The Potential Role of Sulfur Amino Acids in the Acute Inflammatory Response

By

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Human Nutritional Sciences

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Winnipeg

ABSTRACT

The sulfur amino acids (SAA), methionine (Met) and cysteine (Cys) have an important role in the acute inflammatory response, including regulating the production of reactive oxygen species (ROS) and the development of cardiovascular disease (CVD). The objectives of the current study were 1) study alterations in Met cycle on the acute inflammatory response 2) investigate the in vivo temporal changes induced by an intraperitoneal (IP) injection of lipopolysaccharide (LPS), and 3) identify the potential effect of altered dietary SAA on the acute inflammatory response in male Wistar rats. The LPS dose that induced inflammation to a greater extent was 100 μg/kg compared with 50 μg/kg. LPS at 100 μg/kg presented a significant increase in both temperature ($p<0.05$) and cytokine tumor necrosis factor-alpha (TNF-α) ($p<0.001$) post injection compared with control. Additionally, at a dose of 100 μg/kg showed a significant increase in temperature when compared with 50 μg/kg at 120min ($p<0.05$) and 240 min ($p<0.05$). Plasma biochemical analyses revealed that the TNF-α area under the curve in dietary SAA of 50Met:50Cys was significantly higher ($p<0.004$) when compared with a dietary SAA ratio of 100Met:0Cys; however, no significant differences were observed in the percentage of leukocytes that were neutrophils. Additionally, a diet with a balanced Met:Cys ratio increased concentrations of plasma Cys and glutathione (GSH) after 4h. Adequate dietary intake of SAA play an important role in the immune response synthetizing intracellular antioxidant including GSH. Increase in the requirement of SAA or impairment in the SAA metabolism under infection can affect negatively the production of pro-inflammatory cytokines such as TNF-α, IL-6 and neutrophil proliferation. Perturbations in the transmethylation (TM) and transsulfuration (TS) pathways can lead to hyperhomocysteinemia (HHcy; plasma homocysteine >15 μmol/L) increasing the risk of coronary artery disease or kidney failure. Overall, the results presented in

this study serve to highlight potential effect of altered dietary SAA ratio in a model of systemic inflammatory response syndrome in addition to demonstrating the potential negative effect of impairment in the SAA metabolism.

ACKNOWLEDGMENTS

I express my deepest gratitude and appreciation to my supervisor, Dr. James D. House, the person who gave me the opportunity to achieve one of my dreams/goals bringing me into his lab and provide me invaluable knowledge and support throughout my time spent in graduate studies at the Department of Food and Human Nutritional Sciences, University of Manitoba. Dr. House, it has been an incredible and journey, and I will be forever thankful for his patience and guidance.

Similarly, I would like to convey my immense gratitude to my committee members, Dr. Heather Blewett and Dr. Karmin O. Having the opportunity to learn and work with these excellent scientists have made my graduate life a valuable and motivational experience. Thank you both for your patience and for helping me to think critically during my research study.

My gratitude also goes to my fellow graduate students, research assistants and departmental staff, without which this work would not be completed. For sharing innumerable meetings, academic meals and several cups of coffee answering as many questions as I had, I must give thanks to my friend and colleague, Dr. Matthew Nosworthy. Of course, there are friends inside my research group who did their best to help maintain my cool during the graduate process. I thank Adam and Matt for always being one phone call away when I needed it. I also extend my acknowledgement to Jay, Shengnan, Nahid, Neijat, Mingyan, Jason and Shusheng, who helped and enthralled me by providing their time and suggestions every moment we spent in our lab as well as in the experiments.

Finally, without the support of my family, Lilia and David, none of this would have been possible. From their continuous love to always being there to motivate and listen to me even

when I was not at my best. Above all, I would like to thank God and my wife Arely for the constant encouragement and endless love that inspired the best of me.

DEDICATION

I dedicate this thesis to God and my family.

To my wife Arely Rodriguez

To my in-laws David and Lilia

I would have not been able to do it without you

FOREWORD

The thesis begins with a general introduction (chapter 1) and literature review (chapter 2) followed by hypotheses and objectives (chapter 3). Chapter 4 is a systematic review. Manuscript I "Alteration of the dietary methionine:cysteine ratio modulates the inflammatory response to an inter-peritoneal injection of lipopolysaccharide in Wistar rats." (chapter 5 and chapter 6) was submitted on December 18th, 2020 in Journal of Nutritional Biochemistry. The thesis is concluded with an overall summary and discussion (chapter 7). The abstract "Impact of lipopolysaccharide (LPS) administration on markers of inflammation and sulfur amino acid (SAA) metabolism in Wistar rats was presented at the Canadian Nutrition Society Annual Conference. The abstract "Effect of baking, boiling and extrusion on the protein quality of yellow and green split peas (Pisum sativum) as determined by in vitro and in vivo methods" was presented at the Functional Foods and Natural Health Products Symposium, Winnipeg, MB, Canada, May. 2017. Additionally, the manuscript "Effectiveness of a Novel, Rechargeable, Non-leaching Polycationic *N*-halamine Antibacterial Coating on *Listeria Monocytogenes* survival in Food Processing Environments" was published in the Journal of Food Protection.

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ABBREVIATIONS

5-MethylTHF	N^5-methyl-tetrahydrofolate
AP-1	Activator protein 1
APP	Acute phase protein
APR	Acute phase response
BHMT	Betaine homocysteine methyltransferase
CAD	Cardiovascular artery disease
CASP	Colon ascendens stent peritonitis
CBS	Cystathionine beta-synthase
CLP	Caecal ligation and puncture
COMT	Catechol-O-methyltransferase
COX-2	Cyclooxygenase
CRP	C reactive protein
CSE	Cystathionine gamma-lyase
CVD	Cardiovascular disease
Cys	Cysteine
DHFR	Dihydrofolate reductase
ECM	Extracellular matrix of the basement membrane
FPGS	Folypolyglutamate
GCL	L-glutamate cysteine ligase
GCS	Gamma-glutamylcysteine synthase
GNMT	Glycine-N-methyltranferase

GPx	Glutathione peroxidase
GSH	Glutathione
GSS	Glutathione synthase
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HCU	Homocystinuria
Hcy	Homocysteine
HHcy	Hyperhomocysteinemia
HMGB1	High mobility group box 1
HOCl	Hypochlorous acid
IFNγ	Interferon gamma
IL	Interleukin
IP	Intraperitoneally
Iκβα	Inhibitor of nuclear factor kappa beta
LBP	Lipopolysaccharide binding protein
LBT4	Leukotriene B4
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
MAT	Methionine adenosyltransferase
mCD14	Membrane-bound CD14
MDA	Malondialdehyde

Met	Methionine
MetO-R	Methionine-R-sulfoxide
MetO-S	Methionine-S-sulfoxide
MMP	Metalloproteinases
MSR	Methionine sulfoxide reductase system
MTHFR	Methylenetetrahydrofolate reductase
NAC	N-acetylcysteine
NF-κβ	necrosis factor-kappa beta
NGF	Nerve growth factor
NO	Nitric Oxide
NOS	Nitric oxide synthase
NTDs	Neural tube defects
ONOO	Peroxynitrite
PAMPs	Pattern-associated molecular patterns
PGE_2	Prostaglandin E2
PL	Pyridoxal
PLP	Pyridoxal-5-phosphate
PM	Pyridoxamine
PMN	Polymorphonuclear neutrophil
PMNT	Histamine-N-methyltransferase
PN	Pyridoxine
PPP	Pentose phosphate pathway
PRPs	Pattern recognition receptors

RM	Remethylation
ROS	Reactive oxygen species
SAA	Sulfur amino acids
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SAPKs	Stress-activated protein kinases
SIRS	Systemic inflammatory response syndrome
SOD	Superoxide dismutase
SREBP	Sterol regulatory element-binding proteins
TauCl	Taurine chloramine
TGF-1β	Transforming growth factor-1 beta
THF	Tetrahydrofolate
TM	Transmethylation
TNF-α	Tumor necrosis factor-alpha
TRL-4	Toll-like receptor-4
TS	Transsulfuration
VCAM-1	Vascular cell adhesion molecule

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Inflammation is the body's immune response to heal itself after an injury or infection (1). According to its duration, inflammation can be classified as acute or chronic. During acute inflammation, macrophages try to remove injurious agents such as bacteria by producing cytokines including interleukin (IL)-1β , IL-6 and tumor necrosis factor-alpha (TNF-α), as well as other various mediators of inflammation like lipid messengers and proteins (2). Therefore, there is an increase in blood flow to the injured areas resulting in vascular permeability and in the accumulation of serum protein into the damaged tissue. A systemic inflammatory response syndrome (SIRS) can be developed in circumstances such as extreme trauma or in burn victims due to a dangerously unregulated acute inflammatory response in the host/patient, although the primary insult may be quite localized (3). Increased polymorphonuclear neutrophil (PMN) recruitment and activation of several tissues in this type of septic response will contribute to damage in liver, lung and kidney (4). SIRS induces alterations in various macronutrients (fats, carbohydrates, and protein), and promotes cellular breakdown of essential micronutrients (vitamins and minerals) (5). In addition, SIRS stimulates protein and fat breakdown, as well as loss of muscle mass. Tissue failure, especially in the liver, can be caused by SIRS increasing the synthesis of acute phase proteins. These changes increase the requirement of nutrients from food (6).

An acute phase response (APR) can also promote several metabolic changes by increasing the production of C-reactive protein (CRP), as well as the synthesis of several inflammatory markers including TNF-α and PMN proliferation (7). Pro-inflammatory cytokines, such as IL-6 and IL-1β promote alterations in protein and amino acid metabolism (8, 9). Nutritionally essential amino acids released from muscle and other tissues may be insufficient

for the synthesis of acute phase proteins (APP) and essential proteins, increasing the requirement of these amino acids such as arginine, methionine (Met) and cysteine (Cys) (10). Tissue repair following an APR can also raise the demand for the non-essential amino acids like glycine, which is a component of collagen (11).

During inflammatory states, injury or stress the requirement of sulfur amino acid (SAA) requirements increases. Dietary SAA are involved in the synthesis of proteins implicated in the immune system (12), regulation oxidative stress (13) and response to injuries. Additionally, SAA have been shown to play key roles in the acute inflammatory response with putative mechanisms linked to the antioxidant functions of their metabolites (14, 15). Adequate dietary intake of Met has demonstrated to protect cell damage from reactive oxygen species (ROS) through the methionine sulfoxide reductase system (16), as well as increase synthesis of glutathione (GSH) via cystathionine β-synthase (CBS) activity (17). Furthermore, Cys is responsible for the synthesis of antioxidants such as GSH, hydrogen sulfide and taurine (13). Cys also modulates the activity of pro-inflammatory cytokines including nuclear factor- kappa beta (NF-κβ) (18).

Additionally, oxidative stress, particularly ROS, increases the requirements of GSH, which are involved in the synthesis of antioxidant defenses. Antioxidant vitamins and systemic GSH concentrations decrease during inflammatory states (19). For instance, concentrations of vitamins pyridoxal phosphate (B6) and riboflavin (B2), involved in the synthesis of GSH are low in patients with coronary artery disease (CAD) (20). Selenium plays an important role in oxidation-reduction processes, and selenium-dependent GSH enzymes are involved in the reduction phospholipid hydroperoxides to cyclooxygenase (COX) (21).

SIRS affects the metabolism of macronutrients and increases the requirements of key antioxidant micronutrients. Because of the role of amino acids, especially Met and Cys in both the regulation and resolution of inflammation, more studies should focus on the interface between nutrition and inflammation.

CHAPTER 2

LITERATURE REVIEW

2.1 Inflammation

Inflammation is the reaction of the immune system in response to traumatic, infectious, post-ischemic or autoimmune injury (1). The process is responsible for removing injured tissue, offending factors, and restoration of tissues/ healing (22, 23). Inflammation may be considered in terms of five clinical signs (**Fig 2.1**), including redness, fever, edema, pain, and loss of function (24). With lack of proper treatment, inflammation could lead to persistent tissue damage by leukocytes or lymphocytes, as well as metabolic changes (25), the pathogeneses of arthritis, inflammatory bowel disease (26), cancer, cardiovascular disease (CVD) and stroke (24).

The accelerated proliferation of leukocytes into the infected region triggers the inflammatory response. Once the endothelium is activated by inflammatory mediators, rolling leukocytes attach deeply to the stimulated endothelium as they come into contact with a chemokine signal and an upregulated sequence of adhesion molecules, such as intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 (VCAM-1) (27). The capacity to attract immune cells, mostly on inflamed endothelial cells, relies either on the capacity of adhesion molecules (like, selectin) interactions to bind rapidly to its ligands and on the leukocyte rotation speed in post-capillary vessels, wherein active interactions can occur with a lower flow velocity (28, 29).

A series of binding and release events enable the leukocyte to roll around the activated endothelial cell surface until the leukocyte is bound by selectin molecules on the vessel wall (2). First, it is important to alter the principle receptors used by animal cells to bind the cellular matrix called integrins to make a conformation shift that shows the binding site for the particular adhesion molecule (30). Second, to allow the leukocyte to disperse along the active endothelium and sufficient integrin clustering on the leukocyte surface, the density of adhesion molecule

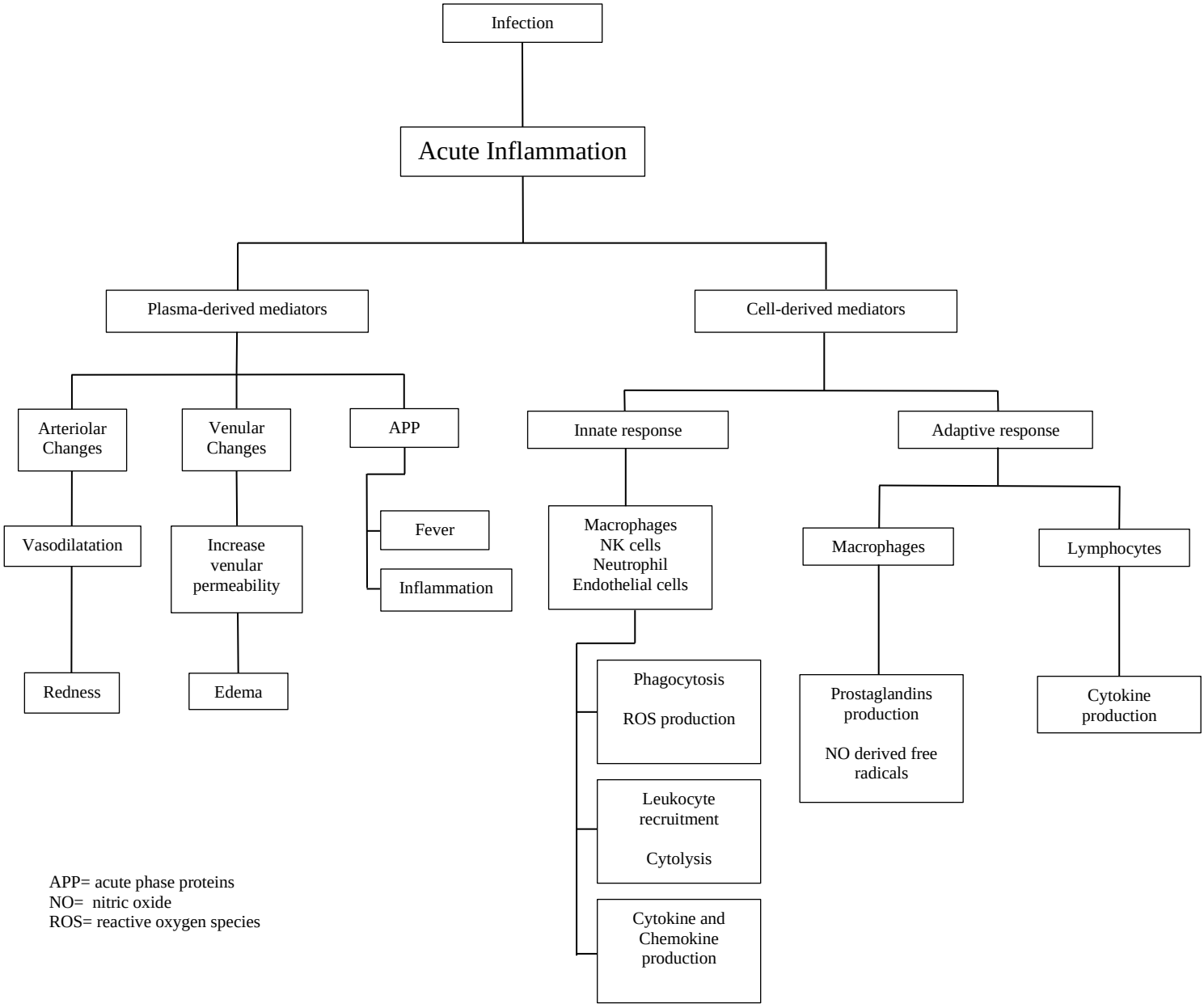

Figure 2.1. Cellular and plasma changes in acute inflammation. In response to infection, acute inflammation develops with its cardinal signs, fever, redness, edema, and pain. If acute inflammation is not regulated, then loss of function may occur.

expression needs to be large enough (2). Finally, an external signal is expected to intensify adhesive interactions across many significant signaling activities, including FGR and HCL, two SRC-like protein tyrosine kinases (30).

The expression of chemoattractants facilitates movement into the junctional area without damaging the surface of the endothelial cell, allowing leukocytes to scatter and move along the perimeter before they enter an endothelial cell junction which has been opened mostly via an inflamed setting (27). Finally, leukocytes infiltrate through the basement membrane into the site of inflammation due to the impact of metalloproteinases (MMPs) that weaken the extracellular matrix of the basement membrane (ECM) (31, 32).

2.1.1 Chemoattractants

The complement system is the most accessible mediator class of chemoattractants studied during inflammation that can participate in the extravasation of leukocytes. Bacterial products or immune complexes mediate the cleavage of C3 and/or C5 into C3a and C5a after activation by alternate or conventional pathways, which can provide an immediate and efficient chemoattractant to trigger neutrophil and monocyte activation. (33-35). The role of the anaphylactic agents C3a and C5a indicates the significance of this early activation event in the biology of mast cells, and the stimulation of neutrophil oxidative metabolism, granule discharge, and adhesiveness to vascular endothelium (36).

Another mediator system involved in leukocyte migration includes a class of lipid mediators, such as leukotrienes, preformed in mast cells or generated through the arachidonic acid pathway (37, 38). Early induction of neutrophil migration, as well as generation of long-term problems during inflammation can be produced by leukotriene B4 (LBT4). LTB4 is

synthesized by phagocytic cells (polymorphonuclear neutrophils [PMN] and macrophages) following stimulation with pathogen products (39, 40). Furthermore, recruitment of lymphocytes that mediate chronic inflammatory diseases, including rodent models of asthma and arthritis is due to the LTB4 receptor.

Mediation of the acute inflammatory response by bacterial infections occurs via the cellular infiltration of neutrophils and the production of CXCR-binding chemokines (41, 42). Additionally, increases in the production of chemokines by immune cytokines, such as interferon (IFN)-γ and interleukin (IL)-4, facilitate the recruitment of mononuclear cells, macrophages, and lymphocytes to the infected site and this occurs when the acute inflammatory mechanisms are not able to control the infectious process (41). When inflamed tissue releases cytokines such as tumor necrosis factor (TNF), chemokines are released, and they function to recruit leukocytes (43). Chemokines correspond to the supergene class of β-thromboglobulin and are distinguished by the inclusion of 4 retained cysteines whose position allows them to be divided into 2 molecular groups CC or CXC (44). During acute inflammatory responses, chemokines moderate the firm adhesion of leukocytes, triggering their β-integrins to the activated endothelial cells and subsequently direct migration of these cells to the site of inflammation (27). A coordinated expression and interaction of chemokines and adhesion of molecules are essential for the increased movement of leukocytes from the endothelial cell border in activated vessels through their arrival at the site of inflammation.

According to the duration and the kinetics of the reaction, the inflammatory response can be classified as either acute or chronic (27, 45). The acute phase of inflammation is the immediate and highly coordinated response of blood granulocytes such as PMN (26, 46, 47) and

adhesion to the vascular endothelium by leukocytes (22, 48), which form an essential part of the innate immune system (49), into the injured tissues from the opening of capillary beds (26).

2.2 Acute inflammatory response

Inhibition of the colonization of microorganisms or isolation of toxic and noxious substances are initiated by a rapid innate immune response to invading pathogens **(Fig 2.1)**. Pathogenic bacteria are capable of multiplying and expanding during infection at a rate that can compromise the capacity of the host to clear and eliminate the bacteria (27). At the beginning, the innate immune response activates edema and releases local fluid to the affected tissue, concurrent with cleavage of C3 and C5 due to the rapid activation of the chemokine complement system. The rapid and effective initiation of PMN and mononuclear phagocyte infiltration to infected sites, are due to the early activation of these inflammatory mediators (50). The recruited phagocytic cells are rapidly activated to produce LTB4 as well as fast response cytokines, such as IL-1 and TNF, and then they engulf invading pathogenic bacteria to enhance phagocytosis and killing (2). Subsequently, the fast response cytokines activate resident cell populations to induce other important mediators of inflammation, such as IL-6 and IL-8 (CXCL8) and promote cytokine cascades that increase leukocyte migration and activation (45). These events are essential for regulation of the intensity of the inflammatory response as well as effective containment of the pathogens and external toxins. Situations such as severe trauma or in burn injury can produce a systemic inflammatory response syndrome (SIRS) due to a dangerously unregulated acute inflammatory response in the host/patient even though the initial insult may be quite localized (45). The SIRS is the result of mediator production (especially TNF and IL-1) that is delivered to multiple tissues, initiating an overproduction of leukocyte chemoattractants in distal tissues

and inducing inflammatory cell influx (3). The SIRS can rapidly damage target tissues including liver, lung, and kidney. In this form of septic response, the increased PMN recruitment and activation to multiple tissues can lead to tissue damage and organ dysfunction (4). The uncontrolled leukocyte infiltration and activation that damages tissues leading to organ dysfunction is due to the ensuing cytokine storm that develops in the affected tissues, resulting from a cascade of cytokine, chemokine and reactive oxygen species (ROS) production (4).

2.2.1 Toll-like receptors

The Toll-like receptors (TLR) are a crucial part for the organism that primarily recognize pattern-associated molecular patterns (PAMPs) from invading pathogens. The TLR family are transmembrane receptors that reside either on the cell surface or within the endosome and that characteristically consist of leucine rich repeats (LRR) for pattern recognition and an intracytoplasmic region for signal transduction (51, 52). Cellular activation signals are transmitted by TLRs via cytoplasmic adapter molecules that initiate a cascade of nuclear factor kappa beta (NF-κβ), IFN regulatory factor 3 (IRF3), IRF7, as well as a link to MAPK pathways (53, 54). These cellular activation pathways allow an effective immune cell activation by providing strong stimuli that alert the host with danger signals. Toll-like receptor 4 (TLR4) primarily recognizes lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria (27). The TLR4 activation pathway is most prominent during sepsis for the strong systemic activation during acute inflammatory responses via multiple adaptor proteins, including MyD88, TRIF, and MD-2, making it the most dynamic TLR within the family (55, 56). Thus, TLR4 pathways through MyD88 are important for the initiation of innate cytokines, including TNF, IL-12, and type I IFN (57, 58).

2.2.2 Cytokines

Numerous cell types are capable of producing cytokines and chemokines, which can modulate immune responses including neutrophils, macrophages, monocytes, eosinophils, basophils, helper T-cells, endothelial cells, stromal cells and fibroblasts (2, 22, 59, 60). Cytokines are involved in biological processes such as inflammation, immunity, and cellular differentiation (61). During an inflammatory reaction, both anti-inflammatory and pro-inflammatory cytokines are released (**Table 2.1**).

2.2.2.1 Interleukin-1β

Interleukin 1β is released during inflammation by monocytes and macrophages (60). Interleukin 1β is part of the interleukin 1 family and has an important role up-regulating the inflammatory response in a variety of cellular activities such as cell proliferation, differentiation, and apoptosis (62, 63). Moreover IL-1β can produce hyperalgesia and raise the production of prostaglandin E2 (PGE_2) by the induction of the neurotrophin nerve growth factor (NGF) (59).

2.2.2.3 Interleukin 6

Interleukin 6 like IL-1β, is another pro-inflammatory cytokine, however IL-6 is secreted by T-cells and macrophages (60). IL-6 has been shown to play a central role in the neuronal reaction to nerve injury during inflammation by stimulating the synthesis of neutrophils (64), growth factor for plasmacytomas, hepatocytes, and mouse hybridomas.

Table 2.1 Summary of selected cytokines and their main function during an inflammatory state.

Cytokine		Cell sources	Function	Ref
Interleukin-1β	IL-1β	Monocytes, lymphocytes, endothelia, keratinocytes, epithelia.	Induction of other cytokines in many cell types; hematopoiesis; co-stimulates T-cells; activates endothelium acute-phase response and fibroblast proliferation.	(59, 60, 62)
Interleukin-6	IL-6	Monocytes/macrophages, fibroblasts, T lymphocytes, Endothelial cells, Epithelial cells and keratinocytes, bone marrow stroma, masts cells.	Stimulates B cells antibody production; stimulates T-cells growth and cytotoxic T lymphocyte (CTL) differentiation; stimulates hematopoiesis; stimulates liver acute-phase proteins.	(60, 64-67)
Interleukin-8	IL-8	Monocytes/macrophages, fibroblasts, Lymphocytes, Endothelial cells, Epithelial cells and keratinocytes, Sinovial cells, Mesangial cells, Smooth-muscle cells.	Neutrophils chemotaxis/activation; triggering of primed basophils, T-cells chemotaxis, T-cells chemotaxis, Keratinocytes mitogenesis/chemotaxis; angiogenesis.	(68, 69)
Interleukin-10	IL-10	Monocytes, T-cells, B-cells, Epithelial cells, Melanoma.	Inhibition pro-inflammatory cytokine production by monocytes, granulocytes endothelial cells and mast cells; inhibition of IL-2 production by T-cells; inhibition antigen-specific T-cells activation and cytokine production; inhibition of nitric oxide (NO) production by monocytes/macrophages; B cell co-stimulator for proliferation and immunoglobulin production; mast cell growth factor.	(70-72)
Interleukin-12	IL-12	Monocytes, B lymphocytes and Epstein-Barr virus-transformed B-lymphoma cell lines.	Stimulates proliferation of activated T and NK cells; enhance lytic ability of NK/lymphocyte-activated killer (LAK) cells; induces cytotoxic T lymphocyte responses to tumor cells; increases INF-y production b T and NK cells; inhibits production of IgE.	(73, 74)
Tumor necrosis factor-alpha	TNF-α	Macrophages, T-cells, many other cells.	Cytotoxic for tumor cells, antiviral activity; antibacterial activity; anti-parasitic activity; growth stimulation; immune modulation; pro-inflammatory.	(75-77)
Interferon gamma	IFNγ	Monocytes-macrophages, dendritic cells, T-cells, NK cells.	Major histocompatibility complex (MHC) class II expression; macrophages activation; NK cell activation; T-cell activation; immunoglobulin isotype regulation; antiviral, antibacterial and anti-parasite host defense.	(78, 79)

IL-6 also induces immunoglobulin production of IL-2 receptor in T-cells and is involved in microglial and astrocytic activation as well as in the regulation of neuronal neuropeptide expression (59, 65, 66). Additionally, IL-6 initiates the synthesis of PGE_2 leading to an increase in infection and injury (67).

2.2.2.4 Tumor necrosis factor-alpha

Tumor necrosis factor, primarily produced as a type II transmembrane protein (75), is an inflammatory cytokine involved in the activation of NF-κβ, stress-activated protein kinases (SAPKs) (60) and tumor regression (76). Additionally, TNF-α mediates hepatotoxicity as a result of producing hepatocellular dysfunction (77). Due to its important pro-inflammatory and immune-stimulatory roles, TNF-α is implicated in multiple organ dysfunction, including liver injury (75).

2.3 Regulation of acute inflammation

As part of a reaction to neutralize acute inflammation, the immune system begins a program that advances in several phases (**Fig 2.2**). The first phase for the regulation of acute inflammation is the influx of PMN, an anti-inflammatory and pro-resolution response to injury or infection (2, 50). Stopping the synthesis of pro-inflammatory mediators, such as cytokines and chemokines, and their subsequent catabolism and decrease of pro-inflammatory signaling pathways, is considered the critical for the resolution of inflammation (26, 47). The second phase occurs after the action of neutrophils at the inflamed site when, under normal circumstances, neutrophils undergo apoptosis and are ingested by macrophages. Clearance of apoptotic neutrophils changes

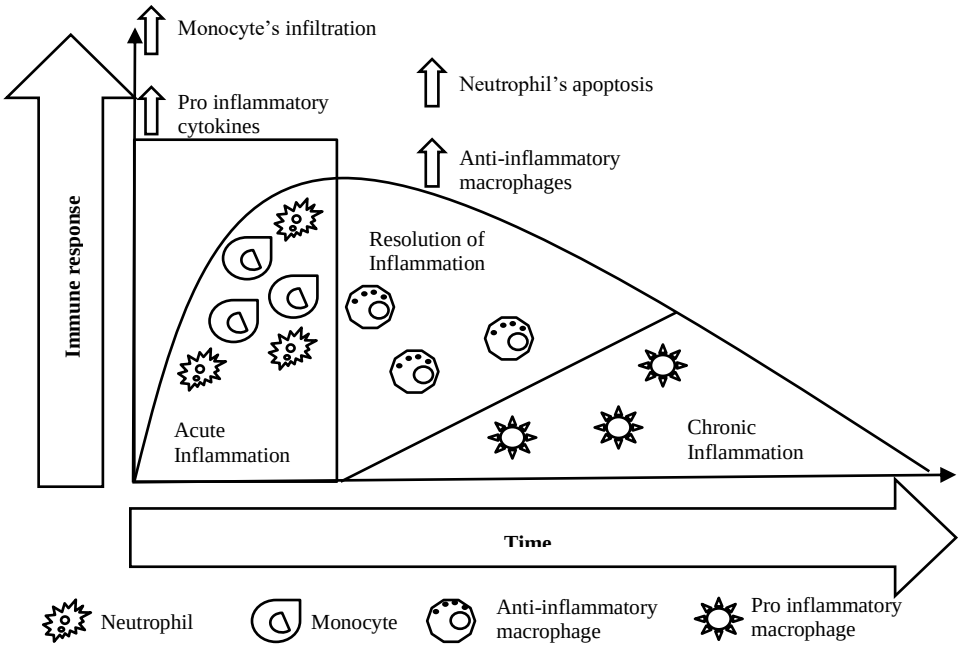

Figure 2.2. The coordinated temporal events of self-limited acute inflammation. The acute inflammatory response is a complex but highly coordinated sequence of events. (A) Onset (acute inflammation) begins with a significant influx of PMN cells to the inflamed tissue and an increased generation of pro-inflammatory mediators by endothelial cells, migrated and resident immune cells. As the inflammatory response progresses (transition phase), there is a switch in pro-inflammatory mediators' production to anti-inflammatory mediators and a reduction of PMN cell migration that parallel an increase in the influx of mononuclear cells. Besides, pro-inflammatory signals induce PMN cell apoptosis and macrophage phagocytosis of apoptotic PMN cells (efferocytosis). During the resolution phase of inflammation, the influx of monocytes prevails over the influx of PMN; the synthesis of anti-inflammatory/pro-resolving mediators is increased, while the levels of pro-inflammatory mediators are decreased. Collectively, these events lead to a successful resolution of acute inflammation. On the other hand, failures in the resolution of inflammation pathways lead to persistent inflammation and maladaptive immune responses.

the phenotype of macrophages from pro-inflammatory to anti-inflammatory (80-82), which leads to the third phase, the migration of macrophages from the inflamed area to the draining lymph node. There, they may play an important role in the presentation of antigens from the inflamed site via the lymphatic vessels promoting a return to tissue homeostasis (82).

Other markers involved in the inflammatory response, which act either by inhibiting or regulating inflammatory responses, include antioxidant enzymes, such as superoxide dismutase (SOD) (EC 1.15.1.1), catalase and glutathione peroxidase (GPx) (EC 1.11.1.9), and physiological gaseous mediators, such as nitric oxide (NO) and hydrogen sulfide (H_2S) (22). In recent years, research has focused on the resolution of inflammation by targeting the initiation of inflammation responses instead of regulating acute inflammation. Some of these early phases that are important in the resolution include: neutralizing TNF-α, blocking leukotriene receptors, inhibiting cyclooxygenase (COX)-2, leukotriene synthetase and 3-hydroxy-3-methylglutaryl coenzyme A reductase activities, and the agonism at protease-activated receptor 1 by activated protein C (23).

2.4 Models of inflammation

Given the variability and difficulties of studying inflammation in humans, animals have been established for the investigation of inflammation pathophysiology. These models of inflammation have often served as preliminary testing grounds for therapeutic agents prior to human trials (83).

On the basis of the initial agent, several models are used to study mechanisms of inflammation (**Table 2.2**), including the Carrageenan Paw model, dextran sulfate sodium

Table 2.2. Summary of some animal models of inflammation.

Name	Disease	Type	Species	Rationale	Strengths	Weaknesses	Ref
Carrageenan Paw Model	Inflammation edema	Inflamed paw	Mouse	Generalized inflammation	NSAIDs model	Non-specific	(84)
Caecal ligation and puncture (CLP)	Ruptured appendicitis or perforated diverticulis.	Creates a bowel perforation with leakage of fecal contents into the peritoneum.	Mouse Rat	Recreates the haemodynamic and metabolic phases of human sepsis.	Ease, general reproducibility and similarity to human disease progression.	A certain percentage of animals successfully contain the infection, do not progress to septic shock and recover fully.	(85, 86)
Transgenic mouse models of autoimmune diseases	Many autoimmune/inflammatory diseases	Can show cellular and tissue features that resemble diseases under study.	Mouse	Allows detailed study of effect of gene depletion or amplification/mutation in vivo.	Can uncover new targets for therapeutics evaluation.	Amplifies specific gain or loss effects of specific genes	(87-91)
Colon ascendens stent peritonitis (CASP)	Acute polymicrobial septic peritonitis	Multiple bacteria florae.	Mouse	Induced by laparotomy and exposure of the colon ascendens portion of the large intestine just distal to the ileocaecal valve.	Rapidly development of systemic inflammatory response syndrome	Less characterized haemodynamic response, less experience to identify possible confounding variables	(92, 93)
Endotoxin induced sepsis	Endotoxic shock, systemic sepsis	Primarily acute in susceptible animal species.	Mouse Rat	Reflects the result of acute bacteremia.	Easy to perform, endotoxin is widely considered as a sepsis response inducer. Induces hemodynamic, hematologic and metabolic changes like those observed in septic patients.	Some mouse strains are resistant- human disease is demonstrably different	(94-97)

induced inflammatory bowel disease, transgenic mouse models of autoimmune diseases, Experimental Autoimmune Encephalomyelitis (EAE), and endotoxin (LPS) induced systemic inflammatory response syndrome. Yet there are several main advantages to the endotoxin animal model, including technical ease and high reproducibility, especially in the acute inflammatory response (98). Endotoxin models can be divided into three broad mechanism-based groups, including the administration of an exogenous toxin, the administration of a viable pathogen, and the destruction of the endogenous defensive barrier of the animal that allows for bacterial invasion (99). Earlier forms of sepsis also required overt administration into the blood, peritoneum, or lung of toxins such as LPS (83).

2.5 Lipopolysaccharides (LPS)

Lipopolysaccharides, glycolipids present in the outer membrane of almost all Gram- negative bacteria such as Escherichia coli, are heat-stable molecules attached to the outer bacterial membrane by a specific carbohydrate-lipid denominated Lipid A. They are capable of stimulating the innate or natural immune response in humans (100, 101). Shortly after LPS administration, the production of pro-inflammatory cytokines increase and they are released to the blood, with the ability to measure their concentration in serum (102). The release of cytokines to the bloodstream contributes to the rapid development of SIRS and the eventual dose-dependent mortality (83, 103). The path whereby LPS activates peripheral monocytes or macrophages is through LPS binding protein (LBP). LPB catalyzes LPS to membrane bound CD14 (mCD14) on the external part of the phagocyte to release endogenous mediators (cytokines and chemokines) via TLR4 (104, 105). In normal circumstances, low concentrations of cytokines and chemokines in combination with biologically active lipids and low levels of

oxygen species lead to immune defense mechanisms (100). However, administration of LPS can increase concentrations of cytokines and chemokines resulting in severe sepsis.

LPS is strongly associated with sepsis, oxidative and inflammatory stress related to an elevated risk of cardiovascular disease, cancer and obesity (106), as well with biochemical changes during inflammation which can increase demand on amino acids (107). The major systemic effect of inflammation or any severe localized inflammation is the acute phase response (APR). Pro-inflammatory cytokines produced in response to local inflammation travel through the blood and stimulate liver cells to synthesize and secrete acute phase proteins (APP) such as C-reactive protein (CRP) (6).

LPS is also involved in the nuclear translocation of NF-κβ and initiates the AP-1 pathway which induces TLR4 signal transduction, enhancing both phagocytosis and cytokine production (108). Similarly, LPS stimulates COX-2 gene expression and prostaglandin production (109). TLR4 generates oxidative stress due to the activation of NF-κβ and AP-1 by myeloid differentiation primary response gene 88 (63). As Toll-like receptor recognize the extracellular component of Gram-negative bacteria like LPS, TLR4 plays a key role in activating the immune system by affecting antioxidant activity (53). An increase in levels of TLR4 on the cell membrane can indicate the beginning of oxidative stress, promoting cell proliferation and apoptosis (108). Furthermore, an increase in COX-2 activity leads to a high production of prostaglandins, which generate an inflammatory response by the growth of the initial signs of acute inflammation.

The effect of LPS in rat models has been well studied (**Table 2.3**). In a study conducted by Hagiwara, et al (110), they found that, after five days, Wistar rats injected intra-peritoneally with 10 mg/kg of LPS significantly increased serum concentrations of pro-inflammatory

Table 2.3. Summary of LPS intervention in animal-based studies evaluating inflammatory markers.

Author	Animal Model	Weight	Administration	Dose	Findings
Wrotek, et al. (111)	Wistar rats	250-350 g	IP injection	50 µg/kg	Induced fever in old rats (38± 0.01°C) and young (38.19± 0.06°C) Increased plasma IL-6 concentration significantly in both young and old rats compared with control.
Wrotek, et al. (112)	Wistar rats	250-300 g	IP injection	50 µg/kg	Induced biphasic fever. First peak recorded 37.87± 0.09°C after 2h post injection and second peak after 5h (38.38± 0.15°C)
Wrotek, et al. (113)	Wistar rats	200-250 g	IP injection	50 µg/kg	Induced fever after 2h (37.8± 0.17°C) and 4.5h (38.2± 0.07°C) Significantly increased plasma TNF-α concentration compared with control.
Ohsaki, et al. (114)	Wistar rats	8 weeks-old	IP injection	0.5 mg/kg	Significantly decreased ALT and AST activity Increased IL-6 expression
Breivik, et al. (115)	Wistar rats	260-300 g	n/a	n/a	Serum IL-10 was 26% lower in rats treated with LPS compared with control. Serum TNF-α was higher in rats treated with LPS although the differences were not significant. Significantly decreased serum TGF-1β
Hagiwara, et al. (110)	Wistar rats	250-300 g	Tail vein injection	10 mg/kg	Significantly increased concentration of serum TNF-α, IL-6 and HMGB1
Turrin, et al. (116)	Wistar rats	250-275 g	IP injection	100 µg/kg	Enhanced expression of IL-1β in liver and spleen TNF-α was significantly higher compared with control
Plata-Salamán, et al. (117)	Wistar Rat	250-275 g	IP injection	100 µg/kg	Enhanced IL-1β and TNF-α expression significantly in the cerebellum, hippocampus and hypothalamus

[1]TGF-1b, transforming growth factor-1b; IL-10, interleukin-10; TNF-α, tumour necrosis factor-alpha; HMGB1, high mobility group box 1; IP, intraperitoneally; LPS, lipopolysaccharide.

cytokines IL-6 and TNF-α in comparison to the control group. A study conducted by Wrotek et al (111, 112) compared the sickness behavior and glutathione levels in healthy old and young rats injected with LPS. Their findings were that pyrogenic dose of LPS enhances sickness behavior in both old and young rats. Moreover, glutathione levels between groups were not significantly different, meaning that changed pattern of fever is not necessarily associated with glutathione level (111).

Furthermore, a study by Humayun et al., (118) showed that high concentrations of LPS (≥100 ng/mL) caused changes in metabolite ratios linked to cellular sulfur amino acid metabolism, such as S-adenosylmethionine: S-adenosylhomocysteine (SAM:SAH) and glutathione:glutathione disulfide (GSH/GSSG). However, the SAM/SAH ratio, which measures the cellular methylation capacity, did not change during the first 6 hours following LPS addition (118). Also, LPS caused a pro-oxidant effect in cells by oxidizing protein thiols through an elevation in cellular cysteine (Cys) and homocysteine (Hcy). Nevertheless, there is no evidence that Cys and Hcy have any involvement in inflammatory signaling (106).

Oxidative stress is defined as an imbalance between pro-oxidants and antioxidants in favour of the former (4). Production of pro-oxidants like reactive oxygen species (ROS) increase under severe trauma and infection caused by LPS through a variety of mechanisms (119). Antioxidants such as SOD, catalase (EC 1.11.1.6), thioredoxin reductase (EC 1.8.1.9), and GPx, prevent or inhibit the development ROS or their interactions with biological structures and/or molecules (120). Consequently, LPS increases antioxidants' requirement, including zinc, copper, selenium and sulfur amino acids (methionine [Met] and Cys) (107, 121). Such an increase in antioxidant requirements can result in a decrease in GSH and taurine concentrations.

The LPS model, however, does not adequately replicate human inflammation; yet, is commonly used as a model of endotoxic shock to research endotoxemia or SIRS pathophysiological processes (122). Interestingly, a significant variable is the route of endotoxin administration, since IP-LPS is expected to hallmark traits of human sepsis, with earlier and higher cytokine activity, which are shorter in length compared to those observed in humans (99, 122). Overall, LPS models are highly used as SIRS models due to the capacity of LPS to reproduce and quantify the activation of the innate immune response (83) and to produce hyperdynamic cardiovascular responses (122).

2.6 Impact of diet on inflammation

Systemic inflammation induces alterations in nutrient requirements, changes the utilization of several macronutrients including fats, carbohydrates and protein, and enhances the absorption of essential micronutrients (vitamins and trace elements) (6) (**Table 2.4**). The systemic inflammatory response also stimulates protein and fat breakdown, muscle mass loss and induces the liver to increase the production of APP (6). These alterations, particularly in malnourished people (121), increase the requirements of different nutrients required for the regulation of the immune response.

2.6.1 Proteins

The amount of protein required by healthy individuals compared with critically ill patients to achieve neutral nitrogen balance differs. A negative nitrogen balance occurs when the amount of nitrogen excreted is greater than the amount of nitrogen consumed. This negative nitrogen balance may be attributable to critical illness or injury such as burns and inflammation. Hence

Table 2.4. Impact of diet on inflammation.

Nutrient		Function on inflammation
Fatty acids	Polyunsaturated fatty acids (PUFA)	Negative correlation with inflammatory markers such as CRP, TNF-α, IL-6 and fibrinogen (123).
	Monounsaturated fatty acids (MUFA)	Anti-inflammatory response. Reduces concentration of IL-6 and CRP (124).
Trace elements	Iron	Iron concentrations reduces during acute inflammation due to synthesis of lactoferrin and ferritin (125).
	Zinc	Involve in growth and function of cell-mediating innate immunity, neutrophils, and natural killer cells (126). Zinc concentration decreases during acute inflammation (127, 128). Zinc supplementation (30 mg) can cause severe fever (128).
	Cooper	Copper concentration increases during acute inflammation (129). Inflammatory conditions increase synthesis of hepatic caeruloplasmin, a major copper binding protein (125). Caeruloplasmin scavenges free radicals and helps to preserve iron (125).
	Selenium	Inverse correlation between plasma selenium and serum CRP concentration (130). Involved in the synthesis of antioxidant GSH (130).
Vitamins	Vitamin A	Bound to retinol-binding protein (RBP) (125). Involve in immune function (131). Reduces CRP concentration (132).
	Vitamin E	Antioxidant properties include limiting ROS activity, inhibiting COX-2 and NF-κβ activation (125).
	Vitamin C	Scavenges ROS (133). Regenerates GSH (125). Reduces IL-6 and fibrinogen (134).
	Vitamin B6	Cofactor in the synthesis and utilization of carbohydrates, lipids and protein. (125). Involved in the SAA metabolism (135). Antioxidant capacity modulating ROS activity (136). Low concentrations of B6 increase risk of CAD (137).
	B12	Cofactor in homocysteine metabolism (125). Deficiency of B12 increases risk of CAD (138).
Proteins	Leucine	Endorses glucose uptake because of an interaction with insulin (139).
	Arginine	Restores endothelial NO synthesis (140). Reduces vascular oxidative damage (141).
	Glycine	Modulates the expression of pro-inflammatory cytokines including IL-1, IL-6 and INFγ (142).
	Cysteine	Precursor of the antioxidants GSH, H_2S and Taurine (13)

[1]CRP, C-reactive protein; TNF-α, tumor necrosis factor-alpha; IL, interleukin; GSH, glutathione; ROS, reactive oxygen species; COX-2, cyclooxygenase-2; NF-κβ, nuclear factor kappa beta; SAA, sulfur amino acids; CAD, coronary artery disease; NO, Nitric oxide; INFγ, interferon gamma; H_2S, hydrogen sulfide.

an increase in protein intake relative to standard requirement estimates for healthy individuals may be needed in order to promote the synthesis of proteins, modulation of immune function and redox status during an inflammatory response.

Different amino acids can exert individual effects on metabolism. Leucine and the other branched chain amino acids (isoleucine and valine) are involved increasing mTOR signaling in human muscle (143) and may endorse glucose uptake as a result of an interaction with insulin (139, 144). Consumption of arginine has beneficial effects such as decreasing serum concentrations of risk factors for CVD like Hcy and excess white fat mass (145). In a study with Zucker diabetic fatty rats, arginine administration demonstrated a recovery of endothelial dysfunction by restoring endothelial NO synthesis and reducing vascular oxidative damage (141).

Moreover, chicks fed with a diet supplemented with glycine reduced mRNA expression of IL-1β, IL-6, IFNγ and TLR4 after an LPS injection when compared with chicks fed the basal diet (142). Glutamine is also related with cytokine synthesis and immune mechanisms. During an inflammatory response, plasma glutamine levels are reduced, and a supplementation of glutamine may lead an improved immune response (142). Cys metabolism is related to the inflammatory response, being a precursor of GSH, H_2S and taurine (see 2.7.2 Cysteine, section 2.7.2). GSH is as well an essential antioxidant and its deficiency is associated with oxidative stress (see 2.7.2.1 Glutathione, section 2.7.2.1)

2.7 Sulfur amino acids

In health and disease, sulfur amino acids and their primary metabolites are of major significance. Met is considered an indispensable amino acid since it can only be obtained from the diet. On

the other hand, Cys is consider a semi-indispensable amino acid because either it is synthetized from Met or provided by dietary supplementation. Met metabolism (**Fig. 2.3**) is involved in the formation of Hcy following the donation of a methyl group during the transmethylation pathway. Hcy is an important product from the Met cycle, being involved in the remethylation pathway to form Met and in the transsulfuration pathway that ultimately leads to the synthesis of Cys.

2.7.1 Methionine

Met is an indispensable amino acid accepted as the precursor of cysteine as well as being required for the synthesis of S-adenosylmethionine (SAM; AdoMet) (146). The metabolism of Met is divided into three phases: 1. - Transmethylation (TM), 2. - Remethylation (RM) and 3. - Transsulfuration (TS). The TM reaction is where the methyl group is donated *in vivo* (147).

2.7.1.1 Transmethylation

The TM pathway involves reactions where first Met is activated in a process involving adenosine triphosphate to form SAM in a reaction catalyzed by methionine adenosyltransferase (MAT) (EC 2.5.1.6). This is then followed by the donation of the methyl group from SAM to a nitrogen, oxygen or sulfur moiety of a methyl acceptor by one of several methyltransferase enzymes (148) such as catechol-O-methyltransferase (COMT) (EC 2.1.1.6.) (149) and histamine-N-methyltransferase (PMNT) (EC 2.1.1.8) (150). S-adenosylhomocysteine (SAH; AdoHcy) is the product of the methyl transfer reactions, and this compound undergoes hydrolysis to yield adenosine and Hcy through the action of the reversible enzyme SAH hydrolase (147, 148). The resulting Hcy can then be metabolized through either the RM or TS pathways, making this partition of Hcy a critical mediating event for the provision of either Cys or Met (147)

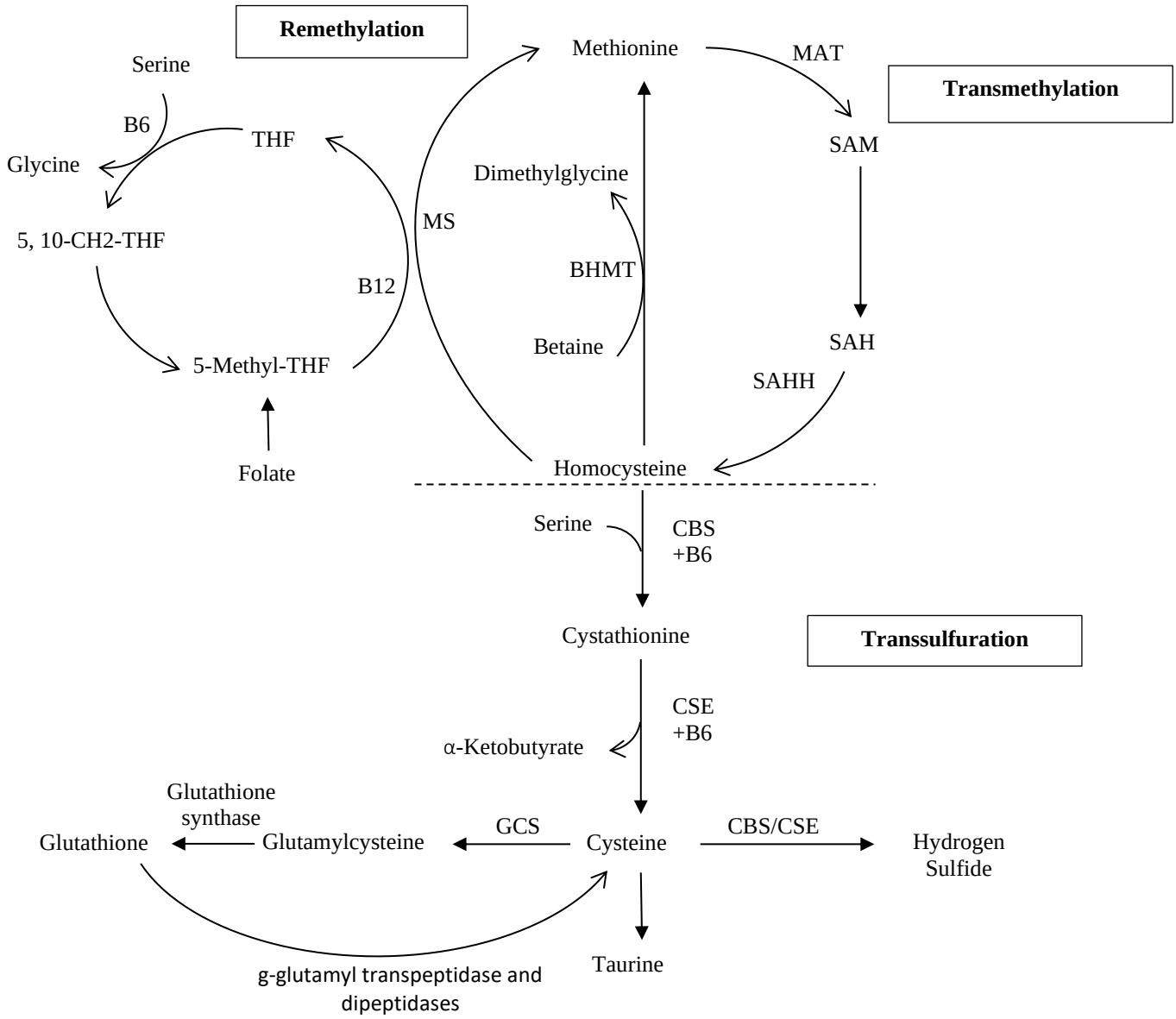

Figure 2.3. Sulfur amino acid metabolism. MAT, methionine adenosyltransferase; SAM, S-Adenosylmethionine; SAH, S-Adenosylhomocysteine; SAHH, S-Adenosylhomocysteine hydrolase; MS, methionine synthase; BHMT, betaine homocysteine methyltranferase; 5-MethylTHF, N^5-methyl-tetrahydrofolate; THF, tetrahydrofolate; CBS, cystathionine beta-synthase; CSE, cystathionine gamma-lyase; GCS, gamma-glutamylcysteine synthase.

2.7.1.2 Remethylation

Hcy is a substrate for the re-synthesis of Met by accepting a methyl group. Hcy receives this methyl group from N^5-methyl-tetrahydrofolate (5-methyl-THF) or from betaine to form methionine (148). The methyl group from 5-methyl-THF is synthesized in the folate coenzyme system catalyzed by methionine synthase (MS) (EC 2.1.1.13), which is regulated by SAM and SAH (147, 151). Betaine (trimethylglycine) acts as a methyl donor to convert Hcy to Met via the enzyme betaine-homocysteine methyltransferase (BHMT) (EC 2.1.1.15), with dimethylglycine released as a by-product. Met metabolism provides a notable example of the role of co-factors in cell chemistry. MS uses methylcobalamin, known to require vitamin B12, as a prosthetic group (151). Methylenetetrahydrofolate reductase (MTHFR) (EC 1.5.1.20), provided from the folic acid 1-carbon pool, reduces 5,10-methylene-THF to 5-methyl-THF (148). As well, cystathionine β-synthase (CBS) (EC 4.2.1.22) and cystathionine γ-lyase (CSE) (EC 4.4.1.10), both enzymes in the TS pathway, contain pyridoxal phosphate. Therefore, deficiency in vitamin B12, folic acid, riboflavin and pyridoxine are associated with increased plasma Hcy concentration (>15 μM/L) (152). Glycine is also directly involved in Met metabolism as a substrate for glycine N-methyltransferase (153).

2.7.1.3 Transsulfuration

Hcy is also involved in the production of Cys via the unidirectional TS pathway. The conversion of Hcy to Cys occurs in two phases by the action of pyridoxal-5-phosphate (PLP)-dependent enzymes (151). First, Hcy is combined with serine by the action of CBS, producing cystathionine. Second, CSE converts cystathione to Cys, alpha- ketobutyrate and ammonia (147, 151). The term TS derives from the fact that the S group of Hcy (and thus of Met) is transferred

to serine to yield Cys. The carbon skeleton of Met is in the alpha-ketobutyrate compound. The TS pathway is highly active in a restricted number of tissues, such as liver, kidney, small intestine and pancreas (146) and plays an indispensable role providing Cys for GSH synthesis, resulting in an increased oxidative defense (147).

The indispensable amino acid Met has many fates including incorporation into polypeptide chains and use in the production of Cys and α-ketobutyrate via the reaction pathway involving the synthesis of SAM and cysteine (154). Additionally, through SAM, Met is involved in methylation of DNA and proteins, the synthesis of spermidine and spermine, and in gene expression via its role as a methyl donor (12, 155).

2.7.2 Cysteine

Cys metabolism is related to the inflammatory response, given its role as a precursor of GSH, H_2S and taurine. Cys consider as a semi-indispensable amino acid as it is obtainable by two pathways: 1) Synthesis from exogenous Met via the combined action of the TM and TS pathways, or 2) dietary intake. Increasing blood and/or intracellular GSH concentrations with Cys derivatives (i.e., N-acetylcysteine; NAC) or Cys-rich dietary proteins have been shown to influence thiol redox status (156, 157). Dietary Cys improves glucose regulation and insulin resistance caused by sucrose (158-160). A few studies have found that Cys supplementation at dietary levels reduces post-exercise oxidative stress in human subjects (161-163). However, an insufficient intake of SAA can result in a lack of Cys which can induce a pro-inflammatory state (155, 164).

2.7.2.1 Glutathione

In 1922, Sir Frederick Gowland Hopkins was the first individual who proposed that GSH is formed by glutamine, Cys and glycine (16, 165). GSH is well known because of its important role in oxidation-reduction reactions. Additionally, GSH is involved in several functional roles such as leukotriene synthesis, modification of proteins, and detoxification of methylglyoxal (16). Thus, GSH most important functions are associated with the prevention of oxidative damage.

GSH is formed by a tripeptide, γ-glutamyl-cysteinyl-glycine and has different roles such as redox buffer in cell, storage of cysteine and regulation of apoptosis. As a redox storage, GSH is oxidized to GSSG by GPx, then GSSG is reduced back to GSH by glutathione reductase (EC 1.6.4.2) (166). In this reaction, one of glutathione's functions is to supply the reductant to preserve an acceptable pool of decreased glutaredoxin, a glutathione-dependent reductase that, for example, catalyzes reversible protein S-glutathionylation to preserve and influence the cellular redox state and redox-dependent signalling pathways (167) for ribonucleotide reductase (EC 1.17.4.1) (166).

In addition, GSH offers an intracellular pool of Cys, which is generally unstable extracellularly in its reduced form, as a result of oxidation of Cys to the disulphide cystine (166). The synthesis of GSH occurs in the liver and begins with the action of L-glutamate cysteine ligase (GCL) (EC 6.3.2.2), which plays a fundamental role in the flux of Cys to GSH (16, 168). After the reaction of GCL, the amino group of glycine is attached to the carboxyl group of Cys by glutathione synthase (GSS) (EC 6.3.2.3), forming GSH. Cys is spared during sepsis to synthesize GSH, which may be more needed in response to sepsis-induced oxidative stress. Given that GSH synthesis is one of the Cys' metabolic uses, a decrease in Cys consumption may increase the synthesis of APP.

2.7.2.2 Hydrogen sulfide

Cysteine has an important role as a precursor of H_2S, a gasotransmitter that has a neuromodulatory function in the central nervous system as well as both anti-inflammatory and antioxidant properties (166). Depending on the tissue, there are different pathways to form H_2S. The first pathway to form H_2S is by the action of CBS in cystathionine synthesis; the second pathway is by the reaction of cystathionase in the synthesis of thiocysteine, pyruvate and ammonium. The third pathway to obtain H_2S is by the action of aspartate transaminase to obtain 3-mercaptopyruvate (168). Recent studies have demonstrated an antioxidant function of H_2S inhibiting cellular damage and cellular oxidation induced by hypochlorous acid (HOCl), peroxynitrite (ONOO) and NO (169).

2.7.2.3 Taurine

Another role of Cys is as a precursor of taurine which can be provided as well in the diet especially by fish and seafood (166). Taurine is synthesized by oxidation of the sulfhydryl group of Cys to form cysteine sulfinic acid, followed by decarboxylation by cysteine sulfinate descarboxylase (EC.4.1.1.29) to form hypotaurine and ending with oxidation by hypotaurine dehydrogenase (EC 1.8.1.3) to taurine (168).

As the most abundant semi-essential sulfonic acid in animal tissues (more than 50% in muscle) (151, 170, 171), taurine has many roles such as antioxidant and membrane stabilizer, an antisympathetic effect in blood pressure, is involved in lowering the reabsorption of bile salts in patients with cystic fibrosis (172) and in immunomodulation (121). Taurine is well known for its antioxidant and anti-inflammatory properties and is therefore able to counter the mechanisms implicated in several diseases (16). Inactivation of HOCl, an oxidant produced by the MPO-

halide mechanism, is the most known antioxidant activity of taurine (173). This behaviour explains taurine's anti-inflammatory properties, as its HOCl reaction results in taurine chloramine (TauCl) generation, a more soluble and less harmful anti-inflammatory mediator (174).

2.8. Sulfur amino acids and inflammation

Sulfur amino acid requirements are increased during injury, stress, or inflammatory states. An adequate intake of dietary SAA is important for the synthesis of proteins of the immune system (12), regulating oxidative stress (13) and the body's response to injuries. The SAA have been shown to play key roles in the acute inflammatory response **(Table 2.5)** with putative mechanisms linked to the antioxidant functions of their metabolites (14, 15). Therefore, the objective of the current review was to investigate the role of SAA in the acute inflammatory response.

2.8.1. Methionine on the acute inflammatory response

Methionine is responsible for the synthesis of succinyl-CoA, Hcy and Cys (175). It is also responsible for the biosynthesis of SAM, which is implicated in polyamine, creatine, and phosphatidylcholine metabolism (176). Met is catalyzed by MAT to form SAM, which it is known as the methyl donor for most of the methyltransferase reactions that alter DNA, RNA, and other compounds, including proteins (175). SAM increases the CBS activity, which is one of the primary enzymes in the TS pathway and participates in the synthesis of Cys, thus increasing GSH concentrations (17). The reduction of oxidative stress by SAM administration have been investigated (12). For instance, Li et al.(12) found that the administration of SAM

Table 2.5. Anti-inflammatory properties of sulfur amino acids.

Sulfur amino acid metabolites	Anti-inflammatory properties
Methionine	Methyltransferases reaction donor (175). Increase synthesis of GSH through CBS activity (17). Protects cell damage from ROS via the methionine sulfoxide reductase system (16). Synthesis of phosphatidylcholine and acetylcholine (12, 46).
Cysteine	Synthesis of GSH, H_2S and taurine (13). Cys metabolite NAC regulates NF-κβ activity (18, 177). Modulates lymphocyte and macrophage functions (178, 179).
Glutathione	Protects cells from ROS (16). Controls cytokine production (TNF-α and NF-κβ) and decreases oxidant molecules (16, 121). Activates AP-1 (180). Modulates antigen-processing T-helper cells (12). Decreases COX-2 gene expression (181).
Hydrogen Sulfide	Reduces oxidative strss by enhancing the activity of γ-glutamylcysteine synthetase and cystine transport (182). Facilitates vasorelaxation through stimulating the activity of KATP channels (183-185). Reduces leukocyte adherence (186, 187) Decreases pro-inflammatory cytokine activity, neutrophil apoptosis and COX-2 expression (186, 188-191).
Taurine	Modulates NO, oxygen radicals and peroxynitrite formation (192). Inhibits production of IL-6, TNF-α, prostaglandins like PGE_2 (193). Reduces ROS production (194). Restores SOD and GPx function (195).
Vitamin B6	Required in the synthesis of cystathionine and Cys (135). Modulates superoxide radicals (136). Reduces Hcy, CRP and MDA concentration (138). Reduces risk of rheumatoid arthritis (125).
Folate	Supplies methyl groups for SAM renewal (196, 197). Assists in the antibody response to antigens (198).

[1]CBS, cystathionine beta-synthase; SAM, S-adenosylmethionine; GSH, glutathione; H2S, hydrogen sulfide; NAC, N-acetylcysteine; NF-κβ, nuclear factor-kappa beta; COX-2, cyclooxygenase-2; IL, interleukin; TNF-α, tumor necrosis factor-alpha; NO, nitric oxide; PGE2, prostaglandin-2; ROS, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxide; Hcy, homocysteine; CRP, C-reactive protein; MDA, malondialehyde

reduced oxidative stress and demonstrated cell protective effects by activating the endogenous antioxidant system induced by amyloid-β. They also observed that SAM administration restored the GSH:GSSG ratio and increased the synthesis of GPx, glutathione-S-transferase (EC 2.5.1.18) (GST), and SOD (17). Under physiological conditions, Met is an efficient scavenger of almost all oxidizing molecules including hydrogen peroxide (H_2O_2), hydroxyl radicals, peroxynitrite, chloramines and hypochlorous acid (199). During the presence of ROS damage, Met is converted to methionine sulfoxide (MetO), which deactivates certain protein activities, resulting in an equal mixture of two stereoisomers methionine-S-sulfoxide (MetO-S) and methionine-R-sulfoxide (MetO-R) (199). The methionine sulfoxide reductase system (MSR) reduces MetO back to methionine, therefore this system has an important role protecting cells from oxidative damage (16). The S and R forms of MetO can be reduced back to Met by methionine sulfoxide reductase A (Msr-A) and B (Msr-B), respectively. Each cycle of Met oxidation and reduction will destroy one equivalent of ROS (200). Furthermore, Yermolaieva et al. (201) observed that in PC12 cells, which is a cell line derived from the rat adrenal medulla, the overexpression of Msr-A maintained normal growth, significantly decreased hypoxia and increased ROS activity.

Met plays an essential role in the immune system through a rise in the synthesis of GSH, taurine, and other metabolites. Met administration can alter the host's defense response to oxidative stress or inflammatory states. On one hand, an increase in Met administration has an impact on the pathway flux, with a priority in TS pathway flux over the TM pathway (202, 203). Campbell et al. (204) observed that increasing Met supplementation altered the oxidative activity in a section of the pentose phosphate pathway (PPP). The PPP controls oxidative stress regenerating GSH from GSSG via the production of nicotinamide adenine dinucleotide

phosphate (NADPH). On the other hand, Met restriction led to a reduction in oxidative stress by enhancing the synthesis of GSH (203, 205).

Furthermore, Met is a precursor to choline and thus phosphatidylcholine and acetylcholine, which are necessary for nerve function and leukocyte metabolism.(12, 46). High dietary intake of Met (between 1.5 and >6 g/day) raises plasma Hcy concentrations (206). Verhoef et al. (207) compared fasting and postprandial plasma Hcy concentrations after healthy men consumed a low-protein diet with 1.7 g of Met accompanied by a high-protein diet (breakfast, lunch, and dinner) with 4.5 g of Met in a dietary controlled crossover study. For several hours after dinner, postprandial Hcy increased steadily on the high-protein diet, from ~9 to 11 μmol/L. The increase of plasma Hcy concentrations have been associated with activation of the immune system due to the fact that Hcy increases the adhesion of monocytes to endothelial cells (107).

2.8.2. Cysteine on the acute inflammatory response

Like the Met metabolites, Cys metabolites are also susceptible to oxidation. The antioxidant properties of Cys metabolites are due to their capacity to react with H_2O_2 (208, 209). The TS pathway of Cys include protein synthesis, as well as synthesis of GSH, H_2S, cysteine sulfinate, taurine, pyruvate, and inorganic sulfur. Through the synthesis of GSH, H_2S, and taurine, Cys can regulate nutrient metabolism, oxidative stress, physiologic signalling pathways, and diseases associated with them (17).

Interestingly, during stress conditions or inflammatory states, Cys is considered as "conditionally indispensable" because the requirement of Cys increases (200). In such situations, the body's capacity to synthesize Cys is incapable to meet Cys demands. For

instance, it was observed that an increase in GSH synthesis (40% of the increase Cys demand) in several tissues (liver, spleen, lung, muscle etc.) occurred in rats with acute infection (200). Furthermore, Cys is also responsible for taurine production and acute-phase proteins implicated in the immune response in the liver of septic rats (15, 210). Cys and cystine (the disulfide molecule formed during the oxidation of two Cys residues) ratio plays an important role in regulating the redox potential, production of other active substrates (i.e., GSH, H_2S, and taurine), ROS, and inflammatory responses (211-213). The administration of SAA alters cell signaling in the postprandial period through regulating intracellular concentrations of Cys and cystine, as well as via changes in the cysteine/cystine redox state (214).

Cys and Cys metabolites like, N-acetylcysteine, or N-acetylcysteine amide (NAC) can regulate the activity of NF-κβ (18, 177, 215, 216), which induces the expression of many proinflammatory genes. The latter genes include those for cytokines and chemokines that are implicated in cell survival and proliferation, and in the control of immune and inflammatory responses (178, 179). The consumption of Cys-rich diets has demonstrated similar results in the production of several APP in comparison to rats fed with methionine-rich diets following injection with TNF-α, due to the fact that Cys has an important role in the immune response (217). The cysteine/cystine redox couple has been proven to work as an extracellular thiol redox buffer (i.e., central plasma redox pool) and a cell function modulator. *In vitro* studies showed that the extracellular cysteine/cystine redox potential influences the development of ROS and expression of cell adhesion molecules in endothelial cells (218) and regulates ROS-induced apoptosis in endothelial cells (219, 220).

The cysteine/cystine redox potential results as a thiol/disulphide redox couple were independent of intracellular GSH status in these experiments, indicating that the extracellular

redox environment plays a unique role in cell function (221). Furthermore, Cys and Cys metabolites such as NAC have been shown to modulate lymphocyte and macrophage functions during *in vivo* studies (222). In postmenopausal women, supplementation of NAC (600 g/day) for 2-4 months significantly improved lymphocyte and neutrophil function, suggesting that NAC has a modulatory effect on the immune system (222). NAC increases intracellular GSH content of postmenopausal women's leukocytes with no changes in plasma concentrations thus decreasing plasma malondialdehyde (MDA) concentrations.

Dietary SAA intake influences plasma GSH concentrations. When rats were fed diets with different SAA contents, 7 molecules of cysteine were incorporated into GSH for every 10 incorporated into protein in the liver, according to an isotopic analysis (5). The ratio dropped to 3:10 when intakes were insufficient. This response to an insufficient intake of SAA is undesirable since antioxidant defences are reduced. In comparison to GSH redox potential, the cysteine/cystine redox potential was affected by both a decrease and an increase in the diet's SAA content in rats (223). These findings provide new evidence of Cys and dietary SAA's importance in controlling cell function through redox regulation of the extracellular environment.

2.8.3. Glutathione (GSH) on the acute inflammatory response

GSH is implicated in inflammatory responses given its role as the main thiol antioxidant (6, 12, 16), because it inhibits the production of several inflammatory cytokines and chemokines, such as TNF-α (224-226). However, GSH also is essential for several immune functions including IL-2 production, cytotoxic T-cell activity (227-230). Furthermore GSH synthesis minimizes excess amounts of endogenous and exogenous electrophilic xenobiotic compounds (231), metabolism of prostaglandins and leukotrienes (232), and acts as a cellular Cys reserve (106).

As an antioxidant, GSH prevents oxidative damage by acting as a free radical scavenger protecting the cells from ROS (16) via scavenging hydroxyl radical and superoxide directly, and serving as a cofactor for the enzyme GPx in metabolizing H_2O_2 and lipid peroxides (233).

Another function of GSH is to decrease the stimulatory influence of oxidant molecules and TNF-α on NF-κβ activity, thereby controlling cytokine production (16, 121). Furthermore, intracellular concentration of GSH prevents the activation of NF-κβ pathway by oxidants produced during the inflammatory response and, during immune challenges, activates activator protein-1 (AP-1) which is strongly associated with cell proliferation (16, 180).

An increase in thiol antioxidants and GSH-repleting agents (i.e., vitamin B6, riboflavin, folic acid, and NAC) increases neutrophil filtration and improves survival during sepsis (234), which ultimately protects the surrounding tissue by reducing the damaging influence of cytokines including IL-1, IL-8 and TNF-α and inhibiting Kupffer cell NF-κβ activation (16). Villa et al (235) observed that in a mouse model of polymicrobial sepsis induced by cecal ligation and puncture (CLP), depletion of GSH reduced peritoneal neutrophil infiltration, and decreased survival. However, the opposite effect was observed with oral supplementation of NAC (1g/kg) 45 minutes before CLP. Glutamine can increase the production of GSH by increasing the activity of intestinal glutaminase (236). As well, there´s evidence that GSH concentrations modulate antigen-processing cells, such as T-helper cells, hence a deficiency in GSH in CD4 cells may lead to a decrease survival in HIV infected individuals (237), which have an important role in the immune system, reducing the production of IFNγ and T-cell activity (12, 16).

Deng, et al (108) studied alterations of the antioxidant activity in transgenic sheep by the TLR4 pathways. They observed a negative correlation between GSH/GSSG ratio and TLR4

copy numbers in transgenic sheep were intradermally injected with LPS in each ear. Moreover, decrease in the GSH/GSSG ratio can cause redox imbalance and is associated with a variety of human diseases including diabetes and renal failure (238). Studies have also shown that a rise in cellular GSH content was accompanied with an increase in IL-2 production, with a decrease in PGE2 in pretreated pulmonary endothelial cells either with NAC or glutathione ethyl ester (GSE) (109, 227-229, 235). This may be due to an inhibition of the mitogen activated protein kinase MAPKs (p42/44) resulting in a decrease of COX-2 gene expression stimulated by LPS (181).

2.8.4. Hydrogen sulfide (H_2S) on the acute inflammatory response

Another product of Cys that has shown an effect on reducing inflammation is H_2S. H_2S, once considered a toxic gas, in recent years has been recognized for its anti-inflammatory properties in the body (239). Cys is one of the main sources that contribute about 70% of H_2S through CBS and CSE enzymes (240). Anti-inflammatory effects of H_2S include reduced leukocyte adherence to the endothelium (186, 187), decreased pro-inflammatory cytokine activity, neutrophil apoptosis, (189) and regulation of COX-2 expression (186, 188, 190, 191). Inhibition of H_2S synthesis contributes to leukocyte adherence because of H_2S functions as a tonic down-regulator of leukocyte adherence (187). Treatment of rats with inhibitors of H_2S synthesis resulted in a significant increase in mucosal inflammation (elevated granulocyte levels) and increased susceptibility to injury (241-243) in the gastrointestinal tract. This may be attributed to lower basal levels of COX-2 expression and, as a result, lower mucosal PGE2 synthesis (244). COX-2 and PGE2 play important roles in maintaining mucosal defence and modulating mucosal inflammation in the digestive tract (188, 190, 191, 244, 245).

H_2S can reduce plasma exudation (edema formation) in addition to modulating leukocyte adhesion and recruitment, while H_2S inhibitors can increase edema formation caused by an inflammatory agent (242). In carrageenan and Freund's adjuvant paw swelling models was observed an increase (40%) in H_2S synthesis activity suppressing edema formation (241, 242). Furthermore, up-regulation of enzymes such as SOD, GPx, and thioredoxin reductase can mediate H_2S's antioxidant function, as demonstrated in rats with intestinal ischemia-reperfusion damage (246) or in Met-treated brain endothelial cells -mediated oxidative stress *in vitro* (247) or even NADPH oxidase (NOX) inhibition, as in the case of osteoblasts exposed to H_2O_2 *in vitro* (248). Additionally, H_2S can serve as a neuromodulator enhancing NMDA receptor-mediated responses (249) and save neurons from oxidative stress in the brain (250-254). In vascular smooth muscle cells and insulin-secreting cells, H_2S has been shown to stimulate KATP channels activity (183-185), thereby facilitating vasorelaxation and preventing insulin secretion (182-185, 255, 256).

2.8.5. Taurine on the acute inflammatory response

Taurine (2-aminoethanesulfonic acid, Tau) is an exceptionally abundant free sulfonic acid in the cytosol of inflammatory cells and especially in neutrophils (257) and it has been shown to have a protective effect against systems involved in inflammation (233). Taurine can act against damage caused to hepatocytes by endotoxins by modulation of NO, oxygen radicals and the formation of peroxynitrite (192). Two major sources in mammals contribute to taurine production: dietary absorption and the metabolism of SAA, mainly Cys. Taurine is synthesized as follow: 1) formation of cysteine sulfinate by formation of cysteine dioxygenase from Cys; 2)

formation of hypotaurine by the removal of cysteine sulfinate through cysteine sulfinic acid decarboxylase; and 3) synthesis of taurine by hypotaurine oxidation (148).

Taurine produces taurine chloramine (Tau-Cl) by the interaction with HOCI. Tau-Cl has been shown to have important immunomodulatory properties including the inhibition of the production of proinflammatory cytokines such as IL-6, TNF-α, prostaglandins like PGE_2 and oxide nitric, and the inhibition of the ability of antigen presenting cells to process and present ovalbumin (193). In several models induced by different oxidants, taurine demonstrates its antioxidant properties (194, 258). Taurine's antioxidant property is correlated with the scavenging of ROS. Chang et al. (194) showed that rats with toxic concentrations of Hcy (133.51±27.91μmol/L) also called hyperhomocysteinemia (HHcy) reduced the production of ROS after taurine supplementation, and Palmi et al. (259) stated that taurine stimulated the mitochondrial Ca^{2+} absorption by inhibiting ROS activity. Moreover, taurine also enhances the activity of certain antioxidant enzymes in oxidant-induced models. Furthermore, in mice mitochondrial taurine has been reported to restore the function of SOD and GPx following intraperitoneal injection with tamoxifen (195). In addition, Choi and Jung (260) reported that in a calcium-deficient mice, taurine supplementation improved hepatic SOD activity, but no significant differences were observed in GPx and catalase activities between control and experimental mice.

SAA metabolites play an important role protecting against oxidative stress including reduction of ROS production and inhibition of proinflammatory cytokines like TNF-α and cell infiltration. Furthermore, SAA decreased NO production and down-regulated COX-2 expression in macrophages. Adequate dietary provision of SAA is necessary for sustaining normal immune

response and protecting the host from a variety of diseases paying special attention to folic acid and vitamins B6 and B12.

2.8.6. B-vitamins on the acute inflammatory response

Numerous studies have shown the antioxidant effect of B-group vitamins, such as vitamins B1 (thiamine), B2 (riboflavin), vitamin B6 (pyridoxal phosphate) and B12 (cobalamin) during the acute phase of inflammation. Furthermore, vitamins B6, B12 and folate are related as cofactors in homocysteine metabolism, with the latter known to act as a pro-inflammatory factor (125).

The activity of vitamin B6, a water-soluble vitamin, is linked to three related compounds, namely pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), and their phosphorylated forms. Vitamin B6 acts as a cofactor in the synthesis and utilization of carbohydrates, lipids and proteins (261). It is also involved in sulfur amino acid metabolism in its active form as PLP (135) and has an anti-oxidative capacity modulating many ROS such as superoxide radicals (136). For instance, in terms of its ability to quench superoxide radicals, PL at a concentration of 1mM was equivalent to one unit of SOD (136).

PLP has an important role in two important pathways within the methionine cycle: Transsulfuration and remethylation. The PLP-dependent enzymes CBS and CSE are implicated in the transsulfuration pathway condensing Hcy and serine to form cystathionine (262) and hydrolyzing cystathionine to form Cys and α-ketobutyrate, respectively. In the remethylation pathway Met is re-synthetized from Hcy by accepting a methyl group from N^5 -methyl-THF in the folate coenzyme system (148). Therefore, a methyl group from serine needs to be transferred to tetrahydrofolate in order to form N^5-methyl-THF. This reaction is mediated by a PLP-dependent serine hydroxymethyltransferase (EC 2.1.2.1) (148, 199).

Increases in vitamin B6 intake have been shown to have Hcy-lowering effects thereby reducing oxidative damage (125). In one study, patients with acute ischemic stroke received an oral supplementation of B-group vitamins for 14 days. As a result, plasma concentration of CRP, Hcy and MDA were significantly lower in patients with oral supplementation of B-group vitamins (138). Besides, vitamin B6 levels are inversely associated with TNF-α production by peripheral blood mononuclear cells in patients with rheumatoid arthritis (125).

Vitamin B12 is closely connected with the metabolism of Hcy. Inadequate concentrations of B12 can result in HHcy (263). Vitamin B12 plays an important role in the immune system as an immunomodulator for cellular immunity, facilitating the production of T-cells (198), such as cytotoxic T-cell (264, 265) and regulating the T-helper cells and cytotoxic T-cells ratio (265). Additionally, endogenous synthesis and metabolism of vitamin B12 is involved in the synthesis of antibodies (198, 265). Reduced CD8+ cell levels, as well as a high CD4/CD8 ratio and suppressed NK cell activity, were found in patients with B12 deficiency (266). Vitamin B12 is required for cell replication and division, which could explain why it affects rapidly proliferating immune cells.

Humans are incapable of synthetizing folate *de novo*. Yet, folate can be converted from dihydrofolate reductase (DHFR) to tetrahydrofolate (THF) and polyglutamylated folates due to activity of the enzymes DHFR and folylpolyglutamate synthase (FPGS) (267, 268). Humans are therefore almost entirely dependent on their folate supply from diet, provided that the intestinal microbiome has a marginal contribution to the folate pool (269, 270). Dietary folate, B12 and betaine supplementation can increase the RM pathway through the folate cycle and BHMT (271). Folate, and its metabolites, have several properties, including their essential role for DNA

synthesis and for the supply of methyl groups to proteins due to their need for SAM renewal (196, 197).

Certainly, the role of SAA in the acute inflammatory response regulating cell infiltration and pro-inflammatory cytokines has been reported. However, studies looking at inborn errors of sulfur amino acid metabolism also offer helpful information regarding both normal metabolic pathways and potential pathological mechanisms when SAA metabolism is impaired.

2.9 Impact of impaired sulfur amino acid metabolism on inflammation

Impairment of SAA metabolism has been shown to impact acute inflammatory responses **(Table 2.6)** due to imbalances in the TM and TS pathways, resulting in an impaired immune response during inflammation (272) and a deficit of certain antioxidant metabolites such GSH (273) or taurine (12). Disorders of Met metabolism are caused by impairments in the synthesis of the essential cobalamin coenzyme, as well as deficiency of methionine synthase reductase or methylenetetrahydrofolate reductase (274). In terms of Met metabolism, all these deficiencies result in an increased homocysteine concentration (HHcy), reduction of Met accumulation, reduced SAM and higher SAH concentrations. An elevated flow through the TS pathway can result in an increase in cystathionine concentrations, but high concentrations of Hcy show that the TS pathway alone is not adequate to eliminate the cystathionine formed (146).

An increased intake of Met, inadequacy of folic acid, vitamins B6 and B12, and a genotype that results in reduced recycling of Hcy to methionine are all conditions that enhance plasma Hcy concentration in the blood and urine (107). One of these errors in the Met pathways is due to a homozygous deficiency in CBS, resulting in homocystinuria (HCU) (146).

2.9.1. Methionine impairment

In adult subjects with reported B12 or folate deficiency, serum Met was not lower than in controls, contrary to low values in those with inborn remethylation errors (275). Compared to the standard upper-normal ranges of ~40 μmol/L, serum Met concentrations are often elevated in

Table 2.6. Impact of impairment sulfur amino acids on the acute inflammatory response.

Sulfur amino acid impairment	Impact on acute inflammatory response
High methionine	Dietary supplementation of Met induces HHcy (146). High serum concentrations of Met found on chronic liver disease (276).
Hyperhomocysteinemia	Increases H_2O_2 (277). Induces production of IL-6, TNF-α, IL-1β and ROS (278). Increases acute phase protein concentration like CRP (279). Increases cholesterol, triglycerides and LDL concentration increasing risk of CAD (280). Increases alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), blood urea nitrogen (BUN) production causing liver damage (281). Promotes development of atherosclerosis, CAD and ischemic stroke (282).
CBS deficiency	Induces homocystinuria (146). Reduces synthesis of antioxidants Cys and sulfate (283). Increases risk of CAD decreasing apolipoprotein A (284, 285). Increases IL-6, TNF-α, IL-1β concentration (286).
MAT I/III deficiency	Induces HHcy (276). Increases Met and cystathionine concentration which enhances the risk of cardiovascular death (287).
GNMT deficiency	Induces moderate HHcy (288). Moderate increases serum transaminases and hepatomegaly (146).
CSE deficiency	Induces cystathionuria (289). Induces mild to moderate HHcy (290). Attenuates liver damage increasing ALT and AST production (289). Increases lung injure (291). Higher risk of developing CAD by increasing cholesterol and LDL concentrations (290).
B6 deficiency	Induces HHcy (292). Increases CRP concentration (152). Increases risk of CAD increasing APP (293). Impaired lymphocyte maturation and growth (294). Decreased IL-2 production (265).
B12 deficiency	Supresses NK cell activity (265, 266, 295). Decreases T-cell proliferation (296). Decreases number of lymphocytes (265).
Folate deficiency	Induces HHcy (292). Reduces erythrocyte development, resulting in megaloblastic anemia (297). Increases risk of CAD and stroke (280, 298, 299). Associates with multiple types of cancer including colorectal, prostate and pancreatic (300-302). Induces neural tube defects (287, 303, 304).

[1]CBS, cystathionine beta-synthase; MAT, methionine adenosyltransferase; GNMT, glycine N-methyltransferase; CSE, cystathionine gamma-lyase; HHcy, hyperhomocysteinemia; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; IL, interleukin; TNF-α, tumor necrosis factor-alpha; CRP, C-reactive protein; ALP, alkaline phosphatase;

ALT, alanine aminotransferase; AMY, amylase; TBIL, total bilirubin; BUN, blood urea nitrogen; CAD, cardiovascular artery disease; LDL, low density lipoprotein; NK, natural killer.

chronic liver disease (305). Mudd et al. (306) analyzed data from 33 studies of chronic liver disease, finding elevated mean values for specific groups and upper concentrations of serum Met (>200 μmol/L) in about a third of the cases. In liver disease, TM and TS are likely to be impaired since the metabolic utilization of a Met load is decreased (307), and mRNA for many of the enzymes involved in Met metabolism is reduced in cirrhotic livers (308-310). High Met diets on infants can become hypermethioninemic (305). In 2003, a group of nine children fed with a protein hydrolysate formula fortified with Met experienced severe hypermethioninemia (>1000 μmol/L) (311). Many of the infants were screened for CBS deficiency, even though the associated Hcy elevations were mild in most cases (311). Extreme hypermethioninemia can lead to edema in the brain. Plasma Met concentrations do not rise in renal failure, unlike other SAA. According to a recent study, Met requirements in children with renal dysfunction are higher, while cysteine requirements are comparable to healthy children (312).

2.9.2. Cysteine impairment

Increased Cys concentrations in the serum/plasma have been associated with chronic renal disease (313, 314). In a population of 1765 Chinese people with normal baseline renal function, higher Cys concentrations showed a higher incidence (p<3.8E-03) of decreased renal function over six years (314). Obesity and metabolic syndromes can also be linked to increased plasma/serum Cys concentrations (315). Patients with Down syndrome with three copies of CBS are often obese (315). Epidemiology from Norway's broad Hordaland cohort, plasma Cys

correlates with fat mass and body mass index (BMI) and predicts weight gain (315). Since CSE activity may be low at birth, Cys concentrations were lower in infants with respiratory distress than in control infants (316).

2.9.3. Cystathionine impairment

Standard amino acid analysis can detect cystathionine, but average concentrations in human serum (44– 342 nmol/L) are below detection limits (317). Most B12- and folate-deficient patients had elevated cystathionine concentrations, according to more sensitive and precise stable isotope dilution GC-MS assays (275). In animal models of vitamin B6 deficiency, cystathionine concentrations increased in a graduated manner (318). Cystathionine levels are higher in alcoholic liver disease and linked to fibrosis in liver biopsy samples (319). This increase may be attributed to pyridoxal phosphate deficiency caused by alcohol or a dietary vitamin B6 deficiency. Cystathionine is a precursor for the synthesis of H_2S and can help the endothelium (320). Renal dysfunction causes a rise in cystathionine (313). While high cystathionine concentrations are linked to an increased risk of cardiovascular death, it is difficult to distinguish the harmful effects of coexisting chronic kidney disease since cystathionine concentrations are significantly affected by renal function (321).

2.9.4. Cystathionine β-synthase deficiency

CBS deficiency has been shown to reduce the synthesis of Cys and sulfate from Met, thereby providing the distinctive function of the TS pathway (283). Additionally, the RM pathway is not able to catalyze the Hcy formed, resulting in a reduction of betaine and methyltetrahydrofolate by MS and BHMT, respectively (146).

2.9.5. Cystathionine γ-lyase (CSE) deficiency

Deficiency in CSE results in primary or congenital cystathionuria (289). CSE-deficiency is a model that presents with mild to moderate HHcy, and has been used as a model to investigate the development of inflammatory responses under several stressors like atherosclerosis (290), hepatitis (322) and chronic heart failure (323). However, inhibition of CSE also has positive impacts on inflammatory states. Pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, and nitric oxide synthase (NOS) synthesis were significantly reduced in the plasma of CSE deficient mice after burn improving liver function (289). CSE deficiency reduced plasma alkaline phosphatase (ALP) and alanine aminotransferase (ALT) concentration after 24 hours, and burn failed to increase amylase concentration after 24 hours in CSE deficient mice (289).

2.9.6. MAT deficiency

Hypermethioninemia is a consistent characteristic of MAT deficiency. MAT II is responsible for the synthesis of SAM, which is needed for critical SAM-dependent reactions in liver and extrahepatic tissues (276). Notably, MAT deficiency alters Met synthesis's mechanism, resulting in the body's inability to keep normal Met concentration in tissues. In patients with MAT I/III deficiency methionine transamination products are increased; however, there is no evidence of damage to the myelin sheath surrounding nerve fibres in the brain, optic nerves and spinal cord (dymyelinating syndrome) (283). Some studies indicate that Met synthesis in MAT II/III deficient patients enhances Met conservation regardless of a marked improvement in Met concentration in tissue (324, 325). In addition, MAT deficient patients demonstrated that Met can enhance plasma cystathionine by inhibition of cystathionase (326).

2.9.7. Glycine N-methyltransferase (GNMT) deficiency

Glycine N-methyltransferase (GNMT) deficiency has an impact on the TS pathway as evident by a marked increase in several SAA metabolites(146). GNMT-deficiency inhibits MAT II in extrahepatic tissues by increasing Met concentration (327). Additionally, cystathionine concentrations are elevated through GNMT-deficiency. Increased cystathionine can derive from SAM activation of CBS along with the Met inhibition of CSE (326, 328). Hcy and SAH concentrations are moderately increased with a deficiency of GNMT; however, this modest increase remains to be clarified (288). Moreover, a few reports observed moderate increases of serum transaminases and hepatomegaly in patients GNMT-deficient when Met intake was 5 times the mean recommended (329, 330).

2.9.8. Vitamin B deficiency

Disorders in the TS pathway may occur when there is a deficiency in the status of B vitamins, particularly vitamins B6, B12 or folate (292), resulting in HHcy. Low vitamin B6 status has been shown to be a risk factor for the development of CAD (293, 331, 332). The enhanced risk associated with CAD may appear as a result of an increased concentration of LDL and CRP in patients suspected of having CAD (152).

Increased plasma cystathionine in humans (333-335) and rats (275) during moderate vitamin B6 deficiency has been shown to influence the TS pathway. In rats, cystathionine concentrations in the liver and muscle both rise simultaneously (336, 337). When isolated cells were cultured at moderate vitamin B6 deficiency, they developed more cystathionine than when cultured at severe deficiency (338). Since CBS and CSE have similar PLP characteristics (20), cystathionine accumulation has been explained by a higher turnover rate for CSE than for CBS

due to loss of CSE apoenzyme at low PLP concentrations (337). The accumulation of cystathionine during vitamin B6 deficiency can maintain Cys flux, ensuring enough Cys to prevent GSH depletion (334, 337). This explains why, during mild to moderate vitamin B6 deficiency, vitamin B6-dependent changes in TS do not reduce GSH content in the liver (337). In contrast, dietary vitamin B6 deficiency increases GSH concentrations in human plasma (334) and rat liver (337).

During dietary vitamin B6 restriction, isolated cells displayed impaired synthesis of H_2S and the H_2S biomarkers homolanthionine and lanthionine (339). Moderate, short-term vitamin B6 deficiency, on the other hand, did not affect plasma homolanthionine and lanthionine concentrations in healthy subjects for unclear reasons (340). The mechanisms by which the TS enzymes' products, H_2S and Cys, modulate the inflammatory response are complicated (22). Sulfhydration (persulfidation), which involves altering Cys groups of various target proteins by converting an -SH group to a more reactive -SSH group, has recently received much attention (341). The sulfhydration of the transcription factor NF-κβ, as well as the sulfhydration of a protein that sequesters Nrf2, can allow H_2S to modulate inflammatory responses (341).

MTHFR deficiency is associated with high plasma Hcy concentrations, low or low-to-normal Met concentration, and several clinical variations, with symptoms including gait disturbances, intellectual deficiencies, and isolated thromboembolic episodes (271). Vitamin B12 deficiency may trigger a demyelinating central and peripheral nervous system disorder that is inversely proportional to the severity of megaloblastic anemia (342, 343). Up to 10% of patients with pernicious anemia present purely neurologic symptoms and are commonly misdiagnosed (343). Hcy was elevated in 36 of 37 patients with neurologic disease due to vitamin B12 deficiency who had a normal hematocrit before treatment and dropped in the 30

patients who had posttreatment measurements (343). The treatment goal is lower Hcy while increasing Met and SAM concentrations, but the clinical benefit is unclear, and many aspects of treatment are still being tested. Oral folates, betaine or methionine, vitamin B12, and riboflavin are essential aspects of treatment (274). Homozygosity for a common polymorphism in the MTHFR gene increases the risk of thromboembolism slightly, mainly if dietary folate levels are low.

Inadequate dietary folate intake can contribute to a deficiency in folate status, a deficiency that has a wide pathophysiology. Folate deficiency causes a reduction of erythrocyte development, resulting in megaloblastic anemia (297). In 434 episodes of clinical folate deficiency, 97.5% of patients with megaloblastic anemia presented increased plasma Hcy concentrations (>58.3 mol/L) (344). Additionally, a deficiency in folate can result in vascular diseases, like CAD and stroke due to high concentrations of plasma Hcy (280, 298, 299). However, the most notable effect from low folate status is its adverse impact on neurulation. The effect of folate deficiency on neurulation is evident by the prevalence of neural tube defects (NTDs) like spina bifida, encephalocele and anencephaly in mothers with low folate status (287, 303, 304). Folate deficiency ha also been associated with multiples types of cancer, including colorectal (300), prostate (301), and pancreatic tumors (302)

2.10 Summary

Inflammation is the reaction of the immune system that involves blood vessels, immunological cells, and inflammatory mediators. Certain inflammatory markers involved in the acute phase are cytokines such as IFNγ, IL-6, IL-2, TNF-α and IL-1β. Cytokines play an important role in inflammation by regulating numerous cell functions including proliferation, differentiation, and

apoptosis. Additionally, cytokines also play a role in the synthesis of neutrophils, growth of neuropathic pain response, activation of NF-κβ, and tumor regression.

The treatment of inflammation requires the decrease of these cytokines; this requirement is considered the most critical one to resolve acute inflammation. However, treatments of inflammation are currently focused on the regulation of inflammation. Regulation of inflammation is an active process that involves mechanisms that lead to the eventual return of macrophages and lymphocytes numbers to basal levels. This requires modulation of cellular signaling pathways such as neutralizing TNF-α, blocking leukotriene receptors, and alteration of enzymatic function including inhibition of COX-2 synthesis, leukotriene synthetase and 3-hydroxy-3-methylglutaryl coenzyme A reductase, and agonism at protease-activated receptor 1 by activated protein C.

The role of dietary factors in the resolution of inflammation has been subject of considerable attention. Part of this attention can be attributed to the direct or indirect effects of nutrients and dietary pattern on components of the inflammatory response itself. For example, during inflammation the requirement for protein increases in order to promote synthesis of APP, modulation of immune function and redox status during an inflammation response.

The synthesis of proteins is, among other factors, mediated by an adequate intake of dietary SAA. Sulfur amino acids (Met and Cys) play an important role regulating ROS reactions and cytokine production through metabolites from transsulfuration pathway including Cys, GSH, H_2S and taurine. GSH is a tripeptide comprised of glutamine, cysteine and glycine that are involved in several anti-inflammatory reactions. These anti-inflammatory reactions include reducing cytokine activity such as TNF-α, inhibiting the activation of NF-κβ pathway by oxidants and activation of AP-1 during an immune challenge. A recent study conducted by Deng

et al (108) observed that the increased expression of γ-GCS is correlated with TLR4 copy numbers. Under oxidative stress conditions, γ-GCS increases GSH synthesis. GSH can be oxidized into GSSG to eliminate free radicals, thus avoiding damage induced by TLR4 pathways. Moreover, intracellular GSH regulates LPS-stimulated COX-2 gene expression and prostaglandin synthesis in cells within the initial activation of MAPKs (345). SAA play an important role in the regulation of pro-inflammatory markers such as production of TNF-α, IL-6, and PMN proliferation and oxidative stress including production of CRP and expression of COX-2. Dietary SAA requirements increase under stress conditions or acute infection; therefore, it is essential to better understand the importance of dietary intake of SAA in the control of oxidative stress and immune response.

CHAPTER 3

HYPOTHESES AND OJECTIVES

3.1. Hypotheses

i. Alteration in the SAA metabolism will affect the acute inflammatory response, induced tissue failure and increase health risks.

ii. Intraperitoneal injection of LPS at a dose of 100 µg/kg in Wistar rats will induce greater temporal changes in inflammatory markers and SAA metabolism compared to an IP LPS injection at a dose of 50 µg/kg.

iii. Dietary Met alone will be sufficient to attenuate the inflammatory response in IP LPS Wistar rats and maintain SAA metabolites concentration.

3.2. Objectives

The main purpose of this research was to investigate the role of SAA in the acute inflammatory response, particularly the Met-sparing effect of Cys via the TS pathway. The current research will also study the potential deleterious effect that impairment in SAA metabolism has on the immune response during SIRS. Therefore, the above hypotheses will be tested through the following objectives:

i. Investigate the impact of impairment in SAA metabolism on the acute inflammatory response, side effect on tissue function and health risks.

ii. Development of a rodent model of systemic inflammatory response syndrome (SIRS) and determine if there were any differences in inflammatory markers due to administration of different LPS doses.

iii. Study the potential effect of altered dietary SAA ratio on pro-inflammatory cytokines, proportion of neutrophils and SAA concentrations in a rodent model of SIRS induced by the application of LPS.

CHAPTER 4

SYSTEMATIC REVIEW

Effect of Impairment in Sulfur Amino Acids Metabolism on the Acute Inflammatory Response: A Systematic Review

4.1. Abstract

Despite several studies looking at the effect of sulfur amino acid (SAA) metabolism on inflammatory markers, no systematic reviews on how SAA impairment may influence immune response have been conducted. The objectives of this systematic review are to: 1) provide a summary of the effects of SAA impairment on pro-inflammatory cytokines, lymphocyte proliferation, and oxidative stress; 2) document side effects on tissue function; 3) discuss any health risks associated with SAA impairment; and 4) highlight research gaps in the literature. Electronic databases (Medline, Embase, Cochrane, Scopus, CAB and CINAHL) were searched up to August 2021. Only randomized placebo-controlled trials in humans and murines assessing the effect of SAA impairment on the acute inflammatory response were included. Of the 2728 papers identified through searches, 44 met the inclusion criteria for analysis. Models for SAA impairment varied in classification from methionine (Met) or homocysteine (Hcy) supplementation to cystathionine gamma-lyase (CSE) and cystathionine beta-synthase (CBS) deficiency. We report consistent effects for SAA impairment on pro-inflammatory cytokines production, acute phase protein secretion, and increase lymphocyte proliferation and lipid profile. As well as clear effects on increasing oxidative stress enhancing tissue failure in heart, liver, lung, and kidney. We also describe the effect of hyperhomocysteinemia on vascular disease development; the most consistent findings were on initiating atherosclerosis and cardiovascular acute disease. We reported various protective effects for CSE deficiency on inflammation. We provide recommendations for future research and discuss the potential risk with SAA impairment.

Keywords: SAA impairment, acute inflammatory response, hyperhomocysteinemia, inflammation, deficiency, B-vitamin

4.2. Introduction

The inflammatory response is initiated by early migration of leukocytes as a reaction to injury or infection. Rapid leukocyte migration into the affected area is associated with different initiating signals like neutrophil and monocyte activation mediated by the complement system (346). The acute phase of inflammation is the immediate and highly coordinated response of blood granulocytes, such as polymorphonuclear neutrophils (PMN), and adhesion to the vascular endothelium by leukocytes, which form an essential part of the innate immune system, into the injured tissues from the opening of capillary beds (27). Situations such as severe trauma, or burn injury, can produce a systemic inflammatory response syndrome (SIRS) due to unregulated acute inflammatory responses (2). The SIRS is the result of mediator production (especially tumor necrosis factor [TNF] and interleukin [IL]-1) that is delivered to multiple tissues, initiating an overproduction of leukocyte chemoattractants in distal tissues and inducing inflammatory cell influx (346). The SIRS can rapidly damage target tissues including liver, lung, and kidney (27). In this form of septic response, the increase PMN recruitment and activation to multiple tissues can lead to tissue damage and organ dysfunction (22, 284). The role of dietary factors in acute inflammatory markers has been the subject of considerable attention. Part of this attention can be attributed to the direct or indirect effects of nutrients and dietary patterns on components of the inflammatory response itself (346).

An adequate intake of dietary sulfur amino acids (SAA), specifically methionine (Met) and cysteine (Cys), is important for the synthesis of proteins of the immune system (12), regulation of oxidative stress (13) and the body's response to injuries. Metabolism of SAA has an important redox regulation function due to the balance of transmethylation (TM), transsulfuration (TS), glutathione (GSH) synthesis, and the γ- glutamyl-cycle (12, 13, 106). A

restrictive consumption of SAA-rich food could lead to an impairment of the SAA metabolism, compromising the immune system. Impairment of the SAA metabolism has been shown to potentiate the acute inflammatory response due to an imbalance in the TM and TS pathways leading to a lower immune response during inflammation (272) and a deficiency of some antioxidant metabolites like GSH (273) or taurine (12). High dietary intakes of Met raise plasma Hcy concentrations (347). An inborn error in metabolic pathways of Met synthesis leads to a large elevation of Hcy (>15 μmol/L) in the blood and urine (146). One of these errors in Met pathways is due to a homozygous deficiency in cystathionine β-synthase (CBS), resulting in homocystinuria (HCU) (146). Other disorders in transsulfuration pathway may occur with an increase of Hcy and Met due to B vitamin deficiency like vitamins B6, B12 or folate deficiency (199) or in a heterozygous defect of CBS or cystathionine γ-lyase (CSE), leading to an inability to metabolize Hcy resulting in a rise in plasma Hcy concentration (266.5± 66.71 μmol/L) known as hyperhomocysteinemia (HHcy) (285). As well, due to the sparing effect of Cys on Met through the transsulfuration pathway, a Cys deficient diet may lead to a lack of methionine-dependent compounds, such as choline, resulting in homocysteine stress (348). Raised plasma Hcy (>15 μmol/L) has been linked with an increased risk of atherosclerosis (282, 349), cardiovascular artery disease (CAD) (350) and acute liver failure (ALF) (351). Recent studies have shown that the inflammatory arm of the immune system is intimately linked to the pathogenesis of atherosclerosis (352). Several studies have reported that HHcy elevated biomarkers that integrate metabolic and pro-inflammatory cytokines, like tumor necrosis factor-alpha (TNF-α), IL-1β and IL-6 (281), are attractive biomarkers for defining the risk of atherosclerotic cardiovascular disease (353).

SAA impairment has been shown to play a role in the acute inflammatory response, revealing potential therapeutic targets in the context of thrombotic disorders. Therefore, in the current review we systematically studied available data derived from randomized placebo-controlled trials with both human and murine subjects. The objective of this systematic review was to investigate the effects of impairment in SAA metabolism on the acute inflammatory response and in the initiation or development of diseases such as atherosclerosis, CAD and ALF.

4.3. Methods

The Preferred Reporting for Systematic Reviews and Meta-Analysis (PRISMA) guidelines was followed. **Figure 4.1** summarizes the study selection process. Electronic searches were performed in Medline, Embase, Cochrane, Scopus, CAB, CINAHL using the search terms "sulfur amino acid impairment" and "acute inflammatory response" with the terms being included in the title, abstract, and/or keywords. To ensure the search strategy captured all relevant studies, searches within the electronic databases were conducted under each of the following specific components "cysteine deficien*" (using PubMed and EMBASE search filters [tw, kw]), "systemic inflammatory response syndrome" and "sulfur amino acid". Titles and abstracts of studies retrieved using the search strategy were screened independently by one review author and two collaborators to identify studies that potentially meet the inclusion criteria. The full text of these potentially eligible studies was retrieved and independently assessed for eligibility by two review team members. Any disagreement between them over the eligibility of studies was resolved through discussion with a third reviewer. The last search was performed in August 12, 2021.

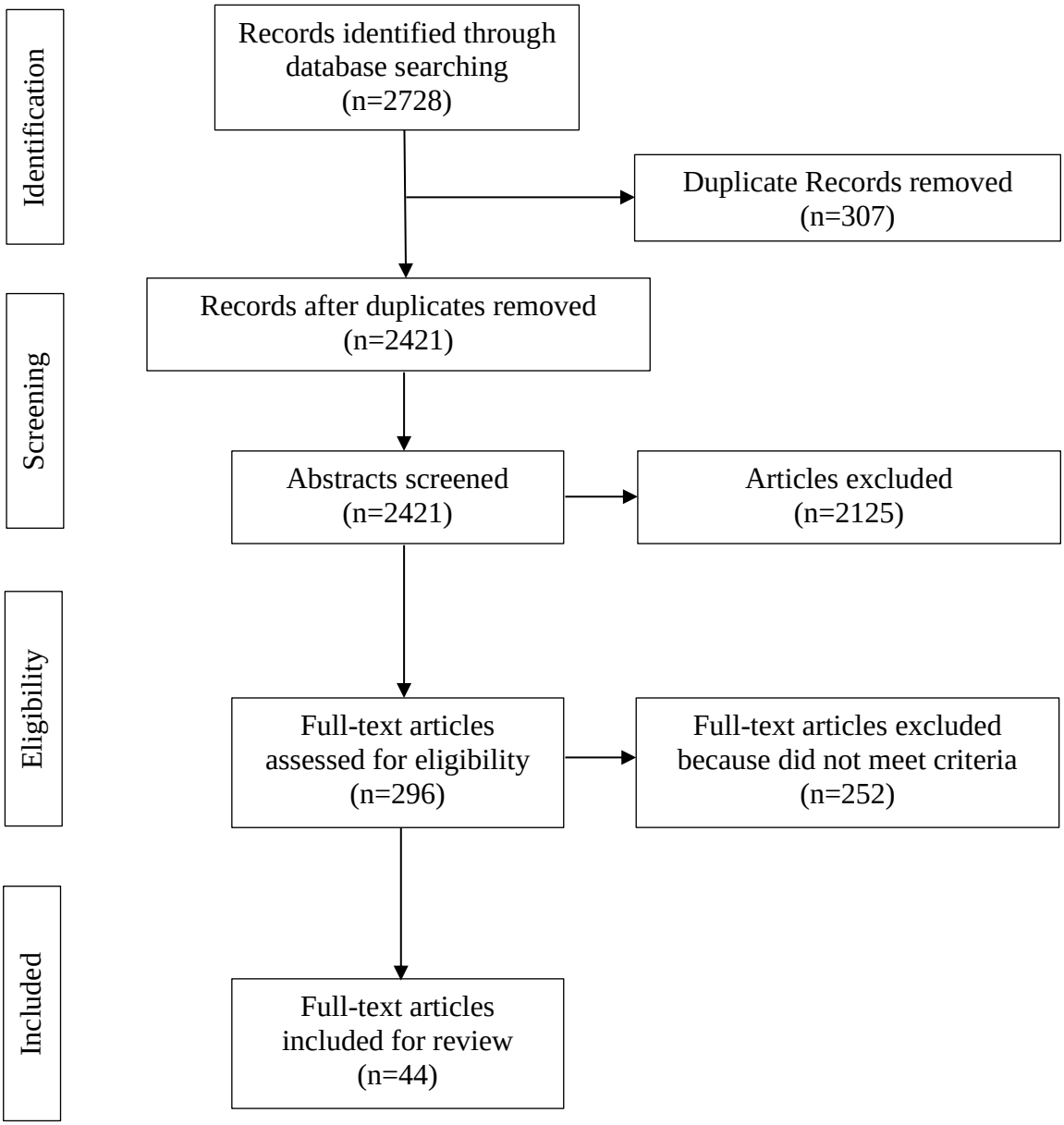

Figure 4.1 Flow diagram depicting the screening and selection of the study**.**

4.3.1. Eligibility criteria for studies

The review included only papers in English. There were no restrictions imposed on date of publication. Randomized controlled studies in humans and murine with impaired SAA metabolism were included. Studies assessing animals in diets/treatment with high Met, high Hcy for at least 3 weeks, CBS or CSE deficient models and their effect on acute inflammatory markers were included. Studies conducted in non-murine species or inflammatory challenge without impaired sulfur amino acid metabolism, as well as animals with less than seven days with an impaired sulfur amino acid metabolism were excluded. Additionally, case studies, cross-over studies, and studies without a separate control group were excluded. The primary outcomes **(Tables 4.1-3 and 4.6-8)** considered in this review are biomarkers of SAA: plasma/serum Hcy, hydrogen sulfide (H_2S); inflammatory markers, cytokines (e.g., TNF-α, IL-6, IL-1β, oxidative stress (e.g., SOD1); and lipid profile. Additionally, damage in tissues like liver, heart, and lung **(Table 4.4 and 4.9)**, as well as the development of cardiovascular diseases such as CAD, ischemia, and atherosclerosis **(Table 4.5 and 4.10)** were also reviewed.

We provided a narrative synthesis of the findings from the included studies, structured around how an impairment in the SAA pathway alters cytokines like IL-6, IL-10, IL-1β, NF-κβ, TNF-α, IL-17, oxidative stress, leukocyte count/neutrophil infiltration and lipid profile. As well, how an impairment in the SAA pathway damages tissues and how an impairment in the SAA pathway initiates CAD, hypertension, vascular dementia, and atherosclerosis.

4.3.2. Identification of studies

Identified studies were screened according to the inclusion criteria selected. One reviewer screened the titles and removed duplicates. Abstracts of selected studies were then reviewed

Table 4.1 Demographic characteristics of the subjects.

Subjects	Age	Weight	BMI	Strain		Sex	
				Mice	Rats	Male	Female
Animal	9 weeks	79 g	--	65%	35%	86.5%	13.5%
Human	46.8 years	--	25.24	--	--	64.5%	35.5%

[1]BMI, body mass index

Table 4.2 Characteristics of impaired sulfur amino acid metabolism among 12 published human trials with plasma indicators of sulfur amino acid metabolites and B vitamins.

Author	Subjects	Study design	Impairment	SAA			Vitamin concentration		
				Cys	Hcy	GSH	B6	B12	Folate
Antoniades et al.(354)	Subjects=30 Case=15 Control=15	Double blind, placebo controlled	HMD	--	↑275% (16.5μM)	--	--	--	--
Cheng et al.(152)	Subjects n=700 Case=184 Control=516	Hospital-based case-control	B6 def	--	↑135% (9.7μM)	--	↓29% (60nM)	--	--
Chernyshov et al.(273)	Sepsis= 24	Controlled trial	GSH def	--	--	↓35% (877mcM)	--	--	--
Davis et al.(334)	Subjects=9 Female=5 Male=4	Controlled trial	B6 def	No change	--	↑138% (6.9μM)	--	--	--
El Oudi et al.(355)	Subjects=202 ACS=122, Control=80	Case-controlled	HHcy	--	↑127% (13.95μM)	--	--	↑139% (259pg/mL)	↑117% (5.6ng/mL)
Erdem et al.(279)	Subjects= 141 Sepsis=71 Control=70	Hospital-based case-control	HHcy	--	↑141% (10.1μM)	--	--	No change	No change
Gokkusu et al.(351)	Subjects= 102 (ACS)	Case-controlled	Folate def	--	↑230% (9.48μM)	--	--	No change	↓11% (7.29ng/mL)
Kelly et al.(356)	Subjects=274 Cases=156 (IS) Control=118	Hospital-based case-control	B6 def	--	No change	--	↓50% (62.4nM)	--	--
Lin et al. (293)	Subjects=700 Case=184 (CAD) Control=516	Hospital-based case-control	B6 def	--	↑133% (9.8μM)	--	↓32% (59.2nM)	--	--
Ploder et al.(281)	Subjects= 18 (sepsis) Survivors=11 No survivors=7	Case-controlled	HHcy	No change	↑700% (5μM)	--	--	--	--

Schroechsnadel et al.(357)	Subjects=1717	Hospital-based case-control	Folate def	--	↑110% (11.7μM)	--	--	No change	↓2% (8.2ng/mL)
Vanzin et al.(285)	Subjects=30 CBS-/-=10 CBS-/- treat=10 Control=10	Case-controlled	CBS def	--	↑4563% (5.84μM)	--	--	--	--

[1] SAA, sulfur amino acid; HMD, high methionine diet; B6 def, vitamin B6 deficiency; HHcy, hyperhomocysteinemia; Folate def, folate deficient diet; GSH def, glutathione deficient diet; CBS def, cystathionine beta-synthase deficiency; SAA, sulfur amino acid; Cys, cysteine; Hcy, homocysteine; GSH, glutathione; B6, vitamin B6; B12, vitamin B12

[2] arrows mean significantly different compared with control.

Table 4.3 Characteristics of impaired sulfur amino acid metabolism among 17 published animal trials with plasma indicators of sulfur amino acid metabolites and B vitamins.

Author	Animal Model	Impairment	SAA						Vitamins		
			Met	Cys	Hcy	GSH	CBS	H_2S	B6	B12	Folate
Ahmad et al.(289)	CSE-/- mise (n=20)	CSE-/-	--	--	--	--	--	↓31% (130μM)	--	--	--
Ansari et al.(350)	Wistar rat (n=20)	HMD	--	--	--	--	--	--	--		
Benight et al.(358)	C57BL/6 mice (n=31)	B def	Plasma: No change Liver: ↓23.25% (615μM)		Plasma: ↑417% (1.2μM) Liver: ↑600% (3 μM)	--	--	--	Plasma: ↓91.3% (230nM) Colon: ↓71% (14nM)	No change	--
Chen et al.(359)	C57BL/6 mice (n=30) HHcy (n=15) Control (n=15)	HMD	--	--	↑357% (7μM)	--	--	--	--	--	--
Dudman et al.(360)	Sprague-Dawley rats (n=12)	HHcy	--	--	↑1125% (8μM)	--	--	--	--	--	--
Durand et al.(292)	Sprague-Dawley rats (n=24)	B def/HDM	↑351% (93.54μM)	↑116% (62.99μM)	↑630% (4.92μM)	↑131% (22.23μM)	--	--	--	--	--
Keating et al.(361)	CBS +/- mice (n=16)	CBS+/-	--	--	↑5595% (4.3μM)	--	--	--	--	--	--
King et al.(362)	CSE-/- mice (n=12)	CSE-/-	--	--	--	--	No change	↓39% (0.13μM)	--	--	--
Liu et al.(363)	Wistar rats (n=32)	HMD	--	--	↑267% (15μM)	--	--	--	--	--	--
Mani et al.(290)	CSE-/- mice (n=32)	CSE-/-	--	--	--	↓14% (35 μM)	--	--	--	--	--
Sharma et al.(364)	Wistar rats (n=30)	HMD	--	--	↑500% (10μM)	--	--	--	--	--	--
Shirozu et al.(365)	CSE-/- mice	CSE-/-	--	No change	↑1000%	--	No change	--	--	--	--

	(n=24)				(10μM)						
Sudduth et al.(366)	C57BL6 mice (n=20)	B def/HMD	--	--	↑1408% (5.89μM)	--	--	--	--	--	--
Wang et al.(291)	CSE-/- mice (n=28)	CSE-/-	--	--	--	No change	--	--	--	--	--
Winchester et al.(282)	Murine J774A.1 Raw 264.7 Mφ	HHcy	--	--	--	--	--	--	--	--	--
Zhang et al.(349)	Ldlr-/- CBS-/+ mice (n=27)	HF+HMD	--	--	--	--	--	--	No change	No change	↓25% (75.3ng/mL)
Zhang et al.(367)	ApoE-KO mice (n=20)	HMD	--	--	↑780% (2.6μM)	--	--	--	--	--	--

[1] SAA, sulfur amino acid; HMD, high methionine diet; B def, vitamin B deficiency; CBS+/-, cystathionine bet-synthase deficient model; CSE-/-, cystathionine gamma-lyase deficient model; HHcy, hyperhomocysteinemia; HF, high folate diet; SAA, sulfur amino acid; Met, methionine; Cys, cysteine; Hcy, homocysteine; GSH, glutathione; B6, vitamin B6; B12, vitamin B12

[2] arrows mean significantly different compared with control.

Table 4.4. Characteristics of impaired sulfur amino acid metabolism among 14 published human trials with plasma inflammatory indicators.

Author	Impairment	Cytokines			Platelet activity		Oxidative stress	APP
		TNF-α	IL-1β	IL-6	Leukocytes	Monocytes	H_2O_2	CRP
Alvarez-Maqueda et. al.(277)	HHcy	--	--	--	--	--	↑213% (8 Δfluorescence)	--
Antoniades et al.(354)	HMD	No change	--	↑121% (1.9pg/mL)	--	--	--	--
Cheng et al.(152)	B6 def	--	--	--	--	--	--	↑3700% (0.1mg/dL)
Chernyshov et al.(273)	GSH def	--	--	--	↑160% (6.92 x10^9/l)	↑161% (0.36 x10^9/l)	--	--
Dalal et al.(278)	HHcy	↑926% (1064pg/mL)	↑186% (3575pg/mL)	↑259% (10776pg/mL)	--	--	--	--
El Oudi et al.(355)	HHcy	↑164% (6.81pg/mL)	No change	↑498% (2.32pg/mL)	--	--	--	↑387% (0.0037mg/dL)
Erdem et al.(279)	HHcy	--	--	--	--	--	--	↑910% (0.0048mg/dL)
Gokkusu et al.(351)	Folate def	--	--	↑1288% (0.25pg/mL)	--	--	--	
Kelly et al.(356)	B6 def	--	--	--	--	--	--	↑544% (0.16mg/dL)
Lin et al.(293)	B6 def	--	--	--	--	--	--	↑550% (0.2mg/dL)
Poddar et al.(368)	HHcy	No change	No change	--	--	--	--	--
Ploder et al.(281)	HHcy	↑3750% (4pg/mL)	--	↑160% (250pg/mL)	--	--	--	--
Schroechsnadel et al.(357)	Folate def	--	--	--	--	--	--	↑150% (2.8mg/dL)
Van Aken et al.(369)	HHcy	--	--	↑50000% (1pg/mL)	--	--	--	--

[1] HMD, high methionine diet; B6 def, vitamin B6 deficiency; HHcy, hyperhomocysteinemia; Folate def, folate deficient diet; GSH def, glutathione deficient diet; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; IL-6, interleukin-6; H_2O_2, hydrogen peroxide; APP, acute protein phase; CRP, C-reactive protein.
[2] arrows mean significantly different compared with control.

Table 4.5. Characteristics of impaired sulfur amino acid metabolism among 7 published human trials with plasma lipid profile.

Author	Impairment	Lipid profile			
		Cholesterol	TG	LDL	HDL
Antoniades et al.(354)	HMD	--	--	↑156% (78 IU/L)	--
Cheng et al.(152)	B6 def	--	↑127% (122.6mg/dL)	↑121% (101.4mg/dL)	↓30% (60.5mg/dL)
El Oudi et al.(355)	HHcy	--	No change	No change	↓8% (41.76 mgl/dL)
Erdem et al.(279)	HHcy	↑115% (177.6mg/dL)	↑238% (76.4 mg/dL)	↑123% (107.1mg/dL)	↓24% (50.7mg/dL)
Gokkusu et al.(351)	Folate def	No change	↑174% (85mg/dL)	No change	↓26% (53mg/dL)
Lin et al.(293)	B6 def	No change	↑124% (123.9mg/dL)	↑122% (101.2mg/dL)	↓31% (60.2mg/dL)
Vanzin et al.(285)	CBS def	↓36% (185.62mg/dL)	--	No change	↓34% (59.35mg/dL)

[1] HDM, high methionine diet; B6 def, vitamin B6 deficient diet; HHcy, hyperhomocysteinemia; Folate def, folate deficient diet; CBS def, cystathionine beta-synthase deficiency; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein.
[2] arrows mean significantly different compared with control.

Table 4.6. Characteristics of impaired sulfur amino acid metabolism among 22 published animal trials with plasma inflammatory indicators.

Author	Impairment	Cytokines			Platelet activity		Oxidative stress			APP
		TNF-α	IL-1β	IL-6	Leukocytes	Lymphocytes	MPO	MDA	ROS	CRP
Ahmad et al.(289)	CSE-/-	↓28.57% (35pg/mL)	↓42.85% (35pg/mL)	↓40% (50pg/mL)	--	--	Heart: ↓33% (150000μM) Lung: ↓37.5% (800000 μM) Liver: ↓27.7% (130000 μM) Kidney: ↓33% (15000 μM)	Heart: ↓50% (8 μM) Lung: ↓61.5% (13 μM) Kidney: ↓40% (10 μM)	--	--
Ahmad et al.(286)	CBS+/-	↑4000% (0pg/mL)	↑4500% (0pg/mL)	↑400% (2000pg/mL)	--	--	↑179% (14000U/g)	↑136% (59μM)	--	--
	CSE-/-	↑4200% (0pg/mL)	↑6000% (0pg/mL)	↑425% (2000pg/mL)	--	--	↑186% (14500U/g)	↑160% (50μM)	--	--
	3MST def	↑4500% (70pg/mL)	↑6000% (0pg/mL)	↑425% (2000pg/mL)	--	--	↑193% (14000U/g)	↑200% (50μM)	--	--
Benight et al.(358)	B def	↑700% (1fold)	--	--	--	--	↑1000% (1 U/g)	--	--	--
Catana et al.(370)	HHcy	--	--	Young: No change Old: ↑136% (101.3pg/mL)	--	--	--	--	--	--
Chaturvedi et al.(371)	HMD/CBS+/-	↑200% (4U)	↑233% (3 U)	↑700% (1 U)		--	--	--	--	--
Chen et al.(359)	HMD	↑400% (1mRNA)	↑200% (1mRNA)	--	↑250% (20 count)	--	--	--	--	--
Ci et al.(322)	CSE-/-	↑225% (.0004 mRNA)	↑200% (0.0035 mRNA)	↑275% (0.0002 mRNA)		--	--	--	--	--
Dudman et al.(360)	HHcy	--	--	--	↑300% (4 count)	--	--	--	--	--
Scherer et al.(372)	HHcy	Hippocampus: ↑300% (20pg/mg) Heart: ↑120% (75pg/mg)	Hippocampus: ↑167% (12pg/mg) Heart: No change	Hippocampus: ↑200% (400pg/mg) Heart: ↑125% (180pg/mg)	--	--	--	--	--	--

Familtseva et al.(284)	CBS+/-	↑600% (1fold)	--	↑700% (1fold)	--	--	--	--	--	--
Fefelova et al.(373)	HHcy	↑3656% (30.2pg/mL)	--	--	--	↑181% (8210 count)	--	--	--	--
Zanin et al.(374)	HHcy	--	↑700% (100pg/mL)	--	--	↑250% (500 count)	--	--	--	--
Keating et al.(361)	CBS+/-	↑140% (10pg/mL)	↑350% (20pg/mL)	--	--	--	--	--	--	↑6831% (0.0009mg/L)
King et al.(362)	CSE-/-	--	--	--	--	--	--	Heart: ↑134% (15μM) Liver: ↑167% (12μM)	--	--
Liu et al.(363)	HMD	--	↑600% (1fold)	--	--	--	--	--	--	--
Mani et al.(290)	CSE-/-	--	--	--	--	--	--	↑160% (50μM)	↑300% (0.4AU)	--
Sharma et al.(364)	HMD	--	--	--	--	--	--	--	--	↑144% (4.8mg/L)
Shirozu et al.(365)	CSE-/-	↓43% (35mRNA)	↓50% (10mRNA)	↓43% (7mRNA)	--	--	--	--	--	--
Sudduth et al.(366)	B def/HMD	↑126% (1fold)	↑177% (1fold)	↑166% (1fold)	--	--	--	--	--	--
Wang et al.(291)	CSE-/-	↑300% (300pg/mL)	↑1000% (10pg/mL)	↑400% (400pg/mL)	--	--	--	--	200% (1 rel ratio)	--
Winchester et al.(282)	HHcy	--	--	--	--	--	--	--	↑150% (4AU)	--
Zhang et al.(367)	HF+HMD	↑131% (90pg/mL)	--	↑145% (59pg/mL)	--	--	--	--	--	--

[1] HMD, high methionine diet; B def, vitamin B deficiency; CBS+/-, cystathionine bet-synthase deficient model; CSE-/-, cystathionine gamma-lyase deficient model; HHcy, hyperhomocysteinemia; HF, high folate diet; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; IL-6, interleukin-6; MPO, myeloperoxidase; MDA, malondialdehyde; ROS, reactive oxidative species; APP, acute protein phase; CRP, C-reactive protein.
[2] arrows mean significantly different compared with control.

Table 4.7. Characteristics of impaired sulfur amino acid metabolism among 4 published rat trials with plasma lipid profile.

Author	Impairment	Lipid profile			
		Cholesterol	TG	LDL	HDL
Mani et al.(290)	CSE-/-	↑167% (232mg/dL)	↓61% (115mg/dL)	↑300% (57.9mg/dL)	↑141% (142.8mg/dL)
Sharma et al.(364)	HMD	↑208% (24mg/dL)	↑190% (100mg/dL)	--	↓40% (10mg/dL)
Sudduth et al.(366)	B def/HMD	--	--	No change	No change
Zhang et al.(367)	HF+HMD	--	No change	--	--

[1] CSE-/-, cystathionine gamma-lyase deficient model; HMD, high methionine diet; B def, vitamin B deficiency; HF, high folate diet; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein.
[2] Arrows mean significantly different compared with control

Table 4.8. Summary of studies of impairment in the sulfur amino acid metabolism in humans related to tissue damage.

Author	Impairment	Tissue
Ploder et al.(281)	HHcy	*Failure:* Liver, Kidney, Lung, and heart

[1]HHcy, hyperhomocysteinemia.

Table 4.9. Summary of studies of impairment in the sulfur amino acid metabolism in animals relating to tissue damage.

Author	Impairment	Tissue
Ahmad et al.(289)	CSE-/-	**Liver function** Decreased ALP, ALT, AMY, TBIL, BUN production
Chaturvedi, et al.(371)	HMD/CBS+/-	**Cardiac function** HMD and CBS+/- +Met: Increased LVIDd and LVPWd **Vascular and matrix modeling** HMD: decreased eNOS and iNOS HMD and CBS+/- +Met: increased MMP9, MMP2 and collagen deposition.
Chen et al.(359)	HMD	**Cardiac function** Increased PCNA and CD68 expression
Ci, et al.(322)	CSE-/-	**Liver function** CCl_4: decreased luciferase activity and albumin production CCl_4: increased ALT and AST production **Histophatological changes** CCl_4: increased hemorrhage and neutrophil infiltration
King et al.(362)	CSE-/-	**Cardiac function** Increased myocardial infarct size **Liver function** Increased ALT and AST production
Liu et al.(363)	HMD	**Cardiac function** Increase LVW and LVW/BW Decreased EF and FS Hypertrophy of cardiomyocytes and disarrangement of myofibers
Shirozu et al.(365)	CSE-/-	**Liver function** Attenuated liver damage (did not altered ALT)
Wang et al.(291)	CSE-/-	**Lung function** Increased lung injury

[1]CSE-/-, cystathionine gamma-lyase deficient model; HMD, high methionine diet; CBS+/-, cystathionine beta-synthase deficient model; Met, methionine; ALP, alkaline phosphatase; ALT alanine aminotransferase; AMY, amylase; TBIL, total bilirubin; BUN, total urea nitrogen; LVIDd, Left Ventricular Internal Diameter, diastole; LVPWd Left Ventricular posterior wall, diastole; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinases; PCNA, proliferating cell nuclear antigen; CCL_4, 4-week carbon tetrachloride; EF, ejection fraction; FS, fractional shortening; LVW, left ventricular weight; LVW/BW, left ventricular weight/body weight.

Table 4.10. Summary of studies of impairment in the sulfur amino acid metabolism in humans related to development of diseases.

Author	Impairment	Disease
Cheng et al.(152)	Low PLP	**CAD** Increased risk
Gokkusu et al.(351)	Low folate	**ACS** Increased risk
Kelly et al.(356)	Low PLP	**Ischemic Stroke** Increased risk
Lin et al.(293)	Low PLP	**CAD** Increased risk Blood pressure: Increased systolic and decreased diastolic
Schroechsnadel et al.(357)	Low folate	**CAD** Increased risk Blood pressure: Increased systolic
Vanzin et al.(285)	CBS def	**CAD** Increased risk Decreased Apolipoprotein A, Increased BuChE

[1]PLP, pyridoxal 5′-phosphate; CBS def, cystathionine beta-synthase deficiency; CAD, cardiovascular disease; ACS, acute coronary syndrome.

independently by one reviewer and two collaborators. A second selection was performed by 1 reviewer. The full text was read to determine whether the article met the inclusion criteria.

4.3.3. Data extraction

A standardized form was used to extract data from the included studies for assessment of study quality and evidence synthesis. The extracted information included: authors, title, study setting; animal strain and baseline characteristics; details of the intervention and control conditions; study methodology; treatment and dose; outcomes and times of measurement (SAA concentration, vitamin concentration, inflammatory markers, tissue damage and disease induced); suggested mechanisms of intervention action; conclusions. One review author extracted data and then scrutinized by two reviewers, discrepancies were identified and resolved through discussion. Missing data was requested from study authors.

4.4. Results

4.4.1. Study selection

We identified a total of 2728 papers using our search strategy, of which 44 articles met the inclusion criteria (**Figure 4.1**). The mean sample size of included animal studies was 26 animals and the mean sample size for human trials was 329 participants. The 44 articles were grouped into the following areas (some studies represented multiple times): inflammatory markers (n=25 animal trials, n=16 human trials); tissue damage (n=8 animal trials, n=2 human trials); and disease induction (n= 8 animal trials, n=6 human trials). Only significant findings are reported in the results and discussed. However, **Tables 4.1-11** provide a summary of both the significant and non-significant findings.

4.4.2. Subject characteristics.

All the investigations in human trials studied both sexes **(Table 4.1)**. Most participants from the articles were men (64.5%). The mean age for participants in the case and control groups across the studies was 46.8 y. Human studies reported different SAA impairment models **(Table 4.2)**, supplementation of Met (n=1), HHcy (n=4), CBS deficiency (n=1) and vitamin deficiency (n=5). Most animal trials (86.5%) investigated only male subjects **(Table 4.1)**. Three studies included females only and one study included both sexes. The mean age for animals in the case and control groups across the investigations was 9 weeks; three studies failed to report age of the animals. Animal studies also reported different models of SAA impairment **(Table 4.3)**, including CSE-/- mice (n=7), supplementation of Met (n=5) or Hcy (n=5), CBS+/- mice (n=3) and vitamin B6 or B12 deficiency (n=1). Other impairments observed were CBS+/- mice +Met (n=1) and vitamin deficient diet + Met (n=2).

4.4.3. Main findings

The results from human clinical trials suggest a negative association between impairment of SAA and acute inflammatory markers, mainly in increasing the concentration of pro-inflammatory cytokines **(Table 4.4)**. Cytokines, such as TNF-α, IL, i.e., IL-1β and IL-6, are upregulated during inflammatory events while IL-8 is inhibited, playing a central role in leukocyte recruitment during inflammation (278). The study performed by Dalal et al. investigated the effects of homocysteine on cytokine production by human endothelial cells treated with different doses of Hcy (278). The researchers founded that IL-6 was significantly stimulated following 24 h treatment with Hcy, while IL-8 concentrations were inhibited after

Table 4.11. Summary of studies of impairment in the sulfur amino acid metabolism in animals related to development of diseases.

Author	Impairment	Disease
Ansari, M., et al.(350)	HMD	**CAD** hemolytic anemia Crenation of RBCs
Benight, N.M., et al.(358)	B def	**Colitis** Histological damage Vit def+DSS: ↑hDAI score
Familtseva, A., et al.(284)	CBS+/-	**CAD** *Blood pressure:* CBS+/-: ↑systolic and diastolic *Cell death:* CBS+/-: ↑caspase-9 and Bax ↓TUNEL-positive cell
Mani et al.(290)	CSE-/-	**Atherosclerosis** Hypertension ↑Atherosclerotic lesion area Atherosclerotic plaques in carotid artery, aortic arch, and abdominal aorta
Sharma et al.(364)	HMD	**CAD and Atherosclerosis** Atherosclerotic plaque grade 2 (0.5, 1 g/kg of met) ↑Caveolin, P2X and P2Y (1 g/kg met)
Sudduth et al.(366)	B def/HMD	**Vascular dementia** ↑Microhemorrhages (coronal T2) *Microglial activation:* ↑FCX, CA3, DG and EnCX *Matrix MMP2 and MMP9 system:* MMP2: ↑MMP14, MMP2 and TIMP2 MMP9: ↑MMP3 and MMP9
Winchester et al.(282)	HHcy	**Atherosclerosis** ↑EMMPRIN RAW 264.7 (500µmol/L) and J774A.1 (100µmol/L) *Matrix MMP9 system:* MMP9 RAW 264.7 (500µmol/L) and J774A.1 (100µmol/L)
Zhang et al.(349)	HF+HMD	**Atherosclerosis** ↑Atherosclerotic lesion area ↑EGFP+ Mφ and MC, EGFP- Mφ and MC, and Mφ maturation rate.

[1] HMD, high methionine diet; B def, vitamin B deficiency; CBS+/-, cystathionine bet-synthase deficient model; CSE-/-, cystathionine gamma-lyase deficient model; HHcy, hyperhomocysteinemia; HF, high folate diet; Vit def, vitamin deficiency; CAD, cardiovascular disease; RBC, red blood cells; DSS, dextran sodium sulfate; hDAI, disease activity index; TUNEL, 3′-OH end-labeling method; FCX, frontal cortex; CA3, cornu ammonis 3; DG, dentate gyrus; EnCX, endothelial cortex; MMP, matrix metalloproteinases; TIMP2, tissue inhibitor of metalloproteinase 2; EMMPRIN, extracellular matrix metalloproteinase inducer; EGFP, enhanced green fluorescent protein; Mφ, macrophage; MC, monocyte;

[2] Arrows mean a significant increase of markers associated with the development of diseases.

both 4 and 24 h treatments (278). Hcy was also found to stimulate IL-1β production by human peripheral blood monocytes and TNF-α production by monocyte-derived macrophages (360). Other studies also reported a significant increase in pro-inflammatory cytokines and chemokines, TNF-α (278, 355), IL-1β (278), IL-2 (351), monocyte chemoattractant protein-1 (MCP1) (368). However, two studies reported no alteration on plasma cytokines IL-8 (369) and IL-1 β (285) with a SAA impairment. A lipid profile was also reported in human studies **(Table 4.5)**. Hcy's atherogenicity may implicate several processes, such as low density lipoprotein (LDL), oxidative cholesterol modification and high density lipoprotein (HDL) cholesterol reduction (285). Low HDL cholesterol is an independent marker of CAD at a dose of <40 mg/dL (375). Subjects with HHcy exhibited significantly increased cholesterol, triglycerides (TG), LDL concentrations when vitamins B6 and B12 were deficient (293). Erdem, et al. (279) also found that serum Hcy was significantly higher ($p<0.05$) in patients with sepsis compared with the healthy control group. When looking at the lipid profile, researchers found that serum TG, high sensitive C-reactive protein (hsCRP), total cholesterol, and the LDL levels of patients with severe sepsis were significantly higher ($p< 0.01$ for LDL and $p< 0.001$ for the other parameters) whereas HDL, and albumin levels were lower as compared to those of the healthy control subjects ($p< 0.001$) (279). These results showed that moderate HHcy in combination with vitamin deficiency may be useful as a prognostic biomarker of the development CAD because it can indicate the severity of illness in patients with sepsis. Additionally, moderate HHcy significantly decreased lymphocyte numbers, lymphocyte subsets and total numbers of NK cells and NKT cells in subjects with GSH deficiency and low concentrations of folate ($p<0.05$) (273). C-reactive protein (CRP) concentration was reported in 7 studies **(Table 4.4)**. Three studies reported an increase in CRP concentration when B6 was deficient (152, 293, 356), three studies observed increased CRP with

HHcy (279, 355, 357), while one study observed no alteration in CRP concentration; however, results from the study could be limited due to the small sample size, the marginal level of deficiency reached, and the subject population under investigation (334).

Animal trials also observed that impairment in the SAA metabolism increased the acute inflammatory response by activating pro-inflammatory cytokines **(Table 4.6)**. For the majority of pro-inflammatory mediators measured, the effects were consistent. For instance, HHcy caused by supplementation of Met or Hcy resulted in an increase in plasma cytokines such as TNF-α, IL-1β and IL-6 (349, 359, 363, 370-374) and an increase in oxidative stress due to enhanced ROS formation (282, 367). Vitamin B12 and B6 deficient diets, and CBS and CSE deficient mice also yielded an increase in pro-inflammatory cytokines (284, 286, 290, 291, 322, 361). Sudduth et al. (366) induced HHcy in twenty C57B16 WT mice by feeding them a low folate, vitamin B6, and B12 diet and supplemented with Met. They observed that neuroinflammatory markers, IL-1β, IL-6, IL-12, and TNF-α exhibited significantly increased (p<0.05) expression in the HHcy mice compared with control mice (366). These results suggest that rats submitted to mild HHcy presented with an increase in cytokine levels in the brain, heart, and serum (366). Under the influence of IL, i.e., IL-1, IL-2, and TNF-α, liver cells synthesize and secrete acute phase proteins (APP) (376). CRP is one of the most important APP (377). Two studies reported the effect of impairment of SAA metabolism on CRP (361, 364). HHcy caused by supplementation of Hcy in Wistar rats significantly increased (p<0.05) CRP concentrations (364). Another pro-inflammatory marker impacted by impairment of the SAA metabolism was the platelet activity (360, 367). Increased leukocyte adhesion and emigration (367) as well as decreased leukocyte rolling velocity (360) were observed with HHcy

in five studies (286, 292, 360, 367, 373). Total lymphocytes (367), T-cells (373), total leukocytes (350, 360), and PMN (350) were increased with supplementation of Hcy.

Among amino acids, SAA are recognized to be some of the most potent modulators of lipid metabolism (378). It has been demonstrated that SAA have a raising effect on HDL and a lowering effect on very low-density lipoprotein-cholesterol (VLDL) (379). Lipid profiles play an important role in cardiac health as they are considered biomarkers for several heart diseases. Lipid profiles were examined across three animal studies (290, 350, 364). In animals supplemented with Hcy (364), Met (350) or CSE deficient mice (290) plasma cholesterol, TG and LDL were increased while a reduction in HDL was observed **(Table 4.7)**. CSE deficient mice fed with a high-fat atherogenic diet exhibited significantly elevated plasma LDL, HDL, and a total cholesterol/HDL ratio (290). Another study also observed that rats treated with Met (1 g/kg by mouth [p.o.]) for 30 days exhibited significantly increased ($p< 0.01$) total cholesterol, TG, LDL and VLDL levels along with significantly decreased ($p< 0.01$) HDL when compared with vehicle control rats (350). Three studies reported significant increases ($p<0.01$) in Hcy concentrations in rats with high intake of methionine in the diet when compared with their control groups. These results suggest that HHcy increases the risk for atherosclerosis development and should be considered as a biomarker due to increase Hcy concentrations (>15 µmol/L) can result in reduction of HDL, which is a strong indicator for atherosclerosis development.

4.4.4. Impact of SAA metabolism in different tissues function

Tissue function in humans with an impairment in SAA metabolism was examined in one study **(Table 4.8)**. The study performed by Ploder et al. (281) investigated the effects of moderate HHcy on plasma cytokines in patients with sepsis alone or post-trauma sepsis. The

researchers found that Hcy concentrations were 12% above the upper limit of normal in adults (15 µmol/L) while, Cys concentrations (347 µmol/L) were below the upper limit (281). At their follow-up, patients presented significantly increased Hcy ($p<0.05$) and Cys ($p<0.05$) compared with baseline. However, only Hcy was significantly higher ($p<0.05$) in patients after trauma with unfavorable outcome. Concentrations of pro-inflammatory cytokines TNF-α and IL-6 concentrations on patients at their follow-up stayed at baseline; however, in non-survivors IL-6 concentrations were significantly higher at day 13 compared with baseline. After 10 days Cys concentrations increased in non-survivors but remained within a normal range compared with baseline. Additionally, patients with trauma and sepsis and sepsis alone also demonstrated increased liver, kidney, lung, and heart failure (281).

Tissue function in animals with a SAA impairment was examined across 8 studies **(Table 4.9)**. Four studies reported negative effects on cardiac function with supplementation of Met or with CBS+/- deficient mice (284, 285, 363, 371). Cardiac function was diminished with a decrease in eNOS, iNOS (371) and the ejection fraction (363). One study reported that CSE-/- mice presented lung injury due to a markedly increase of Nox4 expression, NADPH oxidase activity and ROS production in lung tissues (291). Liver function was also reported in 4 studies. In CSE deficiency, mice exhibited increased pro-inflammatory cytokines in the liver, and exacerbations in acute hepatitis and liver fibrosis; however this exacerbated hepatic injury was largely reversed after H_2S treatment with sodium sulfide (Na_2S) (500 µg/kg) (362). On the other hand, three studies reported that the inhibition of CSE improved tissue function in murine models (289, 322, 365). These studies observed that CSE deficiency attenuated post-burn lung, heart, kidney– but not liver– MDA levels, and reduced lung, heart, kidney, and liver MPO

levels (289). Additionally, inhibition of CSE prevented acute inflammatory liver failure by augmenting thiosulfate levels and led to an attenuated histological liver injury (365).

4.4.5. Impact of SAA metabolism in the development of different diseases

Six studies have examined disease development in humans with impaired SAA metabolism **(Table 4.10)**. Three studies reported that subjects with low B6 or folate experienced a significantly higher risk of developing CAD (152, 293, 357). Two studies observed that subjects with a high risk of CAD and vitamin deficiency also presented with moderate HHcy (293, 357) and increased inflammatory markers such as CRP, neopterin and TNF-α (357). However, one study reported that low plasma pyridoxal 5'-phosphate (PLP) was associated with higher the risk of CAD, but the concentration of Hcy was not affected. An increased risk of acute coronary syndrome (ACS) was reported by one study (351). Subjects with significantly higher (p<0.001) plasma Hcy concentrations and decreased (p<0.05) folate concentrations presented with significantly higher (p<0.001) concentrations of the cytokines IL-2 and IL-6, and an increased risk of ACS (351). One study reported an increased risk of ischemic stroke when subjects had a significantly lower concentration of plasma PLP; however, the concentration of Hcy did not differ between case subjects and control (356).

Few studies have examined disease development in animals with impaired SAA metabolism **(Table 4.11)**. One study reported that mice given a B6/B12-vitamin-deficient diet experienced significantly lower mortality than those fed the control diet when colitis was induced acutely with 3% dextran sodium sulfate (DSS) (358). Colonic MPO (myeloperoxidase) activity, a measure of neutrophil infiltration, was markedly increased by DSS treatment in both diet groups, but this increase was reduced by nearly half in the B6/B12-vitamin-deficient diet +3% DDS compared with the control diet+3% DSS, indicating that the B6/B12-vitamin-deficient

diet led to reduced neutrophil infiltration and subsequent inflammation (358). Atherosclerosis risk was enhanced in animals with SAA impairment (282, 290, 349). Severe HHcy was found in CSE-/- mice, but not in WT mice, fed with either the control or atherogenic diet, and led to an accelerated atherosclerosis development (290). CSE-/- mice showed hypertension, and the aortic root lesion area was significantly greater compared with WT mice (290). Administration of 0.5-1 g/kg Met in rats-initiated atherosclerosis by increasing Hcy concentration (HHcy) and the atherosclerotic lesion area, in comparison to control rats. Increased risk of CAD was reported in 3 animal studies (284, 350, 364). Animals with HHcy induced by Met supplementation (1 g/kg) observed an increased in caveolin, P2X-purinoceptors (produce vasoconstriction) and P2Y-purinoceptors (produce vasodilatation**)** (364) and showed crenation and other morphological changes in the erythrocytic membrane (284).

4.5. Discussion

4.5.1. Summary of key findings

We present, to our knowledge, the first systematic review of SAA metabolism impairment on acute inflammatory responses (**Figure 4.2**). We report evidence from a few studies for an impact of SAA metabolism impairment upon inflammatory mechanisms. The most consistently reported findings to date relate to biomarkers of cytokine production, lipid metabolism and platelet activity. HHcy was the most studied variable in relation to SAA metabolism impairment, with Hcy concentration modifying various acute inflammatory markers such as increasing TNF-α production and leukocyte adhesion and emigration. SAA metabolism

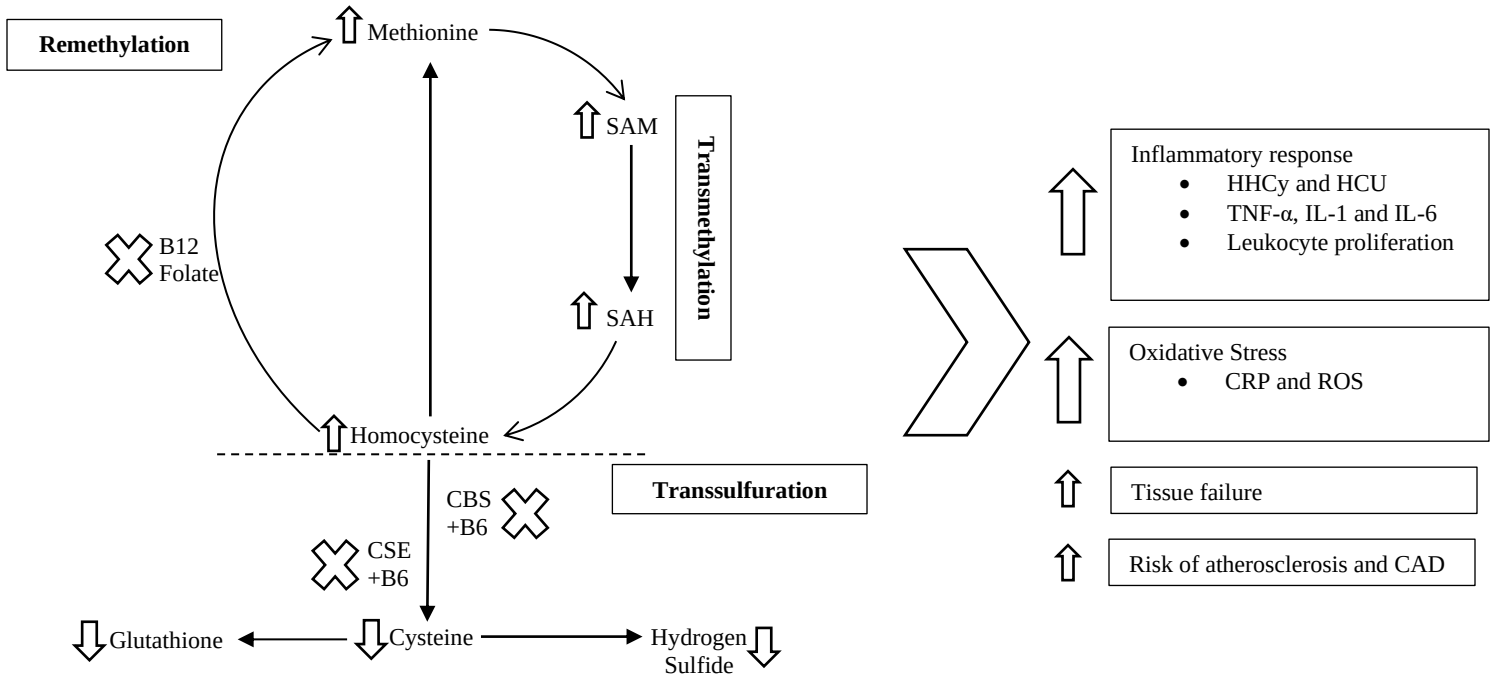

Figure 4.2. Sulfur amino acid metabolism and potential impact on inflammatory markers. When the sulfur amino acid metabolism is disrupted due to deficient cystathionine beta-synthase (CBS), cystathionine gamma-lyase and vitamin B6, B12, and folate activity (cross), leads to the increase of upstream metabolites homocysteine (Hcy), methionine (Met), S-adenosylmethionine (SAM), and S-adenosylhomocysteine (SAH) and reduction of downstream metabolites cystathionine (Cth) and cysteine (Cys) as indicated by arrows. This disruption has a potential impact elevating the inflammatory response, oxidative stress, tissue dysfunction and risk of cardiovascular diseases as indicated by arrows.

impairment also increased tissue failure with the infiltration of inflammatory cells and increased oxidation. A few positive outcomes including decreased production of alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL) and blood urea nitrogen (BUN) were reported in liver function in the presence of CSE deficiency (289, 365).

4.5.2. Impaired SAA metabolism: changes on acute inflammatory markers

The initial phase of the inflammatory response is characterized by a rapid leukocyte migration into the affected tissue (27). Hcy enhances the oxidative stress of neutrophils and underscore the potential role of phagocytic cells in vascular wall injury through superoxide anion release during HHcy conditions (277). We found evidence for a consistent effect of SAA metabolism impairment on increasing platelet activity in the presence of HHcy by Met supplementation (292, 350, 367). A mechanism for this effect to happen is related to the high concentration of Hcy (0.3 mM) with an increase of the total lymphocyte count at the expense of cytotoxic T-cell population and inhibiting lymphocyte apoptosis (367). Indeed, 6 studies (animal studies n=4; human studies n=2) reported an effect of HHcy on increasing lymphocyte proliferation (273, 357, 359, 360, 373, 374). Furthermore, the lymphocyte proliferation was also observed with folate deficient subjects (292, 357). Indeed, folate deficiency led to a prethrombotic state by increasing the activity of macrophages, and platelet aggregation of thrombin and thromboxane (292). These early inflammatory mediators provide a relatively effective and rapid initiation of PMN and mononuclear phagocyte infiltration to sites of infection (27).

Pro-inflammatory cytokines such as interferon gamma (IFNγ), IL-6, IL-2, TNF-α and IL-1 (22, 61), are produced by peripheral nerve tissue, enrolled by macrophages and promote cytokine cascades that lead to continued leukocyte migration and activation (27). For healthy

subjects, the evidence summarized in **Table 4.4** suggests that SAA impairment can modify the pro-inflammatory response, when assessed through changes in cytokines, for example, TNF-α. We identified 25 studies (animals n=16; humans n=9) investigating cytokine production and inflammatory response, in which differences in SAA impairment could account for divergent outcomes (273, 278, 281, 284-286, 289, 290, 322, 349, 351, 355, 357, 359, 361, 363, 365, 366, 368-374). HHcy (>15 μmol/L) induced by Met or Hcy supplementation or CBS inhibition, induced oxidative stress and inflammation by increasing TNF-α (286), IL-6 (285, 371), IL-1β (361) and TLR-4 (284, 371) production. In addition, HHcy has been proven to promote proinflammatory status increasing cytokine concentrations in different tissues like brain (372) and heart (285, 372). When inhibition of CSE was present, the myocardial infarct per area at risk increased due to combination of insufficient NO bioavailability and increased ROS in myocardial ischemia (362). On the other hand, the positive effects on pro-inflammatory production and inflammatory mechanism (CRP) were evident with inhibition of CSE (289, 365). Inhibition of CSE prevents acute inflammatory liver failure by augmenting thiosulfate levels and upregulating antioxidant and anti-apoptotic mechanisms like deficiency markedly attenuated levels of TNF-α, IL-6, IL-1β, and nitric oxide synthase (NOS) (365). Similar results were observed in response to burn (289). In their study Ahmad et al (289) reported positive effects of CSE deficiency in the liver of CSE deficiency rats 24 h after subjected to burn injury at the dorsa with water at 95°C for 10 seconds, with reduced concentrations of MPO, ALT and ALP when compared with control.

Under the influence of IL, i.e., IL-1, IL-2, and TNF-α, liver cells synthesize and secrete APP (376). CRP is one of the most important APP (377). We observed consistent findings for an effect of SAA impairment on APP increasing CRP concentration (152, 279, 293, 355, 357,

361, 364). We found conflicting evidence regarding APP secretion in SAA impairment, with differences in CRP concentration (334), vitamin status and SAA metabolites concentration (355). Some studies reported that low PLP concentrations presented high CRP concentrations; however, no association was observed between low PLP and high CRP (293, 356). In contrast studies with inhibition of CBS and vitamin B6 or folate-deficient diets reported a relationship between inflammation and CRP concentration in the acute phase response (357). Disturbance of the hepatic transsulfuration reactions appears to be responsible for the imbalance between pro-oxidants and antioxidant capacity, which further aggravates the oxidative stress associated with SAA impairment (380). We identified 8 studies (animal n=7; human n=1) that reported the effect of SAA impairment on oxidative stress (277, 282, 289-291, 362, 371). Toxic concentrations of Hcy (HHcy) increase activity of MPO, MDA activity that is a marker of inflammatory and oxidative stress, LOX-1 and SOD-1 and ROS in heart, lung and liver with different SAA impairment models (371). In contrast, one study reported the beneficial effect of inhibition of CSE reducing MPO and MDA concentrations in heart, liver, lung, and kidney after burn (289). These results suggest that a SAA impairment can induce oxidative stress and lead to decreased tissue function. Additionally, inhibition of CSE may be beneficial in attenuating oxidative stress and improving tissue function under inflammatory challenges.

4.5.3. Impaired SAA metabolism: changes on tissue function

There are some reports indicating that SAA impairment is involved in the regulation of certain tissue functions including cardiac injury markers ejection fraction, Left Ventricular Internal Diameter, diastole (LVIDd), Left Ventricular posterior wall, diastole (LVPWd), hepatic injury markers ALP and ALT and renal dysfunction markers creatine and BUN (281, 289, 291, 322,

359, 362, 363, 365, 371). Supplementation of Met seems to be implicated in heart function failure (363, 371). Studies with Met supplementation with or without inhibition of CBS reported an increase in Hcy concentration in plasma (363). It has been reported that decreased cardiac function due to high concentration of Met in diet lead to increased HHcy, which is considered as a cardiovascular risk (371, 381, 382). Animal studies with supplementation of Met observed upregulated iNOS, MMP2 and MMP9 in heart, which showed endothelial dysfunction since these endothelial markers are responsible for vascular remodeling (363). In addition, the ensuing cytokine storm that develops in the affected tissues results from a cascade of cytokine production, leading to increased leukocyte infiltration and activation that damages the tissue leading to organ failure (27). High concentrations of Hcy (>15 μmol/L) have been associated with an increased risk of heart failure, myocardial infarction, and stroke (363, 373). NF-κβ plays an important role in the initiation and development of several diseases and heart failure (383-385). A study conducted by Liu et al (363) supported the effect of HHcy and high NF-κβ concentration. They observed that rats fed with a high methionine (1-3%) diet (HMD) induced phenotypic changes such as inflammatory infiltration and cell apoptosis (363). Moreover, Chatuverdi et al. (371) showed that mice with CBS deficiency fed with a HMD exhibited decreased cardiac function. The authors concluded that a HMD poses a high cardiac threat since HMD can induce vascular remodeling and decreased cardiac function in a way which are not beneficial for the heart (371). In summary, HMD stimulates HHcy and pro-inflammatory markers leading to endothelial dysfunction and heart failure, thus highlighting some of the negative effects of SAA impairment.

Inhibition of CSE has also been recognized as an SAA impairment model to study tissue function **(Tables 4.8 and 4.9)**. CSE is the dominant enzyme for H_2S formation (386). H_2S

exerts potent cytoprotective effects in the setting of cardiovascular disease in various animal model systems (323, 387-390). King et al. (362) reported that inhibition of CSE led to elevated ROS, diminished NO concentrations and exacerbated myocardial injury. The authors concluded that H_2S deficiency from the inhibition of CSE resulted in a more susceptible myocardial injury due to the combination of insufficient NO bioavailability and increased oxidative stress (362). CSE deficiency models have also been used for the study of liver failure (281, 322). CSE deficiency markedly attenuated inflammation and apoptosis, protected hepatic architecture, and improved survival of mice after GalN/LPS challenge (365). Shirozu et al. (365) reported that congenital CSE deficiency increases thiosulfate concentrations in the liver, thus preventing ALF by increasing antioxidant mechanisms. The authors suggested that inhibition of CSE prevents acute inflammatory liver failure by augmenting thiosulfate concentration, attenuating ALT and improving survival of primary hepatocytes after GalN/LPS challenge (365). Ahmad et al. (289) also reported the protective effect of CSE deficiency against burn. In their study, the authors observed that inhibition of CSE resulted in a significant amelioration of burn-induced accumulation of myeloperoxidase levels in heart, lung, liver, and kidney and significantly attenuated inflammatory mediators like TNF-α, IL-1β, IL-6 compare with control (289). In summary, CSE deficiency is a commonly used SAA impairment model to study the development of severe pathophysiological responses when subjected to various stressors, such as tissue ischemia-reperfusion (362, 391), atherosclerosis (290), hepatitis and liver fibrosis (322) or chronic heart failure (323). However, models of CSE deficiency also present an over-activation of the systemic inflammatory response, and often involve some form of single-organ or multiple-organ injury.

4.5.4. Impaired SAA metabolism: effects on disease development

Homocysteine has been widely identified as a risk factor in numerous disease states (392-398). HHcy, plasma Hcy levels >15 μmol/L, initiates atherosclerosis by modulating cholesterol biosynthesis and by significantly inducing the level of other cardiovascular risk factors and markers, which play important roles in initiating atherosclerosis (364). Zhang et al. (367) reported that CBS-/+ mice fed with HMD induced severe HHcy (Hcy= 20.3 ±2.9 μmol/L) and accelerated atherosclerosis. These authors concluded that HHcy may be involved in the pathogenesis of atherosclerosis by significantly enhancing lymphocyte proliferation in response to T-cell mitogen (367). Sharma et al (364) also concluded that HHcy initiates atherosclerosis by modulating the cholesterol biosynthesis and by significantly increasing concentrations of caveolin-2 which cover the aorta with atherosclerotic plaques. Caveolin forms oligomers and associates with cholesterol in certain areas of the cell membrane of cells and plays a role in cancer and vascular diseases (399). These results suggest that HHcy may play an important role initiating and developing atherosclerosis, modulating the metabolism of cholesterol and upregulating P2 receptors and caveolins.

HHcy (400-404) and low vitamin B6 status (405-407) have been demonstrated to be independent risk factors for CAD (152). A study conducted by Schroechksnadel et al. (357) observed that subjects hospitalized for coronary angiography with moderate HHcy were correlated with significantly higher concentrations of neopterin and CRP. Additionally, patients with moderate HHcy showed low folate and vitamin B12 status (357). These authors concluded that moderate HHcy relates to B-vitamin deficiency which may have occurred due to an increased demand for oxidation-sensitive vitamins associated with CAD (357). Neopterin as well as the combination of neopterin and CRP have been shown to be predictive of adverse

outcome in patients with both stable CAD and ACS (357). Lin et al. (293) studied the effect of low PLP associated with an increased risk of CAD. These authors reported that patients suspected of having CAD with low concentrations of PLP had significantly higher concentrations of Hcy and LPL and lower HDL values compared with the control group (293). They concluded that borderline B6 deficiency strongly enhanced the risk of CAD (293). SAA impairment due to CBS deficiency has also been reported to lead to an increased risk of CAD (284, 285). Inhibition of CBS enhanced vascular damage by increasing Hcy or its derivatives (homocysteine-thiolactone) concentrations (284). Patients with CBS deficiency significantly decreased HDL and apolipoprotein A (ApoA-1) concentration compared with control (285). Low HDL cholesterol is a strong independent predictor of CAD, particularly when its level are below 40 mg/dL (375). These results suggest that Hcy can cause important metabolic perturbations in CBS deficient patients, including a decrease in HDL and ApoA-1 concentrations, thus further enhancing the risk of CAD. In summary, SAA impairment can lead to a pro-inflammatory state associated with an increased risk of CAD.

4.5.5. Limitations and future directions

Certain limitations require consideration for the current systematic review. Only clinical and controlled trials were included in the review to minimize selection bias associated with the nonrandomization of the subjects. However, our initial search strategy captured observational studies and other animal models which could offer further insight into this field albeit without the same scientific rigor. With regard to the specific SAA impairment, some studies included additional supplementation of vitamins. Such studies were not excluded from the review. In fact,

by including studies with vitamin supplementation (i.e., B6, folic acid, B12), an effect for antioxidants attenuating inflammatory markers was observed.

As noted above, most human and animal studies have shown that an impairment in SAA metabolism can play a major role in modulating inflammatory markers and maintaining the immune system. Nevertheless, future interventions studies with subjects with chronic inflammation are warranted to evaluate the long-term effects of SAA impairment on inflammatory markers and disease development. Hcy and pro-inflammatory cytokines concentrations, lipid profile and tissue function are the co-factors that should be considered when evaluating differences in response to SAA impairment.

4.6. Conclusion

Accordingly, for the studies examined in this review, an impairment in SAA metabolism affects the acute inflammatory response, induces tissue damage, and plays a key role in the development of vascular diseases. More importantly, pro-inflammatory cytokines and lymphocyte migration can increase following the attainment of toxic (>15 μmol/L) plasma concentrations of Hcy. An increased level of Hcy can be caused by an imbalance in the TM and TS pathways. In summary, SAA impairment could be involved in the regulation of acute inflammatory markers by: 1) stimulating the production and expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 which increases leukocyte proliferation to enhance the inflammatory response; 2) promoting APP secretion (CRP) aggravating oxidative stress; 3) inducing ROS, which leads to failure on several tissues such as heart, liver, kidney and lung; 4) increasing the risk of several vascular diseases like atherosclerosis and CAD. Collectively, these results lend support to advice not restricting consumption of SAA-rich food to maintain a healthy immune system.

Furthermore, the beneficial effects of diets enriched in vitamins involved in SAA metabolism (B6, B12 and folate) regulating Hcy concentrations lead to the conclusion that keeping special attention to B-vitamin concentration should be included in inflammatory-management therapies.

CHAPTER 5

STUDY 1

The temporal and dosage effects of LPS administration on markers of inflammation and SAA metabolism in Wistar rats.

5.1. Abstract.

Inflammation is the response of the immune system to traumatic, infectious, post-ischemic or autoimmune injury. Administration of the bacterial endotoxin lipopolysaccharide (LPS) yields a model to study the pathophysiology of sepsis. While LPS induces hemodynamic, hematologic and metabolic changes similar to those observed in septic patients, multiple dosages of IP LPS have been studied, with no consensus being reached regarding the ideal dosage of LPS to study the role of SAA as mediators of immune responses. The current study was designed to determine whether 50 or 100 μg/kg IP LPS would induce greater temporal changes in inflammatory markers and sulfur amino acid (SAA) metabolism. Male Wistar rats (n=15 ~250g) were fed with an amino acid defined diet for ten days. Following the diet intervention, the rats were randomized to one of three groups, either 0 (saline control), 50, or 100 μg/kg LPS via IP injection, and maintained under general anesthesia for a period of five hours. Venous blood sampling was performed at 0, 30, 120, 240 and 300 min and plasma cysteine (Cys), homocysteine (Hcy), and tumor necrosis factor-alpha (TNF-α) concentrations were determined. Body temperature was measured at regular intervals. LPS concentration did not alter total plasma Cys or Hcy. LPS induced a significant increase in plasma TNF-α ($p< 0.001$) after 120 min (1188 pg/mL ±1590 for 50 μg/kg and 1503 pg/mL ±1706 for 100 μg/kg LPS) in comparison to control; however, there was no significant difference between LPS doses. Moreover, while there was no significant difference between body temperature of animals in the 50μg/kg and control groups, significant differences were found between the 100 μg/kg and control, as well as 100 μg/kg and 50 μg/kg at 120 min ($p<0.05$) and 240 min ($p<0.05$). These results indicated that an IP LPS dose of 100 μg/kg induced inflammation to a greater extent and would be appropriate

for use in the future to investigate the effect of variable dietary SAA content on inflammatory response.

5.2. Introduction.

Inflammation is a protective biological response that involves blood vessels, immunological cells, and inflammatory mediators (22). Hallmarks of inflammation include five clinical signs, including redness, fever, swelling, pain, and loss of function (22, 24). Fever is manifested by a rise in body temperature which represents a physiological, highly regulated, and adaptive response to pathological challenges such as infection, injury trauma and inflammation (408-410). Infectious fever is triggered by several exogenous viral, bacterial, mycobacterial and endogenous pyrogens, including lipopolysaccharides (LPS) (411). With the lack of proper treatment, inflammation could lead to multiple issues including persistent tissue damage by leukocytes or lymphocytes, metabolic changes (25), the development of arthritis, inflammatory bowel disease (26), cancer, cardiovascular disease and stroke (24).

LPS derived from the outer membrane of Gram-negative bacteria have been confirmed to trigger inflammatory responses, fibrosis, and hepatocellular damage (412-414). It is proposed that the interaction of LPS with Toll-like receptors (TLR) (415, 416) induces endotoxemia, which is responsible for the development of hepatic damage (232). TLR consist of a family of pattern recognition receptors (PRPs) that recognise components of bacteria, viruses and fungi (232). Of these PRP, TLR4 reacts to LPS and leads to the activation of Kupffer cells (417). The activation of NF-κβ induces TLR4 signal transduction further enhances the synthesis of tumor necrosis factor-alpha (TNF-α) (418). This contributes to the formation of hepatic steatosis (416, 419), by attenuating sterol regulatory element-binding proteins (SREBP) (232). These proteins are a family of membrane-bond transcription factors that trigger enzyme-encoding genes involved in lipid synthesis (420). TNF-α is also involved in regulation of the expression of the transcriptional factor NF-κβ, which participates in the acute inflammatory response associated

with increased generation of reactive oxygen species (ROS) (411). Agonistic forms of LPS trigger numerous physiological immunostimulatory effects in mammalian organisms, but higher doses can also lead to pathological reactions such as the induction of endotoxin shock. LPS causes an endotoxin shock which is associated with coagulopathy and multi-organ dysfunction (421). It is generally considered that endotoxin shock is induced by numerous pro-inflammatory cytokines including TNF-α, interleukin (IL)-6 and IL-1 that are mainly released by macrophages (422-425). TNF-α and IL-1 represent the archetypal pro-inflammatory cytokines, which are rapidly released upon tissue injury or infection. TNF-α plays an important role triggering the synthesis of endogenous mediators of fever such as endogenous pyrogens, which initiate a chain of biochemical and physiological changes leading to the upward shifting of the thermoregulatory set-point (426).

Sulfur amino acids (SAA) participate in the control of oxidative status since they are involved in the synthesis of intracellular anti-oxidants, particularly glutathione (GSH), and in the methionine sulfoxide reductase antioxidant system (199). Due to the vast number of reducing equivalents supplied by GSH, SAA is the main intracellular redox buffer in different cell types (182, 427). It has been shown that the increase of blood and/or intracellular GSH through Cys derivatives or Cys-rich dietary proteins can impact the redox state of thiols (15). Evolving data shows that SAA influences the secretion of numerous cytokines, such as those related to fever and inflammation (14). The enhancement in the content of GSH in monocytes has been shown to serve as a buffer against pro-oxidants (428), that effectively protects the surrounding tissue by decreasing the negative effects of cytokines like IL-1, IL-8, TNF-α, and retarding the activation of Kupffer cell NF-κβ (16). In addition, the role of glutathione in the production of TNF-α by macrophages treated with LPS was postulated by Salzano et al. (429). In the present study, LPS

was used as a model for systemic inflammation. LPS is reported to exert pro-inflammatory effects in rodent models of systematic inflammation; however, different doses have not been evaluated against specific outcomes in the inflammatory response. For this reason, we initially established whether 50 or 100 μg/kg IP LPS would induce greater temporal changes in inflammatory markers and SAA metabolism.

5.3. Materials and Methods.

All procedures were approved by the Institutional Animal Care Committee of the University of Manitoba, in accordance with the guidelines of the Canadian Council on Animal Care (Protocol Number F19-014).

5.3.1. Study design

For this study, male Wistar rats (n=15, ~250 g) were provided an amino acid-defined diet (**Table 5.1**) formulated to meet the nutrient requirements of rats. Diets were formulated to contain 20% amino acid mix (Dyets, Bethlehem, PA), 7% total fat, and 5% cellulose with the remaining energy derived from corn starch. Vitamins (AIN-93 formulations; Harlan Teklad, Madison, WI) and minerals (AIN-93G Mineral Mix, Dyets.) were added to diets to meet the micronutrient requirements of laboratory rats. The SAA ratio for the study was 50% methionine and 50% cysteine (50Met:50Cys) as percentage of requirement for SAA. All groups were fed the experimental diet ad libitum for 10 days with free access to water. Food intake was recorded daily while body weight was measured after the acclimatization period and at the day of termination. After 10 days on the experimental diet, animals were anesthetized with 5% isoflurane with an oxygen flow at .8-1 L/min and randomized to one of 3 possible groups of IP

Table 5.1. Amino acid defined diet.

	Amino Acid defined diet (g/kg) 50Met:50Cys
L-Alanine	3.5
L-Arginine HCl	12.1
L-Asparagine	6.0
L-Aspartic Acid	3.5
L-Cystine	**5.1**
L-Glutamic Acid	40.0
Glycine	23.3
L-Histidine HCl, monohydrate	4.5
L-Isoleucine	8.2
L-Leucine	11.1
L-Lysine HCl	18.0
L-Methionine	**6.2**
L-Phenylalanine	7.5
L-Proline	3.5
L-Serine	3.5
L-Threonine	8.2
L-Tryptophan	1.8
L-Tyrosine	5.0
L-Valine	8.2
Sucrose	351.68
Corn Starch	150.0
Maltodextrin	150.0
Soybean Oil	80.0
Cellulose	30.0
Mineral Mix, AIN-93M-MX (94049)	35.0
Calcium Phosphate, monobasic, monohydrate	8.2
Vitamin Mix, AIN-93VX (94047)	13.0
Choline Bitartrate	2.5
TBHQ, antioxidant	0.02

[1]Met, methionine; Cys, cysteine.

LPS in 0.9% saline (Escherichia coli, 0111: B4 from Sigma Chemical Co., USA) – 0 (control), 50, or 100 μg/kg (n = 5/group). Immediately before the administration of LPS, rats were maintained under anesthesia and blood samples collected via saphenous vein into heparinized microvette 500 μl tubes at time 0, 30, 90, 120, 180, and 240 min post-injection (total volume of blood withdrawal <10 mL). At 300 min, rats were terminated by exsanguination and blood was collected into heparinized tubes. Body temperature was monitored via a rectal digital thermometer throughout the 5 h procedure. Immediately after collection, blood was rapidly centrifuged (10,000 x g for 15 min), plasma removed and frozen at -80 ºC for later analysis.

5.3.2. Determination of plasma and liver cysteine and homocysteine.

Plasma and liver Cys and Hcy were measure using a method previously described by Mayengbam et al (430). Briefly, 20 μl of 10% tris (2-carboxylethyl) phosphine (TCEP) (Pierce chemical Co, Rockford, IL, USA) was added to plasma and liver samples, and incubated at room temperature for 30 min. After incubation, 125 μl of 0.6 M perchloric acid was added to deproteinize the sample. The samples were centrifuged at 5,000 x g for 10 min and 50 μl of the supernatant was added to 100 μl of potassium borate buffer (pH 10.5), containing 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and 50 μl of 1 mg/mL ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) (Wako Chemicals, Richmond, VA, USA). This solution was incubated for one hour at 60 ºC and then cooled to 4 ºC. 20 μl of the sample was injected onto a $C18_{(2)}$ column to run at a flow rate of 1.0 ml/min. Plasma total Hcy and Cys concentrations were analyzed according to a reverse phase-HPLC (Shimadzu Inc, Nakagyo-82ku, Kyoto, Japan) method with fluorescence detection (λEx= 385 nm, λEm= 515 nm).

5.3.3. Determination of plasma and liver GSH

Plasma and liver GSH concentrations were treated and measured according to a GSH assay kit (Cayman Chemical Co, USA). The kit utilizes an optimized enzymatic recycling method using glutathione reductase, for the quantification of GSH. Briefly, 50 µl of plasma and liver samples were added to each of the sample wells. Then, 150 µl of the assay cocktail was added to each of the wells containing standards and samples using a multichannel pipette. GSH concentration of the samples was determined after reading the plate at 405-414 nm at five minutes intervals for 30 min. All samples and standards were run in duplicates with a detection range of 0.5-16 µM (GSH). Hemolysis was not evident in the plasma samples.

5.3.4. Determination of TNF-α protein concentrations

TNF-α concentrations were measured using a commercially available ELISA kit (# KRC3011, Invitrogen, MA, USA). The rat TNF-α ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of rat TNF-α. Briefly, 50 µl of sample diluent and 50 µl of the samples were added in to the microwell strips. Then, 50 µl of a biotin-conjugated anti-rat TNF-α antibody was added and bound to the rat TNF-α captured by the first antibody. Samples were incubated at room temperature for 2 h. Following incubation unbound biotin-conjugated anti-rat TNF-α antibody was removed during a wash step. A volume of 100 µl of streptavidin-HRP was added to all wells and incubated for 1 h at room temperature. Following incubation unbound Streptavidin-HRP was removed during a wash step, and substrate solution reactive with HRP was added to the wells. A coloured product was formed in proportion to the amount of rat TNF-α present in the sample. The reaction was terminated by addition of acid and absorbance measured at 450 nm.

5.3.5. RNA Isolation and Quantitative Real-Time PCR

RNA expression of TNF-α in spleen was evaluated from total RNA isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of the RNA was assessed by spectrophotometry (DU800 Spectrophotometer, Beckman Coulter Canada Inc.), and the absorbance ratio (wavelength 260/280 nm) was within 1.8 to 2.1. Reverse transcription was done using 1 μg of total RNA and the SuperScript VILO cDNA Synthesis Kit (Bio-Rad, Ca, USA), with the cDNA used for the detection of gene expression. The mRNA abundance of the genes was analyzed by SYBR green real-time PCR, using primer sequences for TNF-α, and *Actb* encoding β-actin as previously described by Jing et al (431). The following conditions for quantitative real-time PCR were used: a) 95 °C for 20 s and 40 cycles of denaturation at 95 °C for 3 s; b) combined annealing and extension at respective annealing temperature (60 °C for both TNF-α and *Actb*) for 30 s; and c) one 3-segment cycle of product melting (95 °C/15 s, 60 °C/1 min, 95°C/15 s) for confirmation of the specific amplification of the 2 genes. Samples were performed in triplicate 20 μl reactions using a StepOne Real-Time PCR System (Life Technologies Inc., ON, Canada). The primer sequences used were as follow: TNF-α (5'-GGCCACCACGCTCTTCTGTC-3', 5'- GGGCTACGGGCTTGTCACTC-3'), GAPDH (5'-CGTGGAGTCTACTGGCGTCTT-3', 5'-CATTGCTGACAATCTTGAGGGA-3'), Beta Actin (5'-GAGATTACTGCCCTGGCTCCTAG-3', 5'-CGGACTCATCGTACTCCTGCTT-3') Data were normalized to *Actb* controls, with results analyzed using the delta-delta Ct method, also known as the $2^{-\Delta\Delta Ct}$ (User Bulletin #2: Relative Quantitation of Gene Expression; Applied Biosystems, MA, USA). For controls, saline injection was used for relative abundance measurements.

5.3.6. Statistics

Data are expressed as mean ±SE. The effect of LPS dose on body temperature and plasma TNF-α over time was determined using repeated measures ANOVA. One-way ANOVA was used to determine the effect of LPS dose on splenic TNF-α gene expression, as well as plasma and liver concentrations of Cys, Hcy and GSH. As a post-hoc test the Tukey's multiple comparison test was used using GraphPad Prism, 7.0. The threshold of statistical significance was $p<0.05$ for all tests.

5.4. Results

5.4.1. Determination of body temperature

We measured the effects of an IP dosage of LPS at either 50 or 100 µg/kg in relation to the induction of fever in rats after 5 h. As shown in **Fig 5.1**., fever was strongly induced ($p<0.01$) by the administration of LPS at 100 µg/kg in a time dependent manner, whereas administration of LPS at 50 µg/kg did not affect body temperature compared with saline-treated rats.

5.4.2. Determination of TNF-α protein concentrations and gene expression

The inflammatory cytokine TNF-α was analyzed in plasma after LPS stimulation. Compared to saline, LPS at 100 µg/kg ($p<0.001$) and LPS at 50 µg/kg ($p<0.01$) dramatically increased TNF-α concentration (**Table 5.2**) in rats after 120 min. No significant difference in TNF-α

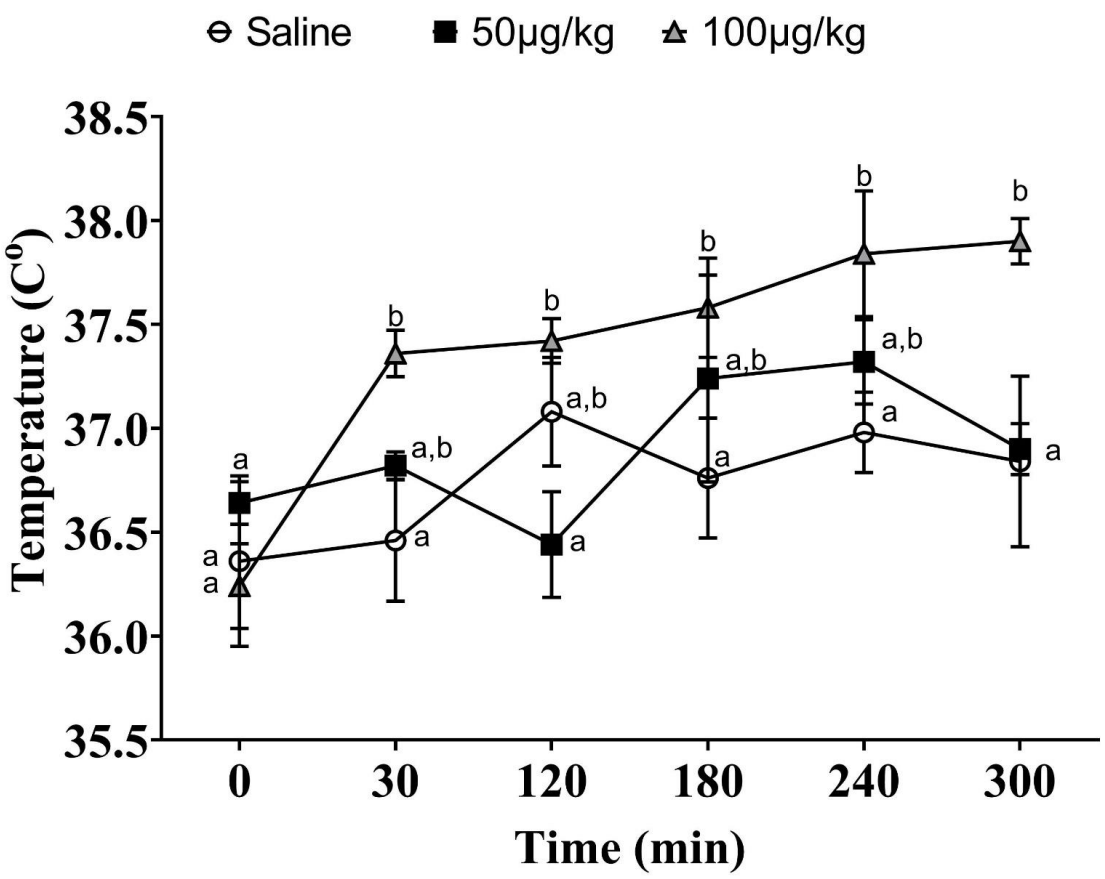

Figure 5.1. Body temperature of Wistar rats over a period of five hours after IP injection of saline, LPS 50 µg/kg and LPS 100 µg/kg. Mean ± SE (n = 5). Data were analyzed via repeated measures one-way ANOVA with Tukey's post-hoc test. Means with different letters are significantly different (p<0.05).

Table 5.2. Plasma TNF-α injected with two different doses of LPS.

TNF-α (pg/mL)	Dose			*p* value		
	Saline	50µg/kg	100 µg/kg	Time	Dose	Time x Dose
0 min	0.002	0.0035	0.004	0.02	0.1	0.02
30 min	0.0042	0.0072	0.0075			
120 min	0.005	1189**	1503****			
180 min	0.005	202.1	84.68			
300 min	0.002	0.0038	0.0055			
SE	0.001	231	297			

[1]Data are presented in mean ± SE (n = 9). Data were analyzed via two way-ANOVA with Tukey's post-hoc test. Significant differences are designated by the symbol **, (p<0.01), **** (p<0.0001)

concentration was found between LPS doses. TNF-α concentration peak was reached within 2h TNF-α mRNA expression (**Fig. 5.2**), a marker of inflammation, was clearly detected in the splenic tissue of rats treated with 50 and 100 μg/kg LPS ($p<0.05$ and $p<0.05$, respectively). However, no significant differences were observed between doses.

5.4.3. Determination of SAA metabolites

The influence of LPS administration in plasma concentrations of Cys and Hcy (**Fig 5.3A, C**) were evaluated. Administration of LPS at 50 and 100 μg/kg did not alter concentrations of Cys and Hcy in plasma. LPS-treated rats with 50 and 100 μg/kg decreased significantly ($p<0.001$ and $p<0.001$, respectively) the concentration of plasma GSH (**Fig 5.3E**). In addition, 50 μg/kg of LPS further reduced ($p<0.001$) plasma concentration of GSH compared to rats treated with LPS at 100 μg/kg after 5 h.

As shown in **Fig 5.3B, D, F**, the effect of LPS on SAA metabolites in the liver was dose=-dependent. Administration of LPS at 50 μg/kg strongly increased liver concentration of Cys ($p<0.0001$), Hcy ($p<0.05$) and GSH ($p<0.0001$) when compared with saline-treated rats. Concentrations in the liver of Cys ($p<0.0001$), Hcy ($p<0.0001$) and GSH ($p<0.0001$) also increased significantly after the administration of LPS at 100 μg/kg. In addition, LPS at 100 μg/kg had a significantly stronger effect increasing concentration of Cys ($p<0.0001$), Hcy ($p<0.0001$) and GSH ($p<0.001$) in liver compared with rats treated with 50 μg/kg LPS.

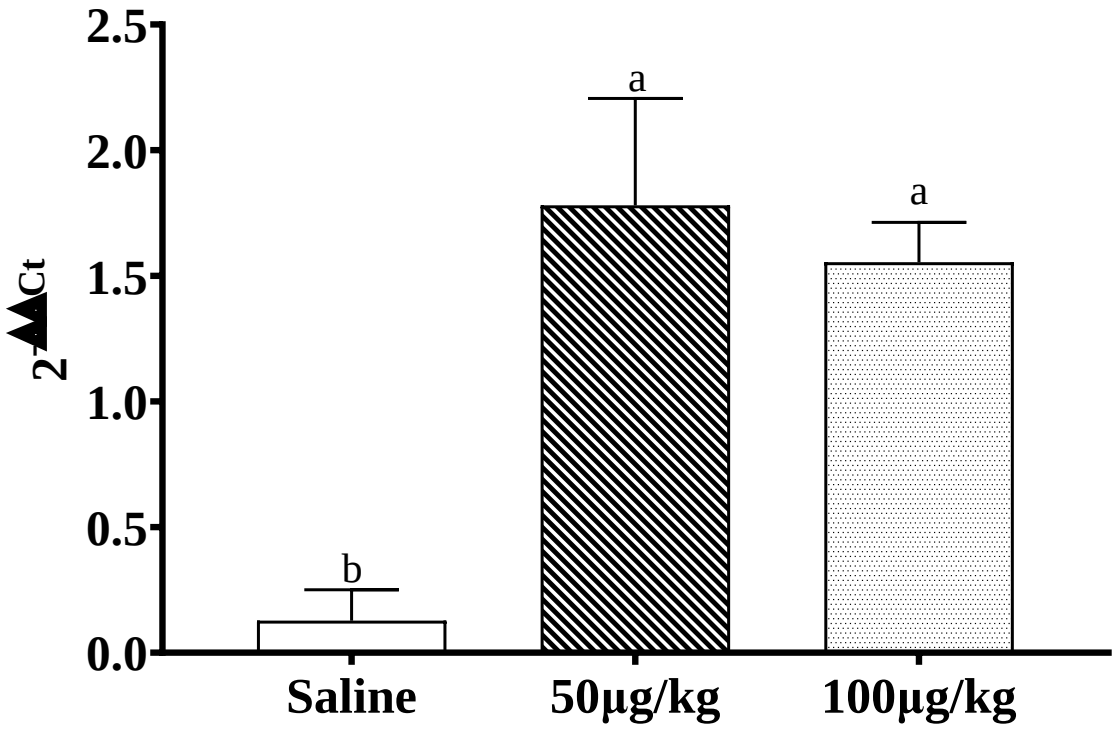

Figure 5.2. Gene expression of TNF-α in spleen of Wistar rats five hours after IP injection of saline, LPS 50 µg/kg and LPS 100 µg/kg. Data are presented as mean ± SE (n = 5). Data were analyzed via One-Way ANOVA with Tukey's post-hoc test. Means with different letters are significantly different (p<0.05).

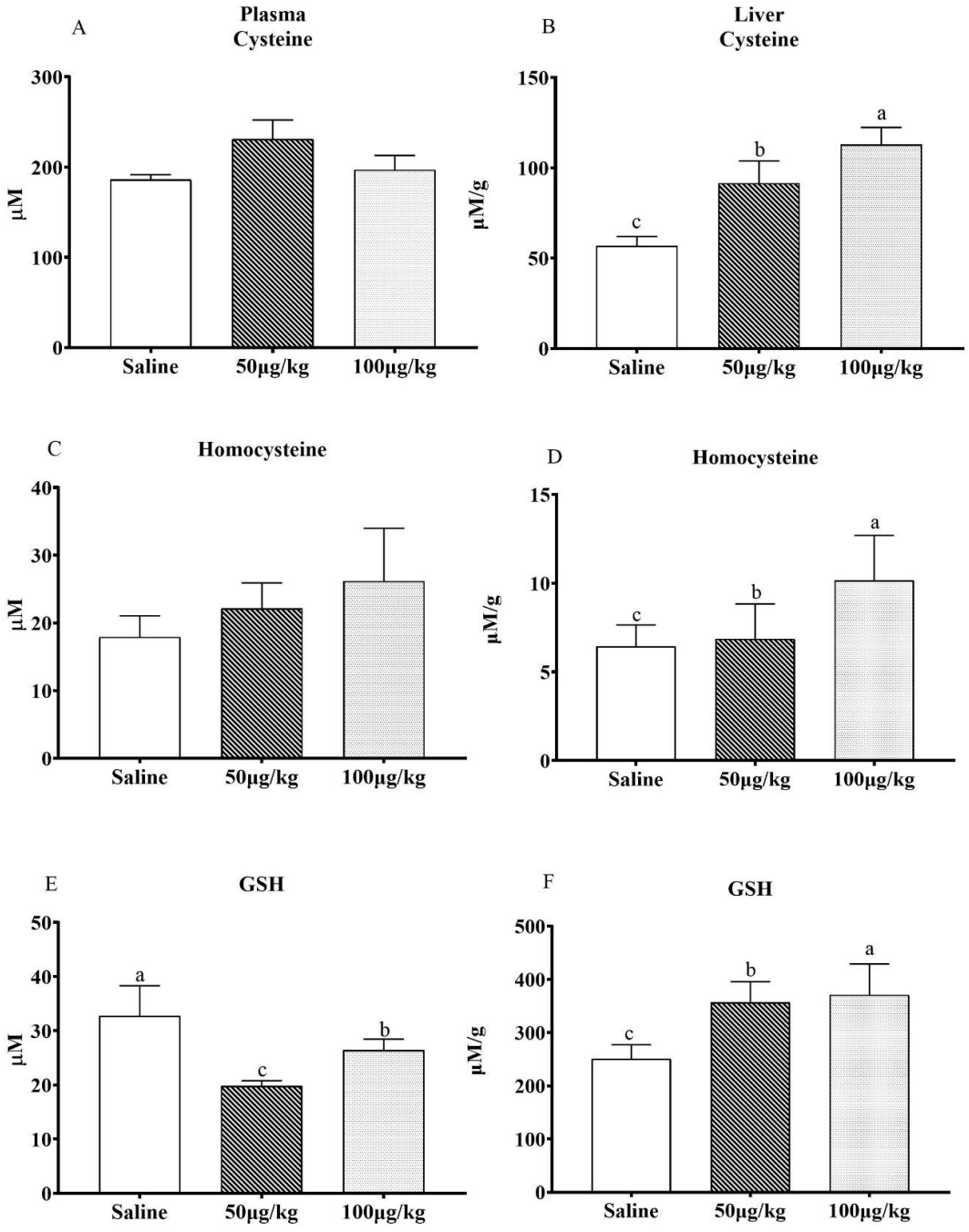

Figure 5.3. Plasma [A, C, E] and liver [B, D, F] concentration of Cysteine, Homocysteine and GSH of Wistar rats after a period of five hours after IP injection of saline, LPS 50 µg/kg and LPS 100 µg/kg. Data are shown in mean ± SE (n = 5). Data were analyzed via One-Way ANOVA with Tukey's post-hoc test. Means with different letters are significantly different (p<0.05)

5.5. Discussion

Our study found that an IP LPS administration of 100 μg/kg induced fever after 5h post injection. Also, TNF-α production was significantly increased after IP LPS injection at 50 and 100 μg/kg; however, no significant differences were observed between doses, and that LPS did alter SAA metabolite concentrations after 5h post injection.

Body temperature data is presented in Figure **5.1**. The effect of LPS in body temperature has been described previously (111, 113). An IP LPS injection at a dose of 100 μg/kg significantly increased temperature compared with control inducing fever after 30 min post injection and remain steadily increasing for the entirety of the procedure (5h). Our results are supported by a study conducted by Wrotek et al. (111) where LPS at a dose of 50 μg/kg induced fever in old rats (38± 0.01°C) and young rats (38.19± 0.06°C). On the other hand, contrary to other studies (113) rats injected with 50 μg/kg IP LPS in our study did not increased body temperature. Our results suggest that.an IP LPS injection at a dose of 100 μg/kg more effectively raised body temperature, effectively inducing fever.

The effect of LPS in cytokine production has been well studied; however, little is known of the effects that different doses of IP LPS may have on pro-inflammatory markers. For the current study, we examined whether different doses of LPS-induced (50 and 100 μg/kg) elicited changes in TNF-α concentrations in rats. The concentration of TNF-α was significantly increased in the experimental groups with IP LPS compared with saline ($p<0.001$). Plasma TNF-α protein concentrations in LPS-injected rats exhibited a significant increase when compared with saline at 120 min (50 μg/kg, $p<0.001$ and100 μg/kg, $p<0.0001$); however, there was no significant difference found between LPS doses. An increase in TNF-α concentrations were expected with an LPS injection since TNF-α is considered the first pro-inflammatory

cytokine-induced after LPS injection (111, 426). Our results are similar to those presented by Turrin et al (116). They showed that Wistar rats injected with 100 μg/kg of IP LPS presented significantly higher TNF-α concentrations compared with control (116).

Administration of both doses (50 and 100 μg/kg) of LPS induced significantly higher TNF-α mRNA expression in splenic tissue compared with control animals (p<0.01). However, no significant differences were observed between doses. High concentrations of TNF-α mRNA after 4 h were likely due to the considerable amounts of TNF-α that are produced and released by macrophages in response to LPS and other bacterial products [34]. A study conducted by Ato et al. (432) in MCP-1 transgenic mice treated with 5 mg/kg of LPS demonstrated the expression of a peak in TNF-α mRNA after two hours with a prompt return to baseline. Results of the current investigation demonstrated that an IP LPS dose of 100 μg/kg induced a greater concentration of TNF-α and the timepoints measured in the splenic tissue were enough to capture a rise in TNF-α expression.

Cys requirements increase during inflammatory states (200). LPS also influence SAA metabolites. In our study, after 5h, no significant differences were observed between IP LPS doses for either cysteine or homocysteine concentrations. However, plasma GSH concentration decreased significantly with IP LPS at both doses. Plasma concentration of GSH in rats treated with 50 μg/kg and 100 μg/kg IP LPS were 39 and 19% lower, respectively, compared with the control group after 5 h. Our study also showed that Cys and Hcy concentrations in liver after an LPS injection of 50 μg/kg and 100 μg/kg were significantly (p< 0.001) higher compared with control. Since GSH is mainly synthesized in the liver, high concentrations of Cys and Hcy could result in the generation of GSH (433, 434). Our results showed that liver GSH concentration was significantly higher in both doses of LPS (50 and 100 μg/kg) compare with control. In contrast,

Minamimaya, et al (435)**,** reported that after 4 h of the administration of 20 mg/kg of LPS, GSH concentration in liver decreased slightly. These data may support the fact that acute infection in the rats significantly increased the synthesis of GSH in liver after 5 h and that the dynamic aspects of GSH metabolism in liver and plasma might vary significantly during the time course of endotoxemia.

5.6. Conclusion

In summary, these results indicated that an LPS dose of 100 μg/kg IP induced inflammation to a greater extent and would be appropriate for use in the future to investigate the effect of variable dietary SAA content on inflammatory response. Interestingly, since a limited number of studies have been conducted on the impact that SAA dietary supply, including the ratio of methionine to cysteine, have on inflammatory processes, further studies are warranted to determine the potential role of SAA in the resolution of inflammation.

CHAPTER 6

STUDY 2

Alteration of the dietary methionine:cysteine ratio modulates the inflammatory response to an inter-peritoneal injection of lipopolysaccharide in Wistar rats.

6.1. Abstract.

Sulfur amino acids (SAA) are essential for multiple physiological/metabolic processes, with the ratio of dietary methionine: cysteine (Met:Cys) being an important contributor to pro-inflammatory responses, including tumor necrosis factor-alpha (TNF-α) activity. The current study was designed to determine the effect an altered dietary SAA ratio, and the resulting reliance on the transsulfuration pathway to supply Cys, will have on the inflammatory response. In the present study, 100 µg/kg of an intraperitoneal (IP) injection of lipopolysaccharide (LPS) was used as a model for systemic inflammation. Male Wistar rats were randomized to one of two amino acid-defined diets, (100Met:0Cys or 50Met:50Cys) and subdivided to receive either IP LPS or saline injections. LPS significantly increased total plasma Cys, homocysteine (Hcy) and glutathione (GSH) 240 min post-IP injection in rats fed a 50Met:50Cys ratio compared to other treatments. The TNF-α area under the curve for LPS-treated rats consuming a dietary 50Met:50Cys ratio was significantly higher ($p< 0.004$) compared to those consuming a dietary 100Met:0Cys ratio. A significant increase in the percentage of leukocytes that were neutrophils was observed in rats injected with LPS when compared to saline with no effect of diet. These results demonstrate that 100Met:0Cys may slow production of TNF-α in response to LPS, but not the proportion of circulating neutrophils. These results demonstrate that an alteration of the dietary Met:Cys ratio did not attenuate the inflammatory response to an IP injection of LPS in Wistar rats; however, a diet with a balanced Met:Cys ratio increased concentrations of Cys and GSH which may result in a more rapid response to an LPS challenge.

Key words: Methionine, Cysteine, SAA ratio, Inflammation, LPS

6.2. Introduction.

Systemic inflammation is a protective biological response that involves blood vessels, immunological cells, and inflammatory mediators (22). Hallmarks of inflammation include five clinical signs, including redness, fever, swelling, pain, and loss of function (22, 436). Systemic inflammation triggers the production of acute phase proteins (APP) in the liver within a few hours after the onset of inflammation (346). Pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1 that are mainly released by macrophages (422-425), play an important role in the production of these acute phase proteins. TNF-α and IL-1 represent the archetypal pro-inflammatory cytokines, which are rapidly released upon tissue injury or infection. TNF-α plays an important role triggering the synthesis of endogenous mediators of fever, such as pyrogens, which initiate a chain of biochemical and physiological changes leading to the upward shifting of the thermoregulatory set-point (426). The metabolic response to infection and injury is mediated by a variety of secondary messengers and cell signaling mechanisms, which offer broad scope for nutritional modulation (121).

One of the adverse effects of inflammation is collateral damage to surrounding tissue caused by oxidative stress. Sulfur amino acids (SAA), methionine (Met) and cysteine (Cys), play an important role in the effectiveness of antioxidant defense. SAA are involved in the regulation of oxidative status since they are involved in the production of intracellular anti-oxidants, specially glutathione (GSH), and in the methionine sulfoxide reductase antioxidant system (199). SAA are the major intracellular redox buffer in various cell types due to the large amount of reducing equivalents supplied by GSH (182, 427). Metabolism of SAA has an important redox regulation function due to the balance of transmethylation (TM), transsulfuration (TS), GSH synthesis, and the γ- glutamyl-cycle (12, 13, 106). Using diets rich in

Cys or its derivatives has been shown to affect the thiol redox status by increasing blood and/or intracellular GSH (15). Accumulating evidence suggests that SAA affects secretion of various cytokines, including those associated with fever and inflammation (14). It has been shown that an increase in GSH content in monocytes acts as a defense against pro-inflammatory insult (428), which ultimately protects the surrounding tissue by reducing the damaging influence of pro-inflammatory cytokines such as IL-1, IL-8, TNF-α, and inhibiting Kupffer cell NF-κβ activation (16). Furthermore, Salzano et al. (429) have reported the participation of GSH in the secretion of TNF-α by macrophages incubated with lipopolysaccharides (LPS).

The Met:Cys ratio has been shown be an important factor in some immune responses such as IL-1β, T-cell (437), TNF-α activity (217, 438), and oxidative stress (348). An extremely important function of Met in animal metabolism is as the precursor of Cys via the TS pathway, primarily in the liver (439). In mixed diets, Met and Cys occur together, with the ratio of Met:Cys varying from 40:60 for most plant-based proteins, to 60:40 or greater with animal-based proteins (440). When Met occurs as the larger contributor to total SAA, endogenous Cys synthesis, via the TS pathway, becomes important for ensuring adequate Cys supply, particularly at lower levels of total protein intake. A few studies examining the impact of alterations in dietary SAA intake on the inflammatory process have been conducted in rats (121); however, little is known about what effect an altered dietary SAA ratio, and the resulting reliance on TS to supply Cys, will have on the inflammatory response. For this reason, we investigated whether different dietary Met:Cys ratios alter plasma SAA concentrations and what impact these ratios have on the inflammatory response.

6.3. Materials and Methods

All procedures were approved by the Institutional Animal Care Committee of the University of Manitoba, in agreement with the guidelines of the Canadian Council on Animal Care (441) (Protocol Number F19-014).

6.3.1. Study design

6.3.1.1. Effect of alteration of the dietary Met:Cys ratio on markers of inflammation

Forty male Wistar rats were randomized to one of two possible diet groups (n=20/group), and subsequently divided into IP LPS or saline treatment groups (n=10/group). All diets were made to meet the nutrient requirements of rats, with a modified sulfur amino composition (**Table 6.1**): 1) ratio of 100Met:0Cys, and 2) ratio of 50Met:50Cys, as percentage of requirement for SAA. All groups were fed experimental diets *ad libitum* for 4 weeks with free access to water. Food intake was recorded daily while body weight was measured every 5 days.

At the end of four weeks of experimental diet feeding, rats were injected IP with either 100 µg/kg LPS in 0.9% saline, or saline alone. They were then anesthetized with isoflurane 5% and 0.8-1.0 L/min oxygen flow, and a 16G IV cannula was inserted into the jugular vein. Rats were maintained under general anaesthesia with isoflurane (2%) throughout the procedure (4h) to avoid any distress.

After 5 minutes of equilibration of body temperature, and immediately before the administration of 100µg/kg of LPS or saline IP, a time 0 blood sample was taken (0.7 mL). Similar volumes were removed at 60, 120 and 180 min after the IP administration of LPS or saline. At 240 minutes rats were terminated by exsanguination and blood (8-10 mL) was collected in heparin tubes. At the end of the experiment, blood, spleen, and liver tissue samples

Table 6.1. Amino acid defined diet.

	Amino Acid defined diet (g/kg)	
	100Met:0Cys	**50Met:50Cys**
L-Alanine	3.5	3.5
L-Arginine HCl	12.1	12.1
L-Asparagine	6.0	6.0
L-Aspartic Acid	3.5	3.5
L-Cystine	**0.0**	**5.1**
L-Glutamic Acid	40.0	40.0
Glycine	23.3	23.3
L-Histidine HCl, monohydrate	4.5	4.5
L-Isoleucine	8.2	8.2
L-Leucine	11.1	11.1
L-Lysine HCl	18.0	18.0
L-Methionine	**11.3**	**6.2**
L-Phenylalanine	7.5	7.5
L-Proline	3.5	3.5
L-Serine	3.5	3.5
L-Threonine	8.2	8.2
L-Tryptophan	1.8	1.8
L-Tyrosine	5.0	5.0
L-Valine	8.2	8.2
Sucrose	351.7	351.7
Corn Starch	150.0	150.0
Maltodextrin	150.0	150.0
Soybean Oil	80.0	80.0
Cellulose	30.0	30.0
Mineral Mix, AIN-93M-MX (94049)	35.0	35.0
Calcium Phosphate, monobasic, monohydrate	8.2	8.2
Vitamin Mix, AIN-93VX (94047)	13.0	13.0
Choline	2.5	2.5
TBHQ, antioxidant	0.02	0.02

[1] Met, methionine; Cys, cysteine

were collected, immediately frozen and stored at -80 °C for later analysis.

6.3.1.2. Determination of plasma and liver cysteine and homocysteine

Plasma and liver Cys and Hcy were measured using a method previously described in Chapter 5 section 5.3.2. Briefly, plasma and liver samples were treated with 20 µl 10% tris (2-carboxylethyl) phosphine (TCEP) (Pierce Chemical Co, USA), and incubated for 30 min at room temperature. Then, samples were deproteinized with 125 µl of 0.6 M perchloric acid. The samples were centrifuged at 5,000 x g for 10 min and 50 µl of the supernatant was added to 100 µl of potassium borate buffer (pH 10.5), containing 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and 50 µl of 1 mg/ml ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) (Wako Chemicals, VA, USA). This solution was incubated for one hour at 60 ºC and then cooled to 4 ºC. 20 µl of the sample was injected onto a $C18_{(2)}$ column at a flow rate of 1.0 mL/min. Plasma and liver total Hcy and Cys concentrations were evaluated using the reverse phase-HPLC (Shimadzu Inc, Nakagyo-82ku, Kyoto, Japan) with fluorescence detection (λEx= 385 nm, λEm= 515 nm).

6.3.1.3. Determination of plasma and liver GSH

Plasma and liver GSH concentrations were also measured with a GSH assay kit from Cayman Chemical as according to Chapter 5 section 5.3.3. Briefly, 50 µl of plasma samples were added to each of the sample wells. Then, 150 µl of the assay cocktail (MES Buffer, reconstituted co-factor Mixture, reconstituted Enzyme Mixture, water, and reconstituted DTNB) was added to each of the wells containing standards and samples using a multichannel pipette. GSH

concentration of the samples was determined after reading the plate at 405-414 nm at five min intervals for 30 min.

6.3.1.4. Determination of TNF-α protein concentrations

TNF-α concentrations were calculated utilizing the method described in Chapter 5 section 5.3.4. Briefly, 50 µl of a biotin-conjugated anti-rat TNF-α antibody was pipetted into 25 µl of plasma samples on each of the sample wells. Then, a volume of 100 µl of streptavidin-HRP was added to all wells and incubated for 1 h. TNF-α concentration of the samples was determined after reading the plate at 450nm. All samples and standards were run in duplicates with a sensitivity of <25 pg/mL and a detection range between 25-20,000 pg/mL.

6.3.1.5. RNA Isolation and Quantitative Real-Time PCR

RNA expression of TNF-α in spleen was evaluated as previously described (Chapter 5 section 5.3.5). The quality of the RNA was assessed with an absorbance ratio (wavelength 260/280 nm) within 1.8 to 2.1 by a spectrophotometry (DU800 Spectrophotometer, Beckman Coulter Canada Inc.). Reverse transcription was performed adding 1 µg of total RNA and the SuperScript VILO cDNA Synthesis Kit (Bio-Rad, CA, USA), to the cDNA utilized for gene expression. The mRNA abundance of the genes was evaluated using SYBR green real-time PCR (Bio-Rad). The primer sequences used were as follow: TNF-α (5'-GGCCACCACGCTCTTCTGTC-3', 5'-GGGCTACGGGCTTGTCACTC-3'), GAPDH (5'-CGTGGAGTCTACTGGCGTCTT-3', 5'-CATTGCTGACAATCTTGAGGGA-3'), Beta Actin (5'-GAGATTACTGCCCTGGCTCCTAG-3', 5'-CGGACTCATCGTACTCCTGCTT-3'). Samples were done in triplicate 20 µl reactions using a StepOne Real-Time PCR System (Life Technologies Inc. ON, Canada). Data were

normalized to *Actb* controls using the delta-delta Ct method, also known as the $2^{-\Delta\Delta Ct}$ (442). For controls, saline injection was used for relative abundance measurements.

6.3.1.6. Plasma leukocytes present as neutrophils.

The proportion of circulating neutrophils were determined by flow cytometry. Erythrocyte lysis and leukocyte fixation was based on a modified version of Skrajnar 2009. First, 50 µl of blood was diluted in 100 µl PBS (Hyclone 0.0067M PO4). A 50 µl aliquot of diluted blood was lysed and fixed by adding 500 µl BD FACS Lysing Solution (BD BioSciences, ON, Canada). The sample was mixed and incubated at room temperature for 10 min. Then 500 µl of PBS was added, mixed, and placed on ice prior to storing at 4 °C overnight. The next day, samples were centrifuged for 5 min at 500xg. Cell pellets were resuspended in 50 µl of PBS containing 2.5 µl of Purified Mouse Anti-Rat CD32 to block non-specific binding and incubated at room temperature for 5 min. Monoclonal antibodies against rat Granulocytes (PE label, clone RP-1, isotype mouse IgG1,κ), and CD45 (Alexa Fluor® 647 label, clone OX-1, isotype mouse IgG1,κ) were obtained from BD. Antibodies were incubated for 30 min at room temperature in the dark. Following incubation, cells were washed with the 1 mL Flow Cytometry Staining Buffer (Invitrogen) and re-suspended in 400 µl PBS.

Samples were measured on a FACSCanto II flow cytometer (BD) equipped with a blue 488nm solid state laser and a red 633 nm Helium-Neon laser. The RP1-PE signal was collected using the PE 585/42 nm band pass (BP) filter while CD45 AlexaFluor 647 using the APC 660/20 nm BP filter. Data collection and analysis was based on 20,000 events satisfying the CD45 positive gate for leukocytes using FACSDiva software v8.0.1 (BD). Doublets and cell clumps

were removed by FSC-H and FSC-W gating. Unstained cells were used to assess auto-fluorescence and single-color stained samples used to set gates.

6.3.1.7. Statistics

Data are expressed as mean ±SE. A repeated measures 2-way ANOVA was used to analyze the effect of diet and LPS challenge over time on food intake, body weight, body temperature, proportion of leukocytes that were neutrophils, plasma TNF-α, Cys, Hcy and GSH concentrations. Two-way ANOVA was used to determine the effect of diet and LPS challenge on liver Cys, Hcy, GSH, splenic TNF-α gene expression levels and area under the curve (AUC) for % neutrophils. A T-test was used to determine differences between dietary treatments in the LPS treated groups for AUC for plasma TNF-α. Differences among means were determined and corrected for multiple comparisons using the Tukey's multiple comparison test in GraphPad Prism, 7.0. The threshold of statistical significance was $p<0.05$ for all tests.

6.4. Results

6.4.1. Effect of alteration of the dietary Met:Cys ratio on markers of inflammation

6.4.1.1. Food Intake, Body Weight and Body Temperature

To evaluate whether different ratios of Met:Cys had an effect on measures of food intake, growth and body temperature, two different SAA ratios (100Met:0Cys and 50Met:50Cys) where evaluated. The SAA ratio did not affect the weight gain (164.2±25.63 g 100Met:0Cys vs 168.5±25.85 g 50Met:50Cys) or the final body weight of rats (278.8.2±29.67 g 100Met:0Cys vs 282.3±30.33 g 50Met:50Cys). Food intakes of rats fed with 50Met:50Cys ratio were higher but

did not reach statistical significance (p<0.06) in comparison to rats consuming the 100Met:0Cys ratio diets (23.2±3.16 g 100Met:0Cys vs 24±3.2 g 50Met:50Cys). Body temperature of fed rats with 50Met:50Cys or 100Met:0Cys did not change after the administration of LPS 100 μg/kg when after 4h when compared with saline-treated rats.

6.4.1.2. Determination of TNF-α protein concentrations and gene expression.

TNF-α protein concentrations are presented in **Figure 6.1A**. Significant increases in plasma TNF-α in both LPS-treated groups when compared to saline at 120 and 180 min (p<.0001) were observed. The peak concentration of plasma TNF-α for the 50Met:50Cys ratio was observed at 120 min post IP LPS-injection (497.66 ±51 SE pg/mL). For the 100Met:0Cys ratio treatment, the peak was reached at 180 min (418.12 ±93 SE pg/mL) after IP LPS-injection. No significant difference was observed between maximum peaks. When comparing the effect of administration of LPS at 100 μg/kg after 120 min for both dietary ratios, a 50Met:50Cys ratio presented a significantly higher (p< 0.001) TNF-α concentration peak compared with the 100Met:0Cys ratio. Meanwhile, the dietary 100Met:0Cys ratio significantly increased (p< 0.001) the plasma TNF-α concentration peak compared with the 50Met:50Cys ratio after 180 min. Additionally, the area under the curve (**Fig. 6.1.B**) for an LPS-treated dietary 50Met:50Cys ratio was significantly higher (p< 0.004) compared to the dietary 100Met:0Cys ratio. TNF-α gene expression (**Fig 6.1.C**) in spleen tissue significantly increased (p<0.0001) in rats injected with LPS 100 μg/kg. No significant difference was observed between diets.

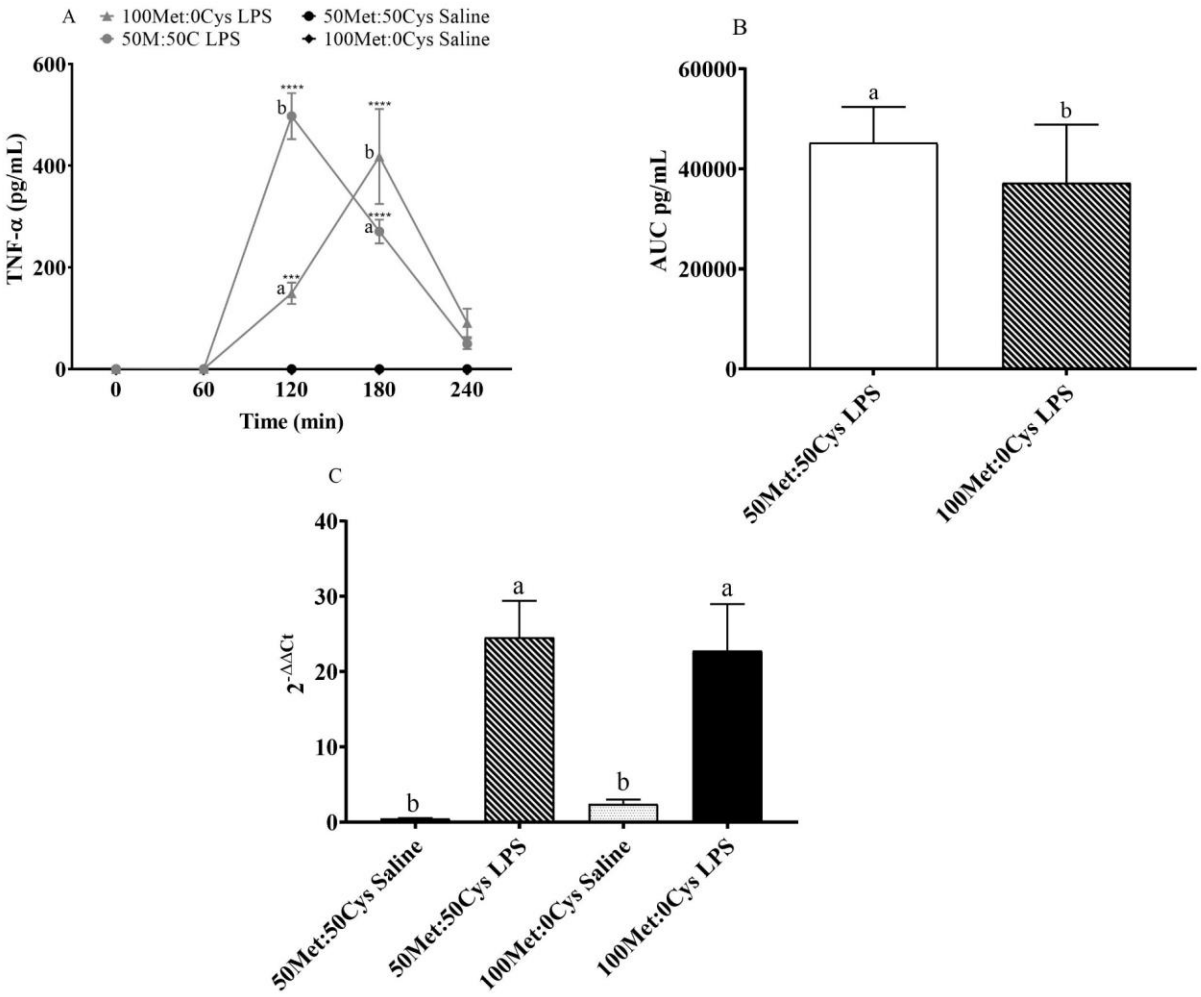

Figure 6.1. Plasma TNF-α concentration (A) of Wistar rats in plasma over a period of four hours after IP injection of saline or LPS 100 µg/kg. Effect of time, diet and LPS determined using repeated measures ANOVA and Tukey's post-hoc test. Plasma TNF-α concentration area under the curve (B) for LPS treated groups only analyzed via T-test. Mean ± SE (n = 9). Spleen TNF-α gene expression (C) effect of diet and LPS determined using two-way ANOVA and Tukey's post-hoc test. Means with different letters are significantly different a $p<0.05$.

6.4.1.3. Determination of plasma SAA metabolites

To determine whether the alteration of SAA ratio had an effect on SAA metabolites, plasma concentrations of Cys, Hcy and GSH (**Table 6.2.**) were evaluated. In the absence of an inflammatory challenge, SAA metabolites (Cys, Hcy, GSH) at baseline showed no differences in concentrations between altered dietary SAA ratios. Similar results were observed with no significance between diets in concentration of Cys, Hcy and GSH after an IP injection with LPS. Additionally, the effect of LPS on SAA metabolites was evaluated. A dietary 50Met:50Cys ratio significantly increased ($p<0.01$) plasma Cys concentration after IP LPS injection at 240 min compared with a 50Met:50Cys ratio injected with IP saline. Similarly, plasma concentration of GSH was significantly higher ($p<0.04$) in rats fed with 50Met:50Cys ratio IP LPS-injected compared with a saline injection with a 50Met:50Cys ratio after 240 min. Plasma Hcy showed a significant increase after 180 min ($p<0.04$) and 240 min ($p<0.05$) in rats injected with LPS with an altered dietary 50Met:50Cys ratio compared with rats injected with saline.

6.4.1.4. Determination of liver SAA metabolites

Our results demonstrated that administration of LPS significantly lowered Cys ($p<0.05$) concentration for both treatments in the liver (**Fig 6.2.**). Additionally, administration of LPS also affected Hcy and GSH concentration in the liver. LPS-treated rats from both dietary treatments exhibited significantly increased concentrations of Hcy ($p<0.05$) and GSH ($p<0.05$) after LPS injection compared with administration of saline after 4 h.

Table 6.2. Plasma Cys, Hcy and GSH of rats fed with two ratios of SAA injected with either LPS or saline.

SAA (μM/L)	100 Met:0Cys		50Met:50Cys		*p* value		
	LPS	Saline	LPS	Saline	Diet	Challenge	Time
Cys							
0 min	164.0±7.8	163.7±6.7	204.8±18.7	172.2±7.4	0.16	0.01	0.0001
60 min	226.3±11.8	201.9±8.8	226.3±15.9	213.8±16.8			
120 min	215.4±7.1	188.2±13.3	235.7±23.8	206.0±14.7			
180 min	210.9±13.6^{a,b}	205.1±15.1^{a,b}	232.8±25.1^{b}	200.5±13.0^{a}			
240 min	205.5±19.2	194.9±13.3	200.5±23.0	203.1±12.4			
Hcy							
0 min	14.0±0.9	15.6±1.1	15.6±1.3	15.3±1.1	0.99	0.0001	0.0001
60 min	34.6±5.5	19.2±1.1	31.1±2.7	18.2±1.0			
120 min	35.4±5.8	20.7±0.8	33.9±3.0	19.4±0.4			
180 min	37.8±5.2^{a,b}	22.8±1.4^{a,b}	38.5±3.5^{b}	20.1±0.8^{a}			
240 min	40.0±4.3^{a,b}	23.7±1.2^{a}	49.2±5.3^{b}	22.6±1.3^{a}			
GSH							
0 min	19.1±1.9	16.1±1.3	20.1±2.0	17.3±0.9	0.54	0.04	0.006
60 min	25.0±1.5	20.8±1.2	22.6±2.0	21.3±0.9			
120 min	22.2±1.6	21.0±1.6	24.1±2.7	21.4±2.1			
180 min	22.4±3.6	23.1±2.1	23.1±2.6	21.9±1.9			
240 min	20.8±4.3^{a,b}	19.6±1.5^{a,b}	30.2±4.6^{b}	19.5±1.6^{a}			

[1] Cys, cysteine; Hcy, homocysteine; GSH, glutathione; LPS, lipopolysaccharide

[2] Data are presented in mean ± SE (n = 9). Data were analyzed via repeated measures ANOVA with Tukey's post-hoc test. Means with different letters are significantly different ($p<0.05$)

6.4.1.5. Plasma leukocytes present as neutrophils.

The proportion of plasma leukocytes present as neutrophils is presented in **Figure 6.3A**. Our study showed that the proportion of neutrophils was significantly increased (p<0.05) by the administration of LPS at 100 µg/kg in a time dependent manner, whereas administration of LPS did not affect neutrophil proportion between diets. Additionally, the area under the curve (**Fig 6.3B**) demonstrated a significant increase (p< 0.05) in the proportion of neutrophils as a percentage of blood leukocytes in rats injected with LPS when compared to saline; however, no significant difference was observed between diets over 240 min.

6.5. Discussion

The main findings of the current study are that a sufficient supply of SAA of Met alone is sufficient for the synthesis of Cys and GSH to regulate TNF-α production and the proportion of neutrophils in rats. Furthermore, a 50Met:50Cys dietary ratio affected the concentrations of SAA metabolites in rats injected with LPS. This is the first study to report that the SAA dietary ratio may play an important role in the response to an LPS challenge.

First, we injected LPS intraperitoneally into the rats which served as a systemic inflammatory response model, to identify the effect of altered dietary SAA ratios (50Met:50Cys and 100Met:0Cys) on the inflammatory response. Results from our study differ to those found in other studies (111-113) where temperature did not rise after the administration of LPS in rats. Wrotek *et al.* (111) reported that LPS induced fever. They observed that rats injected with LPS increased temperature at two different time points, a first peak after 2 h (37.4 ±0.07 ºC) and the second peak at 4 h post-injection (37.6±0.01 ºC). In our preview study rats IP injected with LPS at a dose of 100 µg/kg developed fever (>37 ºC) after 30 min pos-injection and keep increasing

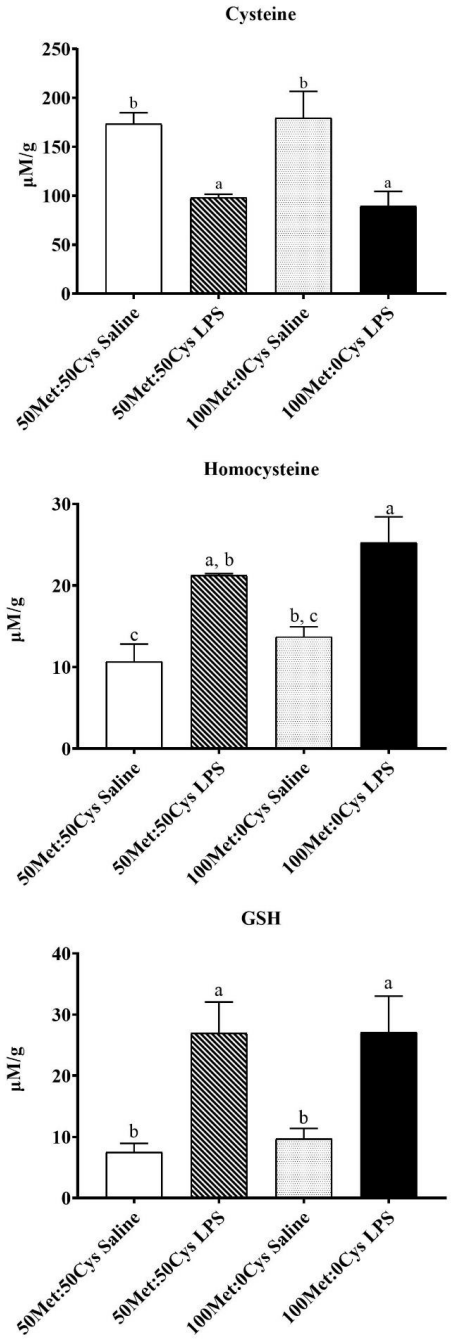

Figure 6.2. Concentration of Cysteine (A), Homocysteine (B) and GSH (C) in the liver of Wistar rats after a period of four hours post IP injection of saline or LPS (100 µg/kg). Data are shown in µM/g. Mean ± SE (n = 9). Data were analyzed via two-way ANOVA with Tukey's post-hoc test. Means with different letters are significantly different (p<0.05)

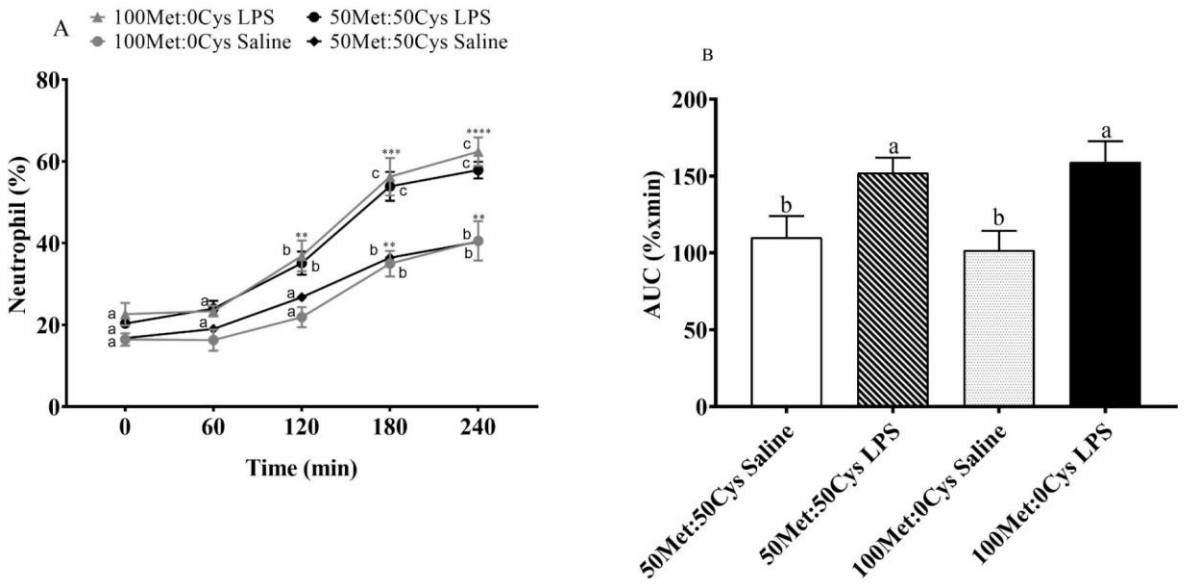

Figure 6.3. Effect of diet and LPS on the percentage of leukocytes as neutrophils over time determined by repeated measures ANOVA (A). Area under the curve for proportion of leucocytes as neutrophil in blood (B) of Wistar rats over a period of four hours after IP injection of saline or LPS 100 μg/kg. Two-way ANOVA with Tukey's post-hoc test. Means with different letters are significantly different (p<0.05). Mean ± SE (n = 9).

after 5h. The variation found in our results could be due to several factors. First, the room used in our second experiment was larger; therefore, it was difficult to maintain a thermoneutral ambient temperature during the experiment. Second, the variability in individual reaction to LPS of the tested subjects to react to the endotoxin challenge.

Although there are reports showing that altered dietary SAA can lead to adaptations in SAA metabolism (433), little is known of the effects that an altered dietary SAA ratio may have on inflammatory markers. Under normal conditions, a diet with sufficient Met provides Cys requirements through the TS pathway; however, a deficit in endogenous Cys supply may occur during infection, sepsis and trauma since the SAA requirement increases (434). We showed that IP-LPS administration significantly increased TNF-α production in the 100Met:0Cys and 50Met:50Cys ratio-fed rats. These results suggest that an IP-LPS injection increases the production of TNF-α regardless of the dietary SAA ratio. The pro-inflammatory cytokine TNF-α triggers metabolic effects such as fever and enhancement of the TS pathway (443). We showed that rats fed with a 50Met:50Cys ratio exhibited a more rapid production of TNF-α, with the latter reaching its peak after 120 min post-injection. In contrast, rats consuming a diet with a 100Met:0Cys ratio reached peak TNF-α after 180 min. Previous studies have described the production of TNF-α in response to LPS in rats with impaired SAA metabolism (408, 444, 445). Kudo et al (444) reported that methionine-choline deficient mice treated with LPS significantly increased hepatic TNF-α production. Our results suggest that the consumption of a diet where Met served as the sole source of SAA significantly affected the total production of TNF-α after an IP-LPS injection. Consumption of a dietary ratio of 50Met:50Cys led to faster TNF-α peaks, which returned to baseline, those rats consuming a dietary ratio of 100Met:0Cys presented a delayed reaction with a peak TNF-α at 180 min. TNF-α activity may be more susceptible to an

IP-LPS injection in rats consuming a diet with a 50Met:50Cys ratio. However, the consumption of a diet with a SAA ratio of 100Met:0Cys led to rats being able to exhibit decreased TNF-α production in a more effective manner as compared to those consuming a balanced SAA ratio over 4h after IP-LPS injection. When looking at the expression of TNF-α on spleen for both groups, TNF-α gene expression (Fig 6.1.C) in spleen tissue significantly increased ($p<0.0001$) in rats injected with LPS 100 μg/kg. However, no significant differences were observed. Despite the literature suggesting that, during an endotoxin challenge such as LPS, Cys requirements increase, our results showed that Met alone is capable of meeting the metabolic requirements for Cys under conditions of an acute endotoxin challenge. However, we anticipate that under conditions where TS activity might be compromised, such as with an inadequacy in vitamin B6 status, a pro-inflammatory responses could be favored due to compromised cellular concentrations of GSH (107).

Large amounts of pro-inflammatory acute-phase protein cytokines such as TNF-α are primarily produced and released by macrophages in response to LPS (432, 444) and drive hepatic acute-phase protein synthesis, endothelial cell activation, and leukocyte extravasation (446). Circulating neutrophils are believed to be significant contributors to tissue damage during the systemic inflammatory response (346). SAA and their metabolic products, especially GSH, have an important role in lymphocyte proliferation (447). In rats fed with low protein diets with reduced GSH tissue concentrations presented with an increase in in the number of lung neutrophils after endotoxin challenge (217). Supplementation of Met increased liver GSH concentration and restored neutrophil counts to numbers seen in unstressed animals (217). In the present study, we demonstrated that the proportion of circulating leucocytes that were neutrophils was not different in rats fed a 100Met:0Cys compared with a 50Met:50Cys ratio after

administration of IP-LPS. Taken together, our results suggest that although IP-LPS increased the proportion of leucocytes that were neutrophils in the blood, the Met:Cys ratio had no effect.

Data derived from studies examining the amount and proportion of dietary Cys have provided evidence of enhanced growth and food intake when Cys supplies 43-47% of the total SAA requirement in broiler chickens (448). However, our results showed that total body weight and weight gain did not differ in the absence of Cys compared with a balanced SAA dietary intake. These findings demonstrated that Met alone can supply enough Cys through TS pathway to maintain growth in rats. Our results are supported by Shannon *et al*. (433). They reported that a diet with a SAA ratio of 100Met:0Cys and 50Met:50Cys did not result in a significant difference in daily food intake, daily weight gain and total carcass nitrogen.

Under circumstances where Cys availability is depleted, synthesis of GSH and taurine will diminish mainly because Cys requirements may not be met by the TS pathway (107). Our results show that rats fed with a 50Met:50Cys ratio after an LPS challenge had significantly higher concentrations of plasma Cys, Hcy and GSH compared with the other treatments. As previously mentioned, during sepsis Cys concentrations could be deficient due to an increase in SAA demand and limited hepatic transsulfuration (438). Therefore, our study looked at the effect of no supplementation of Cys in the diet on SAA metabolites after an endotoxin challenge. We showed in **Table 6.2** and **Fig 6.2** that Cys, Hcy and GSH were significantly affected by LPS treatment. Our results demonstrated that administration of IP-LPS did not lead to differences in Met catabolism in rats fed with a 100Met:0Cys ratio compared with a 50Met:50Cys ratio. However, IP-LPS injection significantly increased liver concentration of Cys and Hcy in comparison to saline-injected controls. These results suggest that a dietary 100Met:0Cys ratio it is capable of maintaining a similar synthesis of SAA metabolites during inflammation.

Additionally, since these metabolites are involved in GSH synthesis, these results indicate that the SAA metabolic pathways are directly impacted by IP-LPS. Previous studies have demonstrated the effect of acute inflammation on cellular SAA metabolite concentrations when SAA metabolism is impaired. Benight et al (358) reported that C57BL/6 mice fed with a diet deficient in B6 and B12 presented with significantly higher plasma and liver concentrations of Hcy when injected with 3%DSS compared with control. Our study reported that after four hours, rats in both diets presented mild (12 to 30 mmol/L) hyperhomocysteinemia (HHcy) in plasma; however, after 4 h rats showed a significantly higher moderate (30 to 100 mmol/L) HHcy in those injected with IP-LPS compared with saline. The elevation of Hcy concentrations exert pro-inflammatory effects in oxidative stress and pro-inflammatory cytokine production**.** Hcy content alters the profile of cytokine/chemokine production by endothelial cells injury and macrophages secretion (284). This altered profile may be important in the inflammatory events that initiate or enhance the development of atherosclerotic lesions (349). Another important observation relates to the synthesis of GSH under stress conditions. Cys and GSH requirements may be difficult to meet during sepsis since the TS pathway may be inadequate due to impairment in the SAA metabolism generally present during inflammation. We reported that the liver concentration of GSH significantly increased after administration of LPS; however, no significant differences were observed between diets. These results are supported by a previous study conducted in septic rats where GSH concentrations were significantly higher in all tissues evaluated except the small intestine (182). Others have also described an increase in total GSH in different tissues after induction of stress in animal models (183, 184). Our results suggest that an adequate intake of SAA, regardless of Met:Cys ratio, is able to support the synthesis of liver GSH and may improve the GSH status and function as a mechanism to protect against oxidative

damage (182). Results reported here also show that the TS pathway may be maintained under inflammatory challenges even in the absence of Cys as long as the overall SAA requirement is met.

6.6. Conclusion

Overall, this study illustrates the effect of altered dietary SAA ratios on the levels of TNF-α, neutrophils and SAA metabolites after an endotoxin challenge. In summary, our study showed that a dietary ratio of 100Met:0Cys could meet Cys requirements during an acute inflammatory state by reducing the total area under the curve of pro-inflammatory cytokine TNF-α compared with a balance SAA ratio; 2) the proportion of neutrophils was equally enhanced in both diets. Additionally, a diet with a 100Met:0Cys ratio was able to meet SAA metabolites requirements under an inflammatory challenge. These results lay the foundation for studies examining inflammatory responses *in vivo* under conditions where Cys synthesis from Met is compromised, including marginal vitamin deficiencies.

CHAPTER 7

OVERALL DISCUSSION AND SUMMARY

7.1. General discussion and summary

7.1.1. General overview

The series of studies presented in this thesis set out to investigate the role of sulfur amino acids in the acute inflammatory response. This was accomplished through *in vivo* experimentation using IP-LPS injections for the study of the impact of SAA in systemic inflammation. Given the variability and difficulties of studying inflammation in humans, animal models of inflammation were used for the investigation of inflammation pathophysiology. There are several main advantages to the endotoxin animal model, including technical ease and high reproducibility, especially in the acute inflammatory response. As such, information on SAA on inflammation in rats has important implications in both human health and rats' experimentation.

7.1.2. Investigation of the effect of impairment in sulfur amino acids metabolism on the acute inflammatory response

7.1.2.1 Overview of the results

To our knowledge, this research was the first systematic analysis to examine the effect on the acute inflammatory reaction of deficiency of the SAA metabolism. We presented data from a few studies of the effect on inflammatory pathways of SAA metabolism impairment. Biomarkers of cytokine production, lipid metabolism and platelet activation are the most consistently documented findings to date. Tissue loss was also enhanced by SAA metabolism impairment. In the presence of CSE deficiency, a few promising findings were recorded for liver function.

Results from our study indicate that SAA impairment can cause oxidative stress and contribute to a decrease in the function of the tissues. In addition, CSE inhibition can be helpful

in reducing oxidative stress and enhancing tissue function in the face of inflammatory difficulties. CSE dysfunction is a common SAA disability model used when exposed to multiple stressors, such as tissue ischemia-reperfusion, atherosclerosis, hepatitis and liver fibrosis or chronic heart failure, to study the production of extreme pathophysiological responses. Even so, CSE inhibition has reported a preventive effect against injury with systemic inflammatory response over-activation and sometimes includes a certain type of single-organ or multiple-organ damage. These findings indicate that atherosclerosis mediating cholesterol metabolism and upregulating P2 receptors and caveolins may play an important role in initiating and producing HHcy.

We also stated that Hcy can induce major changes in the metabolism of patients with CBS deficiency, including a reduction in concentrations of HDL and ApoA-1, further raising the risk of CAD. SAA metabolism deficiency can result in a pro-inflammatory state linked with an increased incidence of CAD. Likewise, SAA metabolism deficiency influences the acute inflammatory response, causes tissue damage, and plays a key role in the progression of vascular diseases, similar to the findings observed. More specifically, pro-inflammatory cytokines and proliferation of lymphocytes can increase following toxic concentrations of Hcy. A rise in Hcy can be caused by a TM and TS pathway imbalance.

Overall, SAA deficiency may be involved in controlling acute inflammatory markers by stimulating the development and expression of pro-inflammatory cytokines including TNF, IL-1 and IL-6 that enhance the migration of leukocytes to increase the inflammatory response, fostering oxidative stress aggravating APP secretion (CRP), inducing ROS that contributes to multiple tissue damage and increasing the possibility of multiple vascular diseases like atherosclerosis and CAD. Collectively, these observations help recommendations not to limit

SAA-rich food intake in order to preserve a balanced immune system. In addition, the beneficial benefits of diets supplemented with vitamins involved in the synthesis of SAA (B6, B12 and folate) controlling Hcy concentrations contribute to the conclusion that inflammatory treatment therapies should require keeping particular attention to B-vitamin concentration.

7.1.2.2. Implications and limitations

In order to eliminate selection bias associated with the nonrandomization of the samples, only clinical and controlled trials were included in the study. However, our initial search strategy captured observational experiments and other animal models that, though without the same research rigour, may provide more insight into this area. Some included extra supplements of vitamins with respect to SAA impairment. The study did not exclude that research. In fact, the influence of antioxidants attenuating inflammatory markers was detected by integrating experiments with vitamin supplementation (e.g., B6, folic acid, B12).

As stated before, most human and animal studies have shown that SAA metabolism deficiency can play a significant role in inflammatory markers and immune system conservation. Future intervention experiments in subjects with chronic inflammation are, however, justified in evaluating the long-term impact of SAA deficiency on inflammatory markers and the progression of diseases. When evaluating differences in response to SAA impairment, co-factors like Hcy and pro-inflammatory cytokines concentrations, lipid profile and tissue function are to be considered. In addition, clinical trials with sample selection based on the assigned single nucleotide polymorphism–related genotypes are needed.

7.1.3. Investigation of the temporal and dosage effects of LPS administration on markers of inflammation and SAA metabolism in Wistar rats

7.1.3.1 Overview of the results

The objective for the first study was to whether 50 or 100 µg/kg IP-LPS would induce greater temporal changes in inflammatory markers and sulfur SAA metabolism. To accomplish this goal, we quantified the induction of pro-inflammatory markers including development of fever by body temperature and production of pro-inflammatory cytokine TNF-α in plasma and spleen. SAA metabolites were also analysed from blood at different time points, as well as samples from liver, to determine if there were any impact in the concentration of some SAA metabolites. Through this model, we determined that the pro-inflammatory response of LPS in Wistar rats is highly induced with a dose of 100 µg/kg. Analysis of Cys, Hcy and GSH revealed that concentration of these metabolites was altered in plasma samples in both 50 and 100 µg/kg IP-LPS doses, after GSH concentration was significantly reduced.

7.1.3.2. Implications and limitations

This study was the first to use the endotoxin model to investigate the dose that would exhort higher temporal changes in acute inflammatory markers. Characterizing the capacity for LPS model in Wistar rats was an important step in my research programme. Knowing the timeline for LPS to enhance TNF-α production could influence strategical decisions during the surgical stage such as prioritizing the timeframe as well as timepoints for blood collection. Similarly, armed with knowledge that systemic inflammation is possible through LPS at a dose of 100 µg/kg, SAA metabolites concentration could be altered to include different SAA ratios due to such as GSH synthesis. The fact that a dose of 100 µg/kg IP LPS developed fever after 30 min

post-injection and kept the temperature kept rising for the entirety of the trial (5h) was also an interesting finding. It is possible that in higher doses of LPS there is a greater potential for the development of acute inflammation than with lower doses.

One of the findings of this work was a decreased concentration of GSH in the blood of rats injected with a dose of 100 µg/kg of LPS. Unfortunately, it cannot be determined whether this occurs over the period of 5h post-injection, only after 5h or the combination of both. The study design could have been altered to increase the amount of blood collected for analysis of GSH at different timepoints. This would have provided a clearer image of the true impact of LPS in SAA metabolism. Additionally, using a different site for blood collection would have been beneficial. We found highly variable quantities of TNF-α protein concentration in plasma in both doses of LPS. Blood collection from the jugular vein through a catheter, would have provided more reliable information regarding the quantity of TNF-α protein concentration as the saphenous vein could have caused further production of TNF-α due to repetitive damage to the tissue area.

Overall, we believe that this initial project was well designed and an excellent starting point for the study which followed. The use of a 100 µg/kg LPS dose allowed for investigation of pro-inflammatory markers at multiple timepoints in the same animal, increasing the amount of data collected. The choice of area of blood collection, saphenous vein, allowed for multiple blood collection without a surgical procedure and inclusion of timepoints provided an easy way to track an inflammatory curve.

The endotoxin model used an IP-LPS dose of 100 µg/kg. As we had previously determined that LPS was important in the development of acute inflammation and impact in SAA metabolism we were interested in moving my research into this optimized model. Was it

possible that inclusion of different dietary Met:Cys ratios alter plasma SAA concentrations and that these ratios have an impact on the inflammatory response?

7.1.4. Investigation of the alteration of the dietary methionine:cysteine ratio on the inflammatory response

7.1.4.1. Overview of results

Continuing our work in SAA on inflammation, our objective was the effect an altered dietary SAA ratio, and the resulting reliance on TS to supply Cys, will have on the inflammatory response. This would provide a better understanding of the interaction between SAA ratios and SIRS, potentially explaining the capacity of Met to synthesize Cys after an LPS injection. After rats were fed for 4 weeks with two different experimental diets with a modified sulfur amino composition: 1) ratio of 100Met:0Cys, and 2) ratio of 50Met:50Cys, as percentage of requirement for SAA, rats were anesthetized with isoflurane and then injected IP with either 100 ug/kg LPS in 0.9% saline, or saline alone. Under anesthesia a 16G IV cannula was inserted into the jugular vein of the rats and maintain anesthetized with isoflurane throughout the procedure (4h) to avoid any distress. After 4 weeks fed with experimental diets, the absence of Cys did not impact total body weight and weight gain compared with a balance SAA dietary intake. These findings showed that rats fed with Met alone can maintain a normal growth due to the ability of Met to supply enough Cys through TS pathway. Total production TNF-α after an IP-LPS injection was significantly reduced in the absence of Cys; however, a dietary 50Met:50Cys ratio induced faster peak of TNF-α concentration in plasma compared with a delayed reaction of a 100Met:0Cys dietary ratio after 4h. On the other hand, expression of TNF-α on spleen showed no significant differences between both diets. Similar results were observed in neutrophil

migration after an IP LPS injection, where no differences were found in proportion of neutrophil regardless of the Met:Cys ratio. Our outcomes measured, including SAA metabolites (Cys, Hcy and GSH), did not differed amongst any dietary regimen. These results suggest that an adequate intake of SAA can meet the requirements for Cys and GSH under an acute inflammatory challenge, regardless of Met:Cys ratio.

7.1.4.2. Implications and limitations

The design of this study developed out of the results of our previous work, hence the focus on the impact of altered dietary SAA ratios on the acute inflammatory response. Similar to the first study, using the endotoxin model allowed for a marked pro-inflammatory response to be investigated in rats; however, with an altered dietary ratio we were able to assess the Met sparing effect of Cys. The use of the jugular vein as the site for blood collection led to more accurate and efficient way to extract blood reducing variability in TNF-α quantity due to external factors besides the endotoxin infection. Although increases in inflammatory markers including cytokine production and neutrophil migration in the endotoxin model, the use of two different dietary ratios of SAA and their impact on inflammation is novel. Results reported here indicate that despite the absence of Cys, the TS pathway may be maintained under inflammatory challenges as long as the overall SAA requirement is met.

Moving from saphenous vein to jugular vein as the site for blood extraction was necessary for several reasons. Most importantly the fragility of the saphenous vein after the first blood collection would cause complications for multiple collection timepoints and accuracy of the amount collected, and the lack of visibility of the vein after IP LPS injections would make this study difficult to complete in the previous model. Unfortunately, the results of this study do

not answer all the questions raised from the first study. We were able to demonstrate that Met alone could impact the production of TNF-α in response to LPS. Whether the mechanism by which the absence of Cys affects the SAA pathway during an inflammatory response is due an impact on the pathway flux, with a priority in TS pathway flux over the TM pathway, the end point selected or a combination of both was not determined by this study. The analyses of other SAA metabolites such as Met, taurine, H_2S and other amino acids in plasma and tissues (e.g., liver, kidneys, and skeletal muscle), additional co-substrates including SAM or SAH, as well as the activity and/or expression of enzymes like CBS and CSE would have provided the necessary information to elucidate the underlying mechanism by which Cys and GSH were increased during inflammation.

Working within the endotoxin model, a few minor alterations to the study design would have provided additional information. Although the previous study demonstrated to induce fever, the present study did not show this outcome. Stabilization of body temperature for a longer time prior injection would have provided information regarding potential increases in body temperature while small intestinal sampling would have indicated any alteration in Met absorption confirming the impact on the pathway flux. While the initial objective of the study was to determine the impact of altered dietary SAA ratios on acute inflammation, investigation of the small intestine would have been relatively easy. Under LPS challenge development of small bowel bacteria overgrowth syndrome and increased intestinal permeability in the small intestine. If adequate dietary intake of SAA induces similar anti-inflammatory responses in the small intestine, the impact of a dietary 100Met:0Cys SAA ratio in that region may be noteworthy in cases where the SAA metabolism was impaired like in deficiency of vitamin B6. During the tissue analysis process, if histological samples for spleen and liver architecture were taken, the

development of hepatic damage through the interaction of endotoxin could have been calculated leading to a more precise measurement of the anti-inflammatory response of SAA.

7.2. Future directions

Due to the effect of SAA on inflammation by modifying metabolic changes in the immune response, further studies should be conducted to determine whether the results of different SAA ratios during inflammation induced by an LPS injection could be described by the outcomes obtained by the proposed study. Moreover, the effect of supplementary SAA needs to be examined during an acute inflammatory phase to recognize the influence of exogenous sources after an LPS injection. Finally, a study with different SAA challenges such as vitamin B6 deficiency may need to be investigated in order to understand the role of SAA during an inflammation.

7.3. Conclusion

SAA play a significant role in controlling the acute inflammatory response, reducing inflammatory markers such as TNF-α, IL-6 and IL-1β, as well as reducing oxidative stress. More specifically, in the prevention of CAD and atherosclerosis, adequate dietary intake of SAA can be beneficial. An elevated concentration of GSH may be helpful for the immune response against tissue damage or inflammation. Impairment of SAA metabolism affects the acute inflammatory response and is responsible for the increased risk of cardiovascular and hepatic diseases. Besides, HHcy enhances pro-inflammatory cytokine production and lymphocyte proliferation due to elevated Met concentration or inborn error in SAA metabolism. The SAA pathway imbalance can be caused by an increase in plasma Hcy concentration. Due to their

function in increasing the development of inflammatory markers such as cytokines, CRP, ROS, a deficit of B vitamins may contribute to a rise in inflammation-causing many harms to the organism. For this cause, adequate food supply and vitamin supplementation during an inflammatory reaction is recommended. Overall, by reducing the synthesis of inflammatory markers such as NF-κβ and TNF-α, which increases neutrophil migration, enhancing inflammatory response, inhibiting ROS activity, reducing oxidative stress. Vitamin B6 deficiency can result in an increased risk of CAD, increasing CRP and homocysteine concentration by altering TS pathway flux. Collectively, these studies provide evidence to encourage adequate dietary intake of SAA, although adequate dietary intake of B vitamins (B6, B12 and folate) is helpful for the healthy metabolism of SAA. Additional studies are needed to lay the basis for studies investigating *in vivo* inflammatory responses under conditions where Cys synthesis from Met is impaired, particularly marginal vitamin B6 deficiencies.

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CHAPTER 8

Appendix

8.1 Supplemental material

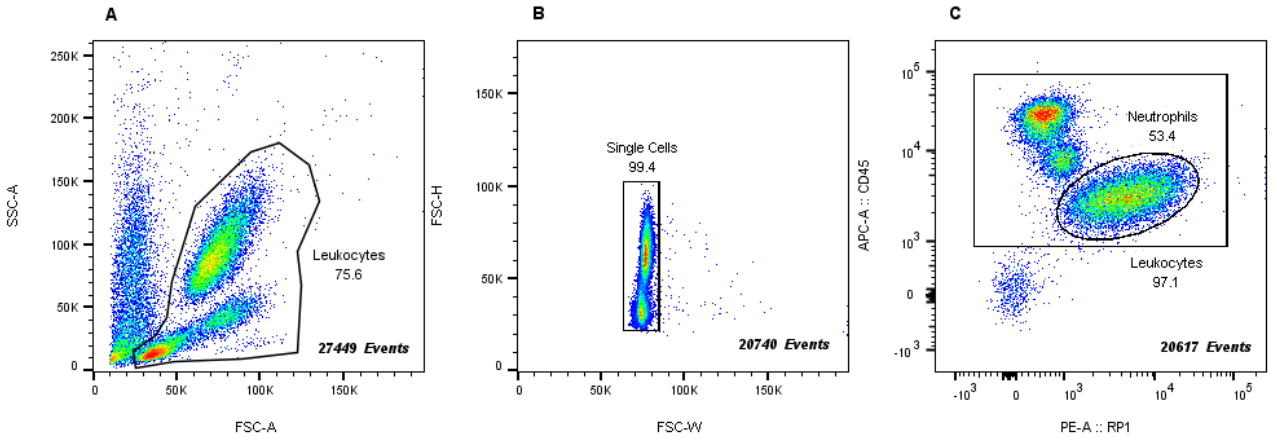

Figure 8.1. Representative flow cytometry plot. Definition of leukocytes based on FSC vs SSC (A); Definition of single cells after gating on FSC vs SSC leukocytes (B); Definition of Neutrophils after gating in single cells (C).

Table 8.1. Database(s): Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations and Daily 1946 to November 06, 2020

Search Strategy:

#	Searches	Results
1	choline deficiency/ or folic acid deficiency/ or vitamin b 6 deficiency/ or vitamin b 12 deficiency/	13298
2	((folic or folate) adj3 deficien*).tw,kw.	4532
3	((vitamin b12 or vitamin b 12) adj3 deficien*).tw,kw.	4477
4	((vitamin b6 or vitamin b 6) adj3 deficien*).tw,kw.	1009
5	(choline adj3 deficien*).tw,kw.	2493
6	hyperhomocysteinemia/ or homocystinuria/	6006
7	(hyperhomocysteinemia or homocystinuria).tw,kw.	7137
8	(cystathioninemia or cystathionuria).tw,kw.	14
9	cysteine/df [deficiency]	90
10	((cysteine or L-cysteine or cystine) adj deficien*).tw,kw.	103
11	(low* adj (cysteine or L-cysteine or cystine)).tw,kw.	138
12	homocysteine/df [deficiency]	19
13	((homocysteine or L-homocysteine or L homocysteine or homocystine) adj deficien*).tw,kw.	7
14	(low* adj (homocysteine or L-homocysteine or L homocysteine or homocystine)).tw,kw.	413
15	glutathione/df [deficiency]	686
16	((glutationine or GSH or gamma-L-Glutamyl-L-Cysteinylglycine or gamma L Glutamyl L Cysteinylglycine or gamma-L-Glu-L-Cys-Gly) adj deficien*).tw,kw.	228
17	(low* adj (glutationine or GSH or gamma-L-Glutamyl-L-Cysteinylglycine or gamma L Glutamyl L Cysteinylglycine or gamma-L-Glu-L-Cys-Gly)).tw,kw.	562
18	((cystathionine beta synthase or cystathionine beta-synthase or cystathionine b-synthase) adj deficien*).tw,kw.	262
19	(low* adj (cystathionine beta synthase or cystathionine beta-synthase or cystathionine b-synthase)).tw,kw.	4
20	cystathionine gamma-lyase/df [deficiency]	55
21	((cystathionine gamma lyase or cystathionine gamma-lyase or cystathionine g-lyase) adj deficien*).tw,kw.	15

22	(low* adj (cystathionine gamma lyase or cystathionine gamma-lyase or cystathionine g-lyase)).tw,kw.	1
23	methionine/df [deficiency]	746
24	((methionine or L-methionine or L methionine) adj deficien*).tw,kw.	301
25	(low* adj (methionine or L-methionine or L methionine)).tw,kw.	249
26	acute-phase reaction/ or systemic inflammatory response syndrome/	8807
27	(acute-phase reaction or systemic inflammatory response syndrome or SIRS or cytokine release syndrome or sepsis syndrome).tw,kw.	10883
28	tumor necrosis factor-alpha/	125421
29	(tumor necrosis factor alpha or tumor necrosis factor-alpha or TNF alpha or TNF-alpha or TNFa).tw,kw.	174151
30	interleukin-6/ or interleukin-8/ or interleukin-10/ or interleukin-17/	111778
31	(interleukin 6 or interleukin-6 or IL-6 or IL 6).tw,kw.	137580
32	(Interleukin 8 or Interleukin-8 or IL 8 or IL-8 or chemokine CXCL8 or CXCL8).tw,kw.	43332
33	(interleukin 10 or interleukin-10 or IL-10 or IL 10).tw,kw.	63040
34	(interleukin 17 or interleukin-17 or IL-17 or IL 17).tw,kw.	18208
35	leukocytes/	67446
36	(leukocyte* or white blood cell*).tw,kw.	180766
37	neutrophils/	87808
38	(neutrophil* or LE cell* or neutrophil band cell* or erythrocyte sedimentation).tw,kw.	160153
39	c-reactive protein/	46504
40	(C reactive protein or C-reactive protein or CRP).tw,kw.	85403
41	macrophages/	131215
42	(bone marrow derived macrophage* or bone marrow-derived macrophage* or macrophage* or monocyte-derived macrophage*).tw,kw.	267043
43	edema/	40240
44	(Edema or luid accumulation or swelling or oedema).tw,kw.	221473
45	hypothermia/ or fever/	53133
46	hypothermia*.tw,kw.	34457
47	(fever or hyperthermia* or pyrexia*).tw,kw.	199623
48	tachycardia/	19837
49	(tachycardia or tachyarrhythmia).tw,kw.	60246

50	tachypnea/	231
51	tachypnea.tw,kw.	3224
52	leukocytosis/	5631
53	(leukocytosis or pleocytosis or pleocytoses).tw,kw.	13264
54	amino acids, sulfur/ or cystathionine/ or cysteic acid/ or cysteine/ or acetylcysteine/ or cystine/ or homocysteine/ or s-adenosylhomocysteine/ or homocystine/ or methionine/ or s-adenosylmethionine/ or methionine sulfoximine/	98384
55	((sulfur or sulphur) adj amino acid*).tw,kw.	1487
56	(cystathionine or L-cystathionine or L cystathionine).tw,kw.	4103
57	(cysteine or L-cysteine or L cysteine or cystine or N-Acetyl-L-cysteine or N Acetyl L cysteine or NAC).tw,kw.	117491
58	(homocysteine or homocystine or L-homocysteine or L homocysteine).tw,kw.	22084
59	(S-Adenosylhomocysteine or S Adenosylhomocysteine).tw,kw.	1945
60	(Methionine or Methionine Sulfoximine or L-Methionine or L Methionine).tw,kw.	50704
61	(S Adenosylmethionine or S-Adenosylmethionine or S-Adenosyl-L-Methionine or S Adenosyl L Methionine or SAM-e).tw,kw.	8952
62	glutathione/	56662
63	(glutationine or GSH or gamma-L-Glutamyl-L-Cysteinylglycine or gamma L Glutamyl L Cysteinylglycine or gamma-L-Glu-L-Cys-Gly).tw,kw.	46826
64	cystathionine beta-synthase/ or cystathionine gamma-lyase/	2496
65	(cystathionine beta synthase or cystathionine beta-synthase or cystathionine b-synthase).tw,kw.	2244
66	(cystathionine gamma lyase or cystathionine gamma-lyase or cystathionine g-lyase).tw,kw.	1348
67	(alpha-ketobutyrate or alpha ketobutyrate).tw,kw.	291
68	taurine/	9694
69	taurine.tw,kw.	13338
70	hydrogen sulfide/	7194
71	(Hydrogen adj (sulfide or sulphide)).tw,kw.	9405
72	or/1-5 [vit b deficiency]	18385
73	or/4,6-25 [impaired transsulfuration]	13232
74	or/1-25 [impaired SAA]	29196
75	or/26-27,43-53 [SIRS]	572925
76	or/26-53 [acute inflammatory response]	1461217

77	or/54-71 [SAA]	309171
78	73 and 75 [impaired transsulfuration SIRS]	111
79	73 and 76 [impaired transsulfuration acute inflammatory response]	911
80	74 and 75 [impaired SAA SIRS]	298
81	74 and 76 [impaired SAA acute inflammatory response]	1850
82	72 and 75 [vit df SIRS]	204
83	72 and 75 and 77 [vit df SIRS SAA]	20
84	72 and 76 [vit df acute inflammatory response]	1130
85	72 and 76 and 77 [vit df acute inflammatory response SAA]	406
86	4 and 75 [vit b6 SIRS]	7
87	4 and 75 and 77 [vit b6 SIRS SAA]	1
88	4 and 76 [vit b6 acute inflammatory response]	37
89	4 and 76 and 77 [vit b6 acute inflammatory response SAA]	9
90	Methionine Adenosyltransferase/df [Deficiency]	56
91	Glycine N-Methyltransferase/df [Deficiency]	43
92	Adenosylhomocysteinase/df [Deficiency]	12
93	S-Adenosylmethionine/df [Deficiency]	28
94	((Methionine Adenosyltransferase or MAT I or MAT II) adj deficien*).tw,kw.	17
95	(Glycine N-Methyltransferase adj deficien*).tw,kw.	11
96	((Adenosylhomocysteinase or S-Adenosylhomocysteine hydrolase or SAHH) adj deficien*).tw,kw.	21
97	((S-Adenosylmethionine or S Adenosylmethionine or SAM-e) adj deficien*).tw,kw.	8
98	90 or 91 or 92 or 93 or 94 or 95 or 96 or 97	157
99	73 or 98	13352
100	74 or 98	29308
101	75 and 99	114
102	76 and 99	920
103	75 and 100	301
104	76 and 100	1858
105	76 and 77 and 100	1013
106	76 and 77 and 99	766

Figure 8.2 Research Ethics and Compliance approval letter

UNIVERSITY OF MANITOBA | Research Ethics and Compliance
Office of the Vice-President (Research and International)

Animal Care & Veterinary Services
208-194 Dafoe Road
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veterinaryservices@umanitoba.ca

4 September 2019

TO: Dr. J. House
Department of Human Nutritional Sciences

FROM: Dr. L. Connor, Chair, Fort Garry Campus Animal Care Committee

RE: Your protocol entitled **"The potential role of sulphur amino acids in the resolution of inflammation"**

Please be advised that your response to the FG ACC's queries (following review of your protocol at their meeting of 27 June 2019) was forwarded to the primary reviewers for their consideration. Upon review of your response, the reviewers agreed that your protocol can now be **APPROVED.**

Protocol Reference Number: **F19-014 (AC11492)**
Animals approved for use:

Number	Species	Common Name	Sex
40	Rats – older than 21 days	wistar	Male only
4	Rats – older than 21 days	any strain	Male and female

Protocol approval is valid from: **September 4 2019 to September 3 2020**
Category of Invasiveness: **B**

The protocol reference number must be used when ordering animals. It is understood that these animals will be used only as described in your protocol. Failure to follow this protocol will result in the termination of your ability to use animals.

The protocol must be kept current. Minor modifications to the protocol must be submitted in the form of an amendment. Major changes would necessitate preparation and submission of a new protocol. Failure to renew this protocol prior to the expiry date will result in the termination of your ability to continue ordering animals.

On behalf of the Fort Garry Campus Animal Care Committee, I would like to extend our best wishes for the successful completion of your research.

LC/tvo

copy: Veterinary Services
Ms. J. Nelson, Department of Biological Sciences
Dr. J. Treberg, Department of Biological Sciences
Ms. C. Edworthy, ORS