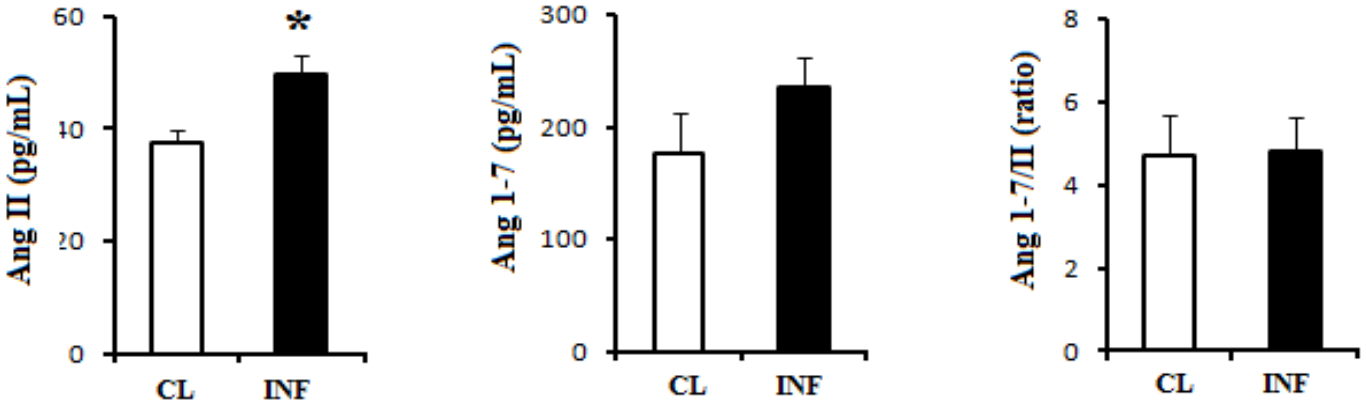

Figure 5.6. Effect of inflammation on Ang peptides concentration in the rat plasma

The plasma concentration of Ang II, Ang 1-7 and their ratio in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

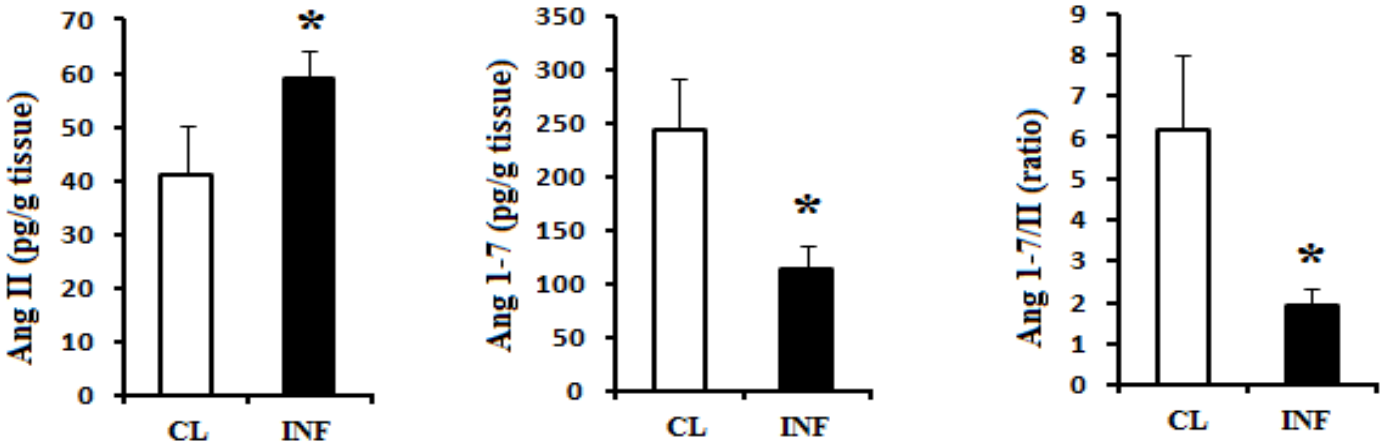

Figure 5.7. Effect of inflammation on Ang peptides concentration in the rat heart

The heart concentration of Ang II, Ang 1-7 and their ratio in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

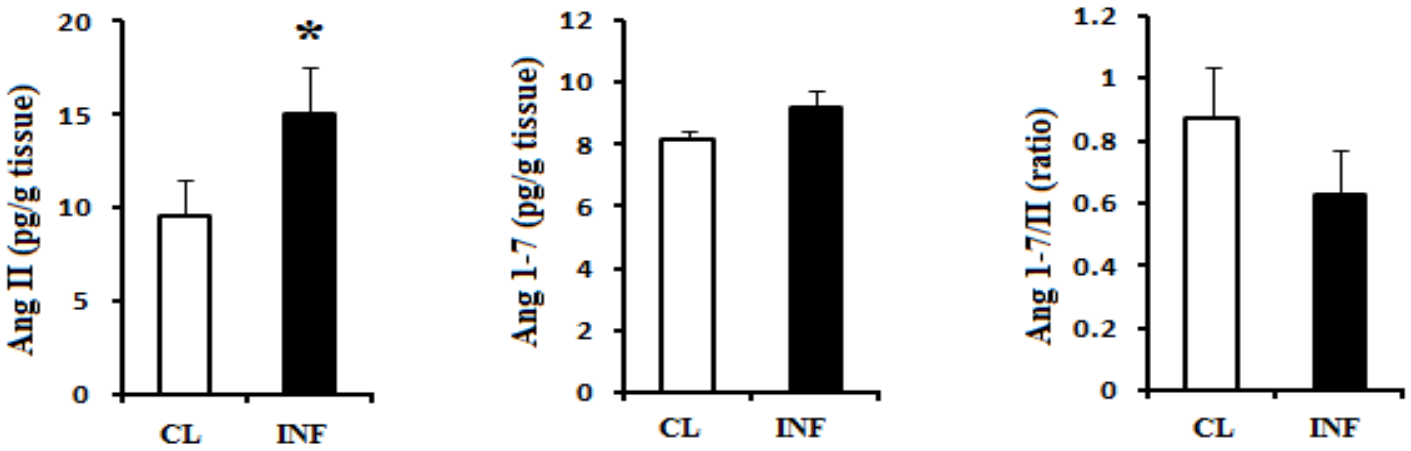

Figure 5.8. Effect of inflammation on Ang peptides concentration in the rat kidney

The kidney concentration of Ang II, Ang 1-7 and their ratio in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

Effect of inflammation on AT1R, AT2R, and Mas receptor protein expression and their ratios are presented in Figures 5.9 and 5.10. In the heart and kidney, AT1R receptor was over-expressed in the INF group. While AT2R receptor expression was significantly higher in the heart of the INF group, it did not reach a significant difference in the kidney tissue. For Mas receptor the trend was different between tissues, as it was significantly over-expressed in the kidney while it was down-regulated in the heart tissue of rats with AA (Figure 5.11 and 5.12). The ratio of Mas/AT1R was lower in both the heart and kidney of animals with AA then control group, but the difference was significant only on the heart tissue. The same trend was observed for Mas/AT2R in the heart but not for the kidney. The ratio of AT2R/AT1R in the heart was not significantly different between groups; however, in the kidney it was significantly lower in the INF group compared with control animals.

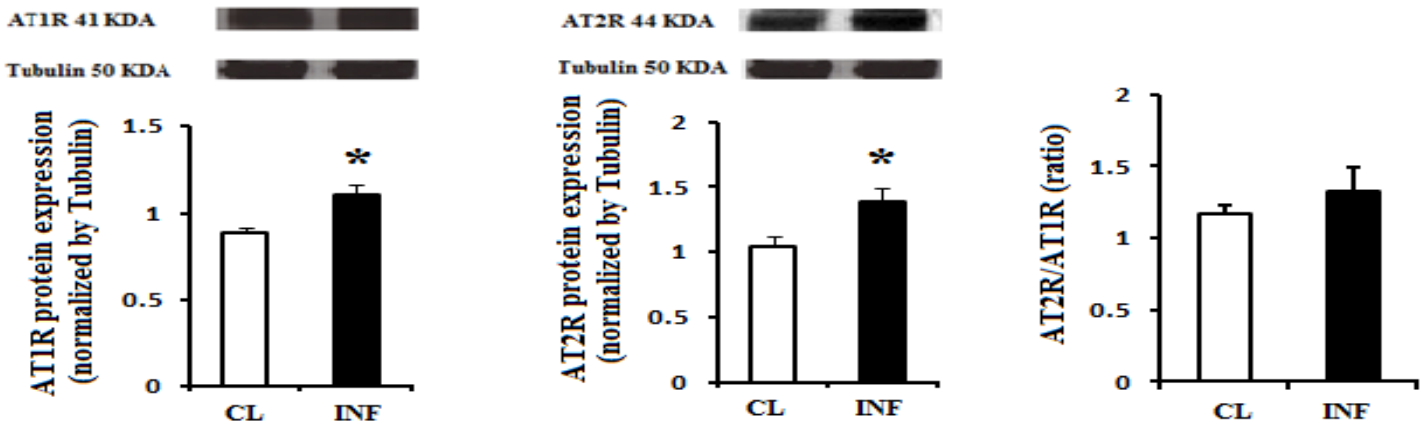

Figure 5.9. Effect of inflammation on Ang II receptor expression level in the rat heart

The heart tissue expression level of AT1R, AT2R and their ratio in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

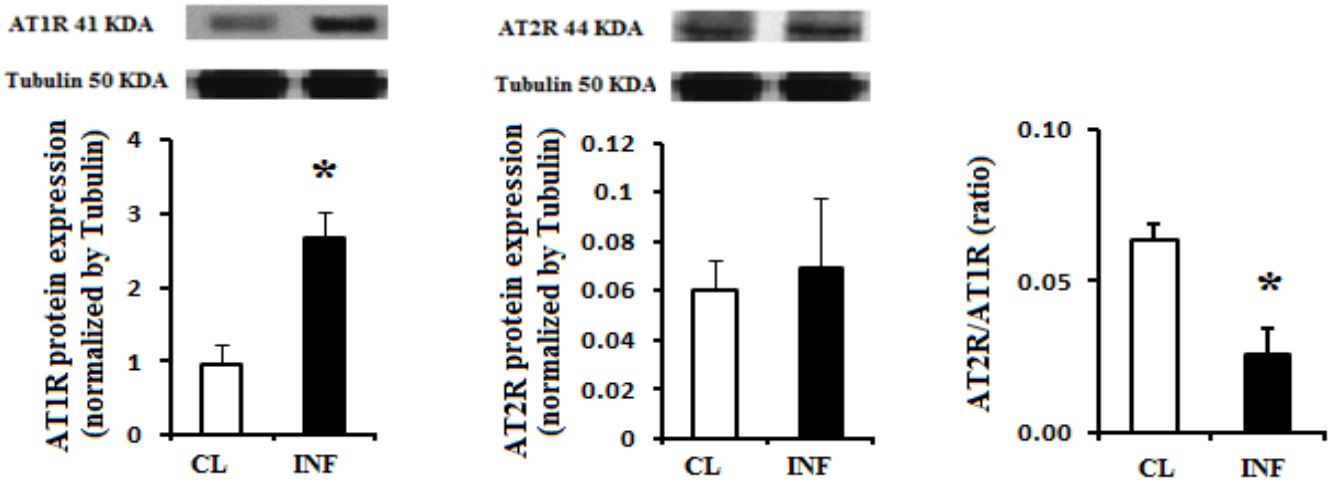

Figure 5.10. Effect of inflammation on Ang II receptor expression level in the rat kidney

The kidney tissue expression level of AT1R, AT2R and their ratio in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

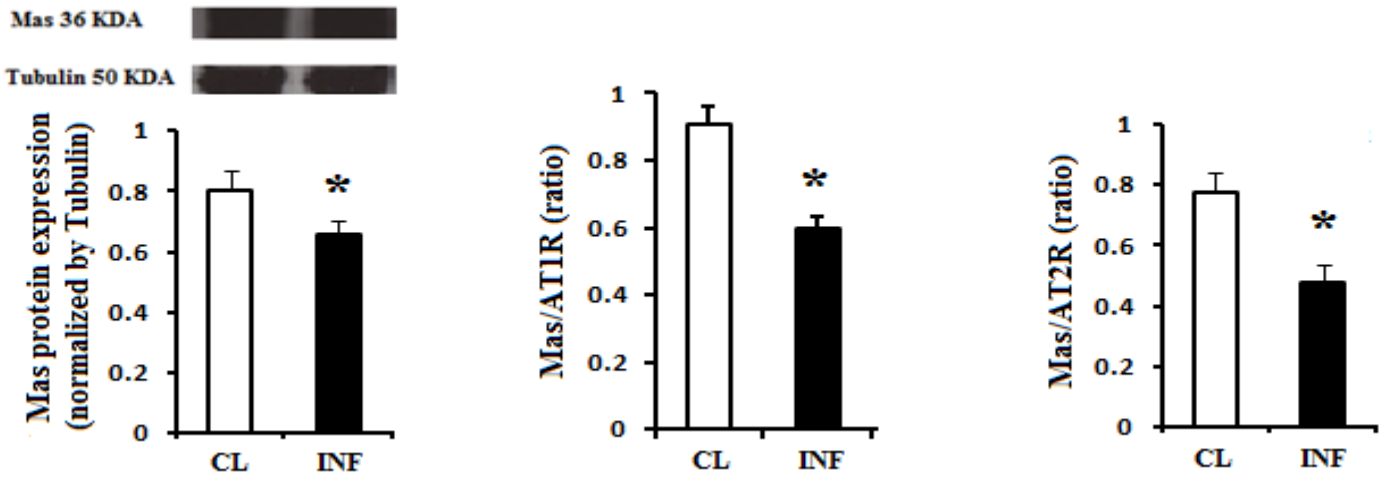

Figure 5.11. Effect of inflammation on Mas receptor expression level in the rat heart

The heart tissue expression level of Mas and its ratio with AT1R and AT2R in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

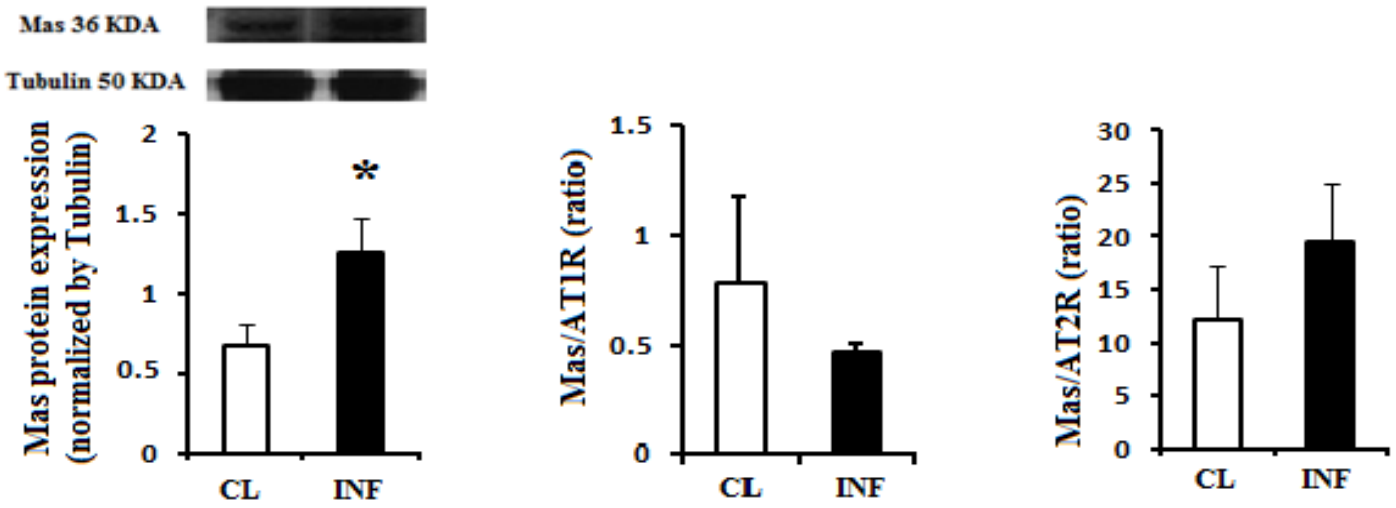

Figure 5.12. Effect of inflammation on Mas receptor expression level in the rat kidney

The kidney tissue expression level of Mas and its ratio with AT1R and AT2R in control (CL) and inflamed (INF); *statistically significant differences ($p < 0.05$), (n=3-4/ group).

5.5 The correlation between the plasma and heart and kidney ArA metabolites concentration

We observed correlation of some ArA metabolites concentration between plasma and heart and kidney (Table 5.2, Figure 5.13). For instance, 20-HETE concentration was correlated between plasma vs. heart ($r = 0.9133$, $p < 0.05$) and kidney ($r = -0.9440$, $p < 0.01$) (Table 5.2). However, some of the plasma ratios of DHTs/EETs, 20-HETE/EETs, 20-HETE/14,15-EET, 20-HETE/8,9-EET, and 14,15-(DHT/EET) were correlated with that of heart or kidney tissues (Table 5.2).

Table 5.2 . Correlations of ArA metabolites and their ratios between plasma vs. the heart and kidney (N=6)

Plasma ArA metabolites	Heart		Kidney	
	r	*p*	r	*p*
14,15-DHT	0.7163	0.109	-0.5371	0.272
11,12-DHT	0.0685	0.896	-0.2308	0.660
8,9-DHT	0.7552	0.083	0.1971	0.708
5,6-DHT	0.1185	0.823	-0.3404	0.509
T-DHT	0.6997	0.122	-0.3845	0.452
20-HETE	0.9133	0.011	-0.9440	0.005
18-HETE	0.5391	0.270	-0.4434	0.379
15-HETE	0.4433	0.379	-0.7997	0.056
12-HETE	0.2290	0.663	-0.1626	0.758
14,15-EET	0.8043	0.058	-0.1790	0.734
11,12-EET	0.3301	0.523	-0.4084	0.421
8,9-EET	-0.4219	0.405	-0.8519	0.067
T-EET	0.1352	0.799	-0.6692	0.146
DHTs+EETs	0.6411	0.170	-0.4093	0.420
DHTs/EETs	0.8290	0.041	-0.4315	0.393
20-HETE/EETs	0.7830	0.066	-0.8546	0.030
20-HETE/DHTs	0.1916	0.716	-0.5328	0.277
20-HETE/EETs+DHTs	0.3596	0.484	-0.5604	0.247
20-HETE/14,15-EET	0.9765	0.001	-0.7551	0.083
20-HETE/11,12-EET	0.7678	0.075	-0.7379	0.094
20-HETE/8,9-EET	0.0959	0. 857	-0.8550	0.030
15-HETE/EETs	0.2166	0.680	-0.7710	0.073
15-HETE/DHTs	0.1601	0.762	-0.5126	0.299
15-HETE/EETs+DHTs	0.1419	0.786	-0.4636	0.354
12-HETE/EETs	0.2264	0.666	-0.2237	0.670
12-HETE/DHTs	-0.5172	0.293	-0.3791	0.459
12-HETE/EETs+DHTs	-0.4549	0.365	-0.3909	0.444
14,15 (DHT/ EET)	0.7572	0.081	-0.8267	0.043

r; correlation coefficient, *p*; *p*-value. Significant correlations ($p < 0.05$)

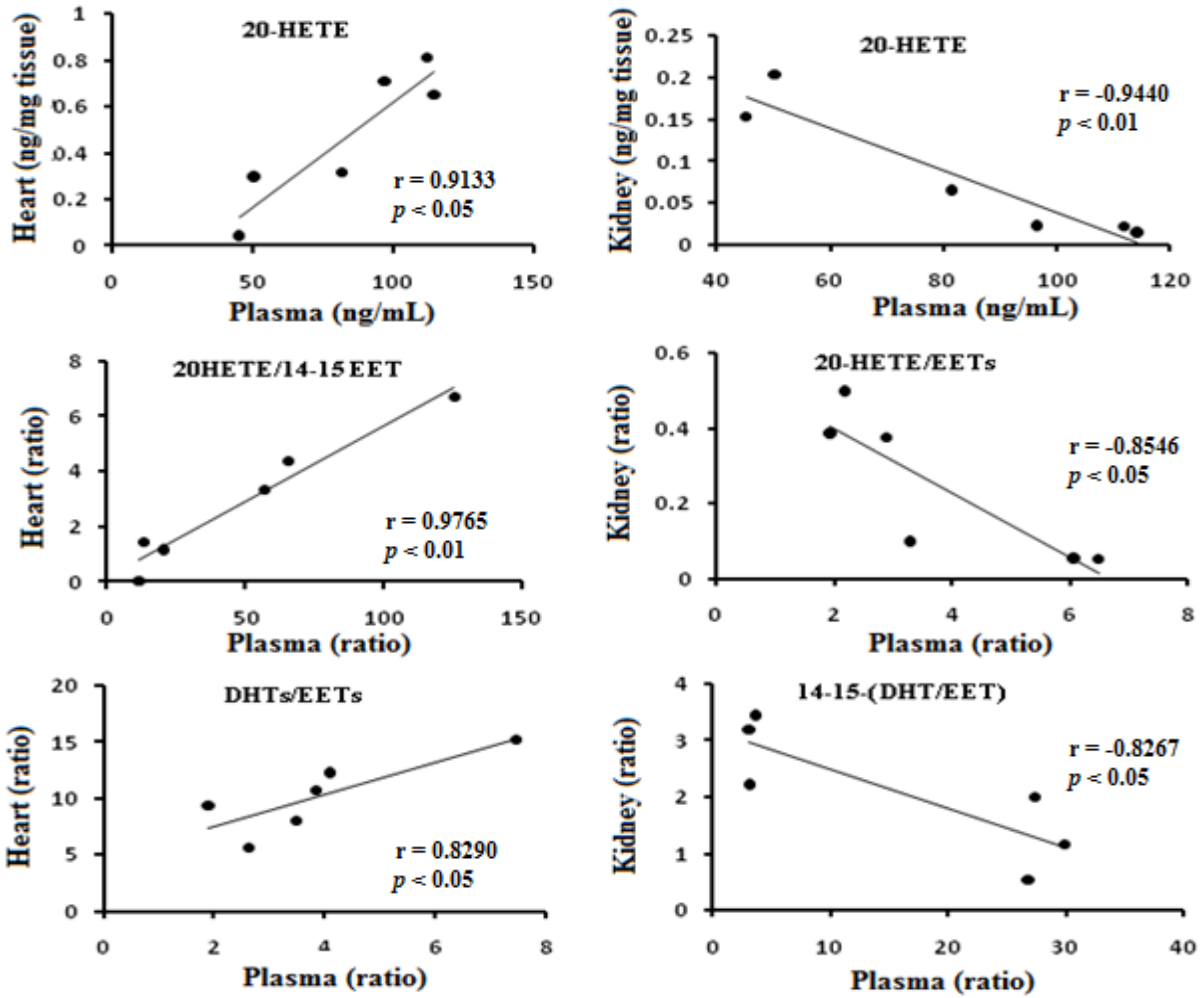

Figure 5.13. Correlation between the plasma (ng/mL) and heart and kidney concentration (ng/mg tissue) of representative ArA metabolites. The relationship between two parameters was analyzed using the Pearson correlation**.**

5.6 The correlation between ArA metabolites concentration and RAS components

5.6.1 Plasma ArA metabolites vs. tissue Ang peptides

5.6.1.1 Plasma Ang peptides

We found correlations between some of plasma ArA metabolites and plasma Ang peptides (Table 5.3, Figure 5-14). 20-HETE (r = 0.8410, $p < 0.05$) and total DHT (r = 0.8411, $p < 0.05$) were positively correlated with Ang II. On the other hand, there was a negative correlation between 14,15-EET and Ang II (r = -0.8791, $p < 0.05$). As an indication of sEH activity, the ratio of DHTs/EETs was not significantly associated with Ang II. However, the ratio of individual 14,15-(DHT/EET) was correlated with Ang II (r = 0.9692, $p < 0.01$). We observed a negative correlation between plasma 14,15-EET (r = -0.8052, $p < 0.05$) and Ang 1-7. At the same time, positive correlations for 15-HETE (r =0.9117, $p < 0.05$), 15-HETE/EETs (r = 0.9380, $p < 0.01$), and 15-HETE/EETs+DHTs (r = 0.8539, $p < 0.05$) with Ang 1-7 were achieved. The only significant correlation for Ang 1-7/II was observed with ratio of 15-HETE/DHTs (r = 0.9009, $p < 0.05$) and 15-HETE/EEts+DHTs (r = 0.8655, $p < 0.05$).

5.6.1.2 Heart Ang peptides

Correlations between plasma ArA metabolites and the heart Ang peptides are presented in Table 5.3, Figure 5-14. The heart Ang II was correlated with plasma 14,15-EET (r = -0.8721, $p < 0.05$),15-HETE/EETs (r = 0.8852, $p < 0.05$), and 14,15-(DHTs/EETs) (r = 0.8187, $p < 0.05$). In terms of the heart Ang 1-7, was not associated plasma ArA metabolites (Table 5.3) nevertheless, correlations were observed only with plasma 14,15-EET (r = 0.8981, $p < 0.05$) and the ratio of

14,15-(DHTs/EETs) (r = -0.8931, $p < 0.05$). The plasma 14,15-DHT, T-DHTs, 8,9-EET,15-HETE/EETs and 12-HETE/DHTs were not associated with Ang 1-7/II ($p < 0.1$), however, correlations were observed between plasma 14,15-EET (r = 0.8992, $p < 0.05$) and 14,15-(DHTs/EETs) (r = -0.8866, $p < 0.05$) with the heart Ang 1-7/II.

5.6.1.3 Kidney Ang peptides

The kidney tissue concentration of Ang II was (r > 0.82, $p < 0.05$) correlated with plasma 18-HETE, 14,15-EET, DHTs/EETs, 20-HETE/EETs, and 14,15-(DHT/EET). The kidney Ang 1-7 concentration was associated with 14,15-DHT (r = 0.9412, $p < 0.01$) and consequently, the ratio of the kidney Ang 1-7/II significantly (r = 0.8297, $p < 0.05$) correlated with total EETs, while was no association between Ang 1-7/II (r > 0.74, $0.05 < p < .1$) and 8-HETE, 14,15-EET, 8,9-EET, 20-HETE/EETs, and 15-HETE/EETs (Table 5.3, Figure 5-14).

Table 5.3 Correlations of plasma ArA metabolites and their ratio vs. the plasma, heart and kidney angiotensin peptides (N=6)

Plasma ArA metabolites	Plasma						Heart						Kidney					
	Ang II		Ang 1-7		1-7 / II		Ang II		Ang 1-7		1-7 / II		Ang II		Ang 1-7		1-7 / II	
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
14,15-DHT	0.9693	0.001	0.4685	0.349	-0.2835	0.586	0.5877	0.220	-0.7675	0.0745	-0.7495	0.086	0.6532	0.156	0.9412	0.005	-0.4857	0.329
11,12-DHT	0.0544	0.9185	-0.4330	0.391	-0.6117	0.197	0.1758	0.739	-0.1763	0.738	-0.3165	0.541	-0.1218	0.818	-0.1243	0.815	-0.3994	0.433
8,9-DHT	0.4502	0.370	-0.1171	0.825	-0.4671	0.350	-0.0146	0.978	-0.0498	0.925	-0.1127	0.832	0.0888	0.867	0.7100	0.114	0.0276	0.959
5,6-DHT	0.5497	0.259	0.6574	0.156	0.2841	0.585	0.3074	0.553	-0.5561	0.252	-0.3131	0.546	0.3599	0.484	0.3713	0.469	-0.1378	0.795
T-DHT	0.8411	0.036	0.1428	0.787	-0.576	0.231	0.5173	0.293	-0.6888	0.130	-0.7276	0.101	0.6241	0.185	0.7546	0.083	-0.5441	0.264
20-HETE	0.8410	0.036	0.6583	0.155	0.0290	0.957	0.5740	0.234	-0.7794	0.068	-0.6237	0.186	0.6935	0.127	0.5896	0.218	-0.4881	0.326
18-HETE	0.9174	0.010	0.5904	0.217	-0.0793	0.881	0.7176	0.108	-0.6743	0.142	-0.7037	0.119	0.8943	0.016	0.7267	0.102	-0.7385	0.094
15-HETE	0.5515	0.257	0.9117	0.011	0.5849	0.223	0.7093	0.115	-0.6591	0.155	-0.6716	0.144	0.5002	0.313	0.5201	0.290	-0.4347	0.389
12-HETE	0.2772	0.595	-0.1128	0.832	-0.2804	0.591	-0.1252	0.813	0.2417	0.645	0.2106	0.689	0.1868	0.723	0.3692	0.471	-0.0266	0.960
14,15-EET	-0.8791	0.021	-0.8052	0.049	-0.1619	0.759	-0.8721	0.024	0.8981	0.015	0.8992	0.015	-0.8594	0.028	-0.6548	0.158	0.7574	0.081
11,12-EET	-0.0495	0.926	0.0626	0.906	0.0853	0.872	-0.2590	0.620	-0.131	0.805	0.0537	0.919	-0.5113	0.300	0.1869	0.723	0.5719	0.236
8,9-EET	-0.4413	0.381	-0.2371	0.651	0.1051	0.843	-0.7234	0.104	0.4148	0.414	0.7337	0.097	-0.6357	0.175	-0.4219	0.405	0.7724	0.072
T-EET	-0.3664	0.475	-0.2166	0.680	0.0431	0.936	-0.5884	0.219	0.2139	0.684	0.4230	0.403	-0.7854	0.064	-0.0974	0.854	0.8297	0.041
DHTs + EETs	0.8277	0.042	0.1118	0.833	-0.6055	0.203	0.4418	0.380	-0.6938	0.126	-0.6964	0.124	0.5190	0.292	0.7854	0.064	-0.4256	0.400
DHTs / EETs	0.7739	0.071	0.1703	0.747	-0.4628	0.355	0.5618	0.246	-0.5400	0.269	-0.6313	0.179	0.7745	0.071	0.5963	0.212	-0.7027	0.120
20-HETE/EETs	0.8017	0.055	0.5779	0.230	-0.0146	0.978	0.6799	0.137	-0.6644	0.150	-0.6403	0.171	0.8734	0.023	0.4967	0.316	-0.7381	0.098
20-HETE/14,15-EET	0.6764	0.140	0.7992	0.056	0.3252	0.529	0.8069	0.052	-0.7652	0.076	-0.7474	0.088	0.8136	0.049	0.3652	0.477	-0.7372	0.095
20-HETE/11,12-EET	0.7647	0.077	0.5184	0.292	-0.0487	0.927	0.6102	0.198	-0.6036	0.205	-0.5651	0.243	0.8354	0.038	0.4572	0.362	-0.6847	0.134
20-HETE/8,9-EET	0.9257	0.008	0.4695	0.348	-0.2474	0.636	0.7609	0.079	-0.7423	0.091	-0.8196	0.046	0.8989	0.016	0.7425	0.091	-0.7982	0.057
20-HETE/DHTs	0.0603	0.910	0.4224	0.404	0.4348	0.389	-0.209	0.969	-0.1358	0.798	0.1229	0.817	0.0485	0.927	-0.1360	0.797	0.1136	0.830
20-HETE/EETs+DHTs	0.3013	0.562	0.5875	0.220	0.4150	0.413	0.25845	0.621	-0.3668	0.475	-0.158	0.765	0.3396	0.510	0.0324	0.952	-0.1793	0.734
15-HETE/EETs	0.6193	0.190	0.9380	0.006	0.5651	0.243	0.8852	0.019	-0.6652	0.149	-0.7488	0.087	0.7997	0.056	0.4300	0.395	-0.7547	0.083
15-HETE/DHTs	0.0236	0.965	0.7709	0.073	0.9009	0.014	0.3648	0.477	-0.1467	0.782	-0.1550	0.769	0.1631	0.756	0.0253	0.962	-0.1386	0.793
15-HETE/EETs+DHTs	0.1682	0.750	0.8539	0.031	0.8655	0.026	0.5331	0.276	-0.3016	0.561	-0.3382	0.512	0.3321	0.520	0.1223	0.818	-0.3146	0.544
12-HETE/EETs	0.4381	0.385	-0.0288	0.957	-0.3324	0.520	0.0827	0.876	0.0586	0.912	-0.0149	0.978	0.4005	0.431	0.4596	0.359	-0.2594	0.620
12-HETE/DHTs	-0.3054	0.556	-0.2547	0.626	0.0637	0.905	-0.5666	0.241	0.709	0.114	0.7482	0.087	-0.3452	0.503	-0.1150	0.828	0.4697	0.347
12-HETE/EETs+DHTs	-0.1459	0.783	-0.2112	0.688	-0.0158	0.976	-0.4339	0.390	0.6181	0.191	0.6134	0.195	-0.1744	0.741	0.0284	0.957	0.3072	0.554
14,15-(DHT/EET)	0.9692	0.001	0.7330	0.098	0.0111	0.983	0.8187	0.046	-0.8931	0.017	-0.8866	0.019	0.8484	0.033	0.7971	0.058	-0.7106	0.114

r; correlation coefficient, *p*; *p*-value, significant correlations ($p < 0.05$).

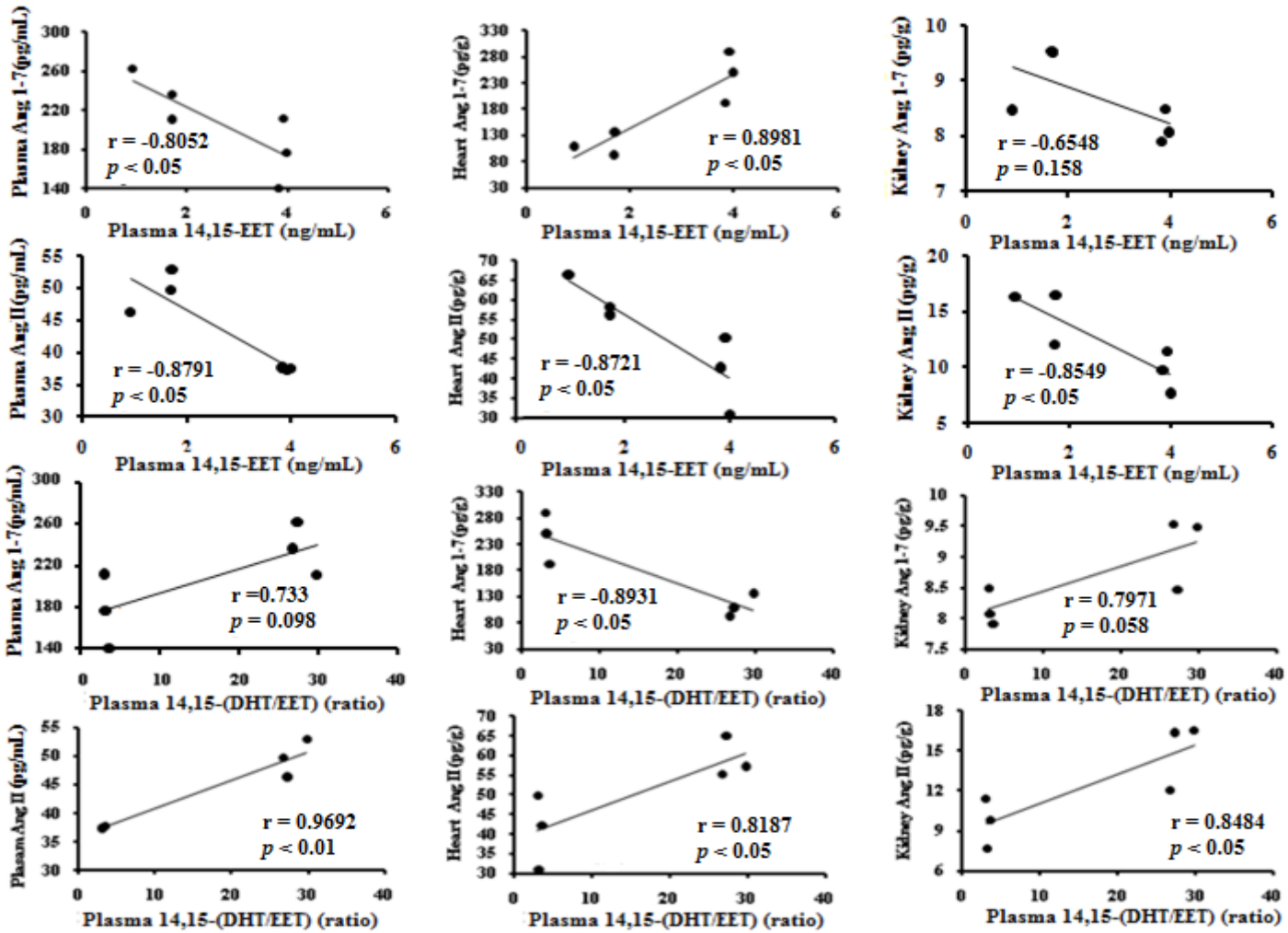

Figure 5.14. Correlation between the plasma representative ArA metabolites and the plasma, heart and kidney Ang peptides concentration. The relationship between two parameters was analyzed using the Pearson correlation.

5.6.2 Plasma ArA metabolites vs. the heart RAS components

The correlations between plasma ArA metabolites and the heart RAS enzyme and receptor expression are presented in Table 5.4 and Figure 5.15. Some major ArA metabolites were positively or negatively correlated with different RAS components. For instance, plasma 20-HETE was negatively correlated with ACE2 (r = -0.7978, p = 0.057), Mas/AT1R (r = -0.8617, $p < 0.05$) and Mas/AT2R (r = -0.8816, $p < 0.05$) and positively correlated with AT1R (r = 0.9354 $p < 0.01$) and AT2R (r = 0.9196, $p < 0.01$) receptor expression in the heart. The same correlation pattern was observed for 18-HETE vs. ACE2 (r = -0.8681, $p < 0.05$, Mas/AT1R (r = -0.8860, $p < 0.05$) and Mas/AT2R (r = -0.8581, $p < 0.05$), AT1R (r = 0.9659, $p < 0.05$), and AT2R (r = 0.9040, $p < 0.05$). Plasma 14,15-DHT was correlated with all except ACE protein expression in the heart. From the family of EETs only 14,15-EET was negatively correlated with AT1R (r = -0.9188, $p < 0.05$) and AT2R (r = -0.8408, $p < 0.05$) and positively with ACE2 (r = 0.9469, $p < 0.01$), Mas/AT1R (r = 0.9165, $p < 0.01$), and Mas/AT2R (r = 0.8720, $p < 0.05$). Plasma 20-HETE/EETs was significantly correlated with AT1R (r = 0.9434 $p < 0.05$) and AT2R(r = 0.8242, $p < 0.05$) receptor expression. There were significant associations between 14,15- (DHT/EET) vs. ACE2 (r = - 0.9830, $p < 0.001$), AT1R (r = 0.9634, $p < 0.01$) , AT2R (r = 0.9355, $p < 0.01$) , Mas (r = -0.8439, $p < 0.05$), Mas/AT1R (r = -0.9723, $p < 0.01$), and Mas/AT2R (r = -0.9472, $p < 0.01$) (Table 5.4, Figure 5.15).

Table 5.4 Correlations of plasma ArA metabolites and their ratio vs. the heart RAS components and their ratio (N=6)

Plasma ArA metabolites	ACE		ACE2		ACE2/ACE		AT1R		AT2		AT2R/AT1R		Mas		Mas/AT1R		Mas/AT2	
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
14,15-DHT	-0.3536	0.492	-0.8931	0.017	*-0.7481*	*0.087*	0.8548	0.030	0.9371	0.006	*0.7341*	*0.097*	-0.8477	0.033	-0.9065	0.013	-0.9214	0.009
11,12-DHT	-0.3551	0.490	-0.0539	0.919	0.2923	0.574	0.0646	0.903	-0.0731	0.891	-0.3540	0.491	0.2901	0.577	0.1560	0.768	0.2349	0.654
8,9-DHT	0.3264	0.528	-0.2890	0.579	-0.6062	0.202	0.2227	0.672	0.4100	0.420	0.5114	0.300	-0.4580	0.361	-0.3436	0.505	-0.3914	0.443
5,6-DHT	-0.2839	0.586	-0.5252	0.285	-0.4175	0.410	0.6573	0.156	0.6903	0.129	0.4932	0.320	-0.5231	0.287	-0.6582	0.155	-0.7154	0.110
T-DHT	-0.3919	0.442	*-0.7510*	*0.085*	-0.5239	0.286	*0.7400*	*0.093*	*0.7652*	*0.076*	0.4719	0.345	-0.5716	0.236	-0.6793	0.138	-0.6661	0.149
20-HETE	-0.4331	0.391	*-0.7978*	*0.057*	-0.5878	0.220	0.9354	0.006	0.9169	0.010	0.4828	0.332	-0.6580	0.155	-0.8617	0.027	-0.8816	0.020
18-HETE	-0.2649	0.612	-0.8681	0.025	*-0.7879*	*0.063*	0.9659	0.002	0.9040	0.013	0.3155	0.542	-0.7101	0.114	-0.8860	0.019	-0.8581	0.029
15-HETE	-0.5574	0.251	-0.7103	0.114	-0.3896	0.445	0.5174	0.293	0.5083	0.303	0.5484	0.260	*-0.7608*	*0.079*	-0.7203	0.106	-0.6891	0.130
12-HETE	0.6708	0.145	-0.0767	0.885	-0.6514	0.161	0.2664	0.610	0.3323	0.520	0.0017	0.997	-0.1740	0.742	-0.2064	0.695	-0.2476	0.636
14,15-EET	0.6649	0.150	0.9469	0.004	0.5530	0.255	-0.9188	0.010	-0.8408	0.036	-0.4512	0.369	*0.7731*	*0.071*	0.9165	0.010	0.8720	0.024
11,12-EET	-0.1726	0.744	0.0365	0.945	0.1498	0.777	-0.2411	0.645	-0.0046	0.993	0.7210	0.106	-0.2278	0.664	-0.0282	0.958	-0.1205	0.820
8,9-EET	0.4269	0.399	0.5567	0.251	0.2484	0.635	-0.3369	0.514	-0.2143	0.684	0.0019	0.997	0.3838	0.453	0.3680	0.473	0.2433	0.642
T-EET	0.1133	0.831	0.3935	0.440	0.3268	0.527	-0.5241	0.286	-0.2803	0.591	0.4845	0.330	0.0905	0.865	0.1876	0.722	0.1562	0.768
DHTs + EETs	-0.3963	0.437	-0.7267	0.102	-0.4973	0.316	0.6908	0.129	*0.7629*	*0.078*	0.5923	0.215	-0.5919	0.216	-0.6678	0.147	-0.6745	0.142
DHTs / EETs	-0.2295	0.666	-0.6858	0.133	-0.5804	0.227	*0.7831*	*0.066*	*0.7100*	*0.066*	0.1285	0.808	-0.4423	0.380	-0.6244	0.185	-0.5817	0.226
20-HETE/EETs	-0.3414	0.508	*-0.7708*	*0.073*	-0.6114	0.197	0.9434	0.005	0.8242	0.044	0.1424	0.788	-0.5346	0.275	*-0.7829*	*0.066*	*-0.7507*	*0.086*
20-HETE/14,15-EET	-0.6270	0.183	*-0.7746*	*0.071*	-0.3857	0.450	0.8190	0.046	0.6704	0.145	0.1812	0.731	-0.5561	0.252	*-0.7498*	*0.086*	-0.6934	0.127
20-HETE/11,12-EET	-0.2690	0.606	-0.7127	0.112	-0.6047	0.204	0.9191	0.010	*0.8033*	*0.054*	0.1010	0.849	-0.4790	0.336	*-0.7376*	*0.094*	-0.7131	0.112
20-HETE/8,9-EET	-0.4047	0.426	-0.8906	0.017	-0.6823	0.135	0.9256	0.008	0.8580	0.029	0.3217	0.534	-0.6775	0.139	-0.8409	0.036	*-0.7944*	*0.059*
20-HETE/DHTs	-0.0383	0.943	-0.0534	0.920	-0.0742	0.889	0.2636	0.614	0.2450	0.640	0.0696	0.896	-0.0646	0.903	-0.2029	0.700	-0.2595	0.620
20-HETE/ET+DT	-0.2217	0.673	-0.3160	0.542	-0.2185	0.678	0.5147	0.296	0.4470	0.374	0.1038	0.845	-0.2398	0.647	-0.4272	0.398	-0.4530	0.367
15-HETE/EETs	-0.5416	0.267	*-0.7782*	*0.068*	-0.4652	0.353	0.7011	0.121	0.5627	0.245	0.2062	0.695	-0.6722	0.144	*-0.7559*	*0.082*	-0.6782	0.139
15-HETE/DHTs	-0.1863	0.724	-0.2103	0.689	-0.1276	0.810	0.0837	0.875	0.0355	0.947	0.1311	0.804	-0.4557	0.364	-0.2755	0.597	-0.2481	0.636
15-HETE/ET+DTs	-0.3250	0.530	-0.3693	0.471	-0.1904	0.718	0.2306	0.660	0.1531	0.772	0.1578	0.765	-0.4488	0.372	-0.4034	0.428	-0.3568	0.488
12-HETE/EETs	0.4913	0.322	-0.2591	0.620	-0.7035	0.119	0.4377	0.385	0.4557	0.364	-0.0048	0.993	-0.2635	0.614	-0.3416	0.508	-0.3542	0.491
12-HETE/DHTs	0.9454	0.004	0.4636	0.354	-0.2727	0.601	-0.2792	0.592	-0.1821	0.730	-0.2020	0.701	0.2028	0.700	0.2687	0.607	0.1958	0.710
12-HETE/ET+DTs	0.9233	0.009	0.3170	0.540	-0.4180	0.410	-0.1297	0.807	-0.4800	0.928	-0.1814	0.731	0.0934	0.860	0.1373	0.795	0.0785	0.883
14,15-(DHT/EET)	0.5673	0.240	-0.9830	0.000	-0.6763	0.140	0.9634	0.002	0.9355	0.006	0.5645	0.243	-0.8439	0.035	-0.9723	0.001	-0.9472	0.004

+r; correlation coefficient, *p*; *p*-value. Significant correlations are in , ($p < 0.05$)

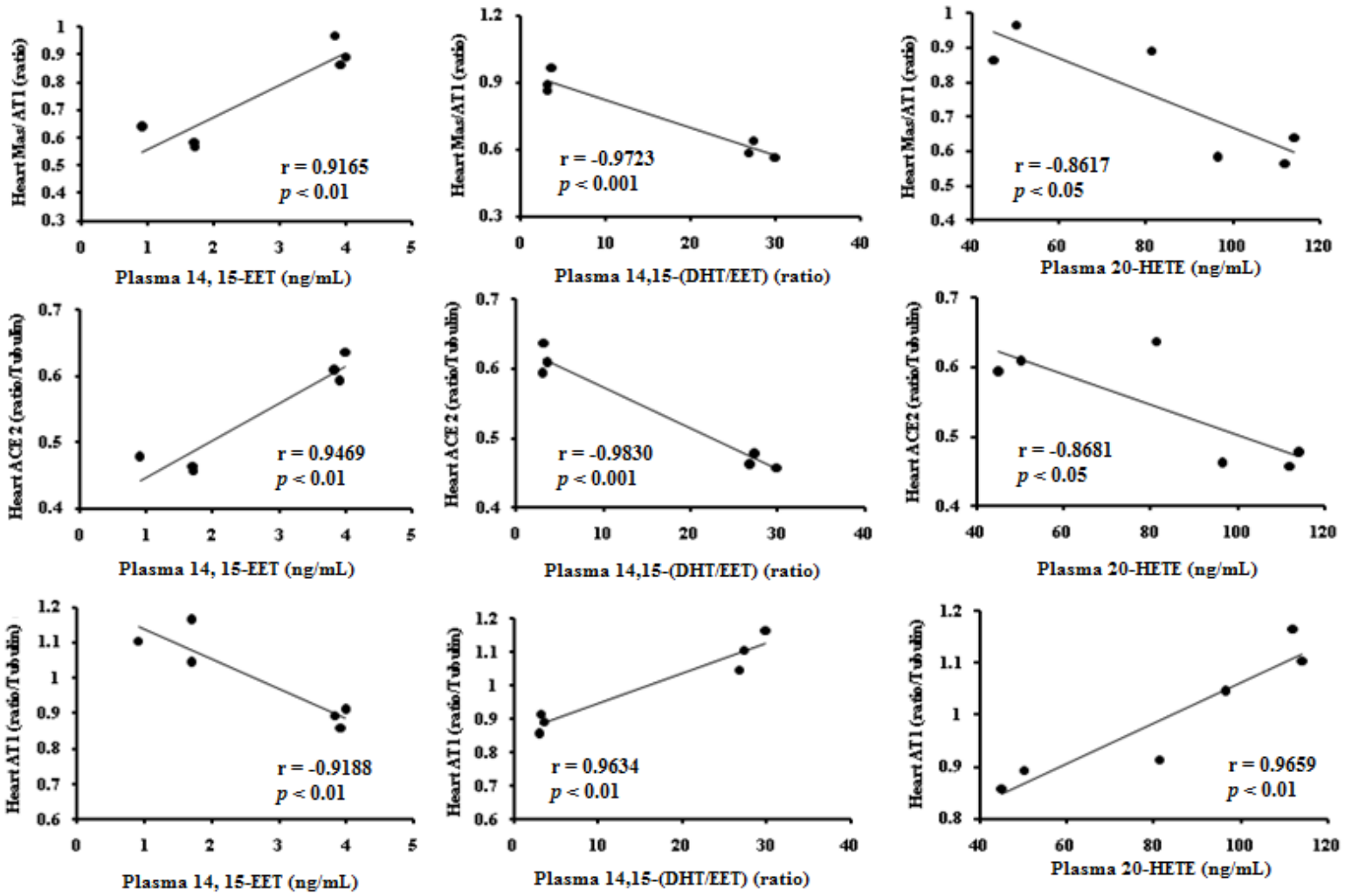

Figure **5.15. Correlation between the plasma representative ArA metabolites and the heart RAS components expression level.**

The relationship between two parameters was analyzed using the Pearson correlation.

5.6.3 Plasma ArA metabolites vs. the kidney RAS components

The correlation between plasma ArA metabolites and the kidney RAS enzyme and receptor expression is presented in Table 5.5 and Figure 5-16. Plasma 20-HETE was negatively correlated with ACE2/ACE (r = -0.8617, $p < 0.05$) and AT2R/AT1R (r = -0.8830, $p < 0.01$); however, it was positively correlated with AT1R (r = 0.8392, $p < 0.05$) receptor expression in the kidney. Plasma 14,15-EET followed an opposite pattern to plasma 20-HETE, as it was positively correlated with the kidney ACE2 (r = 0.9105, $p < 0.05$), ACE2/ACE (r = 0.8946, $p < 0.05$), AT2R/AT1R (r = 0.9240, $p < 0.01$), and Mas/AT1R (r = 0.9412, $p < 0.01$) and negatively correlated with AT1R (r = -0.8908, $p < 0.05$) and Mas (r = -0.8342, $p < 0.05$). Plasma 14,15-DHT was negatively associated with the kidney ACE2/ACE (r =-0.8232, $p < 0.05$), AT2R/AT1R (r = - 0.8649, $p < 0.05$), and Mas/AT1R (r = -0.8383, $p < 0.05$) and positively with AT1R (r = 0.9182, $p < 0.05$) and Mas (r = 0.8650, $p < 0.05$). From the list of different ratios of plasma ArA metabolites, several of them were presented significant associations with the kidney RAS components, namely DHTs/EETs vs. Mas/AT2R (r = 0.8216, $p < 0.05$) and 20-HETE/EETs vs. AT2R/AT1R (r = -0.8968, $p < 0.05$) and 15-HETE/EETs vs. Mas/AT1R (r = -0.8310, $p < 0.05$). The ratio of plasma 14,15-(DHT/EET) was also associated with all but the kidney ACE and AT2R protein expression.

Table 5.5 Correlations of plasma ArA metabolites and their ratio vs. the kidney RAS components and their ratio (N=6)

Plasma ArA metabolites	ACE		ACE2		ACE2/ACE		AT1R		AT2		AT2R/AT1R		Mas		Mas/AT1R		Mas/AT2	
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
14,15-DHT	0.6394	0.172	-0.7289	0.100	-0.8232	0.044	0.9182	0.010	0.2179	0.678	-0.8649	0.026	0.8650	0.026	-0.8383	0.037	0.7001	0.121
11,12-DHT	-0.2749	0.598	-0.2450	0.640	-0.0668	0.900	-0.2003	0.704	-0.6919	0.128	0.0398	0.940	0.0763	0.886	-0.0137	0.980	0.6742	0.142
8,9-DHT	0.3512	0.495	-0.0692	0.896	-0.1283	0.809	0.3710	0.469	0.0768	0.885	-0.2850	0.584	0.3190	0.538	-0.2441	0.641	0.3381	0.512
5,6-DHT	0.6892	0.130	-0.3571	0.487	-0.6599	0.154	0.6684	0.147	0.4319	0.392	-0.6237	0.186	0.4312	0.393	-0.5082	0.303	0.0488	0.927
T-DHT	0.4522	0.368	-0.6727	0.143	-0.6997	0.122	0.6703	0.145	-0.1548	0.774	-0.6975	0.123	*0.7364*	*0.095*	-0.6808	0.137	0.9007	0.014
20-HETE	0.7007	0.121	-0.6309	0.179	-0.8617	0.027	0.8392	0.037	0.1564	0.767	-0.8830	0.020	0.6486	0.164	*-0.7665*	*0.075*	0.5185	0.292
18-HETE	0.4664	0.351	-0.6749	0.141	*-0.7351*	*0.096*	0.8193	0.046	-0.1435	0.786	-0.9642	0.002	0.6295	0.181	0.8601	0.028	0.7850	0.064
15-HETE	0.1992	0.705	*-0.7353*	*0.096*	-0.6330	0.177	*0.7481*	*0.087*	0.6866	0.132	-0.6162	0.193	0.7193	0.107	*-0.7405*	*0.092*	0.0328	0.951
12-HETE	0.2818	0.589	0.2644	0.613	0.1008	0.849	0.1458	0.783	-0.3659	0.476	-0.2922	0.574	-0.1599	0.762	-0.0778	0.884	0.2872	0.581
14,15-EET	-0.4007	0.431	0.9105	0.012	0.8946	0.016	-0.8905	0.017	-0.1989	0.706	0.9240	0.008	-0.8342	0.039	0.9412	0.005	-0.6167	0.192
11,12-EET	0.3402	0.509	-0.0091	0.986	-0.1437	0.786	0.1729	0.743	0.9123	0.011	0.2074	0.693	0.3032	0.559	0.0875	0.869	-0.5335	0.276
8,9-EET	0.3513	0.495	0.6960	0.125	0.2889	0.579	-0.3249	0.530	0.2307	0.660	0.4085	0.421	-0.5267	0.283	0.5769	0.231	-0.6828	0.135
T-EET	0.2313	0.659	0.3667	0.475	0.1817	0.731	-0.1614	0.760	*0.7570*	*0.084*	0.5085	0.303	-0.0670	0.900	0.4385	0.384	*-0.7421*	*0.091*
DHTs + EETs	0.5244	0.286	-0.6483	0.164	-0.7114	0.113	0.6839	0.134	-0.0214	0.968	-0.6485	0.164	*0.7718*	*0.072*	-0.6437	0.168	0.8216	0.045
DHTs / EETs	0.3005	0.563	-0.5635	0.244	-0.5625	0.245	0.5528	0.255	-0.4722	0.344	*-0.7388*	*0.093*	0.5025	0.310	-0.6823	0.160	0.9632	0.002
20-HETE/EETs	0.4361	0.387	-0.6179	0.191	-0.7187	0.108	0.7018	0.120	-0.2412	0.645	-0.8968	0.015	0.5027	0.310	*-0.7669*	*0.075*	*0.7308*	*0.099*
20-HETE/14,15-EET	0.2666	0.610	*-0.7674*	*0.075*	*-0.7559*	*0.082*	0.6982	0.123	0.0739	0.889	*-0.8037*	*0.054*	0.5985	0.210	*-0.7928*	*0.060*	0.4796	0.336
20-HETE/11,12-EET	0.4514	0.369	-0.5385	0.270	-0.6707	0.145	0.6522	0.161	-0.2947	0.571	-0.8629	0.027	0.4297	0.395	-0.7071	0.116	0.7163	0.109
20-HETE/8,9-EET	0.3795	0.458	*-0.7777*	*0.069*	*-0.7594*	*0.080*	*0.7843*	*0.065*	-0.2055	0.696	0.6196	0.186	-0.9124	0.011	-0.8664	0.026	0.9191	0.010
20-HETE/DHTs	0.4118	0.417	0.0709	0.894	-0.2330	0.657	0.1941	0.712	0.2556	0.625	-0.2125	0.686	-0.0739	0.889	-0.0666	0.900	-0.3023	0.560
20-HETE/ET+DT	0.4329	0.391	-0.2003	0.704	-0.4463	0.375	0.3968	0.436	0.1777	0.736	-0.4639	0.354	0.1396	0.792	-0.3306	0.522	-0.0307	0.954
15-HETE/EETs	0.783	0.883	*-0.7854*	*0.064*	-0.6378	0.173	0.7110	0.113	0.2488	0.635	*-0.7720*	*0.072*	0.6125	0.196	-0.8310	0.040	0.3222	0.534
15-HETE/DHTs	-0.1046	0.844	-0.2388	0.649	-0.1306	0.805	0.2826	0.587	0.5093	0.302	-0.2034	0.699	0.1564	0.767	-0.2915	0.575	-0.4576	0.362
15-HETE/ET+DTs	-0.0924	0.862	-0.4153	0.413	-0.2698	0.605	0.4003	0.432	0.5580	0.250	-0.3455	0.502	0.2986	0.565	-0.4476	0.374	-0.2818	0.589
12-HETE/EETs	0.2675	0.608	0.0494	0.926	-0.0571	0.915	0.2644	0.613	-0.4638	0.354	-0.4533	0.367	-0.0026	0.996	-0.2577	0.622	0.5183	0.292
12-HETE/DHTs	0.0636	0.905	*0.7595*	*0.080*	0.5645	0.243	-0.2994	0.564	-0.1482	0.779	0.2294	0.662	-0.6352	0.175	0.4298	0.395	-0.3966	0.436
12-HETE/ET+DTs	0.0888	0.867	0.6396	0.171	0.4721	0.344	-0.1849	0.726	-0.2463	0.638	0.0753	0.887	-0.5309	0.279	0.2848	0.584	-0.1964	0.709
14,15-(DHT/EET)	0.5178	0.293	-0.8855	0.019	-0.9165	0.010	0.9536	0.003	0.1940	0.713	-0.9698	0.001	0.8784	0.021	-0.9608	0.002	0.6919	0.128

+r; correlation coefficient, *p*; *p*-value. Significant correlations are in , ($p < 0.05$)

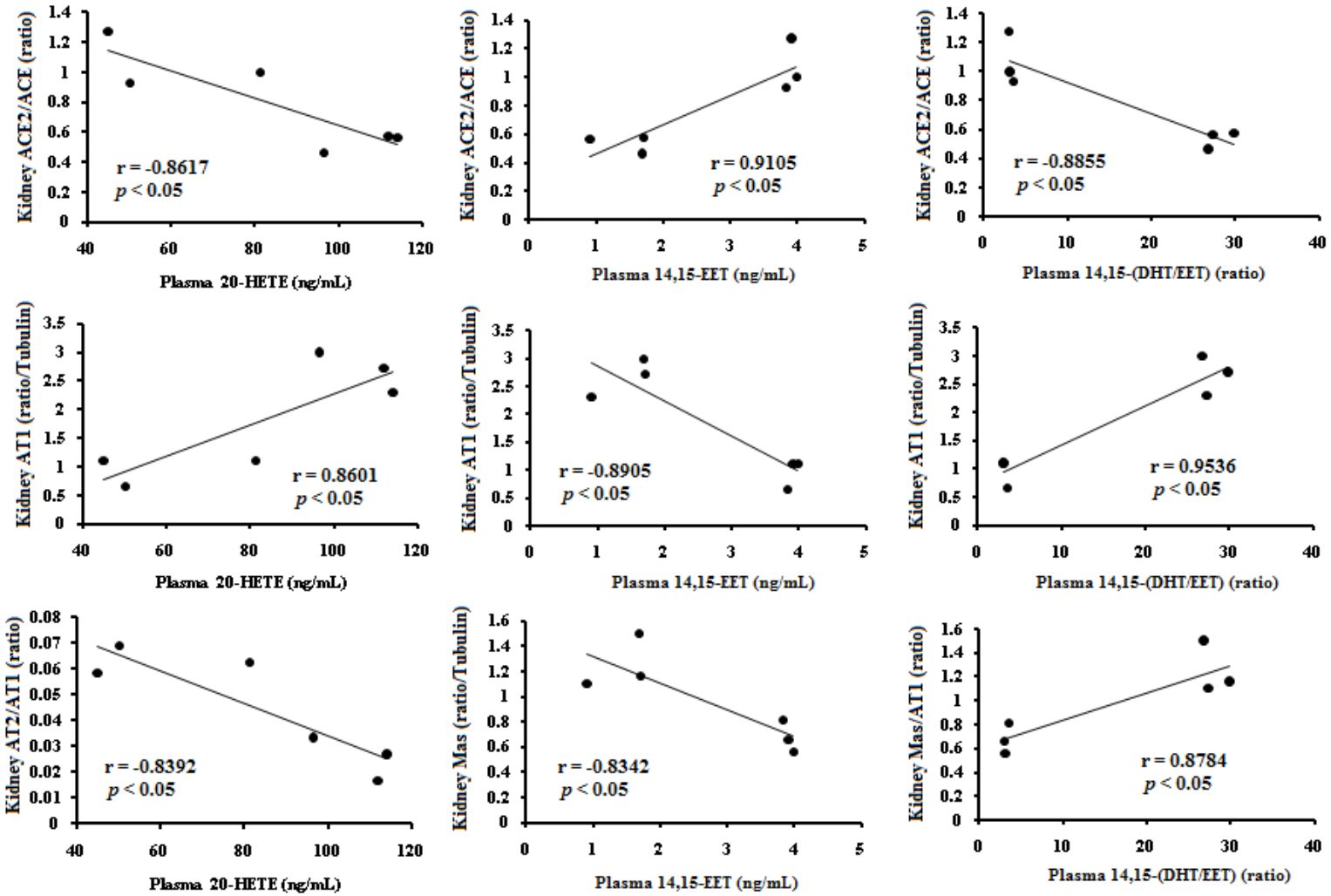

Figure 5.16. Correlation between the plasma representative ArA metabolites and the kidney RAS components expression level. The relationship between two parameters was analyzed using the Pearson correlation.

5.7 Discussion

The present study is the first detailed examination of the effect of inflammation on circulating and tissue levels of ArA metabolites and the RAS components in peptide and receptor levels. The results indicates that: 1) Inflammation alters the ArA and RAS pathways, which consequently changes the balance between anti-inflammatory and proinflammatory ArA metabolites, Ang peptides, and their receptors in adjuvant arthritis (Table 5.1, Figure 5.4 through 5.12). 2) In rats with AA, heart and kidney concentrations of ArA metabolites were associated with plasma concentrations (Table 5.2). 3) Plasma, heart and kidney concentrations of Ang peptides were correlated with plasma concentration of ArA metabolites (Table 5.3). 4) Heart and kidney tissue expression level of the RAS components; i.e. ACE, ACE2, AT1R, AT2R and Mas are associated with plasma concentration of ArA metabolites (Table 5.4 and 5.5).

The present data indicate that the tissue concentrations of key ArA metabolites and Ang peptides, particularly the CV and renal active components, can be predicted from their plasma concentrations. This observation suggests that the use of blood is a useful surrogate biological sample for detecting changes in ArA metabolites and Ang peptides and their receptors concentrations in deep tissues such as heart and kidney. These data were obtained in control rats and ones with arthritis. It is important to note that some of the demonstrated correlations were based on clustered data points which were due to the substantial differences in the value of tested parameters between two groups.

No previous study has examined *in vivo* effect of adjuvant arthritis on ArA metabolites or peptide and reporter level of the RAS. Adjuvant arthritis reduced the ACE2 level and ACE2/ACE ratio in rat heart (20). LPS induced inflammation in rat altered the expression of CYP enzyme and ArA metabolites profiles (32,69). Taking together with the previous studies,

the present study suggest that inflammation has effects on both ArA and RAS pathway which could be translate into changes on blood pressure and cardiac and renal functions which are mainly regulated with these two systems. Assuming that these observations can be extrapolated to humans, we might speculate that concentration profiles of ArA metabolites and Ang peptides from a single blood sample could be used as biomarkers for CV risks in individuals with inflammatory conditions such as RA. Indeed, this hypothesis has to be tested in human clinical trials.

The discovery of ACE2 and Ang 1-7 in the last decade has contributed to the realization that RAS consists of two opposing arms: the pressor and vasoconstrictive arm composed of ACE/Ang II/ and AT1R mediating the proliferative, profibrotic, and prothrombotic actions of Ang II; and the vasorelaxant arm comprised of ACE2/Ang 1-7 and Mas receptor that expresses opposing effects of the other arm by vasodilation, antiproliferation, and antithrombotic actions of Ang 1-7. The modulation of these two arms of RAS is essential for maintaining the cardioprotective-cardiotoxic balance and consequently the CV homeostasis (324). ACE and ACE2 in the RAS are important as they are involved in the metabolism of Ang to two physiologically important peptides, the proinflammatory/cardiotoxic, Ang II and the vasodilator/cardioprotective, Ang 1-7, respectively (24). A change in the concentration of these enzymes *per se*, as previously reported by us (20), however, does not necessarily mean a corresponding alteration on the concentration of their physiologically active products, thereby, a physiological outcome may stays same, because the concentration of product may remain unaffected. The present data (Figure 5.4 and 5.5) confirms the previous observation (20) and suggest, for the first time, that inflammation, indeed, causes significant increases in the Ang II concentrations in all of the examined tissues with corresponding significant decreases in the

cardiac Ang 1-7 (Figure 5.6 through 5.8); i.e., a potential CV risk. This is consistent with the well-known effect of inflammatory conditions on the cardiac function (76,77). Inflammation was not able to alter the expression of ACE at the heart and kidney tissue concentrations (Figure 5.4) (20); however the product of ACE, i.e., Ang II, was elevated in the plasma as well as in the heart and kidney tissues (Figure 5.6). This may contribute to the activity of Chymase as an ACE independent pathway for production of Ang II. Chymase was not detectable in the rat heart. However, its concentration was elevated in the kidney tissue due to inflammation (data not shown). On the other hand, inflammation reduced the ACE2 protein expression in the heart and kidney tissues and consequently lowered the ACE2/ACE ratio as well (Figure 5.4) (20). Therefore, the heart concentration of Ang 1-7 was reduced as was expected from the down regulation of ACE2 protein (Table 5.3). However, Ang 1-7 concentration was not different in the plasma and kidney between INF and CL groups. The similar concentration of Ang 1-7 in animals with AA, despite the low expression of ACE2 enzyme in their tissues, could be partly due to the involvement of other pathways in Ang 1-7 production also influenced by inflammation. For instance, as an ACE independent producer of Ang1-7, neutral endopeptidase protein expression was down-regulated in the kidney but not affected in the heart due to inflammation (data not shown). The overall outcome of these changes translates to lower concentration of vasorelaxant and cardioprotective peptide; i.e., Ang1-7 in the body.

In addition, in this study we report another novel observation that inflammation also alters the target protein concentrations of three physiologically important receptors; i.e., AT1R, AT2R and Mas (Figure 5.9-5.12). The Mas receptor mediates the anti-inflammatory effects of Ang 1-7 and counteracts Ang II proinflammatory actions imposed through the AT1R receptor. Activation of Mas by binding to Ang 1-7 has an inhibitory effect on the downstream cascade of AT1R receptor

(325). Based on our observation, Mas receptor expression was tissue-dependent as it was reduced in the heart and increased in the kidney tissues (Figure 5.11 and 5.12). This is accompanied by increased concentrations of cardiac and renal AT1R and AT2R expressions (Figure 5.9 and 5.10). These changes are also reflected in the protective/toxic ratios of the measure metrics (Figure 5.9-5.12). AT2R is mainly expressed in fetal tissues and hardly measurable in the adult heart (326). Ang II by binding to AT2R exerts counter-regulatory actions to AT1R. The expression of AT2R is increased in inflammatory conditions such as hypertension (327). The results of the current study indicate that a reduced expression of ACE2 and consequently higher Ang II and lower Ang 1-7 due to inflammation disrupt the cardioprotective-cardiotoxic balance of RAS. Additionally, over-expression of AT1R in the heart and kidney tissues along with increased Ang II in the plasma, heart, and kidney could shift the balance even more toward cardiotoxicity. Consistent with previous reports (118,325,327,328), we observed that Ang II, AT1R, and AT2R expression increases in both the heart and kidney of animals with AA. The increased expression of AT2R could be considered as the body's compensatory mechanism in order to modulate inflammation. Supporting this hypothesis, the stimulation of AT2R using agonist have induced vasodilation and inhibited inflammation, proliferation and remodeling (329,330). Altogether, the altered levels of ACE, ACE2, AT1R, AT2R, and Mas (i.e., the RAS pathway) observed here may explain, at least in part, the cardiotoxicity that is caused by inflammation.

The altered RAS pathway was accompanied by an altered ArA metabolism pathway. Among the tested metabolites, a few were affected by inflammation (Table 5.1). The most commonly studied ones were the vasoconstrictor 20-HETE and the vasodilator 14,15-EET. Inflammation significantly influenced 20-HETE concentrations that were reflected in increases in the plasma

and heart but decreases in the kidney (Table 5.1). Inflammation due to AA decreased 14,15-EET concentration which was only significant in plasma. Importantly, however, the 20-HETE/14,15-EET ratio was substantially elevated in the heart and plasma but lowered in the kidney. This observation can be explained by metabolic pathways involved in the production and degradation of 20-HETE. CYP4A as hydroxylase enzymes metabolize ArA to HETEs, with 20-HETE as a major product. The production and physiological action of 20-HETE depends on tissue expression of specific CYP4A isoforms. CYP4A and CYP4F enzymes expressed in renal tubular cells produce 20-HETE that lowers BP by inhibiting sodium reabsorption and inducing natriuresis (33,331). Alternatively, 20-HETE generated by vasculature CYP4A2 promotes vasoconstriction and causes hypertension (331,332). Inflammation induces vascular expression of CYP4A2 and increases 20-HETE production and consequently causes hypertension (333); on the other hand, as it has been reported in Dahl salt sensitive rats, reduced tubular expression of CYP4A and CYP4F and diminished concentration of 20-HETE contributed to the development of hypertension (334). Additionally, lower kidney concentrations of 20-HETE is probably attributed to multiple disposition pathways, mainly COX-2 activity that is enhanced by inflammation in the kidney (39,335,336).

These changes in concentration of ArA metabolites with the known CV effects may provide another explanation for the increased risk of CV events in the presence of inflammatory conditions. It has been previously reported that CV complications are one of the major comorbidities of RA patients, accounting for 35% to 50% of excess mortality in this population (10). The findings of the current study are confirmed by several studies that previously identified HETEs as potent pro-inflammatory agents and considered them as indices of inflammation (63-68). *In vitro* studies using an acute inflammatory model of LPS in mice and rat have reported similar

results (32,69). We observed in the kidney tissues, however, the concentration of all HETEs homologs significantly reduced by inflammation (Table 5.1). 20-HETE as a key eicosanoid in the kidney plays a dual role as pro- and anti-hypertensive agents in vasculature and tubules, respectively. As mentioned above, its production and physiological action in kidney tubules differs from vasculature. It is involved in tubulo-glomerular feedback (TGF) response (337), which is one of several mechanisms that the kidney uses to regulate glomerular filtration rate. It has been reported that the TGF response has been intensified by perfusion of loop of Henle with exogenous ArA, whereas it was blocked by inhibition of 20-HETE production through CYP inhibition (338). On the other hand, in a proximal tubule, 20-HETE reduces sodium transport, while in a thick ascending loop of Henle (TALH) it limits the availability of K^+ for transport by a Na^+-K^+-$2Cl^-$ transporter, which enhances the natriuresis and acts as an anti-hypertensive and tissue protective agent (339,339-341). The decreased concentration of 20-HETE in the kidneys of animals with AA could be an indication of lower natriuresis and an antihypertensive effect. The role of the kidney on long term arterial BP homeostasis is based on the concept of the pressure-natriuretic response and hypertension develops when this response is compromised (342). It has been shown that 20-HETE modulates this response by inhibition of Na+ transport in renal tubule (339,341). In support of our results, there are several reports that indicate a deficiency of 20-HETE production in the kidney results in the development of salt sensitive hypertension (343-345). In a review article, Williams et al. have exclusively provided lines of evidence in support of the hypothesis that lower renal production of 20-HETE contributes to the elevation of Cl^- transport in the TALH and development of hypertension (62).

On the other hand, EETs have been considered as anti-inflammatory mediators (53,346). Inflammation did not alter the plasma, heart, or kidney concentrations of EETs between INF and

CL groups but the ratio of plasma 20-HETE/EETs was significantly increased (Table 5.1). In the body, as a dynamic system, the elevation of plasma and heart 20-HETE concentrations without change in EETs concentrations implies that the balance between pro- and anti-inflammatory ArA metabolites is skewed toward pro-inflammatory mediators in animals with arthritis. Inflammation upregulates the sEH enzyme (347), therefore, the DHTs concentration as sEH-derived metabolites of EETs increases. We observed an elevation of the heart's DHTs concentration in the INF group, but it was not significant. As a result of this alteration, in rats with arthritis the heart ratio of DHTs/EETs was higher when compared with control rats (Table 5.1). The higher DHTs concentrations in the INF group with comparable EETs concentrations between groups suggest that indeed the EETs concentration was elevated as a compensatory mechanism, but due to overexpression of sEH enzyme in response to inflammation, EETs were metabolized to produce higher concentrations of DHTs with lower anti-inflammatory and vasodilatory effects compared with EETs. Therefore, the higher ratios of the plasma and the heart 20-HETE/EETs and DHTs/EETs, as an indication of domination of pro-inflammatory mediators, at least in part, may be considered as an underlying cause for endothelial dysfunction and CV disease incidence in inflammatory situations such as RA.

The present data suggest that inflammation influences both the RAS pathway and the ArA metabolism and possibly increased the CV risks. It is therefore reasonable to assume a correlation between the two physiological processes. This was, indeed, confirmed when we plotted the two metrics against each other using various options. Our attempt to test the possibility of such an association revealed that there are correlations between key elements of the two systems (Table 5.5).

There are several reports in the literature regarding contribution of Ang II to production of 20-HETE (348) and its actions on endothelial dysfunction, essential hypertension (349) and renal disorders (350-358). It has been shown that higher plasma concentrations of 20-HETE were correlated with augmented plasma renin activity in hypertensive humans (335), whereas the production of 20-HETE in isolated glomeruli was reduced due to low expression of CYP4A in high-salt diet–fed rats in which the RAS is suppressed (359). In agreement with these reports, our results present a positive ($r = 0.8410$) correlation between plasma 20-HETE vs. plasma Ang II concentrations. Additionally, plasma 20-HETE was correlated with the heart RAS components such as ACE2 negatively, and AT1R and AT2R expression positively. ACE is responsible for enzymatic production of Ang II; therefore, despite no change in ACE expression, the higher concentrations of Ang II in animals with AA could be attributed to lower levels of ACE2. Sodhi et al. (323) recently reported that 20-HETE induces ACE and AT1R expression and increases Ang II concentrations. Using a transgenic rat over-expressing CYP4A2 producing higher concentrations of 20-HETE, the authors concluded that 20-HETE promotes hypertension through activation of RAS, which is in line with our observation in the current study. The cause and effect relationship between 20-HETE and Ang II is not clear. The elevated BP in transgenic rat was brought back to a normal level as a result of treatments with ACE inhibitor, AT1R blocker, and inhibitor of 20-HETE synthesis or 20-HETE antagonist. These findings suggest that 20-HETE acts as an endogenous regulator of RAS activation (323). The mechanism of RAS activation by 20-HETE is unknown and needs to be explored further. However, it has been shown that 20-HETE reduces bioavailability of nitric oxide by uncoupling of endothelial nitric oxide synthase which leads to activation of NF-κB. The reported *in-vitro* induction of ACE by 20-HETE and increase of Ang II (323) in endothelial cell is also mediated by NF-κB activation

(360). In a recent study, Cheng et al. (361) have shown that 20-HETE-mediated endothelial dysfunction causes hypertension through activation and induction of ACE and AT1R with unknown mechanism.

Despite positive correlations of the plasma 20-HETE concentration (r = 0.5740-0.6935) vs. the heart and kidney Ang II concentrations (Table 5.4 and 5.5) statistical significance were lacking. The failure to reach a significant level could be attributed to the small sample size of the current study; otherwise these result are in concert with previous studies (350-358), which report a direct association of Ang II in CV and renal pathologic conditions resulting from an excess or lack of production of 20-HETE, respectively.

It has been reported that vasoconstrictor effect of Ang II on rabbit afferent renal artery is mediated by AT1R and AT2R. The effect was eliminated or enhanced by blockade of AT1R or AT2R, respectively (362). Arima et al. found that in norepinephrine preconstricted artery treated with an AT1R antagonist, Ang II caused dose-dependent dilation which was abolished by AT2R blockade and an AT2R agonist caused dose-dependent dilation in the same setting. It was concluded that the activation of AT2R is linked to a vasodilatory mechanism that opposes vasoconstriction induced by AT1R activation. The authors proposed that the selective activation of AT2R may cause endothelium-dependent vasodilation via a CYP pathway, possibly through the activity of EETs on endothelium, as inhibition of EETs synthesis abolished this vasodilation completely (362). In our study we found a strong negative correlation between plasma 14,15-EET and the kidney Ang II and AT1R protein expressions and a positive and strong correlation between plasma11,12-EET and the kidney AT2R receptor protein expression. The opposing sign of AT1R and AT2R correlations with 14,15 and 11,12-EET, respectively, is in concert with the opposing action of these receptors. It has been reported that 11,12- and 14,15-EET are the main

EETs produced in rabbit kidney and rat preglomerular microvessels (363,364), and only 11,12-EET was able to cause significant vasodilation in both isolated perfused rabbit kidney (365) and rat preglomerular microvessels in the juxtamedullary nephron preparation (366).Consistent with these findings, Arima et al. reported that 11,12-EET was able to dilate rabbit norepinephrine preconstricted renal artery (362). Nevertheless, in another study both 11,12-EET, 14,15-EET and their hydrated products (DHTs) presented as vasoactive agents and induced relaxation in bovine adrenal artery (321). On the other hand, based on the current study, the plasma 20-HETE was positively correlated with kidney Ang II and AT1R receptor protein expression, while no correlation was observed with the kidney AT2R receptor protein expression. Taken together, these results indicate that an inflammatory condition with higher concentrations of plasma Ang II, 20-HETE and higher expressions of AT1R and at the same time, lower EETs concentration can mediate vasoconstriction and cause hypertension. Negative correlation between plasma14,15-EET and kidney AT1R and positive association between plasma 11,12-EET and kidney AT2R protein expression suggest a compensatory mechanism for BP homeostasis by the kidney.

EETs as cardioprotective ArA metabolites counterbalance the deleterious actions of 20-HETE in the CV system. However, in the kidney 20-HETE as a natriuretic agent and EETs with vasodilator effect may act in line for tissue protection purposes. We observed negative correlations between plasma EETs vs. plasma, heart, and kidney Ang II (Table 5.3). Although these correlations were not significant for all members of EETs, plasma14,15-EET was significantly correlated with plasma and tissue Ang II. The ratio of plasma 20-HETE/EETs as a critical measure of cardiotoxic-cardioprotective balance was positively correlated with plasma and kidney Ang II. These findings suggest that plasma 20-HETE, EETs, and their ratio are useful

surrogate biomarkers for identification of individuals at risk of development of renal and CV disease such as hypertension and atherosclerosis.

It has been previously reported (367) that Ang II and Ang 1–7 can stimulate ArA release from tissue lipids to promote the prostacyclin synthesis via activation of distinct types of AT receptors. The vasodilatory actions of Ang 1–7 could be explained by the observation that it does stimulate AT2R and Mas receptors but not AT1R. As aforementioned, Ang II which has been the subject of several studies regarding its interaction with ArA metabolites, but, such information is lacking for Ang 1-7. It has been reported that Ang 1-7 is more potent than Ang II in stimulating synthesis of vasodilatory PGs probably because of its low potency in stimulating lipoxygenase activity (367). However, correlation of Ang 1-7 with ArA metabolites such as 20-HETE or EETs has not been fully explored. The results of this study indicate that plasma Ang 1-7 is positively correlated with 15-HETE. Considering the opposing actions of HETEs and Ang 1-7 makes this positive correlation sensible in a way that production of a proinflammatory mediator such as 20-HETE is counterbalanced by increased concentrations of Ang 1-7. The same explanation could apply for the negative correlation between plasma 14,15-EETs and plasma Ang 1-7 with same vasodilatory affects. The sign of correlation between plasma ArA metabolites and kidney Ang 1-7 follows the same pattern as plasma; however, the statistical significance was reached only with 14,15-DHT (Table 5.3). The correlation of plasma ArA metabolites with heart Ang 1-7 were contrary to that of plasma ArA metabolites vs. plasma Ang 1-7. It was significant only with 14,15-EET (Table 5.3). This correlation sign discrepancy could be explained by different systemic and local expression of RAS system and tissue dependency of the physiological effects of ArA metabolites.

The ACE2/Ang1-7/Mas axis has shown a counter-regulatory effect against many actions of Ang II through AT1R associated with acute and chronic inflammation (23). Ang II is associated with several molecular signaling pathways involved in inflammation including protein kinase and nuclear transcription factor activation, free radical production, cytokine and chemokine release, and inflammatory cell recruitment (368-374). Recent studies have shown that ACE2/Ang 1-7/Mas receptor negatively alters most of these proinflammatory actions (26-29,375,376). It has also been shown that angiotensin receptor blockers such as telmisartan and olmesartan activate the ACE2/Ang 1-7/Mas axis which resulted in reduction of several proinflammatory mediators and an increase of anti-inflammatory cytokines (375,376). Results of the current study are in line with these finding as inflammation reduced heart ACE2 expression, Ang 1-7 concentration, and Mas receptor expression levels. The negative correlation of plasma 20-HETE with heart ACE2 expression, Ang 1-7 concentrations, and Mas receptor expression, further support the hypothesis that suppression of this axis may be attributed, in part, to CV events in inflammatory conditions such as RA. This maybe happens due to lack cardioprotection in this setting.

The effects of Ang 1-7 in the kidney are complex and may not necessarily always oppose the effects of AngII. Ang II increases efferent arteriolar resistance without having any effects on afferent arterioles; however, Ang 1-7 through Mas receptor relaxes the afferent vessels and increases renal blood flow (377). Ang 1-7 presents different effects dependent on sites of nephron used and peptides concentration (378). It has been shown that Ang 1-7 exerts a diuretic effect on the proximal tubule (379) and in contrast, it has been reported that Ang 1-7 has antidiuretic effects in water loaded animals by acting on Mas receptor in distal tubules (380,381).These conflicting effects of Ang 1-7 on kidney function were attributed to differences

in animal species, Ang 1-7 systemic and local concentrations, nephron segment, sodium and water status and RAS activation level (23). Our results indicate that Ang 1-7 kidney concentration and Mas receptor expression are increased due to inflammation. This may be explained by a compensatory mechanism that the kidney initiates in response to alteration of BP due to increased ACE/AngII/AT1R axis. The Mas receptor dimerizes with AT1R and modulates its interaction with Ang II. Therefore, over-expression of Mas in renal tissue could be considered as a counter-regulatory action of ACE2/Ang 1-7/Mas receptor in inflammatory conditions. A lack of Mas receptor as seen in knockout animals has resulted in a RAS imbalance with unopposed action of inflammatory axis in the kidney (382-384).

Considering the aforementioned discussion about the correlation of plasma 14,15-EET and 14,15-DHT with the plasma, heart, and kidney Ang peptides, it is worth taking into the account the ratio of these two ArA metabolites as a measure of sEH enzyme activity, which up-regulates in inflammatory conditions (347). We observed correlations between the ratio of plasma 14,15-(DHT/EET) and different tissue Ang peptides, enzymes, and receptors expression levels (Table 5.3-5.5). More importantly, the sign of the correlations is in concert with the expected effect of inflammation on the RAS. 14,15-(DHT/EET) is positively associated with the plasma, heart, and kidney Ang II, and the heart and kidney AT1R, and the kidney Mas receptor expression. On the other hand, 14,15-(DHT/EET) was negative correlated with the heart and kidney ACE2, the heart Ang 1-7, the heart Mas, Mas/AT1R and Mas/AT2R, and the kidney Mas/AT1R.The physiological activity of DHT as a vasodilator is much lower than the EET; therefore, the higher ratio of DHT over EET indicates a possible shift of the system toward the proinflammatory axis of the RAS. The correlation of this ratio with positive and negative signs against proinflammatory and anti-inflammatory mediators of the RAS components, respectively, makes

it a good candidate to be used as a biomarker in this inflammatory condition similar to the animals with AA.

5.8 Conclusion

In conclusion, RAS and ArA pathways as two main systems, involved in modulation and homeostasis of physiological and pathophysiological functions of body, are in intimate interaction with each other. They are present in all tissues and systemic circulation, and their balance is essential for controlling the body functions locally and systematically. Pathological conditions alter their balance and better knowledge of their status is critical for identification and planning of an optimal therapeutic approach for individuals at risk of experiencing CV events. In order to achieve this goal, availability of detectable biomarkers within plasma would be very helpful. The findings of the current work suggest plasma Ang II, Ang 1-7, 20-HETE, EETs, 20-HETE/EETs, and 14,15-(DHT/EET) as useful surrogate biomarkers could set the stage for more investigation for identification of individuals at risk of developing CV disease. There were other ArA metabolites and their ratios that could be considered as biomarkers, but the above-mentioned ones were more inclusive than the others.

Chapter 6

6 Effect of Glucosamine on Renin-Angiotensin System and Arachidonic Acid Metabolites in Plasma, Heart and Kidney of in Rats with Adjuvant Arthritis

6.1 Introduction

Glucosamine, a naturally occurring amino monosaccharide, is a constituent of glycosaminoglycan in the cartilage matrix and synovial fluid and is involved in cartilage formation by acting as a precursor of proteoglycan synthesis. It contributes to the strength, flexibility and elasticity of cartilage. GlcN, similarly to DMARDs, is able to inhibit the underlying destructive process caused by MMP-3 and prevent long-term damages (385). *In vitro* and *in vivo* studies have shown that GlcN suppresses PGE2 production in cultured chondrocytes and lowers serum concentrations of MMPs and PGE2 in patients with RA (239,386). These findings are consistent with GlcN clinical effects. GlcN has been demonstrated some symptomatic effects to elicit beneficial effects against the progression of OA (131,132,175,298). In a randomized placebo-controlled study of patients with RA, GlcN administration had no antirheumatic effect measured by conventional methods, however, it decreased the serum concentration of MMP-3 and based on the results of the physicians and patients self- evaluations GlcN treatment seems to have some symptomatic effects in humans as well(130) .

In previous chapter we observed that inflammation alters the ArA and RAS pathways. These two pathways are involved in regulation of CV functions and changes on their component balance may have an impact on CV system (52,53,387-389). GlcN as an anti-inflammatory agent may provide some beneficial effect through these pathways. There is some evidence that GlcN presents a protective role in animal models of atherosclerosis (174,182). In addition, recent meta-

analysis indicates that use of GlcN is associated with reduction of systemic inflammation biomarkers and mortality rate in humans (390-392).

Using the rat AA model, a well established animal model of human RA, our group has recently demonstrated that inflammation affected RAS homeostasis by altering the ACE and ACE2 enzymes expression in the rat heart (20). It also has been reported that inflammation induced by LPS influenced the ArA pathway by changing the CYP enzymes expression and consequently the ArA metabolites concentration (32,69). The aim of this study was to investigate the effect of GlcN on the RAS and ArA pathways as two main regulatory systems of the body. We hypothesized that with the demonstrated suppressive effect on inflammatory mediators and anti-inflammatory actions, GlcN is able to modulate inflammatory induced changes in the RAS and ArA pathways. The observed results indicate that GlcN has an optimum safety profile and comparable anti-inflammatory effects compared with NSAIDs in inflammatory conditions, and probably is able to reduce the elevated CV risk and mortality rate in RA patients (10).

6.2 Hypothesis

GlcN as anti-inflammatory agents is able to correct the harmful effects of inflammation on the RAS by modulating the altered expression of enzymes, receptors, and peptides, and will restore the balance between cardiotoxic and cardioprotective components of the RAS and ArA pathway.

6.3 Objectives

To investigate the effect of GlcN treatment on the RAS components expression level and ArA metabolites concentration in biological tissues.

6.4 Results

6.4.1 Effect of GlcN on ArA metabolite concentrations in the plasma, heart and kidney

In order to investigate whether GlcN treatment is able to modulate that observed effects of inflammation on the ArA pathway, the metabolites concentration in different tissues of rats with AA were measured. Figure 6.1 to 6-3 represents the plasma, heart and kidney concentration of ArA metabolites in INF and INF-GlcN groups, respectively.

In the plasma the elevated concentration of 20-HETE due to AA in the INF group was reduced by GlcN (Figure 6.1). GlcN treatment decreased the EETs concentration in INF-GlcN group which did not reached significant level. The ratio of 20-HETE/T-EETs as cardiac toxicity index (cardiotoxic/cardioprotective metabolite ratio) was significantly elevated in the INF group when compared with CL (see chapter 5, Figure 5.1). The GlcN treatment was able to lower the index to comparable level of CL group, however it was not significantly different from INF group as well. Similarly, in the heart tissue, GlcN treatment not only reduced the elevated 20-HETE concentration in rats with AA, but also diminished T-EET concentration and as result the increased ratio of 20-HETE/T-EET in INF was similar between groups (Figure 6.2)

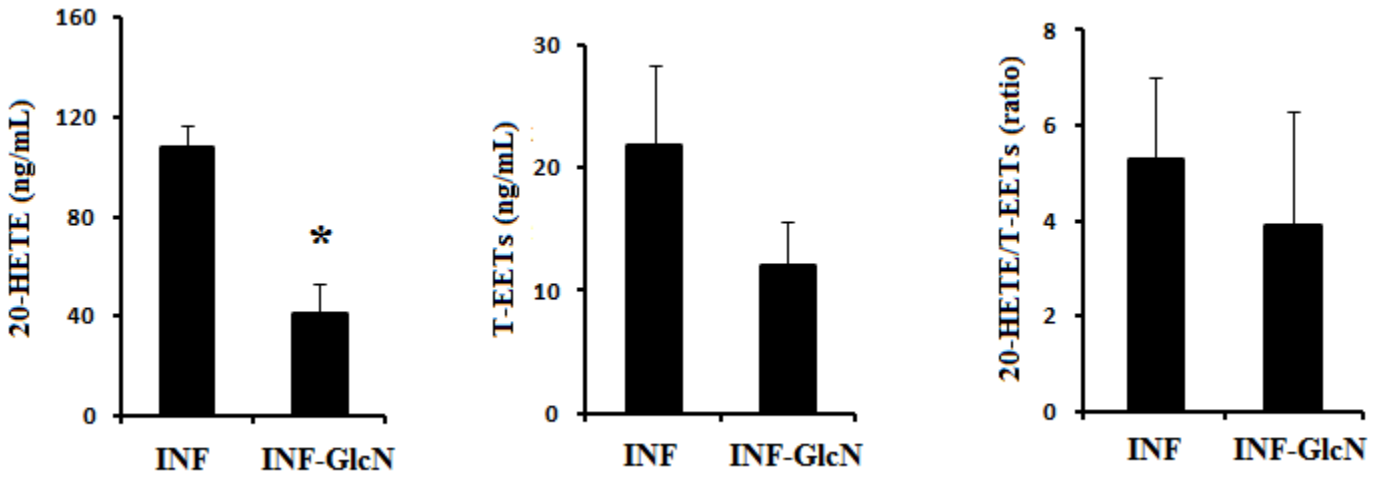

Figure 6.1. Effect of GlcN treatment on ArA metabolites concentration in the plasma of rats with adjuvant arthritis

The plasma concentration of 20-HETE, T-EETs and their ratio in inflamed (INF), and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

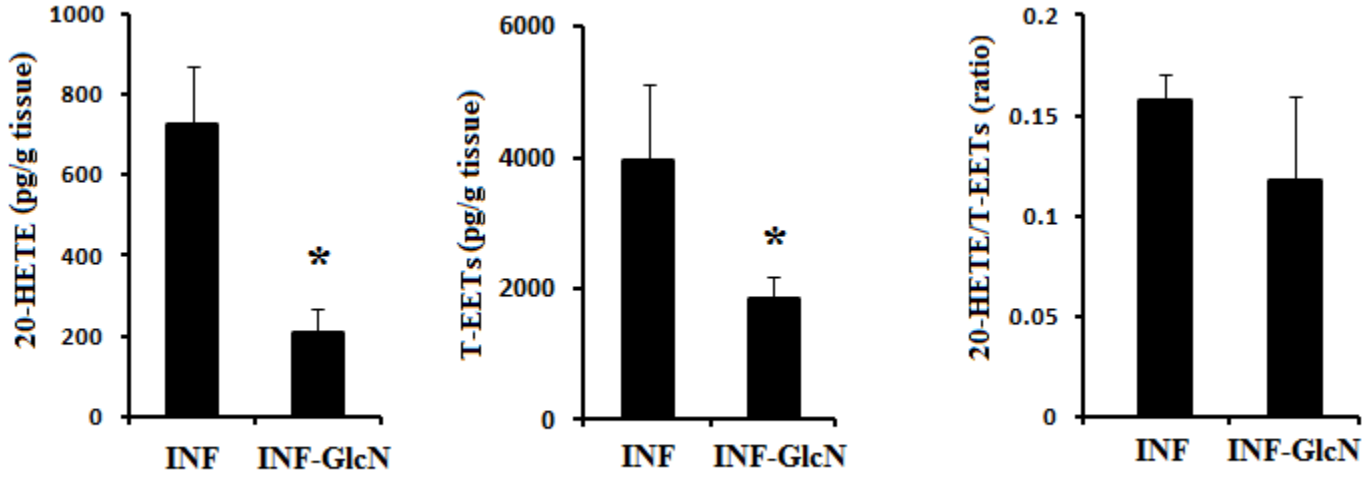

Figure 6.2. Effect of GlcN treatment on ArA metabolites concentration in the heart of rats with adjuvant arthritis

The heart tissue concentration of 20-HETE, T-EETs and their ratio in inflamed (INF), and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group)

As we observed in chapter 5, contrary to the plasma and heart tissues, 20-HETE concentration in kidney was decreased in the INF compared with the CL group, but T-EET concentration stayed the same. GlcN treatment normalized the concentration of 20-HETE concentration without affecting on T-EET level, as a result, the ratio of kidney 20-HETE /T-EETs was elevated when compared with INF group.

The GlcN treatment of control animals had on significant effects on the ArA metabolites concentration in plasma and heart compared with CL group, however, it increased kidney 20-HETE and its ratio over EETs.

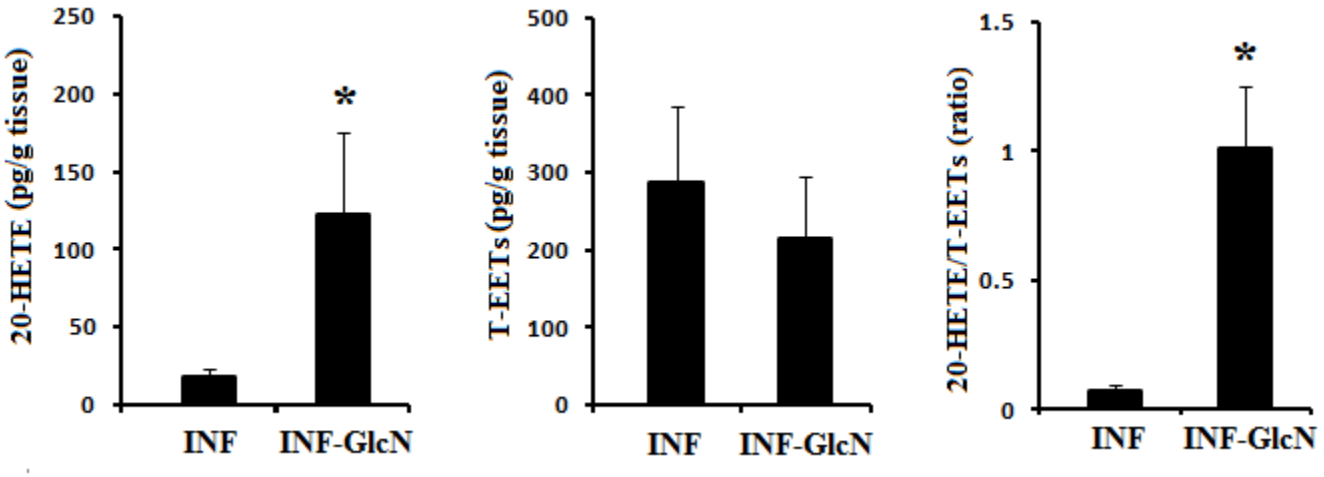

Figure 6.3. Effect of GlcN treatment on ArA metabolites concentration in the kidney of rats with adjuvant arthritis

The kidney tissue concentration of 20-HETE, T-EETs and their ratio in inflamed (INF), and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

6.4.2 Effect of GlcN on RAS component level in the plasma, heart and kidney

The effect of arthritis on the RAS cascade was examined by analysis of tissue concentrations of the two main active peptides of the RAS, Ang II and Ang 1-7, which were measured using a commercial ELISA kit according the manufacturer instructions. This analysis was accompanied with measurement of the expression of the ACE and ACE2 enzymes; the Ang II specific AT1R and AT2R; and the Ang 1-7 specific receptor Mas by western blotting analysis. The heart (Figure 6.4) and kidney (Figure 6.5) samples from INF and INF-GlcN groups showed marked changes of ACE enzymes and Ang peptides receptors expression in response to the inflammation and GlcN treatment. As our group previously reported for both tissues (20), ACE protein expression was not altered due to inflammation. However, ACE2 protein expression was significantly reduced in the INF group and consequently it reduced the ACE2/ACE ratio in both tissues.

In the heart tissue, the GlcN treatment did not alter the ACE, but restored the inflammation induced reduction of the ACE2 enzyme expression. Consequently, the ratio of ACE2/ACE was re-established in the GlcN treated animals with AA and was significantly higher when compared with INF groups.

In the kidney tissue, however, the GlcN treatment of rats with AA resulted in a significant reduction of ACE compared with INF-GlcN group. However, the reduction ACE2 level was not significant. Accordingly, we observed a significant elevation of the ACE2/ACE ratio in the INF-GlcN.

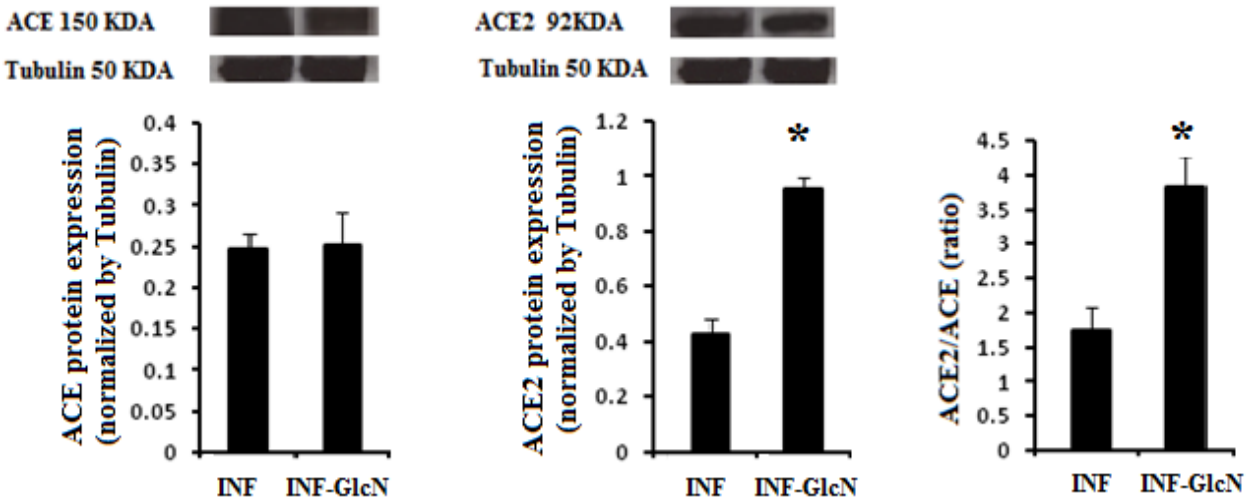

Figure 6.4. Effect of GlcN treatment on ACE, ACE2 protein expression level and their ratio in the heart of rats with adjuvant arthritis.

The heart tissue ACE and ACE2 protein expression levels and their ratio in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/ group).

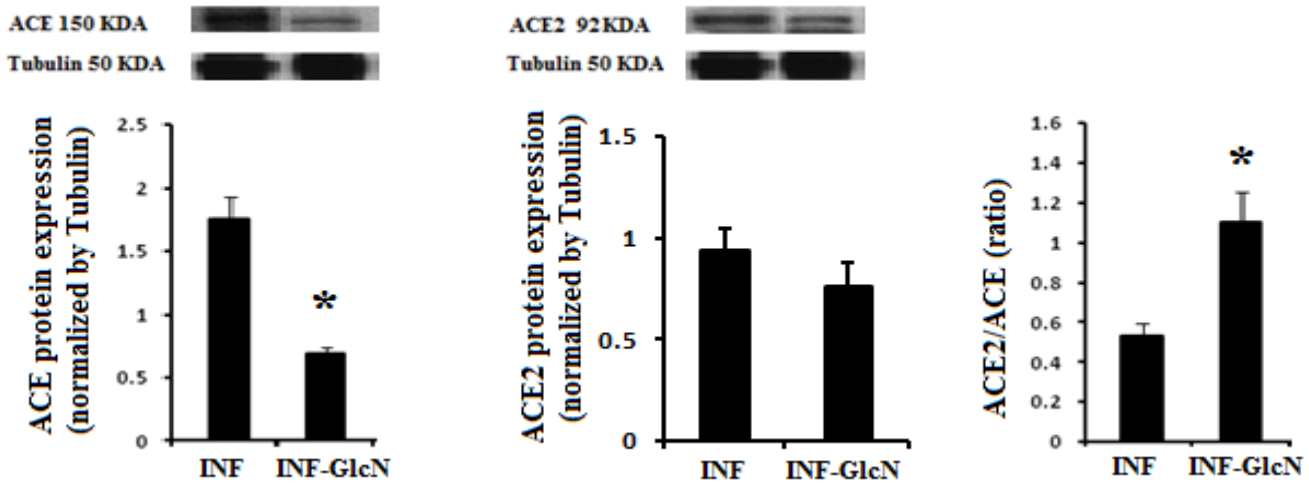

Figure 6.5. Effect of GlcN treatment on ACE, ACE2 protein expression level and their ratio in the kidney of rats with adjuvant arthritis.

The kidney tissue ACE and ACE2 protein expression levels and their ratio in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/ group).

The plasma concentration of Ang peptides or their ratio was comparable between groups (Figure 6.6). However, consistent with ACE enzymes expression in the heart and kidney tissues, the Ang peptides concentrations were altered accordingly.

In the heart tissue of INF group, Ang II was increased, but Ang 1-7 and its ratio with Ang II were decreased significantly when compared with the CL group (see Chapter 5, Figure 5.7). Treatment with GlcN normalized both peptides concentration and increased their ratio significantly (Figure 6.7).

In the kidney tissue, however, Ang II concentration was higher in INF group despite the fact the ACE enzyme was not altered, which it could attributed to a significant decrease of the kidney ACE2 expression (see Chapter 5,Figure 5.5). The Ang 1-7 concentration did not follow the same pattern as of the enzyme responsible for its production. The ratio of kidney Ang 1-7/II was also comparable with other group (see chapter 5, Figure 5.8). GlcN treatment in INF-GlcN group reduced both Ang peptide concentrations with no effect on their ratio. This could be due to down-regulation of both ACE and ACE2 enzymes which could offset the effect of each other on the corresponding substrates concentration without any change in their ratio (Figure 6.8).

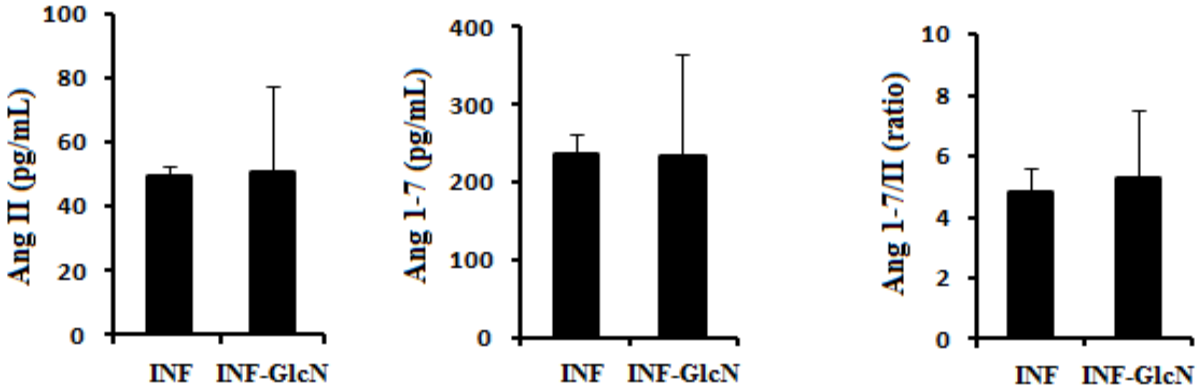

Figure 6.6. Effect of GlcN treatment on angiotensin peptides concentration in the plasma of rats with adjuvant arthritis.

The plasma concentrations of Ang II and Ang 1-7 and their ratio in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

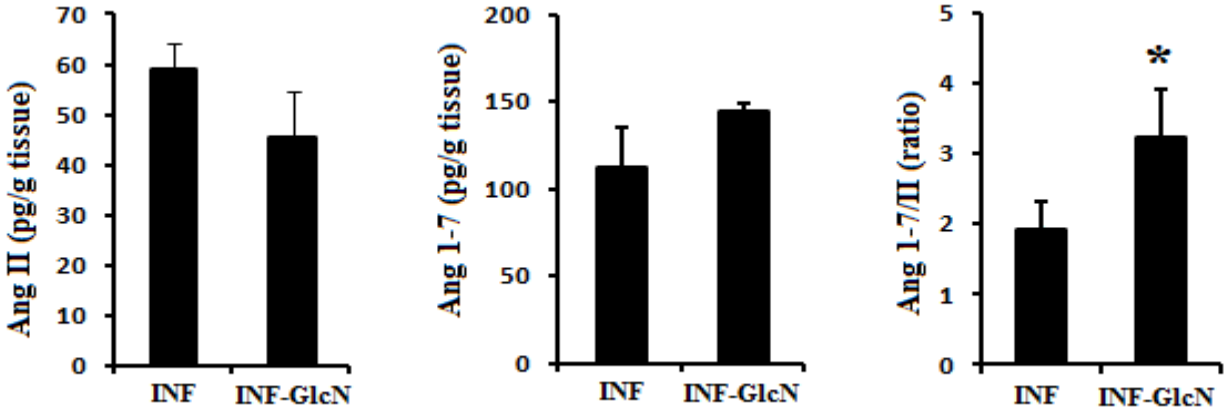

Figure 6.7. Effect GlcN treatment on angiotensin peptides concentration in the heart of rats with adjuvant arthritis.

The heart tissue concentrations of Ang II and Ang 1-7 and their ratio in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

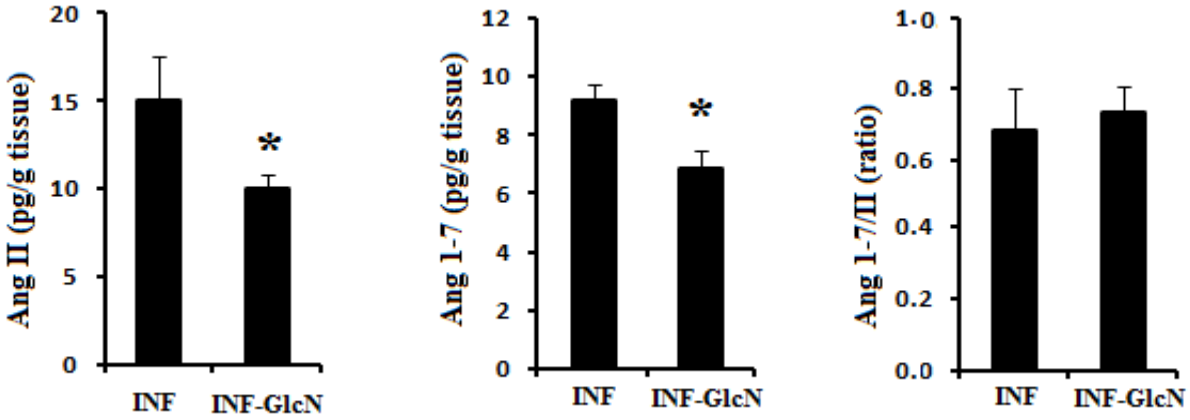

Figure 6.8. Effect of GlcN treatment on angiotensin peptides concentration in the kidney of rats with adjuvant arthritis.

The kidney tissue concentrations of Ang II and Ang 1-7 and their ratio in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

The heart tissue from animals with AA presented significant increase in the expression of the AT1R and AT2 R receptor when compared with control littermates, with no difference on their ratio with AT1R (see, chapter 5, Figure 5.9). Interestingly, the expression of the Mas, the Ang 1-7 specific receptors and its ratio with AT1R were decreased in the heart in response to inflammation (see chapter 5, Figure 5.11). GlcN treatment of rats with AA lowered the expression of AT1R and AT2R. Nevertheless, the AT2R/AT1R ratio was comparable with the other group (Figure 6.9). As GlcN did not alter Mas receptor level, the ratio of Mas/AT1R was increased as a result of reduction of AT1R. This was not the case for the heart Mas/AT2R ratio (Figure 6.11).

On the other hand, in the kidney tissue of rats with AA a significant increase on the AT1R level was detected when compared with control rats, while the AT2R expression was similar between INF and CL groups, the ratio of AT2R/AT1R was insignificantly lower in the kidney of INF group. The expression of the Mas receptor was increased due to inflammation, nevertheless, the ratio of Mas/AT1R or Mas/AT2R was comparable with CL group (see chapter 5, Figure 5.10 and 5.12). GlcN treatment in rats with arthritis lowered the elevated AT1R expression level with no change in AT2R (Figure 6.10). The elevated kidney expression level of Mas receptor due to inflammation was reduced and normalized by GlcN treatment in INF-GlcN group. Due to reduction of the kidney AT1R expression, the ratio of Mas/AT1R was increased in INF-GlcN. The insignificant reduction of AT2R level failed to change Mas/AT2R ratio (Figure 6.12).

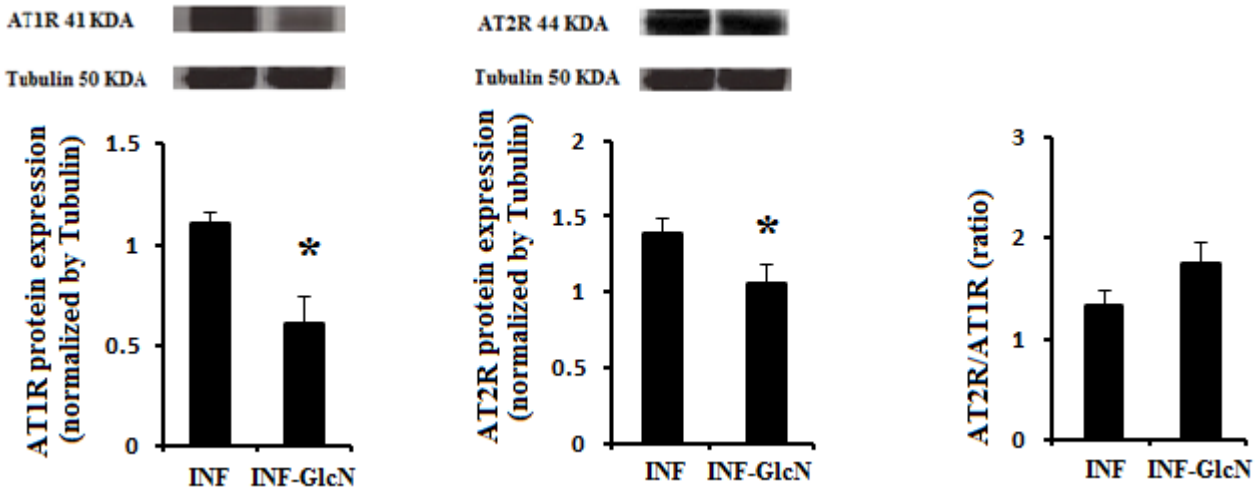

Figure 6.9. Effect of GlcN treatment on AT1R, AT2R protein expression level and their ratio in the heart of rats with adjuvant arthritis

The heart tissue AT1R and AT2R protein expression levels and their ratio in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

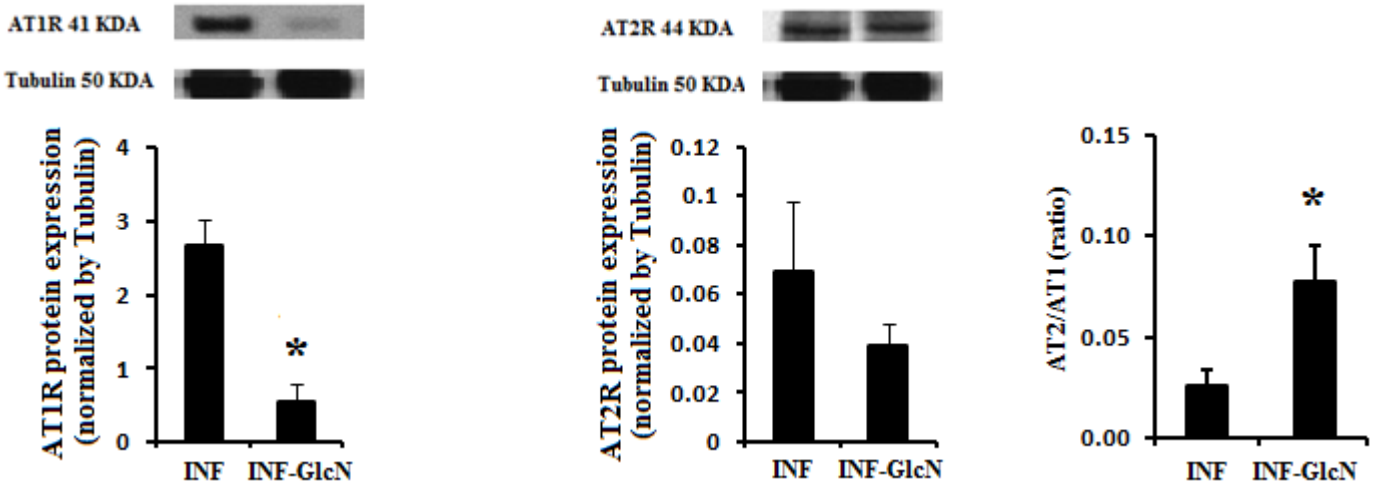

Figure 6.10. Effect of GlcN treatment on AT1R, AT2R protein expression level and their ratio in the kidney of rats with adjuvant arthritis

The kidney tissue AT1R and AT2R protein expression levels and their ratio in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

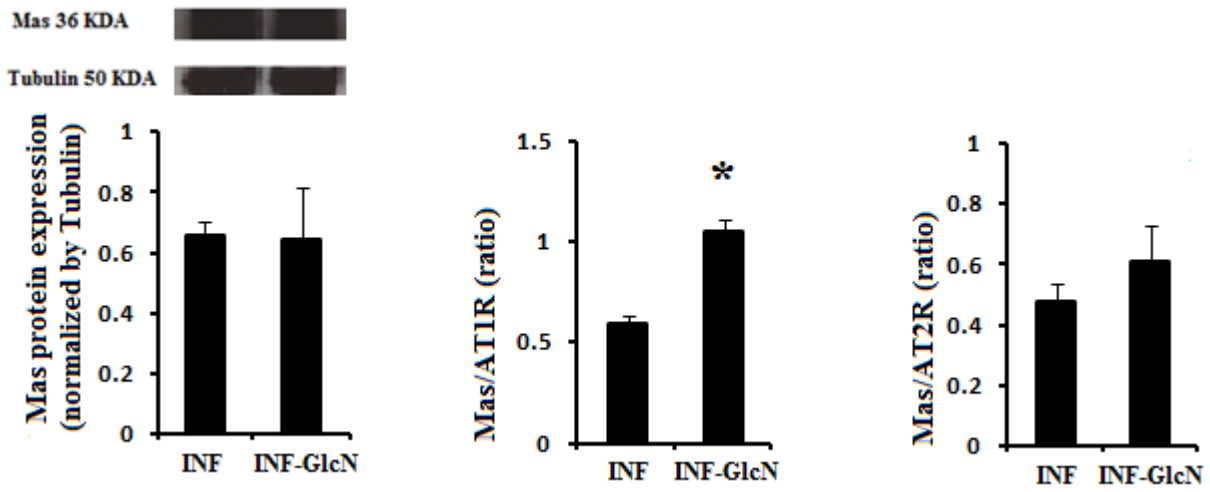

Figure 6.11. Effect of GlcN treatment on Mas receptor protein expression level and its ratio with AT1R and AT2R protein expression in the heart of rats with adjuvant arthritis.

The heart tissue Mas receptor protein expression level and its ratio with AT1R and AT2R protein expression in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

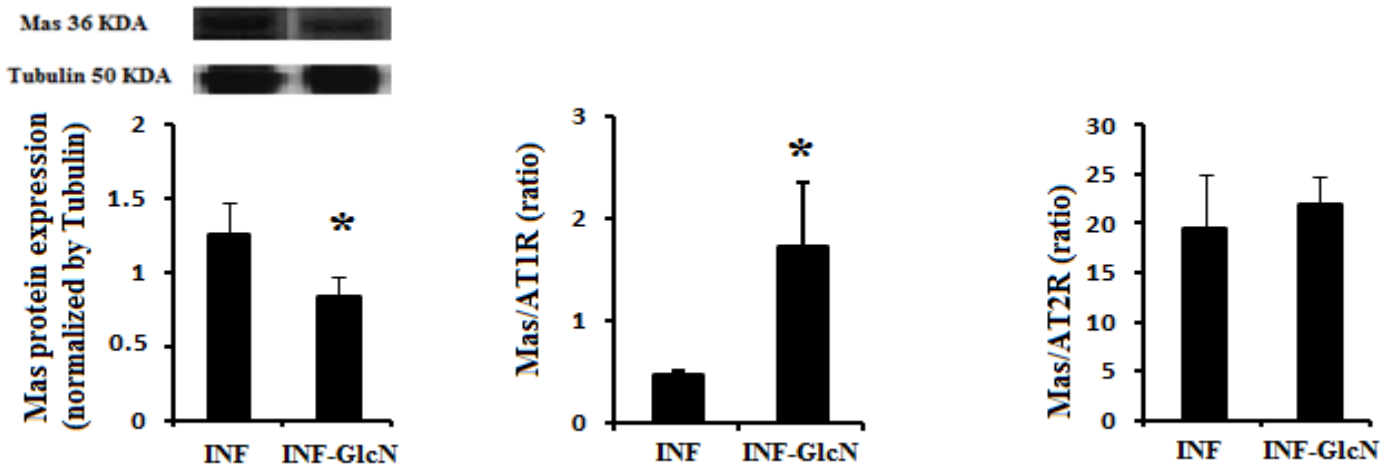

Figure 6.12. Effect of GlcN treatment on Mas receptor protein expression level and its ratio with AT1R and AT2R protein expression in the kidney of rats with adjuvant arthritis.

The kidney tissue Mas receptor protein expression level and its ratio with AT1R and AT2R protein expression in inflamed (INF) and inflamed-GlcN (INF-GlcN) groups; *statistically significant differences ($p < 0.05$, n=3-4/group).

The GlcN treatment of control animals had minimal effects on the RAS. However, ACE2 enzyme level was increased in the heart but decreased in the kidney by GlcN. The ratio of ACE2/ACE followed the same order when compared with not treated control group (CL). The expression level of heart and kidney AT1R, but not AT2R or Mas, was decreased and accordingly the ratio of Mas /AT1R was higher in this group compared with CL group.

6.5 Discussion

We observed in chapter 3, that GlcN dose-dependently presented a preventive and ameliorating anti-inflammatory effect in rats with AA. Furthermore, the results presented in this chapter, suggests that GlcN may modulate the inflammation through RAS and ArA pathways. GlcN was able to reduce the key inflammatory and angiogenic eicosanoid, 20-HETE, concentrations in the plasma and heart which were elevated due inflammation. GlcN normalizes inflammation-induced reduction of 20-HETE, a natriuretic and antihypertensive agent in the kidney. It was able to reinstate the altered balance between cardiotoxic (20-HETE) and cardioprotective (EETs) ArA metabolites in the plasma and heart and as a consequence, to reduce the cardiotoxic index (20-HETE/EETs). Due to the protective effect of 20-HETE in the kidney, the increased ratio of 20-HETE/EETs by GlcN treatment is an indication of its possible natriuretic, vasodilatory and tissue protective property. In addition to ArA pathway, in the local RAS at the heart and kidney tissues, GlcN treatment reestablished the disrupted balances of ACE2/ACE and Ang1-7/II ratios. On the other hand, the expression of AT1R, the main target of Ang II as a proinflammatory mediator, was reduced in the heart by GlcN treatment. The observed higher heart concentration of Ang II along with higher AT1R expression in animals with AA can be considered as activation of cardiotoxic axis of the RAS which is a risk factor for developing CV events in inflammatory conditions (393). GlcN treatment in INF-GlcN group, by restoring the Ang II and AT1R levels, diminishes this risk factor and lowers it to a degree that has been seen in control animals. Alternatively, inflammation suppressed the cardioprotective axis of ACE2/Ang 1-7/Mas receptor expression in the heart of the rats with AA and treatments with GlcN reinstated the ACE2, Ang 1-7 concentration and recovered the reduced Mas receptor level. Moreover, due to reduction of AT1R, the higher ratio of Mas/AT1R compared with INF

group may present beneficial effect toward to stabilization of the disrupted balance between cardioprotective and cardiotoxic axes in rats with AA.

Several studies have already identified 20-HETE as an effective pro-inflammatory agent and considered it as an index of inflammation (66-68). GlcN treatment normalizes the increased plasma and heart concentrations of 20-HETE by an unknown mechanism. *In vitro* studies using an acute inflammatory model of LPS in mice and rats have reported increased concentrations of 20-HETE as a result of CYP enzyme inductions (32). The GlcN action on the plasma and heart 20-HETE concentration cannot be attributed to its effect on the CYP enzymes responsible for synthesis or metabolism of 20-HETE, since GlcN does not cause any inhibition or induction on these enzymes (207). One possible mechanism could be an indirect effect on 20-HETE production as GlcN suppresses inflammatory cells activation through IL-1β and TNF-α (394). It has been reported that activation of the inflammatory cells, such as endothelial progenitor cell, was able to induce the CYP enzymes responsible for production of 20-HETE (395,396). This observation cannot be exclusively explained by GlcN anti-inflammatory effects, and further investigation needed to explain these observations.

The opposing effects of inflammation on 20-HETE concentrations in the plasma and heart vs. the kidney tissue can be explained by metabolic pathways involved in the production and degradation of 20-HETE. We address this issue in chapter 5. The production and physiological action of 20-HETE depends on tissue and expression of specific CYP4A isoforms. CYP4A and CYP4F enzymes expressed in renal tubular cells produce 20-HETE that lowers BP by inhibiting the sodium reabsorption and inducing natriuresis (33,331). On the other hand, as it has been reported in Dahl salt sensitive rats, the reduced tubular expression of CYP4A and CYP4F and diminished concentration of 20-HETE contributed to the development of hypertension (334).

Moreover, lower kidney concentrations of 20-HETE is probably attributed to multiple disposition pathways, mainly the COX-2 enzyme activity that increases by inflammation in the kidney (39,335,336). However, inhibitory effect of rofecoxib on inflammation induced COX-2 in the kidney seems not to be enough to restore the 20-HETE reduced concentration (unpublished results). These results suggest that the lower concentration of the kidney 20-HETE is attributed mainly to its lower production, rather than to its enhanced degradation by the COX-2 enzyme. GlcN treatment of rats with AA neutralizes the effect of inflammation on kidney 20-HETE concentration and restored it to the control level. The mechanism of induction of 20-HETE production in kidney by GlcN is not known.

20-HETE generated by vasculature CYP4A2 promotes vasoconstriction and causes hypertension (331,332). Some pathological conditions such as ischemic cerebrovascular diseases, hypertension, diabetes, kidney diseases and cancer are associated with changes in 20-HETE production (33,43). The development of hypertension and CV diseases are linked to CYP4A activity in the vasculature where the enzyme expression increases as the vessel diameter decreases (56,57).This is in agreement with an increased concentration of 20-HETE level narrower vessel due to higher rate of its synthesis (58). Inflammation also induces the vascular expression of CYP4A2 and increases 20-HETE production and consequently causes hypertension (333). In this study using AA rat model, we observed significant increase in 20-HETE in the plasma and heart samples. This change could partially explain the elevated risk of CV complication in RA patients (10). In attempt to prevent the development of AA by daily administration of GlcN we observed that it reduced AI (see chapter 3, Figure 3.1 and 3.2) and lowered the 20-HETE concentrations in both plasma and heart tissues when compared with non-treated arthritic animals (Figure 6.1 and 6.2).

20-HETE also plays a role in the regulation of vascular tone by assisting vasoconstrictor stimulus such as Ang II, endothelin and phenylephrine to impose their effect on smooth muscle cells. It can activate protein kinase C, mitogen activated protein kinase and tyrosine kinase. After phosphorylation, these proteins inhibit the conductance Ca^{2+}-activated K^{+} channel, leading to depolarization and elevation in cytosolic [Ca^{2+}]. These effects may translate into the increase in BP and development of hypertension seen in experimental models where vascular 20-HETE synthesis is increased (62).

Ang II, as a main component of RAS increases BP by vasoconstriction through AT1R activation in blood vessels as well as increasing sympathetic tone and the release of arginine vasopressin. It is also modulate renal sodium reabsorption by stimulating renal AT1R. Knockout mice of angiotensinogen, renin, ACE and AT_{1A} (murine homolog of human AT1R gene) exhibit marked reduction in BP, indicating the role of RAS in normal BP homeostasis (397).Taking into account the characteristics of the RAS and the ArA pathways, cross-talk between these two systems ,as it presented in chapter 5, are highly plausible. Ang II has been shown to stimulate the release of 20-HETE in isolated preglomerular vessels (356) and the renal synthesis of 20-HETE (333). Increased production of 20-HETE in the peripheral vasculature contributes to the acute vasoconstrictor response to Ang II (398). Acute inhibition of 20-HETE synthesis attenuates the renal pressor response to Ang II (333), and chronic inhibition of 20-HETE biosynthesis attenuates the development of Ang II-dependent hypertension (399). In cultured aortic vascular smooth muscle cells, 20-HETE mediates Ang II-induced mitogenic effects and contributes to the vascular injury, hypertrophy and hypertension caused by Ang II in rats (400-402). These studies also suggest that 20-HETE may contribute to the hypertensive actions of Ang II. To this end, experimental models of hypertension that show increased vascular 20-HETE production, such as

the spontaneous hypertensive rat (403,404) and the androgen-induced hypertension in rats and mice (405-407) are also RAS mediated. Some evidence suggested that the interaction between 20-HETE and the RAS includes induction of vascular ACE. In endothelial cells, 20-HETE is a potent inducer of ACE expression. Increased expression and synthesis of 20-HETE as in the CYP4A2-transduced rats (349) or the androgen-treated rats (54) is associated with increased expression of renal and vascular ACE which is abolished by treatment with 20-HETE synthesis inhibitors. In the model of 20-HETE-dependent hypertension (rats overexpressing the CYP4A2 in vascular endothelium), blood pressure is normalized by ACE inhibition or AT1R blockade (349). This suggests the presence of a feed forward amplification of 20-HETE- induced vascular dysfunction by the RAS. Thus, ACE induction by 20-HETE brings about increases in Ang II levels and its actions through the AT1R in vascular smooth muscle and endothelial cells. Such interactions may constitute at least in part the mechanism by which 20-HETE causes hypertension. Activation of AT1R by Ang II through stimulation of multiple signaling pathways, several growth factor receptors, reactive oxygen species, and other pro-inflammatory responses mimics several cytokine-like actions (107). Ang II production can be regulated by NF-κB and in return it is also capable of activating NF-κB and increasing the expression of NF-κB dependent genes (107). Ang II is also associated with the expression of pro-inflammatory cytokines, such a TNF-α, IL-1β and IL-6 (108). The modulatory effects of GlcN treatment on ArA and RAS could be attributed to cross talk mechanisms between the components these two systems. There are several mechanism of action were purposed for GlcN such as the inhibition of proteoglycan degradation and stimulation of proteoglycan synthesis, suppression of IL-1β-induced COX-2 and MMPs expression, reduction of TNF- α level, suppression of NO and PGE_2 production (134). Anti-inflammatory effect of GlcN is also associated with the inhibition of NF-κB

activation by promoting the acetylation of nuclear components of transcription process (135). These are in line with the result of this study that shows GlcN treatment normalized the inflammation induced changes of ACE2/ACE, Ang1-7/II, AT1R and Mas receptor in different tissues of rats with AA. These changes in expression could be attributed to GlcN interaction on NF-κB activation.

EETs, as ArA epoxygenase produced metabolites, have been considered as anti-inflammatory mediators (53,346). Inflammation did not alter the plasma and heart concentration of EETs. In the body, as a dynamic system, the inflammation-induced elevation of the plasma and heart 20-HETE concentration without a substantial change in the EETs concentrations implies that the balance between pro- and anti-inflammatory ArA metabolites is skewed toward pro-inflammatory mediators. Therefore, the higher ratio of plasma and heart 20-HETE/EETs in rats with AA might be an indication of domination of pro-inflammatory mediators, and at least in part, may be considered as an underlying cause for endothelial dysfunction and CV disease incidence in inflammatory situations such as RA. Treatments with GlcN reduced this ratio and normalize it back to the comparable level with control animals. This reduction did not reach significant difference when compared with INF group, probably due to small size limitation.

The RAS as one of the main systems involved in homeostasis of CV function, constitutively, consists of two opposing axes. The first axis is constituted of ACE/Ang II/AT1R, which is responsible for harmful effects, such as inflammation, vasoconstriction, cell proliferation, and fibrosis (22). Ang II by binding to AT1R in the circulation controls body fluid volume homoeostasis and BP. It is now well known that Ang II by applying a pro-inflammatory effect through AT1R contributes to hypertension, CV disease, and renal disease (388). Additionally, the expression and up-regulation of ACE in the peripheral blood and synovium samples of

patients with RA has been reported previously (408,409). The results of these studies suggest that new therapeutic approaches involving strategies on modulating RAS components, including inhibition of Ang II synthesis or disruption of interaction with its main target, AT1R, should be taken toward treatment RA. Previous reports confirm the beneficial effect of ACE inhibitors and/or ARBs in RA experimental animal models, including AA (106,410). Similar to these reports, the results of the present study indicate that GlcN treatment was able to normalize the inflammation induced increase on production of Ang II. Additionally, GlcN was able to reduce the AT1R expression in the heart and kidney. It is documented that activation of AT1R by Ang II is associated with vasoconstriction, pro-inflammation, pro-fibrosis and growth promotion (324,411,412). Other than AT1R, Ang II binds to AT2R subtype as well, which mostly counteracts the action of AT1R. Therefore, this interaction makes AT2R a potential target for therapeutic intervention. In an animal study with AA rat model, the therapeutic anti-inflammatory effects of losartan, an AT1R blocker, have been correlated and attributed to upregulation of AT2R and downregulation of AT1R (118). In the present study, GlcN also ameliorated the symptoms of arthritis in rats with AA and similarly suppressed AT1R expression with a minimal effect on AT2R expression and consequently increased the ratio of AT2R/AT1R in the heart and kidney. Therefore, it could be concluded that anti-inflammatory effects of GlcN similar to losartan in rats with AA, at least in a part, is associated through its interaction with AT1R expression.

The activation of the second axis of the RAS, which is composed of the ACE2/Ang 1-7/Mas receptor, results in anti-inflammatory, antifibrotic, antiproliferative, and vasodilatory effects (23). The key peptidase activity of ACE2 is degradation of Ang II to Ang 1-7 by which it changes the balance within the RAS cascade from vasoconstriction and proinflammatory to

vasodilatation and anti-inflammatory actions (24). {{}}Several studies have demonstrated that ACE2/Ang 1-7/Mas modulate inflammatory response and Ang 1-7, as the main component of this axis, negatively regulate proinflammatory mediator expression and release and modulate fibrogenic pathways (26-29). Previously, our group reported that inflammation alters the constitutive balance of the RAS enzymes: ACE, ACE2 in the heart (20). In the present study we observed the same results concerning the effect of AA on the RAS. Therefore, the concentration of Ang 1-7 was reduced as it was expected from down regulation of the ACE2 protein in the heart tissue due to inflammation. However, its plasma and kidney concentration was not different between the rats with AA and CL groups (chapter 5, Figure 5.6 and 5-8). The overall outcome of these changes translates into lower concentration of vasodilator and cardioprotective peptide, i.e., Ang1-7 in inflammatory conditions. We did not measure ACE, ACE2 in the plasma. However, plasma Ang II concentration was elevated significantly by inflammation and, due to a slight increase of Ang 1-7, the ratio of Ang 1-7/II was not different between control and INF groups. Although GlcN treatment of inflamed animals did not result in significant changes on peptide concentrations or their ratio, the GlcN-treated control animal showed an increased concentration of Ang 1-7. Lack of significant changes in the plasma concentration of peptides could be due to involvement of several pathways in their production and degradation in systemic circulation.

On the other hand, GlcN treatment was able to reinstate the disrupted balance of ACE2/ACE by increasing the expression of ACE2, which was reduced by inflammation in the heart and the kidney. The changes made by the GlcN at enzyme level were translated to the altered peptide concentration in the heart, i.e., the elevated concentration of Ang II was reduced and the diminished concentration of Ang 1-7 restored to a comparable level with the CL group. More

importantly, the ratio of cardioprotective over cardiotoxic peptides, Ang 1-7/II, shifted toward the cardioprotective side when compared with animals with AA.

AT1R and AT2R and Mas receptor are the targets of Ang peptides in order to exert their pharmacological and physiological response. Inflammation has induced upregulation of AT1R and AT2R. The activation of AT1R by Ang-II in the circulation may cause vasoconstriction, thrombogenesis and mutagenesis in inflammatory conditions. In contrary, the binding of Ang II at AT2R oppose the above mentioned effects. AT2R is not expressed under normal physiological conditions, but in response to pathological changes as a body's defense mechanism expression increases. We observed the same phenomenon in this setting as inflammation increased AT1R and consequently AT2R expression levels increased in the heart and kidney with no changes their ratio of AT2R/AT1R. GlcN treatment significantly lowered AT1R in both tissues when compared with the rats with AA. The GlcN treatment had no significant effect on the AT2R expression; nevertheless, the ratio of AT2R/AT1R was increased.

Collective alteration of several factors in the heart of rats with arthritis such as: a) the reduced level of ACE2 expression and the diminished cardioprotective effect of Ang 1-7 through Mas receptor due suppressed levels of Ang 1-7 and Mas receptor, b) the higher expression of ACE along with exaggerated effect of increased concentration of Ang II, on over-expressed AT1R, may suggest that both axis of the RAS were influenced by inflammation and the balance between these two arms shifted toward the cardiotoxicity. These changes may potentiate the risk of CV complications in inflammatory situations such as RA. GlcN treatment, however, could reduce the risk by normalizing the ACE, ACE2, Ang II, Ang 1-7, AT1R and Mas receptor levels, which collectively could switch the disrupted balance back toward the cardioprotection arm of the RAS.

In the kidney tissue, although the Mas receptor expression was increased by inflammation, the ratio of its ligand Ang 1-7 over Ang II was decreased. The increase in Mas receptor expression could be considered as the body's defense mechanism in order to protect the kidney tissue from proinflammatory actions of Ang II by potentiating the Ang 1-7 effect. GlcN treatment of rats with AA resulted in comparable level of kidney Mas, AT1R, AT2R and their ratio to control animals. This result could be attributed to the anti-inflammatory effect of GlcN on the COX-2 enzymes, which inducibly up-regulated in the kidney of rats with arthritis (134,236). *In-vitro* studies suggest that GlcN may affect inflammation by inhibiting of NF-κB from translocation to the nucleus (179). Activation of NF-κB initiates a series of downstream inflammatory processes involved in several inflammatory diseases, including inflammation-related cancers (413). Inhibitory effect of GlcN on including IL-1β, IL-6, TNF-α, and PGE2, as well as COX-2 expression is attributed to NF-κB inhibition which additionally support for mechanism of its anti-inflammatory effect (179,414-416). However, in order to elucidate the detailed mechanisms of action of GlcN for the observed results further investigation is essential.

In addition, a recent meta-analysis demonstrated an association between use of GlcN and CS with biomarker of systemic inflammation such as the acute-phase reactant, CRP (390). It has been reported that use of GlcN was related to reduction of mortality (391,392). Overall, these observed coordinated effects of GlcN on the ArA pathway and RAS can be considered as a confirmation of its anti-inflammatory and possible cardioprotective property and suggest that GlcN could be safely used as an alternative to the NSAIDs in this setting without any risk of elevation of at least cardiotoxic metabolite, 20-HETE, concentration level which has been reported for rofecoxib (61) .

The present study has a few limitations. First of all, it was a pilot study using a small number (n=3-4/group) of animals per group that therefore did not provide strong statistical power when we compared ArA metabolite or RAS component levels between groups, nevertheless, their profiles were different enough between groups to achieve significant differences. It is obvious that a higher sample size will make these results more reliable. Secondly, in the current study we directly measured the final product of the pathways, as main parameters, considering that the alteration of mRNA and protein expression enzymes responsible for production of ArA metabolites and Ang peptides by inflammation is already established (20,32,69). Third, we used the AA animal model to represent human RA. Although this model is vastly used in studies dealing with chronic inflammations, it may not reflect all aspects of RA inflammatory conditions and may differ in representation of the onset of arthritis, which is much slower in humans compared with rodents. This issue may be addressed by testing the concentrations of these ArA metabolites and RAS components using other animal models for RA. Overall, these results should be interpreted cautiously when they are extrapolated to humans.

6.6 Conclusion

The present study reveals that AA in rat, as a model of chronic systemic inflammation, elevates several pro-inflammatory and diminishes anti-inflammatory ArA metabolites and the RAS components in plasma and tissues. This alteration could disrupt the essential balance between the cardioprotective and cardiotoxic mediator in heart and kidney. The results of this study, by extrapolation to human may suggest that GlcN has a therapeutic value for improvement of inflammation- induced disruption of the RAS and ArA pathway in inflammatory conditions. Furthermore, these results may suggest the mechanism underlying CV side effects of inflammation could be explained through ArA and RAS pathways. In general, the lack of CV

side effects after long term use of GlcN along with its anti-inflammatory effects could be attributed to modulation of the RAS and ArA pathway. This suggest GlcN as an alternative option for treatment of inflammatory conditions, such as RA, obesity, CV disease, inflammatory bowel syndrome, cancer, pulmonary disease, etc.

Chapter 7

7 General Conclusion

Despite the overwhelming evidence generated using experimental animals in favour of beneficial anti-inflammatory effects of GlcN in the treatment of arthritis, randomized human clinical trials are not conclusive as some have observed benefit for both pain and joint function (190,245) and others have seen no or negligible positive effects (246,247). Interestingly, despite the controversy about its effectiveness, GlcN has maintained its popularity among OA patients (142,255). Several reasons for this controversy have been proposed including superiority of a crystalline sulfate salt formulation over GlcN HCl, industry bias, insensitive assessment metrics and poor methodology. In this thesis we ruled out some of these reasons and suggested additional factors; i.e., inconsistency in the active ingredient content of some products used, under-dosing of patients as well as variable and erratic bioavailability indices for the lack of GlcN efficacy observed in some studies. Result of our bioequivalency study between GlcN sulfate and hydrochloride dosage forms indicates that the discrepancy between the reported human clinical data is probably not due the nature of the salt or formulation properties. Regardless of the formulation used, following the commonly used 1500 mg/day doses, no or marginal beneficial effects may be observed because of under-dosing which stems from low GlcN bioavailability and inconsistency in the active ingredient content of commercially available products. Limited and erratic bioavailability of GlcN may also contribute to the problem. In addition, insensitive clinical outcomes and inclusion of patients with low baseline pain might have contributed to the unsatisfactory treatment outcome. The source of the controversy in the efficacy of GlcN seems to be pharmacokinetic in nature as it is generally agreed that the available GlcN formulations yield sub-therapeutic plasma concentrations. The other possible reasons could be listed as poor methodology of the clinical trials and insensitive assessment tools.

Based on the above mentioned discussion, there was an obvious need to determine the minimum effective GlcN dose and/or concentration. We performed preventive and treatment studies in order to determine MED and to show its effectiveness at both setting. Results of this thesis indicate that GlcN possesses preventive and ameliorating effects on AA in a dose and concentration-dependent manner. The MED is approximately about 40 to 80 mg/kg that corresponds with maximum plasma concentrations that are within the range reported for pharmaceutical-grade products used in human trials that have reported beneficial effects. Considering the observed dose dependency of the GlcN effect and the possibility of under-dosing, mainly because of the use of inferior products, future clinical trials should consider using pharmaceutical grade GlcN and, higher than 1500 mg/kg/day doses.

As a chronic, progressive and systemic inflammatory condition, RA leads to joint destruction and considerable pain, and diminished function and disability. RA is associated with higher morbidity and mortality rate from CV complications than the general population without RA (73). Evidence suggests that common proinflammatory cytokines are involved in the development and progression of both atherosclerosis and RA (78). The activation of the RAS plays an important role in the physiology and pathophysiology of the CV system. The balance between two axes of ACE/Ang II/AT1R and ACE2/Ang 1-7/Mas receptor regulate CV functions including BP, fluid and electrolyte homeostasis. Inflammation is prone to disrupt this balance which consequently could result in CV complications. It has been shown that ACEIs and ARBs were able to attenuate the endothelial dysfunction caused be inflammation (25).

The ArA pathway is an important system in regulating body functions. ArA has a central role as a precursor of a variety of mediators produced by the involvement of either constitutive or inducible enzymes or by nonenzymatic pathways. ArA and its metabolites are also involved in

CV system homeostasis. Cytochrome P450 (CYP) metabolizes arachidonic acid to HETEs and EETs. These metabolites play a complex role in the modulation of BP. Recent findings indicate that inflammatory conditions alter the metabolism of eicosanoids (69) and disrupt the balance between cardioprotective (EETs) and cardiotoxic (HETEs) metabolites (32). Recent preclinical and epidemiologic data suggest that modulation of eicosanoids metabolism may be a feasible clinical therapeutic strategy for the management of different pathological disorder and in particular CV disease (33).

Estimation of plasma and tissue concentrations of ArA metabolites is, therefore, important for understanding their role in physiological and pathophysiological processes. In order to study the status of ArA metabolites in inflammatory conditions, we developed an improved LC-FL assay with greater sensitivity and improved selectivity so that is suitable for a wider applicability for simultaneous analysis of ArA metabolites in different biological specimens.

Considering the characteristics of RAS and ArA pathway, the possibility of interaction between these two systems is quite feasible. Using LC-FL, ELISA, western blotting and AA animal model we studied the effect of inflammation on the RAS and ArA pathway and investigated the possible correlations between their components.

The results of this thesis are the first to show the following:

1) Inflammation due to AA alters the ArA and RAS pathways, which consequently changes the balance between anti-inflammatory and proinflammatory ArA metabolites, ACE, ACE2, Ang peptides, and their receptors.

2) Heart and kidney tissues concentration of ArA metabolites are correlated with plasma concentrations in rats with AA.

3) Plasma, heart and kidney concentration of Ang peptides are correlated with plasma concentration of ArA metabolites in rats with AA.

4) Heart and kidney tissues levels of the RAS components: ACE, ACE2, AT1R, AT2R and Mas, are correlated with plasma concentration of ArA metabolites in rats with AA.

These findings, therefore, suggest that the concentrations of key ArA metabolites and Ang peptides, particularly the CV and renal active components, can be predicted with confidence from their plasma concentrations. This validates the use of blood as a useful surrogate biological sample for detecting changes in ArA metabolites and Ang peptides and their receptors levels in the heart and kidney organs due to physiopathological status changes. Therefore, by extrapolating the results of animal studies to humans, the application of through concentration profiles of ArA metabolites and Ang peptides attainable from a single blood sample could be a useful biomarker identification tool in order to categorize individuals at risk in inflammatory conditions such as RA.

Various non-pharmacological treatments such as physical, occupational and nutritional therapy are available for RA, but these approaches appear to have little or no effect on the progression of the disease. As pharmacological approaches, analgesics, disease-modifying antirheumatic drugs, NSAIDs and biologics are used to suppress the symptoms of RA, but these drugs have a wide spectrum of some serious side effects. The most important of them all, was CV complications after administration of selective COX-2 NSAIDs which led to withdrawal of rofecoxib from the market. Therefore, a safe alternative therapeutic option for this population is needed. GlcN, similar to a disease-modifying antirheumatic drug, can inhibit or halt the underlying immune process and prevent long-term damage and has shown pharmacological effect against OA (131,132).

We investigated the effect of GlcN on the RAS and ArA as two regulatory systems of the body. We hypothesized that with the demonstrated suppressive effect on inflammatory mediator and anti-inflammatory actions, GlcN is able to modulate inflammation induced changes in RAS and ArA pathways. The observed result suggests that GlcN with optimum safety profile and anti-inflammatory effects, probably, is able to reduce the elevated CV risk and mortality rate in RA patients.

The results of this thesis suggest that GlcN probably modulates inflammation through RAS and ArA pathways. We observed that, GlcN was able to reduce the key inflammatory and angiogenic eicosanoid, 20-HETE, concentrations in the plasma and heart which were elevated due to inflammation. GlcN normalizes inflammation-induced reduction of 20-HETE, a natriuretic and antihypertensive agent in the kidney. It was able to reinstate the altered balance between cardiotoxic (20-HETE) and cardioprotective (EETs) ArA metabolites in the plasma and heart and to reduce the cardiotoxic index (20-HETE/EETs). Due to the protective effect of 20-HETE in the kidney, the increased kidney concentration of 20-HETE by the GlcN treatment may suggest a tissue protective property for GlcN. Additionally, in the local RAS at the heart and kidney tissues, GlcN treatment reestablished the disrupted balances of ACE2/ACE and Ang1-7/II ratios. The expression of AT1R, the main target of Ang II as a proinflammatory mediator, was reduced in the heart by GlcN treatment. The observed higher concentration of Ang II along with over-expression of AT1R in animals with AA can be considered as a risk factor for developing CV events in chronic inflammatory conditions. However, GlcN treatment, by restoring Ang II and AT1R levels, diminishes this risk factor and lowers it to a degree that has been seen in control animals. On the other hand, inflammation-induced reduction of the cardioprotective axis

of ACE2/Ang 1-7/Mas receptor expression in the heart, reinstated to the control level by GlcN treatment.

The results of this study indicate that GlcN possibly modulates both RAS and ArA pathway and regulate the malicious effects of inflammation imposed on these systems. Based on these findings, GlcN with an optimal safety profile and positive effects on RAS and ArA pathway on higher doses could be considered as an alternative for the NSAIDs in treatment of inflammatory conditions such as RA.

7.1 Future directions and studies

For further elaboration of GlcN mechanism of action through ArA pathway and RAS, several studies can be deigned;

1) A study of GlcN effect on mRAN expression of enzymes and receptors involved in ArA and RAS using suggested MED by this study.

2) A study of GlcN effects on intracellular and nuclear downstream cascade of AT1R, AT2R and Mas receptors

For validation of observed correlations between ArA and RAS components and suggested biomarkers, animal *in vivo* studies using different animal models of CV disease could be conducted with some end point parameter measurements; such as BP, INR, or histological evaluation of heart and kidney tissues.

In order to address the controversy around GlcN effectiveness, a clinical trial with GlcN doses higher than suggested MED in a patient population with moderate to severe OA could be helpful.

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