Inhibitory Effect of Isoflavones on Mouse Inflammatory Mediators and Relevance to Chronic Fatigue Syndrome

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ABSTRACT

Chronic fatigue syndrome (CFS) is a complex, heterogeneous disease affecting more than one million Americans, mostly women. Despite extensive research, no definitive cause has been determined, even though some evidence suggests an infectious etiology. Many CFS patients demonstrate abnormal hypothalamic-pituitary-adrenal (HPA) axis activity and high anxiety. Corticotropin-releasing hormone (CRH) and neurotensin (NT), secreted under stress, could activate mast cells (MC), which are involved in comorbid diseases with CFS, such as fibromyalgia, to release inflammatory mediators contributing to CFS symptoms. Abnormal overexpression of MC has been linked to skin hypersensitivity in fibromyalgia patients.

Most current murine "models" of CFS use forced swim daily for 7-43 days, with or without an immunological trigger, including lipopolysaccharide (LPS) and/or polyinosinic:polycytidylic acid [poly(I:C)], to induce "chronic fatigue," assessed by behavioral and biochemical parameters. I used C57BL/6 and/or BALB/c mice, subjected to forced swim, daily for up to twenty one days, with or without LPS or poly(I:C), which did not result in chronic fatigue, as reported previously by similar studies on albino LACA mice. Fatigue symptoms in either C57BL/6 or BALB/c mice were present only for the first three days and were best documented by decreased locomotor activity.

Poly(I:C) treatment decreased mouse locomotor activity and increased inflammatory mediator levels in the serum, as well as their brain and skin expression. High isoflavone diet (daidzein, genistein) reversed these effects. Poly(I:C) alone increased tumor necrosis factor (TNF) gene expression in cultured MC, and when used together with CRH, NT or substance P (SP), for 24 hours (hr), TNF gene expression was significantly enhanced.

To circumvent the challenge of obtaining skin biopsies from CFS patients, I analyzed skin and serum samples from atopic dermatitis (AD) and psoriasis (PS) patients, who exhibit similar skin sensitivity as CFS patients. I demonstrated that NT and CRH serum levels are increased in patients with both diseases, as is NT gene expression in affected skin from AD patients. NTR-1 skin gene expression in AD patients was unchanged, while NT and NTR-1 skin gene expression in PS patients, as well as CRHR-1 skin gene expression in AD and PS patients, were reduced suggesting downregulation. High expression of NT and CRH in the serum and skin of AD and serum of PS patients, and increase in TNF gene expression after stimulation of human cultured MC with NT and CRH, suggest that these peptides, which are secreted under stress, could activate MC to release inflammatory mediators contributing to CFS symptoms.

In summary, poly(I:C) treatment induced “fatigue behavior” after 24 hr which was reversed by isoflavones known to inhibit MC.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

°C	Degrees Celsius
µl	Microliter
5-HT	Serotonin or Dopamine Receptor Agonist
$5\text{-HT}_{1A,2,3}$	Serotonin Receptor Agonist
D3 RA	Dopamine Receptor Agonist
5-HT syndrome	Described as an ataxic gate and decrease in locomotor activity
5-HTT	Serotonin Transporter
ACTH	Adrenocorticotropic Hormone
AD	Atopic Dermatitis
AMF	Astragalus Membranaceus
ATP synthase	F1-F0 synthase (assessment of mitochondrial complex-V activity)
AUC	Area Under the locomotor activity-time Curve
BA	Brucella Abortus antigen
bp	Base pairs
BT	Body Temperature
BW	Body Weight
CA	Catalase Activity (assessment of oxidative stress)
CBT	Cognitive Behavioral Therapy
CCL	(CC motif) Ligand
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic Acid
CFS	Chronic Fatigue Syndrome
CIT	Citalopram
CMV	Cytomegalovirus
CRH	Corticotropin-Releasing Hormone
CRHR-1	CRH Receptor-1
Ct	Cycle Threshold
CW	Cerebral Weight
CXC	(CXC motif) Ligand

Cyt c oxid	Cytochrome oxidase (assessment of mitochondrial complex-IV activity)
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
EC	Endurance Capacity
EGCG	Epigallocatechin gallate
FDA	U.S. Food and Drug Administration
FI	Food Intake
FST	Forced Swim Test
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GET	Graded Exercise Therapy
GSH	Reduced Glutathione Levels (assessment of oxidative stress)
hr	Hour
HDC	Histidine Decarboxylase
HERV-K18	Human Endogenous Retrovirus-K18
HHV-6	Human Herpesvirus-6
HHV-7	Human Herpesvirus-7
HIV	Human Immunodeficiency Virus
HPA	Hypothalamic-Pituitary-Adrenal
IFN-α	Interferon alpha
IL	Interleukin
Imblt	Immobility time while in water
IMP	Imipramine (Non Selective 5-HT Reuptake Inhibitor)
IO&NS	Inflammatory and Oxidative and Nitrosative Stress
IP-10	Interferon gamma-induced Protein (CXCL10)
KC	Keratinocyte-derived Chemokine (human IL-8/CXCL8 murine homologue protein)
LA	Locomotor activity
L-arg	L-arginine, a NO precursor
L-NAME	(L-NG-Nitroarginine Methyl Ester): selective NOS inhibitor
LPS	Lipopolysaccharide

MAOIs	Monoamine Oxidase Inhibitors
MC	Mast Cell
MCH	Mirror chamber (assessment of anxiety)
MCP1	Monocyte Chemotactic Protein-1 (CCL2)
MDA	Maldodialdehyde content (assessment of oxidative stress)
ME	Myalgic Encephalomyelitis
mg	milligram
MHC	Major Histocompatibility Complex
min	Minute
MIP-1α and β	Macrophage Inflammatory Protein-1α (CCL3) and -1β (CCL4)
ml	Milliliter
MLVs	Murine Leukemia Viruses
mM	Millimolar
MPO	Myeloperoxidase activity
MPP	Mitochondrial membrane potential
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
MTT	Estimation of MTT reduction (assessment of mitochondrial respiration)
NADH	Nicotinamide Adenine Dinucleotide
NADH dehy	NADH dehydrogenase (assessment of mitochondrial complex-I activity)
NFκB	Nuclear Factor-KappaB
ng	Nanogram
NIH	National Institutes of Health
NJE	Nardostachys jatamansi extract
nM	Nanomolar
NS	Nitrosative stress
NT	Neurotensin
OFT	Open Field Test (assessment of locomotor and exploratory behavior in the novel circumstances)

OS-MDA	Malonaldehyde content (assessment of oxidative stress-lipid peroxidation)
p38 MAPK	p38 Mitogen-Activated Protein Kinase
PA	Peak Activity (assessment of normal pace-running wheel)
PCR	Polymerase Chain Reaction
PMT	Plus-Maze Test (assessment of cognitive behavior (PMT_1) or anxiety (PMT_2))
PMV	Polytropic Murine Leukemia Viruses
Poly(I:C)	Polyinosinic:polycytidylic acid
PRP	Radix Pseudostellarie polysaccharide
PSF	Post Swim Fatigue (time to start grooming after FST)
qPCR	Quantitative Polymerase Chain Reaction
RA	Running Activity
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted (CCL5)
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
RRT	Rota Rod Test (assessment of muscle in coordination)
RW	Running Wheel activity
s	Second
SAg	Superantigen
SD	Standard Deviation
SDH	Succinate Dehydrogenase (assessment of mitochondrial complex-II activity)
SDL	Step Down Latency of passive avoidance - number of errors here (assessment of learning activity)
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
SNRIs	Serotonin-Norepinephrine Reuptake Inhibitors
SOD	Cytosolic Superoxide Dismutase enzyme activity (assessment of oxidative stress)

SP	Substance P
SSRIs	Selective Serotonin Reuptake Inhibitors
T	Temperature
TA	Total Activity (assessment of normal duration-running wheel)
TJ-41	Hochu-ekki-to
TNF	Tumor Necrosis Factor
TRD	Trazodone, Serotonin Antagonist and Reuptake Inhibitor (SARI)
TWL	Tail Withdrawal Latency (assessment of stress induced hyperalgesia)
U	Unit
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
XMRV	Xenotropic Murine leukemia-like virus Related Virus

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Introduction

Chapter 1. Chronic Fatigue Syndrome (CFS)

1.1. Demographics/Prevalence

CFS is a complex and heterogeneous disease that affects more than one million Americans (http://www.cfids.org/about-cfids/prevalence-study.asp). Its prevalence may be as high as 1%[1]. Previous reports show a female to male ratio of 1.3 to 1[2]; however, U.S. Centers for Disease Control and Prevention (CDC) report a female:male ratio of 4:1[3]. CFS affects all ethnic groups and can be observed in all socioeconomic groups[4-6]. The estimated prevalence of CFS is significantly lower among children and adolescents than among adults[7-9]. Patients with CFS have overwhelming fatigue that is not improved with bed rest and worsens after physical activity or mental exertion. This excessive fatigue prevents individuals from performing everyday tasks and limits their daily activities. Despite extensive research for over two decades, the causes of CFS still remain a controversial topic among practitioners, scientists, and patients.

1.2. Case Definition

CFS has also been known as "neurasthenia," "post viral fatigue," and "chronic mononucleosis"[10-12]. CFS is often comorbid with other disorders that include fibromyalgia, pelvic bladder syndrome/interstitial cystitis, irritable bowel syndrome, migraines, post-traumatic stress disorder, and temporomandibular joint disorder, all of which are worsened by stress[13-16].

CFS-like cases were noticed throughout the 1960s and 1970s without any advances in diagnostic tools or definition until 1985[17]. The initial CFS definition (Holmes, CDC

USA, 1988) [18] was used during an outbreak of what can only be described as distinct myalgic encephalomyelitis (ME) in Nevada at Lake Tahoe in the mid-1980s [19, 20]. CFS was defined as a syndrome, a complex of potentially related symptoms tending to occur together that may have several causes based on signs and symptoms. The 1988 case definition was intentionally restrictive to delineate a more uniform patient population and maximize the chances that research studies will detect significant associations with causative agents[18]. In the absence of diagnostic tests, CFS was merely a collection of symptoms and a diagnosis of exclusion[18].

Two Australian CFS definitions appeared shortly after the US definition[2, 21], and another came out of Great Britain[22]. All of these latter definitions focused on fatigue without excluding psychiatric illness as a cause of the fatigue. The CDC and the International Chronic Fatigue Syndrome Study Group revised the 1988 CFS definition in 1994 (summarized in Table 1)[23]. The new definition removed all physical signs from the inclusion criteria, because their presence had been unreliably documented in past studies, lowered the required number of symptoms from eight to four, and decreased the list of symptoms from eleven to eight [23]. It is currently in use for research and clinical diagnosis in the US and most countries abroad (http://www.cfids.org/about-cfids/prevalence-study.asp and http://www.cdc.gov/cfs/diagnosis/index.html). Another accepted case-definition is the pediatric case-definition[24]. In 2003, a Canadian clinical case-definition placed more emphasis on the symptoms other than fatigue. The definition makes it compulsory that in order to be diagnosed with CFS, a patient must become symptomatically ill after exercise and must also have neurological, neurocognitive, neuroendocrine, dysautonomic, circulatory, and immune manifestations[25]. Neither the

1988 case-definition nor the 1994 revision were intended to be used by physicians for the clinical diagnosis of CFS[25-27]. However, almost all studies use the 1994 CDC case-definition or the Canadian case-definition[28, 29].

Table 1. The CDC Definition for Chronic Fatigue Syndrome
A case of chronic fatigue syndrome must meet both the following major and minor criteria [23]:
1. Major Criteria
Clinically evaluated, unexplained persistent or relapsing chronic fatigue that: • is of new or definite onset (i.e. not lifelong) • is not the result of ongoing exertion • is not substantially alleviated by rest • results in substantial reduction in previous levels of occupational, educational, social, or personal activities All other medical conditions associated with fatigue must be ruled out.
2. Minor criteria
The concurrent occurrence of four or more of the following symptoms: (These symptoms must have persisted or recurred during six or more consecutive months of illness and must not have predated the fatigue) • substantial impairment in short-term memory or concentration • sore throat • tender lymph nodes • muscle pain • multi-joint pain without swelling or redness • headaches of a new type, pattern, or severity • unrefreshing sleep • post-exertional malaise lasting more than 24 hr *http://www.cdc.gov/cfs/case-definition/1994.html*

Individuals with CFS have been found to differ in functional comorbidity, viral, immunological, neuroendocrine, autonomic and genetic biomarkers, resulting in a

discrepancy among findings from different studies in this field[10]. Therefore, recent studies demonstrate the need to revise or replace this definition in the future, including specific subtypes and case definitions[10, 30].

1.3. Symptoms/Clinical Manifestations

CFS patients meet specific criteria and experience persistent severe fatigue lasting longer than six months that is not alleviated with rest, but most patients also complain of muscle pain and cognitive dysfunction[31]. Symptoms include myalgia, impaired memory or concentration, gastrointestinal problems, headaches, pain in muscles or several joints, dizziness, nausea, anorexia, and night sweats[28]. In almost all cases, symptoms have substantially reduced a person's everyday activities and quality of life [32] (Table 1).

Moreover, according to CDC, patients may also experience brain fog, difficulty maintaining an upright position, irritable bowel, chills and night sweats, visual disturbances and depression or mood swings (http://www.cfids.org/about-cfids/prevalence-study.asp), and (http://www.cdc.gov/cfs/diagnosis/index.html)[33]. CDC studies show that CFS can be as debilitating as multiple sclerosis, lupus erythymatosus and similar chronic conditions. CFS symptoms tend to affect patients in cycles with periods of illness, followed by periods of remission. Although these are the common symptoms among CFS patients, not all patients have every symptom. The severity of CFS varies from patient to patient.

Many CFS patients demonstrate abnormal hypothalamic-pituitary-adrenal (HPA) axis activity[34, 35]. Anxiety symptoms are high in children with CFS[36], who appear to be particularly vulnerable to stress[16], through the possible release of inflammatory molecules

that could affect brain function[37, 38]. Many patients report an acute onset of symptoms after an infectious flu-like illness[31].

1.4. Causes/Possible Etiologies

There is no universal known cause for CFS and multiple causes may result in the same symptoms[3, 28, 31]. Nevertheless, there is a general agreement that there is a triggering physical or psychological event[31], as well as an immune dysfunction [39-42] in CFS. However, the neuroimmune and neuroendocrine interactions involved are still unknown.

1.4.1. Infectious Etiology

Different publications support the theory that attempts to describe CFS as either a post-infectious state or the result of a chronic inflammation. An acute onset of symptoms is often reported after an infectious flu-like illness[31, 43-45]; therefore, clinical and subclinical viral infections have been suspected[46, 47]. A multitude of studies have attempted to link viral infections to CFS {21160, 21160, 21163, 14805}, although the evidence of their association is controversial.

Retroviruses, like xenotropic murine leukemia virus-related virus (XMRV), have been associated with CFS [48]. However, subsequent studies found no association of XMRV with CFS[49, 50]. Furthermore, it was proven that the PCR test for XMRV was positive only if mouse DNA was contaminating the human sample [49, 50] and this was confirmed by other studies {21164, 21165, 21166}. Early studies found high antibody

titers to Epstein-Barr virus (EBV) in patients with CFS[19], but subsequent studies failed to discern a difference in antibody titers between CFS patients and healthy controls[51, 52].

High rates of CFS have also been reported after Q fever and Lyme disease [53] but no causal evidence exists. Other infectious agents considered to have been linked to CFS include borna disease virus[54, 55], enterovirus[56, 57], parvovirus B19[58, 59], glandular fever[60], Nipah virus encephalitis[61], as well as EBV and cytomegalovirus (CMV) leading to infectious mononucleosis[62]. The human herpes viruses 6 and 7 have also been implicated in CFS[20, 62-64], but another study found no association between human herpes virus 6 and 7 viral load and disease state[65].

Although studies have found associations between some of these pathogens and CFS, there is no evidence that any one of these viruses causes CFS.

1.4.2. Genetic Etiology

CFS is sometimes seen in members of the same family [66] and has a higher concordance rate in monozygotic female twins than dizygotic female twins[67]. However, no specific genes or mutations have been linked to CFS. Some studies have isolated different mRNA expression patterns in CFS patients compared to healthy controls[68, 69], but these findings were not confirmed by other studies[70]. Hence, additional work is necessary to determine if there is a true genetic link.

1.4.3. Endocrinology/Metabolism Etiologies

CFS is suspected to have an abnormal immune component, but the neuroimmune and neuroendocrine interactions involved are still unknown. HPA axis abnormalities have

been linked to CFS[3, 34, 35, 71]. Studies have shown both HPA hypoactivity and higher chronic adrenocorticotropic hormone (ACTH) autoantibody levels in CFS[72, 73]. Anxiety symptoms are high in children with CFS[36], who appear to be particularly vulnerable to stress[16, 74], through the possible release of inflammatory molecules that could affect brain function[37, 38, 75]. However, another study showed no role for deficiency in central opioids or the HPA axis in the symptoms of CFS[76]. Nevertheless, HPA dysfunction does occur in some CFS patients and cannot be overlooked as possessing a possible etiological role[77].

1.4.4. Mental/Neurologic Etiology

As stated above, most experts agree that there is a triggering physical or psychological event leading to CFS[32]. Psychosocial factors are frequently thought to contribute to fatigue. Serious life events, such as the loss of a loved one or other stressful situations have been found to precipitate CFS[78, 79]. Although stress by itself cannot cause CFS, it can be a contributing factor to the development and prolonging of CFS[80].

1.4.5. CFS and Inflammation

1.4.5.1. Serum Cytokine Profiles

In an attempt to define specific biomarkers for CFS, studies to profile serum cytokine concentrations in CFS patients have produced contradictory results[45]. Some studies reported increase on specific inflammatory mediator levels, including tumor necrosis factor (TNF)[81-84], Interleukin (IL)-1α[84, 85], IL-1β[83, 86, 87], and IL-6[85], while others reported no changes in TNF[85, 88-94], IL-1α[93], IL-1β[88, 91, 94], and IL-6[89-92, 94-96]. Anti-

inflammatory IL-10 serum levels were shown to remain unchanged[85, 91, 93, 94], while IL-13 was reported in different studies to remain unchanged [91] or to be decreased[85]. These differences in cytokine profiles contribute to the notion that there is a need to define specific patient subgroups in the CFS symptoms spectrum[10, 30].

Studies on cytokines mediating T helper cell (Th) 1 response are controversial as well. Interferon (IFN) γ serum levels were found to remain unchanged[85, 91, 94], while other studies reported IL-12 to be elevated [85] or remain unchanged[91, 94]. The same is true for IL-2; some studies reported increase [84] while others reported no change[85, 88, 91, 94]. Studies on cytokines mediating Th2 response have similar results. IL-4 serum levels are either reported to be elevated [85] or unchanged[88, 91, 92, 94], and IL-5 serum levels are either increased [85] or unchanged[91]. Lastly, IL-18 and IL-23 levels (Th17 response) are unchanged, although upregulation of transforming growth factor (TGF)-β1 gene expression was noted in peripheral blood mononuclear cells of CFS patients[97].

Other evidence for inflammatory status includes increased amyloid β and IL-8 concentrations in the cerebrospinal fluid of CFS patients[98, 99]. Alternatively, the persistence of symptoms in CFS long beyond the initial inflammatory insult could be explained by the persistence of inflammatory mediators due to luck of anti-inflammatory functions in the brain[100, 101]. Moreover, inflammatory hyperalgesia, as part of CFS sickness behavior might be attributed to loss of descending inhibition of pain pathways in CFS. The cytokine IL-1β contributes in hyperalgesia[102], which is mediated through substance P (SP) and increase of nitric oxide (NO) production within the spinal cord[103, 104]. Interestingly, it is also found that cerebrospinal NO fluid concentrations in CFS patients are increased[105].

While there is still an ongoing debate regarding the role of inflammatory cytokines in the etiology of CFS[106], there is a general agreement that chronic inflammation or even an acute inflammatory event would be sufficient to induce long-term pathology through the neuroimmunoregulatory modulation in brain physiology.

IFN-α was shown to cross blood-brain barrier (BBB), leading to the release of other inflammatory cytokines[107], including IL-6 that is known to be related to decreases in a breakdown product of serotonin by inhibiting monoamine oxidase (MAO) enzyme, contributing to symptoms of depression, often present with chronic fatigue[108]. Additionally, people given IFN-α developed insomnia, which is related to fatigue[109]. IFN-α has also been shown to cause changes in the secretion of cortisol, with increased evening levels, similar to the changes in cortisol found in CFS patients and in breast cancer survivors with fatigue[109, 110].

Using brain scans, including magnetic resonance imaging (MRI) and positron emission tomography (PET), IFN-α was shown to affect two separate brain regions, including the basal ganglia and the dorsal anterior cingulate cortex (dACC)[111]. The basal ganglia regulate motor activity and motivation, as well fatigue symptoms and the effect of IFN-α on the basal ganglia has been linked with symptoms of fatigue[111]. Ongoing studies at CDC are currently evaluating whether similar changes in the basal ganglia occur in patients with CFS. The dACC is a brain region associated with arousal and alarm, and changes in this brain region have been found in connection to anxiety[112].

1.4.5.2. Cell Mediated Immunity

Similar to the controversy on the role of inflammatory cytokines in the etiology of CFS, the evidence on the role of cellular immunity in CFS is also conflicting. Natelson et. al. (2002) reviewed in great detail the immunological aspects of CFS and concluded that according to the majority of authors, there is no significant differences between CFS patients and controls regarding total T cell, CD4+ or CD8+ populations, B cells, NK cells or monocytes[113]. Moreover, there was no significant difference in terms of the activity of the cells mentioned above, except for NK cells, whose activity was impaired according to the majority of investigations[113].

1.4.5.3. Oxidative and Nitrosative Stress Pathways

CFS may have an abnormal immune component[39-41], but the interactions of the neuroimmune and neuroendocrine systems involved [114, 115] are still unknown, nor is the identity of any reliable biomarkers [116] known. A recent proposal on the cause of CFS, is the upregulation in the inflammation and oxidative and nitrosative stress pathways following a nonspecific trigger that activates inflammatory responses marked by increased proinflammatory cytokines[117]. The proinflammatory stimuli increase oxygen radicals, such as peroxides and superoxides, which lead to oxidative damage of the cell membranes causing them to be immunogenic. Inflammatory cytokines also activate neutrophils and monocytes that produce nitrogen monoxide and peroxynitrite (ONOO-). The free radicals that cause oxidative stress are also linked to muscle fatigue and muscle pain[118].

Nitration causes chemical modifications of proteins, which render them immunogenic[117]. Systemic inflammation can lead to a central neuroinflammation with increased levels of proinflammatory cytokines, that remain for several months[101]. There is a strong correlation between inflammation and symptoms of depression, sleep disorders and psychomotor retardation[119].

1.4.5.3.1. Studies Demonstrating Increased Oxidation Products in CFS

Increased lipoprotein peroxidation, decreased serum antioxidant activity, decreased vitamin E and A[120], as well as persistent low serum magnesium correlated with low glutathione[121], have been reported in CFS patients. Studies have also shown increased concentrations of protein carbonyls in CFS patients compared to controls [122] and increased lipid peroxidation, as well as increased catalase and glutathione peroxidase activity in muscle biopsy samples from CFS patients[118, 123]. The immune response to antigenic determinants, as a result of damage to lipids and proteins by oxidative and nitrosative stress (OS and NS) in CFS patients, was measured by serum concentrations of plasma peroxides and oxidized LDL [81, 124] and were found to be increased in CFS patients compared to controls[124]. Moreover, it was shown that there is a correlation between CFS symptoms and concentrations of isoprostane and oxidized LDL in CFS patients with no significant cardiac disease risk factors[125].

1.4.5.3.2. Studies demonstrating Antioxidant Depletion in CFS

Several studies have shown increased oxidative stress and decreased antioxidant levels in CFS patients as compared to healthy controls[124, 126]. Richards *et al.* presented

evidence of oxidative damage in the red blood cells of CFS patients by showing statistically significant increases in methemoglobin and malondialdehyde[126]. Urinary concentrations of oxidative DNA damage in CFS patients was shown to be increased[127], while serum concentrations of coenzyme Q10 were low in depressed patients with chronic fatigue[128].

The presence of stress factors (acute infection or physical activity) in the history of CFS patients is associated with severe oxidative stress and the suppression of protective heat shock protein (HSP) HSP27 and HSP70 responses to exercise[129]. Parameters of oxidant/antioxidant balance significantly correlated to muscle fatigue symptoms, including muscle hyperalgesia[130]. Specifically, plasma levels of vitamin E, C, and HSP were found to be decreased in CFS patients[90, 130-132], while lipid peroxidation was increased[90, 130].

1.4.6. Management of CFS

Since there is no cure for CFS, multiple treatment options aim to relieve CFS symptoms and to improve the quality of life for CFS patients. Treatment strategies for CFS include psychological, physical, and pharmacological intervention[108]. Most treatment regimens start with well-balanced nutrition habits and encouragement to exercise mildly as a way to boost their immune system[3, 31, 108, 133-136]. CFS population is very heterogeneous, and all treatment options must be discussed on an individual patient basis instead of using one treatment for all CFS patients.

Vitamins and other supplements can also be helpful in alleviating symptoms[133]. There are a few trials assessing the effectiveness of alternative medicine (fatty acids,

magnesium supplements, Acetyl-L-carnitine and propionyl-L-carnitine, effects of NADH on symptom scores)[137-141]; however, most of these studies are poorly designed and their overall effectiveness cannot be ascertained[142, 143].

Physicians have prescribed drugs to treat various symptoms, such as sleep-aids for insomnia and restless sleep[31, 144], pain-relievers for joint or muscle pain [3] and antidepressants[145-148]. However, these drugs have well-known side effects and are rarely prescribed for CFS patients {21153, 16911} . Potential serious side effects limit the use of antiviral medications in CFS patients, and therefore, are only prescribed when a proven infection is present[149-151]. Currently, there are no approved drugs for the treatment of CFS[152]. More possible treatment approaches will be discussed later.

1.4.7. CFS Murine Models

Several researchers have been focused on establishing a "murine model" for the study of CFS. Previous studies reported either the chronic use (7-42 days) of forced swim (Table 2), either an immunological trigger (Table 3) or combination of both (Table 4) as a way to induce "fatigue behavior." Experimental paradigms varied greatly, with swim stress applied for 7-42 days, and Brucella abortus antigen (BA), lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (polyI:C) used as immunological triggers. Fatigue was established via assessment of post swim fatigue-like behavioral parameters compared to normal conditions, including locomotor activity. Biochemical parameters were also reported, including brain oxidative stress. Typically, mice with "fatigue behavior" would demonstrate reduced activity, accompanied with increased brain oxidative stress.

In the first experimental paradigm, researchers (Sachdeva AK 2010 [153] and 2011 [154] from Chopra K group; Kuo YH 2009[155], Lyle N 2009[156], Singh A 2002 [157] and Dhir A 2008 [158] from Kulkarni SK group; Surapaneni DK 2012[159], and Kumar A 2008 [160] and 2011[161]) reported the chronic use of swim stress (forced swim test, FST) (six minutes to exhaustion time, daily, for one week to six weeks), as a way to induce fatigue (Table 2). Animals used included mice (Albino LACA) and rats (Wistar, Sprague Dawley and Charles Foster albino).

Fatigue was assessed via specific behavioral parameters compared to normal conditions, that included endurance capacity (EC), post swim fatigue as measured by time to start grooming after forced swim (PSF), locomotor activity (LA), assessment of cognitive behavior as measured by plus-maze test (PMT_1), assessment of anxiety as measured by plus-maze test (PMT_2) and mirror chamber (MC) assessment of muscle coordination as measured by rota rod test (RRT), assessment of stress induced hyperalgesia as measured by tail withdrawal test (TWL), body weight (BW), food intake (FI), water intake (WI), immobility time while in water (Imblt), locomotor activity (LA), and fatigue as measured by immobility time in water. Fatigue symptoms would encompass decreased locomotor activity, increased hyperalgesia and anxiety and decreased cognitive behavior.

Once the "chronic fatigue behavior" was established, further biochemical parameters were assayed, including brain oxidative and nitrosative stress as measured by malondialdehyde content (MDA), superoxide dismutase enzyme activity (SOD), reduced glutathione levels (GSH), catalase activity (CA) and myeloperoxidase activity (MPO). Some papers investigated the mitochondrial complex activity measuring NADH

dehydrogenase (NADH dehy, complex I activity), succinate dehydrogenase (SDH, complex II activity), cytochrome oxidase (Cyt c oxid, complex IV activity), and F1-F0 synthase (ATP synthase, complex V activity), as well as mitochondrial membrane potential (MMP) and MTT reduction (MTT, mitochondrial respiration assessment). Lastly, some papers measured specific neurotransmitters in the brain, including norepinephrine (NE), serotonin (Ser) and dopamine (DO), while others measured TNFα serum levels and corticosterone plasma levels.

Table 2. CFS Murine Models using FST

Conditions	Behavioral assessments	Biochemical measurements
[154]Wistar rats male (n=5-6/group) Model: weight loaded FST→until excaustion →for 28d	EC↓ PSF↑ LA↑ PMT_1↓ RRT↓ TWL↑ BW↓ FI↓ WI↓	MDA↑ SOD↓ GSH↓ CA↑ NS↑ TNFα serum↑
Treatment: EGCG (50, 100 mg/kg, p.o.)→1/2h before FST		
[153]Albino LACA mice (n=5-6/group) Model: FST→6 min, for 15d	Imblt↑ PSF↑ LA↑ RRT↓ PMT_1↓ MCH↓ BW↓ FI↓ WI↓	MDA↑ SOD↓ GSH↓ NS↑ TNFα serum↑
Treatment: EGCG (25, 50, 100 mg/kg, p.o.)→1/2h before FST		
[155]Sprague Dawley male rats (n=6/group) Model: weight loaded FST→until excaustion →for 43d+food intake restriction	EC↓	Spleenocytes: Proliferation↓ IL-2(TH1)/IL-4(TH2)↑
Treatment: AMF* (20, 50, 100 mg/kg, p.o.)→1/2h before FST (*11.51±0.03mg/g flavonoid content)		
[156]Wistar rats male (n=7/group) Model: FST→15min for 21d	Imblt↑ LA↓ PMT_2↑	MDA↑ CA↓ SOD↑ NS↑
Treatment: NJE (200, 500 mg/kg, p.o.)→1h before FST (Panax ginseng, as prototype anti-stress agent, 100 mg/kg)		
[157]Albino LACA mice (n=6/group) Model: FST→6 min, for 15d	Imblt↑ ↑	MDA↑ SOD↓ GSH↓ CA↓
Treatment: Carvedilol (5 mg/kg, ip) or melatonin (5 mg/kg, ip) or Withania somnifera (100 mg/kg, p.o.) or quercetin (50 mg/kg, p.o.) or Hypericum perforatum (St.John's wort, 10 mg/kg, p.o.) →daily, 1/2h before FST		
[159]Charles Foster albino rats (n=6/group) Model: FST→15min for 21d	Imblt↑ PMT_2↑	Plasma corticosterone↓ NADH dehy↓ SDH↓ Cyt c oxid↓ ATP Synthase↓ MTT↓ MPP↓ MDA↑ CA↓ SOD↑ NS↑`
Treatment: Shilajit (25,50,100mg/kg) p.o.→1h before FST (Withania somnifera, as positive control, 100 mg/kg)		
[158]Albino LACA mice (n=6-8/group) Model: FST→6 min, for 15d	Imblt↑ LA↑ RRT↓ TWL↓ MCH↓ PMT_1↓	MDA↑ GSH↓ NS↑ MPO↑ Adrenal ascorbic acid↓ NE↓, Ser↓, DO↓
Treatment: Venlafaxine (8, 16mg/kg ip)→daily, 1/2h before FST		
[160]Albino LACA mice (n=6/group) Model: FST→6 min, for 7d	Immobility↑: 1, 3, 5, 7d LA↓ PMT↓ MC↓	MDA↑ NS↑ CA↓ GSH↓
Treatment: TRD (5, 10mg/kg ip)→daily, 1/2h before FST L-arg (100mg/kg) and L-NAME (5mg/kg ip)→ daily, 15min prior TRD	Immobility: L-arg↑ TRD↓ L-NAME↓ TRD+L-NAME↓↓ LA and PMT and MC: L-arg↓ TRD↑ L-NAME↑ TRD+L-NAME↑↑	L-arg: MDA↑ NS↑ CA↓ GSH↓ TRD, L-NAME: MDA↓ NS↓ CA↑ GSH↑ TRD, L-NAME: MDA↓↓ NS↓↓ CA↑↑ GSH↑↑
[161]Albino LACA mice (n=10/group) Model: FST→6 min, for 7d	Immobility↑: 1, 3, 5, 7d LA↓ PMT↓ MCH↓	MDA↑ NS↑ CA↓ GSH↓
Treatment: CIT (5, 10mg/kg ip) and IMP (10, 20mg/kg)→daily, 1/2h before FST L-arg (100mg/kg), L-NAME (5mg/kg ip) and MB (10mg/kg ip)→ daily, 1h prior CIT and IMP	Immobility: L-arg↑ CIT↓ IMP↓ MB↓ L-NAME↓ MB+L-NAME+CIT or IMP ↓↓ LA and PMT and MC: L-arg↓ CIT↑ IMP↑ MB↑L-NAME↑ MB+L-NAME+CIT or IMP ↑↑	L-arg: MDA↑ NS↑ CA↓ GSH↓ CIT, IMP: MDA↓ NS↓ CA↑ GSH↑ MB+L-NAME+CIT or IMP : MDA↓↓ NS↓↓ CA↑↑ GSH↑↑
[162]Albino LACA mice, male (n=6/group) Model: FST→6 min, for 7d	Imblt↑	MDA↑ GSH↓
Polyphenols in green tea extract (GTE) (25, 50mg/kg) and catechin (50,100mg/kg)	Imblt↓	MDA↓ GSH↑

Overall, the papers reported that chronic use of swim stress alone would induce "fatigue behavior" as was evidenced by reduced activity and under these conditions oxidative stress in the brain would be increased. Details on these models and comparison with my experimental protocol will be analyzed extensively in Discussion.

In the second experimental paradigm, researchers (Ottenweller JE 1998[163], Fomicheva EE 2010[164], Katafuchi T 2003[165], 2005 [166] and 2006[167], Cunningham C 2007[168], Chen R 2008 [169] and 2009[170], and Sheng R, 2009[171]) used an immunological trigger systemically or by direct delivery to the brain, once on Day 1 or repetitively to induce fatigue (Table 3). Different concentrations of BA or polyI:C were used. Animals used included mice (BALB/c, ICR, C57BL/6) and rats (Wistar). Fatigue was assessed via behavioral parameters, including running activity (RA), cerebral weight over body weight (CW/BW), FI, forced swim until exhaustion (used here as behavioral assessment), open field test (OFT, assessment of locomotor and exploratory behavior in the novel circumstances), step down latency of passive avoidance (SDL, assessment of learning activity), and running wheel activity (RW), PSF, running wheel activity (RW), along with peak activity (PA, assessment of normal pace-running activity) and total activity (TA, assessment of normal duration-running wheel). Once the chronic fatigue behavior was established, further biochemical parameters were assayed, including NK cell activity, serum corticosterone, CD4+/CD8+ T lymphocytes, spleen T lymphocytes proliferation activity, brain serotonin levels (5-HT), brain gene expression of TNFα, IL-6, IL-1β, IFNα, p38 mitogen activated protein kinase (MAPK), IkB, and COX-2, as well as TNFα, IL-6, IL-1β, and IFNβ, serum levels.

Table 3. CFS Murine Models using Immunological Activation

Conditions	Behavioral assessments	Biochemical measurements
[170]Female BALB/c mice (n=5/group) Model: BA→ 6 injections (0.2ml)/tail vein/2 wk (compare to baseline level)	RA↓	
Treatment: TJ-41 (500mg/kg) p.o./4 wk or TJ-41 ± IFNγ (08.5 millionU/kg) ip /4 wk	RA↑ BW, FI, BT: no change	NK cell activity: only group3↑ Hippocampus: Bcl-2, BDNF expr: no change
[169]Female BALB/c mice (n=5/group) Model: BA→ 6 injections (0.2ml)/tail vein/2 wk (compare to baseline level)	RA↓ CW/BW↓	
Treatment: TJ-41 (500mg/kg) p.o./4 wk	RA↑ BW↑ FC BT no change	Hippocampus: Bcl-2, BDNF expr: no change
[171]ICR male mice (3/group) Model: poly(I:C)→ip (5 mg/kg) on 4d FST on 1, 4, 7, 10, 13d was used as behavioral assessment	FST with tail load until fatigue↓,up to 7d OFT↓ on Day 1 SDL↑ on Day 18	Serum Corticosterone↓on Day 18 CD4+/CD8+ T lymphocyte↑ Spleen T lymphocytes proliferation ability↓
Treatment: PRP (100, 200, 400 mg/kg)→P.O. for 17d	Only PRP 400 mg/kg: FST↑, up to 7d BW↑ OFT↑-1d SDL↓-18d	Only PRP 400 mg/kg: Serum Corticosterone↑ on Day 18 CD4+/CD8+ T lymphocyte↓ Spleen T lymphocytes proliferation ability↑
[166, 167] Wistar rats male (n=4-5/group) Model: poly(I:C)→3 mg/kg ip	RW↓ -1d, 8d	Brain gene expression: TNF, IL6→ no change IFNα↑(On Day 1, 8), p38MAPK↑ (On Day 1, 8) IL-1β↑(only on Day 1) IκB↑ (only on Day 1) 5-HTT↑ (on Day 1, 8) (+ western) Prefrontal 5-HT levels↓ up to 8d
Treatment: IMP → local brain perfusion→10μM, once, before poly(I:C) 5-HT_{1A} agonist→ 0.3mg/kg, ip on 5d, 6d, and 7d	5-HT_{1A} (but not 5-HT_2, 5-HT_3, or D3 agonist) blocks 5-HT syndrome	IMP blocks prefrontal 5-HT levels decrease
[163]Female BALB/c mice (n=5/group) Model: BA (0.1, 2, 4, 8*10^8 particles /0.2ml) / tail vein, on Day1	RW↓(Only max C) PA↓ up to 4d TA↓ up to 23d lower doses recovered earlier	
[164]Wistar rats male (n=?/group) Model: Poly(I:C)→3mg/kg ip with or without cool stress for 30min, 1h before poly(I:C) → up to 14days And ACTH (250 mg/kg) or hydrocortisone (50mg/kg) on Day7,9,14		Corticosterone↑ (1st peak on Day 1, 2nd peak on Day 7) Stress and poly(I:C)→ ↑ HPA axis impairment: up to 14d
[165] Wistar rats male (n=4-9/group) Model: Poly(I:C)→1, 3 mg/kg ip Positive control: heat stress→ 1h, for 3d	RW-TA↓up to 4d (1mg/kg) RW-TA↓up to 9d (3mg/kg) T↑ up to 7h (3mg/kg) OFT↓ on Day1 only BW→no change	Brain IFNα↑-7d Brain IL-1β→no change ACTH → no change Catecholamines→no change
[168]Female C57BL/6 mice (n=3-5/group) Model: poly(I:C)→ip (2, 6, 12mg/kg) Test tolerance: Rechallenge with poly(I:C): 1week or 3 weeks after 1st challenge	1st Poly(I:C) 12mg/kg: OFT↓→ up to 12h FI↓→up to 26h BW↓→up to 48h Rechallenge: no differences-maybe quicker recovery	1st poly(I:C) 12mg/kg: Serum levels: max3h-up to 24h IFNβ↑,-IL-6↑, IL-1β↑, TNFα↑ Brain gene expression: IL-6↑, IFN-β↑: max3h-24h IL-1β↑, TNFα↑: max 24h COX-2↑:max 6h-up to 24h

Different experimental paradigms were reported (Table 3) and results varied considerably, depending on the route of administration, the type of the immunological trigger and the number of challenges. However, in most of the cases the behavioral changes were maintained between 1-7 days.

Table 4. CFS Murine Models using FST and Immunological Activation

Conditions	Behavioral assessments	Biochemical measurements
[172]Albino LACA mice (n=6/group) Model: LPS→ip injection(1mg/kg) or BA→ip (0.2ml) on Day1 + FST→10min for 19d	Imblt↑ TWL↓ PSF↑ BW↓ FI↓ WI↓	MDA↑ NS↑ TNFα serum ↑ TGFβ serum ↑
Treatment: EGCG (50,100mg/kg) p.o. → 1/2h before FST		
[173]Albino LACA mice (n=6-7/group) Model: LPS→ip injection(1mg/kg) or BA→ip (0.2ml) on Day1 + FST→10min for 19d	Imblt↑ TWL↓	MDA↑ GSH↓ NS↑ TNFα serum ↑
Treatment: Naringin (50,100,200mg/kg) p.o. → 1/2h before FST		
[174]Albino LACA mice (n=6-7/group) Model: LPS→ip injection(1mg/kg) or BA→ip (0.2ml) on 1d + FST→10min for 19d	Imblt↑ TWL↓	MDA↑ GSH↓ NS↑ TNFα serum ↑
Treatment: Curcumin (5,30,60mg/kg) p.o. → 1/2h before FST		
[175]Albino LACA mice (n=6-7/group) Model: LPS→ip injection(1mg/kg) or BA→ip (0.2ml) on Day 1 + FST→10min for 19d	Imblt↑ TWL↓	MDA↑ GSH↓ NS↑ TNFα serum ↑
Treatment: Olive extract (50,300,400mg/kg) p.o.→ 1/2h before FST		

Lastly, in the third experimental paradigm, there are some studies published (Sachdeva AK 2009[172], Vij G[173], 2009, Gupta A 2009 [174] and 2010[175], all from Chopra K group) where a combination of forced swim and immunological stimulation was used to induce fatigue behavior (Table 4). Chopra's group used forced swim daily for nineteen days, along with intraperitoneal (ip) injection of LPS and BA on Day 1. Albino LACA mice were used and fatigue was assessed via assessment of behavioral parameters compared to normal conditions. Behavioral parameters included Imblt, TWL, BW, FI,

and WI. Once the "chronic fatigue behavior" was established, further biochemical parameters were assayed, including brain oxidative and nitrosative stress, as measured by MDA, GSH, and NS, as well as TNFα and TGFβ serum levels. Overall, the studies reported a reduction on mouse activity and body weight, as well as an increase on brain oxidative stress and TNFα serum levels.

Overall, in previous studies, "fatigue behavior" was established via assessment of post swim fatigue-like behavioral parameters compared to normal conditions, including decreased locomotor activity and increased hyperalgesia. Moreover, some biochemical studies would show increased oxidative stress in the brain and increased TNF.

In some of these "models," treatment with specific flavonoids with antioxidant and anti-inflammatory properties, like epigallocatechin 3-gallate (EGCG), curcumin and naringin, ameliorated the FST-induced "fatigue" behavior and oxidative stress, showing a potential treatment approach on the fatigue symptoms. In the Discussion I will refer to these papers, find similarities and differences with my experimental protocol and comment on the outcome of each study.

Chapter 2. Flavonoids

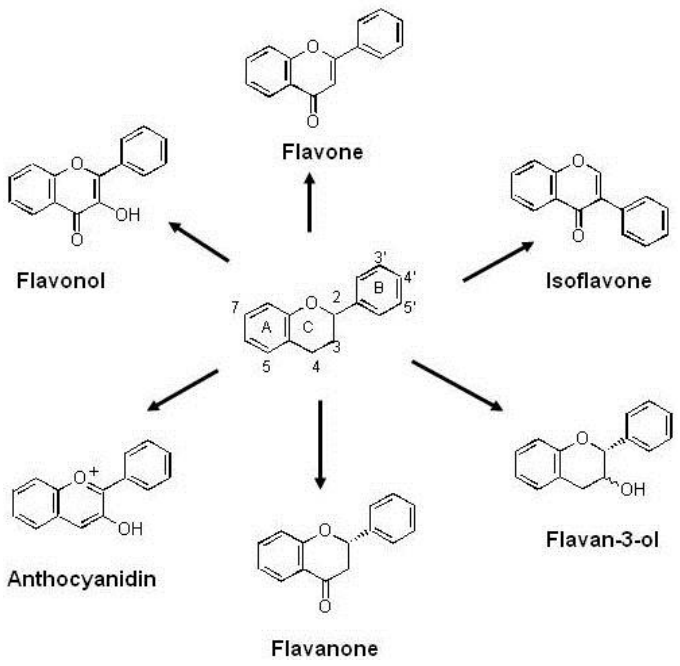
2.1.1. Major Classes of Flavonoids

Flavonoids belong to a large family of polyphenolic coumpounds synthesized by plants and it is a class that shares a common chemical structure. The basic structure of flavonoids consists of two aromatic rings, A and B, and an oxygen-containing heterocyclic ring C (Figure 1). Flavonoids are divided into six subclasses, depending on the oxidation levels of the heterocyclic ring C[176]: flavonols, flavones, isoflavones, flavanols (also called catechins), flavanones, and anthocyanidins. Flavonols, such as quercetin, kaempferol and myricetin, are widely available and are abundant in onions, broccoli, lettuce, blueberries, apples and tea. Flavones, like luteolin and apigenin, are found in high amounts in parsley, celery, thyme and peppers. The isoflavones daidzein and genistein, which are the ones I am using in Chapter 2, are found mainly in legumes, such as soy beans. Flavanols, mainly as monomeric catechins and epicatechins are found in tea, cocoa, grapes and red wine, apples, apricot and beans. Flavanons include naringenin and hesperetin and are abundant in citrus fruits like oranges, lemons and grapefruits. Lastly, the anthocyanidins cyanidin, delphinidin and pelargonidin are abundant in berries and pomegranate.

2.1.2. Absorption and Metabolism

The biological effects of flavonoids in humans depend on the absorption, distribution, metabolism and excretion of these compounds[176-179]. The absorption of flavonoids liberated from food depends on the gastrointestinal metabolism, the molecular size, configuration, lipophilicity, solubility and pKa.

Figure 1. Flavonoid subclasses



Adopted from[179]

Most flavonoids, except catechins, are usually present in the diet in glycosylated forms. Glycosides are more hydrophilic in contrast to aglycones. Flavonoids, in the form of glycosides (glucosides, galactosides, arabinosides, xylosides, and rhamnosides) are therefore hydrolyzed in the small intestine by lactase phloridzin hydrolase (LPH) in the brush border of the small intestine epithelial cells to release aglycones that can enter the cell by passive diffusion[180-182]. Alternatively, glycosides can also enter the cells through the intestinal Na^+-dependent glucose cotransporter (SGLT1) [183]where cytosolic β-glycosidase (CG) hydrolyzes them. Within the cells, UDP-glucuronosyltransferases, sulfotransferases and/or catechol-O-methyltransferases convert the aglycones to their respective glucuronide, sulfate and/or methylated metabolites and enter the circulation. Conversely, multidrug resistance-associated proteins (MRP) or P-glycoproteins are

transporting them back to the intestinal lumen. Glycosides that are not substrates for LPH and SGLT1, and aglycones not absorbed in the small intestine, as well as conjugated metabolites are transported towards the large intestine. In the colon, bacteria are able to hydrolyze flavonoid glycosides, glucuronides, and sulphates, demethylate, reduce double bonds and cleavage rings, resulting in minimal absorption capacity in the colon[180-182].

In contrast with other flavonoids, catechins occur as aglycones and galloylated forms and as such are absorbed from the small intestine[184]. Recent studies suggest that the clinical effectiveness of isoflavones might be due to their ability to produce metabolites in the gut[185, 186]. In particular, daidzein metabolites include dihydrodaidzein, O-desmethylangolensin and equol, while genistein metabolite is dihydrogenistein[186, 187]. In contrast, the isoflavone daidzein 7-O-glycoside has noticeable low hydrolysis rate and is converted into equol by colon bacteria, that can act as an agonist of the estrogen receptor type β in the S enantiomer form[188-190]. Ring fission results in phenolic acids that can be absorbed and subjected to phase II metabolism in the liver.

Importantly, aglycones and other flavonoid metabolites are subjected to Phase I and II metabolism in the liver, including cytochrome P450 mediated oxidation and conjugation reactions. Liver metabolites go through the circulation to the tissues and then reach the kindeys for excretion, while some of the conjugated metabolites are excreted in bile and return to the small intestine, metabolized and reabsorbed. In humans, the main metabolite for isoflavones[191-194], for catechins[195, 196], for flavanones[197], and for anthocyanins [198, 199] are glucuronides. The known concentrations of catechin aglycones that can be present in plasma range from 10% up to 80%[196]. Conjugation percentage for phenolic acids (flavonoid metabolites in the colon from bacteria) ranges from 13% to

100% depending on the type of phenolic acid[200]. Flavonoid aglycones, whose plasma concentration is very low, due to extensive metabolism and conjugation reaction in the small intestine and liver, bind strongly to plasma proteins[201].

Urinary excretion usually correlates with the amount of intact flavonoid metabolite in plasma. Therefore, even though absorption of quercetin glycosides is high, excretion of intact quercetin in urine is low, suggesting lower bioavailability than isoflavones, whose excretion is the highest of all flavonoids[177, 202, 203].

Peak plasma concentration (Cmax) of flavonoids and conjugates after consuming flavonoid-rich foods or drink ranges from 0.06-7.6 μM[176, 204], while absorption can be very rapid to very slow, as Tmax after a single meal dose ranges between < 30 min-9 h[177, 202, 203]. In general, isoflavones have the highest bioavailability, followed by flavonols, flavanones, and flavanols[180, 205]. Isoflavones max concentrations in humans can reach 3-5 μmol/L[206, 207], although concentrations as high as 25 μM have been achieved after supplementation[208]. It is noticeable that the absorption of isoflavones is slow, suggesting absorption from the colon, in accordance with the finding that LPH has a weak affinity for daidzein-7-O-glycoside[180]. Chronic or long-term consumption of flavonoid-rich foods also does not result in accumulation of significant amount of flavonoids in plasma, as shown by steady-state concentration of quercetin in human plasma (less than 1 μM). Plasma concentrations are therefore expected to be in the low μM range, depending on the food content.

Soybeans contain isoflavones at concentrations as high as 1-3mg/g [206] and although consumption of soya in Asian countries is 10-35 g/d, which is equivalent to a mean intake of 25-40 mg/day isoflavones with a maximum of 100 mg/day[209], Americans

consume only a few milligrams/day. Interestingly, in a Spanish-conducted study, mean intake of polyphenols was 8210 mg/day with flavonoids 433 mg/day[210, 211].

2.1.3. Flavonoid Properties

Flavonoids have strong antioxidant activity but also show anti-inflammatory, neuroprotective, anticancer and cardiovascular effects[176, 212].

2.1.3.1. Antioxidant Properties

Flavonoids exert their antioxidant properties by directly scavenging ROS, metal chelating activity, activation of antioxidant enzymes and inhibition of oxidases and ROS-generating enzymes, as well as inhibition of inducible NO synthase activity which leads to oxidative stress by NO and scavenging NO[204, 213, 214].

The antioxidant effects of flavonoids are mediated by the Keap1/Nrf2 pathway. Nrf2 is a member of the nuclear factor-E2 family and upon induction the complex with Keap1 in the cytoplasm is disrupted and Nrf2 translocates to nucleus, where it binds to antioxidant response elements (AREs) and induces detoxifying enzymes an antioxidant proteins[215-217]. Interestingly, Nrf2 can be regulated though multiple signal transduction pathways, including MAPKs, phosphatidylinositol-3-kinase (PI3K/AKT), and protein kinase C (PKC)[218-220].

The protective effects of flavonoids are mainly due to their ability to transfer electrons free radicals, chelate metal catalysis and activate antioxidant enzymes[187]. There is a structure-activity relationship, depending on the substitutions on the rings. Higher Trolox equivalent antioxidant activities (TEAC) values reflect greater antioxidant

capability. A free 3-hydroxyl group and 3′,4′-catechol (dihydroxy) structure, a 2–3 double bond, and a 4-oxo group gives the flavonoid increased activity compared to flavonoids lacking these features. Glycosidic substitution decreases TEAC[187]. For example, quercetin (3,5,7,3', 4'-OH) and epigallocatechin gallate (3,5,7,3',4',5'-OH, 3-gallate) exhibit a TEAC of around 4.7, while catechin (3,5,7,3', 4'-OH) around 2.5, luteolin (5,7,3', 4'-OH) around 2.1, genistein (5,7,4'-OH) around 2.9, daidzein (7,4'-OH) around 1.25, and naringenin (5,7,4'-OH) around 1,53 . Although the flavonoid metabolites themselves are biologically active, metabolism alters their anti-oxidant and anti-inflammatory activity[204].

Genistein reversed the fatigue symptoms by down-regulating oxidative stress level and up-regulating antioxidant enzymatic activity probably through extracellular signal-regulated kinase (ERK) 1/2 signaling pathway[221]. More specifically, genistein reduced and improved muscle fatigue resistance and enzymatic activity of glutathione peroxidase, catalase, and superoxide dismutase. Additionally, quercetin and genistein showed an antioxidant activity towards lipid oxidation and the myofibrillar proteins carbonylation pathway, which involves the oxidative deamination of lysine residues to finally form Schiff bases structures[222].

2.1.3.2. Anti-inflammatory Properties

Flavonoids exert potent anti-inflammatory effects via various pathways, including inhibition of NF-κB pathway at multiple steps, but also of TBK-1 and AP-1 transcription factor[215, 218, 220, 223]. Flavonoids can also interfere with soluble N-ethylmaleimide-sensitive

factor attachment protein receptor (SNARE) proteins inhibiting inflammatory mediator release[224].

In one study, the effects of genistein, daidzein, and soy protein on paraoxonase and arylesterase activity, MDA levels, and lipid profiles of arthritic rats *in vivo* were investigated. Arthritis-induced decreases in paraoxonase and arylesterase activity was restored after treatment with soy protein and isoflavones and MDA concentrations were lower after treatment with all tested compounds[225].

Another study investigated the influence of quercetin on CRH-induced anxiogenic and depressant-like effects in mice, assessed by changes in social interaction time and immobility time in forced swim test. Quercetin increased social interaction time and decreased immobility time indicating anxiolytic- and antidepressant-like effect and produced just opposite effects to that of CRH on these parameters[226].

Chapter 3. Mast Cells (MC)

MC and their mediators have been implicated in CFS [227-233] but also in the comorbid conditions with CFS[234-236], all of which worsen by stress[13-16]. MC derive from a bone marrow progenitor and mature in tissues depending on microenvironmental conditions[237-240]. MC are important for allergic reactions,[241] but also in innate and adaptive immunity[242-244] and many inflammatory diseases[245, 246].

3.1. MC Activation

MC are activated through the classic pathway that involves crosslinking of the immunoglobulin (IgE) receptor (FcεRI) and degranulation, but also through other mechanisms, that result in selective release of mediators[247]. Two alternative ways include activation by neuropeptides, via their G protein coupled receptors (GCPRs) MrgX2[248-250], and by molecules of microbial origin via Toll-like receptors (TLRs)[251, 252].

3.1.1. IgE/Antigen

The most studied pathway of MC activation involves crosslinking of the FcεRI through the IgE binding to a specific antigen[253-255], followed by degranulation[256-258]. IgE is produced by B cells [259] in response to a specific antigen. There is a strong correlation between the IgE levels and the allergic symptoms[260-262]. IgE can also be produced on specific tissues, like lungs, where it initiates local allergic reactions[260, 263].

The MC cell surface membrane has around 500 copies of the high affinity FcεRI, which is also expressed by basophils and other cells of the immune system, but in a lower levels[259]. FcεRI is a tetramer, consisting of a heterodimer of α and β subunit and a

homodimer of γ subunits. A-subunit is located within the cell membrane and carries the region for the IgE binding, while β and γ subunits are responsible for the downstream signaling[259]. Exposure to the antigen causes phosphorylation of Lyn and Syk kinases[264], activation of phospholipase Cγ (PLCγ), followed by formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 leads to endocellular increase of calcium (Ca^{2+})[265], while DAG activates protein kinases (PK), including PKC isoenzymes [266-269] and MAPKs, leading to activation of transcription factors and cytokine production. ERK, c-Jun N-terminal kinase (JNK) και p38 MAPK are all activated after FcεRI activation[254].

3.1.2. Neuropeptides

MC communicate with cells of the nervous system[245, 270-278]. Neuropeptides released from sensory neuronal endings in the periphery activate MC[247, 279]. Neuropeptides, like SP, CRH, calcitonin gene-related peptide (CGRP) and NT can activate MC through their G protein-coupled receptors (GPCR)[280-282], like NTR-1 [283, 284] and CRHR-1[285].

MC also synthesize NT precursor, which is secreted and cleaved to give the active form[286]. MC-derived NT might serve autocrine and paracrine roles and it is tightly regulated since there is rapid degradation of NT from MC-derived carboxypeptidase-like enzymes, modulating the level and form of NT-related peptides in various states involving activation of MC[287]. For example, NT (1-12 amino acids) was effective in releasing histamine from MC in vitro and increasing vascular permeability in vivo, NT (1-11 amino acids) was not.

SP can activate MC through the MrgX2 receptor [288, 288, 289] and the high affinity neurokinin 1 receptor (NK1R), which leads to nuclear factor-kappa B (NF-κB) activation and cytokine production[290]. Depending on the trigger, different subunits of the guanosine nucleotide-binding protein (G protein) are activated, followed by distinct signaling pathways. For example, activation of receptors bound to G_s protein, like CRHR-1, leads to increase of cAMP and although it does not cause degranulation, it activates p38 MAPK causing eventually selective release of specific mediators, like vascular endothelial growth factor (VEGF)[285, 291]. Activation of receptors bound to G_q proteins, like MrgX2, cause intracellular Ca^{2+} increase, although extracellular presence of Ca^{2+} is not necessary for degranulation[280, 288].

3.1.3. Molecules of Microbial Origin

MC have traditionally been known for their roles in allergy and immunity to multi-cellular parasites[292], but currently are also being recognized for their crucial roles in immune defense against bacteria and viruses[251, 293]. MC can be activated by direct and indirect mechanisms as a result of exposure to pathogens[251, 293]. Direct interactions are mediated by Toll-like receptors (TLRs) [252, 294]and usually do not lead to degranulation, but to increased expression of cytokine, chemokine and lipid mediators, as well as mannosylated receptors, like CD48[251, 295, 296].

Indirect activation includes: (1) Fcγ-receptor (FcγR)-mediated activation, either from antigen-specific interactions with antibody or of B-cell superantigens, leading to the degranulation of MC and the production of multiple newly generated mediators [297-302]. Although human MC usually express FcεRI (the high-affinity receptor for IgE) and

FcγRII (a low-affinity receptor for IgG), the expression of FcγRI (the high-affinity receptor for IgG) and FcγRIII (a low affinity receptor for IgG) are induced after exposure to IFN-gamma[303]. FcγR mediated activation leads to the degranulation of MC and the production of multiple newly generated mediators. (2) complement-receptor (CR)-mediated activation, either from complement components (such as CR3) or receptors for split products (such as the C3aR)[297, 304-306]. CR expression level varies depending on the differentiation status of the MC and the microenviroment of each MC subtype[305, 306]. Degranulation seems to occur mainly as a result of the C5aR[304]. (3) Cytokine receptors, like IL-1R, IFN-γR, IL-10R and IL-12R that play a immunoregulatory role[251, 303, 307-310]; (d) chemokine receptors, like CCR3, CCR5 and CXCR4, that mediate MC chemoattraction[251, 293, 311, 312].

3.1.3.1. Toll-like Receptors (TLR)

Molecules of microbial origin are recognized cells of the immune system through TLRs, which are a type of pattern recognition receptors (PRRs). Besides TLRs, other PRR families have been described, including nucleotide-binding oligomerization domain protein (NOD)-like receptors (NLRs)[313, 314], RIG-I-like receptors (RLRs)[315], and the cell-surface C-type lectin receptors (CLRs)[316]. PRR signals trigger intracellular pathways, including those mediated by NF-κB, MAPKs and interferon regulatory factors (IRFs)[317, 318].

NOD1 and NOD2 are cytosolic proteins that recognize bacterial peptidoglycan fragments and activate NF-κB pathway[313], while some NLRs assemble inflammasomes, leading to activation of caspase 1 which cleaves and releases IL-1β and IL-18 [319, 320] and

others regulate type I interferon (IFN) production[321]. RLRs, retinoic acid-inducible gene I (RIG-I), melanoma differentiation gene 5 (MDA5) and LGP2, are cytosolic proteins that sense nucleic acids, such as viral DNA. MDA5 is thought to bind to double-stranded RNA[322].

PPRs are expressed by cells of the immune system and are associated with pathogen-associated molecular patterns (PAMPs) [322]triggering intracellular pathways, including those mediated by MAPKs, NF-κB and interferon regulatory factors (IRFs)[317, 318]. Expression of TLR1–10 with the exception of TLR8 has been identified on human MC, although some studies were unable to identify TLR1, 4, 6, or 9[252, 323-328]. Expression of TLR1–4 and 6–9 was identified at least at the mRNA level in murine MC[323, 329-334], while expression of TLR5 has not been demonstrated yet[329, 331, 332, 335].

Studies have identified TLRs that recognize PAMPS from bacteria, parasites, fungus and viruses[336, 337]. Each TLR has a distinct function in terms of PAMP recognition and immune responses [337] and molecules released following activation of TLR signaling cascades result in innate responses[338-341], as well as the initiation of an adaptive immune response[342, 343]. While the TLRs mainly responsible for the detection of bacterial products (TLR 1, 2, 4, 5, and 6) are expressed on the cell surface, the subset of TLRs that sense viral components (TLR 3, 7, 8 and 9) are located mainly intracellularly on endosomal membranes[343]. Examples include nucleic acid double-stranded RNA from viruses (dsRNA) and poly(I:C), recognized by TLR-3; bacterial lipopolysaccharide (LPS), an endotoxin found on the gram-negative bacterial cell membrane, and BA, specifically recognized by TLR-4; bacterial flagellin, recognized by TLR-5; single-stranded RNA (ssRNA) from viruses, recognized from TLR-7 and 8; and bacterial and viral CpG DNA

motifs, recognized by TLR-9. In addition, TLRs can be activated by endogenous ligands such as heatshock proteins, hyaluronate and single-stranded RNA, the detection of which may contribute to the development of autoimmune disorders[344].

Signaling pathways involved can generally be grouped into the MyD88-dependent pathway, used by all TLRs but TLR3, and MyD88-independent/TRIF-dependent pathway, mainly used by TLR3[345]. Downstream events lead to the activation of NF-κB and members of the IRF family of transcription factors, ultimately resulting in the production of pro-inflammatory cytokines and type-1 interferons[346].

In the case of TLR4, activated pathways involve (1) activation of IKK complex, degradation of IkB, and translocation of NF-kB to the nucleus to produce proinflammatory cytokines; (2) activation of JNK and p38, leading to AP-1 activation and the production of proinflammatory cytokines; (3) activation of IRF5. Additionally, there is the alternative MyD88 pathway that occurs in plasmacytoid dendritic cells with the activation of TLR-7 and TLR-9 leading to the activation of IRF7, which translocates to the nucleus to produce IFN-α and IFN-inducible genes[345]. In the case of TLR3, MyD88-independent/TRIF-dependent pathway involves the either of TBK-1 leading to the activation of IRF3, a transcription factor that translocates to the nucleus to produce IFN-inducible genes or activates of RIP1 feeding into the MyD88 pathway and eventually activating NFκ-B and AP-1 to produce proinflammatory cytokines[345, 347].

3.2. MC Mediators and Inflammation

Depending on the localization and role, MC present great diversity on the size and content of their granules, as well as in the cytokine and receptor expression, since they

can produce and release many different mediators[247, 348-351]. Such mediators include preformed mediators, which are stored in the secretory granules, newly formed lipid mediators (eicosanoids), and a wide variety of cytokines and chemokines[352-358].

3.2.1. Preformed Mediators

MC granules contain proteases, vasoactive amines and proteoglycans[247, 251, 293]. MC release NO, superoxide radicals, and other antimicrobial peptides, that play a bactericidal role[359-362]. MC store serine proteases, mainly of trypsin family [363-365] including tryptase and chymase, and these have been shown to have various roles in tissue remodeling and cellular recruitment[366-370].

It is estimated that lung and skin MC contain 12-35 pg serine proteases per cell[371]. Tryptase contributes to allergic asthma and rhinitis, urticaria and angiogenesis[363]. Some of its action is mediated through activation of protease-activated receptor 2 (PAR-2)[372]. MC chymase offers protection against external venoms [373] and have anti-inflammatory role as they degrade cytokines and chemokines, such as IL-3, IL-5, IL-6, TNF, as well as peptides that can stimulate MC, such as calcitonin gene-related peptide-CGRP), SP and NT (18162, 5120}. Chymase acts mainly in the cardiocascular system via convertion of angiotensin I to II[365], but also contributes to the rupture of atheromatous plaques[374, 375].

MC, along with basophils and enterochromaffin cells, contain large quantities of vasoactive amines, like histamine and serotonin[376]. They have potent effects on vascular permeability and have been implicated in many of the symptoms of acute allergic diseases[377]. Histamine receptor (H) activation, mainly H1, leads to increased vascular permeability, urticaria and smooth muscle cell contraction[378-380]. H1 antagonists are used

for allergic rhinitis, atopic dermatitis, conjunctivitis, PS, and urticaria[381]. MC granules also contain highly sulfated glycosaminoglycan heparin or other highly sulfated structures[382, 383]. MC can increase vascular permeability by heparin-initiated bradykinin formation *in vivo*, as shown in mice and humans[384].

3.2.2. Eicosanoids

Eicosanoids are produced rapidly, *de novo* from arachidonic acid, in response to MC activation [385] through ERK activation [386] and include mainly PGD_2, PGE_2, and leukotrienes B_4 and C_4 (LTB_4, LTC_4). These recruit effector cells, regulate immune responses, and promote angiogenesis, edema and bronchoconstriction contributing to the pathogenesis of many diseases, like asthma[387].

MC are the main source of PGD_2[385, 388-390], which worsens the hypersensitivity of the airways and causes erythematous skin reactions[385]. The PGD_2 receptors, DP1 and DP2, mediate lung allergic inflammation [391] and have chemoattractant action to eosinophils and Th2 cells respectively[392]. LTB release is energy dependent[393]. Activated mast cell induced chemotaxis of effector, but not central memory, CD8+ T cells through production of leukotriene B4 (LTB4)[394]. LTB_4 levels are found to be increased in lungs of patients with asthma or chronic lung diseases[395, 396], while LTB_4 endodermal injection induces erythymatous skin rush, accompanied with neutrophil increase[397]. LTC4 is one of the most bronchoconstrictive substances known and it is involved in the pathogenesis of asthma[398].

3.2.3. Cytokines/Chemokines/Growth Factors

Cytokines and chemokines that are produced by MC include classical pro-inflammatory mediators, such as TNF, IL-1α, IL-1β, IL-6, IL-18, GM-CSF, LIF, IFN-α and IFN-β[399-401], as well as cytokines that are associated with immunomodulatory effects and angiogenesis, such as IL-10, TGF-β and VEGF[402]. Some cytokines, like TNF-α, are found stored in the granules [403] and play an important role in the initiation of an immune response. However, most of the cytokines are formed *de novo*, depending on the MC subtype, location, and trigger.

Although frequently described as a source of T helper 2 (TH2)-type cytokines[251], including IL-3, IL-4, IL-5, IL-9, IL-13, IL-15 and IL-16[404-406], MC can also produce TH1-type cytokines, such as interferon-γ (IFN-γ), IL-12 and IL-18[309, 407], all of which participate in chronic inflammatory conditions. TNF-α can be released along with histamine from rat brain MC,[408] and is involved in both brain inflammation[409, 410] and increased vascular permeability[356].

MC can be an important source of several chemokines, including those associated with TH2-type responses, such as CCL2, CCL3, CCL4, CCL5, CCL11 and CCL20 that are shown to recruit effector cells, including dendritic cells, and regulate immune responses[411-415]. MC can be an important source of several chemokines, including those associated with TH1-type responses, CXCL1, CXCL2, CXCL8, CXCL9, CXCL10 and CXCL11 that are shown to recruit effector cells and regulate immune responses[416, 417]. More specifically, CXCL8 and CXCL10, are important for the recruit of neutrophils, eosinophils, T, B, dendritic cells and mononuclear cells[405, 418, 419].

Lastly, MC can release growth factors[405], like VEGF[291, 420], an isoform of which is particularly vasodilatory[421, 422]. VEGF is involved in wound healing[423], cancer [424] and inflammatory diseases[425].

3.3. MC in Clinical Syndromes

The range of diseases in which MC participate, is wide. First, MC play an important role in allergic diseases, like anaphylaxis[363, 371, 405, 426], allergic rhinitis[427], asthma [428-432] and atopic dermatitis (AD)[433-437], but also in autoimmune diseases, [438], like rheumatoid arthritis[439-442], PS [443-447] and multiple sclerosis (MS)[448-452]. MC and their mediators have been implicated in the comorbid conditions with CFS[234-236]. Previous studies reported abnormal increase and expression of MC[231, 232, 453], as well as increased hypersensitivity skin reaction in patients with CFS[229].

Even though there is skin hypersensitivity in many CFS patients, skin lesions are not apparent in these patients. However, since AD patients also have similar skin hypersensitivity and lesions, I obtained and analyzed biopsies and serum from patients with AD. I focused my studies on NT and CRH serum levels, as well as gene expression in those patients. Another group of patients that has skin hypersensitivity and inflammation that involves MC is psoriasis (PS). Additionally I analyzed biopsies and serum from patients with PS.

AD is a chronic allergic skin disease, characterized by inflammation and severe itching. Serum levels of IgE are increased in 80% of AD patients[405], and moreover MC number is increased in AD lesions[437]. Although MC are located in the papillary dermis, in AD skin are found to translocate to epidermis[433]. Histamine levels are not always

increased in AD patients and antihistamines are not very effective even though they are used extensively[405]. Nevertheless, tryptase contributes to urticaria, via PAR-2 receptors [436] and there is a polymorphism in the promoter of *chymase* that is related to AD[434]. Chymase can weaken skin barrier, allowing allergen permeability[435]. Also, in the AD skin there exists an increased number of contacts between MC and neuronal cells [454] allowing neuropeptides including SP, vasoactive intestinal peptide (VIP) and nerve growth factor (NGF) to activate MC[405].

PS is a chronic inflammatory skin disease characterized by excessive proliferation of keratinocytes and increased epidermal vasculature, associated with high skin VEGF expression[455]. Neuropeptides have been implicated in PS[444]; however, their mechanism of action is not well understood. Studies show increased MC number in lesions from PS patient biopsies in comparison to non-lesion skin from PS patients or biopsies from healthy subjects[443, 446]. MC number is also increased in patients with psoriatic arthritis[456].

Neuropeptides play an important role in PS [444] and stimulation of MC with neuropeptides leads to release or pro-inflammatory mediators, as mentioned above. Moreover, studies show increased number of contacts between SP-positive neuronal cells with MC in PS[447], and there is increased number of neuronal endings that contain SP in close proximity to perivascular areas with increased degranulated MC in PS patients with intense itching-urticaria in comparison with PS patients without itching[445]. NT is a vasoactive peptide[457] which increases histamine release from rodent skin in a MC-dependent manner[458]. NT also augments the effect of CRH, secreted under stress, to increase MC-dependent skin vascular permeability[459].

T cells are important players in autoimmune diseases, but MC interact and regulate T cell responses. Close interaction between the two cell types is noticed in specific tissues[460]. MC can attract T cells and induce their activation as shown with the use of rodent [461-464] and human cells[465]. These interaction can be mediated by TNF-α and costimulatory molecules, like OX-40 ligand (CD134)[438, 464].

MC and their mediators have been implicated in the comorbid conditions with CFS[234-236], including fibromyalgia, painful bladder syndrome/interstitial cystitis, irritable bowel syndrome, migraines, post-traumatic stress disorder, and temporomandibular joint disorder, all of which worsen by stress[13-16]. Some papers suggest there may be altered MC function in bronchial and oral tissues of CFS patients[227, 228]. Additionally, immediate hypersensitivity skin reactions are noted in patients with CFS[229], while there are dermal IgG deposits and increase of MC with abnormal overexpression of MC in skin biopsies [453] of CFS and fibromyalgia patients[231, 232], and such patients also show increased skin hypersensitivity[229]. Moreover, CU that involves MC, is usually associated with fibromyalgia[233, 466]. MC are implicated in CFS, but the mechanism and the triggers remain unknown.

Working Hypothesis

The main focus of this dissertation is to investigate the effects of isoflavones on poly(I:C) and/or swim-treated mouse fatigue-related behavioral parameters and inflammatory mediators in the brain, serum and skin.

Our hypothesis is that using an immunological trigger alone or in combination with a stress trigger would induce “fatigue behavior”, as assessed with the use of specific behavioral parameters, as well as increased inflammation in the brain and the periphery, as measured with inflammatory mediators in the brain, serum and skin. The use of isoflavones with antioxidant and anti-inflammatory properties would ameliorate the “fatigue behavior” and decrease the inflammatory related parameters.

Materials and Methods

Chemicals and Reagents

Polyinosinic-polycytidylic acid-TLR3-based adjuvant (Poly(I:C)), HMW VacciGrade, Cat# vac-pic) was purchased from Invivogen (San Diego, CA, USA) and aliquots were prepared according to the manufacturer's instructions. Lipopolysaccharide from Escherichia coli (LPS, Cat# L4391) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and aliquots were prepared according to the product sheet instructions.

Neurotensin (NT, Cat# N6383), and Corticotropin-Releasing Factor (CRF, Cat# C3042) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and aliquots were prepared according to the manufacturer's instructions. IL-33 (3625-IL) was purchased from R&D Systems, Minneapolis, MN, USA. Teklad lab animal diets (Cat# 2918X, and 2920X) were purchased from Harlan (Indianapolis, IN, USA).

Chapter 1. Mouse Studies - CFS Mouse Model

1.1. Animals

C57BL/6 and BALB/c female mice, 20-25 mg (Jackson Laboratories, Bar Harbor, ME) were kept in virus-free sections of a modern animal facility and were allowed *ad libitum* access to food and water. They were maintained on a 14:10 hr light-dark cycle.

1.2. Treatment Conditions

Mice were provided with chow containing low to non-detectable isoflavone levels (non-detectable-20 mg/kg, Teklad 2920X) or chow high in isoflavones (150-250 mg/kg expected range of daidzein plus genistein, Teklad 2918X) *ad libitum* -standard chow used by DLAM for at least two weeks before experiments. Both diets were purchased by Harlan (South Easton, MA, USA).

Mice were divided into the following groups: control/no swim (saline ip injection), control/swim, LPS/no swim, LPS/swim, poly(I:C)/no swim, and poly(I:C)/swim. Mice were injected, ip, with LPS (1 mg/kg) or poly(I:C) (10 and 20 mg/kg or normal saline on Day 1. Subsequently, they were subject to swim for 15 min, daily, individually in a transparent plastic cylindrical jar (17 cm × 25 cm) containing 15 cm-deep water at room temperature (23 ± 1 °C) for up to 21 days. This approach reflects both exercise and the stress of water immersion. A warm pad underneath the cages was used for mice when taken out of the forced swim test to keep them warm.

Ethovision software from Noldus Inform. Technol. (Leesburg, VA, USA) was used to videotape mice while swimming. Percent weight change for each mouse was calculated on specific days in comparison to the weight of that specific mouse on day 1. I

assessed behavioral parameters, including time to immobility, immobility time, time to start grooming and locomotor activity.

Mice were euthanized at the end of the experiments using isoflurane overdose and thoracotomy and samples were collected. Blood was collected by cardiac puncture. Brain samples were snap-frozen in liquid nitrogen and stored at -80C until analyzed for oxidative stress.

1.3. Behavioral Parameter Assessment

***Immobility time*:** Defined as the total time the mouse does minimal movements to sustain its nose out of the water over the 15 min of swimming.

Time to start grooming: Defined as the time for mice to start grooming their fur for more than 5 seconds, after the forced swim is over. A warm pad underneath the cages was used for mice when taken out of the forced swim test to keep them warm. Cut-off time was 600 seconds.

Locomotor activity: Defined as "Total Distance Travelled" (in cm). After the experiments, animals were placed individually into standard plastic housing cages with food and water available *ad libitum* and overnight locomotor activity (16 hr) was monitored with the Neuroscience Behavior Core's mouse SmartFrame® Cage Rack System (Kinder Scientific, Poway CA, USA). This system consists of 20 PC-interfaced horizontal photobeam frames. The frame (containing 12 photocells; 8Lx4W) surrounds 1 home cage environment and continuously tracks the animal's movement. This fully automated system allows the user to quantify horizontal ambulation by counting breaks in infrared photocell beams arranged on a 4 x 8 (X x Y) grid using MotorMonitor®

software (Hamilton/Kinder). Data was collected and subsequently analyzed in time bins (e.g. every hour) or as a total over the course of collection to the "Total Distance Travelled" (in cm) parameter for each zone.

1.4. Biochemical Parameter Assessment

1.4.1. Assessment of oxidative stress

Estimation of lipid peroxidation. Determination of MDA content, a measure of lipid peroxidation, was performed in Dr. Blumberg's lab (Director of Human Nutrition Research Center, Tufts University), according to a previously published method [467]. The method uses reverse phase HPLC, in which a thiobarbituric acid-MDA conjugate derivative was injected onto a C18 column and fluorometrically quantified at an excitation wavelength of 515 nm and emission wavelength of 553 nm. MDA concentration was calculated using calibration curves of an authentic standard. Intra- and inter-assay CV in brains was 3.8 and 4.7%, respectively.

1.5. Statistics

I present behavioral parameters data in the figures as mean ± SEM. Data from immobility time and time to groom assessments was analyzed by one-way ANOVA, followed by Dunnett's test. Data from percent weight change assessments were analyzed using two-way ANOVA, followed by Bonferroni post-test for multiple comparisons. Data from locomotor activity was analyzed using one-way ANOVA, followed by Dunnett's test and non-parametric Mann-Whitney U test.

In the oxidative stress assessment, each value represents the MDA content (nmol/g protein) in the whole brain of an individual mouse. Data was analyzed using two-way ANOVA, followed by Bonferroni post-test for multiple comparisons. Significance of comparisons is denoted by $p<0.05$. I used GraphPad Prism 5 for the graphs and statistical analysis.

Chapter 2. Mouse Studies - Poly(I:C) and Isoflavones Effect

2.1. Animals

C57BL/6, CRHR-1 KO and their wild type (WT) littermates female mice, 20-25mg (Jackson Laboratories, Bar Harbor, ME) were kept in virus-free sections of a modern animal facility and were allowed *ad libitum* access to food and water. They were maintained on a 14:10 hr light-dark cycle. Female mice were chosen as CFS occurs more often in females than males.

2.1.1. Knockout (KO) Animals

CRHR-1 breeding pairs were generously given to us by Dr. Douglas Vetter (Tufts University, Boston, MA). The mice were bred in-house and genotyped by PCR analysis of tail DNA. The KO mice and their wild-type littermates were used to explore the role of CRH in the swim and poly(I:C)-induced fatigue parameters.

2.1.1.1. Mouse Genotyping

2.1.1.1.1. Isolation of Ear DNA for Genotyping

At the time of weaning (i.e., 21 days), ear tissue was harvested by ear punching. The tissue sample was stored in an Eppendorf tube at -80°C. Mice had their tail tattooed to identify individual mice. DNA was isolated by incubating the ear tissue sample in 150 µl DirectPCR Lysis Reagent (Cat# 401-E , Viagen Viotech, Los Angeles, CA, USA) with 0.4 mg/ml Proteinase K (Cat# P6556, Sigma, St Louis, MI, USA) at 55°C for 5 h, and 85°C for 45min. For the PCR reaction, 1 µl from the supernatant was used.

2.1.1.1.2. PCR Conditions for CRHR-1 Genotyping

Two PCR reactions were performed each in a total volume of 20 µl. The first reaction used primers CR1-1 and CR1-2, whereas the second reaction used primers CR1-1 and PGK (Table 5). Both reactions contained 0.8µM of the appropriate primers (10µM), 2X GoTaq Green Master Mix (Cat# 9PIM712, Promega, Madison, WI, USA) and H_2O up to 20 µl. PCR conditions: 95 °C for 2 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30s, 72°C for 30s with a final extension at 72°C for 10 minutes. The PCR products were run on an agarose gel and visualized under UV light. The wild type band is 200bp and the mutant band is 400bp.

Table 5. Oligonucleotide primers used for *CRHR-1* genotyping	
CR1-1	5'-ACTGCTAGTGTGATGTCCTGC-3'
CR1-2	5'-TCTCAGGATTGCTAAGTTCAG-3'
PGK	5'-GGGAACTTCCTGACTAGGGG-3'

2.2. Treatment Conditions

Mice were provided with chow containing low to non-detectable isoflavone levels (non-detectable-20 mg/kg) or chow high in isoflavones (150-250 mg/kg expected range of daidzein plus genistein) *ad libitum* -standard chow used by DLAM for at least 2 weeks before experiments.

Mice were injected ip with poly(I:C), 20 mg/kg for the C57BL/6 mice and 15 mg/kg for the CRHR-1 KO mice, or normal saline on Day 1. Subsequently, they were subject to swim for 15 min, individually in a transparent plastic cylindrical jar (17 cm × 25 cm) containing 15 cm-deep water at room temperature (23 ± 1 °C). This approach reflects

both exercise and the stress of water immersion. A warm pad underneath the cages was used for mice when taken out of the forced swim test to keep them warm. Following the forced swim test, mice were individually placed into specific cages and locomotor activity overnight was monitored.

Mice were euthanized the following day, using isoflurane overdose and thoracotomy and samples were collected. Blood was collected by cardiac puncture. Brain (diencephalon), skin (back) and serum samples were collected and stored at -80C until analyzed for gene expression in the brain, gene expression in the skin, as well as inflammatory mediator levels in the serum.

2.3. Behavioral Parameter Assessment

Locomotor activity was measured as "Total Distance Travelled" (in cm) parameter for each zone, as described above, in Materials and Methods, Chapter 1.

2.4. Inflammatory Mediator Measurements

2.4.1. Estimation of Inflammatory Mediator Serum Levels

TNFα, VEGFα, IL-1α, IL-1β, IL-4, IL-6, IL-9, IL-10, IL-12p70, IL-17, KC (human IL-8/CXCL8 murine homologue), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), IP-10 (CXCL10), IFNγ mouse serum levels were determined using the Milliplex microbead assay and the measurements were performed blindly by Millipore, St. Charles, MI, USA.

2.4.2. Estimation of Inflammatory Mediator Gene Expression in Brain and Skin

Brain diencephalon and skin (back) from mice were isolated and immersed into RNAlater (Cat# AM7021, Invitrogen, Grand Island, NY, USA). Total RNA from mouse tissues was extracted using RNeasy Plus Mini kit (Cat # 74134) and RNeasy Fibrous Tissue Mini Kit (Cat # 74704), QIAGEN, Valencia, CA, USA. Reverse transcription was performed with 300 ng of total RNA using the iScript cDNA synthesis kit (Cat # 170-8891, BIO-RAD, Hercules, CA).

Real-time quantitative polymerase chain reaction (qPCR) was carried out in a 7300 Sequence Detector, according to TaqMan Gene Expression Assay instructions (Applied Biosystems, Grand Island, NY, U.S.A.) using Taqman primer/probe sets (Table 6). Brain and skin mouse gene expression of TNFα, IL-6, KC, MCP-1, MIP-1β, RANTES, IP-10, HDC, NT, NTR-1, NTR-2, IFNγ, IP-9 (CXCL11), IL-4, and CRHR-1 was tested.

Thermal cycling proceeded at 50 ^{o}C for 2 min, 95 ^{o}C for 10 min, 95 ^{o}C for 15 s, for 40 cycles, and 60 ^{o}C for 1 min. Negative controls included samples with water instead of template. Assays were performed in triplicate for each data point. Results were normalized against the endogenous gene, GAPDH, and were expressed relative to the mean of the control for each gene.

2.5. Statistics

I present locomotor activity data in the figures as mean ± SEM. Inflammatory mediator serum levels and gene expression in brain and skin data are presented as individual values from each mouse. Data was analyzed using the non-parametric Mann-Whitney U test. Significance of comparisons is denoted by $p<0.05$. GraphPad Prism 5 was used for the graphs and statistical analysis.

Table 6. Taqman primer/probes for mouse studies	
Gene of interest	**Taqman primer/probe set**
TNF	Mm00443258_m1
VEGFα	Mm01281449_m1
IL-1β	Mm00434228_m1
IL-4	Mm00445259_m1
IL-6	Mm00446190_m1
KC	Mm04207460_m1
MCP-1 (CCL2)	Mm00441242_m1
MIP-1β (CCL4)	Mm01443111_m1
RANTES (CCL5)	Mm01302427_m1
IP-10 (CXCL10)	Mm00445235_m1
CXCL11	Mm04207460_m1
NT	Mm00481140_ml
NTR-1	Mm00444459_m1
NTR-2	Mm00435426_m1
HDC	Mm00456104_m1
CRHR-1	Mm00456104_m1
IFNγ	Mm01168134_m1
GAPDH	4352339E-1207040

Chapter 3. Human Studies - *In vitro*

3.1. Human MC Culture

LAD2 cells (kindly provided by Dr. A.S. Kirshenbaum, National Institutes of Health, NIH) were derived from a single patient with human MC leukemia. LAD2 cells were cultured in StemPro®-34 SFM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin/streptomycin and 100 ng/mL recombinant human stem cell factor (rhSCF) (Kindly supplied by Sweden Orphan Biovitrum AB, Stockholm, Sweden).

3.2. Mediator Assays

3.2.1. Beta-Hexosaminidase Assay

Beta-hexosaminidase (β-hex), as an index of MC degranulation, was assayed using a fluorometric assay. Briefly, LAD2 cells (0.5×10^5/tube) were stimulated for 30min, supernatant fluids were collected and cell pellets were lysed with 1% Triton X-100. Supernatant fluids and cell lysates were incubated in reaction buffer (p-nitrophenyl-N-acetyl-β-D-glucosaminide from Sigma-Aldrich) for 90 min and then 0.2 M glycine was added to stop the reaction. Absorbance was measured at 405 nm in an enzyme-linked immunosorbent assay reader, and the results are expressed as the percentage of β-hex released over the total.

3.2.2. ELISA

LAD2 cells were treated with poly(I:C) (10 μg/ml), with or without SP (1μM), (NT (1, and 10 μM) and CRH (1 μM) for 24 h. Alternatively, LAD2 cells were treated for 24 h either with NT alone (1 μM) or together with interleukin IL-33 (10 ng/mL). TNF,

IL-8, VEGFα, IL-6, and MCP-1 release was measured by ELISA (R&D Systems, Minneapolis, MN, USA) in the supernatant fluid of control and stimulated LAD2 cells.

3.2.3. Gene Expression

Total RNA from human MC was extracted using RNeasy Plus Mini kit (Cat # 74134, QIAGEN, Valencia, CA, USA). Reverse transcription was performed with 300 ng of total RNA using the iScript cDNA synthesis kit (Cat # 170-8891, BIO-RAD, Hercules, CA). Real-time quantitative polymerase chain reaction (qPCR) was carried out in a 7300 Sequence Detector, according to TaqMan Gene Expression Assay instructions (Applied Biosystems, Grand Island, NY, USA) using Taqman primer/probe sets (Table 7). TNFα, VEGFα and IL-8 gene expression was tested.

Thermal cycling proceeded at 50 ^{o}C for 2 min, 95 ^{o}C for 10 min, 95 ^{o}C for 15 s, for 40 cycles, and 60 ^{o}C for 1 min. Negative controls included samples with water instead of template. Assays were performed in triplicate for each data point. Results were normalized against the endogenous gene, GAPDH, and were expressed relative to the mean of the control for each gene.

Table 7. Taqman primer/probes for *in vitro* human studies

Gene of interest	**Taqman primer/probe set**
TNF	Hs99999043_m1
VEGFα	Hs00173626_m1
IL8 (CXCL8)	Hs00174103_m1
GAPDH	4352339E-1009033

3.3. Statistics

All *in vitro* conditions were performed in triplicate, and all experiments were repeated at least three times (n = 3). I present the results as mean ± SD. *In vitro* data from stimulated and control samples were compared using the non-parametric Mann-Whitney U test. Significance of comparisons is denoted by $p<0.05$. I used GraphPad Prism 5 for the graphs and statistical analysis.

Chapter 4. Human Studies - Human Samples

4.1. Sample Collection

4.1.1. Biopsies and Serum

Serum and full-length 3-mm^3 punch skin biopsies were collected from non-exposed skin (back and gluteal) for diagnostic purposes, out of allergy season, from subjects with PS and AD patients. Samples of unaffected skin were obtained from sites at least 15 cm away from the lesional skin. Skin biopsies were immediately placed in RNAlater® solution (Ambion Inc., Austin, TX, USA) for quantitative polymerase chain reaction (qPCR), or in freezing medium (Triangle Biomedical Sciences, Durham, NC, USA) for immunohistochemistry, and were stored along with the serum at −80 °C.

Patients had not received any medication for 15 days prior to biopsy and were free from any systemic allergic or inflammatory disease and from controls, at least 2 h after food. Patients with contact dermatitis were excluded by history. Two patients with AD had asthma and one had allergic rhinitis. Total serum IgE was within the normal range in all but two patients with AD. Patients were selected from the Attikon and A. Sygros Hospitals of the Athens University Medical School (Athens, Greece). The institutional review boards of both hospitals approved this protocol. All participants gave their written informed consent according to the Declaration of Helsinki principles. The Psoriasis Area and Severity Index (PASI) score was recorded for some of the patients with psoriasis; no severity index score was obtained from the patients with AD.

There were fewer skin biopsy samples than serum samples because it was more difficult to obtain consent. Control (healthy individuals) skin samples from abdominal reductions were obtained from the Agios Savas Oncology Clinic (Athens, Greece), while

control serum samples were obtained from Agios Savas and the Ano Toumba IKA Polyclinic (Thessaloniki, Greece).

All human samples had no identifiers except for age and sex. There was no statistical difference in the mean age between patients with psoriasis and controls.

4.1.2. Certified Stress Questionnaires

Some patients with PS and AD filled the State-Trait Anxiety Inventory (STAI), which has been validated for the Greek population[468], to investigate the extent of stress. The STAI measures separate constructs of psychosocial stress and clearly differentiates the temporary condition of "state anxiety" (STATE now) from the more general and long-standing quality of "trait anxiety" (STATE trait).

The SCORing Atopic Dermatitis (SCORAD) index was calculated using the SCORAD Index application by LinkWave Inc. (http://linkwave.com/). There was no statistically significant difference among the SCORAD scores of any subgroups.

4.2. Inflammatory Mediator Measurements

4.2.1. Estimation of Inflammatory Mediator Serum Levels

CRH serum levels were measured using ELISA (Phoenix Pharmaceutical, Belmont, CA, USA). NT serum levels were measured using a Milliplex array, which was performed blind by Millipore (Millipore, St. Charles, MI, USA).

4.2.2. Estimation of Inflammatory Mediator Skin Gene Expression

Total skin RNA was extracted using the Qiagen Fibrous Tissue mini kit (Qiagen, Valencia, CA, USA) and cDNA synthesis was performed using the iScript cDNA

synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time quantitative polymerase chain reaction (PCR) was carried out in a 7300 Sequence Detector, according to TaqMan Gene Expression Assay instructions (Applied Biosystems, Foster City, CA, USA) using Taqman primer/probe sets (Table 8).

Thermal cycling proceeded at 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, for 40 cycles, and 60 °C for 1 min. Negative controls included samples with water instead of template. Human pituitary cDNA was used as a positive control for NT gene expression (NTS), while cDNA from LAD2 human MC was used as a positive control for NTR-1 gene expression (NTSR1). Experiments were performed in triplicate for each data point. Results were normalized against the endogenous gene, GAPDH, and were expressed relative to the mean of the control for each gene.

Table 8. Taqman primer/probes for human samples studies

Gene of interest	**Taqman primer/probe set**
CRHR-1	Hs00366363_m1
NT	Hs00175048_m1
NTR-1	Hs00901551_m1
VEGFα	Hs00900055_m1
GAPDH	4352339E-1009033

4.3. Immunohistochemistry-Biopsies

4.3.1. NT Immunohistochemistry

Paraffin sections (4 μm) were cut, and immunohistochemistry was performed using an indirect biotin streptavidin detection system (Ventana Medical Systems, Roche, Basel, Switzerland) with the following primary antibodies: rabbit polyclonal for NTR-1 (Cat # ab117592, 1 : 500 dilution; Abcam Inc., Cambridge, MA, USA); mouse monoclonal for NT (E5) (Cat # sc-377503, 1 : 50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal for c-kit (CD117, 1 : 200 dilution; Dako Cytomation, Carpinteria, CA, U.S.A.).

4.3.2. CRH Immunohistochemistry

Cryostat sections were prepared and fixed with acetone for three minutes and incubated with normal blocking serum for twenty minutes. Sections were then incubated with primary antibodies (goat polyclonal antibody to CRHR-1; Cat # ab59023, Abcam Inc, Cambridge, MA, USA) diluted to 1:100 for thirty minutes and then immunostained with Vectastain ABC AP kit (Vectastain Lab, Burlingame, CA, USA) and Vector Red Alkaline Phosphatase Substrate kit (Vector Lab) as per the kit's directions. Presence of red color indicates a positive reaction for CRHR-1.

4.4. Statistics

Results of NT, CRH serum levels and NT, NTR-1, CRHR-1 gene expression in the skin of patients with PS and controls were compared using the Mann–Whitney nonparametric U-test. Lesional and nonlesional skin gene expression results from patients with psoriasis were compared with those of controls using the Kruskal–Wallis test,

followed by Dunnet's test for multiple comparisons, while the Wilcoxon signed rank test was used to compare lesional and nonlesional psoriasis skin gene expression from the same patients. Significance is denoted by $p<0.05$.

Results

Chapter 1. Mouse Studies / CFS Mouse Model

1.1. Experiments using C57BL/6 Female Mice

C57BL/6 female mice were provided with chow containing low to non-detectable isoflavone levels (non-detectable-20 mg/kg) or chow high in isoflavones (150-250 mg/kg expected range of daidzein plus genistein) *ad libitum* -standard chow used by DLAM- for at least 2 weeks before experiments. Mice were randomly divided into 6 groups; control/no-swim, control/swim, LPS/no-swim, LPS/swim, poly(I:C)/no swim, poly(I:C)/swim (n=5-7/group). Mice were injected with LPS, 1 mg/kg or poly(I:C), 10 mg/kg on Day 1 and they were subjected to forced swim test for 15 min, daily for 21 days. Ethovision software was used to videotape mice while swimming.

Behavioral assessments included immobility time, time to groom and percent change in weight. Brain samples were collected and analyzed for malondialdehyde content.

This protocol was repeated four times and results from a representative experiment with chow low isoflavone diet and high isoflavone diet are presented.

1.1.1. Behavioral Assessments (21-Days experiments)

Figure 2. Immobility time (21-Days).

Total time of immobility-over the 15 min of forced swim was noted daily, for 21 days.

Figure 2a. Mice were provided with low in isoflavone diet.

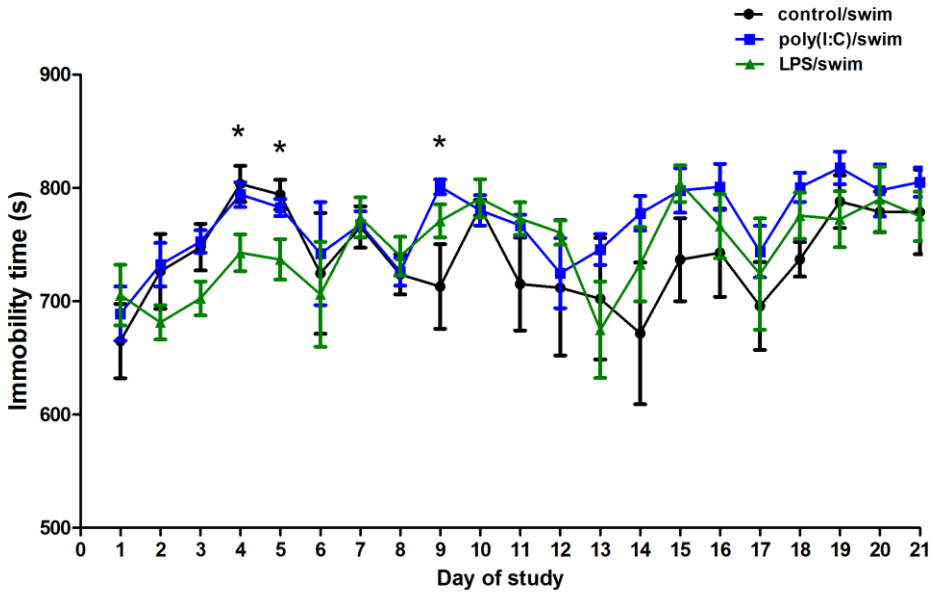
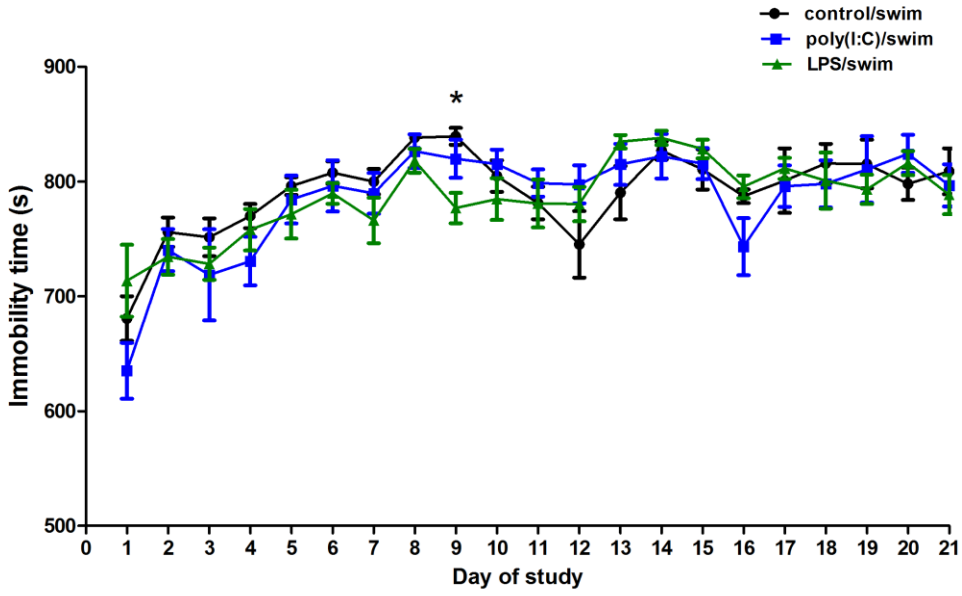


Figure 2b. Mice were provided with high in isoflavone diet.



Total time of immobility, over the 15 min of forced swim test, was noted daily over the course of 21-days experiment with low isoflavone diet (Figure 2a). Statistical analysis of the data (1-way ANOVA followed by Dunnett's multiple comparison test) showed significant differences between the LPS group and the control group at days 4 and 50 ($p=0.0177$ and 0.0193, respectively) and poly(I:C) group and control group at day 9 ($p=0.0342$).

Total time of immobility -over the 15 min of forced swim test- was noted daily over the course of 21-days experiment with high isoflavone diet (Figure 2b). Statistical analysis of the data showed a significant difference between the LPS group and the control group at day 9 ($p=0.0199$).

Figure 3. Time to groom (21-Days).

Time to start grooming once forced swim test was over was noted daily, for 21 days.

Figure 3a. Mice were provided with low in isoflavone diet.

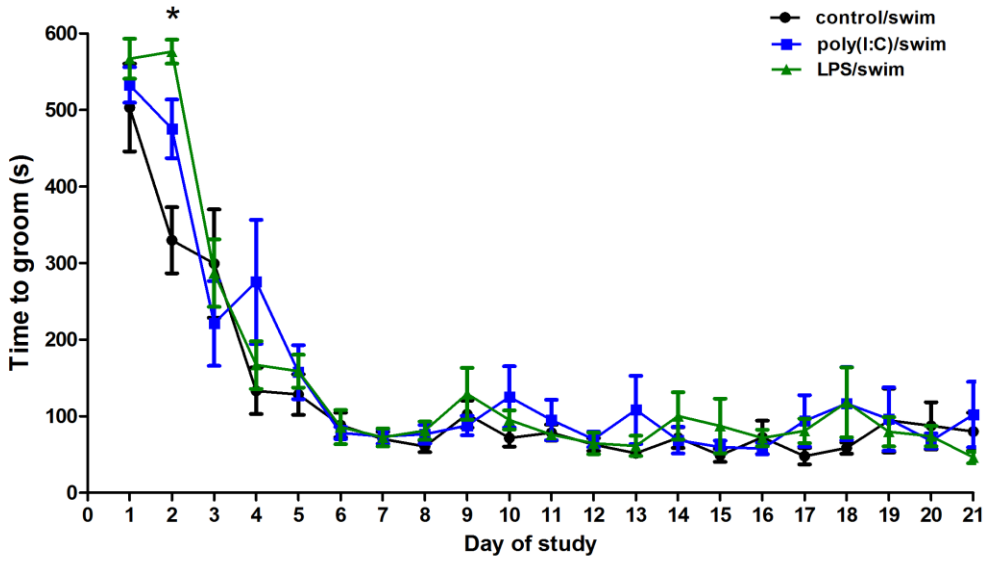
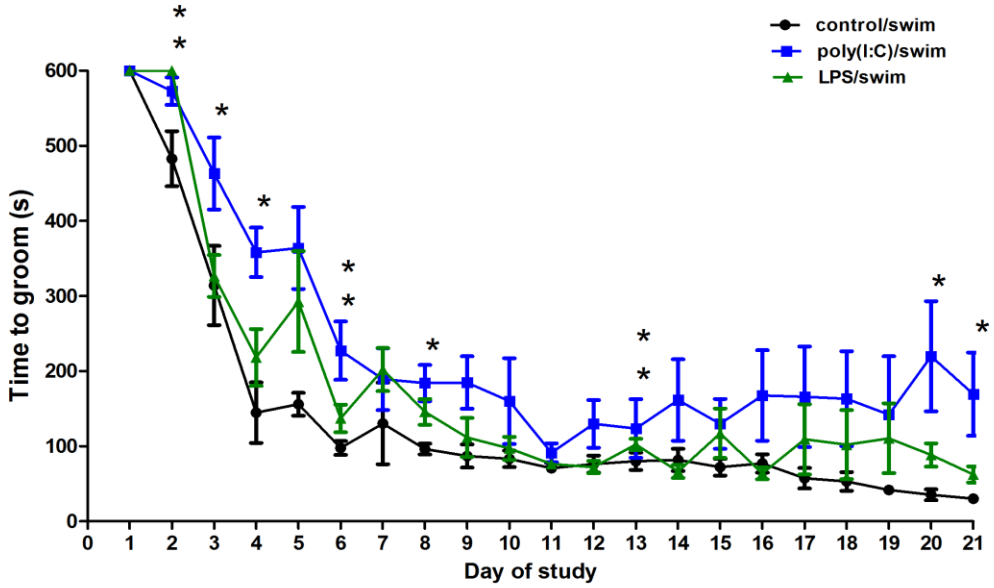


Figure 3b. Mice were provided with high in isoflavone diet.



I also noted the time to start grooming once forced swim test was over, daily over the course of 21-days experiment with low isoflavone diet (Figure 3a). On Day 2 there was statistically significant difference between the LPS group and the control group ($p<0.0001$).

I also noted the time to start grooming once the forced swim test was over, daily over the course of 21-days experiment with high isoflavone diet (Figure 3b). The total area under the curve (AUC) for the poly(I:C) group, but not for the LPS group was statistically significant different from the control group over the course of 21 days ($p=0.0145$). Further analysis showed that there was statistically significant difference for the days noted by the asterisk: On Day 2 there was a statistically significant difference between both poly(I:C), and LPS groups and control groups (two asterisks, $p=0.035$), while on Day 3, 4, 6, 8, 13, 20, and 21 there was a statistically significant difference between the poly(I:C) group and the control group ($p=0.0426$, $p=0.0032$, $p<0.0001$, $p=0.0229$, $p<0.0001$, $p=0.0446$, and $p=0.0388$, respectively).

Figure 4. Percentage weight change (21-Days).

Percentage change for each mouse was calculated in comparison to the weight of that specific mouse on day 1.

Figure 4a. Mice were provided with low isoflavone diet.

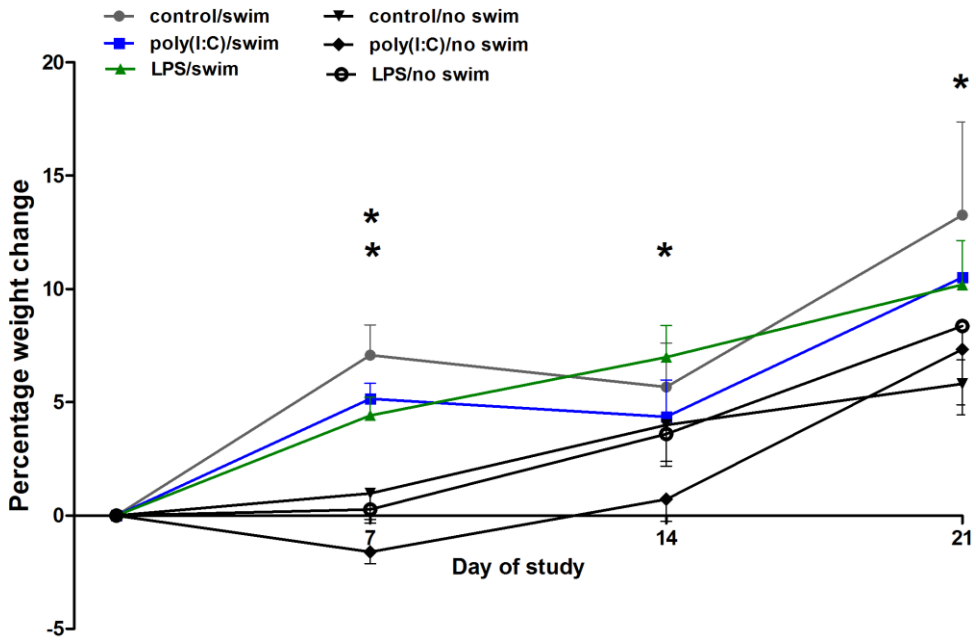
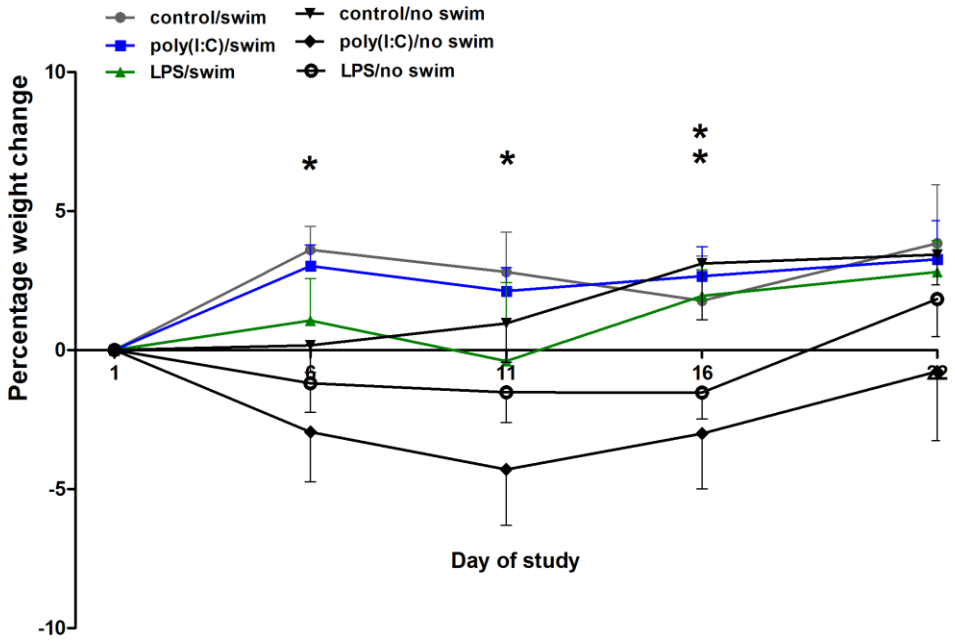


Figure 4b. Mice were provided with high isoflavone diet.



During the low isoflavone diet experiment, weight of the mice was noted on days 1, 7, 14, and 21 and percentage weight change for each mouse was calculated in comparison to the weight of that specific mouse on day 1 (Figure 4a). Overall, percentage weight change over the 21 days increased for all groups. There was a statistical significant increase on the percentage weight change in all the experimental treatment groups that were forced to swim in comparison with the groups that were not forced to swim for Day 7, 14, and 21 (2 way ANOVA followed by Bonferroni, multiple comparisons, $p<0.0001$, $p=0.0232$, $p=0.0263$, respectively). Also, on Day 7 there is a significant smaller percentage weight change in the group that were immunologically treated and underwent swim stress, compared to control/swim mice ($p=0.0414$).

During the high isoflavone diet experiment, weight of the mice was noted on days 1, 6, 11, 16 and 22, and the percentage weight change for each mouse was calculated in comparison to the weight of that specific mouse on day 1 (Figure 4b). There was statistically significant increase in the percentage weight change in all the experimental treatment groups that were forced to swim in comparison with the groups that were not forced to swim for Day 6, 11, and 16 ($p=0.0007$, $p=0.0402$, $p=0.0387$, respectively). On Day 16, poly(I:C)/no swim group had significant decrease in weight compared to control/no swim group ($p=0.05$).

1.1.2. Biochemical Assessment (21-Days experiments)

Table 9. Brain MDA content

Each value represents the MDA content in the whole brain of an individual mouse (nmol/g protein)

	Mice stayed in cages			Forced swim		
	Poly(I:C)	**Poly(I:C)**	**Poly(I:C)**	**Poly(I:C)**	**LPS**	**Control**
Low isoflavone diet	1223	179	1311	218	246	240
	187	610	273	389	916	2063
	182	221	262	196	220	259
	132	166	133	223	151	251
	796	203	199	2486	278	184
	206	211	162	262	1062	185
				201	236	
High isoflavone diet	257	324	220	160	216	387
	221	203	223	266	309	187
	271	357	209	322	279	200
	230	227	173	424	236	391
	178	259	276	253	222	269
	251	176	131	266	376	
	245	237				

Figure 5. Brain MDA content (21-Days).

Figure 5a. Mice were provided with low isoflavone diet.

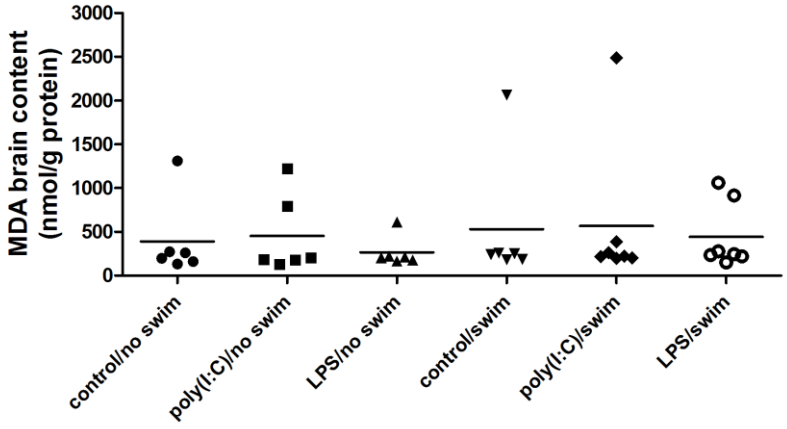
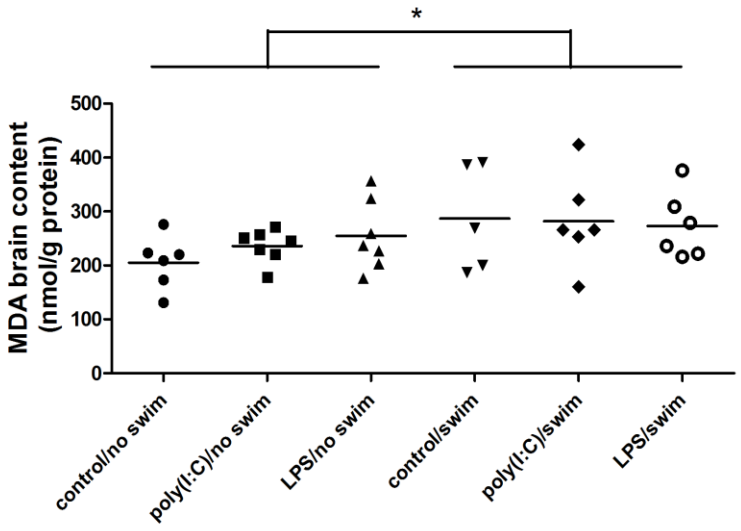


Figure 5b. Mice were provided with high isoflavone diet.



Statistical analysis of the MDA content data from the brain of mice that were provided with high isoflavone diet (2 way ANOVA, followed by Bonferroni for multiple comparisons) showed that there was a significant increase in brain oxidative stress in mice the underwent swim stress compared to mice that did not. Nevertheless, there were no further differences between any of the groups (2 way ANOVA followed by

Bonferroni post-test for multiple comparisons, p=0362) (Figure 5b). There were no differences noted in low isoflavone diet (Figure 5a).

1.1.2.1. Conclusion

There were statistical significant differences in immobility time and time to start grooming on specific days of experiments with low and high isoflavone diet, between the different treatment groups. More specifically, poly(I:C) treatment increased immobility time at day 9 in experiment with low isoflavone diet, while it did not have such an effect in high isoflavone diet experiment. Moreover, differences in time to groom during experiment with high isoflavone diet were noted, even though ameliorated fatigue related behavioral parameters were expected because of the use of a high isoflavone diet. Instead, experiment with low isoflavone diet showed increased time to groom on days 1 and 3, but the average time frames were similar with experiment with high isoflavone diet. Unexpectedly, forced swim test resulted in positive percentage weight change in almost all groups during experiments with high and low isoflavone diet.

Given the fact that there was no significant difference of any kind post day 10 and that other studies have reported induction of CFS in mice within shorter period of time (Tables 2, 3, and 4) I decided to focus on the assessment of the parameters on the first week of experiments. Moreover, given the variability in the outcome of the behavioral parameters, I decided to incorporate daily locomotor activity, an alternative, more objective mean to quantify the potential fatigue the mice experience under my treatment. Lastly, I decided to focus on the poly(I:C) effect and test higher poly(I:C) concentrations in a number of experiments.

1.1.3. Behavioral Assessments (7-Days experiments)

C57BL/6 female mice were provided with chow containing low to non-detectable isoflavone levels (non-detectable-20 mg/kg, Teklad 2920X) *ad libitum* -standard chow used by DLAM- for at least 2 weeks before experiments. Mice were randomly divided into the following groups (n=5/group); control/no-swim, control/swim, poly(I:C)/no swim, poly(I:C)/swim. Mice were injected with poly(I:C), 20 and 40 mg/kg on Day 1 and they were subjected to forced swim test for 15 min, daily for 7 days. Ethovision software was used to videotape mice while swimming. Behavioral assessments included immobility time, time to groom and overnight locomotor activity. Mice were maintained on a 14:10 hr light-dark cycle. The black bar in locomotor activity indicates the 10 hr dark cycle.

Figure 6. Immobility time (7-Days).

Total time of immobility-over the 15 min of forced swim test was noted daily, for 7 days.

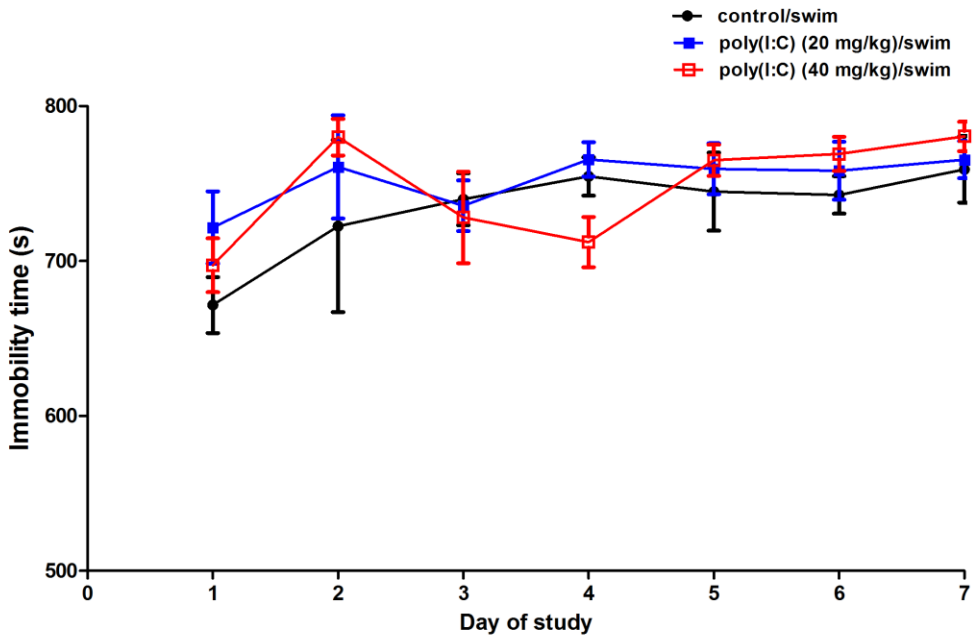
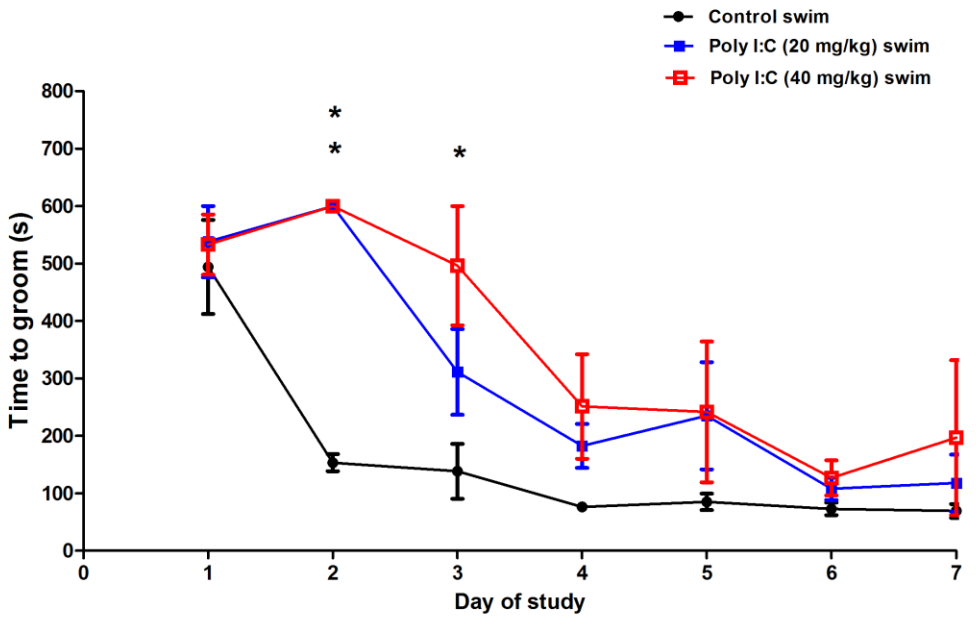


Figure 7. Time to groom (7-Days).

Time to start grooming once forced swim test was over was noted daily, for 7 days.



Total time of immobility, over the 15 min of forced swim test, was noted daily over the course of 7-days experiment with low isoflavone diet (Figure 6). Statistical analysis of the data (one-way ANOVA followed by Dunnett's multiple comparison test) showed no significant differences between the groups.

Time to start grooming once forced swim test was over was noted daily over the course of 7-days experiment with low isoflavone diet (Figure 7). On Day 3 there was statistically significant difference between poly(I:C) group (40mg/kg) as compared to control group (one asterisk, $p=0.0223$) and on Day 2 between both poly(I:C) (20 mg/kg) and poly(I:C) (40 mg/kg) groups as compared to control group (two asterisks, both $p<0.0001$).

Figure 8. Locomotor activity (7-Days).

Figure 8a. Locomotor activity - Day 1

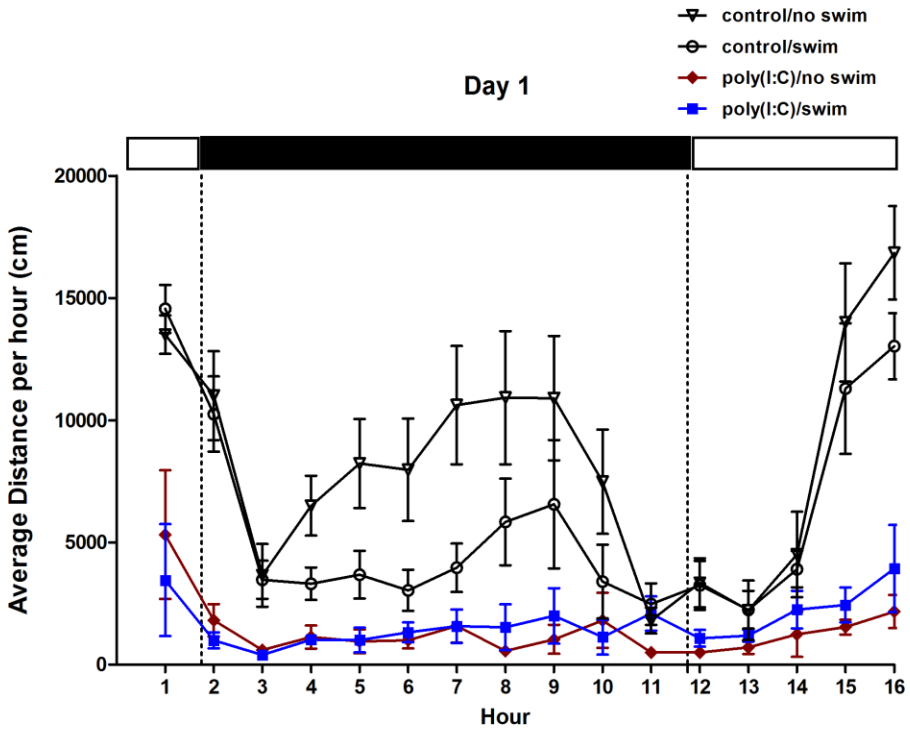


Figure 8b. Locomotor activity - Day 2

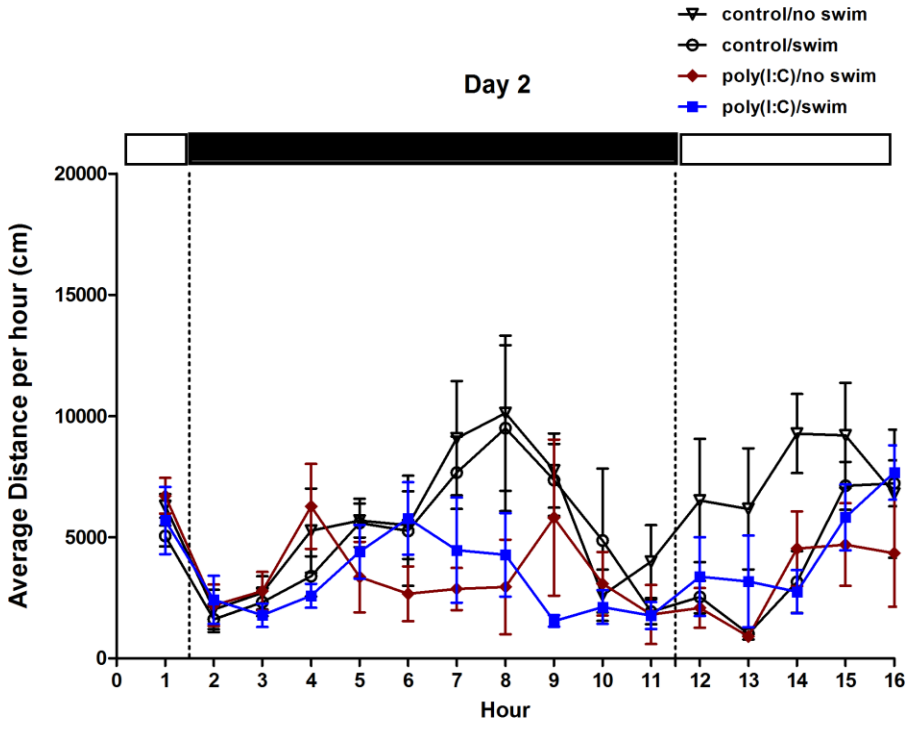


Figure 8c. Locomotor activity - Day 3

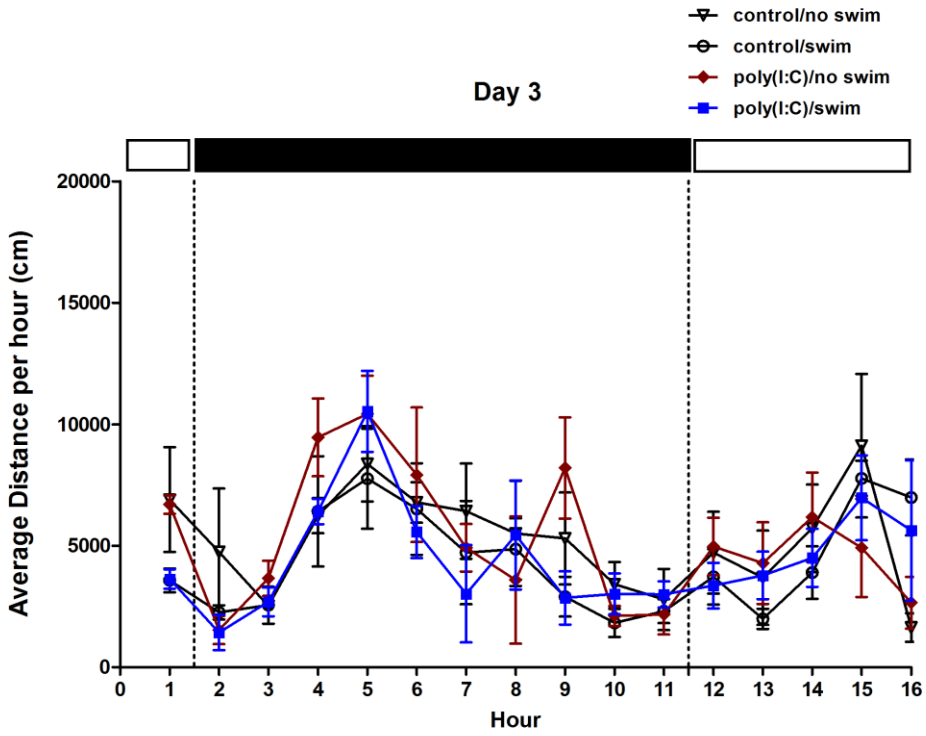


Figure 8d. Locomotor activity - Day 4

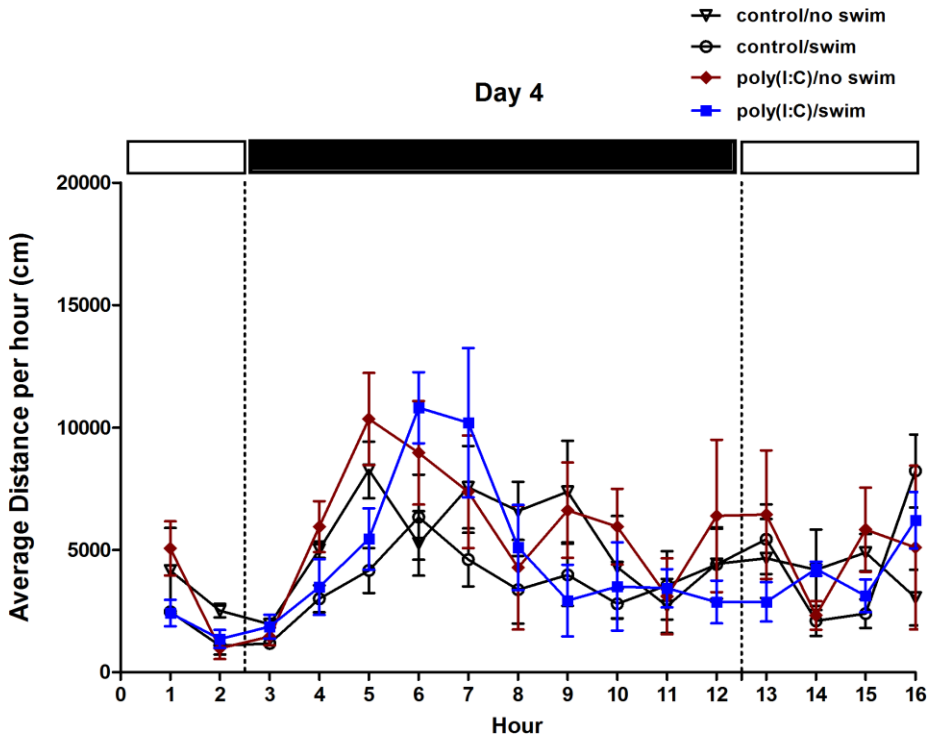


Figure 8e. Locomotor activity - Day 5

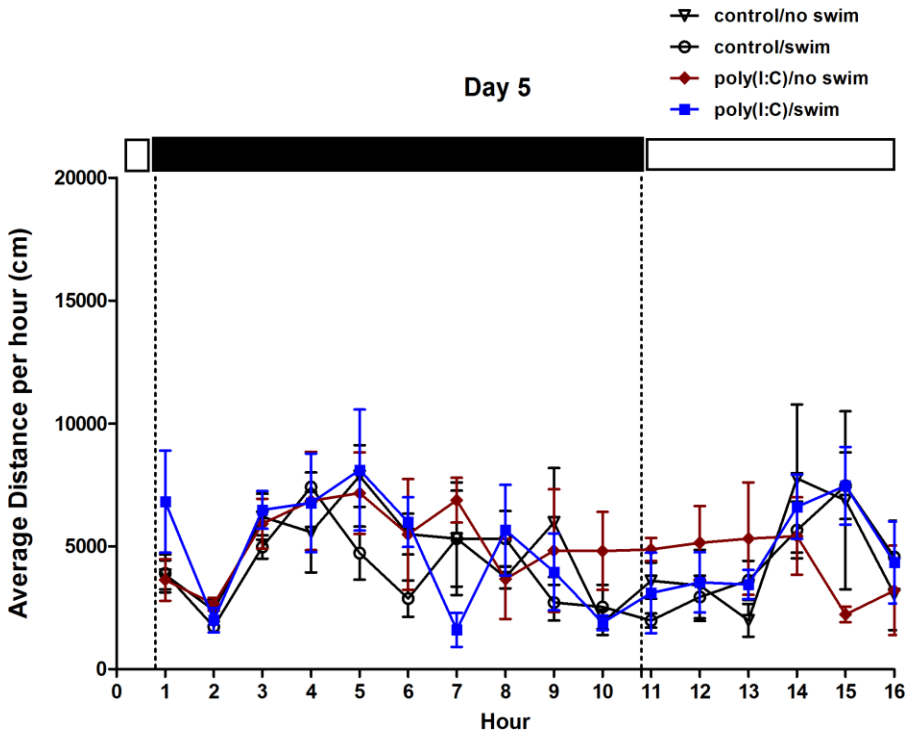
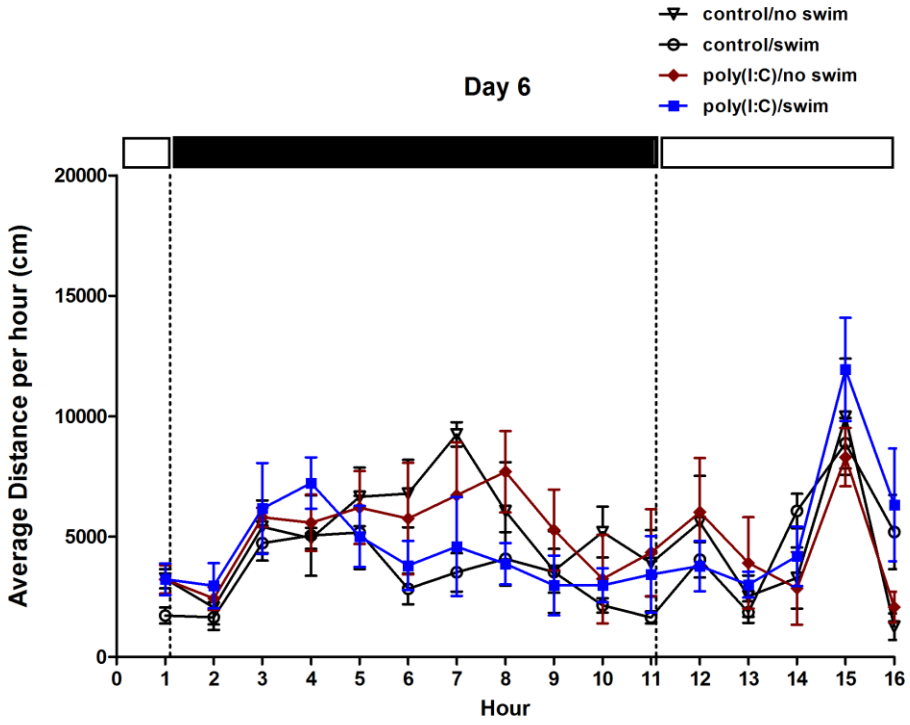


Figure 8f. Locomotor activity - Day 6



Overnight (16 hr) locomotor activity was monitored daily over the course of 7-days experiment with low isoflavone diet (Figure 8). AUC and maximum locomotor activity over the course of the 16 hr monitoring (AUC-16 hr, max loco-16 hr) and only during the 10 hr dark cycle (AUC-10 hr, max loco-10 hr) were calculated.

Statistical analysis of the AUC-16 hr and AUC-10 hr on Day 1, using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and the control/swim, poly(I:C)/no swim and poly(I:C)/swim groups ($p<0.0001$). Poly(I:C) treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the AUC-16 hr on Day 1 (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0159$) and the control/swim ($p=0.0079$) respectively.

Comparison of the max loco-16 hr and max loco-10 hr on Day 1, using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and the control/swim, poly(I:C)/no swim and poly(I:C)/swim groups ($p<0.0001$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the max loco-16 hr on Day 1 (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/swim groups had reduced locomotor activity compared to the control/swim ($p=0.0159$), but there was not difference between the poly(I:C)/no swim and the control/no swim group ($p=0.0635$) respectively.

Moreover, further analysis of the max loco-10 hr on Day 1 (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0159$), but there was not difference between the poly(I:C)/swim and the control/swim group ($p=0.0556$) respectively.

On Day 2, most of the statistical differences subsided and only comparison of the AUC-16 hr, using one-way ANOVA followed by Dunnett's multiple comparison test showed statistical significant differences between the means of the control/no swim and the poly(I:C)/no swim and poly(I:C)/swim groups ($p=0246$). By Day 3, there was no difference noted between any of the groups.

1.1.3.1. Conclusion

Previous experiments (21 days each) were inconclusive as the protocol used did not seem to significantly affect mouse activity level. As a result, I decided to focus on poly(I:C) and increase the amount administered from 10 mg/kg to 40 mg/kg. Unlike before, where mice injected with 10 mg/kg were not affected, mice injected with 20 and 40 mg/kg had reduced mobility lasting for several days, as it was assessed by the time to start grooming, although immobility time was not different. Administration of 80 mg/kg was lethal, while administration of 40 mg/kg was lethal for two out of ten mice (n=5/group, 6 groups). Therefore, I proposed to use this paradigm in the subsequent experiments.

Unfortunately, when I tried to repeat the experiment with the highest dose of poly(I:C) (40 mg/kg), eight out of ten mice died n=5/group, 6 groups). I then tried to repeat with the lowest dose of poly(I:C) (20 mg/kg) used, followed by forced swim test,

for 15 min, for 6 consecutive days and I monitored the locomotor activity of the mice overnight. As shown in Figure 8, differences were only obvious the first two days, while forced swim test seems to have an effect on Day 1.

These results do not agree with some previous publications (Table 3) that reported significant changes in the behavioral parameters that I studied. Our results indicate no dramatic alterations in behavioral parameters attributable to forced swim either/and immunological trigger.

One potential explanation is that they used either BALB/c or Albino LACA mice and in some cases they used male mice. I used C57BL/6 female mice because I have proposed to use CRHR knockout (KO) mice, of a similar background. Therefore, I considered the possibility of using another strain of mice and use CRHR-antagonists instead. In the following experiment, I used BALB/c mice and locomotor activity as behavioral assessment as it provides a more objective and representative way to assess fatigue in mice.

1.2. Experiments using BALB/c Female Mice

BALB/c female mice were provided with chow containing low to non-detectable isoflavone levels (non-detectable-20 mg/kg, Teklad 2920X) *ad libitum* -standard chow used by DLAM- for at least 2 weeks before experiments. Mice were randomly divided into the following groups (n=5/group); control/no-swim, control/swim, poly(I:C)/no swim, poly(I:C)/swim. Mice were injected with poly(I:C), 20 mg/kg on Day 1 and they were subjected to forced swim test for 15 min, daily for 4 days. Overnight locomotor

activity (16 hr) was monitored. Mice were maintained on a 14:10 hr light-dark cycle. The black bar in locomotor activity indicates the 10 hr dark cycle.

Figure 9. Locomotor activity (BALB/c).

Figure 9a. Locomotor activity - Day 1

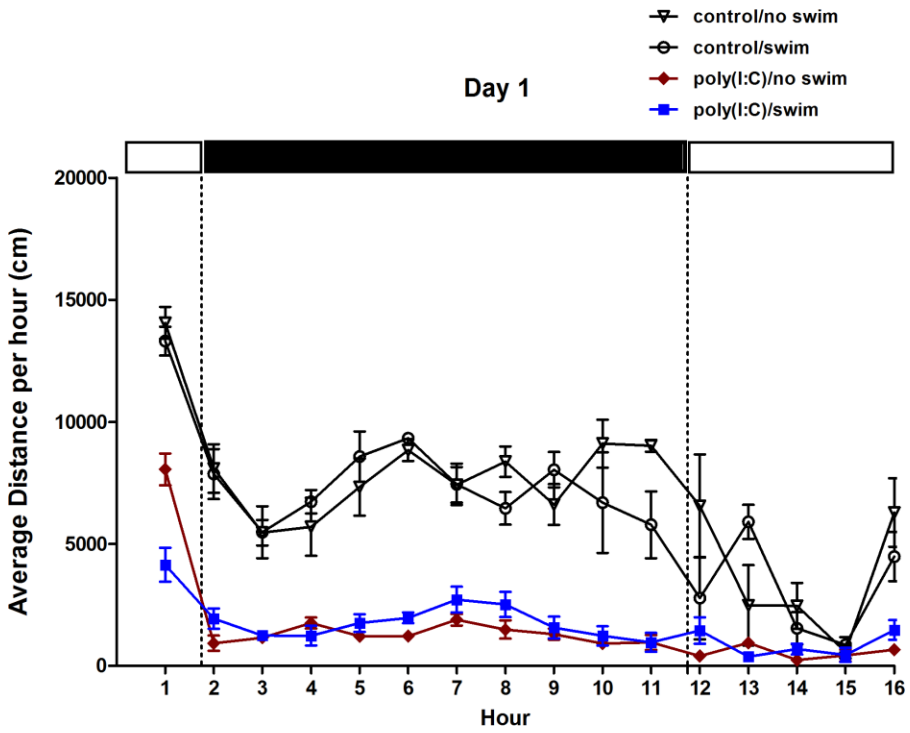


Figure 9b. Locomotor activity - Day 2

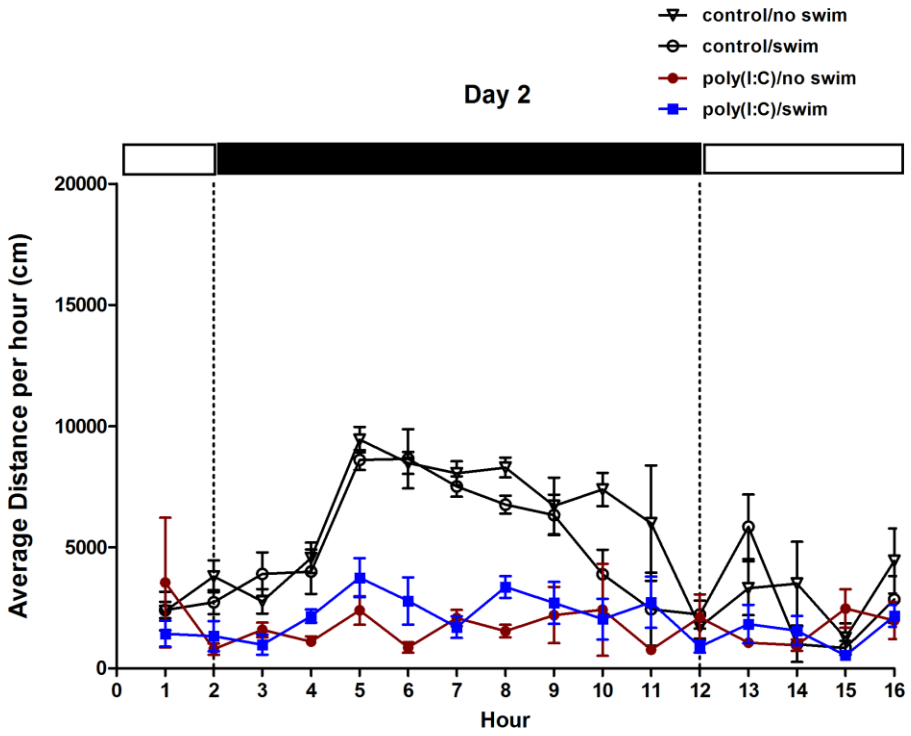


Figure 9c. Locomotor activity - Day 3

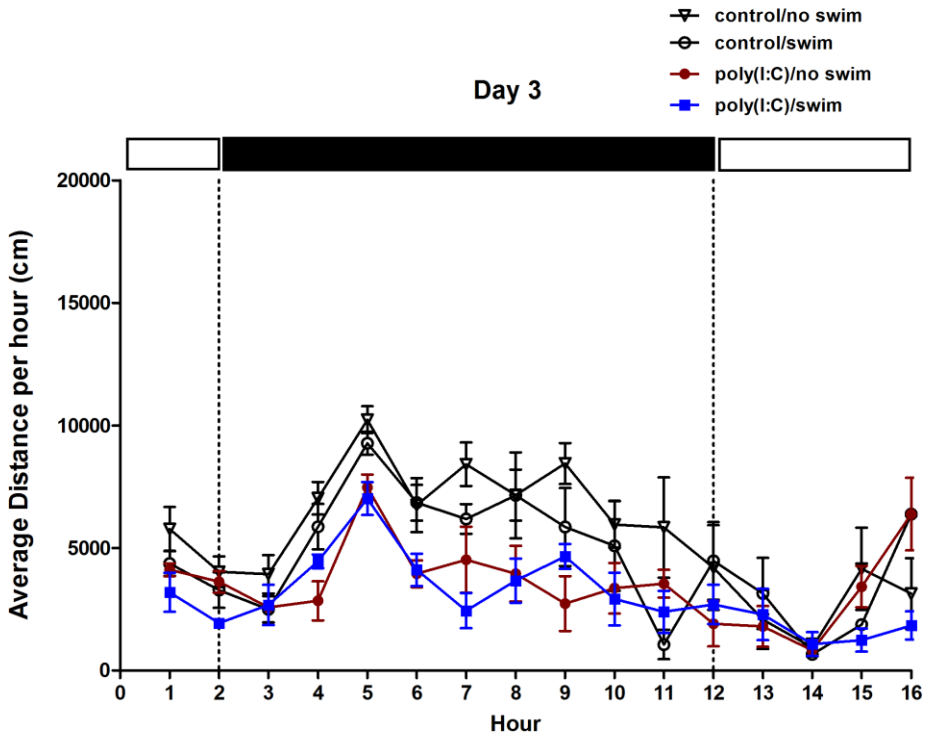
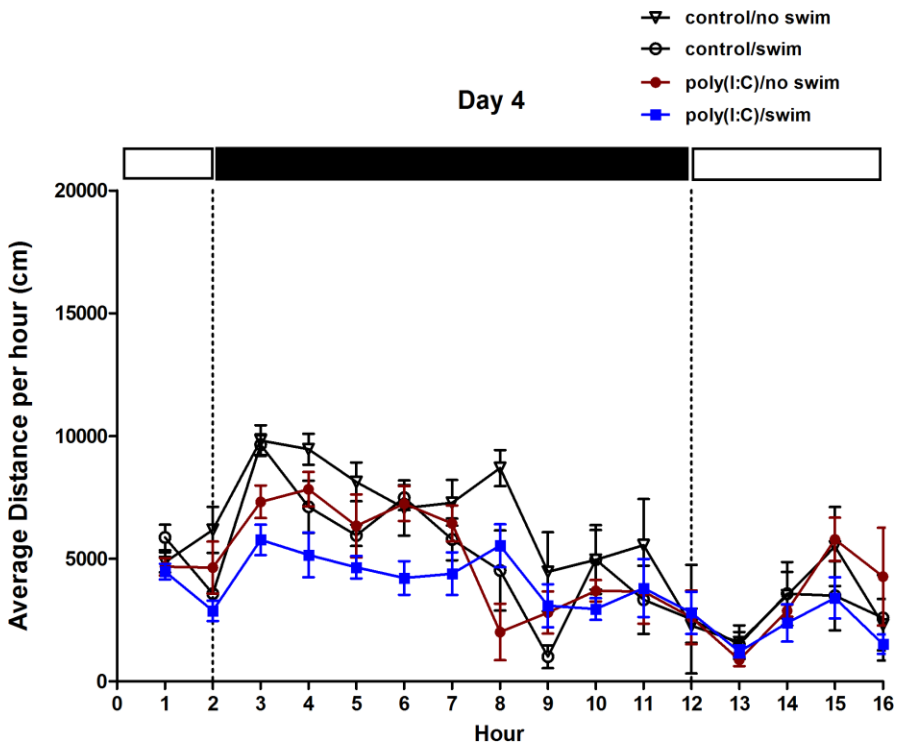


Figure 9d. Locomotor activity - Day 4



Overnight (16 hr) locomotor activity was monitored daily over the course of 7-days experiment with low isoflavone diet (Figure 9). Statistical analysis of the AUC-16 hr on Day 1 and 2, using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and the control/swim, poly(I:C)/no swim and poly(I:C)/swim groups ($p<0.0001$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the AUC-16 hr on Day 1 and 2 (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0357$) and the control/swim ($p=0.0159$), respectively.

Statistical analysis of the AUC-10 hr on Day 1 and 2, using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and the control/swim, poly(I:C)/no swim and poly(I:C)/swim groups ($p<0.0001$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the AUC-10 hr on Day 1 and 2 (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0357$) and the control/swim ($p=0.0159$) respectively.

Comparison of the max loco-16 hr on Day 1 and 2, using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and the control/swim, poly(I:C)/no swim and poly(I:C)/swim groups ($p<0.0001$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the of the max loco-16 hr on Day 1 and 2 (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0357$) and the control/swim ($p=0.0159$) respectively.

Comparison of the max loco-10 hr on Day 1 and 2, using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and the control/swim, poly(I:C)/no swim and poly(I:C)/swim groups ($p<0.0001$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of

the of the max loco-10 hr on Day 1 and 2 (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0357$) and the control/swim ($p=0.0159$) respectively.

Most of the statistical differences were maintained through Days 2 and 3 and subsided on Day 4.

1.2.1. Conclusion

These results do not agree with some previous publications that reported significant changes in the behavioral parameters that I studied. One potential explanation is that they used either BALB/c or Albino LACA mice. I used C57BL/6 because I had proposed to use similar background CRHR-1 KO mice, and because one paper stated that C57BL/6 mice are susceptible to stress[469]. Therefore, I considered the possibility of using another strain of mice and testing the same end points. I used BALB/c female mice with ip injection of the lowest dose of poly(I:C) (20 mg/kg) on Day 1, followed by forced swim test, for 15 min, for 4 consecutive days and I monitored locomotor activity of the mice overnight. As shown in Figure 9, the difference in locomotor activity was back to normal by day 3. There seems to be no difference on the locomotor activity regarding on the mouse strain (C57BL/6 versus BALB/c strain).

Additionally, I utilized CRHR-1 KO mice that I have been breeding. I repeated the protocol with female CRHR-1 KO animals and the corresponding control animals. Unfortunately, even though most of the C57BL/6 mice survived in the early experiment with the highest dose of poly(I:C) (40 mg/kg), seven out of nine CRHR-1 KO mice died.

Moreover, in order to investigate the potential effect of NT, C57BL/6 mice were treated with ip injection of 10 mg/kg poly(I:C) and/or 1-10 mg/kg NT (n=5/group) and low in isoflavone chow. They were monitored for one day. There were no obvious changes in the behavior of the mice, as NT is rapidly degraded once injected.

Taking into consideration the results above, it is clear from the experiments so far that the protocols copied from previous publications do not produce "chronic fatigue". Therefore, I decided to focus on the first day of the experiments, use female C57BL/6 mice and locomotor activity in order to investigate the effect of poly(I:C)(20mg/kg) and isoflavone diet on inflammatory mediator serum levels and gene expression in brain and skin.

Chapter 2. Mouse Studies - Poly(I:C) and the Effect of Isoflavones

2.1. Experiments using C57BL/6 Female Mice

C57BL/6 female mice were provided with chow containing low to non-detectable isoflavone levels (non-detectable-20 mg/kg, Teklad 2920X) *ad libitum* -standard chow used by DLAM- for at least 2 weeks before experiments. Mice were randomly divided into the following groups (n=5-7/group); control/no-swim, control/swim, poly(I:C)/no swim, poly(I:C)/swim. Mice were injected ip with 20 mg/kg poly(I:C) or saline on Day 1. Subsequently, they were subject to swim for 15 min and then they were individually placed into specific cages for overnight locomotor activity to be monitored.

Mice were euthanized the following day and serum, brain and skin samples were collected. Assessment of inflammatory mediators in the serum included estimation of TNFα, VEGFα, IL-1α, IL-1β, IL-4, IL-6, IL-9, IL-10, IL-12p70, IL-17, KC (human IL-8/CXCL8 murine homologue) [470], MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), IP-10 (CXCL10), and IFNγ mouse serum levels, using the Milliplex microbead assay, and was performed blindly by Millipore (St. Charles, MI, USA). Assessment of inflammatory mediators in the brain and skin included testing for brain and skin mouse gene expression of TNFα, IL-6, KC, MCP-1, MIP-1β, RANTES, IP-10, HDC, NT, NTR-1, NTR-2, IFNγ, IP-9 (CXCL11), IL-4, and CRHR-1.

Three separate experiments were performed with n=5/group, 4 groups. Some of the serum values were excluded as there was no result reported due to low bead count per Millipore's procedures (NR). NR means that no result was obtained for a specific sample as the bead count was too low to get a valid result. The instrument is set to count 50 beads for each analyte, however sometimes something interferes, they are not sure what,

with a particular sample with a particular analyte and the instrument is unable to reach 50 beads. They have determined that results with as few as 30 beads are acceptable, but they do not report results obtained with fewer than 30 beads. This is to be distinguished from values listed as <X.0 or >Y.0, that represent Millipore's evaluation of samples outside of the reporting range of the assay and were considered equal with the detection limit of each assay. Here I present representative results with n=5-7/ group.

Overall, poly(I:C)-treated mice with or without forced swim have decreased locomotor activity, as well as increased serum levels of TNF, L-6, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and IP-10. Moreover, brain and skin gene expression of TNF, IL-6, KC, MCP-1, MIP-1β, RANTES, and IP-10 was increased in poly(I:C)-treated mice, while HDC and NT gene expression was only increased in the skin.

High isoflavone diet reverses the reduced locomotor activity and minimizes or reduces the serum level increase of the above inflammatory markers, as well as their increased gene expression in the brain and the skin noted in the poly(I:C)-treated mice.

Swim stress does not increase any parameter over that of poly(I:C) but augments the poly(I:C) effect on TNFα, MIP-1β, and RANTES. CRHR-1 KO mice showed no reductions in any parameter.

IFNγ, IL-1β, IL-4, IL-9, IL-10, IL-12p70, IL-17 and VEGF serum levels were below the detection limit, while IL-1α serum levels were similar between the different treatment groups.

2.1.1. Behavioral Assessment

Mice were maintained on a 14:10 hr light-dark cycle. The black bar in locomotor activity indicates the 10 hr dark cycle.

Figure 10. Locomotor activity - Low isoflavone diet

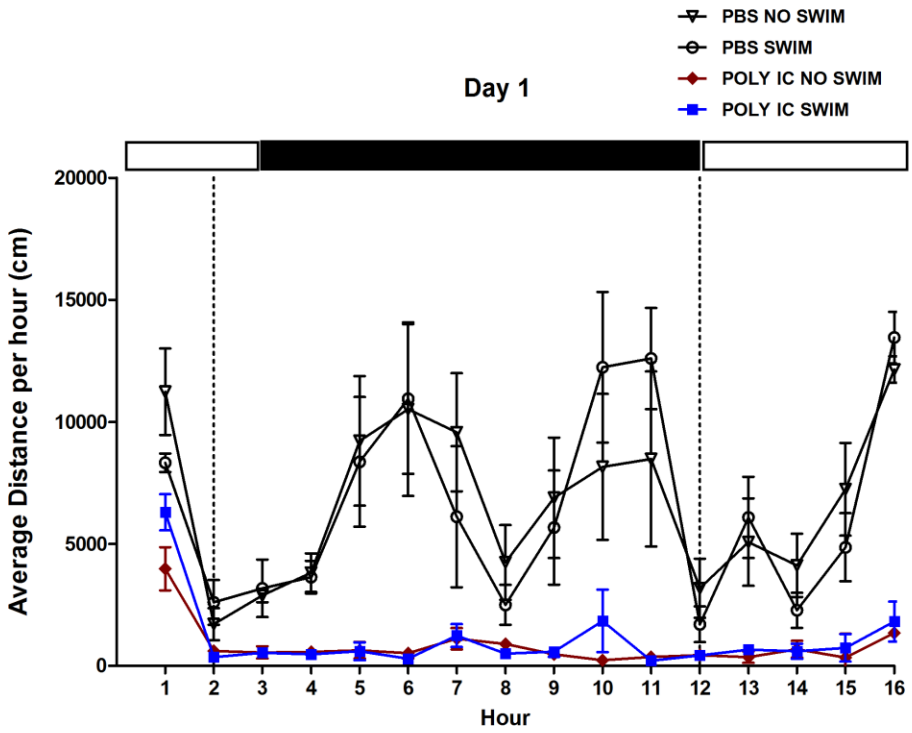
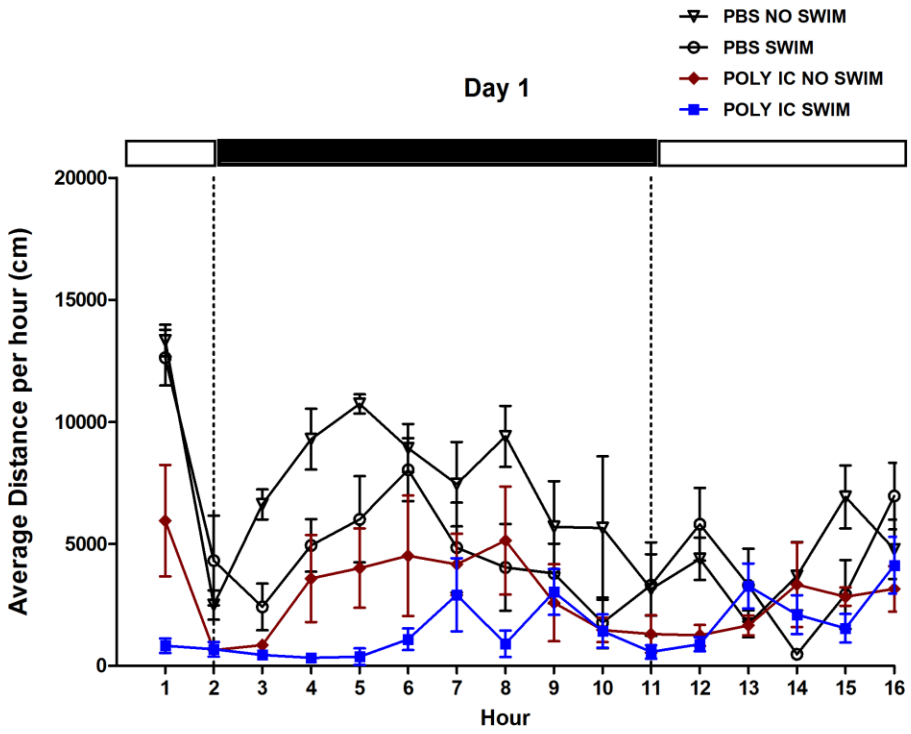


Figure 11. Locomotor activity - High isoflavone diet



Statistical analysis of the AUC-16 hr and AUC-10 hr in the low isoflavone experiments (Figure 10), using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and poly(I:C)/no swim and poly(I:C)/swim group ($p<0.0001$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the AUC-16 hr and AUC-10 hr (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0079$) and the control/swim ($p=0.0079$), respectively.

Comparison of the max loco-16 hr and max loco-10 hr in the low isoflavone experiments (Figure 10), using one-way ANOVA followed by Dunnett's multiple

comparison test, showed statistical significant differences between the means of the control/no swim and poly(I:C)/no swim and poly(I:C)/swim ($p<0.0001$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the max loco-16 hr and max loco-10 hr (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0079$) and the control/swim ($p=0.0079$) respectively.

Statistical analysis of the AUC-16 hr and AUC-10 hr in the high isoflavone diet experiments (Figure 11), using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the control/no swim and poly(I:C)/no swim and poly(I:C)/swim group ($p<0.0001$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the AUC-16 hr and AUC-10 hr (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0079$) and the control/swim ($p=0.0079$) respectively.

Comparison of the max loco-16 hr in the high isoflavone diet experiments (Figure 11), using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant between control/no swim and poly(I:C)/no swim and poly(I:C)/swim ($p=0.0012$). Poly(I:C)-treated groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the max loco-16 hr (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/swim group had reduced locomotor activity compared to the control/swim

(p=0.0079), but there was no difference in the locomotor actitivity of the poly(I:C)/no swim group in comparison to the control/swim group (p=0.0556).

Moreover, comparison of the max loco-10 hr in the high isoflavone diet experiments (Figure 11), using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant difference between the means of the control/no swim and poly(I:C)/no swim and poly(I:C)/swim (p=0.244), but Dunnett's test showed that only the poly(I:C)/swim group had reduced locomotor activity($p<0.05$), while there was no statistical difference with the poly(I:C)/no swim group. Further analysis of the max loco-10 hr in the high isoflavone diet experiments (Figure 11), using the non-parametric Mann Whitney U test showed that the poly(I:C)/no swim and poly(I:C)/swim groups did not have significant reduced locomotor activity in comparison to the control/no swim (0.2222) and control/swim respectively (p=0.0556).

Direct comparison of the AUC-16 hr and max loco-16 hr of the groups treated the same way between the low and high isoflavone diet experiments (Figure 10 vs 11) showed that poly(I:C)/no swim-treated group in the low isoflavone diet had statistically significant lower locomotor activity in comparison with the same group in the high isoflavone diet (non-parametric Mann Whitney U test, p=0.0317). Moreover, direct comparison of the max loco-10 hr showed that poly(I:C)/no swim-treated group in the low isoflavone diet had statistically significant lower locomotor activity in comparison with the same group in the high isoflavone diet (non-parametric Mann Whitney U test, p=0.0317).

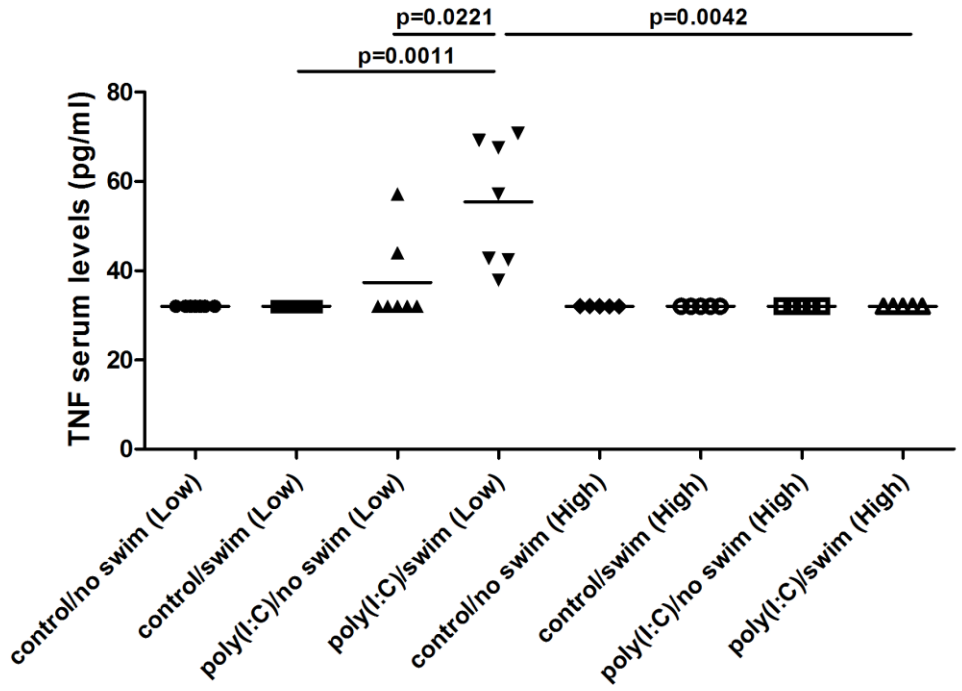
2.1.1.1. Conclusion

Poly(I:C)-treated mice with or without forced swim show reduced max locomotor activity in comparison to the control mice. High isoflavone diet reverses the statistically significant reduced max locomotor activity of poly IC/no swim and poly(I:C)/swim-treated mice (there is no statistical significant difference in high isoflavone diet). Direct comparison of the poly(I:C)-treated groups between low and high isoflavone diet reveals that in the low isoflavone diet the locomotor activity is significantly reduced. Swim stress does not increase any parameter over that of poly(I:C) over the 21 days, although there are some differences on specific days, and CRHR-1 KO mice showed no reductions in any parameter.

2.1.2. Biochemical Assessment

2.1.2.1. Serum Levels of Inflammatory Mediators

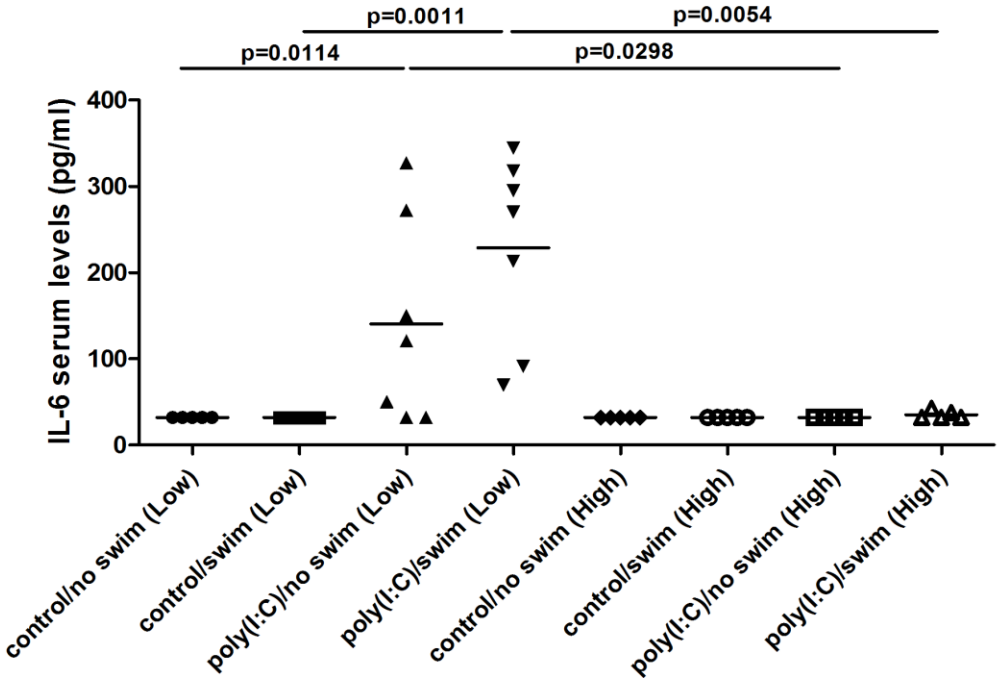
Figure 12. TNF serum levels – Low vs High isoflavone diet.
(TNF serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)/swim-treated mice have increased TNF serum levels when they are provided with low isoflavone diet. Moreover, high isoflavone diet reduces the increased TNF serum levels noted in poly(I:C)/swim-treated mice. Lastly, forced swim contributes to poly(I:C) effect on increasing TNF serum levels (Figure 12).

Figure 13. IL-6 serum levels - Low vs High isoflavone diet.

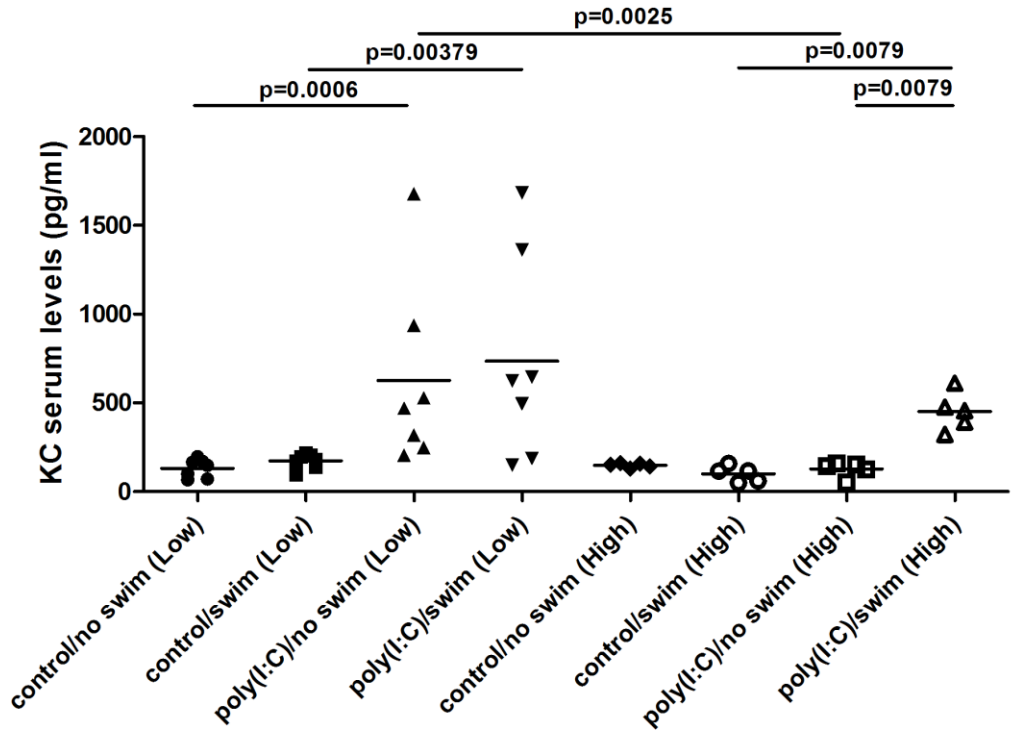
(IL-6 serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased IL-6 serum levels when they are provided with low isoflavone diet. Moreover, high isoflavone diet reduces the increased IL-6 serum levels noted in both poly(I:C)-treated groups. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on increasing IL-6 serum levels (Figure 13).

Figure 14. KC serum levels - Low vs High isoflavone diet.

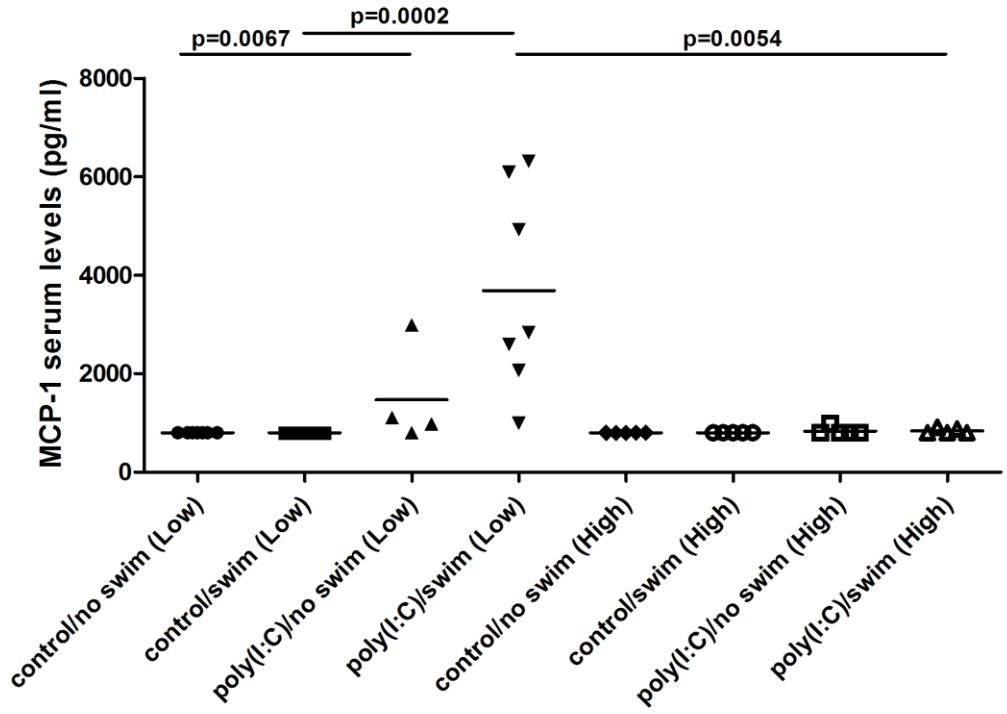
(KC serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased KC serum levels when they are provided with low isoflavone diet. In contrast, when they are provided with high isoflavone diet only poly(I:C)/swim-treated mice show significant increase in KC serum levels. Moreover, high isoflavone diet reduces the increased KC serum levels noted in poly(I:C)/no swim-treated groups. Lastly, forced swim contributes to poly(I:C) effect on increasing KC serum levels only in the high isoflavone diet (Figure 14).

Figure 15. MCP-1 serum levels - Low vs High isoflavone diet.

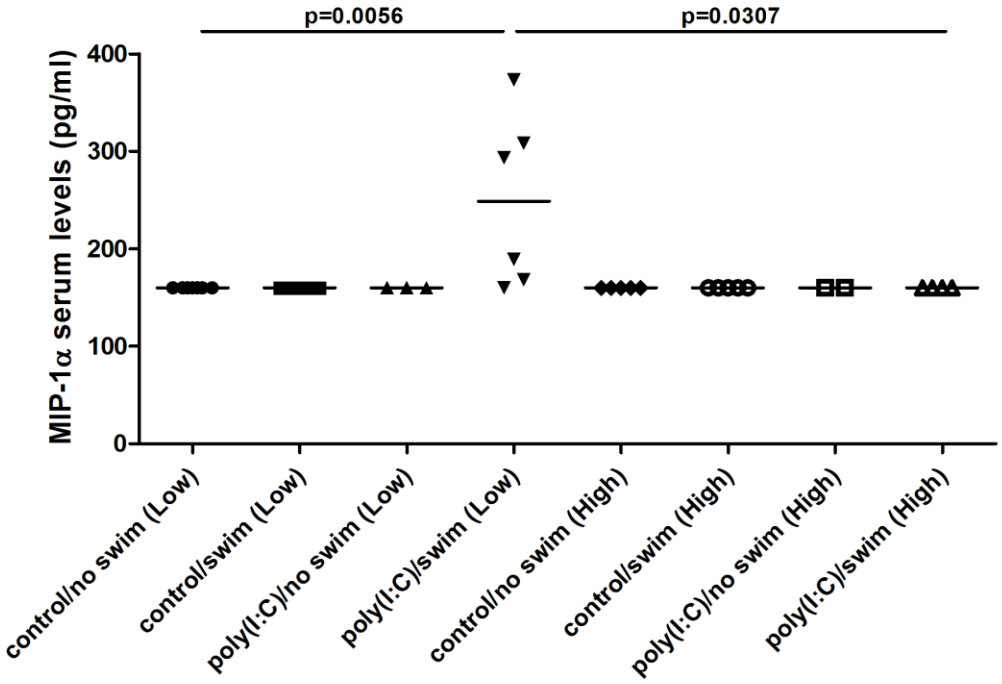
(MCP1 serum levels below the detection limit, <800 pg/ml, are considered as 800 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased MCP-1 serum levels when they are provided with low isoflavone diet. Moreover, high isoflavone diet reduces the increased MCP-1 serum levels noted in the poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on increasing MCP-1 serum levels (Figure 15).

Figure 16. MIP-1α serum levels - Low vs High isoflavone diet.

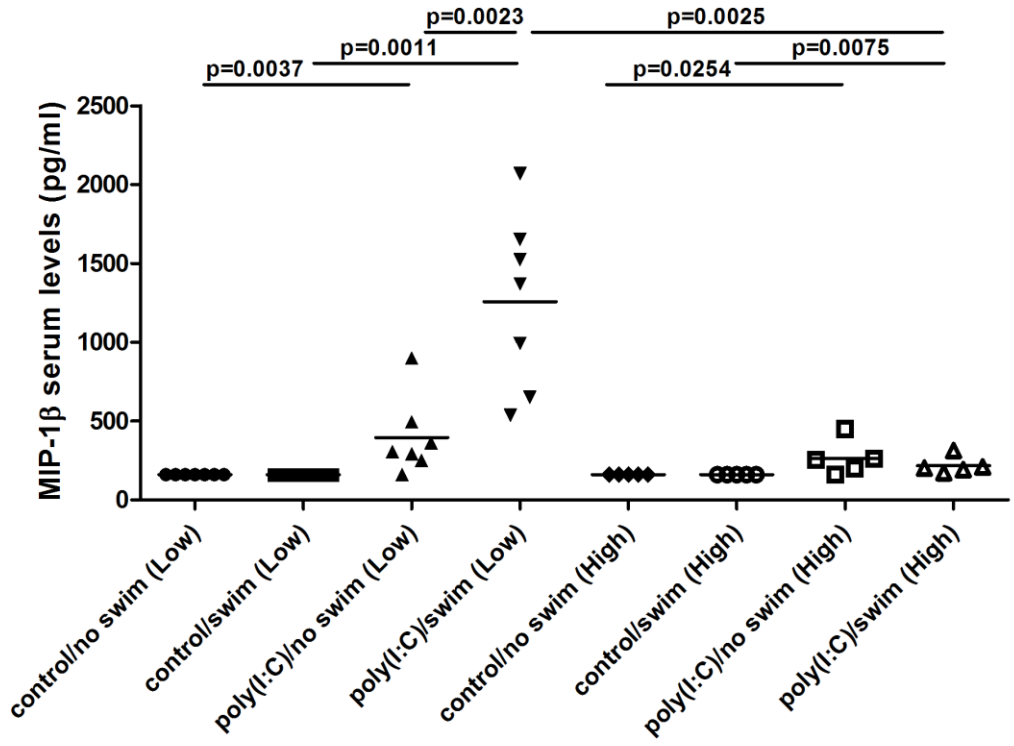
(MIP1α serum levels below the detection limit, <160 pg/ml, are considered as 160 pg/ml)



Statistical analysis showed that poly(I:C)/swim-treated mice have increased MIP-1α serum levels when they are provided with low isoflavone diet. Moreover, high isoflavone diet reduces the increased MIP-1α serum levels noted in the poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on increasing MIP-1α serum levels (Figure 16).

Figure 17. MIP-1β serum levels - Low vs High isoflavone diet.

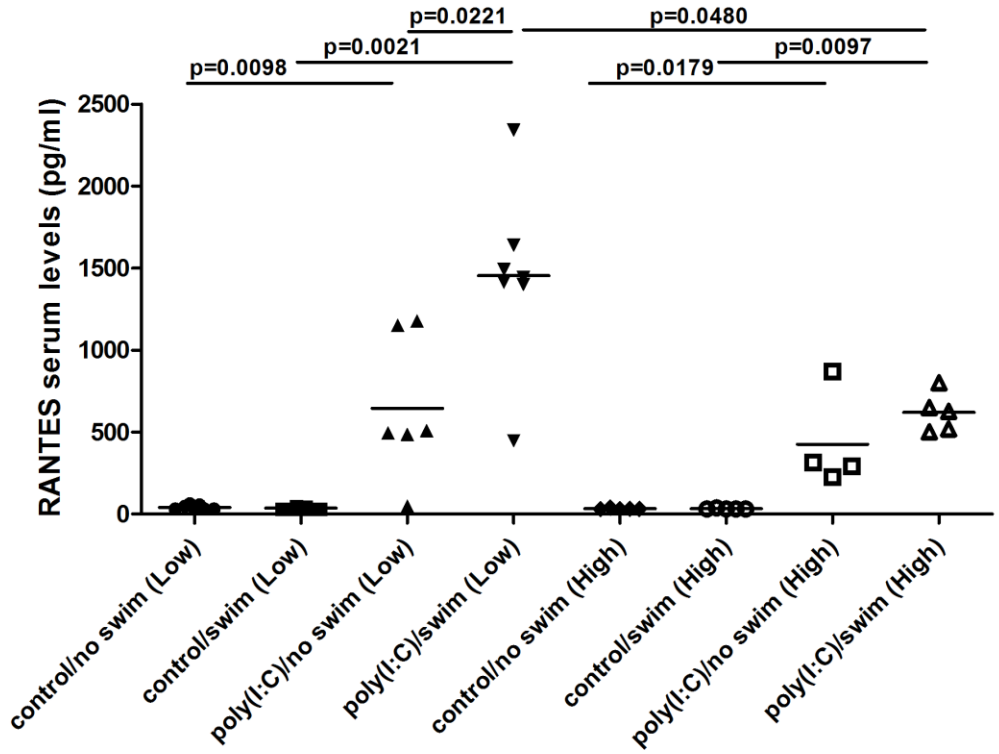
(MIP1β serum levels below the detection limit, <160 pg/ml, are considered as 160 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased MIP-1β serum levels when they are provided with either low or high isoflavone diet. However, high isoflavone diet reduces the increased MIP-1β serum levels noted in poly(I:C)/swim-treated group. Lastly, forced swim contributes to poly(I:C) effect on increasing MIP-1β serum levels when mice are provided with low isoflavone diet (Figure 17).

Figure 18. RANTES serum levels - Low vs High isoflavone diet.

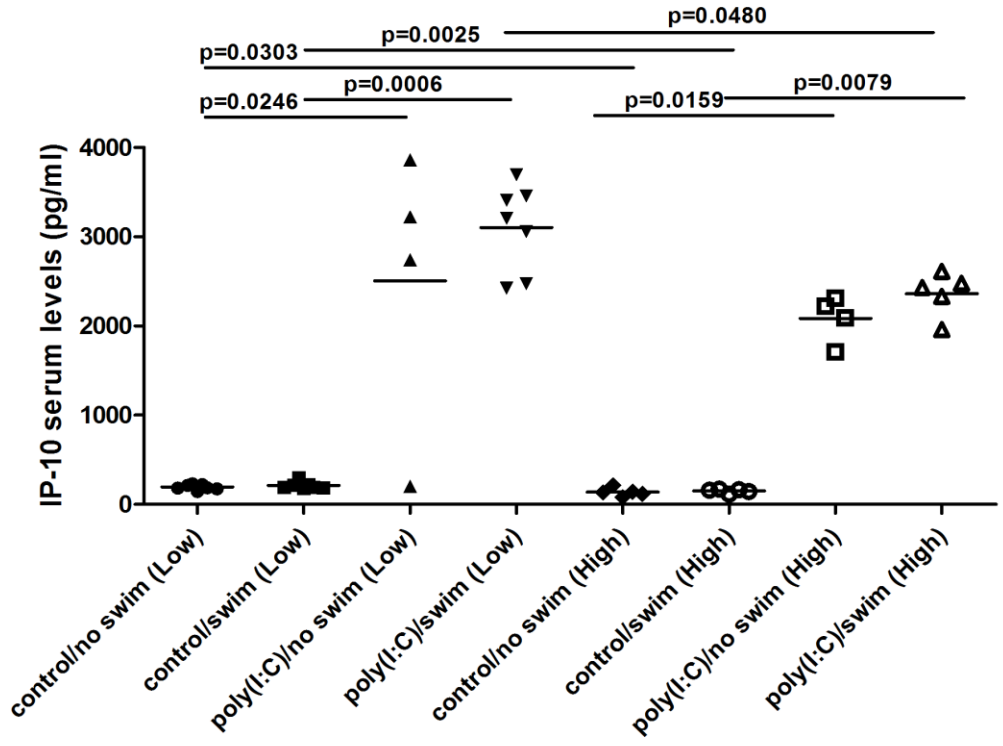
(RANTES serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased RANTES serum levels when they are provided with either low or high isoflavone diet. However, high isoflavone diet reduces the increased RANTES serum levels noted in poly(I:C)/swim-treated group. Lastly, forced swim contributes to poly(I:C) effect on increasing RANTES serum levels when mice are provided with low isoflavone diet (Figure 18).

Figure 19. IP-10 serum levels - Low vs High isoflavone diet.

(IP-10 serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased IP-10 serum levels when they are provided with either low or high isoflavone diet. However, high isoflavone diet reduces the increased RANTES serum levels noted incontrol/no swim, control/swim and poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on increasing IP-10 serum levels (Figure 19).

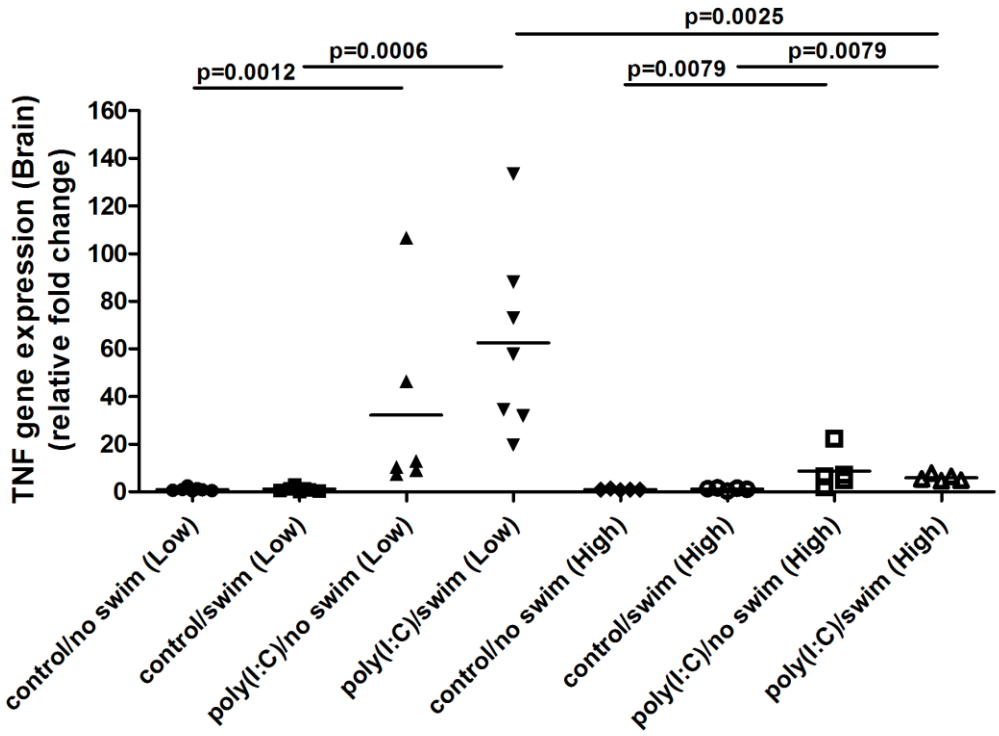
2.1.2.1.1. Conclusion

Overall, poly(I:C)-treated mice with or without forced swim have increased serum levels of TNF, IL-6, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and IP-10.

High isoflavone diet minimizes or reduces the serum level increase of the above inflammatory markers. Swim stress contributes to the poly(I:C) effect on increasing TNF, MIP-1β and RANTES serum levels in mice provided with low, but not high, isoflavone diet.

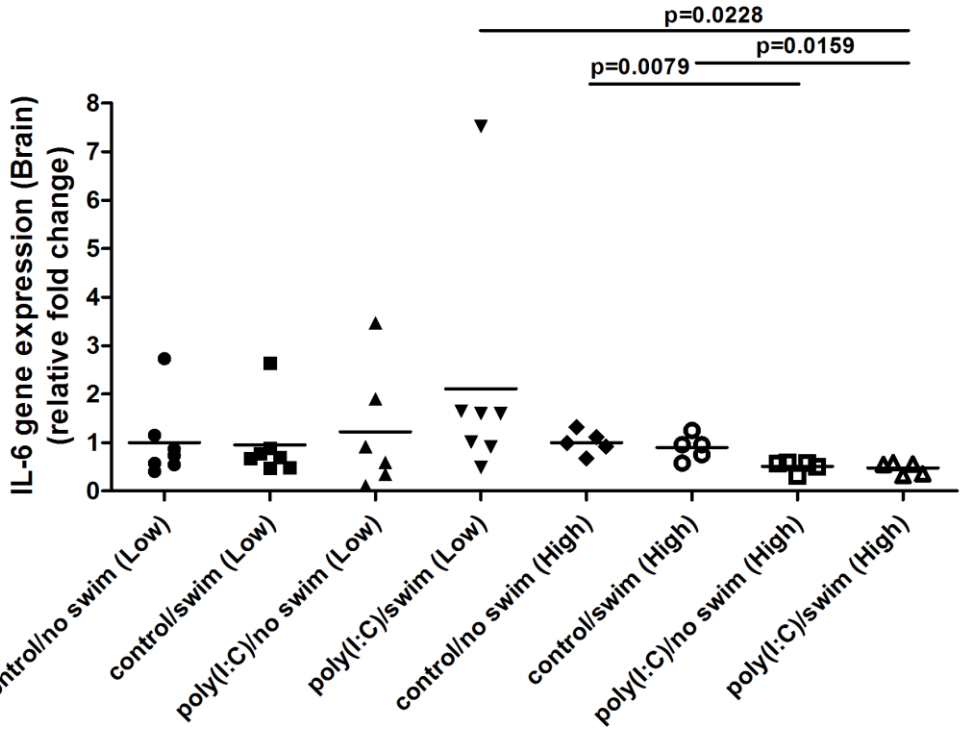
2.1.2.2. Brain gene expression of inflammatory mediators

Figure 20. TNF gene expression (Brain) - Low vs High isoflavone diet.



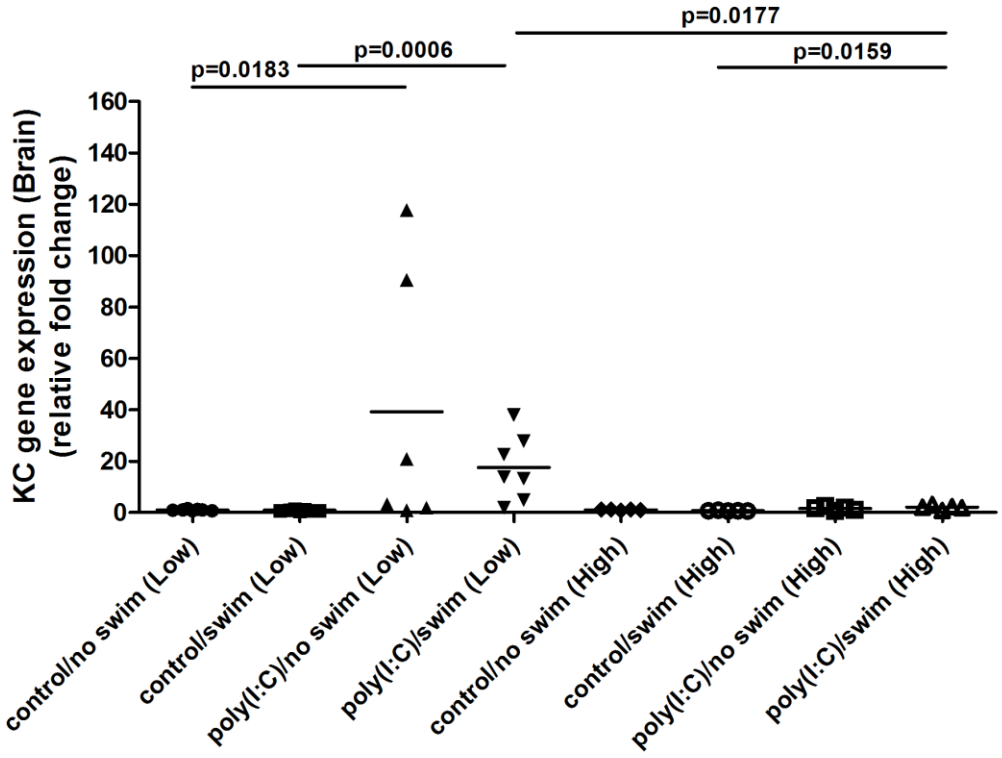
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased brain TNF gene expression when they are provided with either low or high isoflavone diet. However, high isoflavone diet reduces the increased TNF gene expression noted in poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on brain TNF gene expression (Figure 20).

Figure 21. IL-6 gene expression (Brain) - Low vs High isoflavone diet



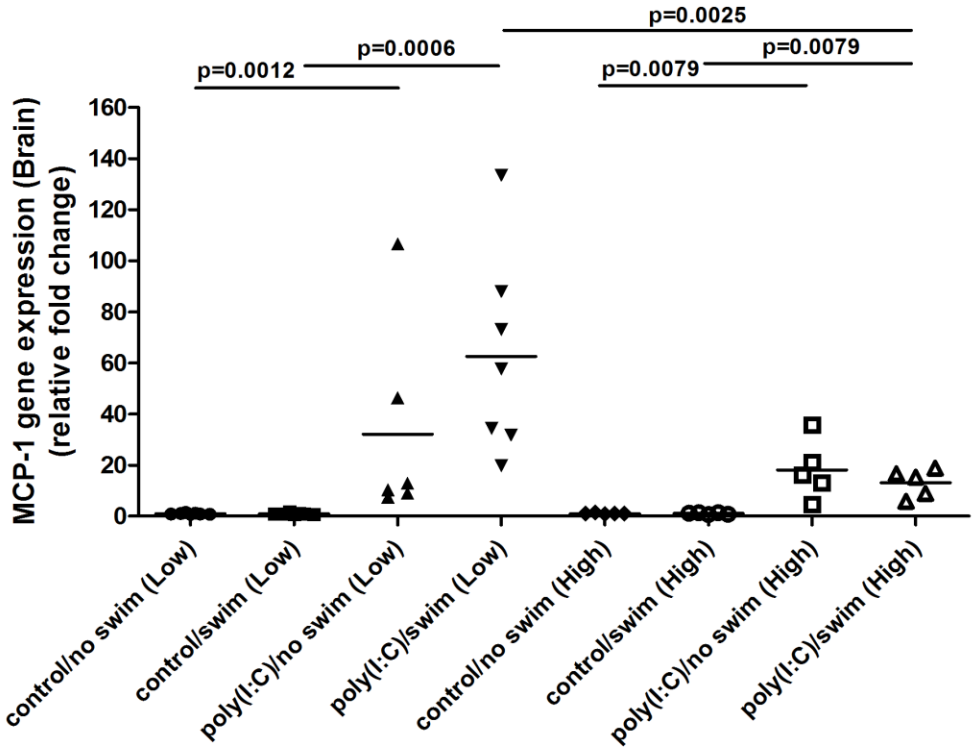
Statistical analysis showed that poly(I:C)-treated mice on high isoflavone diet have decreased IL-6 brain gene expression in comparison with mice on low isoflavone diet. Forced swim does not seem to have any additional effect to on brain IL-6 gene expression (Figure 21).

Figure 22. KC gene expression (Brain) - Low vs High isoflavone diet



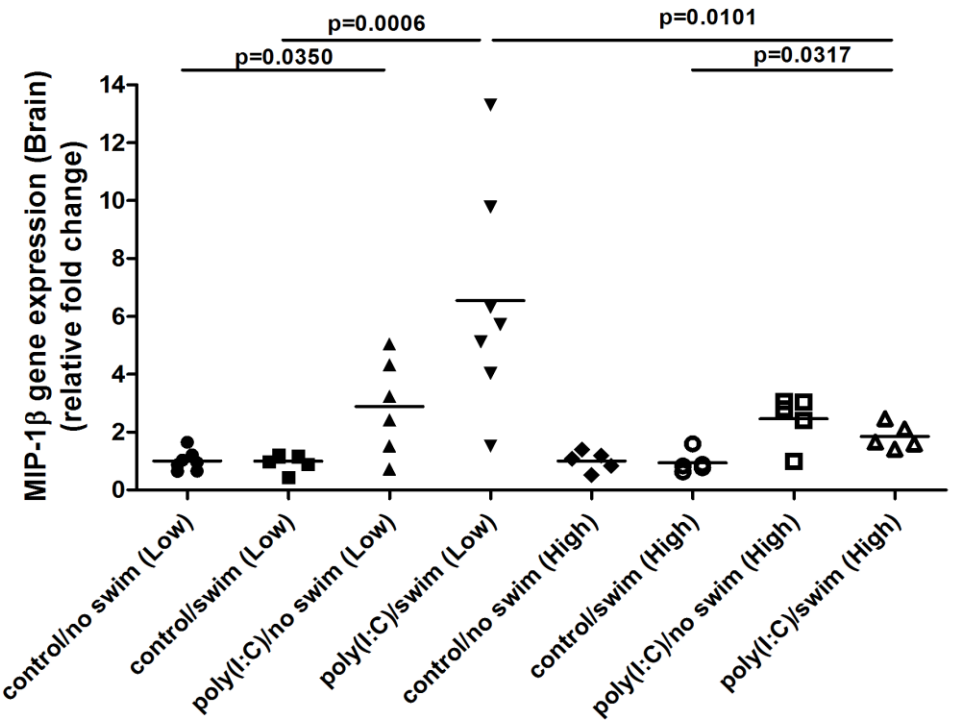
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased brain KC gene expression when they are provided with low isoflavone diet, while only poly(I:C)/swim-treated mice have increased KC brain gene expression in the case of high isoflavone diet. Moreover, high isoflavone diet reduces the increased KC gene expression noted in poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on brain KC gene expression (Figure 22).

Figure 23. MCP-1 gene expression (Brain) - Low vs High isoflavone diet



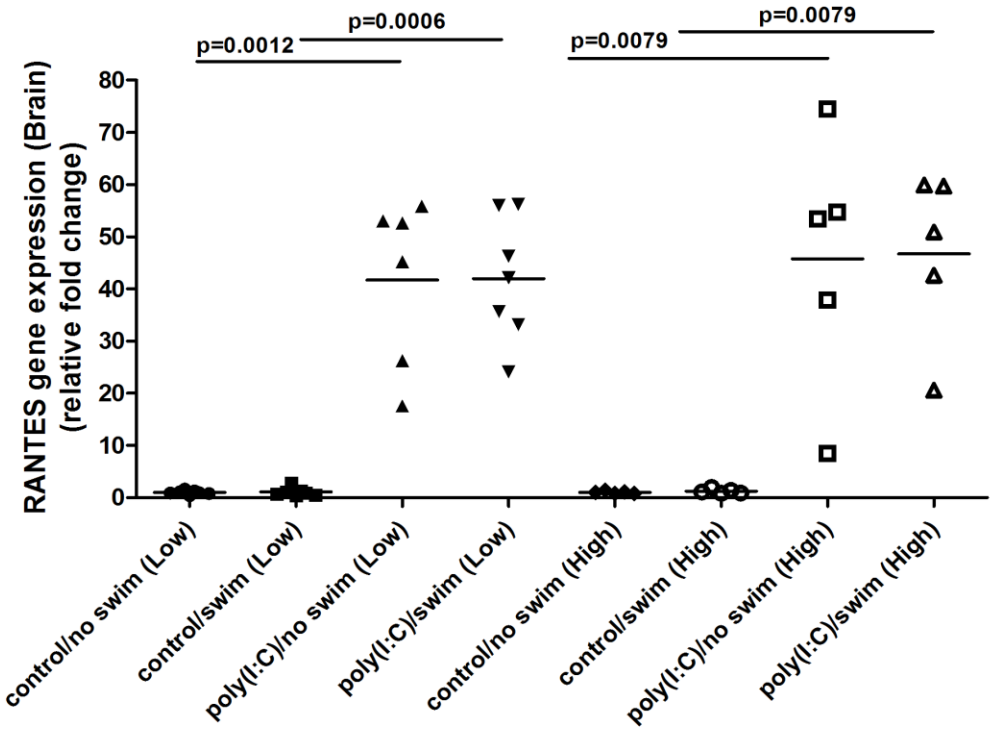
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased brain MCP-1 gene expression when they are provided with either low or high isoflavone diet. However, high isoflavone diet reduces the increased MCP-1 gene expression noted in poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on brain MCP-1 gene expression (Figure 23).

Figure 24. MIP-1β gene expression (Brain) - Low vs High isoflavone diet



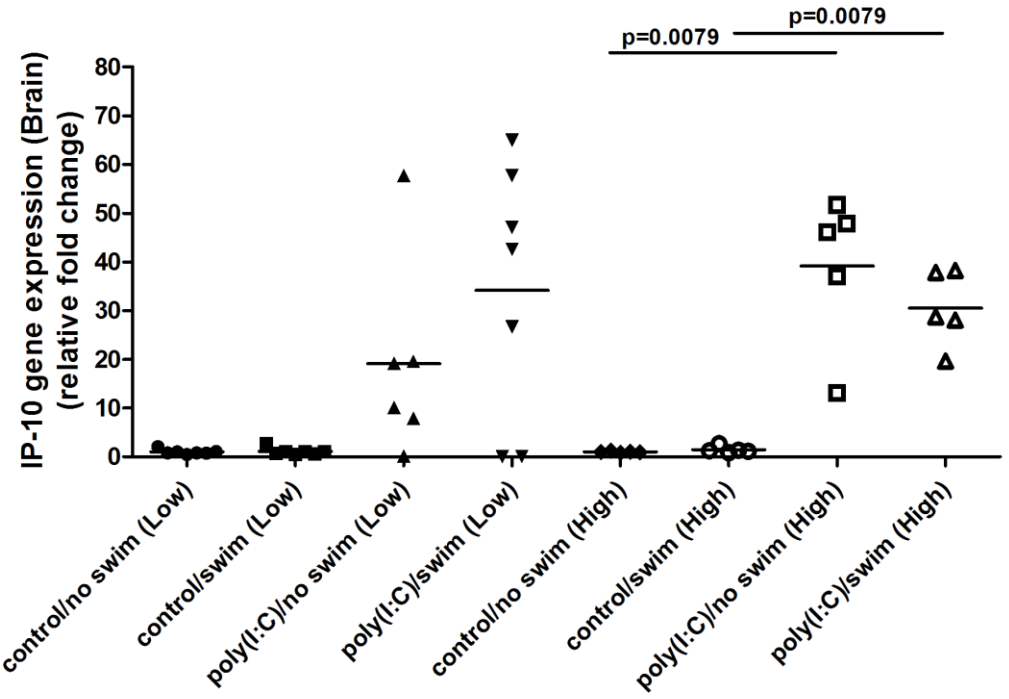
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased brain MIP-1β gene expression when they are provided with low or high isoflavone diet, while only poly(I:C)/swim-treated mice have increased MIP-1β brain gene expression in the case of high isoflavone diet. However, high isoflavone diet reduces the increased MIP-1β gene expression noted in poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on brain MIP-1β gene expression (Figure 24).

Figure 25. RANTES gene expression (Brain) - Low vs High isoflavone diet



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased brain RANTES gene expression when they are provided with either low or high isoflavone diet. High isoflavone diet and forced swim do not seem to have any additional effect to the poly(I:C) effect on brain RANTES gene expression (Figure 25).

Figure 26. IP-10 gene expression (Brain) - Low vs High isoflavone diet



It seems that poly(I:C)-treated, with or without swim, mice have increased brain IP-10 gene expression when they are provided with either low or high isoflavone diet. However, this increase was statistical significant only in the case of high isoflavone diet. High isoflavone diet and forced swim do not seem to have any additional effect to the poly(I:C) effect on brain IP-10 gene expression (Figure 26).

Figure 27. HDC gene expression (Brain) - Low vs High isoflavone diet

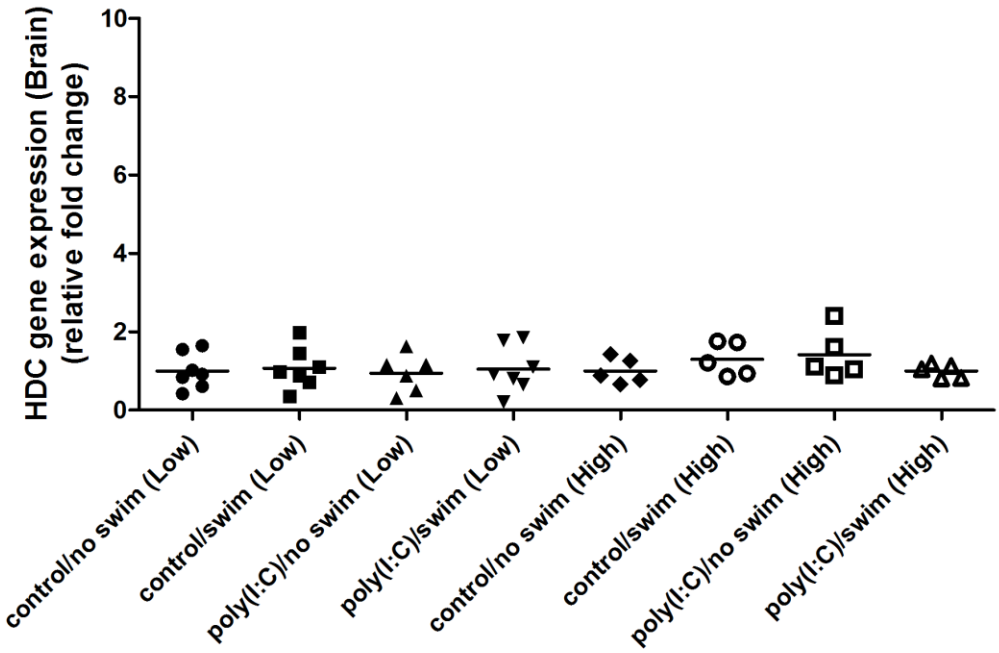
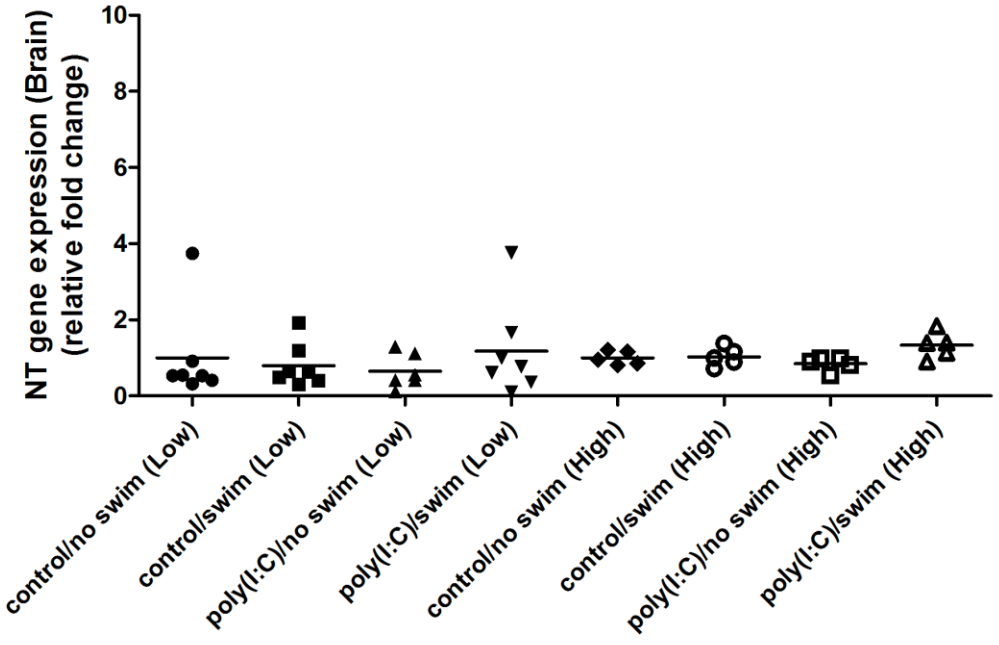


Figure 28. NT brain gene expression - Low vs High isoflavone diet



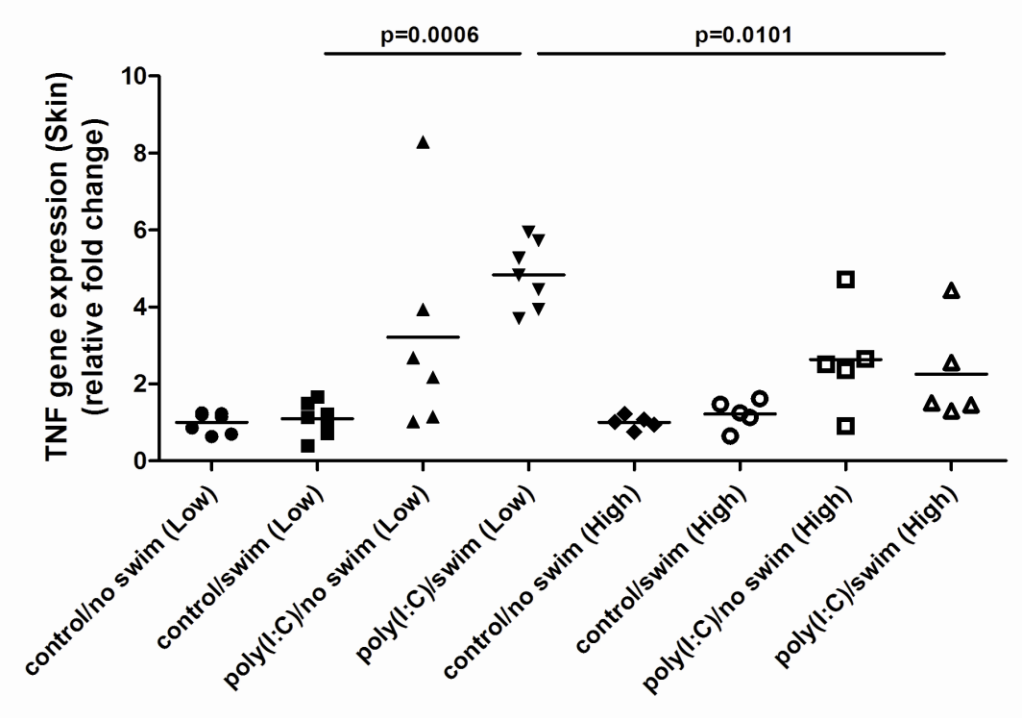
There was no difference regarding the brain HDC and NT gene expression between the groups either on low or high isoflavone diet (Figures 27 and 28).

2.1.2.2.1. Conclusion

Brain gene expression of TNF, IL-6, KC, MCP-1, MIP-1β, RANTES, and IP-10 was increased in poly(I:C)-treated mice. High isoflavone diet minimizes or reduces the brain gene expression increase of the TNF, IL-6, KC, MCP-1 and MIP-1β noted in the poly(I:C)-treated mice. Swim stress does not seem to have any additional effect on any parameter over that of poly(I:C).

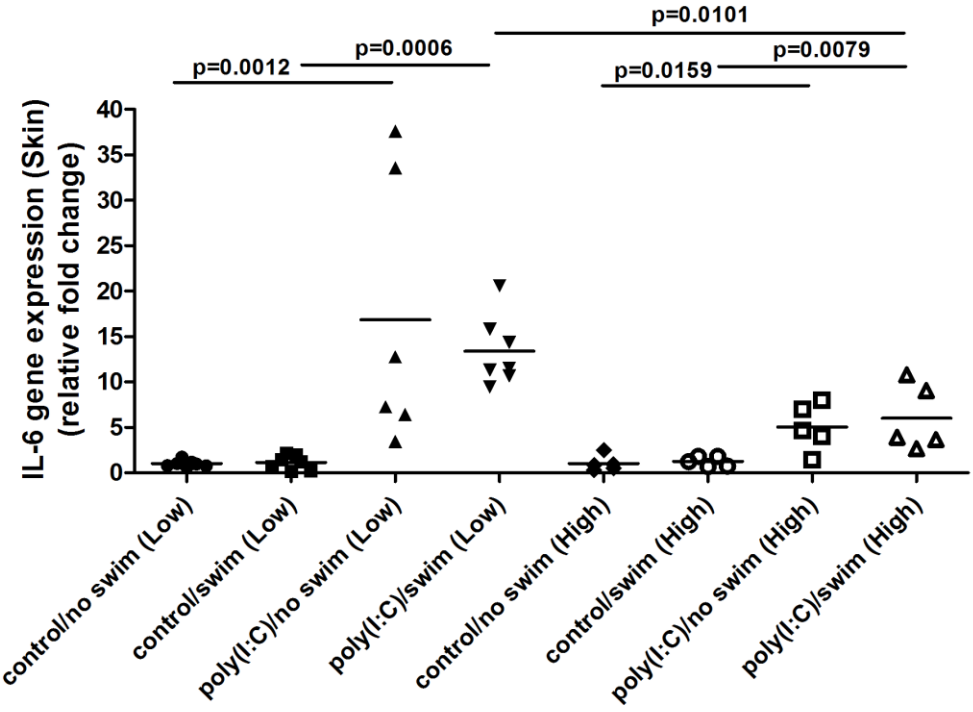
2.1.2.3. Skin gene expression of inflammatory mediators

Figure 29. TNF gene expression (Skin) - Low vs High isoflavone diet



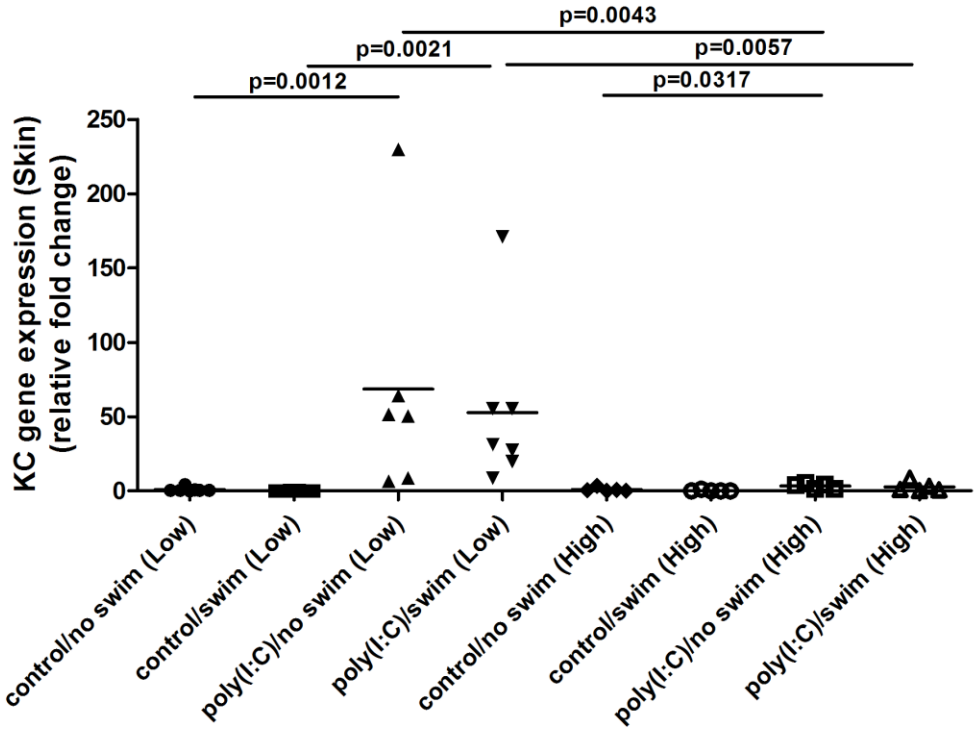
Statistical analysis showed that poly(I:C)/swim-treated mice have increased skin TNF gene expression when they are provided with low isoflavone diet. However, high isoflavone diet reduces the increased TNF gene expression noted in poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on skin TNF gene expression (Figure 29).

Figure 30. IL-6 gene expression (Skin) - Low vs High isoflavone diet



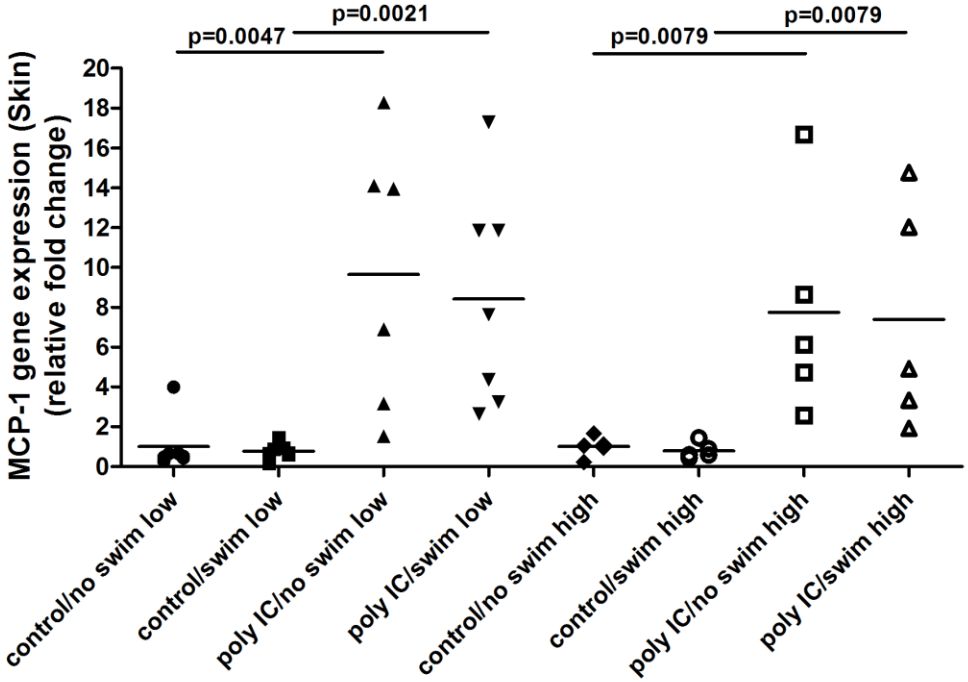
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased skin IL-6 gene expression when they are provided with either low or high isoflavone diet. However, high isoflavone diet reduces the increased IL-6 gene expression noted in poly(I:C)/swim-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on skin IL-6 gene expression (Figure 30).

Figure 31. KC gene expression (Skin) - Low vs High isoflavone diet



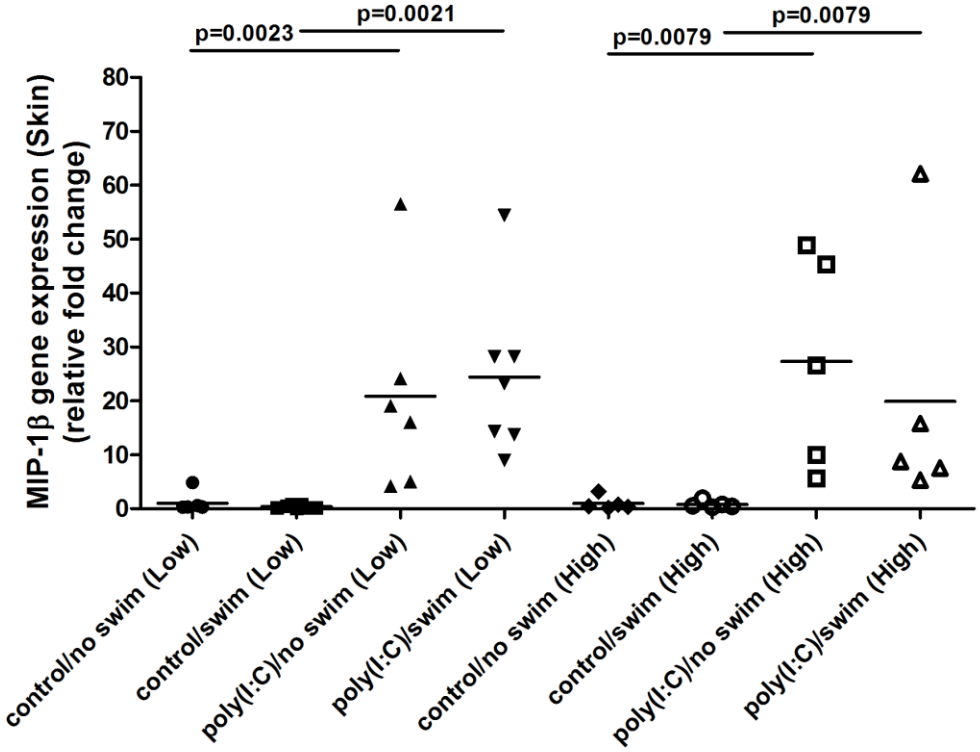
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased skin KC gene expression when they are provided with low isoflavone diet, while only poly(I:C)/no swim-treated mice have increased KC skin gene expression in the case of high isoflavone diet. However, high isoflavone diet reduces the increased KC gene expression noted in both the poly(I:C)-treated group. Lastly, forced swim does not seem to have any additional effect to poly(I:C) effect on skin KC gene expression (Figure 31).

Figure 32. MCP-1 gene expression (Skin) - Low vs High isoflavone diet



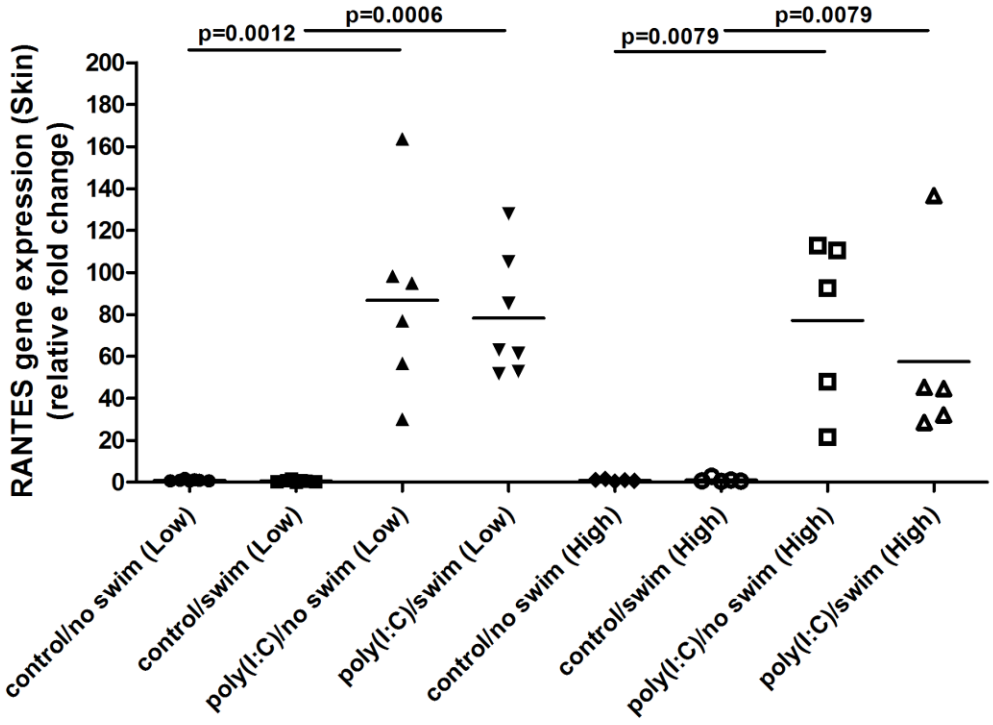
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased skin MCP-1 gene expression when they are provided with either low or high isoflavone diet. High isoflavone diet and forced swim do not seem to have any additional effect to the poly(I:C) effect on skin MCP-1 gene expression (Figure 32).

Figure 33. MIP-1β gene expression (Skin) - Low vs High isoflavone diet



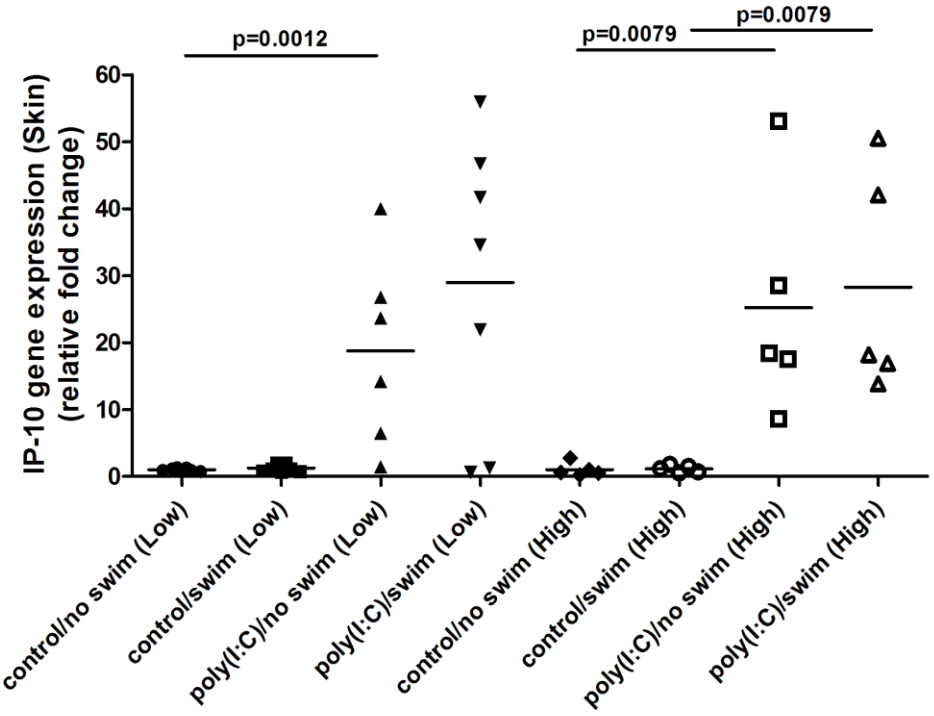
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased skin MIP-1β gene expression when they are provided with either low or high isoflavone diet. High isoflavone diet and forced swim do not seem to have any additional effect to the poly(I:C) effect on skin MIP-1β gene expression (Figure 33).

Figure 34. RANTES expression (Skin) - Low vs High isoflavone diet



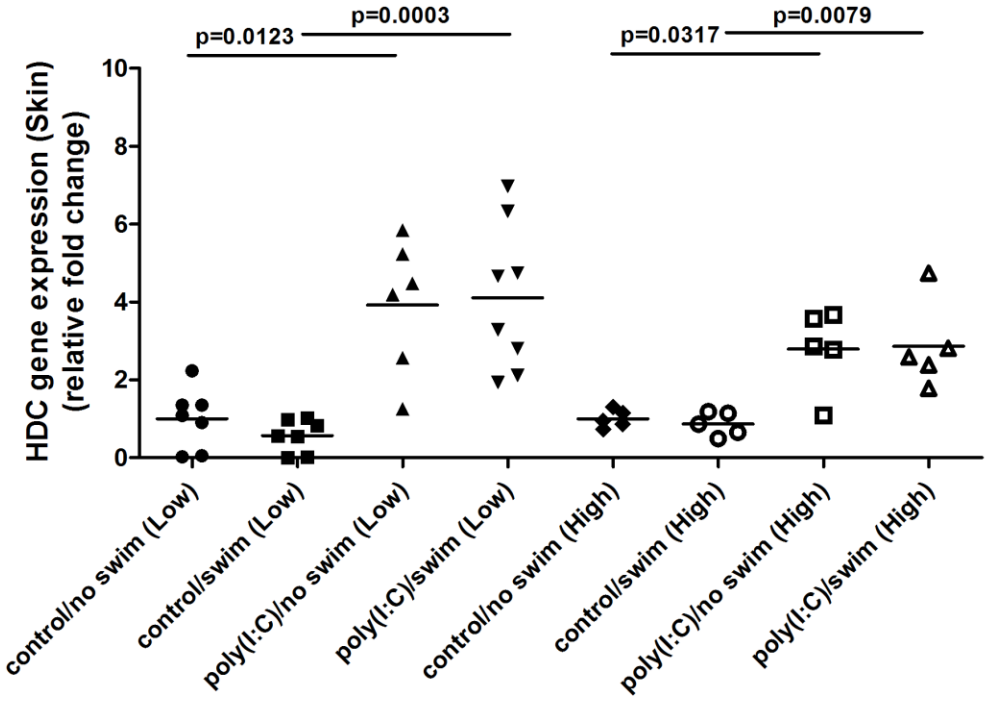
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased skin RANTES gene expression when they are provided with either low or high isoflavone diet. High isoflavone diet and forced swim do not seem to have any additional effect to the poly(I:C) effect on skin RANTES gene expression (Figure 34).

Figure 35. IP-10 gene expression (Skin) - Low vs High isoflavone diet



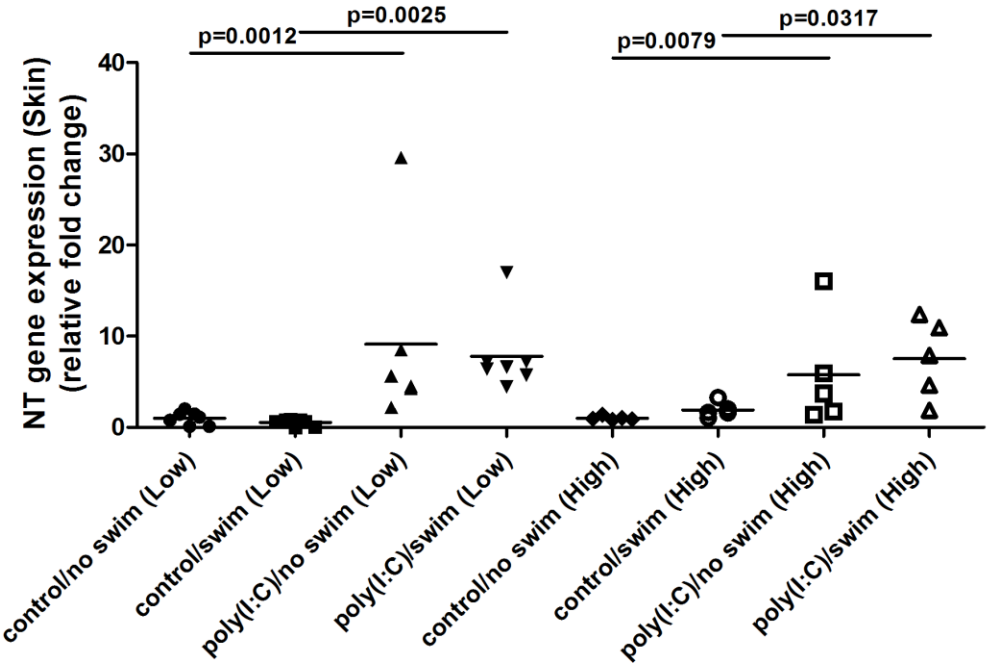
Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased skin IP-10 gene expression when they are provided with either low or high isoflavone diet, with the exception of poly(I:C)/swim-treated mice on low isoflavone diet. High isoflavone diet and forced swim do not seem to have any additional effect to the poly(I:C) effect on skin IP-10 gene expression (Figure 35).

Figure 36. HDC expression (Skin) - Low vs High isoflavone diet



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased skin HDC gene expression when they are provided with either low or high isoflavone diet. High isoflavone diet and forced swim do not seem to have any additional effect to the poly(I:C) effect on skin HDC gene expression (Figure 36).

Figure 37. NT expression (Skin) - Low vs High isoflavone diet



Statistical analysis showed that poly(I:C)-treated, with or without swim, mice have increased skin NT gene expression when they are provided with either low or high isoflavone diet. High isoflavone diet and forced swim do not seem to have any additional effect to the poly(I:C) effect on skin NT gene expression (Figure 37).

2.1.2.3.1. Conclusion

Skin gene expression of TNF, I L-6, KC, MCP-1, MIP-1β, RANTES, and IP-10 was increased in poly(I:C)-treated mice, similar to the results from the brain, while HDC and NT gene expression was only increased in the skin.

High isoflavone diet minimizes or reduces the skin gene expression increase of the TNF, IL-6, and KC noted in the poly(I:C)-treated mice.

Swim stress does not seem to have any additional effect on any parameter over that of poly(I:C).

2.2. Experiments using CRHR-1 KO Female Mice

CRHR-1 KO female mice and their WT littermates were provided with chow containing low to non-detectable isoflavone levels (non-detectable-20 mg/kg) *ad libitum* - standard chow used by DLAM- for at least 2 weeks before experiments. Mice were randomly divided into the following groups (n=5/group); control/no-swim, control/swim, poly(I:C)/no swim, poly(I:C)/swim. Mice were injected ip with 15 mg/kg poly(I:C) or saline on Day 1. Subsequently, they were subject to swim for 15 min and then they were individually placed into specific cages for overnight locomotor activity to be monitored. One mouse in the poly(I:C)/no swim group and two mice in the poly(I:C)/swim group of the CRHR-1 KO animals died.

Mice were euthanized the following day and serum, brain and skin samples were collected. Assessment of inflammatory mediators in the serum included estimation of TNFα, VEGFαα, IL-1α, IL-1β, IL-4, IL-6, IL-9, IL-10, IL-12p70, IL-17, KC (human IL-8/CXCL8 murine homologue), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), IP-10 (CXCL10), and IFNγ mouse serum levels, using the Milliplex microbead assay, and was performed blindly by Millipore (St. Charles, MI, USA). Assessment of inflammatory mediators in the brain and skin included testing for brain and skin mouse gene expression of TNFα, IL-6, KC, MCP-1, MIP-1β, RANTES, IP-10, HDC, NT, NTR-1, NTR-2, IFNγ, IP-9 (CXCL11), IL-4, and CRHR-1.

Some of the serum values were excluded as there was no result reported due to low bead count per Millipore's procedures (NR). NR means that no result was obtained for a specific sample as the bead count was too low to get a valid result. The instrument is set to count 50 beads for each analyte, however sometimes something interferes, they are not

sure what, with a particular sample with a particular analyte and the instrument is unable to reach 50 beads. They have determined that results with as few as 30 beads are acceptable, but they do not report results obtained with fewer than 30 beads. This is to be distinguished from values listed as <X.0 or >Y.0, that represent Millipore's evaluation of samples outside of the reporting range of the assay and were considered equal with the detection limit of each assay.

Overall, poly(I:C)-treated WT mice with or without forced swim have decreased locomotor activity, while there were no differences in the KO mice. Poly(I:C)/with or without swim-treated WT mice had increased IL-6, MIP-1β, RANTES and IP-10 serum levels and poly(I:C)/no swim-treated WT mice had increased KC and MCP-1 serum levels in comparison with the corresponding control WT mice.

Additionally, poly(I:C)/with or without swim-treated WT mice have increased TNF and KC brain gene expression, while poly(I:C)/with or without swim-treated KO mice have increased TNF brain gene expression and poly(I:C)/swim-treated KO mice have increased HDC brain gene expression. Moreover, poly(I:C)/swim-treated KO mice have higher TNF and lower NT brain gene expression when compared to the corresponding WT mice.

Lastly, poly(I:C)/with or without swim-treated WT mice have increased TNF, IL-6 and HDC brain gene expression, and poly(I:C)/no swim-treated WT mice have increased KC and NT brain gene expression. On the other hand, poly(I:C)/with or without swim-treated KO mice have increased IL-6 and HDC skin gene expression, poly(I:C)/swim-treated KO mice have increased TNF and KC skin gene expression and poly(I:C)/no swim KO mice have increased NT skin gene expression. Moreover, poly(I:C)/swim-

treated KO mice have higher NT skin gene expression compared to the corresponding WT mice. Due to the small number of measurements further studies are needed to investigate this effect further. In summary, CRHR-1 knockout (KO) mice show no reductions in any parameter.

IFNγ, IL-1β, IL-4, IL-9, IL-10, IL-12p70, IL-17 and VEGFα serum levels were below the detection limit, while IL-1α serum levels were similar between the different treatment groups.

2.2.1. Behavioral Assessment

Mice were maintained on a 14:10 hr light-dark cycle. The black bar in locomotor activity indicates the 10 hr dark cycle.

Figure 38. Locomotor activity – WT littermates

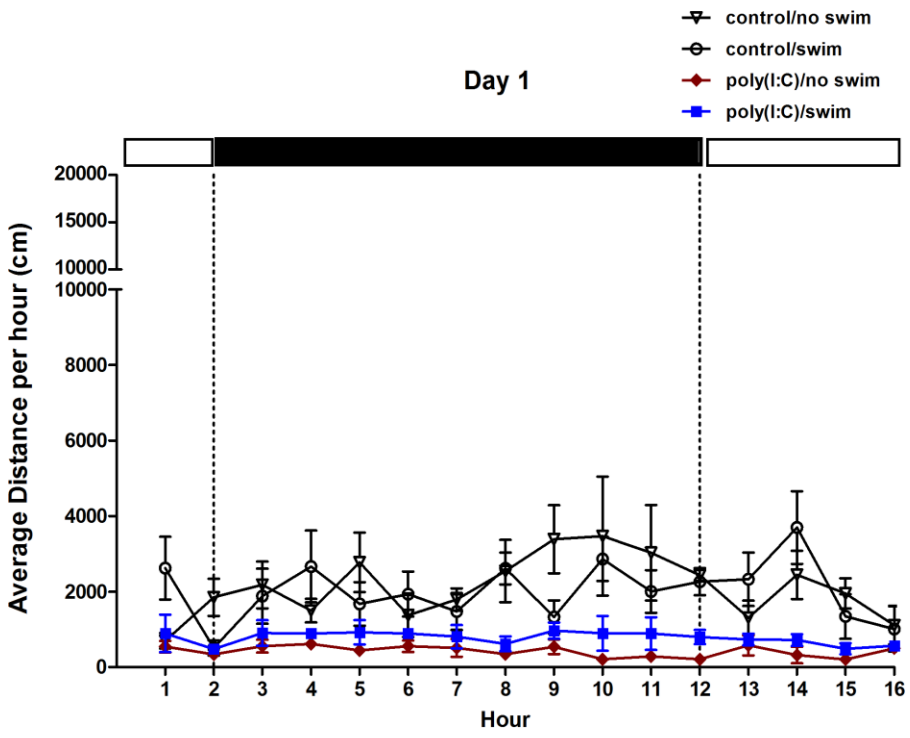
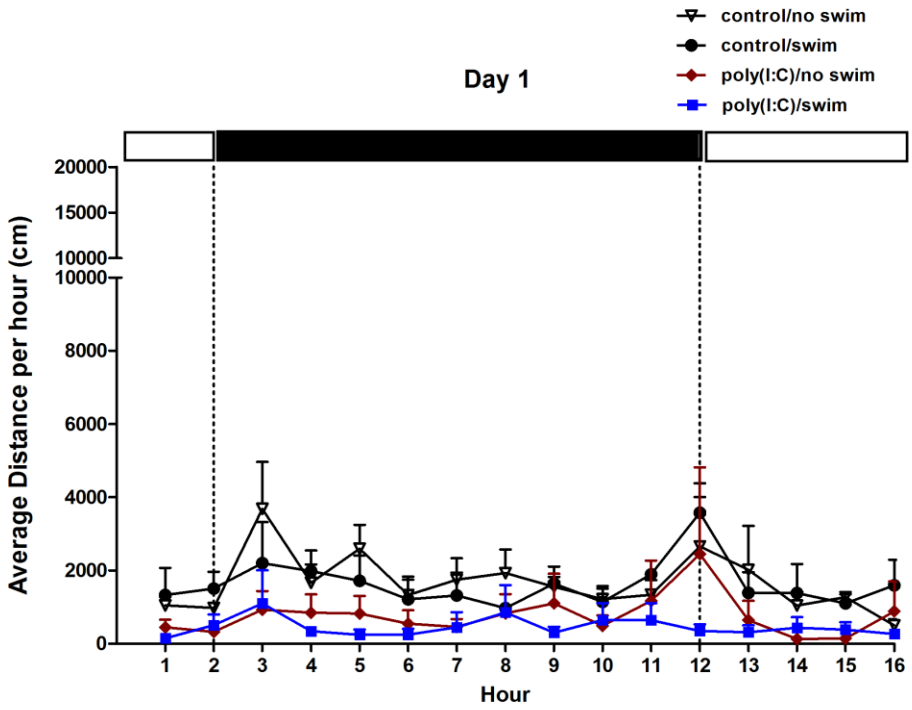


Figure 39. Locomotor activity – CRHR-1 KO



Statistical analysis of the AUC-16 hr and AUC-10 hr in the locomotor activity of the WT littermates (Figure 38), using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and poly(I:C)/no swim and poly(I:C)/swim group ($p<0.0001$ and $p=0.0030$ respectively). Poly(I:C)-treated, with and without swim, groups showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both). Further analysis of the AUC-16 hr and AUC-10 hr (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0079$) and the control/swim ($p=0.0079$), respectively.

Comparison of the max loco-16 hr and max loco-10 hr in the locomotor activity of the WT littermates (Figure 38), using one-way ANOVA followed by Dunnett's multiple comparison test, showed statistical significant differences between the means of the control/no swim and poly(I:C)/no swim and poly(I:C)/swim ($p=0.0060$ and $p=0.0260$ respectively). Poly(I:C)-treated groups, with or without swim, showed reduced locomotor activity in comparison to the control/no swim group ($p<0.05$ for both), except for the poly(I:C)/ swim for the max loco-10 hr . Further analysis of the max loco-16 hr and max loco-10 hr (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups had reduced locomotor activity compared to the control/no swim ($p=0.0079$) and the control/swim ($p=0.0159$) respectively.

Statistical analysis of the AUC-16 hr and AUC-10 hr in the locomotor activity of the CRHR-1 KO mice (Figure 39), using one-way ANOVA followed by Dunnett's multiple comparison test, showed no statistical significant differences between the control/no swim and poly(I:C)/no swim and poly(I:C)/swim group. Poly(I:C)-treated groups did not show reduced locomotor activity in comparison to the control/no swim group. Further analysis of the AUC-16 hr and AUC-10 hr (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and the poly(I:C)/swim groups did not have reduced locomotor activity compared to the control/no swim and the control/swim respectively, except for the AUC-10 hr of the poly(I:C)/swim compared to control/swim group ($p=0.0357$) .

Comparison of the max loco-16 hr and max loco-10 hr in the locomotor activity of the CRHR-1 KO mice (Figure 39), using one-way ANOVA followed by Dunnett's multiple comparison test, showed no statistical significant differences between control/no

swim and poly(I:C)/no swim and poly(I:C)/swim. Poly(I:C)-treated groups did not show reduced maximum locomotor activity in comparison to the control/no swim group. Further analysis of the max loco-16 hr and 10 hr (using the non-parametric Mann Whitney U test) showed that the poly(I:C)/no swim and poly(I:C)/swim group did not have reduced locomotor activity compared to the control/no swim and control/swim, respectively, except for the max loco-10 hr of the poly(I:C)/swim compared to control/swim group (0.0357).

Direct comparison of the AUC-16 hr, AUC-10 hr, max loco-16 hr and max loco-10 hr of the groups treated the same way between the CRHR-1 KO mice and their WT littermates did not yield any statistical significant differences.

2.2.1.1. Conclusion

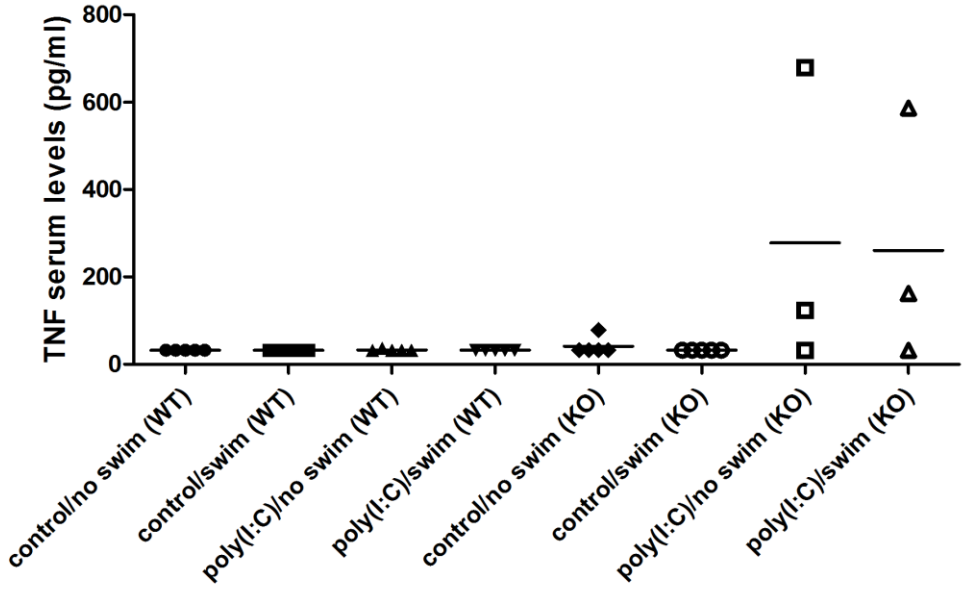
Poly(I:C)-treated, with or without swim, WT mice have decreased locomotor activity when compared to the corresponding control groups of the WT mice, while in the CRHR-1 KO mice these differences were not present .

2.2.2. Biochemical Assessment

2.2.2.1. Serum Levels of Inflammatory Mediators

Figure 40. TNF serum levels - WT vs CRHR-1 KO

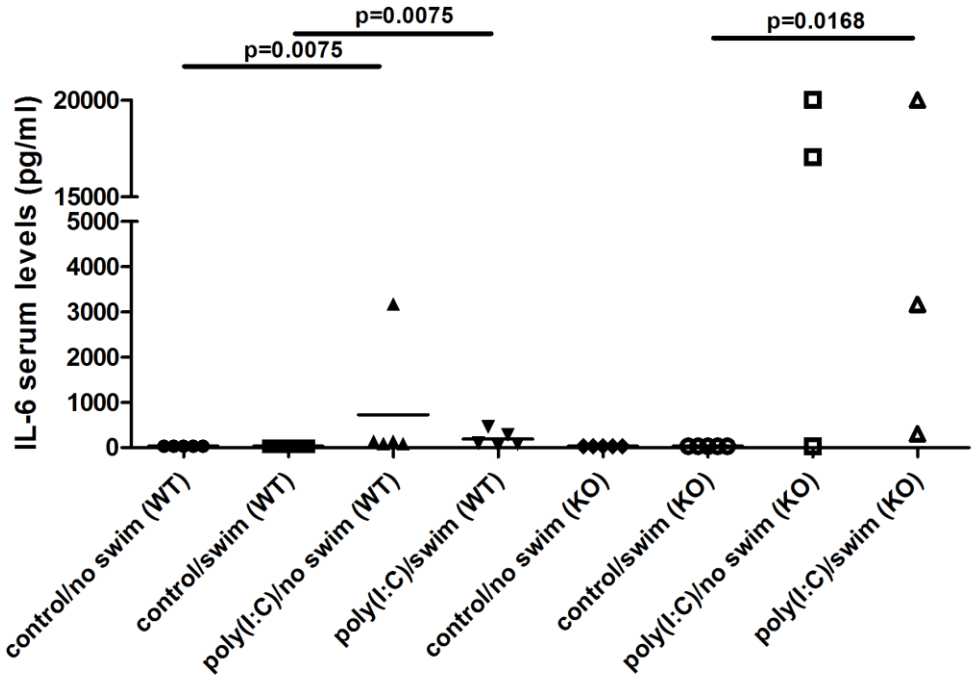
(TNF serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis between the different treatment groups within each category of mice, but also between the same treatment groups of the different categories of mice did not show any differences in the TNF serum levels. There seems to be a tendency for the poly(I:C)-treated mice in the CRHR-1 KO category to have increased TNF serum levels, but further studies need to take place to investigate this further (Figure 40).

Figure 41. IL-6 serum levels - WT vs CRHR-1 KO

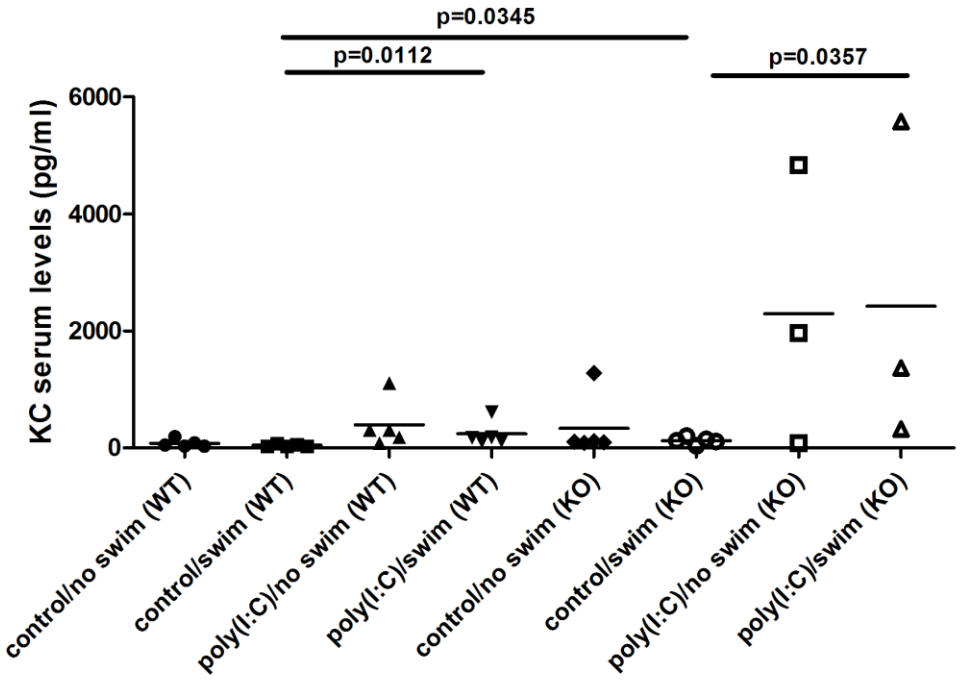
(IL-6 serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, WT mice have increased IL-6 serum levels. Moreover. poly(I:C)-treated/swim CRHR-1 KO mice have increased IL-6 serum levels (Figure 41).

Figure 42. KC serum levels - WT vs CRHR-1 KO

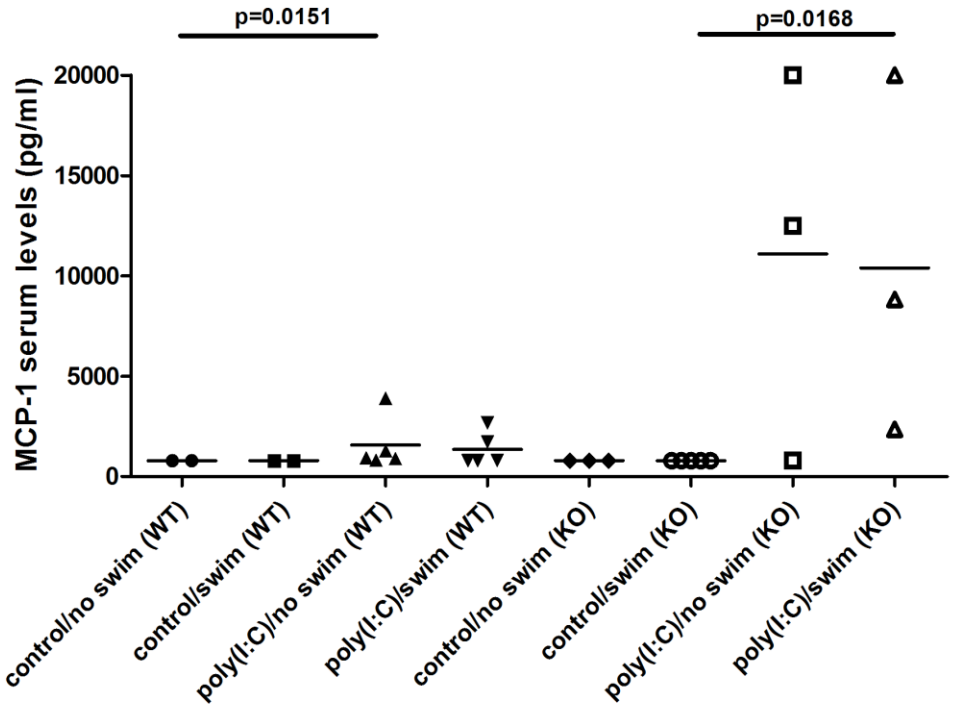
(KC serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)/swim-treated WT and KO mice have increased KC serum levels. Moreover, control/swim KO mice had increased KC serum levels, compared to the corresponding WT (Figure 42).

Figure 43. MCP-1 serum levels - WT vs CRHR-1 KO

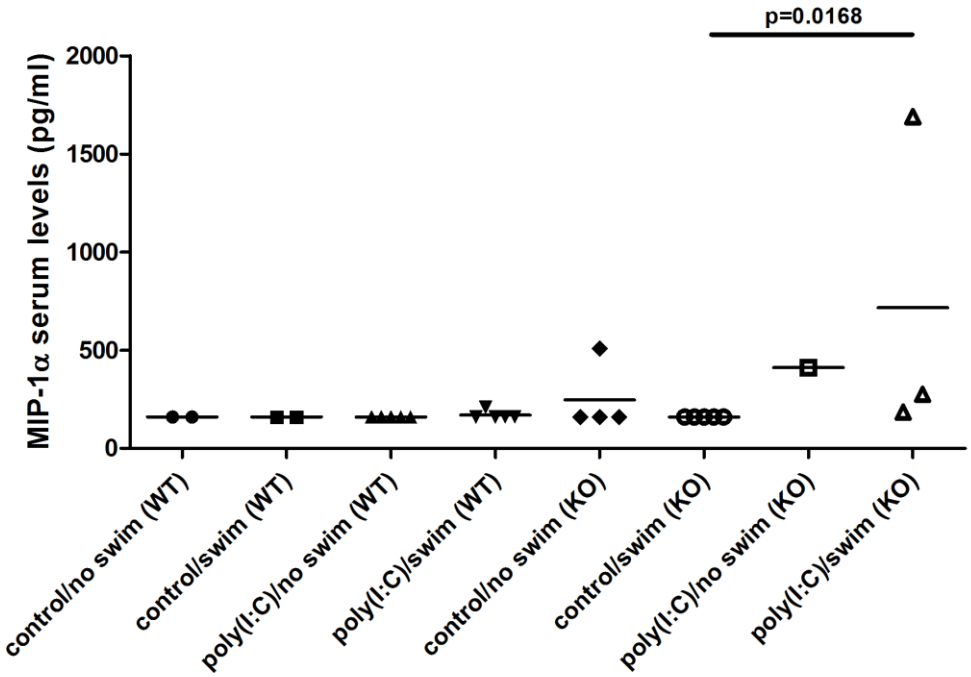
(MCP1 serum levels below the detection limit, <800 pg/ml, are considered as 800 pg/ml)



Statistical analysis showed that poly(I:C)/no swim-treated WT have increased MCP-1 serum levels, while poly(I:C)/swim-treated CRHR-1 KO have increased MCP-1 serum levels (Figure 43).

Figure 44. MIP-1α serum levels - WT vs CRHR-1 KO

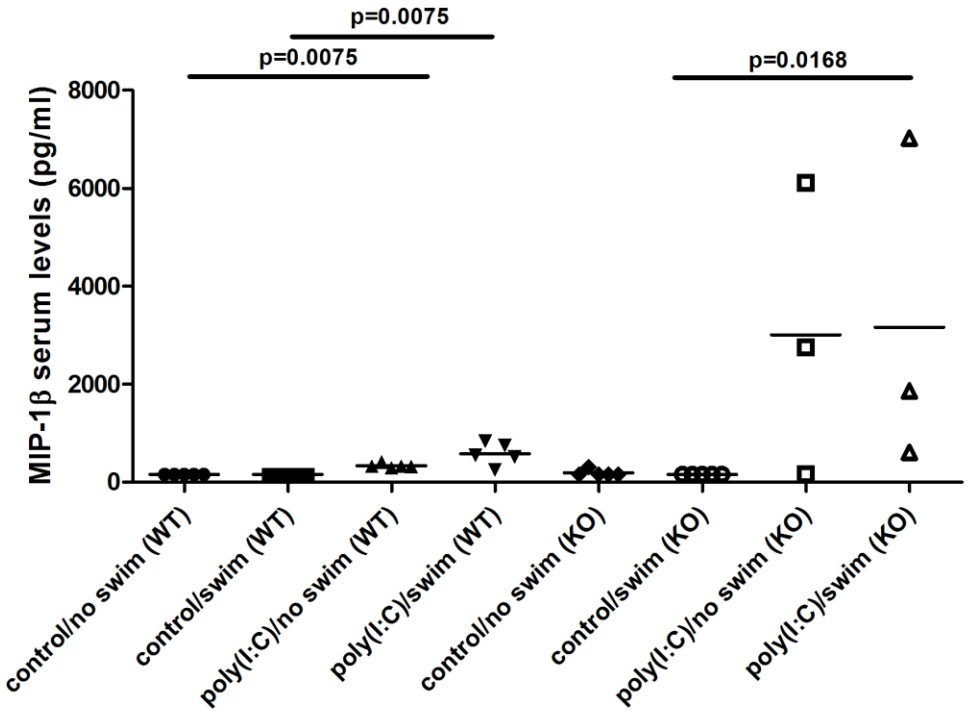
(MIP1α serum levels below the detection limit, <160 pg/ml, are considered as 160 pg/ml)



Statistical analysis showed that poly(I:C)/swim-treated KO mice have increased MIP-1α serum levels when they are provided with low isoflavone diet (Figure 44).

Figure 45. MIP-1β serum levels - WT vs CRHR-1 KO

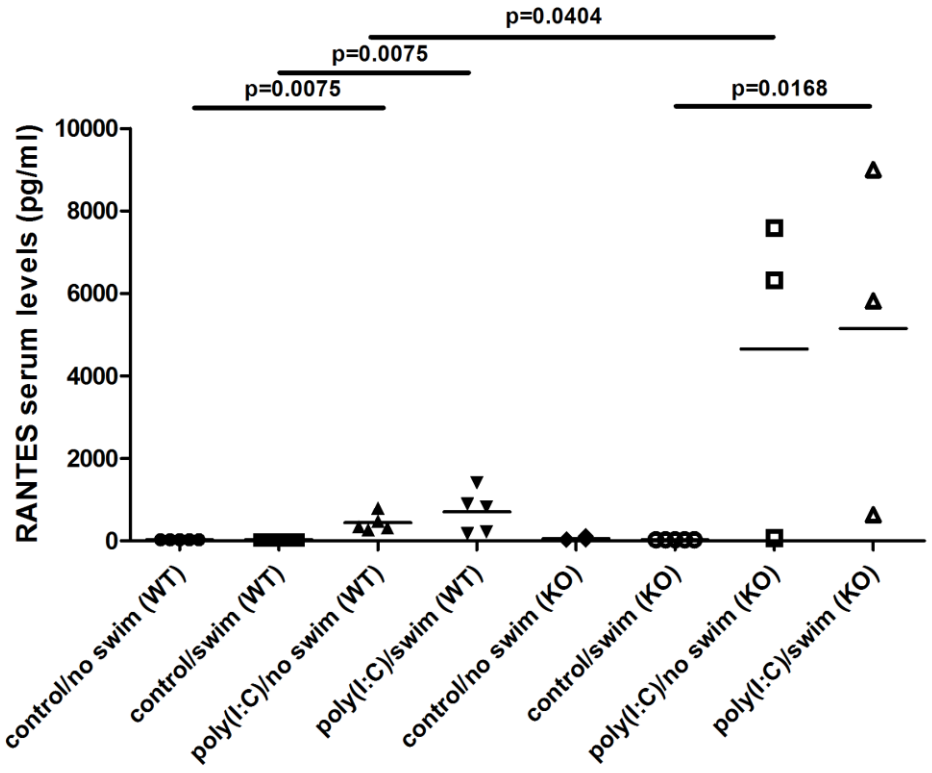
(MIP1β serum levels below the detection limit, <160 pg/ml, are considered as 160 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, WT mice have increased MIP-1β serum levels Moreover, poly(I:C)/swim-treated KO mice have increased MIP-1β serum levels (Figure 45).

Figure 46. RANTES serum levels - WT vs CRHR-1 KO

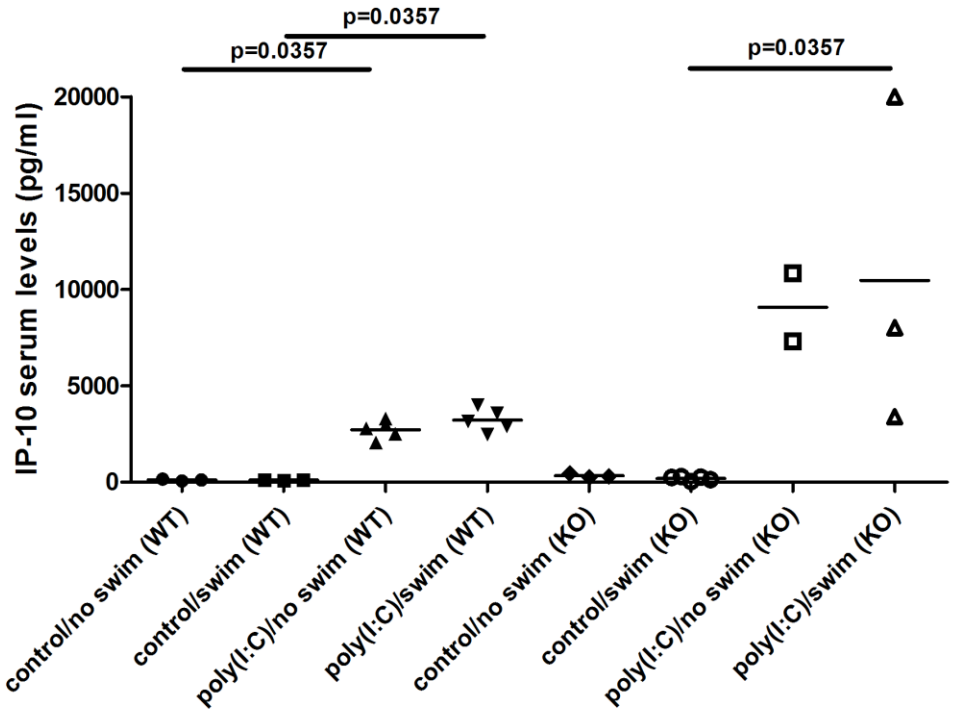
(RANTES serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, WT mice and poly(I:C)/swim KO mice have increased RANTES serum levels. Moreover, poly(I:C)/no swim KO mice have increased RANTES serum levels in comparison with the same group of the WT mice (Figure 46).

Figure 47. IP-10 serum levels - WT vs CRHR-1 KO

(IP-10 serum levels below the detection limit, <32 pg/ml, are considered as 32 pg/ml)



Statistical analysis showed that poly(I:C)-treated, with or without swim, WT and poly(I:C)/swim KO mice have increased IP-10 serum levels (Figure 47).

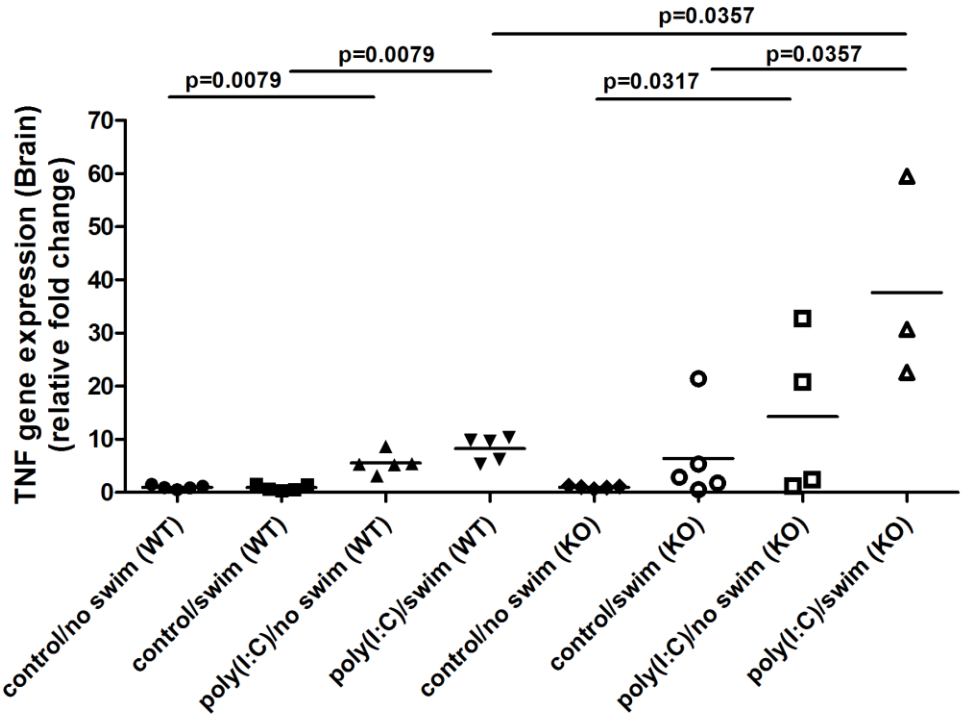
2.2.2.1.1. Conclusion

Overall, poly(I:C)/with or without swim-treated WT mice have increased serum levels of IL-6, MIP-1β, RANTES, and IP-10, while poly(I:C)/no swim-treated WT mice have increased KC and MCP1 serum levels. On the other hand, poly(I:C)/with or without swim-treated KO mice have increased IL-6 serum levels, while poly(I:C)/swim-treated KO mice have increased MCP-1, MIP1α, MIP1β, RANTES and IP-10. Moreover, poly(I:C)/no swim-treated KO mice have higher RANTES serum levels compared to the corresponding WT, while control/swim KO mice have higher KC serum levels compared to the corresponding WT mice.

There seems to be an increase in the TNF serum levels of the poly(I:C)/treated KO animals, but due to the small number of measurements further studies need to take place to investigate this further.

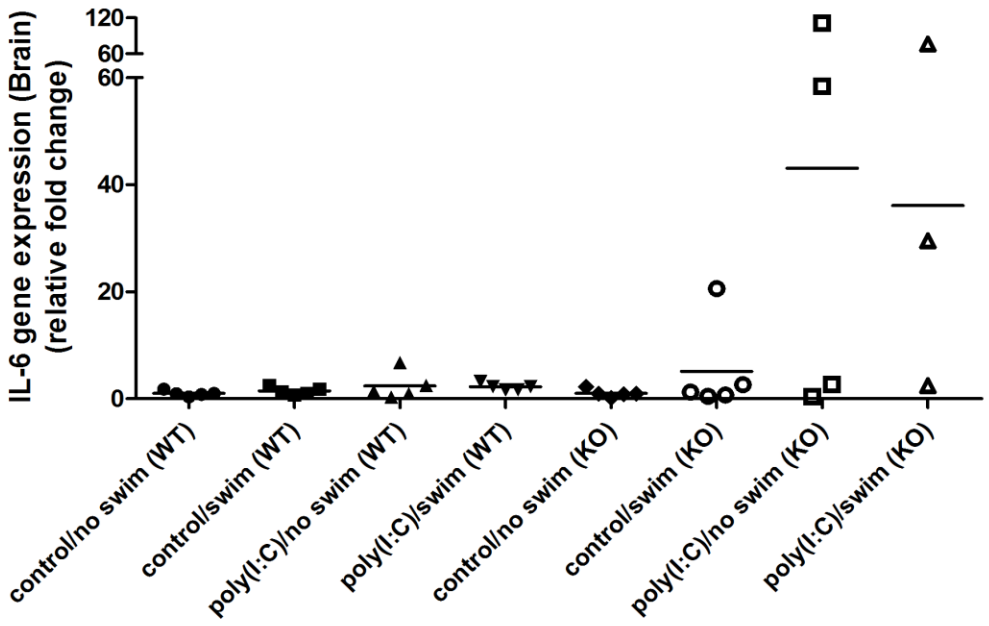
2.2.2.2. Brain gene expression of inflammatory mediators

Figure 48. TNF gene expression (Brain) - WT vs CRHR-1 KO



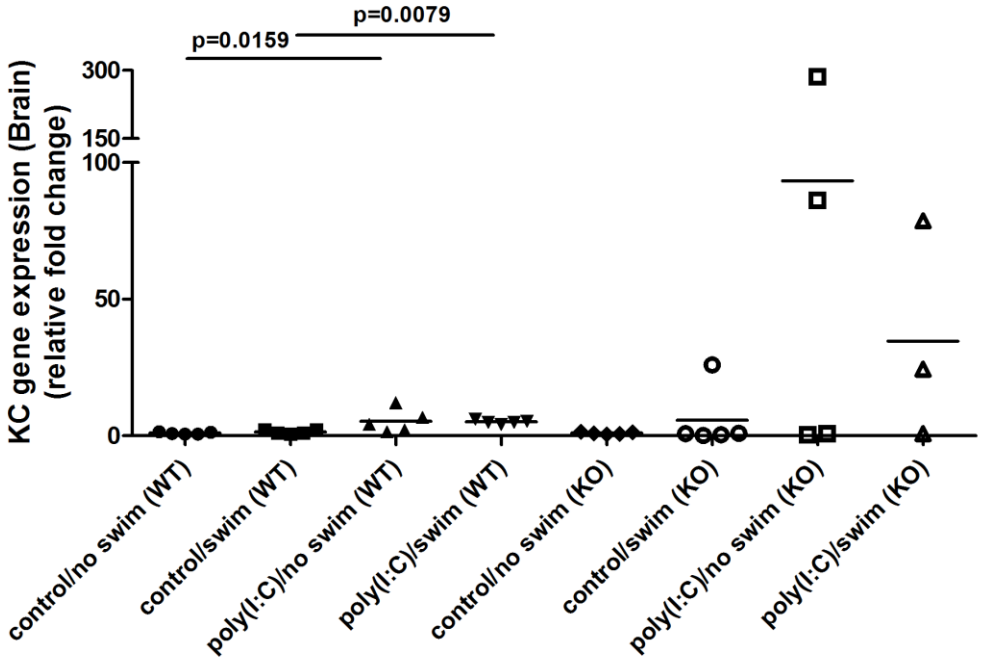
Statistical analysis showed that poly(I:C)-treated, with or without swim, WT and KO mice have increased brain TNF gene expression. Moreover, poly(I:C)/swim-treated KO mice have increased TNF brain gene expression compared to the corresponding WT mice (Figure 48).

Figure 49. IL-6 gene expression (Brain) - WT vs CRHR-1 KO



Statistical analysis between the different treatment groups within each category of mice, but also between the same treatment groups of the different categories of mice did not show any differences in the TNF serum levels. There seems to be a tendency for the poly(I:C)-treated mice in the CRHR-1 KO category to have increased IL-6 brain gene expression, but further studies need to take place to investigate this further (Figure 49).

Figure 50. KC gene expression (Brain) - WT vs CRHR-1 KO



Statistical analysis showed that poly(I:C)-treated, with or without swim, WT mice have increased brain KC gene expression. There seems to be a tendency for the poly(I:C)-treated mice in the CRHR-1 KO category to have increased KC brain gene expression, but further studies need to take place to investigate this further (Figure 50).

Figure 51. HDC gene expression (Brain) - WT vs CRHR-1 KO

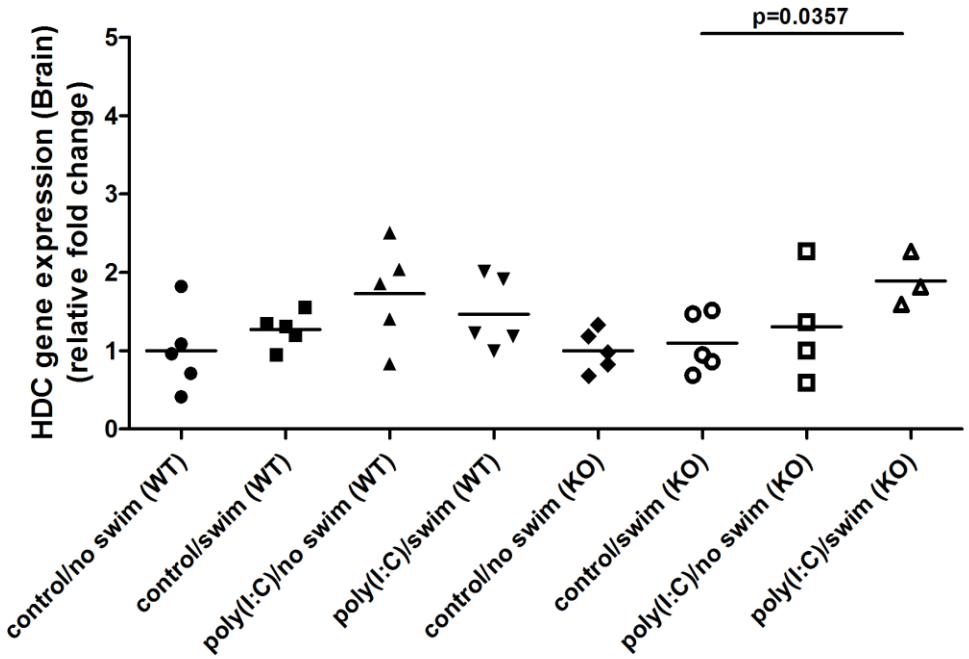
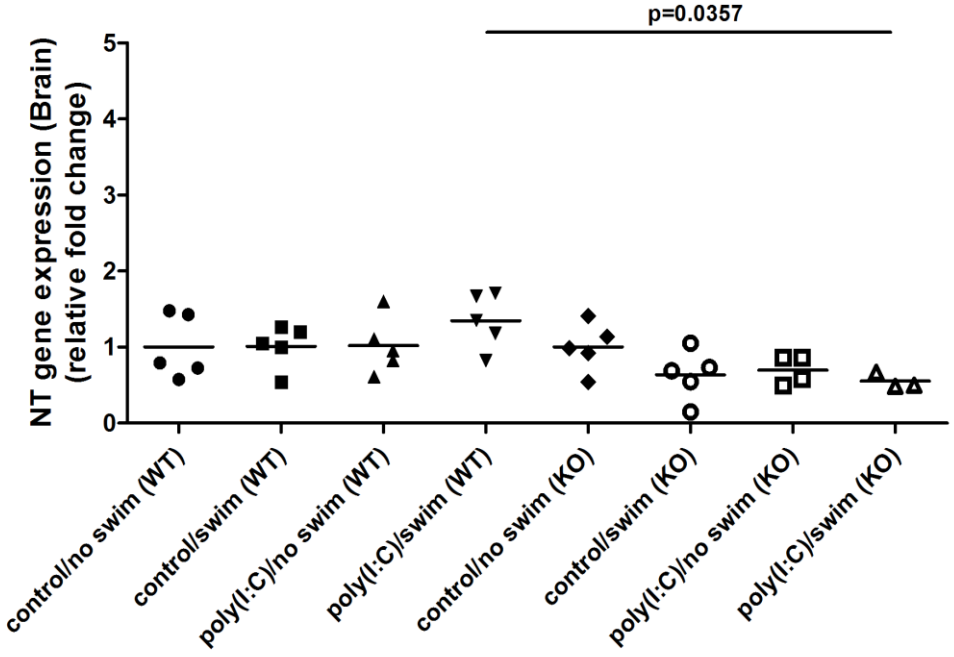


Figure 52. NT gene expression (Brain) - WT vs CRHR-1 KO



Statistical analysis for the brain HDC gene expression showed that poly(I:C)/swim-treated KO mice have increased brain HDC gene expression (Figure 51).

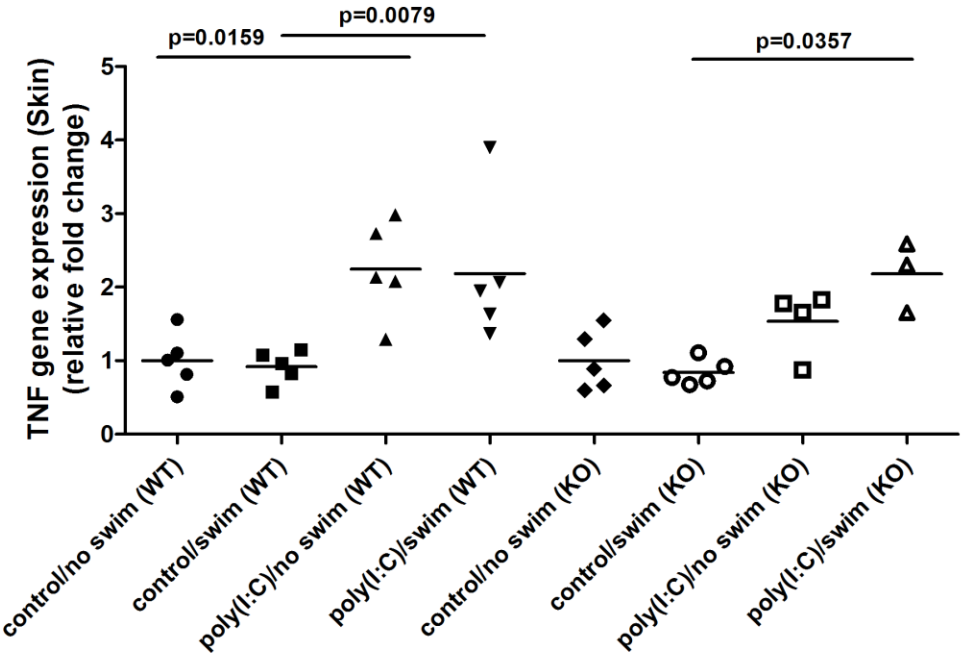
Statistical analysis for the brain NT gene expression showed that poly(I:C)/swim-treated KO mice have increased brain HDC gene expression compared to the corresponding WT (Figure 52).

2.2.2.2.1. Conclusion

Overall, poly(I:C)/with or without swim-treated WT mice have increased TNF and KC brain gene expression, while poly(I:C)/with or without swim-treated KO mice have increased TNF brain gene expression and poly(I:C)/swim-treated KO mice have increased HDC brain gene expression. Moreover, poly(I:C)/swim-treated KO mice have higher TNF and lower NT brain gene expression when compared to the corresponding WT mice.

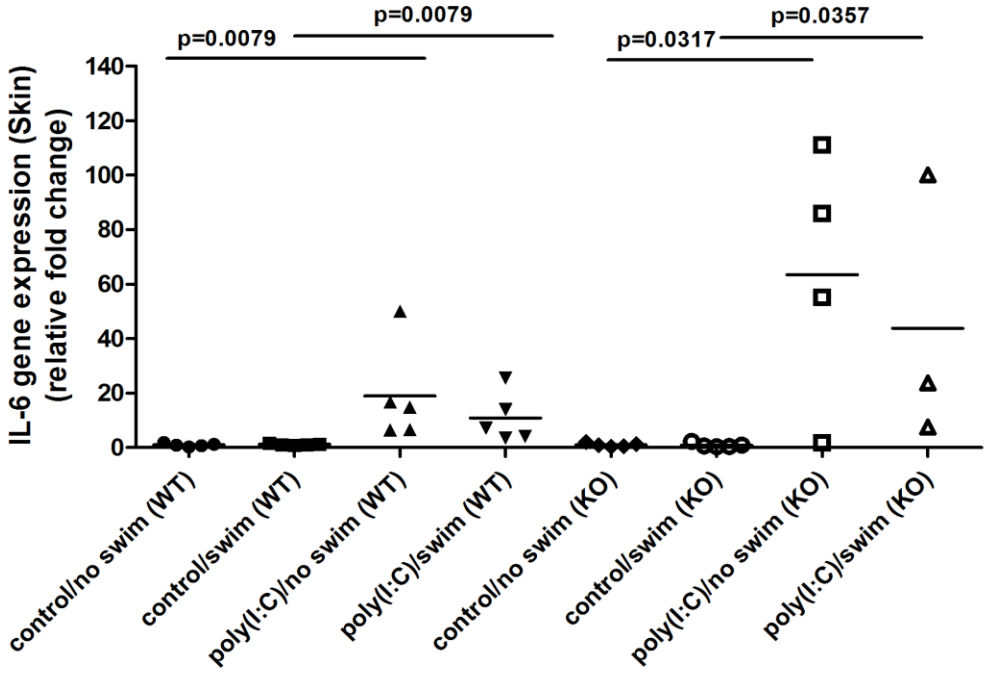
2.2.2.3. Skin gene expression of inflammatory mediators

Figure 53. TNF gene expression (Skin) - WT vs CRHR-1 KO



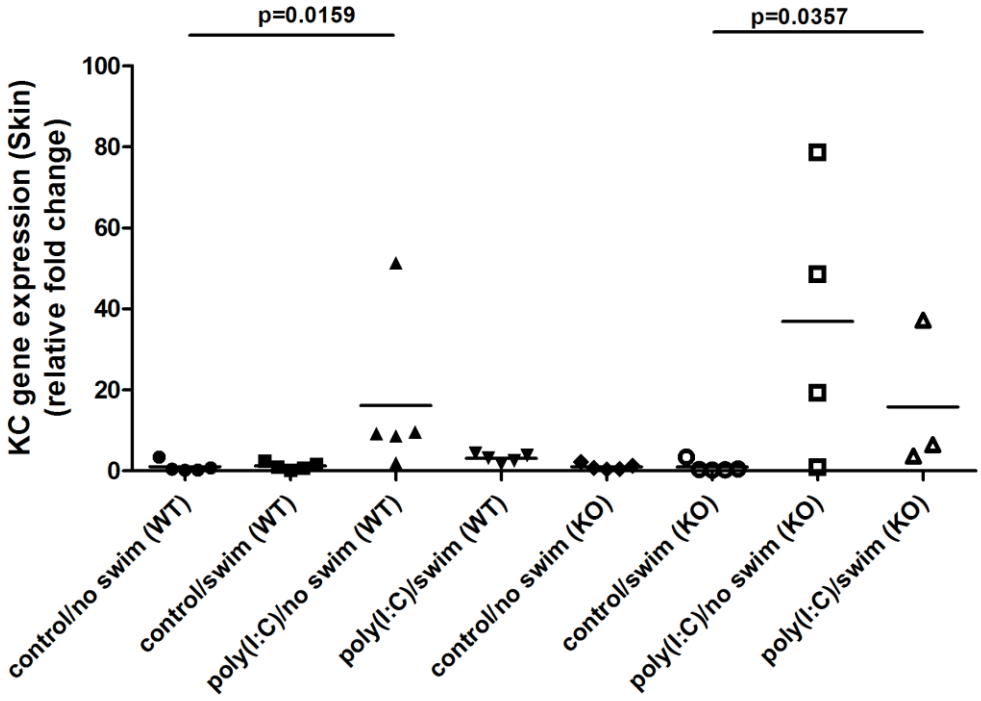
Statistical analysis showed that poly(I:C)-treated, with or without swim WT mice and poly(I:C)/swim KO mice have increased skin TNF gene expression (Figure 53).

Figure 54. IL-6 gene expression (Skin) - WT vs CRHR-1 KO



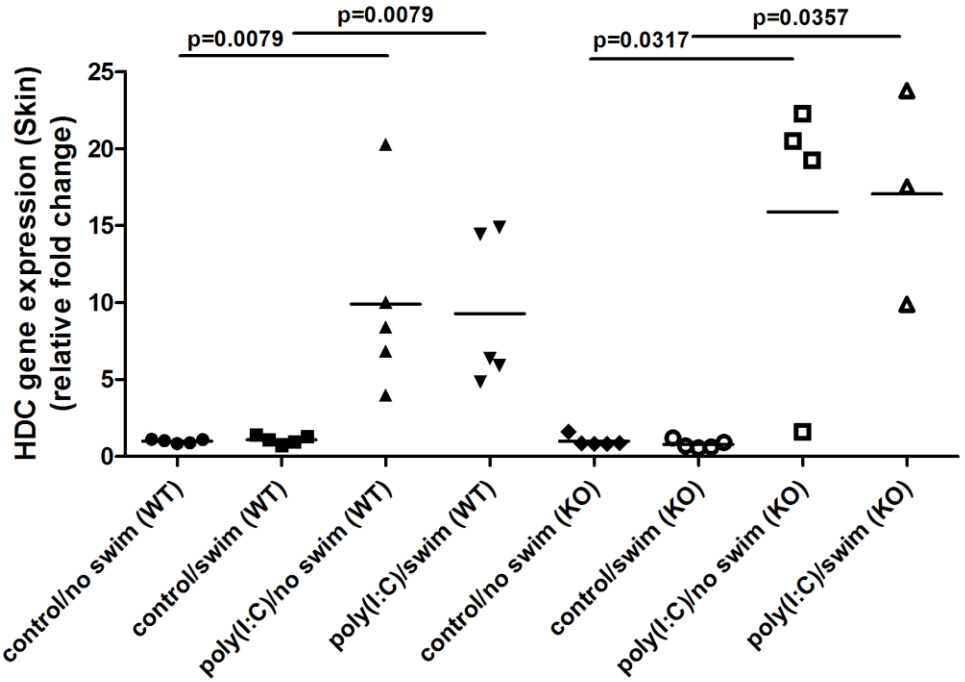
Statistical analysis showed that poly(I:C)-treated, with or without swim, WT and KO mice have increased skin IL-6 gene expression (Figure 54).

Figure 55. KC gene expression (Skin) - WT vs CRHR-1 KO



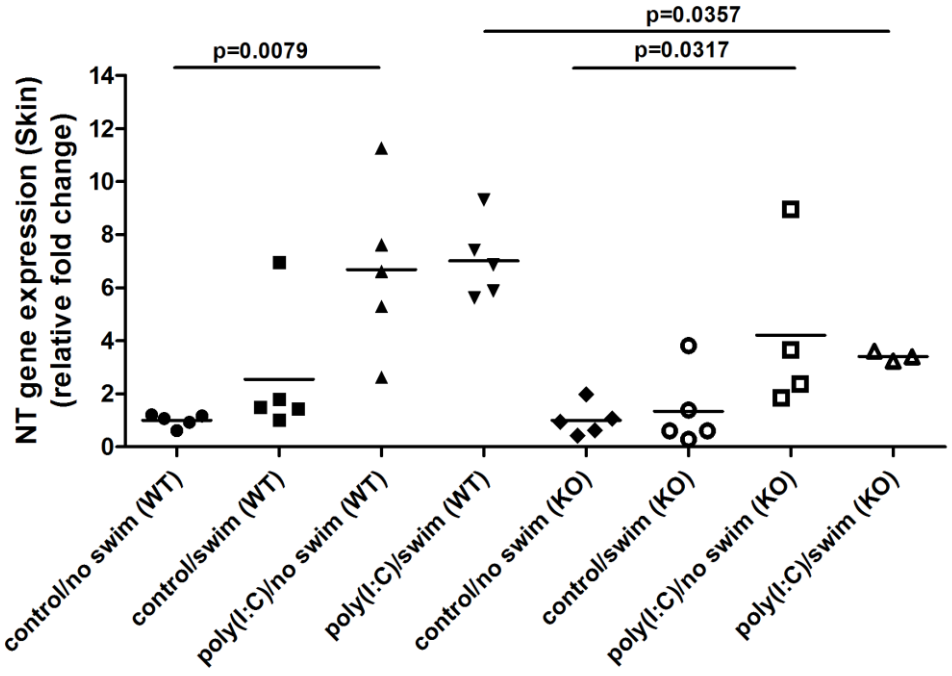
Statistical analysis showed that poly(I:C)/no swim-treated WT mice and poly(I:C)/swim-treated mice have increased skin KC gene expression (Figure 54).

Figure 56. HDC expression (Skin) - WT vs CRHR-1 KO



Statistical analysis showed that poly(I:C)-treated, with or without swim, WT and KO mice have increased skin HDC gene expression (Figure 56).

Figure 57. NT expression (Skin) - WT vs CRHR-1 KO



Statistical analysis showed that poly(I:C)/no swim-treated WT and KO mice have increased skin NT gene expression. Moreover, poly(I:C)/swim KO mice have increase NT skin gene expression compared to the corresponding WT mice (Figure 57).

2.2.2.3.1. Conclusion

Overall, poly(I:C)/with or without swim-treated WT mice have increased TNF, IL-6 and HDC brain gene expression, and poly(I:C)/no swim-treated WT mice have increased KC and NT brain gene expression. On the other hand, poly(I:C)/with or without swim-treated KO mice have increased IL-6 and HDC skin gene expression, poly(I:C)/swim-treated KO mice have increased TNF and KC skin gene expression and poly(I:C)/no swim KO mice have increased NT skin gene expression. Moreover, poly(I:C)/swim-treated KO mice have higher NT skin gene expression compared to the corresponding WT mice.

In summary, CRHR-1 knockout (KO) mice show no differences in any parameter, which is no surprising as the swim stress did not change any parameters over and beyond that with poly(I:C).

Chapter 3. Human Studies - *In vitro*

3.1. Poly(I:C) Effect on Human LAD2 MC

To investigate the effect of poly(I:C) *in vitro* and the role of MC in the parameters studied *in vivo* above, I treated human leukemic LAD2 MC with poly(I:C) ± NT ± CRH.

LAD2 cells were either stimulated with poly(I:C) (10, 25, and 50 μg/ml) alone or with poly(I:C) (10 μg/ml) with or without NT (1, and 10 μM) or CRH (1 μM) for 30 min or 24 h. Supernatant fluids were collected and analyzed for β-hexosaminidase release (30 min), or TNF, IL-8, and VEGFα levels (24 h) (measured by ELISA). Cell pellets were collected and analyzed for TNF, IL-8, MCP1, and VEGFα gene expression (measured by qPCR). Treatment with SP (1 μM) was used as a positive control of MC stimulation.

In vitro experiments using human cultured MC show that poly(I:C) alone (10, 25 and 50 μg/ml) does not cause degranulation or release of TNF, VEGFα, or IL-8. However, poly(I:C) (10 μg/ml) significantly increases TNF gene expression and when used together with CRH, NT or SP at 24 h, TNF gene expression is augmented. The effect of poly(I:C) *in vivo* may be due to a synergistic action with NT or SP, as suggested by the *in vitro* experiments (Figures 58-64).

Figure 58. Beta-hex Release – Poly(I:C) different concentrations

Results are expressed as the percentage of β-hexosaminidase released over the total in control and stimulated LAD2 cells.

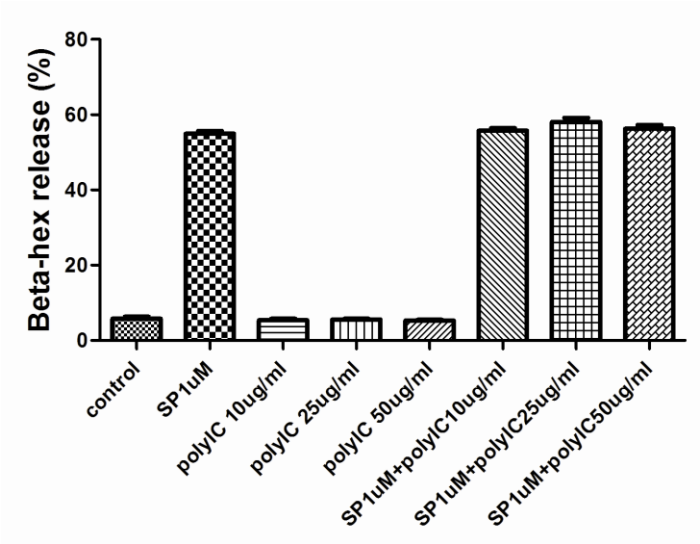


Figure 59. TNF Release – Poly(I:C) different concentrations

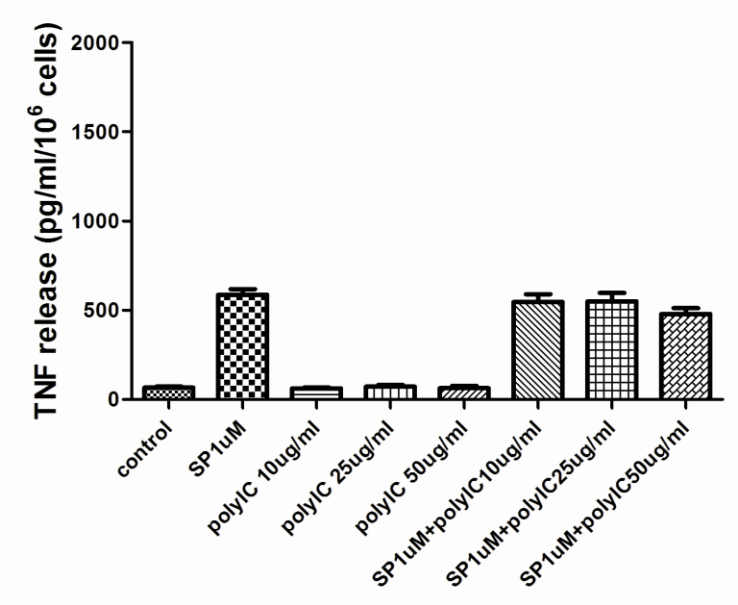


Figure 60. VEGF Release – Poly(I:C) different concentrations

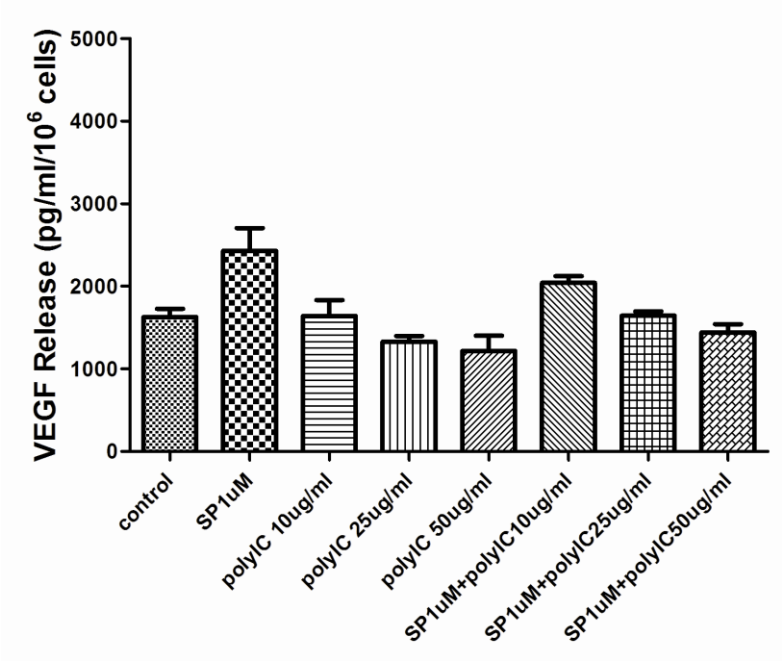


Figure 61. IL-8 Release – Poly(I:C) different concentrations

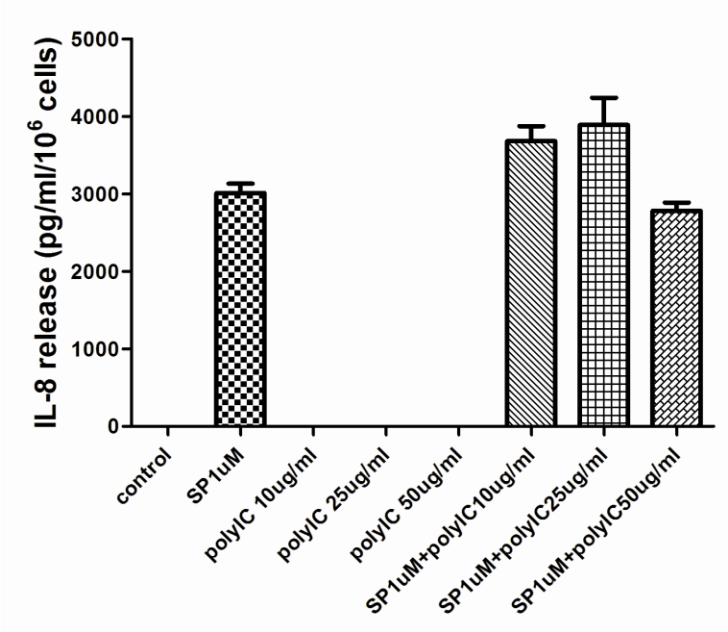


Figure 62. B-hex release - Poly(I:C) +/- NT +/- CRH

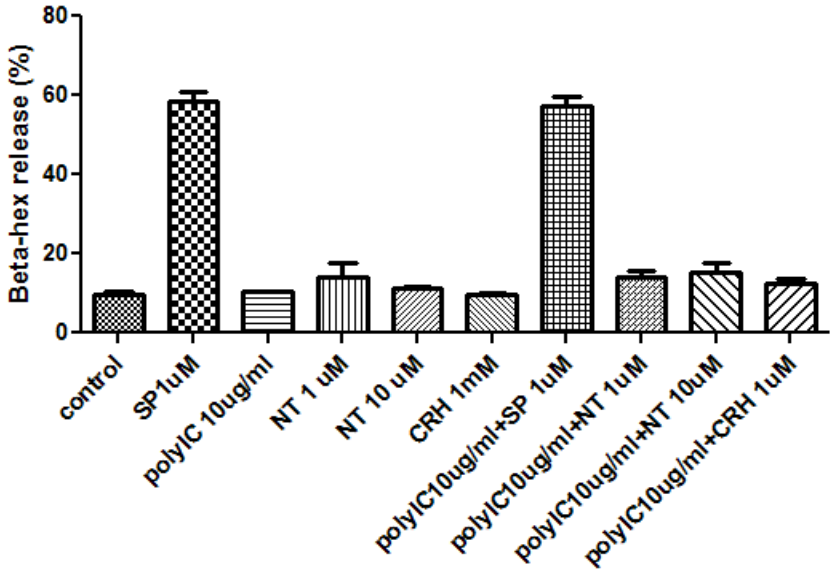


Figure 63. TNF release – Poly(I:C) +/- NT +/- CRH

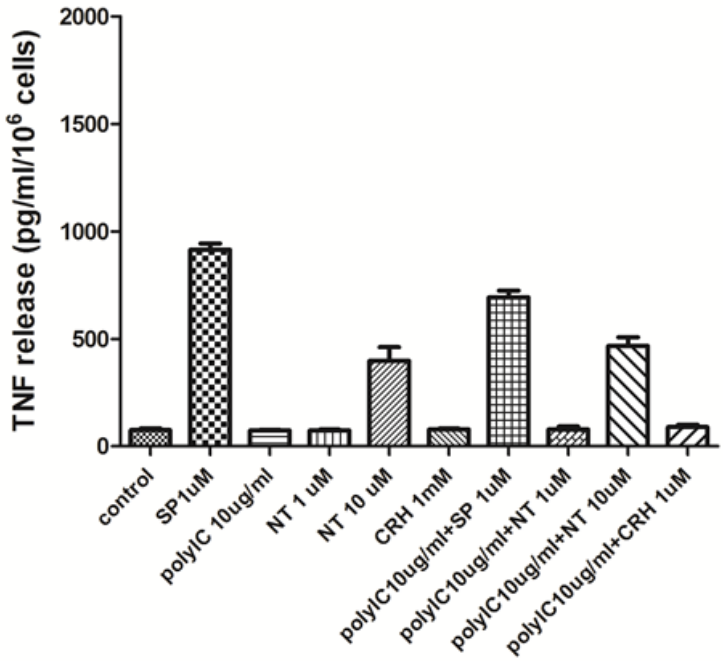
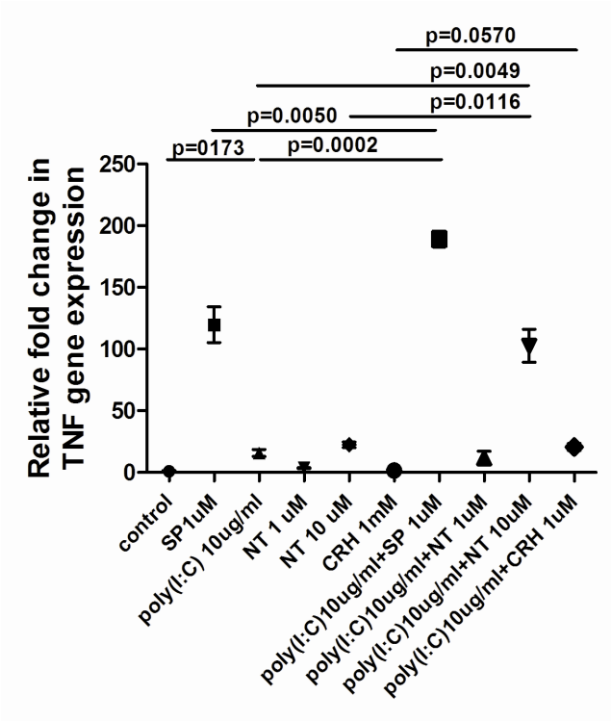


Figure 64. TNF gene expression– Poly(I:C) +/- NT +/- CRH

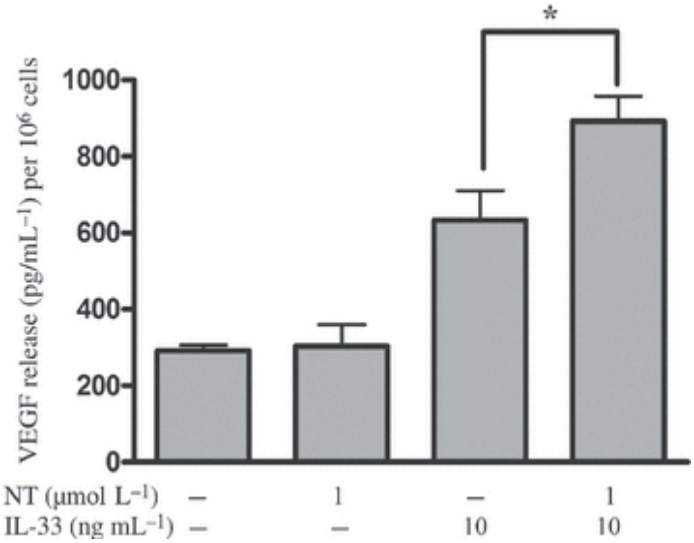
Results were normalized against the endogenous gene, GAPDH, were expressed relative to the mean of the control for each gene, and are presented as mean +/- SD.



3.2. NT and IL-33 Effect on Human LAD2 MC

To investigate the effect of NT and IL-33 *in vitro* and the role of MC in the parameters studied *in vivo* above, I treated human leukemic LAD2 MC with either NT (1 μmol/L) alone or together with interleukin IL-33 (10 ng/mL) for 24 h. NT augmented the IL-33-induced VEGF release from about 600 pg/ml to 800 pg/ml ($n = 3$, $p<0.05$) (Figure 65).

Figure 65. VEGF release from MC - NT +/- IL-33



Chapter 4. Human Studies – Human Samples

Immediate hypersensitivity skin reaction are noted in patients with CFS[229], while there are dermal IgG deposits and increase of MC [231, 232] with abnormal overexpression of MC in skin biopsies [453] of fibromyalgia patients, a disease comorbid to CFS. Moreover, CU, known to involve MV, is usually associated with fibromyalgia[233, 466]. Since it was difficult to identify skin lesion from CFS patients, I obtained and analyzed biopsies and serum from patients with AD, suggestive of similar skin hypersensitivity, focusing my studies in the NT and CRH expression in those patients. Additionally I analyzed biopsies and serum from patients with PS.

Serum and full-length 3-mm^3 punch skin biopsies were collected from AD, PS and CU patients, according to the Methods, Chapter 4. CRH serum levels were measured using ELISA, while NT and cytokine serum levels were measured blindly by Millipore, using Milliplex assay. Skin biopsies were analyzed for gene expression using qPCR or immunohistochemistry.

Overall, NT serum levels and skin gene expression were increased in AD patients[471]. Additionally, NT serum levels were increased in PS patients, while NT and NTR-1 gene expression in lesional PS skin was decreased compared to controls[472]. Moreover, serum CRH levels were increased in AD and PS patients, while CRHR-1 skin gene expression was decreased[473].

4.1. Inflammatory Mediator Measurements in Serum of AD and PS Patients

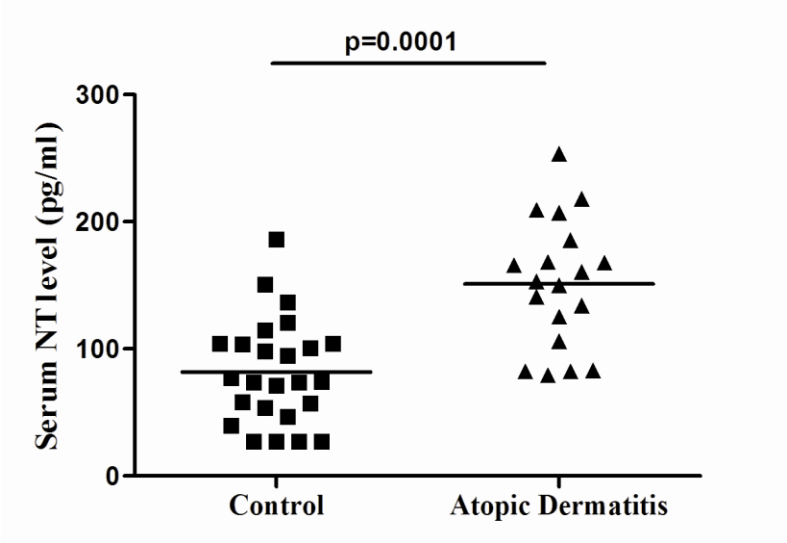
For inflammatory mediator serum measurements, the mean age for patients with AD (n = 18; 12 women and 6 men) was 38 ± 20 years, while the mean age for controls (n = 33; 14 women and 19 men) was 48 ± 18 years. The mean age for patients with PS (n = 56; 26 women and 30 men, 31 of whom had PASI scores of 10-50) was 40 ± 14 years, while the mean age for controls (n = 33; 14 women and 19 men) was 48 ± 18 years. There was no statistical difference in the mean age between controls and patients.

In AD and PS patients, serum levels of IL-6, IL-9, IL-33, TNFβ, and thymic stromal lymphopoietin were undetectable, except for VEGFα and IL-8. Serum VEGFα levels were not different in patients with AD (291.4 ± 280.1 pg/ml) as compared with those in controls (257.2 ± 123.8 pg/ml). Serum IL-8 levels were lower (p=0.0171) in patients with AD (19.7 ± 17.2 pg/ml) than in controls (31.3 ± 32.2 pg/ml). There was a reasonable correlation between serum CRH levels and STAI (STATE now) scores (Pearson r = 0.55; p=0.041; n = 14) for patients with psoriasis with PASI scores of more than 10, but there was no correlation with STAI (STATE trait). There was no correlation between STAI scores and CRH serum measurements or VEGFα gene expression for the patients with AD.

Serum VEGFα levels were not different in patients with PS (411.9 ± 280 pg/ml) as compared with those in controls (257 ± 123.8 pg/ml). However, serum VEGFα levels in patients with PS with PASI scores of more than 10 were significantly increased (p=0.0286), while in patients with PS with PASI scores of less than 10 were not. There was no difference between serum IL-8 levels in patients with PS (25.7 ± 27.5 pg/ml) and those in controls (31.3 ± 31.2 pg/ml).

NT serum levels were measured in AD patients (*n* = 19; six male, mean age 28 ± 10 years and 13 female, mean age 41 ± 23 years) compared with controls (*n* = 25; 15 male, mean age 49 ± 15 years and 10 female, mean age 43 ± 12 years).

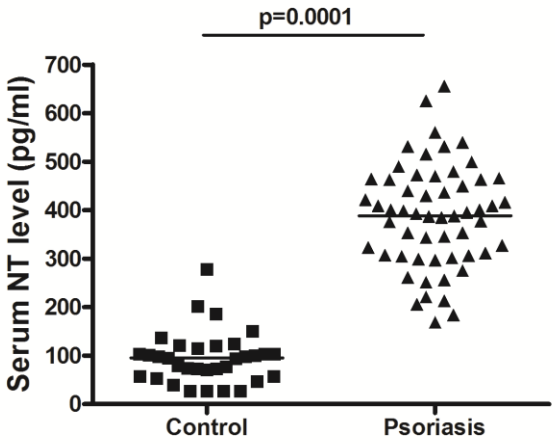
Figure 66. NT serum levels in AD patients and controls.
Horizontal bars indicate the means



Serum levels of NT were significantly increased at 151.2 ± 50.5 pg/ml in AD patients compared to 81.9 ± 41.2 pg/ml in controls (p=0.0001). There was no apparent correlation between NT levels and clinical status (Figure 66).

Serum levels of NT were measured in PS patients (n=56; 30 male, mean age 48 ± 19 years and 26 female, mean age 40 ± 14 years) compared to controls (n=33; 14 male, mean age 53 ± 16 years and 19 female, mean age 49 ± 19 years).

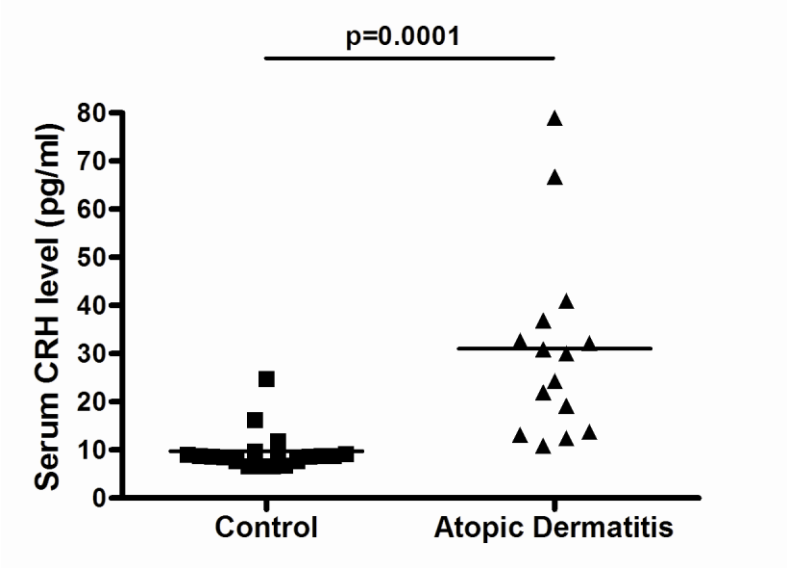
Figure 67. NT serum levels in PS patients and controls.
Horizontal bars indicate the means.



There was a statistically significant increase of serum NT levels in PS patients (411.9 ± 280 pg/ml) compared to controls (95.4 ± 53.6 pg/ml) (Figure 67). It is interesting that the mean levels in PS patients are apparently more than double than those seen in AD patients. This difference may reflect the degree of inflammation seen in these two diseases.

For CRH serum measurements in AD patients, the mean age for patients with AD (n = 15; 9 women and 6 men) was 36 ± 18 years, while the mean age for controls (n = 19; 10 women and 9 men) was 47 ± 16 years.

Figure 68. CRH serum levels in AD patients and controls.



Serum CRH levels were higher (p=0.0001) in patients with AD (31.0 ± 19.5 pg/mL) than in controls (9.7 ± 4.2 pg/mL) (Figure 68).

For CRH serum measurements in PS patients, the mean age for patients with PS (n = 48; 23 women and 25 men, 27 of whom had a PASI score of 10-50) was 46 ± 17 years, while the mean age for controls (n = 19; 10 women and 9 men) was 47 ± 16 years.

Figure 69. CRH serum levels in PS patients and controls

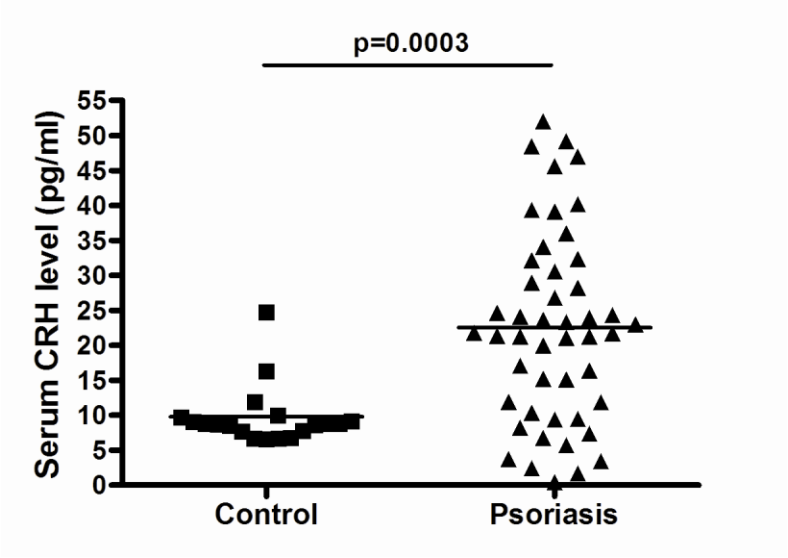
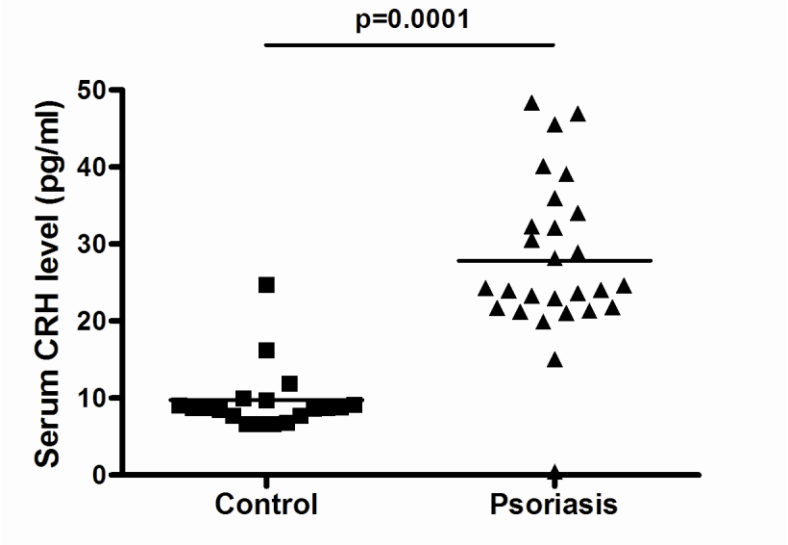


Figure 70. CRH serum levels in PS patients with PASI score>10



Serum CRH levels were higher (p=0.0001) in patients with PS (22.5 ± 13.7 pg/ml) than in controls (9.7 ± 4.2 pg/ml) (Figure 70). When patients with PS were separated into

those with PASI scores of less than 10 (Figure 69)and those with PASI scores of more than 10 (Figure 70), there was a statistically significant increase (p=0.0001) in patients with PS with PASI scores of more than 10 (Figure 71) but not in those with PASI scores of less than 10 (data not shown).

4.2. Gene Expression in Skin of PS and AD Patients

For CRHR-1 gene expression assays, the mean age for patients with AD (n = 16; 10 women and 6 men) was 35 ± 21 years, while the mean age for controls (n = 30; 22 women and 8 men) was 43 ± 15 years. Skin CRHR-1 gene expression was lower (p=0.0001) in affected samples from patients with AD (0.2 ± 0.1) than in those from controls (1 ± 0.7). Skin VEGFα gene expression was lower (p=0.0001) in affected samples from patients with AD (0.2 ± 0.1) than in those from controls (1.0 ± 0.7).

For CRHR-1 gene expression assays, the mean age for patients with PS (n = 40; 19 women and 21 men) was 46 ± 17 years, while the mean age for controls (n = 30; 22 women and 8 men) was 43 ± 15 years. Skin CRHR-1 gene expression was lower (p=0.0001) in affected skin samples from patients with PS (0.1 ± .01) than in those from controls (1 ± 0.7). There was also statistically significant lower (p=0.0001) CRHR-1 gene expression in the samples obtained from affected versus unaffected PS skin, while there was no statistically significant difference in CRHR-1 gene expression between the control and the non-lesion samples. Lesional skin CRH gene expression was undetectable in PS. Skin VEGFα gene expression was lower (p=0.0121) in affected samples from patients with PS (0.6 ± 0.4) than in those from controls (1 ± 0.7). There was statistically

significant lower VEGFα gene expression (p=0.0009) among the samples obtained from affected and from unaffected PS skin.

There was a positive correlation between CRH serum levels and VEGFα gene expression in patients with PS with PASI scores of more than 10 (Pearson $r = 0.44$; p=0.021; n = 22), which is known to be associated with increased skin vascularization. This finding implies that high serum CRH level correlates best only in patients with severe PS.

Skin NT gene expression was significantly increased in affected skin samples from patients with AD (2.2 ± 2.5; n=16; 10 male, mean age 28 ± 10 years and six female, mean age 40 ± 24 years) compared with controls (1 ± 1.2; n=25; six male, mean age 35 ± 16 years and 19 female, mean age 52 ± 17 years; p=0.0194). There was no significant difference in skin NTR-1 gene expression between affected skin samples from patients with AD (1.6 ± 1.6) and controls (1 ± 0.8).

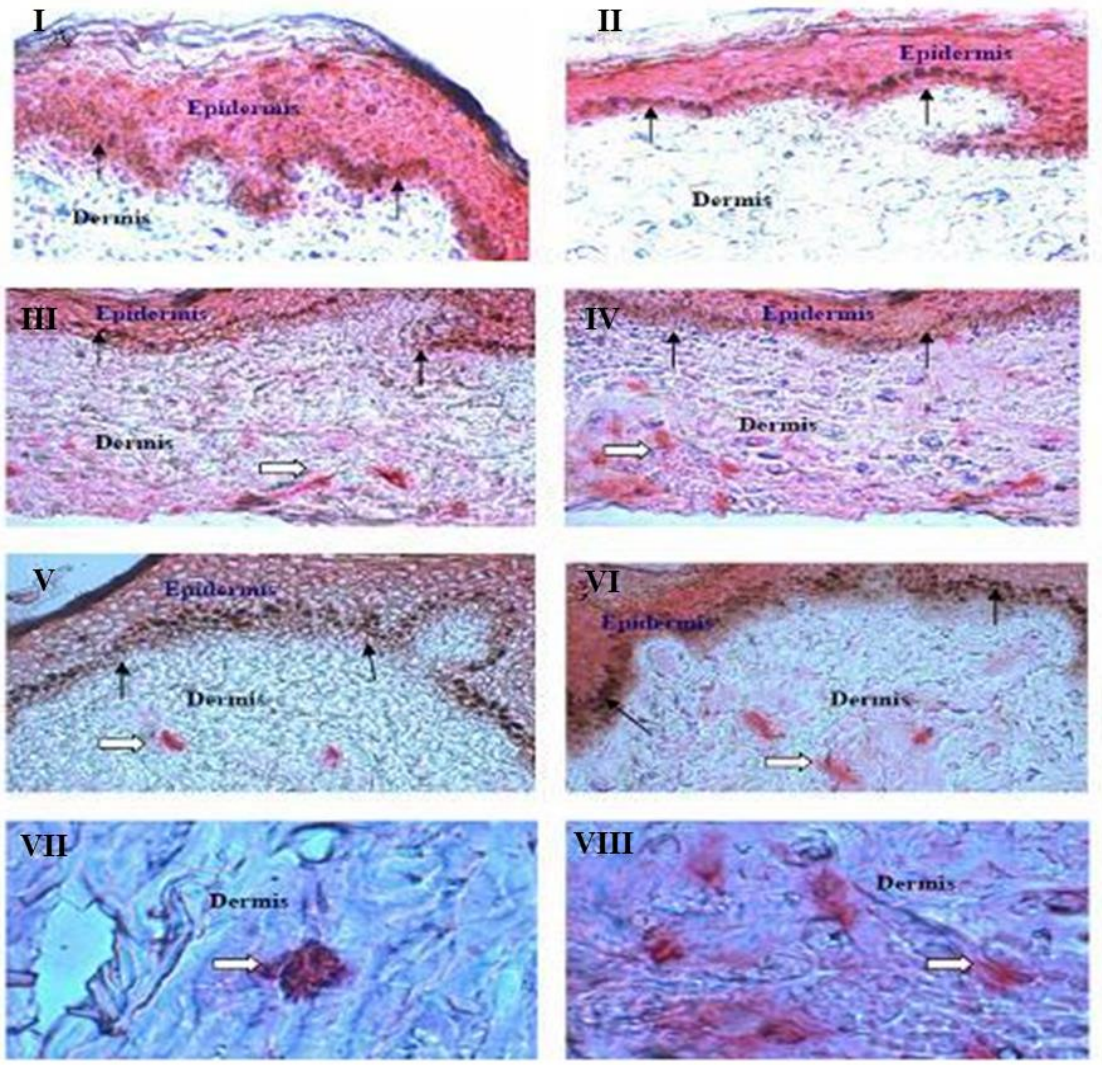
Gene expression of NT and NTR-1 was also measured in PS patients (n=40; 21 male, mean age 48 ± 18 years and 19 female, mean age 42 ± 15 years) compared to controls (n=30; 8 male, mean age 40 ± 16 years and 22 female, mean age 51 ± 19 years). Relative expression of the gene for NT in the skin was lower (p=0.0363) as was expression of the gene for NTR-1 (p=0.0007) in affected skin samples from patients with PS compared with controls. In contrast, there was no statistically significant difference in NT mRNA expression (n = 15), but there was a statistically significant increase in NTR-1 mRNA expression (n = 15, p=0.0023) in samples obtained from unaffected compared with affected PS skin.

4.3. Immunohistochemistry - PS Skin

Cryostat sections were prepared and fixed with acetone for 3 min and incubated with normal blocking serum for 20 min. Sections were then incubated with primary antibodies (goat polyclonal antibody to CRHR-1) diluted to 1:100 for 30 min and then immunostained with Vectastain ABC AP kit and Vector Red Alkaline Phosphatase Substrate kit as per the kit's directions. Presence of red color indicates a positive reaction for CRHR-1.

Figure 71. Immunohistochemistry of CRHR-1 and MC in PS skin

Epidermis and MC (I and II) in dermis (III-VI) and only MC (VII and VIII). Solid arrow shows epidermis, and hollow arrow shows MC.



CRHR-1 expression was documented in lesional PS skin by immunohistochemistry and appeared to colocalize with MC (Figure 71).

4.3.1. Conclusion

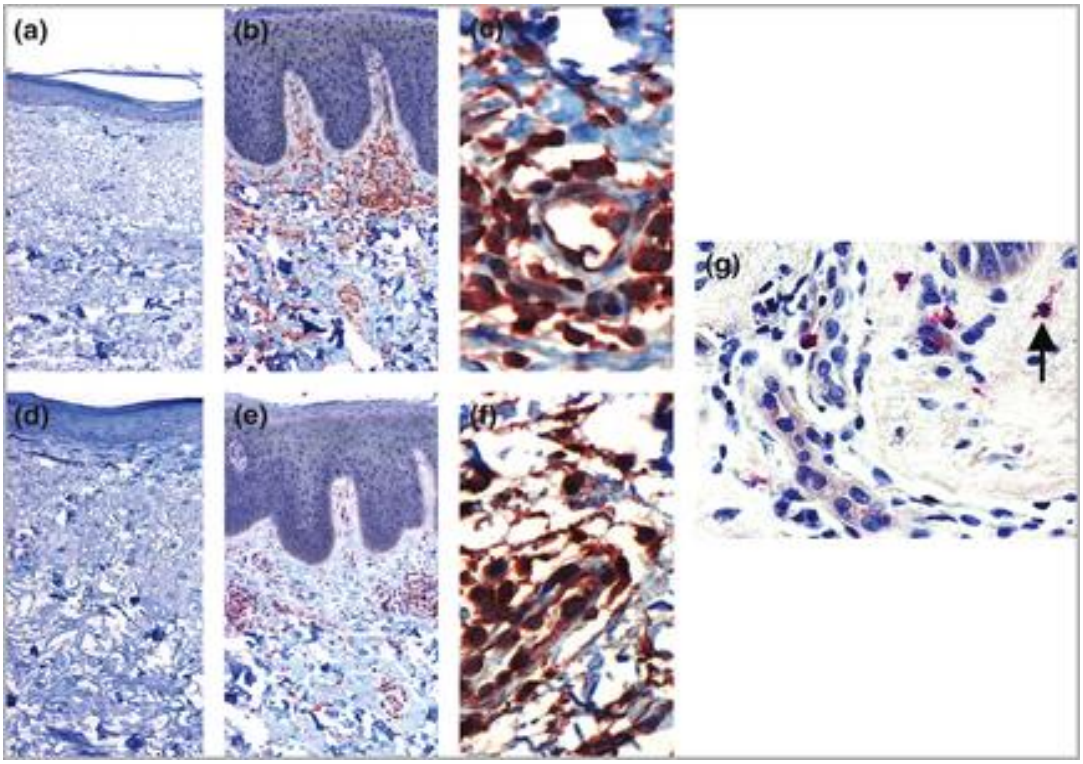
Our findings suggest that overstimulation by the increased serum levels of CRH and VEGFα, possibly in response to stress, leads to decreased gene expression of skin CRH and CRHR-1, as well as skin VEGFα gene expression, respectively. The positive correlation between serum CRH levels and the STAI-now scores in patients with severe PS supports this possibility. The lack of a similar correlation in patients with AD may be because I did not have access to severity index scores for these patients, unlike in patients with PS, in order to carry out a subgroup analysis.

The present findings suggest that high serum CRH level, possibly in response to stress, stimulates skin MC to release VEGFα and contribute to skin inflammation evident in patients with severe PS. Continuous or repeated stimulation may lead to decreased expression of skin CRHR-1, as I showed recently with cultured MC[474] and hence decreased VEGFα, as was evident in patients with mild disease. CRH and CRHR-1 may therefore participate in the pathogenesis of PS and AD, especially when worsened with stress, through mast-cell activation to release VEGFα. MC blockers may provide novel treatment approaches.

4.4. Immunohistochemistry-AD Skin

The presence of NT and NTR-1 proteins in AD skin was confirmed by immunohistochemistry (Figure 72). (a) Healthy skin stained for NT (magnification 5×). (b, c) Skin of patient with AD stained for NT (b, 20×; c, 40×). (d) Healthy skin stained for NTR-1 (5×). (e, f) AD skin stained for NTR-1 (e, 20×; f, 40×). (g) MC identified by naphthol AS-D chloroacetate esterase (Leder) staining (20×, solid arrow) and confirmed by c-kit (not shown).

Figure 72. Immunohistochemistry of NT, NTR-1 and MC in AD skin.



Control tissue showed no positivity for either NT or NTR-1 (a, d). Semiquantitative analysis of the cases studied (n = 5) revealed high expression of NT (b, c) and even higher NTR-1 (e, f), ranging from 26% to 50% of the dermal infiltrate. MC (g, solid

arrow) were characteristically the most positive cells, located mainly in the papillary dermis, as evidenced by dark brown staining.

4.4.1. Conclusion

Levels of NT and expression of its gene, *NTS*, are increased in the serum and lesional skin, respectively, of patients with AD compared with healthy controls. Immunohistochemistry confirmed the presence of NT peptide in lesional AD skin, but not in healthy skin. A possible explanation may be that NT is not present in skin under normal conditions, but is released in a precursor form from nerve endings and is generated by proteases, as I have shown in rodent skin[459].

I showed that NT is increased also in the serum of patients with PS, and it stimulates release of VEGFα from human MC[472]. It is interesting that NT was also elevated in the serum of children with autism[475], which has been associated with AD[476], and who are born to mothers who had PS during pregnancy[477], implying a possible causal connection.

I also show decreased expression of genes for NT and NTR-1 in psoriatic skin. Similarly, I previously reported that CRH is increased in the serum, while there is decreased CRHR-1 gene expression in the lesional skin of patients with PS[478]. Expression of the gene for NTR-1 was increased in nonlesional psoriasis skin. The reason for this finding is not clear, but suggests nonlesional skin may not as yet have been maximally affected by the circulating NT.

I also show that NT induces VEGFα release from MC. Also, MC express NTR[479] stimulation of which results in histamine release[480] and NT is rapidly degraded by MC proteases[287] implying tight regulation[480]. I previously showed that NT has a

synergistic action with CRH, increasing skin vascular permeability in rodents through MC activation[459]. Interestingly, NT increases expression of CRH receptor 1 in human cultured MC[250]. Moreover, CRH is also increased in the serum of patients with AD[473]. These findings imply that NT–CRH interactions may be important for the effect of stress on dermatoses and are relevant in view of evidence showing increased numbers of skin MC in PS[481]. NT may be involved in the pathogenesis of PS by acting together with CRH secreted under stress in the skin[482], which has its own equivalent of the hypothalamic–pituitary–adrenal axis[483]. The stimulatory effects of NT and IL-33 on VEGFα release may be in addition to a similar action by local skin SP. IL-33 is a potent inducer of inflammatory mediators by mast cells[484]. I have shown that SP stimulates human MC to release VEGFα[485] and that this action is augmented by IL-33[485]. However, contrary to my findings, there is increased expression of SP and its receptor neurokinin-1 in lesional PS skin[486], but plasma concentrations of SP in patients with PS did not differ from controls[487].

MC are now considered critical in allergy and innate immunity in the skin[488]. The present findings suggest that NT may participate in the pathogenesis of PS, especially when worsened by stress, through MC involvement in a 'brain–skin' connection[489]. Interactions between NT, CRH and MC may be involved in skin inflammation[489], which worsens with stress, and may provide new targets for therapy. NT and MC blockers may provide novel treatment approaches.

Discussion

Overall

I report the inhibitory effect of the natural occurring antioxidant and antinflammatory isoflavones (daidzein and genistein) on the mouse inflammatory mediators gene expression in the brain, and skin, as well as their serum levels. Although I was unable to validate a "mouse model" for chronic fatigue, I showed that using poly(I:C) alone or in combination with swim stress induced "fatigue behavior" for 24 h, as assessed by reduced locomotor activity. Moreover, poly(I:C)-treated mice showed increased inflammation in the brain and the periphery, as measured with increased inflammatory mediator serum levels, and gene expression in the brain and skin. Treating mice with high isoflavone diet ameliorated or diminished the poly(I:C)-induced effects, such as decreased locomotor activity, as well as increased inflammatory mediators in the brain, serum and skin. Hence, my results suggest using isoflavones for possibly treating fatigue symptoms.

Although I could not establish a "mouse model" for CFS, I feel it is important to discuss other such "models" reported in order to highlight difficulties, inconsistencies, dependence on specific mice species, as well as the fact that data obtained from such limited publications.

1. A "Mouse Model" for CFS

My initial studies tried to use an "established mouse model" for the study of chronic fatigue (Table 2, 3, 4). Previous studies used swim stress (7-42 days), and/or an immunological trigger (poly(I:C) or LPS or BA) to induce "fatigue behavior" in certain

types of mice, assessed by specific behavioral parameters. Some studies showed that under these conditions brain oxidative stress was increased and use of flavonoids, including EGCG, curcumin and naringin, ameliorated this induced “fatigue” behavior and brain oxidative stress.

Thus, I used swim stress (15 min for 21 days, daily) alone or in combination with poly(I:C) or LPS (10 mg/kg, and 1 mg/kg respectively, ip, on Day 1) or combinations of the above to induce “fatigue behavior”. As behavioral assessments for “fatigue behavior”, I used immobility time of mice while they were swimming, time to start grooming once they finished the swim test and percent weight change. Moreover, I repeated the protocol providing my mice with low or high isoflavone diet (daidzein and genistein, 0-20 and 150-250 mg/kg, respectively).

Statistical analysis of the behavioral data showed that poly(I:C)-treated mice had increased immobility time and time to start grooming, but only on specific days of experiments (Figure 2a, 3a, 4a). When I calculated the area under the curve (AUC) for immobility time and time to groom, to compare the behavioral parameters over the 21 days, there was no significant effect of any treatment on either behavioral parameter after 21 days. Additionally, I measured the MDA content in the brain of mice on the 22nd day and treated mice (swim stress with or without poly(I:C) or LPS) did not show increased MDA brain content after 21 days (Figure 5a). Results were similar when mice were provided low or high isoflavone diet. There was only a small effect of swim stress in the mice participating in the high isoflavone diet, which was not present in mice provided with low isoflavone diet, contradictory to my initial hypothesis.

Our results indicate no significant alterations in behavioral parameters attributable to swim stress and/or immunological trigger after one week. Furthermore, I noticed that mice were acclimated to the procedures quickly and efficiently. During the first week, immobility time during the 15 min of daily swim was low, as mice were trying vigorously to get out of the water, while the time to start grooming was high as mice were obviously recovering from stress. This behavior rapidly shifted and mice after the first week exhibited minimum mobility and only to keep their heads out of the water. This behavior would be interpreted as "fatigue" according to previous publications, but it should also be accompanied with increased time to start grooming after the end of the swim test. However, in contrast to the first few days, mice began grooming almost immediately after they were taken out of the water, in my studies. This indicates that mice did not develop "fatigue" behavior, but only adjusted their behavior to the specific procedures used.

In contrast to previous studies, percentage weight change was increased slightly over the 21 days experiments (Figure 4), while others reported a decrease. This observed effect was also similar after a low and high isoflavone diet. Another paper showed that stressed C57BL/6 mice that underwent social defeat increased their body weight, in contrast to BALB/c, showing that there are differences between species[147].

Since there were no differences of any kind after the first week and that other studies have reported induction of CFS in mice within shorter period of time (Table 2, 3, and 4) I decided to proceed with a shorter duration (one week) for experiments. Moreover, given the variability in the outcome of the behavioral parameters, I decided to incorporate an alternative, more objective mean to quantify the potential fatigue the mice experience

under my treatment, locomotor activity daily monitoring. Lastly, I decided to focus on the poly(I:C) effect and use higher poly(I:C) concentrations in the following experiments (20 mg/kg to 40 mg/kg).

Unlike before, where mice injected with 10 mg/kg exhibited no changes, mice injected with 20 and 40 mg/kg of poly(I:C) had reduced mobility lasting for several days, as it was assessed by the time to start grooming, although immobility time was not different (Figure 6 and 7). Administration of 40 mg/kg was lethal to mice (eight out of ten mice did not survive (n=5/group, 6 groups). Therefore, I decided to proceed with use of the poly(I:C) (20 mg/kg) dose, followed by forced swim test, for 15 min, for 6 consecutive days. After each experimental day, I monitored locomotor activity of the mice for 16 hr. Analysis of the locomotor activity results involved calculation of the total locomotor activity (AUC) and the maximum locomotor activity over the 16 hr monitoring and only over the night (10 hr). As shown in Figure 8, poly(I:C)-treated mice with or without swim stress had significantly lower locomotor activity, while differences were only obvious the first two days. Forced swim test seems to have an effect on Day 1.

These results are not in accordance with previous publications (Table 3) that reported significant changes in the behavioral parameters under study. Our results indicate no significant alterations in behavioral parameters attributable to forced swim and/or immunological trigger that lasted more than a few days. Looking closely to other papers, there were some discrepancies among the results of the same behavioral and biochemical parameters across various studies. For example, in the behavioral parameters, some papers reported an increase in locomotor activity in “fatigued” albino LACA mice and Wistar rats[153, 154, 158], while other papers with similar treatment protocols reported a

decrease using the same rodents[156, 160, 161]. In the biochemical parameters, one paper reported a decrease of SOD levels in the brain of "fatigued" albino LACA mice[154], while another reported an increase[156]. However, in these murine "models" for CFS it was generally reported that chronic use of forced swim resulted in reduction of mouse activity and increase of oxidative stress.

Taking this into consideration, I was very detail-oriented with my experimental protocol. I used Ethovision software with a Noldus apparatus to videotape mice while swimming individually and analyze the results over the course of the 15 min swim test, for every day, up to 21 days. Locomotor activity was monitored in the fully automated Neuroscience Behavior Core's mouse SmartFrame® Cage Rack System that continuously tracks the animal's movement. Data were collected and subsequently analyzed in time bins (e.g. every hour) or as a total over the course of collection to the "Total Distance Travelled" (in cm). Moreover, analysis of the behavioral parameters and brain MDA content was performed blindly and I used corresponding control group of mice for each condition with n=5-7/group. In contrast to my results, did not mention the use of any specific standardized automated system, reported fragmented time periods for monitoring, and/or reporting results by researchers, without even mentioning if the experiments were performed blindly. Nevertheless, I was unable to develop a "chronically fatigue mouse model".

One potential explanation is that other investigators used a different mouse strain. The impact of the stressors vary on the nature of the immune challenge and the stress response, as these vary greatly between different strains with respect to both behavioral and neurochemical outcomes[490]. I used C57BL/6 mice because I had planned to use

CRHR-1 KO mice, of a similar background. Since I could not validate the "model" using C57BL/6 mice, I decided to test my experimental protocol on BALB/c mice, as others have done, and use CRHR-antagonists instead. I used the same protocol with 15 min swim stress, daily for one week and poly(I:C) (20 mg/kg, ip) as immunological trigger on Day 1 and monitored locomotor activity of female BALB/c mice for the next 16 hr. Similar to the experiments with the C57BL/6 mice, locomotor activity of the mice returned to levels similar to control groups after the first 3 days (Figure 9).

Studies using BABL/c mice [163, 169, 170] (Table 3) showed induced fatigue after injecting BA via the tail vein, six times every two weeks and reduced running activity, which was noticed for the first several days; but quickly returned to normal behavior. However, there were no other significant behavioral or biochemical differences reported[169, 170], while the peak running activity was significantly reduced only for the first four days[163].

In general, C57BL/6 mice were reported to exhibit greater Th1 responses[491, 492], whereas BALB/c were highly Th2 responsive. According to one paper C57BL/6 mice are more susceptible to immunologically mediated fatigue than BALB/c mice[493], as C57BL/6 mice treated with a single injection of Corynebacterium parvum showed greater reduction in running activity and slower recovery than BALB/c, compared to their baseline. In the same paper, antibodies specific to either TNFα or IL-1β administered at the time of challenge with the trigger, did not alter the immunologically induced fatigue, while C57BL/6 mice had increased TNFα and IL-1β brain gene expression lasted up to 15d post injection, which corresponded to fatigue, suggesting an important role for the central nervous system[493].

In contrast, another publication showed that BALB/c mice are more sensitive to restraint stress than C57BL/6 mice[494], while others showed that challenging C57BL/6 and BALB/c mice with LPS and poly(I:C) yielded divergent results [495] on specific increases of inflammatory mediators. More specifically, both strains showed increase in corticosterone levels with higher levels noticed in BABL/c. When mice were treated with poly(I:C), TNF serum levels were equally increased, but C57BL/6 mice exhibited increases in IL-6 and IL-10 serum levels and TNF gene expression in the hippocampus, while BALB/c did not. At the same time though increase in IL-1β, and IL-6 gene expression in the hippocampus was lower in C57BL/6 mice than BALB/c.

Treatment of mice with LPS yielded similar increases in serum corticosterone results, but higher increases of IL-6, TNF serum levels and TNF, IL-6 and IL-1β gene expression in hippocampus of C57BL/6 mice compared to BALB/c. Overall, the impact of a social stressor varied with the immune trigger, the mouse strain and the mediator measured [495] and C57BL/6 mice can be a good model to study poly(I:C)-mediated inflammatory responses.

One study reported "fatigue behavior" in ICR (Institute of Cancer Research) male mice [171] (Table 3), which are outbred mice produced in 1947 by Hauschkaat the Institute of Cancer Research, Fox Chase. They originally derived from Swiss mice of the Rockerfeller Institute and are now widely distributed[496]. These mice, when treated with poly(I:C) (5 mg/kg, ip, on Day 1) demonstrated "fatigue behavior", as was assessed with forced swim test with tail load every 2-3 days, for 2 weeks. The behavioral assessment used included time until the mice could not keep their head over the water surface, was noted every 2-3 days and a decrease reflected "fatigue behavior". In this mouse model,

"fatigue behavior" lasted up to 7 days, similar to my results on the behavioral parameters. Noteworthy, in this study they also measured serum corticosterone levels (represents stress levels) after 18 days, which unexpectedly was found to be lower than the control mice.

Other papers that reported mouse "models" for chronic fatigue used albino LACA (Laboratory Animal Centre Albino) mice induced with swim stress alone [153, 157, 158, 160-162] or together with ip LPS injection[172-175]. LACA outbred mice derive from a Swiss Webster stock with 20 generations of brother-sister mating at the Rockefeller Institute; went first to Carworth Farms in 1937, where it became Carworth Farms White (CFW), and then to the Laboratory Animals Centre, Carshalton, Surrey, UK, where the name changed to LACA in the 1970s.

As general research animals, outbred stocks, including ICR and LACA mice, in contrast to inbred strain like C57BL/6 usually have an unknown extent of genetic variation depending on the history of the stock. This might be a useful tool for a toxicology study, where the chosen inbred strain may be genetically resistant to the test chemical and thereby give misleading results, but this unknown genetic variation may be a problem in other studies, like behavioral analysis, if the same endpoints cannot be validated in another type of mouse, outbred or inbred[496].

In order to be able to compare behavioral and biochemical parameters with outbred stocks each colony of the outbred stocks should be characterized extensively, origin recorded, as well as other characteristics such as degree of inbreeding, and genotypic and phenotypic characteristics should be taken into consideration. Notably, a systematic study

evaluation revealed that acute inflammatory responses to stress from different etiologies poorly correlate between mouse models[497].

Moreover, male mice were used in some mice models, while the gender specification was lacking in other studies, making these publications questionable. I used female mice, as chronic fatigue syndrome has a higher prevalence in women. There are clearly sex differences in incidence and onset of stress-related disorders in humans. Yet, rodent model studies are predominantly based on male animals. The strongest argument for not using female rodents is their estrous cycle and the fluctuating sex hormones per phase that increases the number of animals to be tested.

Female sex hormones often affect emotions, and the immune system, contributing to behavioral differences. For example, female rats and mice express less anxiety than males in a novel environment and this is related to the phase of the estrous cycle with proestrus females to be even less anxious. On the other hand, even though males perform in spatial tasks superior to females, stress impairs their spatial memory, while females improve their spatial abilities, depending on the task and kind of stressor[498].

Therefore, another explanation for my findings could be that the female mice I used quickly adjusted to the repetitive forced swim trigger, as was indicated by the behavioral parameters I studied. After the first week, mice quit struggling to get out of the water over the 15 min period time, while they decreased the time to start grooming, implying that decreased time of immobility was just an indication of behavioral adjustment.

Taking into consideration the results above and previous studies, I decided to shift my experimental protocol to investigate the effects of swim stress and poly(I:C) on fatigue-like behavior and inflammatory parameters on Day 1.

2. Effect of Poly(I:C) in Mice and Inhibitory Effect of Isoflavones

I have analyzed, in detail, the "fatigue" behavior (24 h) induced by ip challenge with poly(I:C) (20mg/kg), with or without 15 min swim stress, in C57BL/6 female mice by monitoring their locomotor activity for 16 hr after the experiment. C57BL/6 female mice were provided with chow containing low or high detectable isoflavone levels (0-20 and 150-250 mg/kg respectively).

Here I showed that poly(I:C), with or without swim stress, significantly reduced locomotor activity in comparison to control mice (Figure 10). Further analysis of mouse samples obtained the following day, showed that poly(I:C) also increased molecules that have been associated with inflammation and fatigue. In particular, poly(I:C) increased serum levels of TNF, IL-6, KC, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5), and IP-10 (CXCL10) (Figure 12-19). Importantly, my serum measurements were performed blindly my Millipore. Moreover, poly(I:C) also increased brain and skin gene expression of TNF, IL-6, KC, MCP-1, MIP-1β, and RANTES, IP-10. In addition, HDC and NT gene expression was increased in the skin (Figure 20-28 for brain and 29-37 for skin), suggesting MC involvement. Forced swim did not have any effect on its own, but augmented the effect of poly(I:C) on TNF, MIP1β and RANTES serum levels (Figure 12, 17 and 18).

In support of my findings, another study used female C57BL/6 mice that were treated with lower concentrations of poly(I:C) (2, 6, 12 mg/kg) by ip, alone (no swim stress), on Day 1 [168] (Table 2), were also not able to cause "fatigue behavior" lasted for more than 3 days. In this paper they report that only the poly(I:C) (12 mg/kg) increased serum and brain inflammatory mediators, including TNF, IL-6 IL-1β and IFNβ that lasted

up to 24h[168]. Importantly, rechallenging these mice one week or three weeks after the first challenge did not result in augmented response, but accelerated the recovery process. Also Chopra et al. (Table 3) showed increases in TNFα serum levels following similar experimental paradigm. Our studies investigated a plethora of chemokine levels in the serum, but also chemokine brain gene expression, cytokine and chemokine skin gene expression, as well as the inhibitory effect of isoflavones.

Here, I report for the first time in my knowledge that isoflavones genistein and daidzein ameliorate the poly(I:C)-effects on "fatigue behavior" in C57BL/6 female mice. Poly(I:C)-treated mice that were provided with high isoflavone diet showed higher locomotor activity compared to mice provided with low isoflavone diet (Figure 11). Moreover, isoflavones, which are natural occurring MC inhibitors, minimized or reduced the serum level increase of the above inflammatory markers and the corresponding brain and skin gene expression (Figure 12-19 for serum, 20-28 for brain and 29-37 for skin).

MC are an important source of the chemokines that were found increased in my studies[411-417]. Activated MC are likely to contribute to CFS symptoms through the release of a number of chemokines and cytokines[482, 499]. MC have traditionally been known for their roles in allergy and immunity to multi-cellular parasites[292], but only recently are being recognized for their crucial roles in immune defense against viruses[251, 293]. Double-stranded RNA from viruses (dsRNA) and poly(I:C) are recognized by TLR-3, leading to activation of transcription factors, and production inflammatory mediators[346, 347, 500].

Several studies showed that murine MC have been shown to respond to poly(I:C)-TLR3 activation in multiple ways[325, 332]. FcεRI-mediated MC reactivity is amplified

through prolonged TLR-ligand treatment[501]. Fetal rat skin-derived MC were shown to express TLR-3 and activation induced release of TNF-α and IL-6, as well as RANTES and MIP, but without degranulation[332]. Another study showed that poly(I:C) caused IFNα and β secretion from human peripheral blood derived (PBDMC) and murine bone marrow derived MC (BMMC)[325]. Murine fetal skin-derived MC (FSDMC) were shown to release IL-6, TNFα, MIP-1α, MIP-2, and RANTES upon poly(I:C)-TLR3 activation[252]. Moreover, in support of my serum results, another study showed that peritoneal MC from C57BL/6 mice activated by poly(I:C)-TLR3 have increased IP-10 and RANTES expression, and additionally they stimulate CD8+ T cell recruitment in vitro and in vivo[502].

MyD88-independent/TRIF-dependent signaling pathway, used by TLR3, involves the recruitment of TRIF, activation of TBK-1 leading to the activation of IRF3, a transcription factor that translocates to the nucleus to produce IFN-inducible genes. Alternatively, TRIF activates RIP1 and this feeds into the MyD88 pathway by activating TRAF6 [345-347] and eventually activating NFκ-B and AP-1 to produce proinflammatory cytokines. Additionally, RIG-I, MDA5 (RLR family) and NALP3 (NLR family), are cytosolic proteins that sense viral RNA[503]. MDA5 is thought to bind to double-stranded RNA[322]. NALP3 (NLR family) assemble inflammasomes, leading to activation of caspase 1 which cleaves and releases IL-1β and IL-18 [319, 320] and others regulate type I interferon (IFNα and β) production[321].

Rodent, monkey, and human MC are able to detect Dengue virus (DENV), a lymphotropic, enveloped, single-stranded, positive-sense RNA virus that results in MC activation and degranulation. It was shown that the response of MC to DENV involves

the activation of MDA5 and RIG-I antiviral intracellular host response pathways, and the de novo transcription of cytokines, including TNF-α and IFN-α, and chemokines, such as RANTES (CCL5), CXCL12, and CX3CL1. Moreover, after subcutaneous infection, MC-deficient mice show increased viral burden within draining lymph nodes compared with MC-sufficient mice. This containment of DENV is linked to the MC-driven recruitment of natural killer and natural killer T cells into the infected skin[504].

Analysis of the expression of dsRNA recognition receptors in BMMCs revealed that they express MDA5, RIG-I and TLR-3. The expression levels of these receptors were found to increase upon stimulation of MC with synthetic poly(I:C) as well as with vesicular stomatitis virus (VSV). Moreover, both RIG-I and MDA5 were involved in cytokine production, but not in the degranulation of MC[505]. Primary human cord blood-derived mast cell (CBMCs) and HMC-1 MC responded to poly(I:C) or antibody-enhanced dengue virus infection treatment with the production of type I interferons and the rapid and potent production of chemokines including MIP-1β (CCL4), RANTES (CCL5) and IP-10 (CXCL10).

Multiple interferon-stimulated genes were also upregulated, as well as mRNA and protein for the RNA sensors RIG-I and MDA5. These findings support a role for tissue-resident MC in the early detection of antibody-enhanced dengue virus infection via RNA sensors, the protection of neighboring cells through interferon production and the potential recruitment of leukocytes via chemokine production[506].

In a paper studying respiratory Influenza A virus (IAV) infection, they found that significant disease and immunopathology is caused in C57BL/6 mice, but not in B6.Cg-Kit(W-sh) mice, which lack MC. Moreover, when MC were infected with IAV, the virus

did not replicate within MC. Importantly, human H1N1, H3N2, IAV and influenza B virus isolates could also activate MC *in vitro* to produce cytokines and chemokines, through a RIG-I dependent mechanism, offering a unique inflammatory cascade could potentially be targeted to limit morbidity virus infection[507].

2.1 Flavonoid Effects on Signaling Pathways

Flavonoids exert their strong antioxidant and anti-inflammatory activity [176, 212] through multiple mechanisms, including inhibition of the pathways mentioned above. Most importantly, flavonoids are shown inhibit TLR-3and TLR-4 signaling pathways by inhibiting TBK1 kinase activity and IRF3 transcription factor, AP-1 transcription factor pathways[508], as well as suppress signaling by RIG-1[509], inhibiting the poly(I:C)-TLR3 induced pathway. Also, flavonoids inhibit the NF-κB pathway at multiple steps[176], as well as the NOD2-mediated NF-κB activation[508]. Importantly, genistein was shown to selectively inhibited human topoisomerase II activity[510].

The flavone luteolin suppressed symptoms of a rat experiments allergic encephalitis by preventing monocyte migration across the brain endothelium, through modulating the activity of Rho GTPases involved in transendothelial migration signaling[511]. Interestingly, luteolin inhibited myelin basic protein-induced human MC activation to release histamine, tryptase, IL-6, IL-8, TGFβ, TNF and VEGFα and MC-dependent stimulation of Jurkat T cells[465]. Also, luteolin consumption restored expression of inflammatory markers in the hippocampus compared with that of young adults and improved spatial working memory[512].

Luteolin suppressed the expression of TNF-alpha, IL-8, IL-6, GM-CSF, and COX-2 through a decrease in the intracellular Ca2+ levels, and also showed a suppression of the MAPKs ERK 1/2, JNK 1/2, and NF-κB activation in HMC-1[513]. Luteolin-7-O-glucoside inhibited LTC4 production and degranulation of activated mouse bone marrow-derived MC (BMMCs) by inhibiting the activation of MAPKs ERK1/2, p38, JNK and phospholipase Cγ1[514].

Another study showed that quercetin reduced CRH-induced anxiogenic and depressant-like effects, as assessed by increased social interaction time and decreased immobility time during forced swim test.[226]. Moreover, quercetin inhibited p38, PKC-theta, and NF-κB activation in a dose-dependent manner[515, 516], resulting in inhibition of TNFα, IL-1β, IL-6 and IL-8 gene expression and production. Quercetin also inhibited histamine, tryptase and MCP-1 production and histidine decarboxylase transcription in human MC[517-520].

EGCG prevented and reversed disability in EAE in an experimental mouse model by both limiting brain inflammation and reducing neuronal damage[521]. EGCG inhibited MC degranulation, LTC_4 secretion and Ca^{+2} influx[522], but also TNF-α, IL-6 and IL-8 expression and production from stimulated HMC-1 cells, through attenuation of NF-κB and ERK1/2 activation, but not of JNK or p38 MAPK[523]. Genistein also blocked LTC_4 and PGD_2 production from cultured murine MC[524]. EGCG inhibited the mRNA expression and production of thymic stromal lymphopoietin (TSLP), and the activation of caspase-1 in HMC-1 cells[525]. Caspase 1 is an enzyme that proteolytically cleaves other proteins, such as the precursor forms of the inflammatory cytokines IL-1β and IL-18, into active mature peptides.

Fisetin, kaempferol, myricetin, quercetin, and rutin inhibited histamine release in RBL-2H3 cells and decreased gene expression and production of proinflammatory cytokines, including TNFα, IL-1β, IL-6 and IL-8, in HMC-1 MC, while suppressing activation of NF-κB[518]. Apigenin inhibited TNF-α, IL-8, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) production and cyclooxygenase (COX)-2 expression by decreasing the intracellular Ca^{+2} level and inhibiting NF-κB activation in HMC-1 stimulated cells[526]. Another study showed that hesperin has similar effect, inhibiting the hypoxia-inducible factor-1alpha (HIF-1alpha) expression and inflammatory cytokine production TNFα, IL-1β, IL-8, and VEGFα in HMC-1 cells[527].

3. Additional Flavonoid Inhibitory Mechanisms

An additional mechanism of the inhibitory effect of flavonoids on MC is though inhibition of FcεRI-mediated activation pathway. Isoflavones genistein and daidzein reduce the cell surface expression and mRNA levels of high-affinity FcεRI, as it was shown on mouse bone-marrow-derived MC[528]. Furthermore, studies in mice also showed this effect *in vivo* with genistein and daidzein reducing the anaphylactic symptoms and MC degranulation in mice after peanut challenge. Isoflavones also inhibited cholera toxin-induced DC maturation in the mesenteric lymph [529] nodes and human monocyte-derived dendritic cells (MDDCs) and subsequent DC-mediated CD4(+) T-cell function *in vitro*[529].

Quercetin and the structurally similar luteolin also inhibited the release of histamine, leukotrienes and PGD_2 from human cultured MC in response to cross-linkage of FcεRI[530]. Quercetin effectively suppressed the development of IgE-mediated allergic

inflammation of intestinal cell models. More specifically, quercetin inhibited the secretion of allergic mediators in RBL-2H3 cells and suppressed the CD23 mRNA expression and p38 MAPK activation in IL-4-stimulated Caco-2 cells, as well as extra signal-regulated protein kinase (ERK) activation and chemokine release[531].

Flavonoids can also inhibit directly the release of inflammatory mediators in MC. It was shown that the release of mediators contained in distinct subsets of granules of MC, is specifically mediated by two sets of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, and it was shown that different polyphenols selectively interfered with ternary complex formation of SNARE complexes, thereby stopping membrane fusion between granules and plasma membrane[224], and inhibiting inflammatory mediator release from MC[215, 218, 220, 223].

Interestingly, EGCG treatment of HMC-1 cells resulted in a lower adhesion of MC, which was associated with a decreased potential to produce signals eliciting monocyte recruitment, including two integrins (alpha5 and beta3) and a chemokine (MCP1). These effects on gene expression levels were functionally validated by showing inhibitory effects in adhesion, aggregation, migration and recruitment assays[532]. Moreover, genistein at physiologically relevant concentrations (0.1-10 μM) significantly inhibited thrombin-induced increase in endothelial monolayer permeability, through PKA-mediated suppression of RhoA signaling, suggesting it can improve endothelial barrier dysfunction[533].

3.1. Effect of Flavonoids on Oxidative Stress

Additionally, the strong antioxidant effects of flavonoids are mediated by the Keap1/Nrf2 pathway[215-217]. Interestingly, Nrf2 can be regulated though multiple signal transduction pathways, including MAPKs, PI3K/AKT, and PKC[218-220].

It was shown that forced swimming stress produced a severe oxidative damage in the rat hypothalamus, as was measured by oxidative biomarkers (lipid hydroperoxides, antioxidant enzymes and total antioxidants, and increase in serum corticosterone. Treatment with quercetin markedly attenuated these stress-induced changes[534]. Furthermore, quercetin administration to rats significantly suppressed water-immersion-restraint stress-induced increase of plasma corticosterone and adrenocorticotropic hormone levels as well as the mRNA expression of CRH in the hypothalamic region. HPA axis activation is a major component of stress response. This effect was mediated by modulation of the DNA binding activities of glucocorticoid receptor, phosphorylation of cyclic adenosine 3', 5'-monophosphate (cAMP) response element binding protein, as well as phosphorylation of the extracellular signal-regulated kinase 1/2 in the hypothalamic region, all of which are known to regulate the expression of CRF mRNA[535].

Genistein mediated induction of fatty acid oxidation genes through the JAK2-mediated phosphorylation and activation of AMPK in skeletal muscle[631]. Quercetin and genistein showed an antioxidant activity towards lipid oxidation and the myofibrillar proteins carbonylation pathway, The carbonylation pathway involves the oxidative deamination of lysine residues to yield a carbonyl compound (α-aminoadipic

semialdehyde) that can be further oxidised to α-aminoadipic acid and form Schiff bases structures[222].

Flavonoid antioxidant properties were also shown to improve mitochondrial function[536, 537]. Findings on quercetin effect on mouse markers of mitochondrial biogenesis in skeletal muscle and brain, and on endurance exercise tolerance showed that quercetin increased mRNA expression of peroxisome proliferator-activated receptor-gamma coactivator (PGC-1alpha) and sirtuin 1 (SIRT1), as well a mitochondrial DNA (mtDNA) and cytochrome c concentration. Moreover, these changes in markers of mitochondrial biogenesis were associated with an increase in both maximal endurance capacity and voluntary wheel-running activity, showing the capability of quercetin to prevent and/or alter chronic diseases[538].

Epicatechin was shown to be associated with increased angiogenic and mitochondrial signalling in the hindlimb of rats selectively bred for innate low running capacity [539] and to attenuate mitochondrial damage by enhancing mitochondrial multi-marker enzymes, adenosine triphosphate and lowering calcium in isoproterenol induced myocardial infarcted rats[540]. Quercetin supplementation was also shown to be effective in improving mitochondrial dysfunctions[541]. Moreover, quercetin-related flavonoids and tea catechins have inhibitory effects of on the monoamine oxidase-A reaction in mouse brain mitochondria[542].

Additionally, polyphenols were shown to prevent ageing-related impairment in skeletal muscle mitochondrial function through decreased reactive oxygen species production[543]. One study using genistein and daidzein, showed arthritis-induced decreases in paraoxonase and arylesterase activity to be restored after treatment with

isoflavones and MDA concentrations to be lower after treatment with all tested compounds[225]. Also, isoflavones were shown to attenuate brain mitochondrial oxidative stress induced by beta-amyloid peptides injection in lateral cerebral ventricle[544].

4. MC Important Role in Innate and Adaptive Immunity

MC are essential for initiating both innate and adaptive immune responses[293, 545-547]. MC are located strategically in tissues that come in contact with the environment, like in skin, lungs, lymphatic tissues and the gastrointestinal track[548-550], but also in the brain, affecting neuroimmune processes. Brain MC are located perivascularly in close proximity to neurons[489], especially in the diencephalon, cerebellum and leptomengines[551, 552]. MC can influence the behavior of nerve cells through products such as histamine (and serotonin in the case of mouse MC)[553]. MC are also localized around the cerebral microvasculature[554], and have been identified close to CRH positive neurons in the rat median eminence [555] where functional MC-neuron interactions take place[552, 556].

This is important, because another study has shown that there may be specialized TLR3/double-stranded RNA interactions at the cerebral endothelium since recent studies show that West Nile virus appears to use to TLR3 to facilitate its entry into the brain[557]. It has been shown that histamine release from MC can occur in response to Sendai virus [558] and that the gp120 envelope protein of HIV interacts with the VH3 region of IgE to induce the release of IL-4 and IL-13 from human FcεRI(+) cells, like MC[298]. MC can also comprise a long-lived inducible reservoir of persistent HIV in infected persons[559], possibly contributing to a subclinical HIV infection. It was shown that signaling through TLRs triggers HIV replication in latently infected MC[560].

Both stress and CRH could precipitate or worsen CFS through activation of brain MC[482], and functional MC-neuron interactions in the brain [552, 556] and the GI tract[561, 562], that could mediate brain inflammation[246, 563]. I showed that MC could be unique targets of CRH and related peptides contributing to neurogenic inflammation[246, 482]. There is little information on CNS responses to poly(I:C) *in vivo*, but two independent studies have shown that direct application of poly(I:C) to the brain demonstrates that CNS cell populations are capable of mounting an *in vivo* response to poly(I:C)[564, 565].

However, systemic exposure to poly(I:C) is more likely to activate an indirect response in the brain. In both rodents and humans, MC number in the lungs is higher than uninfected controls following respiratory infections[566]. Peripherally induced cytokines may also have direct effects on neurons in the CNS through direct activation of vagal afferents or by action on neurons in the circumventricular organs. Similarly it is possible that either poly(I:C) itself or the systemic cytokines induced by it act on the cerebral endothelium to induce lipophilic mediators such as PGE_2 or NO, which are secreted by MC, and transduce the signal into the brain in this way. Restraint stress is reported to activate dura MC and lead to CFS elevation of rat MC protease, effects abolished by pretreatment with polyclonal antiserum to CRH and the CRHR-1 antagonist Antalarmin[567]. Acute stress increased BBB permeability in rats and mice only in brain areas containing MC[568]. Moreover, the MC secretagogue compound 48/80 stimulated brain MC in rats[569], and increased BBB permeability in doves[570]. Once brain MC get activated by poly(I:C), and CRH or NT, they can selectively release CRH [571] and IL-6 [572] that could further stimulate this process leading to a self-sustaining cyclic reaction.

The strategic location of MC and their release of specific mediators upon stimulation, as mentioned above, facilitate immune cell recruitment to sites of infection[573]. For example, MC are located proximal to blood vessels, and when they release factors, such as histamine, TNF, VEGFα and proteases, they contribute to increased vascular permeability and edema at the site of infection[400, 420, 574]. As it was shown in bacterial peritonitis models [367, 575] and other inflamed tissues[576], MC-derived TNF and MCP-6 promote neutrophil recruitment. Human cord blood-derived MC can also be stimulated with poly(I:C) and produce a wide range of chemokines, including CCL-11 (eotaxin) and IL-8 (CXCL-8), which in the case of a viral infection could recruit eosinophils and natural killer (NK) cells, respectively[577].

MC are also crucial for adaptive immunity[547]. MC themselves can function as antigen-presenting cells (APCs), as it was shown that activated MC upregulated MHC class II and co-stimulatory molecule expression and have been visualized to physically interact with T cells in vivo[292]. MC can also function efficiently as APCs for MHC class I-restricted CD8+ T cells in vivo[578], and promote CD8+ T cell activation, proliferation and production of T cell products, such as IL-2 and granzyme B, describing for MC an important functional role not only a Th2, but also in Th1 response. Moreover it has been shown that specifically TLR3-induced activation of MC can modulate CD8+ T-cell recruitment[502].

Moreover, MC/DC interaction resulted in DC maturation and in the release of the T-cell modulating cytokines IFN-γ, IL-2, IL-6 and TGF-β into coculture supernatants, while it also increased the IL-12p70, IFN-γ, IL-6 and TGF-β secretion of LPS-matured DCs.

MC-primed DCs stimulated CD4+ T cells proliferation and release of high levels of IFN-γ and IL-17, demonstrating that MC promote Th1 and Th17 responses[579].

Conversely, T cells can modulate MC, through the production of chemokines such as CLL3/MIP-1α and CCL2, as well as through physical contact between MC and T cells leading to release of histamine and TNF[124, 580], suggesting that another feedback mechanism exists, by which the adaptive immune system might regulate MC function during an ongoing inflammatory process or infection.

TNF release from MC eventually promotes the influx of monocyte-derived DCs, through E-selectin expression upregulation by the local vascular endothelium, which are subsequently increased in draining lymph nodes[581]. Additionally, MC-derived CCL20 probably contributes to the recruitment of DC precursors from the blood and into the tissues[582]. In addition, MC products can directly modulate DC activation and antigen presentation [583] and the upregulation of co-stimulatory molecules required for T cell activation[584]. Furthermore, MC products can promote DC to acquire a Th2 cell-inducing phenotype[585].

MC can also promote activation of Langerhans cells, a skin-resident DC subset, in response to the bacterial product peptidoglycan [586] or Gram-negative bacteria[581], which lead to increased numbers of Langerhans cells in the draining lymph nodes[581, 586]. MC can also modulate cell trafficking to draining lymph nodes, as MC-derived TNF is required for normal lymph node hypertrophy due to retention of lymphocytes in an *Escherichia coli* infection model[545], contributing to adaptive responses.

In chapter 3, I demonstrate that poly(I:C) with or without NT or CRH does not have any in vitro effect on β-hex, TNF, IL-8 and VEGFα release from human cultured LAD2

MC, but increases only TNF gene expression and when used together with CRH, NT, or SP, TNF gene expression is augmented. This may be relevant to my results with human samples in chapter 4, where CRH and NT serum levels were shown to be increased in patients with AD, a skin condition involving MC activation often present in CFS patients[473, 587].

NT is a vasoactive peptide originally isolated from the brain[457, 588], which increases histamine release from rodent skin in a MC-dependent manner [458] and has been implicated in MC activation through specific NT receptors (NTR)[286]. NT is increased in the skin following acute stress, stimulates skin MC and increases vascular permeability in rodents[459].

MC synthesize and secrete the NT precursor, which is cleaved to give the active form[286]. NT from MC might serve autocrine and paracrine roles and it is tightly regulated since there is rapid degradation of NT from MC-derived carboxypeptidase-like enzymes, modulating the level and form of NT-related peptides in various conditions involving activation of MC[287, 589]. MC activation leads to the release of multiple mediators with potent vasodilatory, inflammatory and nociceptive properties through which they participate in innate and acquired immunity[418], as well as in inflammation[590], especially in the skin[591]. NT also augments the effect of CRH, secreted under stress, to increase MC-dependent skin vascular permeability[459].

There is considerable evidence that stress worsens AD, a skin disease with chronic inflammation and severe pruritus[592], lesions of which contain increased MC close to nerve endings[454], containing neuropeptides, such as SP[593]. PS is a chronic inflammatory skin disorder characterized by keratinocyte hyperproliferation and increased epidermal

vascularization, associated with high skin VEGFα [455]. Neuropeptides have been implicated in PS[444], but their mechanism is not well understood. I therefore investigated serum NT, as well as skin NT and NTR-1 gene expression in patients with AD and PS and controls. I also studied the effect of NT on human cultured MC VEGFα release.

NT serum levels were increased in AD patients[587]. NT gene expression was also increased in lesional AD as compared to normal skin. Immunohistochemistry confirmed the presence of NT in lesional AD skin, but was absent in normal skin. A possible explanation may be the fact that as I had shown, NT is not present in rat skin under normal conditions, but is released in a precursor form from nerve endings and is generated by proteases in the skin[459].

Additionally, I showed that NT is also increased in the serum of PS patients and stimulates VEGFα release from human MC[472]. NT was also elevated in the serum of children with autism[475], which has been associated with AD and with mothers who had psoriasis during pregnancy[477]. I also showed decreased PS skin NT and NTR-1 gene expression. NTR-1 gene expression was increased in non-lesional PS skin. The reason for this finding is not clear, but suggests non-lesional skin may not as yet have been maximally affected by the circulating NT.

I also showed that NT induces MC VEGFα release. MC express NT receptors [479] stimulation of which results in histamine release [458] and NT is rapidly degraded by MC proteases [287] implying tight regulation[458]. I had previously shown that NT has synergistic action with CRH to increase skin vascular permeability in rodents through MC activation[459]. This finding is relevant in view of evidence showing increased number of skin MC in Ps[446].

Similarly, I reported that CRH is increased in the serum of AD and PS patients, while there is decreased CRHR-1 gene expression in lesional skin of patients with Ps[478]. Autoimmune skin diseases, such as PS and AD, are chronic inflammatory skin disorders mediated primarily by T cells[594], but MC are also implicated[595]. Symptoms of both PS and AD worsen with stress[246]. Acute stress leads to increased vascular permeability and inflammation of skin, through mast-cell activation by CRH both in rodents [596] and in humans[597]. CRH and CRH receptor 1 (CRHR-1) are both expressed in human skin[483], leading to the hypothesis that CRH may be involved in the pathophysiology of skin diseases[598]. Our findings suggest that overstimulation by the increased serum levels of CRH and VEGFα, possibly in response to stress, leads to decreased gene expression of skin CRH and CRHR-1, as well as skin VEGFα gene expression, respectively. The positive correlation between serum CRH levels and the STAI-now scores in patients with severe PS supports this possibility. The lack of a similar correlation in patients with AD may be because I did not have access to severity index scores for these patients, unlike in patients with PS, in order to carry out a subgroup analysis.

The present findings suggest that high serum CRH level, possibly in response to stress, stimulates skin MC to release VEGFα and contribute to skin inflammation evident in patients with severe PS. Continuous or repeated stimulation may lead to decreased expression of skin CRHR-1, as I showed recently with cultured MC [474] and hence decreased VEGFα, as was evident in patients with mild disease. CRH and CRHR-1 may therefore participate in the pathogenesis of PS and AD, especially when worsened with stress, through mast-cell activation to release VEGFα. Mast-cell blockers may provide novel treatment approaches.

This is the first report to my knowledge of increased serum levels of NT in any autoimmune or inflammatory disease. NT can be released from sensory nerve endings[459], and MC[286]. NT can stimulate VEGFα release from human MC [472] and augments the ability of CRH to increase skin vascular permeability in rodents[459]. I also recently showed that NT increases CRHR-1 expression in human cultured MC [250] and that CRH is increased in the serum of AD patients[473].

NT may be involved in the pathogenesis of PS by acting together with CRH secreted under stress in the skin[482], which has its own equivalent of the HPA axis[483]. The stimulatory effects of NT and IL-33 on VEGFα release may be in addition to a similar action by local skin SP. I had shown that SP stimulated human MC to release VEGFα [485] and that this action was augmented by IL-33[485].

MC are now considered critical in allergy and innate immunity in the skin[488, 591]. Interactions among NT, CRH, and MC may mediate the effect of stress on skin inflammation in AD, as part of a "brain-skin" connection[489]. Moreover, the present findings suggest that NT may participate in the pathogenesis of PS, especially when worsened with stress, through MC involvement in a "brain-skin" connection[489]. NT and MC blockers may provide novel treatment approaches.

5. Mechanisms for Flavonoids in CFS

A study looking at the production of NF-κB p50 subunit in peripheral blood lymphocytes of 18 unmedicated patients with CFS and 18 matched controls showed significantly higher levels in CFS patients than controls. The level of NF-κB was also

highly correlated with the severity of illness as measured with the FibroFatigue scale (Fibromyalgia and chronic fatigue syndrome rating scale)[599].

Morris and Maes hypothesized that increased NF-κB (upstream regulator of the oxidative and nitrosative (IO&NS) pathway[599, 600] and/or loss of p53, their association with elevated ROS production, along with mitochondrial dysfunction in skeletal muscles or the brain may explain the symptoms of CFS including muscle pain, fatigue and neurocognitive disorders. The ATP profile test carried out in neutrophils of CFS patients showed mitochondrial dysfunction[601-603]. These findings suggest that future studies should look at inhibiting NF-κB activation and translocation, as well as antioxidant treatment as strategies for alleviating the symptoms of CFS. However, these are relatively new findings and more independent studies must be done to confirm them.

Flavonoids exert their strong antioxidant and anti-inflammatory activity [176, 212] through multiple mechanisms, including inhibition of the pathways mentioned above. Although studies with flavonoids *in vitro* are generally examined at concentrations that are higher than physiologically achievable levels[176], in general, isoflavones have the highest bioavailability, followed by flavonols, flavanones and flavanols[180, 205].

Isoflavones serum max concentrations in humans can reach 3-5 μM[206, 207], although concentrations as high as 25 μM have been achieved after supplementation. One study showed that isoflavone aglycones are absorbed faster and in higher amounts than their glycosides in humans[208]. Another study has shown that commercial rodent-diets can lead to steady-state isoflavone serum concentrations in adult rats around 2.6 μM and mice around 2.3 μM[604]. In another study, rats provided with diet of 0.2 g/kg genistein for 19 weeks had plasma levels around 5 μM, while it could also go as high as 10-11 μM[605-607].

I provided my mice with the commercially available 2018 Teklad Global 18% Protein Rodent Diet, a fixed formula that does not contain alfalfa thus lowering the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein and genistein aglycone equivalents) range from 150 to 250 mg/kg. Absence of animal protein and fish meal minimizes the presence of nitrosamines. If the food intake of typical C57BL/6 mouse is around 3-5 g/d, then the daily isoflavone oral intake would be around 0.8 mg, giving a plasma levels in the scale of μM, according to the papers mentioned above.

The capacity to conjugate isoflavones differs between mice and humans. The steady state percentage of unconjugated genistein concentration in plasma from C57BL/6 mice was about 4.6% and was about 23 times that in humans (the mean percentage of genistein in human plasma is 0.2% of steady state)[608]. Using human liver microsomes, the isoflavone daidzein was shown to be metabolized by CYP1A2 [609] and the same was shown for genistein in rats and humans[610-612]. Moreover, the extra-hepatic CYP1A1 and CYP1B1 may contribute to localized metabolism[609]. This is important in tissues such as breast and prostate, which express these isoforms, especially because one study showed that *in vitro* there was an enhancement of biological anti-cancer activity in a CYP-mediated daidzein metabolite[609].

There are some discrepancies between *in vitro* and *in vivo* studies regarding interactions of isoflavones with P450 cytochromes (CYP) activity. *In vitro* studies demonstrated that genistein and daidzein strongly inhibit CYP3A activity[613, 614]. However, in healthy Chinese individuals, oral administration of genistein (1000 mg once daily for 14 days) modestly (about 10%) induced CYP3A. Moreover, induction of

CYP3A was probably mainly in the wall of the small intestine[615, 616]. The mechanism of CYP3A induction by genistein remains to be investigated, but it was shown that human CYP3A4 and murine Cyp3A11 mRNA are up regulated by genistein and equol via the pregnane X receptor (PXR) in a species-specific manner.

Flavonoids and BBB

Studies have shown that flavonoids can cross BBB[632]. Quercetin was shown to accumulate in rat brain after repeated oral doses and effectively increased the forced swim-reduced locomotor activity and the open field test parameter[632]. Moreover, oral administration of quercetin attenuated the increased oxidative stress in the hippocampus and striatum of rats exposed to chronic forced swimming, as was measured by thiobarbituric acid-reactive substances (TBARS) and reduced glutathione (GSH) levels. Transport of quercetin derivatives into the brain tissue was reproduced in vitro by using a rat brain capillary endothelial cell line, RBEC1, a model of the blood-brain barrier[633].

Flavonoid naringein and quercetin permeability of BBB was shown using an *in situ* model of rat brain[634]. Further *in vitro* studies using ECV304/C6 coculture and immortalized rat brain endothelial cells (RBE4) showed that the permeability of flavonoids is influenced by their lipophilicity and interactions with efflux transporters[634]. Another study showed the transmembrane transport of flavonoids catechin and quercetin across RBE-4 cells (an immortalized cell line of rat cerebral capillary endothelial cells), a blood-brain barrier model[635].

6. New Treatment Approaches for CFS

Currently, there are no approved drugs for the treatment of CFS[152]. Treatment strategies for CFS include psychological, physical, and pharmacological intervention[108]. Most treatment regimens start with well-balanced nutrition habits and encouragement to exercise mildly as a way to boost their immune system[3, 31, 108, 133-136]. A new drug candidate, Ampligen®, is a mismatched double-stranded RNA with immunomodulatory and antiviral properties that acts as a TLR-3 agonist[617]. A phase III prospective, double-blind, randomized, placebo-controlled trial compared twice-weekly Ampligen® to a placebo in 234 subjects with long-standing, debilitating CFS. The 40 week study showed improvement in exercise tolerance, as well as reduction in other medication usage[618]. In 2009, the FDA rejected the application for approval of Ampligen® as a CFS treatment and recommended another trial be conducted in more than 300 CFS patients for proving more sufficient efficacy and safety data. The initial study was released in early 2012, and patients are currently being recruited for a clinical trial of Ampligen® in CFS patients.

Unfortunately, there is no effective human MC inhibitor clinically available either. Disodium cromoglycate (cromolyn) improved only gastrointestinal symptoms in patients with mastocytosis[619]. Interestingly, cromolyn inhibits histamine secretion from rat peritoneal MC[620], but not intestinal MC[621, 622]. It also does not inhibit human gastrointestinal and lung mucosal MC[623], or human umbilical cord blood-derived cultured MC (hCBMC)[624, 625]. More recently, cromolyn was reported to not even inhibit mouse MC[624, 625]. Therefore, there is reason to investigate new effective MC blockers, like flavonoids, that are potent anti-oxidant and anti-inflammatory compounds with MC inhibitory actions.

7. Future Directions

7.1 *In vitro* Studies

Our *in vitro* results from poly(I:C)-treated human LAD2 MC indicate that LAD2 MC do not exhibit strong antiviral responses. Although TLR-3 expression on the MC lines LAD2, and HMC-1 has been assessed in different studies[325-327, 330, 335, 626], studies show that poly(I:C)-induced antiviral response depends on the MC type[325]. Use of another cell line like human umbilical cord blood-derived MC might be more appropriate, as the cytokine generation is more efficient in primary cultures of human MC derived from CD34+ progenitor cells[627]. Moreover, *in vitro* use of a live virus, instead of poly(I:C), could trigger a stronger response.

Increasing evidence implicates CNS inflammation, as well as MC-microglia interactions, in neurophychiatric diseases [628, 629] and TLRs have been implicated in stress-induced central nervous system (CNS) dysfunction through MC and glial activation[628]. Therefore, isolation of brain MC using a recently published novel isolation method, would enable us to study MC function *in vivo*, identify their environment-specific phenotype, study their interaction with other neural cells *in vitro*, and investigate the pathways mediating the inhibitory effect of isoflavones. This method adapted a previously described technique of coupling an antibody to the MC-specific FcεRI receptor to a MACS microbead for the selective removal of intact MC from a neonatal rat brain preparation[630].

7.2 *In vivo* studies

There is still need to establish a murine model for chronic fatigue. Infection of MC with live Sendai virus induced stronger anti-viral response than extracellular poly(I:C)[558]. Future studies including treatment with Sendai virus, could be proven helpful.

Further studies need to take place to explore the role of MC in fatigue symptoms. *In vivo* studies towards this direction could include the use of MC-deficient mice, as a study showed that respiratory Influenza A virus (IAV) infection caused significant disease and immunopathology in C57BL/6 mice, but not in B6.Cg-Kit(W-sh) mice, which lack MC[636].

7.3 Human Studies

A systematic study evaluating how well murine models mimic human inflammatory diseases showed that although acute inflammatory stress from different etiologies result in highly similar genomic responses in human, the responses in corresponding mouse models correlate poorly with the human conditions[497]. Therefore, there is need for studies in humans.

7.3.1. Subgroups of CFS patients

Patients with CFS have increased and dysregulated MC in their skin, as well as hypersensitivity. NT is a vasoactive peptide [457] which increases histamine release from rodent skin in a MC-dependent manner[458]. NT also augments the effect of CRH, secreted under stress, to increase MC-dependent skin vascular permeability[459]. There is plenty of evidence that there may be subgroups of CFS patients, especially those with depression.

Serum biomarkers should therefore be measured in appropriately defined cohorts of patients, and any clinical studies should also address such subgroups.

7.3.2. Clinical trials with isoflavones and other flavonoids

Effects of isoflavones may not only be present in the brain, but also in the periphery and muscles. *In vitro* studies showed that genistein reduced and improved muscle fatigue resistance and enzymatic activity of glutathione peroxidase, catalase, and superoxide dismutase after chronic intermittent hypoxia conditions. Genistein reversed the fatigue symptoms by down-regulating oxidative stress level and up-regulating antioxidant enzymatic activity probably through ERK1/2 signaling pathway[221].

However, one study failed to show that quercetin supplementation improves repeated-sprint performance in fifteen active young adult men (drink of 500 mg of quercetin-3-glucoside, consumed twice a day (1,000 mg/d), for one week)[637]. Another randomized, double-blind, study using repeated-measures, placebo-controlled design, with 58 healthy, moderately trained men and women, failed to show that quercetin influenced energy, fatigue or sleep quality in young individuals conducting military physical training (42 to 54 days of supplementation with 1 g/day of quercetin in a soft chew)[638].

Flavonoids have strong antioxidant activity, but also show antinflammatory, neuroprotective, anticancer and cardiovascular effects[212]. Several in vitro studies examined the anti-carcinogenic function of these compounds, that are be attributed to a combination of their cytoprotective effect on normal cells and their cytotoxic effect on pre-neoplastic and/or neoplastic cells[639].

A phase I pharmacokinetic and pharmacodynamics analysis of unconjugated soy isoflavones administered to individuals with cancer showed that oral administration of genistein gives plasma concentrations of genistein that have been associated with antimetastatic activity in vitro (Cmax: 4-16 μM). Estimates of average daily genistein consumption by soy consumers range from 0.3-1 mg/kg. Thus participants in the current study were given 2-8x the max average dietary intake. No toxicities were observed[640]. A Phase II randomized, placebo-controlled clinical trial showed that purified isoflavones failed to modulate steroid hormones in men diagnosed with localized prostate cancer, although significant increases in plasma isoflavones were observed with no toxicity[641].

Whether polyphenols exert any direct antioxidant effects in the brain or rather act by evoking alterations in regulatory systems of the brain or even the body periphery is still unclear[536]. Concentrations of polyphenols in animal brains following oral administration are low; moreover, flavonoids in vitro are generally examined at concentrations that are much higher than physiologically achievable levels[176]. However, a review of human randomized controlled trial studies (2009) summarized some significant benefits to cognitive function after flavonoid supplementation[642]. Papers have reported improvements mainly in executive function, working memory, other memory functions and more general measures such as processing speed[642]. Specifically, in two studies they found significant treatment effects with 60 mg/day isoflavones in processing speed or psychomotor speed[643, 644].

Certain pilot clinical trials, used dietary supplements containing quercetin and luteolin (in olive seed oil microspheres to increase oral absorption) and showed

significant benefit in bladder pain syndrome/interstitial cystitis [645, 646] and autism [647, 648] patients.

7.3.3. Increasing oral absorption

Finally, the structure-activity relationship, with respect to CFS has not been defined yet. Obviously, improving oral bioavailability is very important. Methylation appears to be a simple and effective way to improve flavonoid intestinal absorption and hepatic metabolic stability[649]. One study showed that methylation increased hydrophobic interaction and genistein binding affinity for human serum albumin by two to sixteen times, that can facilitate transportation ability[650].

The sugar moiety increases absorption of quercetin in humans as was shown by a study where there was a twenty-fold increase in Cmax, but maybe this is not the case for isoflavones. A study carried out on the bioavailability of genistein (aglycone) and its glycoside genistin, confirmed that the bioavailability of the aglycone was higher compared to its glycoside form[651]. The experiment was carried out on unanesthetized rats and after ingestion of the compounds, blood was collected using a permanently inserted cannula into the portal vein. The isoflavone profile from the portal vein plasma provided an insight into deglycosylation of isoflavones in the small intestine before undergoing hepatic metabolism.

Improving oral bioavailability is obviously important, as is improving metabolic stability, changing the site of absorption (from colon to small intestine). To achieve these goals microencapsulation, nano-delivery systems, microemulsions, enzymatic methylation of flavonoids are among some of the techniques used[652].

A study reported enhanced intestinal absorption of daidzein by borneol/menthol eutectic mixture and microemulsion[653], while a self-assembled nanodelivery system was shown to enhance the oral bioavailability of daidzein in rats[654].

Another paper showed that formulated fabricating solid dispersion granules (SDGs) increased the intestinal absorption and antiasthmatic efficacy of curcumin. They showed that compared to curcumin, SDG-curcumin showed a 9.1- and 13.1-fold increase in AUC and Cmax, respectively. Further, SDG-curcumin effectively alleviated airway hyperresponsiveness and levels of Th2 cytokines in a murine model of asthma[655]. Currently, an oral enhanced-absorbed curcumin, Enhansa ®, is used employed by physicians and pharmacists by prescription and over-the-counter for years with an excellent safety profile.

Studies with isoflavone supplementation in CFS patients might be helpful.

List of original manuscripts

This dissertation work was published as follows:

Vasiadi M, Newman J, Theoharides TC. Inhibitory effect of isoflavones on poly(I:C)-induced inflammatory markers and relevance to chronic fatigue syndrome. *Journal of Neuroinflammation*, submitted.

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Vasiadi M, Therianou A, Alysandratos KD, Katsarou-Katsari A, Petrakopoulou T, Theoharides A, Papadavid E, Stavrianeas N, Antoniou C, Kalogeromitros D, Theoharides TC. Serum neurotensin (NT) is increased in psoriasis and NT induces vascular endothelial growth factor release from human MC. *Br J Dermatol*. 2012 Jun;166(6):1349-52.

Vasiadi M, Mondolfi AP, Alysandratos KD, Therianou A, Katsarou-Katsari A, Petrakopoulou T, Theoharidis A, Miniati A, Theoharides TC. Neurotensin serum levels and skin gene expression are increased in atopic dermatitis. *Br J Dermatol.* 2013 169: pp695-699.

Articles written during, but not directly related to, this dissertation:

Alevizos M, Karagkouni A, Panagiotidou S, **Vasiadi M**, Theoharides TC. Stress triggers coronary mast cells leading to cardiac events. *Ann Allergy Asthma Immunol.* 2013 Oct 10. [Epub ahead of print]

Alevizos M, Karagkouni A, **Vasiadi M**, Sismanopoulos N, Makris M, Kalogeromitros D, Theoharides TC. Rupatadine inhibits inflammatory mediator release from human laboratory of allergic diseases 2 cultured mast cells stimulated by platelet-activating factor. *Ann Allergy Asthma Immunol.* 2013 Dec;111(6):542-7.

Court MH, Peter I, Hazarika S, **Vasiadi M**, Greenblatt DJ, Lee W. Candidate Gene Polymorphisms in Patients with Acetaminophen-Induced Acute Liver Failure. *Drug Metab Dispos*. 2014 Jan;42(1):28-32.

Miniati A, Weng Z, Zhang B, Therianou A, **Vasiadi M**, Nicolaidou E, Stratigos AJ, Antoniou C, Theoharides TC. Stimulated human melanocytes express and release interleukin-8, which is inhibited by luteolin: relevance to early vitiligo. *Clin Exp Dermatol. 2014 Jan;39(1):54-7.*

Sismanopoulos N, Delivanis DA, Alysandratos KD, Angelidou A, **Vasiadi M**, Therianou A, Theoharides TC. IL-9 induces VEGFα secretion from human mast cells and IL-9/IL-9 receptor genes are overexpressed in atopic dermatitis. *PLoS One.* 2012;7(3):e33271.

Asadi S, Alysandratos KD, Angelidou A, Miniati A, Sismanopoulos N, **Vasiadi M**, Zhang B, Kalogeromitros D, Theoharides TC. Substance P (SP) induces expression of functional corticotropin-releasing hormone receptor-1 (CRHR-1) in human mast cells. *J Invest Dermatol*. 2012 Feb;132(2):324-9.

Clemons A, **Vasiadi M**, Kempuraj D, Kourelis T, Vandoros G, Theoharides TC. Amitriptyline and prochlorperazine inhibit proinflammatory mediator relase from human mast cells: possible relevance to chronic fatigue syndrome. *J Clin Psychopharmacol*. 2011 Jun;31(3):385-7.

Zhang B, Alysandratos KD, Angelidou A, Asadi S, Sismanopoulos N, Delivanis DA, Weng Z, Miniati A, **Vasiadi M**, Katsarou-Katsari A, Miao B, Leeman SE, Kalogeromitros D, Theoharides TC. Human mast cell degranulation and preformed TNF secretion require mitochondrial translocation to exocytosis sites: relevance to atopic dermatitis. *J Allergy Clin Immunol.* 2011 Jun;127(6):1522-31.e8.

Angelidou A, Alysandratos KD, Asadi S, Zhang B, Francis K, **Vasiadi M**, Kalogeromitros D, Theoharides TC. Brief report: "allergic symptoms" in children with Autism Spectrum Disorders. More than meets the eye? *J Autism Dev Disord.* 2011 Nov;41(11):1579-85.

Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, Asadi S, **Vasiadi M**, Weng Z, Miniati A, Kalogeromitros D. Mast cells and inflammation. *Biochim Biophys Acta*. 2012 Jan;1822(1):21-33.

Zhang B, Angelidou A, Alysandratos KD, **Vasiadi M**, Francis K, Asadi S, Theoharides A, Sideri K, Lykouras L, Kalogeromitros D, Theoharides TC. Mitochondrial DNA and anti-mitochondrial antibodies in serum of autistic children. *J Neuroinflammation.* 2010 Nov 17;7:80.

Alysandratos KD, Angelidou A, **Vasiadi M**, Zhang B, Kalogeromitros D, Katsarou-Katsari A, Theoharides TC. Increased affected skin gene expression and serum levels of

thymic stromal lymphopoietin in atopic dermatitis. *Ann Allergy Asthma Immunol.* 2010 Nov;105(5):403-4.

Kempuraj D, Conti P, **Vasiadi M**, Alysandratos KD, Tagen M, Kalogeromitros D, Kourelis T, Gregoriou S, Makris M, Stavrianeas NG, Theoharides TC. IL-32 is increased along with tryptase in lesional psoriatic skin and is up-regulated by substance P in human mast cells. *Eur J Dermatol.* 2010 Nov-Dec;20(6):865-7.

Angelidou A, Francis K, **Vasiadi M**, Alysandratos KD, Zhang B, Theoharides A, Lykouras L, Sideri K, Kalogeromitros D, Theoharides TC. Neurotensin is increased in serum of young children with autistic disorder. *J Neuroinflammation*. 2010 Aug 23;7:48.

Theoharides TC, Zhang B, Kempuraj D, Tagen M, **Vasiadi M**, Angelidou A, Alysandratos KD, Kalogeromitros D, Asadi S, Stavrianeas N, Peterson E, Leeman S, Conti P. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. *Proc Natl Acad Sci U S A.* 2010 Mar 2;107(9):4448-53.

Vasiadi M, Kalogeromitros D, Kempuraj D, Clemons A, Zhang B, Chliva C, Makris M, Wolfberg A, House M, Theoharides TC. Rupatadine inhibits proinflammatory mediator secretion from human mast cells triggered by different stimuli. *Int Arch Allergy Immunol*. 2010;151(1):38-45.

Theoharides TC, Kempuraj D, Marchand J, Tzianoumis L, **Vasiadi M**, Katsarou-Katsari A, Makris M, Kalogeromitros D. Urticaria pigmentosa associated with acute stress and lesional skin mast-cell expression of CRF-R1. *Clin Exp Dermatol.* 2009 Jul;34(5):e163-6.

Bibliography

1. Van't LM, Zielhuis GA, van der Meer JW, Verbeek AL, Bleijenberg G. Fatigue and chronic fatigue syndrome-like complaints in the general population. ***Eur J Public Health*** 20(3): 251-257, 2010.

2. Lloyd AR, Hickie I, Boughton CR, Spencer O, Wakefield D. Prevalence of chronic fatigue syndrome in an Australian population. ***Med J Aust*** 153(9): 522-528, 1990.

3. Griffith JP, Zarrouf FA. A systematic review of chronic fatigue syndrome: don't assume it's depression. ***Prim Care Companion J Clin Psychiatry*** 10(2): 120-128, 2008.

4. Jason LA, Richman JA, Rademaker AW, Jordan KM, Plioplys AV, Taylor RR, McCready W, Huang CF, Plioplys S. A community-based study of chronic fatigue syndrome. ***Arch Intern Med*** 159(18): 2129-2137, 1999.

5. Reyes M, Nisenbaum R, Hoaglin DC, Unger ER, Emmons C, Randall B, Stewart JA, Abbey S, Jones JF, Gantz N, Minden S, Reeves WC. Prevalence and incidence of chronic fatigue syndrome in Wichita, Kansas. ***Arch Intern Med*** 163(13): 1530-1536, 2003.

6. Steele L, Dobbins JG, Fukuda K, Reyes M, Randall B, Koppelman M, Reeves WC. The epidemiology of chronic fatigue in San Francisco. ***Am J Med*** 105(3A): 83S-90S, 1998.

7. Jordan KM, Landis DA, Downey MC, Osterman SL, Thurm AE, Jason LA. Chronic fatigue syndrome in children and adolescents: a review. ***J Adolesc Health*** 22(1): 4-18, 1998.

8. Chalder T, Goodman R, Wessely S, Hotopf M, Meltzer H. Epidemiology of chronic fatigue syndrome and self reported myalgic encephalomyelitis in 5-15 year olds: cross sectional study. ***BMJ*** 327(7416): 654-655, 2003.

9. Jones JF, Nisenbaum R, Solomon L, Reyes M, Reeves WC. Chronic fatigue syndrome and other fatiguing illnesses in adolescents: a population-based study. ***J Adolesc Health*** 35(1): 34-40, 2004.

10. Jason LA, Corradi K, Torres-Harding S, Taylor RR, King C. Chronic fatigue syndrome: the need for subtypes. ***Neuropsychol Rev*** 15(1): 29-58, 2005.

11. Jahan F, Nanji K, Qidwai W, Qasim R. Fibromyalgia syndrome: an overview of pathophysiology, diagnosis and management. ***Oman Med J*** 27(3): 192-195, 2012.

12. Anderson VR, Jason LA, Hlavaty LE, Porter N, Cudia J. A review and meta-synthesis of qualitative studies on myalgic encephalomyelitis/chronic fatigue syndrome. ***Patient Educ Couns*** 86(2): 147-155, 2012.

13. Aaron LA, Buchwald D. Fibromyalgia and other unexplained clinical conditions. ***Curr Rheumatol*** 3: 116-122, 2001.

14. Abbi B, Natelson BH. Is chronic fatigue syndrome the same illness as fibromyalgia: evaluating the 'single syndrome' hypothesis. ***QJM*** 106(1): 3-9, 2013.

15. Aaron LA, Buchwald D. Chronic diffuse musculoskeletal pain, fibromyalgia and co-morbid unexplained clinical conditions. ***Best Prac Res Clin Rheumatol*** 17(4): 563-574, 2003.

16. Smith MS, Martin-Herz SP, Womack WM, Marsigan JL. Comparative study of anxiety, depression, somatization, functional disability, and illness attribution in adolescents with chronic fatigue or migraine. ***Pediatrics*** 111(4 Pt 1): e376-e381, 2003.

17. Patarca-Montero R. Medical Etiology, Assessment, and Treatment of Chronic Fatigue and Malaise: Clinical Differentiation and Intervention; What Does the Research Say? Binghamtom, NY: The Hawthorne Medical Press, 2004

18. Holmes GP, Kaplan JE, Gantz NM, Komaroff AL, Schonberger LB, Straus SE, Jones JF, Dubois RE, Cunningham-Rundles C, Pahwa S, . Chronic fatigue syndrome: a working case definition. ***Ann Intern Med*** 108(3): 387-389, 1988.

19. Barnes DM. Mystery disease at Lake Tahoe challenges virologists and clinicians. ***Science*** 234(4776): 541-542, 1986.

20. Buchwald D, Cheney PR, Peterson DL, Henry B, Wormsley SB, Geiger A, Ablashi DV, Salahuddin SZ, Saxinger C, Biddle R, . A chronic illness characterized by fatigue, neurologic and immunologic disorders, and active human herpesvirus type 6 infection. ***Ann Intern Med*** 116(2): 103-113, 1992.

21. Lloyd AR, Wakefield D, Boughton C, Dwyer J. What is myalgic encephalomyelitis? ***Lancet*** 1(8597): 1286-1287, 1988.

22. Sharpe MC, Archard LC, Banatvala JE, Borysiewicz LK, Clare AW, David A, Edwards RH, Hawton KE, Lambert HP, Lane RJ, . A report--chronic fatigue syndrome: guidelines for research. ***J R Soc Med*** 84(2): 118-121, 1991.

23. Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, Komaroff A, Intnl.Chron.Fatigue Synd.Study Group. The chronic fatigue syndrome: a comprehensive approach to its definition and study. ***Ann Intern Med*** 121: 953-959, 1994.

24. Jason LA, Jordan K, Miike T, Bell DS, Lapp C, Torres-Harding S, Rowe K, Gurwitt A, De Meirleir K, Van Hoof ELS, Psych C. A pediatric case definition for myalgic encephalomyelitis and chronic fatigue syndrome. ***Journal of Chronic Fatigue Syndrome*** 13(No. 2/3): 1-44, 2006.

25. Jason LA, Torres-Harding SR, Jurgens A, Helgerson J. Comparing the Fukuda et al. criteria and the Canadian case definition for chronic fatigue syndrome. ***Journal of Chronic Fatigue Syndrome*** 12: 37-52, 2004.

26. Carruthers BM, Jain AK, DE Meirleir KL, Peterson DL, Klimas NG, Lerner AM, Bested AC, Flor-Henry P, Joshi P, Powles ACP, Sherkey JA, van de Sande MI. Myalgic encephalomyelitis/chronic fatigue syndrome: clinical working case definition, diagnostic and treatment protocols. ***Journal of Chronic Fatigue Syndrome*** 11(1)2003.

27. King C, Jason LA. Improving the diagnostic criteria and procedures fro chronic fatigue syndrome. ***Biological Psychology*** 68: 87-106, 2005.

28. Holgate ST, Komaroff AL, Mangan D, Wessely S. Chronic fatigue syndrome: understanding a complex illness. ***Nat Rev Neurosci*** 12(9): 539-544, 2011.

29. Christley Y, Duffy T, Martin CR. A review of the definitional criteria for chronic fatigue syndrome. ***J Eval Clin Pract*** 18(1): 25-31, 2012.

30. Morris G, Maes M. Case definitions and diagnostic criteria for Myalgic Encephalomyelitis and Chronic fatigue Syndrome: from clinical-consensus to evidence-based case definitions. ***Neuro Endocrinol Lett*** 34(3): 185-199, 2013.

31. Prins JB, van der Meer JW, Bleijenberg G. Chronic fatigue syndrome. ***Lancet*** 367(9507): 346-355, 2006.

32. Nijs J, Almond F, De BP, Truijen S, Paul L. Can exercise limits prevent post-exertional malaise in chronic fatigue syndrome? An uncontrolled clinical trial. ***Clin Rehabil*** 22(5): 426-435, 2008.

33. Buchwald D. An overview of chronic fatigue syndrome. ***NIH, Bethesda, MD*** 04-5497: 7-10, 2003.

34. Gur A, Oktayoglu P. Central nervous system abnormalities in fibromyalgia and chronic fatigue syndrome: new concepts in treatment. ***Curr Pharm Des*** 14(13): 1274-1294, 2008.

35. Cleare AJ, Miell J, Heap E, Sookdeo S, Young L, Malhi GS, O'Keane V. Hypothalamo-pituitary-adrenal axis dysfunction in chronic fatigue syndrome, and the effects of low-dose hydrocortisone therapy. ***J Clin Endocrinol Metab*** 86(8): 3545-3554, 2001.

36. Crawley E, Hunt L, Stallard P. Anxiety in children with CFS/ME. ***Eur Child Adolesc Psychiatry*** 18(11): 683-689, 2009.

37. Theoharides TC, Weinkauf C, Conti P. Brain cytokines and neuropsychiatric disorders. ***J Clin Psychopharmacol*** 24(6): 577-581, 2004.

38. Maes M, Twisk FN, Ringel K. Inflammatory and cell-mediated immune biomarkers in myalgic encephalomyelitis/chronic fatigue syndrome and depression: inflammatory markers are higher in myalgic encephalomyelitis/chronic fatigue syndrome than in depression. ***Psychother Psychosom*** 81(5): 286-295, 2012.

39. Bansal AS, Bradley AS, Bishop KN, Kiani-Alikhan S, Ford B. Chronic fatigue syndrome, the immune system and viral infection. ***Brain Behav Immun*** 26(1): 24-31, 2012.

40. Dietert RR, Dietert JM. Possible role for early-life immune insult including developmental immunotoxicity in chronic fatigue syndrome (CFS) or myalgic encephalomyelitis (ME). ***Toxicology*** 247(1): 61-72, 2008.

41. Bower JE. Fatigue, brain, behavior, and immunity: summary of the 2012 Named Series on fatigue. ***Brain Behav Immun*** 26(8): 1220-1223, 2012.

42. Lorusso L, Mikhaylova SV, Capelli E, Ferrari D, Ngonga GK, Ricevuti G. Immunological aspects of chronic fatigue syndrome. ***Autoimmun Rev*** 8(4): 287-291, 2009.

43. Avellaneda FA, Perez MA, Izquierdo MM, Arruti BM, Barbado Hernandez FJ, de la Cruz LJ, az-Delgado PR, Gutierrez RE, Palacin DC, Rivera RJ, Ramon G, Jr. Chronic fatigue syndrome: aetiology, diagnosis and treatment. ***BMC Psychiatry*** 9 Suppl 1: S1, 2009.

44. Chaudhuri A, Behan PO. Fatigue in neurological disorders. ***Lancet*** 363(9413): 978-988, 2004.

45. Arnett SV, Clark IA. Inflammatory fatigue and sickness behaviour - lessons for the diagnosis and management of chronic fatigue syndrome. ***J Affect Disord*** 141(2-3): 130-142, 2012.

46. Katz BZ, Shiraishi Y, Mears CJ, Binns HJ, Taylor R. Chronic fatigue syndrome after infectious mononucleosis in adolescents. ***Pediatrics*** 124(1): 189-193, 2009.

47. Fremont M, Metzger K, Rady H, Hulstaert J, De MK. Detection of herpesviruses and parvovirus B19 in gastric and intestinal mucosa of chronic fatigue syndrome patients. ***In vivo*** 23(2): 209-213, 2009.

48. Lombardi VC, Ruscetti FW, Das GJ, Pfost MA, Hagen KS, Peterson DL, Ruscetti SK, Bagni RK, Petrow-Sadowski C, Gold B, Dean M, Silverman RH, Mikovits JA. Detection of an Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome. ***Science***2009.

49. Oakes B, Tai AK, Cingoz O, Henefield MH, Levine S, Coffin JM, Huber BT. Contamination of human DNA samples with mouse DNA can lead to false detection of XMRV-like sequences. ***Retrovirology*** 7: 109, 2010.

50. Oakes B, Qiu X, Levine S, Hackett J, Jr., Huber BT. Failure to Detect XMRV-Specific Antibodies in the Plasma of CFS Patients Using Highly Sensitive Chemiluminescence Immunoassays. ***Adv Virol*** 2011: 854540, 2011.

51. Swanink CM, van der Meer JW, Vercoulen JH, Bleijenberg G, Fennis JF, Galama JM. Epstein-Barr virus (EBV) and the chronic fatigue syndrome: normal virus load in blood and normal immunologic reactivity in the EBV regression assay. ***Clin Infect Dis*** 20(5): 1390-1392, 1995.

52. Swanink CM, Vercoulen JH, Bazelmans E, Fennis JF, Bleijenberg G, van der Meer JW, Galama JM. Viral antibodies in chronic fatigue syndrome. ***Clin Infect Dis*** 21(3): 708-709, 1995.

53. Lloyd A. Postinfective fatigue. In: Jason LA, Taylor RR, eds. Handbook of chronic fatigue syndrome.Hoboken, NJ: 2003:108-123.

54. Nakaya T, Takahashi H, Nakamura Y, Asahi S, Tobiume M, Kuratsune H, Kitani T, Yamanishi K, Ikuta K. Demonstration of Borna disease virus RNA in peripheral blood mononuclear cells derived from Japanese patients with chronic fatigue syndrome. ***FEBS Lett*** 378(2): 145-149, 1996.

55. Li YJ, Wang DX, Bai XL, Chen J, Liu ZD, Feng ZJ, Zhao YM. [Clinical characteristics of patients with chronic fatigue syndrome: analysis of 82 cases]. ***Zhonghua Yi Xue Za Zhi*** 85(10): 701-704, 2005.

56. Chia JK. The role of enterovirus in chronic fatigue syndrome. ***J Clin Pathol*** 58(11): 1126-1132, 2005.

57. Chia JK, Chia AY. Chronic fatigue syndrome is associated with chronic enterovirus infection of the stomach. ***J Clin Pathol*** 61(1): 43-48, 2008.

58. Matano S, Kinoshita H, Tanigawa K, Terahata S, Sugimoto T. Acute parvovirus B19 infection mimicking chronic fatigue syndrome. ***Intern Med*** 42(9): 903-905, 2003.

59. Kerr JR. Pathogenesis of parvovirus B19 infection: host gene variability, and possible means and effects of virus persistence. ***J Vet Med B Infect Dis Vet Public Health*** 52(7-8): 335-339, 2005.

60. White PD, Thomas JM, Amess J, Crawford DH, Grover SA, Kangro HO, Clare AW. Incidence, risk and prognosis of acute and chronic fatigue syndromes and psychiatric disorders after glandular fever. ***Br J Psychiatry*** 173: 475-481, 1998.

61. Ng BY, Lim CC, Yeoh A, Lee WL. Neuropsychiatric sequelae of Nipah virus encephalitis. ***J Neuropsychiatry Clin Neurosci*** 16(4): 500-504, 2004.

62. White PD, Thomas JM, Sullivan PF, Buchwald D. The nosology of sub-acute and chronic fatigue syndromes that follow infectious mononucleosis. ***Psychol Med*** 34(3): 499-507, 2004.

63. Kondo K. [Human herpesvirus latency and fatigue]. ***Uirusu*** 55(1): 9-17, 2005.

64. Komaroff AL. Chronic fatigue syndromes: a preliminary overview. ***Can Dis Wkly Rep*** 17 Suppl 1E: 23-28, 1991.

65. Oakes B, Hoagland-Henefield M, Komaroff AL, Erickson JL, Huber BT. Human endogenous retrovirus-k18 superantigen expression and human herpesvirus-6 and human herpesvirus-7 viral loads in chronic fatigue patients. ***Clin Infect Dis*** 56(10): 1394-1400, 2013.

66. Rangel L, Garralda ME, Jeffs J, Rose G. Family health and characteristics in chronic fatigue syndrome, juvenile rheumatoid arthritis, and emotional disorders of childhood. ***J Am Acad Child Adolesc Psychiatry*** 44(2): 150-158, 2005.

67. Buchwald D, Herrell R, Ashton S, Belcourt M, Schmaling K, Sullivan P, Neale M, Goldberg J. A twin study of chronic fatigue. ***Psychosom Med*** 63(6): 936-943, 2001.

68. Kaushik N, Fear D, Richards SC, McDermott CR, Nuwaysir EF, Kellam P, Harrison TJ, Wilkinson RJ, Tyrrell DA, Holgate ST, Kerr JR. Gene expression in peripheral blood mononuclear cells from patients with chronic fatigue syndrome. ***J Clin Pathol*** 58(8): 826-832, 2005.

69. Kerr JR, Petty R, Burke B, Gough J, Fear D, Sinclair LI, Mattey DL, Richards SC, Montgomery J, Baldwin DA, Kellam P, Harrison TJ, Griffin GE, Main J, Enlander D, Nutt DJ, Holgate ST. Gene expression subtypes in patients with chronic fatigue syndrome/myalgic encephalomyelitis. ***J Infect Dis*** 197(8): 1171-1184, 2008.

70. Frampton D, Kerr J, Harrison TJ, Kellam P. Assessment of a 44 gene classifier for the evaluation of chronic fatigue syndrome from peripheral blood mononuclear cell gene expression. ***PloS One*** 6(3): e16872, 2011.

71. Sternberg EM. Health consequences of a dysregulated stress response. 2003. Bethesda, MD, *NIH*.

72. Wheatland R. Chronic ACTH autoantibodies are a significant pathological factor in the disruption of the hypothalamic-pituitary-adrenal axis in chronic fatigue syndrome, anorexia nervosa and major depression. ***Med Hypotheses*** 65(2): 287-295, 2005.

73. Wessely S. The neuropsychiatry of chronic fatigue syndrome. ***Ciba Found Symp*** 173: 212-229, 1993.

74. Richardson GS. Corticotropin-releasing hormone, insomnia and depression. ***NIH, Bethesda, MD*** 04-5497: 45-48, 2003.

75. Krueger J. Mechanisms underlying the central effects of cytokines. ***NIH, Bethesda, MD*** 04-5497: 63-68, 2003.

76. Inder WJ, Prickett TC, Mulder RT. Normal opioid tone and hypothalamic-pituitary-adrenal axis function in chronic fatigue syndrome despite marked functional impairment. ***Clin Endocrinol (Oxf)*** 62(3): 343-348, 2005.

77. Cleare AJ. The HPA axis and the genesis of chronic fatigue syndrome. ***Trends Endocrinol Metab*** 15(2): 55-59, 2004.

78. Hatcher S, House A. Life events, difficulties and dilemmas in the onset of chronic fatigue syndrome: a case-control study. ***Psychol Med*** 33(7): 1185-1192, 2003.

79. Theorell T, Blomkvist V, Lindh G, Evengard B. Critical life events, infections, and symptoms during the year preceding chronic fatigue syndrome (CFS): an examination of CFS patients and subjects with a nonspecific life crisis. ***Psychosom Med*** 61(3): 304-310, 1999.

80. Prins JB, Bos E, Huibers MJ, Servaes P, van der Werf SP, van der Meer JW, Bleijenberg G. Social support and the persistence of complaints in chronic fatigue syndrome. ***Psychother Psychosom*** 73(3): 174-182, 2004.

81. Maes M, Kubera M, Uytterhoeven M, Vrydags N, Bosmans E. Increased plasma peroxides as a marker of oxidative stress in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). ***Med Sci Monit*** 17(4): SC11-SC15, 2011.

82. Maes M, Twisk FN, Kubera M, Ringel K. Evidence for inflammation and activation of cell-mediated immunity in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS): Increased interleukin-1, tumor necrosis factor-alpha, PMN-elastase, lysozyme and neopterin. ***J Affect Disord***2011.

83. Maes M, Twisk FN, Kubera M, Ringel K, Leunis JC, Geffard M. Increased IgA responses to the LPS of commensal bacteria is associated with inflammation and activation of cell-mediated immunity in chronic fatigue syndrome. ***J Affect Disord*** 136(3): 909-917, 2012.

84. Patarca R, Klimas NG, Lugtendorf S, Antoni M, Fletcher MA. Dysregulated expression of tumor necrosis factor in chronic fatigue syndrome: interrelations with cellular sources and patterns of soluble immune mediator expression. ***Clin Infect Dis*** 18 Suppl 1: S147-S153, 1994.

85. Fletcher MA, Zeng XR, Barnes Z, Levis S, Klimas NG. Plasma cytokines in women with chronic fatigue syndrome. ***J Transl Med*** 7: 96, 2009.

86. Kim YI, Neher E. IgG from patients with Lambert-Eaton Syndrome blocks voltage- dependent calcium channels. ***Science*** 239: 405-408, 1988.

87. Nugraha B, Karst M, Engeli S, Gutenbrunner C. Brain-derived neurotrophic factor and exercise in fibromyalgia syndrome patients: a mini review. ***Rheumatol Int*** 32(9): 2593-2599, 2012.

88. Chao CC, Janoff EN, Hu SX, Thomas K, Gallagher M, Tsang M, Peterson PK. Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. ***Cytokine*** 3(4): 292-298, 1991.

89. Gaab J, Rohleder N, Heitz V, Engert V, Schad T, Schurmeyer TH, Ehlert U. Stress-induced changes in LPS-induced pro-inflammatory cytokine production in chronic fatigue syndrome. ***Psychoneuroendocrinology*** 30(2): 188-198, 2005.

90. Jammes Y, Steinberg JG, Delliaux S, Bregeon F. Chronic fatigue syndrome combines increased exercise-induced oxidative stress and reduced cytokine and Hsp responses. ***J Intern Med*** 266(2): 196-206, 2009.

91. White AT, Light AR, Hughen RW, Bateman L, Martins TB, Hill HR, Light KC. Severity of symptom flare after moderate exercise is linked to cytokine activity in chronic fatigue syndrome. ***Psychophysiology*** 47(4): 615-624, 2010.

92. Kerr JR, Barah F, Mattey DL, Laing I, Hopkins SJ, Hutchinson IV, Tyrrell DA. Circulating tumour necrosis factor-alpha and interferon-gamma are detectable during acute and convalescent parvovirus B19 infection and are associated with prolonged and chronic fatigue. ***J Gen Virol*** 82(Pt 12): 3011-3019, 2001.

93. Amel Kashipaz MR, Swinden D, Todd I, Powell RJ. Normal production of inflammatory cytokines in chronic fatigue and fibromyalgia syndromes determined by intracellular cytokine staining in short-term cultured blood mononuclear cells. ***Clin Exp Immunol*** 132(2): 360-365, 2003.

94. Vollmer-Conna U, Cameron B, Hadzi-Pavlovic D, Singletary K, Davenport T, Vernon S, Reeves WC, Hickie I, Wakefield D, Lloyd AR. Postinfective fatigue syndrome is not associated with altered cytokine production. ***Clin Infect Dis*** 45(6): 732-735, 2007.

95. Nas K, Cevik R, Batum S, Sarac AJ, Acar S, Kalkanli S. Immunologic and psychosocial status in chronic fatigue syndrome. ***Bratisl Lek Listy*** 112(4): 208-212, 2011.

96. Tomoda A, Joudoi T, Rabab e, Matsumoto T, Park TH, Miike T. Cytokine production and modulation: comparison of patients with chronic fatigue syndrome and normal controls. ***Psychiatry Res*** 134(1): 101-104, 2005.

97. Zhang HY, Liu ZD, Hu CJ, Wang DX, Zhang YB, Li YZ. Up-regulation of TGF-beta1 mRNA expression in peripheral blood mononuclear cells of patients with chronic fatigue syndrome. ***J Formos Med Assoc*** 110(11): 701-704, 2011.

98. Baraniuk JN, Casado B, Maibach H, Clauw DJ, Pannell LK, Hess SS. A Chronic Fatigue Syndrome - related proteome in human cerebrospinal fluid. ***BMC Neurol*** 5: 22, 2005.

99. Natelson BH, Weaver SA, Tseng CL, Ottenweller JE. Spinal fluid abnormalities in patients with chronic fatigue syndrome. ***Clin Diagn Lab Immunol*** 12(1): 52-55, 2005.

100. Schwartz M, Shechter R. Protective autoimmunity functions by intracranial immunosurveillance to support the mind: The missing link between health and disease. ***Mol Psychiatry*** 15(4): 342-354, 2010.

101. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. ***GLIA*** 55(5): 453-462, 2007.

102. Maier SF, Wiertelak EP, Martin D, Watkins LR. Interleukin-1 mediates the behavioral hyperalgesia produced by lithium chloride and endotoxin. ***Brain Res*** 623(2): 321-324, 1993.

103. Wiertelak EP, Furness LE, Watkins LR, Maier SF. Illness-induced hyperalgesia is mediated by a spinal NMDA-nitric oxide cascade. ***Brain Res*** 664(1-2): 9-16, 1994.

104. Watkins LR, Wiertelak EP, Furness LE, Maier SF. Illness-induced hyperalgesia is mediated by spinal neuropeptides and excitatory amino acids. ***Brain Res*** 664(1-2): 17-24, 1994.

105. Nijs J, van d, V, De MK. Pain in patients with chronic fatigue syndrome: does nitric oxide trigger central sensitisation? ***Med Hypotheses*** 64(3): 558-562, 2005.

106. Arnett SV, Alleva LM, Korossy-Horwood R, Clark IA. Chronic fatigue syndrome--a neuroimmunological model. ***Med Hypotheses*** 77(1): 77-83, 2011.

107. Raison CL, Borisov AS, Majer M, Drake DF, Pagnoni G, Woolwine BJ, Vogt GJ, Massung B, Miller AH. Activation of central nervous system inflammatory pathways by interferon-alpha: relationship to monoamines and depression. ***Biol Psychiatry*** 65(4): 296-303, 2009.

108. Huang L, Frampton G, Rao A, Zhang KS, Chen W, Lai JM, Yin XY, Walker K, Culbreath B, Leyva-Illades D, Quinn M, McMillin M, Bradley M, Liang LJ, DeMorrow S. Monoamine oxidase

A expression is suppressed in human cholangiocarcinoma via coordinated epigenetic and IL-6-driven events. ***Lab Invest*** 92(10): 1451-1460, 2012.

109. Raison CL, Rye DB, Woolwine BJ, Vogt GJ, Bautista BM, Spivey JR, Miller AH. Chronic interferon-alpha administration disrupts sleep continuity and depth in patients with hepatitis C: association with fatigue, motor slowing, and increased evening cortisol. ***Biol Psychiatry*** 68(10): 942-949, 2010.

110. Raison CL, Borisov AS, Woolwine BJ, Massung B, Vogt G, Miller AH. Interferon-alpha effects on diurnal hypothalamic-pituitary-adrenal axis activity: relationship with proinflammatory cytokines and behavior. ***Mol Psychiatry*** 15(5): 535-547, 2010.

111. Capuron L, Pagnoni G, Demetrashvili MF, Lawson DH, Fornwalt FB, Woolwine B, Berns GS, Nemeroff CB, Miller AH. Basal ganglia hypermetabolism and symptoms of fatigue during interferon-alpha therapy. ***Neuropsychopharmacology*** 32(11): 2384-2392, 2007.

112. Capuron L, Pagnoni G, Demetrashvili M, Woolwine BJ, Nemeroff CB, Berns GS, Miller AH. Anterior cingulate activation and error processing during interferon-alpha treatment. ***Biol Psychiatry*** 58(3): 190-196, 2005.

113. Natelson BH, Haghighi MH, Ponzio NM. Evidence for the presence of immune dysfunction in chronic fatigue syndrome. ***Clin Diagn Lab Immunol*** 9(4): 747-752, 2002.

114. Dhabhar FS. Stress and the augmentation of immune function. ***NIH, Bethesda, MD*** 04-5497: 17-21, 2003.

115. Klimas N. Evaluating immune function in CFS. ***NIH, Bethesda, MD*** 04-5497: 23-26, 2003.

116. Klimas NG, Broderick G, Fletcher MA. Biomarkers for chronic fatigue. ***Brain Behav Immun*** 26(8): 1202-1210, 2012.

117. Maes M. Inflammatory and oxidative and nitrosative stress pathways underpinning chronic fatigue, somatization and psychosomatic symptoms. ***Curr Opin Psychiatry*** 22(1): 75-83, 2009.

118. Fulle S, Pietrangelo T, Mancinelli R, Saggini R, Fano G. Specific correlations between muscle oxidative stress and chronic fatigue syndrome: a working hypothesis. ***J Muscle Res Cell Motil*** 28(6): 355-362, 2007.

119. Anderson G, Berk M, Dean O, Moylan S, Maes M. Role of Immune-Inflammatory and Oxidative and Nitrosative Stress Pathways in the Etiology of Depression: Therapeutic Implications. ***CNS Drugs***2013.

120. Keenoy B, Moorkens G, Vertommen J, De L, I. Antioxidant status and lipoprotein peroxidation in chronic fatigue syndrome. ***Life Sci*** 68(17): 2037-2049, 2001.

121. Keenoy B, Moorkens G, Vertommen J, Noe M, Neve J, De L, I. Magnesium status and parameters of the oxidant-antioxidant balance in patients with chronic fatigue: effects of supplementation with magnesium. ***J Am Coll Nutr*** 19(3): 374-382, 2000.

122. Smirnova IV, Pall ML. Elevated levels of protein carbonyls in sera of chronic fatigue syndrome patients. ***Mol Cell Biochem*** 248(1-2): 93-95, 2003.

123. Fulle S, Mecocci P, Fano G, Vecchiet I, Vecchini A, Racciotti D, Cherubini A, Pizzigallo E, Vecchiet L, Senin U, Beal MF. Specific oxidative alterations in vastus lateralis muscle of patients with the diagnosis of chronic fatigue syndrome. ***Free Radic Biol Med*** 29(12): 1252-1259, 2000.

124. Maes M, Mihaylova I, Leunis JC. Chronic fatigue syndrome is accompanied by an IgM-related immune response directed against neopitopes formed by oxidative or nitrosative damage to lipids and proteins. ***Neuro Endocrinol Lett*** 27(5): 615-621, 2006.

125. Kennedy G, Spence VA, McLaren M, Hill A, Underwood C, Belch JJ. Oxidative stress levels are raised in chronic fatigue syndrome and are associated with clinical symptoms. ***Free Radic Biol Med*** 39(5): 584-589, 2005.

126. Richards RS, Wang L, Jelinek H. Erythrocyte oxidative damage in chronic fatigue syndrome. ***Arch Med Res*** 38(1): 94-98, 2007.

127. Maes M, Mihaylova I, Kubera M, Uytterhoeven M, Vrydags N, Bosmans E. Increased 8-hydroxy-deoxyguanosine, a marker of oxidative damage to DNA, in major depression and myalgic encephalomyelitis / chronic fatigue syndrome. ***Neuro Endocrinol Lett*** 30(6): 715-722, 2009.

128. Maes M, Mihaylova I, Kubera M, Uytterhoeven M, Vrydags N, Bosmans E. Lower plasma Coenzyme Q10 in depression: a marker for treatment resistance and chronic fatigue in depression and a risk factor to cardiovascular disorder in that illness. ***Neuro Endocrinol Lett*** 30(4): 462-469, 2009.

129. Jammes Y, Steinberg JG, Delliaux S. Chronic fatigue syndrome: acute infection and history of physical activity affect resting levels and response to exercise of plasma oxidant/antioxidant status and heat shock proteins. ***J Intern Med*** 272(1): 74-84, 2012.

130. Vecchiet J, Cipollone F, Falasca K, Mezzetti A, Pizzigallo E, Bucciarelli T, De Laurentis S, Affaitati G, De CD, Giamberardino MA. Relationship between musculoskeletal symptoms and blood markers of oxidative stress in patients with chronic fatigue syndrome. ***Neurosci Lett*** 335(3): 151-154, 2003.

131. Miwa K, Fujita M. Fluctuation of serum vitamin E (alpha-tocopherol) concentrations during exacerbation and remission phases in patients with chronic fatigue syndrome. ***Heart Vessels*** 25(4): 319-323, 2010.

132. Kennedy G, Khan F, Hill A, Underwood C, Belch JJ. Biochemical and vascular aspects of pediatric chronic fatigue syndrome. ***Arch Pediatr Adolesc Med*** 164(9): 817-823, 2010.

133. Werbach MR. Nutritional strategies for treating chronic fatigue syndrome. ***Altern Med Rev*** 5(2): 93-108, 2000.

134. Edmonds M, McGuire H, Price J. Exercise therapy for chronic fatigue syndrome. ***Cochrane Database Syst Rev***(3): CD003200, 2004.

135. Nunez M, Fernandez-Sola J, Nunez E, Fernandez-Huerta JM, Godas-Sieso T, Gomez-Gil E. Health-related quality of life in patients with chronic fatigue syndrome: group cognitive behavioural therapy and graded exercise versus usual treatment. A randomised controlled trial with 1 year of follow-up. ***Clin Rheumatol*** 30(3): 381-389, 2011.

136. Light AR, White AT, Hughen RW, Light KC. Moderate exercise increases expression for sensory, adrenergic, and immune genes in chronic fatigue syndrome patients but not in normal subjects. ***J Pain*** 10(10): 1099-1112, 2009.

137. Behan PO, Behan WM, Horrobin D. Effect of high doses of essential fatty acids on the postviral fatigue syndrome. ***Acta Neurol Scand*** 82(3): 209-216, 1990.

138. Cox IM, Campbell MJ, Dowson D. Red blood cell magnesium and chronic fatigue syndrome. ***Lancet*** 337(8744): 757-760, 1991.

139. Vermeulen RC, Scholte HR. Exploratory open label, randomized study of acetyl- and propionylcarnitine in chronic fatigue syndrome. ***Psychosom Med*** 66(2): 276-282, 2004.

140. Forsyth LM, Preuss HG, MacDowell AL, Chiazze L, Jr., Birkmayer GD, Bellanti JA. Therapeutic effects of oral NADH on the symptoms of patients with chronic fatigue syndrome. ***Ann Allergy Asthma Immunol*** 82(2): 185-191, 1999.

141. Santaella ML, Font I, Disdier OM. Comparison of oral nicotinamide adenine dinucleotide (NADH) versus conventional therapy for chronic fatigue syndrome. ***P R Health Sci J*** 23(2): 89-93, 2004.

142. Alraek T, Lee MS, Choi TY, Cao H, Liu J. Complementary and alternative medicine for patients with chronic fatigue syndrome: a systematic review. ***BMC Complement Altern Med*** 11: 87, 2011.

143. Theoharides TC, Asadi S, Panagiotidou S, Weng Z. The "missing link" in autoimmunity and autism: Extracellular mitochondrial components secreted from activated live mast cells. ***Autoimmun Rev*** 12(12): 1136-1142, 2013.

144. Mariman A, Vogelaers D, Hanoulle I, Delesie L, Pevernagie D. Subjective sleep quality and daytime sleepiness in a large sample of patients with chronic fatigue syndrome (CFS). ***Acta Clin Belg*** 67(1): 19-24, 2012.

145. Spotila L. Pharmacotherapy. ***CFIDS Chronicle*** . 2006.

146. Natelson BH, Cheu J, Pareja J, Ellis SP, Policastro T, Findley TW. Randomized, double blind, controlled placebo-phase in trial of low dose phenelzine in the chronic fatigue syndrome. ***Psychopharmacology (Berl)*** 124(3): 226-230, 1996.

147. Goodnick PJ. Treatment of chronic fatigue syndrome with venlafaxine. ***Am J Psychiatry*** 153(2): 294, 1996.

148. White PD, Cleary KJ. An open study of the efficacy and adverse effects of moclobemide in patients with the chronic fatigue syndrome. ***Int Clin Psychopharmacol*** 12(1): 47-52, 1997.

149. Straus SE, Dale JK, Tobi M, Lawley T, Preble O, Blaese RM, Hallahan C, Henle W. Acyclovir treatment of the chronic fatigue syndrome. Lack of efficacy in a placebo-controlled trial. ***N Engl J Med*** 319(26): 1692-1698, 1988.

150. Lerner AM, Zervos M, Chang CH, Beqaj S, Goldstein J, O'Neill W, Dworkin H, Fitgerald T, Deeter RG. A small, randomized, placebo-controlled trial of the use of antiviral therapy for patients with chronic fatigue syndrome. ***Clin Infect Dis*** 32(11): 1657-1658, 2001.

151. Lerner AM, Beqaj SH, Deeter RG, Fitzgerald JT. Valacyclovir treatment in Epstein-Barr virus subset chronic fatigue syndrome: thirty-six months follow-up. ***In vivo*** 21(5): 707-713, 2007.

152. Pae CU, Marks DM, Patkar AA, Masand PS, Luyten P, Serretti A. Pharmacological treatment of chronic fatigue syndrome: focusing on the role of antidepressants. ***Expert Opin Pharmacother*** 10(10): 1561-1570, 2009.

153. Sachdeva AK, Kuhad A, Tiwari V, Arora V, Chopra K. Protective Effect of Epigallocatechin Gallate in Murine Water-Immersion Stress Model of Chronic Fatigue Syndrome. ***Basic Clin Pharmacol Toxicol***2010.

154. Sachdeva AK, Kuhad A, Chopra K. Epigallocatechin gallate ameliorates behavioral and biochemical deficits in rat model of load-induced chronic fatigue syndrome. ***Brain Res Bull*** 86(3-4): 165-172, 2011.

155. Kuo YH, Tsai WJ, Loke SH, Wu TS, Chiou WF. Astragalus membranaceus flavonoids (AMF) ameliorate chronic fatigue syndrome induced by food intake restriction plus forced swimming. ***J Ethnopharmacol*** 122(1): 28-34, 2009.

156. Lyle N, Gomes A, Sur T, Munshi S, Paul S, Chatterjee S, Bhattacharyya D. The role of antioxidant properties of Nardostachys jatamansi in alleviation of the symptoms of the chronic fatigue syndrome. ***Behav Brain Res*** 202(2): 285-290, 2009.

157. Singh A, Naidu PS, Gupta S, Kulkarni SK. Effect of natural and synthetic antioxidants in a mouse model of chronic fatigue syndrome. ***J Med Food*** 5(4): 211-220, 2002.

158. Dhir A, Kulkarni SK. Venlafaxine reverses chronic fatigue-induced behavioral, biochemical and neurochemical alterations in mice. ***Pharmacol Biochem Behav*** 89(4): 563-571, 2008.

159. Surapaneni DK, Adapa SR, Preeti K, Teja GR, Veeraragavan M, Krishnamurthy S. Shilajit attenuates behavioral symptoms of chronic fatigue syndrome by modulating the hypothalamic-pituitary-adrenal axis and mitochondrial bioenergetics in rats. ***J Ethnopharmacol*** 143(1): 91-99, 2012.

160. Kumar A, Garg R, Kumar P. Nitric oxide modulation mediates the protective effect of trazodone in a mouse model of chronic fatigue syndrome. ***Pharmacol Rep*** 60(5): 664-672, 2008.

161. Kumar A, Garg R, Gaur V, Kumar P. Nitric oxide modulation in protective role of antidepressants against chronic fatigue syndrome in mice. ***Indian J Pharmacol*** 43(3): 324-329, 2011.

162. Singal A, Kaur S, Tirkey N, Chopra K. Green tea extract and catechin ameliorate chronic fatigue-induced oxidative stress in mice. ***J Med Food*** 8(1): 47-52, 2005.

163. Ottenweller JE, Natelson BH, Gause WC, Carroll KK, Beldowicz D, Zhou XD, LaManca JJ. Mouse running activity is lowered by Brucella abortus treatment: a potential model to study chronic fatigue. ***Physiol Behav*** 63(5): 795-801, 1998.

164. Fomicheva EE, Filatenkova TA, Rybakina EG. Activity in the hypothalamo-hypophyseal-adrenocortical system on experimental induction of chronic fatigue syndrome. ***Neurosci Behav Physiol*** 40(3): 245-250, 2010.

165. Katafuchi T, Kondo T, Yasaka T, Kubo K, Take S, Yoshimura M. Prolonged effects of polyriboinosinic:polyribocytidylic acid on spontaneous running wheel activity and brain interferon-alpha mRNA in rats: a model for immunologically induced fatigue. ***Neuroscience*** 120(3): 837-845, 2003.

166. Katafuchi T, Kondo T, Take S, Yoshimura M. Enhanced expression of brain interferon-alpha and serotonin transporter in immunologically induced fatigue in rats. ***Eur J Neurosci*** 22(11): 2817-2826, 2005.

167. Katafuchi T, Kondo T, Take S, Yoshimura M. Brain cytokines and the 5-HT system during poly I:C-induced fatigue. ***Ann N Y Acad Sci*** 1088: 230-237, 2006.

168. Cunningham C, Campion S, Teeling J, Felton L, Perry VH. The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C). ***Brain Behav Immun*** 21(4): 490-502, 2007.

169. Chen R, Moriya J, Yamakawa J, Takahashi T, Li Q, Morimoto S, Iwai K, Sumino H, Yamaguchi N, Kanda T. Brain atrophy in a murine model of chronic fatigue syndrome and beneficial effect of Hochu-ekki-to (TJ-41). ***Neurochem Res*** 33(9): 1759-1767, 2008.

170. Chen R, Moriya J, Luo X, Yamakawa J, Takahashi T, Sasaki K, Yoshizaki F. Hochu-ekki-to combined with interferon-gamma moderately enhances daily activity of chronic fatigue syndrome mice by increasing NK cell activity, but not neuroprotection. ***Immunopharmacol Immunotoxicol*** 31(2): 238-245, 2009.

171. Sheng R, Xu X, Tang Q, Bian D, Li Y, Qian C, He X, Gao X, Pan R, Wang C, Luo Y, Xia Y, Dai Y. Polysaccharide of Radix Pseudostellariae Improves Chronic Fatigue Syndrome Induced by Poly I:C in Mice. ***Evid Based Complement Alternat Med***2009.

172. Sachdeva AK, Kuhad A, Tiwari V, Chopra K. Epigallocatechin gallate ameliorates chronic fatigue syndrome in mice: Behavioral and biochemical evidence. ***Behav Brain Res*** 205(2): 414-420, 2009.

173. Vij G, Gupta A, Chopra K. Modulation of antigen-induced chronic fatigue in mouse model of water immersion stress by naringin, a polyphenolic antioxidant. ***Fundam Clin Pharmacol*** 23(3): 331-337, 2009.

174. Gupta A, Vij G, Sharma S, Tirkey N, Rishi P, Chopra K. Curcumin, a polyphenolic antioxidant, attenuates chronic fatigue syndrome in murine water immersion stress model. ***Immunobiology*** 214(1): 33-39, 2009.

175. Gupta A, Vij G, Chopra K. Possible role of oxidative stress and immunological activation in mouse model of chronic fatigue syndrome and its attenuation by olive extract. ***J Neuroimmunol***2010.

176. Miranda CL, Maier CS, Stevens JF. Flavonoids. ***In: eLS John Wiley & Sons, Ltd: Chichester DOI: 10 1002/9780470015902 a0003068 pub2***2012.

177. Hollman PC. Absorption, Bioavailability, and Metabolism of flavonoids. ***Pharmaceutical Biology*** 42(Supplement): 74-83, 2004.

178. Del RD, Borges G, Crozier A. Berry flavonoids and phenolics: bioavailability and evidence of protective effects. ***Br J Nutr*** 104 Suppl 3: S67-S90, 2010.

179. Nishiumi S, Miyamoto S, Kawabata K, Ohnishi K, Mukai R, Murakami A, Ashida H, Terao J. Dietary flavonoids as cancer-preventive and therapeutic biofactors. ***Front Biosci (Schol Ed)*** 3: 1332-1362, 2011.

180. Nemeth K, Plumb GW, Berrin JG, Juge N, Jacob R, Naim HY, Williamson G, Swallow DM, Kroon PA. Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. ***Eur J Nutr*** 42(1): 29-42, 2003.

181. Day AJ, Canada FJ, Diaz JC, Kroon PA, Mclauchlan R, Faulds CB, Plumb GW, Morgan MR, Williamson G. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. ***FEBS Lett*** 468(2-3): 166-170, 2000.

182. Sesink AL, Arts IC, Faassen-Peters M, Hollman PC. Intestinal uptake of quercetin-3-glucoside in rats involves hydrolysis by lactase phlorizin hydrolase. ***J Nutr*** 133(3): 773-776, 2003.

183. Hollman PC, Bijsman MN, van GY, Cnossen EP, de Vries JH, Katan MB. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. ***Free Radic Res*** 31(6): 569-573, 1999.

184. Lee MJ, Wang ZY, Li H, Chen L, Sun Y, Gobbo S, Balentine DA, Yang CS. Analysis of plasma and urinary tea polyphenols in human subjects. ***Cancer Epidemiol Biomarkers Prev*** 4(4): 393-399, 1995.

185. Heinonen S, Wahala K, Adlercreutz H. Identification of isoflavone metabolites dihydrodaidzein, dihydrogenistein, 6'-OH-O-dma, and cis-4-OH-equol in human urine by gas chromatography-mass spectroscopy using authentic reference compounds. ***Anal Biochem*** 274(2): 211-219, 1999.

186. Uehara M. Isoflavone metabolism and bone-sparing effects of daidzein-metabolites. ***J Clin Biochem Nutr*** 52(3): 193-201, 2013.

187. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. ***J Nutr Biochem*** 13(10): 572-584, 2002.

188. Setchell KD, Clerici C. Equol: history, chemistry, and formation. ***J Nutr*** 140(7): 1355S-1362S, 2010.

189. Setchell KD, Clerici C. Equol: pharmacokinetics and biological actions. ***J Nutr*** 140(7): 1363S-1368S, 2010.

190. Yuan JP, Wang JH, Liu X. Metabolism of dietary soy isoflavones to equol by human intestinal microflora--implications for health. ***Mol Nutr Food Res*** 51(7): 765-781, 2007.

191. Doerge DR, Chang HC, Churchwell MI, Holder CL. Analysis of soy isoflavone conjugation in vitro and in human blood using liquid chromatography-mass spectrometry. ***Drug Metab Dispos*** 28(3): 298-307, 2000.

192. Setchell KD, Brown NM, Desai P, Zimmer-Nechemias L, Wolfe BE, Brashear WT, Kirschner AS, Cassidy A, Heubi JE. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. ***J Nutr*** 131(4 Suppl): 1362S-1375S, 2001.

193. Shelnutt SR, Cimino CO, Wiggins PA, Ronis MJ, Badger TM. Pharmacokinetics of the glucuronide and sulfate conjugates of genistein and daidzein in men and women after consumption of a soy beverage. ***Am J Clin Nutr*** 76(3): 588-594, 2002.

194. Zhang Y, Hendrich S, Murphy PA. Glucuronides are the main isoflavone metabolites in women. ***J Nutr*** 133(2): 399-404, 2003.

195. Baba S, Osakabe N, Yasuda A, Natsume M, Takizawa T, Nakamura T, Terao J. Bioavailability of (-)-epicatechin upon intake of chocolate and cocoa in human volunteers. ***Free Radic Res*** 33(5): 635-641, 2000.

196. Lee MJ, Maliakal P, Chen L, Meng X, Bondoc FY, Prabhu S, Lambert G, Mohr S, Yang CS. Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. ***Cancer Epidemiol Biomarkers Prev*** 11(10 Pt 1): 1025-1032, 2002.

197. Manach C, Morand C, Gil-Izquierdo A, Bouteloup-Demange C, Remesy C. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. ***Eur J Clin Nutr*** 57(2): 235-242, 2003.

198. Wu X, Cao G, Prior RL. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. ***J Nutr*** 132(7): 1865-1871, 2002.

199. Felgines C, Talavera S, Gonthier MP, Texier O, Scalbert A, Lamaison JL, Remesy C. Strawberry anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans. ***J Nutr*** 133(5): 1296-1301, 2003.

200. Olthof MR, Hollman PC, Buijsman MN, van Amelsvoort JM, Katan MB. Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. ***J Nutr*** 133(6): 1806-1814, 2003.

201. Xiao J, Wu M, Kai G, Wang F, Cao H, Yu X. ZnO-ZnS QDs interfacial heterostructure for drug and food delivery application: enhancement of the binding affinities of flavonoid aglycones to bovine serum albumin. ***Nanomedicine*** 7(6): 850-858, 2011.

202. Xu X, Wang HJ, Murphy PA, Cook L, Hendrich S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. ***J Nutr*** 124(6): 825-832, 1994.

203. Setchell KD, Faughnan MS, Avades T, Zimmer-Nechemias L, Brown NM, Wolfe BE, Brashear WT, Desai P, Oldfield MF, Botting NP, Cassidy A. Comparing the pharmacokinetics of daidzein and genistein with the use of 13C-labeled tracers in premenopausal women. ***Am J Clin Nutr*** 77(2): 411-419, 2003.

204. Lotito SB, Zhang WJ, Yang CS, Crozier A, Frei B. Metabolic conversion of dietary flavonoids alters their anti-inflammatory and antioxidant properties. ***Free Radic Biol Med*** 51(2): 454-463, 2011.

205. Hollman PC, Cassidy A, Comte B, Heinonen M, Richelle M, Richling E, Serafini M, Scalbert A, Sies H, Vidry S. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. ***J Nutr*** 141(5): 989S-1009S, 2011.

206. King RA, Bursill DB. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. ***Am J Clin Nutr*** 67(5): 867-872, 1998.

207. Piazza C, Privitera MG, Melilli B, Incognito T, Marano MR, Leggio GM, Roxas MA, Drago F. Influence of inulin on plasma isoflavone concentrations in healthy postmenopausal women. ***Am J Clin Nutr*** 86(3): 775-780, 2007.

208. Izumi T, Piskula MK, Osawa S, Obata A, Tobe K, Saito M, Kataoka S, Kubota Y, Kikuchi M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. ***J Nutr*** 130(7): 1695-1699, 2000.

209. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. ***Am J Clin Nutr*** 79(5): 727-747, 2004.

210. Tangney CC, Rasmussen HE. Polyphenols, inflammation, and cardiovascular disease. ***Curr Atheroscler Rep*** 15(5): 324, 2013.

211. Tresserra-Rimbau A, Medina-Remon A, Perez-Jimenez J, Martinez-Gonzalez MA, Covas MI, Corella D, Salas-Salvado J, Gomez-Gracia E, Lapetra J, Aros F, Fiol M, Ros E, Serra-Majem L, Pinto X, Munoz MA, Saez GT, Ruiz-Gutierrez V, Warnberg J, Estruch R, Lamuela-Raventos RM. Dietary intake and major food sources of polyphenols in a Spanish population at high cardiovascular risk: The PREDIMED study. ***Nutr Metab Cardiovasc Dis*** 23(10): 953-959, 2013.

212. Middleton EJ, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. ***Pharmacol Rev*** 52(4): 673-751, 2000.

213. Gutierrez-Merino C, Lopez-Sanchez C, Lagoa R, Samhan-Arias AK, Bueno C, Garcia-Martinez V. Neuroprotective actions of flavonoids. ***Curr Med Chem*** 18(8): 1195-1212, 2011.

214. Prochazkova D, Bousova I, Wilhelmova N. Antioxidant and prooxidant properties of flavonoids. ***Fitoterapia*** 82(4): 513-523, 2011.

215. Fraga CG, Oteiza PI. Dietary flavonoids: Role of (-)-epicatechin and related procyanidins in cell signaling. ***Free Radic Biol Med*** 51(4): 813-823, 2011.

216. Mann GE, Bonacasa B, Ishii T, Siow RC. Targeting the redox sensitive Nrf2-Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones. ***Curr Opin Pharmacol*** 9(2): 139-145, 2009.

217. Surh YJ, Kundu JK, Na HK, LEE JS. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. ***J Nutr*** 135(12 Suppl): 2993S-3001S, 2005.

218. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. ***Nat Cell Biol*** 4(5): E131-E136, 2002.

219. Naugler WE, Karin M. NF-kappaB and cancer-identifying targets and mechanisms. ***Curr Opin Genet Dev*** 18(1): 19-26, 2008.

220. Hess J, Angel P, Schorpp-Kistner M. AP-1 subunits: quarrel and harmony among siblings. ***J Cell Sci*** 117(Pt 25): 5965-5973, 2004.

221. Ding W, Liu Y. Genistein attenuates genioglossus muscle fatigue under chronic intermittent hypoxia by down-regulation of oxidative stress level and up-regulation of antioxidant enzyme activity through ERK1/2 signaling pathway. ***Oral Dis*** 17(7): 677-684, 2011.

222. Utrera M, Estevez M. Impact of trolox, quercetin, genistein and gallic acid on the oxidative damage to myofibrillar proteins: the carbonylation pathway. ***Food Chem*** 141(4): 4000-4009, 2013.

223. Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. ***Planta Med*** 74(13): 1526-1539, 2008.

224. Yang Y, Oh JM, Heo P, Shin JY, Kong B, Shin J, Lee JC, Oh JS, Park KW, Lee CH, Shin YK, Kweon DH. Polyphenols differentially inhibit degranulation of distinct subsets of vesicles in mast cells by specific interaction with granule-type-dependent SNARE complexes. ***Biochem J*** 450(3): 537-546, 2013.

225. Mohammadshahi M, Haidari F, Saei AA, Rashidi B, Mahboob S, Rashidi MR. Soy protein, genistein, and daidzein improve serum paraoxonase activity and lipid profiles in rheumatoid arthritis in rats. ***J Med Food*** 16(2): 147-154, 2013.

226. Bhutada P, Mundhada Y, Bansod K, Ubgade A, Quazi M, Umathe S, Mundhada D. Reversal by quercetin of corticotrophin releasing factor induced anxiety- and depression-like effect in mice. ***Prog Neuropsychopharmacol Biol Psychiatry*** 34(6): 955-960, 2010.

227. Nijs J, De BP, De MK, Demanet C, Vincken W, Schuermans D, McGregor N. Associations between bronchial hyperresponsiveness and immune cell parameters in patients with chronic fatigue syndrome. ***Chest*** 123(4): 998-1007, 2003.

228. Woo SB, Schacterle RS, Komaroff AL, Gallagher GT. Salivary gland changes in chronic fatigue syndrome: a case-controlled preliminary histologic study. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod*** 90(1): 82-87, 2000.

229. Steinberg P, Pheley A, Peterson PK. Influence of immediate hypersensitivity skin reactions on delayed reactions in patients with chronic fatigue syndrome. ***J Allergy Clin Immunol*** 98(6 Pt 1): 1126-1128, 1996.

230. Kurup VP, Alenius H, Kelly KJ, Castillo L, Fink JN. A two-dimensional electrophoretic analysis of latex peptides reacting with IgE and IgG antibodies from patients with latex allergy. ***Int Arch Allergy Immunol*** 109: 58-67, 1996.

231. Enestrom S, Bengtsson A, Frodin T. Dermal IgG deposits and increase of mast cells in patients with fibromyalgia-relevant findings or epiphenomena? ***Scand J Rheumatol*** 26(4): 308-313, 1997.

232. Enestrom S, Bengtson A, Lindstrom F, Johan K. Attachment of IgG to dermal extracellular matrix in patients with fibromyalgia. ***Clin Exp Rheumatol*** 8(2): 127-135, 1990.

233. Torresani C, Bellafiore S, De PG. Chronic urticaria is usually associated with fibromyalgia syndrome. ***Acta Derm Venereol*** 89(4): 389-392, 2009.

234. Theoharides TC, Donelan J, Kandere-Grzybowska K, Konstantinidou A. The role of mast cells in migraine pathophysiology. ***Brain Res Rev*** 49: 65-76, 2005.

235. Theoharides TC, Kempuraj D, Sant GR. Mast cell involvement in interstitial cystitis: a review of human and experimental evidence. ***Urology*** 57(Suppl 6A): 47-55, 2001.

236. Theoharides TC. Atopic conditions in search of pathogenesis and therapy. ***Clin Ther*** 35(5): 544-547, 2013.

237. Tsai M, Shih L-S, Newlands GFJ, Takeishi T, Langley KE, Zsebo KM, Miller HRP, Geissler EN, Galli SJ. The rat c-*kit* ligand, stem cell factor, induces the development of connective tissue-type and mucosal mast cells *in vivo*. Analysis by anatomical distribution, histochemistry and protease phenotype. ***J Exp Med*** 174: 125-131, 1991.

238. Rodewald HR, Dessing M, Dvorak AM, Galli SJ. Identification of a committed precursor for the mast cell lineage. ***Science*** 271: 818-822, 1996.

239. Kitamura Y, Ito A. Mast cell-committed progenitors. ***Proc Natl Acad Sci U S A*** 102(32): 11129-11130, 2005.

240. Chen CC, Grimbaldeston MA, Tsai M, Weissman IL, Galli SJ. Identification of mast cell progenitors in adult mice. ***Proc Natl Acad Sci U S A*** 102(32): 11408-11413, 2005.

241. Metcalfe DD, Kaliner M, Donlon MA. The mast cell. ***CRC Crit Rev Immunol*** 3: 23-74, 1981.

242. Wedemeyer J, Tsai M, Galli SJ. Roles of mast cells and basophils in innate and acquired immunity. ***Curr Opin Immunol*** 12: 624-631, 2000.

243. Puxeddu I, Piliponsky AM, Bachelet I, Levi-Schaffer F. Mast cells in allergy and beyond. ***Int J Biochem Cell Biol*** 35: 1601-1607, 2003.

244. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. ***Annu Rev Immunol*** 23: 749-786, 2005.

245. Theoharides TC. Mast cell: a neuroimmunoendocrine master player. ***Int J Tissue React*** 18(1): 1-21, 1996.

246. Theoharides TC, Cochrane DE. Critical role of mast cells in inflammatory diseases and the effect of acute stress. ***J Neuroimmunol*** 146((1-2)): 1-12, 2004.

247. Theoharides TC, Kempuraj D, Tagen M, Conti P, Kalogeromitros D. Differential release of mast cell mediators and the pathogenesis of inflammation. ***Immunol Rev*** 217: 65-78, 2007.

248. Tatemoto K, Nozaki Y, Tsuda R, Konno S, Tomura K, Furuno M, Ogasawara H, Edamura K, Takagi H, Iwamura H, Noguchi M, Naito T. Immunoglobulin E-independent activation of mast cell is mediated by Mrg receptors. ***Biochem Biophys Res Commun*** 349(4): 1322-1328, 2006.

249. Okayama Y, Saito H, Ra C. Targeting human mast cells expressing g-protein-coupled receptors in allergic diseases. ***Allergol Int*** 57(3): 197-203, 2008.

250. Alysandratos KD, Asadi S, Angelidou A, Zhang B, Sismanopoulos N, Yang H, Critchfield A, Theoharides TC. Neurotensin and CRH interactions augment human mast cell activation. ***PloS One*** 7(11): e48934, 2012.

251. Marshall JS. Mast-cell responses to pathogens. ***Nat Rev Immunol*** 4(10): 787-799, 2004.

252. Sandig H, Bulfone-Paus S. TLR signaling in mast cells: common and unique features. ***Front Immunol*** 3: 185, 2012.

253. Metzger H, Eglite S, Haleem-Smith H, Reischl I, Torigoe C. Quantitative aspects of signal transduction by the receptor with high affinity for IgE. ***Mol Immunol*** 38: 1207-1211, 2002.

254. Turner H, Kinet JP. Signalling through the high-affinity IgE receptor Fc epsilonRI. ***Nature*** 402(6760 Suppl): B24-B30, 1999.

255. Rivera J, Olivera A. A current understanding of Fc epsilon RI-dependent mast cell activation. ***Curr Allergy Asthma Rep*** 8(1): 14-20, 2008.

256. Blank U, Rivera J. The ins and outs of IgE-dependent mast-cell exocytosis. ***Trends Immunol*** 25: 266-273, 2004.

257. Kinet JP. The essential role of mast cells in orchestrating inflammation. ***Immunol Rev*** 217: 5-7, 2007.

258. Metzger H, Alcazar G, Hohman R, Kinet J-P, Pribluda V, Quarto R. The receptor with high affinity for immunoglobulin E. ***Annu Rev Immunol*** 4: 419-470, 1986.

259. Gould HJ, Sutton BJ, Beavil AJ, Beavil RL, McCloskey N, Coker HA, Fear D, Smurthwaite L. The biology of IGE and the basis of allergic disease. ***Annu Rev Immunol*** 21: 579-628, 2003.

260. Smurthwaite L, Walker SN, Wilson DR, Birch DS, Merrett TG, Durham SR, Gould HJ. Persistent IgE synthesis in the nasal mucosa of hay fever patients. ***Eur J Immunol*** 31(12): 3422-3431, 2001.

261. Theoharides TC, Kalogeromitros D. The critical role of mast cell in allergy and inflammation. ***Ann NY Acad Sci*** 1088: 78-99, 2006.

262. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. ***J Allergy Clin Immunol*** 125(2 Suppl 2): S73-S80, 2010.

263. Wilson DR, Merrett TG, Varga EM, Smurthwaite L, Gould HJ, Kemp M, Hooper J, Till SJ, Durham SR. Increases in allergen-specific IgE in BAL after segmental allergen challenge in atopic asthmatics. ***Am J Respir Crit Care Med*** 165(1): 22-26, 2002.

264. Kambayashi T, Koretzky GA. Proximal signaling events in Fc epsilon RI-mediated mast cell activation. ***J Allergy Clin Immunol*** 119(3): 544-552, 2007.

265. Hoth M, Penner R. Depletion of intracellular calcium stores activates a calcium current in mast cells. ***Nature*** 355: 353-358, 1992.

266. Theoharides TC, Kotkow KJ. Calcium ion levels during differential mast cell secretion. ***FASEB J*** 45: 387, 1986.

267. Siraganian RP. Mast cell signal transduction from the high-affinity IgE receptor. ***Curr Opin Immunol*** 15: 639-646, 2003.

268. Rivera J, Fierro NA, Olivera A, Suzuki R. New insights on mast cell activation via the high affinity receptor for IgE. ***Adv Immunol*** 98: 85-120, 2008.

269. Vig M, Kinet JP. Calcium signaling in immune cells. ***Nat Immunol*** 10(1): 21-27, 2009.

270. Bienenstock J, Tomioka M, Matsuda H, Stead RH, Quinonez G, Simon GT, Coughlin MD, Denburg JA. The role of mast cells in inflammatory processes: evidence for nerve mast cell interactions. ***Int Arch Allergy Appl Immunol*** 82: 238-243, 1987.

271. Dimitriadou V, Aubineau P, Taxi J, Seylaz J. Ultrastructural evidence for a functional unit between nerve fibers and type II cerebral mast cells in the cerebral vascular wall. ***Neuroscience*** 22: 621-630, 1987.

272. Skofitsch G, Savitt JM, Jacobowitz DM. Suggestive evidence for a functional unit between mast cells and substance P fibers in the rat diaphragm and mesentery. ***Histochemistry*** 82: 5-8, 1985.

273. Newson B, Dahlström A, Enerbäck L, Ahlman H. Suggestive evidence for a direct innervation of mucosal mast cells. ***Neuroscience*** 10: 565-570, 1983.

274. Stead RH, Tomioka M, Quinonez G, Simon GT, Felten SY, Bienenstock J. Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. ***Proc Natl Acad Sci USA*** 84: 2975-2979, 1987.

275. Pang X, Marchand J, Sant GR, Kream RM, Theoharides TC. Increased number of substance P positive nerve fibers in interstitial cystitis. ***Br J Urol*** 75: 744-750, 1995.

276. Dvorak AM, McLeod RS, Onderdonk AB, Monahan-Earley RA, Cullen JB, Antonioli DA, Morgan E, Blair JE, Estrella P, Cisneros RL, Cohen Z, Silen W. Human gut mucosal mast cells: ultrastructural observations and anatomic variation in mast cell-nerve associations *in vivo*. ***Int Arch Allergy Immunol*** 98: 158-168, 1992.

277. Letourneau R, Pang X, Sant GR, Theoharides TC. Intragranular activation of bladder mast cells and their association with nerve processes in interstitial cystitis. ***Br J Urol*** 77: 41-54, 1996.

278. Skofitsch G, Zamir N, Helke CJ, Savitt JM, Jacobowitz DM. Corticotropin-releasing factor-like immunoreactivity in sensory ganglia and capsaicin sensitive neurons of the rat central nervous system: colocalization with other neuropeptides. ***Peptides*** 6: 307-318, 1985.

279. Roth S, Kummer W. A quantitative ultrastructural investigation of tyrosine hydroxylase-immunoreactive axons in the hairy skin of the guinea pig. ***Anat Embryol (Berl)*** 190(2): 155-162, 1994.

280. Ferry X, Brehin S, Kamel R, Landry Y. G protein-dependent activation of mast cell by peptides and basic secretagogues. ***Peptides*** 23(8): 1507-1515, 2002.

281. Janiszewski J, Bienenstock J, Blennerhassett MG. Picomolar doses of substance P trigger electrical responses in mast cells without degranulation. ***Am J Physiol*** 267: C138-C145, 1994.

282. Tore F, Tuncel N. Mast cells: target and source of neuropeptides. ***Curr Pharm Des*** 15(29): 3433-3445, 2009.

283. Barrocas AM, Cochrane DE, Carraway RE, Feldberg RS. Neurotensin stimulation of mast cell secretion is receptor-mediated, pertussis-toxin sensitive and requires activation of phospholipase C. ***Immunopharmacology*** 41: 131-137, 1999.

284. Miller LA, Cochrane DE, Carraway RE, Feldberg RS. Blockade of mast cell histamine secretion in response to neurotensin by SR 48692, a nonpeptide antagonist of the neurotensin brain receptor. ***Br J Pharmacol*** 114: 1466-1470, 1995.

285. Cao J, Papadopoulou N, Kempuraj D, Boucher WS, Sugimoto K, Cetrulo CL, Theoharides TC. Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. ***J Immunol*** 174(12): 7665-7675, 2005.

286. Cochrane DE, Carraway RE, Harrington K, Laudano M, Rawlings S, Feldberg RS. HMC-1 human mast cells synthesize neurotensin (NT) precursor, secrete bioactive NT-like peptide(s) and express NT receptor NTS1. ***Inflamm Res*** 60(12): 1139-1151, 2011.

287. Cochrane DE, Carraway RE, Boucher W, Feldberg RS. Rapid degradation of neutotensin by stimulated rat mast cells. ***Peptides*** 12: 1187-1194, 1991.

288. Tatemoto K, Nozaki Y, Tsuda R, Konno S, Tomura K, Furuno M, Ogasawara H, Edamura K, Takagi H, Iwamura H, Noguchi M, Naito T. Immunoglobulin E-independent activation of mast cell is mediated by Mrg receptors. ***Biochem Biophys Res Commun*** 349(4): 1322-1328, 2006.

289. Ogawa K, Nabe T, Yamamura H, Kohno S. Nanomolar concentrations of neuropeptides induce histamine release from peritoneal mast cells of a substrain of Wistar rats. ***Eur J Pharmacol*** 374(2): 285-291, 1999.

290. Douglas SD, Leeman SE. Neurokinin-1 receptor: functional significance in the immune system in reference to selected infections and inflammation. ***Ann N Y Acad Sci*** 1217: 83-95, 2011.

291. Cao J, Curtis CL, Theoharides TC. Corticotropin-releasing hormone induces vascular endothelial growth factor release from human mast cells via the cAMP/protein kinase A/p38 mitogen-activated protein kinase pathway. ***Mol Pharmacol*** 69(3): 998-1006, 2006.

292. Metcalfe DD, Baram D, Mekori YA. Mast cells. ***Physiol Rev*** 77: 1033-1079, 1997.

293. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. ***Nat Rev Immunol*** 10(6): 440-452, 2010.

294. Vercammen E, Staal J, Beyaert R. Sensing of viral infection and activation of innate immunity by toll-like receptor 3. ***Clin Microbiol Rev*** 21(1): 13-25, 2008.

295. Munoz S, Hernandez-Pando R, Abraham SN, Enciso JA. Mast cell activation by Mycobacterium tuberculosis: mediator release and role of CD48. ***J Immunol*** 170(11): 5590-5596, 2003.

296. Malaviya R, Gao Z, Thankavel K, van der Merwe PA, Abraham SN. The mast cell tumor necrosis factor α response to FimH-expressing *Escherichia coli* is mediated by the glycosylphosphatidylinositol-anchored molecule CD48. ***Proc Natl Acad Sci USA*** 96: 8110-8115, 1999.

297. Woolhiser MR, Brockow K, Metcalfe DD. Activation of human mast cells by aggregated IgG through FcγRI: additive effects of C3a. ***Clin Immunol*** 110: 172-180, 2004.

298. Patella V, Florio G, Petraroli A, Marone G. HIV-1 gp120 induces IL-4 and IL-13 release from human Fc epsilon RI+ cells through interaction with the VH3 region of IgE. ***J Immunol*** 164(2): 589-595, 2000.

299. Genovese A, Borgia G, Bouvet JP, Detoraki A, de Paulis A, Piazza M, Marone G. Protein Fv produced during viral hepatitis is an endogenous immunoglobulin superantigen activating human heart mast cells. ***Int Arch Allergy Immunol*** 132: 336-345, 2003.

300. Jarrett EE, Miller HR. Production and activities of IgE in helminth infection. ***Prog Allergy*** 31: 178-233, 1982.

301. Verwaerde C, Joseph M, Capron M, Pierce RJ, Damonneville M, Velge F, Auriault C, Capron A. Functional properties of a rat monoclonal IgE antibody specific for Schistosoma mansoni. ***J Immunol*** 138(12): 4441-4446, 1987.

302. Gurish MF, Bryce PJ, Tao H, Kisselgof AB, Thornton EM, Miller HR, Friend DS, Oettgen HC. IgE enhances parasite clearance and regulates mast cell responses in mice infected with Trichinella spiralis. ***J Immunol*** 172(2): 1139-1145, 2004.

303. Woolhiser MR, Okayama Y, Gilfillan AM, Metcalfe DD. IgG-dependent activation of human mast cells following up-regulation of FcgammaRI by IFN-gamma. ***Eur J Immunol*** 31(11): 3298-3307, 2001.

304. Nilsson G, Johnell M, Hammer CH, Tiffany HL, Nilsson K, Metcalfe DD, Siegbahn A, Murphy PM. C3a and C5a are chemotaxins for human mast cells and act through distinct receptors via a pertussis toxin-sensitive signal transduction pathway. ***J Immunol*** 157: 1693-1698, 1996.

305. Fureder W, Agis H, Willheim M, Bankl HC, Maier U, Kishi K, Muller MR, Czerwenka K, Radaszkiewicz T, Butterfield JH, Klappacher GW, Sperr WR, Oppermann M, Lechner K, Valent P. Differential expression of complement receptors on human basophils and mast cells. Evidence for mast cell heterogeneity and CD88/C5aR expression on skin mast cells. ***J Immunol*** 155(6): 3152-3160, 1995.

306. Weber S, Babina M, Feller G, Henz BM. Human leukaemic (HMC-1) and normal skin mast cells express beta 2-integrins: characterization of beta 2-integrins and ICAM-1 on HMC-1 cells. ***Scand J Immunol*** 45(5): 471-481, 1997.

307. Kandere-Grzybowska K, Kempuraj D, Letourneau L, Asare A, Athanasiou A, Theoharides TC. IL-1 induces differential release of IL-6 form human mast cells. ***FASEB J*** 16: A332. 2002.

308. Love KS, Lakshmanan RR, Butterfield JH, Fox CC. IFN-gamma-stimulated enhancement of MHC class II antigen expression by the human mast cell line HMC-1. ***Cell Immunol*** 170(1): 85-90, 1996.

309. Gupta AA, Leal-Berumen I, Croitoru K, Marshall JS. Rat peritoneal mast cells produce IFN-y following IL-12 treatment but not in response to IgE-mediated activation. ***J Immunol*** 157: 2123-2128, 1996.

310. Lin TJ, Befus AD. Differential regulation of mast cell function by IL-10 and stem cell factor. ***J Immunol*** 159: 4015-4023, 1997.

311. Ochi H, Hirani WM, Yuan Q, Friend DS, Austen KF, Boyce JA. T helper cell type 2 cytokine-mediated comitogenic responses and CCR3 expression during differentiation of human mast cells in vitro. ***J Exp Med*** 190(2): 267-280, 1999.

312. Lin TJ, Issekutz TB, Marshall JS. Human mast cells transmigrate through human umbilical vein endothelial monolayers and selectively produce IL-8 in response to stromal cell-derived factor-1 alpha. ***J Immunol*** 165(1): 211-220, 2000.

313. Inohara N, Ogura Y, Nunez G. Nods: a family of cytosolic proteins that regulate the host response to pathogens. ***Curr Opin Microbiol*** 5(1): 76-80, 2002.

314. Philpott DJ, Girardin SE. Nod-like receptors: sentinels at host membranes. ***Curr Opin Immunol*** 22(4): 428-434, 2010.

315. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. ***Nat Immunol*** 5(7): 730-737, 2004.

316. Osorio F, Reis e Sousa. Myeloid C-type lectin receptors in pathogen recognition and host defense. ***Immunity*** 34(5): 651-664, 2011.

317. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. ***Int Rev Immunol*** 30(1): 16-34, 2011.

318. Kawai T, Akira S. TLR signaling. ***Semin Immunol*** 19(1): 24-32, 2007.

319. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. ***Mol Cell*** 10(2): 417-426, 2002.

320. Schroder K, Tschopp J. The inflammasomes. ***Cell*** 140(6): 821-832, 2010.

321. Kufer TA, Sansonetti PJ. NLR functions beyond pathogen recognition. ***Nat Immunol*** 12(2): 121-128, 2011.

322. Blander JM, Sander LE. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. ***Nat Rev Immunol*** 12(3): 215-225, 2012.

323. McCurdy JD, Olynych TJ, Maher LH, Marshall JS. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. ***J Immunol*** 170: 1625-1629, 2003.

324. Varadaradjalou S, Feger F, Thieblemont N, Hamouda NB, Pleau JM, Dy M, Arock M. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. ***Eur J Immunol*** 33: 899-906, 2003.

325. Kulka M, Alexopoulou L, Flavell RA, Metcalfe DD. Activation of mast cells by double-stranded RNA: evidence for activation through Toll-like receptor 3. ***J Allergy Clin Immunol*** 114(1): 174-182, 2004.

326. Kulka M, Metcalfe DD. TLR3 activation inhibits human mast cell attachment to fibronectin and vitronectin. ***Mol Immunol***2005.

327. Yoshioka M, Fukuishi N, Iriguchi S, Ohsaki K, Yamanobe H, Inukai A, Kurihara D, Imajo N, Yasui Y, Matsui N, Tsujita T, Ishii A, Seya T, Takahama M, Akagi M. Lipoteichoic acid downregulates FcepsilonRI expression on human mast cells through Toll-like receptor 2. ***J Allergy Clin Immunol*** 120(2): 452-461, 2007.

328. Okumura S, Kashiwakura J, Tomita H, Matsumoto K, Nakajima T, Saito H, Okayama Y. Identification of specific gene expression profiles in human mast cells mediated by Toll-like receptor 4 and FcepsilonRI. ***Blood*** 102: 2547-2554, 2003.

329. Supajatura V, Ushio H, Nakao A, Akira S, Okumura K, Ra C, Ogawa H. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. ***J Clin Invest*** 109: 1351-1359, 2002.

330. Masuda A, Yoshikai Y, Aiba K, Matsuguchi T. Th2 cytokine production from mast cells is directly induced by lipopolysaccharide and distinctly regulated by c-Jun N-terminal kinase and p38 pathways. ***J Immunol*** 169: 3801-3810, 2002.

331. Ikeda T, Funaba M. Altered function of murine mast cells in response to lipopolysaccharide and peptidoglycan. ***Immunol Lett*** 88(1): 21-26, 2003.

332. Matsushima H, Yamada N, Matsue H, Shimada S. TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells. ***J Immunol*** 173: 531-541, 2004.

333. Mrabet-Dahbi S, Metz M, Dudeck A, Zuberbier T, Maurer M. Murine mast cells secrete a unique profile of cytokines and prostaglandins in response to distinct TLR2 ligands. ***Exp Dermatol*** 18(5): 437-444, 2009.

334. Li G, Domenico J, Jia Y, Lucas JJ, Gelfand EW. NF-kappaB-dependent induction of cathelicidin-related antimicrobial peptide in murine mast cells by lipopolysaccharide. ***Int Arch Allergy Immunol*** 150(2): 122-132, 2009.

335. McCurdy JD, Lin TJ, Marshall JS. Toll-like receptor 4-mediated activation of murine mast cells. ***J Leukoc Biol*** 70: 977-984, 2001.

336. Albiger B, Dahlberg S, Henriques-Normark B, Normark S. Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. ***J Intern Med*** 261(6): 511-528, 2007.

337. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. ***Cell*** 124(4): 783-801, 2006.

338. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. ***Nat Immunol*** 2: 675-680, 2001.

339. Akira S, Hoshino K, Kaisho T. The role of Toll-like receptors and MyD88 in innate immune responses. ***J Endotoxin Res*** 6(5): 383-387, 2000.

340. Okayama Y. Mast cell-derived cytokine expression induced via Fc receptors and Toll-like receptors. ***Chem Immunol Allergy*** 87: 101-110, 2005.

341. Doyle SL, O'Neill LA. Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. ***Biochem Pharmacol*** 72(9): 1102-1113, 2006.

342. Heine H, Lien E. Toll-like receptors and their function in innate and adaptive immunity. ***Int Arch Allergy Immunol*** 130: 180-192, 2003.

343. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. ***Nat Immunol*** 5(10): 987-995, 2004.

344. Ehlers M, Ravetch JV. Opposing effects of Toll-like receptor stimulation induce autoimmunity or tolerance. ***Trends Immunol*** 28(2): 74-79, 2007.

345. Watters TM, Kenny EF, O'Neill LA. Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. ***Immunol Cell Biol*** 85(6): 411-419, 2007.

346. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. ***Nat Rev Immunol*** 7(5): 353-364, 2007.

347. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. ***Nat Immunol*** 11(5): 373-384, 2010.

348. Tainsh KR, Pearce FL. Mast cell heterogeneity: evidence that mast cells isolated from various connective tissue locations in the rat display markedly graded phenotypes. ***Int Arch Allergy Immunol*** 98: 26-34, 1992.

349. Bradding P, Okayama Y, Howarth PH, Church MK, Holgate ST. Heterogeneity of human mast cells based on cytokine content. ***J Immunol*** 155: 297-307, 1995.

350. Levi-Schaffer F, Austen KF, Gravallese PM, Stevens RL. Co-culture of interleukin 3-dependent mouse mast cells with fibroblasts results in a phenotypic change of the mast cells. ***Proc Natl Acad Sci USA*** 83: 6485-6488, 1986.

351. Bischoff SC, Sellge G, Lorentz A, Sebald W, Raab R, Manns MP. IL-4 enhances proliferation and mediator release in mature human mast cells. ***Proc Natl Acad Sci USA*** 96: 8080-8085, 1999.

352. Lagunoff D, Pritzl P. Characterization of rat mast cell granule proteins. ***Arch Biochem Biophys*** 173: 554-563, 1976.

353. Dvorak AM. New aspects of mast cell biology. ***Int Arch Allergy Immunol*** 114: 1-9, 1997.

354. Hogan AD, Schwartz LB. Markers of mast cell degranulation. ***Methods Enzymol*** 13: 43-52, 1997.

355. Theoharides TC, Doyle R, Francis K, Conti P, Kalogeromitros D. Novel therapeutic targets for autism. ***Trends Pharmacol Sci*** 29(8): 375-382, 2008.

356. Kim KS, Wass CA, Cross AS, Opal SM. Modulation of blood-brain barrier permeability by tumor necrosis factor and antibody to tumor necrosis factor in the rat. ***Lymphokine Cytokine Res*** 11: 293-298, 1992.

357. de Boer AG, Breimer DD. Cytokines and blood-brain barrier permeability. ***Prog Brain Res*** 115: 425-451, 1998.

358. Abbott NJ. Inflammatory mediators and modulation of blood-brain barrier permeability. ***Cell Mol Neurobiol*** 20: 131-147, 2000.

359. Heavey D, Ernst P, Stevens R, Befus A, Bienenstock J, Austen K. Generation of leukotriene C4, leukotriene B4 and prostaglandin D2 by immunobiologically activated rat intestinal mucosa mast cells. ***J Immunol*** 140: 1953-1957, 1988.

360. Bissonnette EY, Hogaboam CM, Wallace JL, Befus AD. Potentiation of tumor necrosis factor-alpha-mediated cytotoxicity of mast cells by their production of nitric oxide. ***J Immunol*** 147: 3060-3065, 1991.

361. Gilchrist M, McCauley SD, Befus AD. Expression, localization, and regulation of NOS in human mast cell lines: effects on leukotriene production. ***Blood*** 104(2): 462-469, 2004.

362. Malaviya R, Ross EA, MacGregor JI, Ikeda T, Little JR, Jakschik BA, Abraham SN. Mast cell phagocytosis of FimH-expressing enterobacteria. ***J Immunol*** 152: 1907-1914, 1994.

363. Caughey GH. Tryptase genetics and anaphylaxis. ***J Allergy Clin Immunol*** 117(6): 1411-1414, 2006.

364. Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. ***Immunol Rev*** 217: 141-154, 2007.

365. Trivedi NN, Caughey GH. Mast cell peptidases: chameleons of innate immunity and host defense. ***Am J Respir Cell Mol Biol*** 42(3): 257-267, 2010.

366. Compton SJ, Cairns JA, Holgate ST, Walls AF. The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. ***J Immunol*** 161(4): 1939-1946, 1998.

367. Huang C, Friend DS, Qiu WT, Wong GW, Morales G, Hunt J, Stevens RL. Induction of a selective and persistent extravasation of neutrophils into the peritoneal cavity by tryptase mouse mast cell protease 6. ***J Immunol*** 160(4): 1910-1919, 1998.

368. Algermissen B, Hermes B, Feldmann-Boeddeker I, Bauer F, Henz BM. Mast cell chymase and tryptase during tissue turnover: analysis on in vitro mitogenesis of fibroblasts and keratinocytes and alterations in cutaneous scars. ***Exp Dermatol*** 8(3): 193-198, 1999.

369. Muramatsu M, Katada J, Hayashi I, Majima M. Chymase as a proangiogenic factor. A possible involvement of chymase-angiotensin-dependent pathway in the hamster sponge angiogenesis model. ***J Biol Chem*** 275(8): 5545-5552, 2000.

370. Compton SJ, Cairns JA, Holgate ST, Walls AF. Human mast cell tryptase stimulates the release of an IL-8-dependent neutrophil chemotactic activity from human umbilical vein endothelial cells (HUVEC). ***Clin Exp Immunol*** 121(1): 31-36, 2000.

371. Schwartz LB, Lewis RA, Austen KF. Tryptase from human pulmonary mast cells. Purification and characterization. ***J Biol Chem*** 256(22): 11939-11943, 1981.

372. McNeil HP, Adachi R, Stevens RL. Mast cell-restricted tryptases: structure and function in inflammation and pathogen defense. ***J Biol Chem*** 282(29): 20785-20789, 2007.

373. Metz M, Piliponsky AM, Chen CC, Lammel V, Abrink M, Pejler G, Tsai M, Galli SJ. Mast cells can enhance resistance to snake and honeybee venoms. ***Science*** 313(5786): 526-530, 2006.

374. Lindstedt KA, Kovanen PT. Mast cells in vulnerable coronary plaques: potential mechanisms linking mast cell activation to plaque erosion and rupture
1. ***Curr Opin Lipidol*** 15(5): 567-573, 2004.

375. Johnson JL, Jackson CL, Angelini GD, George SJ. Activation of matrix-degrading metalloproteinases by mast cell proteases in atherosclerotic plaques. ***Arterioscler Thromb Vasc Biol*** 18: 1707-1715, 1998.

376. Razin E, Mencia-Huerta J-M, Stevens RL, Lewis RA, Liu F-T, Corey E, Austen KF. IgE-mediated release of leukotriene C4, chondroitin sulfate E proteoglycan, beta-hexosaminidase, and histamine from cultured bone marrow-derived mouse mast cells. ***J Exp Med*** 157: 189-201, 1983.

377. MacGlashan D, Jr. Histamine: A mediator of inflammation. ***J Allergy Clin Immunol*** 112(4 Suppl): S53-S59, 2003.

378. Ohtsu H, Watanabe T. New functions of histamine found in histidine decarboxylase gene knockout mice. ***Biochem Biophys Res Commun*** 305(3): 443-447, 2003.

379. O'Donoghue M, Tharp MD. Antihistamines and their role as antipruritics. ***Dermatol Ther*** 18(4): 333-340, 2005.

380. Thurmond RL, Gelfand EW, Dunford PJ. The role of histamine H1 and H4 receptors in allergic inflammation: the search for new antihistamines. ***Nat Rev Drug Discov*** 7(1): 41-53, 2008.

381. Saravanan C, Bharti SK, Jaggi S, Singh SK. Histamine H receptor: a novel target for inflammation therapy. ***Mini Rev Med Chem*** 11(2): 143-158, 2011.

382. Davidson S, Gilead L, Amira M, Ginsburg H, Razin E. Synthesis of chondroitin sulfate D and heparin proteoglycans in murine lymph node-derived mast cells. The dependence on fibroblasts. ***J Biol Chem*** 265(21): 12324-12330, 1990.

383. Gilead L, Livni N, Eliakim R, Ligumsky M, Fich A, Okon E, Rachmilewitz D, Razin E. Human gastric mucosal mast cells are chondroitin sulphate E-containing mast cells. ***Immunology*** 62(1): 23-28, 1987.

384. Oschatz C, Maas C, Lecher B, Jansen T, Bjorkqvist J, Tradler T, Sedlmeier R, Burfeind P, Cichon S, Hammerschmidt S, Muller-Esterl W, Wuillemin WA, Nilsson G, Renne T. Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo. ***Immunity*** 34(2): 258-268, 2011.

385. Boyce JA. Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation. ***Immunol Rev*** 217: 168-185, 2007.

386. Clark JD, Lin LL, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milona N, Knopf JL. A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. ***Cell*** 65(6): 1043-1051, 1991.

387. Boyce JA. Eicosanoid mediators of mast cells: receptors, regulation of synthesis, and pathobiologic implications. ***Chem Immunol Allergy*** 87: 59-79, 2005.

388. Murakami M, Matsumoto R, Austen KF, Arm JP. Prostaglandin endoperoxide synthase-1 and -2 couple to different transmembrane stimuli to generate prostaglandin D2 in mouse bone marrow-derived mast cells. ***J Biol Chem*** 269(35): 22269-22275, 1994.

389. Urade Y, Ujihara M, Horiguchi Y, Igarashi M, Nagata A, Ikai K, Hayaishi O. Mast cells contain spleen-type prostaglandin D synthetase. ***J Biol Chem*** 265(1): 371-375, 1990.

390. Lu R, Kanai N, Bao Y, Schuster VL. Cloning, in vitro expression, and tissue distribution of a human prostaglandin transporter cDNA(hPGT). ***J Clin Invest*** 98(5): 1142-1149, 1996.

391. Matsuoka T, Hirata M, Tanaka H, Takahashi Y, Murata T, Kabashima K, Sugimoto Y, Kobayashi T, Ushikubi F, Aze Y, Eguchi N, Urade Y, Yoshida N, Kimura K, Mizoguchi A, Honda Y, Nagai H, Narumiya S. Prostaglandin D2 as a mediator of allergic asthma. ***Science*** 287(5460): 2013-2017, 2000.

392. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S, Nagata K. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. ***J Exp Med*** 193(2): 255-261, 2001.

393. Murphy RC, Gijon MA. Biosynthesis and metabolism of leukotrienes. ***Biochem J*** 405(3): 379-395, 2007.

394. Ott VL, Cambier JC, Kappler J, Marrack P, Swanson BJ. Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4. ***Nat Immunol*** 4(10): 974-981, 2003.

395. Csoma Z, Kharitonov SA, Balint B, Bush A, Wilson NM, Barnes PJ. Increased leukotrienes in exhaled breath condensate in childhood asthma. ***Am J Respir Crit Care Med*** 166(10): 1345-1349, 2002.

396. Vachier I, Bonnans C, Chavis C, Farce M, Godard P, Bousquet J, Chanez P. Severe asthma is associated with a loss of LX4, an endogenous anti-inflammatory compound. ***J Allergy Clin Immunol*** 115(1): 55-60, 2005.

397. Soter NA, Lewis RA, Corey EJ, Austen KF. Local effects of synthetic leukotrienes (LTC4, LTD4, LTE4, and LTB4) in human skin. ***J Invest Dermatol*** 80(2): 115-119, 1983.

398. O'Byrne PM. Leukotrienes in the pathogenesis of asthma. ***Chest*** 111(2 Suppl): 27S-34S, 1997.

399. Williams CM, Coleman JW. Induced expression of mRNA for IL-5, IL-6, TNF-alpha, MIP-2 and IFN-gamma in immunologically activated rat peritoneal mast cells: inhibition by dexamethasone and cyclosporin A. ***Immunology*** 86(2): 244-249, 1995.

400. Gordon JR, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. ***Nature*** 346(6281): 274-276, 1990.

401. Gordon JR, Burd PR, Galli SJ. Mast cells as a source of multifunctional cytokines. ***Immunol Today*** 11: 458-464, 1990.

402. Bissonnette EY, Enciso JA, Befus AD. TGF-β_1 inhibits the release of histamine and tumor necrosis factor-α from mast cells through an autocrine pathway. ***Am J Respir Cell Mol Biol*** 16: 275-282, 1997.

403. Gordon JR, Galli SJ. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. ***J Exp Med*** 174(1): 103-107, 1991.

404. Stassen M, Muller C, Arnold M, Hultner L, Klein-Hessling S, Neudorfl C, Reineke T, Serfling E, Schmitt E. IL-9 and IL-13 production by activated mast cells is strongly enhanced in the presence of lipopolysaccharide: NF-kappa B is decisively involved in the expression of IL-9. ***J Immunol*** 166(7): 4391-4398, 2001.

405. Brown JM, Wilson TM, Metcalfe DD. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. ***Clin Exp Allergy*** 38(1): 4-18, 2008.

406. Rumsaeng V, Cruikshank WW, Foster B, Prussin C, Kirshenbaum AS, Davis TA, Kornfeld H, Center DM, Metcalfe DD. Human mast cells produce the CD4+ T lymphocyte chemoattractant factor, IL-16. ***J Immunol*** 159(6): 2904-2910, 1997.

407. Smith TJ, Ducharme LA, Weis JH. Preferential expression of interleukin-12 or interleukin-4 by murine bone marrow mast cells derived in mast cell growth factor or interleukin-3. ***Eur J Immunol*** 24: 822-826, 1994.

408. Cocchiara R, Bongiovanni A, Albeggiani G, Azzolina A, Geraci D. Evidence that brain mast cells can modulate neuroinflammatory responses by tumor necrosis factor-α production. ***Neuroreport*** 9: 95-98, 1998.

409. Probert L, Akassoglou K, Kassiotis G, Pasparakis M, Alexopoulou L, Kollias G. TNF-α transgenic and knockout models of CNS inflammation and degeneration. ***J Neuroimmunol*** 72: 137-141, 1997.

410. Klinkert WEF, Kojima K, Lesslauer W, Rinner W, Lassmann H, Wekerle H. TNF-α receptor fusion protein prevents experimental auto- immune encephalomyelitis and demyelination in Lewis rats: An overview. ***J Neuroimmunol*** 72: 163-168, 1997.

411. King CA, Anderson R, Marshall JS. Dengue virus selectively induces human mast cell chemokine production. ***J Virol*** 76(16): 8408-8419, 2002.

412. Lin TJ, Maher LH, Gomi K, McCurdy JD, Garduno R, Marshall JS. Selective early production of CCL20, or macrophage inflammatory protein 3alpha, by human mast cells in response to Pseudomonas aeruginosa. ***Infect Immun*** 71(1): 365-373, 2003.

413. Rajakulasingam K, Hamid Q, O'Brien F, Shotman E, Jose PJ, Williams TJ, Jacobson M, Barkans J, Durham SR. RANTES in human allergen-induced rhinitis: cellular source and relation to tissue eosinophilia. ***Am J Respir Crit Care Med*** 155(2): 696-703, 1997.

414. Selvan RS, Butterfield JH, Krangel MS. Expression of multiple chemokine genes by a human mast cell leukemia. ***J Biol Chem*** 269: 13893-13898, 1994.

415. Jia GQ, Gonzalo JA, Lloyd C, Kremer L, Lu L, Martinez A, Wershil BK, Gutierrez-Ramos JC. Distinct expression and function of the novel mouse chemokine monocyte chemotactic protein-5 in lung allergic inflammation. ***J Exp Med*** 184(5): 1939-1951, 1996.

416. Moller A, Lippert U, Lessmann D, Kolde G, Hamann K, Welker P, Schadendorf D, Rosenbach T, Luger T, Czarnetzki BM. Human mast cells produce IL-8. ***J Immunol*** 151: 3261-3266, 1993.

417. Mori Y, Hirose K, Suzuki K, Nakajima H, Seto Y, Ikeda K, Shimoda K, Nakayama K, Saito Y, Iwamoto I. Tyk2 is essential for IFN-alpha-induced gene expression in mast cells. ***Int Arch Allergy Immunol*** 134 Suppl 1: 25-29, 2004.

418. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. ***Nat Rev Immunol*** 8(6): 478-486, 2008.

419. Galli SJ, Tsai M. Mast cells in allergy and infection: versatile effector and regulatory cells in innate and adaptive immunity. ***Eur J Immunol*** 40(7): 1843-1851, 2010.

420. Boesiger J, Tsai M, Maurer M, Yamaguchi M, Brown LF, Claffey KP, Dvorak HF, Galli SJ. Mast cells can secrete vascular permeability factor/vascular endothelial cell growth factor and exhibit enhanced release after immunoglobulin E-dependent upregulation of Fcε receptor I expression. ***J Exp Med*** 188: 1135-1145, 1998.

421. Grutzkau A, Kruger-Krasagakes S, Baumeister H, Schwarz C, Kogel H, Welker P, Lippert U, Henz BM, Moller A. Synthesis, storage and release of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) by human mast cells: Implications for the biological significance of $VEGF_{206}$. ***Mol Biol Cell*** 9: 875-884, 1998.

422. Laham RJ, Li J, Tofukuji M, Post M, Simons M, Sellke FW. Spatial heterogeneity in VEGF-induced vasodilation: VEGF dilates microvessels but not epicardial and systemic arteries and veins. ***Ann Vasc Surg*** 17: 245-252, 2003.

423. Artuc M, Hermes B, Steckelings UM, Grutzkau A, Henz BM. Mast cells and their mediators in cutaneous wound healing--active participants or innocent bystanders? ***Exp Dermatol*** 8: 1-16, 1999.

424. Conti P, Castellani ML, Kempuraj D, Salini V, Vecchiet J, Tete S, Mastrangelo F, Perrella A, De Lutiis MA, Tagen M, Theoharides TC. Role of mast cells in tumor growth. ***Ann Clin Lab Sci*** 37(4): 315-322, 2007.

425. Puxeddu I, Ribatti D, Crivellato E, Levi-Schaffer F. Mast cells and eosinophils: a novel link between inflammation and angiogenesis in allergic diseases. ***J Allergy Clin Immunol*** 116(3): 531-536, 2005.

426. Montrucchio G, Alloatti G, Camussi G. Role of platelet-activating factor in cardiovascular pathophysiology. ***Physiol Rev*** 80(4): 1669-1699, 2000.

427. Kawabori S, Kanai N, Tosho T. Proliferative activity of mast cells in allergic nasal mucosa. ***Clin Exp Allergy*** 25: 173-178, 1995.

428. Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. ***Thorax*** 62(1): 8-15, 2007.

429. Bradding P. The role of the mast cell in asthma: a reassessment. ***Curr Opin Allergy Clin Immunol*** 3: 45-50, 2003.

430. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. ***N Engl J Med*** 346: 1699-1705, 2002.

431. Carroll NG, Mutavdzic S, James AL. Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma. ***Thorax*** 57(8): 677-682, 2002.

432. Pesci A, Foresi A, Bertorelli G, Chetta A, Olivieri D. Histochemical characteristics and degranulation of mast cells in epithelium and lamina propria of bronchial biopsies from asthmatic and normal subjects. ***Am Rev Respir Dis*** 147(3): 684-689, 1993.

433. Groneberg DA, Bester C, Grutzkau A, Serowka F, Fischer A, Henz BM, Welker P. Mast cells and vasculature in atopic dermatitis--potential stimulus of neoangiogenesis. ***Allergy*** 60(1): 90-97, 2005.

434. Weidinger S, Rummler L, Klopp N, Wagenpfeil S, Baurecht HJ, Fischer G, Holle R, Gauger A, Schafer T, Jakob T, Ollert M, Behrendt H, Wichmann HE, Ring J, Illig T. Association study of mast cell chymase polymorphisms with atopy. ***Allergy*** 60(10): 1256-1261, 2005.

435. Badertscher K, Bronnimann M, Karlen S, Braathen LR, Yawalkar N. Mast cell chymase is increased in chronic atopic dermatitis but not in psoriasis. ***Arch Dermatol Res*** 296(10): 503-506, 2005.

436. Steinhoff M, Buddenkotte J, Shpacovitch V, Rattenholl A, Moormann C, Vergnolle N, Luger TA, Hollenberg MD. Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. ***Endocr Rev*** 26(1): 1-43, 2005.

437. Damsgaard TE, Olesen AB, Srensen FB, Thestrup-Pedersen K, Schitz PO. Mast cells and atopic dermatitis. Stereological quantification of mast cells in atopic dermatitis and normal human skin. ***Arch Dermatol Res*** 289(5): 256-260, 1997.

438. Christy AL, Brown MA. The multitasking mast cell: positive and negative roles in the progression of autoimmunity. ***J Immunol*** 179(5): 2673-2679, 2007.

439. Maruotti N, Crivellato E, Cantatore FP, Vacca A, Ribatti D. Mast cells in rheumatoid arthritis. ***Clin Rheumatol*** 26(1): 1-4, 2007.

440. Nigrovic PA, Lee DM. Mast cells in inflammatory arthritis. ***Arthritis Res Ther*** 7: 1-11, 2005.

441. Hiromatsu Y, Toda S. Mast cells and angiogenesis. ***Microsc Res Tech*** 60: 64-69, 2003.

442. Yamada T, Sawatsubashi M, Yakushiji H, Itoh Y, Edakuni G, Mori M, robert L, Miyazaki K. Localization of vascular endothelial growth factor in synovial membrane mast cells: examination with "multi-labelling subtraction immunostaining". ***Virchows Arch*** 433(6): 567-570, 1998.

443. Harvima IT, Nilsson G, Suttle MM, Naukkarinen A. Is there a role for mast cells in psoriasis? ***Arch Dermatol Res*** 300(9): 461-476, 2008.

444. Saraceno R, Kleyn CE, Terenghi G, Griffiths CE. The role of neuropeptides in psoriasis. ***Br J Dermatol*** 155(5): 876-882, 2006.

445. Nakamura M, Toyoda M, Morohashi M. Pruritogenic mediators in psoriasis vulgaris: comparative evaluation of itch-associated cutaneous factors. ***Br J Dermatol*** 149(4): 718-730, 2003.

446. Jiang WY, Chattedee AD, Raychaudhuri SP, Raychaudhuri SK, Farber EM. Mast cell density and IL-8 expression in nonlesional and lesional psoriatic skin. ***Int J Dermatol*** 40(11): 699-703, 2001.

447. Naukkarinen A, Jarvikallio A, Lakkakorpi J, Harvima IT, Harvima RJ, Horsmanheimo M. Quantitative histochemical analysis of mast cells and sensory nerves in psoriatic skin. ***J Pathol*** 180: 200-205, 1996.

448. Bartosik-Psujek H, Stelmasiak Z. The levels of chemokines CXCL8, CCL2 and CCL5 in multiple sclerosis patients are linked to the activity of the disease. ***Eur J Neurol*** 12(1): 49-54, 2005.

449. Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, Langer-Gould A, Strober S, Cannella B, Allard J, Klonowski P, Austin A, Lad N, Kaminski N, Galli SJ, Oksenberg JR, Raine CS, Heller R, Steinman L. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. ***Nat Med*** 8: 500-508, 2002.

450. Krüger PG. Mast cells and multiple sclerosis: a quantitative analysis. ***Neuropathol Appl Neurobiol*** 27: 275-280, 2001.

451. Carrieri PB, Provitera V, DeRosa T, Tartaglia G, Gorga F, Perrella O. Profile of cerebrospinal fluid and serum cytokines in patients with relapsing-remitting multiple sclerosis: A correlation with clinical activity. ***Immunopharmacol Immunotoxicol*** 20: 373-382, 1998.

452. Ibrahim MZ, Reder AT, Lawand R, Takash W, Sallouh-Khatib S. The mast cells of the multiple sclerosis brain. ***J Neuroimmunol*** 70: 131-138, 1996.

453. Blanco I, Beritze N, Arguelles M, Carcaba V, Fernandez F, Janciauskiene S, Oikonomopoulou K, de Serres FJ, Fernandez-Bustillo E, Hollenberg MD. Abnormal overexpression of mastocytes in skin biopsies of fibromyalgia patients. ***Clin Rheumatol*** 29(12): 1403-1412, 2010.

454. Jarvikallio A, Harvima IT, Naukkarinen A. Mast cells, nerves and neuropeptides in atopic dermatitis and nummular eczema. ***Arch Dermatol Res*** 295(1): 2-7, 2003.

455. Gottlieb AB. Psoriasis: emerging therapeutic strategies. ***Nat Rev Drug Discov*** 4(1): 19-34, 2005.

456. Naidu PS, Singh A, Kulkarni SK. Quercetin, a bioflavonoid, attenuates haloperidol-induced orofacial dyskinesia. ***Neuropharmacology*** 44(8): 1100-1106, 2003.

457. Mustain WC, Rychahou PG, Evers BM. The role of neurotensin in physiologic and pathologic processes. ***Curr Opin Endocrinol Diabetes Obes*** 18(1): 75-82, 2011.

458. Cochrane DE, Boucher W, Bibb P. Neurotensin stimulates histamine release in *in vivo* skin 'blisters' in rats: an effect inhibited by cromolyn or somatostatin. ***Int Arch Allergy Appl Immunol*** 80: 225-230, 1986.

459. Donelan J, Boucher W, Papadopoulou N, Lytinas M, Papaliodis D, Theoharides TC. Corticotropin-releasing hormone induces skin vascular permeability through a neurotensin-dependent process. ***Proc Natl Acad Sci USA*** 103: 7759-7764, 2006.

460. Powe DG, Huskisson RS, Carney AS, Jenkins D, McEuen AR, Walls AF, Jones NS. Mucosal T-cell phenotypes in persistent atopic and nonatopic rhinitis show an association with mast cells. ***Allergy*** 59(2): 204-212, 2004.

461. Nakajima T, Inagaki N, Tanaka H, Tanaka A, Yoshikawa M, Tamari M, Hasegawa K, Matsumoto K, Tachimoto H, Ebisawa M, Tsujimoto G, Matsuda H, Nagai H, Saito H. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcepsilon receptor I cross-linking: an interspecies comparison. ***Blood*** 100(12): 3861-3868, 2002.

462. Kashiwakura J, Yokoi H, Saito H, Okayama Y. T cell proliferation by direct cross-talk between OX40 ligand on human mast cells and OX40 on human T cells: comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. ***J Immunol*** 173(8): 5247-5257, 2004.

463. Nakae S, Suto H, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cells enhance T cell activation: Importance of mast cell-derived TNF. ***Proc Natl Acad Sci U S A*** 102(18): 6467-6472, 2005.

464. Nakae S, Suto H, Iikura M, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. ***J Immunol*** 176(4): 2238-2248, 2006.

465. Kempuraj D, Tagen M, Iliopoulou BP, Clemons A, Vasiadi M, Boucher W, House M, Wolferg A, Theoharides TC. Luteolin inhibits myelin basic protein-induced human mast cell activation and mast cell dependent stimulation of Jurkat T cells. ***Br J Pharmacol*** 155(7): 1076-1084, 2008.

466. Yener M, Erturan I, Ceyhan AM, Inal EE, Kozanoglu OO. The evaluation of prevalence of fibromyalgia in patients with chronic urticaria. ***Med Sci Monit*** 19: 757-761, 2013.

467. Volpi N, Tarugi P. Improvement in the high-performance liquid chromatography malondialdehyde level determination in normal human plasma. ***J Chromatogr B Biomed Sci Appl*** 713(2): 433-437, 1998.

468. Fountoulakis KN, Papadopoulou M, Kleanthous S, Papadopoulou A, Bizeli V, Nimatoudis I, Iacovides A, Kaprinis GS. Reliability and psychometric properties of the Greek translation of the State-Trait Anxiety Inventory form Y: Preliminary data. ***Ann Gen Psychiatry*** 5: 2, 2006.

469. Sheng WS, Hu S, Lamkin A, Peterson PK, Chao CC. Susceptibility to immunologically mediated fatigue in C57BL/6 versus Balb/c mice. ***Clin Immunol Immunopathol*** 81(2): 161-167, 1996.

470. Hol J, Wilhelmsen L, Haraldsen G. The murine IL-8 homologues KC, MIP-2, and LIX are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies. ***J Leukoc Biol*** 87(3): 501-508, 2010.

471. Vasiadi M, Mondolfi A, Alysandratos K-D, Therianou A, Katsarou-Katsari A, Petrakopoulou D, Theoharides A, Miniati A., Theoharides T.C. Neurotensin serum levels and skin gene expression are increased in atopic dermatitis. ***Br J Dermatol*** 166(6): 1349-1352, 2013.

472. Vasiadi M, Therianou A, Alysandratos KD, Katsarou-Katsari A, Petrakopoulou T, Theoharides A, Papadavid E, Stavrianeas N, Antoniou C, Kalogeromitros D, Theoharides TC. Serum neurotensin (NT) is increased in psoriasis and NT induces VEGF release from human mast cells. ***Br J Dermatol*** 166(6): 1349-1352, 2012.

473. Vasiadi M, Therianou A, Sideri K, Smyrnioti M, Delivani D, Sismanopoulos N, Asadi S, Katsarou-Katsari A, Petrakopoulou D, Theoharides A, Antoniou C, Stavrianeas N, Kalogeromitros D, Theoharides TC. Increased serum CRH levels with decreased skin CRH-R1 gene expression in psoriasis and atopic dermatitis. ***J Allergy Clin Immunol*** 129(5): 1410-1413, 2012.

474. Asadi S, Alysandratos KD, Angelidou A, Miniati A, Sismanopoulos N, Vasiadi M, Zhang B, Kalogeromitros D, Theoharides TC. Substance P (SP) induces expression of functional corticotropin-releasing hormone receptor-1 (CRHR-1) in human mast cells. ***J Invest Dermatol*** 132(2): 324-329, 2012.

475. Angelidou A, Francis K, Vasiadi M, Alysandratos K-D, Zhang B, Theoharides A, Lykouras L, Kalogeromitros D, Theoharides T. Neurotensin is increased in serum of young children with autistic disorder. ***J Neuroinflam*** 7: 48, 2010.

476. Yaghmaie P, Koudelka CW, Simpson EL. Mental health comorbidity in patients with atopic dermatitis. ***J Allergy Clin Immunol*** 131(2): 428-433, 2013.

477. Angelidou A, Asadi S, Alysandratos KD, Karagkouni A, Kourembanas S, Theoharides TC. Perinatal stress, brain inflammation and risk of autism-Review and proposal. ***BMC Pediatr*** 12(1): 89, 2012.

478. Tagen M, Stiles L, Kalogeromitros D, Gregoriou S, Kempuraj D, Makris M, Donelan J, Vasiadi M, Staurianeas NG, Theoharides TC. Skin corticotropin-releasing hormone receptor expression in psoriasis. ***J Invest Dermatol*** 127(7): 1789-1791, 2007.

479. Feldberg RS, Cochrane DE, Carraway RE, Brown EB, Sawyer R, Hartunian M, Wentworth D. Evidence for a neurotensin receptor in rat serosal mast cells. ***Inflamm Res*** 47: 245-250, 1998.

480. Cochrane DE, Emigh C, Levine G, Carraway RE, Leeman SE. Neurotensin alters cutaneous vascular permeability and stimulates histamine release from isolated skin. ***Ann NY Acad Sci***: 396-397, 1982.

481. Jiang YA, Zhang YY, Luo HS, Xing SF. Mast cell density and the context of clinicopathological parameters and expression of p185, estrogen receptor, and proliferating cell nuclear antigen in gastric carcinoma. ***World J Gastroenterol*** 8: 1005-1008, 2002.

482. Theoharides TC, Donelan JM, Papadopoulou N, Cao J, Kempuraj D, Conti P. Mast cells as targets of corticotropin-releasing factor and related peptides. ***Trends Pharmacol Sci*** 25(11): 563-568, 2004.

483. Slominski A, Wortsman J. Neuroendocrinology of the skin. ***Endocr Rev*** 21: 457-487, 2000.

484. Sabatino G, Nicoletti M, Neri G, Saggini A, Rosati M, Conti F, Cianchetti E, Toniato E, Fulcheri M, Caraffa A, Antinolfi P, Frydas S, Pandolfi F, Potalivo G, Galzio R, Conti P, Theoharides TC. Impact of IL -9 and IL-33 in mast cells. ***J Biol Regul Homeost Agents*** 26(4): 577-586, 2012.

485. Theoharides TC, Zhang B, Kempuraj D, Tagen M, Vasiadi M, Angelidou A, Alysandratos KD, Kalogeromitros D, Asadi S, Stavrianeas N, Peterson E, Leeman S, Conti P. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. ***Proc Natl Acad Sci U S A*** 107(9): 4448-4453, 2010.

486. Amatya B, El-Nour H, Holst M, Theodorsson E, Nordlind K. Expression of tachykinins and their receptors in plaque psoriasis with pruritus. ***Br J Dermatol*** 164(5): 1023-1029, 2011.

487. Reich A, Orda A, Wisnicka B, Szepietowski JC. Plasma concentration of selected neuropeptides in patients suffering from psoriasis. ***Exp Dermatol*** 16(5): 421-428, 2007.

488. Metz M, Maurer M. Innate immunity and allergy in the skin. ***Curr Opin Immunol*** 21(6): 687-693, 2009.

489. Paus R, Theoharides TC, Arck PC. Neuroimmunoendocrine circuitry of the 'brain-skin connection'. ***Trends Immunol*** 27(1): 32-39, 2006.

490. Anisman H, Merali Z, Stead JD. Experiential and genetic contributions to depressive- and anxiety-like disorders: clinical and experimental studies. ***Neurosci Biobehav Rev*** 32(6): 1185-1206, 2008.

491. Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A. Innate immune response in Th1- and Th2-dominant mouse strains. ***Shock*** 22(5): 460-466, 2004.

492. Manetti R, Annunziato F, Tomasevic L, Gianno V, Parronchi P, Romagnani S, Maggi E. Polyinosinic acid: polycytidylic acid promotes T helper type 1-specific immune responses by stimulating macrophage production of interferon-alpha and interleukin-12. ***Eur J Immunol*** 25(9): 2656-2660, 1995.

493. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. ***J Clin Periodontol*** 25(2): 134-144, 1998.

494. Anisman H, Prakash P, Merali Z, Poulter MO. Corticotropin releasing hormone receptor alterations elicited by acute and chronic unpredictable stressor challenges in stressor-susceptible and resilient strains of mice. ***Behav Brain Res*** 181(2): 180-190, 2007.

495. Gibb J, Hayley S, Poulter MO, Anisman H. Effects of stressors and immune activating agents on peripheral and central cytokines in mouse strains that differ in stressor responsivity. ***Brain Behav Immun*** 25(3): 468-482, 2011.

496. Chia R, Achilli F, Festing MF, Fisher EM. The origins and uses of mouse outbred stocks. ***Nat Genet*** 37(11): 1181-1186, 2005.

497. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, Donald-Smith GP, Gao H, Hennessy L, Finnerty CC, Lopez CM, Honari S, Moore EE, Minei JP, Cuschieri J, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Jeschke MG, Klein MB, Gamelli RL, Gibran NS, Brownstein BH, Miller-Graziano C, Calvano SE, Mason PH, Cobb JP, Rahme LG, Lowry SF, Maier RV, Moldawer LL, Herndon DN, Davis RW, Xiao W, Tompkins RG. Genomic responses in mouse models poorly mimic human inflammatory diseases. ***PNAS*** 110(9): 3507-3512, 2013.

498. ter Horst JP, de Kloet ER, Schachinger H, Oitzl MS. Relevance of stress and female sex hormones for emotion and cognition. ***Cell Mol Neurobiol*** 32(5): 725-735, 2012.

499. Theoharides TC, Papaliodis D, Tagen M, Konstantinidou A, Kempuraj D, Clemons A. Chronic fatigue syndrome, mast cells, and tricyclic antidepressants. ***J Clin Psychopharmacol*** 25(6): 515-520, 2005.

500. Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. ***Biochem Biophys Res Commun*** 388(4): 621-625, 2009.

501. Saluja R, Delin I, Nilsson GP, Adner M. FcepsilonR1-mediated mast cell reactivity is amplified through prolonged Toll-like receptor-ligand treatment. ***PloS One*** 7(8): e43547, 2012.

502. Orinska Z, Bulanova E, Budagian V, Metz M, Maurer M, Bulfone-Paus S. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. ***Blood*** 106(3): 978-987, 2005.

503. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. ***Immunity*** 34(5): 637-650, 2011.

504. St John AL, Rathore AP, Yap H, Ng ML, Metcalfe DD, Vasudevan SG, Abraham SN. Immune surveillance by mast cells during dengue infection promotes natural killer (NK) and NKT-cell recruitment and viral clearance. ***Proc Natl Acad Sci U S A*** 108(22): 9190-9195, 2011.

505. Fukuda M, Ushio H, Kawasaki J, Niyonsaba F, Takeuchi M, Baba T, Hiramatsu K, Okumura K, Ogawa H. Expression and functional characterization of retinoic acid-inducible gene-I-like receptors of mast cells in response to viral infection. ***J Innate Immun*** 5(2): 163-173, 2013.

506. Brown MG, McAlpine SM, Huang YY, Haidl ID, Al-Afif A, Marshall JS, Anderson R. RNA sensors enable human mast cell anti-viral chemokine production and IFN-mediated protection in response to antibody-enhanced dengue virus infection. ***PloS One*** 7(3): e34055, 2012.

507. Graham AC, Hilmer KM, Zickovich JM, Obar JJ. Inflammatory response of mast cells during influenza A virus infection is mediated by active infection and RIG-I signaling. ***J Immunol*** 190(9): 4676-4684, 2013.

508. Zhao L, Lee JY, Hwang DH. Inhibition of pattern recognition receptor-mediated inflammation by bioactive phytochemicals. ***Nutr Rev*** 69(6): 310-320, 2011.

509. Ranjith-Kumar CT, Lai Y, Sarisky RT, Cheng KC. Green tea catechin, epigallocatechin gallate, suppresses signaling by the dsRNA innate immune receptor RIG-I. ***PloS One*** 5(9): e12878, 2010.

510. Mizushina Y, Shiomi K, Kuriyama I, Takahashi Y, Yoshida H. Inhibitory effects of a major soy isoflavone, genistein, on human DNA topoisomerase II activity and cancer cell proliferation. ***Int J Oncol*** 43(4): 1117-1124, 2013.

511. Hendriks JJ, Alblas J, van der Pol SM, van Tol EA, Dijkstra CD, de Vries HE. Flavonoids influence monocytic GTPase activity and are protective in experimental allergic encephalitis. ***J Exp Med*** 200(12): 1667-1672, 2004.

512. Jang S, Dilger RN, Johnson RW. Luteolin inhibits microglia and alters hippocampal-dependent spatial working memory in aged mice. ***J Nutr*** 140(10): 1892-1898, 2010.

513. Kang OH, Choi JG, Lee JH, Kwon DY. Luteolin isolated from the flowers of Lonicera japonica suppresses inflammatory mediator release by blocking NF-kappaB and MAPKs activation pathways in HMC-1 cells. ***Molecules*** 15(1): 385-398, 2010.

514. Jin M, Son KH, Chang HW. Luteolin-7-O-glucoside suppresses leukotriene C(4) production and degranulation by inhibiting the phosphorylation of mitogen activated protein kinases and phospholipase Cgamma1 in activated mouse bone marrow-derived mast cells. ***Biol Pharm Bull*** 34(7): 1032-1036, 2011.

515. Kandere-Grzybowska K, Kempuraj D, Cao J, Cetrulo CL, Theoharides TC. Regulation of IL-1-induced selective IL-6 release from human mast cells and inhibition by quercetin. ***Br J Pharmacol*** 148: 208-215, 2006.

516. Min YD, Choi CH, Bark H, Son HY, Park HH, Lee S, Park JW, Park EK, Shin HI, Kim SH. Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-kappaB and p38 MAPK in HMC-1 human mast cell line. ***Inflamm Res*** 56(5): 210-215, 2007.

517. Kempuraj D, Madhappan B, Christodoulou S, Boucher W, Cao J, Papadopoulou N, Cetrulo CL, Theoharides TC. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. ***Br J Pharmacol*** 145: 934-944, 2005.

518. Park HH, Lee S, Son HY, Park SB, Kim MS, Choi EJ, Singh TS, Ha JH, Lee MG, Kim JE, Hyun MC, Kwon TK, Kim YH, Kim SH. Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. ***Arch Pharm Res*** 31(10): 1303-1311, 2008.

519. Castellani ML, Kempuraj D, Frydas S, Theoharides TC, Simeonidou I, Conti P, Vecchiet J. Inhibitory Effect of Quercetin on Tryptase and MCP-1 Chemokine Release, and Histidine Decarboxylase mRNA Transcription by Human Mast Cell-1 Cell Line. ***Neuroimmunomodulation*** 13(3): 109-116, 2006.

520. Kempuraj D, Castellani ML, Petrarca C, Frydas S, Conti P, Theoharides TC, Vecchiet J. Inhibitory effect of quercetin on tryptase and interleukin-6 release, and histidine decarboxylase mRNA transcription by human mast cell-1 cell line. ***Clin Exp Med*** 6(4): 150-156, 2006.

521. Aktas O, Prozorovski T, Smorodchenko A, Savaskan NE, Lauster R, Kloetzel PM, Infante-Duarte C, Brocke S, Zipp F. Green tea epigallocatechin-3-gallate mediates T cellular NF-kappa B inhibition and exerts neuroprotection in autoimmune encephalomyelitis. ***J Immunol*** 173(9): 5794-5800, 2004.

522. Inoue T, Suzuki Y, Ra C. Epigallocatechin-3-gallate inhibits mast cell degranulation, leukotriene C4 secretion, and calcium influx via mitochondrial calcium dysfunction. ***Free Radic Biol Med*** 49(4): 632-640, 2010.

523. Shin HY, Kim SH, Jeong HJ, Kim SY, Shin TY, Um JY, Hong SH, Kim HM. Epigallocatechin-3-gallate inhibits secretion of TNF-alpha, IL-6 and IL-8 through the attenuation of ERK and NF-kappaB in HMC-1 cells. ***Int Arch Allergy Immunol*** 142(4): 335-344, 2007.

524. Karimi K, Kool M, Nijkamp FP, Redegeld FA. Substance P can stimulate prostaglandin D2 and leukotriene C4 generation without granule exocytosis in murine mast cells. ***Eur J Pharmacol*** 489(1-2): 49-54, 2004.

525. Moon PD, Choi IH, Kim HM. Epigallocatechin-3-O-gallate inhibits the production of thymic stromal lymphopoietin by the blockade of caspase-1/NF-kappaB pathway in mast cells. ***Amino Acids***2011.

526. Kang HK, Ecklund D, Liu M, Datta SK. Apigenin, a non-mutagenic dietary flavonoid, suppresses lupus by inhibiting autoantigen presentation for expansion of autoreactive Th1 and Th17 cells. ***Arthritis Res Ther*** 11(2): R59, 2009.

527. Choi IY, Kim SJ, Jeong HJ, Park SH, Song YS, Lee JH, Kang TH, Park JH, Hwang GS, Lee EJ, Hong SH, Kim HM, Um JY. Hesperidin inhibits expression of hypoxia inducible factor-1 alpha and inflammatory cytokine production from mast cells. ***Mol Cell Biochem*** 305(1-2): 153-161, 2007.

528. Yamashita S, Tsukamoto S, Kumazoe M, Kim YH, Yamada K, Tachibana H. Isoflavones Suppress the Expression of the FcepsilonRI High-Affinity Immunoglobulin E Receptor Independent of the Estrogen Receptor. ***J Agric Food Chem***2012.

529. Masilamani M, Wei J, Bhatt S, Paul M, Yakir S, Sampson HA. Soybean isoflavones regulate dendritic cell function and suppress allergic sensitization to peanut. ***J Allergy Clin Immunol*** 128(6): 1242-1250, 2011.

530. Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. ***Clin Exp Allergy*** 30: 501-508, 2000.

531. Lee EJ, Ji GE, Sung MK. Quercetin and kaempferol suppress immunoglobulin E-mediated allergic inflammation in RBL-2H3 and Caco-2 cells. ***Inflamm Res*** 59(10): 847-854, 2010.

532. Melgarejo E, Medina MA, Sanchez-Jimenez F, Botana LM, Dominguez M, Escribano L, Orfao A, Urdiales JL. (-)-Epigallocatechin-3-gallate interferes with mast cell adhesiveness, migration and its potential to recruit monocytes. ***Cell Mol Life Sci*** 64(19-20): 2690-2701, 2007.

533. Jia Z, Zhen W, Velayutham Anandh BP, Liu D. Phytoestrogen genistein protects against endothelial barrier dysfunction in vascular endothelial cells through PKA-mediated suppression of RhoA signaling. ***Endocrinology*** 154(2): 727-737, 2013.

534. Haleagrahara N, Radhakrishnan A, Lee N, Kumar P. Flavonoid quercetin protects against swimming stress-induced changes in oxidative biomarkers in the hypothalamus of rats. ***Eur J Pharmacol*** 621(1-3): 46-52, 2009.

535. Kawabata K, Kawai Y, Terao J. Suppressive effect of quercetin on acute stress-induced hypothalamic-pituitary-adrenal axis response in Wistar rats. ***J Nutr Biochem*** 21(5): 374-380, 2010.

536. Schaffer S, Asseburg H, Kuntz S, Muller WE, Eckert GP. Effects of polyphenols on brain ageing and Alzheimer's disease: focus on mitochondria. ***Mol Neurobiol*** 46(1): 161-178, 2012.

537. Duluc L, Soleti R, Clere N, Andriantsitohaina R, Simard G. Mitochondria as potential targets of flavonoids: focus on adipocytes and endothelial cells. ***Curr Med Chem*** 19(26): 4462-4474, 2012.

538. Davis JM, Murphy EA, Carmichael MD, Davis B. Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. ***Am J Physiol Regul Integr Comp Physiol*** 296(4): R1071-R1077, 2009.

539. Huttemann M, Lee I, Perkins GA, Britton SL, Koch LG, Malek MH. (-)-Epicatechin is associated with increased angiogenic and mitochondrial signalling in the hindlimb of rats selectively bred for innate low running capacity. ***Clin Sci (Lond)*** 124(11): 663-674, 2013.

540. Stanely Mainzen PP. (-) Epicatechin attenuates mitochondrial damage by enhancing mitochondrial multi-marker enzymes, adenosine triphosphate and lowering calcium in isoproterenol induced myocardial infarcted rats. ***Food Chem Toxicol*** 53: 409-416, 2013.

541. Sandhir R, Mehrotra A. Quercetin supplementation is effective in improving mitochondrial dysfunctions induced by 3-nitropropionic acid: implications in Huntington's disease. ***Biochim Biophys Acta*** 1832(3): 421-430, 2013.

542. Bandaruk Y, Mukai R, Kawamura T, Nemoto H, Terao J. Evaluation of the inhibitory effects of quercetin-related flavonoids and tea catechins on the monoamine oxidase-A reaction in mouse brain mitochondria. ***J Agric Food Chem*** 60(41): 10270-10277, 2012.

543. Charles AL, Meyer A, Dal-Ros S, Auger C, Keller N, Ramamoorthy TG, Zoll J, Metzger D, Schini-Kerth V, Geny B. Polyphenols prevent ageing-related impairment in skeletal muscle mitochondrial function through decreased reactive oxygen species production. ***Exp Physiol*** 98(2): 536-545, 2013.

544. Ding J, Yu HL, Ma WW, Xi YD, Zhao X, Yuan LH, Feng JF, Xiao R. Soy isoflavone attenuates brain mitochondrial oxidative stress induced by beta-amyloid peptides 1-42 injection in lateral cerebral ventricle. ***J Neurosci Res*** 91(4): 562-567, 2013.

545. Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α. ***Nature*** 381: 77-80, 1996.

546. Echtenacher B, Männel DN, Hültner L. Critical protective role of mast cells in a model of acute septic peritonitis. ***Nature*** 381: 75-79, 1996.

547. McLachlan JB, Hart JP, Pizzo SV, Shelburne CP, Staats HF, Gunn MD, Abraham SN. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. ***Nat Immunol*** 4: 1199-1205, 2003.

548. Schmolke B, Amon U, Zemcke N, Wolff HH. Immunohistochemical studies with skin mast cells. ***Agents Actions*** 41: C49-C50, 1994.

549. Maurer M, Theoharides TC, Granstein RD, Bischoff SC, Bienenstock J, Henz B, Kovanen P, Piliponsky AM, Kambe N, Vliagoftis H, Levi-Schaffer F, Metz M, Miyachi Y, Befus D, Forsythe P, Kitamura Y, Galli S. What is the physiological function of mast cells? ***Exp Dermatol*** 12: 886-910, 2003.

550. Krishnaswamy G, Kelley J, Johnson D, Youngberg G, Stone W, Huang SK, Bieber J, Chi DS. The human mast cell: functions in physiology and disease. ***Front Biosci*** 6: D1109-D1127, 2001.

551. Theoharides TC, Konstantinidou A. Corticotropin-releasing hormone and the blood-brain-barrier. ***Front Biosci*** 12: 1615-1628, 2007.

552. Rozniecki JJ, Dimitriadou V, Lambracht-Hall M, Pang X, Theoharides TC. Morphological and functional demonstration of rat dura mast cell-neuron interactions *in vitro* and *in vivo*. ***Brain Res*** 849: 1-15, 1999.

553. Johnson D, Krenger W. Interactions of mast cells with the nervous system--recent advances. ***Neurochem Res*** 17(9): 939-951, 1992.

554. Robinson-White A, Beaven MA. Presence of histamine and histamine-metabolizing enzyme in rat and guinea-pig microvascular endothelial cells. ***J Pharmacol Exp Ther*** 223: 440-445, 1982.

555. Theoharides TC, Spanos CP, Pang X, Alferes L, Ligris K, Letourneau R, Rozniecki JJ, Webster E, Chrousos G. Stress-induced intracranial mast cell degranulation. A corticotropin releasing hormone-mediated effect. ***Endocrinology*** 136: 5745-5750, 1995.

556. Dimitriadou V, Rouleau A, Trung Tuong MD, Newlands GJF, Miller HRP, Luffau G, Schwartz J-C, Garbarg M. Functional relationships between sensory nerve fibers and mast cells of dura mater in normal and inflammatory conditions. ***Neuroscience*** 77: 829-839, 1997.

557. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. ***Nat Med*** 10(12): 1366-1373, 2004.

558. Lappalainen J, Rintahaka J, Kovanen PT, Matikainen S, Eklund KK. Intracellular RNA recognition pathway activates strong anti-viral response in human mast cells. ***Clin Exp Immunol*** 172(1): 121-128, 2013.

559. Sundstrom JB, Ellis JE, Hair GA, Kirshenbaum AS, Metcalfe DD, Yi H, Cardona AC, Lindsay MK, Ansari AA. Human tissue mast cells are an inducible reservoir of persistent HIV infection. ***Blood*** 109(12): 5293-5300, 2007.

560. Sundstrom JB, Little DM, Villinger F, Ellis JE, Ansari AA. Signaling through Toll-like receptors triggers HIV-1 replication in latently infected mast cells. ***J Immunol*** 172(7): 4391-4401, 2004.

561. McKay DM, Bienenstock J. The interaction between mast cells and nerves in the gastrointestinal tract. ***Immunol Today*** 15: 533-538, 1994.

562. Stead RH, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J. Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. ***Gastroenterology*** 97: 575-585, 1989.

563. Purcell WM, Atterwill CK. Mast cells in neuroimmune function: neurotoxicological and neuropharmacological perspectives. ***Neurochem Res*** 20: 521-532, 1995.

564. Melton LM, Keith AB, Davis S, Oakley AE, Edwardson JA, Morris CM. Chronic glial activation, neurodegeneration, and APP immunoreactive deposits following acute administration of double-stranded RNA. ***GLIA*** 44(1): 1-12, 2003.

565. Town T, Jeng D, Alexopoulou L, Tan J, Flavell RA. Microglia recognize double-stranded RNA via TLR3. ***J Immunol*** 176(6): 3804-3812, 2006.

566. Sundstrom JB, Ellis JE, Hair GA, Kirshenbaum AS, Metcalfe DD, Yi H, Cardona AC, Lindsay MK, Ansari AA. Human tissue mast cells are an inducible reservoir of persistent HIV infection. ***Blood*** 109(12): 5293-5300, 2007.

567. Theoharides TC, Letourneau R, Patra P, Hesse L, Pang X, Boucher W, Mompoint C, Harrington B. Stress-induced rat intestinal mast cell intragranular activation and inhibitory effect of sulfated proteoglycans. ***Dig Dis Sci*** 44: 87S-93S, 1999.

568. Esposito P, Gheorghe D, Kandere K, Pang X, Conally R, Jacobson S, Theoharides TC. Acute stress increases permeability of the blood-brain-barrier through activation of brain mast cells. ***Brain Res*** 888: 117-127, 2001.

569. Silver R, Curley JP. Mast cells on the mind: new insights and opportunities. ***Trends Neurosci***2013.

570. Zhuang X, Silverman A-J, Silver R. Brain mast cell degranulation regulates blood-brain barrier. ***J Neurobiol*** 31: 393-403, 1996.

571. Kempuraj D, Lytinas M, Madhappan B, Christodoulou S, Athanasiou A, Theoharides TC. Human mast cells synthesize and secrete corticotropin-releasing hormone (CRH) and urocortin (Ucn). ***The American Association of Immunology, 90th Anniversary Meeting, May 6-10, 2003 Denver, CO*** Abstract # 9216. 2003.

572. Kandere-Grzybowska K, Letourneau R, Kempuraj D, Donelan J, Poplawski S, Boucher W, Athanassiou A, Theoharides TC. IL-1 induces vesicular secretion of IL-6 without degranulation from human mast cells. ***J Immunol*** 171(9): 4830-4836, 2003.

573. Urb M, Sheppard DC. The role of mast cells in the defence against pathogens. ***PLoS Pathog*** 8(4): e1002619, 2012.

574. Sendo T, Sumimura T, Itoh Y, Goromaru T, Aki K, Yano T, Oike M, Ito Y, Mori S, Nishibori M, Oishi R. Involvement of proteinase-activated receptor-2 in mast cell tryptase-induced barrier dysfunction in bovine aortic endothelial cells. ***Cell Signal*** 15(8): 773-781, 2003.

575. Sutherland RE, Olsen JS, McKinstry A, Villalta SA, Wolters PJ. Mast cell IL-6 improves survival from Klebsiella pneumonia and sepsis by enhancing neutrophil killing. ***J Immunol*** 181(8): 5598-5605, 2008.

576. Biedermann T, Kneilling M, Mailhammer R, Maier K, Sander CA, Kollias G, Kunkel SL, Hultner L, Rocken M. Mast cells control neutrophil recruitment during T cell-mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. ***J Exp Med*** 192: 1441-1452, 2000.

577. Burke SM, Issekutz TB, Mohan K, Lee PW, Shmulevitz M, Marshall JS. Human mast cell activation with virus-associated stimuli leads to the selective chemotaxis of natural killer cells by a CXCL8-dependent mechanism. ***Blood*** 111(12): 5467-5476, 2008.

578. Stelekati E, Bahri R, D'Orlando O, Orinska Z, Mittrucker HW, Langenhaun R, Glatzel M, Bollinger A, Paus R, Bulfone-Paus S. Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions. ***Immunity*** 31(4): 665-676, 2009.

579. Dudeck A, Suender CA, Kostka SL, von SE, Maurer M. Mast cells promote Th1 and Th17 responses by modulating dendritic cell maturation and function. ***Eur J Immunol*** 41(7): 1883-1893, 2011.

580. Mekori YA, Metcalfe DD. Mast cell-T cell interactions. ***J Allergy Clin Immunol*** 104(3 Pt 1): 517-523, 1999.

581. Shelburne CP, Nakano H, St John AL, Chan C, McLachlan JB, Gunn MD, Staats HF, Abraham SN. Mast cells augment adaptive immunity by orchestrating dendritic cell trafficking through infected tissues. ***Cell Host Microbe*** 6(4): 331-342, 2009.

582. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. ***Nat Immunol*** 6(2): 135-142, 2005.

583. Amaral MM, Davio C, Ceballos A, Salamone G, Canones C, Geffner J, Vermeulen M. Histamine improves antigen uptake and cross-presentation by dendritic cells. ***J Immunol*** 179(6): 3425-3433, 2007.

584. Caron G, Delneste Y, Roelandts E, Duez C, Herbault N, Magistrelli G, Bonnefoy JY, Pestel J, Jeannin P. Histamine induces CD86 expression and chemokine production by human immature dendritic cells. ***J Immunol*** 166(10): 6000-6006, 2001.

585. Mazzoni A, Siraganian RP, Leifer CA, Segal DM. Dendritic cell modulation by mast cells controls the Th1/Th2 balance in responding T cells. ***J Immunol*** 177(6): 3577-3581, 2006.

586. Jawdat DM, Rowden G, Marshall JS. Mast cells have a pivotal role in TNF-independent lymph node hypertrophy and the mobilization of Langerhans cells in response to bacterial peptidoglycan. ***J Immunol*** 177(3): 1755-1762, 2006.

587. Vasiadi M, Mondolfi AP, Alysandratos KD, Therianou A, Katsarou-Katsari A, Petrakopoulou T, Theoharidis A, Miniati A, Theoharides TC. Neurotensin serum levels and skin gene expression are increased in atopic dermatitis. ***Br J Dermatol*** 169(3): 695-699, 2013.

588. Carraway R, Leeman SE. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. ***J Biol Chem*** 248: 6854-6861, 1973.

589. Piliponsky AM, Chen CC, Nishimura T, Metz M, Rios EJ, Dobner PR, Wada E, Wada K, Zacharias S, Mohanasundaram UM, Faix JD, Abrink M, Pejler G, Pearl RG, Tsai M, Galli SJ. Neurotensin increases mortality and mast cells reduce neurotensin levels in a mouse model of sepsis. ***Nat Med*** 14(4): 392-398, 2008.

590. Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, Asadi S, Vasiadi M, Weng Z, Miniati A, Kalogeromitros D. Mast cells and inflammation. ***Biochim Biophys Acta*** 1822(1): 21-33, 2010.

591. Metz M, Maurer M. Mast cells--key effector cells in immune responses. ***Trends Immunol*** 28(5): 234-241, 2007.

592. Bieber T. Atopic dermatitis. ***N Engl J Med*** 358(14): 1483-1494, 2008.

593. Pincelli C, Fantini F, Massimi P, Girolomoni G, Seidenari S, Giannetti A. Neuropeptides in skin from patients with atopic dermatitis: an immunohistochemical study. ***Br J Dermatol*** 122(6): 745-750, 1990.

594. Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting pathogenesis of atopic dermatitis and psoriasis-Part I: Clinical and pathologic concepts. ***J Allergy Clin Immunol*** 127(5): 1110-1118, 2011.

595. Metz M, Grimbaldeston MA, Nakae S, Piliponsky AM, Tsai M, Galli SJ. Mast cells in the promotion and limitation of chronic inflammation. ***Immunol Rev*** 217: 304-328, 2007.

596. Theoharides TC, Singh LK, Boucher W, Pang X, Letourneau R, Webster E, Chrousos G. Corticotropin-releasing hormone induces skin mast cell degranulation and increased vascular permeability, a possible explanation for its pro-inflammatory effects. ***Endocrinology*** 139: 403-413, 1998.

597. Crompton R, Clifton VL, Bisits AT, Read MA, Smith R, Wright IM. Corticotropin-releasing hormone causes vasodilation in human skin via mast cell-dependent pathways. ***J Clin Endocrinol Metab*** 88: 5427-5432, 2003.

598. Slominski A. On the role of the corticotropin-releasing hormone signalling system in the aetiology of inflammatory skin disorders. ***Br J Dermatol*** 160(2): 229-232, 2009.

599. Maes M, Mihaylova I, Bosmans E. Not in the mind of neurasthenic lazybones but in the cell nucleus: patients with chronic fatigue syndrome have increased production of nuclear factor kappa beta. ***Neuro Endocrinol Lett*** 28(4): 456-462, 2007.

600. Morris G, Maes M. Increased nuclear factor-kappaB and loss of p53 are key mechanisms in Myalgic Encephalomyelitis/chronic fatigue syndrome (ME/CFS). ***Med Hypotheses*** 79(5): 607-613, 2012.

601. Booth NE, Myhill S, Laren-Howard J. Mitochondrial dysfunction and the pathophysiology of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS). ***Int J Clin Exp Med*** 5(3): 208-220, 2012.

602. Myhill S, Booth NE, Laren-Howard J. Targeting mitochondrial dysfunction in the treatment of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) - a clinical audit. ***Int J Clin Exp Med*** 6(1): 1-15, 2013.

603. Myhill S, Booth NE, Laren-Howard J. Chronic fatigue syndrome and mitochondrial dysfunction. ***Int J Clin Exp Med*** 2(1): 1-16, 2009.

604. Brown NM, Setchell KD. Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. ***Lab Invest*** 81: 735-747, 2001.

605. Si H, Liu D. Genistein, a soy phytoestrogen, upregulates the expression of human endothelial nitric oxide synthase and lowers blood pressure in spontaneously hypertensive rats. ***J Nutr*** 138(2): 297-304, 2008.

606. King RA, Broadbent JL, Head RJ. Absorption and excretion of the soy isoflavone genistein in rats. ***J Nutr*** 126(1): 176-182, 1996.

607. King RA. Daidzein conjugates are more bioavailable than genistein conjugates in rats. ***Am J Clin Nutr*** 68(6 Suppl): 1496S-1499S, 1998.

608. Setchell KD, Brown NM, Zhao X, Lindley SL, Heubi JE, King EC, Messina MJ. Soy isoflavone phase II metabolism differs between rodents and humans: implications for the effect on breast cancer risk. ***Am J Clin Nutr*** 94(5): 1284-1294, 2011.

609. Atherton KM, Mutch E, Ford D. Metabolism of the soyabean isoflavone daidzein by CYP1A2 and the extra-hepatic CYPs 1A1 and 1B1 affects biological activity. ***Biochem Pharmacol*** 72(5): 624-631, 2006.

610. Roberts-Kirchhoff ES, Crowley JR, Hollenberg PF, Kim H. Metabolism of genistein by rat and human cytochrome P450s. ***Chem Res Toxicol*** 12(7): 610-616, 1999.

611. Hu M, Krausz K, Chen J, Ge X, Li J, Gelboin HL, Gonzalez FJ. Identification of CYP1A2 as the main isoform for the phase I hydroxylated metabolism of genistein and a prodrug converting enzyme of methylated isoflavones. ***Drug Metab Dispos*** 31(7): 924-931, 2003.

612. Breinholt VM, Rasmussen SE, Brosen K, Friedberg TH. In vitro metabolism of genistein and tangeretin by human and murine cytochrome P450s. ***Pharmacol Toxicol*** 93(1): 14-22, 2003.

613. Foster BC, Vandenhoek S, Hana J, Krantis A, Akhtar MH, Bryan M, Budzinski JW, Ramputh A, Arnason JT. In vitro inhibition of human cytochrome P450-mediated metabolism of marker substrates by natural products. ***Phytomedicine*** 10(4): 334-342, 2003.

614. Tsujimoto M, Horie M, Honda H, Takara K, Nishiguchi K. The structure-activity correlation on the inhibitory effects of flavonoids on cytochrome P450 3A activity. ***Biol Pharm Bull*** 32(4): 671-676, 2009.

615. Xiao CQ, Chen R, Lin J, Wang G, Chen Y, Tan ZR, Zhou HH. Effect of genistein on the activities of cytochrome P450 3A and P-glycoprotein in Chinese healthy participants. ***Xenobiotica*** 42(2): 173-178, 2012.

616. Thelen K, Dressman JB. Cytochrome P450-mediated metabolism in the human gut wall. ***J Pharm Pharmacol*** 61(5): 541-558, 2009.

617. Mismatched double-stranded RNA: polyI:polyC12U. ***Drugs R D*** 5(5): 297-304, 2004.

618. Strayer DR, Carter WA, Stouch BC, Stevens SR, Bateman L, Cimoch PJ, Lapp CW, Peterson DL, Mitchell WM. A double-blind, placebo-controlled, randomized, clinical trial of the TLR-3 agonist rintatolimod in severe cases of chronic fatigue syndrome. ***PloS One*** 7(3): e31334, 2012.

619. Horan RF, Sheffer AL, Austen KF. Cromolyn sodium in the management of systemic mastocytosis. ***J Allergy Clin Immunol*** 85(5): 852-855, 1990.

620. Theoharides TC, Sieghart W, Greengard P, Douglas WW. Antiallergic drug cromolyn may inhibit histamine secretion by regulating phosphorylation of a mast cell protein. ***Science*** 207(4426): 80-82, 1980.

621. Barrett KE, Metcalfe DD. The histologic and functional characterization of enzymatically dispersed intestinal mast cells of nonhuman primates: effects of secretagogues and anti-allergic drugs on histamine secretion. ***J Immunol*** 135: 2020-2026, 1985.

622. Pearce FL, Befus AD, Gauldie J, Bienenstock J. Mucosal mast cells. II: Effects of anti-allergic compounds on histamine secretion by isolated intestinal mast cells. ***J Immunol*** 128: 2481-2486, 1982.

623. Fox CC, Wolf EJ, Kagey-Sobotka A, Lichtenstein LM. Comparison of human lung and intestinal mast cells. ***J Allergy Clin Immunol*** 81: 89-94, 1988.

624. Oka T, Kalesnikoff J, Starkl P, Tsai M, Galli SJ. Evidence questioning cromolyn's effectiveness and selectivity as a 'mast cell stabilizer' in mice. ***Lab Invest*** 92(10): 1472-1482, 2012.

625. Weng Z, Zhang B, Asadi S, Sismanopoulos N, Butcher A, Fu X, Katsarou-Katsari A, Antoniou C, Theoharides T.C. Quercetin is more effective than cromolyn in blocking human mast cell cytokine release and inhibits contact dermatitis and photosensitivity in humans. ***PloS One*** 7(3)2012.

626. Kubo Y, Fukuishi N, Yoshioka M, Iriguchi S, Imajo S, Yasui Y, Matsui N, Akagi M. Bacterial components regulate the exxpression of Toll-like receptor 4 on human mast cells. ***Inflamm Res*** 56(2): 70-75, 2007.

627. Radinger M, Jensen BM, Kuehn HS, Kirshenbaum A, Gilfillan AM. Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. ***Curr Protoc Immunol*** Chapter 7: Unit, 2010.

628. Aguirre A, Maturana CJ, Harcha PA, Saez JC. Possible Involvement of TLRs and Hemichannels in Stress-Induced CNS Dysfunction via Mastocytes, and Glia Activation. ***Mediators Inflamm*** 2013: 893521, 2013.

629. Skaper SD, Giusti P, Facci L. Microglia and mast cells: two tracks on the road to neuroinflammation. ***FASEB J*** 26(8): 3103-3117, 2012.

630. Patel SD, Brennan G, Brazin J, Ciardiello AJ, Silver RB, Vannucci SJ. Mast cell isolation from the immature rat brain. ***Dev Neurosci*** 35(2-3): 265-271, 2013.

631. Palacios-Gonzalez B, Zarain-Herzberg A, Flores-Galicia I, Noriega LG, eman-Escondrillas G, Zarinan T, Ulloa-Aguirre A, Torres N, Tovar AR. Genistein stimulates fatty acid oxidation in a leptin receptor-independent manner through the JAK2-mediated phosphorylation and activation of AMPK in skeletal muscle. ***Biochim Biophys Acta*** 1841(1): 132-140, 2013.

632. Paulke A, Noldner M, Schubert-Zsilavecz M, Wurglics M. St. John's wort flavonoids and their metabolites show antidepressant activity and accumulate in brain after multiple oral doses. ***Pharmazie*** 63(4): 296-302, 2008.

633. Ishisaka A, Ichikawa S, Sakakibara H, Piskula MK, Nakamura T, Kato Y, Ito M, Miyamoto K, Tsuji A, Kawai Y, Terao J. Accumulation of orally administered quercetin in brain tissue and its antioxidative effects in rats. ***Free Radic Biol Med*** 51(7): 1329-1336, 2011.

634. Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, Abbott NJ. Flavonoid permeability across an in situ model of the blood-brain barrier. ***Free Radic Biol Med*** 36: 592-604, 2004.

635. Faria A, Pestana D, Teixeira D, Azevedo J, De F, V, Mateus N, Calhau C. Flavonoid transport across RBE4 cells: A blood-brain barrier model. ***Cell Mol Biol Lett*** 15(2): 234-241, 2010.

636. Graham AC, Hilmer KM, Zickovich JM, Obar JJ. Inflammatory Response of Mast Cells during Influenza A Virus Infection Is Mediated by Active Infection and RIG-I Signaling. ***J Immunol*** 190(9): 4676-4684, 2013.

637. Abbey EL, Rankin JW. Effect of quercetin supplementation on repeated-sprint performance, xanthine oxidase activity, and inflammation. ***Int J Sport Nutr Exerc Metab*** 21(2): 91-96, 2011.

638. Bigelman KA, Chapman DP, Freese EC, Trilk JL, Cureton KJ. Effects of 6 weeks of quercetin supplementation on energy, fatigue, and sleep in ROTC cadets. ***Mil Med*** 176(5): 565-572, 2011.

639. Chen C, Kong AN. Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects. ***Trends Pharmacol Sci*** 26(6): 318-326, 2005.

640. Takimoto CH, Glover K, Huang X, Hayes SA, Gallot L, Quinn M, Jovanovic BD, Shapiro A, Hernandez L, Goetz A, Llorens V, Lieberman R, Crowell JA, Poisson BA, Bergan RC. Phase I pharmacokinetic and pharmacodynamic analysis of unconjugated soy isoflavones administered to individuals with cancer. ***Cancer Epidemiol Biomarkers Prev*** 12(11 Pt 1): 1213-1221, 2003.

641. Kumar NB, Krischer JP, Allen K, Riccardi D, Besterman-Dahan K, Salup R, Kang L, Xu P, Pow-Sang J. A Phase II randomized, placebo-controlled clinical trial of purified isoflavones in modulating steroid hormones in men diagnosed with localized prostate cancer. ***Nutr Cancer*** 59(2): 163-168, 2007.

642. Macready AL, Kennedy OB, Ellis JA, Williams CM, Spencer JP, Butler LT. Flavonoids and cognitive function: a review of human randomized controlled trial studies and recommendations for future studies. ***Genes Nutr*** 4(4): 227-242, 2009.

643. Casini ML, Marelli G, Papaleo E, Ferrari A, D'Ambrosio F, Unfer V. Psychological assessment of the effects of treatment with phytoestrogens on postmenopausal women: a randomized, double-blind, crossover, placebo-controlled study. ***Fertil Steril*** 85(4): 972-978, 2006.

644. Duffy R, Wiseman H, File SE. Improved cognitive function in postmenopausal women after 12 weeks of consumption of a soya extract containing isoflavones. ***Pharmacol Biochem Behav*** 75(3): 721-729, 2003.

645. Theoharides TC, Sant GR. A pilot open label of CystoProtek® in interstitial cystitis. ***Intl J Immunopathol Pharmacol*** 18: 183-188, 2005.

646. Theoharides TC, Kempuraj D, Vakali S, Sant GR. Treatment of refractory interstitial cystitis/painful bladder syndrome with CystoProtek - an oral multi-agent natural supplement. ***The Canadian Journal of Urology*** 15: 4410-4414, 2008.

647. Theoharides TC, Asadi S, Panagiotidou S. A case series of a luteolin formulation (NeuroProtek(R)) in children with autism spectrum disorders. ***Int J Immunopathol Pharmacol*** 25(2): 317-323, 2012.

648. Taliou A, Zintzaras E, Lykouras L, Francis K. An open-label pilot study of a formulation containing the anti-inflammatory flavonoid luteolin and its effects on behavior in children with autism spectrum disorders. ***Clin Ther*** 35(5): 592-602, 2013.

649. Walle T. Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption. ***Mol Pharm*** 4(6): 826-832, 2007.

650. Cao H, Jing X, Wu D, Shi Y. Methylation of genistein and kaempferol improves their affinities for proteins. ***Int J Food Sci Nutr*** 64(4): 437-443, 2013.

651. Steensma A, Faassen-Peters MA, Noteborn HP, Rietjens IM. Bioavailability of genistein and its glycoside genistin as measured in the portal vein of freely moving unanesthetized rats. ***J Agric Food Chem*** 54(21): 8006-8012, 2006.

652. Thilakarathna SH, Rupasinghe HP. Flavonoid bioavailability and attempts for bioavailability enhancement. ***Nutrients*** 5(9): 3367-3387, 2013.

653. Shen Q, Li X, Li W, Zhao X. Enhanced intestinal absorption of daidzein by borneol/menthol eutectic mixture and microemulsion. ***AAPS PharmSciTech*** 12(4): 1044-1049, 2011.

654. Zhang Z, Huang Y, Gao F, Gao Z, Bu H, Gu W, Li Y. A self-assembled nanodelivery system enhances the oral bioavailability of daidzein: in vitro characteristics and in vivo performance. ***Nanomedicine (Lond)*** 6(8): 1365-1379, 2011.

655. Jang DJ, Kim ST, Lee K, Oh E. Improved bioavailability and antiasthmatic efficacy of poorly soluble curcumin-solid dispersion granules obtained using fluid bed granulation. ***Biomed Mater Eng*** 24(1): 413-429, 2014.