CHAPTER FIVE

VALIDATION OF RADIOLABELLED PK11195 AS AN INFLAMMATORY TRACER IN
MULTIPLE SCLEROSIS
MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is the prototype inflammatory autoimmune disorder of the central nervous system and, with a lifetime risk of one in 400, potentially the most common cause of neurological disability in young adults. As with all complex traits, the disorder results from an interplay between as yet unidentified environmental factors and susceptibility genes. Together, these factors trigger a cascade of events, involving engagement of the immune system, acute inflammatory injury of axons and glia, recovery of function and structural repair, post-inflammatory gliosis, and neurodegeneration. The sequential involvement of these processes underlies the clinical course characterised by episodes with recovery, episodes leaving persistent deficits, and secondary progression. The aim of treatment is to reduce the frequency, and limit the lasting effects, of relapses, relieve symptoms, prevent disability arising from disease progression, and promote tissue repair. Despite limited success in each of these categories, everyone touched by multiple sclerosis looks for a better dividend from applying an improved understanding of the pathogenesis to clinical management.

The present part reviews the history, epidemiology, pathophysiology, clinical features and disease course, and treatment of MS.

HISTORY AND EPIDEMIOLOGY

Previously unrecognised, multiple sclerosis makes a fleeting appearance in the early 19th century before taking centre stage as clinical neurology began to flourish in the 1860s. By the beginning of the 20th century, a disease only a few years earlier meriting individual case reports had become one of the most common reasons for admission to a neurological ward. The incidence of MS is greatest at the extremes of latitude in both the northern and southern hemispheres (hence rarely observed in Asians or Africans) and affects almost twice as many women as it does men (at least in the most common relapsing remitting type of MS); this unexplained bias mimics that seen in other putative autoimmune diseases. The disease has an incidence of about seven per 100 000 every year, prevalence of around 50 to 150 per 100 000 for Caucasians, and a lifetime risk ranging from one in 1000 to one in 250. Now, multiple sclerosis is recognised throughout the world, with around 2.5 million affected individuals. These crude statistics conceal the harsh reality of a frightening and potentially disabling disease for young adults.

PATHOPHYSIOLOGY

The oligodendrocyte, a principal target of immune attack in multiple sclerosis, synthesises and maintains the myelin sheath of up to 40 neighbouring nerve axons in the central nervous system. Compact myelin consists of a condensed membrane, spiralled around axons to form the insulating segmented sheath needed for saltatory axonal conduction: voltage-gated sodium channels cluster at the unmyelinated nodes of Ranvier, between myelin segments, from where the action potential is
propagated and spreads passively down the myelinated nerve segment to trigger another action potential at the next node.

The consequences of demyelination for saltatory conduction explain many clinical and laboratory features of multiple sclerosis. Partially demyelinated axons conduct impulses at reduced velocity - explaining the characteristic delays in conduction of evoked potentials. Demyelinated axons can discharge spontaneously and show increased mechanical sensitivity - accounting for the flashes of light on eye movement (phosphenes) and electrical sensation running down the spine or limbs on neck flexion (Lhermitte's sign). Partially demyelinated axons, whose safety factor for conduction is compromised, cannot sustain the fall in membrane capacitance induced by a rise in temperature, and conduction fails - leading to the characteristic appearance of symptoms and signs after exercise or a hot bath (Uthhoff's phenomenon). Ephaptic transmission (cross-talk) can arise between neighbouring demyelinated axons, resulting in paroxysmal symptoms - trigeminal neuralgia, ataxia, and dysarthria, or painful tetanic posturing of the limbs, lasting one or two minutes and often triggered by touch or movement. Individuals with multiple sclerosis characteristically tire during physical and cognitive tasks, and take longer to recover: although poorly understood, and probably multifactorial, fatigue in multiple sclerosis can be very disabling, even in isolation.

The symptoms and signs of multiple sclerosis merely reflect the functional anatomy of impaired saltatory conduction at affected sites. The cerebrum is almost always involved when assessed with magnetic resonance imaging (MRI), but most white matter abnormalities cannot be linked to specific events or clinical symptoms, anyway, new lesions appear to occur at least seven to ten times more frequently than clinical attacks, where especially the correlation between T2 lesions and disability is poor (clinicoradiological paradox) [1]. Involvement of the anterior visual pathway is the rule. Lesions of the brainstem and cerebellar pathways produce precise clinicopathological correlations; typically, coordinated movement of the eyes, limbs, bulbar musculature, and axial muscles is disrupted. The spinal cord is frequently affected, leading to alterations in motor, sensory, and autonomic functions.

One hypothesis to explain the breakdown of immune regulation in autoimmune diseases is molecular mimicry, which suggests that a peptide (the environmental factor), presented in the groove of specific class II molecules (one component of inherited risk), is immunologically indistinguishable from self-antigen and, hence, an appropriate response to infection generates inappropriate inflammation against some component of the oligodendrocyte-myelin unit. Also, damage to normal tissue may expose novel antigens to the immune system that would otherwise not be contacted and further direct attack against nearby self-antigens, i.e. epitope spreading. In common with all organ-specific autoimmune diseases, this systemic defect results not in a sustained autoimmune attack on the entire target organ but, rather, in inflammatory lesions that are temporally and spatially segregated.
MOLECULAR GENETICS AND ENVIRONMENTAL FACTORS

Multiple sclerosis is caused by an interplay between genes and environment. There is a familial recurrence rate of about 15%. The age-adjusted risk is higher for siblings (3-5%), parents (2%), and children (2%) than for second-degree and third-degree relatives. Recurrence in monozygotic twins is around 25-35%. Recurrence is higher in the children of conjugal pairs with multiple sclerosis (20%) than in the offspring of single affecteds (2%). Conversely, the risk is not increased either for individuals adopted into a family with an affected individual or in the non-biological relatives of adoptees who themselves develop multiple sclerosis.

Multiple sclerosis seems to be genuinely polygenic in which the genes responsible for the complex traits are not mutations coding for aberrant gene products but normal polymorphisms [2]. They act independently or through epistasis, and each polymorphism can exert a small contributory effect on some as yet undefined structure or physiological function. Extensive searches have yielded few secure candidate regions. Results of population studies suggest an association between the human leucocyte antigen (HLA) class II MHC alleles which code for molecules that participate in antigen recognition by T lymphocytes and the gene for TNF-α encoded within the same linkage group. The list of candidate genes that have been screened includes many adhesion molecules, immune receptors or accessory molecules, cytokines and their receptors or antagonists, chemokines, growth promoting molecules, and structural genes of the myelin-oligodendrocyte unit. Disappointingly, the low yield from this trawl is not definitively advanced by eight whole genome linkage screens done in several countries [3]. In common with most other complex traits, no major susceptibility gene has yet been identified, although several promising chromosomal linkages are provisionally linked and associated with multiple sclerosis - at 1p, 6p, 10p, 17q, and 19q [4].

The distribution of multiple sclerosis cannot be explained on the basis of population genetics alone. Outside Europe, prevalence rates among white people are half those documented for many parts of northern Europe. In Australia and New Zealand, there are gradients in frequency that do not follow genetic clines. The risk is higher for English-speaking white people who migrate into South Africa as adults than as children. The low frequency of multiple sclerosis in Africans increases substantially for first-generation descendants raised in the UK. Results of surveys of multiple sclerosis have prompted speculation on the occurrence of epidemics in Iceland, the Orkney and Shetland Islands, and the Faroes (where MS was unknown until 1940 when British soldiers landed on its shores), although others prefer the interpretation that these merely indicate improved case recognition. There is age-linked susceptibility to viral exposure in those who are constitutionally at risk of developing the disease. Attempts to reliably implicate specific environmental agents are frustrating. Recent, yet unsubstantiated candidates, include Chlamydia pneumoniae [5], Ebstein-Barr Virus, and human herpesvirus type 6 [6].
Revised diagnostic criteria classify individuals in the categories of multiple sclerosis, not multiple sclerosis, or possible multiple sclerosis, and incorporate evidence from MRI. As with the previous diagnostic criteria, individuals must have a minimum of two attacks, affecting more than one anatomical site, but, assuming an initial presentation suggestive of multiple sclerosis, the second lesion need not necessarily be clinically expressed [7].

Investigations are done for four main reasons in patients with multiple sclerosis: they allow to see the anatomical dissemination of lesions in time and space (imaging); they permit the assessment of intrathecal inflammation (spinal fluid analysis); they show that conduction has altered in a pattern consistent with demyelination (evoked potentials); and they allow the exclusion of conditions that mimic the disease.

More than 95% of patients with multiple sclerosis have T2-weighted white matter abnormalities, but these are not diagnostic. They occur about 10 times more frequently than new clinical events. Imaging is not necessary for diagnostic purposes in patients with a history of relapsing disease, affecting multiple sites within the central nervous system. The major practical use is in the investigation of individuals with clinically isolated lesions or progressive disease at a single site. There is less cerebral involvement in patients with primary progressive multiple sclerosis than in those who have comparable disability from secondary progression. Variations in imaging protocols are beginning to distinguish separate components of the underlying pathological process - inflammation (gadolinium DTPA enhancement of T1-weighted lesions, reflecting a breach in the normal blood-brain barrier and indicating that the lesion is of recent origin), demyelination (magnetisation transfer ratio), astrocytosis (T2-weighted lesions, the signal arising from increased water content), and axonal damage (reduction in diffusion tensor imaging anisotrophy and N-acetyl-aspartate spectra with chemical shift imaging, or the presence of focal atrophy and T1-weighted black holes) [8].

Cerebrospinal fluid protein electrophoresis shows oligoclonal IgG bands in more than 90% of cases. Their role in the pathogenesis of multiple sclerosis is unresolved. Screening spinal fluid against cDNA expression, phage display, or random peptide libraries has not distinguished common antigen specificities; some antibodies are directed against components of the oligodendrocyte or its myelin membranes, and others recognise extrinsic antigens including viruses, but collectively these specificities only account for a minority of the bands. Diagnostically, spinal fluid oligoclonal bands confirm that the underlying pathology is inflammatory, which can be useful in excluding alternative explanations, especially in the context of progressive spinal cord syndromes and in elderly patients in whom imaging abnormalities are not discriminatory.

Demyelination characteristically delays the latencies of visual, auditory, and somatosensory evoked potentials, as well as central motor conduction times, leaving the amplitude of responses unchanged. Before the advent of MRI, these abnormalities provided evidence for clinically silent lesions; now, their
role is confined to the provision of circumstantial evidence for demyelination in diagnostically difficult situations, such as syndromes that progress from onset.

**NATURAL HISTORY AND EVOLUTION OF THE PLAQUES**

80-90% of patients present with relapsing - remitting disease and, typically, the illness passes through phases of relapse with full recovery, relapse with persistent deficit, and secondary progression. In about a quarter of patients, multiple sclerosis never affects activities of daily living; conversely, up to 15% become severely disabled within a short time. Episodes happen at random intervals, but initially average about one per year, decreasing steadily thereafter. In 10-20% of patients, the disease is progressive from onset, hence termed primary progressive - affecting the spinal cord and, less frequently, the optic nerve, cerebrum, or cerebellum. Disease onset is usually in the third or fourth decade, but 2% of patients with multiple sclerosis present before the age of 10 years, and 5% before age 16 years. In children, the distinction from acute disseminated encephalomyelitis can often only be established by observing the subsequent natural history. Overall, life expectancy is at least 25 years from disease onset with most patients dying from unrelated causes.

The prognosis is relatively good when sensory or visual symptoms dominate the course of multiple sclerosis in adults, and there is complete recovery from individual episodes. This pattern is most common in young women. Conversely, motor involvement, especially when coordination or balance are disturbed, has a less positive prognosis. The outlook is also poor in older men who develop the disease. Frequent and prolonged relapses with incomplete recovery at onset and a short interval between the initial episode and first relapse are adverse prognostic features [9], but the main determinant of disability is onset of the progressive phase [10].

Fixed disability in multiple sclerosis is acquired through two distinct mechanisms: incomplete recovery from relapse and disease progression. Patients with relapsing-remitting multiple sclerosis accumulate disability from disease onset more slowly than those with primary progressive multiple sclerosis. However, beyond a degree of disability sufficient to limit walking to less than 500 m without aid or rest (Kurtzke expanded disability status scale [EDSS] of 4.0), subsequent accumulation no longer correlates with mode of presentation, suggesting that the pathological substrate for progression determines disability at this stage of the disease [11].

Prospective studies show that around 10% of upper respiratory (adenovirus) and gastrointestinal infections, arising in patients with multiple sclerosis, are followed by relapse, and about 30% of new episodes relate to infection. There is no evidence that trauma causes multiple sclerosis, triggers latent disease in someone who has the underlying disease process, or alters the course in individuals who have already experienced symptoms. People with multiple sclerosis cope less well with symptoms while exposed to stress, but psychological factors do not directly affect disease activity.
Maturation of the individual lesion involves several stages: a) immune engagement, b) acute inflammatory injury of axons and glia, c) recovery of function and structural repair and d) post-inflammatory gliosis and neurodegeneration. Healthy individuals harbour autoreactive myelin T cells, normally kept in check by regulatory T cells. Failure of regulation leads to proliferation, activation, and entry into the circulation of autoreactive T cells; they express adhesion molecules and induce reciprocal changes in endothelia, allowing access across the blood-brain barrier into the central nervous system. There, activated T cells re-encounter antigen and activate microglia (the CNS macrophage); they, in turn, express HLA class II molecules, re-present antigen to T cells, and set up a proinflammatory loop, which provides an infiltrate rich in activated T cells and microglia with some neutrophils. On the figure below perivascular inflammation is shown (panel 1) which causes acute axonal transection (panel 2), and microglia-mediated removal of myelin (panel 3) with persistent demyelination despite some remyelination (panel 4); chronic lesions show further axonal loss (panel 5) and gliosis (panel 6). This scheme does not depict primary progressive multiple sclerosis in which there is pronounced axonal degeneration with or without a preceding inflammatory phase [12].

Toxic inflammatory mediators are released, sustaining breakdown of the blood-brain barrier and leading to injury of axons and glia. Nitric oxide might act directly on normal or hypomyelinated axons, transiently blocking conduction [13] and reversibly increasing deficits arising from already compromised pathways. Symptoms also improve as surviving functional pathways are reorganised at
the cellular [14] and systems level [15]. Together, these mechanisms account for remission early in the disease. But tissue vulnerability is easily exposed. When compounded by high axonal firing frequency, nitric oxide causes structural (and hence irreversible) changes to axons [16]. Axonal transection in acute inflammatory plaques is shown histologically [17] and radiologically through reduction in the neuronal spectroscopic marker, N-acetyl aspartate (NAA) [18]. These transected axons undergo Wallerian degeneration during the subsequent 18 months [19], but this action does not seem to extend the lesion or shape the clinical deficit.

Cytokines and growth-promoting factors released by reactive astrocytes and microglia as part of the acute inflammatory process promote endogenous remyelination. But, over time, astrocyte reactivity seals the lesion and gliosis causes a physical barrier to further remyelination, reducing the capacity to accommodate cumulative deficits, and marking transition to the stage of persistent deficit.

Most axonal loss is seen in secondary progressive multiple sclerosis [20]. It is proposed that chronic axonopathy is not due directly to inflammation nor does it occur only colocalised with discrete pathological lesions, but results from loss of trophic support normally provided to axons by myelin or glia, acting directly or through the maintenance of electrical activity, or both [21,22]. As such, chronic axonal degeneration might slowly increase the clinical deficit, decaying a compromised but functioning pathway and leading to disease progression.

**TREATMENT**

The aims of treatment are to: a) reduce relapse rates, b) prevent fixed disability directly attributable to relapse, c) provide symptomatic management of fixed neurological deficit, d) prevent disability acquired through progression, e) treat established progression.

*Reducing relapse rates in multiple sclerosis*

Since permanent disability can be caused by incomplete recovery from episodes, relapse frequency is bound to correlate with accumulation of disability during the relapsing-remitting phase of multiple sclerosis. The dividend from reducing the relapse rate is best shown by use of the β-interferons: interferon β-1a, and interferon β-1b. These type-1 interferons were first used in multiple sclerosis for their antiviral action, in view of the known propensity of viral infections to trigger relapses. Their predominant mechanism of action is unknown, but they have been found to suppress T cell proliferation, reduce T cell migration from the systemic circulation into the CNS, alter the T cell cytokine secretion repertoire from relatively proinflammatory Th1 to relatively anti-inflammatory Th2 response, and downregulate class II MHC antigen expression [23]. The annual relapse rate for individuals treated with interferon-β is significantly reduced by 30-37%, however, only with interferon β-1a this change in relapse rate is also accompanied by a reduction in the accumulation of disability [24].
Three other agents reduce relapse frequency, and the accumulation of disability, in relapsing-remitting multiple sclerosis; each has similar efficacy to the β-interferons and acceptable adverse effects profiles. Glatiramer acetate was noted serendipitously to suppress experimental allergic encephalomyelitis, the animal model of demyelinating disease, perhaps by inhibiting the binding of myelin basic protein (MBP) to the T cell receptor or by altering the phenotype of myelin-autoreactive T cells [25], and is able to reduce the annual relapse rate by 25% [26]. Secondly, azathioprine inhibits lymphocyte proliferation by inhibiting purine synthesis, and probably has similar efficacy to the β-interferons, although trial data were less clear-cut [27]. Thirdly, mitoxantrone inhibits DNA repair and synthesis in dividing and non-dividing cells through inhibition of DNA topoisomerase II; it is potentially much more toxic than the β-interferons, and is mainly used for the treatment of aggressive relapsing disease, including patients with high relapse frequency in the progressive phase [28].

Prevention of disability attributable to relapse
Mild attacks causing little or no functional impairment may require no treatment other than rest and often resolve spontaneously. Relapses causing functional impairment (e.g., visual loss, weakness, significant gait impairment) are typically treated with corticosteroids where they reduce the duration of relapses and hence their short-term morbidity, but not the ensuing permanent deficits. Corticosteroids, bound to their cytoplasmic receptors, enter the cell nucleus and inhibit transcription of proinflammatory cytokines, such as interleukin-1, interleukin-2, tumour necrosis factor-α (TNF-α) and proinflammatory enzymes, including collagenase, elastase, and plasminogen activator [29].

Symptomatic management of fixed neurological deficits
Fixed neurological deficits in multiple sclerosis are best managed by a multidisciplinary team, attending to physical therapies, psychological, and social interventions supplemented by medical treatments. The benefits of intense inpatient rehabilitation outlast the duration of therapy by up to 9 months [30]. The symptoms that are most amenable to treatment are spasticity and sphincter dysfunction. Spasticity causes discomfort and hinders care and is usefully treated by baclofen, which acts on spinal cord GABA-B receptors to suppress reflex arcs that have been released from higher inhibitory control; or tizanidine, which acts through spinal cord α9 receptors to modulate presynaptic release of excitatory aminoacids. Bladder symptoms are most easily categorised by measuring the postmicturition bladder volume. If greater than 100 mL, there is primarily failure to empty and the treatment is ideally intermittent self-catheterisation; if the bladder empties fully but stores poorly, the detrusor might be inhibited by anti-cholinergics such as oxybutynin [31]. In fact, most patients have a combination and experience the urge frequently to empty a partially filled bladder against a closed sphincter. Erectile impotence is successfully treated with sildenafil citrate, a phosphodiesterase inhibitor that acts predominantly on nitric oxide within the penile vasculature. Paroxysmal attacks respond well to membrane-stabilising drugs - typically carbamazepine. No pharmacological treatment has shown a useful effect on the tremor of multiple sclerosis. There are advocates for thalamotomy and thalamic stimulation in highly selected patients. Fatigue cannot be satisfactorily treated; lowering body temperature might help and small trials report some benefit from amantadine and modafani [32].
**Prospects for improved treatment of disease activity**

In view of the fact that the ability to suppress relapses and limit their consequences is partial, no informed analyst could reasonably conclude that (despite their achievements) the β-interferons are a definitive therapy in multiple sclerosis. The pharmaceutical industry has responded by sponsoring studies with combinations of established drugs (such as β-interferon and cyclophosphamide) without compelling evidence for synergistic benefit to date, together with a significant investment in novel immunotherapeutic strategies.

There are two approaches to reduce the activation and proliferation of autoreactive T cells. One is to search for new agents that suppress immune activity non-specifically and have acceptable safety profiles. Past attempts (with cyclophosphamide, ciclosporin, lymphoid irradiation, cladribine) have shown evidence for efficacy but with major side-effects; examples of this legacy are paclitaxel, teriflunomide, and autologous bone marrow transplantation, in which the limited efficacy seen to date must be weighed against the procedural mortality of around 5% [33]. The second strategy is to assume that the specific interaction between MBP, T cells, and antigen-presenting cells is the pivotal event driving multiple sclerosis. Several drugs have been designed to manipulate this interaction - for instance, vaccination with T cell receptor subtypes [34], MBP specific T cell clones [35], or disrupted MBP peptides. Although the results were disappointing, it would be premature to judge whether the strategy is wrong or the reagents insufficiently active. The hope was that, by minor changes in the presumed immunodominant peptide, autoaggressive MBP-reactive T cells might be tolerised. Alternatively, there are treatment strategies to reduce the effect of activated T cells; by blocking their entry into the brain (with an antibody against the α-4 integrin) or by neutralising putative toxic products [36].

**Prevention of disability acquired through progression**

Despite several attempts, immunomodulatory drugs are of little use once axonal degeneration has reached a critical threshold and clinical progression is established [37]. It follows that there might be an opportunity, early in the disease course, to suppress those components of the inflammatory process that initiate the cascade leading to delayed progression. Thus, the aim of immunotherapies is not only to reduce relapse frequency, but also to prevent transition to the secondary progressive phase of the illness. These essential issues in the therapy of multiple sclerosis - does early effective anti-inflammatory therapy reduce the proportion of patients who ever enter the secondary progressive phase, or usefully affect the slope of that progression? - are now being addressed in trial design [38].

**Can surviving axons be remyelinated?**

The informed patient often expresses disappointment that management aims merely to limit further damage without seeking to restore the neurological past. Endogenous remyelination is limited to the acute inflammatory phase, and this timing raises the issue of whether, paradoxically, anti-inflammatory treatment might contribute to the failure of repair. For those axons that degenerate early as a direct result of the inflammatory process, efforts at remyelination might have little to offer; conversely, if the
naked axon is resistant to the inflammatory milieu but has poor survival properties, remyelination might be neuroprotective and its timing important.

The therapeutic challenge is whether to enhance endogenous remyelination or develop exogenous cell-based therapies. Experimentally, endogenous remyelination restores conduction and function in young and adult nervous systems [39]. The lesions of multiple sclerosis do contain oligodendrocyte progenitors, but these seem unable to usefully engage naked axons [40]. Manipulation of mechanisms involved in receptor-ligand growth factor interactions during the inflammatory phase of tissue injury might energise these indolent progenitors and improve remyelination. Thus, one option is to wait until a therapy is available that can be given systemically and delivered simultaneously to all affected parts of the central nervous system. The alternative is first to prove that structure and function can usefully be restored in a single informative lesion before tackling the secondary task of making this intervention diffusely available in the central nervous system. The initial proof of principle will almost certainly involve cell implantation; at present, the most promising candidates are autologous peripheral nerve Schwann cells [41] or olfactory bulb ensheathing cells [42]. How best to plan the difficult transition from experimental to clinical science in the context of a multifocal and multiphasic disease has been much discussed. The ideal lesion would be accessible, responsible for clinically significant and stable deficits, resulting from persistent demyelination, and at a site where the risks of failure would be acceptable (perhaps through the presence of an intact paired structure or pathway) and where tissue was shown to be undergoing progressive axonal degeneration in the absence of active inflammation. The optic nerve is perhaps the best candidate, because the symptoms are clinically eloquent, physiological assessment and imaging are well developed, and serial atrophy is seen after unilateral optic neuritis despite recovery of vision [43]; this combination suggests postinflammatory axonal degeneration consistent with loss of trophic support from myelin [22].

CHALLENGES
For the pathologist, multiple sclerosis is a disorder of the central nervous system, manifesting as acute focal inflammatory demyelination and axonal loss with limited remyelination, culminating in the chronic multifocal sclerotic plaques from which the disease gets its name. For the patient, multiple sclerosis threatens an apparently infinite variety of symptoms but with certain recurring themes and an unpredictable course. For the neurologist, multiple sclerosis is a disorder of young adults diagnosed on the basis of clinical and paraclinical evidence for at least two demyelinating lesions, affecting different sites within the brain or spinal cord, separated in time. For the clinical scientist, multiple sclerosis is the prototype inflammatory autoimmune disease of the central nervous system in which knowledge gained across a range of basic and clinical neuroscience disciplines has already allowed rational strategies for treatment. For all these groups, multiple sclerosis remains a difficult disease for which solutions seem attainable yet remain elusive.
A major part of future studies will be to resolve the question of disease heterogeneity [44,45]. Because primary progressive multiple sclerosis affects an older (predominantly male) population, has a less favourable prognosis, and is associated with fewer radiological and histological inflammatory lesions - such that these patients are disenfranchised with respect to clinical trials of immunomodulatory drugs - this type of multiple sclerosis is considered by many to be a separate disorder [46]. Genetic analyses suggest specifically different MHC associations in northern Europeans and the Mediterranean (especially Sardinians), and (perhaps) between primary progressive and relapsing-remitting multiple sclerosis. This notion of heterogeneity is further developed in pathological studies with biopsy and necropsy material, in which four distinct but overlapping histological types are described. The histopathological appearances are generally similar between lesions from each patient, but the nature of necropsy or biopsy material makes it more difficult to show subtype consistency over time [45].

Within 40 years of its first depiction, the clinical and pathological details of multiple sclerosis had been adequately characterised. Over the past 120 years, ideas have consolidated on the cause and mechanisms of inflammatory demyelination and axonopathy. In the past 10 years, therapies have emerged that modestly affect the course of the illness. Current research is increasingly seen as coherent and focused on the hot topics that need to be solved to limit, repair, and prevent the damage caused by multiple sclerosis.


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**PET VISUALISATION OF MICROGLIA IN MULTIPLE SCLEROSIS PATIENTS**

**USING [{\textsuperscript{11}}C]PK11195**

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**ABSTRACT**

Activated microglia are involved in the immune response of multiple sclerosis (MS). The peripheral benzodiazepine receptor (PBR) is expressed on microglia and upregulated after neuronal injury. [{\textsuperscript{11}}C]PK11195 is a positron emission tomography (PET) radioligand for the PBR. The objective of the present study was to investigate [{\textsuperscript{11}}C]PK11195 imaging in MS patients and its additional value over MRI concerning the immuno-pathophysiological process. Seven healthy and 22 MS subjects were included. Semiquantitative [{\textsuperscript{11}}C]PK11195 uptake values were assessed with normalisation on cortical gray matter. Uptake in Gadolinium-lesions was significantly increased compared to normal white matter. Uptake in T2-lesions was generally decreased, suggesting a PBR downregulation. However, uptake values increased whenever a clinical or MR-relapse was present, suggestive for a dynamic process with a transient PBR upregulation. During disease progression, an increase of normal-appearing white matter (NAWM) uptake was found, propagating NAWM as the possible real burden of disease. In conclusion, [{\textsuperscript{11}}C]PK11195 and PET are able to demonstrate inflammatory processes with microglial involvement in MS.
INTRODUCTION

Microglia, the brain’s intrinsic macrophages, play a major role in the inflammatory process of autoimmune demyelinating diseases and particularly multiple sclerosis (MS). They initiate the immunological process in the early stages of the disease. More specifically, microglial activation in experimental allergic encephalomyelitis (EAE) and MS contributes to CNS damage through several mechanisms such as the production of proinflammatory cytokines (e.g. TNF-α), matrix metalloproteinases and free radicals, even as still ramified but activated cells (Benveniste, 1997). Indeed, by releasing myelinotoxic factors with direct injury to the oligodendrocyte-myelin unit, activated microglia stimulate this demyelinating process (Sriram and Rodriguez, 1997). Additionally, during relapses when the blood-brain barrier (BBB) opens, they are thought to act as antigen presenting cells having the capacity of activating perivascular myelin-reactive T lymphocytes by costimulatory molecules on their surface (Dangond et al., 1997; Vowinckel et al., 1997).

One of the characteristic features of microglia is their rapid activation in response to brain injury with a concomitant higher expression of the peripheral benzodiazepine receptor (PBR) (Banati et al., 2000). Visualisation of this PBR is possible by means of radiolabeled PK11195, a selective and specific high affinity ligand, allowing as such the assessment of microglial activity and providing more specific in vivo information concerning the underlying histopathological features. Indeed, high resolution microautoradiography combined with immunohistochemical cell identification in MS and EAE tissue demonstrated that in focal inflammation, when the BBB is disrupted, the PBR is expressed on invading blood-borne cells such as macrophages (Banati et al., 2000). However in brain lesions without direct damage to the BBB, the predominant cell type expressing PBR binding sites is the activated microglial cell (Banati et al., 1997).

PK11195 has been labeled with $^{11}$C for the study of the lesioned brain with positron emission tomography (PET) in various neuroinflammatory conditions like stroke, Rasmussen’s encephalitis and cerebral vasculitis (Goerres et al., 2001; Ramsay et al., 1992). A previous feasibility study showed the value of a straightforward semiquantitative approach by means of defining an accurate confidence interval for the $^{[11]}$C]PK11195 uptake values in controls for the eventual detection of microglial activation in acute and chronic neuroinflammatory diseases (Debruyne et al., 2002).

Up till now, magnetic resonance imaging (MRI) has been widely used in MS to evaluate disease activity and to monitor clinical trials or the natural course of the disease process itself. Although MRI is of paramount value for diagnostic procedures, there remains a weak correlation between MRI parameters and disability as measured with the expanded disability status scale (EDSS). The reason for this clinico-radiological paradox may be the lack of histological specificity of conventional MRI lesions and the underestimation of disease burden in the normal-appearing white matter (NAWM) and spinal cord (Barkhof and van Walderveen, 1999; Nijeholt et al., 1998). The aims of the present study are, firstly, to
assess the $[^{11}\text{C}]$PK11195 uptake in MS patient subgroups with chronic and active white matter lesions on MRI and, secondly, to investigate the additional value of $[^{11}\text{C}]$PK11195 imaging over MRI concerning the immuno-pathophysiological process during the disease course in this cohort of patients with different clinical manifestations and invalidity.
Radiochemistry
PK11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide] was obtained from RBI (Natick, MA, USA). \([^{11}\text{C}]\text{PK11195}\) was synthesized according to a procedure described by Camsonne et al. (Camsonne et al., 1984). Briefly, 3 µmol N-desmethyl PK11195 was dissolved in 150 µL dimethylsulphoxide, containing 3 µmol tetrabutylammonium hydroxide. After trapping of \([^{11}\text{C}]\text{CH}_3\), the vial was closed and heated at 80°C for 3 min. Purification was done by HPLC using a RP-C18 column (Econosil, 250 mm x 10 mm, 10 µm particle size) and an ethanol/water (70/30) mixture as mobile phase. Radiochemical yield towards \([^{11}\text{C}]\text{CH}_3\) was 57 ± 2 %. Finally, 3.7 GBq \([^{11}\text{C}]\text{PK11195}\) was obtained with a specific activity of 25 GBq/µmol. Chemical and radiochemical purity were higher than 99%. All subjects were injected intravenously with 370 ± 10 % MBq \([^{11}\text{C}]\text{PK11195}\) with a slow bolus in a time course of 30 seconds.

Data acquisition and processing
All subjects underwent MR and PET scanning on the same day.

MR imaging was performed on a 1.5 T commercial MR scanner (Siemens, Magnetom 1.5 T, SP4000; Erlangen, Germany). Prior to the administration of gadolinium (Gd), standard spin-echo imaging was carried out in 5 mm thick axial planes (pixel size=0.9 x 0.9 mm\(^2\)) with proton density- (TR/TE/NEX=2170/20/1), T2- (TR/TE/NEX=2170/80/1) and T1-weighted (TR/TE/NEX=600/12/1) contrast. Five minutes after Gd injection, the axial slices were rescanned with T1-weighting (TR/TE/NEX=800/20/1).

PET studies were performed on a Siemens ECAT 951/31 PET scanner (Siemens, Knoxville, TN, USA) with a transaxial and axial resolution (FWHM) of 5.8 and 5 mm respectively, operating in 2D mode. All subjects were placed in supine position with dimmed lights and low ambient noise. Reconstruction was done using filtered backprojection with a Hanning filter with a (cut-off 0.5 cycles/cm). Sequential transmission scanning was performed using a germanium - 68 / gallium - 68 ring source. Correction for scatter was done using the standard software provided by the manufacturer (CTI). Subsequently, regional cerebral blood flow (rCBF) images were acquired by \([^{15}\text{O}]\text{CO}_2\) inhalation at 900 MBq/min. For this, the steady state technique using \([^{15}\text{O}]\text{CO}_2\) was applied (Frackowiak et al., 1980). Eight min later, a one-hour dynamic PET study was conducted following the injection of 370 MBq \([^{11}\text{C}]\text{PK11195}\). Nineteen frames each consisting of thirty-one planes of 3.375 mm thickness were recorded over 60 min with an increasing duration of 2 * 5 s, 5 * 10 s, 4 * 1 min, 2 * 3 min, 1 * 4 min, 1 * 5 min and 4 * 10 min. No partial volume correction was applied. Since a previous feasibility study showed that the time-activity curves for both volunteers and patients had a similar decline with a steady-state period from 40 min postinjection, data obtained from 40-60 min postinjection were summed (static scans). Automatic radioactive decay correction was applied to all images. Individual automatic coregistration of MR and perfusion PET data was achieved using SPM99 (Statistical Parametric Mapping, Functional Imaging Laboratory, Wellcome Department of Cognitive Neurology, Institute of Neurology, London, UK) (Friston et al., 1991). Spatial normalisation was done on the normal templates for T1-weighted MR in MNI space (Montreal Neurological Institute), using non-linear warping with 7x8x7 basic functions (5 iterations), and to a voxel size of 3x3x3 mm\(^3\). T2-, Gd- and coregistered PET data underwent the same transformation as the original T1-image. The same spatial transformation as for the perfusion PET studies was applied to all \([^{11}\text{C}]\text{PK11195}\) data. Five patients were excluded because of head movement and subsequent incorrect coregistration between the perfusion and receptor PET scan.
PATIENTS AND METHODS

Subjects
The study was approved by the Ethical Committee of the Ghent University Hospital and written informed consent was obtained from each subject. Seven healthy volunteers (3 males, mean age 33 ± 8, range 23-41 yrs) and 22 out of 27 initially randomly included clinical definite and laboratory proven MS patients referring to the Poser criteria (Poser et al., 1983) (mean age 43 ± 11, range 26-64 yrs) were finally included. Demographic and clinical parameters of the MS patients are described in table 1. Four MS patients were treated with IFN-ß at the time of inclusion. Thirteen patients had the relapsing-remitting type (RR) of the disease while 7 patients were secondary progressive (SP), having a continuous worsening of invalidity over a follow-up period of at least 6 months. Two patients had a primary progressive course (PP) without relapses from the very beginning of the disease. Six patients had a clinical relapse during the time of scanning, defined as the occurrence or worsening of neurological dysfunction lasting more than 24 hours. In 3 of them, corticosteroids needed to be administered after all imaging was performed. Ten patients showed Gd-enhancing lesions on the MR scan, 5 of them with a concomitant clinical relapse. One patient with the RR type of disease was scanned twice within a time interval of 4 months, the first imaging session at the time of a clinical relapse with Gd-lesions and the second session at the time of remission. The average disease duration was 9.3 ± 8.3, range 0.3-32.7 yrs. The average EDSS was 4.0 ± 2.0, range 1.0-7.0. To assess differences between moderate and severe impairment, patients were, for some analyses, subdivided into a low and high invalidity category (cut-off EDSS 4.0).

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Table 1 Clinical characteristics of the MS patients.
Volumetric interest and semiquantification
Since no generally accepted model of absolute quantification of $[^{11}]$C PK11195 specific binding in normal brain has been widely validated, and moreover, as no dynamic data for all volunteers were available and no arterial sampling was carried out, a straightforward semiquantitative approach was performed on the static scans. Volumes of interest (VOIs, on average 400 voxels ranging from 5 to more than 3000) were manually defined on the reoriented high-resolution MR scans in white and gray matter for volunteers and NAWM, gray matter, T2- and T1-Gd-lesions for MS patients and automatically transferred onto the reoriented $[^{11}]$C PK11195 scans by means of PMOD, a kinetic modeling and image fusion environment tool in JAVA (Zurich, Switzerland) (Mikolajczyk et al., 1998). When drawing VOIs, care was taken to avoid the carotid and major cerebral arteries as well as the ventricles and venous sinuses. For all controls, 52 VOIs were sampled in white matter and 264 in gray matter. For all MS patients, 153 VOIs were sampled in NAWM and 84 in gray matter. Gray matter was divided in cortical and central gray matter (thalamus and striatum) because higher binding of $[^{3}]$H PK11195 was described in diencephalic structures notably in several thalamic nuclei (Doble et al., 1987). The normalised specific uptake for a particular VOI was defined as the mean number of counts per volume unit in this VOI divided by the mean number of counts per volume unit in cortical gray matter. Since there was a significant age difference between patients and controls, the effect of age in controls for white matter, cortical and subcortical gray matter, and thalamic $[^{11}]$C PK11195 uptake values was studied.

Statistical analysis
Data were analysed by means of SPSS v10.0 for Windows (Chicago, IL, USA). For normality testing, a Kolmogorov-Smirnov test statistic was applied. For differences between groups, an independent samples t or a Mann-Whitney U test was applied when appropriate. A p-value lower than 0.05 was considered as significant while a value lower than 0.10 was considered as a meaningful trend. No Bonferroni correction was performed. Data are given as means ± one standard deviation.
RESULTS

[\textsuperscript{11}C]PK11195 uptake values for MS patients versus controls and effect of age

A [\textsuperscript{11}C]PK11195 uptake value of 100.3 ± 9.9 in white matter and 100.5 ± 9.4 in gray matter for controls was found. In patients, NAWM and gray matter uptake was respectively 101.7 ± 11.1 and 101.0 ± 9.4, both higher, however, not statistically significant different from white and gray matter in controls (p=0.3 and 0.7 respectively). No effect of age was found in controls for white, cortical and subcortical gray matter, or thalamic [\textsuperscript{11}C]PK11195 uptake values. In T2-lesions, the uptake tended to be lower compared to white matter in controls (97.3 ± 13.8, p=0.06). Regarding Gd-lesions, [\textsuperscript{11}C]PK11195 uptake was significantly higher (p=0.001) compared to normal white matter (110.9 ± 14.7). Figure 1A shows the raised [\textsuperscript{11}C]PK11195 uptake corresponding with a Gd-lesion on MRI.

![Figure 1: Illustration of the remote projected neuroinflammatory response in the wake of a primary lesion elsewhere. Gd-lesion located in the right periventricular parietal region in a patient with active relapsing-remitting MS with the corresponding focally increased uptake on the [\textsuperscript{11}C]PK11195 image (A). On a slightly higher level, the patient has a T2-weighted MR-lesion in the left periventricular parietal region corresponding with the concomitantly raised uptake on the [\textsuperscript{11}C]PK11195 image (B). On the same image, the increased uptake of the upper border of the Gd-lesion in the right parietal region is still visible. Note the uptake in the frontal meningeal area on both [\textsuperscript{11}C]PK11195 images, indicating the aspecific signal of [\textsuperscript{11}C]PK11195. Both [\textsuperscript{11}C]PK11195 images are superimposed on the corresponding proton density-weighted MR scan.](image)

Figure 2 shows the mean [\textsuperscript{11}C]PK11195 normalised uptake values for different types of MRI-lesions in MS patients.

![Figure 2: Boxplot of [\textsuperscript{11}C]PK11195 normalized uptake values in different types of MRI-lesions.](image)
$[^{11}C]PK11195$ uptake values for MRI-lesions subdivided according to the presence of a relapse (clinical or MR-relapse)

A higher $[^{11}C]PK11195$ uptake for T2-lesions was found in patients presenting at the time of scanning with an attack, defined as a clinical relapse or the presence of Gd-lesions on MRI ($100.7 \pm 14.2$ versus $93.7 \pm 12.5$; $p=0.01$, figure 3). Also, when subdivided according to the presence of Gd-lesions on MRI or a clinical relapse separately, $[^{11}C]PK11195$ uptake values were higher in both situations (respectively $p=0.05$ and $0.02$). Table 2 shows the T2-lesional $[^{11}C]PK11195$ uptake values for the distinct conditions. The single patient with the RR type of disease, which was scanned twice, had a mean T2-lesional uptake value of 130.3, which dropped to 100.9 four months later at the time of remission. Figure 1 shows the raised $[^{11}C]PK11195$ uptake corresponding with a T2-lesion in a patient with the RR type of disease during a clinical and MR-relapse.

![Boxplot of $[^{11}C]PK11195$ uptake values for all T2-lesions subdivided according to the presence of an attack (clinical or MR-relapse).](image)

$^{=}$ outlier value (cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range); * = extreme value (cases with values more than 3 box lengths from the upper or lower edge of the box)

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Table 2 $[^{11}C]PK11195$ uptake values for T2-lesions, subdivided according to the presence of a relapse (clinical or MR-relapse).

When the $[^{11}C]PK11195$ uptake for Gd-lesions was subdivided according to the coinciding presence of a clinical relapse, uptake was higher when a relapse was present ($104.4 \pm 17.0$ versus $115.4 \pm 11.3$; $p = 0.05$).
No significantly different NAWM $[^{11}C]$PK11195 uptake was found when a relapse was present.

**Correlation of lesional $[^{11}C]$PK11195 uptake values with disease duration and invalidity**

Concerning the relationship between NAWM $[^{11}C]$PK11195 uptake values and disease duration, a steady rising slope during the progression of the disease was found ($r=0.4$; $p=0.05$; figure 4). Without the outlier value (see figure 4), the positive correlation remained, however, statistically non-significant ($p>0.1$). Concerning the correlation between gray matter $[^{11}C]$PK11195 uptake and disease duration, subdivided according to high and low invalidity category patients, significance was reached for the high invalidity patients only ($r=0.6$; $p=0.05$). The correlations between $[^{11}C]$PK11195 uptake values for both T2-weighted lesions and Gd-lesions on one side and EDSS or disease duration on the other side did not reach statistical significance.

**Thalamic and NAWM $[^{11}C]$PK11195 uptake in PP MS patients**

For the two patients with the PP type of MS, a substantially higher uptake was found both in the thalamus (mean values respectively 109.9 for PP, 104.0 for RR and 104.3 for SP; p-value of 0.3 PP versus RR) and the NAWM (mean values for all lesions respectively 104.1 for PP, 99.8 for RR and 100.7 for SP; p-value of 0.1 PP versus RR).

![Graph of NAWM uptake value vs. disease duration](image)

**FIGURE 4**: Correlation of NAWM $[^{11}C]$PK11195 uptake and disease duration.
DISCUSSION

Radiolabeled PK11195 has been validated as a marker of microglial proliferation, with its ensuing increased PBR density, in ample neuropathological and animal experimental work involving lesions with or without BBB damage. Moreover, the full transformation of microglia into parenchymal phagocytes, absent in areas with chronic or subtle brain pathology, is not necessary to reach maximal levels of PK11195 binding (Banati et al., 1997; Benavides et al., 1988). An equal observation was made in a previous study in MS patients, where a high $^{11}$C]PK11195 uptake occurred in active white matter inflammation defined by MR, reflecting the presence of activated microglia (Vowinckel et al., 1997). The latter findings were confirmed in the present study where significantly increased $^{11}$C]PK11195 uptake values were found in patients with active focal inflammatory lesions defined by Gd-enhanced T1-MRI (figure 1 and 2). The increased uptake in Gd-lesions is in accordance with the known histology of these conventional MR lesions. Indeed, in focal Gd-enhanced T1-weighted MRI lesions, the BBB breakdown is related to an extensive inflammatory response including macrophages and T-cells (Nesbit et al., 1991).

Microglia occupy 5 to 12% of the total number of cells in the normal brain. Gray matter contains more microglia than white matter, especially in sensory, limbic and subcortical gray matter structures (Lawson et al., 1990). The latter was also demonstrated in a previous feasibility study where a higher mean gray matter $^{11}$C]PK11195 uptake was detected in seven controls (Debruynne et al., 2002). Although a larger group of MS patients of the PP type is needed to make a clearer statement, the higher thalamic $^{11}$C]PK11195 uptake found in the present study could be explained by a projected neuroinflammatory response secondary to descending corticothalamic tract lesions but, alternatively, also due ascending to spinal cord long tract lesions such as in myelopathetic syndromes, typically seen in this subgroup of MS patients (Andersson et al., 1999; Banati et al., 2000; Sorensen et al., 1996). This hypothesis of a remote neuroinflammatory response in the wake of primary lesions elsewhere along a neural pathway was already formulated by Banati et al. where an increased thalamic $^{11}$C]PK11195 uptake was found in a patient with a spinal cord MRI lesion, and also in a case of EAE where PBR expression was induced in the midbrain caused by an injured spinothalamic tract (Banati et al., 2000).

T2-lesions on MRI are known for their high sensitivity but low specificity with regard to histopathology. The $^{11}$C]PK11195 uptake for T2-weighted lesions in the present study was low (figure 2). As such, these findings may be related to a PBR downregulation of chronic, immunologically less triggered microglia due to the disappearance of myelin fibers, which are over time replaced by axonal scars (Johnson et al., 1986; Schlumpf et al., 1993; Trapp et al., 1998). Then, whenever a temporarily reactivation of the autoimmune process occurs, PBR expression will be higher compared to lesions in an existing steady state, as demonstrated in the present study where an overall global T2-lesional upgrading of $^{11}$C]PK11195 uptake was noticed when imaging was performed during a clinical or MR-relapse (figure 3). This upgrading of $^{11}$C]PK11195 uptake is illustrated in figure 1 and was also seen in the single RR patient who was scanned twice and showed a much larger mean T2-lesional $^{11}$C]PK11195 uptake value at the time of
relapse compared to the time of remission. These findings confirm earlier in vitro and in vivo observations showing activated microglia remote from the primary inflammatory site (Banati et al., 2000). As such, the molecular analogue of the natural disease course in which clinical and MR relapses are followed by periods of a relative stagnation is a waxing and waning phenomenon for the PBR expression in the T2-weighted lesions.

In the present study, a slightly higher value of NAWM \[^{11}\text{C}]\text{PK11195}\) uptake compared to white matter of controls was found, without any significantly different radioligand uptake when a relapse was present. This is also in agreement with histopathological studies, where an increased number of MHC class II microglia attributing to a loss of myelin extending far beyond visible detectable inflammatory foci was found (Hayes et al., 1987), as well as with autoradiographic studies both in EAE and human post-mortem tissue demonstrating regions of an increased number of non-phagocytic, still ramified, but activated microglia along NAWM tracts (Banati et al., 2000). Also, a tendency to a rising of the NAWM \[^{11}\text{C}]\text{PK11195}\) uptake during disease progression was found (figure 4). These findings are in line with the hypothesis that the demyelinating process is initiated by microglia from the very beginning of the disease, unrelated to BBB disruption and autoreactive T-cell function (Kesselring, 1990), the latter being involved in clinical and MR-relapses (Miller et al., 1995). As such, NAWM microglia constitutes a real burden of the disease, causing invisible demyelinisation independent from relapses. With this regard, Confavreux et al. recently concluded already that relapses do not significantly influence the progression of irreversible disability (Confavreux et al., 2000).

In conclusion, with \[^{11}\text{C}]\text{PK11195}\) PET as a marker of the immuno-pathophysiological process, we in vivo raise evidence to the hypothesis that microglia may play an important role in the immunopathogenesis of multiple sclerosis. For T1-Gadolinium-weighted MR lesions, the \[^{11}\text{C}]\text{PK11195}\) uptake was significantly increased. Concerning T2-weighted MR lesions, the microglial activity was decreased suggesting a PBR downregulation related to the gradual vanishing of myelin fibers towards the chronic degenerative phase. An increased uptake in these lesions was found in patients with a clinical relapse or the presence of remote Gd-lesions, indicating a dynamic process with a transient upregulation following the natural course of the disease. In NAWM, the increase of \[^{11}\text{C}]\text{PK11195}\) uptake with disease duration is in line with the theory proposing NAWM microglia as a real burden of the disease, causing invisible demyelinisation independent from relapses.
REFERENCES


Validation of radiolabelled PK11195 as an inflammatory tracer in Multiple Sclerosis
IMAGING OF MICROGLIAL ACTIVATION WITH PET AND ATROPHY WITH MRI IN MULTIPLE SCLEROSIS: INTERRELATIONSHIP AND CLINICAL CORRELATES

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ABSTRACT

Objective: The objectives of the present study were to assess brain atrophy in MS patients during different disease stages and to investigate by PET and [¹¹C]PK11195, a marker of microglial activation, the relationship between inflammation, atrophy, and clinical relevant measures.

Methods: Eight healthy subjects and 22 MS patients were included. Semiquantitative [¹¹C]PK11195 uptake values, with normalization on cortical gray matter, were measured for magnetic resonance imaging T₂⁻ and T₁-lesions and normal appearing white matter (NAWM). As atrophy index we used the ratio of the amount of white and gray matter divided by the ventricular size, using an optimized a priori based segmentation algorithm (SPM99).

Results: Atrophy was significantly greater in MS patients compared to age-matched controls. A significant correlation was found between brain atrophy and both disease duration and disability, as measured with the expanded disability status scale (EDSS). For NAWM, [¹¹C]PK11195 uptake increased with the amount of atrophy, while T₂-lesional [¹¹C]PK11195 uptake values decreased according to increasing brain atrophy.

Conclusions: Brain atrophy, correlating with disease duration and disability, is directly related to NAWM and T₂-lesional inflammation as measured by microglial activation.
INTRODUCTION

In MS, neuroinflammation is the pivotal event that causes demyelination and oligodendrocyte cell death [1]. Whereas over the past decade most attention was focused on the theory of a T-cell mediated autoimmune process [2], in vitro studies however showed that microglia also can be activated by various stimuli resulting in the expression of proinflammatory cytokines, matrix metalloproteinases, and free radicals [3]. In addition, activated microglia serve as the major antigen-presenting cell in the CNS, likely contributing to aberrant immune reactivity at this site. Accordingly, it has been hypothesized that demyelination may, at least partly, be the result of microglial activation leading to the release of myelinotoxic factors that directly injure the oligodendrocyte-myelin unit [4]. As a consequence, it is believed that microglia play a prominent role in autoimmune mediated demyelinating disorders of the central nervous system.

One of the characteristic features of microglia is their rapid activation in response to brain injury with a concomitant higher expression of the peripheral benzodiazepine receptor (PBR) [5]. Visualization of the PBR can be performed by means of radiolabeled PK11195. This specific high-affinity ligand, allows as such the assessment of microglial activation giving more specific in vivo information concerning the underlying histopathological features due to neuroinflammation in general and to MS in particular [6]. PK11195 has been labeled with $^{11}$C for PET and applied in various neuroinflammatory conditions like Rasmussen’s encephalitis [7], cerebral vasculitis [8], and Herpes simplex encephalitis [9].

Previously, we demonstrated characteristic $[^{11}\text{C}]$PK11195 uptake patterns for chronic and active white matter lesions and normal appearing white matter (NAWM) in subgroups of MS patients, exemplifying an additional value of $[^{11}\text{C}]$PK11195 imaging over MRI concerning the assessment and monitoring of the immuno-pathophysiological process in MS. Moreover, a significant steady rising amount of NAWM $[^{11}\text{C}]$PK11195 uptake during disease progression was found, propagating NAWM as a major burden of the disease [10]. Concordingly with the hypothesis that microglia can directly induce myelin damage through the release of proinflammatory mediators [11], this study suggested that during the disease process invisible demyelination is an increasingly ongoing NAWM process.

On the other hand, atrophy of the brain and spinal cord at postmortem examination is considered to be a hallmark of irreversible CNS damage. Indeed, MS involves substantial loss of both axonal density and volume, in addition to the well-recognized loss of myelin within lesions [12,13]. Whereas in healthy controls, the progressive loss of brain parenchyma occurs with magnitudes ranging from 0.1 to 0.3 % per year, for MS patients the reduction in brain volume is estimated at 0.4 to 5 % per year where most published patient cohorts narrow this annual loss range to 0.6 to 1 % per year [13-15], starting already in the early stage of the disease, and being associated with short-term clinical disease activity [16,17]. Although the pathologic basis of MS-related atrophy is still unclear, there seems to be a connection with the NAWM inflammatory process. As such, a rather global process, such as (MRI-)invisible demyelination with consequently a steady axonal loss might underlie this brain atrophy [18]. This was
also demonstrated by Trapp et al. in a postmortem study showing axonal transection as a consistent feature in NAWM [19]. Newer MR techniques, such as magnetization transfer imaging and magnetization resonance spectroscopy have confirmed this loss of brain parenchyma in the NAWM as being a major burden of disease contributing to whole brain atrophy [20,21].

The objectives of the present study were threefold: firstly, the assessment of brain atrophy in MS patients compared to age-matched normal controls, secondly, the correlation of brain atrophy with clinical measures, and thirdly, the correlation of $[^{11}C]PK11195$ uptake with brain atrophy measures.
PATIENTS AND METHODS

Subjects
The study was approved by the Ethical Committee of the Ghent University Hospital and written informed consent was obtained from each subject. Eight controls (3 men, five women; mean age 37.2 ± 13, range 23-65 y) and 27 clinical definite and laboratory proven MS patients referring to the criteria of the International Panel on the Diagnosis of Multiple Sclerosis were randomly included [22]. Five patients had to be removed from the study because of head movement and subsequent coregistration errors between perfusion and receptor PET scanning. Finally, 22 patients were eligible (9 men, 13 women; mean age 42.6 ± 11, range 26-64 y). There was no statistically significant age difference between patients and controls (p = 0.3). Demographic and clinical parameters of the MS patients are described in table 1. Four MS patients were treated with IFN-β at the time of inclusion. Thirteen patients had the relapsing-remitting type (RR) of the disease while 7 patients were secondary progressive (SP), having a continuous worsening of invalidity over a follow-up period of at least 6 months. Two patients had a primary progressive course (PP) without relapses from the very beginning of the disease. Six patients had a clinical relapse at the time of scanning, defined as the occurrence or worsening of neurological dysfunction lasting more than 24 hours. In 3 of them, corticosteroids needed to be administered after all imaging was performed. Ten patients showed Gd-enhancing lesions on the MRI scan, 5 of them with a concomitant clinical relapse. The average disease duration was 9.3 ± 8.3 y (range 0.3-33 y). The average expanded disability status scale (EDSS) was 4.0 ± 2.0 (range 1.0-7.0).

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Type</th>
<th>disease duration (y)</th>
<th>EDSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>M</td>
<td>RR</td>
<td>2.3</td>
<td>3.0</td>
</tr>
<tr>
<td>37</td>
<td>F</td>
<td>RR</td>
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<td>1.5</td>
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<td>RR</td>
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<td>2.5</td>
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<tr>
<td>64</td>
<td>F</td>
<td>PP</td>
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<td>7.0</td>
</tr>
<tr>
<td>45</td>
<td>F</td>
<td>PP</td>
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<tr>
<td>32</td>
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<td>RR</td>
<td>3.1</td>
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</tr>
<tr>
<td>45</td>
<td>F</td>
<td>RR</td>
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</tr>
<tr>
<td>27</td>
<td>M</td>
<td>RR</td>
<td>3.8</td>
<td>1.0</td>
</tr>
<tr>
<td>49</td>
<td>M</td>
<td>SP</td>
<td>8.8</td>
<td>7.0</td>
</tr>
<tr>
<td>50</td>
<td>F</td>
<td>SP</td>
<td>8.7</td>
<td>5.5</td>
</tr>
<tr>
<td>38</td>
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<td>RR</td>
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<td>1.5</td>
</tr>
<tr>
<td>51</td>
<td>F</td>
<td>SP</td>
<td>23.7</td>
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<td>36</td>
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</tr>
<tr>
<td>31</td>
<td>M</td>
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<tr>
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<td>RR</td>
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<td>4.5</td>
</tr>
<tr>
<td>60</td>
<td>M</td>
<td>SP</td>
<td>32.7</td>
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<td>SP</td>
<td>7.6</td>
<td>4.5</td>
</tr>
<tr>
<td>26</td>
<td>M</td>
<td>RR</td>
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<td>1.5</td>
</tr>
<tr>
<td>45</td>
<td>F</td>
<td>SP</td>
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</tr>
<tr>
<td>42</td>
<td>M</td>
<td>RR</td>
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<td>1.0</td>
</tr>
<tr>
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<td>M</td>
<td>RR</td>
<td>6.9</td>
<td>5.5</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>RR</td>
<td>3.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

RR = relapsing-remitting; SP = secondary progressive; PP = primary progressive; EDSS = expanded disability status scale

Table 1 Clinical characteristics of the MS patients
Radiochemistry
PK11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide] was obtained from RBI (Natick, MA, USA). $^{[11}C]$PK11195 was synthesized according to a procedure described by Camsonne et al [23]. Briefly, 3 µmol N-desmethyl PK11195 was dissolved in 150 µL dimethylsulphoxide, containing 3 µmol tetrabutylammonium hydroxide. After trapping of $^{[11}C]$CH$_3$I, the vial was closed and heated at 80°C for 3 min. Purification was done by HPLC using a RP-C18 column (Econsil, 250 mm x 10 mm, 10 µm particle size) and an ethanol/water (70/30) mixture as mobile phase. Radiochemical yield towards $^{[11}C]$CH$_3$I was 57% (confidence interval 2 %, 30 experiments). Finally, 3.7 GBq $^{[11}C]$PK11195 was obtained with a specific activity of 25 GBq/µmol. Chemical and radiochemical purity were higher than 99%. All subjects were injected intravenously with $370 \pm 10 \%$ MBq $^{[11}C]$PK11195 with a slow bolus in a time course of 30 seconds.

Data acquisition and processing
All subjects underwent MR and PET scanning on the same day. MR imaging was performed on a 1.5 T MR scanner (Siemens, Magnetom SP4000; Erlangen, Germany). Prior to the administration of gadolinium, standard spin-echo imaging was carried out in 5 mm thick axial planes (pixel size of 0.9 x 0.9 mm$^2$) with proton density- (TR/TE/NEX=2170/20/1), $T_2$- (TR/TE/NEX=2170/80/1) and $T_1$-weighted (TR/TE/NEX=600/12/1) contrast. Five minutes after gadolinium injection, the axial slices were rescanned with $T_1$-weighting (TR/TE/NEX=800/20/1).

PET studies were performed on a Siemens ECAT 951/31 PET scanner (Siemens, Knoxville, TN, USA) with a transaxial and axial resolution (FWHM) of 5.8 and 5 mm respectively (values provided by Siemens, operating in 2D mode). All subjects were placed in supine position with dimmed lights and low ambient noise. Realignment of the head to the orbitomeatal line was performed by laser guidance. Reconstruction was done using filtered backprojection with a Hanning filter (cut-off of 0.5 cycles/cm). Sequential transmission scanning was performed using a $^{68}$Ge/$^{68}$Ga ring source. Correction for scatter was done using the standard software provided by the manufacturer.

Volumes of interest and $^{[11}C]$PK11195 uptake semiquantification
Since no generally accepted model of absolute quantification of $^{[11}C]$PK11195 specific binding in normal brain has been widely validated and no dynamic data for all volunteers were obtained neither was arterial sampling carried out, a validated semiquantitative approach was carried out on the static images obtained during the steady-state period of the $^{[11}C]$PK11195 brain uptake [25]. Volumes of interest (VOIs) were defined on the individual’s spatially standardized high-resolution MR scan in white and gray matter for volunteers and in NAWM, gray matter, $T_2$- and $T_1$-
Gd-lesions for MS patients and automatically transferred onto the coregistered \[^{11}C\]PK11195 scan by means of PMOD (University Hospital Zurich, Switzerland), a kinetic modeling and image fusion environment tool in Java [27]. When drawing VOIs, care was taken to avoid the carotid and major cerebral arteries as well as the ventricles or venous sinuses. Three global regions were defined namely white matter, cortical and central gray matter (thalamus and striatum, this was done because high labeling of \[^{3}H\]PK11195 was described in diencephalic structures notably in several thalamic nuclei [28]). The normalized specific uptake for a particular area of interest was defined as the mean activity per volume unit in this area divided by the mean activity per volume unit in cortical gray matter.

**Atrophy measures**

Relative parenchymal fractions were assessed with the whole-brain probabilistic segmentation and volumetry module incorporated within SPM99. T1-weighted images were used. Since relative brain atrophy measures have shown to be superior to absolute measures for cross-sectional studies [29], images were first normalized to the MNI template using the default SPM spatial pre-processing method (normalization with bilinear interpolation, 7×8×7 nonlinear basic functions, 12 iterations and medium nonlinear regularization). Then, images were segmented, without lesion extraction using the ‘lots of inhomogeneity correction’ option into gray and white matter and CSF maps for which the respective volume was determined as the proportion of voxels, worked out from a subregion of the volume that encloses the brain [30]. Finally, the brain atrophy index was defined as the relative CSF volume divided by the relative white and gray matter volume. Figure one shows an image of a segmented axial MR slice of a 37-year old MS patient (panel A) and a 39-year old healthy control subject (panel B).

**Statistical analysis**

Data were analyzed by means of SPSS v10.0 for Windows (Chicago, IL, USA). For normality testing, a Kolmogorov-Smirnov test statistic was applied. For differences between groups, an independent samples T test or a Mann-Whitney U test was applied whereas for the correlational analysis, a Pearson or Spearman correlation coefficient was calculated, when appropriate. A p value lower than or equal to 0.05 was considered as significant. Data are given as means ± one standard deviation.

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Figure 1. Example of a segmented axial MR slice into gray and white matter and cerebrospinal fluid for a 37-year old MS patient (panel A) and a 39-year old healthy control (panel B), after anatomical standardization
RESULTS

Atrophy measures for MS patients versus age-matched controls

Table 2 shows the atrophy measures for the different brain compartments. Atrophy as measured by the atrophy index was significantly greater in MS patients compared to age-matched controls \((p = 0.04)\). When considering the brain compartments separately, the relative amount of gray matter, (normal appearing) white matter and CSF were all indicative of the loss of brain tissue for MS patients, however, only the difference in ventricular size (CSF) was statistically significant \((p = 0.04)\). Whereas for controls a significant correlation was found between age and the amount of gray matter, CSF and the atrophy index \((p = 0.009, p = 0.03\) and \(p = 0.02\) respectively), no statistically significant correlation was found between age and any atrophy measure for patients.

<table>
<thead>
<tr>
<th></th>
<th>MS patients</th>
<th>age-matched controls</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>gray matter</td>
<td>0.183 ± 0.011</td>
<td>0.186 ± 0.012</td>
<td>0.6</td>
</tr>
<tr>
<td>(normal appearing) white matter</td>
<td>0.102 ± 0.009</td>
<td>0.106 ± 0.007</td>
<td>0.3</td>
</tr>
<tr>
<td>CSF</td>
<td>0.089 ± 0.012</td>
<td>0.080 ± 0.007</td>
<td>0.04</td>
</tr>
<tr>
<td>CSF</td>
<td>0.314 ± 0.047</td>
<td>0.275 ± 0.034</td>
<td>0.04</td>
</tr>
<tr>
<td>gray + white</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Atrophy comparison between MS patients and age-matched controls

Correlation of the atrophy index with disease duration and disability

As shown in figure two, there was a statistically significant correlation between disease duration and brain atrophy \((r = 0.5; p = 0.03)\). The correlation between atrophy and disability as measured by the EDSS yielded also statistical significance \((r = 0.4, p = 0.05; \text{figure three})\).

Correlation of lesional \([^{11}\text{C}]\)PK11195 uptake values with atrophy

Figure four demonstrates the relation of NAWM \([^{11}\text{C}]\)PK11195 uptake with atrophy, indicating that the amount of NAWM microglial activation as measured with \([^{11}\text{C}]\)PK11195 PET scanning is statistically
significant correlated to the amount of atrophy ($r = 0.2; p = 0.03$). It was also shown that the $[^{11}]$C]PK11195 uptake for T2-lesions decreases significantly with increasing brain atrophy ($r = -0.3, p = 0.03$; Figure five). There was no relation between $[^{11}]$C]PK11195 uptake in gadolinium-enhancing lesions and brain atrophy ($r = -0.01; p = 0.9$).

![Figure 4](image1.png)

**Figure 4.** Scatterplot of the NAWM $[^{11}]$C]PK11195 uptake value with atrophy as measured by the atrophy index

![Figure 5](image2.png)

**Figure 5.** Scatterplot of the T2-lesional $[^{11}]$C]PK11195 uptake value with atrophy as measured by the atrophy index
DISCUSSION

Recent MRI studies in MS have emphasized that brain atrophy is a robust and pivotal marker of disease progression, reflecting the destructive processes as a result of extensive axonal damage [15,16]. However, the assessment of cerebral atrophy by MRI is difficult due to general factors such as (small) inter-individual variations in head size and intracranial volume, inconsistencies in the acquisition sequence like differences in the measured voxel size or slice thickness, movement artifacts, magnetic field inhomogeneities and variations in MR image intensity scales. In addition, also disease-specific aspects like fluctuations of tissue water content such as in vasogenic edema coupled to active lesions or the administration of ‘anti-inflammatory’ treatment are potential sources of error [31,32]. Atrophy has been measured with MRI both by manual techniques as well as by semi-automated contour or thresholding segmentation techniques. Actual atrophy measures ranged from linear atrophy measures like the intercaudate distance, whole brain or ventricular width to absolute or relative volumes of ventricles, whole brain, hemisphere or some adherent slices, corpus callosum, or more specific brain structures like the cerebellum or brainstem [16,33-35].

Ideally however, the measurement technique should be fully reproducible, automated, accurate and very sensitive. In the present study the statistical parametric mapping software package (SPM99) was used for the assessment of brain atrophy which is a relatively robust method and requires no manual intervention [36]. SPM software uses prior spatial information from a database of normal brain images to classify voxels, according to their location and signal intensity characteristics, as gray matter, white matter or CSF and can be applied to both 2D or 3D images. This method was recently introduced by Chard et al. for the measurement of brain parenchymal, white and gray matter fractions in early relapsing-remitting MS patients making use of three-dimensional fast spoiled gradient recall MR scans with a slice thickness of 1.5 mm [37].

Measures of brain atrophy have been found to correlate to varying degrees with intellectual and memory dysfunction, dementia, and scores for neuropsychological tests [38,39]. Concerning the relationship between atrophy and disability, several conflicting results have been reported, most likely also enhanced by varying atrophy measures throughout different studies. The present study found a significant correlation between atrophy and disability, as measured by the EDSS, which is in agreement with most previously reported findings [40-47]. However, Wilson et al found no correlation between cerebral volume and clinical disability [48] while Ge et al as well found no significant correlation between fractional white nor gray matter volume and disability in a group of 27 RR MS patients [49].

In a previous study, we demonstrated a slightly higher NAWM \(^{11}\)C\(\text{PK11195}\) uptake compared to normal white matter where the NAWM \(^{11}\)C\(\text{PK11195}\) uptake showed a steady rising slope with disease progression indicating the growing amount of activated microglia, undetectable for MRI in this brain area [10]. The present study demonstrates the weak, however significant correlation between this rising NAWM \(^{11}\)C\(\text{PK11195}\) uptake during disease progression and the decrease in brain parenchymal volume.
It has to be noted however that this study was not a longitudinal study but rather a snapshot study of patients with a different disease duration, making it rather difficult to draw formal conclusions about disease progression and as such necessitating extrapolations. Yet, this is the first study to reveal in vivo the direct relationship between NAWM inflammation or microglial activation as measured with radiolabeled PK11195 on one hand and atrophy on the other hand and demonstrates that abnormalities in the NAWM are one factor, if not one of the most important, contributing to neurological impairment [50,51]. The mechanism of neuroinflammation leading to atrophy is most probably mediated by a steady ongoing NAWM process of invisible demyelination by activated microglia. Indeed, a study by Yin et al. with myelin-associated glycoprotein (MAG) deficient mice demonstrated already this relationship between demyelination and atrophy where the absence of MAG lead to chronic atrophy of myelinated axons and axonal degeneration [52]. This process of (hypo)myelination seems pivotal in the evolution of brain atrophy and is maintained by the lack of neurotrophic factors like MAG in the myelin sheat which are important for the protection of the cytoplasmic collar and the periaxonal space [53].

The present study did not reveal any correlation between T_1-Gadolinium-enhanced lesional [^{11}C]PK11195 uptake and brain atrophy. This is in concordance with Saindane et al. where the relationship between the Gadolinium-enhancing T_1 lesion load and the development of whole brain atrophy over a 2-years period was investigated and no correlation was found [18]. This suggests that the breakdown in the blood-brain barrier may be considered as an epiphenomenon in the process of brain atrophy giving rise to localized inflammation with reversible edema and some degree of focal axonal damage. Moreover, both magnetization transfer and magnetic resonance spectroscopy studies established that a rather global process underlies atrophy in MS [18,54,55], which was confirmed in the present study indicating the NAWM as a substantial contributor to brain atrophy.

The results of this study implicate the role of activated microglia in the overall demyelinating process especially in NAWM and emphasize the important long-term relationship between neuroinflammation and brain atrophy, the latter evolving by mechanisms that are at least partly independent of those mechanisms responsible for MRI lesions.
REFERENCES


