uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Wafa M. Juma

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Neuroscience)

GRADE / DEGREE

Department of Neuroscience

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

White Matter Lesions, C-Reactive Protein and Microglia: A Putative Relation

TITRE DE LA THÈSE / TITLE OF THESIS

Antoine Hakim

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Antonio Colavita

Ruth Slack

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

White Matter Lesions, C-reactive protein and Microglia: A Putative Relation.

Wafa M. Juma
4698919

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the MSc degree in Neuroscience Program

Neuroscience Depatment
Faculty of Medicine

Your file Votre référence
ISBN: 978-0-494-61206-4
Our file Notre référence
ISBN: 978-0-494-61206-4

ABSTRACT

White matter lesions (WML) are a clinically significant, common feature of the ageing brain associated with cognitive decline and depression. Recent research has been focused on identifying parameters which may have a correlation with WML. To this effect the acute phase protein, C-reactive protein (CRP) was shown to have an increased plasma level with progressive WML in human studies. However, due to the limitations of human-based research the expression of CRP from brain tissue demonstrating progressive WML over a given period has not been reported. Our results have shown elevated mRNA and protein expression of CRP from rat brain tissue manifesting features of WML. Moreover, we have demonstrated that microglia, cultured from rat brain, are a source of mRNA and protein expression of CRP. We suggest that this key novel relation could be a target for future therapeutic interventions in diseases where WML and microglial activity are prominent features.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

2 VO 2 Vessel Occlusion

ARIC Atherosclerosis Risk In communities Study

A-β Amyloid Beta

BBB Blood Brain Barrier

BCCAL Bi-Lateral Common Carotid Artery Ligation

BDNF Brain Derived Neurotrophic Factor

BI Brain Infarct

CAA Cerebral Amyloid Angiopathy

CgA Chromogranin A

cGMP cyclic Guanosine Monophosphate

CI Confidence Interval

CNS Central Nervous System

CRP C-reactive protein

CRP 3 Complement Receptor 3

DSCL Deep Subcortical Lesions

eNOS endothelial Nicotine Oxide Synthase

EP E Prostaniod

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GDNF Glial Derived Neurotrophic Factor

GFAP Glial fibrillary Acidic Protein

HRP Horse Radish Peroxidase

LA Leukoariosis

MHC Major Histocompatibility Complex

MRI Magnetic Resonance Imaging

NGF Nerve Growth Factor

OR	Odds Ratio
PG	Prostaglandin
PVH	Periventricular Hyper intensities
RSS	Rotterdam Scan Study
SBI	Silent Brain Infarct
SVD	Small Vessel Disease
SWML	Sub cortical White Matter lesions
TLR4	Toll Like Receptor 4
TP	T Prostaniod
WM	White Matter
WMD	White Matter Disease
WMH	White Matter Hyperintensities
WML	White Matter Lesions

ACKNOWLEDGEMENTS

My appreciation is first directed to my supervisors Dr. C.S. Thompson and Dr. A. M. Hakim for providing me with the opportunity to perform this exciting study in their laboratory. Their encouragement and support have allowed for the completion of my Master's thesis. I would also like to acknowledge my committee members Dr. A. Colavita and Dr. M. Schlossmacher for their constructive criticism and comments.

I would like to further express my gratitude to all my colleagues, students and laboratory members for their hospitality and greatly appreciated assistance with various aspects of my study namely: Arman Lira, Juliet Rashidian, Heather Boston, Zohreh Galehdar, Maxime Rousseaux, Jack Nehme, Julianna Ng, Paul Manninger, Hiba Tu-Allah Yousef, Ali Marzuk, Reema Kamal, Fatemah Kamkar, Nasrin H.Babadi, Zaynab Marzuk, Tatiana Souslova and Reza Hamid Edrissi. My sincere appreciation to Head Technician, Kim Wong and to the Histopathology Department members: Chief Technical Officer, Louise Pelletier and Technologist, Li Dong for their outstanding assistance.

My deepest thanks go to my family Ali, Zaynab, Mohammed and Dr. Wahib O. Marzouk for their patience and unconditional support throughout my years of study. Thank you.

INTRODUCTION

The Cerebral White Matter

Neuroanatomy

In recent years white matter lesions (WML) have become a highly prominent clinical issue due to their impact on neurological function and the consequent associated cognitive decline and depression. Knowledge of the essential anatomy of the cerebral white matter is important in the understanding of the neurological and neuropsychiatric diseases accompanying WML.

At the microscopic level, cerebral white matter consists of collections of closely apposed axons that are wrapped concentrically in myelin, the insulation of nerve cells made up of roughly 70% lipid and 30% protein. Oligodendrocytes, the glial cells that myelinate the axons, are numerous, as are astrocytes, ependymal cells, and blood vessels. Macroscopically, the white matter can be seen to form fiber collections known as tracts, fasciculi, bundles, peduncles, and lemnisci (Filley CM, 2005).

Galen's (AD 129–130 to 200–201) identification of the corpus callosum (Garrison FH, 1969) was perhaps the first recognition of a major fiber bundle, but it was not until the scientific renaissance of the 17th century that it became apparent that the WM was not an amorphous mass but rather consisted of distinct fibers (Schmahmann JD & Pandya DN, 2006, 2007).

The clinical relevance of association pathways was introduced by Carl Wernicke's (1848–1900) description of conduction aphasia from what he believed to be the arcuate fasciculus, and Joseph Jules Dejerine's (1849–1917) account of alexia without agraphia

from lesions that involved the left occipital pole in addition to the splenium of the corpus callosum. Disconnection syndromes were first emphasized in the modern era by Norman Geschwind (1926–1984) and provided clinical and neuroanatomical impetus to the emergence of behavioral neurology as a discipline (Schmahmann JD *et al.*, 2008).

The great complexity of connections and pathways arising from the cerebral cortex can be reduced to a relatively simple schema (Fig. 1). There is a general principle of brain organization (Schmahmann JD & Pandya DN, 2006) that every area of the neocortex is linked with other cortical and subcortical areas by pathways grouped into five fiber bundles, identified as follows (Schmahmann JD *et al.*,2008) :

1. Association fibers travel to other ipsilateral cortical areas.

2. Striatal fibers course to the basal ganglia. There is a confluence of fibers (termed the cord) that divides into:

3. Commissural fibers (composed of anterior commissure, corpus callosum, and hippocampal commissures) that pass to the contralateral hemisphere, and another contingent of the cord, the subcortical bundle of projection fibers (composed of internal capsule, sagittal stratum, and thalamic peduncles), that segregates into

4. Thalamic fibers, and

5. Pontine fibers that descend to the diencephalon, pons, and other brain stem structures, and/or the spinal cord (Schmahmann JD *et al.*, 2008).

According to Schmahmann JD *et al.* (2008), and from what has been stated it is clear that the distributed neural circuits that subserve behavior are topographically linked in a highly precise manner by the above mentioned five major groupings of fiber tracts. Lesions of association fibers prevent communication between cerebral cortical areas engaged in

different domains of behavior. Lesions of subcortical structures, or the projection/striatal fibers that link them with the cerebral cortex, disrupt the contribution of subcortical nodes to the ultimate behavior. Disconnection syndromes may thus be regarded as resulting not only from lesions of the cerebral cortex but also from lesions of subcortical structures themselves, and of the WM tracts that link the nodes that make up the distributed circuits. The nature and the severity of the clinical manifestations of subcortical and WM lesions are determined, in large part, by the location, extent, and timing of onset of the underlying pathology. Discrete neurological and neuropsychiatric symptoms result from focal WM lesions. Cognitive impairment across multiple domains—WMD—is now recognized in the setting of diffuse WM disease (Schmahmann JD *et al.*, 2008).

From this brief anatomical review it can be clearly understood how the disruption of the fiber pathways that constitute the cerebral WM can produce neurological and neuropsychiatric diseases.

Cerebral White Matter Disorders

The highly susceptible cerebral WM is the centre of a wide variety of neuropathological disorders. The history in a particular patient, the results of the clinical examination, and specifically targeted laboratory investigations will often lead to the correct diagnosis and MRI has proven to be invaluable in the study of these disorders because it discloses structural aspects of WM systems *in vivo* with great clarity (Schmahmann JD *et al.*, 2008). The most useful means of classifying WM disorders is by careful analysis of the specific neuropathology, which reveals an impressive range of diseases, injuries, and intoxications to which the WM is vulnerable (Schmahmann JD *et al.*, 2008). The cerebral white matter

disorders can be classified as genetic, demyelinative, infectious, inflammatory, toxic, metabolic, vascular, traumatic, neoplastic, or hydrocephalic (Filley CM, 2001). Each category involves a fundamentally distinct pathophysiologic basis, and even the entities within these categories can vary in many clinical and neuropathologic respects, nevertheless, a consideration of the white matter disorders as whole points out commonalities in their effects on neurobehavioral function (Filley CM, 2001).

Few disorders damage only the WM, and there is usually some combination of gray matter (GM) and WM neuropathology (Schmahmann JD *et al.*, 2008). Remarkably, after careful review of available clinical literature, all of these disorders -of which there are more than 100-can be seen to be associated with some form of cognitive or emotional dysfunction (Filley CM, 2001). This observation alone validates the emphasis on white matter; in addition, similarities in neurobehavioral dysfunction cut across all etiologic categories, further justifying an emphasis on the white matter as a substrate of higher function in humans (Filley CM, 2005).

Since our research interest lies in WM lesions as a manifestation of cerebral small vessel disease our focus will be on the association between vascular disease, aging and WM lesions.

WM Lesions, Aging and Cerebral Small Vessel Disease

Cerebral small vessel disease (SVD) is a major cause of vascular cognitive impairment and dementia (Roman GC *et al.*,2002).White matter hyperintensities (WMH) and lacunes are frequently observed in elderly subjects and are considered to be the main MRI representatives of SVD (De Leeuw FE *et al.*,2001). WM lesions referred to radiologically

as "Leukoariosis"(LA) can be visualized on CT as areas of hypoattenuation (Fig. 2),whereas MRI has greater sensitivity and reveals WM lesions that may not be identified on CT and appear as hyperintensity (WMH) on T2-weighted and FLAIR images. These WMHs are distinguished from infarction by the absence of well-defined hypointensity on T1-weighted images (Schmahmann JD *et al.*, 2008). Periventricular regions are most commonly affected, particularly around the frontal and occipital horns and in severe cases there is a halo of WMH surrounding the lateral ventricles (DeCarli C *et al.*, 2005).

The white matter manifests a unique pattern of development through the life span. In early life, brain myelination is not complete for many years, perhaps past the second decade, in contrast to the full complement of brain neurons, which are present at birth (Filley CM, 2001). Conversely, in the later decades of life, there is slow but steady loss of white matter that may in fact be more crucial than the loss of neurons, which now is thought to be not as pronounced as once believed (Filley CM, 2001).

Important clinical implications of this developmental trajectory may soon emerge. In children and adolescents, for example, the maturation of white matter, particularly in the frontal lobes, has been proposed to correlate with the acquisition of mature aspects of personality such as motivation, comportment, and executive function (Filley CM, 1998, 2001). These attributes, well known to reflect the function of the frontal lobes, may signify the myelination of white matter tracts connecting the frontal lobes to other brain regions and thus contributing to the mature behavioral repertoire of adults (Filley CM, 1998, 2001). In addition, a variety of psychiatric disorders in childhood may be influenced by abnormal white matter maturation (Durston S, 2001).

In contrast, the aging process may involve a progressive diminution of white matter. In an autopsy study of normal brains from age 20 to 90 years, white matter volume loss was 28%, whereas neocortical volume loss was just 12% (Pakkenberg B, 1997). This selective tissue loss may account for normal cognitive changes in aging such as slowed speed of information processing, diminished attentional capacity, and forgetfulness (Filley CM, 1994). Normal age-related changes in white matter loss may also contribute to the development of mild cognitive impairment (Petersen RC *et al.*, 1999), a controversial concept recently proposed to represent a transitional stage between normal aging and dementia. Although mild cognitive impairment most often is considered a precursor of Alzheimer's disease (Petersen RC *et al.*, 1999) the neuropathological basis of mild cognitive impairment is unclear and may be explained by white matter changes in some individuals (Maruyama M *et al.*, 2004). Filley CM (2005) suggests that even if mild cognitive impairment becomes established as a harbinger of Alzheimer's disease, age-related changes in white matter cannot be ignored as having a potential impact on cognitive function in older persons. Normal aging changes in myelin must also be considered when the impact of superimposed disease or injury, such as infarction or traumatic brain injury, further damages the white matter (Filley CM, 2005).

Pathophysiology

Cerebral small-vessel disease is thought to cause ischemia through vascular stenosis, occlusion, or impaired reactivity producing the WM changes (Schmahmann JD *et al.*, 2008). Histopathology shows demyelination with various degrees of axonal loss and gliosis, consistent with injury to the myelin or oligodendrocyte, but this has not helped

determine the underlying causes (Fernando MS *et al.*, 2004). The tissue pathology consists only of nonspecific injury without evidence of frank infarction, although lesions show immunoreactivity for hypoxia-inducible factor 1, which is expressed in the presence of ischemia (Fernando MS *et al.*, 2006).

Hemispheric WM blood supply is derived predominantly from penetrating branches of the middle cerebral artery stem or from penetrating branches of circumferential arteries coursing over the hemispheric surface (Pantoni L & JH Garcia, 1997).The few millimeters of WM adjacent to the wall of the lateral ventricle represent a distal endzone territory of blood supply from the choroidal arteries (Schmahmann JD *et al.*, 2008). Blood flow studies show this to be a low perfusion region, and the fact that it is the most frequent site of WMH involvement possibly reflects a vulnerability to blood flow reduction (Holland CM *et al.*, 2008). Brain regions with higher burden of WMH in demented subjects show decreased blood flow and metabolism, as well as increased oxygen extraction indicative of hypoperfusion (De Reuck JD *et al.*, 1998, Tohgi H *et al.*, 1998). Blood flow disturbances are less severe in the non demented (ten Dam VH *et al.*, 2007).

Relevance of WML

WML have been identified as a predictor of stroke (Kuller LH *et al.*, 2004).Moreover, marked periventricular hyperintensity (PVH) and marked subcortical white matter lesions (SWML) are significant risk factors for subsequent stroke, but only marked PVH is a strong risk factor for mortality (Bokura H *et al.*, 2006).

Population-based studies indicate that the occurrence of WML is high. In the Atherosclerosis Risk in Communities Study (ARIC) of persons 55-72 years, the prevalence

of any white-matter lesions increased from 88% in 55-year-olds to 92.2% in 65-year-olds. Severe lesions were already present in 6.5% of 55-year olds and 19.5% in 65-year-olds (Liao *et al.*, 1997). In the Rotterdam Scan Study (RSS) of persons 60-90 years of age, 95% of the participants had some WML. There was a steady increase in white matter lesion load with age, with a higher load in the frontal lobe compared to the other lobes (de Leeuw FE *et al.*, 2001). Based on the available studies, it is unclear as to whether there are sex differences in WML. In the RSS, there was a slightly higher load in women, and in the ARIC study, there was a slightly higher prevalence in men (Liao D *et al.*, 1997).

Consequently, studies of WMH in older persons are focused on determining variability in extent of WM lesions rather than their presence or absence. The strongest risk factors for a greater extent of WMH are age, hypertension, diabetes, and smoking (de Leeuw FE *et al.*, 2002, Longstreth WT Jr. *et al.*, 1996), whereas systemic measures of atherosclerosis, such as internal carotid artery plaques, are weakly associated. Retinal vascular changes (Longstreth WT Jr. *et al.*, 2007) and indices of renal function (Khatri M *et al.*, 2007) are closely associated with WMH, possibly reflecting the presence of shared risk factors for small vessel disease. Serum studies show associations between WMH, or their progression, and markers of endothelial dysfunction [serum homocysteine and intercellular adhesion molecule 1] (Hassan A *et al.*, 2004), thrombogenesis [thrombomodulin and fibrinogen] (Breteler MM *et al.*,1994; Schmidt R *et al.*, 1997), inflammation [C-reactive protein] (Hassan A *et al.*, 2004) and antioxidant levels (Schmidt R *et al.*, 1997).A link with β-amyloid metabolism is shown by associations with either increased serum A-β211 (van Dijk EJ *et al.*, 2004), or decreased cerebrospinal fluid A-β (Stenset V *et al.*, 2006). The basis for these findings is unknown but might be related to the presence of cerebral amyloid

angiopathy (CAA) (Gurol ME *et al.*, 2006). Despite these known risk factors, much of the variance in age-related WMH remains unexplained and may be accounted for by genetic factors (Carmelli D *et al.*, 1998).

An Animal Model of Chronic Hypoperfusion Exhibiting WML

One of the first reports to demonstrate WM changes after chronic hypoperfusion due to stenosis of the common carotid arteries in a gerbil was by Hattori H *et al.* (1992).From these and other findings Wakita H *et al.* (1994) described glial activation and WM changes (rarefaction of the WM) in a rat model of chronic hypoperfusion induced by permanent bilateral ligation of the common carotid arteries (BCCAL).

Bilateral common carotid artery ligation (also referred to as'2-VO') in rats produced a chronic reduction in cerebral blood flow by 50% to 70%; (Ni J *et al*, 1994; Wakita H *et al*, 1995, 2002; Ohta H *et al.*, 1997; Ueno M *et al.*, 2002). Lesions were predominantly in white matter, with vacuolation of myelin, axonal damage, and demyelination in corpus callosum, internal capsule, and caudate putamen. Lesions were apparent from 7 days after occlusion and were persistent (Wakita H *et al*, 1995, 2002; Farkas E *et al*, 2004), preceded by temporary blood-brain barrier (BBB) opening in white matter areas with collagen deposition in vessel walls (Ueno *et al.*, 2002). Cognitive impairment was seen, with persistent learning deficit in Morris water maze and eight arm radial maze tasks (Ni J *et al.*, 1994; Ohta H *et al.*, 1997).

Hainsworth and Markus (2008) stated after a review of literature that rat BCCAL models are potentially useful in studying diffuse WML. For the purpose of our research and since our interests lie in correlating certain indicators (microglial activation, CRP) with WML we

have reproduced a rat model of chronic hypoperfusion through BCCAL as previously described by Wakita H *et al.*, 1994 and demonstrated through immunohistochemistry some of the relevant histopathological findings associated with it.

C - reactive protein

CRP: An Acute Phase Plasma Protein

The discovery of C-reactive protein (CRP) was reported in1930 by Tillett and Francis in Oswald Avery's laboratory (Tillet WS *et al.*, 1930).They were investigating serological reactions in pneumonia with various extracts of pneumococci and observed that a non-type-specific somatic polysaccharide fraction, which they designated fraction C, was precipitated by the sera of acutely ill patients. After the crisis the capacity of the patients' sera to precipitate C polysaccharide (CPS) rapidly disappeared, and the C-reactive material was not found in sera from normal healthy individuals (Pepys MB, 1981).

Avery and his collaborators characterized the C-reactive material as a protein which required calcium ions for its reaction with CPS and introduced the term "acute phase" to refer to serum from patients acutely ill with infectious disease and containing the C-reactive protein (Abernethy TJ *et al.*, 1941; MacLeod CM *et al.*, 1941). In 1971, Volanakis and Kaplan identified the specific ligand for CRP in the pneumococcal C polysaccharide as phosphocholine, part of the techoic acid of the pneumococcal cell wall (Volanakis JE *et al.*, 1971). Although phosphocholine was the first defined ligand for CRP, a number of other ligands have since been identified. In addition to interacting with various ligands, CRP can activate the classical complement pathway, stimulate phagocytosis, and bind to immunoglobulin receptors (FcγR), (Black S *et al.*, 2004).

In humans, plasma levels of CRP may rise rapidly and markedly, as much as 1000-fold or more, after an acute inflammatory stimulus (Black S *et al.*, 2004), largely reflecting increased synthesis by hepatocytes (Hurlimann J *et al.*, 1966).

CRP induction is part of a larger picture of reorchestration of liver gene expression during inflammatory states, the *acute phase response*, in which synthesis of many plasma proteins is increased, whereas that of a smaller number, notably albumin, is decreased. At least 40 plasma proteins are defined as acute phase proteins, based on changes in circulating concentration of at least 25% after an inflammatory stimulus (Black S *et al.*, 2004). This group includes clotting proteins, complement factors, anti-proteases, and transport proteins (Samols D *et al.*, 2002). These changes presumably contribute to defensive or adaptive capabilities (Black S *et al.*, 2004).

CRP Structure

Human CRP consists of five identical, noncovalently associated protomers ≈23-kDa arranged symmetrically around a central pore (Fig.3). The term "pentraxins" has been used to describe the family of related proteins with this structure. Each protomer has been found by x-ray crystallography to be folded into two antiparallel β sheets with a flattened jellyroll topology similar to that of lectins such as concanavalin A (Shrive AK *et al.*, 1996; Thompson D *et al.*, 1999). Human CRP is not glycosylated, and circulates at a median concentration of approximately 0.8mg/L in health, increasing to as much as 400 to 500mg/L at the peak of the most intense acute phase response (Pepys and Hirschfield, 2003).

In contrast, rat C-reactive protein (CRP) is unique among mammalian CRPs in being a glycoprotein and in containing a covalently linked dimer in its pentameric structure rat CRP is composed of five monomeric units, two of which exist as a dimer (≈ 52 kDa) while the remaining three exist as monomers (≈26 kDa) (Rassouli M *et al.*, 1992). Furthermore, rat CRP is glycosylated and circulates at concentrations of approximately 300mg/L, increasing to approximately 900mg/L in the acute phase response (de Beer *et al.*, 1982*a*).This increment is low in comparison to the dramatic rise seen in the acute phase response in humans, however it is advantageous when using a rat as an animal model since it is measurable at these levels and provides a basis for what can be expected to happen in humans on a larger scale. It has been reported that a mouse animal model is useless regarding the study of CRP since it is not an acute phase reactant in mice (Torzewski J, 2005).Moreover, in contrast to humans, plasma levels of mouse CRP rarely exceed 2μg/ml following inflammatory stimuli (Black S *et al.*, 2004).

CRP Production and Regulation

Plasma CRP is produced only by hepatocytes (Pepys and Hirschfield, 2003). Extrahepatic synthesis of CRP has also been reported in neurons in Alzheimer patients (Yasojima K *et al.*, 2000) , atherosclerotic plaques (Jialal I *et al.*, 2004), lymphocytes (Kuta AE *et al.*, 1986), alveolar macrophages(Dong Q *et al.*, 1996), aortic endothelial cells(Venugopal SK *et al.*, 2005) , coronary artery smooth muscle cells (Calabro P *et al.*, 2003), and adipocytes (Calabro P *et al.*, 2005) .Induction of CRP in hepatocytes is principally regulated at the transcriptional level by the cytokine interleukin-6 (IL-6), an effect which can be enhanced by interleukin-1β (IL-1β) (Kushner I *et al.*, 1995). De novo hepatic synthesis starts very

rapidly after a single stimulus, serum concentrations rising above 5 mg/l by about 6 hours and peaking around 48 hours (Pepys and Hirschfield, 2003). The plasma half-life of CRP is about 19 hours and is constant under all conditions of health and disease, so that the sole determinant of circulating CRP concentration is the synthesis rate (Vigushin DM *et al.*, 1993). Therefore CRP concentration directly reflects the intensity of the pathological process (es) stimulating CRP production and when the stimulus for increased production completely ceases, the circulating CRP concentration falls rapidly, at almost the rate of plasma CRP clearance (Pepys and Hirschfield, 2003). However, in view of the sensitivity, speed, and range of the CRP response, subjects in the general population tend to have stable CRP concentrations characteristic for each individual, apart from occasional spikes presumably related to minor or subclinical infections, inflammation, or trauma and no significant seasonal variation in base-line CRP concentration is found (Pepys and Hirschfield, 2003).

In most disease entities such as infections, inflammatory diseases, trauma, malignancy, necrosis, and allergic complications of infection the circulating value of CRP reflects ongoing inflammation and/or tissue damage much more accurately than do other laboratory parameters of the acute-phase response, such as plasma viscosity and the erythrocyte sedimentation rate (Pepys and Hirschfield, 2003). More importantly, acute-phase CRP, values show no diurnal variation and are unaffected by eating. However, systemic lupus erythematosus, scleroderma, dermatomyositis, ulcerative colitis, leukemia, graft-versus-host disease show moderate or absent CRP response (Pepys and Hirschfield, 2003). Liver failure impairs CRP production, but no other intercurrent pathologies and very few drugs reduce CRP values unless they also affect the underlying pathology providing the acute-

phase stimulus, therefore CRP concentration is thus a very useful nonspecific biochemical marker of inflammation, measurement of which contributes importantly to (a) screening for organic disease, (b) monitoring of the response to treatment of inflammation and infection, and (c) detection of intercurrent infection in immunocompromised individuals, and in the few specific diseases characterized by modest or absent acute-phase responses (Pepys MB *et al.*, 1983).

It is not known why systemic lupus erythematosus and the other conditions similar to it fail to elicit major CRP production despite evident inflammation and tissue damage, nor why the CRP responses to intercurrent infection are apparently intact in patients with such conditions (Pepys and Hirschfield, 2003).Intriguingly it has been demonstrated that CRP may exert an ameliorative effect upon murine models of systemic lupus erythematosus (SLE). Two reports have shown that injection or transgenic expression of CRP in a murine strain prone to development of a disease resembling human SLE resulted in a slight delay in mortality (Szalai AJ *et al.*, 2003; Du Clos TW *et al.*, 1994). In addition to these mouse models, a polymorphism in the human CRP gene resulting in a lower basal level of CRP has been associated with an increased risk of developing systemic lupus erythematosus (Russell AI *et al.*, 2004). These findings raise the possibility that decreased amounts of CRP may contribute to the pathogenesis of SLE since it has long been held that an important function of CRP is to target for clearance of the cellular debris of necrotic and apoptotic cells by binding to damaged cell membranes and nuclear material, therefore, decreased clearance of such material might well enhance development of autoantibodies to them (Black S *et al.*, 2004).

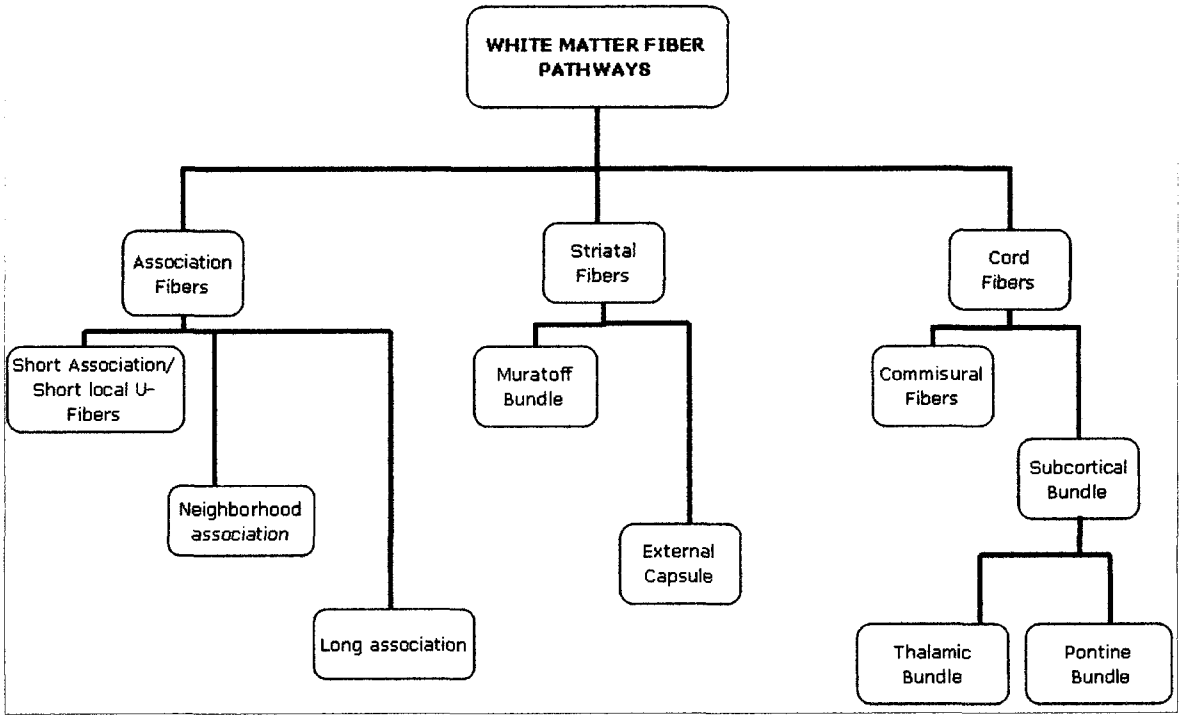


Fig.1 - Schematic diagram of the white matter pathways originating from the cerebral cortex. This figure has been modified from Schmahmann JD *et al.*, 2008.

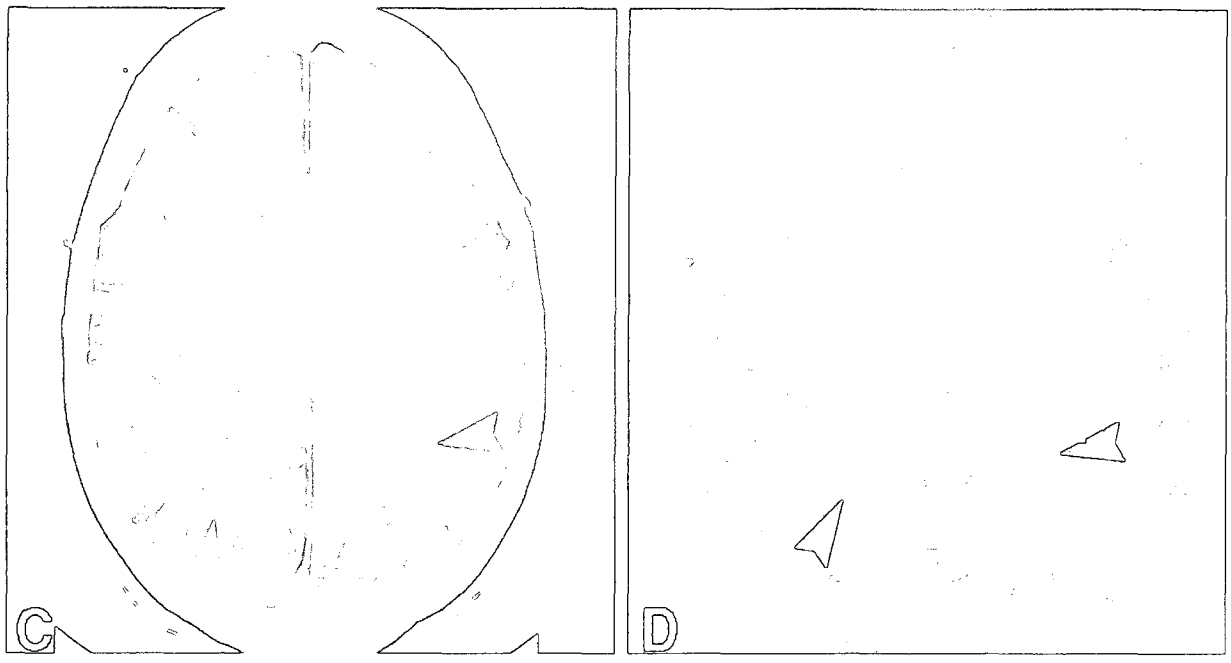


Fig.2 - A pair of images (CT/MRI) of the same patient. The lesions are chosen from matching slices. Hypodense lesions are seen on CT (C) (see arrow); on MRI (D), lesions show as hyperintense areas.

http://stroke.ahajournals.org/cgi/figsearch

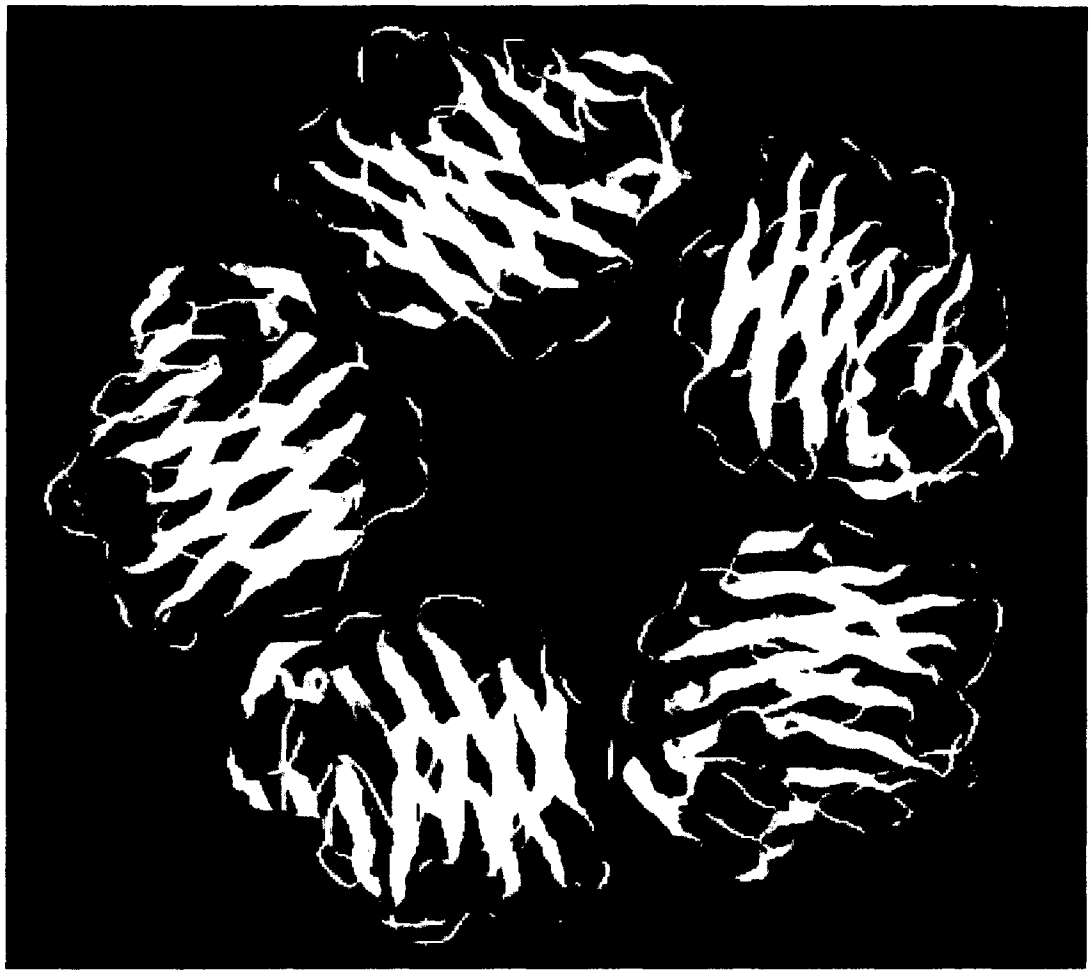


Fig.3 - Ribbon structure image of C-reactive protein adapted from original by Paul Ridker.

http://focus.hms.harvard.edu/2002/Nov22_2002/pathology.html

The Biological Role of CRP

Human CRP binds with highest affinity to phosphocholine residues, but it also binds to a variety of other autologous and extrinsic ligands, and it aggregates or precipitates the cellular, particulate, or molecular structures bearing these ligands (Pepys and Hirschfield, 2003). Autologous ligands include native and modified plasma lipoproteins (Pepys MB *et al.*, 1985), damaged cell membranes (Volanakis JE *et al.*, 1979), small nuclear ribonucleoprotein particles (Du Clos TW, 1989), and apoptotic cells (Gershov D *et al.*, 2000). Extrinsic ligands include many glycan, phospholipid, and other constituents of microorganisms, such as capsular and somatic components of bacteria, fungi, and parasites, as well as plant products (Pepys and Hirschfield, 2003). When aggregated or bound to macromolecular ligands, human CRP is recognized by C1q and potently activates the classical complement pathway, engaging C3, the main adhesion molecule of the complement system, and the terminal membrane attack complex, C5–C9 (Mold C *et al.*, 1999; Volanakis JE *et al.*, 1982). Bound CRP may provide secondary binding sites for factor H and thereby regulate alternative-pathway amplification and C5 convertases (Volanakis JE, 1982; Pepys and Hirschfield, 2003).

Mediators of inflammatory processes have been known to have pleiotropic effects and CRP is no exception as it exhibits both anti- and pro- inflammatory activities. Concerning its anti-inflammatory effects CRP has been shown to induce the expression of interleukin-1 receptor antagonist (Tilg H *et al.*, 1993) and increase release of the anti-inflammatory cytokine interleukin-10 (Mold C et al., 2002; Szalai AJ et al., 2002) while repressing synthesis of interferon-γ (Szalai AJ et al., 2002). However, many other functions that can be regarded as pro-inflammatory are recognized. For example, human CRP activates

complement and enhances phagocytosis (Pepys and Hirschfield, 2003) and contrary to previous belief rat CRP also can activate autologous complement (Padilla ND *et al.*, 2003).CRP up-regulates the expression of adhesion molecules in endothelial cells (Kawanami D *et al.*, 2005), inhibits endothelial nitric-oxide synthase expression in aortic endothelial cells (Venugopal SK *et al.*, 2002), stimulates IL-8 release from several cell types (Khreiss T *et al.*, 2005; Devaraj S *et al.*, 2004) ,increases plasminogen activator inhibitor-1 expression and activity(Devaraj S *et al.*, 2003), and increases the release of IL-1, IL-6, IL-18, and tumor necrosis factor-α from monocytes (Ballou SP *et al.*, 1992).

It is likely that the activity of CRP in humans, either pro- or anti-inflammatory is dependent on the context in which it is acting (Black S *et al.*, 2004). Moreover, even though recent data have raised the possibility that CRP may participate in the pathogenesis of atherosclerosis, this is far from established (Black S *et al.*, 2004) and a recent viewpoint has considered CRP neither a marker nor a mediator of atherosclerosis (Pepys M., 2008).However in contrast to the absence of rigorous evidence for a pathogenic role of human CRP in atherosclerosis, there is robust evidence that high concentrations of human CRP can exacerbate ischemic infarction via a complement dependent mechanism (Pepys MB *et al.*, 2006) . This process is completely different from the direct cellular signaling postulated by the proponents of CRP *per se* as a pathogenic mediator, and it could well operate in many different clinical situations in which high CRP concentrations are associated with pre-existing tissue damage. Abundant CRP is required, rather than just trivially increased baseline values, and the enhanced tissue damage is prevented by a specific drug that inhibits CRP ligand binding and thus inhibits complement activation by CRP (Pepys MB *et al.*, 2006).

Recently the reliability of results concerning CRP obtained in vitro has been questioned because commercial CRP preparations contain the biologically active bacteriostatic preservative sodium azide (Swafford AN *et al.*, 2005; Van den Berg CW *et al.*, 2004; Liu C *et al.*, 2005; Lafuente N *et al.*, 2005) and the endotoxin LPS (lipopolysacharide). In particular, the contaminating presence of sodium azide has been considered responsible for the proapoptotic, antimigratory, antiproliferative, antiangiogenetic, and vasodilating effects previously attributed to CRP (Doronzo G *et al.*, 2005; Swafford AN *et al.*, 2005; Van den Berg CW *et al.*, 2004; Liu C *et al.*, 2005). In response, Doronzo G *et al.* (2005) demonstrated that purified CRP increased MMP-2 secretion, expression, and activity, with no difference compared with the nonpurified preparation and that sodium azide does not influence MMP-2 synthesis and secretion from human vascular smooth muscle cells (Doronzo G *et al.*, 2006). Furthermore, Dasu MR *et al.*(2007) provided evidence that TLR4 knockdown had no influence on CRP biological effects via interleukin-8 (IL-8), IL-6, IL-1ß, PAI-1, and cGMP levels as well as eNOS activity, convincingly demonstrating that these effects are attributable to the native pentameric protein and not to endotoxin contamination (Dasu MR *et al.*, 2007).

Conclusively, the absence of any known deficiency or protein polymorphism of human CRP, and the phylogenetic conservation of CRP structure with homologs in vertebrates and many invertebrates and its ligand-binding specificity for phosphocholine and related substances, suggest that this protein must have had survival value (Pepys and Hirschfield, 2003).

CRP, Cerebral Small Vessel Disease and WML

The burden of brain vascular disease is far greater than that of the clinically-recognizable acute neurological event referred to as stroke (Longstreth WT Jr., 2005). In addition, although the majority of MRI-detectable abnormalities e.g. WML, silent brain infarcts (SBI) do not produce acute clinical symptoms, they cannot be considered benign as they are often associated with an increased risk for cognitive deficit (Pantoni L *et al.*, 1999; Mosley TH Jr. *et al.*, 2005) motor function impairment (Rosano C *et al.*, 2003; Starr JM *et al.*, 2003) and future stroke (Bernick C *et al.*, 2001; Kuller LH *et al.*, 2004; Longstreth WT Jr. *et al.*, 2005). Canadian statistics suggest that 4.1% of individuals over the age of 65 years have suffered a stroke (Hodgson C, 1998), twice as many (8%) being demented, and that for every person demented, 2 (16.8%) have cognitive impairment short of dementia (Graham JE *et al.*, 1997).

Numerous prospective studies have shown associations of serum levels of biomarkers of inflammation such as C-reactive protein (CRP), interleukin 6 (IL6), and fibrinogen with myocardial infarction, stroke, cardiovascular death, and peripheral arterial disease (Ridker PM *et al.*, 2000, 2001; Ridker PM, 2003; Cao JJ *et al.*, 2003).Little is known, however, about the relationship between inflammation and small vessel disease of the brain (Di Napoli M *et al.*, 2005).Previous studies have shown evidence of inflammatory activation and endothelial dysfunction in individuals with lacunar infarction and WML (Fassbender K *et al.*, 1999; Hassan A *et al.*, 2003).More recently some population based studies have explored the relation between CRP and WML :

The Rotterdam Study (van Dijk EJ et al., 2005)

In this study van Dijk and colleagues demonstrated the relationship of C-reactive protein (CRP) with the severity and progression of cerebral WML/LA on MRI scans. They based their study on 1033 participants of the population-based Rotterdam Scan Study for whom complete data on CRP levels were available and who underwent brain MRI scanning. Subjects were 60 to 90 years of age and free of dementia at baseline. Six hundred thirty-six subjects had a second MRI scan on average 3.3 years later. Multivariate regression models were used to assess the associations between CRP levels and markers of small-vessel disease. Higher CRP levels were associated with presence and progression of WML, particularly with marked lesion progression (ORs for highest versus lowest quartile of CRP 3.1 [95% CI 1.3 to 7.2] and 2.5 [95% CI 1.1 to 5.6] for periventricular and subcortical white matter lesion progression, respectively). These associations persisted after adjustment for cardiovascular risk factors and carotid atherosclerosis. Persons with higher CRP levels tended to have more prevalent and incident lacunar infarcts. Conclusively they stated that inflammatory processes may be involved in the pathogenesis of cerebral small-vessel disease, in particular, the development of white matter lesions (van Dijk EJ et al., 2005).

The Austrian Stroke Prevention Study (Schmidt R et al., 2006)

In the community-based Austrian Stroke Prevention Study, CRP concentrations were measured by a highly sensitive assay in 700 participants at baseline, all of whom underwent carotid duplex scanning, and a subset of 505 subjects underwent brain magnetic resonance imaging which was repeated after 3 and 6 years (Schmidt R et al., 2006). The volume of white matter lesions and the number of lacunes were considered small vessel disease–

related brain abnormalities. In reference to their results the associations between severity and progression of small vessel disease–related brain abnormalities and CRP were non significant and they concluded that CRP was a marker for active carotid atherosclerosis but not for small vessel disease–related brain lesions (Schmidt R *et al.*, 2006).

This study contrasts with previous results from the Rotterdam Scan Study (van Dijk EJ *et al.*, 2005) and as will be mentioned the Cardiovascular Health Study (Fornage M *et al.*, 2008). Schmidt R *et al.* (2006) provide the following causes as an explanation for this controversy:

- The Austrian Stroke Prevention study included participants that were younger and apparently healthier than those of the Rotterdam cohort. Patients with a history of symptomatic strokes or transient ischemic attacks or evidence of dementia were not included (Schmidt R *et al.*, 2006).
- Fewer subjects with hypertension and fewer smokers were in the Austrian cohort. Consequently, it is likely that earlier stages of cerebral small-vessel disease were studied (Schmidt R *et al.*, 2006).
- Unlike in large vessel atherosclerosis, in which CRP seems to be involved in very early disease stages (Balletshofer B *et al.*, 2005), the situation in cerebral small-vessel disease may be different. Increases in CRP could rather be the epiphenomenon of brain damage attributable to small-vessel disease rather than being related to the development of arteriolosclerosis per se (Schmidt R *et al.*, 2006).
- In addition subjects who participated in the follow-up were younger and had fewer risk factors for stroke than did those who did not participate in the follow-up

examinations (Schmidt R et al., 2005).This might have resulted in lower progression rates of large- and small-vessel disease than in the general population and most likely has reduced the statistical power of the respective analyses (Schmidt R *et al.*, 2006).

- Treatment and management of study participants was according to the treating general practitioners, and thus, it cannot be excluded that treatment might have had an effect on study results (Schmidt R *et al.*, 2006).

The Cardiovascular Health Study (Fornage M et al., 2008).

The aim of this study was to investigate the association between common variation in the C-reactive protein (CRP) and interleukin (IL)-6 genes, plasma CRP and IL6 levels, and presence of MRI-defined white matter lesions (WML) and brain infarcts (BI) in elderly participants of the Cardiovascular Health Study (Fornage M et al., 2008).

The authors selected Tag single nucleotide polymorphisms (SNPs) in the CRP and IL6 genes from the SeattleSNPs database and using cross-sectional analyses, logistic regression models adjusting for known cardiovascular disease risk factors were constructed to assess the associations of plasma CRP and IL6 levels and common CRP and IL6 gene haplotypes with presence of WML or BI in Blacks (n=532) and Whites (n=2905), (Fornage M et al., 2008).

Their results showed that plasma IL6 and CRP levels were associated with presence of WML and BI in both races. In Whites, common haplotypes of the IL6 gene were significantly associated with WML and BI and the common haplotype tagged by the -174G/C promoter polymorphism was associated with an increased risk of WML (OR=1.14;

95% CI: [1.02; 1.28]), whereas the common haplotype tagged by the -572G/C promoter polymorphism was associated with an increased risk of BI (OR=1.57; 95% CI: [1.15; 2.14]), (Fornage M *et al.*, 2008).

Significant associations were lacking for WML or BI with IL6 gene variation in Blacks or with CRP gene variation in either race, the latter is in agreement with recent findings from the Rotterdam Scan study and the MEMO study (Reitz C *et al.*, 2007). The Cardiovascular Health Study provides evidence of a genetic basis underlying the relationship between plasma biomarkers of inflammation and small vessel disease of the brain (Fornage M *et al.*, 2008).

Community-based Japanese Study (Wada M. *et al.*, 2008)

This community–based study by Wada M. *et al.* assessed whether higher CRP levels were associated with an increased number of lacunar infarcts or severity of white matter lesions, both pathologies being cerebral SVD related brain lesions. In a community-based group of Japanese elderly (n=689), CRP concentrations were measured using a highly sensitive assay and all participants underwent magnetic resonance imaging (MRI). Cerebral small vessel disease-related lesions (lacunar infarcts and white matter hyperintensity) were subsequently evaluated and carotid atherosclerosis was also assessed with ultrasonography (Wada M. *et al.*, 2008). As the grades of white matter hyperintensity and the numbers of lacunes were considered small vessel disease-related lesions, Wada M. *et al.* (2008), evaluated the relationships between CRP levels and small vessel disease-related brain lesions. Interestingly, the median CRP concentration of their participants was remarkably lower, being approximately one third or one quarter of the value of Western populations,

moreover subjects with higher CRP levels tended to have more SVD-related lesions; however, these associations were not seen after adjustment for cardiovascular risk factors and carotid atherosclerosis (Wada M. *et al.*, 2008).

From their results it seems that the relationship between CRP levels and small vessel disease-related lesions was not apparent in the community-based Japanese elderly, therefore the impact of inflammation in the pathogenesis of small vessel disease-related brain lesions seems to be weak among the Japanese elderly (Wada M. *et al.*, 2008).

The results of this study support previous epidemiological investigations which have revealed that CRP levels vary remarkably with ethnic status (Albert MA *et al.*, 2004; Khera A *et al.*, 2005), and that Asian populations seem to have low levels of CRP (Ichikawa K *et al.*, 2004).

To conclude, the findings in most population–based studies need to be explored further. To the best of our knowledge the study of CRP in an animal model of chronic hypoperfusion manifesting WML has not been documented previously.

Microglia

Historical Background

The early years of research on the nature of microglia, the resident macrophages of the nervous system, are noteworthy for the remarkable insights of many illustrious anatomists and neuropsychiatrists (reviewed by Rezaie P& Male D, 2002), including Gluge (who in 1841 identified phagocytic cells of mesodermal origin in the damaged brain), Virchow (who in 1846 observed phagocytes ["foam cells"] contributing to a disease process termed

congenital encephalitis), His (who in 1890 described amoeboid mesodermic corpuscles which entered the developing brain of human embryos in the second month, colonized both grey and white matter, and emitted protoplasmic radiations), Nissl (who in 1899 suggested that glial cells in the brain have similar functions to macrophages in other tissues), Robertson (who in 1900 distinguished "neuroglia" and "mesoglia," the latter cells, derived from mesoderm, displaying phagocytic activity in pathological conditions such as chronic brain degeneration), Alzheimer (who in 1904 believed that glial cells became amoeboid in certain acute infections and were destined to combat the infection), and Cajal (who in 1913 recognized mesoglia as the "third element" of the central nervous system [CNS]), (Rock RB *et al.*, 2004).However, it was the Spanish neuroanatomist Pio del Rio-Hortega who in 1932 earned the title "father of microglia biology." He was the first to demonstrate (in 1919 to 1922) that mesoglia were composed of microglia, which are of mesodermal origin, and oligodendroglia, which, along with astroglia and neurons, are of neuroectodermal lineage. In his classic treatise published in 1932 (del Rio-Hortega P, 1932), Rio-Hortega framed a "modern conception of microglia" that remains relevant to this day (Rock RB *et al.*, 2004).

Microglia: The Resident Macrophage of the CNS

The term "microglia" refers to cells that reside within the parenchyma of the nervous system, that share many if not all the properties of macrophages in other tissues, but that in their non activated or resting state have a characteristic "ramified" morphology not seen in resident macrophages of other organ systems (Rock RB *et al.*, 2004). Although microglia are "brain macrophages," they are distinguished by their parenchymal location and certain

functional differences from other types of brain macrophages such as meningeal and perivascular macrophages (Nguyen MD *et al.*, 2002; Polfliet MMJ *et al.*, 2001, 2002) and perivascular cells or pericytes (Thomas EW, 1999; Williams E *et al.*, 2001), which are enclosed by a perivascular basement membrane within blood vessels and are not part of the CNS parenchyma (Rock RB *et al.*, 2004).

In rodents and humans, postnatal microglia is thought to arise from two different pools of myeloid cells that successively colonize the developing CNS (Davoust N *et al.*, 2008). The first wave of microglial progenitors invades the embryonic and fetal CNS and derives essentially from extramedullary sources of hematopoiesis, including the yolk sac (Rezaie P *et al.*, 1999; Kaur, C. *et al.*, 2001). The second wave of microglial progenitors is formed by bone marrow– derived monocytic cells that colonize the CNS during the early postnatal period (P0–P15), in rodents, or before birth, in humans (Rezaie P *et al.*, 1999; Kaur, C. *et al.*, 2001; Cuadros MA *et al.*, 1998). As Rio-Hortega recognized, the penetration and migration of microglia takes place very quickly, and postnatally, microglia are to be found in every location within the nervous system (del Rio-Hortega P, 1932). Often not appreciated, however, is the fact that the brain is composed primarily of glial cells. While about 15% of the cells in the brain are neurons, it is estimated that microglia are found in roughly equivalent numbers (Streit WJ, 1995). In a recent study of the local density of microglial cells in the normal adult brain, ramified microglia bearing markers such as CD68 and major histocompatibility complex (MHC) class II antigen were found to be more concentrated in white matter than in grey matter, and significant regional differences were observed, with microglia ranging from 0.5 to 16.6% of all the cells within various areas of the brain parenchyma (Mittelbronn M et al., 2001). Grey matter of the cerebellum had the

lowest density of microglia, while the highest level of CD68- and MHC class II-positive cells was found in the medulla (Rock RB *et al.*, 2004).

Microglia: Active Sensors

Ramified morphology and the sparse expression of molecules associated with macrophage function in microglia of the healthy adult CNS have been associated with a 'resting' phenotype (Hanisch UK *et al.*, 2007).However, resting microglia are not dormant. Studies based on *in vivo* two-photon microscopy in transgenic mice expressing enhanced green fluorescent protein in the *Cx3cr1* locus (encoding CX3CR1, the receptor for the chemokine CX3CL1, also known as fractalkine) revealed that microglial processes and arborizations are highly mobile(Nimmerjahn A *et al.*, 2005; Davalos D *et al.*, 2005) . Time-lapse imaging showed that processes are continually rebuilt, with *de novo* formation and withdrawal of processes as well as motile filopodium-like protrusions and such dynamic and careful reorganization may enable the otherwise stationary microglia to thoroughly scan their environment without disturbing fine-wired neuronal structures (Hanisch UK *et al.*, 2007). Estimates are that the complete brain parenchyma could be monitored every few hours and neighboring microglial cells take turns scanning shared regions, guaranteeing exhaustive screening while avoiding contact (Hanisch UK *et al.*, 2007).The random scanning by processes rapidly changes to a targeted movement toward the site of an injury when microlesions are induced and this response and its directional guidance apparently depend on purinoreceptor stimulation and may involve assistance from astrocytes (Haynes SE *et al.*, 2006; Davalos D *et al.*, 2005).

Microglial Cell Membrane Receptors

Microglia share phenotypic markers of monocyte–macrophage lineage and many of them are surface antigens/receptors with significant functional properties (Williams *et al.*, 1992; McGeer and McGeer, 1995). They are b2-integrins (CD11a, CD11b, CD11c, and CD18), leukocyte common antigen (LCA; CD45), immunoglobulin Fcγ receptors, major histocompatibility complex (MHC) class I glycoproteins (HLAABC; b-2 microglobulin), and MHC class II glycoproteins (HLA-DR, HLA-DP, and HLA-DQ), (reviewed by Kim SU *et al.*, 2005).

Human microglial cells express mRNA transcripts for cytokine receptors IL-1RI, IL-1RII, IL-5R, IL-6R, IL-8R, IL-9R, IL-10R, IL-12R, IL-13R, IL-15R, TNFRI, and TNFRII, whereas expression of IL-2R, IL-3R, IL-4R, IL-7R or IL-11R was not detected (Kim SU *et al.*, 2005). In addition, microglia express gp130, a common receptor component for IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and oncostatin M, as well as chemokine receptor CXCR4, which has drawn considerable attention as a co receptor for human immunodeficiency virus (HIV) entry into human microglia (Doms and Peiper, 1997). In addition, it is important that human microglia express receptors for immunomodulatory cytokines, IL-5R, IL-12R and IL-15R, and receptors for anti-inflammatory cytokines IL-10R and IL-13R. Although an earlier study in mouse microglia reported the expression of transcripts for IL-2R, IL-3R, IL-4R, and IL-7R (Sawada et al., 1993), none of these receptors was found in human microglia (Kim SU *et al.*, 2005).

Microglial Secretory Products

One of the major functions of microglia is to send signals to other cells that will regulate the inflammatory response following exposure to a specific insult or infection (Garden GA *et al.*, 2006). Four major classes of molecules are responsible for communicating signals from microglia to surrounding cells and invading leukocytes and these include cytokines, chemokines, trophic factors, and small molecule mediators of inflammation such as prostaglandins (Garden GA *et al.*, 2006):

(1) Cytokines are immunomodulatory peptides that include interleukins, IFNs, TNFα, and TGFβ. Cultured human microglia express mRNA for IL-1a/b, IL-6, IL-10, IL-12, IL-15, and TNF α (Kim and de Vellis, 2005), whereas additional studies have documented expression of IL-3, IL-18, IFNs, and TNF α in rodent microglia (Hanisch UK, 2002). Microglia activation generally increases cytokine expression, and proinflammatory cytokines are the first to be released and tend to have both toxic effects on surrounding cells as well as CNS effects that are separate from their role in stimulating the immune response and as mentioned microglia also elaborate receptors for most of these cytokines resulting in autocrine feedback loops that are likely to be crucial for the eventual down regulation of an inflammatory response (Garden GA *et al.*, 2006).

Anti-inflammatory cytokines IL-4/IL-10/IL-13 and TGFβ may not be constitutively expressed or available for release until the proinflammatory response is well underway (Garden GA *et al.*, 2006). Generally, the anti-inflammatory cytokines have neuroprotective properties (Hanisch, 2002). Both IL-4 and IL-13 appear to support eventual microglia apoptosis (Yang *et al.*, 2002) days to weeks following a discreet inflammatory stimulus and

have been demonstrated to act as an important feedback mechanism to eventually turn off an inflammatory response in vivo (Shin *et al.*, 2004).

(2) Chemokines are a family of peptide chemoattractant molecules that interact with a specific family of Gprotein-coupled receptors (Garden GA *et al.*, 2006). Activated microglia have been shown to express many of the receptors and ligands belonging to the three major chemokine families, i.e., the CC (CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES), CXC(CXCL8/IL-8, CXCL9/MIG, CXCL10/IP-10, CXCL12/SDF-1α), and CX3C (CX3CL1/fractalkine) families (reviewed in Rock RB *et al.*, 2004). Many of these receptors and chemokines can also be expressed in astrocytes, suggesting that chemokines may serve as communication signals between microglia and astrocytes; it has been proposed that CX3CR1 and its ligand (CX3CL1/fractalkine), which are also expressed in neurons, play an important role in neuronal signaling of microglia (reviewed in Rock RB *et al.*, 2004). Microglia also express receptors for many of the microglia-elaborated chemokines, suggesting that one of the main functions of chemokine release is to attract additional microglia to the site of the insult, however this assertion may be difficult to prove using in vitro methods because cultured microglia have very low level of surface receptor expression of the CXCR4 and CCR5 chemokine receptors compared to microglia cultured with neurons (Garden *et al.*, 2004). Using an alternate approach, it was demonstrated that chronic exposure to MCP-1 via transgenic overexpression led to chronic microglia activation (Takahashi *et al.*, 2005).

(3) Trophic factors that promote neuronal survival are frequently synthesized in and released from microglia (Garden GA *et al.*, 2006). Classical neurotrophins, including NGF, BDNF, and NT-3, basic fibroblast growth factor, and glial-derived neurotrophic factor (GDNF) are synthesized by microglia (Presta M *et al.*, 1995; Elkabes E *et al.*, 1996; Honda S *et al.*, 1999). GDNF is also expressed by microglia and, in conjunction with BDNF, may be responsible for guiding regenerating axons toward a lesion (Batchelor PE *et al.*, 2002). In addition to providing trophic support to surrounding neurons and neuronal processes, trophic factors from microglia may also act in an autocrine fashion to regulate the ability of microglia to sustain the proinflammatory state (Garden GA *et al.*, 2006), for example, NGF treatment causes a reduction in MHC class II expression on microglia (Neumann H *et al.*, 1998).

(4) Activated microglia release small molecule lipid inflammatory mediators including arachidonic acid, prostaglandins D2, E2 and F2α, platelet-activating factor, thromboxane B2, and leukotriene B4 (Minghetti L and Levi G, 1998). Arachidonic acid released by phospholipase activity is the substrate for generation of these metabolites of lipoxygenase and cyclooxygenase activities (Garden GA *et al.*, 2006). Microglia appear to be an important source of prostaglandins in the CNS, secreting significantly more prostanoids than astrocytes in response to CD14/TLR4 stimulation (Minghetti L and Levi G, 1998). An important point to be mentioned is that prostanoids include prostaglandins, prostacyclins, thromboxanes, and related substances, but "prostaglandins" is often used loosely to include all prostanoids. Microglia also respond to the prostanoids they secrete, expressing several prostaglandin receptors (Garden GA *et al.*, 2006). Microglia cells have been demonstrated

to express the TP, EP2, and EP3 prostaglandin receptors that recognize thromboxane, PGE2, and PGE2/PGE1, respectively (Kitanaka J *et al.*, 1996). The EP2 receptor, in particular, appears to play an important role in regulating microglia activation and mice deficient in the EP2 receptor are protected from LPS-induced neurotoxicity, and EP2-deficient microglia fail to develop a neurotoxic phenotype following Aβ exposure, but nevertheless have enhanced Aβ phagocytic activity (Shie FS *et al.*, 2005 a, b). These reports suggest that specific antagonism of the microglia EP2 receptor might be a potential therapeutic target to lessen the impact of neuron inflammation in AD (Garden GA *et al.*, 2006).

Conclusively an intriguing study by Aaron Y. Lai & Kathryn G. Todd (2008) demonstrated, by using an in vitro hypoxia model, that the severity of neuronal injury is an important factor in determining microglial release of ''toxic'' versus ''protective'' effectors and the resulting neurotoxicity versus neuroprotection (Lai AY & Todd KG, 2008).

Microglia, Aging and WML

The aging brain is characterized by a demonstrable decrease in weight and volume, particularly after the age of 50. This atrophy, which affects both grey and white matter, is presumed to result from a loss of neurons and myelinated axons. Glial cells, on the other hand, appear to increase in the aging brain, which exhibits greater immunoreactivity with both astrocytic and microglial markers (Conde JR *et al.*, 2006). The effects of aging on microglia are conceivably 2- fold: microglia may react to aging-related changes in their environment, or microglia may be directly affected by the aging process (Conde JR *et al.*,

2006). Because the CNS parenchyma is subject to some known aging-related changes such as reduction of extracellular volume, increased astrogliosis, and neuronal loss or shrinkage (Duffy PE *et al.*, 1980; Sykova E *et al.*, 1998; Terry RD *et al.*, 1987; Anderson JM *et al.*, 1983), it is possible that these changes may affect microglia secondarily and just as aging related astrogliosis has been interpreted as a secondary reactive glial cell response to neuronal changes, the microglial activation that reportedly occurs with aging (Rogers J *et al.*, 1988; Streit WJ *et al.*, 1997; DiPatre PL *et al.*, 1997) may also be considered a secondary glial response (Conde JR *et al.*, 2006).

The other possibility, that aging has a direct impact on microglia, seems more likely. That is, why would aging not affect microglia when it affects all other cells and tissues in the body? However, current textbooks of neuropathology do not even mention such a possibility, likely because relatively little is known about the effects of aging on microglia. What is known, specifically from histopathologic studies, concerns aging-related changes in microglial morphology and immunophenotype (Conde JR *et al.*, 2006), and I will briefly mention some of these changes in relation to the WM.

The vast majority of studies of aging-related immunophenotypic changes in microglia have demonstrated a steady increase in the expression of markers usually found to be upregulated on activated microglia after acute CNS injuries (Conde JR *et al.*, 2006). Most notable is an aging-related increase in microglial expression of MHC class II antigens, which has been reported in humans (Rogers J *et al.*, 1988; Streit WJ *et al.*, 1997; DiPatre PL *et al.*, 1997), monkeys (Sheffield LG *et al.*, 1998; Sloane JA *et al.*, 1999), and rats (Ogura K *et al.*, 1994; Morgan TE *et al.*, 1999; Perry VH *et al.*, 1993). The increases in MHC II expression very likely represent immunophenotypic changes in the existing

microglial population, because there is no clear evidence of increased microglial cell numbers in old compared with young rat brains (Long JM *et al.*, 1998). A recent study also demonstrated that activated microglia in the white matter of rhesus monkeys had an aging-related increase in the expression of active calpain-1 (Hinman JD *et al.*, 2004), a proteolytic enzyme that was previously proposed to have a role in aging-related myelin protein degradation (Sloane JA *et al.*, 2003); thus, age-dependent increases in calpain-1 expression may be a reflection of increased degradation of phagocytosed myelin fragments by microglia. Aging-related increases in the breakdown of myelin and other cellular components, and the subsequent phagocytosis of these substances by microglia, may account for the substantial increase in inclusions within microglia reported in aged monkeys (Peters A *et al.*, 2002, 1991; Sandell JH *et al.*, 2002) and rats (Peinado MA *et al.*, 1998).

In addition, several studies have reported significant changes in microglial morphology, including the presence of cytosolic inclusions already mentioned. Perry *et al.* (1993) reported vacuolated processes in MHC II-positive (OX-6 antibody) microglia in aging rats, and although they found no aging related difference in the density of CR3 (complement receptor-3)-positive (antibody OX-42) microglia, they described CR3-positive microglia in old rats as having abnormal morphology and a higher incidence of clumping, particularly in and around white matter (Perry VH *et al.*, 1993). Other studies have also confirmed that aging produces dramatic changes in the morphology of so-called resting microglia (i.e. cells present in non pathologic normal brain tissue) and showed that microglia in the aged brain are characterized by significant hypertrophy of their cytoplasm and dense lectin staining (Conde JR *et al.*, 2005; Streit WJ *et al.*, 2005).

In an attempt to define microglial response in WML associated with aging Simpson JE *et al.* (2007), have recently provided novel evidence that microglia within control WM from lesional cases express significantly higher levels of MHC II expression than control WM from nonlesional ageing brain. In their study, WML of brains from an unbiased population-based autopsy cohort (Medical Research Council's Cognitive Function and Ageing Study) were identified by *post mortem* magnetic resonance imaging and sampled for histology (Simpson JE *et al.*, 2007). PVL (periventricular lesions) contain significantly more activated microglia, expressing major histocompatibility complex (MHC) class II and the costimulatory molecules B7-2 and CD40, than either control white matter WM or DSCL (deep subcortical lesions), and they show that significantly more microglia express the replication licensing protein minichromosome maintenance protein 2 within PVL, suggesting this is a more proliferation-permissive environment than DSCL (Simpson JE *et al.*, 2007). Although microglial activation occurs in both PVL and DSCL, their findings suggest a difference in pathogenesis between these lesion-types: the ramified, activated microglia associated with PVL may reflect immune activation resulting from disruption of the blood brain barrier, while the microglia within DSCL may reflect an innate, amoeboid phagocytic phenotype (Simpson JE *et al.*, 2007).Since microglia in control WM from lesional cases express significantly more MHC II than control WM from nonlesional ageing brain they have proposed that the pathogenesis of WML is a generalized process within the cerebral WM (Simpson *et al.*, 2007).

In accordance to animal models, experimental cerebral hypoperfusion through BCCAL has been shown to induce white matter injury and microglial activation in the rat brain (Farkas E *et al.*, 2004; Wakita H *et al.*, 1994). Microglial activation was observed after the

first day of ligation, peaked at 7-14 and persisted till 90 days after ligation (Wakita H *et al.*, 1994).Interestingly, minocycline, a microglial inhibitor was shown to attenuate white matter damage in a rat model of cerebral hypoperfusion (Cho KO *et al.*, 2006). The direct relation between microglial activity and WML/aging proposes an important role for the known and "unknown" effectors of microglia in these processes.

By reproducing an animal model of WML we hope to define the relation between WML, CRP and the accompanying microglial activation.

AIM

Define the relation between CRP and WML in an animal model of white matter injury, and how this may be associated with microglial activation.

HYPOTHESIS

CRP correlates with WM injury and microglial activation plays a role in this correlation.

MATERIALS & METHODS

Chronic Hypoperfusion Model

All experiments conformed to the guidelines set forth by the Canadian Council for the Use and Care of Animals in Research (CCAC) with approval from the Ottawa Health Research Institute Animal Care Facility.

An animal model of chronic hypoperfusion, which experimentally induces WML, was reproduced through a bilateral common carotid artery ligation [BCCAL] (Wakita H *et al.*, 1994) procedure conducted on Long Evans rats (weighing 350-450g, Charles River, Saint-

Constant, Quebec, Canada).Animals were anaesthetized with isoflurane (5% induction, 2% maintenance) in a 70:30 mixture of oxygen and nitrous oxide. Rats were placed on heating pads and through a midline cervical incision; both common carotid arteries were exposed and double-ligated with silk sutures. The rectal temperature was maintained between 36.5°C and 37.5° c during the surgical procedure. After the operation, the rats were kept in cages with food and water ad libitum. Sham operated rats underwent the same surgical procedures except for ligation of the arteries. At 1, 3,7,14 and 30 days after ligation the animals were deeply anaesthetized, blood samples for serum analysis of C-reactive protein obtained from the heart and then the animals were perfused transcardially with 0.9% normal saline. Brain samples intended for immunohistochemistry (n=3for BCCAL, n=3for sham for each time period) were further perfused with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), whereas those obtained for PCR and Western blotting (n=3for BCCAL, n=3for sham for each time period) were quick frozen in liquid nitrogen and kept at -80 °C until used.

Cell Culture

Microglia cells were cultured from brain tissue of 2-3 day postnatal rat pups according to previous protocols (Mount MP & Lira A et *al.*, 2007). Postnatal 2–3day old rat pups were used for microglia cultures. Brains were dissociated using a tissue grinder kit and plated in flasks with minimum essential medium supplemented with 5% heat-inactivated fetal bovine plasma, 5% heat-inactivated horse plasma, and 0.05 mg/ml gentamycin. Two days later, the medium was replenished, and the culture reached confluency ~8 d later, these confluent mixed-glia cultures were shaken for 5 h at 180 rpm at 37°C to separate microglia from

astrocytes (Mount MP & Lira A, et *al.*, 2007). This produced a yield of >95% enriched microglia .After confluency the cells were plated in 12 well plates on cover slips or on 6 cm^2 dishes at a density of $2X10^5$ /cm^2 and treated with IL-1β, IL-6 or a combination of both. After 24hrs cells were used for RNA and protein extraction.

Haematoxylin & Eosin Staining

Coronal brain blocks including the optic nerves and corpus callosum (Bregma 1.88mm-2.8mm) were embedded in paraffin for histological examination. Four micrometer-thick paraffin sections were cut on a microtome, and stained with a basic Haematoxylin and Eosin stain.

Immunohistochemistry

Paraffin embedded rat brain sections were deparaffinised and antigen retrieval step using citrate buffer (PH 6.0) was performed in a rapid microwave histoprocessor (model 5, Milestone). Following antigen retrieval, sections were rinsed with 1X PBS for 5 minutes. Non-specific binding sites were blocked with 10%TritonX-100 and donkey serum diluted in 1X PBS. Sections were then incubated with monoclonal anti-rat CD 68(Abcam, USA, 1:100),a marker for microglial activity, and glial fibrillary acidic protein (GFAP) (1:200),an astrocyte marker, diluted in 1X PBS with 10 % Triton X-100 overnight at 4°C in order to detect microglia cell activity and astrocytes respectively.Cy-3 conjugated donkey anti-mouse IgG antibody (1 : 200) and FITC (1:200) donkey anti-mouse IgG antibody were used for visualization of immunolabeling. DAPI was used to visualize nuclei.

Also following antigen retrieval other sections were incubated with rabbit polyclonal Chromogranin A antibody (Abcam, USA, 1:100), a marker for axonal injury, diluted in TBS with 1% BSA. Subsequently sections were revealed using a highly sensitive biotin free polymer detection system (Cat#M4U534Biocare Medical); slides were incubated with anti-Rabbit MACH 4 Polymer HRP for 20 minutes at room. After each incubation the sections were rinsed for 5 min. with 0.01M TBS pH7.6. Finally the immunoreaction complex was visualized using a solution of Chromogen DAB (0.02% 3, 3′-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer, pH 7.6) with a substrate of hydrogen peroxide (0.001% H2O2) to catalyze the enzymatic reaction of the HRP.

Immunocytochemistry

Microglia cells cultured on cover slips in 12 well plates were used to demonstrate Cd11B (Serotec, USA, 1:200) and CRP (ICL [Immunology Consultants Laboratory Inc.], Newberg, USA, 1:200) immunostaining. Non-specific binding sites were blocked with 10%TritonX-100 and donkey serum diluted in 1X PBS. Sections were then incubated with mouse anti-rat Cd11b (a marker for microglia) and rabbit anti-rat C-reactive protein (for detection of C-reactive protein expression) diluted in 1X PBS with 10 % Triton X-100 overnight at 4°C. Cy-3 conjugated donkey anti-rabbit IgG antibody (Chemicon,1 : 200) and FITC (Chemicon,1:200) donkey anti-mouse IgG antibody were used for visualization of immunolabeling. DAPI was used to visualize nuclei.

Quantification of Immunohistochemistry and H&E

To quantify immune cells in the optic tract and corpus callosum, digital images of CD68 stained sections (Bregma −1.8 to −2.8) were acquired using Axioskop 2 Mot microscope (Zeiss, Toronto, Canada) and Northern Eclipse software (Empix Imaging Inc., Mississauga, Canada) under 20× objective. Images were captured as gray scale (8 bit) with a fixed exposure, gain and offset and the intensity measured as % object area of immunoreactive cells of the total selected area. Two bilateral measures, one for each optic tract were made on each section. The corpus callosum did not show significant immunoreactivity and measures were not done for it. For H&E basic stain, images were captured as gray scale (16 bit) and areas within the selected, demarcated optic tracts and corpus callosum which showed rarefaction and did not take up the basic H&E stain were measured by threshold to the background and recorded as % object area of vacuoles of the total selected area. Two bilateral measures, one for each optic tract, and three measures for the corpus callosum (1 middle and 2 lateral periventricular areas) per section were made.

CRP mRNA Expression

RNA was extracted from the cells/tissue using TRIzol (Invitrogen, CAN.) reagent. The first strand of cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, CAN.) at a total RNA (3μg/reaction for RNA extracted from brain tissue samples or 2μg/reaction for RNA extracted from microglia cultures) and according to manufacturer's protocol. cDNA was amplified using primers (Invitrogen, CAN) specific for CRP (forward: 5′-CACGCTGATGTGAGCCGAAG-3′ and reverse: 5′-

ACTTCAGTGCCCGCCAGTTC-3′). CRP was amplified for 35 cycles and yielded a band at 450 bp on1% agarose gels. GAPDH was used as a loading control.

CRP Protein Expression

The cells were collected in lysis buffer containing1M Tris 62.5 ml/L,100mM EDTA25ml /L, 100mM EGTA 25ml/L, 50% glycerol 200ml/L, 20% SDS 100ml/L and 1% Bromophenol Blue 10ml/L. β-Mercaptoethanol 5% was added to the final solution at the time of use. The samples were sonicated briefly for 5 seconds and then boiled for 5 min.Tissue samples were grinded in a lysis buffer containing RIPA buffer and the protein loaded at a concentration of 30μg/lane. All protein samples were loaded on ready prepared gels (Biorad) transferred to nitrocellulose membranes and blocked using 5% milk solution. Rabbit anti-rat CRP (ICL, USA) antibody was used as primary antibody, and after washing with 1X TBS-Tween 20 (3 times) the membranes were incubated with anti-rabbit HRP secondary antibody and developed using chemiluscent reagents. In all Western blot experiments, actin was used as an internal control and samples normalized to it.

CRP Assay

CRP levels in the serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit specific for rat CRP (ICL, USA) according to the manufacturer's instructions. Because of the limitation of availability of a highly sensitive rat CRP ELISA kit, CRP levels could not be measured in the media obtained from microglial cultures.

Stastical Analysis

All results taken from 3 independent experiments for each group are presented as values ± SEM (Standard Error of Mean). Probability based on Student T-Test is shown to be significant at* $p \leq 0.05$, ** $p \leq 0.005$ and *** $p \leq 0.00005$ accordingly.

RESULTS

An Animal Model Exhibiting WML

The first step in our study was to reproduce an animal model exhibiting WML and from there correlate our findings to these lesions. Cerebrovascular WM lesions are reported to be induced experimentally in the rat brain under chronic cerebral hypoperfusion by the permanent occlusion of both common carotid arteries (Wakita H *et al.*, 1994). We therefore implemented the surgical method of bi-lateral common carotid artery ligation (BCCAL) as described in the materials and method section to reproduce an animal model exhibiting WML. In addition previous studies have shown that the pathology of WML includes characteristic features represented by WM rarefaction (vacuolation), axonal disruption, gliosis and microglial activation in lesional areas (Wakita H et al., 1994, 2002; Simpson JE *et al.*, 2007) .To establish these findings in our model H&E staining was used to show areas of vacuolation, Chromogranin A immunostain for axonal disruption, GFAP for gliosis and CD68 immunostain for microglial activation. The following results were obtained:

H&E stain: Vacuolation

The anatomical arrangement of the WM in the rat brain: the corpus callosum, the internal capsule, and the optic tract serve as the most common and easily delineated regions of

interest for WM research (Farkas E *et al.*, 2004). Therefore, we intended to demonstrate lesions in these areas within the 30 day survival period of our BCCAL rat model.

H&E staining showed WM lesions such as vacuolation in the optic tract 3 days after BCCAL and they increased and persisted up to 30 days after ligation in comparison to the sham operated animals (Fig.4, 5) with values reaching 8.92±0.73 % *vs.* 1.16±0.2% for BCCAL vs. sham animals respectively at 30 days of survival. The optic tract showed early, intense and prominent vacuolations over the 30 day survival period in comparison to other WM regions. In the corpus callosum intense vacuolations were observed gradually from day 7-30 after BCCAL in comparison to the sham operated animals (Fig.6, 7) with values of 4.97±0.44% *vs.* 2.9±0.35% for BCCAL vs. sham animals respectively at 30 days of survival. The latter values in comparison to those for optic tract vacuolations at the same time of survival present that the greater lesional area was seen in the optic tracts. The periventricular area of the corpus callosum showed the most vacuolations. The internal capsule showed no vacuolations. These observations correlate with previously reported studies (T. Suenaga *et al.*, 1994; Wakita *et al.*, 1994, 2002; Farkas E *et al.*, 2004, 2007) which indicate that the first and most affected area to show vacuolation is the optic tract and consequently the corpus callosum while the internal capsule is well preserved.

Quantification of the vacuolated areas in the optic tract (Fig.5) and the corpus callosum (Fig.7) at the specified time points after ligation in comparison to the sham operated animals served as a guide to show the increase in the severity and progression of lesions over our specified survival time and how this may correlate with further proposed experiments regarding rat brain tissue expression of CRP, serum CRP levels and microglial activation.

Chromogranin A immunostain: Axonal disruption

In the 30 day sham-operated animals, a few fibers immunoreactive for CgA (a marker for axonal disruption) were observed however a more intense immunoreactivity was observed in animals subjected to BCCAL after 30 days of ligation (Fig.8). Chromogranin A (CgA) is a glycoprotein stored in the matrix of large dense-core vesicles in neurons and endocrine cells (Winkler H *et al.*, 1992). This protein is transported by an anterograde axonal flow and can be used as a marker of axonal injury (Akiguchi I *et al.*, 1997). Compared to the optic tract, which had the most severe vacuolation (rarefaction), the CgA-immunopositive fibers were most numerous in the internal capsule which was well preserved and this corresponds with previously reported studies (Suenaga T *et al.*, 1994; Wakita H *et al.*, 2002) which demonstrate that CgA-immunopositive fibers increase most markedly in the internal capsule.

GFAP immunostain: Gliosis

As for gliosis the regions with the most intense glial activation exhibited greater loss of white matter , and this is similar to previous reports (Wakita H *et al.*, 1994; Farkas E *et al.*, 2004, 2007) which demonstrated that glial activation and white matter changes were most pronounced and early in the optic tract and only moderate in the corpus callosum.

Immunoreactivity of GFAP (an astrocyte marker) showed increased staining in BCCAL animals in comparison to sham after 30 days of survival (Fig.9) demonstrating another characteristic feature associated with WML. An interesting observation was the appearance of large glial cells positive for GFAP which were not seen in CD68 immunostained

sections. This would indicate that they are of astrocytic origin. Large astrocytes known as clasmatodendritic astrocytes have been previously reported (*Simpson JE *et al.*, 2007; Kawamoto Y *et al.*, 2006; Tomimoto H *et al.*, 1997), (Fig.9B).

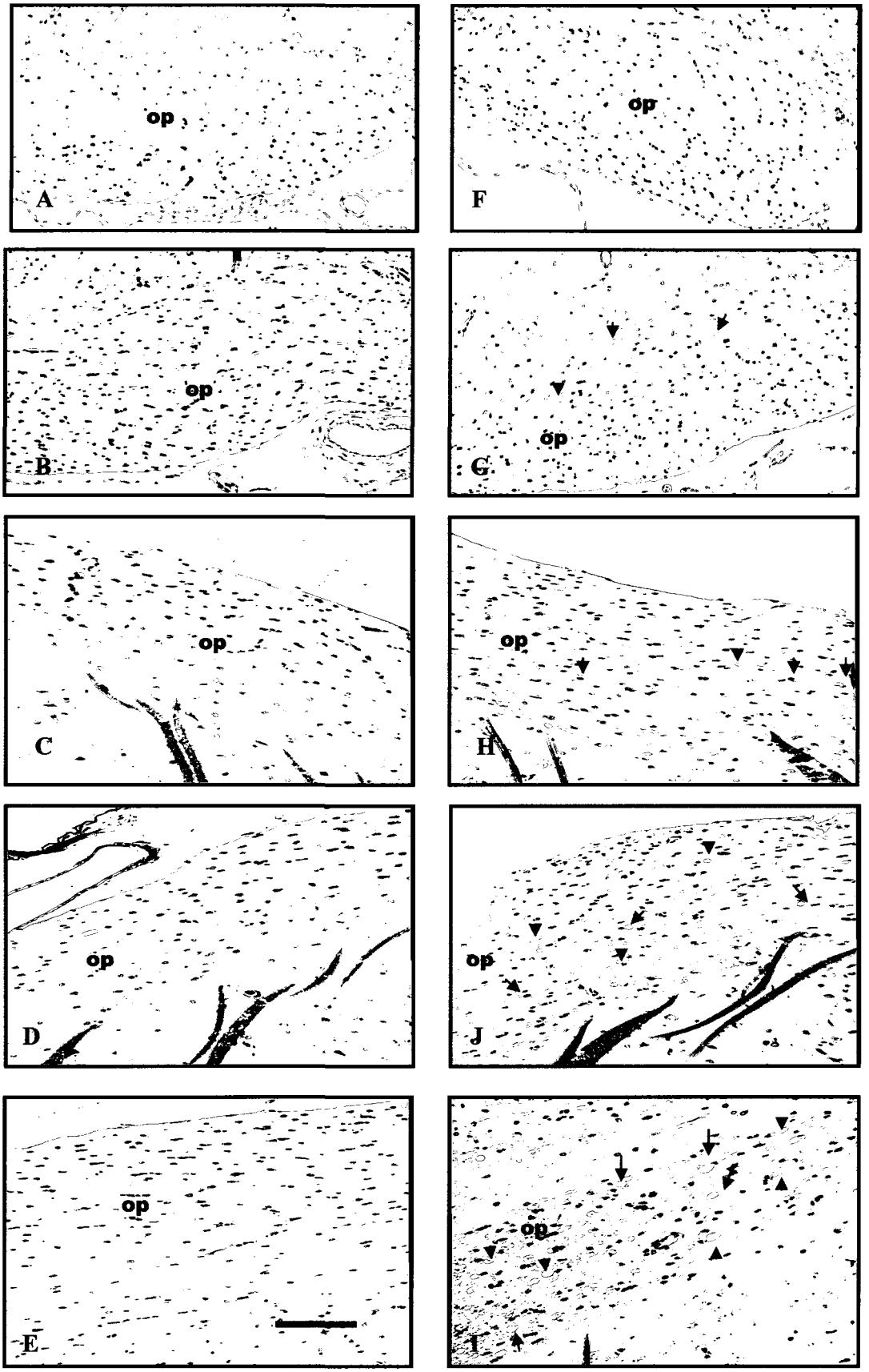
CD68 immunostaining: Microglial activity

CD68 immunostain (a marker for microglial activity) was markedly intense in areas of greater WM vacuolation as seen in the optic tracts, demonstrating the strong association between active microglia and lesional areas in WM injury as previously reported (Wakita H *et al.*, 1994; Farkas E *et al.*, 2004, 2007; Simpson JE *et al.*, 2007). CD68 immunostain in animals subjected to BCCAL was more intense compared to sham operated animals (Fig.10) at the same time point, and there was a specific, relevant increase in CD68 immunoreactivity in the lesional optic tract from day 3 of survival and became significant from day 7 up to day 30 in comparison to the corpus callosum or internal capsule which both showed slight immunoreactivity. It has been reported that Cd68 could play a role in phagocytic activities of tissue macrophages, both in intracellular lysosomal metabolism and extracellular cell-cell and cell-pathogen interactions as it binds to tissue- and organ-specific lectins or selectins, allowing homing of macrophage subsets to particular sites (Holness CL et *al.*, 1993). This would support the initial increase in CD68 immunoreactivity in the optic tract at day 3 of BCCAL when WM lesions have began to appear and then the significant increase in CD68 immunoreactivity as microglia move towards the microlesions and show enhanced activity. In addition the near null immunoreactivity for CD68 in the optic tract of sham operated animals as compared to the increasingly intense immunoreactivity in the optic tract of animals subjected to BCCAL (Fig.11) with values of 5.93±1.53% vs.

0.016±0.008% for BCCAL *vs.* sham animals respectively at 30 days of survival would suggest the importance and significance of microglial activity and their subsequent effect in lesional areas.

To this point we can state that an animal model exhibiting features of white matter damage was established and reproduced in Long Evans rats. A prominent finding in this model was the significant association between severity of optic tract vacuolation and the predominant microglia activity in the optic tract.

Fig.4- Photomicrographs of the H&E staining in the optic tract. Prominent characteristic WML represented by vacuolation in the optic tract are shown and are indicated to demonstrate the early onset, progression and severity of lesions in the optic tracts over the 30 day survival period for BCCAL animals. The animals were subjected to a sham operation (A, B, C, D, E) or a BCCAL (F, G, H, I, J) for 1, 3, 7, 14, 30 days respectively. The most severe WM vacuolation was observed in the optic tracts of BCCAL animals in comparison to other WM regions and arrow heads point to some of the vacuolations observed from day 3-30 of survival. Sham operated animals showed no vacuolations indicating that the BCCAL animal model is presenting a characteristic feature of WML due to the implemented surgical procedure and as reported in previous literature .Scale bar 100μm. op=optic tract.


op
A
op
F
op
B
op
G
op
C
op
H
op
D
op
J
op
E
op
I

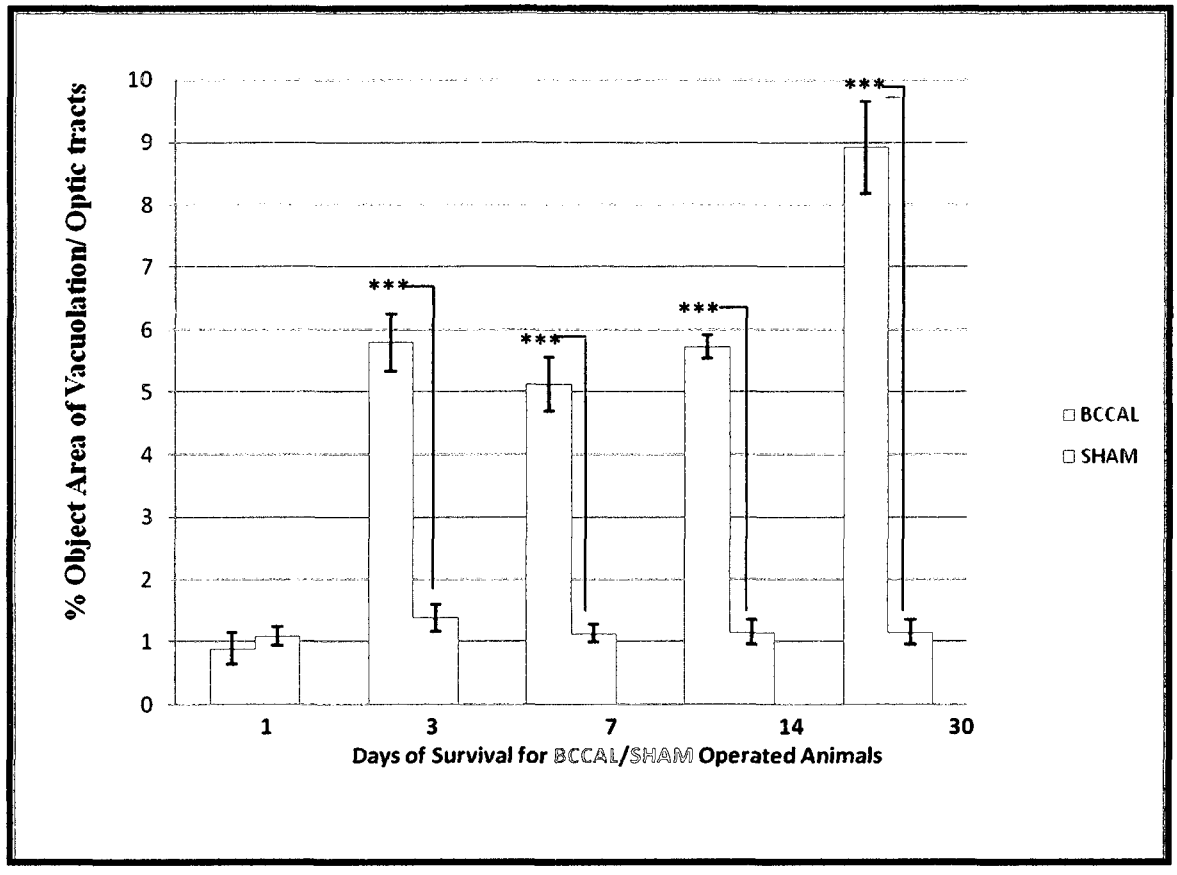


Fig.5- Quantification of % Object Area of Vacuolation /Optic tracts .Intense significant vacuolation is noticed in BCCAL animals compared to SHAM at specified time points of survival, indicating a characteristic feature of WML and the severity of affection of the optic tract in comparison to other WM regions. Intense optic tract vacuolation, a characteristic feature of WML, was noticed in BCCAL animals from day 3-30 of survival. Vacuolations were thresholded against background on H&E stained brain sections obtained from BCCAL/SHAM operated animals after 1,3,7,14,30 days of survival. Data is presented as mean ± SEM (n=3 for BCCAL or Sham operated animals at each time interval). Highly significant (***p <0.00005) increase in vacuolations is presented.

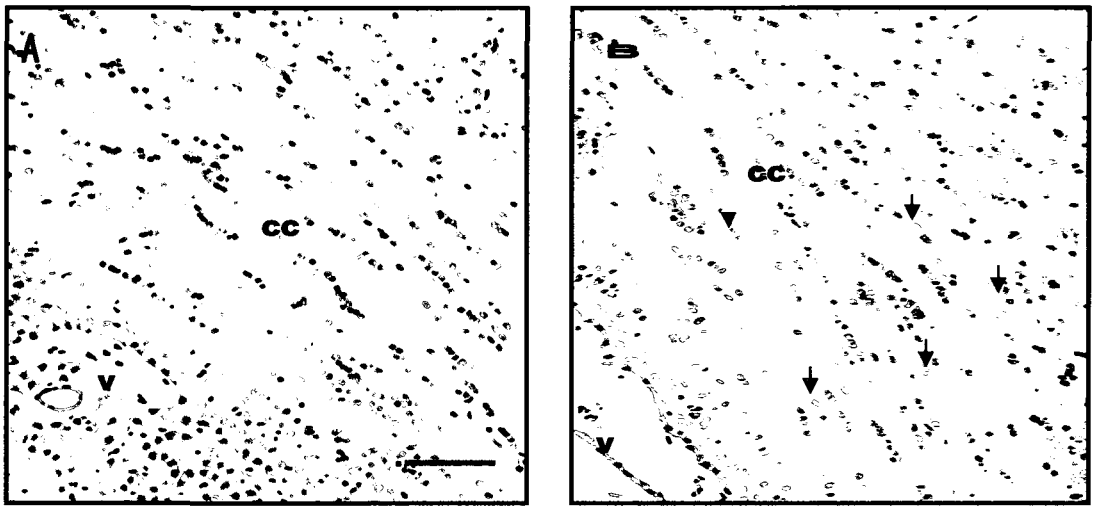


Fig.6- Photomicrographs of the H&E staining in the corpus callosum. WML represented by vacuolation in the corpus callosum are shown. The animals were subjected to a sham operation (A) or a bilateral ligation of the carotid arteries (B) for 30 days. The intensity of changes were less marked in comparison to the optic tract and mostly observed in the periventricular area of the corpus callosum. Arrows point to areas of vacuolation. Scale bar 100μm. cc=corpus callosum, v=ventricle.

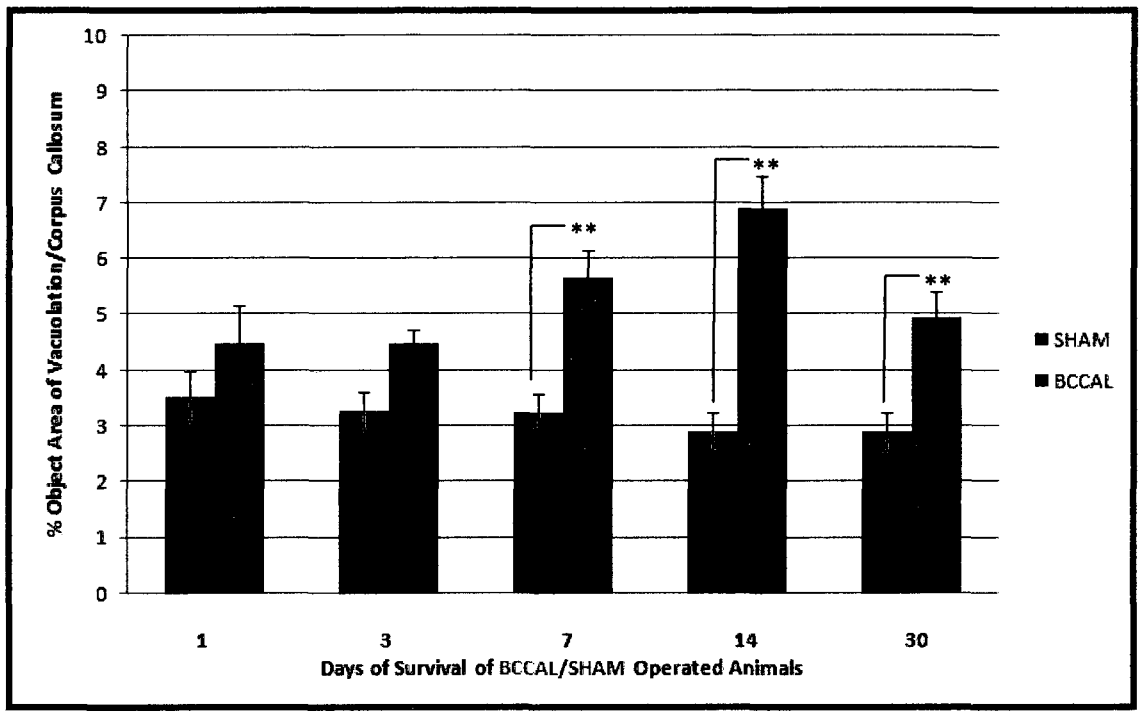


Fig.7- Quantification of % Object Area of Vacuolation /Corpus callosum. Vacuolation was noticed in BCCAL animals from day 7-30 of survival compared to SHAM. Vacuolations were less marked and later in appearance in the corpus callosum than in the optic tracts. Vacuolations were thresholded against background on H&E stained brain sections obtained from BCCAL/SHAM operated animals after 1,3,7,14,30 days of survival. Data is presented as mean ± SEM (n=3 for BCCAL or Sham operated animals at each time interval). Significant (**p <0.005) increase in vacuolations is presented.

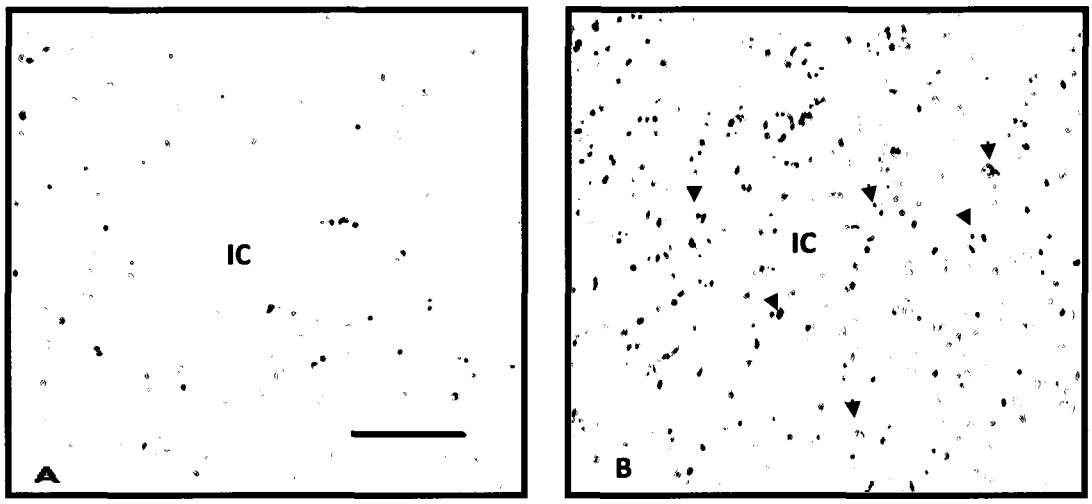


Fig.8- Photomicrographs of the immunohistochemical staining for CgA (a marker for axonal disruption) in the internal capsule. Axonal disruption is a characteristic feature associated with WML. The rats were subjected to a sham operation (A) or a bilateral ligation of the carotid arteries for 30 days (B). In the sham-operated animals, very few fibers immunoreactive for CgA were observed while immunoreactivity increased markedly in the internal capsule of BCCAL animals at 30 days of survival. Arrow heads point to some fibers positive for CgA immunoreactivity. Scale bar, 100μm. IC=internal capsule.

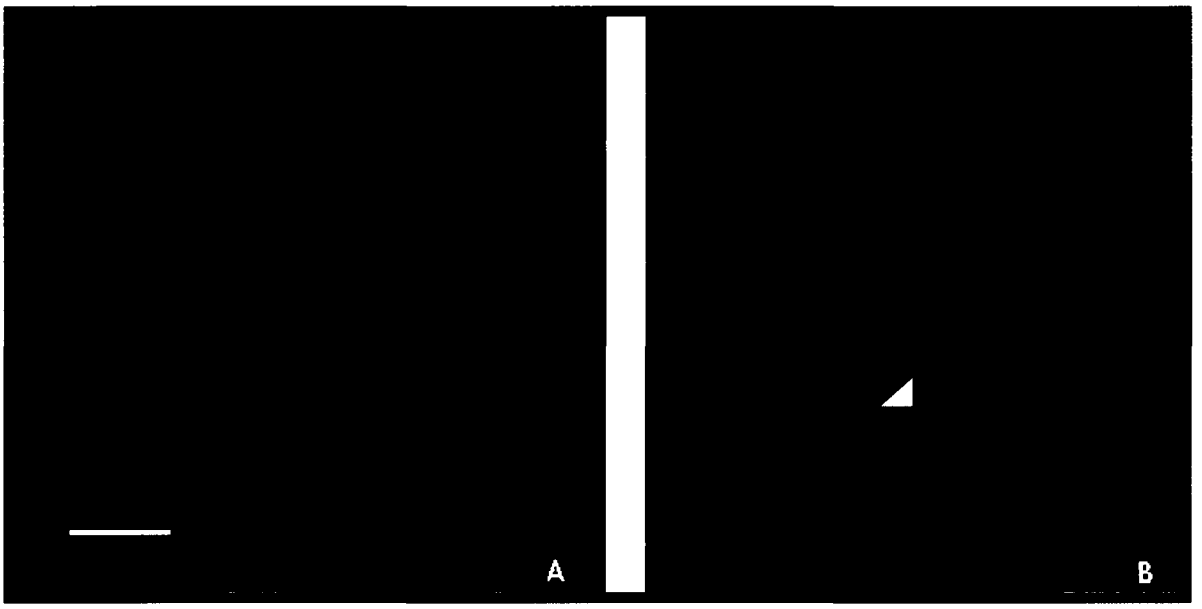
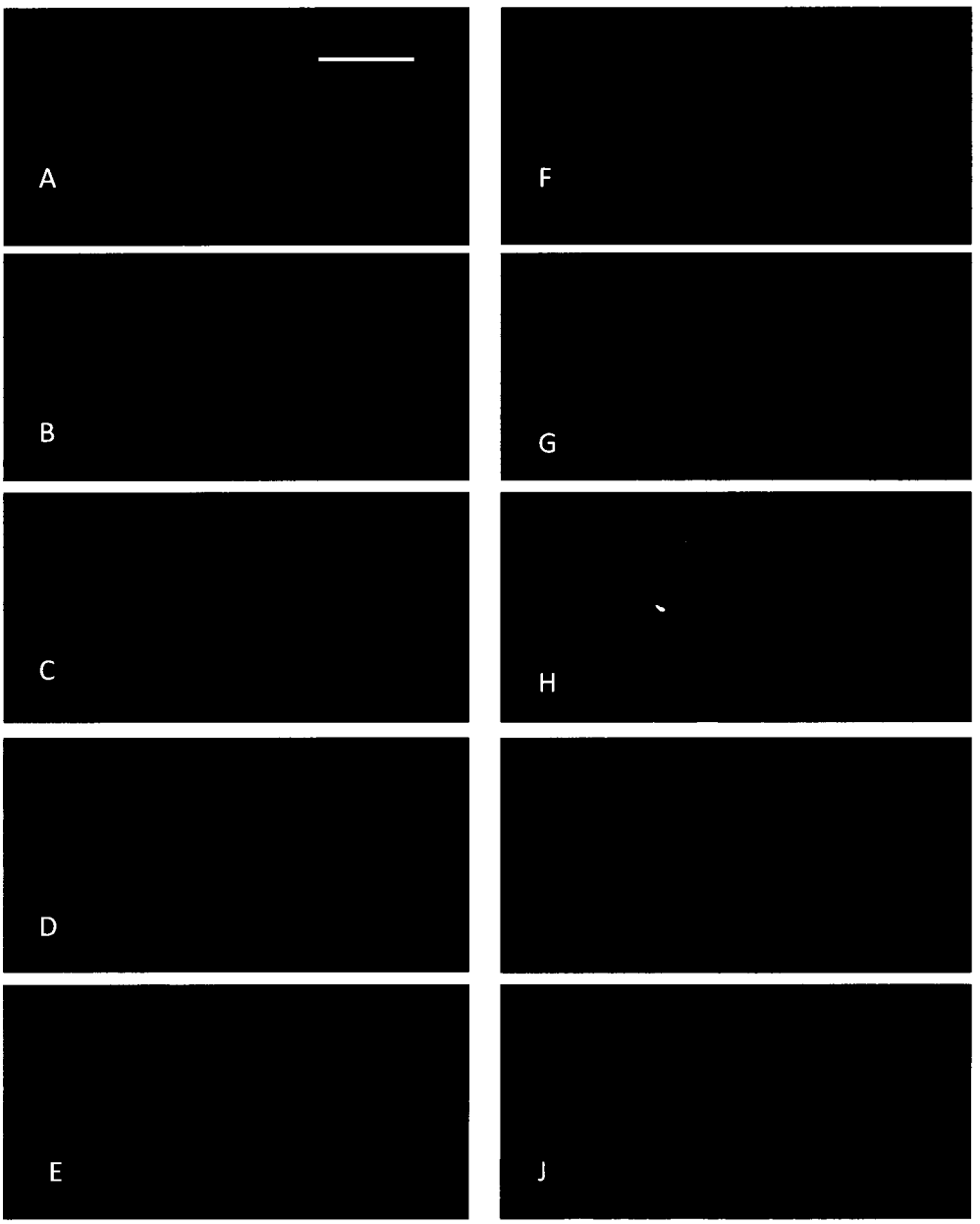


Fig.9- Photomicrographs of immunohistochemical staining for GFAP. Astrocytosis as a feature associated with WM injury is demonstrated. Animals were subjected to a sham operation (A) or bilateral ligation of the carotid arteries for 30 days (B). Large glial cells positive for GFAP were observed in 30 day BCCAL animals. Such cells were not seen with CD68 immunostaining. White arrow head points to large glial cell. Scale bar, 50μm.

Fig.10 -Photomicrographs of immunohistochemical staining for CD68. A significant increase in microglial activity as a feature associated with WM injury is demonstrated. Sections show optic tracts for SHAM (A, B, C, D, E) versus BCCAL (F, G, H, I, J) at 1, 3, 7, 14, 30 days after surgery respectively. Immunoreactivity of CD68 in animals subjected to BCCAL was significantly more intense compared to sham operated animals at the same time point, and there was an increase in CD68 immunoreactivity in the lesional optic tract at day 3 of survival and this increased significantly and persisted until day 30 of survival in BCCAL. Scale bar, 50μm.


A
F
B
G
C
H
D
E
J

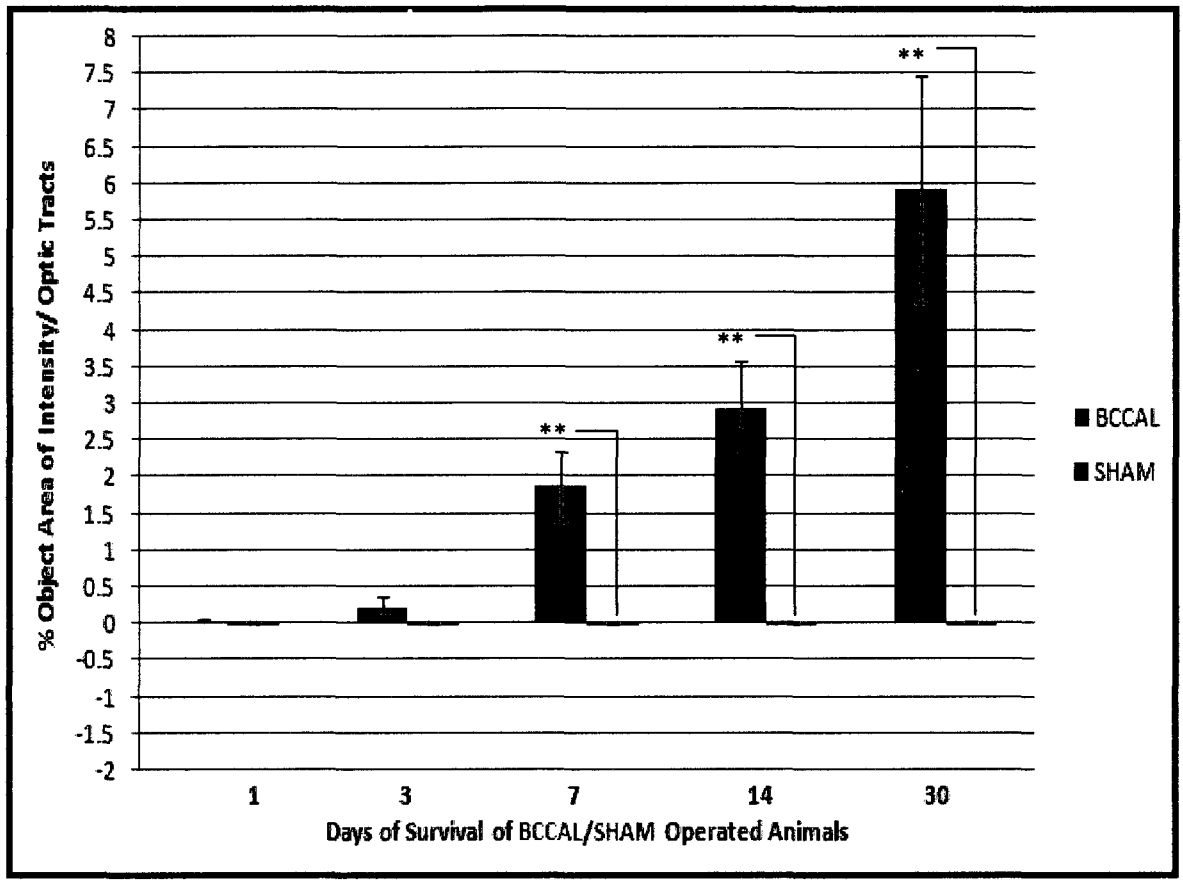


Fig.11- Quantification of immunohistochemical staining for CD68 at 1, 3, 7, 14, 30 days after BCCAL/SHAM surgery. An increase for CD68 (a marker for microglial activity) intensity was noticed from day 3 of survival and became significant from days 7-30 of survival in the optic tracts of BCCAL versus Sham animals (**p <0.005). Values are presented as Mean ± SEM (n=3 for BCCAL or Sham operated animals at each time interval).

WML and CRP

One of the objectives in this study was to show the presence of CRP in samples of brain tissue obtained from animals subjected to BCCAL demonstrating features of WML and how this would compare to sham operated animals. By this we would try to define if CRP is found in brain tissue and if it is how this may correlate with WML induced in the BCCAL model. Western blot analysis (Fig.12A) revealed a CRP protein band identical to that found in rat liver tissue, our positive control and the main site of CRP production as stated in previous literature (Black S et al., 2004; Pepys and Hirschfield, 2003; Pepys MB *et al.*, 1983).Sham operated animals showed much less intense bands (Fig.12B), however, a few occasional thicker bands were noticed. Quantitative analysis of protein CRP expression for BCCAL vs. SHAM animals showed an increase in BCCAL samples in comparison to Sham samples for all 1, 3, 7, 14, 30 days of survival. However an intriguing finding was the significant increase of protein expression for CRP in BCCAL vs. SHAM animals at day 3 of survival with values of 0.122±0.012 vs. 0.046±0.016 for BCCAL vs. SHAM respectively (Fig.12C). This increase coincides with the appearance of WM vacuolations and microglial activity (CD68) seen in our animal model.

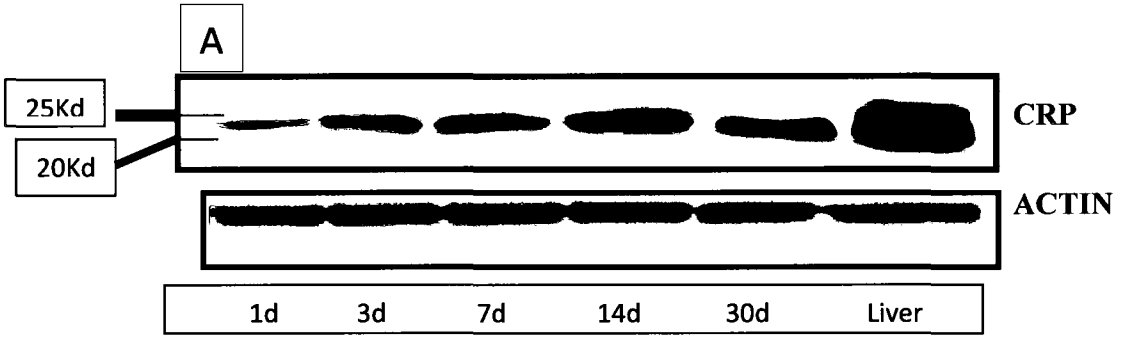
It could be argued that plasma CRP carried within the blood vessels due to the acute inflammatory state imposed by surgery for both groups may account for the increased presence of CRP in some samples and this has to be considered. In addition it should be noted that a baseline level for CRP production from cells within the rat brain tissue and from the cerebral microvasculature cannot be ruled out and this would account for the sham

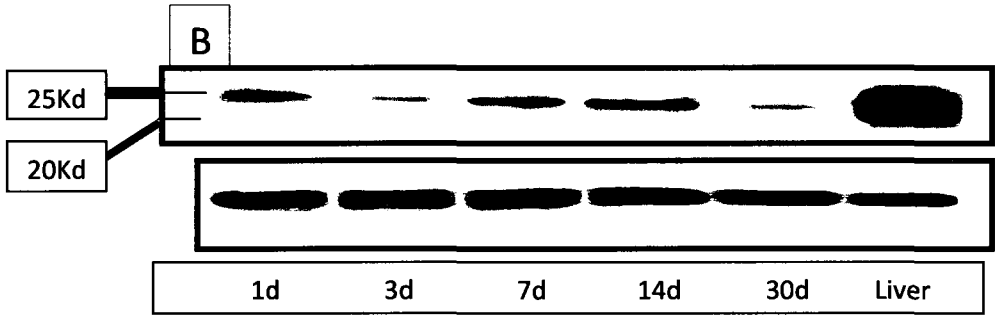
animals also expressing CRP bands. To answer these proposals and emphasize that the CRP protein expression in BCCAL animals is mainly from brain tissue we went on to demonstrate mRNA CRP expression. The expression of mRNA would be of tissue origin i.e. from the cells within the brain tissue and is not affected by plasma CRP carried within the blood vessels. Therefore, expression of mRNA CRP was demonstrated in RNA extracted from animals subjected to BCCAL and this also revealed a band identical to that found in rat liver tissue (Fig.13A).This mRNA expression of CRP would consequently cause protein expression of CRP in the brain tissue irrespective of what may be carried within the blood vessels as plasma CRP. Sham operated animals mostly showed no detectable bands however a few very faint bands for CRP mRNA production were seen in some time points (Fig.13B) and this would propose that a baseline level for CRP production from within the rat brain tissue and other cell types within the cerebral microvasculature is a point to be considered. Quantitative analysis of CRP mRNA expression for BCCAL vs. SHAM animals showed an increase in BCCAL samples in comparison to Sham samples for all 1, 3, 7, 14, 30 days of survival. However a significant increase of mRNA expression for CRP in BCCAL vs. SHAM animals was initially noticed at day 3 of survival (values of 1.183±0.351 vs. 0.027±0.011 respectively) and day 7 of survival (values of 0.849±0.273 vs. 0.053±0.028 respectively) and then became less significant after day 7 of survival (Fig.13C). This increase coincides with the significant increase in protein expression of CRP, appearance of WM vacuolations and microglial activity (CD68) in our animal model.

To the best of our knowledge no previous literature has identified protein and mRNA expression of CRP from brain tissue in a rat model of chronic hypoperfusion exhibiting WML.

Elisa assay of serum CRP levels from BCCAL animals compared to Sham operated animals did not show a significant difference. This may well be attributed to the fact that both groups of animals are in a state of acute inflammation due to the surgical procedure. Moreover, an acute inflammatory state due to surgical trauma followed by consequent wound healing would overshadow the subtle chronic effect of cerebral hypoperfusion on plasma CRP concentration. A longer time for survival may show some differences in CRP serum levels between the two groups (Fig.14).

Fig.12 - CRP/Actin western blots (*in vivo*). Brain tissue as a source for protein expression of CRP is demonstrated. Expression of CRP is found in all BCCAL brain tissue protein extracts. **(A)** ***Western blot (BCCAL)*** shows protein expression for CRP at 1,3,7,14,30 days of survival after BCCAL. Bands correlate to MW of ~25 KD as shown on ladder. Adjacent blot shows actin as a loading control. Lanes 1, 2, 3, 4, 5 from the same blot correspond to 1,3,7,14,30 days after BCCAL. Lane 6 shows CRP in liver tissue as a positive control. **(B)** ***Western blot (SHAM)*** shows protein expression for CRP at 1, 3, 7, 14, 30 days after surgery for SHAM animals. Adjacent blot shows B-actin as a loading control. CRP expression in SHAM animals was less in comparison to BCCAL animals at the same day of survival. Lanes 1, 2, 3, 4, 5 from the same blot correspond to 1,3,7,14,30 days of survival after sham surgery. Lane 6 shows CRP in liver tissue as a positive control. **(C)** ***Quantitative Analysis*** of protein bands for CRP in BCCAL /SHAM operated animals. All BCCAL protein samples showed a relative increase in comparison to their SHAM counterparts for the specified day of survival. However a very interesting finding was the significant increase of protein expression for CRP in BCCAL vs. SHAM animals at day 3 of survival. This increase coincides with the appearance of WM vacuolations and microglial activity (CD68) in our animal model. Values are shown as Mean ± SEM (n=3 for each BCCAL/SHAM group). $*p \leq 0.05$ denotes significance. All values were normalized to actin.


A
25Kd
20Kd
CRP
ACTIN
1d
3d
7d
14d
30d
Liver


B
25Kd
20Kd
1d
3d
7d
14d
30d
Liver

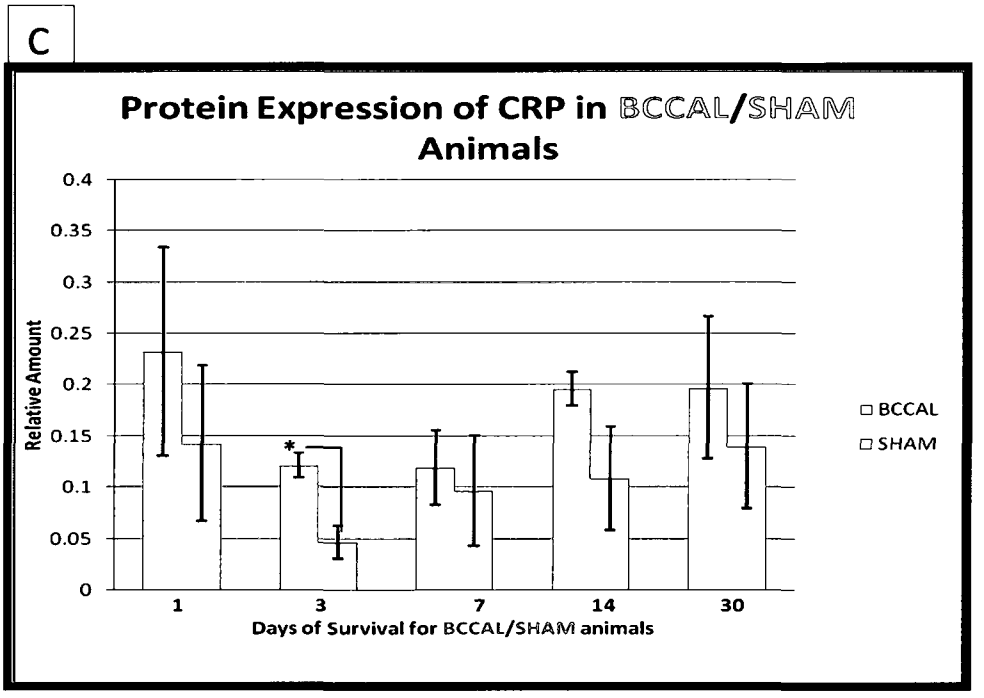
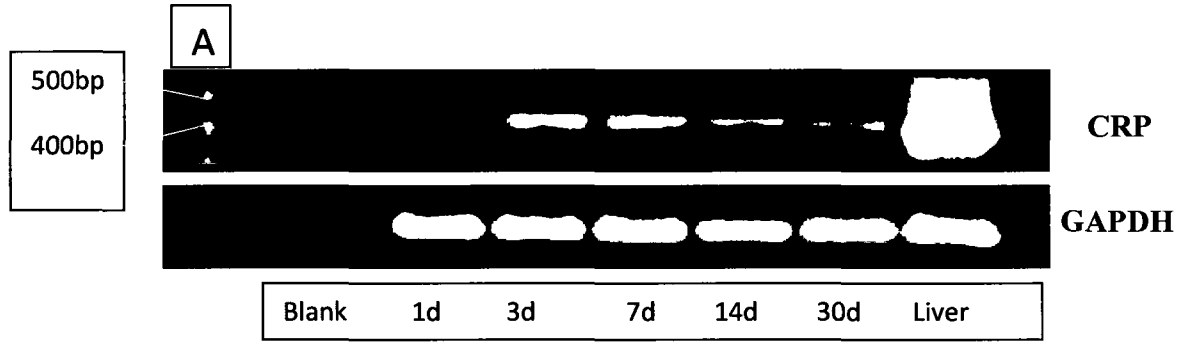
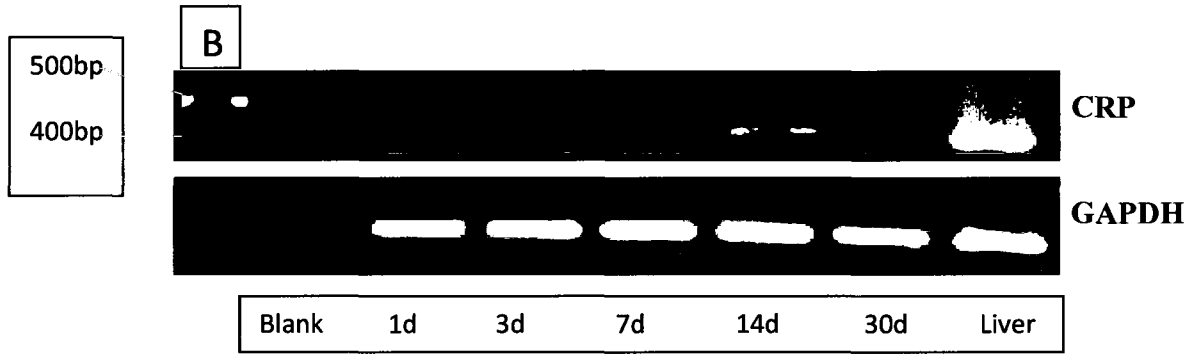
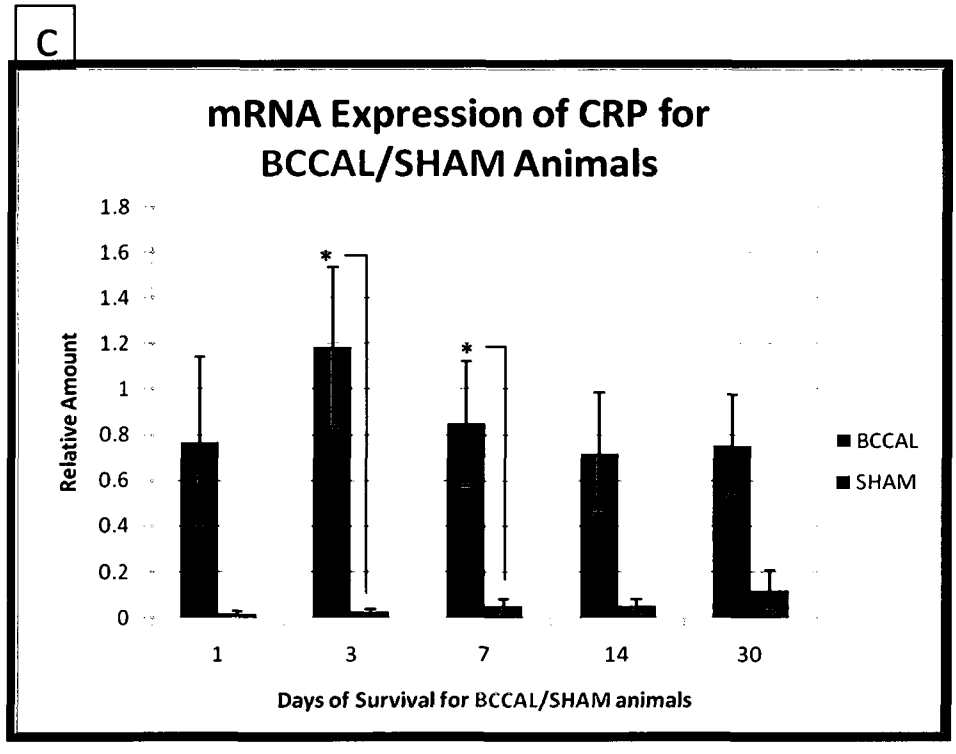

C
Protein Expression of CRP in BCCAL/SHAM Animals
Relative Amount
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
1
3
7
14
30
Days of Survival for BCCAL/SHAM animals
BCCAL
SHAM

Fig.13- mRNA CRP/GAPDH gels (*in vivo*). Brain tissue as a source for CRP mRNA expression is demonstrated. This would emphasize that rat brain tissue is a source for CRP synthesis and expression irrespective of plasma CRP carried within the cerebral blood vessels. (**A*) mRNA CRP/GAPDH gels (BCCAL)*** shows mRNA expression for CRP at 1,3,7,14,30 days of survival after BCCAL. Bands correlate to ~450 bp as shown on ladder. Adjacent blot shows GAPDH as a loading control. Lanes 1, 2, 3, 4, 5 from the same blot correspond to 1,3,7,14,30 days after BCCAL. Lane 6 shows CRP in liver tissue as a positive control. Expression of mRNA CRP bands was found in BCCAL brain tissue RNA extracts specifically from day 3 of survival up to day 30 of survival. **(B) *mRNA CRP/GAPDH gels (SHAM)*** shows mRNA expression for CRP at 1, 3, 7, 14, 30 days of survival after SHAM surgery. Most RNA extracts from brain tissue of SHAM animals showed faint (as shown) or no CRP mRNA bands. This would indicate that a baseline level for CRP expression is a point for consideration. Bands correlate to ~450 bp as shown on ladder. Adjacent blot shows GAPDH as a loading control. Lanes 1, 2, 3, 4, 5 from the same blot correspond to 1,3,7,14,30 days of survival after sham surgery. Lane 6 shows CRP in liver tissue as a positive control. **(C) *Quantitative Analysis*** of mRNA bands for CRP in BCCAL /SHAM operated animals. All BCCAL protein samples showed a relative increase in comparison to their SHAM counterparts for the specified day of survival. However a significant increase of mRNA expression for CRP in BCCAL vs. SHAM animals was noticed initially at day 3 and became less significant after day 7 of survival. This increase coincides with the appearance of WM vacuolations and microglial activity (CD68) in our animal model. Values are shown as Mean ± SEM (n=3 for each BCCAL/SHAM group). $*p \leq 0.05$ denotes significance. All values were normalized to GAPDH.


A
500bp
400bp
CRP
GAPDH
Blank
1d
3d
7d
14d
30d
Liver


B
500bp
400bp
CRP
GAPDH
Blank
1d
3d
7d
14d
30d
Liver


C
mRNA Expression of CRP for BCCAL/SHAM Animals
Relative Amount
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
1
3
7
14
30
Days of Survival for BCCAL/SHAM animals
BCCAL
SHAM

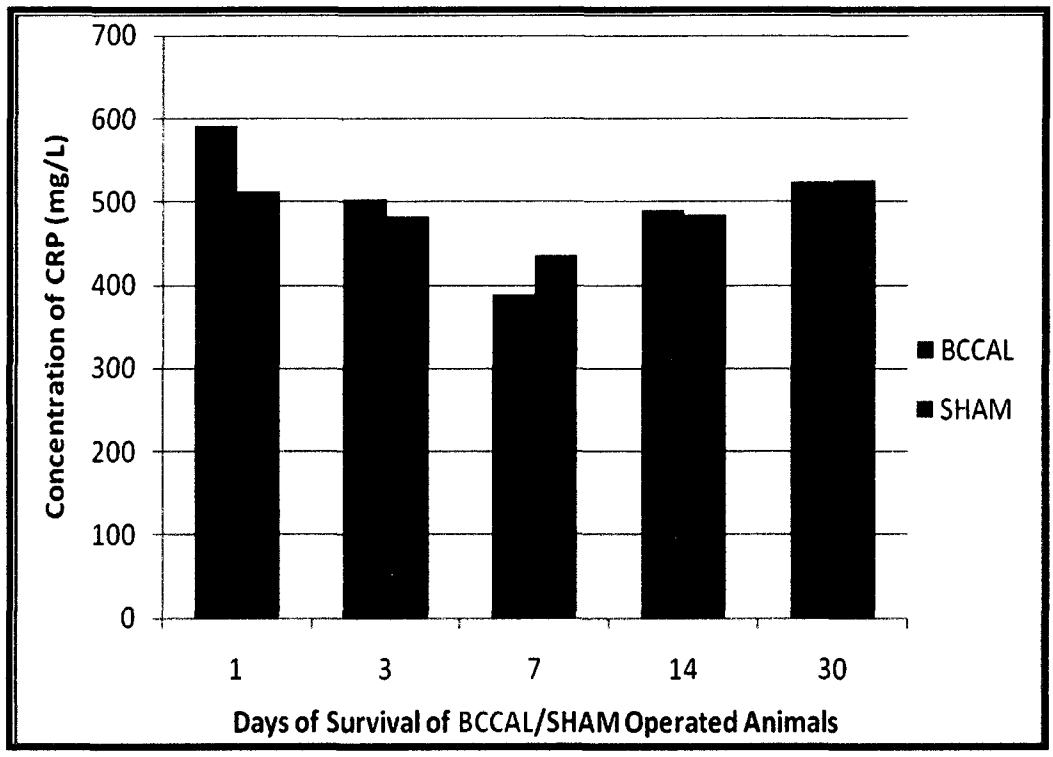


Fig.14- Quantification of serum levels of CRP by ELISA. Concentration of serum CRP obtained from blood of BCCAL/Sham animals after 1, 3, 7, 14, 30 days after surgery is shown. No relevant increase in plasma CRP was detected between the two groups. The state of acute inflammation inflicted due to the surgical procedures on BCCAL/ SHAM animals could be the cause for this result. An acute inflammatory state followed by consequent wound healing would overshadow the subtle chronic effect of cerebral hypoperfusion. A longer time for survival may show some differences in serum levels. Values are presented as Mean± SEM for 3 independent assays for BCCAL/SHAM at each time interval.

CRP and Microglia

The above observations state that rat brain tissue is a source for CRP mRNA and protein expression. The next question to introduce itself is "From where within the brain is CRP expressed". Any cell type contained by the brain tissue or cerebral microvasculature could be a candidate. We chose to study the "Surveillance cells of the brain: The Microglia". We based our choice on previous literature and our own above mentioned findings.

Previous literature has shown that in states of chronic cerebral hypoperfusion, the activation of microglia appeared from day 3 of survival onwards (Watanabe T *et al.*, 2006; Shibata M *et al.*, 2004; Wakita H *et al.*, 1994). In our study an intriguing observation was that the expression of CRP protein and mRNA was significant from day 3 of survival in BCCAL animals coinciding with the appearance of WM vacuolations and microglial activity (CD68) in the optic tracts at the same time in our animal model. We therefore hypothesized that microglia could play a role in CRP expression and we proceeded to *in-vitro* studies of microglial cultures.

Our intention was to not use any bacterial endotoxin as this would not mimic the in vivo environment found in WML which is due to chronic hypoperfusion. We needed to induce CRP through a stimulant for which microglia possesses receptors and which would be present in the *in vivo* environment associated with WML. Induction of CRP in hepatocytes (the main site of CRP production in the body) is principally regulated at the transcriptional level by the cytokine interleukin-6 (IL-6), an effect which can be enhanced by interleukin-1β (IL-1β) (Kushner I *et al.*, 1995). Therefore we hypothesized that microglia may also result in

CRP induction by the same mentioned interleukins since it elaborates receptors for and secretes IL-6 and IL-1β (Kim SU *et al.*, 2005; Kim and de Vellis, 2005; Hanisch UK, 2002). In addition, in response to any tissue damage (which in our study is WML) an acute phase response is initiated (Baumann H *et al.*, 1994). This response is mediated by the action of cytokines, mainly interleukin 6-(IL6-) and interleukin 1-(IL1-) type cytokines (Ramadori G *et al.*, 1999), therefore they would be present in the *in vivo* environment associated with WML. For the mentioned reasons we chose IL-6 (at30ng/ml or 50ng/ml), IL-1β (at30ng/ml or 50ng/ml) or a combination of IL-6+ IL-1β (at30ng/ml or 50ng/ml) to show various levels of induction of CRP expression in microglia. The different concentrations used were based on previous literature which applied approximately the same range of interleukin concentrations to induce expression of CRP from human aortic endothelial cells (Venugopal SK *et al.*, 2005).

Our initial experiments were with microglia cultures at the control level and those treated with IL-6 (at30ng/ml or 50ng/ml) since it is reported to be the principal regulator of CRP (Kushner I *et al.*, 1995). Surprisingly and in accordance with our presumption, RNA extraction from cultured microglia showed faint expression of CRP mRNA at the control level and with an increasing intensity in treated cultures of IL-6 (Fig.15A).mRNA band was similar to that found in the liver (our positive control) at ~ 450bp. Consequently we went on to demonstrate and compare cultures treated with IL- 1β (at30ng/ml or 50ng/ml), IL-6 (at30ng/ml or 50ng/ml) and a combination of both interleukins IL-β+ IL-6 (at30ng/ml or 50ng/ml). All treated cultures showed increased mRNA CRP expression in comparison to control cells (Fig.15B). This expression however was not greatly affected by the different concentrations used in each treatment. All values obtained from 3 independent experiments

for each group were normalized to GAPDH and then to control to show relevance of treatments in stimulating CRP expression (Fig.15C).

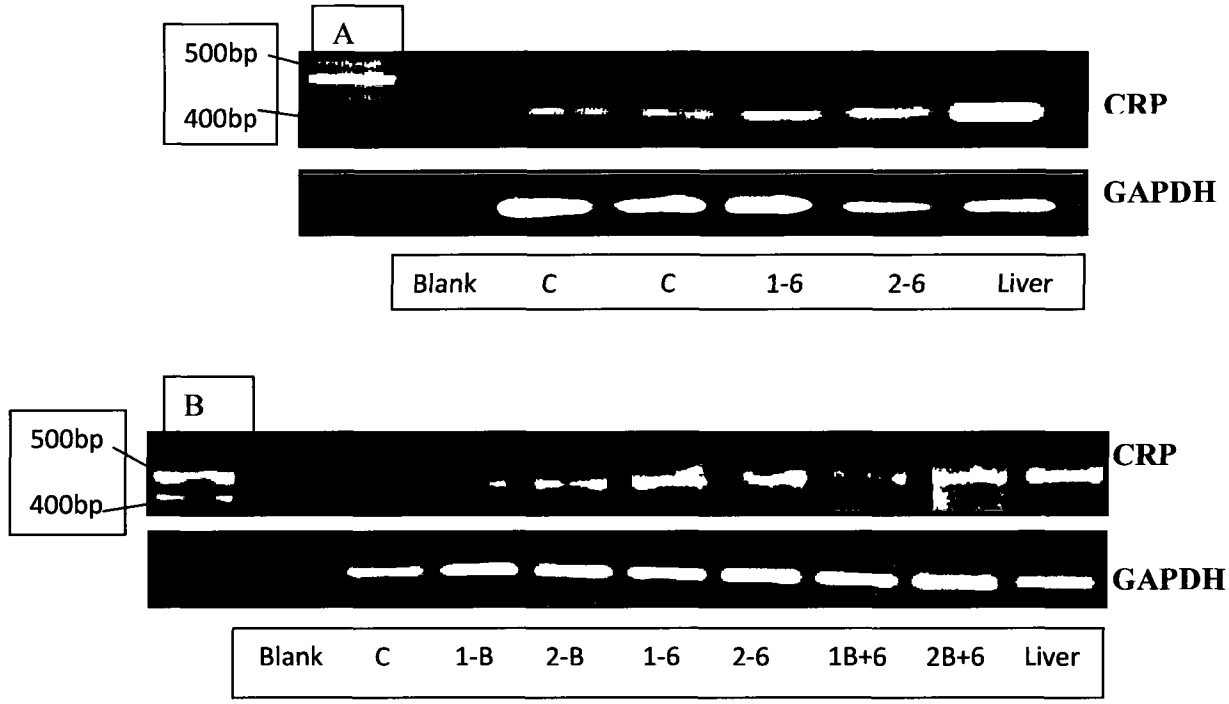
In addition protein extracted from cultured microglia revealed a similar band to that found in liver tissue at~25Kd (Fig.16A). Moreover microglial cultures treated with IL- 1β (at30ng/ml or 50ng/ml), IL-6 (at30ng/ml or 50ng/ml) and a combination of both interleukins IL-β+ IL-6 (at30ng/ml or 50ng/ml) showed a slight increase in CRP protein expression in comparison to control (Fig.16A). As previously mentioned microglia cells themselves also secrete different interleukins including IL-1β and IL-6 and this would assumingly enable the expression of CRP in untreated cultures. CRP expression however was not greatly affected by the different concentrations used in each treatment. All values obtained from 3 independent experiments for each group were normalized to actin and then to control to show relevance of treatments in stimulating CRP expression (Fig.16B).

Because of the limitation of availability of a highly sensitive rat CRP ELISA kit, CRP levels could not be measured in the media obtained from microglial cultures.

To demonstrate microglia in culture and to show if they posses affinity for CRP, immunocytochemistry of the cultured cells was performed. Immunocytochemistry revealed a positive immunoreactivity for the microglial marker Cd11b and cultures showed more than 95% purity (Fig.17A). Significantly, microglia also showed immunoreactivity for anti-rat CRP antibody and the latter co-localized with Cd11b immunostained microglia (Fig.17A, D). These results would additionally demonstrate that CRP expression is from microglia cells. A clear difference in CRP intensity was not noted with increased IL-1β or IL-6 concentrations (Fig.17C, D).

To this point, the relation between microglia and CRP expression has not been demonstrated in previous literature and further research may well elucidate a role for this novel relation in the pathologies of chronic hypoperfusion and WML affecting the brain.

Fig.15- mRNA CRP/GAPDH gels (*in vitro*). Microglia as a source for CRP mRNA expression is demonstrated. **(A*) mRNA CRP/GAPDH gels*** show mRNA expression for CRP in control and IL-6 treated cultures. Control microglia cultures show a faint band for CRP expression, this was intensified with IL-6 treated cultures at concentrations of 30ng/ml or 50ng/ml. Bands correlate to ~450 bp as shown on ladder. Adjacent blot shows GAPDH as a loading control. Lanes 2&3 (C) correspond to control cultures; lane 4 (1-6) is microglia cultures treated with IL-6 at 30ng/ml; lane 5 (2-6) is microglia cultures treated with IL-6 at 50ng/ml; lane 6 shows CRP in liver tissue as a positive control. Lane one is a blank. **(B*) mRNA CRP/GAPDH gels*** show mRNA expression for CRP in control (C); IL-1β at 30ng/ml (1-B); IL-1β at 50ng/ml (2-B); IL-6 at 30ng/ml (1-6) ; IL-6 at 50ng/ml (2-6); a combination of IL- 1β + IL-6 at 30ng/ml each (1B+6); a combination of IL- 1β + IL-6 at 50ng/ml each (2B+6); liver tissue as a positive control, in lanes 2, 3, 4, 5, 6, 7, 8, 9 respectively. Lane one is a blank. All treated cultures showed intensified bands for mRNA expression of CRP in comparison to control cultures in this batch of experiments. Bands correlate to ~450 bp as shown on ladder. Adjacent blot shows GAPDH as a loading control. **(C) *Quantitative Analysis*** of mRNA bands for CRP in microglia cultures. All cultures treated with IL-1β at 30ng/ml (1 IL-1b); IL-1β at 50ng/ml(2 IL-1b); IL-6 at 30ng/ml (1 IL-6) ; IL-6 at 50ng/ml (2 IL-6); a combination of IL-1β + IL-6 at 30ng/ml each (1 IL-1b+6); a combination of IL- 1β + IL-6 at 50ng/ml each (2 IL-1b+6); showed a relative increase in mRNA CRP expression in comparison to control (C). However this expression was not greatly affected by the different concentrations used for each treatment. Values are shown as Mean ± SEM for 3 independent experiments for each group. All values were normalized to GAPDH and then normalized to control to show relevance of treatments on mRNA CRP expression.


A
500bp
400bp
CRP
GAPDH
Blank C C 1-6 2-6 Liver
B
500bp
400bp
CRP
GAPDH
Blank C 1-B 2-B 1-6 2-6 1B+6 2B+6 Liver

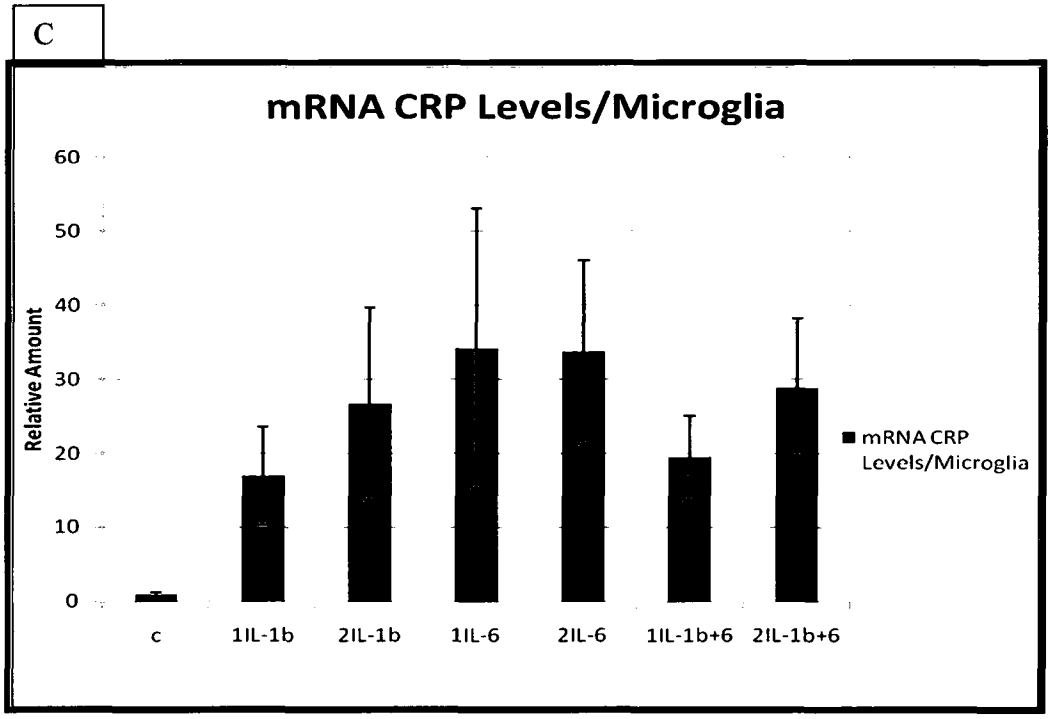
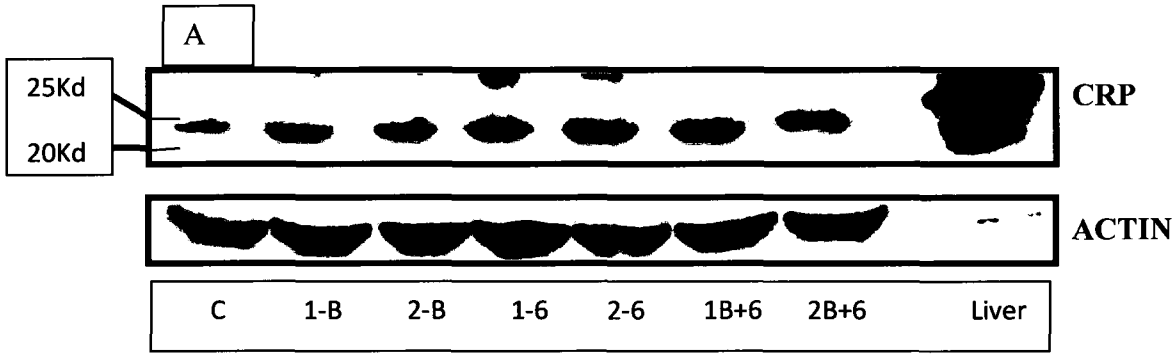

C
mRNA CRP Levels/Microglia
Relative Amount
60
50
40
30
20
10
0
c
1IL-1b
2IL-1b
1IL-6
2IL-6
1IL-1b+6
2IL-1b+6
mRNA CRP Levels/Microglia

Fig.16 - CRP/Actin western blots. Microglia as a source for protein expression of CRP is demonstrated. Expression of CRP is found in control and treated microglia cultures. **(A)** ***Western blot (Microglia Cultures)*** shows protein expression for CRP in control;1-B: IL-1β at 30ng/ml ; 2-B: IL-1β at 50ng/ml; 1-6: IL-6 at 30ng/ml ; 2-6: IL-6 at 50ng/ml; 1B+6: a combination of IL- 1β + IL-6 at 30ng/ml each; 2B+6: a combination of IL- 1β + IL-6 at 50ng/ml each; liver tissue as a positive control(loading amount of liver tissue protein sample was decreased so as to produce a discrete band to correlate with cell cultures in this experiment) in lanes 1, 2, 3, 4, 5, 6, 7, 8 respectively. Bands correlate to MW of ~25 KD. Adjacent blot shows actin as a loading control. **(B)** ***Quantitative Analysis*** of protein bands for CRP in microglia cultures. All cultures treated with IL-1β at 30ng/ml (1 IL-1b); IL-1β at 50ng/ml(2 IL-1b); IL-6 at 30ng/ml (1 IL-6) ; IL-6 at 50ng/ml (2 IL-6); a combination of IL-1β + IL-6 at 30ng/ml each (1 IL-1b+6); a combination of IL- 1β + IL-6 at 50ng/ml each (2 IL-1b+6); showed a slight increase in mRNA CRP expression in comparison to control (C). However this expression was not greatly affected by the different concentrations used for each treatment. Values are shown as Mean ± SEM for 3 independent experiments for each group. All values were normalized to actin and then normalized to control to show relevance of treatments on protein CRP expression.


A
25Kd
20Kd
CRP
ACTIN
C
1-B
2-B
1-6
2-6
1B+6
2B+6
Liver

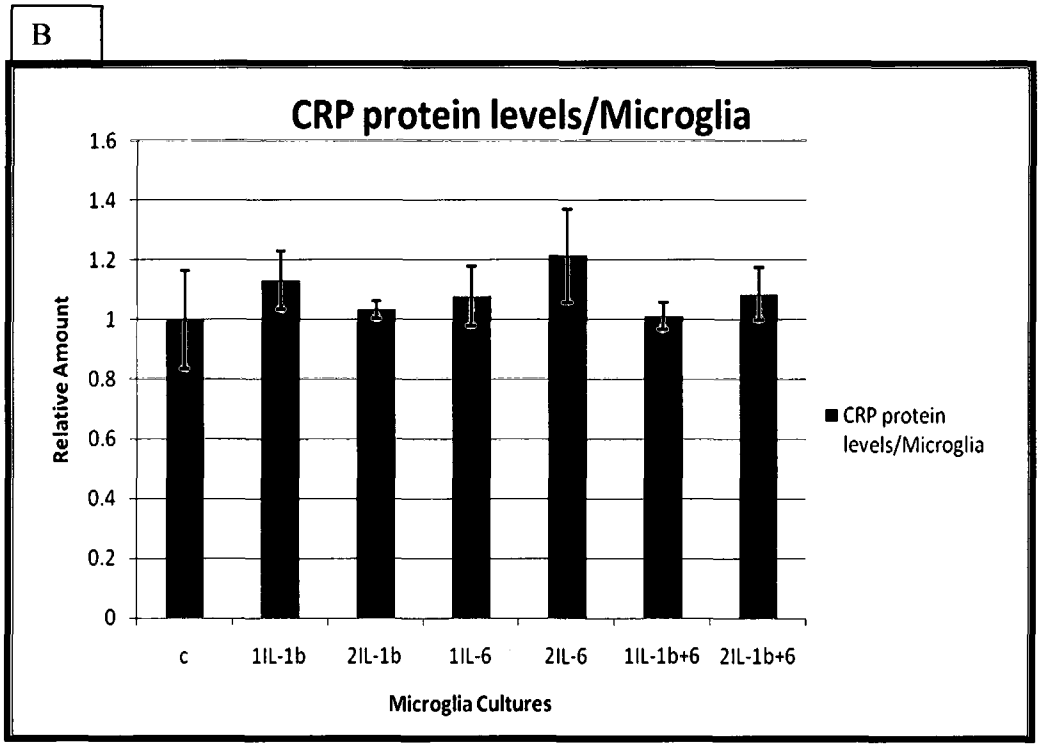
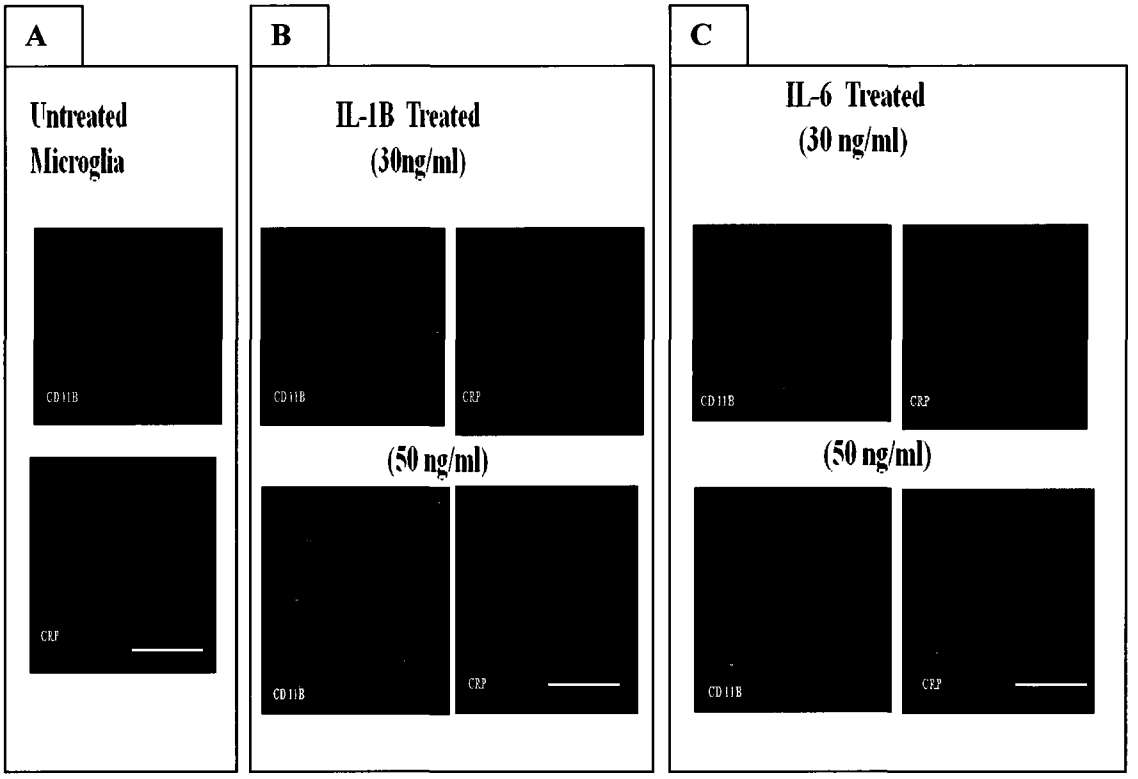
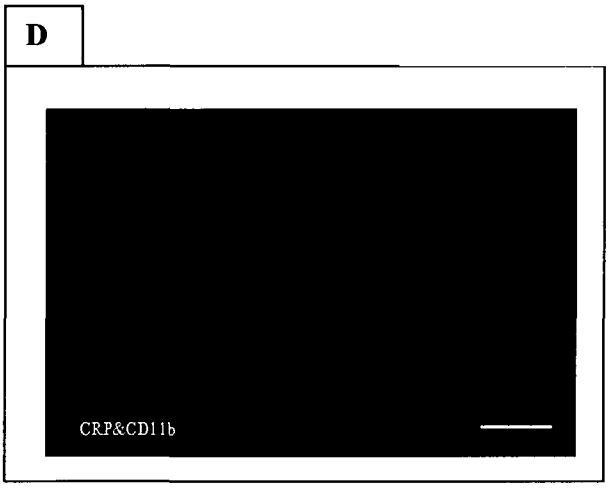

B
CRP protein levels/Microglia
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative Amount
c
1IL-1b
2IL-1b
1IL-6
2IL-6
1IL-1b+6
2IL-1b+6
Microglia Cultures
CRP protein levels/Microglia

Fig.17 -Photomicrographs of microglia cultures. A positive affinity for CRP immunostain is demonstrated. Photomicrographs show immunohistochemical staining for Cd 11b (a microglia marker), anti-rat CRP and their co-localization in microglia cultures. This finding supports that CRP expression is associated with microglia cells in culture. Cell cultures were > 95% pure microglia. **(A)** ***Untreated Microglia Cultures:*** Untreated (control) microglia in culture is shown. Cells were immunopositive for Cd11b and anti-rat CRP. Scale bar, 20μm. **(B)** ***IL-1β Treated Microglia Cultures:*** Cultures were treated with IL-1β at 30ng/ml or 50ng/ml to enhance CRP expression. A difference in CRP intensity was not noted with increased IL-1β concentration. Scale bar, 20μm. **(C)** ***IL-6 Treated Microglia Cultures:*** Cultures were treated with IL-6 at 30ng/ml or 50ng/ml to induce CRP expression. A clear difference in CRP intensity was not noted with increased IL-6 concentration. Scale bar, 20μm. **(D)** ***CRP & Cd11b Co-localized:*** Microglia cells in culture were double labeled for Cd11b and CRP. Both immunostain showed co-localization within the microglia cells. Scale bar, 50μm.


A
Untreated
Microglia
CD11B
CRP
B
IL-1B Treated
(30ng/ml)
CD11B
CRP
(50 ng/ml)
CD11B
CRP
C
IL-6 Treated
(30 ng/ml)
CD11B
CRP
(50 ng/ml)
CD11B
CRP


D
CRP&CD11b

DISCUSSION

The aim of this research was to identify the relation between WML, CRP and microglial activity in an animal model of chronic hypoperfusion. To this effect the reproduced model in Long Evans rats showed the characteristic features of rarefaction (vacuolation) and associated features of gliosis, microglia activity and axonal damage as reported in previous literature (Farkas E *et al.*, 2004; Simpson JE *et al.*, 2007; Wakita H *et al.*, 1994, 2002).The sham operated animals showed a significant contrast in the characteristic features of WML especially in the intensity of involvement of the optic tracts and microglial activity and our results are similar to others in this context (Farkas E *et al.*, 2004; Wakita H *et al.*, 1994, 2002; Watanabe T *et al.*, 2006).

The cause for the intense involvement of the optic tract can be explained on the basis of the rat brain angioarchitecture. Blood supply through the circle of Willis in the rat is similar to that in the human, and there is a well established communication between the carotid and vertebral circulation via the prominent posterior communicating artery (Takamatsu J *et al.*, 1984). Anatomically the rat optic nerve is supplied from the carotid circulation via branches of the ophthalmic artery and possibly by the anterior cerebral artery and an insufficient blood supply to both the retina and optic nerve would be the cause for the lesions of the optic nerve in a model of chronic hypoperfusion (Takamatsu J *et al.*, 1984). The vascular supply for these structures is farthest from the vertebral circulation; therefore, they may be more involved without any other significant brain lesion after BCCAL (Takamatsu J *et al.*, 1984).

The corpus callosum demonstrated a lesser degree of vacuolation in comparison to the optic tracts. Within the corpus callosum the periventricular area showed more vacuolations.

This can be accounted for by knowing that WM adjacent to the wall of the ventricle represents a distal endzone territory of blood supply from the choroidal arteries and hence reflects a vulnerability to blood flow reduction (Schmahmann *et al.*, 2008).

Interestingly there was no relation between the severity of involvement of WM and the occurrence of CgA immunolabeled fibers. Previous literature reported the same findings in human cerebrovascular WM lesions (Suenaga T *et al.*, 1994). One possible explanation for this discrepancy is that under severe ischemic conditions, CgA does not accumulate in spite of axonal injury, since the transport of these proteins is an energy-requiring process (Sherriff FE *et al.*, 1994; Ferreira A et al., 1993). Thus, the number of CgA-immuno-positive fibers in the optic nerve and optic tract may represent an underestimation of the real extent of axonal injury in these areas (Wakita H *et al.*, 2002).

Astrogliosis correlated with areas demonstrating greater WM injury. It is reported that reactive astrogliosis is a prominent feature of astrocytes adjacent to and extending far beyond the site of injury and is characterized by astrocyte proliferation and extensive hypertrophy of the cell body and cytoplasmic processes (Eng LF *et al.*, 1994).Some astrocytes which maybe clasmatodendritic astroglia were noticed. The role of clasmatodendritic astroglia in the pathogenesis of WM lesions is not entirely clear but they may clean the lesion sites by incorporating edema fluid and phagocytizing cell debris (Tomimoto H *et al.*, 1997).It has been suggested that these astroglia eventually disappear, resulting in a numerical decrease in astroglia in the severe WM lesions as compared to the slight WM lesions (Tomimoto H *et al.*, 1996).

Microglia activity as demonstrated by CD68 immunolabeled microglia showed a relevant increase in the lesional optic tract from day 3 of survival in comparison to the near null immunoreactivity demonstrated in SHAM animals. This increase became significant from day 7-30 and was highly associated with the progressive significant appearance of vacuolations in the lesional optic tracts from day 3-30 of survival. Chronic cerebral hypoperfusion has been suggested to contribute to WM pathology (Fernando MS *et al.*, 2006; Irving EA *et al.*, 2001), and it has been shown to induce WM injury and microglial activation in the rat brain (Farkas E *et al.*, 2004). Microglial activation has been reported in the rat optic tract following the onset of ischemia (Ihara M *et al.*, 2001) and in the WM of patients with vascular dementia (Rosenberg GA et al., 2001) and AD (Lue LF *et al.*, 2001). Our findings complement these previous observations and demonstrate the significant microglial activity immunostaining in the rat optic tract in an animal model exhibiting WML due to chronic hypoperfusion. Microglia may contribute to WM pathology by secreting an array of proinflammatory and neurotoxic molecules (Lue LF *et al.*, 2001). Our results are in line with previous mentioned reports and would suggest the importance of microglial activity and their subsequent effect in lesional areas.

The similar levels of serum CRP detected by ELISA can be explained on the basis that the effects of surgery are similar in both groups and this results in an inflammatory state causing increased plasma levels of CRP. Moreover chronic hypoperfusion is a long term, subtle event and animal trials for longer periods may provide a better insight once the effects of surgery and subsequent inflammation have subsided in compared groups.

C-reactive protein (CRP) is a pentraxin, acute phase plasma protein produced predominantly by the liver and adipocytes .It can activate the classical complement

pathway, stimulate phagocytosis, and bind to immunoglobulin receptors (Fc γR) (Black S *et al.*, 2004) . Its activity in humans, either pro- or anti- inflammatory is dependent on the context in which it is acting (Black S *et al.*, 2004). Our results demonstrated the intriguing finding of increased CRP expression at the protein and more significantly the mRNA level in the BCCAL animal groups in comparison to the Sham groups. To the best of our knowledge this has not been reported in previous literature concerning animal models of chronic hypoperfusion. The expression of mRNA CRP in our study supports that the consequent protein expression is mainly from rat brain tissue and that this is irrespective of what is carried in the blood vessels as plasma CRP. This important finding of the significant increase in mRNA and protein expression coincided with the appearance of WM vacuolations and microglial activity (CD68) seen in the optic tract in our animal model. These results proposed that a correlation may exist between microglia and CRP within areas of WML.

Previous literature has shown that in states of chronic cerebral hypoperfusion, the activation of microglia appeared from day 3 of survival onwards (Watanabe T *et al.*, 2006; Shibata M *et al.*, 2004; Wakita H *et al.*, 1994, 2002). Our study revealed an intriguing observation in that the expression of CRP protein and mRNA was significant from day 3 of survival in BCCAL animals coinciding with the appearance of WM vacuolations and increased microglial activity (CD68) in the optic tracts at the same time. We therefore hypothesized that microglia could play a role in CRP expression and our *in-vitro* studies of microglial cultures support this proposition. Surprisingly microglia cultures expressed CRP at both the mRNA and protein level in control and treated cultures. In addition double labeling of microglia cultures with Cd11b (a microglia marker for active and ramified microglia) and

anti-rat CRP demonstrated their co-localization in microglia cells. Our results would therefore suggest that microglia are a source for CRP expression.

An important fact concerning microglia in culture should be noted: cultured microglia due to the inevitable use of proteolytic and mechanical means in dissociating cells from CNS tissue become activated (Garden GA *et al.*, 2006). Therefore control microglia cultures would also show a varying level of CRP expression. Moreover microglia also secrete varying levels of IL-1β and IL-6 (Kim SU *et al.*, 2005; Kim and de Vellis, 2005; Hanisch UK, 2002) and this would also have an effect on their ability to express CRP.

Our aim in treating microglia cultures with interleukins IL-6 and IL-1β was to induce further CRP expression and observe if increasing their concentrations or their combination at different concentrations resulted in a change in CRP expression. We found no relevant increase in mRNA or protein expression of CRP with different concentrations of interleukins. A possible assumption in regard to these results can be based on the fact that microglia activation generally increases cytokine expression, and proinflammatory cytokines are the first to be released including IL-6 and IL-1β (Kim and de Vellis, 2005; Garden GA *et al.*, 2006).However and as mentioned previously microglia also elaborate receptors for IL-6 and IL-1β and for most of the proinflammatory cytokines resulting in autocrine feedback loops that are likely to be crucial for the eventual down regulation of an inflammatory response (Garden GA *et al.*, 2006).We suggest that this explanation could be the cause for the results seen in microglia cultures in respect to CRP expression. It could be that the increased concentrations of IL-6 and IL-1β would result in no further expression of CRP because of auto regulatory feedback loops which causes stabilization of all products expressed by microglia. This assumption could also be true for our *in-vivo* findings in which significant CRP expression was noticed with the initiation of WML and microglial

activity and this seemed to stabilize over time. Future studies are indicated so as to reveal the mechanisms which affect CRP production from microglia.

In conclusion, our results have shown a relation between WML and CRP expression in rat brain tissue in a model of chronic hypoperfusion exhibiting characteristic features of WML. In addition we have demonstrated cultured rat microglia as a novel source for CRP expression. Further research could prove this to be a key finding in WML and all diseases associated with increased microglia activity e.g. Alzheimer's, Parkinson and most neuroinflammatory diseases of the brain.

FUTURE STUDIES

The important association of microglial activity with WML has been emphasized through previous studies which have demonstrated attenuation of WML by minocycline, a microglial inhibitor, in a rat model of chronic hypoperfusion (Cho K *et al.*, 2006).In addition i*n vitro* studies have demonstrated that minocycline suppressed microglial production of IL-1β, IL-6, TNF, and NGF (Seabrook TJ *et al.*, 2006).Our observations have shown that addition of IL-1β and IL-6 to the media of cultured microglia further induced the expression of CRP from these cells. Therefore it might well be rendered that the induced suppression of microglial production of IL-1β and IL-6 by minocycline may well affect the expression of CRP which itself maybe a key factor in the pathogenesis of WML. This point can be clarified by future studies which may introduce microglial inhibitors and block Il-1β and IL-6 receptors to demonstrate their effects on CRP expression.

How relevant is the noticed relation between microglia and CRP in an *in vivo* context? Intraperitoneal injection of human CRP into rats has been reported to increase cerebral infarct size after middle cerebral artery occlusion in adult rats and also enhance ischemic tissue damage by a complement-dependent mechanism (Gill R et al., 2004). However, no previous studies on the effect and mechanism of CRP in a model of chronic hypoperfusion demonstrating WML have been reported. To the best of our knowledge this research is the first to show the expression of CRP in rat brain tissue from a model of chronic hypoperfusion, and further studies are indicated to dissect the pathway by which CRP may play a role in WML.

In addition studies to reveal other sources for CRP from within the brain tissue are indicated. CRP expression and production from human aortic endothelial cells and its up-regulation with the cytokines IL-1 and IL-6 in combination has been documented and it was proposed that local concentrations of CRP that are far in excess of plasma concentrations could contribute to proinflammatory and proatherogenic effects ((Venugopal SK *et al.*, 2004).In line with these reports, brain endothelial cells may exhibit similar expression of CRP and add to the cause of cerebral small vessel disease as manifested by WML.

An interesting trial would be to show if there is any association between plasma CRP and progressive WML in models of chronic hypoperfusion conducted over prolonged periods. Previous population based studies, as mentioned earlier, have shown a positive relation between plasma CRP levels and progression of WML, while others did not find such a relation. Animal studies would help to provide an insight on this controversy.

CONCLUSION

Our results have demonstrated a relation between WML and CRP expression in a rat model of chronic hypoperfusion and have also detected the expression of CRP mRNA and protein in rat microglial cultures. We propose that these findings will be beneficial in targeting the role and effect of microglia and CRP in WML of the brain.

REFERENCES

Abernethy TJ, Avery OT. 1941. The occurrence during acute infections of a protein not normally present in the blood: I. Distribution of the reactive protein in patients' sera and the effect of calcium on the flocculation reaction with C polysaccharide of pneumococcus. J Exp Med, 73: 173 -182.

Akiguchi I, Tomimoto H, Suenaga T, Wakita H, Budka H. 1997. Alterations in glia and axons in the brains of Binswanger's disease patients. Stroke 28, 1423–1429.

Albert MA, Glynn RJ, Buring J, Ridker PM. 2004. C-reactive protein levels among women of various ethnic groups living in the United States (from the Women's Health Study). Am J Cardiol 93:1238–42.

Anderson JM, Hubbard BM, Coghill GR, et al. 1983. The effect of advanced old age on the neuron content of the cerebral cortex. Observations with an automatic image analyser point counting method. J Neurol Sci 58:235Y46.

Balletshofer B, Haap M, Rittig K, Stock J, Lehn-Stefan A, Haring H. 2005. Early carotid atherosclerosis in overweight non-diabetic individuals is associated with subclinical chronic inflammation independent of underlying insulin resistance. Horm Metab Res. 37:331–335.

Ballou SP, Lozanski G. 1992. Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein. Cytokine 4:361-8.

Batchelor PE, Porritt MJ, Martinello P, Parish CL, Liberatore GT, Donnan GA, Howells DW. 2002. Macrophages and microglia produce local trophic gradients that stimulate

axonal sprouting toward but not beyond the wound edge. Mol Cell Neurosci. Nov; 21(3):436-53.

Baumann H, Gauldie J. 1994. The acute phase response. Immunol Today. Feb; 15(2):74-80.

Bernick C, Kuller L, Dulberg C, Longstreth WT Jr, Manolio T, Beauchamp N, Price T. 2001. Silent MRI infarcts and the risk of future stroke: the Cardiovascular Health Study. Neurology 57:1222–1229.

Black S, Kushner I, Samols D. 2004. C-reactive protein. J Biol Chem. Nov 19;279 (47):48487-90.

Black SE. 2005. Links Vascular dementia. Stroke risk and sequelae define therapeutic approaches. Postgrad Med. Jan; 117(1):15-6, 19-25.

Bokura H, Kobayashi S, Yamaguchi S, Iijima K, Nagai A, Toyoda G, Oguro H, Takahashi K. 2006. Silent brain infarction and subcortical white matter lesions increase the risk of stroke and mortality: a prospective cohort study. J Stroke Cerebrovasc Dis. Mar-Apr; 15(2):57-63.

Breteler MM, van Swieten JC, Bots ML, et al. 1994. Cerebral white matter lesions, vascular risk factors, and cognitive function in a populationbased study: the Rotterdam Study. Neurology 44: 1246–1252.

Bryan Rock R, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, and Peterson PK. 2004.Role of Microglia in Central Nervous System Infections. Clinical microbiology reviews, Oct., p. 942–964.

Calabro P, Chang DW, Willerson JT, Yeh ET. 2005. Release of C-reactive protein in response to inflammatory cytokines by human adipocytes: linking obesity to vascular inflammation. J Am Coll Cardiol. 46:1112–1113.

Calabro P, Willerson JT, Yeh ET. 2003. Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells. Circulation. 108:1930–1932.

Cao JJ, Thach C, Manolio TA, Psaty BM, Kuller LH, Chaves PH, Polak JF, Sutton-Tyrrell K, Herrington DM, Price TR, Cushman M. 2003.C-reactive protein, carotid intima-media thickness, and incidence of ischemic stroke in the elderly: the Cardiovascular Health Study. Circulation. 108:166–170.

Carmelli D, DeCarli C, Swan GE, et al. 1998. Evidence for genetic variance in white matter hyperintensity volume in normal elderly male twins. Stroke 29: 1177–1181.

Cho KO, La HO, Cho YJ, Sung KW, Kim SY. 2006. Minocycline attenuates white matter damage in a rat model of chronic cerebral hypoperfusion. J Neurosci Res. Feb 1;83(2):285-91.

Conde JR and Streit WJ. Microglia in the Aging Brain. J Neuropathol Exp Neurol. 2006 Mar;65(3):199-203. Review.

Conde JR, Streit WJ. 2005. Effect of aging on the microglial response to peripheral nerve injury. Neurobiol Aging. 2006 Oct;27(10):1451-61. Epub 2005 Sep 12.

Cuadros, M.A. and Navascues, J. 1998.The origin and differentiation of microglial cells during development. Prog. Neurobiol. 56, 173–189.

Dasu MR, Devaraj S, Du Clos TW, Jialal I. 2007. The biological effects of CRP are not attributable to endotoxin contamination: evidence from TLR4 knockdown human aortic endothelial cells. J Lipid Res. Mar;48(3):509-12.

Davalos D et al. 2005.ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci. Jun;8(6):752-8. Epub 2005 May 15.

de Beer FC, Baltz ML, Munn EA, Feinstein A, Taylor J, Bruton C, Clamp JR, Pepys MB. 1982. Isolation and characterisation of C-reactive protein and serum amyloid P component in the rat. Immunology 45:55–70

de Leeuw FE, de Groot JC, Oudkerk M, et al. 2002. Hypertension and cerebral white matter lesions in a prospective cohort study. Brain 125(Pt 4): 765–772.

De Reuck J, Decoo D, Marchau M, et al. 1998. Positron emission tomography in vascular dementia. J. Neurol. Sci. 154: 55–61.

del Rio-Hortega P. 1932. Microglia, p. 483–534. In W. Penfield (ed.), Cytology and cellular pathology of the nervous system. P. B. Hoebaer, New York, N.Y

Devaraj S, Xu DY, Jialal I. 2003. C-reactive protein increases plasminogen activator inhibitor-1 expression and activity in human aortic endothelial cells: implications for the metabolic syndrome and atherothrombosis. Circulation. Jan 28;107(3):398-404.

Di Napoli M, Papa F. 2005. C-reactive protein and cerebral small-vessel disease: an opportunity to reassess small-vessel disease physiopathology? Circulation. 112:781–785.

DiPatre PL, Gelman BB. 1997. Microglial cell activation in aging and Alzheimer disease: partial linkage with neurofibrillary tangle burden in the hippocampus. J Neuropathol Exp Neurol 56:143-49.

Doms RW, Peiper SC. 1997. Unwelcomed guest with master keys: how HIV uses cytokine receptors for cellular entry. Virology 235:179–190.

Dong Q, Wright JR. 1996. Expression of C-reactive protein by alveolar macrophages. J Immunol. 156:4815–4820.

Doronzo G, Russo I, Mattiello L, Trovati M, Anfossi G. 2005.C-reactive protein increases matrix metalloproteinase-2 expression and activity in cultured human vascular smooth muscle cells. J Lab Clin Med 146:287-298.

Doronzo G, Russo I, Trovati M and Anfossi G. 2006.Sodium Azide in Commercially Available C-Reactive Protein Preparations Does Not Influence Matrix Metalloproteinase-2 Synthesis and Release in Cultured Human Aortic Vascular Smooth Muscle Cells. Clinical Chemistry. 52:1200-1201.

Du Clos TW, Zlock LT, Hicks PS, and Mold C. 1994. Clin. Immunol. Immunopathol. 70, 22–27.

Du Clos, T.W. 1989. C-reactive protein reacts with the U1 small nuclear ribonucleoprotein. J. Immunol. 143:2553–2559.

Duffy PE, Rapport M, Graf L. 1980. Glial fibrillary acidic protein and Alzheimer-type senile dementia. Neurology 30:778-82.

Elkabes S, DiCicco-Bloom EM, Black IB. 1996. Brain microglia/ macrophages express neurotrophins that selectively regulate microglial proliferation and function. J Neurosci 16: 2508–2521.

Eng LF, Ghirnikar RS. 1994. GFAP and Astrogliosis. Brain Pathology 4: 229-237.

Farkas E, Donka G, de Vos RAI, Mihály A, Bari F and Luiten PGM. 2004. Experimental cerebral hypoperfusion induces white matter injury and microglial activation in the rat brain. Acta Neuropathologica Volume 108, Number 1/ July, 57-64.

Farkas E, Donka G, de Vos RA, Mihaly A, Bari F, Luiten PG. 2004. Experimental cerebral hypoperfusion induces white matter injury and microglial activation in the rat brain. Acta Neuropathol 108:57–64.

Fassbender K, Bertsch T, Mielke O, Muhlhauser F, Hennerici M. 1999. Adhesion molecules in cerebrovascular diseases. Evidence for an inflammatory endothelial activation in cerebral large- and small-vessel disease. Stroke. 30:1647–1650.

Fernando MS, Simpson JE, Matthews F, Brayne C, LewisCE, Barber R, Kalaria RN, Forster G, Esteves F, WhartonSB, Shaw PJ, O'Brien JT, Ince PG, MRC CFANS Group.. 2006. White matter lesions in an unselected cohort of the elderly: molecular pathology suggests origin from chronic hypoperfusion injury. Stroke 37: 1391–1398.

Ferreira A, Caceres A, Kosik KS. 1993. Intraneuronal compartments of the amyloid precursor protein. J Neurosci. 13 3112–3123.

Garden GA, Mo¨ller T. 2006. Microglia Biology in Health and Disease. J Neuroimmune Pharmacol 1: 127–137.

Garden GA, Guo W, Jayadev S, Tun C, Balcaitis S, Choi J, Montine TJ, Moller T, Morrison RS. 2004. HIV associated neurodegeneration requires p53 in neurons and microglia. FASEB J Jul; 18(10):1141-3. Epub 2004 May 20.

Garrison, F.H. 1969. History of neurology. Revised and enlarged with a bibliography of classical, original, and standard works in neurology. By Lawrence C. McHenry Jr. with a foreword by Derek E. Denny-Brown. Thomas. Springfield, IL.

Gershov D, Kim S, Brot N, and Elkon KB. 2000. C-reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. J Exp Med. Nov 6;192(9):1353-64. Erratum in: J Exp Med 2001 Jun 18;193(12):1439.

Gill R, Kemp JA, Sabin C, Pepys MB. 2004. Human C-reactive protein increases cerebral infarct size after middle cerebral artery occlusion in adult rats. J Cereb Blood Flow Metab. Nov; 24(11):1214-8.

Graham JE, Rockwood K, Beattie BL, Eastwood R, Gauthier S, Tuokko H, McDowell I. 1997.Prevalence and severity of cognitive impairment with and without dementia in an elderly population. Lancet. 349: 1793–1796.

Gurol ME, Irizarry MC, Smith EE, et al. 2006. Plasma beta-amyloid and white matter lesions in AD, MCI, and cerebral amyloid angiopathy. Neurology 66: 23–29.

Hanisch UK, Kettenmann H. 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci. Nov.10 (11):1387-94.

Hanisch UK. 2002. Microglia as a source and target of cytokines. Glia 40:140–155.

Hassan A, Hunt BJ, O'Sullivan M, Parmar K, Bamford JM, Briley D, Brown MM, Thomas DJ, Markus HS. 2003. Markers of endothelial dysfunction in lacunar infarction and ischaemic leukoaraiosis. Brain. 126: 424–432.

Hassan, A., B.J. Hunt, M. O'Sullivan, et al. 2004. Homocysteine is a risk factor for cerebral small vessel disease, acting via endothelial dysfunction. Brain 127(Pt 1): 212–219.

Haynes SE et al. 2006.The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat. Neurosci. 9, 1512–1519

Hinman JD, Duce JA, Siman RA, et al. 2004.Activation of calpain-1 in myelin and microglia in the white matter of the aged rhesus monkey. J Neurochem 89:430-41

Hirschfield GM, Pepys MB. 2003. C-reactive protein and cardiovascular disease: new insights from an old molecule .Q J Med 96: 793-807.

Hodgson C. 1998. Prevalence and disabilities of community/living seniors who report the effects of Stroke. Can Med J. 159 (suppl 6): S9–S14.

Holland CM, Smith EE, Csapo I, et al. 2008.Spatial distribution of white-matter hyperintensities in Alzheimer disease, cerebral amyloid angiopathy, and healthy aging. Stroke 39: 1127–1133.

Holness CL, Simmons DL. 1993. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood. Mar 15;81(6):1607-13.

Honda S, Nakajima K, Nakamura Y, Imai Y, Kohsaka S. 1999. Rat primary cultured microglia express glial cell line-derived neurotrophic factor receptors. Neurosci Lett 275:203–206

Hurlimann J, Thorbecke G, Hochwald G. 1966.The liver as the site of C-reactive protein formation. J Exp Med 123: 365-78.

Ichikawa K, Itoh Y, Min WK, Yap SF, Lam CW, Kong XT, et al. 2004.Diagnosis and epidemiological implication of regional differences in serum concentrations of proteins observed in six Asian cities. Clin Chem Lab Med 42:800–9.

Ihara M, Tomimoto H, Kinoshita M, Oh J, Noda M,Wakita H, Akiguchi I, Shibasaki H. 2001. Chronic cerebral hypoperfusion induces MMP-2 but not MMP-9 expression in the microglia and vascular endothelium of white matter. J Cereb Blood Flow Metab **21**: 828–34.

Irving EA, Bentley DL, Parsons AA. 2001. Assessment of white matter injury following prolonged focal cerebral ischaemia in the rat. Acta Neuropathol (Berl) **102**: 627–35

Jeremy D. Schmahmann , Eric E. Smith , Florian S. Eichler , and Christopher M. Filley. 2008. Cerebral White Matter Neuroanatomy, Clinical Neurology, and Neurobehavioral Correlates. Annals of the New York Academy of Sciences, Volume 1142 , Pages 266 - 309

Jialal I, Devaraj S, and Venugopal SK. 2004. C-reactive protein: risk marker or mediator in atherothrombosis? Hypertension Jul; 44(1):6-11. Epub 2004 May 17. Review.

Kawanami D, Maemura K, Takeda N, Harada T, Nojiri T, Saito T, Manabe I, Imai Y, Nagai R. 2005. C-reactive protein induces VCAM-1 gene expression through NF-κB activation in vascular endothelial cells. Atherosclerosis, Volume 185, Issue 1, Pages 39-46.

Kaur C. et al. 2001. Origin of microglia. Microsc. Res. Tech. 54, 2–9.

Kawamoto Y, Akiguchi I, Tomimoto H, Shirakashi Y, Honjo Y, Budka H. 2006. Upregulated expression of 14-3-3 proteins in astrocytes from human cerebrovascular ischemic lesions.StrokeMar;37(3):830-5. Epub 2006 Jan 19.

Khatri M, Wright CB , Nickolas TL, et al. 2007. Chronic kidney disease is associated with white matter hyperintensity volume: the Northern Manhattan Study (NOMAS). Stroke 38: 3121–3126.

Khera A, McGuire DK, Murphy SA, Stanek HG, Das SR, Vongpatanasin W, et al. 2005. Race and gender differences in C-reactive protein levels. J Am Coll Cardiol 46:464–9.

Kim SU, de Vellis J. 2005. Microglia in health and disease. J Neurosci Res. Aug 1;81(3):302-13. Review.

Kim YS, Joh TH. 2006. Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease.Exp Mol Med. Aug 31;38(4):333-47. Review.

Kitanaka J, Hashimoto H, Gotoh M, Kondo K, Sakata K, Hirasawa Y, Sawada M, Suzumura A, Marunouchi T, Matsuda T, Baba A. 1996. Expression pattern of messenger RNAs for prostanoid receptors in glial cell cultures. Brain Res. Jan 29;707(2):282-7.

Koji Yasojima, Claudia Schwab, Edith G. McGeer and Patrick L. McGeer. 2000. Human neurons generate C-reactive protein and amyloid P: upregulation in Alzheimer's disease. Brain Research Volume 887, Issue 1, 22 December, Pages 80-89

Kuller LH, Longstreth WT Jr, Arnold AM, Bernick C, Bryan RN, Beauchamp NJ Jr. 2004. White matter hyperintensity on cranial magnetic resonance imaging: a predictor of stroke. Stroke. 35:1821–1825.

Kushner I, Jiang SL, Zhang D, Lozanski G, and Samols D. 1995. Do post-transcriptional mechanisms participate in induction of C-reactive protein and serum amyloid A by IL-6 and IL-1? Ann. N. Y. Acad. Sci. 762, 102–107.

Kuta AE and Baum LL. 1986. C-reactive protein is produced by a small number of normal human peripheral blood lymphocytes. J Exp Med. 164, 321–326.

Lafuente N, Azcutia V, Matesanz N, Cercas E, Rodriguez-Manas L, Sanchez-Ferrer LF, et al. 2005. Evidence for sodium azide as an artifact mediating the modulation of inducible nitric oxide synthase by C-reactive protein. J Cardiovasc Pharmacol 45:193-196.

Lai AY, Todd KG. 2008. Differential Regulation of Trophic and Proinflammatory Microglial Effectors is Dependent on Severity of Neuronal Injury. GLIA 56:259–270.

Liao D, Cooper L, Cai J, Toole J, Bryan N, et al. 1997. The prevalence and severity of white matter lesions, their relationship with age, ethnicity, gender, and cardiovascular disease risk factors: Neuroepidemiology 16(3):149-62.

Liu C, Wang S, Deb A, Nath KA, Katusic ZS, McConnell JP, et al. 2005. Proapoptotic, antimigratory, antiproliferative, and antiangiogenic effects of commercial C-reactive

protein on various human endothelial cell types in vitro: implications of contaminating presence of sodium azide in commercial preparations. Circ Res 97:135-143.

Long JM, Kalehua AN, Muth NJ, et al. 1998. Stereological analysis of astrocyte and microglia in aging mouse hippocampus. Neurobiol Aging 19:497-503.

Longstreth WT Jr, Arnold AM, Beauchamp NJ Jr, Manolio TA, Lefkowitz D, Jungreis C, Hirsch CH, O'Leary DH, Furberg CD. 2005. Incidence, manifestations, and predictors of worsening white matter on serial cranial magnetic resonance imaging in the elderly: the Cardiovascular Health Study. Stroke 36:56–61.

Longstreth WT Jr. 2005. Brain vascular disease overt and covert. Stroke 36:2062–2063.

Longstreth,W. Jr., E.K. Larsen,R. Klein, et al. 2007. Associations between findings on cranial magnetic resonance imaging and retinal photography in the elderly: the Cardiovascular Health Study. Am. J. Epidemiol. 165: 78–84.

Longstreth,W.T. Jr., T.A. Manolio, A. Arnold, et al. 1996. Clinical correlates of whitematter findings on cranial magnetic resonance imaging of 3301 elderly people.The CardiovascularHealth Study. Stroke 27: 1274–1282.

Lue LF, Rydel R, Brigham EF, Yang LB, Hampel H, Murphy GM Jr., Brachova L, Yan SD,Walker DG, Shen Y, Rogers J.2001. Inflammatory repertoire of Alzheimer's disease

and nondemented elderly microglia in vitro. *Glia* **35**: 72–9

MacLeod CM, Avery OT. 1941. The occurrence during acute infections of a protein not normally present in the blood. II. Isolation and properties of the reactive protein J Exp Med 73: 183.

Manabu Wada , Hikaru Nagasawa, Keiji Kurita, Shingo Koyama, Shigeki Arawaka, Toru Kawanami, Katsushi Tajima, Makoto Daimon, Takeo Kato. 2008. Cerebral small vessel disease and C-reactive protein: Results of a cross-sectional study in community-based Japanese elderly. Journal of the Neurological Sciences 264, 43–49.

McGeer PL, McGeer EG. 1995. The inflammatory response system of brain: implication for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 21:195–218.

Minghetti L, Levi G. 1998. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. Prog Neurobiol 54:99–125.

Mittelbronn, M., K. Dietz, H. J. Schluesener, and R. Meyermann. 2001. Local distribution of microglia in the normal adult human central nervous system differs by up to one order of magniture. Acta Neuropathol. 101:249–255.

Mohammad Rassouli, Hari Sambasivam, Parastoo Azadij, Anne Dell, Howard R. Morris, Arun Nagpurkar , Sailen Mookerjea, and Robert K. Murray. 1992. Derivation of the Amino Acid Sequence of Rat C-reactive Protein from cDNA Cloning with Additional Studies on the Nature of Its Dimeric Component. The journal of biological chemistry , Vol. 267. No. 5, Issue of February 15, pp. 2947-2954.

Mold C, Rodriguez W, Rodic-Polic B, and Du Clos TW. 2002. C-reactive protein mediates protection from lipopolysaccharide through interactions with Fc gamma R. J. Immunol. 169, 7019–7025.

Mold C, Gewurz H, and Du Clos TW. 1999. Regulation of complement activation by C-reactive protein. *Immunopharmacology.* **42**:23–30.

Morgan TE, Xie Z, Goldsmith S, et al. 1999. The mosaic of brain glial hyperactivity during normal ageing and its attenuation by food restriction. Neuroscience 89:687-99

Mosley TH Jr, Knopman DS, Catellier DJ, Bryan N, Hutchinson RG, Grothues CA, Folsom AR, Cooper LS, Burke GL, Liao D, Szklo M. 2005. Cerebral MRI findings and cognitive functioning: the Atherosclerosis Risk in Communities study. Neurology 64:2056 –2062.

Mount MP, Lira A, Grimes D, Smith PD, Faucher S, Slack R, Anisman H, Hayley S, Park DS. 2007. Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons.J Neurosci. Mar 21;27(12):3328-37.

Nathalie Davoust, Carine Vuaillat, Geraldine Androdias and Serge Nataf. 2008. From bone marrow to microglia: barriers and avenues. Trends Immunol. May;29(5):227-34.

Neumann H, Misgeld T, Matsumuro K, Wekerle H. 1998. Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. Proc Natl Acad Sci USA 95:5779–5784.

Nguyen MD, Julien JP, Rivest S. 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? Nat. Rev. Neurosci. 3:216–227.

Ni J, Ohta H, Matsumoto K, Watanabe H. 1994. Progressive cognitive impairment following chronic cerebral hypoperfusion induced by permanent occlusion of bilateral carotid arteries in rats. Brain Res 653:231–6.

Ogura K, Ogawa M, Yoshida M. 1994. Effects of ageing on microglia in the normal rat brain: Immunohistochemical observations. Neuroreport 5:1224-26

Ohta H, Nishikawa H, Kimura H, Anayama H, Miyamoto M. 1997. Chronic cerebral hypoperfusion by permanent internal carotid ligation produces learning impairment without brain damage in rats. Neuroscience 79:1039–50.

Padilla ND, Bleeker WK, Lubbers Y, Rigter GM, van Mierlo GJ, Daha MR, and Hack CE. 2003. Rat C-reactive protein activates the autologous complement system. Immunology. August; 109(4): 564–571.

Pantoni L, Leys D, Fazekas F, Longstreth WT Jr., Inzitari D, Wallin A, Filippi M, Scheltens P, Erkinjuntti T, Hachinski V. 1999. Role of white matter lesions in cognitive impairment of vascular origin. Alzheimer Dis Assoc Disord. 13:S49 –S54.

Pantoni, L. & J.H. Garcia. 1997. Pathogenesis of leukoaraiosis: a review. Stroke 28: 652–659.

Peinado MA, Quesada A, Pedrosa JA, et al. 1998. Quantitative and ultrastructural changes in glia and pericytes in the parietal cortex of the aging rat. Microsc Res Tech 43:34-42.

Pepys MB et al. 2006. Targeting C-reactive protein for the treatment of cardiovascular disease. Nature 440: 1217–1221.

Pepys MB, Hirschfield GM. 2003. C-reactive protein: A critical update. J Clin Invest 111:1805–1812.

Pepys MB. 1981. C-reactive protein fifty years on. Lancet, Mar 21;1(8221):653-7.

Pepys MB. 2008. C-reactive protein is neither a marker nor a mediator of atherosclerosis.Nat Clin Pract Nephrol. May;4(5):234-5. Epub 2008 Mar 4. Review.

Pepys, M.B., and Baltz, M.L. 1983. Acute phase proteins with special reference to C-reactive protein and related proteins (pentraxins) and serum amyloid A protein. Adv. Immunol. 34:141–212.

Pepys, M.B., Rowe, I.F., and Baltz, M.L. 1985. C-reactive protein: binding to lipids and lipoproteins. Int. Rev. Exp. Pathol. 27:83–111.

Perry VH, Matyszak MK, Fearn S. 1993. Altered antigen expression of microglia in the aged rodent CNS. Glia 7:60-67.

Peters A, Josephson K, Vincent SL. 1991. Effects of aging on the neuroglial cells and pericytes within area 17 of the rhesus monkey cerebral cortex. Anat Rec 229:384-98.

Peters A, Sethares C. 2002. The effects of age on the cells in layer 1 of primate cerebral cortex. Cereb Cortex 12:27-36.

Pohjasraara T, Mäntylä R, Ylikoski MA, Kaste M, Erkinjuntti T. 2000. Comparison of different clinical criteria (DSM-III, ADDTC, ICD-10, NINDS-AIREN, DSM-IV) for the diagnosis of vascular dementia. Stroke 31: 2952–2957.

Polfliet, M. M. J., F. van de Veerdonk, E. A. Dopp, E. M. L. van Kesteren- Hendrikx, N. van Rooijen, C. D. Dijkstra, and T. K. van den Berg. 2002. The role of perivascular and meningeal macrophages in experimental allergic encephalomyelitis. J. Neuroimmunol. 122:1–8.

Polfliet, M. M. J., P. J. G. Zwijnenburg, A. M. van Furth, T. van der Poll, E. A. Dopp, C. R. de Lavalette, E. M. L. van Kesteren-Hendrikx, N. van Rooijen, C. D. Dijkstra, and T. K. van den Berg. 2001. Meningeal and perivascular macrophages of the central nervous system play a progective role during bacterial meningitis. J. Immunol. 167:4644–4650.

Presta M, Urbinati C, Dell'era P, Lauro GM, Sogos V, Balaci L, Ennas MG, Gremo F. 1995. Expression of basic fibroblast growth factor and its receptors in human fetal microglia cells.

Ramadori G, Christ B. 1999. Cytokines and the hepatic acute-phase response. Semin Liver Dis.,19(2):141-55.

Reinhold Schmidt, Helena Schmidt, Martin Pichler, Christian Enzinger, Katja Petrovic, Kurt Niederkorn, Susanna Horner, Stefan Ropele, Norbert Watzinger, Martin Schumacher, Andrea Berghold, Gerhard M. Kostner and Franz Fazekas. 2006. C-Reactive Protein, Carotid Atherosclerosis, and Cerebral Small-Vessel Disease:Results of the Austrian Stroke Prevention Study. Stroke 37;2910-2916.

Reitz C, Berger K, de Maat MP, Stoll M, Friedrichs F, Kardys I, Witteman JC, Breteler MM. 2007. CRP Gene Haplotypes, Serum CRP, and Cerebral Small-Vessel Disease. The Rotterdam Scan Study and the MEMO Study. Stroke. 38:2356 –2359.

Rezaie P, Male D. 2002. Mesoglia & microglia--a historical review of the concept of mononuclear phagocytes within the central nervous system. J Hist Neurosci. Dec;11(4):325-74.

Rezaie, P. and Male, D. 1999. Colonisation of the developing human brain and spinal cord by microglia: a review. Microsc. Res. Tech. 45, 359–382

Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. 2000. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation. 101:1767–1772.

Ridker PM, Stampfer MJ, Rifai N. 2001. Novel risk factors for systemic atherosclerosis: a comparison of C-reactive protein, fibrinogen, homocysteine, lipoprotein (a), and standard cholesterol screening as predictors of peripheral arterial disease. JAMA. 285:2481–2485.

Ridker PM. 2003. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation. 107:363–369.

Robey F.A. and T.Y. Liu. 1981. Limulin: a C-reactive protein from Limulus polyphemus, J Biol Chem 256, pp. 969–975.

Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, Peterson PK. 2004. Role of microglia in central nervous system infections. Clin Microbiol .Rev. Oct; 17(4):942-64

Rogers J, Luber-Narod J, Styren SD, et al. 1988. Expression of immune system-associated antigens by cells of the human central nervous system: Relationship to the pathology of Alzheimer's disease. Neurobiol Aging 9:339-49.

Rosano C, Brach J, Longstreth WT Jr, Newman AB. 2005. Quantitative measures of gait characteristics indicate prevalence of underlying subclinical structural brain abnormalities in high-functioning older adults. Neuroepidemiology. 26:52– 60.

Rosenberg GA, Sullivan N, Esiri MM.2001. White matter damage is associated with matrix metalloproteinases in vascular dementia. Stroke **32**: 1162–8

Russell AI, Cunninghame Graham DS, Shepherd C, Roberton CA, Whittaker J, Meeks J, Powell RJ, Isenberg DA, Walport M J, and Vyse TJ. 2004. Polymorphism at the C-reactive protein locus influences gene expression and predisposes to systemic lupus erythematosus. Hum. Mol. Genet. 13, 137–147.

Samols D, Agrawal A, and Kushner I. 2002. In *Cytokine Reference On-Line* (Feldman, M., and Oppenheim, J. J., eds) Academic Press, London.

Sawada M, Itoh Y, Suzumura A, Marunouchi T. 1993. Expression of cytokine receptors in cultured neuronal and glial cells. Neurosci Lett. 160:131–134.

Schmahmann JD, Smith EE, Eichler FS, Filley CM. 2008. Cerebral white matter: neuroanatomy, clinical neurology, and neurobehavioral correlates. Ann N Y Acad Sci. Oct;1142:266-309.

Schmidt R, Ropele S, Enzinger C, Petrovic K, Smith S, Schmidt H, Matthews P, Fazekas F. 2005. White matter lesion progression, brain atrophy, and cognitive decline: the Austrian Stroke Prevention Study. Ann Neurol. 58:610–616.

Schmidt, R., F. Fazekas, M. Hayn, et al. 1997. Risk factors for microangiopathy-related cerebral damage in the Austrian stroke prevention study. J. Neurol. Sci. 152: 15–21.

Seabrook TJ, Jiang L, Maier M, Lemere C. 2006. Minocycline affects microglia activation, Abeta deposition, and behavior in APP-tg mice. Glia 53: 776–782.

Sheffield LG, Berman NE. 1998. Microglial expression of MHC class II increases in normal aging of nonhuman primates. Neurobiol Aging 19:47-55.

Sherriff FE, Bridges LR, Sivaloganathan S. 1994. Early detection of axonal injury after human head trauma using immunocytochemistry for b-amyloid precursor protein, Acta Neuropathol. (Berl.) 87, 55–62.

Shibata M, Ohtani R, Ihara M, Tomimoto H. 2004. White matter lesions and glial activation in a novel mouse model of chronic cerebral hypoperfusion.Stroke. Nov; 35(11):2598-603.

Shie FS, Breyer RM, Montine TJ. 2005a. Microglia lacking E Prostanoid Receptor subtype 2 have enhanced Abeta phagocytosis yet lack Abeta-activated neurotoxicity. Am J Pathol

Shie FS, Montine KS, Breyer RM, Montine TJ. 2005b. Microglial EP2 is critical to neurotoxicity from activated cerebral innate immunity. Glia 52:70 –77.

Shin WH, Lee DY, Park KW, Kim SU, Yang MS, Joe EH, Jin BK. 2004. Microglia expressing interleukin-13 undergo cell death and contribute to neuronal survival in vivo. Glia 46:142–152.

Shrive AK, Cheetham G M, Holden D, Myles DA, Turnell WG, Volanakis JE, Pepys MB, Bloomer AC and Greenhough TJ. 1996. Three dimensional structure of human C-reactive protein. Nat. Struct. Biol. 3, 346–354.

*Simpson JE, Fernando MS, Clark L, Ince PG, Matthews F, Forster G, O'Brien JT, Barber R, Kalaria RN, Brayne C, Shaw PJ, Lewis CE, Wharton SB; MRC Cognitive Function and Ageing Neuropathology Study Group. 2006. White matter lesions in an unselected cohort of the elderly: astrocytic, microglial and oligodendrocyte precursor cell responses. Stroke Jun; 37(6):1391-8. Epub 2006 Apr 20.

Simpson JE, Ince PG, Higham CE, Gelsthorpe CH, Fernando MS, Matthews F, Forster G, O'Brien JT, Barber R, Kalaria RN, Brayne C, Shaw PJ, Stoeber K, Williams GH, Lewis CE, Wharton SB; MRC Cognitive Function and Ageing Neuropathology Study Group. 2007. Microglial activation in white matter lesions and nonlesional white matter of ageing brains.Neuropathol ApplNeurobiol. Aug;33(4):410-9. Epub 2007 Apr 18.

Sloane JA, Hinman JD, Lubonia M, et al. 2003. Age-dependent myelin degeneration and proteolysis of oligodendrocyte proteins is associated with the activation of calpain-1 in the rhesus monkey. J Neurochem 84:157-68

Sloane JA, Hollander W, Moss MB, et al. 1999. Increased microglial activation and protein nitration in white matter of the aging monkey. Neurobiol Aging 20:395-40.

Sridevi Devaraj , Pappanaicken R. Kumaresan and Ishwarlal Jialal. 2004. Effect of C-reactive protein on chemokine expression in human aortic endothelial cells. Journal of Molecular and Cellular Cardiology Volume 36, Issue 3, March, Pages 405-410.

Starr JM, Leaper SA, Murray AD, Lemmon HA, Staff RT, Deary IJ, Whalley LJ. 2003. Brain white matter lesions detected by magnetic resonance imaging are associated with balance and gait speed. J Neurol Neurosurg Psychiatry 74:94 –98.

Stenset, V., L. Johnsen, D. Kocot, et al. 2006. Associations between white matter lesions, cerebrovascular risk factors, and low CSF Abeta42. Neurology 67: 830–833.

Streit WJ, Conde JR, Fendrick SE, et al. 2005. Role of microglia in the central nervous system's immune response. Neurol Res 27:685-91.

Streit WJ, Sparks DL. 1997. Activation of microglia in the brains of humans with heart disease and hypercholesterolemic rabbits. J Mol Med 75: 130-38

Streit, W. J. 1995. Microglial cells, p. 85–96. In H. Kettenmann and B. R. Ransom (ed.), Neuroglia. Oxford University Press, New York, N.Y.

Swafford AN, Bratz IN, Knudson JD, Rogers PA, Timmerman JM, Tune JD, et al. 2005. C-reactive protein does not relax vascular smooth muscle: effects mediated by sodium azide in commercially available preparations. Am J Physiol Heart Circ Physiol 288:H1786-H1795.

Sykova E, Mazel T, Simonova Z. 1998. Diffusion constraints and neuronYglia interaction during aging. Exp Gerontol 33:837-51.

Szalai, A. J., Nataf, S., Hu, X. Z., and Barnum, S. R. 2002. J. Immunol. 168, 5792–5797

Szalai, A. J., Weaver, C. T., McCrory, M. A., van Ginkel, F. W., Reiman, R. M., Kearney, J. F., Marion, T. N., and Volanakis, J. E. 2003. Arthritis Rheum. 48, 1602–1611

T. Suenaga, K. Ohnishi, M. Nishimura, S. Nakamura, I. Akiguchi, J. Kimura. 1994. Bundles of amyloid precursor protein-immunoreactive axons in human cerebrovascular white matter lesions, Acta Neuro- pathol. (Berl.) 87 , 450–455.

Takahashi K, Rochford CD, Neumann H. 2005. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J Exp Med 201:

Takamatsu J., Hirano A., Levy D. & Henkind P. Experimental bilateral carotid artery occlusion: a study of the optic nerve in the rat. 1984. Neuropathology and Applied Neurobiology 10, 42-28

Tarek Khreiss, Levente József, Lawrence A. Potempa, János G. Filep. 2005. Loss of Pentameric Symmetry in C-Reactive Protein Induces Interleukin-8 Secretion Through Peroxynitrite Signaling in Human Neutrophils . Circulation Research. 97:690-697

tenDam,V.H.,D.M. van den Heuvel,A.J. deCraen, et al. 2007. Decline in total cerebral blood flow is linked with increase in periventricular but not deep white matter hyperintensities. Radiology 243: 198–203.The ARIC Study. Neuroepidemiology, 16,149-162

Terry RD, DeTeresa R, Hansen LA. 1987. Neocortical cell counts in normal human adult aging. Ann Neurol 21:530-39.

Terubumi Watanabe, Ning Zhang, Meizi Liu, Ryota Tanaka, Yoshikuni Mizuno and Thomas, E. W. 1999. Brain macrophages: on the role of pericytes and perivascular cells. Brain Res. Rev. 31:42–57.

Thompson D, Pepys MB, and Wood SP. 1999. The physiological structure of human C-reactive protein and its complex with phosphocholine .Structure 7, 169–177.

Tilg H, Vannier E, Vachino G, Dinarello CA, and Mier JW. 1993. Anti-inflammatory properties of hepatic acute phase proteins: preferential induction of interleukin 1 (IL-1) receptor antagonist over IL-1 beta synthesis by human peripheral blood mononuclear cells. J. Exp. Med. 178, 1629–1636.

Tillett WS, Francis T. 1930. Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. J Exp Med 52: 561-71.

Tohgi, H., H. Yonezawa, S. Takahashi, et al. 1998. Cerebral blood flow and oxygen metabolism in senile dementia of Alzheimer's type and vascular dementia with deep white matter changes. Neuroradiology 40: 131–137.

Tomimoto H, Akiguchi I, Wakita H, Suenaga T, Nakamura S, Kimura J. 1997. Regressive changes of astroglia in white matter lesions in cerebrovascular disease and Alzheimer's disease patients.Acta Neuropathol. Aug;94(2):146-52.

Tomimoto H, Akiguchi I, Suenaga T, Nishimura M, Wakita H, Nakamura S, Kimura J.1996. Alterations of the blood-brain barrier and glial cells in white matter lesions in cerebrovascular and Alzheimer's disease patients. Stroke 27: 2069–2074

Torzewski J. C-reactive protein and atherogenesis: new insights from established animal models. 2005. Am J Pathol. Oct;167(4):1139-48.

Ueno M, Tomimoto H, Akiguchi I, Wakita H, Sakamoto H. 2002. Blood-brain barrier disruption in white matter lesions in a rat model of chronic cerebral hypoperfusion. J Cereb Blood Flow Metab 22:97–104.

Van den Berg CW, Taylor KE, Lang D. 2004. C-reactive protein-induced in vitro vasorelaxation is an artifact caused by the presence of sodium azide in commercial preparations. Arterioscler Thromb Vasc Biol 24:168-171.

van Dijk EJ, Prins ND, Vermeer SE, Vrooman HA, Hofman A, Koudstaal PJ, Breteler MMB. 2005. C-reactive protein and cerebral small-vessel disease: the Rotterdam Scan Study. Circulation 112:900 –905.

van Dijk, E.J., N.D. Prins, S.E. Vermeer, et al. 2004. Plasma amyloid beta, apolipoprotein E, lacunar infarcts, andwhite matter lesions. Ann. Neurol. 55: 570– 575.

Venugopal, S. K., Devaraj, S., Yuhanna, I., Shaul, P., and Jialal, I. 2002. Circulation 106, 1439–1441.

Venugopal, SK; Devaraj, S; Jialal, I. 2005. Macrophage conditioned medium induces the expression of C-reactive protein in human aortic endothelial cells: potential for paracrine/autocrine effects. Am J Pathol. 166:1265–1271.

Vigushin DM, Pepys MB, Hawkins PN. 1993. Metabolic and scintigraphic studies of radioiodinated human C-reactive protein in health and disease. *J Clin Invest* 91:1351–7.

Volanakis JE. 1982. Complement activation by C-reactive protein complexes. Ann. N. Y. Acad. Sci. 389:235–250.

Volanakis JE, Wirtz KWA. 1979. Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. Nature. 281:155–157.

Vyse TJ. 2004. Polymorphism at the C-reactive protein locus influences gene expression and predisposes to systemic lupus erythematosus. Hum. Mol. Genet. 13, 137–147.192:1353–1363.

Wakita H, Tomimoto H, Akiguchi I, Matsuo A, Lin J, Ihara M and McGeer PL. 2002. Axonal damage and demyelination in the white matter after chronic cerebral hypoperfusion in the rat. Brain Research, Volume 924, Issue 1, 4 January, Pages 63-70.

Wakita H, Tomimoto H, Akiguchi I, Kimura J .1995 .Protective effect of cyclosporin A on white matter changes in the rat brain after chronic cerebral hypoperfusion. Stroke 26:1415–22

Wakita H, Tomimoto H, Akiguchi I, Kimura J. 1994. Glial activation and white matter changes in the rat brain induced by chronic cerebral hypoperfusion: an immunohistochemical study. Acta Neuropathol, 87(5):484-92.

Wakita H, Tomimoto H, Akiguchi I, Lin JX, Miyamoto K, Oka N. 1999. A cyclooxygenase-2 inhibitor attenuates white matter damage in chronic cerebral ischemia. Neuroreport 10:1461–5

Williams K, Bar-Or A, Ulvestad E, Olivier A, Antel JP, Yong V. 1992. Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. J Neuropath Exp Neurol 51:538–549.

Williams E, Alvarez X, and Lackner AA. 2001. Central nervous system perivascular cells are immunoregulatory cells that connect the CNS with the peripheral immune system. Glia 36:156–164.

Winkler H, Fisher-Colabrie R. 1992. The chromogranins A and B: the first 25 years and future perspectives. Neuroscience 49 , 497–528.

Yang MS, Park EJ, Sohn S, Kwon HJ, Shin WH, Pyo HK, Jin B,Choi KS, Jou I, Joe EH.2002. Interleukin-13 and -4 induce death of activated microglia. Glia 38:273–280.