Role of neopterin on inflammasome activation in the nervous system

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Declaration of originality

I hereby declare that the work presented in this thesis is originated from a cotutelle PhD realized at Macquarie University, Australia, and at Federal University of Santa Catarina, Brazil. Therefore, the Portuguese version of this thesis has been submitted to the Graduate Program in Biochemistry, Department of Biochemistry, Federal University of Santa Catarina, Brazil. To the best of my knowledge this submission contains no material previously published or written by another person, except where due reference is stated otherwise. Any contribution made to the research by others is explicitly acknowledged.

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Publications

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Abstract

The inflammasome is a protein complex responsible for the maturation of the pro-inflammatory cytokines, pro-IL-1β and pro-IL-18. It represents a key event in the pathogenesis of inflammatory diseases which can be triggered by reactive oxygen species (ROS). Neopterin, an endogenous pteridin, is considered an early and sensitive biomarker of immune system activation. Although elevated neopterin levels have been associated with oxidative stress and inflammation for decades, the role of the pteridine in these conditions also remains unclear. Therefore, we investigated neopterin production in the central nervous system (CNS) under inflammatory conditions and the effect of neopterin preconditioning on inflammasome activation in nervous cells. A single lipopolysaccharide (LPS) (0.33 mg/kg; intraperitoneal) injection elicited an early hippocampal and serum increase of neopterin levels in adult Swiss mice, which occurred in parallel with inflammasome activation. It was also observed that rat cortical astrocytes produced and released neopterin under mitochondrial stress. In addition, extracellular neopterin (50 nM) inhibited ROS production and increased heme-oxygenase-1 (HO-1) content, an inducible antioxidant enzyme controlled by Nrf2 (nuclear factor (erythroid-derived 2)-like 2), which was also increased by the pteridine. Aiming to better understand the role of neopterin, we evaluated the effect of neopterin preconditioning on LPS-induced inflammasome activation in human primary nerve cells. Neopterin inhibited the inflammasome activation in astrocytes and neurons. Moreover, neopterin increased the anti-inflammatory cytokines IL-10 and IL-1Ra astrocytic release. Finally, we analysed the levels of neopterin and cytokines in the serum of patients diagnosed with Autism Spectrum Disorders (ASD), a disease whose the pathophysiology remains virtually unknown. Higher neopterin and IL-10 and IL-1Ra levels were observed in ASD patients, while IL-1β, TNF-α and IL-6 did not change. In conclusion, neopterin appears to induce neuroprotective functions, when produced before or in parallel with the inflammatory stimulus in the CNS, by favoring oxidative stress resistance and inhibiting inflammasome assembly. The proposed molecular mechanism for this effect is throught the activation of the Nrf2/HO-1 cytoprotective pathway.

Keywords: Neopterin, inflammasome, oxidative stress, central nervous system, Autism Spectrum Disorders.

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List of Abbreviations

ADI-R	Autism Diagnostic Interview-Revised
AGMO	Alkylglycerol monooxygenase
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
APC	Allophycocyanin
ARE	Antioxidant responsive element
ASC	Apoptosis-associated speck-like protein containing a CARD
ASD	Autism Spectrum Disorder
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
B2M	β-2-microglobulin
BBB	Blood-brain barrier
BH4	Tetrahydrobiopterin
BLAST	Basic Local Alignment Search Tool
Ca^{2+}	Calcium ions
CAPS	Cryopyrin-associated periodic syndromes
CARD	Caspase recruitment domain
CARS	Childhood Autism Rating Scale
CASP	Protease pro-domain consisting of p20 and p10.
CDC	Cholesterol-dependent cytolysin
cDNA	Complementary DNA
CEUA	Ethics Committee for Animal Research
CNS	Central nervous system
CIITA	Class II, MHC, transactivator
CINCA	Chronic infantile neurological cutaneous and articular syndrome
CLRs	C-type lectin receptors
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
DAMPs	Danger-associated molecular patterns
DCF	2‘–7‘-dichlorofluorescein
DCFH-DA	2‘–7‘-dichlorofluorescein diacetate
DMEM	Dulbecco’s modified Eagle’s medium

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FCAS	Familial cold autoinflammatory syndrome
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FIIND	Function to find domain
GABA	γ-aminobutyric acid
GFAP	Glial fibrilar acid protein
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GTP	Guanosine triphosphate
GTPCH	Guanosine triphosphate cyclohydrolase I
HET-E	Incompatibility protein of *Podospora anserina*
HIN	Hematopoietic expression, interferon-inducible nature, and nuclear localization
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
i.c.v.	Intracerebroventricular
IDO-1	Indoleamine-2,3-dioxygenase 1
IDO-2	Indoleamine-2,3-dioxygenase 2
IFN-α	Interferon- α
IFN-β	Interferon- β
IFN-γ	Interferon- γ
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-18	Interleukin-18
IL-1Ra	Interleukin-1 receptor antagonist

IκB	κB protein inhibitor
i.p,	Intraperitoneal
IPAF	IL-1-converting enzyme protease-activation factor
IRAK	IL-1R associated kinase
JAK	Janus kinases
K^+	Potassium ions
Keap1	Kelch-like ECH-associated protein 1
LAMEB	Multiuser Laboratory for Biological Studies
LDL	Low density lipoprotein
LPS	Bacterial lipopolysaccharide
LRR	Leucine-rich repeat
LTP	Long term potential
MAMPs	Microorganism-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridine
mRNA	Messenger RNA
MWS	Muckle-Wells syndrome
MYD88	Myeloid differentiation factor 88
7,8-NH2	7,8-dihydroneopterin
NACHT	NAIP, CIITA, HET-E and TP1
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Reduced form of Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NAIP	Neuronal apoptosis inhibitor protein
NALP	NACHT, LRR and PYD domains-containing protein
NaOH	Sodium hydroxide
Na_3VO_4	Sodium orthovanadate
NF-κB	Kappa B nuclear factor
NGF	Nerve growth factor
NLRC4	NLR family, CARD domain containing 4

NLRP1	NLR family protein, containing pyrin domain 1
NLRP2	NLR family protein, containing pyrin domain 2
NLRP3	NLR family protein, containing pyrin domain 3
NLRP5	NLR family protein, containing pyrin domain 5
NLRs	NOD-like receptors
NO	Nitric Oxide
NOD	Nucleotide-binding oligomerization domain NOD
NOMID	Neonatal-onset multisystem autoinflammatory disease
NOS	Nitric oxide synthase
NOS1	Neuronal NOS
NOS2	Inducible NOS
NOS3	Endothelial NOS
NMDA	N-methyl-D-aspartate
Nrf2	Nuclear factor erythroid 2-related factor 2
$P2X_7R$	$P2X_7$ receptor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PHA	Phenylalanine hydroxylase
PMSF	Phenylmethylsulfonyl fluoride
PRRs	Pattern recognition receptors
PTPS	6-pyruvoyltetrahydropterin synthase
PYD	Pyrin domain
qRT-PCR	Quantitative real-time polymerase chain reaction
RIG	Retinoic acid-inducible gene-I
RIPA	Radioimmunoprecipitation assay lysis buffer
RLRs	RIG I - like receptors
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
sIL-1Ra	IL-1Ra soluble isoform
SPSS	Statistical Package for the Social Sciences
SDS	Sodium dodecyl sulphate

SR	Sepiapterin reductase
STATs	Signal transducers and activators of transcription
TCA	Trichloroacetic acid
TDO	Tryptophan dioxygenase
TGF-β	Transforming growth factor-β
TH	Tyrosine hydroxylase
Th1	T helper 1 cells
TIR	Toll/IL-1 receptor domain
TIRAP	TIR domain-containing adaptor protein
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TNF-β	Tumor necrosis factor-β
TP1	Telomerase-associated protein 1
TPH	Tryptophan hydroxylase
TXNIP	Thioredoxin-interacting protein
TRIF	TIR domain-containing adapter inducing IFN-β
TRX1	Thioredoxin 1
TRX2	Thioredoxin 2

Chapter 1

Literature Review

Inflammation is a protective response that aims to eliminate pathogens or molecules that cause cell and tissue damage. The persistence and/or imbalance of this response can trigger a state of chronic inflammation, which will be prejudicial to the already affected tissue (for review see Hsieh and Yang, 2013). Thus, the neuroinflammation and the innate immune response are considered to be important events in the pathogenesis of many diseases of the central nervous system (CNS) such as the Alzheimer's disease, Parkinson's disease, autism, and others (Vargas et al., 2005, Sriram et al., 2006, Gubandru et al., 2013). Among the pro-inflammatory cytokines involved in the pathogenesis of CNS diseases are interleukin (IL) -1β and IL-18 which are synthesized as precursor proteins and depend on the processing executed by caspase-1 enzyme to become mature and perform their functions (Thornberry et al., 1992). Likewise, the activation of caspase-1 protease is dependent on the proteolytic cleavage performed by a multiprotein complex called inflammasome. This complex is composed of *i*) a sensor/receptor protein located in the cytosol that serves as a platform for the formation of the complex, ii) an adapter protein, ASC [apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain)], and iii) an effector protein, caspase-1 (Martinon et al., 2002). The inflammasome formation facilitates self-cleavage and the activation of caspase-1, which proteolytically cleaves pro-IL-1β and pro-IL-18 cytokines in their mature forms, favoring pro-inflammatory and anti-microbial responses (Broz et al., 2010, Liu et al., 2012).

Although the inflammasome activation is essential in host defense, recent studies have shown that the activation of this complex is involved in the pathogenesis of various diseases with inflammatory component, including peripheral chronic conditions such as type 2

diabetes and CNS disorders such as neurodegenerative, Parkinson's and Alzheimer's disease, and developmental, such as autism, for example (Halle et al., 2008, Masters et al., 2010, Siniscalco et al., 2012, Codolo et al., 2013, Heneka et al., 2013). Therefore, investigating potential inhibitors of the inflammasome activation will allow the development of effective therapies for the treatment of diseases characterized by chronic inflammation.

The use of peripheral biomarkers contributes for the early disease detection, the assessment of the progression, as well as, the monitoring of the palliative treatment in systemic and/or neurological processes (Brodacki et al., 2008, Molero-Luis et al., 2013, Wissmann et al., 2013, Suh et al., 2014). In this context, the measurement of neopterin has been related to the development and progression of diseases with neurological impairment such as Parkinson's and Alzheimer's disease (Hull et al., 2000, Widner et al., 2002a, Azumagawa et al., 2003, Frick et al., 2004, Molero-Luis et al., 2013, Parker et al., 2013, Wissmann et al., 2013).

Neopterin is a byproduct of the *de novo* synthesis pathway of tetrahydrobiopterin (BH4), synthesized under conditions of cellular stress induced by inflammatory stimuli, including IFN-γ (interferon-γ), LPS (bacterial lipopolysaccharide), hydrogen peroxide (H_2O_2), among others (Troppmair et al., 1988, Franscini et al., 2003, Ishii et al., 2005). The literature suggests that in humans, macrophages and monocytes would be the main producers of neopterin (Werner et al., 1990), in order to exacerbate the inflammatory response mediated by them. In this manner, an increase in neopterin levels in biological fluids has been regarded as a sensitive marker of the immune system activation. For example, plasma concentrations of this pteridine may increase 2-3 times in subjects affected by viral infections, autoimmune diseases, peripheral diseases of chronic progression or the CNS diseases (Fredrikson et al., 1987, Fuchs et al., 1989a, Parker et al., 2013). Moreover, neopterin levels can reach up to 500 nmol/L in the cerebrospinal fluid (CSF) in inherited metabolic diseases characterized by the deficiency of 6-pyruvoyltetrahydropterin synthase (PTPS) (Blau et al., 1996).

The investigations related to the production of neopterin have generally been focused on the peripheral myeloid cells lineage (Franscini et al., 2003). However, Kuehne et al. (2013) recently suggested that neopterin would also be produced in the CNS based on *i*) estimated low quotient (1/40) for neopterin to cross the blood-brain barrier (BBB) (Hagberg, et al., 1993); and *ii*) the neopterin levels found in CSF being higher than the plasma or serum, for example, in patients with neurological diseases with intact BBB (Kuehne et al., 2013). Furthermore, previous results of our group are consistent with this proposal of neopterin central production, since the pteridine secretion was observed from hippocampal slices and striatal astrocytes in conditions of mitochondrial toxicity. On the other hand, little is known

about the extracellular functions of neopterin in the CNS. Some studies have associated high concentrations of neopterin (higher than those encountered in pathological conditions) with oxidative stress, inflammation, and apoptosis (Weiss et al., 1993, Hoffmann et al., 1996, Schobersberger et al., 1996, Cirillo et al., 2006). Conversely, recent studies have demonstrated that, when intracerebroventricularly (i.c.v.) administered, neopterin (at concentrations slightly above those found at baseline levels) enhances the antioxidant status in mice cerebral cortex, reduces reactive oxygen species (ROS) production (Ghisoni and Latini, 2015), besides facilitating the acquisition of aversive memory and the generation of hippocampal long term potential (LTP) and reducing the IL-6 levels induced by peripheral administration of LPS in mice brain tissue (Ghisoni et al.,2016).

Taking into consideration that both the inflammasome activation and the neopterin production occur in inflammatory conditions and that neopterin should be produced and accumulate in the nervous tissue, it was sought to investigate the role of neopterin in the inflammasome activation in the CNS.

1.1 Neuroinflammation

The maintenance of CNS homeostasis is essential for the functioning of neuronal cells. The inflammatory process plays a crucial role in the homeostasis mainly through the active protection against various noxious stimuli such as neurotropic viral infections and/or traumatic injury, promoting tissue regeneration (Butovsky et al., 2006, Das et al., 2014). The CNS has developed strategies to limit the entry of immune elements as well as immune tissue activation, a phenomenon called immune privilege. Such condition is partially dependent on the presence and integrity of the BBB, which limits the entry of solutes and ions (Carson et al., 2006). While the peripheral immune access to the CNS is restricted and finely controlled, it is able to generate acute or chronic inflammatory and immune responses to various harmful stimuli, for example, infections, trauma, stroke, toxins and others (Xiong et al., 2009, Fu et al., 2014, Wang et al., 2014). The acute neuroinflammatory response in the CNS includes the activation of resident immune cells, such as microglia, resulting in a phagocyte phenotype with the release of inflammatory mediators such as cytokines and chemokines (Henry et al., 2009, Puntener et al., 2012). While an acute stimulation can induce cellular stress, this is typically of short duration and, if solved, it does not affect the neuronal survival. Thus, it is believed that an acute neuroinflammatory response is, in general, beneficial to the CNS, provided that it tends to minimize injury and contribute to damaged tissue repair.

When the brain homeostasis is not restored after acute local inflammatory response,

neuroinflammation becomes a chronic condition which can lead to neuronal death, representing one of several pathological events induced by pro-inflammatory factors (for review see Hsieh and Yang, 2013). In addition to persistent microglia activation and subsequent inflammatory mediators release, the chronic neuroinflammation also involves the increase of oxidative and/or nitrosative stress (Tronel et al., 2013, Vasconcelos et al., 2015). The constant release of inflammatory mediators propagates the inflammatory cycle, activating more microglial cells, promoting their proliferation and resulting in the release of more inflammatory factors. Due to the chronic and persistent nature of the inflammation, it can compromise the integrity of the BBB increasing its permeability with consequent infiltration of peripheral macrophages in brain parenchyma (Yang et al., 2014, Maggioli et al., 2015). Generically, chronic inflammation can be defined as a harmful condition that damages the nervous tissue. As a consequence, neuroinflammation has beneficial and deleterious consequences critically depending on the resolution of the inflammatory response.

Microglial cells are macrophages resident in the nervous tissue and are the first cells to respond to a noxious stimulus. They play a critical role in the tissue repair, neuronal regeneration, regulation of the synapses number during development, (Wakselman et al., 2008) and removal of apoptotic neurons (Takahashi et al., 2005). The microglia can be activated by acute stimuli or under chronic conditions such as aging and CNS disorders (McGeer et al., 1988). In the activated state, microglia shares functions with macrophages from other tissues destroying and phagocytosing viruses, bacteria and other invaders, possibly presenting antigens to infiltrated lymphocytes in the brain tissue, and removing cellular debris as part of tissue repair. Microglia can modulate neuroinflammation, restore homeostasis and protect the nervous tissue by producing anti-inflammatory cytokines and tissue repair factors such as glucocorticoids and IFN type 1 α and β (Park et al., 2007, Khorooshi and Owens, 2010). Nevertheless, when the neuroinflammation becomes chronic and/or under secondary stimulation, microglia produces excessive amounts of pro-inflammatory cytokines such as tumor necrosis factor (TNF) -α, IL-1β and IL-6, and ROS (Godbout et al., 2005), exerting deleterious effects, for example, contributing to the development of Parkinson's disease (Lazzarini et al., 2013).

Astrocytes also maintain the brain tissue homeostasis and perform functions that assist in the development and migration of neurons during the CNS development, production of growth factors, maintenance of the BBB integrity and participation of immune and repair responses (for review see Pěkný and Pěkná, 2014). In recent decades, additional functions of astrocytes have emerged, for example, its critical role in synaptic function. Specifically, astrocytic processes involve most of the synapses in the brain and express receptors for

various neurotransmitters which mediate increases in astrocytic calcium concentration (Ca^{2+}), resulting in regulated secretion of gliotransmitters, which modulate neuronal excitability, and synaptic strength (Perea and Araque, 2007). As well as microglia, astrocytes present numerous receptors involved in innate immunity, including Toll-like receptors (TLR), mainly TLRs 2 and 4 (Bowman et al., 2003) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Sterka et al., 2006). Although not considered immune cells, astrocytes also have immune-like properties, such as expression of cytokines (IL-1, IL-6, IL-10, IFN-α and IFN-β, TNF-α and TNF-β) and chemokines (Choi et al., 2014) and phagocytosis of cellular debris (Bechmann and Nitsch, 1997).

The cells with a role in the immune response are activated by the recognition of noxious stimuli to the tissue, which can be originated from pathogens or the host itself. The pathogenic stimuli are preserved microbial structures such as the LPS, lipoproteins, flagellin, peptidoglycan or bacterial and viral nucleic acids classified as pathogen-associated molecular patterns (PAMPs) or microorganism-associated molecular patterns (MAMPs) (Poltorak et al., 1998, Ozinsky et al., 2000, Souza et al., 2004). When invading tissues and/or the circulatory system, PAMPs/MAMPs can initiate an inflammatory response mediated by the innate immunity cells (Figure 1.1) with consequent release of DAMPs (danger-associated molecular patterns). DAMPs are endogenous stimuli that can be damaged cells (debris) or molecules released during cell death such as ATP (adenosine triphosphate) which induce the production of pro-inflammatory cytokines in response to injury or stress (Perregaux and Gabel, 1994). DAMPs can synergistically act with PAMPs/MAMPs and contribute to tissue damage. When the inflammatory response is triggered exclusively by the sensing of DAMPs, it is called sterile inflammation, as there is no involvement of microorganisms (Codolo et al., 2013, Heneka et al., 2013).

The induction of the pro-inflammatory response mediated by PAMPs, MAMPs or DAMPs involves the presence of molecular sensors, collectively called pattern recognition receptors (PRRs). There are several PRRs subfamilies including TLRs, NLRs, C-type lectin receptors (CLRs) and RIG I (retinoic acid-inducible gene-I)- like receptors (RLRs). Nonetheless, NLRs have drawn more attention due to the ability to form multiprotein complex called inflammasomes. The inflammasome activation has already been linked to the physiopathogeny of several chronic diseases with an inflammatory component, such as neurodegenerative diseases (Chin et al., 2008, Heneka et al., 2013, Terrill-Usery et al., 2014, Wang et al., 2014).

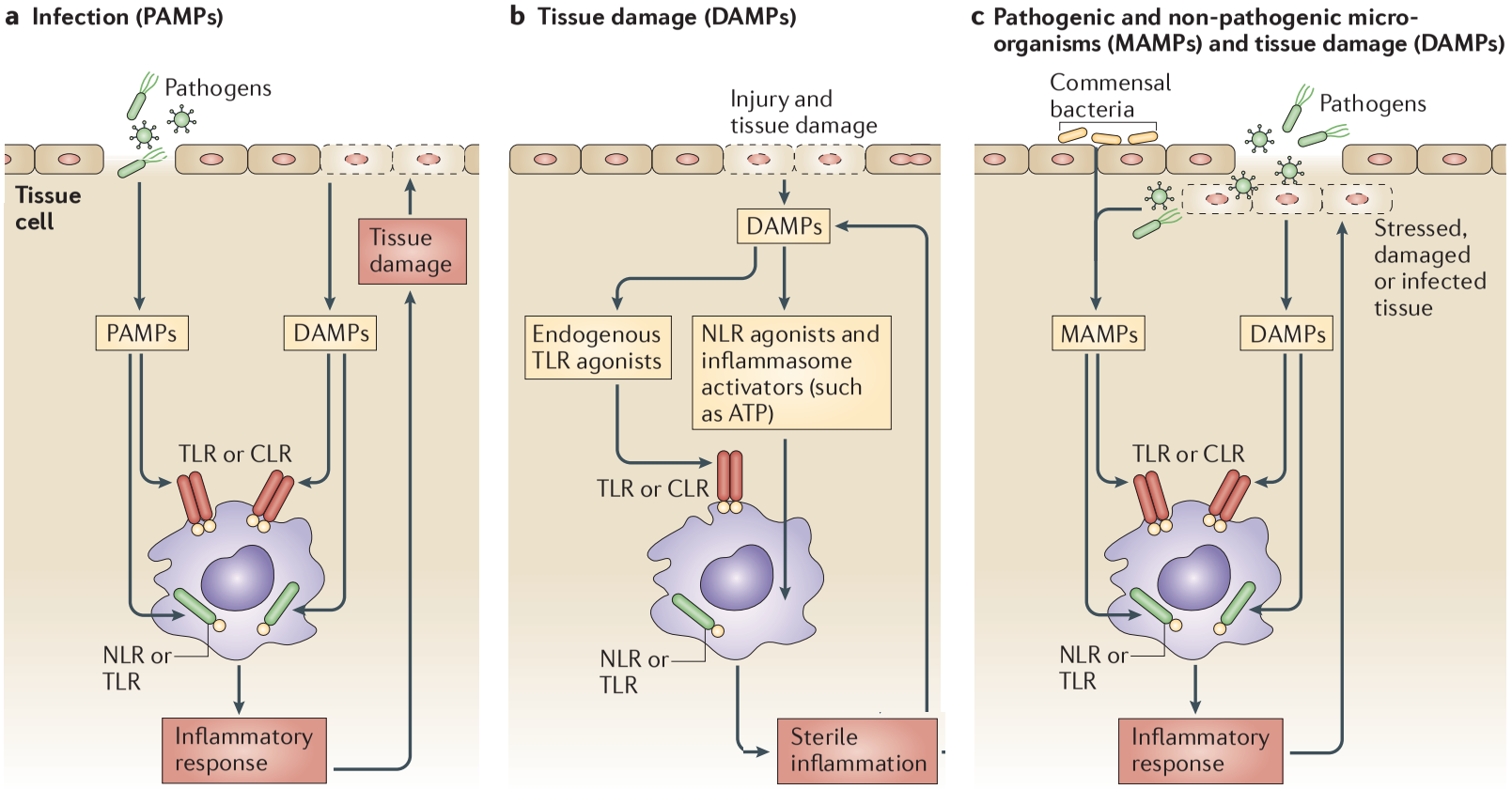

a Infection (PAMPs)
Pathogens
Tissue cell
Tissue damage
PAMPs
DAMPs
TLR or CLR
NLR or TLR
Inflammatory response
b Tissue damage (DAMPs)
Injury and tissue damage
DAMPs
Endogenous TLR agonists
NLR agonists and inflammasome activators (such as ATP)
TLR or CLR
NLR or TLR
Sterile inflammation
c Pathogenic and non-pathogenic micro-organisms (MAMPs) and tissue damage (DAMPs)
Commensal bacteria
Pathogens
Stressed, damaged or infected tissue
MAMPs
DAMPs
TLR or CLR
NLR or TLR
Inflammatory response

Figure 1.1. Activation of the innate immune response mediated by PAMPs, MAMPs and/or DAMPs. (**a**) PAMPs released from pathogens during an infection and/or (c) MAMPs from commensal bacteria activate PRRs located on the cell membrane, such as TLR and CLR, or in the cytosol of immune cells, such as NLR and some subtypes of TLRs, leading to inflammatory response. Further, the infectious process can lead to tissue damage and consequent release of DAMPs which may also activate the PRRs, contributing to inflammation. (**b**) In case of tissue injury, the released DAMPs (e.g., ATP) can be endogenous agonists of TLR and/or NLR receptors and inflammasome activators, or even directly activate membrane and cytosolic PRRs and lead to a sterile inflammation, given that there is no involvement of microorganisms (Figure from Mills, 2011). PAMPs: pathogen-associated molecular patterns; MAMPs: microorganisms-associated molecular patterns; DAMPs: danger-associated molecular patterns; PRRs: pattern recognition receptors; TLRs: Toll-like receptors; CLRs: C-type lectin receptors; NLRs: NOD-like receptors, RLRs: RIG-like receptor.

1.2 Inflammasome

The inflammasome is a complex of signalling proteins that promotes the processing of pro-IL-1β and pro-IL-18 (Martinon et al., 2002), cytokines known for their ability to cause biological effects associated with infection, inflammation and autoimmune processes (Bohn et al., 1998, CASAMENTI et al., 1999, Lachmann et al., 2009b, Palotai et al., 2014). The processing of IL-1β and IL-18 precursors is mediated by caspase-1 (Thornberry et al., 1992), protease synthesized as an inactive zymogen of 45 kDa consisting of a CARD and two subunits, p20 (20 kDa) and p10 (10 kDa), which together form the protease pro-domain. The inflammasome functions as a platform that enables the approaching of two or more pro-caspases, allowing its proteolytic self-cleavage and the formation of an enzymatically active heterodimer composed of the subunits p20 and p10 (Salvesen and Dixit, 1999, Martinon et al., 2002, Boatright et al., 2003).

There are four conventional or canonical inflammasomes which process pro-IL-1β and pro-IL-18 via caspase-1 (Figure 1.2), they are: NLRP1 (NLR family protein, containing pyrin domain 1), NLRP3, IPAF (IL-1-converting enzyme protease-activation factor) and the AIM2 protein (absent in melanoma 2) induced by IFN and belonging to the HIN (hematopoietic expression, interferon-inducible nature, and nuclear localization) family domain. These inflammasomes differ primarily in structure, requirement of the accessory protein ASC and potential activators. The ASC protein is composed of a pyrin domain (PYD) and a CARD domain and it functions as an adapter for the interaction between proteins containing PYD and proteins containing CARD, such as pro-caspase-1 (Masumoto et al., 1999, Conway et al., 2000).

AIM2 is formed by a HIN domain and a PYD domain, which mediates the association

of the receptor with the adapter protein ASC, allowing the recruitment of pro-caspase-1 and the formation of inflammasome (DeYoung et al., 1997). This receptor can be activated either by bacterial or viral deoxyribonucleic acid (DNA) (Hornung et al., 2009, Roberts et al., 2009).

NLRP1, NLRP3 and IPAF are cytosolic receptors of the NLR family characterized by the outstanding structural homology to disease resistance proteins of plants (Dixon et al., 2000). They are consisted of three domains: *i*) LRR (leucine-rich repeat) whose function is related to PAMPs recognition (Kajava, 1998); *ii*) NACHT [NAIP (neuronal apoptosis inhibitor protein), CIITA (class II, major histocompatibility complex (MHC), transactivator), HET-E (incompatibility protein of *Podospora anserina*) and TP1 (telomerase-associated protein)] responsible for the oligomerization of the receptor (Pop et al., 2006, Faustin et al., 2007); and *iii)* CARD or PYD, which will permit the association with ASC or pro-caspase-1 (Zou et al., 1997, Bertin et al., 1999, Inohara et al., 1999) (Figure 1.2).

IPAF, also known as NLRC4 (NLR family, CARD domain containing 4), contains a CARD domain and may interact directly and specifically with the pro-caspase-1 through homotypic interaction CARD-CARD (Poyet et al., 2001). It can be activated by an entire bacteria such as *Salmonella typhimurium, Pseudomonas aeruginosa* or *Legionella pneumophila* as well as bacterial components, for instance, the flagellin (Franchi et al., 2006, Miao et al., 2008, Case et al., 2009b). However, the exact mechanism of bacterial compounds detection by IPAF and of caspase-1 activation is not yet fully elucidated.

NLRP, also known as NALP (NACHT, LRR and PYD domains-containing protein) is the largest subfamily of NLR receptors, containing 14 members in humans and 12 in mice. Although the classical members are NLRP1 and NLRP3 (Martinon et al., 2002, Mariathasan et al., 2004), NLRP2 also forms functional inflammasomes (Minkiewicz et al., 2013). NLRP1 contains the domains mentioned above and also the FIIND (function to find) domain followed by a CARD. Consequently, NLRP1 can directly recruit pro-caspase-1. The NLRP1 activation has been demonstrated by *Bacillus anthracis* and muramyl dipeptide, constituent of the bacterial cell wall (Hsu et al., 2008). NLRP2 has not been widely investigated, but some studies have shown that this receptor can be activated by LPS, IFN-α and IFN-γ (Bruey et al., 2004). The NLRP3 inflammasome is the most studied and best characterized, probably due to its ability to detect both PAMPs and DAMPs. When activated, it is desubiquitinated (preventing its degradation by autophagy) and self-oligomerizes in an ATP-dependent manner (Duncan et al., 2007). The NLRP3 and NLRP2 complexes, different from NLRP1, do not contain the CARD domain, requiring the adapter protein ASC for the recruitment of pro-caspase-1. Thus, these inflammasomes are formed by homotypic interactions PYD-PYD between the receptor and ASC and CARD-CARD between ASC and pro-caspase-1. The

recruitment of pro-caspase-1 for the protein complex will lead to its self-cleavage and activation (Figure 1.2) (Agostini et al., 2004, Bruey et al., 2004).

The inflammasome components are commonly expressed under physiological conditions in myeloid lineage cells and/or tissues with high content of cells involved in innate immunity (Kummer et al., 2007, Guard et al., 2011). Yet, it has been demonstrated that nervous cells also express the proteins that constitutively form the inflammasomes. Thus, NLRP1 has been detected in culture of human neurons (Kaushal et al., 2015) and in neurons from mice spinal cord (Rivero Vaccari et al., 2008). AIM2 and IPAF were also detected in human neuronal cells culture, but not as significantly as NLRP1 (Kaushal et al., 2015). The NLRP2 protein has been described in human primary astrocytes culture (Minkiewicz et al., 2013). In addition, IPAF, NLRP1, AIM2 and NLRP3 mRNA have also been described in mice cortical astrocytes culture, the latter being the most abundant (Alfonso-Loeches et al., 2014). Therefore, though the activation of the inflammasome in the CNS is not known in detail, there is evidence of the presence of several proteins involved in the process.

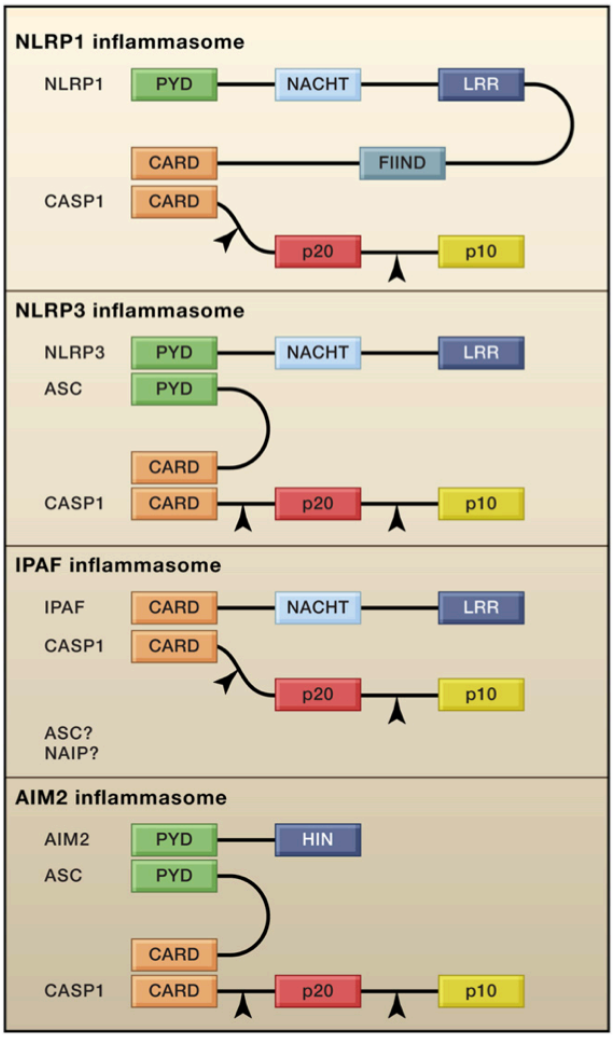


Figure 1.2. Structure of conventional inflammasomes. All conventional inflammasomes are constituted by a receptor and the effector protein pro-caspase-1, differing regarding the requirement of the adapter protein ASC. The NLRP1 and IPAF receptors have a CARD domain, allowing the recruitment of caspase-1 directly, while NLRP3 and AIM2 have a PYD domain and need ASC for the inflammasome formation (Figure from Schroder and Tschopp, 2010). LRR: leucine-rich repeat; NACHT: NAIP, CIITA, HET-E, and TP1; PYD: pyrin domain; CARD: caspase recruitment domain; FIIND: function to find domain; HIN: hematopoietic expression, interferon-inducible nature, and nuclear localization domain.

1.2.1 Conventional activation of NLRP3 inflammasome

The NLRP3 inflammasome is considered unique among the innate immune system sensors given that it can be activated by PAMPs and DAMPs with great structural variability (Mariathasan et al., 2004). NLRP3 can be activated by high concentrations of ATP, glucose, ROS, sphingosine, ceramides, oxidized LDL (low density lipoprotein), β-amyloid peptide, uric acid crystals and cholesterol, apart from environmental irritants, toxic chemicals like particles of asbestos, biomaterials, nanoparticles, among others (Figure 1.3) (Halle et al., 2008, Duewell et al., 2010, Jiang et al., 2012, Luheshi et al., 2012). Because of the great chemical and structural diversity of the activators (Petrilli et al., 2007, Dostert et al., 2008, Halle et al., 2008), it is likely that both NLRP3 agonists and the signalling pathways activated by it, alter cell homeostasis or the function of any organelle that integrates the activation signals and, as a result, causes the activation of the inflammasome. It is important to stress that inflammasomes can only induce the processing and secretion of IL-1β and IL-18 previously stored in the cells. Subsequently, the NLRP3 inflammasome requires two signals to be activated. The first signal or priming is provided by TLR ligands or by kappa B nuclear factor (NF-kB) activators, which will induce the expression of pro-IL-1β and also NLRP3. The second signal will directly activate NLRP3 and will stimulate caspase-1 activation and pro-IL-1β and pro-IL-18 cleavage with subsequent release of mature cytokines.

There are three main NLRP3 inflammasome activation models (Figure 1.3): the potassium (K^+) efflux induced by ATP, the lysosomal rupture and the ROS production.

It may be cited among the NLRP3 inflammasome activators, agents that induce intracellular K^+ efflux, as high extracellular concentrations of ATP and/or pore-forming toxins such as nigericin. Initially, it was shown that these compounds, ATP and nigericin, induce the secretion of mature IL-1β through K^+ depletion in LPS-primed macrophages (Hogquist et al., 1991, Perregaux and Gabel, 1994). In addition, Walev et al. (1995) showed

that K^+ addition to the extracellular medium, blocking the ion efflux, inhibits the secretion of active IL-1β in monocytes culture.

The *in vitro* stimulation of murine macrophages with high concentrations of ATP (from 100 μM up) activates the purinergic receptor $P2X_7$ ($P2X_7R$) which is associated with a cation channel and induces the rapid efflux of K^+ (Surprenant et al., 1996). This leads to the opening of a large pore permeable to molecules of 900 Da or greater, and consisting of pannexin-1, a connexin family protein present in the communicating junctions (Pelegrin and Surprenant, 2006). There are speculations that NLRP3 activators could cross such pore and activate the receptor present in the cytosol, however, the role of pannexin-1 in inflammasome activation is still unclear.

Regarding the activation of $P2X_7R$, this happens exclusively in the ATP-induced NLRP3 activation. In murine macrophages, the activation of $P2X_7R$ by exogenous ATP is strictly necessary for the processing and secretion of IL-1β in response to exogenous stimuli (Franchi et al., 2007). However, depending on the cell type and species, the first signal or priming induces cell disorders (such as ATP secretion) sufficient to activate NLRP3, for example, the secretion of IL-1β via NLRP3 was dependent on the activation of $P2X_7R$ in human monocytes stimulated with LPS (Piccini et al., 2008). The activation of the NLRP3 inflammasome via K^+ efflux has also been demonstrated for other activators in addition to ATP, such as monosodium urate crystals, peptidoglycan of the bacterial cell wall (Petrilli et al., 2007), asbestos (Dostert et al., 2008), among others.

The second conventional inflammasome activation model has been proposed especially for particulate and/or crystalline activators. When these compounds are endocyted, they would cause the destabilization of the lysosomal acid compartment with subsequent release of cathepsin B that would activate the NLRP3 receptor (Hornung et al., 2008). In this regard, it was shown that cathepsin B inhibitors can prevent caspase-1 activation induced by certain microorganisms like *Neisseria gonorrhoeae*, and bacterial toxins such as cholesterol-dependent cytolysin (CDC), in human monocytes and murine macrophages, respectively (Chu et al., 2009, Duncan et al., 2009). Additionally, the lysosomal destabilization can lead to the release of its Ca^{2+} content, with subsequent activation of Ca^{2+} release from the endoplasmic reticulum and subsequent NLRP3 activation (Murakami et al., 2012). The role of Ca^{2+} signalling and flux in the inflammasome activation is still not fully understood, since there is no consensus in the literature about this subject.

In the third model, the production of ROS is considered a critical event for the activation of the inflammasome. Initially, it was suggested that the activation of the inflammasome was induced by ROS produced by NADPH (nicotinamide adenine

dinucleotide phosphate) oxidase in macrophages (Dostert et al., 2008). But, as the deficiency in four of the seven isoforms of the enzyme did not affect the activation of the inflammasome in human macrophages (Meissner et al., 2010), studies were directed to the involvement of mitochondrial ROS (Nakahira et al., 2011, Alfonso-Loeches et al., 2014).

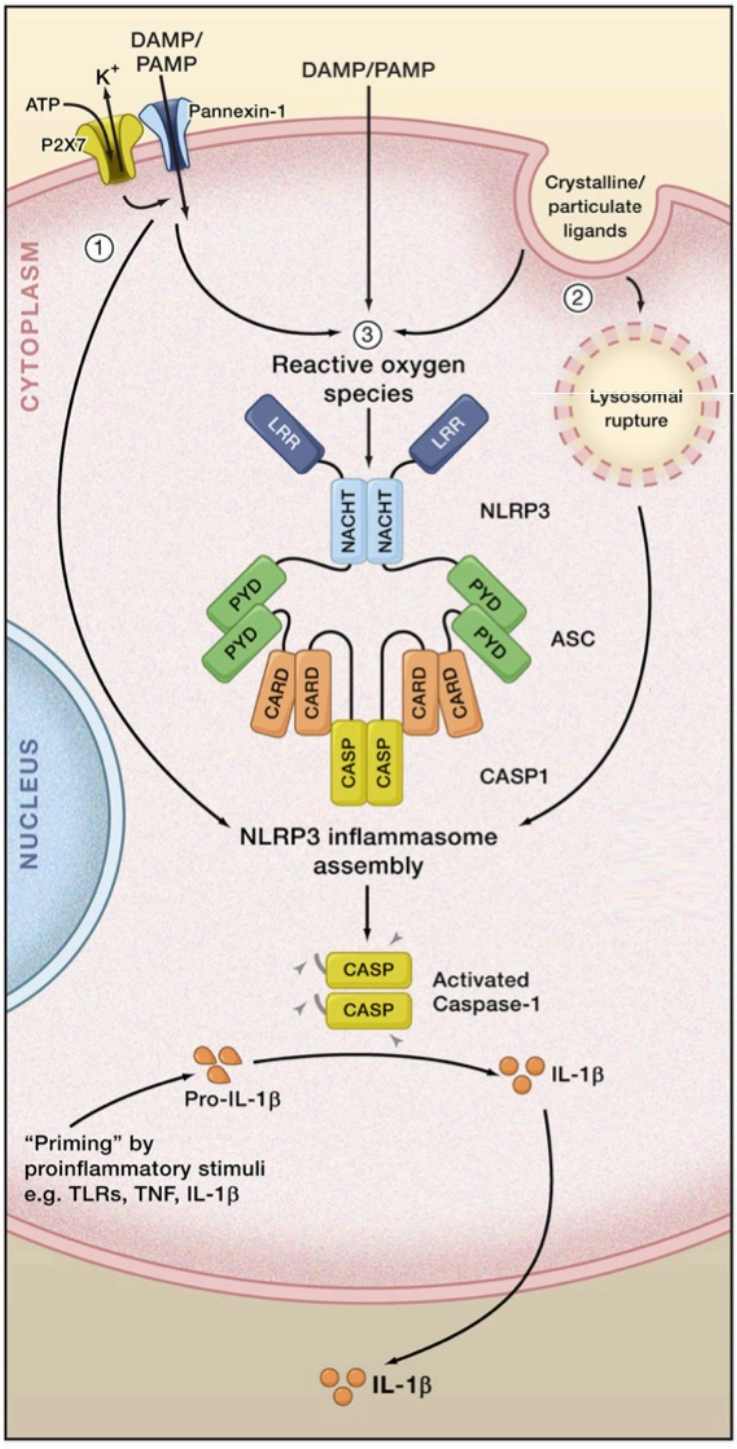


Figure 1.3. Main conventional NLRP3 inflammasome activation pathways. In general, the cells do not store pro-IL-1β. Thus, the cell may be primed to induce the synthesis of pro-IL-1β by the transcription factor NF-kB activation. Subsequently, the NLRP3 inflammasome activators should induce cellular changes that lead to its activation as, for example, the efflux of K^+ induced by ATP, ROS production or lysosomal rupture induced by particulate ligands, for example, uric acid crystals, asbestos and silica. These changes lead to the NLRP3 receptor oligomerization, ASC and pro-caspase-1 recruitment, facilitating its self-cleavage and activation. Activated caspase-1 promotes the proteolytic cleavage of pro-IL-1β into mature IL-1β with subsequent secretion (Figure adapted from Schroder and Tschopp, 2010). PAMPs: pathogens-associated molecular patterns; DAMPs: danger-associated molecular patterns; ROS: reactive oxygen species; LRR: leucine-rich repeats; NACHT: NAIP, CIITA, HET-E, and TP1; PYD: pyrin domain; CARD: caspase recruitment domain; CASP: protease pro-domain consisting of p20 and p10.

Under physiological conditions, the mitochondria are the main ROS source during the electrons transfer to the complete oxygen reduction in water (Barja, 1998). Yet, changes in the activity of the mitochondrial electron transport chain can lead to inflammasome activation by the overproduction of ROS. For example, the complex I inhibitor of the electron transport chain, rotenone, induces the production of mitochondrial ROS together with the NLRP3 inflammasome priming in murine NLRP3 and MyD88/TRIF (myeloid differentiation factor 88/ Toll/IL-1 receptor (TIR) domain-containing adapter inducing IFN-β) knockout macrophages (proteins involved in LPS signalling pathway) (Julien et al., 2012). Moreover, rotenone exposure induces NLRP3 oligomerization in human kidney cells (Park et al., 2013), and NLRP3 activation in murine microglial cells lineage (Liang et al., 2015). Further, antimycin A, respiratory chain complex III inhibitor, and FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) uncoupler of oxidative phosphorylation in mitochondria, significantly increase IL-1β secretion and ROS production in murine macrophages (Jabaut et al., 2013). On the other hand, the use of mitochondrial antioxidants inhibits the inflammasome activation. For example, MitoTEMPO, specific antioxidant against mitochondrial ROS, reduces the its production and the inflammasome activation in inducible nitric oxide synthase (NOS2) knockout macrophages stimulated with LPS and ATP (Mao et al., 2013) and murine macrophages exposed to LPS and palmitate (Weber and Schilling, 2014).

The exact mechanism by which ROS activates the inflammasome is still not well defined. The most plausible or accepted hypothesis is that ROS induces the inflammasome activation via thioredoxin-interacting protein (TXNIP) (Figure 1.4). TXNIP is located in the cell nucleus and, under oxidative stress conditions translocates to the cytosol and mitochondria. In the cytosol, TXNIP associates with the thioredoxin 1 (TRX1) protein and in the mitochondria with the TRX2. The association with TXNIP, inhibits the antioxidant

activity of both TRXs (Nishiyama et al., 1999, Saxena et al., 2010). When ROS production increases, TRX1 is oxidized and the TXNIP/TRX1 complex dissociates, allowing the interaction of TXNIP with the LRR domain of NLRP3 receptor, leading to inflammasome activation (Zhou et al., 2010). In the mitochondria, TRX2 is linked to the apoptosis signal-regulating kinase (ASK) 1 protein. When TXNIP translocates to the mitochondria, it decouples the complex TRX2/ASK and associates with TRX2. Consequently, TXNIP inhibits the TRX2 antioxidant activity and ASK1 is released and activated by phosphorylation, activating caspase-3 and inducing apoptosis (Bhattacharyya et al., 2003).

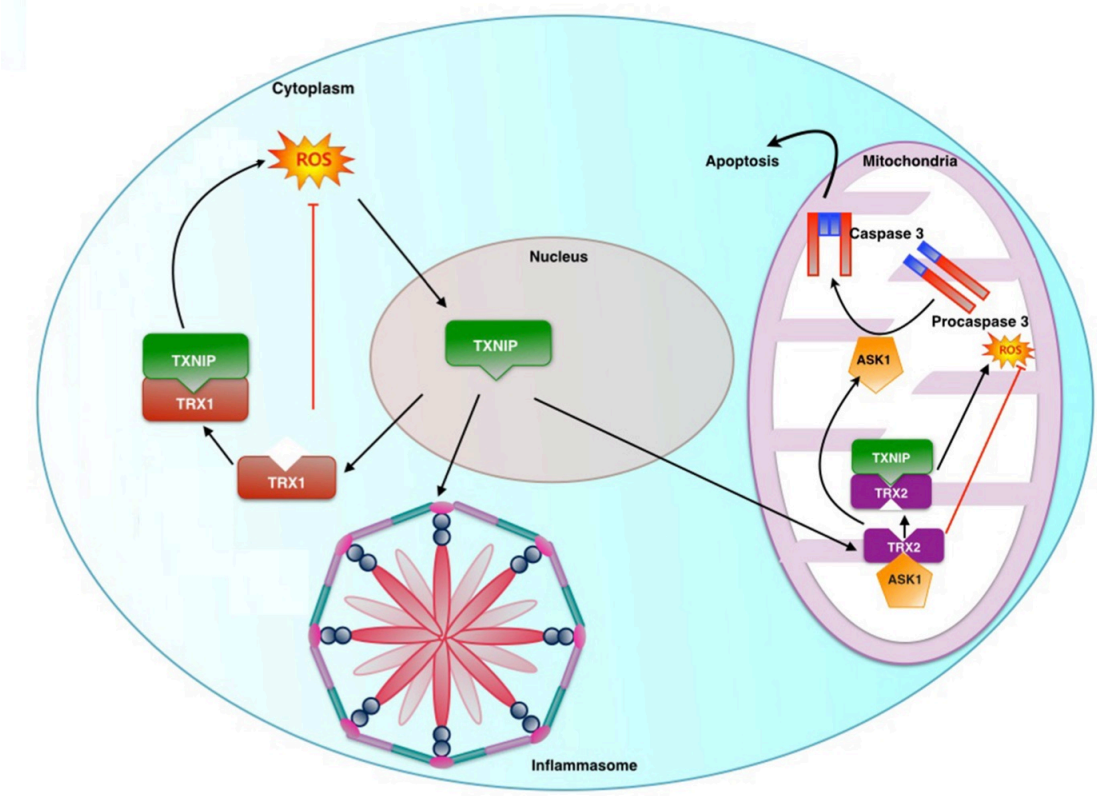


Figure 1.4: ROS induces the inflammasome activation through TXNIP. Under stress conditions, TXNIP can translocate from the nucleus to the cytosol or mitochondria. In the cytosol, TXNIP associates with TRX1. In oxidative stress conditions, TRX1 is oxidized and the association is undone, allowing TXNIP activate the NLRP3 receptor. In mitochondria, TXNIP undoes the complex formed by TRX2 and ASK1, binding to TRX2. The free ASK1 can be phosphorylated and activated, mediating the activation of caspase-3 and inducing apoptosis (Figure adapted from Harijith et al., 2014). TXNIP: thioredoxin-interacting protein; TRX: thioredoxin; ASK1: apoptosis signal-regulating kinase 1 protein; ROS: reactive oxygen species.

Besides these activation mechanisms, recent research found that the NLRP3 inflammasome can also be activated by unconventional ways involving other caspases, called inflammatory.

1.2.2 Unconventional activation of the NLRP3 inflammasome

The unconventional/non-canonical NLRP3 inflammasome activation is mediated by caspase-11 in mice and caspase-4 and -5 in humans (Lamkanfi et al., 2002), which can induce pyroptosis in response to bacterial infections independently of caspase-1. Kayagaki et al. (2011) showed that the secretion of IL-1β and IL-18 via caspase-11 was induced only by gram-negative bacteria and not by other classic NLRP3 inflammasome activators. In 2013, Hagar et al. reported for the first time that the activation of caspase-11 is LPS-dependent, while there is no NLRP3 activation mediated by gram-positive bacteria. In the cytosol, LPS may bind to pro-caspase-11 (or 4/5 in human) promoting its self-cleavage and activation. The activated enzyme can induce pyroptosis or activate NLRP3 inflammasome after a priming signal (Figure 1.5) (Hagar et al., 2013).

The LPS is widely used to induce inflammatory *in vitro* and *in vivo* responses (Hurme and Seppala, 1988, Henry et al., 2009). It binds to the TLR4 membrane receptor (Poltorak et al., 1998), activating a signalling cascade with consequent activation of transcription factors such as NF-kB, controlling the production of pro and anti-inflammatory cytokines (Fitzgerald et al., 2003). *In vivo*, the intraperitoneal administration of high doses of LPS increases the serum concentration of IL-1β dependently on caspase-11 and 1 (Kayagaki et al., 2011) and NLRP3 (He et al., 2013).

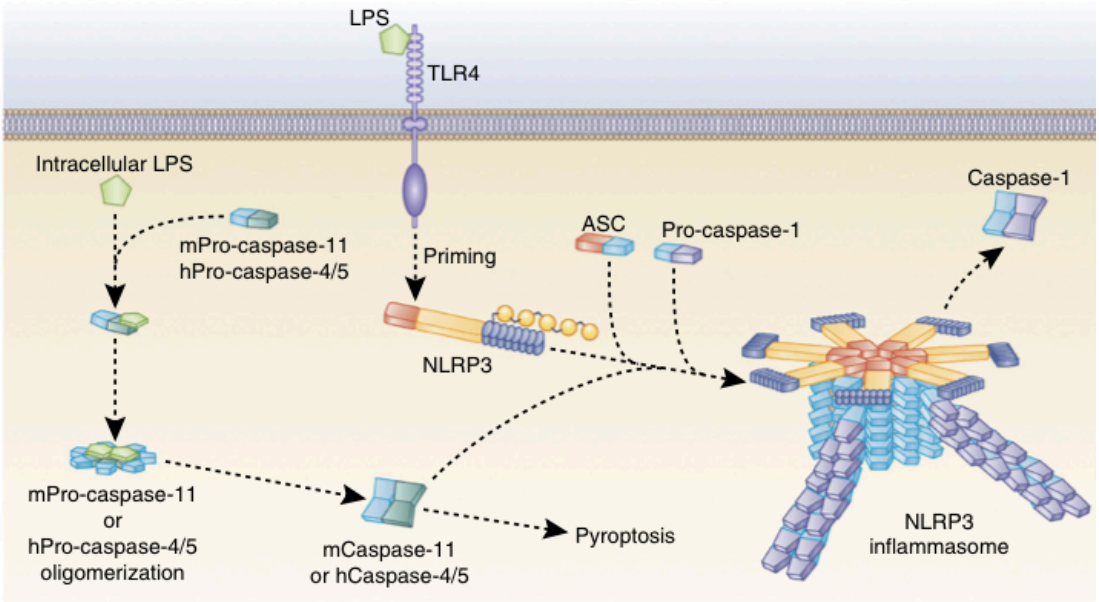


Figure 1.5. Unconventional activation of the NLRP3 inflammasome. The murine pro-caspase-11 enzyme (mPro-caspase-11) and human pro-caspase 4/5 (hPro-caspase 4/5) can directly bind to intracellular LPS, which induces oligomerization of these pro-caspases and its auto-cleavage and activation. This is sufficient for pyroptosis induction but not for IL-1β processing. However, mCaspase-11 and hCaspase-4/5 may promote the oligomerization and activation of NLRP3 inflammasome constituting the unconventional NLRP3 inflammasome activation (Figure from Guo et al., 2015). LPS: bacterial lipopolysaccharide; TLR4: Toll-like receptor 4; NLRP3: NLR protein family, containing pyrin domain 3; ASC: apoptosis-associated speck-like protein containing a CARD.

1.2.3 Pyroptotic cell death

The inflammasome activation induces the formation of ASC oligomerized complexes, nominated pyroptosome (Fernandes-Alnemri et al., 2007). The formation of pyroptosome can lead to pyroptosis, a highly inflammatory cell death and dependent on caspase-1 activity (Cookson and Fink, 2006). The pyroptosis often occurs in response to infection by intracellular pathogens (Suzuki et al., 2007, Fink, 2008, Case et al., 2009) and is part of the antimicrobial response. Although it is considered a way of programmed cell death, it is different from apoptosis, a type of cell death immunologically silent. The apoptosis is characterized by the occurrence of morphological changes in affected cells, which include the translocation of phosphatidylserine from the inner to the outer layer of the cellular lipid bilayer, condensation and aggregation of the chromatin and nuclear fragmentation, ending with the formation of apoptotic bodies (Kerr et al., 1972).

The pyroptosis, in turn, is accompanied by DNA cleavage and nuclear condensation, water influx causing cell swelling and consequent disruption of the cell membrane and release of pro-inflammatory cell content (Fink and Cookson, 2006, Molofsky et al., 2006). Thus, the pyroptosome and caspase-1, IL-1β and IL-18 can be released into the extracellular medium where pyroptosome can be phagocytosed by neighboring cells, or remain active leading to the caspase-1 activation, cytokines release and propagation of the inflammation (Balci-Peynircioglu et al., 2008, Franklin et al., 2014) (Figure 1.6).

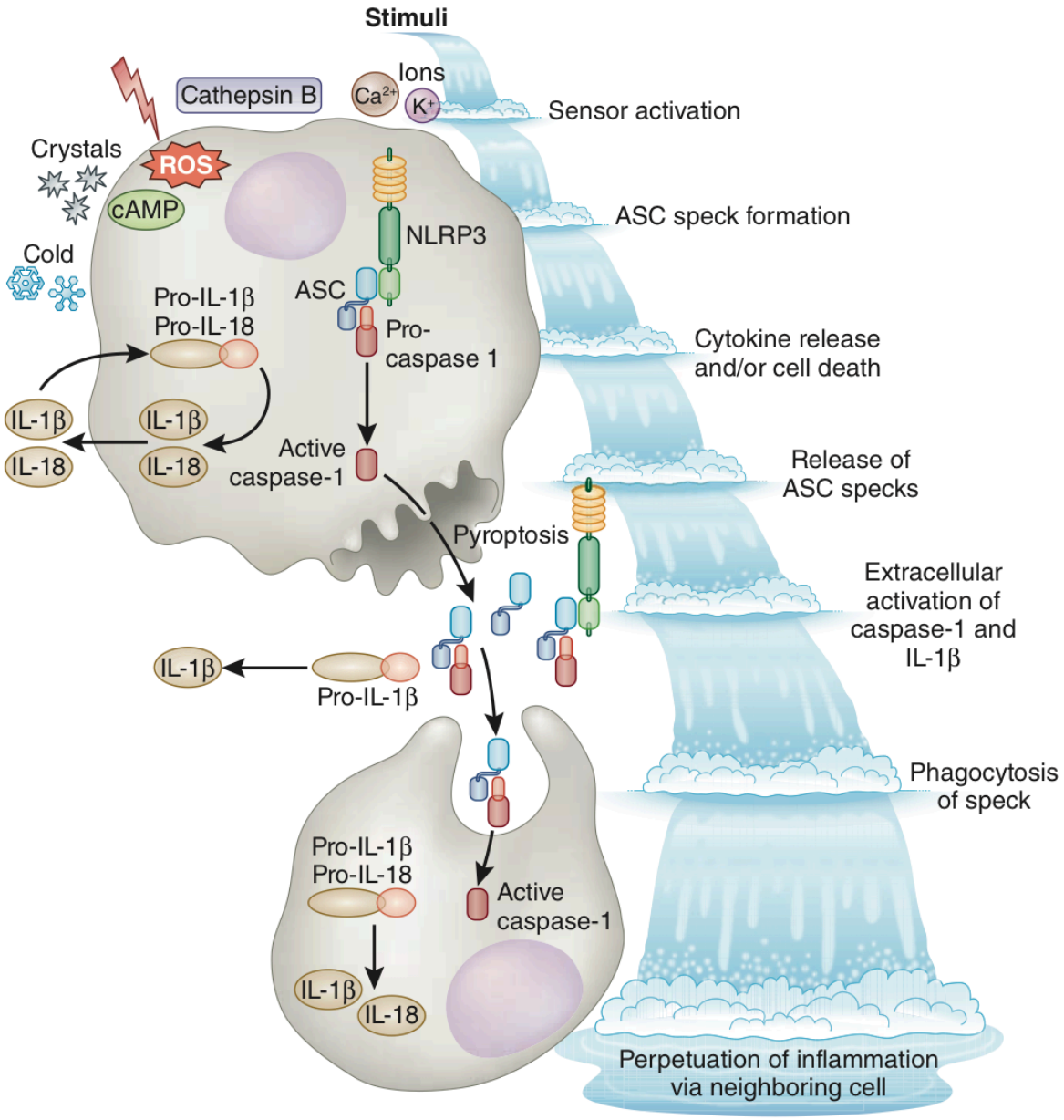


Figure 1.6: The inflammasome activation contributes to the pyroptosis. The activation of caspase-1 leads to cell death by pyroptosis, characterized by rupturing the cell membrane and inflammatory contents extravasation: oligomerized ASC (pyroptosome), activated caspase-1, IL-1β and IL-18. The pyroptosome can be endocytosed by neighboring cells, activating caspase-1 and spreading inflammation (Figure from Broderick and Hoffman, 2014). ROS: reactive oxygen species; NLRP3: NLR protein family, containing pyrin domain 3; ASC: apoptosis-associated speck-like protein containing a CARD; IL-1β: interleukin 1β, IL-18: interleukin 18.

1.2.4. Inflammasome activation in diseases

Despite its importance in the immune response, as already mentioned, the inflammasome activation is also involved in the pathophysiology of several diseases. For example, mutations that generate functional gain of function in the NACHT domain of NLRP3 are involved in cryopyrin-associated periodic syndromes (CAPS), named after the initial designation of the NLRP3 encoding gene. Among these diseases there are the familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multi-system auto-inflammatory disease/ chronic infantile neurological cutaneous and articular syndrome (NOMID/CINCA) (Aganna et al., 2002). These syndromes constitute a spectrum of disorders with varying severity, with FCAS, MWS and NOMID/CINCA representing moderate, intermediate and severe levels respectively. Clinically, they are characterized by recurrent fever, urticaria, ocular and joint inflammation, amyloidosis, and, in the case of NOMID/CINCA, neurological complications (Muckle and Wellsm, 1962, Lampert, 1986, Hoffman et al., 2001, Aksentijevich et al., 2002).

The inappropriate activation of the NLRP3 inflammasome is also involved in most chronic diseases. For example, accumulated metabolites in chronic disorders, such as monosodium urate crystals in the gout (Martinon et al., 2006), β-amyloid plaques in Alzheimer's disease (Halle et al., 2008, Heneka et al., 2013), pancreatic islets β-amyloid polypeptide in type 2 diabetes (Masters et al., 2010) or cholesterol crystals in atherosclerosis (Duewell et al., 2010), cause the activation of the inflammasome.

1.2.5. Activation of the NLRP3 inflammasome in CNS diseases

The role of the inflammasome in CNS diseases has only been recently investigated. The activation of the inflammasome in the CNS is considered one of the primary and critical steps in neuroinflammation induced by PAMPs and DAMPs, followed by the production and secretion of chemokines and cytokines (Rivero Vaccari et al., 2009, Jha et al., 2010). Activated inflammasomes have been identified in the brain tissue of patients and animal models of CNS diseases. It was also identified in nervous cells stimulated with metabolic products involved in the pathophysiology of CNS diseases, for example, the β-amyloid polypeptide (Halle et al., 2008, Heneka et al., 2013).

NLRP3 inflammasome activation has already been identified in the white matter of patients with human immunodeficiency virus (HIV) infection, as evidenced by an increase in IL-1β expression, caspase-1 and ASC (Walsh et al., 2014). Experimentally, the NLRP3 inflammasome activation was observed in the brain structure of animals subjected to chronic mild stress (Pan et al., 2014), diabetes associated with parkinsonism induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Wang et al., 2014), and epilepsy induced by electrical stimulation (Meng et al., 2014). Furthermore, it was observed in the nervous tissue from β-amyloid precursor proteins and presenilin 1 knockout mice, genetic model of Alzheimer's disease that mimics the deposition of amyloid plaques (Alloza-Garcia et al., 2006).

There are also evidences showing that β-amyloid peptide and α-synuclein involved in the physiopathology of Alzheimer's and Parkinson's diseases, respectively, induce the assembly of NLRP3 in cultured murine microglia (Halle et al., 2008, Codolo et al., 2013). Additionally, compounds used to induce experimental models of Parkinson's disease such as MPP^+ (1-methyl-4-phenylpyridine), also induce the activation of NLRP3 inflammasome, evidenced by the high content of NLRP3, caspase-1 and IL-1β in astrocyte culture of neonatal mice cortex (Lu et al., 2014).

1.2.6. Treatment of inflammasome-associated diseases

The treatment of conditions associated with excessive activation of the inflammasome aim at blocking the cytokine IL-1β, the main pyogenic product of the inflammasome activity, or their effects by blocking its receptor. For example, the inhibition of IL-1β activity by IL-1-directed antibody, canakinumab, is used as a therapeutic strategy for the treatment of MWS and FCAS in clinical trials (Lachmann et al., 2009a). The inhibition of the IL-1 receptor by the human IL-1 receptor antagonist, Anakinra, has also been used in the treatment of rheumatoid arthritis and prevents cartilage degeneration associated with this pathology (Kone-paut and Galeotti, 2014). Clinical trials with GSK1070806, antibody for IL-18, have also been performed. Tests on healthy and obese subjects have presented that the antibody was effective in neutralizing IL-18 and showed moderate adverse effects (Mistry et al., 2014), but, it is not used in clinic yet. In summary, the inflammasome inhibition alone would be more effective than inhibitors of cytokines, as it would inhibit the induction of cell death by pyroptosis in addition to blocking the secretion of IL-1β and IL-18, instead of inhibiting only its effects.

1.2.7. NLRP3 inflammasome inhibitors

The inflammasome inhibitors can act in all essential steps for the inflammasome formation, such as in the NF-kB and NLRP3 receptor activation, in the ASC oligomerization or in the caspase-1 activation, as shown in Table 1.1. Some of these compounds have already shown clinical outcome, while others were tested only in animal models and in *in vitro* systems.

Table 1.1: Compounds which inhibit NLRP3 inflammasome activation and their mechanisms of action.

Compound	Mechanism of NLRP3 inflammasome inhibition	References
Parthenolide	Inhibits NF-κB activation, NLRP3 ATPase activity and caspase-1 activity through cysteine residues alkylation	Juliana et al., 2010.
AZD9056	Inhibits K^+ efflux and P2X7R	Keystone et al., 2012.
β-hydroxybutyrate	Inhibits K^+ efflux	Youm et al., 2015.
Curcumin	Inhibits K^+ efflux, lisossomal rupture and ROS production	Gong et al., 2015.
Resveratrol	Induces NLRP3 ubiquitination and autophagy and inhibits ROS production	Chang et al., 2015.
Dopamine	Induces NLRP3 ubiquitination and autophagy	Yam et al., 2015.
Glybenclamide	Not elucidated	Lamkanfi et al., 2009.
MCC950	Inhibits ASC oligomerization	Coll et al., 2015.

Among the compounds mentioned, the only one used in patients was AZD9056, which reduced joint inflammation in patients with rheumatoid arthritis when orally administered (Keystone et al., 2012). All the other compounds showed inhibition of NLRP3 inflammasome activation in murine or human macrophages or monocytes culture (Lamkanfi et al., 2009, Julian et al., 2010, Chang et al., 2015, Coll et al., 2015, Gong et al., 2015, Yan et al., 2015). Particularly, parthenolide and curcumin also inhibit the NLRP3 inflammasome activation induced, respectively, by ethanol in organotypic hippocampal culture and by glutamate in hippocampal slices, besides reducing the deleterious effects of ethanol in hippocampal neurogenesis (Zou and Crews, 2012).

Besides the effects already mentioned, most of these compounds also exert anti-inflammatory *in vivo* effects. For example, β-hydroxybutyrate and resveratrol attenuate the immune cells infiltration and IL-1β secretion induced, respectively, by uric acid crystals in the peritoneum and progressive nephropathy model in mice renal tissue (Chang et al., 2015, Youm et al., 2015). Resveratrol also reduces the infarct volume and myocardial fibrosis

induced by ischemia/reperfusion injury in rats (Dong et al., 2015). Yet, dopamine lowers the serum secretion of NLRP3 inflammasome-dependent cytokines (Yan et al., 2015), while curcumin inhibits the IL-1β secretion in peritoneal cavity and hepatic alterations induced by LPS intraperitoneal (i.p.) administration, besides increasing the survival of mice (Gong et al., 2015). In addition, MCC950, compound containing diarylsulphonylurea, attenuates the severity of symptoms in animal models of NLRP3-associated diseases, as experimental autoimmune encephalomyelitis, which mimics multiple sclerosis in humans, and CAPS family auto-inflammatory diseases (Coll et al., 2015).

1.3 Neopterin: Biomarker in Inflammatory Processes (Review)[1]

Inflammatory processes are characterized by an increase in the number of activated immune cells, besides the levels of cytokines and chemokines (Henry et al., 2009, Suh et al., 2014). Some molecules synthesized during inflammation, such as C-reactive protein and neopterin, may be measured and used as peripheral biomarkers in order to early characterize, assess the progression, as well as monitor the palliative treatment, in systemic and/or neurological processes (Brodacki et al., 2008, Molero-Luis et al., 2013, Wissmann et al., 2013, Suh et al., 2014). In this context, the measurement of neopterin has been related to the development and progression of diseases with neurological impairment, such as Alzheimer's and Parkinson's disease (Hull et al., 2000, Widner et al., 2002, Azumagawa et al., 2003, Frick et al., 2004, Molero-Luis et al., 2013, Parker et al., 2013, Wissmann et al., 2013).

1.3.1 Neopterin synthesis

Neopterin is a pteridine which is formed during the synthesis of BH4, an essential cofactor required for the enzymes phenylalanine, tyrosine and tryptophan hydroxylases, alkylglycerol monooxygenase (for review see Werner et al., 2011), and for the three isoforms of nitric oxide synthase (NOS; NOS1 or neuronal, NOS2 and NOS3 or endothelial) (Mayer et al., 1990). Intracellular concentrations of BH4 are maintained by multiple metabolic routes called *de novo*, salvage and recycling pathways. The *de novo* pathway produces BH4 from guanosine triphosphate (GTP) by the action of the enzymes guanosine triphosphate

[1] This review is adapted and modified from the following journal:

GHISONI, K.; **MARTINS, R.P**.; BARBEITO, L.; LATINI, A. (2015) Neopterin as a Potential Cytoprotective Brain Molecule. *Journal of Psychiatric Research*, v. 71, p. 134-139.

cyclohydrolase I (EC 3.5.4.16; GTPCH), PTPS (EC 4.6.1.12) and sepiapterin reductase (EC 1.1.1.153; SR) (Nichol et al., 1985). The GTPCH catalyzes the conversion of GTP in 7,8-dihydroneopterin triphosphate, which phosphates are removed by PTPS to generate 6-pyruvoyltetrahydropterin, which will give rise to BH4 in a reaction catalyzed by SR (Werner et al., 1990) (Figure 1.7).

GTPCH is the rate-limiting enzyme of the *de novo* pathway (Levine et al., 1990), and is transcriptionally regulated by IFN-γ, IL-1β, TNF-α, nerve growth factor (NGF), H_2O_2, among others (Huber et al., 1984, D'Sa et al., 1996, Bauer et al., 2002, Franscini et al., 2003, Ishii et al., 2005). While the GTPCH activity can be stimulated up to 100 times during inflammatory conditions, PTPS and SR activity is minimally increased (Kerler et al., 1989, Werner et al., 1990, Werner-Felmayer et al., 1993a, Kaneko et al., 2003). Therefore, during inflammatory states, PTPS becomes the rate-limiting enzyme in the BH4 *de novo* biosynthesis (Werner et al., 1990, Werner-Felmayer et al., 1993b), particularly in monocytes and human macrophages, as long as these cells have low expression of PTPS (Werner et al., 1990). For that reason, the intermediate metabolite 7,8-dihydroneopterin triphosphate will be dephosphorylated by alkaline phosphatases generating 7,8-dihydroneopterin that will originate neopterin through non-enzymatic oxidation. On the other hand, the production of biopterin (BH4 highest oxidation state) will be favored in monocytes/murine macrophages or human neuronal cells, which express PTPS in larger quantities where low neopterin concentrations will be accumulated (Werner-Felmayer et al., 1993b).

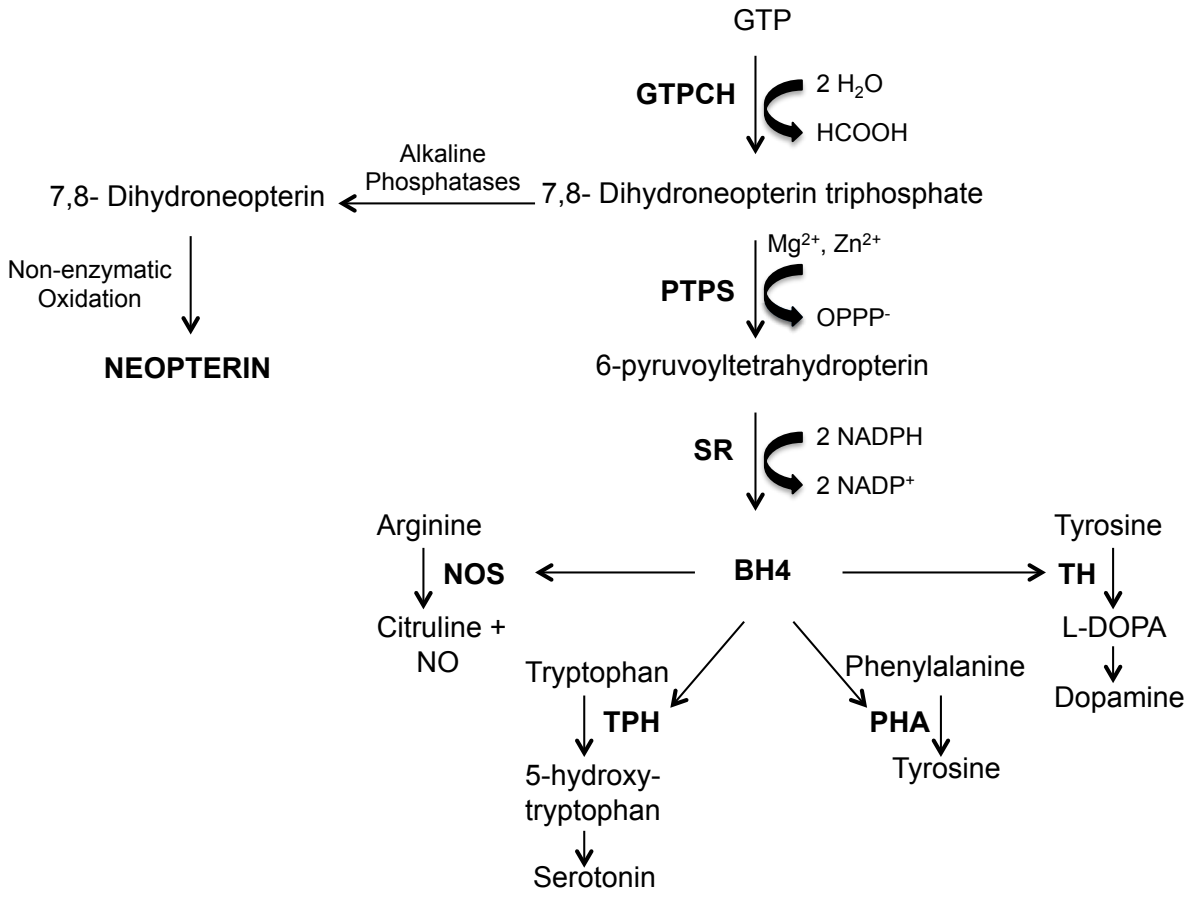


Figure 1.7. Neopterin synthesis from the BH4 *de novo* biosynthetic pathway. Neopterin is formed when the metabolic intermediate 7,8-dihydroneopterin triphosphate accumulates. This will be dephosphorylated by alkaline phosphatases generating 7,8-dihydroneopterin that, by non-enzymatic oxidation, will originate neopterin (Figure from Ghisoni et al., 2015). BH4: tetrahydrobiopterin; GTP: Guanosine triphosphate; GTPCH: guanosine triphosphate cyclohydrolase I; PTPS : 6-pyruvoyl tetrahydrobiopterin synthase; SR: sepiapterin reductase; NOS: all nitric oxide synthase isoforms; TPH: tryptophan hydroxylase; PHA: phenylalanine hydroxylase, TH: tyrosine hydroxylase; AGMO: alkylglycerol monooxygenases; NO: nitric oxide.

1.3.2 Neopterin is a peripheral biomarker of cellular immune response

Neopterin is a recognized biomarker of the immune system activation. IFN-γ, released from T helper 1 (Th1) cells activated during the initiation of the cellular immune response, is a major stimulus of neopterin synthesis in monocytes, macrophages and dendritic cells (Fuchs et al., 1989b, Cano et al., 2008, Molero-Luis et al., 2013). In addition, in order to stimulate the nitric oxide (NO) synthesis, IFN-γ also positively modulates NOS2 expression in activated immune cells (Salvemini et al., 1992), increasing NOS2 activity and requiring high cofactors levels, including the essential cofactor BH4. In consequence, GTPCH is positively modulated,

overlapping the PTPS capacity and leading to the accumulation of 7,8-dihydroneopterin triphosphate, with consequent non-enzymatic conversion to neopterin (Schoedon et al., 1987, Werner et al., 1990) (Figure 1.7).

Once produced, the neopterin levels rises in the plasma and in CSF, being, therefore, considered a sensitive and early biomarker of the immune system activation (Huber et al., 1984, Wirleitner et al., 2002). In healthy adults, it is expected to find neopterin values between 5 and 8 nmol/L in the plasma or serum and also in CSF (Hagberg, et al., 1993, Widner et al., 2002, Casal et al., 2003, Kuehne et al., 2013, Hytonen et al., 2014). These values are at least 2 to 3 times higher in pathological conditions characterized by the immune system activation. For example, the levels of neopterin reach more than 15 nmol/L in the plasma and 10 nmol/L in the CSF of untreated HIV patients (Suh et al., 2014), and around 16 nmol/L in the plasma of patients in advanced stages of Alzheimer's dementia (Wissmann et al., 2013). Moreover, the metabolite may reach approximately 500 nmol/L in the CSF in inherited metabolic diseases characterized by the deficiency of PTPS (Niederwieser et al., 1979, Blau et al., 1996, Opladen et al., 2012). Also, the neopterin concentration reaches up to 30 nmol/L in the plasma of athletes after physical exercise resistance (Lucas et al., 2014).

Several studies use the relationship between the concentrations of neopterin and tryptophan as a marker of the immune system activation in diseases characterized by inflammation (Fuchs et al., 1990, Widner et al., 2000, Lim et al., 2015). The tryptophan, an essential amino acid, can be used for protein synthesis (less than 1 %) or be metabolized into 4 metabolic pathways, being the kynurenine pathway, quantitatively, the most important. The hepatic kynurenine pathway metabolizes more than 95 % of dietary tryptophan (Bender, 1983) and contains the necessary set of enzymes for the nicotinamide and NAD^+ (nicotinamide adenine dinucleotide) synthesis and the complete oxidation of tryptophan to carbon dioxide (CO_2) and water. During inflammatory conditions, increased levels of IFN-γ positively modulate the gene expression and the activity of indoleamine-2,3-dioxygenase (IDO-1), the rate-limiting enzyme of the kynurenine biosynthetic pathway (Figure 1.8) (Takikawa et al., 1986, Dai and Gupta, 1990). There are other rate-limiting enzymes, such as tryptophan dioxygenase (TDO), expressed in the liver (Takikawa et al., 1986, Rafice et al., 2009), and IDO-2, recently described in the kidneys, liver and male and female reproductive systems (Ball et al., 2009). The kynurenine pathway represents the main pathway of tryptophan metabolism in the CNS, where IDO-1 is the rate-limiting enzyme of the pathway. In the kynurenine pathway, tryptophan is metabolized to N-formyl-L-kynurenine, which is converted to kynurenine, reaction catalyzed by formamidases or arylformamidases. Kynurenine can be catabolized by several enzymes forming neuroactive metabolites such as

quinolinic acid, kynurenic acid, and picolinic acid (Stone, 1993). The determination of the IDO-1 activity and metabolites levels of the kynurenine pathway are also indicators of the immune system activation.

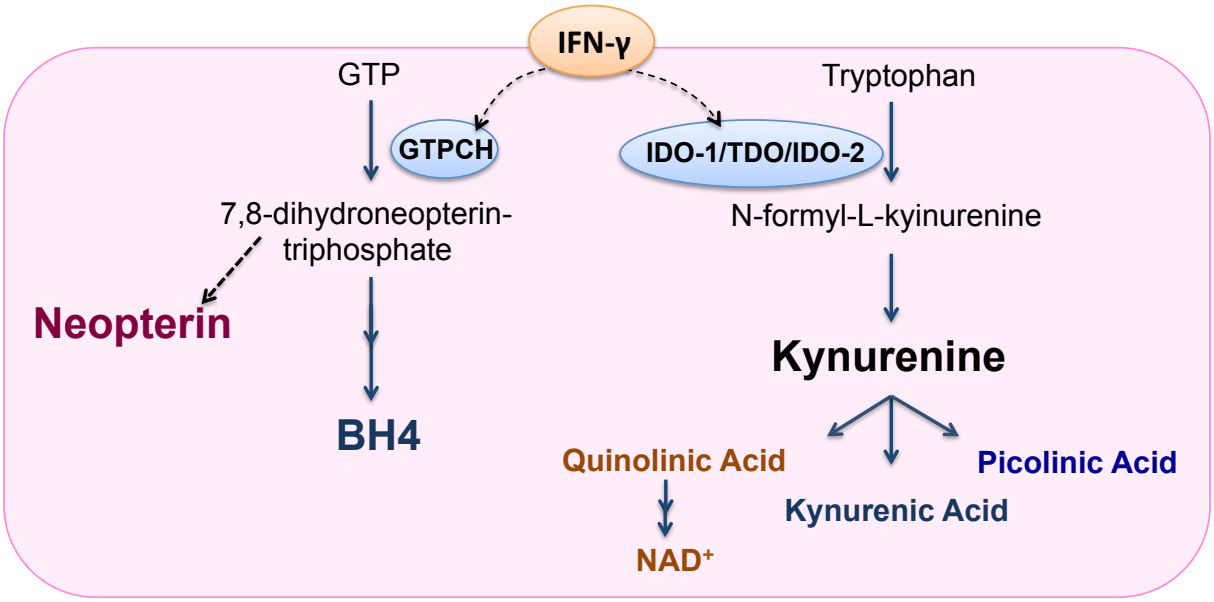


Figure 1.8. The role of IFN-γ in neopterin and kynurenine synthesis. The IFN-γ positively modulates GTPCH and IDO-1 gene transcription and activity, limiting enzymes in the BH4 *de novo* synthesis pathway and the kynurenine pathway respectively. In the kynurenine pathway, IDO-1, TDO or IDO-2 catalyze the conversion of the amino acid tryptophan to N-formyl-L-kynurenine, which is metabolized to kynurenine. This intermediate can give rise to several metabolites such as quinolinic acid, kynurenic acid, and picolinic acid. The final product of the pathway is NAD^+ synthesized from the quinolinic acid. IFN-γ: interferon-γ; GTPCH: guanosine triphosphate cyclohydrolase I; IDO: indoleamine-2,3-dioxygenase; TDO: tryptophan dioxygenase; NAD^+: nicotinamide adenine dinucleotide.

1.3.3 Neopterin production in the CNS

The source of neopterin in the CNS is still not well understood. According to the literature, neopterin could cross the BBB and its concentration in the CSF would reflect the serum or plasma levels (Fuchs et al., 1989a). Although this process occurs at a very low quotient (1:40) (Hagberg et al., 1993), the neopterin levels are higher in CSF than in plasma or serum under some conditions, such as traumatic brain injury (Lenzlinger et al., 2001) and cerebral meninges infection (Yoshida et al., 1999 Azumagawa et al., 2003), indicating that the pteridine could be produced by nervous cells. Regarding the hypothesis of CNS neopterin production, Kuehne et al. (2013) reported that the neopterin levels in the CSF is higher than the levels observed in the serum of psychiatric patients with normal BBB function (CSF 7.4 nmol/L, serum 4.9 nmol/L) and compromised BBB (CSF 8.9 nmol/L, serum 6.6 nmol/L).

Further, our research group has showed that neopterin can be produced by nervous cells under stress conditions using an experimental system based on the use of mytotoxic compounds in rodent brain tissue (Ghisoni and Latini, 2015).

Taking into consideration that astrocytes and microglia are the most abundant nervous cells in the CNS, and that both express NOS in response to IFN-γ (Salvemini et al., 1992, Zielasek et al., 1992), glial cells can be considered good candidates for the neopterin synthesis in the CNS. Furthermore, since neopterin is a byproduct of the *de novo* pathway for the biosynthesis of BH4, it is expected that BH4-dependent brain regions present relative high levels of the pteridine. In agreement, Sawada and coworkers (1987) showed that neopterin levels are higher than biopterin (the full oxidized form of BH4) content in different brain regions, including the hippocampus, amygdala, locus coeruleus, substantia nigra, raphe nucleus, accumbens nucleus, caudate nucleus and the putamen from individuals with no neurological diseases, pointing out to the local production of the molecule.

1.3.4 Biological effects of neopterin

Although there are more than three thousand publications registered in the medical literature search, *PubMed*, about the identification of this molecule in different pathological situations, the physiological role of neopterin is still virtually unknown (search on www.PubMed.com 01.18.2016) both in the CNS and in the peripheral nervous system. Although few studies have reported some extracellular effects of neopterin, as shown in Table 1.2, data are contradictory and difficult to extrapolate to human physiology, especially because, in general, the concentrations used in these scientific contributions reach levels that exceed more than two thousand times the neopterin levels in plasma or CSF from healthy subjects (Hagberg, et al., 1993, Widner et al., 2002, Casal et al., 2003, Kuehne et al., 2013, Hytonen et al., 2014). These few studies show that neopterin and its dephosphorylated precursor, 7,8-NH2, induce similar *in vitro* effects, such as the translocation of NF-kB, intracellular Ca^{2+} increase, ROS production, high expression of proto-oncogenes, apoptosis, and reduced cell viability in different rodent and human cells (Tables 1.2 and 1.3). Nevertheless, when neural cells were exposed to the metabolites, 7,8-NH2 exerted toxic action in concentrations from 400 nM to 5 mM, in which neopterin was protective or had no effect (Tables 1.2 and 1.3). It should be reinforced that there are no reports of 7,8-NH2 in biological fluids, even in situations where the pathway is completely impaired, as PTPS deficiency, that is, the accumulation of 7,8-NH2 appears not to occur *in vivo* (Leuzzi et al., 2010).

Table 1.2. Neopterin extracellular effects in *in vitro* cellular systems

Species	Cell type	Treatment	Effect
Rodents	Vascular smooth muscle cells	20 μM; 2 hours	Up-regulation of nuclear factor kappa B (NF-ϰB). Increased of inducible nitric oxide synthase (NOS2) . (Hoffmann et al., 1996).
	Alveolar epithelial cell line	1 - 1000 μM; 24 hours	Induced apoptosis (Schobersberger et al., 1996).
		1 - 1000 μM; 6 - 24 hours	Induced intercellular adhesion molecule 1 (ICAM-1) expression (Hoffmann et al., 1999).
		1 - 1000 μM; 1 hour	Upregulation of NF-ϰB (Hoffmann et al., 1999).
		100 nM; 10 min	Inhibited ATP-induced calcium release (Hoffmann et al., 2002).
	Embryonic fibroblast cell line	400 nM; 18 hours	Induced *c-fos* gene expression in the presence of cGMP (Uberall et al., 1994).
Humans	Myelonocytic cell tine THP-1	1 μM; 1 min	Increased intracellular Ca^{2+} concentration (Woll et al., 1993).
	Coronary artery endothelial cells	10 - 100 nM; 30 min	Upregulation of NF-ϰB (Cirillo et al., 2006).
	Primary neutrophils	400 μM; 3 min	Inhibited singlet oxygen release induced by opsonized zymosan (Mori et al., 2010).
	U937 - Histiocytic lymphoma cell line	0.5 - 1000 μM; 24 hours	Induced programmed cell death (Baier-Bitterlich et al., 1995).
	Astroglioma U373MG cell line	1 mM; 2 or 5 days	Neopterin did not alter morphology and cell proliferation (Speth et al., 2000).
	Microglial CHME cell line	0.1 - 1 mM; 5 days	Neopterin did not alter cell proliferation (Speth et al., 2000).
	SK-N-SH - neural cell line	0.1 - 1 mM; 5 days	Neopterin did not alter cell proliferation (Speth et al., 2000).

Table adapted from Ghisoni et al, 2015.

Table 1.3. Comparison of the neopterin *in vitro* extracellular effects (NEO) and 7,8-dihydroneopterin (7,8-NH2) in different cell systems

Pterine-induced effect	NEO	7,8-NH2
Induced apoptosis in bacteria (200 μM; 24 h) (Weiss et al., 1993).	+	-
Apoptosis in rat alveolar epithelial (200μM; 24h) (Schobersberger et al., 1996).	+	+
Apoptosis in pheochromocytoma cells (PC12) (5 and 1mM; 48 h) (Enzinger et al., 2002).	-	+
Compromised viability and cell morphology alteration in astrocytic (U373MG), microglial (CHME) and neuronal (SK-N-SH) cell lines (0.1 to 5 mM; 5 days) (Speth et al., 2000).	-	+
Induction of genes related to cell growth in fibroblasts (NIH3T3) (NEO or 7,8-NH2 400 nM in combination with cyclic guanosine monophosphate (cGMP) 20 μM) (Uberall et al., 1994).	+	+

Table adapted from Ghisoni et al, 2015.

Despite the evidence listed in Table 1.2, and the known role as a peripheral biomarker of inflammatory processes, data from our research group have suggested that neopterin can exert neuroprotective effects (Ghisoni and Latini, 2015). Recently, it was demonstrated that

neopterin increases resistance to oxidative stress in the mice cerebral cortex (Ghisoni and Latini, 2015), in addition to facilitate the acquisition of aversive memory, the generation of hippocampal LTP and anti-inflammatory action when intracerebroventricularly administered (Ghisoni et al., 2016), mainly in the dose of 4 picomol (pmol) which would generate a concentration of approximately 150 nM of neopterin in the CSF.

The increased resistance to oxidative stress induced by neopterin administration was characterized by reduced lipid peroxidation and increased total thiol content, glutathione levels, and activities of the two main glutathione-metabolizing enzymes, glutathione peroxidase and glutathione reductase in the mouse brain (Ghisoni and Latini, 2015). The transcription of these antioxidant enzymes is dependent on the activation of the antioxidant responsive element (ARE), common region in the promoter regions in genes that encode antioxidant enzymes, which is principally regulated by the binding of transcription factors, such as the nuclear factor erythroid 2-related factor 2 (Nrf2) (Shih et al., 2003). Under physiological conditions, inactivated Nrf2 is bound to Keap1 (Kelch-like ECH-associated protein 1) in the cytoplasm. On the other hand, under oxidative stress, Nrf2 is released by Keap1 and translocated to the nucleus, where it associates with a member of the Maf family protein, forming a dimer (Figure 1.9) (Itoh et al., 2003). Besides GR and GPx, the ARE activation regulates the transcription of other antioxidant enzymes such as heme oxygenase-1 (HO-1), and stage II detoxification enzymes as glutathione transferases (Prestera et al., 1995).

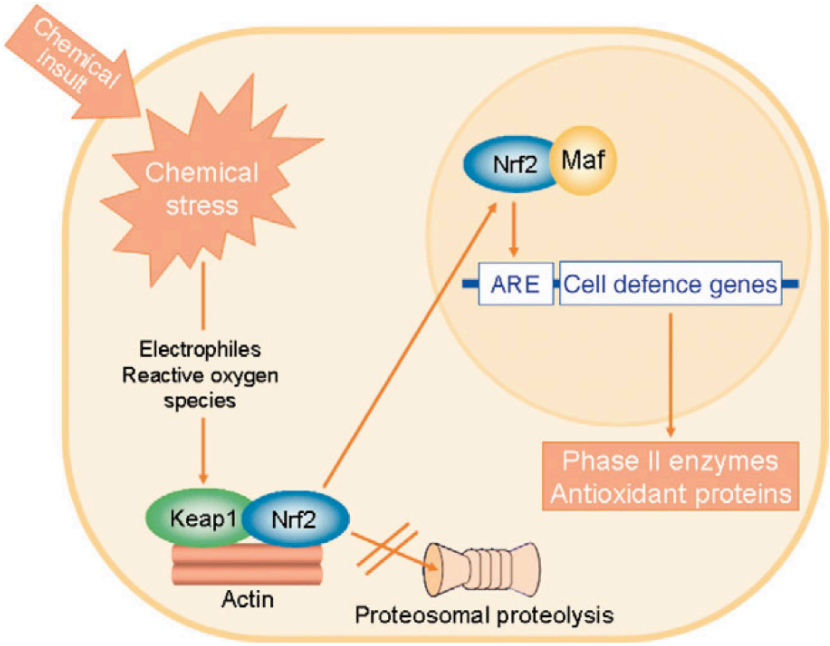


Figure 1.9. The role of the Nrf2-Keap1-ARE system in the regulation of the antioxidant response. The reactive species generated during the electrophilic stress oxidize the cysteine residue essential for the Nrf2-Keap1 complex binding, preventing its proteasome degradation. The free Nrf2 translocates to the nucleus where it forms a dimer with Maf family proteins, activating the ARE region and the transcription of antioxidant enzymes (Figure from Goldring et al., 2006). ROS: reactive oxygen species; RNS: reactive nitrogen species; Keap1: Kelch-like ECH-associated protein 1; Nrf2: nuclear factor erythroid 2-related factor-2; ARE: antioxidant responsive element.

Thus, neopterin seems to have a dual role, that is, high concentrations would be associated with a pro-toxic role (as shown in Table 1.2), while concentrations slightly above baseline concentrations, would exert a neuroprotective role, as has been described for numerous biomolecules, e.g., IL-6 (Li et al., 2009b), IL-1β (Lampa et al., 2012), dopamine (Filloux and Townsend, 1993), among others.

Diseases characterized by neuroinflammation typically present high concentrations of neopterin and cytokines in biological fluids (Azumagawa et al., 2003, Parker et al., 2013). However, the role of neopterin production in these diseases is still unknown. Cytokines, besides serving as immune regulators, are essential to paracrine and autocrine signaling in neural and non-neural nervous cells that are part of the adult or in development CNS. Thus, alterations in the immune regulation, such as maternal infections can affect normal developmental processes (for review see Goines and Ashwood, 2013). Therefore, studying the biology, functions and relationship of neopterin with other inflammatory mediators in the CNS may help in understanding pathological mechanisms in developmental disorders such as autism.

1.4. Autism Spectrum Disorder

The term autism spectrum disorder (ASD) comprises a group of developmental disorders, which are associated with impairments in emotional function and/or social interaction, abnormalities in oral communication and repetitive behavior (APA, 2014). The ASD term includes autism, Asperger syndrome and pervasive developmental disorder - not otherwise specified. In general, autism symptoms start at the age of 6 months old, settling between 2 and 3 years and tend to remain during life (for review see Rapin and Tuchman, 2008). According to the World Health Organization (2013), the prevalence of ASD globally corresponds to 1 in every 160 children. Additionally, it is more prevalent in men, with a ratio of prevalence men: women 4.1: 1 (Fombonne, 2009). About 70 % of autistic individuals do

not present physical abnormalities, but in 30 % of cases, autism is associated with dysmorphic features as microencephaly and/or malformation of the brain structure (Miles et al., 2005). In 5-10 % of the cases, autism is diagnosed in association with other disorders such as Fragile X syndrome, Rett and Down (Rutter et al., 1994).

In recent years, advances in research on the neurobiological basis of ASD has presented some neuropathological evidences related to autism, such as accelerated and abnormal brain growth in early childhood (Redcay and Courchesne, 2005), restricted neuronal development with evidence of abnormalities in brain cytoarchitecture (Bailey et al., 1998, van Kooten et al., 2008, Wegiel et al., 2010), metabolic changes with abnormal processing of amyloid precursor protein (Bailey et al., 2008), oxidative stress induction (James et al., 2009), and activation of glial cells (Vargas et al., 2005).

Although the etiology of ASD is not well defined, it appears to involve genetic predisposition and environmental factors. The relevance of genetic predisposition to the development of ASD is a much discussed topic. Some studies evaluating ASD patients and their families show that individuals with normal development present immune alterations similar to siblings diagnosed with ASD (Saresella et al., 2009, Napolioni et al., 2013), suggesting that heredity contributes to the disease development. On the other hand, genomic studies have shown that children diagnosed with ASD present *de novo* mutations (not inherited) not observed in the DNA of the parents, unaffected siblings, or controls (Miller et al., 2009, Krumm et al., 2015, Tammimies et al., 2015), suggesting that these alterations are due to environmental factors.

Among the environmental factors able to hinder the CNS development, it can be mentioned exposure to heavy metals, drug abuse, and prenatal infections (Atladottir et al., 2010, Hashimoto-Torii et al., 2011). In particular, evidences show a strong correlation between ASD and maternal viral infections during the first trimester of pregnancy (Atladottir et al., 2010), when the BBB is not yet formed. The ASD development has also been associated with familial autoimmune diseases (Comi et al., 1999) and the presence of autoantibodies against essential proteins in the homeostasis of the CNS, such as the myelin basic protein, a constituent of myelin sheath which surrounds and protects the axons (Mostafa and Al-Ayadhi, 2011). Thus, the immune response and inflammation appear to be key components in the pathogenesis of this syndrome.

Studies have already shown that patients with ASD exhibit immune alterations such as natural killer cells dysfunction (Enstrom et al., 2009) and an increase in the number of T cells, which can be reflected in elevated concentrations of cytokines in the patients plasma or serum and in the nervous tissue (Harrison and Pheasant, 1995, Vargas et al., 2005, Li et al., 2009a,

Ashwood et al., 2011). Moreover, the activation of microglial and astrocytic cells has been described in some brain regions of patients diagnosed with ASD, such as cortex and cerebellum (Vargas et al., 2005). The alterations in the inflammatory response can be observed in young and adult patients (5-44 years). In general, ASD patients have elevated concentrations of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, and reduced concentration of anti-inflammatory cytokines such as IL-10 and TGF-β (transforming growth factor β) in the nervous tissue, CSF, serum or plasma (Jyonouchi et al., 2001, Vargas et al., 2005, Li et al., 2009a, Ashwood et al., 2011). Some studies have also evaluated the ability of monocytic cells of ASD patients to produce cytokines towards pro-inflammatory stimuli. Jyonouchi et al. (2014) reported that monocytic cells of patients with more severe symptoms produce higher amounts of cytokines, mainly IL-1β and IL-6 when stimulated with agonists for receptors TLR1, 2, 4, 6, 7 and 8. However, not all studies show a consistent pattern according to the concentration of cytokines, which may vary depending on the severity of symptoms and association with other pathologies, e.g., gastrointestinal tract impairment, very common in patients with autism (Napolioni et al., 2013).

Taking into consideration that the inflammatory response participates in the ASD pathophysiology, the measurement of neopterin can be a reliable biomarker of the immune system activation. Nonetheless, few studies have investigated the concentration of neopterin as a biomarker in patients with autism (Messahel et al., 1998, Sweeten et al., 2003, Zimmerman et al., 2005). Some studies have already reported elevated neopterin levels in urine, plasma and CSF from ASD patients (Harrison and Pheasant, 1995, Messahel et al., 1998, Sweten et al., 2003), while others have not found differences in neopterin levels in comparison with healthy controls (Eto et al., 1992).

Besides the elevated serum and central levels of IL-1β in ASD patients, some evidences suggest that the inflammasome activation is involved in the pathogenesis of the disease. For example, mononuclear cells from ASD patients peripheral blood present a higher gene expression of caspases including inflammasome-dependent caspase-1 (Siniscalco et al., 2012). Furthermore, a recent study has shown that genetic variations in *Nlrp5*, essential gene for embryonic development, are associated with developmental syndromes, including autism (Docherty et al., 2015). Initially, it was believed that *Nlrp5* was exclusively expressed in human and murine monocytes (Tong et al., 2002, Tong et al., 2004), but, recently, it has been shown that this receptor is also expressed in human brain endothelial cell line (Nagyoszi et al., 2015). So far, it is unclear whether the *Nlrp5* receptor is able to form functional inflammasomes, but it can be suggested that this receptor exerts a role in the CNS homeostasis and is related to the ASD pathogenesis.

As mentioned before, neopterin has properties of cognitive enhancer, anti-inflammatory and antioxidant activities. As a consequence, we hypothesized that neopterin production in the CNS during inflammatory conditions would represent a protective mechanism for neighboring cells to processes with active inflammation, whether acute or chronic, by negatively modulating the inflammasome activation.

Chapter 2
Research objectives

2.1 Problem statement

The inflammasome activation is essential for host defense; however, several studies have suggested it may be a key determinant in the pathogenesis of neuroinflammatory conditions. Inflammasomes are macromolecular protein complex responsible for the activation of the pro-inflammatory cytokines IL-1β and IL-18 via caspase-1. ROS have been suggested to be critical for inflammasome activation triggering. Neopterin is an immune system biomarker commonly observed in a large proportion of neurodegenerative diseases patients. It is a metabolite of the *de novo* BH4 pathway, mainly produced by macrophages and monocytes under an inflammatory stimulus and has been considered an inert metabolite. It has been recently proposed that CSF neopterin levels may not correlate with plasma levels, suggesting that neopterin may be centrally produced. In addition, our research group has recently shown that neopterin improves the resistance to oxidative stress in mice brain. Nevertheless, the neopterin role is virtually unknown. In order to better understand neopterin biology and its role in physiology and pathology, it is necessary to further elucidate the following questions: (1) Is neopterin produced in the CNS under inflammatory stimulus? (2) Does neopterin promote cytoprotective responses *in vitro*? If the response is affirmative in these cases, then (3) Does neopterin affect inflammasome activation *in vitro*? (4) Is neopterin an immune system activation biomaker in ASD?

2.2 Main Hypothesis

In response to the questions raised in the problem statement, we hypothesize that inflammatory stimulus induce neopterin production and inflammasome activation in the CNS. In addition, neopterin synthesis may be a protective response in order to control oxidative stress and inflammasome activation in acute and chronic inflammatory conditions.

2.3 Specific Aims

We first focused on neopterin production in the periphery and CNS and its relation to inflammasome activation under inflammatory stimulus. We then studied neopterin cytoprotective effects on mitotoxic conditions. In addition, we evaluated the neopterin role in inflammasome activation. Finally, we analysed neopterin and inflammatory mediators' production in ASD.

AIM 1: Is neopterin produced in the CNS under acute neuroinflammatory stimulus?

Neopterin is used as a biomarker in the CSF, plasma, and urine; however, recent studies suggest that CSF neopterin levels may not correlate with plasma levels. In addition, our group recently showed that hippocampal slices release neopterin under cellular stress. Our aim was to study the peripheral and central neopterin production. We have used adult Swiss mice to determine whether neopterin is produced in the CNS under an acute inflammatory stimulus and rat primary astrocytes to analyse this cell type contribution to neopterin synthesis under cellular stress induced by azide-linked mitotoxicity.

AIM 2: Does neopterin promote cytoprotective responses *in vitro*?

High neopterin levels are generally associated with neurological diseases and have been associated with oxidative stress and inflammation *in vitro*. On the other hand, it was recently shown that neopterin improves the resistance to oxidative stress in mice cerebral cortex. Our aim was to study the potential cytoprotective roles of exogenous neopterin in oxidative stress and mitochondrial dysfunction induced by azide-linked mitotoxicity. This will aid in elucidating neopterin production role during cellular stress. To achieve this goal, we used rat primary astrocytes.

AIM 3: To investigate whether neopterin affects inflammasome activation *in vitro*.

Inflammasome activation has been identified as an important feature in the pathogenesis of many neurodegenerative diseases in which neopterin has been used as a biomarker. Neopterin has been considered an inert compound and whether its production can exert any effects on neuroinflammatory conditions is still unclear. Our aim was to investigate if neopterin pre-conditioning could affect LPS-induced inflammasome activation in human primary astrocytes.

AIM 4: To analyse neopterin and inflammatory mediators secretion in ASD patients.

We showed, with the completion of the previous aims, that neopterin central production may exert beneficial effects inhibiting oxidative stress and inflammasome activation in nervous cells. Thus, this aim is to evaluate neopterin and inflammatory mediators in a chronic inflammatory disorder, ASD. To investigate this objective, we used serum from ASD patients and healthy controls.

2.4 Research approach

To achieve the purpose of the thesis the chapters are as outlined:

Chapter 1 summarises the literature regarding neuroinflammation and NLRP3 inflammasome activation focusing on the CNS. In addition, it reviews neopterin synthesis, its use as a biomarker and suggested effects. Moreover, it is reviewed key information about ASD pathogenesis and neopterin. Therefore, giving a better understanding on how inflammasome is activated and how neopterin could affect its activation.

Chapter 3 describes the general methods used on this study.

Chapter 4 portrays the results obtained during the PhD. It is divided into three sections:

Section 4.1. studies neopterin production in the CNS under inflammatory stimulus and neopterin production and potential cytoprotective effects in rat primary astrocytes.

Section 4.2. analyses neopterin effects on LPS-induced inflammasome activation in human primary astrocytes and neurons.

Section 4.3. investigates neopterin and inflammatory cytokines production in the serum of ASD patients.

Chapter 5 discusses the results of the same sections organized on chapter 4.

Chapter 6 states the overall conclusions.

Chapter 3
Material and Methods

3.1 *In vivo* Experiments

3.1.1. Animals

Adult male Swiss albino mice were obtained from the Central Animal House of the Federal University of Santa Catarina, Florianopolis-SC, Brazil. The animals were maintained in a 12-h light/dark cycle in a constant temperature (22 ± 1 °C) colony room, with free access to water and protein commercial chow (Nuvital-PR, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research (PP00760/CEUA) of the Federal University of Santa Catarina, Florianopolis-SC, Brazil, and followed the Guide for Care and Use of Laboratory Animals (8^{th}), adopted by the National Institutes of Health in January 2012 (available in http://www.nap.edu). All efforts were made to minimize the number of animals used and their suffering.

3.1.2. Neuroinflammation model induced by LPS

The experimental protocol used 50 animals, 25 Swiss mice for experiment 1 and 25 Swiss mice for experiment 2. In each experiment, the animals were randomly divided in five experimental groups: control (N=5), LPS 5 min (N=5), LPS 15 min (N=5), LPS 30 min (N=5) and LPS 60 min (N=5). The animals were intraperitoneally injected with saline or Escherichia coli lipopolysaccharide (LPS; 0.33 mg/kg; serotype 0111:B4, Sigma, St. Louis, MO) and were euthanized by decapitation after 0, 5, 15, 30 or 60 min. LPS was diluted in saline.

Control animals received vehicle injections (saline; i.p.). The LPS dosage was selected because of the already known pro-inflammatory cytokine response in the adult mice brain (Berg et al., 2004, Henry et al., 2009). After euthanasia, mice blood was collected and centrifuged at 3,000 x g for 5 min to obtain the serum. In addition, the brain was dissected to collect the hippocampus. Samples were stored at -80 °C for subsequent analyses of neopterin levels, Gch1 and IL1B gene expression (GTPCH and pro-IL-1β proteins, respectively) and IL- 1β and pro-caspase-1 content.

3.2 *In vitro* Experiments

3.2.1. Rat cortical primary astrocytes

Primary astrocytes culture were prepared from the cerebral cortex collected from Wistar rats 1 – 2 days old, according to Olivera et al. (2008). All procedures performed were previously approved by the Ethics Committee for Animal Research (PP00425/CEUA) of the Federal University of Santa Catarina, Florianopolis-SC, Brazil. Astrocytes were seeded in 24-well plates (2 x 10^4 cells/well) and maintained in Dulbecco's modified Eagle's medium (DMEM) medium containing 10 % heat-inactivated Fetal bovine serum (FBS), 3 g/L HEPES, 100 UI/mL penicillin and 100 µg/mL streptomycin, in a 95 % O_2 and 5 % CO_2 humidified atmosphere, at 37 °C. Purity of astrocyte preparations was checked with a GFAP (glial fibrilar acid protein) antibody and was above 95 %. When cells achieved confluence, the culture medium was removed by suction and the astrocytes were exposed to sodium azide (5 mM) for 3 h and the supernatant was collected for neopterin measurement. In addition, the cells were treated with azide and/ or neopterin (50 and 500 nM) for 3 h to evaluate lactate release in the supernatant, ROS production and HO-1 content in the cells.

3.2.2. Maintenance and treatment of C6 cell line

The astroglioma C6 cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). The cells were seeded in flasks and cultured in DMEM (pH 7.4) containing 5 % FBS, sterile antimycotic solution 100x: 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin, in a 95 % O_2 and 5 % CO_2 humidified atmosphere, at 37 °C. Exponentially growing cells were detached from the culture flasks using 0.05 % trypsin/ethylene-diaminetetracetic acid (EDTA) and seeded in 24-well plates

(10 x 10^3 cells/well). After cells reached confluence, the culture medium was removed by suction and the cells were treated with neopterin (0 - 5000 nM) for 1, 3 and 24 h when cell viability was evaluated through MTT reduction method. Also, the cells were treated with neopterin (0 and 50 nM) for 3 h or for 0.5 to 4 h to evaluate, respectively, Nrf2 content by flow cytometer or Nrf2 activation by Western blot. In addition, the cells were seeded in Petri dishes (10 x 10^5 cells/dish) and exposed to LPS 1 and 5 μg/mL for 24 h. Then, the supernatant and the cell lysate were collected and prepared to analyse caspase-1 content.

3.2.3. Maintenance and treatment of Neuro2a cell line

The murine neuroblastoma cell line, Neuro2a, was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were cultured in flasks in DMEM supplemented with 10 % FCS, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 UI/mL penicillin, and 0.1 mg/mL streptomycin in a 95 % O_2 and 5 % CO_2 humidified atmosphere, at 37 °C. To induce neurite outgrowth, cells were plated at a 1.8 x 10^4 cells/cm^2 and, 48 h after seeding, the medium was replaced with 2% FCS-DMEM containing 20 μM retinoic acid. After 48 h, the cells were treated with neopterin (0 - 5000 nM) for 1, 3 and 24 h when cell viability was evaluated through MTT reduction method.

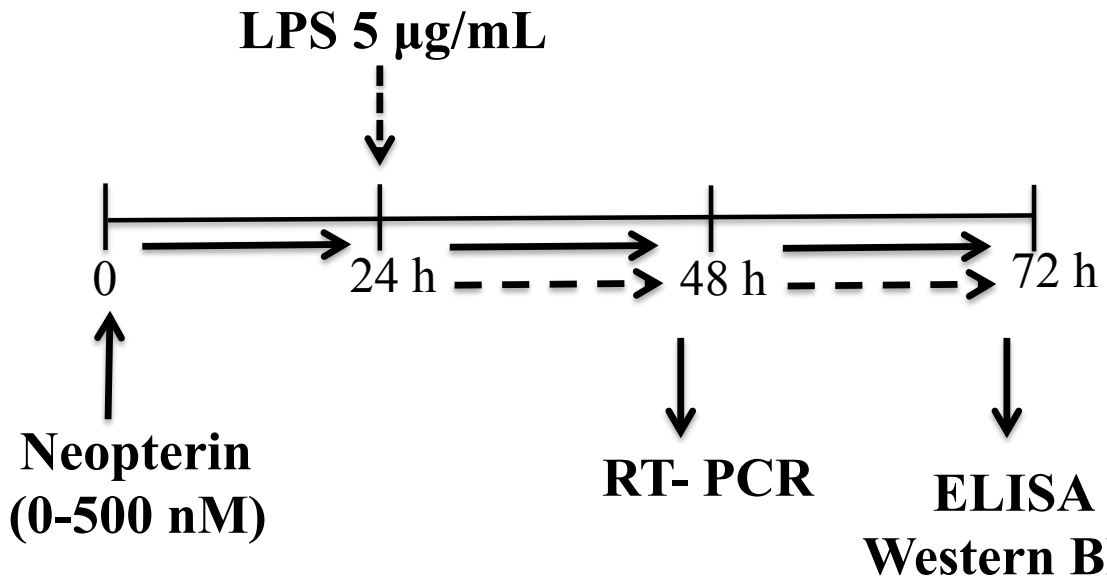
3.2.4. Human nervous cells

3.2.4.1. Human primary astrocytes

Human primary astrocytes were obtained from 16–19 week old fetuses collected following therapeutic termination with informed consent. All experiments followed the ethical standards in accordance with the Australian National Statement on Ethical Conduct in Human Research (2007) (HREC Application REF 5201300330). Primary astrocytes culture was prepared from the mixed brain cell cultures, and maintained using a protocol previously described by our group (Guillemin et al., 2005). Cerebral portions were washed with Phosphate Buffer Saline (PBS) and dissociated by repeated pipettings.

The suspension was centrifuged at 500 *x g* for 5 min and the cell pellet suspended again in RPMI 1640 medium containing 10 % heat-inactivated FBS, 1 % Glutamax-1, 1 % antibiotic-antimicrobial solution and 0.5 % glucose. Then, the cells were plated onto 75 cm^2

culture flasks and incubated at 37 °C. The medium was changed on the 3rd, 5th and 10th day. Microglia was detached from the cultures by mechanically shaking the flasks for 2 h at 220 rpm at room temperature. The astrocytes were trypsinized and plated again at least three times to further purify and isolate astrocytes from contaminating microglia and neurons. Astrocytes were left to recover for 3 days after each passage. Cell cultures at 3–4 weeks were used in all the experiments. Exponentially growing cells were detached from the culture flasks using 0.05 % trypsin/EDTA and seeded in 6-well plates (1 x 10^5 cells/well). Initially, the cells were treated with LPS 5 µg/mL for 6, 12 and 24 h and collected for *IL1B* and *CASP1* gene expression analysis (pro-IL-1β and pro-caspase-1 proteins, respectively). In subsequent experiments, the cells were preconditioned with neopterin (5-500 nM) for 24 h and then stimulated with LPS 5 µg/mL without removing the neopterin (Scheme 3.1). After 24 h of co-exposure to neopterin and LPS, the cells were collected for *IL1B* and *CASP1* gene expression analysis. After 48 h of neopterin and LPS co-treatment, the cells were collected to analyse pro-caspase-1 and ASC content and the supernatant used to measure cytokine levels.



Scheme 3.1. Schematic representation of the *in vitro* experimental protocol used to evaluate neopterin preconditioning effects on LPS-induced inflammasome activation in human primary nervous cells.

3.2.4.2. Human primary neurons

The neuronal cell culture was prepared from the same tissue used for the astrocytes culture using a protocol adapted from Kerr et al. (1998). Neurons were cultured by plating the mixed cell suspension in culture dishes coated with Matrigel (1:20 in Neurobasal) and maintained in Neurobasal medium supplemented with 1 % B-27 supplement, 2 mM Glutamax, 1x antibiotic-antimycotic, 5 mM HEPES and 2 mM glucose for 7-10 days at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was subsequently changed 2 days after plating and then every 3 days thereafter (Guillemin et al., 2004). Cells were treated according to Scheme 3.1, but the samples were exclusively used to analyse *CASP1* gene expression.

3.3 Participants on the Autism Spectrum Disorder Study

Blood samples were collected from Omani Children between 3–13 years old and their age-matched newly diagnosed children with ASD recruited from 15 different families from the pediatric clinic in Sultan Qaboos University Hospital. The study followed the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects. Written consent was obtained from the parents, according to the guidelines and approval of the Ethical Committee of Sultan Qaboos University, Oman (EC 158/2010). Participants were initially screened and excluded based on history of tuberous sclerosis, Fragile X disorder, phenylketonuria, Lesch–Nyhan syndrome, foetal alcohol syndrome, middle ear infection, or a history of maternal illicit drug use. Other demographic data are shown in Table 3.1. None of the participants had any changes in diet or treatment (if any) prior to commencing the study.

Diagnosis of ASD in children were in accordance with the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (APA, 2000), and confirmed by completion of a standardized and validated Arabic version of the Childhood Autism Rating Scale (CARS) questionnaire. A CARS scores greater than 30 was used to confirm all ASD diagnoses. After an overnight fast, blood samples from both the ASD cohort and healthy control children were collected at the Sultan Qaboos University Hospital and stored at -80 °C until biochemical analysis. All biochemical analyses were conducted at Macquarie University, Faculty of Medicine and Health Sciences in compliance with institutional approval for the use of human biomaterials (HREC 5201300330). These analyses included neopterin and cytokines measurement.

Table 3.1. Participant Demographics

	Healthy controls	Autism
N	12	15
Gender (female/male)	2/10	5/10
Age (mean ± SEM), years (range)	9.61 ± 2.9 (4 - 13)	8.47 ± 2.36 (3 – 10)
Childhood Autism Rating Scale (CARS) (mean ± SEM)	17.44 ± 2.89	36.19 ± 4.51

Lim et al., 2016.

3.4. Biochemical parameters

3.4.1. Analysis of neopterin levels

3.4.1.1. Preparation and analysis of samples from the in vivo and in vitro experiments

Mice serum and cell culture supernatant samples were precipitated by adding one volume (1:1, v/v) of trichloroacetic acid (TCA) 5 % and the hippocampus was homogenized in 100 µL of the same solution (1:5 dilution, p/v). Samples were centrifuged at 14,000 *x g* for 10 min at 4 °C. Extracted samples (20 µL) were transferred to a high performance liquid chromatography (HPLC) (Alliance e2695, Waters, Milford, USA) vial for analysis and injected into a SUPELCOSIL^{TM} LC-18T reversed phase, 4.6 x 150 mm column with pore size of 5 µm. The mobile phase consisted of 15 mM potassium phosphate buffer pH 6.4 and was delivered at 0.7 mL/min. The identification and quantification of neopterin was performed by a multi-wavelength fluorescence detector (module 2475, Waters, Milford, USA) with excitation wavelength of 350 nm and emission of 430 nm (Adapted from Lucas et al., 2014). Under these conditions the retention time of neopterin was approximately 4.68 min, with a total running time of 8 min. The results were expressed in nmol/L for serum and pmol/mg protein for tissue.

Blood neopterin levels assessed by HPLC were confirmed by enzyme-linked immunosorbent assay (ELISA) by using a commercial kit (IBL international GmbH, Hamburg, Germany), according to the manufacturer's instructions. Briefly, the levels of neopterin were estimated by interpolation from a standard curve by colorimetric

measurements at 450 nm on an ELISA plate reader (Infinite® 200 PRO TECAN, Männedorf, Switzerland). Results were expressed in nmol/L.

3.4.1.2. Preparation and analysis of samples from the participants of the ASD study

Children serum samples were precipitated by adding one volume (1:1, v/v) of TCA 10% and filtered through a 0.22 μm PTFE syringe filter (Merck-Millipore, CA, USA) in accordance with previous studies (Guillemin et al., 2007). Samples were centrifuged at 14,000 *x g* for 10 min at 4° C. Extracted samples (20 μL) were transferred to HPLC (Agilent Technologies, CA, USA) vial for analysis and injected into a Poroshell® C18 reversed phase, 2.1 x 150 mm column with pore size of 1.8 μm. The mobile phase consisted of 0.1 M sodium acetate buffer pH 4.65 and was delivered at 0.75 mL/min. The identification and quantification of neopterin was performed by a multi-wavelength fluorescence detector (G1321B, Agilent Technologies, CA, USA) with excitation wavelength of 370 nm and emission of 438 nm (Adapted from Lim et al., 2013). Under these conditions the retention time of neopterin was approximately 5 min, with a total running time of 8 min. The results were expressed in nmol/L.

3.4.2. Analysis of Nrf2, IL-1β, caspase-1 and ASC content

3.4.2.1. Preparation and analysis of samples from the in vivo and in vitro (C6 cells) experiments

The hippocampi were homogenized in 2.5 volumes (1:2.5, p/v) of 50 mM Tris, pH 7.0, containing 1 mM EDTA, 100 mM sodium fluoride (NaF), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium orthovanadate (Na_3VO_4), 1 % Triton X- 100, 10 % glycerol and protease inhibitor cocktail (Roche, Mannheim, Germany). Then, the samples were centrifuged at 10,000 x *g* for 10 min at 4 °C and the supernatant was collected and diluted in one volume (1:1, v/v) of 100 mM Tris buffer pH 6.8 containing 4 mM EDTA and 8 % sodium dodecyl sulphate (SDS). The samples were boiled at 100 °C for 5 min. The protein content was quantified by Lowry's method (1951). Samples were diluted in 25 % 100 mM Tris Buffer (pH 6.8, with 40 % glycerol and bromophenol blue) and 8 % β-mercaptoethanol.

For caspase-1 detection, supernatants and cell lysates of C6 astroglioma cell line were collected after 24 h of LPS exposure. To investigate the translocation of Nrf2 induced by neopterin, C6 astroglioma cell line were collected after 0.5 to 4 h of neopterin treatment and

cytosolic and nuclear fractions were prepared. Cell culture supernatants were precipitated by the addition of an equal volume of methanol and 0.25 volumes of chloroform, followed by vortexing and centrifugation for 10 min at 20,000 *x g*. The upper phase was discarded and 500 µL methanol was added to the interphase. This mixture was centrifuged for 10 min at 20,000 x *g* and the protein pellet was dried at 55 °C, suspended in 100 mM Tris Buffer and boiled for 5 min at 99 °C. Cell lysates were prepared in 50 mM Tris, pH 7.0, containing 1 mM EDTA, 100 mM NaF, 0.1 mM PMSF, 2 mM Na_3VO_4, 1 % Triton X- 100, 10 % glycerol and protease inhibitor cocktail (Roche, Mannheim, Germany). Supernatants obtained after centrifugation (12,000 *x g*, 10 min; 4 ºC) were recovered, and the protein content was quantified by Lowry's method (1951). Samples were diluted in 25 % 100 mM Tris Buffer pH 6.8 and 8 % β-mercaptoethanol.

An aliquot of 50 µg of total protein or 10 µL of serum was size-separated by electrophoresis in 15 % SDS–polyacrylamide gel, under reducing conditions, and transferred to a nitrocellulose membrane. After washing and blocking the membranes were incubated overnight with anti-caspase-1 (1:5,000; Cell Signalling; Danvers, USA), anti-IL-1β (1:1,000; Cell Signalling; Danvers, USA) and anti-Nrf2 (1:1,000; Cell signalling; Danvers, USA) primary antibodies. Afterwards, membranes were exposed to the anti-rabbit (1:2,000; Cell Signalling; Danvers, USA) or anti-mouse (1:4,000; Cell Signalling; Danvers, USA) secondary antibodies. The immunocomplexes were visualized using the ECL chemiluminescence detection system (GE Healthcare, São Paulo, SP, Brazil). Protein bands were detected in a ChemiDoc XRS detection system (Bio-Rad) driven by Quantity One software. Membranes were stained with Pounceau S in order to verify loading evenness.

3.4.2.2. Preparation and analysis of samples from human primary astrocytes

Cell lysates of human primary astrocytes were collected after 48 h of LPS exposure. Cell lysates were prepared in radioimmunoprecipitation assay lysis buffer (RIPA) containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % Nonidet P-40 and 50 µL Protease inhibitor cocktail (Roche, Mannheim, Germany). Supernatants obtained after centrifugation (12,000 x *g*, 10 min; 4 ºC) were recovered, and the protein content was quantified by using the BCA Protein Assay kit (Thermo Scientific; Waltham, USA). Samples were diluted in 25 % Laemmni buffer (Bio-Rad; Hercules, USA) and 8 % β-mercaptoethanol.

An aliquot of 15 µg of total protein was size-separated by electrophoresis in 4-15 % Mini-Protean® TGXTM gel (Bio-Rad; Hercules, USA). Proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad; Hercules, USA) equilibrated in blotting

buffer (Bio-Rad; Hercules, USA), using the Trans-Blot Turbo system (Bio-Rad; Hercules, USA) at 25 V for 7 min. After washing and blocking, the membranes were incubated overnight with anti-ASC (1:500; Abcam; Cambridge, USA) and anti-caspase-1 (1:5,000; Abcam; Cambridge, USA) primary antibodies. Afterwards, membranes were exposed to the anti-rabbit or anti-mouse secondary antibodies (1:10,000; Dako; Glostrup, Denmark). The immunocomplexes were detected using the Clarity^{TM} Western ECL chemiluminescence detection system (Bio-Rad; Hercules, USA). Protein bands were detected in a ChemiDoc XRS detection system (Bio-Rad) driven by Quantity One software. Membranes were stripped by washing in ddH_2O for 5-10 min in 0.2 M NaOH (sodium hydroxide) and a further 5 min in ddH_2O at room temperature. After blocking, the content of GADPH (1:1,000; Cell signalling; Danvers, USA) was performed for verifying loading evenness.

3.4.3. Evaluation of Cell Viability

Cell viability of Neuro2a and C6 glioma cells was assessed by following the MTT (3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolic bromide) reduction. Active mitochondrial dehydrogenases cleavage and reduce the soluble yellow MTT dye into the insoluble purple formazan. Cells were incubated for 1, 3 and 24 h with neopterin (0-5000 nM). At the end of the incubation period, MTT test was performed. The formazan formation was spectrophotometrically assayed at 570 nm and 630 nm, and the net $\Delta A_{(570–630)}$ was taken as an index of cell viability. Results were expressed as optical density (O.D.).

3.4.4. Measurement of ROS production

Intracellular ROS production was detected using the non-fluorescent cell permeating compound, 2‘–7‘-dichlorofluorescein diacetate (H_2DCFH-DA). H_2DCFH-DA is hydrolyzed by intracellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent 2‘–7‘-dichlorofluorescein (DCF) by action of cellular oxidants. Rat cortical primary astrocytes exposed to azide (5 mM) and/ or neopterin (50 and 500 nM) for 3 h were treated with H_2DCFH-DA (50 μM) for 30 min at 37 °C. Afterwards, the cells were visualized using the Olympus BX41 fluorescence microscope and photographed using Q-capture Pro 5.1 software. The number of positive cells was counted in 5-8 fields and the results were expressed as number of positive cells.

3.4.5. Lactate quantification

The synthesis and release of lactate was measured in rat primary astrocytes supernatant using the specific analyser YSI 2700 STAT (Yellow Springs, Ohio, USA). Results were expressed as nmol/L.

3.4.6. Analysis of Nrf2 content

Cell lysates of C6 astroglioma cell line were collected after 3 h of neopterin 50 nM exposure and resuspended in 500 μL of PBS with 16 % formaldehyde for fixation. After 10 min at 37 °C, cells were washed with PBS and permeabilized with 4% saponin for 30 min. Then, cells were washed with PBS and incubated with anti-Nrf2 primary antibody (1:1,000; Cell signalling; Danvers, USA) for 1 h. After PBS washing, cells were incubated with anti-rabbit allophycocyanin (APC)-conjugated secondary antibody (1:1,000; Invitrogen; Carlsbad, USA) for 30 min at room temperature in the dark. Cells were washed with PBS, resuspended in 500 μL of the same solution and transferred to specific flow cytometry tubes for fluorescence measurement. Samples (10,000 events for sample) were analysed in FACSCanto II (BD Biosciences, San Jose, USA) flow cytometer using the program CellQuest 2.8 (BD Biosciences, San Jose, USA) in the Multiuser Laboratory for Biological Studies (LAMEB), Federal University of Santa Catarina. The results were analysed using the ModFit 3 (Verity Software House) (BD Biosciences, San Jose, USA) program.

3.4.7. Multiplexing cytokine analysis

Quantification of multiplexing cytokines was performed in human primary astrocytes culture supernatant and the serum from the participants on the ASD study using 27-plex magnetic bead based immunoassay kits (Bio-Rad, CA, USA). The multiplex kit was used in order to measure human cytokines including IL-1β, IL-6, TNF-α, IL-10 and IL-1Ra in the samples (50 μL). The assay was performed in accordance to manufacturer's instruction provided and carried out using the setup described by Khan (2012). All pipetting and mixing steps of the standards and samples (50 μL) with the assay reagents outlined in the manufacturer's protocol were performed with a programmed workflow using the robotic liquid handling workstation, epMotion 5075 (Eppendorf, Hamburg, Germany) in order to minimize pipetting errors. Subsequent washing and read out was carried out with Bio-Plex

Pro II wash station and Bio-Plex system 200, respectively (Bio-Rad, Hercules, USA). Final readout of the samples concentration was calculated based on the standard curves integrated in the assay using the Bio-Plex Manager v5.0 and expressed as percentage of controls.

3.4.8. Protein determination

Sample protein content was determined by the method of Lowry using bovine serum albumin as the standard (Lowry et al., 1951).

3.5. Molecular parameters

3.5.1. Gene expression analysis of *Gch1, IL1B* and *CASP1*

3.5.1.1. Preparation and analysis of samples from the in vivo experiment

Total ribonucleic acid (RNA) was isolated from mouse hippocampus by using a commercial kit (Tri-reagent protocol; Sigma; Saint Louis, USA), and according to the technical recommendations of the manufacturer. The concentration and purity of the extracted RNA was determined on a NanoDrop spectrophotometer at 260 nm and 280 nm. cDNA (complementary DNA) was synthesized from up to 1 µg of total RNA using M-MLV Reverse Transcriptase kit (Sigma; Saint Louis, USA). Briefly, for a single reaction, the following components were combined: M-MLV Reverse Transcriptase Buffer, 0.1 M DTT, RNAse OUT, Oligodt, 10 mM dNTP and the enzyme M-MLV Reverse Transcriptase (and DEPC water for a final volume of 20 µL). A separate tube named as No-RT (no reaction tube), consisted of all of the above components with omission of the Enzyme Mix. This was used as a control to confirm that no genomic DNA was present in the sample. All the tubes were gently mixed and then placed on a Mastercycler gradient PCR machine for the cDNA synthesis reaction with the following protocol: incubation at 25 °C for 10 min, incubation at 37 °C for 50 min, and termination of the reaction at 4 °C for 10 min (Jiang et al., 2010). The resulting cDNA was then stored at -80 °C until further use.

Five µL of cDNA product (equivalent to 5 ng RNA) was used *per* 15 µL reaction mix in each well, with 8.5 µL express SYBR green qPCR super mix universal (Invitrogen; Carlsbad, USA) and 1 µL of each primer (0.3 µM). Each sample was run in triplicate and two PCR controls were included for each running step using 5 µL aliquot of Reverse

Transcription negative control and/or water instead of the sample. The quantitative real time PCR (qRT-PCR) was performed in the ABI PRISM 7900HT (Applied Biosystems; Foster City, USA) using the following protocol: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 min and 60 °C for 1 min. The melting curve was run at the end to confirm the identity and purity of the PCR. The data was normalized by the gene expression of the housekeeping protein β-Actin (*ACTB* gene) and expressed as mRNA expression relative to a control sample.

The oligonucleotide sequences of the primers for mice samples used for qRT-PCR were designed using the Primer "BLAST" (*Basic Local Alignment Search Tool)* available on http://blast.ncbi.nlm.nih.gov/Blast.cgi, according to the specific exons for each protein and are described in Table 3.2.

Table 3.2. Sequence of the primers used to determine gene expression in mice samples

Genes	Forward	Reverse
Gch1	TGCTTACTCGTCCATTCTGC	CCTTCACAATCACCATCTCG
IL1B	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG
ACTB	GCCCTGAGGCTCTTTTCCAG	TGCCACAGGATTCCATACCC

3.5.1.2. Preparation and analysis of samples from the in vitro experiments

Total RNA was isolated from human primary astrocytes and neurons after 24 h of LPS stimulation. The supernatant was discarded and the cells were washed three times with PBS (1x) and the RNA was extracted using RNeasy mini kit (Qiagen; Valencia, USA). The concentration and purity of the extracted RNA was determined on a NanoDrop spectrophotometer at 260 nm and 280 nm. cDNA was synthesized from up to 1 µg of total RNA using superscript® VILO™ MasterMix (Invitrogen; Carlsbad, USA). Briefly, for a single reaction, the following components were combined: 4 µl of 5X VILO Reaction Mix, 2 µl of 10X SuperScript Enzyme Mix, x µl (1.5 µg) of RNA and DEPC-treated water to a final volume of 20 µl. A No-RT reaction tube was also prepared in order to confirm that no genomic DNA was present in the sample. All the tubes were gently mixed and then placed on a Mastercycler gradient PCR machine for the cDNA synthesis reaction with the following protocol: incubation at 25 °C for 10 min, incubation at 37 °C for 50 min, and termination of the reaction at 4 °C for 10 min. The resulting cDNA was then stored at -80°C until further use.

Five µL cDNA product (equivalent to 5 ng RNA) was used per 20 µL reaction mix in each well, with 10 µL express SYBR green qPCR super mix universal, and 1 µL of each primer (0.3 µM). Each sample was run in triplicate and two PCR controls were included as described on the previous section. The real time PCR was performed in the ViiA^{TM} 7 (Life technologies/ Applied Biosystems, Carlsbad, USA) instrument. After 2 min incubation at both 50 °C and 95 °C, DNA amplification occurred over 40 cycles (about 10 min) at 15 sec incubation at 95 °C, followed by 1 min incubation at 60 °C. The melting curve was run at the end to confirm the identity and purity of the PCR. mRNA levels were expressed as fold change from the control using the delta CT method, after adjustment according to levels of the reference housekeeping gene, β-2-microglobulin (*B2M*).

The oligonucleotide sequences of the primers for mice samples used for qRT-PCR were designed using the Primer "BLAST" available on http://blast.ncbi.nlm.nih.gov/Blast.cgi, according to the specific exons for each protein and are described in Table 3.3.

Table 3.3. Sequence of the primers used to determine gene expression in human samples

Genes	Forward	Reverse
CASP1	CTCAGGCTCAGAAGGGAATG	CGCTGTACCCCAGATTTTGT
IL1B	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
B2M	AGGCTATCCAGCGTACTCCA	CGGCAGGCATACTCATCTTT

3.6. Statistical analysis

Results are presented as mean ± standard SEM. Data was analysed using one or two-way ANOVA followed by the Dunnett's or Tukey's *post hoc*, when *F* was significant. Only significant *F* values are given in the text. When comparing two independent or paired groups Student's *t* test for independent or paired samples, respectively, was used. In comparing means, the equal variances assumption for *t*-tests was checked. If not satisfied, a non-equal variance *t*-test was performed. Differences between the groups were rated significant at $P < 0.05$. Statistics were performed using SPSS (Statistical Package for the Social Sciences software; version 16.0 for Windows). All graphs were performed by using GraphPad Prism 5®.

Chapter 4
Results

The results obtained during the PhD were organized into three sections:

Section I: Evaluation of neopterin and inflammatory mediators' synthesis in inflammatory conditions

We organized a first manuscript in the format of a mini-review, which included the available information about neopterin synthesis by nerve cells in the literature and previous data generated by our group. The title of this manuscript was "Neopterin as a potential cytoprotective brain molecule" and it was published in the Journal of Psychiatric Research in December, 2015 (Ghisoni et al., 2015). Consequently, we showed that neopterin is synthesized and released from glial cells after an inflammatory stimulus involving mitochondrial toxicity. In addition, we also showed that neopterin *per se* has antioxidant properties. These results are presented on Figures 4.5 and 4.9 on this section.

Section II: Neopterin role on inflammasome activation in nervous cells

The results presented on this section are organized on the manuscript "*Neopterin prevents inflammasome activation in human primary astrocytes*" submitted to the *Journal of Experimental Medicine*, on 10 October, 2016.

Section III: Evaluation of neopterin and inflammatory mediators' secretion in ASD patients' serum

The results presented on this section integrate the paper "Altered Kynurenine Pathway Metabolism in Autism: Implication for Immune-Induced Glutamatergic Activity" published in the *Autism Research Journal* in October, 2015 (Lim et al., 2015).

4.1 Section I: Evaluation of neopterin and inflammatory mediators' synthesis in inflammatory conditions

4.1.1. Temporal profile of neopterin and the pro-inflammatory cytokine IL-1β synthesis in Swiss mice subjected to the LPS-induced neuroinflammation

The results presented on the next sections were performed at the Bioenergetics and Oxidative Stress Laboratory, Federal University of Santa Catarina, Brazil, and supervised by Dr. Alexandra Latini under a cotutelle agreement.

Figure 4.1 shows a curve of neopterin and IL-1β production overtime in Swiss mice serum in response to systemic administration of LPS (0.33 mg/kg), a classic stimulus to induce inflammation. Serum neopterin levels rapidly increased reaching a peak in 15 min and remaining increased for at least 60 min after the inflammatory challenge (Figure 4.1A) [$F_{(4.9)}$=39.87; P<0.001]. These data were quantified by HPLC and were confirmed by ELISA [$F_{(2,5)}$=14.17; P<0.01], as shown in Figure 4.2. Figure 4.1B shows the classical peak of IL-1β in the serum at 4 h (240 min) after the inflammatory stimulus [$F_{(5,12)}$=315.60; P<0.0001]. In addition, it could also be observed a linear increase of the cytokine release into the serum up to 1 h [β=0.56; P<0.05].

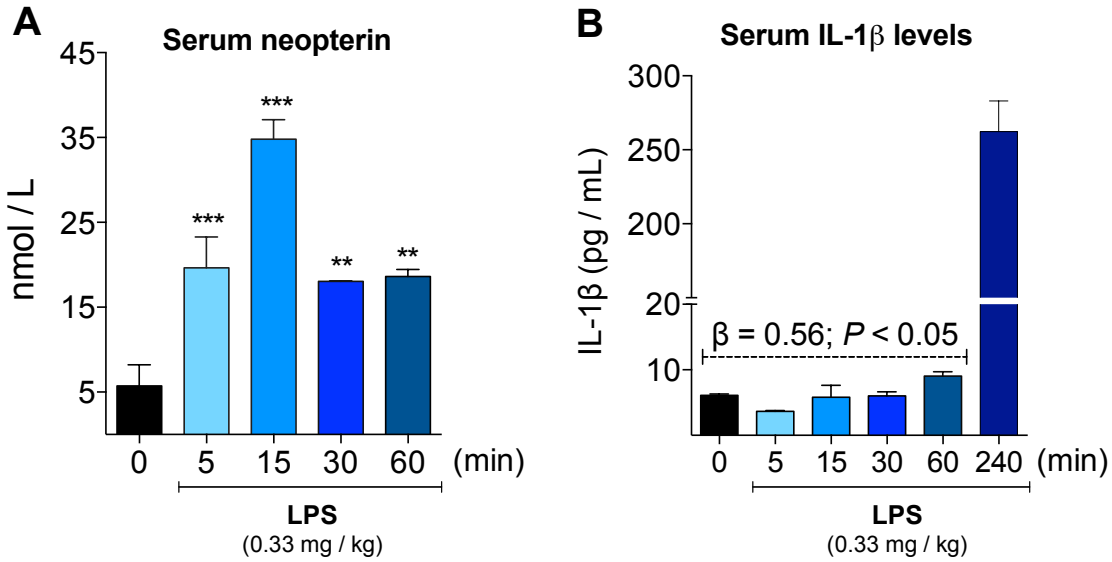


Figure 4.1. LPS systemic administration rapidly increases peripheral neopterin and IL-1β production. Sixty-day old mice received a single LPS intraperitoneal injection (0.33 mg/kg) and the serum was collected for biochemical analyses after 5 to 60 or 240 min. Neopterin levels (**A**) were measured by HPLC and IL-1β levels (**B**) were quantified by ELISA. Mean ± SEM values (n = 5 animals per group). **P<0.01, ***P <0.001, *vs.* controls (One way ANOVA followed by Dunnett's post-hoc test).

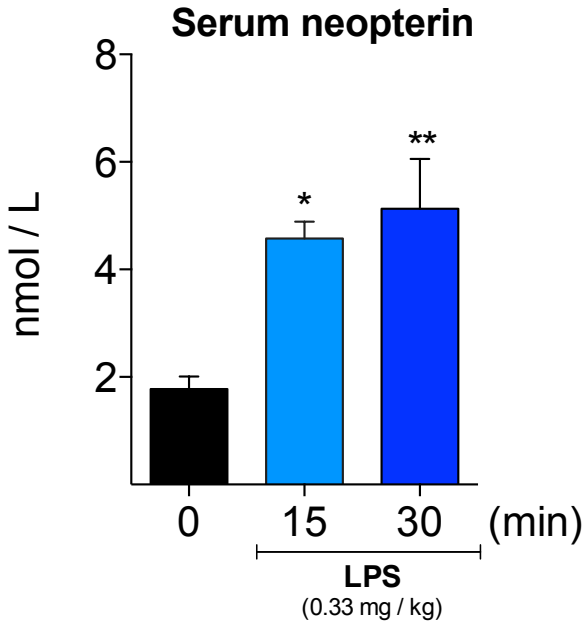


Figure 4.2. Evaluation of serum neopterin levels in LPS-exposed Swiss mice. Sixty-day old mice received a single LPS intraperitoneal injection (0.33 mg/kg) and the serum was collected after 15 to 30 min to measure neopterin levels by ELISA. Mean ± SEM values (n = 5 animals per group). **P*<0.05, ***P*<0.01, *vs*. controls (One way ANOVA followed by Dunnett's post-hoc test).

Figure 4.3 shows neopterin synthesis in LPS-exposed mice hippocampus. It can be observed on Figure 4.3A that neopterin levels significantly increased in mice hippocampus 15 min after LPS systemic administration [$t_{(2)}$=7.80; P<0.01]. It can also be seen that the increase in neopterin coincides with the increase in *Gch1* expression, the enzyme that transcriptionally controls neopterin formation [$t_{(6)}$=3.23; P<0.01] (Figure 4.3B). Increased neopterin production occurred in parallel to increased *Gch1* expression.

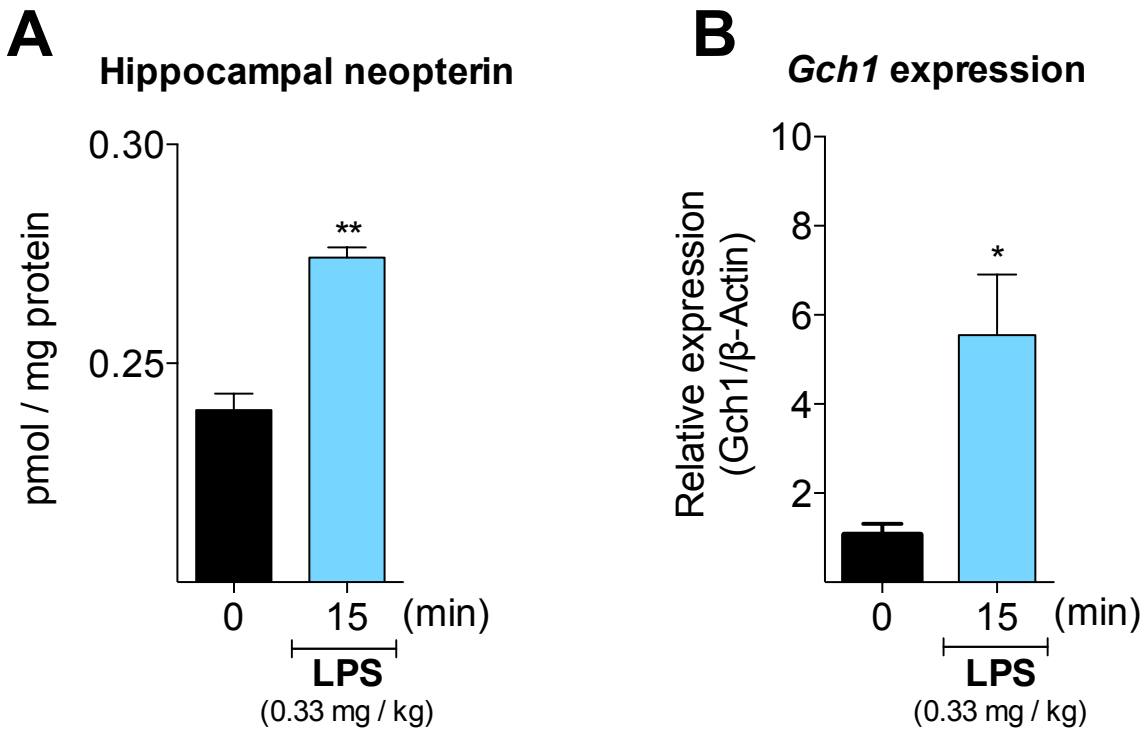


Figure 4.3. LPS systemic administration rapidly induces central neopterin and *Gch1* expression. Sixty-day old mice received a single LPS intraperitoneal injection (0.33 mg/kg) and after 15 min the hippocampi were collected for biochemical analyses. Neopterin levels (**A**) were measured by HPLC and *Gch1* expression (**B**) was analysed by qRT-PCR. Gene expression is shown as the ratio of the studied transcripts relative to β-Actin measured in triplicate. Mean ± SEM values (n = 2-4 animals per group). **P<0.01 *vs*. controls (Student's *t* test for independent samples).

Figure 4.4 shows *IL1B* gene expression and activated caspase-1 (p20) content in LPS-exposed Swiss mice hippocampus. Figures 4.4A and B show an increase in both cytokine expression [$F_{(3,4)}$= 6.88; P<0.05], and caspase-1 p20 content in the mice hippocampus after 5 to 30 min of LPS administration [$F_{(4,7)}$=12.39; P<0.01], suggesting that there is inflammasome activation.

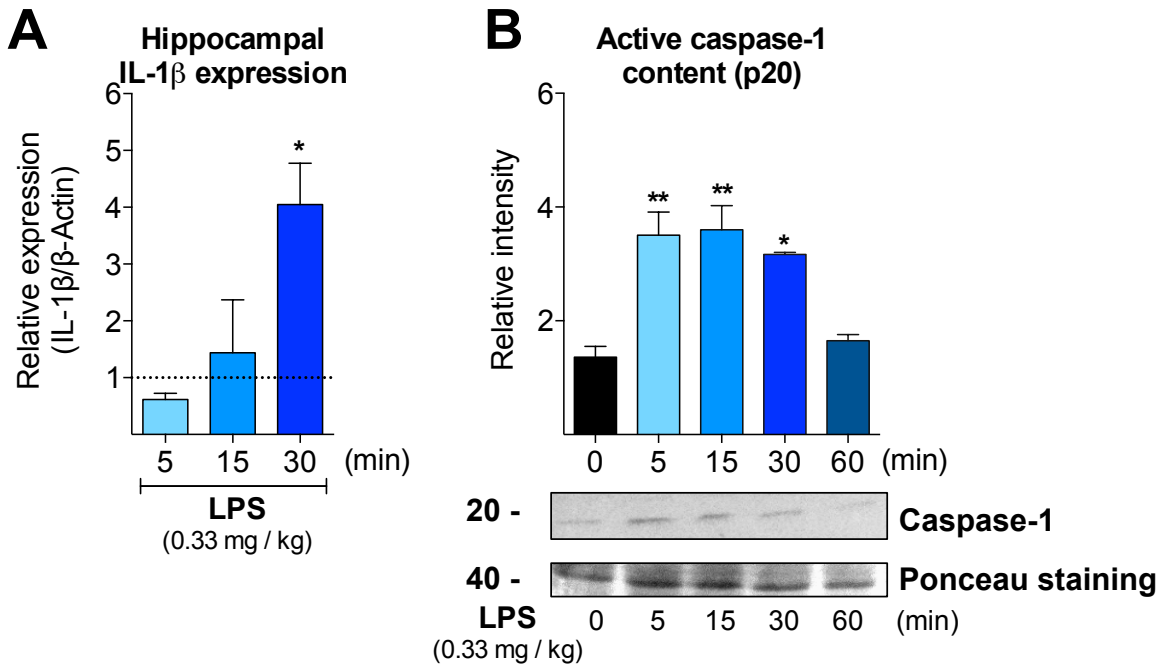


Figure 4.4. LPS systemic administration rapidly increases IL-1β production and caspase-1 activation in mice CNS. Sixty-day old mice received a single LPS intraperitoneal injection (0.33 mg/kg) and after 5 to 60 min the hippocampi were collected for biochemical analyses. *IL1B* gene expression (**A**) was analysed by qRT-PCR. Gene expression is shown as the ratio of the studied transcripts relative to β-Actin measured in triplicate. Active caspase-1 (p20) (**B**) content was assessed by Western blot technique and quantified by densitometry. Ponceau staining was used to control the amount of protein sample. Mean ± SEM values (n = 5 animals per group) and one representative Western blot is shown. *P<0.05; **P<0.01 *vs*. controls (One way ANOVA followed by Dunnett's post-hoc test).

4.1.2. Evaluation of neopterin secretion in conditions of mitotoxicity in rat primary astrocytes

After demonstrating that the inflammatory LPS treatment elicited the synthesis and release of neopterin in rodents, the next set of data showed that increased neopterin was cytoprotective under mitochondrial oxidative stress conditions.

Figure 4.5 shows the quantification of neopterin in the supernatant of murine primary astrocytes culture after 3 h of exposure to sodium azide (5 mM). It can be observed that the levels of secreted neopterin reached up to 35 nmol/L.

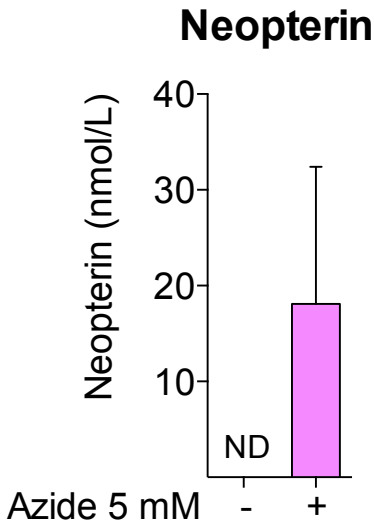


Figure 4.5. Sodium azide induces neopterin secretion in astrocytes. Rat neonatal astrocytes were exposed to sodium azide (5 mM) for 3 h. The supernatant was collected and neopterin release measured by HPLC. ND: non-detected. Mean ± SEM values from three independent experiments.

4.1.3. Evaluation of neopterin effects on cellular viability, oxidative stress and/ or metabolic parameters in neurons and astrocytes

Figure 4.6 shows the effects of neopterin *per se* on cell viability in Neuro2a neurons and C6 astroglioma cell lines. It can be observed that neopterin did not affected the cellular viability in both cell types. In addition, neopterin *per se* did not induce ROS production in C6 astroglioma cell lines, except when the highest concentration was used (5000 nM) (Figure 4.7).

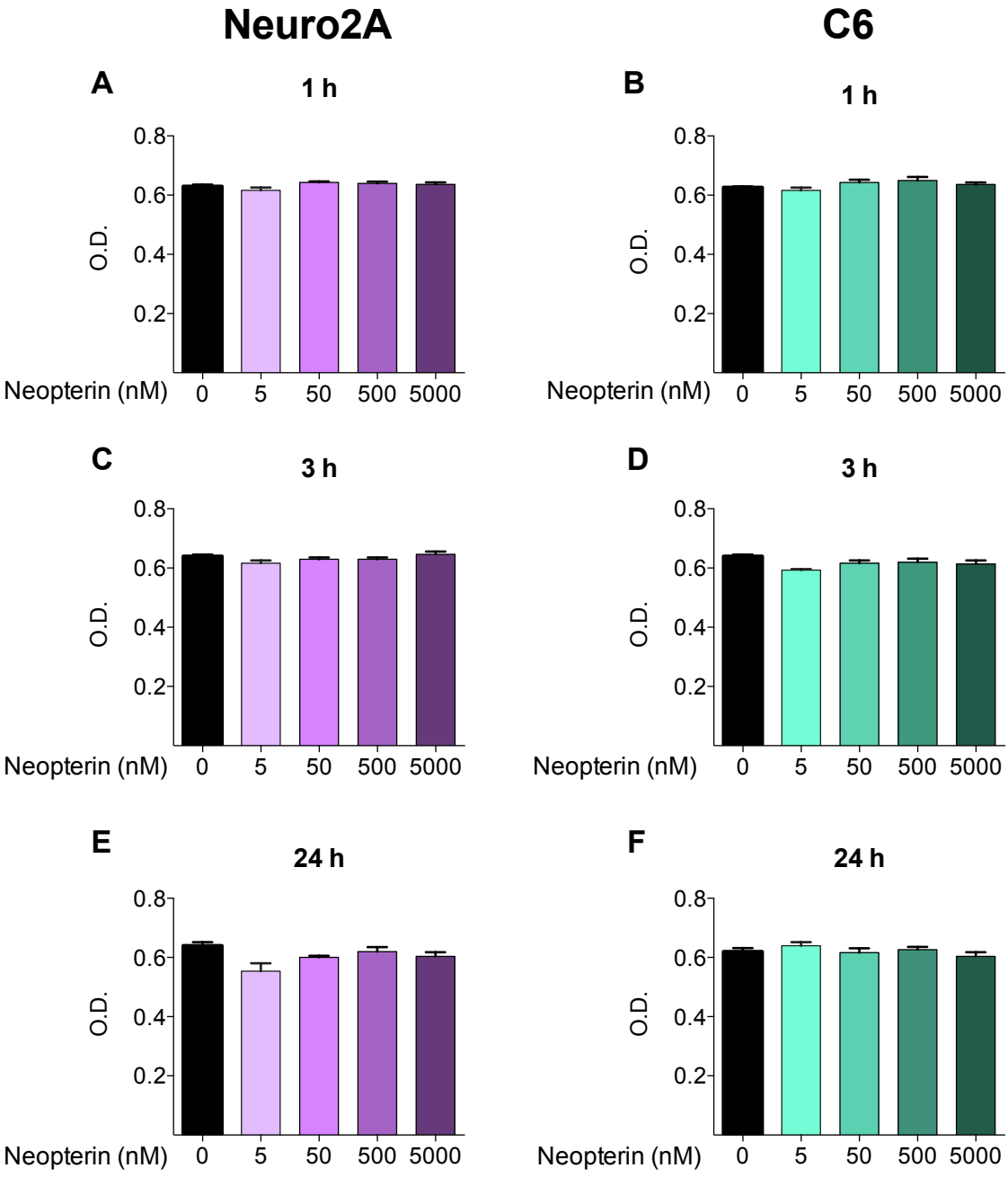


Figure 4.6. Neopterin does not affect cell viability. Neuro2a neurons and C6 astroglioma cell lines were exposed to several concentrations of neopterin (0-5000 nM) for 1, 3 and 24 h (Figures A, C and E correspond to the respectively cited times for Neuro2a neurons and Figures B, D and F for C6 astroglioma cell line). Cell viability was evaluated through MTT reduction. Mean ± SEM values from three independent experiments.

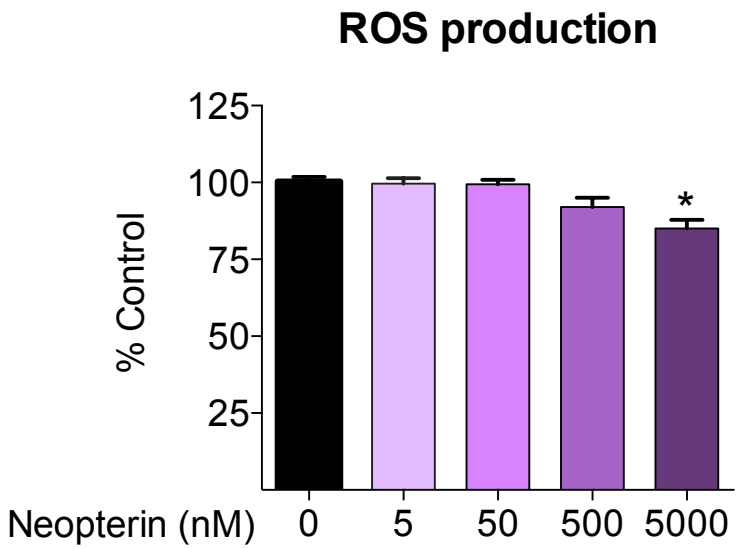
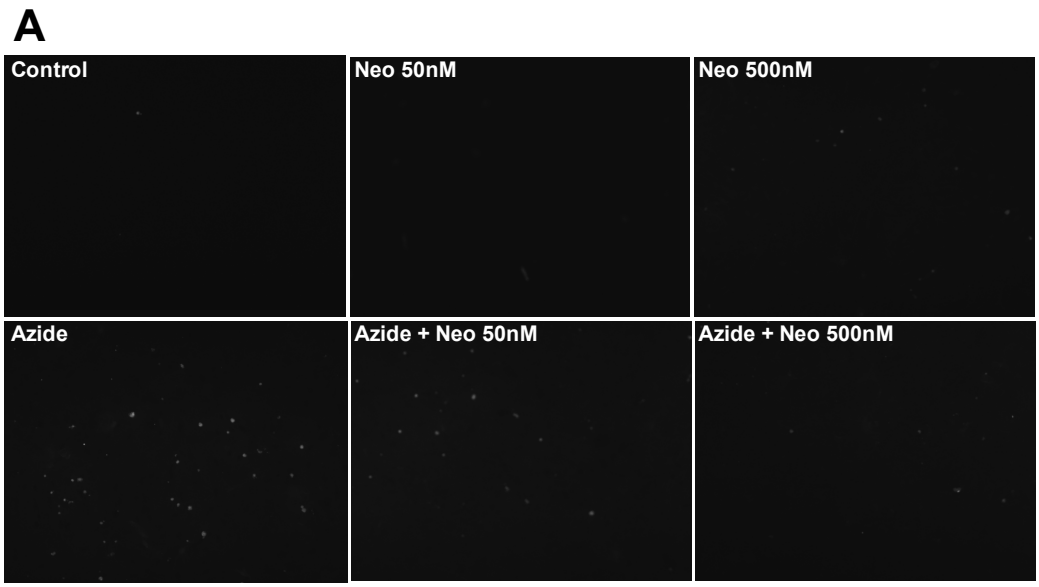


Figure 4.7. Neopterin does not induce ROS production. C6 astroglioma cell lines were treated with crescent concentrations of neopterin (0- 5000 nM) for 3 h. ROS production was evaluated by measuring the oxidation of H_2DCFH-DA. Mean ± SEM values from three independent experiments. **P*<0.05 *vs*. controls (One-way ANOVA followed by Tukey's post-hoc test).

Figure 4.8A and B shows that sodium azide (5 mM) increased ROS production [$F_{(1,14)}$=25.43; P<0.001], while the co-exposure with neopterin (50 and 500 nM) reduced this effect [$F_{(2,14)}$=6.30; P<0.05], indicating that the pteridine inhibits azide-induced ROS production. In addition, it can be observed in Figure 4.9A that despite attenuating ROS production, neopterin did not change the lactate release, an evidence of compensatory glycolysis activation in response to azide-induced oxidative stress. Figure 4.9B shows that oxidative stress attenuation occurs in parallel with the increase on the antioxidant enzyme, HO-1, content. Both neopterin and azide exposure increased HO-1, with no significant interaction between the treatments (neopterin: [$F_{(2,6)}$= 16.45; P<0.01]; sozium azide: [$F_{(1,6)}$= 87.36; P<0.001].



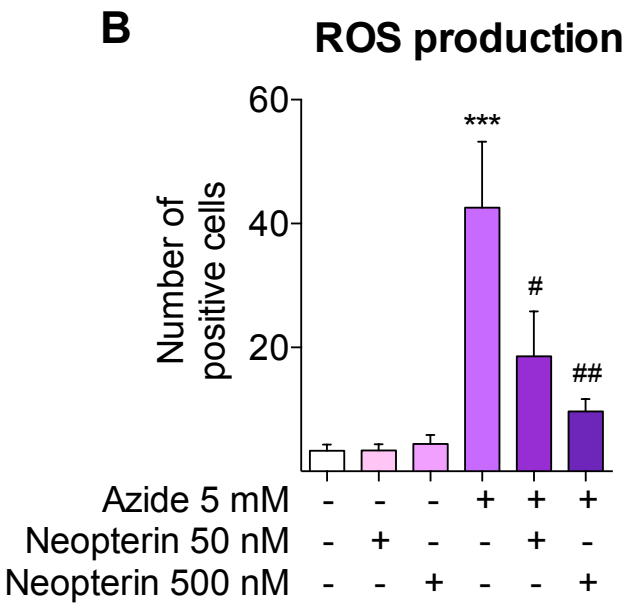


Figure 4.8. Neopterin inhibits azide-induced oxidative stress in astrocytes. Rat neonatal astrocytes were exposed to 5 mM sodium azide and/or neopterin (50 and 500nM) for 3 h. ROS production was evaluated by measuring the oxidation of H_2DCFH-DA (**A**). Fluorescence intensity was quantified and expressed as % of controls (**B**). Mean ± SEM values from three independent experiments. ***P<0.001 *vs*. controls; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs*. azide (Two-way ANOVA followed by Tukey's post-hoc test).

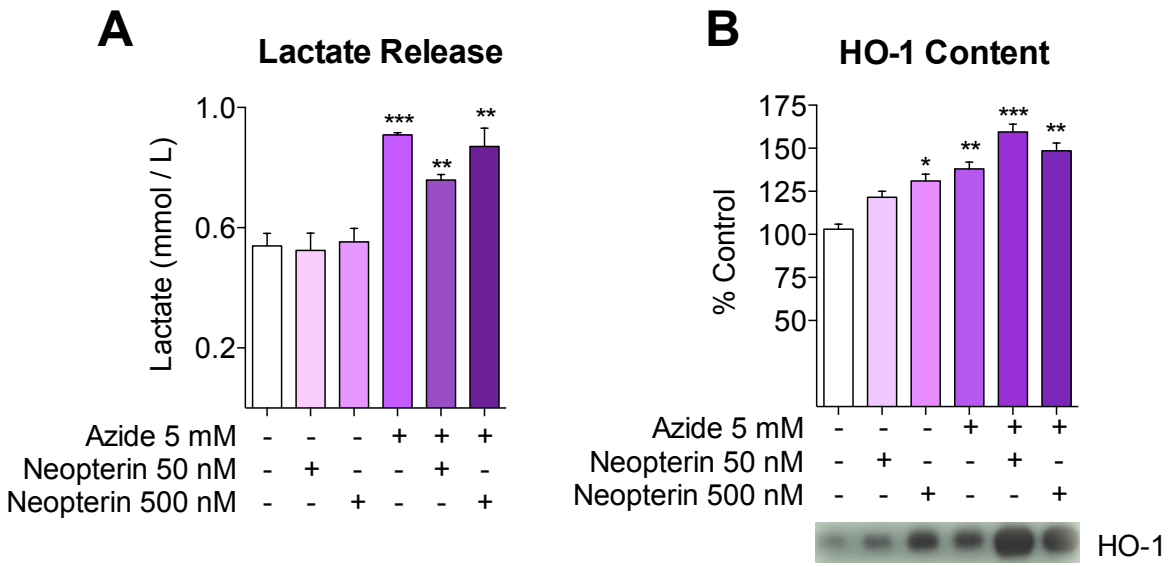


Figure 4.9. Neopterin positively modulates HO-1 content in astrocytes. Rat neonatal astrocytes were exposed to 5 mM sodium azide and/or neopterin (50 and 500nM) for 3 h. The supernatant was collected and used to measure the lactate release (**A**) and the cells collected and prepared to determine HO-1 content (**B**) by Western blot technique and quantified by densitometry. GAPDH expression was used to control the amount of protein sample. Mean ± SEM values from three independent experiments and one representative Western blot is shown. *P<0.05, **P<0.01, ***P<0.001 *vs*. controls (Two-way ANOVA followed by Tukey's post-hoc test).

4.1.4. Neopterin effect on Nrf2 content and activation in C6 astrocytic cell line

Figures 4.10A and B show the effect of neopterin on the cellular content of the transcription factor Nrf2 in C6 astroglial cell line. It can be seen that the content of Nrf2 increased significantly after 3 h of exposure to 50 nM neopterin [$t_{(3)}$=5.81; P<0.01] (Ghisoni et al, 2015; PhD in Neuroscience, 2015). In addition, it can be observed on Figure 4.10C that neopterin treatment (50 nM; 0.5 to 4 h) also significantly increased the translocation of Nrf2 from the cytosol to the nucleus in C6 rat cell line. This result confirm that neopterin induces Nrf2 activation ($F_{(4,10)}$ = 18.21; P<0.001).

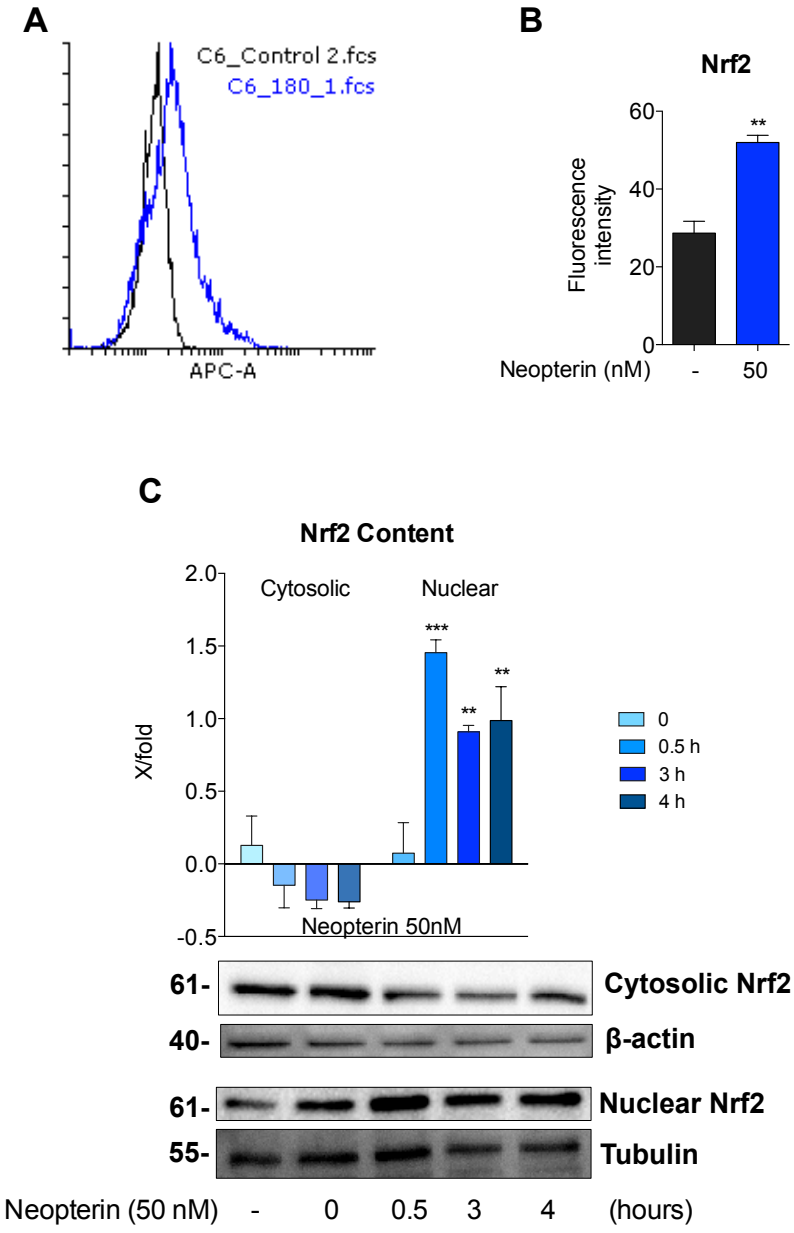


Figure 4.10. Neopterin exposure increases Nrf2 content and activation in astrocytes. C6 rat astrocytic cell line was exposed to neopterin (50 nM). After 3 h, the cells were collected and fixed for Nrf2 staining measurement by flow cytometry. Results were obtained from three independent experiments. It is shown one representative diagram (A) and the data quantification (B). After 0.5 to 4 h, the whole cell lysate was collected and nuclear and cytosolic fractions prepared to determine Nrf2 translocation by Western blot techniques. The results were quantified by densitometry and β-Actin and tubulin expression were used to control the amount of protein sample in the cytosolic and nuclear fractions, respectively. Values are presented as mean ± SEM of three independent experiments. One representative Western blot is shown. ***P*<0.01, ****P* <0.001, *vs*. control (Student's *t* test for dependent samples or One-Way ANOVA followed by the Dunnett's post-hoc test).

4.2 Section II: Neopterin effect on inflammasome activation in nervous cells

4.2.1. LPS-induced inflammasome activation in C6 astrocytic cell line

In order to validate the LPS stimuli to induce inflammasome activation on the next experiments *in vitro*, it was investigated the minimum concentration of LPS that induces active caspase-1 release, a marker for inflammasome activation, in C6 astrocytic lineage. Figure 4.11 shows that the release of active caspase-1 (p20) in the supernatant was only observed when LPS was used at 5 μg/mL concentration.

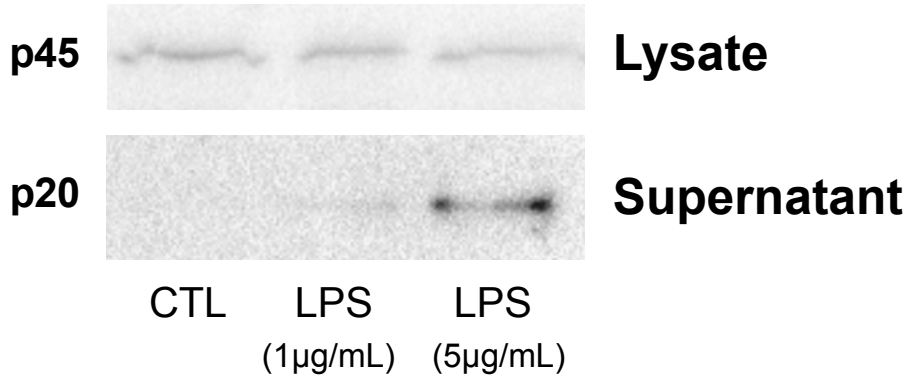


Figure 4.11. Active caspase-1 release in the supernatant of astrocytes treated with crescent concentrations of LPS (1 and 5 μg/mL). C6 rat astrocytic cell line was exposed to LPS for 24h. Afterwards, the whole cell lysate and the supernatant were collected and prepared to determine the content of mature (p45) and active (p20) caspase-1 by Western blot techniques. Ponceau staining was used to control the amount of protein sample on the lysate. Results were obtained from three independent experiments and one representative Western blot is shown.

4.2.2. Temporal gene expression of LPS-induced inflammasome proteins in human primary astrocytes

The results presented on the next sections were performed at the Neuroinflammation Laboratory, Macquarie University, Australia, and supervised by Dr. Gilles Guillemin under a cotutelle agreement. The human cells used in the experiments were obtained following the ethics principles and the methodology used was appropriate as described on chapter 4 (Material and Methods).

Figures 4.12A and B show that LPS 5 µg/mL (the same concentration used on C6 astrocytic cell line) significantly induces *IL1B* and *CASP1* gene expression (*IL1B*: [$F_{(3,4)}$=7.912; P<0.05]; *CASP1*: [$F_{(3,5)}$=32.50; P<0.001]), reaching the maximum effect after 24 h of LPS exposure.

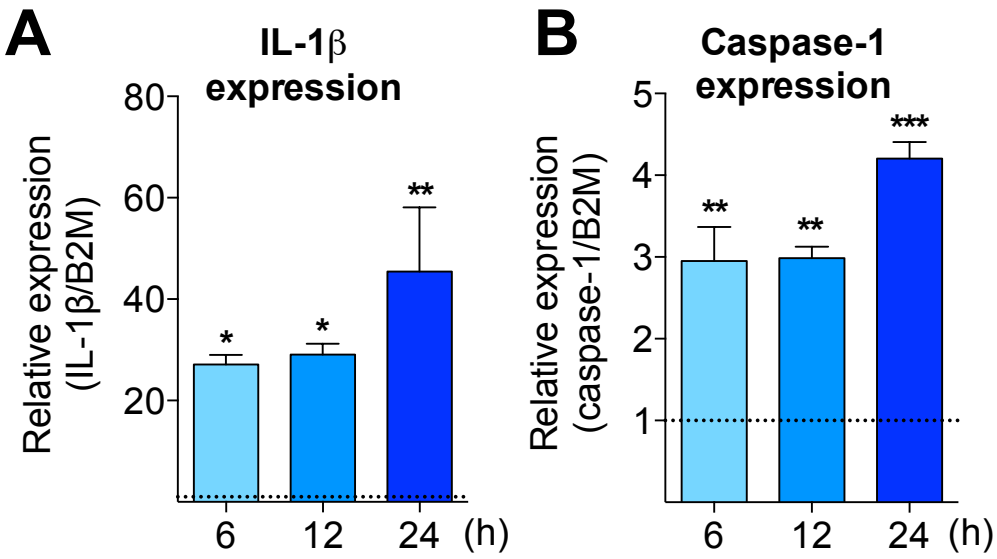


Figure 4.12. LPS increases *IL1B* and *CASP1* gene expression in human primary astrocytes. The cells were stimulated with LPS (5 µg/mL) for 6, 12 and 24 h. Then, the RNA was purified and the transcription of *IL1B* (**A**) and *CASP1* (**B**) were determined by quantitative qRT-PCR. Gene expression is shown as the ratio of the studied transcripts relative to *B2M* measured in triplicate. Values are presented as mean ± SEM of three independent experiments performed in triplicate. *P<0.05, **P<0.01, ***P <0.001, *vs*. basal (One-Way ANOVA followed by Dunnett's post-hoc test).

4.2.3. Neopterin effects on LPS-induced inflammasome activation in human primary astrocytes

Figures 4.13A and B show neopterin effects on *IL1B* and *CASP1* gene expression. It can be observed that neopterin (5-500 nM) preconditioning (24 h) did not affect the LPS-induced *IL1B* gene expression, the *primer* step in inflammasome activation. (Scheme 3.1: experimental design in Material and Methods; page: 40). However, neopterin preconditioning inhibited the increase on *CASP1* gene expression and ASC and pro-caspase-1 content induced by LPS in human primary astrocytes, suggesting that the pteridin inhibits the inflammasome activation (*CASP1* gene expression: [$F_{(3,6)}$=10.27; P<0.01]; pro-caspase-1 content [$F_{(4,7)}$=21.69; P<0.001]; ASC content [$F_{(4,5)}$=71.38; P<0001]) (Figures 4.13C, D and E).

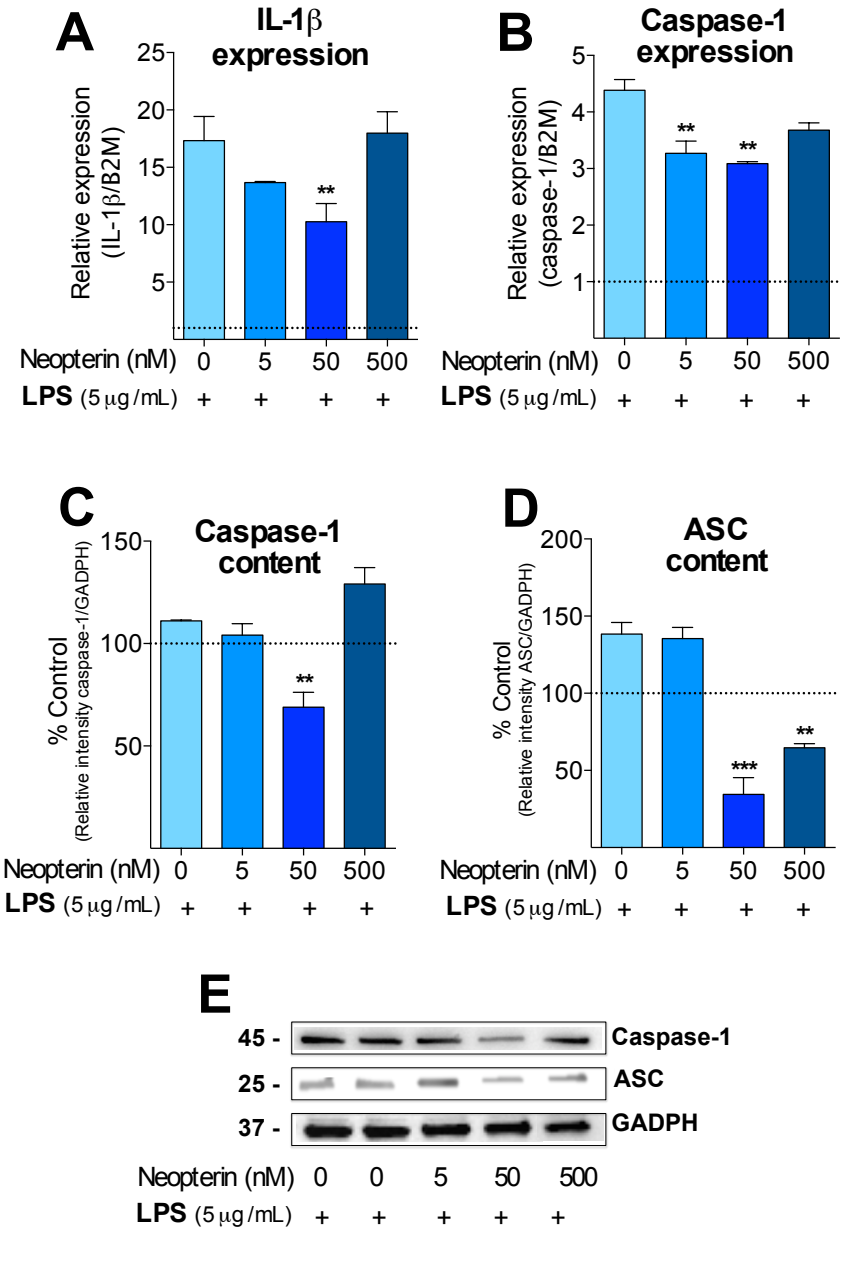


Figure 4.13. Neopterin negatively modulates inflammasome activation in human primary astrocytes exposed to LPS. The cells were pre-treated with neopterin (0-500 nM) for 24 h and then activated with LPS (5 µg/mL). After 24 h of LPS stimulation, RNA was purified and the transcription of *IL1B* (**A**) and *CASP1* (**B**) were determined by quantitative qRT-PCR. Gene expression is shown as the ratio of the studied transcripts relative to *B2M* measured in triplicate. After 24 h of neopterin exposure followed by 48 h of co-exposure toneopterin and LPS (Scheme 3.1: experimental design in Material and Methods; page: 40), the whole cell lysate was collected and prepared to determine pro-caspase-1 (**C**) and ASC (**D**) content by Western blot techniques and quantified by densitometry. GADPH expression was used to control the amount of protein sample. Values are presented as mean ± SEM of three independent experiments. One representative Western blot is shown (**E**). ***P*<0.01, ****P* <0.001, *vs*. LPS (One-Way ANOVA followed by the Dunnett's post-hoc test).

Figure 4.14 shows that neopterin preconditioning significantly inhibited IL-1β secretion, which its maturation depends on inflammasome activation, without affecting IL-6 and TNF-α, inflammasome-independent cytokines [$F_{(3,4)}$=23.49; P<0.01]. In addition, neopterin preconditioning (50 nM) increased the anti-inflammatory cytokines IL-10 and IL-1Ra secretion in comparison to cells exposed to LPS only, favoring an anti-inflammatory environment (IL-10: [$F_{(3,4)}$=7.626; P<0.05]; IL-Ra: [$F_{(3,4)}$=11.09; P<0.05]) (Figures 4.15A and B).

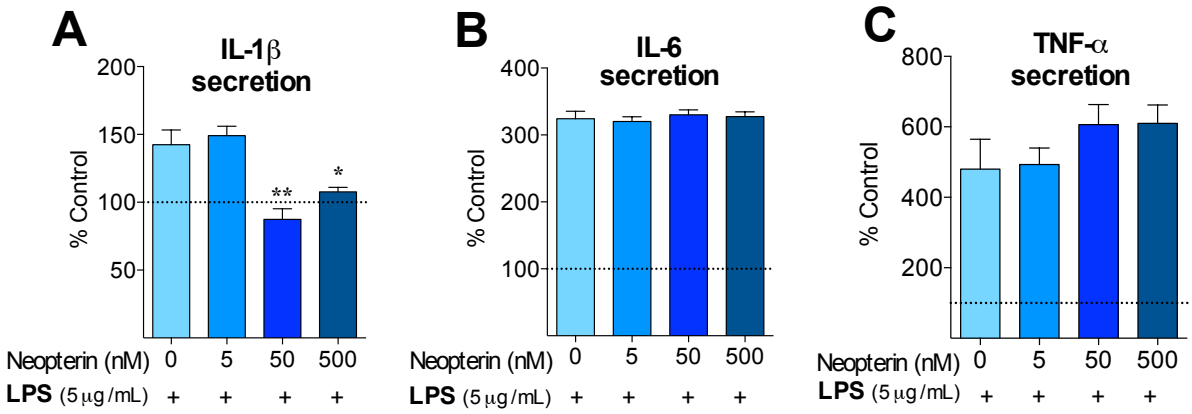


Figure 4.14. Neopterin inhibits IL-1β secretion in human primary astrocytes exposed to LPS. The cells were pre-treated with neopterin (0-500 nM) for 24 h and then activated with LPS (5 µg/mL). After 24 h of neopterin exposure followed by 48 h of co-exposure to neopterin and LPS (Scheme 3.1: experimental design in Material and Methods; page: 40), the supernatant was collected and IL-1β (**A**), IL-6 (**B**), and TNF-α (**C**) secretion were determined by using ELISA commercial kits. Values are presented as mean ± SEM of three independent experiments. **P*<0.05; ***P*<0.01 *vs*. LPS (One-Way ANOVA followed by Dunnett's post-hoc test).

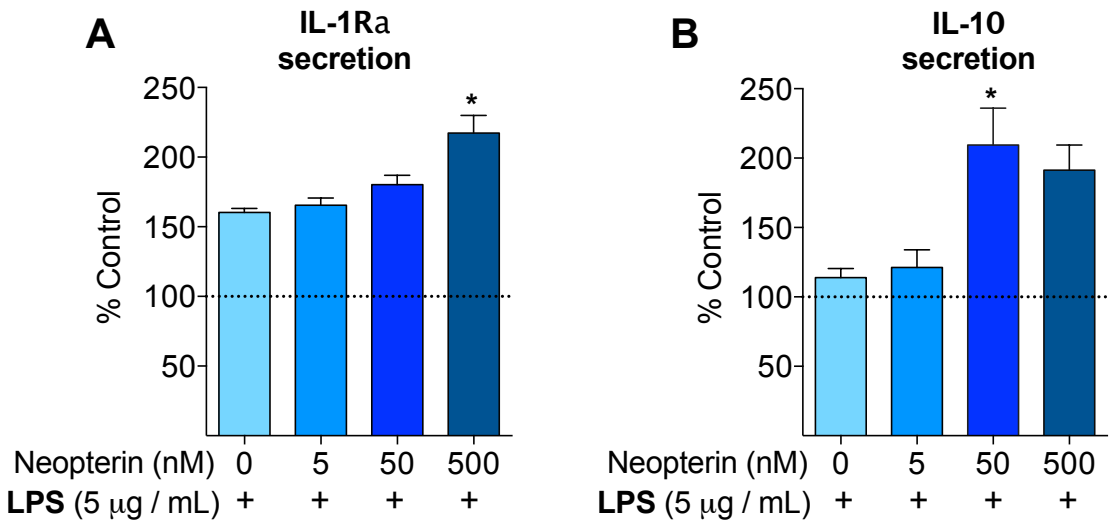


Figure 4.15. Neopterin induces anti-inflammatory cytokines secretion in human primary astrocytes exposed to LPS. The cells were pre-treated with neopterin (0-500 nM) for 24 h and then activated with LPS (5 μg/mL). After 24 h of neopterin exposure followed by 48 h of co-exposure to neopterin and LPS (Scheme 3.1: experimental design in Material and Methods; page: 40), the supernatant was collected and IL-10 (**A**) and IL-1Ra (**B**) secretion were determined by using ELISA commercial kits. Values are presented as mean ± SEM of three independent experiments. **P*<0.05 *vs*. LPS (One-Way ANOVA followed by Dunnett's post-hoc test).

4.2.4. Neopterin effects on inflammasome activation-related parameters induced by LPS in human primary neurons

In order to investigate if neopterin would exert similar inhibitory effects on inflammasome activation in other nervous cells, we analysed the neopterin effects on LPS-induced *CASP1* gene expression in neurons. Figure 4.16 shows that neopterin preconditioning also inhibited the increase in *CASP1* gene expression in human primary neurons [$F_{(3,5)}$=7.902; P<0.05], suggesting that neopterin inhibits inflammasome activation through a general and non cell-restricted mechanism.

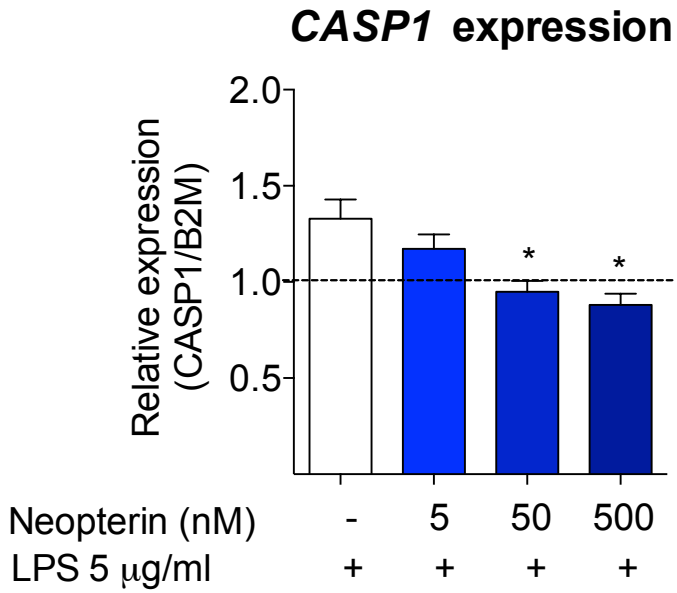


Figure 4.16. Neopterin negatively modulates *CASP1* gene expression in human primary neurons exposed to LPS. The cells were pre-treated with neopterin (0-500 nM) for 24 h and then activated with LPS (5 μg/mL). After 24 h of LPS stimulation (Scheme 3.1: experimental design in Material and Methods; page: 40), RNA was purified and the transcription of *CASP1* was determined by quantitative qRT-PCR. Gene expression is shown as the ratio of the studied transcripts relative to *B2M* measured in triplicate. Values are presented as mean ± SEM of three independent experiments. **P*<0.05 *vs*. LPS (One-Way ANOVA followed by the Dunnett's post-hoc test).

4.3. Evaluation of neopterin and inflammatory mediators' secretion in the serum from ASD patients

The data presented on this section was also performed at the Neuroinflammation Laboratory, Macquarie University, Australia, under the supervision of Dr. Gilles Guillemin. The human samples used in this section were provided by the Sultan Qabbos University Hospital, Oman.

Figure 4.17 shows that elevated neopterin levels were detected in ASD patients' serum in comparison with healthy controls, reaching up to 35 nM [$t_{(14,83)}$=2.45; P<0.05]. Interestingly, the two patients that presented substantially higher neopterin levels had their symptoms classified as severe. It was also observed increased levels of the anti-inflammatory cytokines, IL-1Ra and IL-10, in the ASD patients samples (Figures 4.18A and B). On the other hand, the serum levels of IL-1β, IL-6, and TNF-α, pro-inflammatory cytokines did not significantly change, presenting a trend towards up regulation (Figures 4.18 C - E) (IL-1Ra: $t_{(18,36)}$=2.3; P<0.05; IL-10: $t_{(16,15)}$= 2.33; P<0.05).

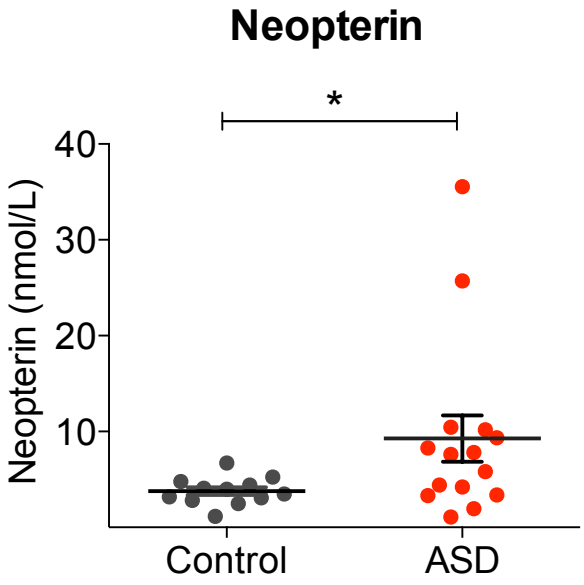


Figure 4.17: Neopterin levels in ASD patients and healthy controls. Neopterin levels were measured in the serum from ASD patients (•) and healthy controls (•) by HPLC. Values are expressed as nmol/L. Horizontal lines represent mean ± SEM of healthy controls (n = 12) and ASD patients (n = 15). *P<0.05 *vs*. controls (In comparing means, the equal variances assumption for t-tests was checked. If not satisfied, a non-equal variance t-test was performed).

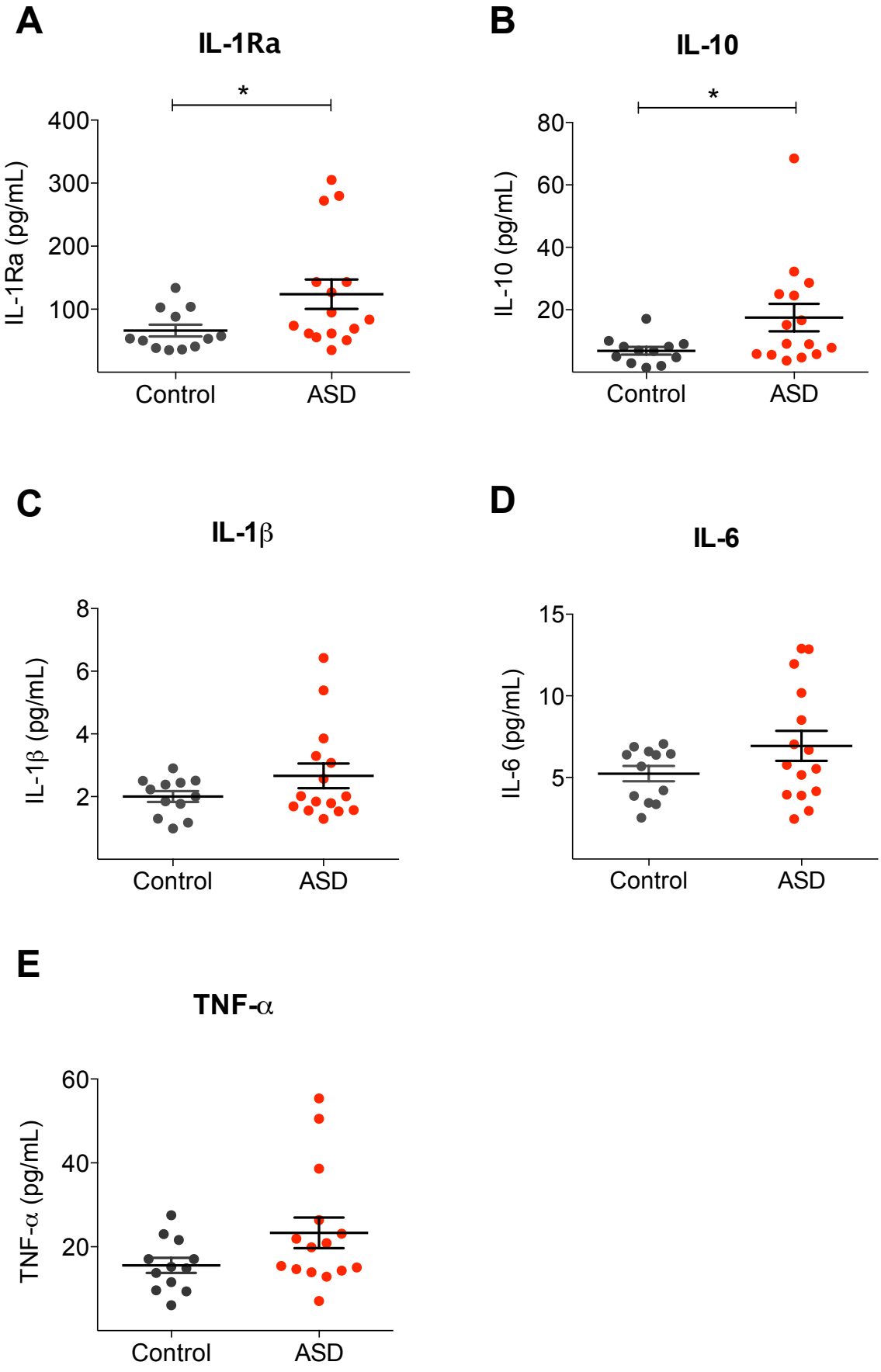

A
IL-1Ra
*
400
300
200
100
IL-1Ra (pg/mL)
Control
ASD
B
IL-10
*
80
60
40
20
IL-10 (pg/mL)
Control
ASD
C
IL-1β
8
6
4
2
IL-1β (pg/mL)
Control
ASD
D
IL-6
15
10
5
IL-6 (pg/mL)
Control
ASD
E
TNF-α
60
40
20
TNF-α (pg/mL)
Control
ASD

Figure 4.18: Inflammatory cytokines levels in ASD patients and healthy controls. The anti-inflammatory cytokines, IL-1Ra (**A**) and IL-10 (**B**), and the pro-inflammatory, IL-1β (**C**), IL-6 (**D**) and TNF-α (**E**), levels were measured in the serum from ASD patients (•) and healthy controls (•) by ELISA commercial kit. Values are expressed as pg/mL. Horizontal lines represent mean ± SEM of healthy controls (n = 12) and ASD patients (n = 15). *P<0.05 *vs*. controls (In comparing means, the equal variances assumption for t-tests was checked. If not satisfied, a non-equal variance t-test was performed).

Chapter 5
Discussion

5.1 Neopterin production under inflammatory conditions and its potential cytoprotective role

The results obtained on this section show that neopterin is produced both in the periphery and in CNS under pro-inflammatory stimuli and mitochondrial toxicity. In addition, they show that neopterin positively modulates cytoprotective systems under cellular stress.

The first results present that the time course of neopterin synthesis occurs in parallel with the pro-inflammatory cytokine IL-1β secretion in the periphery. Under a pro-inflammatory stimulus, activated Th1 cells secrete IFN-γ which activates neutrophils and macrophages to synthesize a series of products during the respiratory burst, including neopterin, NO and ROS, creating an oxidative environment in order to remove pathogens and/or damaged tissues (Huber et al., 1984, Albina et al., 1991, Vaissiere et al., 1999).

Neopterin measurement in biological fluids by HPLC has been performed for the last thirty years and is considered a sensitive and fast method for separation and quantification of this pteridine (Hausen et al., 1982), being considered the gold standard method for neopterin measurement in biological fluids. Therefore, neopterin quantification in the serum was initially performed by HPLC, using a method developed by Werner et al. (1987) with minor modifications (de Lucas et al., 2014), where the maximum serum neopterin secretion was observed 15 min after LPS injection. Even though this method is sensitive, neopterin quantification by HPLC in serum samples can be difficult due to the presence of other analytes that can interfere in the analysis specificity. Thereby, in order to confirm the HPLC

results, neopterin levels in mice serum were also analysed by ELISA, a specific method which involves the use of antibodies against neopterin. Previous studies have already validated neopterin measurement in serum samples by ELISA as a reliable method which provides similar results to HPLC (Nagatsu et al., 1984, Ogiwara et al., 1992, Turgan et al., 2001). Thus, the ELISA results confirm the increase in neopterin synthesis overtime, reaching the maximum peak in 15 min.

Taking into consideration that IL-1β is one of the first cytokines to be secreted in systemic inflammatory conditions (Jiang et al., 1997), we have used this cytokine as a parameter of comparison in order to evaluate neopterin secretion. Neopterin secretion rapidly increased in the serum, before the significant increase in IL-1β secretion (4 h). The slight increase in IL-1β secretion in the serum mice observed in the first time points can be due to the short time of animal's exposure to LPS, agreeing with previous studies, where the maximum concentration of IL-1β in the plasma is observed 4 h after LPS i.p. administration (0.33 mg/kg) (Godbout et al., 2005, Henry et al., 2008, Henry et al., 2009, Ghisoni et al., 2016).

The increase in plasma/serum neopterin and cytokine levels under inflammatory conditions is well established on the literature (Maes et al., 2012, Parker et al., 2013). Considering that recent studies suggest the central neopterin production (Kuehne et al., 2013, Ghisoni and Latini, 2015), we also analysed the LPS-induced neopterin and pro-IL-1β synthesis in mice CNS. Based on previous results from our group which show that the intracerebroventricular administration of neopterin facilitates aversive memory acquisition and the generation of hippocampal LTP (Ghisoni et al., 2016), we decided to focus the investigation on the hippocampus. The results obtained show the rapid neopterin production and increase in *Gch1* expression, suggesting that elevated neopterin levels are due to the increase in the enzyme expression. Previous studies have already shown *Gch1* expression and GTPCH content increase in the mice *locus coeruleus* after 4 and 6 h of LPS systemic administration, respectively (Kaneko et al., 2001, Kaneko et al., 2003), followed by increased BH4 production (Kaneko et al., 2001). According to the literature, neopterin peripherically produced can cross the BBB and the pteridin accumulated in the CSF may reflect serum or plasma levels (Fuchs et al., 1989). However, the temporal profile of neopterin production and secretion in the periphery and hippocampus observed in our study contradict the mechanism of simple difusion proposed for BBB neopterin cross, supporting the hypothesis that the central neopterin production is independent of the periphery. Considering that systemic inflammatory stimuli positively modulate *Gch1* expression in the cerebral tissue without

influencing the PTPS expression (constitutive) (Kaneko et al., 2003), we can conclude that neopterin synthesis is favored in the CNS under inflammatory conditions.

Our results also show that the LPS-induced increase in neopterin levels occured slightly before pro-IL-1β expression increase and in parallel with caspase-1 activation, enzyme responsible for pro-IL-1β proteolytic cleavage and maturation. It was shown, for the first time, that LPS i.p. injection (0.33 mg/kg) induces caspase-1 activation, an indicator of inflammasome activation, in mice CNS. The LPS-induced inflammasome assembly in the brain has already been demonstrated in mice by the increased content of the proteins IL-1 β, active caspase-1 (p20) and NLRP1 in the cerebral cortex (Kaushal et al., 2015), and by increased serum IL-1 β levels (He et al., 2013; Kayagaki et al., 2011). These studies used higher doses of LPS in their experiments (5 – 25 mg / kg; i.p.), around 15 to 75 times higher than those used in our study; however, they also elicited a significant increase of serum IL-1b after 3 h of the LPS administration (25 mg / kg) (He et al., 2013). In addition, the inflammasome activation in the brain has only been investigated after 6 h of mice being challenged to LPS 5 mg / kg (Kaushal et al., 2015), thus this is the first report to show that a peripheral inflammatory stimulus rapidly triggers the complex activation in the brain.

Neopterin levels and *Gch1* expression increased after caspase-1 activation. The increase in neopterin levels in biological fluids have been used as a biomarker in metabolic and/or neurological diseases with an inflammatory component, such as type 2 diabetes, Parkinson's and Alzheimer's disease, among other neurological/neurodegenerative conditions (Widner et al., 2002, Parker et al., 2013, Grossmann et al., 2015). However, the role of peripheral and/or central neopterin synthesis under these conditions is virtually unknown. Recent studies from our research group have shown that neopterin i.c.v. administration improves the resistance to oxidative stress in healthy mice cerebral cortex (Ghisoni and Latini, 2015) and has anti-inflammatory effects in the brain of LPS-treated mice (Ghisoni et al., 2016). Moreover, neopterin (i.c.v.) facilitates aversive memory acquisition and the generation of hippocampal LTP (Ghisoni et al., 2016), described as a persistent increase in synaptic efficiency, which is important for memory formation (Abraham et al., 2002). NLRP3 and NLRP1 inflammasomes activation seems to play a role in the cognitive impairment observed in experimental models caracterized by neurinflammation (Mawhinney et al., 2011, Heneka et al., 2013, Tan et al., 2014). In addition, previous studies have revealed that both caspase-1 and ASC are involved in cognitive impairments induced, respectively, by *Escherichia coli* neonatal infection followed by LPS exposure in the adulthood, and by mutations in β-amyloid precursor protein and presenilin 1, mimicking Alzheimer's disease in mice (Bilbo et al., 2005, Couturier et al., 2016). Considering neopterin beneficial effects

regarding cellular stress, inflammation, and memory and that inflammasome activation is triggered by cellular stress and contributes to inflammation and cognitive impairments, we can hypothesize that neopterin central production may exert an inhibitory role on the inflammasome activation in the CNS.

The consequences of increased synthesis of inflammatory mediators include mitochondrial dysfunction and excessive ROS production (Bolanos et al., 1994, Motori et al., 2013), which may result in inflammasome activation (Zhou et al., 2010).

Therefore, taking into account that (*i*) neopterin was produced in mice hippocampus under an acute inflammatory stimulus; and (*iii*) astrocytes are the most abundant nervous cell in the CNS and express higher content of NOS2 in response to IFN-γ (Salvemini et al., 1992), this study investigated whether astrocytes contribute to neopterin synthesis under cellular stress. Sodium azide was chosen as mitochondrial stressor based on previous data from our group showing that the cellular stress induced by the mitotoxin leads to neopterin secretion in hippocampal slices from neonate rats and rat primary astrocytes supernatants (Ghisoni and Latini, 2015). In addition, this polar toxicant is used at the millimolar range to induce ATP depletion in cultured cells (Schwoebel et al., 2002), mainly because of the week diffusion through the membranes. We evidenced that astrocytes produce and secrete neopterin during conditions of mitochondrial dysfunction, corroborating with the data previously discussed and with previous studies in which this cell type secreted neopterin when stimulated with IFN-γ (Cano et al., 2008). Consequently, we can suggest that astrocytes contribute to neopterin production in the CNS.

The intra and extracellular neopterin effects are not elucidated yet. Some studies have suggested that high concentrations of neopterin contribute to oxidative stress and inflammation in cells from peripheral tissues. Considering the reference values found in healthy population, the concentrations of neopterin used in these studies were 10 to 100.000 times higher and exceed by up to 2000 times the concentrations used in our work. For example, the reference values are between 5 and 8 nmol/L in the CSF and serum (Hagberg et al., 1993, Widner et al., 2002, Casal et al., 2003, Kuehne et al., 2013), while most of these studies used neopterin concentrations ranging from 1 to 1000 μM (Baier-Bitterlich et al., 1995, Schobersberger et al., 1995, Schobersberger et al., 1996, Widner et al., 1998, Hoffmann et al., 1999). According to these studies, neopterin may induce NF-κB activation in murine vascular smooth cells and human coronary endothelial cells (Hoffmann et al., 1996, Cirillo et al., 2006), and NOS2 expression in murine vascular smooth cells contributing to NO production and eventually peroxynitrite synthesis (Schobersberger et al., 1995, Widner et al., 1998). Again, neopterin concentrations used in these works ranged from 1 to 1000 μM.

Finally, neopterin has also been associated with programmed cell death in monocytic cell line and murine alveolar epithelium exposed to concentrations of neopterin up to 1000 μM (Baier-Bitterlich et al., 1995, Schobersberger et al., 1996). Some studies suggest that both neopterin and NO and ROS produced by activated immune cells contribute to host defense, creating an oxidative environment in order to eliminate pathogens and/or remove damaged tissues (Huber et al., 1984, Albina et al., 1991, Vaissiere et al., 1999). Therefore, based on these contradictory results from the literature, neopterin seems to exert a dual role, meaning that high concentrations of the pteridine would be related to pro-inflammatory effects while concentrations slightly above the secreted in pro-inflammatory conditions would exert a neuroprotective role. Other molecules have been described as exerting dual effect such as IL-6, IL-1β, dopamine, among others (Li et al., 2009b, Lampa et al., 2012, Filloux and Townsend, 1993).

In order to investigate the consequences of neopterin secretion, we evaluated neopterin effects in conditions of mitochondrial toxicity in murine primary astrocytes culture. Taking into account the neopterin levels secreted under azide mitotoxicity (35 nmol/L), two concentrations of the pteridine were used in the *in vitro* experiments. One slightly above the neopterin measured in the extracellular medium (50 nM) and one higher (500 nM), which corresponds to the maximum levels of neopterin detected in pathological conditions (Blau et al., 1996, Opladen et al., 2012). The concentrations choice was based on our results showing that crescent neopterin concentrations (0 – 5000 nM) do not change cell viability in C6 astroglial cells or Neuro2a neurons, and on previous results from our group in which neopterin antioxidant properties followed an inverted U curve having its maximal activity at 50 and 500 nM, when tested in vitro in C6 astroglial cells or in rat primary astrocytes. For example, C6 cells co-exposed to 0.1 mM hydrogen peroxide (H_2O_2) plus neopterin (5 – 5000 nM) during 3h showed a significant reduction in the oxidation of the cell-permeant H_2DCFDA, a probe used as an indicator for reactive oxygen species (ROS) in cells. The significant effect was observed only at the intermediate neopterin concentrations 50 and 500nM. Additionally, these two concentrations would correlate with i) 50 nM: the highest value of neopterin within the normal CSF range, and ii) 500 nM: concentrations found in the CSF from individuals affected by inherited metabolic disorders of the tetrahydrobiopterin biosynthesis where neopterin accumulates (deficiency of 6-pyruvoyl-tetrahydropterin synthase deficiency) (Niederwieser et al., 1979, Hagberg et al., 1993, Blau et al., 1996, Widner et al., 2002, Casal et al., 2003, Opladen et al., 2012, Kuehne et al., 2013, Hytonen et al., 2014). The results show that neopterin (50 nM) had a cytoprotective role through the inhibition of ROS production, but did not affect azide-induced mitochondrial dysfunction,

indirectly evaluated by the measurement of lactate production. It is well stablished that azide is an inespecific inhibitor blocking the activities of complexes III and IV and the antioxidant enzymes superoxide dismutase and catalase (Leary et al., 2002; Misra and Fridovich, 1978; Sanchis-Segura et al., 1999). In addition, it is known that mitochondrial dysfunction in astrocytes is efficiently compensated by a rapid activation of the anaerobic glycolysis (Almeida et al., 2004). Thus, we can conclude that neopterin preferably modulates the activity of antioxidant enzymes as a compensatory mechanism to oxidative stress than prevent the respiratory chain inhibition mediated by azide. In addition, both neopterin and azide treatment increased the content of the antioxidant enzyme HO-1, a marker for Nrf2 transcription factor activation. However, they acted by distinct mechanisms. While azide-induced HO-1 content increase was clearly induced by mitochondrial oxidative stress, evidenced by increased lactate and ROS production (Almeida et al., 2004), neopterin exposure only increased HO-1 content. In this context, other highly hydrophilic compounds (like neopterin) such as resveratrol, acetyl-L-carnitine and curcumin have also been associated with protection against oxidative stress and neurotoxicity through increased HO-1 content and/or activity in *in vitro* experimental systems (Calabrese et al., 2005, Gonzalez-Reyes et al., 2013, Quincozes-Santos et al., 2013).

The gene expression of some antioxidant enxymes, including HO-1 and the enzymes involved in GSH synthesis and its redox cycle, GR and GPx, are regulated by the transcription factor Nrf2 through its binding to the ARE sequence (Shih et al., 2003). Besides the increase in HO-1 content, neopterin *per se* also increased the content and activation of Nrf2 in C6 astroglioma culture. This results are in agreement with some data previously published by our group demonstrating that Nrf2 targets are also increased by neopterin. Ghisoni et al. (2016) showed that neopterin intracerebroventricular (0.4 pmol; 1 μL) administration increased the activity of glutathione metabolizing enzymes, including glutathione reductase and glutathione peroxidase in the rodent brain (Ghisoni et al., 2016). As a transcription factor sensitive to the redox status, Nrf2 activation depends on the level of oxidative stress in order to coordinate several biological responses to maintain cellular homeostasis (Shih et al., 2003). In consequence, we can suggest that neopterin production during the respiratory burst in inflammatory conditions may contribute to oxidative stress in immune cells assisting in fighting the pathogen or damaged tissue, but it may also exert beneficial effects in neighbour cells exposed to stress by, for example, activating the Nrf2/ARE pathway. For instance, patients with metastatic colorectal cancer have elevated urine levels of neopterin which is generally associated with an unfavorable prognosis. However, patients who presented reduced neopterin levels at the beginning of the study, had

the neopterin urinary levels raised over the treatment with cetuximab (monoclonal antibody against antigens present in the tumor cells surface, like the epidermal growth factor receptor (Goldstein et al., 1995)), suggesting that the increased immune system activation contributes to cetuximab antitumor effect (Melichar et al., 2014). On the other hand, neopterin also inhibits NADPH oxidase activity and the consequent superoxide production in murine macrophages (Kojima et al., 1993).

The results presented on this section showed that neopterin is synthesized in both periphery and CNS under pro-inflammatory conditions and that astrocytes contribute to the pteridine synthesis. In addition to previous data in which azide induced neopterin secretion in hippocampal slices (Ghisoni and Latini, 2015), the increase in neopterin production and *Gch1* expression in LPS-treated mice hippocampus and azide-treated astrocytes suggest that inflammatory stimulus induces central neopterin production. Moreover, LPS-induced neopterin synthesis in the hippocampus occurred in parallel with caspase-1 activation, suggesting that neopterin synthesis during neuroinflammation may play an inhibitory role in inflammasome activation in the CNS.

Inflammasome activation is essential for anti-microbial responses (Broz et al., 2010, Liu et al., 2012, Hanamsagar et al., 2014); however, controlling its activation is also essencial for cellular homeostasis recovery after an inflammatory response since its persistent activation can trigger the pyroptotic cell death and subsequent tissue degeneration (Fink and Cookson, 2006).

Neopterin has been used as a disease biomarker for the last forty years and is considered an inert excretion product of the *de novo* BH4 pathway. This work demonstrated that neopterin may exert cytoprotective effects under cellular stress induced by mitotoxicity, for instance, reducing ROS production and increasing the antioxidant enzyme HO-1 content through the modulation of Nrf2/ARE pathway in murine primary astrocytes culture. Therefore, it can be concluded that neopterin production and secretion in the CNS under pro-inflammatory conditions exert neuroprotective functions by modulating antioxidants and cytoprotective systems, which can affect inflammasome activation.

5.2. Neopterin effects on inflammasome activation in nervous cells

Considering that *i)* neopterin is produced by cells in the CNS, *ii)* positively modulates antioxidants systems and *iii)* its synthesis is stimulated by inflammatory conditions, we investigated the neopterin preconditioning role (produced previously to the inflammatory stimulus) in inflammasome activation. The results obtained on this section show that the pretreatment with neopterin inhibited the LPS-induced inflammasome activation, evidenced by reduced ASC and pro-caspase-1 content and IL-1β secretion, in human primary nervous cells. Neopterin also favored an anti-inflammatory environment by increasing the anti-inflammatory cytokines IL-10 and IL-1Ra secretion.

Inflammasome activation was induced by LPS (5 μg/mL) in human nervous cells. This concentration corresponds to the minimum concentration required to induce active caspase-1 (p20) secretion in astroglioma C6 supernatant. It is known that LPS can trigger NLRP3 inflammasome activation by a canonical pathway, through ATP secretion induction and subsequent activation of $P2X_7R$ (Piccini et al., 2008), or non-canonical pathway, through caspase-11 activation in mice or its homologs caspase-4/5 in humans (Hagar et al., 2013). Both ASC and caspase-1 activation are essential for pro-IL-1β and pro-IL-18 processing and maturation independently of the NLRP3 inflammasome activation pathway (Yamamoto et al., 2004). Neopterin preconditioning (50 nM) reduced the LPS-induced ASC and pro-caspase-1 content in human primary astrocytes, suggesting that the pteridine may inhibit inflammasome activation *in vitro*. In addition, neopterin also inhibited *CASP1* gene expression induced by LPS in human primary astrocytes and neurons showing that its protective effects would correspond to a common mechanism and would not be restricted to a single cell type. The increased content of ASC and/or pro- and active caspase-1 in human brain tissue has been shown in several diseases characterized by increased levels of neopterin, such as stroke, Alzheimer's disease and AIDS (Fann et al., 2013, Heneka et al., 2013, Kaushal et al., 2015, Walsh et al., 2014). Moreover, caspase-1 expression is involved in neurons and astrocytes death induced by, respectively, glucose deprivation and LPS exposure (Zhang et al., 2003, Alfonso-Loeches et al., 2014). Besides its role in inflammasome activation, caspase-1 can also cleave and activate caspase-6 in human neurons (Guo et al., 2006), enzyme responsible for tau protein cleavage and intracellular neurofibrillary tangles formation (Horowitz et al., 2004), features of Alzheimer's disease (Ishino and Otsuki, 1975). Consequently, inflammasome activation inhibition mediated by neopterin preconditioning in human neurons and astrocytes can be important for tissue homeostasis in pathological conditions.

The inflammasome activation final product is the secretion of active IL-1β and IL-18.

In addition to reducing the content of inflammasome components, neopterin preconditioning also inhibited LPS-induced IL-1β secretion, consistent with the inhibition of inflammasome activation. Other compounds with antioxidant and anti-inflammatory activities were also shown to inhibit inflammasome activation, such as resveratrol, curcumin and hemin (Li et al., 2014, Chang et al., 2015, Gong et al., 2015b, Li et al., 2015). Like neopterin, hemin inhibits NLRP3 inflammasome activation induced by LPS, evidenced by reduced content of NLRP3, ASC and pro-caspase-1 and IL-1β secretion, in human gingival epithelial cells (Li et al., 2014). Resveratrol and curcumin inhibit NLRP3 inflammasome activation, evidenced by reduced caspase-1 activation and IL-1β secretion, in murine macrophages (Chang et al., 2015, Gong et al., 2015). In addition, curcumin also reduces TXNIP expression and inhibits glutamate-induced NLRP3 inflammasome activation, evidenced by reduced NLRP3 and active caspase-1 content and IL-1β secretion, in rat hippocampal slices (Li et al., 2015). We can notice that the common feature between these compounds and neopterin effect in inflammasome activation is the inhibition of IL-1β secretion, reaching around 90 % and 50 % of inhibition, respectively.

The inflammasome only processes the pro-IL-1β previously synthesized in the cell. The activation of TLR4 by LPS triggers the kinases TIRAP (TIR domain-containing adaptor protein), MyD88 and IRAK (IL-1R associated kinase) recruitment, and subsequent NF-κB activation. NF-κB is usually bind to its inhibitor, IκB (κB protein inhibitor), in the cytoplasm. IκB phosphorylation induces its proteasomal degradation and NF-κB release, which translocates to the nucleus, binding to the promoter region of target genes for pro-oxidant and pro-inflammatory mediators (DiDonato et al., 1997), activating the transcription of chemokines and cytokines, receptors and adhesion molecules expression, among other cellular events (Vallabhapurapu and Karin, 2009). Therefore, LPS induces pro-IL-1β, TNF-α and IL-6 expression.

Neopterin effect on LPS-induced cytokines was restricted to IL-1β secretion without affecting TNF-α or IL-6 secretion, cytokines which release does not require processing by the inflammasome. The evidences that *(i)* neopterin only reduced IL-1β and did not change the inflammasome-independent cytokines, TNF-α and IL-6 and *(ii)* neopterin did not significantly affected pro-IL-1β gene expression induced by LPS confirm that neopterin exert its effects modulating inflammasome activation instead of inhibiting NF-κB activation or pro-IL-1β expression. The adaptor protein ASC is essential for both NLRP3 and AIM2 inflammasomes (Schroder and Tschopp, 2010). LPS can induce IL-1β secretion through $P2X_7R$ activation (mediated by secreted ATP) and/or ASC and NLRP3 protein expression in human and/or murine monocytes and dendritic cells (Piccini et al., 2008, Netea et al., 2009, He et al.,

2013a). As a consequence, we can suggest that neopterin inhibits NLRP3 inflammasome.

In addition to inflammasome inhibition, neopterin also increased the anti-inflammatory cytokines IL-10 e IL-1Ra secretion from human primary astrocytes. IL-10 binds to its receptor and activates the Janus kinases (JAK) and subsequently the STATs (signal transducers and activators of transcription) inhibiting TNF-α, IL-1 and IL-6 production (Lai et al., 1996). IL-10 also reduces pro-inflammatory cytokines synthesis in murine microglia and inhibits neuronal death *in vitro* (Lodge and Sriram, 1996, Zhu et al., 2016), probably through the inhibition of LPS-induced NF-κB and MAPK (mitogen-activated protein kinase) activation (Wang et al., 1995, Kontoyiannis et al., 2001). There are three IL-1Ra intracellular isoforms, which can be released during cell death, and one soluble isoform (sIL-1Ra), which can be secreted. All isoforms work as competitive antagonists that bind IL-1 receptor, inhibiting IL-1β and IL-1α binding, without triggering signal transduction (Malyak et al., 1998). The inhibitory properties of sIL-1Ra have already been used for drug development. For instance, Anakinra, a drug used for treatment of diseases in which inflammasome activation plays a role, such as rheumatoid arthritis and auto-inflammatory diseases, like CAPS (Kone-Paut and Galeotti, 2014). Experimentally, Anakinra attenuates the inflammation induced by chronic ethanol ingestion, decreasing cytokines expression and NLRP3-dependent caspase-1 activity in mice cerebellum (Lippai et al., 2013). In addition, IL-1Ra also reduces the number of activated microglial cells and neuronal damage induced by NMDA (N-methyl-D-aspartate) excitotoxicity in hippocampal organotypical culture from newborn rats (Vogt et al., 2008), besides inhibiting LPS-induced caspase-3 activation in neonate rat brain (Cai et al., 2003). It has been demonstrated that both IL-10 and IL-1Ra inhibit inflammasome activation *in vitro* and *in vivo*, respectively. IL-10 inhibits NLRP3 expression, caspase-1 activation and IL-1β secretion in murine macrophages stimulated with LPS+ATP (Gurung et al., 2015), while commercial IL-1Ra (Anakinra) reduces the caspase-1 activation and NLRP3-dependent pro-inflammatory cytokines expression induced by chronic ethanol ingestion in mice cerebellum (Lippai et al., 2013).

Even though the mechanism by which neopterin inhibits inflammasome activation and induces anti-inflammatory cytokines secretion is unclear, Nrf2/ARE pathway activation and subsequent ROS production inhibition may be involved. All known NLRP3 activators, including increased levels of extracellular ATP, cholesterol, increased glucose levels, amyloid-β, oxidized LDL or particulate activators, such as asbestos and silica, trigger the generation of ROS (Petrilli et al., 2007, Halle et al., 2008, Dostert et al., 2009, Duewell et al., 2010, Estruch et al., 2015). The mechanism by which ROS triggers NLRP3 inflammasome activation is not fully elucidated, but most of the studies suggest it may be through TXNIP.

This protein is bound and inhibited by TRX1 in the cytosol, which can be oxidized by ROS, dissociating from and releasing TXNIP to activate NLRP3 receptor leading to its activation (Zhou et al., 2010).

Oxidative stress triggers several cellular responses including Nrf2 activation, which positively modulates the transcription of antioxidant enzymes in order to maintain the cellular homeostasis. However, its role in inflammasome activation is ambiguous. According to Zhao et al. (2014), Nrf2 is essential for NLRP3 and AIM2 inflammasomes activation in murine macrophages and for inflammatory responses induced by peritonitis in mice. Nevertheless, other studies show that Nrf2 activators inhibit inflammasome activation. For example, the bioactive compounds from the green tea and the Chinese medical plant *Litsea cubeba,* respectively, epigallocatechin-3-gallate and citral, activate Nrf2 and inhibit NLRP3 inflammasome activation induced by lupic nephritis in mice glomerular tissue (Tsai et al., 2011, Ka et al., 2015). Moreover, several antioxidants and/or HO-1 inducers have been described with inhibitory activities in NLRP3 inflammasome activation, including ebselen, resveratrol, curcumin and hemin (Jabaut et al., 2013, Chang et al., 2014, Li et al., 2014, Gong et al., 2015a). Furthermore, previous studies have shown that HO-1 triggers IL-10 and IL-1Ra expression in mice liver and hepatocytes and macrophage lineages in an Nrf2 dependent way (Piantadosi et al., 2011). HO-1 induction by hemin also induces IL-10 secretion and prevents oxidative stress in mice hippocampus induced by liver transplant (Wang et al., 2015). Therefore, Nrf2/ARE pathway activation may be a mechanism by which neopterin increases the resistance to oxidative stress in mice brain (Ghisoni and Latini, 2015), the HO-1 content and the anti-inflammatory cytokines secretion, and inhibits azide-induced ROS production and LPS-induced inflammasome activation in astrocytes.

The results presented on this section show that 50 nM was the most effective concentration of neopterin in all parameters analysed, including inhibition of LPS- induced inflammasome activation. This concentration is slightly above the detected on primary astrocytes supernatant (35 nmol/L) under mitotoxicity and neopterin levels in mice CSF when injected i.c.v. (15 nmol/L) (Ghisoni and Latini, 2015, Ghisoni et al., 2016). Therefore, we can suggest that neopterin cytoprotective and cognitive effects can be related to the inhibition of inflammasome activation. In addition, considering that ROS production is a critical event for NLRP3 inflammasome activation, we suggest that Nrf2/HO-1 pathway may be the mechanism by which neopterin induces IL-10 and IL-1Ra secretion, reduces the oxidative stress, and inhibits inflammasome activation.

Nrf2 activation is closely related to the activation of another transcription factor, NF-κB, which modulates chemokines and cytokines production, receptors and adhesion molecules expression, among other important cellular events for the inflammatory response (Vallabhapurapu and Karin, 2009). In order to effectively respond to acute inflammation, NF-κB also induces an increase in mitochondrial activity and NADPH oxidases expression, the main sources of endogenous ROS (Manea et al., 2007, Mauro et al., 2011). Moreover, it has been shown that the NF-κB canonical subunit, p65, induces an increase in Keap1 content, the signalling protein for Nrf2 degradation through the proteasome (Yu et al., 2011). On the other hand, the *Nrf2* gene promoter region has several binding regions for κB, suggesting that NF-κB can activate Nrf2 transcription (Rushworth et al., 2012), which in turn activates the transcription of antioxidant enzymes and reduces pro-inflammatory mediators production (Itoh et al., 1997, Lin et al., 2008). Thus, the coordinated activity of both Nrf2 and NF-κB is essential for the inflammatory status resolution and homeostasis maintenance.

5.3. Neopterin and inflammatory mediators secretion in ASD patients' samples

As we observed, neopterin synthesis overtime is similar to the pro-inflammatory cytokine IL-1β secretion during LPS-induced acute inflammation. Thus, we also analysed the levels of secreted neopterin and anti- and pro-inflammatory mediators in ASD patients' serum.

Our results show that two patients diagnosed with ASD between 3 and 10 years old have substantially elevated serum neopterin levels reaching up to 35 nmol/L. The increased neopterin may be due to immune cells activation and subsequent secretion of IFN-γ and IL-1β, inducers of GTPCH expression and activity (Huber et al., 1984, Franscini et al., 2003). There are few studies on the literature regarding neopterin levels in ASD patients' biological fluids. Sweeten et al. (2003) described elevated neopterin plasma levels in ASD patients around 6 years old (up to 14 nmol/L); but they observed much lower levels of neopterin than we observed in our study, while other studies did not observe differences between ASD patients and healthy controls (Eto et al., 1992). In addition, neopterin levels in ASD patients' CSF are lower (up to 12 nmol/L) in comparison to controls (up to 35 nmol/L). Particularly, this work used patients with other CNS diseases, such as headaches, development delay, among others, as controls (Zimmerman et al., 2005). Considering that these controls were not healthy and that neopterin CSF levels in the normal population are around 5 nmol/L (Kuehne

et al., 2013), we can conclude that neopterin levels in ASD patients' CSF were increased. In contrast, all studies analysing neopterin levels in the urine found increased levels in ASD patients compared with healthy siblings or controls (Harrison and Pheasant, 1995, Messahel et al., 1998). Therefore, our work is one of the first studies to detect elevated neopterin levels in ASD patients' serum. The difference between plasma or serum neopterin values observed in our results (up to 35 nmol/L) compared with others (up to 14 nmol/L) may be due to the late onset of ASD symptoms. When we collected the samples, the participants had recently been diagnosed (3 – 10 years old), while other studies evaluated children in which the symptoms appeared earlier (1 - 2,5 years old) (Zimmerman et al., 2005). According to Ashwood et al. (2011a), children with late symptoms onset have significantly higher levels of inflammatory mediators, like IL-1β, in comparison with children that had autism symptoms since the early childhood. However, the explanation for these observations is still unclear.

Besides neopterin synthesis, IFN-γ produced in inflammatory conditions also induces IDO-1 activity, enzyme that catalyses tryptophan metabolism through the kynurenine pathway (Carlin et al., 1989). The metabolites of this pathway, for instance, quinolinic acid and kynurenic and picolinic acids can have potentially neurotoxic or neuroprotective effects, respectively (Ganong et al., 1983, Lehmann et al., 1983). In addition to the results shown in this thesis, Lim et al. (2015) also observed elevated quinolinic acid and reduced picolinic acid levels in ASD patients' serum, suggesting that both neopterin synthesis and kynurenine pathway may be involved in ASD pathogenesis.

The increase in the kynurenine/tryptophan ratio expresses tryptophan metabolism through the kynurenine pathway. Considering that tryptophan is an essential aminoacid for serotonin and melatonin synthesis, its reduced levels can lead to changes on the serotoninergic synthetic pathway, becoming a potential contributor for developmental alterations. Indeed, serotonin and melatonin levels in ASD patients' serum are lower than in healthy controls (Makkonen et al., 2008), and dietary tryptophan deprivation potentiates autism symptoms (McDougle et al., 1996).

Furthermore, the reduced picolinic acid levels suggest an imbalance on the kynurenine pathway, in which quinolinic acid production is favored. Increased quinolinic acid levels in the CNS, an NMDA receptor agonist able to induce signaling pathway in a glutamate similar way (Stone, 1993), can change the balance between excitatory and inhibitory neurotransmission. It has been suggested that ASD patients have a 'noise' cortical network due to unbalanced excitatory and inhibitory neurotransmission, mediated, respectively, by glutamate and γ-aminobutyric acid (GABA), resulting in alterations on language and social behaviour (Rubenstein and Merzenich, 2003). Indeed, ASD patients have increased glutamate

levels in the serum (Shinohe et al., 2006) and in brain regions related to learning, language and social behaviour, such as the hippocampus-amygdala complex and auditive cortex (Brown et al., 2013), in addition to reduced number of GABA receptors subunits in the frontal cortex (Fatemi et al., 2009). Moreover, ASD patients have increased susceptibility to epileptic events, one consequence of the intrahippocampal lesion induced by quinolinic acid in mice (Schwarcz et al., 1984). Thus, increased quinolinic acid production through the kynurenine pathway can function as an excitotoxin, eventually contributing to ASD pathogenesis, especially during neuroinflammation.

Aside from the neopterin potential anti-inflammatory effects described in our work, inhibition of the inflammasome-dependent IL-1β secretion and increase in IL-10 and IL-1Ra secretion, a recent study has shown that exogenous neopterin also inhibits IDO-1 activity in human mononuclear cells (Schroecksnadel et al., 2013). This study suggests that neopterin synthesis can exert regulatory functions in the immune response, for instance, inhibition of the kynurenine pathway metabolites production and subsequent deleterious effects.

Accordingly with the evidences of inflammation, we also observed elevated anti-inflammatory cytokines, IL-1Ra and IL-10, serum levels in parallel with increased neopterin, and a trend of the pro-inflammatory IL-1β, TNF-α and IL-6 increase on ASD patients' serum. Previous studies suggest that ASD patients have an unbalanced inflammatory mediators' production, with predominant synthesis of pro-inflammatory cytokines (Jyonouchi et al., 2001, Ashwood et al., 2011a). ASD patients have increased plasma levels of IL-6, IL-1β and/or TNF-α, with no changes in IL-10 levels, in comparison with healthy or development delayed controls (Jyonouchi et al., 2001, Ashwood et al., 2011a). Interestingly, patients with late onset of ASD symptoms had increased IL-1β levels compared with patients with early onset (Ashwood et al., 2011a). Moreover, it has been shown elevated caspase-1 expression in blood mononuclear cells from ASD patients (Siniscalco et al., 2012), suggesting that inflammasome activation may be involved in the disease pathogenesis. Furthermore, activated microglia and astrocytes have been observed in ASD patients' (4-55 years old) cerebral tissue, in addition to increased gene expression of IL-6 and IL-10 in the anterior cingulate gyrus and IFN-γ levels in the CSF (Vargas et al., 2005). Elevated levels of IL-6 and TNF-α have also been detected in ASD patients' cerebral cortex compared with other patients or healthy controls (Li et al., 2009a); demonstrating that different ASD patients can have heterogeneous inflammatory responses. This difference on the profile of anti- and pro-inflammatory mediators' production is considered common among studies, since their levels can change depending on the disease severity and association with other pathologies, for example, gastrointestinal problems, substantially common in ASD patients (Napolioni et al., 2013).

The increase in neopterin, IL-10 and IL-1Ra observed in our study may be negatively modulating IL-1β, TNF-α and IL-6 synthesis. Other studies have demonstrated elevated plasma IL-1Ra levels in ASD patients (Zimmerman et al., 2005), suggesting that it can represent a response against chronic inflammation (Suzuki et al., 2011). Moreover, the production of anti- and pro-inflammatory cytokines seems to be associated, respectively, with less or more severe behavioural symptoms of autism. For instance, it has been shown that, when stimulated with pro-inflamamtory triggers, cells from ASD patients produce more IL-10, IFN-γ and TNF-α than cells from healthy controls. Furthermore, patients who produced more IL-10 were characterized by better cognitive development, evidenced by improvement on oral communication, while patients who produced more IFN-γ and TNF-α were characterized by more intense stereotype behaviour determined by the Autism Diagnostic Interview-Revised (ADI-R) (Ashwood et al., 2011b).

On the other hand, we could suggest that there is an exacerbated anti-inflammatory response represented by increased IL-10. This is in line with previous reports showing that IL-10 is abnormally high in other neurological conditions and can be deleterious when overproduced. For instance, excessive levels of IL-10 were found in brain tissue from Alzheimer's disease patients and its overproduction impaired the phagocytosis of amyloid-β peptides by microglia, leading to an unbalanced immune response (Guillot-Sestier et al, 2015). The marked anti-inflammatory response in these patients might be induced by a solid inflammatory status, since the production of pro-inflammatory cytokines in autism individuals appears to be associated with more severe behavioral symptoms of autism (Ashwood et al., 2011). In agreement, subjects included in this study presented a high Childhood Autism Rating Scale score.

The results presented in this section show that ASD patients have increased anti-inflammatory cytokines serum levels and that two patients classified as severe presented substantially high neopterin levels. Unfortunately, the small sample size of the study limited the assessment of the correlation between neopterin levels and autism severity. However, those results can be related to a response against or improvement of the inflammatory response under neopterin-induced cellular stress.

Chapter 6
Final Conclusions

It has been observed that inflammatory conditions lead to rapid increase in peripheral and central neopterin synthesis in parallel with inflammasome activation in the hippocampus. Moreover, it was observed that astrocytes contribute to neopterin synthesis in the CNS.

Besides being an immune system biomarker, neopterin production may be a response against inflammation, reducing ROS production and increasing Nrf2 activation and subsequent HO-1 content. Therefore, the modulation of antioxidant systems may be the mechanism by which neopterin inhibits inflammasome activation in nervous cells. Taking into account that astrocytes are resident nervous cells essential for synaptic formation and function, and neuronal survival and death, controlling cytokine secretion from astrocytes may be an important tool in neuroinflammatory conditions. Furthermore, increased neopterin and anti-inflammatory mediators were observed in ASD patients' serum, probably representing a beneficial response to inflammation. Considering that neopterin inhibited inflammasome activation and increased anti-inflammatory cytokines secretion *in vitro*, we can suggest that neopterin production in chronic inflammatory conditions, like ASD, may exert neuroprotective effects.

Chapter 7
Future Directions

In order to improve our understanding on neopterin biology and effect on inflammasome activation, we will further:

- Investigate whether the NLRP3 inflammasome is activated in blood mononuclear cells from ASD patients and if it is affected by neopterin production. To achieve this objective, we will analyse NLRP3, ASC and caspase-1 content and IL-1β and neopterin secretion and evaluate their correlation.

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Appendix

Office of the Deputy Vice-Chancellor
(Research)

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ABN 90 952 801 237

MACQUARIE University
SYDNEY·AUSTRALIA

15 December 2017

Dear Professor Guillemin

Reference No: 5201600719

Title: *Human foetal tissue biobank for the investigation of physiological and pathological mechanisms involved in human development and diseases*

Thank you for submitting the above application for ethical and scientific review. Your application was considered by the Macquarie University Human Research Ethics Committee (HREC (Medical Sciences)).

I am pleased to advise that ethical and scientific approval has been granted for this project to be conducted at:

- Macquarie University

This approval is subject to the following conditions as determined by the HREC (Medical Sciences) Executive:

- The approved sources of Foetal Tissue are:
  - Clinic66 31 Bertram St, Chatswood
  - All other sources of Foetal tissue must be reviewed and approved by the HREC
- For Macquarie University Researchers (internal) accessing samples from the Foetal Tissue Biobank to conduct research that fits within the scope of current HREC approval, approval will be sought from the Foetal Tissue Biobank Committee. The Biobank committee will provide quarterly reports to the HREC on the research being conducted using the samples and the investigators involved.

- For Macquarie University Researchers (internal) accessing samples from the Foetal Tissue Biobank to conduct research that does not fit within the scope of current HREC approval, ethics approval must be sought from the MDS HREC.

- For External researchers wishing to access samples from the Foetal Tissue Biobank, Ethics approval will be sought. The Biobank committee will provide the MDS HREC with:

  - Information on the research being conducted using the samples

  - An MTA covering transfer of samples

- A copy of the external institutions ethics application and ethics approval covering use of samples.
- This will be done for each request made by an external researcher and research will not commence using the samples until approval by the MDS HREC has been finalised.

This research meets the requirements set out in the *National Statement on Ethical Conduct in Human Research* (2007 – Updated May 2015) (the *National Statement*).

Standard Conditions of Approval:

1. Continuing compliance with the requirements of the *National Statement,* which is available at the following website:

http://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research

2. This approval is valid for five (5) years, subject to the submission of annual reports. Please submit your reports on the anniversary of the approval for this protocol.

3. All adverse events, including events which might affect the continued ethical and scientific acceptability of the project, must be reported to the HREC within 72 hours.

4. Proposed changes to the protocol and associated documents must be submitted to the Committee for approval before implementation.

It is the responsibility of the Chief investigator to retain a copy of all documentation related to this project and to forward a copy of this approval letter to all personnel listed on the project.

Should you have any queries regarding your project, please contact the Ethics Secretariat on 9850 4194 or by email ethics.secretariat@mq.edu.au

The HREC (Medical Sciences) Terms of Reference and Standard Operating Procedures are available from the Research Office website at:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics

The HREC (Medical Sciences) wishes you every success in your research.

Yours sincerely

Professor Tony Eyers
Chair, Macquarie University Human Research Ethics Committee (Medical Sciences)

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research* (2007) and the *CPMP/ICH Note for Guidance on Good Clinical Practice*.

Details of this approval are as follows:

Approval Date: XX

The following documentation has been reviewed and approved by the HREC (Medical Sciences):

Documents reviewed	Version no.	Date
Macquarie University Ethics Application Form		Revised version received 28/10/17
Correspondence responding to the issues raised by the HREC (Medical Sciences)		Received 28/10/16, 25/11/17
MQ Participant Information and Consent Form (PICF) entitled Human foetal tissue biobank	7	12/12/16
HFT Biobank Proposal Template	1	Received 28/10/16
Members of HFT Biobank Committee	1	Received 28/10/16
Workflow for HREC		Received 28/10/16
Letter confirming Clinic66 as source of tissue		Received 21/11/16

MACQUARIE University

ROBERTA DE PAULA MARTINS <roberta.de-paula-martins@students.mq.edu.au>

Animal Ethics Application 5201700249 - Outcome of AEC Meeting<%=Approval Date%>

animal.ethics@mq.edu.au <animal.ethics@mq.edu.au> Wed, Apr 12, 2017 at 3:05 AM
To: roberta.de-paula-martins@students.mq.edu.au

Dear ,

Your notification of collaboration for the following project was considered and noted by the Animal Ethics Committee at the meeting of 12/04/2017

5201700249 - (Collaboration)(PP00760) Modulation of pterines cerebral metabolism as neuroprotective tool in neurodegenerative diseases

Decision
The Committee noted the collaborative report.

This email serves as official notification of the AEC decision. Please keep a copy for your records. Should you have any queries or require clarification, please contact the AEC Secretariat.

Regards,

Chair/Deputy Chair
Animal Ethics Committee

Office of the Deputy Vice Chancellor (Research)
Animal Ethics Secretariat
Research Office
Level 3 C5C Research Hub East
Macquarie University NSW 2109

+61 2 9850 7758
animal.ethics@mq.edu.au

Resultado de Solicitação de Protocolo

Protocolo
PP00760
Título
Modulação do metabolismo cerebral das pterinas como ferramenta neuroprotetora em doenças neurodegenarativas
Data de Entrada
08/05/2012

Resultado:
Aprovado
Data/Prazo
16/10/2015

Considerações
Oficio nº 89/CEUA/PROPESQ/2015
Do: Presidente da Comissão de Ética no Uso de Animais- CEUA
Ao(à): Prof(a) Dr(a), Alexandra Susana Latini, Departamento de Bioquímica - CCB

Prezado(a) Professor(a),
Em relação ao protocolo de pesquisa sob sua responsabilidade a CEUA deliberou o seguinte:

- APROVADO o adendo de suplementação de oito ratos (Rattus Norvegicus).

Adita-se o Oficio nº 007/CEUA/PROPESQ/2015
Do: Presidente da Comissão de Ética no Uso de Animais- CEUA
Ao(à): Prof(a) Dr(a), Alexandra Susana Latini, Departamento de Bioquímica - CCB

Prezado(a) Professor(a),
Em relação ao protocolo de pesquisa sob sua responsabilidade a CEUA deliberou o seguinte:

- APROVADO o adendo com as novas metodologias e suplementação de trinta ratos (Rattus Norvegicus).

Adita-se o Oficio nº 83/CEUA/PRPE/2012
Do: Presidente da Comissão de Ética no Uso de Animais-CEUA
Ao(à): Prof(a) Dr(a) Alexandra Susana Latini, Departamento de Bioquímica - CCB

Prezado(a) Professor(a),

Em relação ao protocolo de pesquisa sob sua responsabilidade a CEUA deliberou o seguinte:

-- APROVADO ad referendum, por quatro anos, para a utilização de seiscentos e trinta e seis camundongos (Mus musculus) e trezentos e quatorze ratos (Rattus Norvegicus).

- Procedência do animal: Biotério Central da UFSC

Por ocasião do término desse protocolo, DEVERÁ SER APRESENTADO RELATÓRIO detalhado relacionando o uso de animais no Projeto desenvolvido aos resultados obtidos, conforme formulário ON LINE CEUA.

Atenciosamente,

Relatório Final previsto para (90 dias após término da vigência do protocolo ou no momento da apresentação de um novo protocolo)
Data 02/01/2015

Data 16/10/2015

Parecer(es):

Abrir Solicitação | Criar Relatório

Parecer1_PP00760.pdf Parecer2_PP00760.pdf Resposta_pesquisador_PP00760.pdf Parecer_final_PP00760.pdf Adendo_PP00760.pdf Parecer_PP00760.pdf

Adendo_Solicitação_Pesquisador_PP00760.pdf Adendo_Parecer_PP00760.pdf