METAL BINDING TO TRANSFERRIN AND IMMUNE REACTIONS IN PARKINSON'S DISEASE

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The binding of iron (^{59}Fe) and gallium (^{67}Ga) to the plasma protein transferrin (Tf) was investigated by G75 gel filtration chromatography in control patients and treated and untreated patients with Parkinson's disease (PD). Fe-Tf binding was 100% in all controls and PD patients suggesting that a defect in Fe-Tf binding was not involved in the aetiology of PD. Ga-Tf binding was significantly reduced in both untreated and treated PD patients compared to controls. In addition, treated PD patients had significantly higher Ga-Tf binding than untreated patients. A reduction in metal binding to Tf could result in the increase of a low molecular weight species which may more readily enter the CNS. Alternatively, it could lead to a decrease in the transport of essential metals into the brain via the Tf receptor system.

A significant elevation in neopterin was demonstrated within the plasma of untreated PD patients compared to controls suggesting the activation of a cellular immune response. Furthermore, plasma neopterin was lower in treated compared to untreated PD patients, although the difference was not significant. There was no evidence for the activation of the humoral immune response in untreated or treated PD patients as measured by circulating immune complex (CIC) levels within the plasma.

An inverse relationship between Ga-Tf binding and neopterin was observed in untreated PD patients. The addition of oxidants in the form of potassium permanganate and activated manganese dioxide reduced Ga-Tf binding in control plasma. However, relatively little response was observed using monocyte preparations. The results suggest that oxidants produced by activation of the cellular immune system could damage the Tf molecule thereby reducing its ability to bind metals.

Key Words : Parkinson's disease; Transferrin binding; Neopterin; Circulating immune complexes; Immune activation.

DEDICATION

I would like to dedicate this thesis to my parents who have provided endless support and encouragement throughout my education. I would also like to thank Paul for his love and understanding throughout the last three years.

ACKNOWLEDGEMENTS

I am indebted to Dr Richard Armstrong, I could not have hoped for a better supervisor. I would also like to thank Professor John Blair who was always available for guidance and advice.

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CHAPTER 1: INTRODUCTION TO PARKINSON'S DISEASE

1.1 INTRODUCTION

Parkinson's disease (PD) is a movement disorder believed to be caused by the degeneration of the extrapyramidal nervous system. The disease was first described by the physician James Parkinson (1817) in a publication entitled an "Essay on the Shaking Palsy". Parkinson originally named the disease "Paralysis Agitans" but later the term Parkinson's disease was suggested by the French neurologist Charcot (1892) as a more appropriate name.

The incidence of PD increases with age, the average age of onset being 58-62 years (Marttila 1987). The disease develops slowly in most patients with a mean duration of 8-10 years increasing to 13-14 years following the introduction of levodopa therapy (Marttila and Rinne 1991).

The prevalance of PD in north America has been estimated at approximately 300 per 100 000 of the population (Rajput 1992), although within the white population values between 66-187 per 100 000 (Marttila 1983) have been reported. Rates may be lower in China (Wang 1991) and amongst the black population (McKeigue and Marmot 1990). There is an increase in prevalence with age, the peak prevalence (300-1800 per 100 000) occuring in the age range 70-79 years (Martilla 1983). Prevalance has been reported to be the same for both men and women (Schoenberg *et al* 1985, Rajput 1984).

Several studies have reported an increased mortality rate in PD patients compared with age matched controls (Joseph *et al* 1978, Rajput 1984, Uitti *et al* 1993). A study by Hoehn and Yahr (1967) found that before the introduction of levodopa, mortality rate was 2.9 times higher in PD sufferers compared to age and sex matched controls resulting in an average reduction in life expectancy of 6 years. Levodopa therapy has been reported to reduce excess mortality in PD (Joseph *et al* 1978, Rajput 1984, Uitti *et al* 1993). However, the extent of this reduction depends first, on the duration of therapy as mortality rates rise after

prolonged treatment (Hardie *et al* 1984) and second, on the severity of the disease when levodopa therapy begins (Martilla *et al* 1977).

1.2 SYMPTOMS OF PARKINSON'S DISEASE

PD is characterised by a number of symptoms which vary in individual patients and are dependent upon the severity of the disease. The three cardinal symptoms are tremor, rigidity and impairment of motor function. However, not all symptoms are present in every case. Patients may be classified into classical PD (all 3 symptoms are expressed), tremor dominant PD (mild akinesia and rigidity) and akinetic-rigid PD (little or no tremor) (Gerstenbrand and Poewe 1990). Initially, symptoms may be unilateral and confined to the upper body but as the disorder worsens the rest of the body may become involved (Shimomura and Headley 1988).

As a rule, the first symptom to be recognised is tremor, a rhythmic alternating contraction and relaxation occuring in opposing muscle groups. Tremor is not specific to PD and may be seen in a number of other conditions including hyperthyroidism, essential tremor, multiple sclerosis and cerebellar damage (Walton 1985). However, Parkinsonian tremor can generally be distinguished as it occurs at a relatively slow frequency (4-8 Hz) and is seen at rest, although it may persist during movement in severe cases (Yahr 1989). In the hands, tremor results in a characteristic "pill-rolling" movement involving the thumb and forefinger. Tremor may then spread to the wrist, elbow and the lower limbs. At a later stage, both sides of the body may be affected. In addition, tremor may occur in the mandibular muscles, tongue and very occasionally in the head (Selby 1990). Parkinsonian tremor may take the form of a rotatory movement or, as in the case of the wrist and ankle tremor, a flexion and extension (Walton 1985). Tremor can be a highly visible and embarrasing symptom of the condition which is worsened by emotional stress and anxiety.

Rigidity is partially responsible for the mask-like face and impairment of movement seen in PD. It can be defined as a resistance to passive movements and occurs in opposing muscle groups. Rigidity can be detected by grasping the arm at the elbow and rotating the shoulder or by alternately flexing and extending the wrist (Walshe 1958). In PD, two forms

may occur. Firstly, lead pipe rigidity which is smooth and uniform throughout the range of movement and secondly, cog wheel rigidity where the muscles yield in a series of jerks as the increased muscle tone is interrupted by tremor (Selby 1990).

In addition to tremor and rigidity, an impairment of motor performance is encountered in PD. This can take three forms depending upon severity:- 1) hypokinesia, a decreased range of motion, 2) bradykinesia, slowness during a movement and 3) akinesia, a lack of spontaneous movement (Selby 1990). A number of typical symptoms of PD can be attributed to these disorder of movement (for a review see Yahr 1989). Initially, movements become slow and restricted in range. The small muscle groups are particularly affected resulting in a loss of dexterity. As the disease worsens, the patient may experience difficulty carrying out daily tasks such as washing, dressing and rising from a chair. Changes in handwriting are often an early sign and include micrographia, a condition in which the patients writing becomes progressively smaller. Bradykinesia and akinesia lead to a "poverty of movement" including the lack of facial expressions and reduction in blinking which result in the mask-like face often seen in early cases. Furthermore, a reduction in fidgeting, a lack of postural adjustments and a loss of associated movement, for example swinging of the arms when walking, may be seen. The muscles used for speech may be affected and voice disturbances occur which are characterised by a reduction in amplitude, lack of pronunciation and variation in pitch. Dribbling from the mouth may occur due to a reduction in the automatic swallowing of saliva. Motor disturbances are also responsible for the short stepped, shuffling gait characteristic of PD. In severe cases, akinesia may cause the patient to "freeze" particularly if there is an obstruction to forward progress.

Disorders of posture and balance occur at a later stage of the disease (Selby 1990). Typically, the head is bent forward and the trunk bowed. The upper limbs are flexed at the elbows and the arms adducted so that the hands are held in front of the body. The hand itself may be affected, the metacarpophalangeal joints are flexed and the interphalangeal joints are hyperextended resulting in"striatal hand". There may also be flexion at the knees, soles of the feet and toes with a tendency for the big toe to be hyper-extended. Disorders of

balance include propulsion and retropulsion where the patient is unable to stop if pushed forwards or backwards. An effect of propulsion is the typical "festinating" gait in which the patient is compelled to accelerate with small steps as if he were chasing his own centre of gravity. In addition, there is an impaired righting reflex and the sufferer may have difficulty correcting a trip resulting in frequent falls.

Autonomic dysfunction is a more uncommon symptom of PD. Such effects may include constipation, thermal paresthesias, postural hypotension, urine retention and excessive sweating (Yahr 1989).

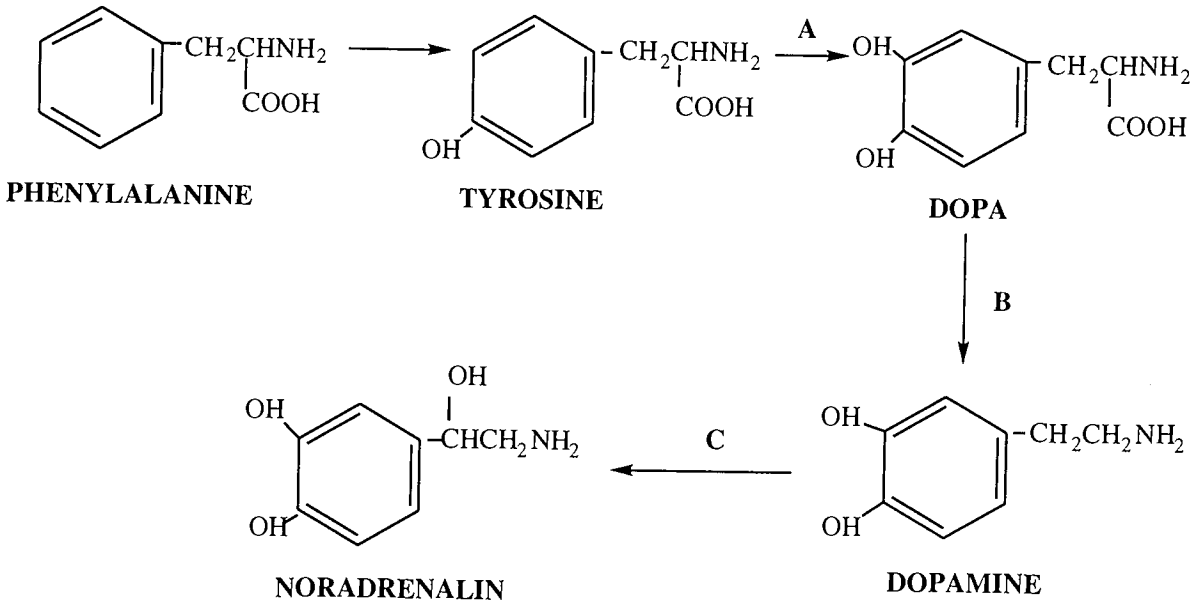
It is now accepted that the mental state of the patient is affected in PD. Initially, there may be a mild decline in memory, attention span and cognitive function. In addition, depression has been found to occur more commonly in PD patients than controls (Gotham *et al* 1986), although it has been suggested that this may be a reaction to the illness rather than a separate physical symptom. Dementia in PD is well documented and occurs at a later stage of the disease. The incidence of dementia in PD patients has been found to be significantly higher than in age matched controls (Rajput *et al* 1984, El-Awar *et al* 1987). However, estimated incidence rates vary from 10% to 81% (Gerstenbrand and Poewe 1990). It is possible that dementia is common in a distinct subset of patients in which the disease process is more rapid, and the patient less responsive to levodopa (Lieberman *et al* 1979). It has also been suggested that demented PD patients actually represent a condition which combines the features of Lewy body disease and Alzheimer's disease (Hakim and Mathieson 1979).

1.3 DOPAMINE

The major symptoms of PD can be attributed to a degeneration of the nigrostriatal pathway which uses dopamine as a neurotransmitter. Dopamine and noradrenalin are catecholamines and consist of a catechol nucleus (a benzene ring plus two hydroxyl groups) and an amino group.

1.3.1 SYNTHESIS OF DOPAMINE

Dopamine and noradrenalin are produced by the same synthetic pathway (figure 1.1) from phenylalanine and tyrosine, both of which are obtainable from the diet (Bradford 1986). Dopaminergic neurons have only the first two enzymes of this pathway ensuring that dopamine is the end product. Neurones that use noradrenalin as a neurotransmitter have an additional enzyme called dopamine-B-hydroxylase which converts dopamine to noradrenalin. The rate limiting step in this pathway is tyrosine hydroxylase and its concentration will determine how much dopamine or noradrenalin is produced.



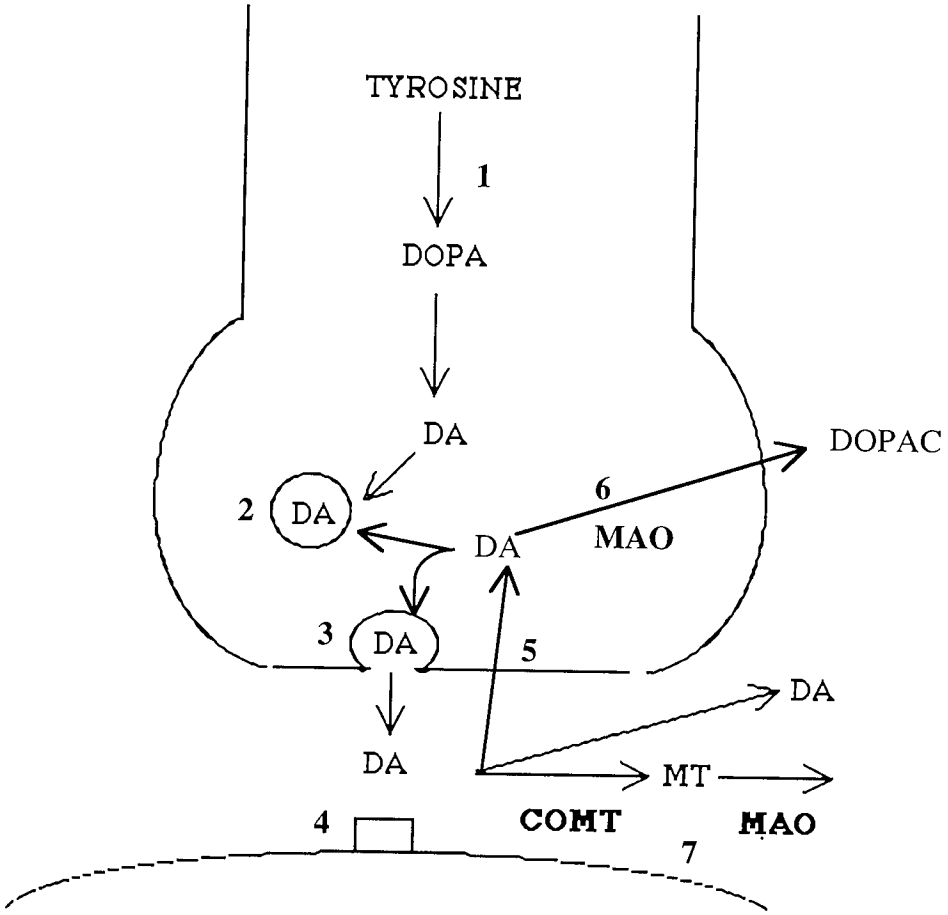
A= Tyrosine hydroxylase
B= L-Aromatic acid decarboxylase
C= Dopamineβ Hydroxylase

Figure 1.1 Synthesis of dopamine.
Adapted from Bradford (1986).

1.3.2 STORAGE AND RELEASE OF DOPAMINE

The structure of the dopamine synapse is shown in figure 1.2. Tyrosine is transported down the axon to the nerve terminal where it is converted into dopamine. The neurotransmitter is synthesised and stored in vesicles within the axon terminal. An action potential, proceeded by an influx of calcium into the terminal, causes the release of dopamine into the synaptic cleft. The dopamine then diffuses across the cleft and attaches

to dopamine receptors on the postsynaptic membrane. This causes a change in the postsynaptic membrane via the activation of a second messanger system thus generating an action potential in the postsynaptic neuron.



1. Synthesis of dopamine
2. Storage of dopamine
3. Release of dopamine
4. Interaction with receptor
5. Re-uptake of dopamine
6. Degredation of dopamine by MAO to form Dihydroxyphenylacetic acid (DOPAC)
7. Inactivation of dopamine by COMT, with further degredation of the product by MAO

Figure 1.2 Dopamine synapse.

Adapted from Thompson (1993).

1.3.3 METABOLISM OF DOPAMINE

Two enzymes are involved in the inactivation of dopamine namely monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). Inactivation occurs mainly via re-uptake into the surrounding neurons and glial cells. MAO is located within the

mitochondria of both neurons and glial cells. It metabolises dopamine resulting from re-uptake or leakage from the storage vescicles. In addition, the enzyme COMT brings about the methylation of dopamine to produce inactive compounds that can enter the blood stream and be excreted. The products of methylation may also be metabolised by MAO. Although COMT is found in the cytoplasm of neurons and glia, extraneuronal COMT is thought to be the major site for the inactivation of dopamine released from the nerve terminal. The metabolites of dopamine are shown in figure 1.3.

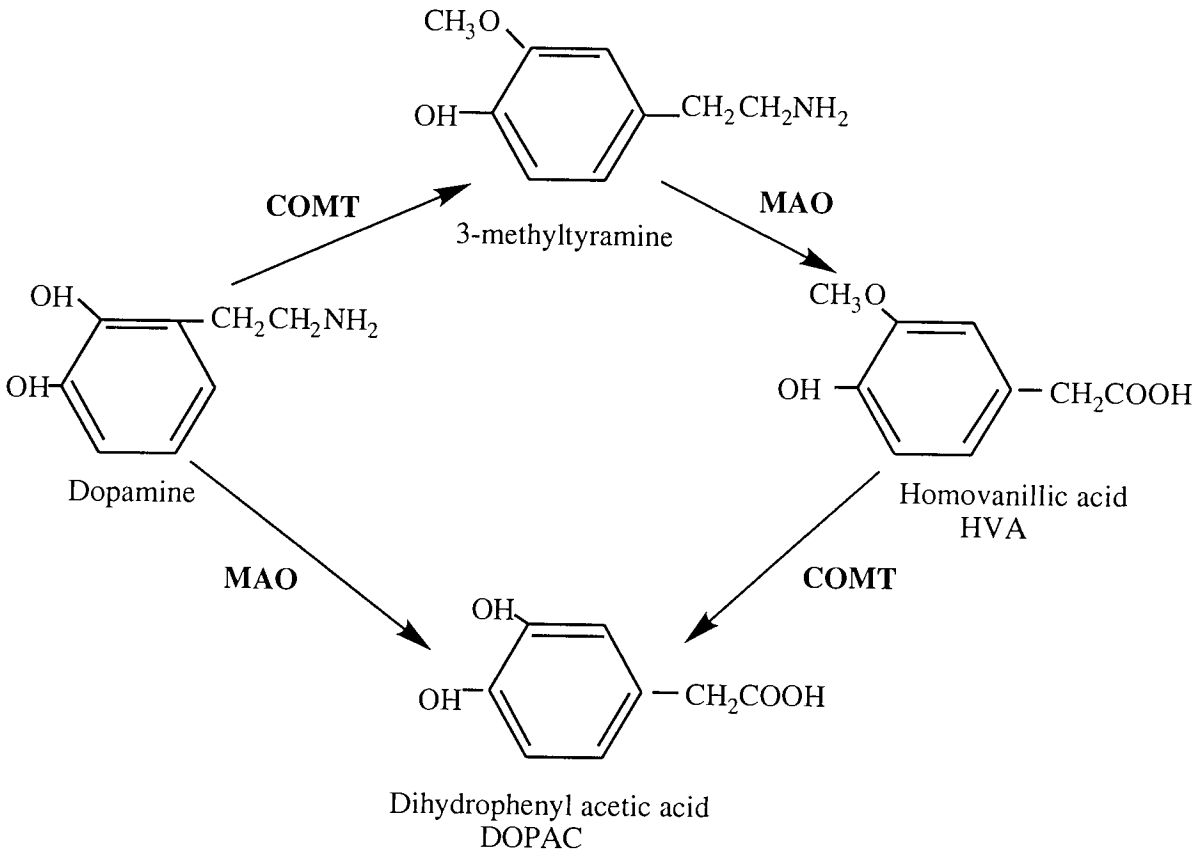


Figure 1.3 Metabolites of dopamine.
Adapted from Bradford (1986)

1.3.4 DOPAMINE CIRCUITS IN THE BRAIN

There are 3 main dopamine pathways in the brain (figure 1.4) (Bradford 1986). Firstly, the tubero-infundibular pathway, a local circuit consisting of cell bodies located in the hypothalamus and projecting to the pituitary gland. This pathway is involved in endocrine control, dopamine being released as a hypothalmic hormone. Secondly, the mesocorticolimbic pathway which involves projections from the midbrain to the frontal cortex and limbic system. Its functions are not fully understood, but it is thought to be

affected in patients with schizophrenia. Finally, the nigrostriatal pathway which arises in the pars compacta of the substantia nigra and terminates in the basal ganglia. This pathway is involved in the control of movement and is of particular relevance to PD. In addition to these three pathways, dopamine is found in the cells of the retina and the sympathetic ganglia of the peripheral nervous system.

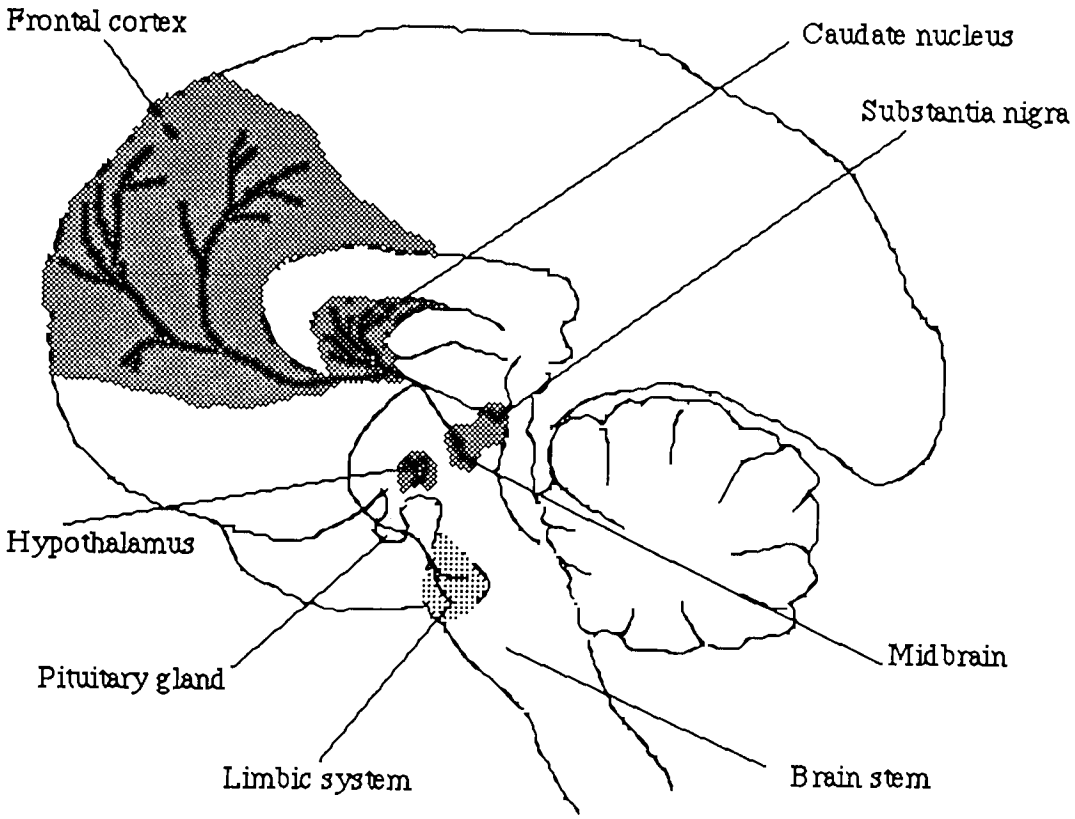


Figure 1.4 Dopamine pathways in the brain.
Adapted from Thompson (1993)

1.4 BASAL GANGLIA

The basal ganglia comprises a group of subcortical structures the largest of which is the corpus striatum (Carpenter 1985, Bradford 1986). The corpus striatum consists of a series of interconnected structures including the caudate nucleus and putamen (collectively known as the neostriatum) and the globus pallidus. In addition, the subthalamic nucleus, the red nucleus and the substantia nigra may be considered to be part of the basal ganglia. Projections exist between the basal ganglia and the thalamus, hypothalamus and cerebral cortex. The main receptive areas of the basal ganglia are the caudate nucleus and putamen

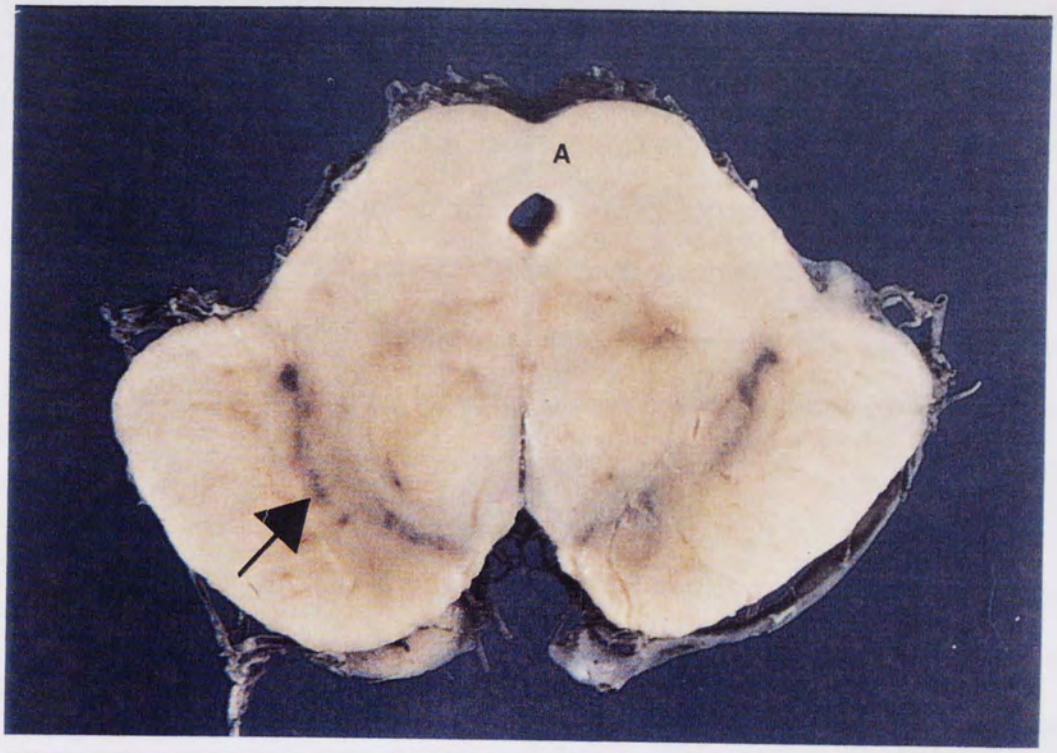
which receive sensory input from the thalamus and cortex and then project to the globus pallidus. The major output of the basal ganglia is from the globus pallidus to the motor cortex via the thalamus. Information then passes via the pyramidal system to the motor neurons of the spinal cord.

The substantia nigra is a pigmented nucleus located in the midbrain. It consists of two layers, an inner layer containing the dopaminergic cell bodies known as the pars compacta and an outer layer called the pars reticulata. The axons from these dopaminergic cells project as far as the neostriatum and constitute the nigrostriatal pathway. This pathway has an inhibitory effect that reduces the activity of the cholinergic interneurones of the corpus striatum. The nigrostriatal pathway is thought to be responsible for the smooth, co-ordinated output from the neostriatum to the globus pallidus. In addition to dopaminergic neurons, the basal ganglia also contain cholinergic and serotonergic neurotransmitter systems, and neurons containing GABA and peptides.

The function of the basal ganglia is believed to be the planning, initiation and co-ordination of movement. The neostriatum controls large subconscious movements of skeletal muscle whilst the globus pallidus regulates muscle tone and body position. This explains why damage to the basal ganglia results in a number of different movement disorders. For example, Huntington's chorea involves damage to the striatum and is characterised by involuntary, irregular contractions called chorea (Fahn 1989a). Ballismus is a form of chorea that involves involuntary flinging of the limbs and can occur due to lesions of the subthalamus (Fahn 1989b).

1.5 DEGENERATION IN PARKINSON'S DISEASE

In PD, degeneration of the dopaminergic neurons of the substantia nigra leads to a depletion of dopamine at the site of projection of these neurons. This degeneration is responsible for the typical loss of pigment within the substantia nigra seen in Parkinsonian brains at post mortem (figure 1.5). Within the Parkinsonian brain, dopamine levels in the neostriatum may be decreased by 80-90% (Bernheimer *et al* 1973) with a greater reduction in the putamen compared with the caudate nucleus (Kish *et al* 1988). This is because most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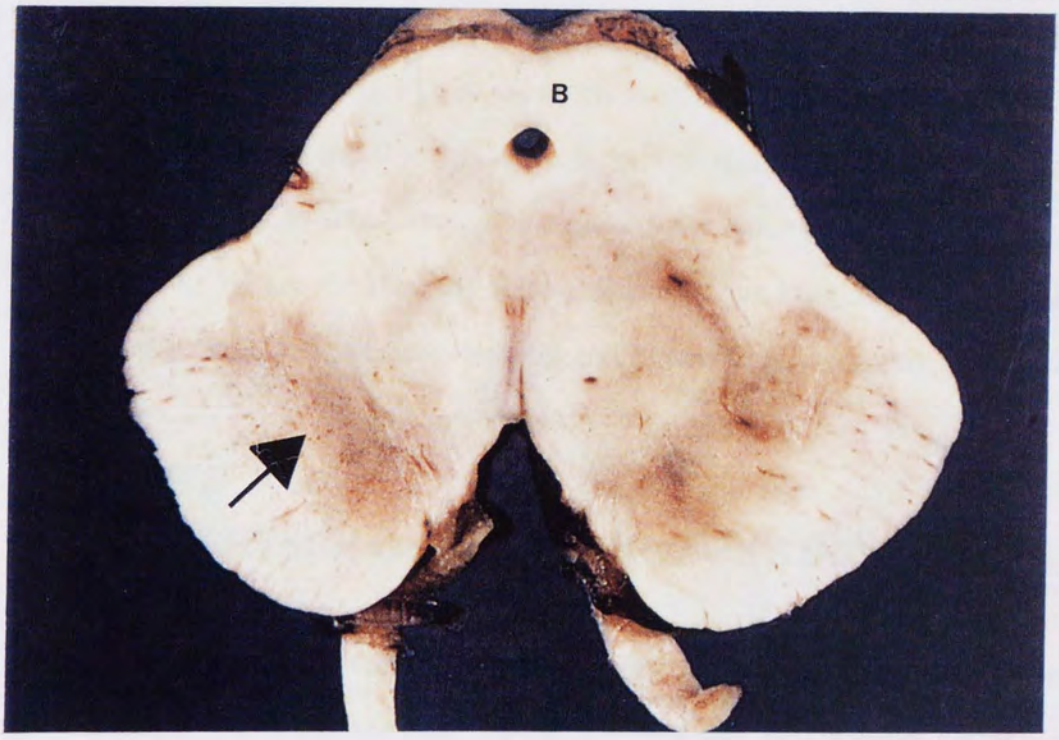
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cell loss occurs in the lateral zona compacta of the substantia nigra which projects primarily to the putamen (Bernheimer 1973). Some cell loss is also seen in the globus pallidus. In addition to loss of dopaminergic neurons, there is a reduction in the enzymes that synthesis dopamine, namely tyrosine hydroxylase (McGeer and McGeer 1976) and dopa decarboxylase (Lloyd *et al* 1975). Initially, an increase in the activity of the remaining dopaminergic neurons and hypersensitivity of the post synaptic dopamine receptors compensates for neuronal cell loss (Agid *et al* 1987). This explains why 80-85% of substantia nigra neurons must be lost before the symptoms of the disease appear (Marsden 1982).

Cell loss in PD is not confined to the nigrostriatal pathway. Dopaminergic neurons of the tuberoinfundibular (Rinne and Sonninen 1973) and mesocorticolimbic pathways (Javoy-Agid and Agid 1980) degenerate but to a lesser extent. However, those projecting to the spinal cord appear to be spared (Agid *et al* 1987). In addition, other neurotransmitter systems may be affected in PD. A reduction in noradrenalin (Rinne and Sonninen 1973) and GABA (Diamond and Borison 1978) have been reported in the substantia nigra of PD patients. Furthermore, noradrenergic neurones in the locus coeruleus, cholinergic neurons in the nucleus basalis of Meynert and serotonergic neurons of the raphe nuclei (all of which project from subcortical to cortical and limbic structures) may be affected (Agid *et al* 1987).

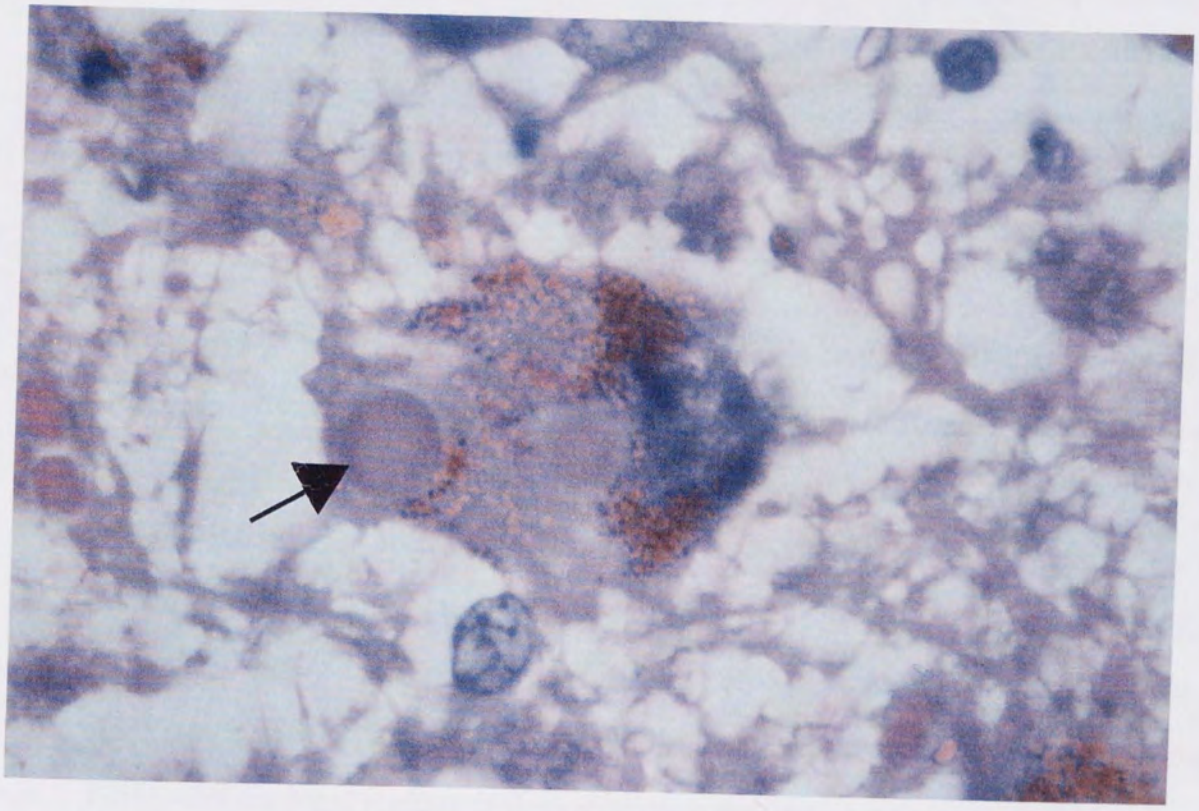
1.6 PATHOPHYSIOLOGY OF PARKINSON'S DISEASE

The classic motor symptoms of PD are believed to arise as a result of the degeneration of dopaminergic systems (Langston 1989a). Rigidity and hypokinesia are associated with dopamine depletion in the putamen. This is the area of the striatum mainly concerned with motor function as oppose to the caudate nucleus, which is involved in psychomotor function and motivation. By contrast, postural and gait defects are most likely to be due to the degeneration of non-dopaminergic neurons (Agid *et al* 1987). Tremor may also arise due to the degeneration of areas other than the nigrostriatal tract. This could explain why tremor is not related to neuronal loss in the substantia nigra (Rinne *et al* 1989). Cognitive

defects such as bradyphrenia (a slowness of information processing that may result in slowness of thought, impaired attention and apathy), depression, memory disorders and dementia are thought to be partly due to subcortical lesions. Degeneration of dopaminergic systems may affect mental state in PD since dementia was found to be correlated with cell loss in the medial substantia nigra (Rinne *et al* 1989). The medial substantia nigra projects to the caudate nucleus which in turn has connections with the frontal cortex. It has also been suggested that dementia in PD patients may arise due to dysfunction of the ascending cholinergic system which originates in the subcortical nucleus basalis of Meynert. Neuronal loss in this nucleus is known to occur to a greater degree in demented patients (Gaspar and Gray 1984). Alternatively, dementia in PD may be due to the presence of the lesions of Alzheimer's disease, namely senile plaques (SP) and neurofibrillary tangles (NFT), within the cortex (Hakim and Mathieson 1979). This is supported by the fact that the severity of dementia in PD is correlated with the extent of Alzheimer type pathology (Jellinger 1986b).

1.7 NEUROPATHOLOGY OF PARKINSON'S DISEASE

In addition to neurochemical changes, neuropathological changes also occur within the Parkinsonian brain. The most important of these changes is a form of cytoskeletal abnormality known as the Lewy body (LB), a cytoplasmic inclusion found within nerve cells (figure 1.6). LBs are said to be the pathological hallmark of PD, although they are not specific to PD and may occur in Alzheimer patients (Morris *et al* 1989) and in elderly controls who have not developed the symptoms of PD (Gibb and Lees 1989). In PD patients, LBs are mainly located in the substantia nigra although they may also be present in the locus coeruleus, dorsal vagal nuclei and nucleus basalis of Meynert (Jellinger 1986b). In a subset of patients, LBs have been reported to be abundant throughout the cortex in a condition known as diffuse Lewy body disease (Yoshimura 1983). This type of pathology has been found to be associated with cognitive defects and dementia (Yagashita *et al* 1980).



The structure of the LB may vary depending upon location. Subcortical or classical LBs consist of a central core surrounded by a less dense zone and an outer halo of radiating filaments. Cortical LBs do not have a definite core and are not distinct from the cytoplasm. The exact composition of LBs is unclear but they are known to have antigenic sites in common with neurofilaments such as tubulin, microtubule associated polypeptides and ubiquitin (Jellinger 1990). They may also contain sulphur, calcium and phosphorus (Kimula *et al* 1983). The pathogenesis of the LB is unknown but it has been suggested that they may arise as a result of free radical damage to organelles and membranes (Barbeau 1984) or due to the degeneration of proteins (Kimula 1983).

In a subset of PD patients, a neuropathology almost indistinguishable from that of AD occurs, ie NFTs and SPs being abundant throughout the neocortex. This type of pathology has been reported to be associated with dementia in PD (Boller *et al* 1980). NFTs are cytoskeletal abnormalities that occur in the perikarya of neurones. They consist of numerous pairs of filaments wound in a helical manner known as paired helical filaments (PHF). The major constituent of the PHF is believed to be hyperphosphorylated tau, a cytoskelatal protein, together with ubiquitin and other unidentified proteins (Iqbal *et al* 1989). SPs are focal regions of degeneration comprising amyloid, in the form of a central core, and abnormal neurites filled with PHFs.

1.8 TREATMENT OF PARKINSON'S DISEASE

The dopamine pathway has an inhibitory effect upon the cholinergic interneurons of the substantia nigra. Hence in PD, dopamine deficiency results in an overactivity of acetylcholine. The treatment of PD involves two approaches. Dopaminergic activity within the brain can be increased or cholinergic transmission can be reduced. Both methods have the effect of restoring the balance between dopamine and acetylcholine within the basal ganglia. The treatment of PD by these methods does not halt the progression of the disease it merely treats the symptoms.

Anticholinergic agents represent the oldest pharmacological therapy for PD and are currently used only in the early stages of the disease. These drugs are acetylcholine

receptor antagonists and act by reducing cholinergic transmission thereby correcting the neurotransmitter imbalance within the striatum. Anticholinergics are effective in the treatment of tremor but have little or no effect upon rigidity or bradykinesia (Calne 1993). Side effects include dry mouth, vasodilation, blurred vision, constipation and cognitive impairment (Calne 1993).

The antiviral drug amantadine may also be prescribed in milder cases of PD. It is thought to increase the presynaptic release of dopamine, inhibit dopamine re-uptake and may also have weak anticholinergic properties (Nastuk *et al* 1976, Stromberg and Svensson 1971)

As the condition progresses and more dopaminergic neurons are lost, it is no longer sufficient to merely enhance existing striatal dopamine levels. Levodopa therapy is undeniably the most effective treatment for moderate to severe PD. Its efficacy in the treatment of PD was first demonstrated in 1967 (Cotzias *et al* 1967). Levodopa is converted into dopamine within the dopaminergic nerve terminals where it is stored and then released. It is used in the place of dopamine due to its ability to cross the blood brain barrier. However, levodopa alone was found to have a number of side effects including nausea, hypotension, cardiac arrhythmias and involuntary movements which result from overstimulation of dopamine receptors. To overcome this problem, levodopa is given in combination with an extracerebral decarboxylase inhibitor. This has the effect of reducing the peripheral conversion of levodopa to dopamine and reducing the dose of levodopa required, minimising peripheral and CNS side effects. Two preparations are commonly given:- 1) Sinemet (levodopa and carbidopa) and 2) Madopar (levodopa and benserazide). A further development in levodopa treatment, is the use of controlled release drug delivery systems which liberate levodopa at a constant rate (Coleman 1992). This prevents surges in brain dopamine levels and therefore minimises accompanying side effects. Problems can arise after 2-3 years of treatment when levodopa preparations become less effective and side effects become more apparent. When brain dopamine levels are high, drug induced dyskinesias (abnormal involuntary movements) may occur. By contrast, when dopamine levels fall, as at the end of a dose or early in the morning, akinesia can result. In addition, random "on-off" episodes may occur, the patient fluctuating between being symptom free

and having full blown Parkinsonism. These episodes are not related to levodopa levels and may occur due to a reduction in the storage capacity of dopamine in the presynaptic terminals or an alteration in the postsynaptic receptors.

There has been some suggestion that levodopa therapy may speed up the progression of the disease by contributing to oxidative stress brought about by the metabolism of dopamine and the subsequent generation of free radicals (Calne 1993). However, levodopa toxicity is unlikely as levodopa therapy was found to increase average life expectancy (Uitti *et al* 1993) and a large dose of levodopa does not induce toxicity in the substantia nigra of mice (Hefti *et al* 1981).

Selegiline is now widely used in conjunction with a levodopa preparation, as it potentiates and prolongs the effects of levodopa, thereby reducing the dose required (Heinonen and Lammintausta 1991). Selegiline inhibits the actions of monoamine oxidase B, the enzyme that catalyses the degradation of dopamine. In addition, selegiline is thought to inhibit dopamine reuptake and increase its synthesis and release from presynaptic terminals. It has been suggested that selegiline may have a neuroprotective effect perhaps by reducing dopamine metabolism which could, in turn, contribute to oxidative stress (Parkinson Study Group 1989). Birkmayer *et al* (1985) reported that selegiline given in combination with levodopa increased life expectancy in PD patients. Furthermore, patients who had received selegiline had significantly more neurons remaining in the medial substantia nigra at postmortem (Rinne *et al* 1991). The Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) study reported promising improvements in motor symptoms with selegiline. However, the patients deteriorated when the drug was removed, suggesting a symptomatic rather than a neuroprotective effect (Shults and the Parkinson Study Group 1993).

At a later stage of the disease, due to a reduction in dopaminergic neurons, the brain loses its ability to convert levodopa to dopamine and a different strategy is usually employed. Dopamine agonists may be used to stimulate the postsynaptic dopamine receptors directly. Side effects of these drugs may include nausea, hypotension and psychiatric complications.

Dopamine agonists are generally reserved for more severe cases that no longer respond to levodopa alone.

A promising area of current research is the use of nigral transplants in the treatment of PD. In the early 1980s, the first attempts were made to transplant tissue from the adrenal medulla, an area rich in dopamine, into the striatum of PD patients (Backlund *et al* 1985). Tissue from the patient was used to avoid ethical problems and to prevent graft rejection. These and subsequent transplantations have resulted in a modest, often short term improvement in Parkinsonian symptoms but are accompanied by unacceptable morbidity and mortality rates (Goetz *et al* 1989). Further work has involved the transplantation of dopaminergic neurons derived from an aborted foetus. Neurons were found to survive within the putamen and restore dopamine synthesis resulting in a marked relief of symptoms (Lindvall *et al* 1990). The availability of foetal tissue and ethical problems may however limit the usefulness of this technique.

1.9 AETIOLOGY OF PARKINSON'S DISEASE

The majority of PD cases are idiopathic, i.e. of unknown aetiology. In addition, the term Parkinsonism is used to describe a variety of disorders that are caused by known factors and are collectively described as secondary Parkinsonism. Parkinsonism may also be apparent in a number of multiple system degenerations where damage to the substantia nigra occurs as part of a more widespread degeneration of the brain (table 1.1). The causes of secondary Parkinsonism are shown in table 1.2.

Table 1.1 Parkinsonism in multiple system degenerations.

Huntington's disease	Striatonigral degeneration
Progressive supranuclear palsy	Shy-Drager syndrome
Olivo-Ponto-cerebellar atropy	Creutzfeld-Jacob disease
Pallidonigral degeneration	Hallervorden-Spatz disease

Table 1.2 The causes of secondary Parkinsonism.

drugs	**infections**
phenothiazines	encephalitis lethargica
butyrophenones	syphilis
reserpine	poliomyelitis
tricyclic anti-depressants	
	malaria
toxins	**metabolic**
MPTP	hypoparathyroidism
manganese, lead, mercury	basal ganglia calcification
carbon monoxide	hepatocerebral degeneration
cyanide	Wilson's disease
head trauma	**brain tumour**
cerebral arteriosclerosis	**Syringomesencephalia**

There are several hypothesis that could explain the aetiology of idiopathic PD, including ageing, genetic defects, environmental toxins and an immune reaction.

The incidence of PD is known to increase with age until the 6th or 7th decade (Langston 1989b). Furthermore, there is a decline in striatal dopamine levels with age (Carlsson and Winblad 1976). These observations have led to the hypothesis that PD arises due to an acceleration of the normal ageing process. It is unlikely however, that ageing plays a major role in PD for two reasons. First, the pattern of neuronal loss in the substantia nigra differs in PD to that seen with ageing (Fearnely And Lees 1991). Second, only 4.7-6% of pigmented nigral cells are lost with age per decade between the ages of 50 and 90 (Gibb and Lees 1991). Alternatively, dopaminergic cell loss may be due to a single insult early in life followed by an age related decline resulting in the appearance of the clinical symptoms of PD.

Evidence suggests that a genetic factor alone is not responsible for the aetiology of idiopathic PD. The concordance rates for PD in monozygotic and dizygotic twins are similar (Ward *et al* 1983, Martilla *et al* 1988) suggesting that simple Mendellian

inheritance is not involved. In addition, the prevalence of the disease amongst first degree relatives is the same in PD patients as in controls (Duvoisin 1986). However, in a small percentage of cases a genetic factor has been implicated. In the early-onset form of the disease, familial cases are more frequent (Alonso *et al* 1986). Furthermore, several families which exhibit an autosomal dominant pattern of transmission of the disease have been described (Golbe *et al* 1990, Maraganore *et al* 1991). Although it is unlikely that a genetic factor alone is important, it has been suggested that an inherited trait plus an environmental factor could be involved. For example, a genetic defect could result in an inability to detoxify an environmental agent.

Infectious agents and exogenous toxins have been implicated in the aetiology of PD. The pandemic of "encephalitis lethargica" between 1917 and 1925 left up to 80% of survivors with a depletion of striatal dopamine and postencephalitic Parkinsonism (Duvoisin 1981). Furthermore, a number of other infections such as poliomyelitis may cause secondary Parkinsonism in man. However, there is little evidence for the involvement of an infectious agent in idiopathic PD. The disease could not be linked to a specific infection (Elizan and Casals 1983) and the injection of Parkinsonian brain tissue has failed to induce the disease in primates (Gibbs and Gajdusek 1982).

A number of exogenous toxins are known to induce Parkinsonism in man. Chronic exposure to a series of metals including manganese, lead and mercury has been found to induce secondary Parkinsonism. In addition, the intravenous injection or chronic exposure to the meperidine derivative MPTP (1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine) has been found to cause the selective destruction of the dopaminergic neurones of the substantia nigra (Langston *et al* 1983). This toxic insult results in an irreversible form of Parkinsonism that responds to levodopa. Many analogues of MPTP exist and it has been suggested that an environmental toxin with a similar chemical structure could be responsible for PD. It has also been reported that an excitatory amino acid in the seeds of a plant called *Cycas circinalis* induces some of the neuropathological features of ALS-Parkinsonism-dementia complex in primates (Spencer *et al* 1987). This condition is common to the Chamorro population of Guam who use the *Cycas* plant as both a food

source and medicine. Epidemiological studies further support the role of an environmental toxin in idiopathic PD. The incidence of PD has been found to be associated with wood pulp and steel industries and with the consumption of well water (Tanner 1989). It is unclear how a toxin could lead to nigral cell death in idiopathic PD but it has been suggested that a similar mechanism to MPTP toxicity could be involved. The toxic effects of MPTP are mediated via MPP+ (1-methyl-4-phenyl-pyridinium) which is thought to act in a number of ways. Firstly, MPP+ may act via the inhibition of the mitochondrial enzyme complex 1 resulting in the depletion of ATP and neuronal cell death (Nicklas *et al* 1985). Reduced activity of complex 1 has been reported in the substantia nigra of PD patients (Schapiro *et al* 1990). Secondly, MPP+ has been reported to induce the formation of free radicals thereby contributing to oxidative stress that could result in lipid peroxidation and cell lysis (Rossetti *et al* 1988). Free radicals may also act by inactivating proteins and enzymes and damaging nucleic acids. Lipid peroxidation is selectively increased in the substantia nigra of PD patients (Dexter *et al* 1989a) providing further evidence for an increase in oxidative stress within the Parkinsonian brain. Furthermore, iron levels are increased in the substantia nigra and could act as a catalyst for the production of reactive oxygen species (Dexter *et al* 1989b).

Alternatively, metabolism within the body may result in the generation of free radicals that could contribute to oxidative stress. The auto-oxidation of dopamine and its enzymatic metabolism by MAO B results in the production of hydrogen peroxide which, in the presence of transition metals, may generate free radicals (Uitti and Calne 1993). A genetic defect could conceivably be responsible for the excessive production of free radicals or could result in an inability to detoxify these products of metabolism using antioxidant defences. MAO B activity has been reported to be increased in PD and could contribute to increased oxidative stress (Gotz *et al* 1990). Furthermore, glutathione peroxidase, catalase and superoxide dismutase, the enzymes responsible for detoxifying free radicals, are reported to be reduced in the substantia nigra of PD patients (Perry *et al* 1982, Ambani 1975 *et al*, Saggu *et al* 1989).

Finally, the activity of the immune system can also provide a source of free radicals and the role of the immune response in the aetiology of neurological diseases in general and PD in particular will be described in chapter 3.

CHAPTER 2: METAL BINDING TO TRANSFERRIN

2.1 METALS AND NEUROLOGICAL DISEASES

A number of different metals have been implicated in the aetiology of neurological diseases. For example, Wilson's Disease is a hereditary disorder that involves abnormalities of copper (Cu) metabolism. A reduction in the incorporation of Cu into the glycoprotein ceruloplasmin and a subsequent increase in non-ceruloplasmin Cu occurs within the serum (Danks 1982). This results in Cu deposition within the liver and basal ganglia of the brain leading to their degeneration. Symptoms of the disorder include liver disease, muscular rigidity and tremor (Menkes 1989).

Aluminium (Al) is the third most abundant metal in the earth's crust and may be involved in a distinct neurological disorder known as dialysis encephalopathy. This condition occurred in patients receiving kidney dialysis who were exposed to high levels of Al within the dialysing fluid. The result was a substantial increase of Al within the brain (Alfrey 1986). Symptoms of the condition included myoclonus (brief muscle jerks), gait disorders, impairment of memory and concentration, hallucinations, dementia and eventually death (Garrett *et al* 1988). In addition, Al has been implicated in the aetiology of Alzheimer's Disease (Birchall and Chappell 1988), although this is controversial and not established with certainty.

Other trace metals may also be involved in neurological diseases (Goldblatt 1989). The ingestion of mercury was responsible for outbreaks of "Minamata's disease" which occurred in a region of Japan where methyl mercury was present as a river pollutant. The consequences of the mercury poisoning included widespread neuronal damage to the cerebellum and neocortex of the brain. The symptoms of the disorder include parasthesias, tremor, dysarthria and incoordination. The ingestion of lead may also induce neurological symptoms, such as ataxia, hemiplegia, rigidity and dementia, collectively known as "Plumbism". Furthermore, a condition resembling PD can be induced by manganese (Mn) intoxication. This condition occurred especially in miners who absorbed Mn dioxide dust

via the respiratory tract. In these cases, widespread damage to the basal ganglia resulted in disorders of gait and posture, hypokinesia, tremor and rigidity.

2.1.1 METALS AND PARKINSON'S DISEASE

Several lines of evidence have implicated metals as being involved in PD. First, environmental toxins and trace metals have been implicated in the aetiology of idiopathic PD (Dexter *et al* 1993). Second, exposure to metals may induce Parkinsonism in man (Goldblatt 1989) and third, epidemiological studies have linked the incidence of PD to the industrial use of heavy metals (Rybicki *et al* 1993).

2.1.1.1 IRON AND PARKINSON'S DISEASE

Iron (Fe) is the metal most commonly discussed as a factor in PD. Firstly within the normal brain, the highest concentrations of Fe occur in the extrapyramidal regions (Dexter *et al* 1989b), the regions that are primarily affected in PD. Secondly, Fe levels have been found to be selectively elevated in the zona compacta of the substantia nigra in PD patients compared to controls (Dexter *et al* 1989b, Sofic *et al* 1991). Thirdly, Hirch *et al* (1991) reported that high levels of Fe were present within Lewy bodies, although this finding was not supported by subsequent studies using a different method of detection (Jellinger *et al* 1993). Finally, the intranigral injection of Fe in rats was found to induce a 95% decrease in striatal dopamine resulting in a reduction in spontaneous movements and episodes of motor freezing (Ben-Shachar and Youdim 1991). A role for Fe in the pathogenesis of PD is further supported by the ability of Fe to contribute to oxidative stress. Fe is a catalyst in the Fenton reaction which generates oxygen radicals from hydrogen peroxide produced during the catabolism of dopamine. In addition, Fe may facilitate the auto-oxidation of dopamine thereby generating hydrogen peroxide and superoxide anions (Halliwell and Gutteridge 1986). The ability of excess Fe to induce oxidative stress will depend upon the form in which it is present. A high proportion of Fe in the tissues is bound to ferritin and is in an inert form. Dexter *et al* (1990) reported that ferritin levels were reduced in the substantia nigra of PD patients. However, this finding is controversial, previous studies

indicating a significant rise in nigral ferritin (Riederer *et al* 1988). A reduction in ferritin levels within the Parkinsonian brain could result in an elevation of reactive Fe and a subsequent increase in oxidative stress. Indeed, the toxic effects of 6 hydroxy-dopamine have been linked to the release of Fe from ferritin (Monteiro and Winterborn 1989). However, the extent to which Fe accumulation is involved in the disease process is unclear. An elevation in nigral Fe has been reported in Progressive Supranuclear Palsy (PSP) and Multiple System Atrophy (Dexter *et al* 1993) suggesting that the increase in Fe is a secondary effect and does not initiate neuronal degeneration. However, these findings were not supported by Hirsch *et al* (1991) who reported that Fe levels were not affected in PSP compared to controls. Further evidence to suggest that Fe accumulation occurs at a later stage of the disease is the fact that Fe levels in the substantia nigra of patients with incidental Lewy body disease were unchanged (Jenner 1993). These patients show some cell loss in the substantia nigra and the presence of Lewy bodies but have no symptoms and are thought to represent early cases of PD. An important consideration is the specific cell populations which actually accumulate Fe within the substantia nigra. It appears that Fe is accumulated mainly within glial cells (Jellinger 1990). Hence, the accumulation of Fe could be a result of a glial cell reaction to the disease pathology. In addition, Fe has been detected within intact dopaminergic neurons of the substantia nigra (Youdim *et al* 1993). The reason for an accumulation of Fe in the Parkinsonian substantia nigra is unknown. A change in the form of Fe present, for example the decompartmentalisation of Fe from ferritin or the binding of Fe to a low molecular weight species, is one possibility. Alternatively, there could be an increased entry of Fe into the brain due to a change in uptake mechanisms, an alteration of the blood brain barrier or increased translocation of Fe from other areas. It is also possible that Fe is liberated at the end stages of the disease by the degeneration of neurons or glial cells (Youdim *et al* 1993).

2.1.1.2 OTHER METALS AND PARKINSON'S DISEASE

In addition to Fe, several other metals have also been implicated in the aetiology of PD. A role for Al in PD is supported by several lines of evidence. Firstly, Al has been found to be elevated in the substantia nigra, caudate nucleus and globus pallidus of PD patients

compared to controls (Yasui *et al* 1992a). Secondly, Al is present within the Lewy bodies of PD patients (Hirsch *et al* 1991) and the NFT bearing neurons of patients with Parkinsonism-Dementia of Guam (Garruto *et al* 1984). Al could act as a neurotoxin in a number of ways including inhibiting enzyme activity (Yates *et al* 1980), interfering with the synaptosomal uptake of neurotransmitters (Lai 1980) or contributing to the oxidative damage of the brain (Oteiza *et al* 1993).

Cu like Fe is a transition metal that can catalyse the Fenton reaction. However, evidence suggests that Cu is not involved in PD. Cu was found to be present at a similar level in the extrapyramidal system compared with other brain regions (Riederer *et al* 1989). Furthermore within the PD substantia nigra, Cu has been reported to be unchanged (Riederer *et al* 1989) or reduced (Dexter 1989b) in comparison to controls. Mn and lead were also found to be unchanged in Parkinsonian brains (Riederer *et al* 1989, Dexter *et al* 1989b) indicating that they too are not involved in the aetiology of PD. By contrast, zinc (Zn) was reported to be increased in the substantia nigra of PD patients (Dexter *et al* 1989b). However, Zn does not have the ability to generate oxidative stress so is a less likely candidate to contribute to the pathogenesis of PD. It is also possible that a deficiency of an essential metal could have an adverse effect upon brain function. Magnesium (Mg) has been reported to be lowered in the substantia nigra, globus pallidus, caudate nucleus and cortex of PD patients (Yasui *et al* 1992a). Mg is an essential element for CNS function and a reduction in Mg is associated with changes in the storage, release and action of neurotransmitters. Yasui *et al* (1990) demonstrated that a Mg deficient diet resulted in CNS degeneration in the rat. In addition, low Mg levels in the CNS tissue, perhaps as a consequence of low Mg in the drinking water, has been implicated in the aetiology of amyotrophic lateral sclerosis (ALS) (Yasui 1992b).

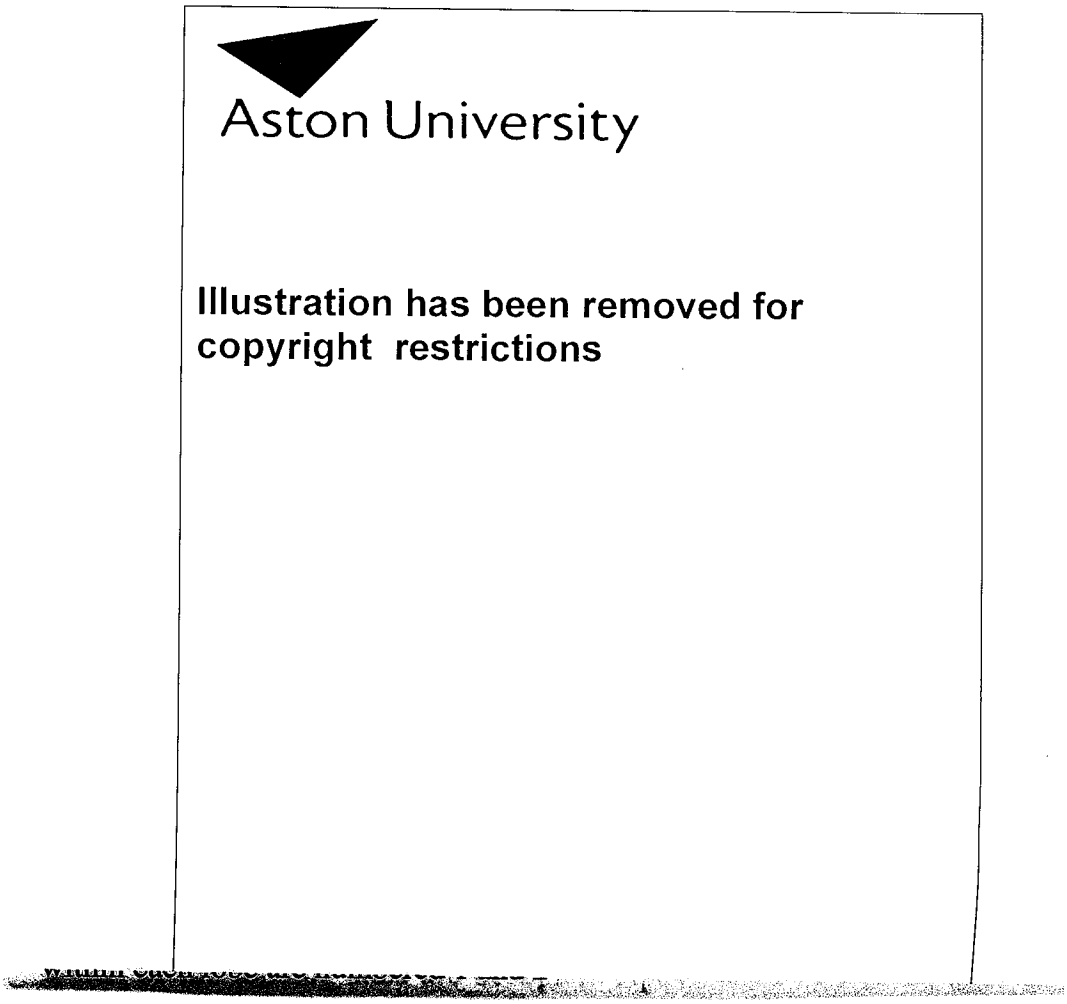
2.2 TRANSFERRIN

The transferrins are a class of proteins concerned with the transport of Fe within the body. Three forms of transferrin (Tf) have been identified. First, ovotransferrin is an Fe binding protein in hen's egg white. Second, lactoferrin refers to Tf found within milk, tears and

other body secretions. Third serotransferrin, often referred to simply as transferrin, is the form found within blood serum and a number of other mammalian fluids including the cerebrospinal fluid, bile, amniotic fluid and lymph (Harris and Aisen 1989). The liver is the major site of Tf synthesis although it is also produced in the mammary glands, lymphocytes, muscle and in the brain (Bomford and Munro 1985).

2.2.1 STRUCTURE OF TRANSFERRIN

The Tf molecule is a glycoprotein consisting of a single polypeptide chain of 679 amino acids with two asparagine linked oligosaccharide chains. The estimated molecular weight of the Tf molecule is 79 570 D (MacGillivray *et al* 1983).



wo domains

Figure 2.1 Simplified diagram of the human lactoferrin molecule.
Taken from Baker *et al* (1987)

Crystallographical studies have provided information about the structure of human lactoferrin which may be extended to the other Tf molecules (Aisen 1989). The polypeptide chain of the protein is arranged in two lobes, an N-terminal lobe and a C-terminal lobe, related by a 2 fold screw axis (figure 2.1). Each lobe is further divided into two domains surrounding a cleft in which the Fe binding site is located.

2.2.2 TRANSFERRIN AND IRON BINDING

There is an Fe binding site within both lobes of the Tf enabling one molecule to bind two atoms of Fe. The amino acids involved in Fe binding are the same for both binding sites although they may be located in different positions. Each Fe atom is co-ordinated by two tyrosines, one aspartic acid, one histidine, a synergistic anion (in vivo HCO_3-) and either a hydroxyl ion or a water molecule (Thorstensen and Romslo 1990). Controversy exists as to whether the two Fe binding sites of Tf are functionally identical. Zak and Aisen (1986) reported that the N-terminal site was predominantly occupied by Fe although it is the weaker of the two binding sites. However, this finding was not supported by a subsequent study which suggested that the two sites were equally occupied (Beguin *et al* 1988).

Physical and chemical differences are believed to exist between the two Fe binding sites of Tf. The C-terminal site has been shown to have a greater affinity for Fe than the N-terminal site (Harris 1983). Furthermore, the C-terminal site is acid stable and only looses its Fe when the pH is reduced to between 5 and 6. The N-terminal site, on the other hand, is acid labile and looses its Fe between pH 6 and 7 (Lestas 1976). Metal binding to Tf involves the ferric form of Fe and the Tf molecule itself may oxidise the ferrous form to enable binding to occur. A unique property of Tf metal binding is the concomitant binding of the Tf molecule to an anion. *In vivo*, the anion is thought to be either HCO^{3-} or CO^{3-} (Harris and Aisen 1989). Four forms of Tf may exist within the serum depending upon which of the binding sites are occupied. ApoTf has two free Fe sites and is the most abundant form of seroTf. Monoferric-A and monferric-B Tf have either the C-terminal site or N-terminal site occupied respectively. Finally, the form of Tf in which Fe is bound to both sites is known as diferric Tf.

2.2.3 TRANSFERRIN AND OTHER METALS

Under normal circumstances only 30% of the binding sites of Tf within the plasma are occupied by Fe (Martin *et al* 1987) leaving plenty of capacity for the binding of Tf to a range of other metals. Tf has been demonstrated to bind to a variety of metal ions including ions of the first transition series, main group elements, lanthanides and actinides. A number of the metal ions that are known to bind to Tf include Al^{3+}, Ga^{3+}, Mn^{3+}, $Cr,^{3+}$, Co^{3+}, Ni^{2+}, Zn^{2+}, Cu^{2+}. In addition to Fe transport, Tf is believed to play a role in the transport of Zn (Evans 1976, Harris and Keen 1989), Mn (Aschen and Gannon 1994) and Al (Roskams and Connor 1990) within the body.

2.2.4 TRANSFERRIN RECEPTORS

The Tf receptor is a dimer of two identical subunits linked together by two disulphide bridges (figure 2.2). Each subunit consists of three domains, an N-terminal cytoplasmic tail, a transmembrane section and an extracellular C-terminal region to which the Tf molecule is bound. In addition, three N-asparginine linked carbohydrate chains are bound to the extracellular portion of the receptor. A cysteine in each transmembrane section anchors the receptor to the cell membrane (Thorstensen and Romslo 1990).

Transferrin receptors are embedded in the cell membranes of a variety of Fe requiring cells including reticulocytes, fibroblasts, hepatocytes and macrophages (Harris and Aisen 1989). In addition, the brain possesses Tf receptors on the vascular endothelium (Jefferies *et al* 1984) and on individual neurons (Morris *et al* 1994).

The number of Tf receptors present on the cell surface is determined by the levels of Fe available. The receptor density on the surface of cultured cells was found to decrease as the availability of Fe increased. Conversely, the number of cell surface Tf receptors was found to increase if Fe was deficient (Rudolph *et al* 1985). Hence, alteration in Fe levels results in a change in the total number of Tf receptors due to an alteration in the rate of receptor synthesis. In addition, the number of cell surface receptors may be altered by the translocation of receptors between the cell surface and the internal environment, although

this occurs more frequently in response to changes in cell differentiation (Bomford and Munro 1985).

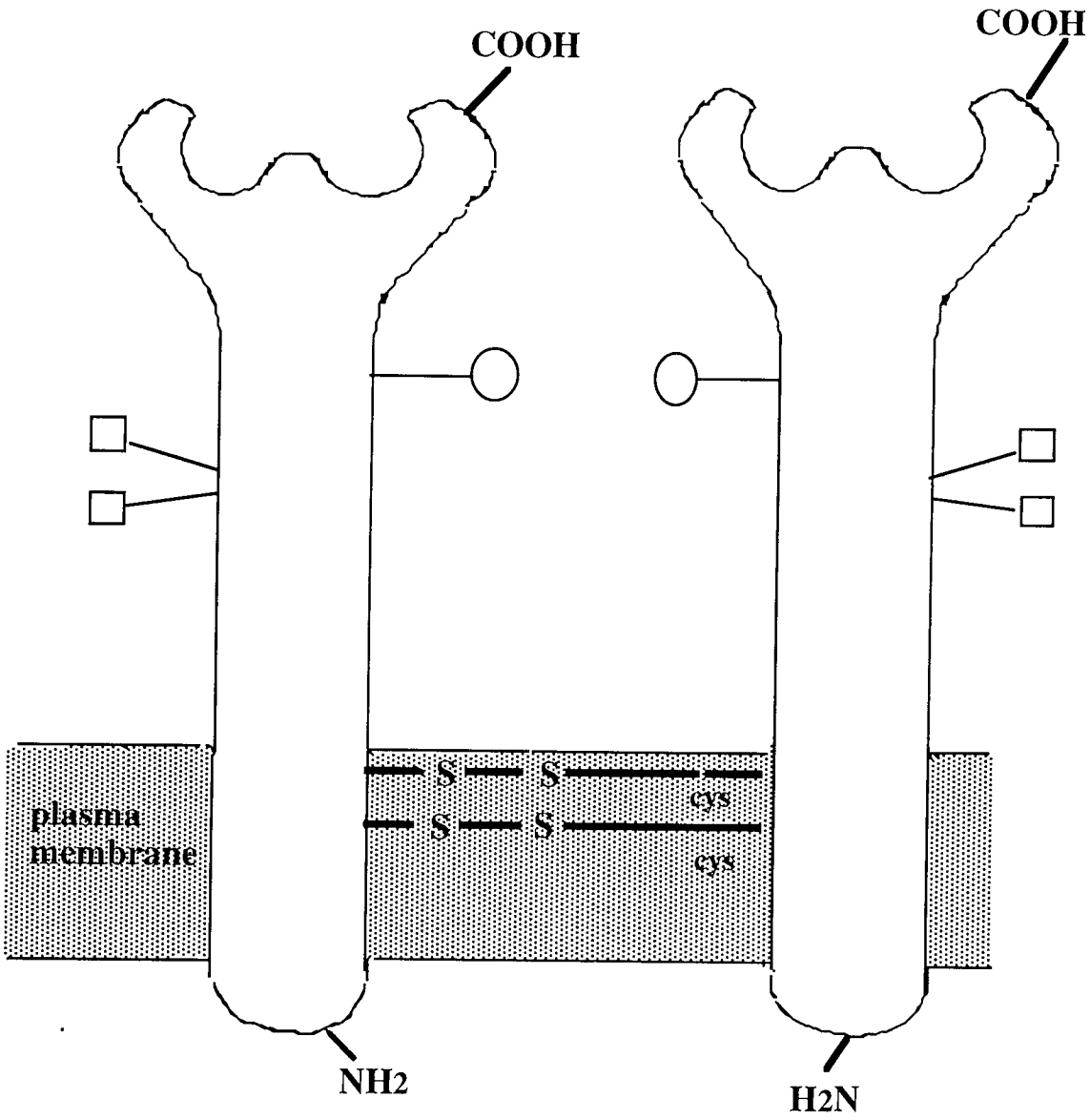


Figure 2.2 Transferrin receptor.
Adapted from Aisen (1989)

2.2.5 CELLULAR UPTAKE OF IRON BOUND TO TRANSFERRIN

The cellular uptake of Fe is generally thought to occur via a process referred to as receptor mediated endocytosis (RME) (Thorstensen and Romslo 1990). Initially, the Fe bound to Tf attaches to the Tf receptor present on the cell surface. The Fe-Tf-receptor complex is then internalised via the formation of an endocytotic vesicle known as an endosome. Within the endosome a proton pump lowers the pH resulting in the release of Fe from the complex.

The Fe is subsequently transported across the endosomal membrane into the cytosol. The apoTf bound to the receptor is then returned to the cell surface in an exocytotic vesicle which fuses with the plasma membrane to release the Tf-receptor complex. The extracellular pH of 7.4 causes the dissociation of the apoTf from the receptor and the Tf molecule re-enters the circulation where it can participate further in the transport of Fe (Thorstensen and Romslo 1990). Additional mechanisms of Fe uptake to RME may exist in the hepatocyte. The Redox model also involves the binding of Tf to its receptor. However, following receptor binding it has been suggested that the actions of protons and reducing factors at the cell membrane destabilize the Tf-Fe bond and bring about a reduction of Fe. Ferrous ions are liberated and are transported across the cell membrane independently of the Tf system via a specific Fe carrier (Thorstensen and Romslo 1988). In addition, the hepatocyte may exhibit a non specific binding of Tf to the cell membrane followed by internalisation of the complex with the pH dependent release of Fe (Morgan and Baker 1986).

2.2.6 TRANSFERRIN AND THE ENTRY OF METALS INTO THE BRAIN

The Tf system is believed to be involved in the entry of Fe into the brain. Tf receptors have been found to be present on the endothelium of brain vasculature and the endocytosis of Tf is known to occur within these capillaries (Pardridge *et al* 1987). The ferroTf may pass directly through the capillary endothelium to deliver Fe directly to those cells possessing Tf receptors (Fishman *et al* 1987). An alternative hypothesis suggests that Fe gains access to the brain via the internalisation of the Tf-Tf receptor complex by the cerebral capillaries. Fe may then dissociate from the seroTf resulting in deposition within the endothelial cells of the capillaries. The deposited Fe may then be taken up by brain derived Tf and delivered to cells of the CNS possessing Tf receptors where it is internalised by RME (Morris *et al* 1992). This theory is supported by the observation that following its systemic administration, uptake of Tf bound Fe in the rat brain was found to reflect the distribution of Tf receptors (Morris *et al* 1992). The highest density of Tf receptors, and hence the greatest uptake of Fe, occurs in the neocortex, hippocampus and brain stem. However, Tf mediated uptake of Fe cannot account for the accumulation of Fe within the brain as the

density of Tf receptors does not correspond to the distribution of brain Fe (Morris *et al* 1992, Hill *et al* 1985) which is highest in the substantia nigra and globus pallidus (Dexter *et al* 1989b). In order to explain this anomaly, it has been proposed that Fe may be transported within neurons from areas with a high density to areas with a low density of Tf receptors (Hill *et al* 1985). It has however, been reported that the levels of Tf itself are highest in the Fe rich areas such as the globus pallidus and substantia nigra in the rat brain (Roskams and Connor 1994).

In addition to Fe, the entry of other metals into the brain may be regulated via the Tf system. It has been reported that the Tf receptor present in the rat brain has a very high affinity for Al bound to Tf. Furthermore, Al has been reported to gain entry into the CNS via the Tf receptor system (Roskams and Connor 1990). Further studies have suggested that Mn may enter the brain via a Tf conjugated Mn transport system (Aschner and Gannon 1994).

2.2.7 METAL-TRANSFERRIN BINDING WITHIN THE PLASMA

Speciation refers to the chemical form in which a metal is present within the plasma. Most studies have been made on Al, a metal that is known to be toxic and has been implicated in a number of neurological disorders. Within the plasma, Al is believed to be bound predominantly to Tf (Fatemi *et al* 1991, Harris 1992), although Al has also been found to bind to serum albumen (Trapp 1983, Fatemi *et al* 1991). Further studies have reported that following an increase in serum Al levels, additional serum proteins, for example immunoglobulins, can compete with Tf and albumen to bind Al (Favarato *et al* 1991). In addition to plasma proteins, a number of low molecular weight (LMW) species have also been reported to bind to Al within the plasma.

In the absence of a suitable radio-isotope of Al, ^{67}Gallium (^{67}Ga) has been employed as a marker to study the binding of Al within the plasma. Ga and Al are both group III metals. ^{67}Ga has been shown to be a suitable analogue of Al in investigations of Al speciation within the plasma (Cochran *et al* 1983, Farrar *et al* 1990) and as a marker for Al transport in the rat brain (Pullen *et al* 1990). Furthermore, Hodgkins *et al* (1993) demonstrated that

Ga-Tf binding was significantly correlated with Al-Tf binding. Ga-Tf binding was initially used as a model to study Al speciation (Farrar *et al* 1990) but can be extended as a marker for the binding of Tf to a variety of other metals.

2.3 REDUCED GA-TF BINDING IN NEUROLOGICAL DISEASES

The binding of Ga to Tf within the plasma has been investigated in a number of neurological disease. Farrar *et al* (1990) reported that the percentage of Ga bound to Tf within the plasma of Alzheimer's Disease and Down's syndrome patients was significantly reduced in comparison with controls. However, these studies have been criticised for a number of reasons. Firstly, the percentage of Ga bound to Tf was lower than would be expected. This could be attributed to a lack of bicarbonate in the medium which is known to be required for Tf binding to metals (Candy *et al* 1990). Secondly, the reduction in binding seen in both patient groups could be a reflection of reduced Tf levels (McGregor *et al* 1991). The studies of Farrar *et al* were also criticised on the grounds that the increased Tf saturation with Fe seen in the AD group could account for the reduction in Ga-Tf binding observed. However, a defect that results in a reduced binding of Tf to Ga could also reduce the amount of Tf available thus increasing Tf-Fe saturation. Further studies have also reported a reduced binding of Ga to Tf in patients with neurological diseases. Forstl *et al* (1991) and Brammer *et al* (1990) reported a significant reduction in the percentage of Ga bound to plasma proteins in Alzheimer patients compared to age and sex matched controls. However, these findings are controversial and could not be confirmed by others (Taylor *et al* 1991, McGregor *et al* 1991). In addition, Ga-Tf binding has been reported to be significantly reduced in Down's syndrome patients compared to controls. Furthermore, binding was significantly lower in demented compared to non demented Down's patients (Hodgkins *et al* 1993). Preliminary studies have also noted a reduction in Ga-Tf binding in PD patients compared to controls (Hodgkins 1992, Winsper *et al* 1994).

2.3.1 BINDING OF METALS TO A LOW MOLECULAR WEIGHT SPECIES

Within control plasma, the binding of Ga to Tf has been found to be less than 100 percent. Hence, a proportion of the Ga (and by implication Al) may be present as a LMW species

and not bound to Tf (Hodgkins *et al* 1993, Winsper *et al* 1994). Subsequently, a reduction in the percentage of Ga bound to Tf may result in a corresponding increase in a LMW species within the plasma. Most studies have been made on Al although there is controversy as to the identity of the LMW species binding to this metal. Chromatographic techniques have produced varying results. VanGinkle *et al* (1990) reported that in rat serum Al was associated with a LMW citrate species. These findings were further supported by Martin *et al* (1987) who suggests that citrate was the most likely LMW carrier of Al within human plasma. Furthermore, Fatemi *et al* (1991) demonstrated that in the presence of high plasma Al levels, appreciable to those found in haemodialysis patients, some of the Al was bound to citrate. Computer models have also been used to predict the LMW forms of Al present within the plasma. Jackson (1990) reported that at physiological pH, the LMW Al would exist predominantly as hydrolysed phosphate complexes with some binding to a citrate species. This finding was supported by Harris (1992). By contrast, Hodgkins *et al* (1993) identified the LMW Ga species (used as a marker for Al) present in the plasma as silicate or phosphate. It has also been suggested that a LMW protein transports Al in the case of haemodialysis patients who have high levels of Al within their plasma (Khalil-Manesh *et al* 1989).

2.3.2 IMPLICATIONS OF REDUCED METAL TRANSFERRIN BINDING

The decreased binding of Ga to Tf may reflect a reduction in Tf binding of many metals within the plasma. The extent to which a particular metal would exhibit a reduction in binding depends upon its stability constant with Tf, those metals with lower stability constant being displaced from Tf first.

Table 2.1 Stability constants of metal-transferrin complexes.

	log K1	log K2
Fe-Tf	22.5	21.4
Ga-Tf	20.3	19.3
Al-Tf	12.9	12.3
Zn-Tf	7.42	6.0

Fe-Tf is known to have the greatest estimated stability constant followed by Ga, Al (Martin 1987) and Zn (Harris 1992) (table 2.1).

A reduction in the binding of metals to Tf could have two possible effects both of which could have implications for a number of neurological diseases. Firstly, it has been suggested that a reduction in Tf mediated transport could have a damaging effect by limiting the brain entry of essential metals (Hodgkins *et al* 1993). The Tf system has been found to be involved in the uptake of both Al and Fe into the brain (Roskams and Connor 1990). A number of metals such as Mg, Zn and Fe are known to be required for brain functioning and a deficiency of essential metals has been implicated in the aetiology of neurological diseases. For example, the level of Zn in the hippocampus has been reported to be decreased in AD and a deficiency of Zn enzymes may play a role in the pathology of this disorder (Constantinidis 1991). In addition, Mg deficiency has been implicated in the aetiology of PD (Yasui *et al* 1992a) and ALS (Yasui *et al* 1992b). Secondly, a reduction in metal Tf binding may facilitate the entry of toxic metals into the brain. Tf may have a protective function by regulating the uptake of certain metals. For example, the uptake of Mg across the BBB of the rat is limited by the binding of the metal to Tf (Rabin *et al* 1993). A reduction in Tf metal binding increases the availability of the LMW form of the metal which could then cross the BBB unhindered, resulting in deposition within the brain (Farrar *et al* 1990). Indeed in hypotransferrinaemic mice, there was a greatly enhanced uptake of iron into the brain indicating that non-Tf bound iron readily crosses the BBB (Ueda *et al* 1993). Furthermore, in haemodialysis patients there is an elevation in plasma Al and a subsequent increase in a LMW form of the metal. It has been suggested that this LMW species is neurotoxic and crosses the BBB more easily (King *et al* 1982). Hence, the identity of the LMW species may be important in determining whether brain entry of the metal is increased. Citrate has been reported to enhance the bioavailability of Al by increasing its absorption and tissue accumulation (Slanina *et al* 1985). On the other hand, silicon, which can react with Al to form Al silicates, may possibly protect against Al accumulation in the brain (Carlisle 1986).

It is also possible that reduced binding is a consequence of earlier pathogenic events in the brain e.g. oxidation or immune activation within the plasma. This hypothesis will be discussed in chapter 7.

2.3.3 WHY IS GALLIUM TRANSFERRIN BINDING REDUCED?

A number of factors could be responsible for a reduction in the binding of Tf to metals. Biochemical alterations within the blood plasma have been shown to result in changes in Ga-Tf binding. The bicarbonate anion is known to be required for Tf binding (Aisen *et al* 1967). As expected, the binding of Ga to Tf *in vitro* was found to depend upon the level of bicarbonate present, maximum binding occurring in the presence of physiological levels of HCO^{3-} (Hodgkins 1992). In addition, deviations from the physiological pH of 7.4 have been shown to reduce Ga-Tf binding and, to a lesser extent, Fe-Tf binding with the liberation of a LMW species (Hodgkins 1992, McGregor and Brock 1992). Changes in the electrolytic composition of plasma can also affect Tf binding. The addition of the metals Fe and Al to plasma was found to bring about a reduction in Ga-Tf binding, although Mg, Zn and Cu had little effect (Hodgkins 1992). Furthermore, the presence of a high affinity LMW ligand may reduce the binding of Tf to metals by competing with the Tf molecule. An increase in the level of citrate within serum was found to markedly reduce the binding of Ga to Tf *in vitro* (McGregor and Brock 1992).

A reduction in the levels of Tf will obviously reduce its availability to bind to metals. Tf levels are depressed in response to a number of events including an increase in body iron stores, nephrosis, cirrhosis of the liver, protein malnutrition, haemolytic anaemia and inflammation (Morgan 1974).

Farrar *et al* (1990) suggested that a functional defect in the Tf molecule could result in a reduction in Ga-Tf binding. A number of variants of the Tf molecule have been reported (Kirk 1967). Furthermore, Evans *et al* (1982) described a variant of Tf which bound to iron abnormally at the C-terminal site.

Alternatively, increased oxidation within the circulation could provide a mechanism whereby the Tf molecule is damaged thus reducing its metal binding capacity. The addition of oxidising agents to plasma was found to induce a reduction in Ga-Tf binding *in vitro* (Hodgkins 1992). A possible source of oxidising species would be an immune reaction within the body. Hence, it has been suggested that an increase in oxidation, due to the activity of macrophages, results in the reduction in Ga-Tf binding seen in PD patients (Winsper *et al* 1994). It is also possible that the components of the immune system attack the Tf molecule directly. An autoimmune form of atransferrinemia has been described involving the production of auto-antibodies specific for the Tf molecule with the generation of circulating immune complexes of Tf and IgG (Westerhausen and Meurret 1977). The Tf molecule may also be responsive to inflammation. The inflammatory response can stimulate changes in the concentration of a series of plasma proteins called Acute Phase Proteins (APP) (Kushner 1988). Tf is believed to be a negative acute phase protein thereby its levels may fall in response to inflammation (Maes *et al* 1992). The possible relationship between the immune response and metal binding will be discussed further in chapter 7.

CHAPTER 3: NEOPTERIN AND THE IMMUNE RESPONSE

3.1 THE IMMUNE RESPONSE

The immune system (IS) is the bodies means of defence against infection. Two types of immune response occur, the innate and the adaptive immune response (Sell 1987, Totora and Anagnostakos 1989).

The innate (or non-specific) IS is inherited and involves a general response to a variety of infectious agents. In this response, phagocytic cells play an important role in engulfing and destroying foreign cells and debris. The phagocytic cell involved in the initial immune reaction is the neutrophil, a form of polymorphonuclear leukocyte. In addition, macrophages, formed from blood monocytes, are recruited at a later stage of the response. The non-specific IS also includes a group of up to 20 proteins collectively referred to as complement. Activation of complement involves a cascade of reactions resulting in the generation of activated molecules. The classical pathway for complement activation is initiated via the binding of the complement protein C1q to an antibody molecule that has reacted with an antigen. In addition, an alternative complement pathway exists that can be activated by bacterial components such as lipopolysaccharide. The complement system can attack foreign invaders in a number of ways. Firstly, the activated complement component C3b may act as an opsonin to attract phagocytic cells which may then engulf and destroy the invader. Secondly, a membrane attack complex (MAC), composed of complement proteins C5-C9, may be formed. This complex can insert itself into the lipid membrane where it forms a transmembrane channel resulting in cell lysis and death.

In contrast to the innate IS, the adaptive (or specific) IS develops in response to a specific antigen. In this case, a particular cell (T-cell) or molecule (antibody) is generated that recognises a specific antigen. Antigens comprise several substances that are recognised by the body as being foreign and include microbial structures, toxins and transplanted tissue. The adaptive IS consists of both cellular and humoral immunity.

Cellular immunity relies upon the actions of sensitised T-lymphocytes and is effective against parasites, viruses, cancer cells and transplanted tissue. The T-cell alone cannot recognise an antigen and requires the help of an antigen presenting cell (APC). APCs are generally macrophages, although a number of other cells can fulfil this role. The APC internalises and processes antigens, fragmenting them into antigenic peptides. These peptides are then displayed upon the surface of the APC in combination with a self-marker known as a major histocompatibility complex (MHC) molecule. T-cells are equipped with cell surface receptors that allow them to react to specific antigens providing they are in combination with an MHC molecule. Two forms of MHC molecule exist. MHC class I antigens which are present on the surface of virtually all nucleated cells and MHC Class II antigens which are only expressed on certain cells such as macrophages and B-lymphocytes. When a T-cell is activated by its specific antigen in combination with an MHC molecule, it divides and differentiates to form a clone consisting of sub-populations of cells each with a different function. T-helper cells aid antibody formation by secreting lymphokines that stimulate B-cells. T-helper/inducer cells can be identified by the presence of CD4 cell surface markers and recognise antigens displayed with MHC class II antigens. T-cytotoxic lymphocytes kill target cells by causing cell lysis and T-suppressor cells are responsible for limiting the immune response. T-suppressor/cytotoxic cells can be detected by CD8 markers and recognise antigen in combination with MHC class I molecules.

Humoral immunity involves the production of antibodies i.e. protein molecules that react specifically to antigens. This system is involved in defence mainly against bacterial and viral pathogens. The B-lymphocyte has a surface receptor that enables it to recognise a specific antigen in a process that does not require the presence of an MHC molecule. Following antigenic stimulation, the B-lymphocyte divides and differentiates into plasma cells which synthesis and secrete antibody. Antibodies can act by binding to antigens to neutralise them or by precipitating the actions of complement or phagocytic cells.

The components of the immune response are influenced by a number of chemical mediators known as cytokines. Lymphokines are a type of cytokine which are produced by

lymphocytes and include the interleukins and γ interferons. Interleukins are the products of activated T-cells and macrophages and are capable of stimulating the activation and differentiation of lymphocytes. By contrast, γ interferons are produced by T-cells and are involved in regulating the expression of MHC molecules and stimulating the differentiation of cytotoxic T-cells.

3.2 THE IMMUNE SYSTEM OF THE BRAIN

Until recently the possible role of the immune response in neurological diseases has been limited by the concept that the brain was "immunologically privileged". This refers to a number of characteristics of the brain-: 1) the existence of the blood brain barrier (BBB) preventing antibodies and immunocompetant cells from entering the brain; 2) the absence of lymphatic drainage of the central nervous system (CNS); 3) the inability of neurons to express MHC molecules and 4) the fact that inflammation is atypical of the brain (McGeer *et al* 1991). However, more recent evidence has brought into question the concept of immunological privilege of the brain. Firstly, in certain circumstances the BBB may not act as a complete barrier to immune system mediators. For example, the BBB is known to be almost absent in the olfactory epithelium, with additional weaknesses detected in regions of the temporal lobe, hypothalamus and brain stem (Balin *et al* 1986). In addition, head trauma and natural ageing can result in a deterioration of the BBB (Wisniewski and Kozlowski 1982). Furthermore, it has been reported that immune cells may penetrate the intact BBB. For example, activated T-cells have been shown to bind to endothelial cells and pass through the BBB (Savion *et al* 1984). Activated T-cells may also secrete mediators that increase vascular permeability thereby allowing antibodies and immunocompetant cells to pass through the BBB (Leibowitz and Hughes 1983). Secondly, recent studies have demonstrated a significant infiltration of lymphocytes into the brain (Wekerle *et al* 1986). Thirdly, it now appears that a variety of cells originating within the CNS are capable of expressing MHC antigens upon their surface and presenting antigen to T-cells in culture. These include microglia (McGeer *et al* 1991, Krzanowski 1993), astrocytes (Fontana *et al* 1984), oligodendrocytes (Cashman and Noronha 1986) and endothelial cells (Fabry *et al* 1990). Finally, it is now generally accepted that in the case of

viral infections, head trauma and certain neurological diseases, inflammatory responses occur within the CNS (Rogers and Luber-Narod 1988, McGeer *et al* 1991, Aisen and Davis 1994).

3.3 THE IMMUNE RESPONSE AND NEUROLOGICAL DISEASES

Immune mechanisms are known to play a role in the pathogenesis of the neurological disease myasthenia gravis. In this disorder, auto-antibodies are directed against acetyl-choline receptors resulting in an inhibition of neuromuscular transmission (Drachman *et al* 1982). In addition, the immune response has been implicated in a variety of disorders affecting the peripheral nervous system. The formation of immune complexes has been reported in Guillain-Barre syndrome in which demyelination of peripheral nerves occurs (Tachovsky *et al* 1976). Amyotrophic lateral sclerosis (ALS) involves degeneration of the motor neurons of the spinal cord. Elements consistent with a cellular immune response, i.e. T-helper/inducer cells, cytotoxic T-cells and MHC positive microglia, have been detected within the spinal cord of ALS patients (McGeer *et al* 1991). Multiple sclerosis, depression, Down's syndrome and Alzheimer's Disease (AD) represent diseases of the CNS in which immune mechanisms have been implicated. In multiple sclerosis, demyelination of the white matter of the CNS occurs. The pathological process is believed to involve the generation of auto-antibodies which attack the oligodendrocytes that synthesis myelin. In addition, T-cells appear to recognise a component of myelin, presented in combination with an MHC molecule by macrophages, leading to the destruction of the myelin sheath of neurons (Steinman 1993). Major depression also appears to be accompanied by immune activation including T-cell activation, B-cell proliferation, generation of auto-antibodies and an increase in the production of IL-1B and IL-6 (Maes 1995). Finally, elevation of urinary neopterin (Armstrong *et al* 1994) and the presence of circulating immune complexes (Heinonen *et al* 1993) provides evidence for the activation of both cellular and humoral immunity in AD and Down's syndrome patients.

Several lines of evidence exist to support a role for the IR in the aetiology of AD. Complement proteins of the classical pathway are associated with amyloid plaques

(Kalaria *et al* 1991, McGeer et al 1989b, Eikelenboom and Stam 1982), dystrophic neurons, neuropil threads and some neurons (McGeer 1989b). In addition, immunoreactivity for the complement components C5b-C9 (i.e. the MAC) has been found to be associated with dystrophic neurons and NFTs in AD brains (McGeer *et al* 1989b, Itagaki *et al* 1994). The presence of T-lymphocytes and cells possessing MHC glycoproteins provides evidence that is consistent with a cell mediated immune response within the AD brain. The presence of both T-helper/inducer (CD4) and T-cytotoxic/suppressor (CD8) cells has been reported within the AD hippocampus (McGeer *et al* 1989a, 1991). Furthermore, Itagaski *et al* (1988) reported an increase in the number of T-cytotoxic cells in AD brain tissue compared to age matched controls. However, these findings were not supported by Rozemuller *et al* (1992a) who failed to identify T-cell subsets in AD brains. Further evidence for a T-cell response in AD brains is the presence of CNS cells expressing both MHC class I (in humans known as HLA-A,B,C) and MHC class II (HLA-DR) molecules which may enable them to present antigens to T-cells. Large numbers of HLA-DR positive microglia have been detected within the AD brain (McGeer *et al* 1988, Rozemuller *et al* 1992a). Furthermore, HLA-DR positive microglia have been reported to be associated with amyloid plaques (McGeer *et al* 1989a, Rogers *et al* 1988) although only the classical and not the diffuse plaques appear to be involved(Rozemuller *et al* 1992a). In addition to acting as APCs, microglia are involved in the mediation of the immune response and in AD brains have been found to express a number of cytokines and their receptors such as Il1, IL6, TNFα (Dickson *et al* 1993) and the IL2 receptor (Rogers and Luber-Narod 1988). Several lines of evidence suggests that the humoral immune response may also be activated in AD. The presence of circulating immune complexes (CICs) has been demonstrated within the serum of patients with AD (Heinonen *et al* 1993). In addition, auto-antibodies that specifically bind to cholinergic neurons have been detected in the sera and cerebrospinal fluid (CSF) of AD patients (Chapman *et al* 1988, McRae Deguerce *et al* 1987). Furthermore, Bradford *et al* (1989) demonstrated that the sera from patients with AD caused complement mediated immunolysis of cholinergic nerve terminals from the rat cerebral cortex. Serum antibodies that recognise the vascular basement membrane have also been reported to occur in AD patients but not ageing controls (Fillit *et al* 1987). The presence of IgG has been reported in senile plaques (Ishii

and Haga 1976, Ishii *et al* 1988) although subsequent attempts to stain for immunoglobulins have proved inconsistent (McGeer *et al* 1989a).

3.4 THE IMMUNE RESPONSE IN PARKINSON'S DISEASE

Several lines of evidence suggest that abnormalities of the immune response may occur in patients with PD. It has been proposed that activation of the complement system may contribute to the pathogenesis of PD. Antibodies to the components of the classical complement pathway, C3d, C4d, C7 and C9, have been found to stain Lewy bodies within the Parkinsonian substantia nigra (Yamada *et al* 1992). In addition, oligodendroglia identified by anti-C3d and anti-C4d were present in increased numbers in the substantia nigra of PD patients compared to controls (Yamada *et al* 1992). It has been suggested that these complement activated oligodendroglia, consisting of swollen processes of degenerating myelin to which complement proteins are attached, may represent opsonised oligodendrocytes. The complement component C1q, the first step of the cascade, was not detected. This may be explained by the fact that C1q is present in small amounts and is loosely bound to the target and may therefore become detached and dissipate within the tissue. The trigger for the activation of the classical complement pathway is unknown but in addition to IgG and IgM, trypsin like enzymes, myelin and viruses are capable of initiating the cascade (Vanguri *et al* 1982). The alternative complement pathway does not appear to be activated in the brains of PD patients as staining for properdin and fraction Bb of factor B was negative (Yamada *et al* 1992).

Disturbances in cellular immunity may also occur in PD patient. Reduced levels of peripheral T-cells and defective T-cell mitogenic responses have been reported in patients with advanced PD compared to age matched controls (Martilla *et al* 1984). In addition, the number of CD4+ T-helper/inducer cells as a proportion of peripheral blood mononuclear cells was reduced in PD patients compared to controls (Martilla *et al* 1985). Kuhn *et al* (1988) reported a reduction in both the T-suppressor cell and B-cell count in PD patients. However, Martilla *et al* (1984) reported that the number of B-cells was similar in PD patients and neurological controls. The cellular immune response has also been found to be

increased in PD patients. Fiszer *et al* (1994) reported that a sub-population of T-cells involved in infection and autoimmunity known as gamma delta T+ cells were increased in the blood of PD patients compared to other neurological disease patients. There was also an increase in the proportion of these cells present in the CSF of PD patients. In addition, cells possessing MHC molecules on their surface, enabling them to present antigen to T-cells, have been identified in PD brains. McGeer *et al* (1988) detected large numbers of HLA-DR positive reactive microglia in the substantia nigra of all PD cases studied. Furthermore, these microglia could be visualised phagocytosing dopaminergic neurons and their processes. In addition, PD cases with dementia had large numbers of HLA-DR positive microglia within the hippocampus (McGeer *et al* 1988). It has been hypothesised that a form of non-specific cell mediated immunity known as antibody dependent cell mediated cytotoxicity (ADCC) may be involved in the pathological processes in PD. ADCC is a type of lymphocyte-mediated target cell killing involving the attachment of antibody to a target cell followed by the binding of a killer lymphocyte through Fc receptors. Killer T-cells can act without prior immunisation and are not MHC restricted. Bokor *et al* (1993) reported that killer cell activity was increased in PD patients with severe symptoms compared to milder cases and it has been hypothesised that an ADCC reaction is mounted against dopaminergic cells.

The humoral IR may also be involved in the aetiology of PD. Pouplard and Emile (1984) detected the presence of auto-antibodies to human sympathetic ganglion neurons in the sera of 70.5% of PD cases studied. These antibodies appeared to cross react with CNS structures namely the neuronal cells of the rat locus ceruleus and the pigmented neurons of the human substantia nigra. In addition, antibodies that recognise specific epitopes of dopaminergic neurons have been detected in the serum and CSF of PD patients. McRae-Deguerce *et al* (1986) reported that the CSF from PD patients produced immunocytochemical staining of the rat substantia nigra and Husby *et al* (1977) detected antibodies against human caudate nucleus in 33% of PD cases. Furthermore, PD serum has been found to cause complement dependent damage to cultured rat dopaminergic neurons and it has been suggested that complement mediated humoral immunity may be involved in the degeneration of dopaminergic neurons in PD (Defazio *et al* 1994).

It has also been suggested that the cytokine tumour necrosis factor (TNF) is involved in the degeneration of dopaminergic neurons in PD. This is supported by several lines of evidence. Firstly, the concentration of TNFα has been reported to be significantly increased within the striatum and CSF of PD patients compared to controls (Mogi *et al* 1994). Secondly, activated microglia and astrocytes are known to release TNF (Sawada *et al* 1989) and TNF-immunoreactive glia have been detected within the substantia nigra of PD patients (Boka *et al* 1994). Finally, immunoreactivity for TNF receptors has been demonstrated in the cell bodies and processes of dopaminergic neurons which suggests that they may be sensitive to TNF (Boka *et al* 1994). TNF is known to exhibit cytotoxic activity against myelin and oligodendrocytes *in vitro* (Selmaj and Raine 1989) and may also be toxic to neurons. TNF has been found to inhibit the respiratory chain (Schulze-Osthoff *et al* 1992) and increase the production or oxygen free radicals (Asoh *et al* 1989). Both of these mechanisms could result in nerve cell death. Alternatively, increased levels of TNF may be a secondary event resulting from gliosis occurring in the substantia nigra in PD.

In the present study, it was proposed to use various markers of the immune response to investigate the plasma of PD patients. Neopterin has been measured as a marker of cell mediated immunity and circulating immune complexes (CICS) as a marker of humoral immunity.

3.5 NEOPTERIN

3.5.1 STRUCTURE OF NEOPTERIN

Neopterin belongs to the class of compounds known as pteridines. The pteridine structure consists of a bicyclic nitrogenous ring system derived from a pyrazine and pyrimidine to form a pyrazino-pyrimidine. Neopterin and biopterin are derivatives of the pyrazino-pyrimidine compound with only small substitutions and are therefore referred to as unconjugated pteridines. Pteridines can be further classified as pterins (derivatives of 2-amino-4-oxo-3,4 dihydropteridine) including neopterin and biopterin and lumazines

(derivatives of 2,4-dioxo-1,2,3,4-tetrahydropteridine) (Wachter *et al* 1992). The structure of neopterin is shown in figure 3.1.

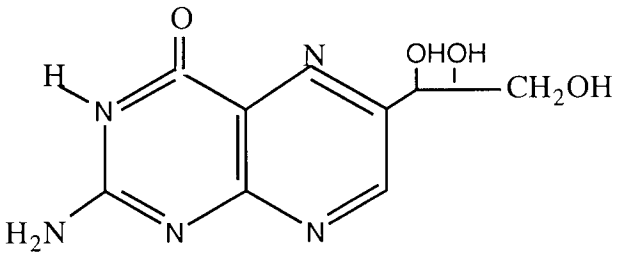


Figure 3.1 Structure of neopterin.

3.5.2 SYNTHESIS OF NEOPTERIN

Neopterin is a metabolite of guanosine triphosphate (GTP) and is formed during the biosynthetic pathway of biopterin (a co-factor for neurotransmitter synthesis) (figure 3.2).

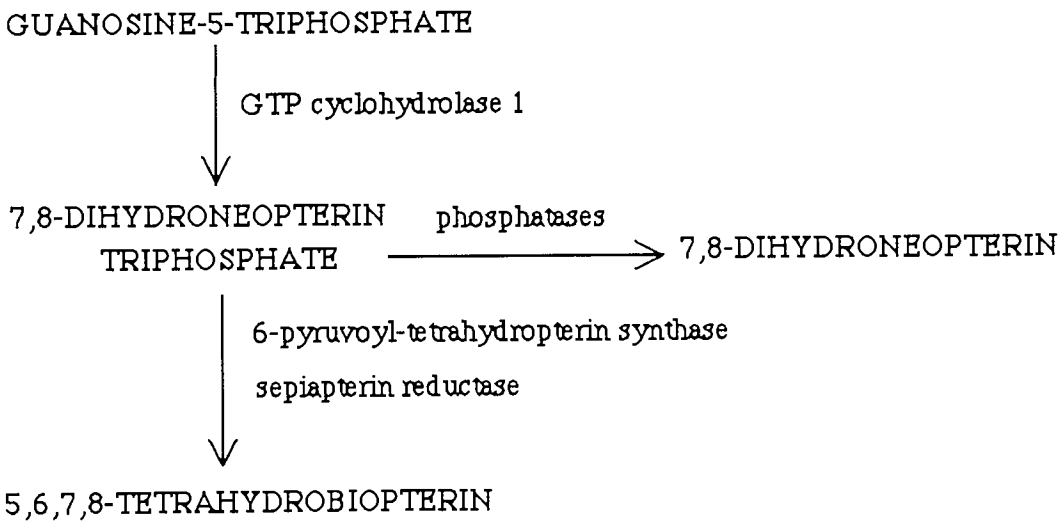


Figure 3.2 Pteridine biosynthesis from guanosine triphosphate.

The first step of this pathway is catalysed by the enzyme GTP cyclohydrolase 1 with the formation of 7,8,-dihydroneopterin triphosphate. This intermediate is then converted to tetrahydrobiopterin by the enzymes 6-pyruvoyl-tetrahydropterin and sepiapterin reductase, and dihydroneopterin by phosphatases. Interferon gamma is known to activate GTP cyclohydrolase 1 in a variety of human cells (Werner *et al* 1990). Macrophages are unique in that the activity of GTP cyclohydrolase 1 exceeds the activity of 6-pyruvoyl-

tetrahydropterin synthase. Subsequently macrophages convert only small quantities of 7,8,-dihydroneopterin triphosphate to tetrahydrobiopterin but they produce and release large amounts of neopterin (Fuchs *et al* 1993a).

The physiological function of neopterin is unknown. Neopterin may influence cytokines and has been found to enhance the release of TNF from monocytes (Barak and Gruener 1991). In addition, neopterin may play a part in the cytotoxic actions of macrophages and oxidised neopterin has been found to increase the toxicity mediated by oxygen free radicals (Weiss *et al* 1993).

3.5.3 NEOPTERIN AS A MARKER

Neopterin is released in large quantities by macrophages stimulated by interferon γ secreted by activated T cells and may therefore be an indicator of cell mediated immunity (Huber *et al* 1984). Indeed, neopterin is elevated in the body fluids in a variety of conditions in which the cellular immune response is activated i.e viral infections, malignancies, allograft rejection and auto-immune disorders (Wachter *et al* 1992).

Neopterin exists as fully oxidised neopterin and its reduced form, dihydroneopterin. Both forms are excreted by activated macrophages. Oxidised neopterin accounts for 43% and 45% of total neopterin in the serum and urine respectively (Levine and Milstein 1984) but less than 30% in the CSF (Howells *et al* 1986). Oxidised neopterin or total neopterin (oxidised + reduced forms) serve equally well as a diagnostic marker of an immune response although it may be preferable to measure oxidised neopterin as dihydroneopterin is easily degraded (Wachter *et al* 1992).

3.5.4 NEOPTERIN AND NEUROLOGICAL DISEASES

Previous studies have reported an increase in neopterin levels in a number of neurological diseases. In multiple sclerosis patients, CSF neopterin was found to be increased during clinical exacerbations compared to levels in the same patient during remission (Fredrikson *et al* 1987). Plasma and urinary neopterin have been found to be elevated in patients with

major depression (Dunbar *et al* 1992, Duch *et al* 1984). Urinary neopterin levels were also found to be significantly increased in Down's syndrome and AD patients compared to age matched controls (Armstrong *et al* 1994). In addition, Winsper *et al* (1994) demonstrated that serum neopterin levels were significantly elevated in PD patients compared to controls. However previous studies reported that neopterin was reduced (Fujishiro *et al* 1990) or unchanged (Furukawa *et al* 1992) in the CSF of PD patients.

3.6 CIRCULATING IMMUNE COMPLEXES

Immune complexes are macromolecules formed by the interaction of antibody with a specific antigen. The production of immune complexes by the IS is a benign and ongoing process. Circulating immune complexes (CICs) are normally cleared from the circulation by a series of biochemical, enzymatic and cellular processes. Prolonged antigenic stimulation can however, result in the deposition of CICs within small blood vessels and tissue leading to the activation of complement and subsequent damage to the host.

Increased levels of CICs occur in auto-immune diseases, malignancies and infections (Theofilopoulos and Dixon 1980). In addition, CICs have been found to be present in the sera of both AD and Down's syndrome patients more frequently than in controls (Soininen *et al* 1993, Heinonen *et al* 1993). Furthermore, cognitive decline in AD patients (as measured by the mini-mental status examination) was correlated with the level of CICs present. (Heinonen *et al* 1993). Elevated CICs have been detected in a number of other neurological disease including multiple sclerosis (Tachovsky *et al* 1976, Cojocaru *et al* 1992) and ALS (Oldstone *et al* 1976, Westarp *et al* 1993). Previous studies have stated that serum and CSF CIC levels did not differ in PD patients compared to patients with progressive supranuclear palsy and cervical spondylosis (Yamada *et al* 1994b).

3.7 ROLE OF THE IMMUNE RESPONSE IN THE PATHOGENESIS OF PARKINSON'S DISEASE

The role of an immune response in the aetiology of PD remains to be established. Three possibilities exist. First, the immune response is the primary process responsible for the

lesions of the disease. In this case an auto-immune response, where B or T lymphocytes are capable of recognising self antigens, could be generated against the dopaminergic neurons resulting in the degeneration of the substantia nigra. Autoimmunity can occur in response to a number of circumstances including damage to the BBB, the presence of an environmental toxin or infectious agent, or the abnormal expression of MHC antigens. Second, the immune response is a secondary event involved in removing debris produced by degeneration due to some other cause. In this case, the immune response could be a more general inflammation occuring in response to the degeneration of the substantia nigra. However, even if the immune response is a secondary event it may play a role by exacerbating neuronal damage. Third, there is a further process that results in both the observed lesions and independently stimulates the immune response.

3.8 METAL BINDING AND THE IMMUNE RESPONSE

A defect in metal binding to transferrin (Tf) and the generation of an immune response may both be involved in the pathogenesis of PD. Furthermore, evidence exists for a relationship between Tf binding and the immune response. In patients with immune activation (as measured by neopterin levels), disturbances in iron metabolism have been observed with a transfer of iron towards storage sites. Neopterin levels were found to be positively correlated with ferritin levels and inversely related to serum iron and Tf levels (Denz *et al* 1992, Fuchs *et al* 1993b). Winsper *et al* (1994) reported an inverse correlation between Ga-Tf binding and serum neopterin (as a marker of a cell mediated response) levels in PD patients. This could be explained by a number of possibilities. Firstly, defects in metal binding to Tf could generate an immune response. An increase in the free metal, due to reduced binding to Tf, could result in the stimulation of the IS by a metal or metal complex. A number of metals have the ability to stimulate an immune response and it has been hypothesised that a metal induced immune response within the brain could contribute to the pathology of AD (Armstrong *et al* 1995). Aluminium compounds have a long history of use as vaccine adjuvants such as in the diptheria toxoid (Edelman 1980). Adjuvants are substances that nonspecifically enhance the immune response to an antigen. The disorder known as chronic beryllium disease (CBD) is a lung disease arising due to

industrial exposure to beryllium dust. In this condition, a cell mediated immunity to beryllium occurs with the formation of granulomas or clusters of immune cells surrounding beryllium particles in the walls of the alveoli (Mroz *et al* 1991). In addition, mercury has been found to induce antibodies against renal antigens (Kosuda *et al* 1993).

Secondly, immune activation could remove metals from Tf. Oxidation has been shown to reduce plasma Ga-Tf binding *in vitro* (Hodgkins 1992). Hence, macrophages, that release a number of oxidants, could result in oxidation within the periphery and a subsequent fall in Ga-Tf binding. It is also possible that a specific immune attack of Tf occurs, damaging the molecule with the release of metals. A Tf immune complex disease has been described involving the production of auto-antibodies specific for the Tf molecule that results in the generation of a CIC of Tf and IgG (Westerhausen and Meuret 1977).

Finally, a third independant factor could be responsible for both immune activation and a reduction in Tf binding.

CHAPTER4:GALLIUM-TRANSFERRIN BINDING IN PARKINSON'S DISEASE

4.1 INTRODUCTION

Metals have been known to be involved in the aetiology of idiopathic PD since the demonstration that the metal manganese induces secondary Parkinsonism in man (Chandra *et al* 1974). Evidence suggests that both iron (Fe) and aluminium (Al) could be involved in the disease. Fe is increased within the substantia nigra of PD patients (Dexter *et al* 1989b, Sofic *et al* 1991) and is present at high levels within substantia nigral Lewy bodies (Hirch *et al* 1991). Furthermore, the intranigral injection of Fe in rats induced a depletion of striatal dopamine resulting in a reduction in spontaneous movements and episodes of motor freezing (Ben-Shachar and Youdim 1991). In addition, Al has been reported to be elevated in the substantia nigra, caudate nucleus and globus pallidus of PD patients compared to controls (Yasui *et al* 1992a) and is present within the Lewy bodies of PD patients (Hirch *et al* 1991). It is also possible that a deficiency of an essential metal could be involved in the pathogenesis of PD. Magnesium (Mg) levels are reduced in the substantia nigra, globus pallidus and caudate nucleus in PD patients compared to controls (Yasui *et al* 1992a) and it has been reported that a deficiency of Mg may result in CNS degeneration in rodents (Yasui *et al* 1990).

The binding of ^{67}Gallium (^{67}Ga) to the plasma protein transferrin has been employed initially as a marker to study Tf binding to Al (Cochran *et al* 1983, Farrar *et al* 1990) but may also reflect Tf binding to other metals. Ga-Tf binding has been investigated in a number of neurological disorders. The binding of Ga to Tf has been reported to be reduced in the plasma of Alzheimer's disease (AD) and Down's syndrome patients (Farrar *et al* 1990, Hodgkins *et al* 1993). Ga-Tf binding has also been found to be depressed in PD patients, especially prior to drug therapy (Hodgkins 1992, Winsper *et al* 1994).

A reduction in Tf binding may result in increased binding of the metal to a low molecular weight (LMW) molecule. Reductions in metal binding to Tf could have several neurological consequences and hence be involved in the pathogenesis of the disease. A

decrease in Tf binding could lead to the increased entry of a toxic LMW species into the brain. Alternatively, a decrease in binding to Tf could limit the transport of an essential metal into the brain via the Tf receptor system. It is also possible that reduced Ga-Tf binding occurs as a consequence rather than a primary cause of the disease process.

4.1.1 AIMS OF THE CHAPTER

The objectives of this study were to test the hypothesis that defective metal binding to Tf is involved in the pathogenesis of PD by studying the binding of Fe and Ga to Tf in plasma taken from controls, untreated and treated PD patients.

4.2 METHODS

4.2.1 MATERIALS

All chemicals used were of general grade and were supplied by: Sigma Chemical Company Ltd (Poole, UK); Aldrich Chemical Company (Gillingham, UK); BDH Chemicals Ltd (Poole, UK). Radio-isotopes were supplied by Amersham International PLC (Aylesbury, UK). Distilled water was used to make up all solutions.

4.2.1.1 RADIO-ISOTOPES

^{67}Ga (half life 78 hours) was supplied as $^{67}GaCl_3$ in 0.4 M HCl. ^{59}Fe was supplied as $^{59}FeCl_3$ in 0.1 M HCl. ^{67}Ga activity was measured by counting gamma emissions over the energy range 50-420 KeV for 60 seconds with automatic correction for radioactive decay. ^{59}Fe was also counted for gamma emissions over the energy range 970-1450 KeV for 60 seconds (LKB 1282 Compugamma counter, Pharmacia-LKB, Milton Kenes, UK).

4.2.2 CONTROL AND PATIENT STUDY GROUPS

A control group without neurological disease comprised 12 healthy volunteers living in the community and 5 cancer patients courtesy of Dr Leeming (General Hospital, Birmingham).

Plasma from untreated PD patients was obtained from Dr. H. Pall (Consultant Neurologist, Queen Elizabeth Hospital, Birmingham) at the time of clinical diagnosis before the start of drug therapy. All patients included in the experimental group displayed at least two of the three classical symptoms of PD (tremor, rigidity and bradykinesia) and subsequently responded to levodopa therapy. The severity of disease varied between stages 1-4 according to the Hoehn and Yahr's classification (Hoehn and Yahr 1967).

In addition, blood samples from treated PD patients, who were receiving anti-Parkinsonian drugs, were obtained from an outpatient clinic courtesy of Dr. H. Pall. For the Fe-Tf binding studies, the 4 treated patients were receiving L-Dopa. For the Ga-Tf binding studies, 26 of the 28 treated patients were receiving L-Dopa therapy (sinemet or madopar), 15 of these were also receiving a MAO B inhibitor (selegiline) and 2 patients were receiving benzhexol alone. Details of the patients and controls used in the Fe and Ga-Tf binding studies are given in tables 4.1 and 4.2.

Table 4.1 Composition of the Fe-Tf study group.

patient group	N	male: female	age (mean ± SD)	age range
controls	6	2:4	30.3 ± 8.3	22-41
untreated PD	6	5:1	64.8 ± 5.9	58-73
treated PD	4	3:1	62.5 ± 6.7	57-72

Table 4.2 Composition of the Ga-Tf study group.

patient group	N	male: female	age (mean ± SD)	age range
controls	17	4:13	38.6 ± 18.7	17-81
untreated PD	26	13:13	63.2 ± 10.5	40-80
all treated PD	28	11:17	66.3 ± 10.9	43-89
PD treated with L-Dopa	11	2:9	70.3 ± 11.3	52-89
PD treated with L-Dopa + selegiline	15	8:7	65.2 ± 9.1	46-76

4.2.2.1 THE UNIFIED PARKINSON'S DISEASE RATING SCORE (UPDRS)

The untreated PD patients were assesed according to the first stage of the unified Parkinson's disease rating score (UPDRS). The first section of the UPDRS consists of 31 items referring to symptoms and signs of the disease and assesses 3 aspects; 1) Mentation, behaviour and mood; 2) Activities of daily living and 3) Motor function. Each item is given a score between 0 and 4 (4 referring to the most severe cases) giving a possible total score of 124. 15 of the untreated PD patients were scored according to section 1 of the UPDRS. The UPDRS system is included in appendix 1.

4.2.3 PLASMA PREPARATION

Blood samples were collected in lithium heparin tubes and plasma obtained by centrifugation at 2000g for 15 minutes at room temperature (MSE Minor centrifuge, MSE Ltd, Crawley, UK). All plasma samples were stored at -20^{o}C prior to use.

4.2.4 GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography is a technique employed to separate solutes depending upon molecular size. Gel filtration chromatography has been demonstrated to be a suitable technique to study the binding of Al (and Ga) to serum proteins (Cochran *et al* 1983, Trapp 1983, Favarato *et al* 1991).

The binding of ^{67}Ga and ^{59}Fe to transferrin in human plasma was analysed by gel filtration chromatography using Sephadex G75 beads (Sigma Chemical Company Ltd, Poole, UK) in a 60cm by 1.6cm glass column (Amicon Ltd, Stonehouse, UK). The dry beads were swollen in excess elution buffer (25mM Tris-HCl, 100mM NaCl, 25mM Na_2HCO_3, pH 7.4) for 48 hours and then degassed by purging hellium through for 30 minutes. The column was packed at a flow rate of 2mls/min and equilibriated by running elution buffer through for 3 hours at a rate of 1ml/min. Due to the dissociation of bicarbonate, it was necessary to replace the buffer with a fresh solution every 3 hours. Fresh buffer was run through the column for 1 hour prior to the experiment to replace the bicarbonate and to

clear the columns of any remaining activity.

4.2.4.1 PROCEDURE

To investigate Ga-Tf binding, 900µl of plasma was incubated with 1-5µCi of ^{67}Ga and a "cold" $Ga(NO_3)_3$ solution giving a final concentration of Ga equal to 10µg/l, a concentration similar to the level of Al present in the serum (Shore and Wyatt 1983). This was placed in a shaking water bath (60 osillations per min) at 37°C for 1 hour then applied to the Sephadex G75 column and eluted upwards at a rate of 1ml/min using the elution buffer. Fifty 6ml fractions were collected from the column (LKB Redirac fraction collector, LKB Pharmacia Ltd, Milton Keynes, UK) and counted for gamma emissions.

To investigate Fe-Tf binding, 900µl of plasma was incubated with 1-5µCi of ^{59}Fe and applied to the G75 column following the same procedure as for ^{67}Ga.

4.2.5 EFFECT OF % SATURATION ON FE-TF BINDING

The estimated % saturation of transferrin with iron was increased from 30% to 50%, 75% and 100% by the addition of $FeCl_3$ to the plasma prior to incubation with ^{59}Fe and separation on a G75 column as described in section 4.2.4.1.

4.2.6 STATISTICAL ANALYSIS

Data were presented as the mean of each patient group ± standard deviation (SD). Data analysis was by analysis of variance (ANOVA) followed by a Fischer t-test to compare the differences between patient groups. This uses a slightly higher level of probability than a conventional t-test because the analysis make multiple comparisons between groups (Snedecor and Cochran 1980). The variance ratio was used to test the difference in variance between patient groups. Differences between males and females within a patient group were analysed via a two-tailed unpaired Student's t-test. The degree of correlation between metal binding and patient age was assessed by correlation and regression methods.

4.3 RESULTS

4.3.1 FE-TF BINDING

4.3.1.1 FE-TF BINDING IN CONTROL PLASMA

Figure 4.1 shows a typical elution profile obtained when control plasma incubated with ^{59}Fe was applied to a G75 column. A single peak, ≥ 75 kDa, was eluted in the void volume (fractions 9-10) and is believed to correspond to Fe bound to Tf. In the control group, Fe-Tf binding was 100% in all individuals. The mean % of radioactivity recovered from the column was 82.3 ± 5.63, with a range of 72-88%.

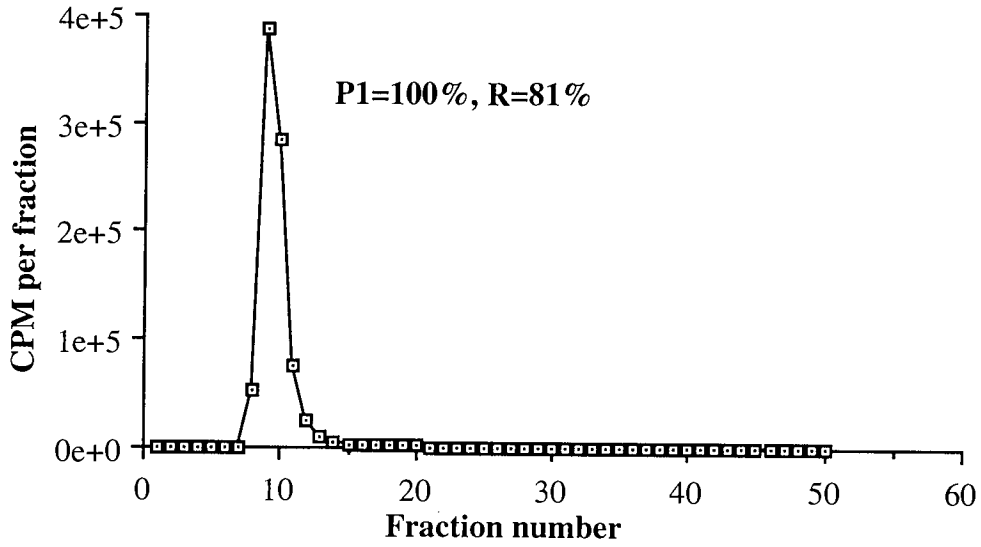


Figure 4.1 Fe-Tf binding in control plasma.

4.3.1.2 FE-TF BINDING IN PARKINSONIAN PLASMA

Figures 4.2 and 4.3 show the speciation of Fe in untreated and treated Parkinsonian plasma. In all untreated and treated PD patients Fe-Tf binding was 100%. The estimated radioactivity recovered from the columns was between 80% and 90%, the mean recovery was 84.8 ± 3.08.

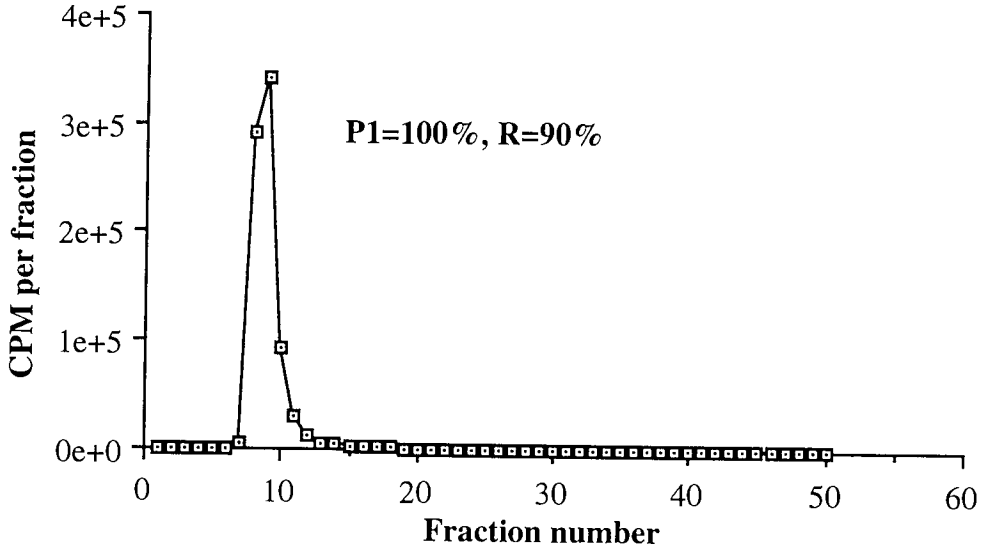


Figure 4.2 Fe-Tf binding in untreated PD plasma.

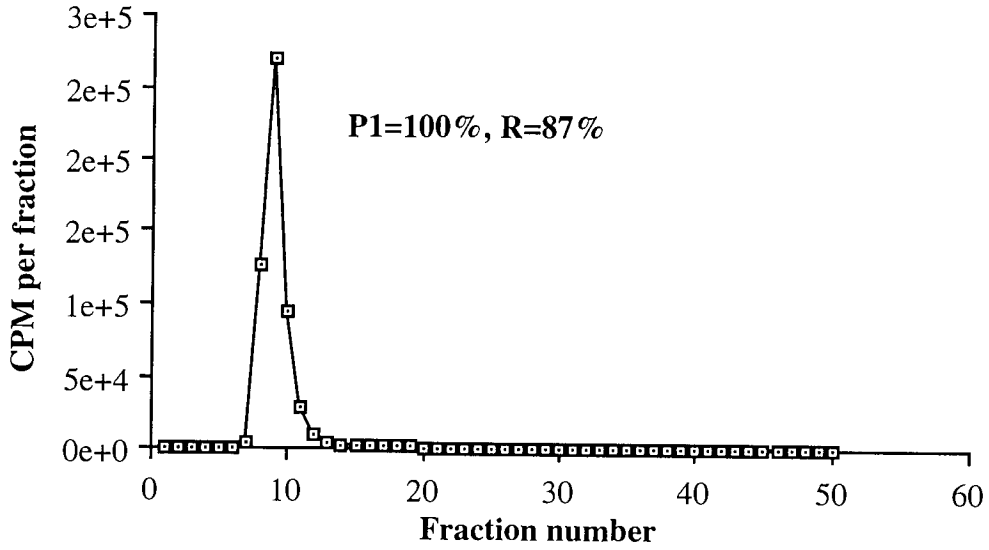


Figure 4.3 Fe-Tf binding in treated PD plasma.

4.3.1.3 EFFECT OF INCREASING FE-TF SATURATION

The estimated saturation of Tf with Fe was increased to 100% in 3 controls and 3 PD patients. Fe-Tf binding remained 100% in two of the controls. In the third control, some binding to a low molecular weight species was observed, Fe-Tf binding equalling 93%. In all 3 PD patients Fe-Tf binding remained 100%.

4.3.2 GA-TF BINDING

4.3.2.1 CHANGES IN GA-TF BINDING WITH TIME

Table 4.3 Changes in Ga-Tf binding over time.

	sample 1	sample 2
control 1	27.5.94 : 60%	27.7.94 : 59%
control 2	25.5.94 : 73%	7.7.94 : 74%
control 3	23.5.94 : 75%	2.8.94 : 68%
PD 1	3.9.93 : 63%	7.6.94 : 71%
PD 2	7.5.93 : 62%	7.6.94 : 76%
PD 3	7.5.93 : 72%	5.11.93 : 72%
PD 4	26.10.92 : 40%	3.9.93 : 32%

Table 4.3 shows changes in Ga-Tf binding with time within individual control and PD patients. Patients 1, 2 and 3 were receiving therapy at both sample dates. Patient 4 was untreated at the first sample date but was receiving therapy at the second date. The results indicate some variation in Ga-Tf binding with time. In control subjects, Ga-Tf binding values varied by as little as 1%. The PD patients who had received therapy at both sample dates showed an increase in binding (PD1 and PD2) or no change in binding (PD3).

4.3.2.2 GA-TF BINDING IN CONTROL PLASMA

Figure 4.4 shows a typical elution profile obtained when control plasma was applied to a G75 column. Peak 1, a high molecular weight form (≥75KD) eluted at fractions 9-10, is believed to be Ga-Tf. Binding activity was also observed between fractions 20 and 24 and represents a low molecular weight species that remains unidentified. The data confirm the results of Farrar *et al* (1990) and Hodgkins (1992). The % recovered radioactivity was between 77 and 95%, mean recovery was 84.5±6.4.

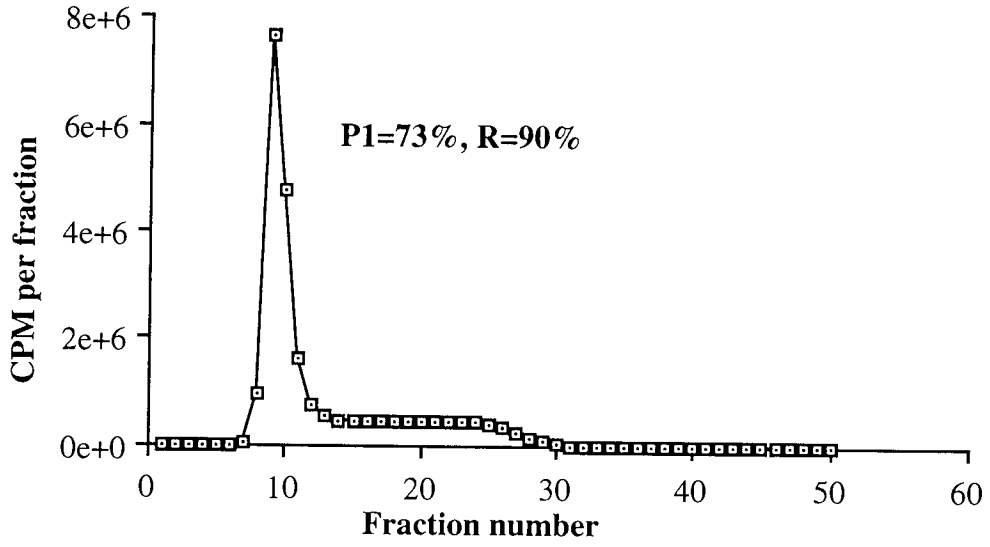


Figure 4.4 Ga speciation in control plasma.

The mean % binding of Ga to Tf (peak 1 as a % of total activity) in the control group was 73.16 ± 5.77. % Ga-Tf binding ranged from 60-82%.

4.3.2.3 GA-TF BINDING IN UNTREATED PD PLASMA

In all untreated PD plasma, a peak of radioactivity occured at fractions 9-10 and is believed to be Ga-Tf. In the majority of individuals, a second region of activity occured between fractions 20-24. However, in a number of PD patients there were low levels of binding to Tf (in 10 patients less than 50% of the Ga was bound to Tf) and a second peak of activity was observed corresponding to high levels of binding to a LMW species. This peak occured between fractions 14 and 24, the exact location of the peak varied between patients. In 3 PD patients, two distinct LMW peaks were observed. Figure 4.5 shows the elution profiles obtained when the plasma from two untreated PD patients incubated with ^{67}Ga was applied to a G75 column. A large variation in % binding was observed in the PD group and it was possible to separate experimental subjects into "high" and "low" binders. Figure 4.6 shows an elution profile obtained with a low Tf binder in which two LMW peaks occured at fractions 14 and 23. The mean recovered radioactivity in the untreated PD group was 89.5 ± 6.2 with a range of 81-99%.

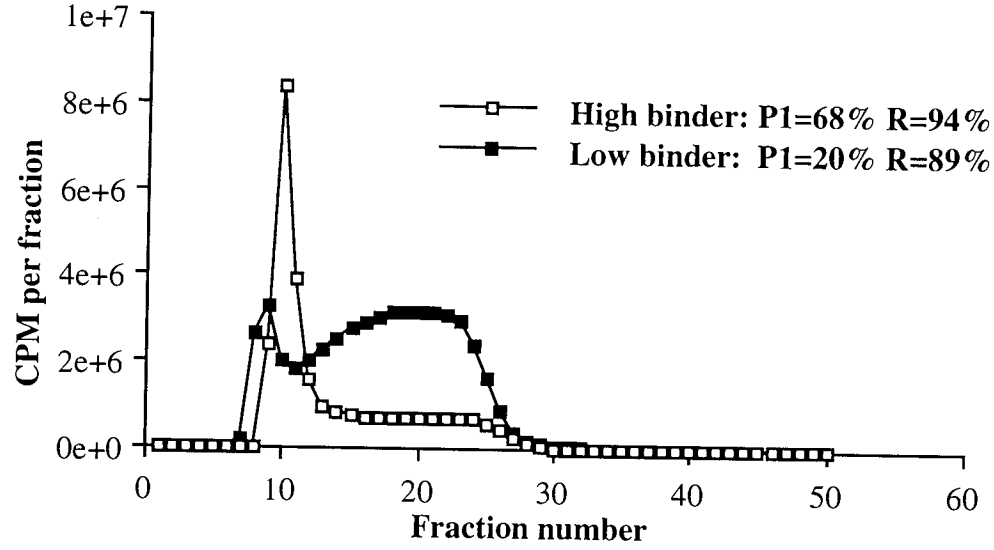


Figure 4.5 Ga speciation in untreated PD plasma.

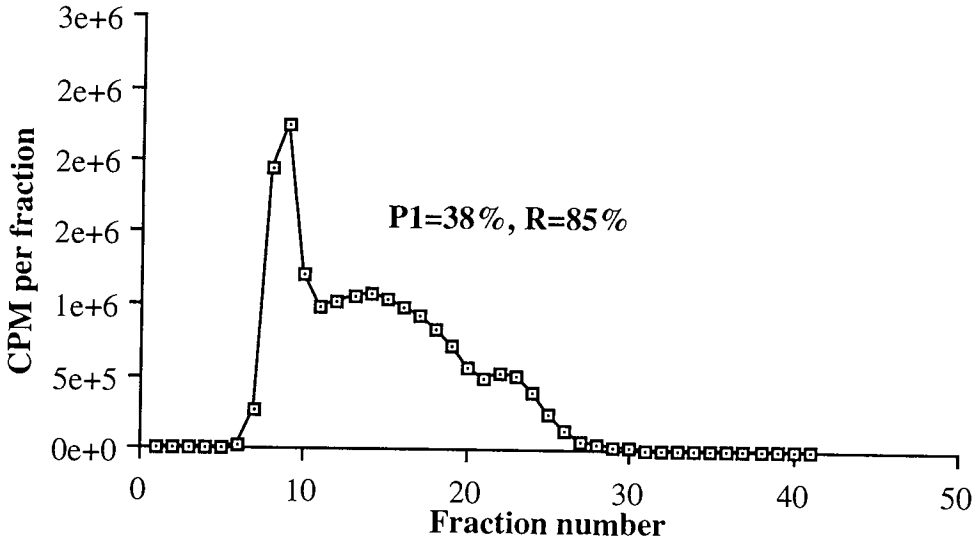


Figure 4.6 Ga speciation in an untreated PD patient with "low binding".
Peak 1 represents Ga binding to Tf, peaks 2 and 3 represent binding to a LMW species.

Mean % Ga-Tf binding in untreated Parkinsonian plasma was 52.15 ± 15.73. Values ranged between 25 and 78%.

4.3.2.4 THE RELATIONSHIP BETWEEN UPDRS AND GA-TF BINDING

The unified Parkinson's disease rating score (UPDRS) was found to be negatively correlated with % Ga-Tf binding in 16 untreated PD patients ($r=-0.61$, $p<0.02$) (figure 4.7).

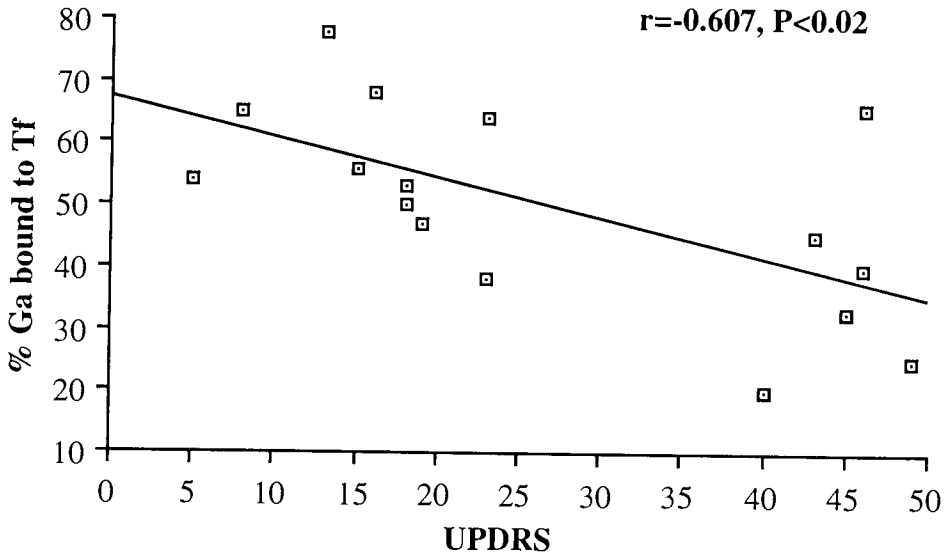


Figure 4.7 The relationship between Ga-Tf binding and UPDRS in untreated PD patients.

4.3.2.5 GA-TF BINDING IN TREATED PD PLASMA

A peak corresponding to Ga-Tf was eluted at fractions 9-10 in all treated PD patients. In the majority of patients, binding activity was also observed between fractions 20 and 24 representing a LMW species. However, in one patient with low binding to Tf, a LMW peaks was seen between fractions 14 and 16. Recovered radioactivity ranged from 81% to 99%, the mean % recovery was 87.9 ± 9.0.

The mean % binding of Ga to Tf in treated PD patients was 65.48 ± 9.02. Binding values ranged from 32% to 76%.

4.3.2.6 COMPARISONS BETWEEN PATIENT GROUPS

Differences in mean % Ga-Tf binding over the study population in table 4.4 (i.e. controls, untreated and treated PD patients) were significant ($p<0.001$).

Ga-Tf binding was found to be significantly reduced ($p<0.05$) in the untreated PD patients (52.2 ± 15.7, n=26) compared to the control group (73.2 ± 5.8, n=17). Ga-Tf binding was also found to be significantly depressed ($P<0.05$) in the treated PD group (65.5 ± 9.0, n=28) compared to the controls (73.2 ± 5.8, n=17). However, there was a significant

increase in Ga-Tf binding in treated PD patients compared to untreated PD patients ($p<0.05$).

Table 4.4 Ga-Tf binding in controls, untreated and treated PD patients.

	control	untreated PD	treated PD
% Ga-Tf binding	73.2±5.8 (n=17)	52.2±15.7 (n=26)*	65.5±9.0 (n=28)*#
Binding range	60-82	32-76	25-78

Values represent means ± SD, n=number of observations, *$p<0.05$ compared to controls; #$p<0.05$ compared to untreated PD.
Statistical analysis: ANOVA (1 way): Effects of treatment, $F=18.94$, $p<0.001$. Comparison between means (Fisher's t-test): Untreated PD vs control $t=7.13$ $p<0.05$; Treated PD vs control $t=7.03$, $p<0.05$; Treated PD vs untreated PD $t=6.22$, $p<0.05$.

In addition, the data was analysed by dividing the treated PD patients into those receiving L-Dopa (sinemet/madopar) and those receiving L-Dopa plus selegiline (table 4.5).

Table 4.5 Ga-Tf binding in PD patients treated with L-Dopa only and L-Dopa plus selegiline.

	control	untreated PD	PD treated with L-Dopa	PD treated with L-Dopa + selegiline
% Ga-Tf binding	73.2 ± 5.8 (n=17)	52.2 ± 15.7 * (n=2)	65.6 ± 6.3 # (n=11)	64.9 ± 11.9 * # (n=15)

Values represent means ± SD, n=number of observations, *$p<0.05$ compared to controls; #$p<0.05$ compared to untreated PD.
Statistical analysis: ANOVA (1 way): Effects of treatment, $F=11.96$, $p<0.001$. Comparison between means (Fisher's t-test): Untreated PD vs control $t=7.28$ $p<0.05$; L-Dopa vs control $t=9.03$, $p>0.05$; L-Dopa + selegiline vs control $t=8.26$, $p<0.05$. L-Dopa vs untreated PD $t=8.39$, $p<0.05$; L-Dopa + selegiline vs untreated PD $t=7.56$, $p<0.05$. L-Dopa vs L-Dopa + selegiline $t=9.26$, $p>0.05$.

Mean Ga-Tf binding values were found to differ significantly ($p<0.001$) between the patient groups shown in table 4.5 (i.e. controls, untreated PD, PD treated with L-Dopa and PD treated with L-Dopa + selegiline). However, there was no significant difference in Ga-

Tf binding between PD patients treated with L-Dopa alone (65.5 ± 6.3, n=11) and patients treated with L-Dopa plus selegiline (64.9 ± 11.9, n=15).

4.3.2.7 VARIATION WITHIN PATIENT GROUPS

As shown in table 4.4 the range of % Ga-Tf binding values varied between the patient groups. The most limited range was seen in the control group with binding figures of 60-82%. In treated PD patients, values varied from 32-76% while the untreated PD group demonstrated the greatest range of binding figures from 25-78%. Figure 4.8 shows the variation in % binding within each patient group.

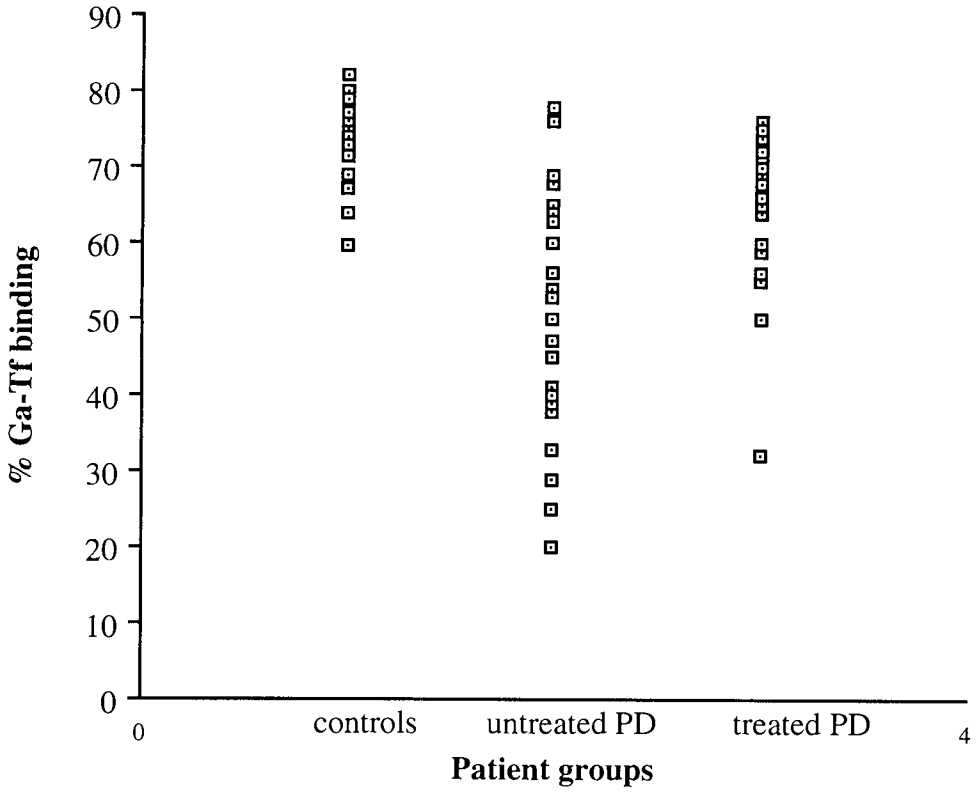


Figure 4.8 Variation in % Ga-Tf binding within the patient groups.

The untreated PD group was found to have significantly different variance compared to the controls ($F=7.43$, $p<0.05$). The variance of the treated PD patients did not differ significantly in comparison to controls ($F=2.44$, $P>0.05$).

4.3.2.8 EFFFECT OF AGE AND SEX UPON GA-TF BINDING

There was no significance difference in Ga-Tf binding between males and females in controls, untreated PD or treated PD patients (table 4.6).

Table 4.6 Ga-Tf binding in males and females in controls, untreated and treated PD patients.

	% Ga-Tf binding males	% Ga-Tf binding females
controls	70.4 ± 8.6 (n=4)	74.02 ± 4.8 (n=13)
untreated PD	53.8 ± 11.2 (n=13)	50.5 ± 19.6 (n=13)
treated PD	64.6 ± 6.4 (n=11)	66.1 ± 10.5 (n=17)

Values represent means ± SD, n=number of observations.
Statistical analysis: Comparison between males and females, unpaired Student's t-test: Controls t= 0.81, p>0.05; Untreated PD t= 0.516, p>0.05; Treated PD t= 0.48, p>0.05.

Ga-Tf binding was not correlated with age in controls, untreated PD or treated PD patients (figures 4.9 a, b, c).

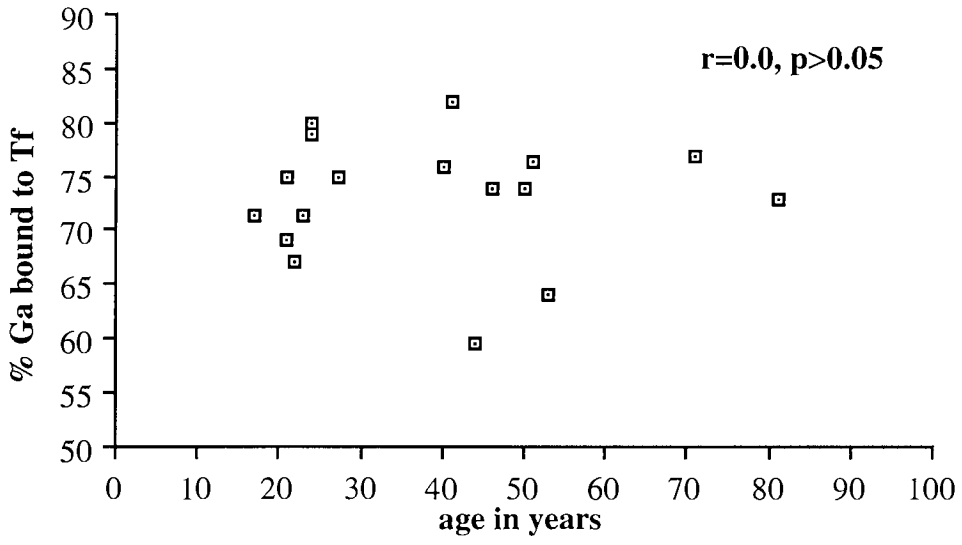


Figure 4.9a The relationship between Ga-Tf binding and age in controls.

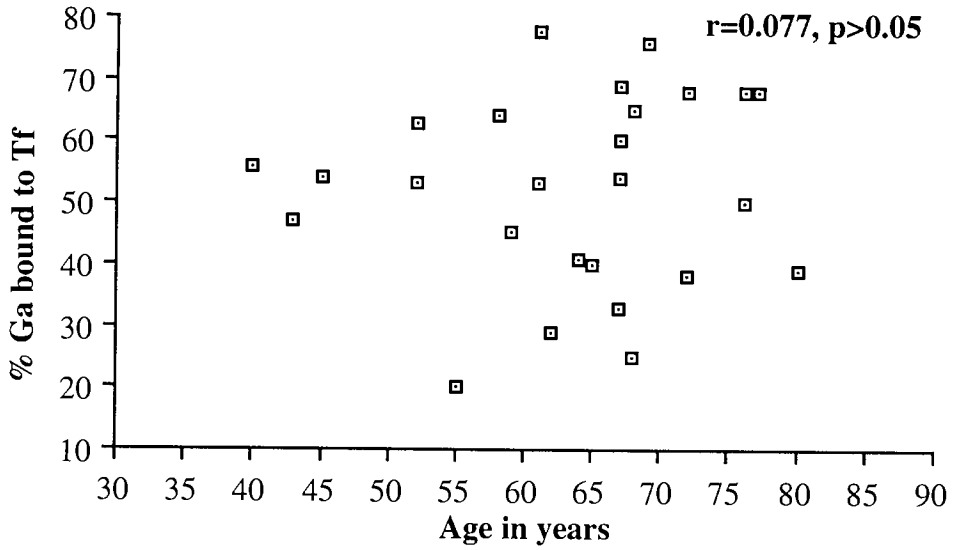


Figure 4.9b The relationship between Ga-Tf binding and age in untreated PD patients.

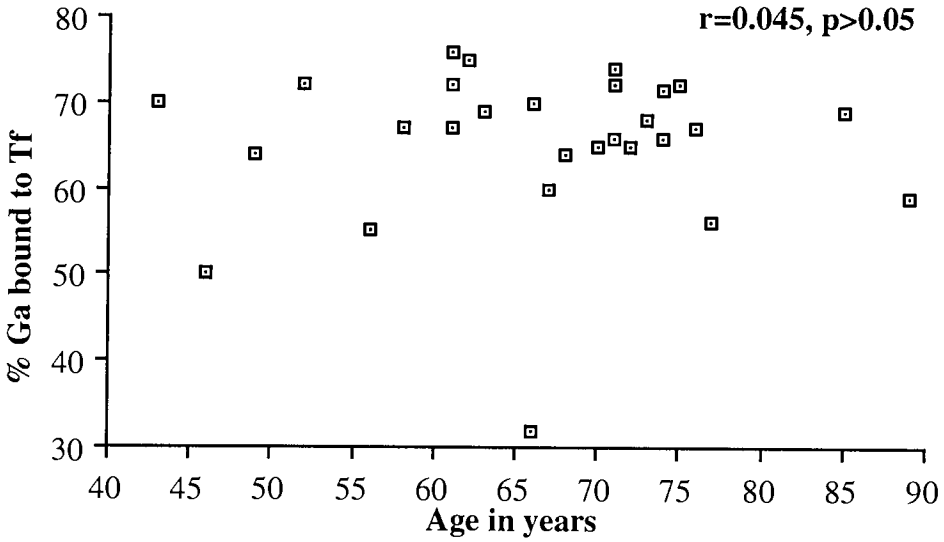


Figure 4.9c The relationship between Ga-Tf binding and age in treated PD patients

4.4 DISCUSSION

4.4.1 FE-TF STUDIES

The binding of Fe to Tf was found to be 100% in all controls, untreated and treated PD patients. Furthermore, increasing the saturation of Tf with Fe to approximately 100% by adding $FeCl_3$ to the plasma, had little or no effect upon Fe-Tf binding in any of the patient

groups. The results support the findings of Winsper *et al* (1994) and Hodgkins (1992) who previously reported that Fe-Tf binding was 100% in both controls and PD patients. In addition, Fe-Tf binding was found to exceeded 97% in controls and AD patients and was unaffected by the degree of Tf saturation with Fe, changes in pH and the addition of citrate to the serum (McGregor and Brock 1992, McGregor *et al* 1991). The results suggest that within Parkinsonian plasma, Fe exists largely in complex with Tf to which it is very tightly bound. These findings would not be consistent with the hypothesis that brain accumulation of Fe, as a result of an increase in a LMW species, is involved in the aetiology of PD.

4.4.2 GA-TF STUDIES

In the absence of a suitable isotope of aluminium, ^{67}Ga was used as a marker for aluminium binding in plasma. ^{67}Ga has been found to be a suitable analogue of Al in investigations of Al speciation within the plasma (Cochran *et al* 1983, Farrar *et al* 1990) and Ga-Tf binding has been found to correlate significantly with Al-Tf binding (Hodgkins *et al* 1993).

Repetition of measurements in individual controls showed relatively little variation in Ga-Tf binding values although there was a time lapse of several months between samples and the runs were carried out on different columns. This indicates that the measurement was reliable and did not vary greatly over time.

In all controls and PD patients, a high molecular weight species was eluted at fractions 9-10 on a G75 column. This species is believed to be Ga bound to Tf, the principle binding protein for both Ga and Al (Trapp 1983, Martin *et al* 1987, Harris 1992). A region of activity corresponding to a LMW Ga species was also observed between fractions 20 and 24 in all controls and the majority of PD patients. The binding of Ga to Tf and a LMW species within the plasma has been previously reported by Hodgkins *et al* (1993) and Farrar *et al* (1990). In a number of PD patients, who demonstrated low Ga-Tf binding values, a defined peak was seen between fractions 14 and 24 corresponding to high levels of binding to a LMW ligand. The location of this LMW peak varied between patients. At present the LMW species obtained have not been identified. Citrate has been implicated as

the most probable LMW species to bind Al within the blood (Van Ginkel *et al* 1990, Fatemi *et al* 1991, Martin *et al* 1987). However, Hodgkins *et al* (1993) reported that Al bound citrate species were absent in control and Down's syndrome plasma. Using a computer model, Harris (1992) predicted that the primary LMW ligand to bind Al would be phosphate with minor amounts binding to citrate and hydroxide. In addition, Hodgkins *et al* (1993) identified phosphate and silicate as the predominant non-Tf bound Ga species within control and Down's syndrome plasma. It is possible that Ga (and by implication Al) is bound to a number of different LMW ligands. In low binders for example, the LMW species was eluted at several different positions and in a few individuals two distinct LMW peaks were observed. The identity of the LMW species formed as a consequence of reduced binding could be important in determining whether the metal is transported into the brain.

In control plasma, Ga was found to exist mainly as a Tf bound species with minimal binding to a LMW ligand. This supports the findings of Hodgkins *et al* (1993) and Farrar *et al* (1990). The control group included healthy individuals and cancer patients. The cancer patients were included in the control group as they had no neurological symptoms and there is no evidence that cancer affects Ga-Tf binding. There was little variation in Ga-Tf binding within the control group indicating that illness or hospitalisation (as in the case of cancer patients) alone did not affect Ga-Tf binding.

In plasma from untreated PD patients, Ga-Tf binding was significantly reduced compared with controls. Previous studies have reported a decrease in Ga-Tf binding in untreated PD patients (Winsper *et al* 1994, Hodgkins 1992). Reduced Ga-Tf binding has also been reported in Down's Syndrome (Hodgkins *et al* 1993) and Alzheimer's Disease patients (Farrar *et al* 1990, Brammer *et al* 1990), although the later finding is controversial and could not be confirmed by McGregor *et al* (1991) and Taylor *et al* (1991).

Within the untreated PD group there was a wide range of binding values, the variance being significantly different from controls. Subsequently, individuals could be classified into those with a high or low binding of Ga to Tf. This phenomenon may occur for a number of reasons. Firstly, PD could arise from a number of different causes. In low

binders, reduced metal binding could play a role in the aetiology of the disease while in high binders some other cause could be involved. Secondly, if reduced binding is merely a consequence of the underlying disease process, binding levels could be related to the progression of the disease, low binders being at a later stage than high binders. However, the lack of correlation between binding and age would not support the later hypothesis.

Ga-Tf binding was also found to be significantly decreased in treated PD patients compared to controls. However, treatment appeared to increase binding towards control levels, Ga-Tf binding being significantly greater in the treated PD patients compared to the untreated PD group. Furthermore, two patients receiving therapy showed an increase in binding over time which could have occurred due to the effects of treatment. The results are supported by the findings of Hodgkins (1992) who demonstrated significantly higher levels of Ga-Tf in treated PD compared to untreated PD patients. The fact that therapy may affect binding suggests that Ga-Tf binding could be involved in the pathogenesis of PD. Farrar (1991) demonstrated the inhibition of the oxidation of 5-methyltetrahydrofolate in the presence of L-Dopa which was proposed to be due to the ability of L-Dopa to combine with free radicals to preventing further oxidation. Increased oxidation within the circulation has been implicated as a cause of reduced Ga-Tf binding (Hodgkins *et al* 1993, Winsper *et al* 1994)) therefore if L-Dopa inhibited oxidative stress it would be expected to improved Ga-Tf binding. In addition, the drug selegiline is an inhibitor of the enzyme monoamine oxidase B (MAO B) and therefore has the effect of reducing the production of oxidising species. L-Dopa has also been reported to influence the immune response. PD patients treated with L-Dopa were found to demonstrate increased IL-1 synthesis and IgM and IgG levels within the plasma (Fiszer *et al* 1994). In addition, the intra-peritoneal administration of dopamine in mice depressed the antibody response and delayed hypersensitivity reaction to sheep red blood cells and resulted in a decrease in spleen T-cell numbers. *In vitro*, lymphocyte proliferation and the generation of cytotoxic T-cells in response to allogenic stimulator cells were decreased by L-Dopa (Boukhris *et al* 1987, Kouassi *et al* 1987). Therefore if the immune response is responsible for increased oxidation, L-Dopa could act by reducing the degree of the immune reaction. However, it is also possible that the level of Tf binding relates to the disease stage.

No significant differences in the effects of L-Dopa and selegiline upon Ga-Tf binding were observed. This supports the finding that the long-term use of selegiline has minimal benefits compared with L-Dopa alone (Brannan and Yahr 1995).

4.4.2.1 IMPLICATIONS OF REDUCED GA-TF BINDING

Throughout these studies ^{67}Ga has been used as a marker for Al. Thus we are making implications about Al-Tf binding from results obtained with ^{67}Ga. The affinity of a metal will influence its binding to Tf. For example, Ga is bound more weakly to Tf than Fe as the stability constants for Ga-Tf (log K_1=20.3; log K_2=19.3) are less than those for Fe-Tf (K_1=22.5; log K_2=21.4) (Martin *et al* 1987). As a result, Ga-Tf binding is more labile than Fe-Tf binding and is susceptible to changes in pH and competing chelators (McGregor and Brock 1992). The lower stability constants of Ga-Tf explains why Ga-Tf binding was reduced in PD plasma, but Fe-Tf binding remained unaltered. The stability constants of Al-Tf are log K_1=12.9, log K_2=12.3 (Martin *et al* 1987). Consequently, Al is more likely to be liberated from Tf than Ga and Al-Tf binding may be considerably lower than the values stated for Ga-Tf binding. If the capacity of Tf to bind metals is reduced, those metals with lower binding constants that Fe, namely Ga and Al, would be expected to be released first.

The reduced binding of Ga to Tf in PD may reflect a decrease in the binding of Tf to a variety of metals within the plasma. A decrease in Tf metal binding and a subsequent increase in a LMW form could have a number of implications. It has been suggested that reduced Tf binding leads to a decrease in the transport of essential metals into the brain via the Tf system (Hodgkins *et al* 1993). A deficiency of essential metals has been implicated in a number of neurological diseases. For example, in AD a depression of zinc (Zn) within the hippocampus has been reported (Constantinidis 1991). Tf is believed to play a considerable role in the serum transport of Zn (Harris and Keen 1989). Furthermore, Zn is known to have a relatively low Tf binding constant (log K_1=7.42, log K_2=6.0) (Harris 1992) so would be displaced from Tf relatively easily (Hodgkins *et al* 1993). However, a defect in brain uptake of zinc is not supported by the finding that Zn is increased in the substantia nigra, caudate nucleus and putamen in PD (Dexter *et al* 1989b, Dexter *et al*

1993), although this refers to total zinc and may not reflect Zn levels within specific types of cell. In addition, a reduction in brain magnesium (Mg) has been implicated in the pathogenesis of PD (Yasui *et al* 1992a) and amyotrophic lateral sclerosis (ALS) (Yasui *et al* 1992b). However, there is little evidence that plasma Mg is transported by Tf as within the serum the protein bound Mg is mainly attached to albumen with the rest being bound to globulins (Kroll and Elin 1985). Therefore reduced Tf binding is unlikely to account for a Mg deficiency within the brain.

Conversely, a reduction in Tf binding could enhance the entry of toxic metals into the brain. Tf may have a protective effect by regulating the brain uptake of certain metals. For example, in the rat, the binding of manganese (Mn) to Tf limits the transport of the metal across the BBB (Rabin *et al* 1993). Reduced metal Tf binding results in an increase in a LMW species which could enter the CNS unhindered providing a mechanism for the accumulation of the metal within the brain (Farrar *et al* 1990). Indeed, in hypotransferrinaemic mice, there was greatly enhanced brain uptake of iron indicating that non-Tf bound Fe readily crosses the BBB (Ueda *et al* 1993). Furthermore, it has been suggested that a LMW form of Al, present in increased amounts within the plasma of haemodialysis patients, is neurotoxic and readily crosses the BBB (King *et al* 1982). The identity of the LMW species may determine whether brain entry of the metal is increased. Slanina *et al* (1985) demonstrated that citrate, a candidate for a LMW Al species, increased Al absorption from the gut and resulted in its accumulation within the tissues. It has been suggested that the Al citrate complex in serum may be involved in the intracellular accumulation and toxicity of Al (Van Ginkle 1990). By contrast, the binding of Al to silicon may possibly protect against Al accumulation in the brain (Carlisle 1986). The results indicated that a number of different LMW species may occur within Parkinsonian plasma. In low binders, an additional toxic LMW species could occur which is not present in high binders.

It is also possible that reduced Tf binding is secondary to an earlier pathogenic event, for example, increased oxidation or immune activation. However, the fact that in untreated PD patients Ga-Tf binding was found to be negatively correlated with the UPDRS (a measure

of disability) indicates that a reduction in Tf binding may be an important factor in the pathogenic process.

4.4.2.2 WHY IS GALLIUM TRANSFERRIN BINDING REDUCED IN THE PLASMA OF PD PATIENTS ?

The results demonstrated that Ga-Tf binding was not correlated with age in controls or PD patients. Furthermore, there were no differences in Ga-Tf binding values between the sexes. Therefore, the reduced Tf binding observed in PD patients was not due to an age or sex effect.

A number of factors have been found to influence Ga-Tf binding. Biochemical alterations within the plasma may result in a reduction in Tf binding, although there is no evidence that such changes occur in PD. In order to bind metals optimally, Tf requires the bicarbonate anion (Aisen *et al* 1967). Ga-Tf binding is dependent upon the level of bicarbonate available with maximum binding occurring at the normal physiological level of 25mM (Hodgkins 1992). Deviation from physiological pH has been shown to reduce Ga-Tf binding in plasma (Hodgkins 1992, McGregor and Brock 1992). In addition, changes in the electrolytic composition of plasma can have an effect. The addition of Fe and Al to plasma was found to result in a reduction in Ga-Tf binding (Hodgkins 1992). The presence of a high affinity LMW ligand such as citrate may also reducing metal Tf binding by competing with the Tf molecule (McGregor and Brock 1992).

A reduction in Tf levels within the plasma would reduce the availability of Tf to bind to metals. Tf levels are depressed in response to a number of circumstances including increased body iron stores, cirrhosis of the liver, protein malnutrition and haemolytic anaemia (Morgan 1974). In addition, Tf levels could be depressed in response to inflammation. The inflammatory response can stimulate changes in the concentration of a series of plasma proteins known as acute phase proteins (Kushner 1988). Tf is believed to be a negative acute phase protein therefore its plasma levels may fall in response to inflammation (Maes *et al* 1992). However, serum/plasma Tf levels have been reported to be unchanged in both AD (Corrigan *et al* 1992) and PD (Hodgkins 1992, Cabrera-Valdivia

et al 1994). It has also been suggested that a reduction in plasma Ga-Tf binding arises due to an increase in Tf saturation with Fe (Schulman and Ponka 1990). This is unlikely to be the case in PD as plasma Tf saturation did not differ in PD patients compared to controls. (Chen and Shih 1992, Hodgkins 1992).

It is also possible that an inherent defect in the Tf molecule is responsible for reduced Ga-Tf binding in PD. A variant of Tf has been described in human serum which binds iron abnormally at one site (Evans *et al* 1982). In addition, Hodgkins (1992) described a subject with Tf that was unable to bind iron on its C-terminal lobe due to the absence of tyrosine residues. It is possible that a functional defect of Tf could result in reduced metal binding (Farrar *et al* 1990). However, is unlikely that an inherent defect of Tf occurs in PD due to the lack of evidence for a genetic basis to the disease.

Alternatively, increased oxidation within the circulation could provide a mechanism whereby the Tf molecule is damaged thus reducing its metal binding capacity. Hence, the addition of oxidising agents to plasma has been found to decrease Ga-Tf binding *in vitro* (Hodgkins 1992). The excessive formation of reactive oxygen species within the substantia nigra in PD could occur due to increased metabolism of dopamine or by the actions of a neurotoxin (Jenner 1992). An alternative source of oxidising species is the immune response. It has been proposed that the activation of macrophages results in increased oxidative activity thereby reducing Ga-Tf binding in Parkinsonian plasma (Winsper *et al* 1994). Hodgkins *et al* (1993) also suggested that oxidation of Tf, occurring as a consequence of an immune response, results in reduced metal Tf binding in Down's syndrome. It is also possible that the immune response could be directed against the Tf molecule itself. An auto-immune form of atransferrinaemia has been described in which auto-antibodies against the Tf molecule are produced with the generation of circulating immune complexes of Tf and IgG (Westerhausen and Meurret 1977). The possible relationship between the immune response and Tf binding will be investigated in chapter 7.

CHAPTER 5: NEOPTERIN IN PARKINSON'S DISEASE

5.1 INTRODUCTION

Immunological factors may be important in the aetiology of a number of neurological diseases including Alzheimer's disease (Chapman *et al* 1988, McGeer *et al* 1991, Aisen and Davis 1994), Down's syndrome (Hodgkins *et al* 1993, Heinonen *et al* 1993, Armstrong *et al* 1994), amyotrophic lateral sclerosis (McGeer *et al* 1991), multiple sclerosis (Steinman 1993) and depression (Maes 1995).

Considerable evidence suggests that the immune response is involved in the aetiology of PD. The classical complement pathway appears to be activated within the substantia nigra of PD patients (Yamada *et al* 1992). Components of the cellular immune system have also been demonstrated within the Parkinsonian brain. T-cytotoxic/suppressor lymphocytes and HLA-DR positive microglia, which are capable of presenting antigens to T cells, have been detected within the substantia nigra of PD patients (Yamada *et al* 1992, McGeer *et al* 1988). In addition, gamma delta T+ cells (a sub-population of T-cells involved in infection and auto-immunity) have been found to be increased within the blood of PD patients compared to other neurological disease patients (Fiszer *et al* 1994). The humoral immune response has also been implicated in the pathogenesis of PD as anti-neuronal antibodies have been detected in the serum and CSF of PD patients (Husby *et al* 1977, Pouplard and Emile 1984, McRae-Deguerce *et al* 1986). Finally, tumour necrosis factor (TNF) α has been reported to be increased in the striatum and CSF of PD patients (Mogi *et al* 1994) and TNF immunoreactive glia have been detected in the Parkinsonian substantia nigra (Boka *et al* 1994).

Neopterin is a pteridine derived from guanosine triphosphate (GTP) during the biosynthetic pathway of biopterin. Large quantities of neopterin are released by macrophages on stimulation with interferon γ secreted by activated T cells (Huber *et al* 1984). Furthermore, neopterin is elevated in body fluids in a number of conditions in which the cellular immune response is involved i.e. viral infections, malignancies, allograft

rejections and auto-immune disorders (Wachter *et al* 1992). Neopterin can therefore be employed as a marker of a cell mediated immune response.

Neopterin has previously been reported to be elevated within body fluids in a number of neurological diseases. These include Alzheimer's disease, Down's syndrome (Armstrong *et al* 1994) and depression (Duch *et al* 1984, Dunbar *et al* 1992, Maes *et al* 1994). Furthermore, in a preliminary report, Winsper *et al* (1994) reported that serum neopterin levels were significantly elevated in untreated PD patients compared to controls.

5.1.1 AIMS OF THE CHAPTER

The objectives of this study were to extend the findings of Winsper *et al* (1994) and to determine whether levels of neopterin were influenced by treatment of PD patients.

5.2 METHODS

5.2.1 CONTROL AND PATIENT STUDY GROUPS

The control group comprised 20 healthy volunteers living in the community and a cancer patient courtesy of Dr Leeming (Department of Haematology, General Hospital, Birmingham). A second control group was also obtained from blood bank samples age and sex matched with the untreated PD patients courtesy of Professor Fuchs (Institute of Medicinal Chemistry and Biochemistry, University of Innsbruck, Austria).

Plasma from untreated PD patients was obtained from Dr. H. Pall (consultant neurologist, Queen Elizabeth Hospital, Birmingham) at the time of clinical diagnosis before the start of drug therapy. All patients in the experimental group displayed at least two of the three classic symptoms of PD (tremor, rigidity and bradykinesia) and subsequently responded to levodopa therapy. The severity of the disease varied between stages 1-4 according to the Hoehn and Yahr's classification (Hoehn and Yahr 1967).

Blood samples were also obtained from treated PD patients, who were receiving anti-Parkinsonian drugs, from an outpatient clinic courtesy of Dr. H. Pall. 28 of the 30 treated patients were receiving L-Dopa therapy (sinemet or madopar), 12 of these were also receiving a MAO B inhibitor (selegiline). In addition, one patient was receiving selegiline alone and another benzhexol alone.

The same selection criteria were applied to controls and PD patients. The study excluded individuals known to have any condition that could activate the immune response and thereby possibly increase neopterin levels i.e. inflammatory disorders, infections, allergies, auto-immune disorders and types of malignancy. In addition, individuals receiving medication that could affect neopterin levels, for example anti-inflammatory drugs, were not included in the study group. A cancer patient who was in remission however was included as neopterin is believed to return to normal levels during the remission phase (Wachter 1992). Details of the patients and controls used in the neopterin study are given in table 5.1.

Table 5.1 Composition of the neopterin study group.

patient group	number of subjects	male: female	age (mean ±SD)	age range
controls	21	9:12	38.1±17.9	17-81
untreated PD	17	6:11	64.0±10.4	43-80
all treated PD	30	15:15	66.7±10.7	43-89
PD treated with L-Dopa	16	7:9	68.6±11.2	52-89
PD treated with L-Dopa + selegiline	12	7:5	65.3±9.0	49-78

5.2.2 PLASMA PREPARATION

Blood samples were collected in lithium heparin tubes and plasma obtained by centrifugation at 2000g for 15 minutes at room temperature (MSE Minor centrifuge, MSE Ltd, Crawley, UK). All plasma samples were stored at -20°C prior to use.

5.2.3 MEASUREMENT OF NEOPTERIN

Oxidised plasma neopterin was measured by radioimmunoassay (Fuchs *et al* 1992) courtesy of Professor Fuchs (Institute of Medicinal Chemistry and Biochemistry, University of Innsbruck, Austria).

5.2.4 STATISTICAL ANALYSIS

Data were presented as the mean of each patient group ± standard deviation (SD). Data analysis was by analysis of variance (ANOVA) followed by a Fisher's t-test to compare the differences between patient groups. For the comparison of PD patients with age and sex matched controls a paired Student's t-test was employed. Differences between males and females within a patient group were analysed using a two-tailed unpaired Student's t-test. The degree of correlation between neopterin and age was tested by regression methods.

5.3 RESULTS

5.3.1 CHANGES IN PLASMA NEOPTERIN OVER TIME

Table 5.2 Changes in oxidised plasma neopterin in nmol/l over time.

	sample 1	sample 2	sample 3
control 1	27.5.94: 5.2	27.7.94: 5.0	
control 2	5.1.94: 5.0	25.5.94: 5.2	7.7.94: 4.6
PD 1	3.9.93: 5.9	7.6.94: 5.3	
PD 2	7.5.93: 7.1	7.6.94: 6.9	
PD 3	7.5.93: 5.8	5.11.93: 4.6	7.1.94: 4.3
PD 4	26.10.92: 6.4	3.9.93: 9.2	
PD 5	28.5.92: 5.6	5.11.93: 3.9	

Table 5.2 shows changes in plasma neopterin over time. PD patients 1, 2, and 3 were receiving treatment at all sample dates. In the case of patients 4 and 5, the first sample was prior to treatment and the second sample post-treatment.

5.3.2 COMPARISONS BETWEEN PATIENT GROUPS

Table 5.3 Oxidised plasma neopterin in controls, untreated and treated PD patients.

	controls	untreated PD	treated PD
oxidised plasma neopterin (nmol/l)	5.52±2.38 (n=21)	8.39±3.29 (n=17)*	7.2±3.87 (n=30)
neopterin range (nmol/l)	2.3-13.7	4.4-17	4.0-23.8

Values represent means ± SD, n=number of observations, *p<0.05 compared to controls. Statistical analysis: ANOVA (1 way): Effects of treatment, F=3.61, p<0.05. Comparison between means (Fisher's t-test): Control vs untreated PD t=2.17 p<0.05; Control vs treated PD t=1.89, p>0.05; Treated vs untreated PD t=2.02, p>0.05. Paired Student's t-test: Untreated PD vs age and sex matched controls t=2.12, p=0.05

Differences in mean oxidised plasma neopterin over the study population in table 5.2 (i.e. controls untreated PD and treated PD) were significant (p<0.05). Plasma neopterin was found to be significantly elevated in untreated PD patients (8.39±3.29, n=17) compared to controls (5.52±2.38, n=21) (p<0.05). A reduction in neopterin was observed in treated PD patients (7.2±3.87, n=30) compared to the untreated PD patients (8.39±3.29, n=17), although this was not significant. There was no statistical difference between neopterin levels in treated PD patients compared with controls. Plasma neopterin was also found to be significantly elevated in the untreated PD group compared to age and sex matched controls (p=0.05)

In addition, subjects could be classified according to whether their neopterin values were within the normal range. For healthy controls below the age of 75 years the upper normal limit of plasma neopterin was taken as 8.7nmol/l (Wachter *et al* 1992). 7 (41.2%) of the untreated PD patients, 6 (20%) of the treated PD patients and 1 (4.8%) of the controls had neopterin levels above the upper normal limit of 8.7nmol/l. In both PD groups only two of the patients with neopterin above 8.7nmol/l were in excess of 75 years of age. In the control group, the patient with neopterin above the normal upper limit was 50 years of age.

The data were also analysed by dividing the treated PD patients into those receiving L-Dopa (sinemet/madopar) and those receiving L-Dopa plus selegiline (table 5.3).

Table 5.4 Oxidised plasma neopterin in PD patients treated with L-Dopa only and L-Dopa plus selegiline.

	controls	**untreated PD**	**PD treated with L-Dopa**	**PD treated with L-Dopa + selegiline**
oxidised plasma neopterin (nmol/l)	5.52±2.38 (n=21)	8.39±3.29* (n=17)	7.26±2.48 (n=16)	7.63±5.4 (n=12)

Values represent means ± SD, n=number of observations, *p<0.05 compared to controls. Statistical analysis: ANOVA (1 way): Effects of treatment, F=2.50, p>0.05. Comparison between means (Fisher's t-test): Control vs untreated PD t=2.19 p<0.05; Control vs treated with L-Dopa t=2.28, p>0.05; Control vs treated with L-Dopa + selegiline t=2.43, p>0.05; Untreated PD vs treated with L-Dopa t=2.34, p>0.05; Untreated PD vs treated with L-Dopa + selegiline t=2.53, p>0.05; L-Dopa vs L-Dopa + selegiline t=2.56, p>0.05.

Plasma neopterin was not found to differ significantly over the study population shown in table 5.4, i.e. controls, untreated PD patients, PD patients treated with L-Dopa and PD patients treated with L-Dopa plus selegiline. Within this study group, neopterin was found to be significantly increased in the untreated PD patients compared to controls (P<0.05). No statistical difference was observed between the PD patients treated with L-Dopa and those treated with L-Dopa plus selegiline. Neopterin did not differ significantly in either of the treatment groups compared with controls or untreated PD.

5.3.3 VARIATION WITHIN PATIENT GROUPS

As shown in table 5.3 the range of plasma neopterin values varied between the patient groups. The control group demonstrated the smallest range of values, neopterin varying between 2.3 and 13.7nmo/l. In the untreated PD patients neopterin varied between 4.4-17nmol/l. The greatest range of neopterin was seen in the treated PD group with values ranging from 4.0-23.8nmol/l. Figure 5.1 shows the variation in plasma neopterin within the patient groups. The treated PD group was found to have significantly different variance

compared with the control group ($F=2.65$, $p<0.05$). The variance of the untreated PD group did not differ significantly from that of the control group ($F=1.91$, $p>0.05$).

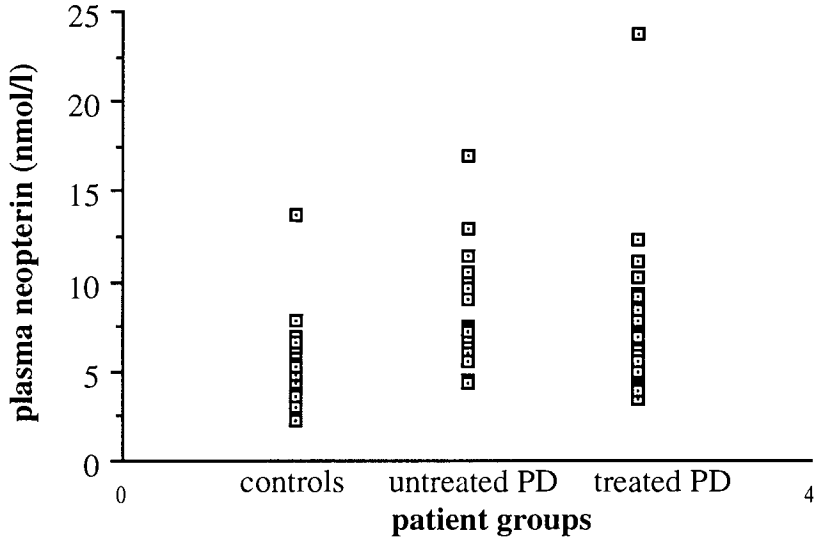


Figure 5.1 Variation in plasma neopterin within the patient groups

5.3.4 EFFECT OF AGE AND SEX UPON NEOPTERIN

Plasma neopterin did not differ significantly between males and females in controls, untreated PD and treated PD patients (table 5.5).

Table 5.5 Plasma neopterin in males and females in controls, untreated and treated PD.

	plasma neopterin (nmo/l) males	plasma neopterin (nmo/l) females
controls	4.48±1.74 (n=9)	6.3±2.57 (n=12)
untreated PD	8.43±2.72 (n=6)	8.36±3.68 (n=11)
treated PD	7.72±5.15 (n=15)	6.68±1.98 (n=15)

Values represent means ± SD, n=number of observations

Statistical analysis: Comparison between males and females, unpaired Student's t-test: Controls $t=1.94$, $p>0.05$; Untreated PD $t=0.045$, $p>0.05$; Treated PD $t=0.73$, $p>0.05$

Plasma neopterin was not correlated with age in controls or untreated PD patients. In treated PD patients a positive correlation between age and serum neopterin was observed ($p<0.05$) (figures 5.2 a, b, c).

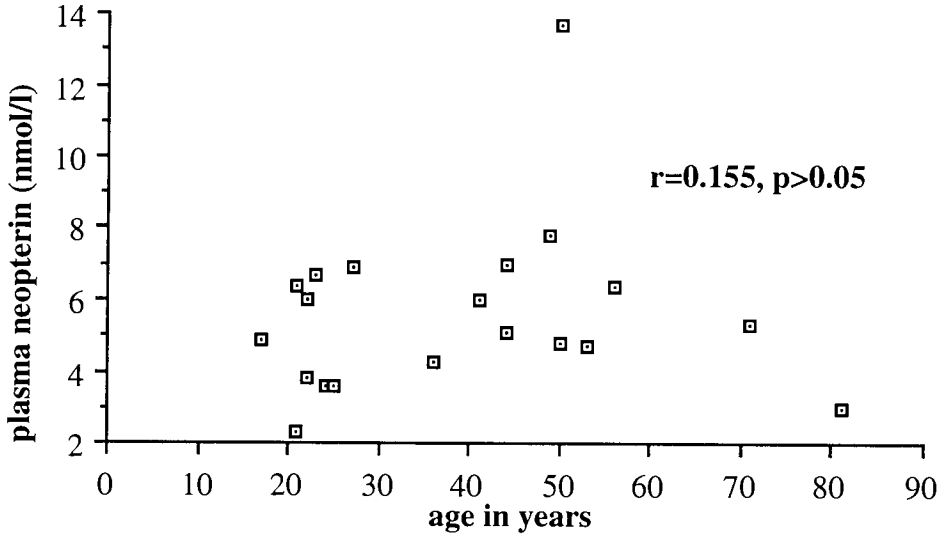


Figure 5.2a The relationship between plasma neopterin and age in controls

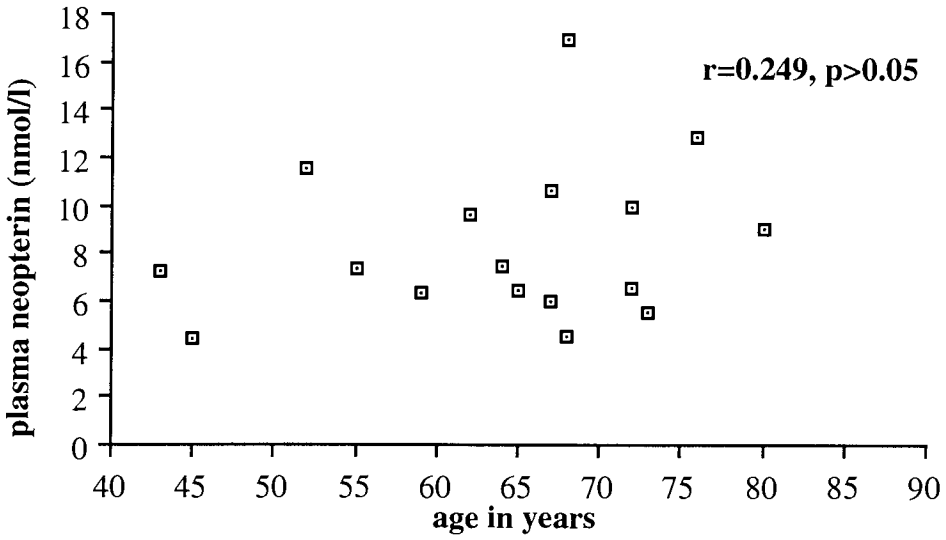


Figure 5.2b The relationship between plasma neopterin and age in untreated PD patients.

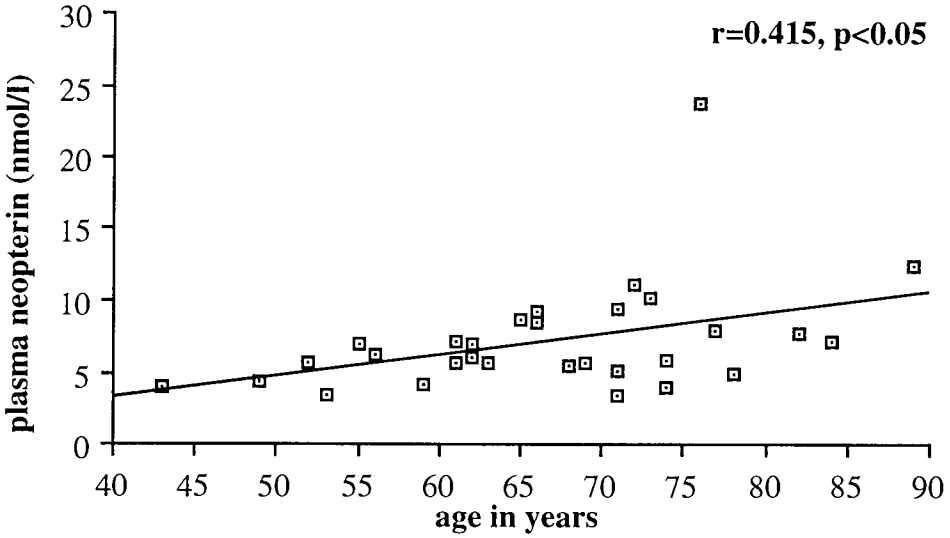


Figure 5.2c The relationship between plasma neopterin and age in treated PD patients

5.4 DISCUSSION

During this study the level of oxidised neopterin as oppose to total neopterin (i.e. oxidised neopterin + 7-8-dihydroneopterin) was determined. It is recommended that oxidised neopterin is measured in preference to total neopterin due to the lability of 7-8-dihydroneopterin to oxidative reactions. However, the diagnostic information provided by the measurement of neopterin does not depend upon whether oxidised or total neopterin is determined (Wachter *et al* 1992).

The results demonstrated that variation in neopterin with time within a single control subject was small, the values remaining within the normal range. Hence, plasma neopterin levels within the blood are stable under normal circumstances and the method of determining neopterin was reliable. A previous study by Haas and Gerstner (1986) reported that when serum neopterin was determined in individuals daily over one month variations in neopterin were small. The 3 PD patients receiving therapy at both sample dates showed a reduction in neopterin with time which may be attributed to the effects of treatment. In addition, one of the patients demonstrated a decrease in neopterin post-treatment compared to pre-treatment, although the reverse was true in another patient. The

effect of treatment upon neopterin levels could be dependent upon the severity of the disease and the duration of treatment.

Mean oxidised plasma neopterin levels in controls (5.52±2.38nmol/l) were similar to serum control values reported by Wachter *et al* (1992) (5.34±0.14) and Hagberg *et al* (1993) (6.0).

Oxidised plasma neopterin was found to be significantly elevated in untreated PD patients compared to controls. Furthermore, the untreated PD group had a greater percentage of individuals with neopterin levels above the normal upper limit compared to the treated PD and control group. Urinary neopterin has previously been reported to be increased in a number of neurological diseases including Alzheimer's disease and Down's syndrome (Armstrong *et al* 1994). However Milstien *et al* (1994) reported that neopterin within the CSF was not significantly different in AD patients compared to controls. In depressed patients, neopterin has been reported to be significantly increased within the plasma (Maes *et al* 1994), serum (Dunbar *et al* 1992) and urine (Duch *et al* 1984). An increase in serum neopterin has also been reported in untreated PD patients compared to healthy controls (Winsper *et al* 1994). However, CSF neopterin has been found to be decreased (Fujishiro *et al* 1990) or unchanged in PD patients (Furukawa *et al* 1992).

The range of plasma neopterin values in control subjects (2.3-13.7nmol/l) was similar to the range demonstrated for plasma neopterin in healthy individuals (3.4-12.8nmol/l) by Maes *et al* (1994). The range of neopterin values in the control sample was increased considerably by 1 patient with a value well above the normal upper limit given by Wachter *et al* (1992). The treated PD group showed the greatest range of neopterin values, the variance being significantly different from that of the controls. The wide range of values could be explained by the fact that treated patients varied according to the disease stage and therefore the duration of treatment.

Treated PD patients were found to have reduced neopterin compared to untreated patients, although the difference was not significant. The treated PD patients also demonstrated a smaller percentage of individuals with plasma neopterin above the upper normal limit

compared to the untreated PD patients. Furthermore, patients receiving therapy showed a reduction in neopterin over time which could have resulted from the effects of treatment. Treatment with L-Dopa has been reported to influence the immune system. Interleukin-1 synthesis and IgM and IgA plasma levels have been found to be increased in PD patients receiving L-Dopa compared to untreated PD patients (Fiszer *et al* 1991). In addition, L-Dopa has been reported to selectively depress T-dependent immune responses in mice. Administration of L-Dopa reduced the antibody response and delayed hypersensitivity reaction to sheep red blood cells (a T-dependent antigen). *In vitro*, L-Dopa decreased lymphocyte proliferation and the generation of cytotoxic T-cells in response to allogeneic stimulator cells. Furthermore, L-Dopa treatment of mice resulted in a decrease in spleen T-cell numbers (Boukhris *et al* 1987). L-Dopa could produce selective T-cell defects either by direct action upon subsets of T-lymphocytes or via neuro-endocrine interactions (Kouassi *et al* 1987, Boukhris *et al* 1987). It is therefore possible that in treated PD patients L-Dopa could reduce neopterin levels by depressing the T-cell response. Alternatively, the level of neopterin could reflect the progression of the disease. The immune response could be more intense during the early stages when the majority of dopamine neurons are destroyed, the response later being less intense. Treated PD patients may have lower neopterin levels as they are generally at a later stage of the disease process than untreated patients.

No statistical difference was observed between the PD patients treated with L-Dopa and those treated with L-Dopa plus selegiline. This supports the finding that the long-term use of selegiline has minimal benefits for the clinical condition of the patient over L-Dopa (Brannan and Yahr 1995).

Plasma neopterin was not found to differ between males and females. Previous studies have also reported that there were no difference in plasma or serum neopterin between the sexes (Werner *et al* 1987, Wachter *et al* 1992, Maes *et al* 1994).

A weak positive correlation between neopterin and age was observed in the treated PD group but not the controls or untreated PD patients. Plasma neopterin has been reported to be positively correlated with age in controls and depressed patients (Maes 1994). In

addition, Wachter *et al* (1992) reported an increase in serum neopterin in healthy controls aged above 75 years compared to controls aged below 75 years. The relationship between neopterin and age in treated PD patients could also be related to the duration of treatment. Older patients would be expected to have been receiving treatment for a longer period. The efficacy of L-Dopa has been reported to be reduced after a maintained period of treatment. This could mean that in older patients L-Dopa has less effect in reducing neopterin levels. A relationship between age and neopterin was not observed in the untreated PD group, although they had a similar age range and mean age as the treated group. An age effect may occur in untreated PD patients but may be hidden as the effect of age may be less pronounced than the effect of the disease upon neopterin levels. Plasma neopterin was increased in untreated PD patients compared to age matched controls thus elevated neopterin cannot be explained by an age effect alone. An increase in neopterin with age may only occur above a certain age. This may explained why there was no relationship between age and neopterin in the control group which had a lower mean age than the treated and untreated PD patients.

5.4.1 IMPLICATIONS OF ELEVATED NEOPTERIN

Neopterin is generally accepted as a marker of a cellular immune response (Wachter *et al* 1992). However, elevated neopterin is not specific to any one disorder and occurs in a variety of conditions in which the cellular immune system is activated. Individuals were excluded from the present study population if they were known to be suffering from conditions or were receiving drug treatment which could modify the immune response and therefore neopterin levels. However, it is possible that individuals could have elevated neopterin due to a sub-clinical condition. This could explain why one of the healthy controls had a neopterin value above the normal upper limit.

The increase in plasma neopterin suggests that a cellular immune response occurs in untreated PD patients. A role for cell mediated immunity in PD is supported by several lines of evidence. Yamada *et al* (1992) and McGeer *et al* (1988) reported the presence of T-cytotoxic/suppressor lymphocytes and HLA-DR positive microglia within the substantia

nigra of PD patients. In addition, gamma delta T+ cells (a sub-population of T-cells involved in infection and auto-immunity) have been found to be increased in the blood of PD patients compared to other neurological disease patients. An increase in the proportion of these cells within the CSF was also reported (Fiszer *et al* 1994).

During this study neopterin was measured within the plasma. An important consideration is whether a marker of immune activation within the periphery reflects processes occurring within the brain. CNS disorders may be accompanied by an elevation of neopterin within the periphery. Neopterin has previously been reported to be elevated within the urine in Alzheimer's disease and Down's syndrome patients (Armstrong *et al* 1994) and in the plasma (Maes *et al* 1994), serum (Dunbar *et al* 1992) and urine (Duch *et al* 1984) of depressed patients. In addition, serum neopterin was found to be significantly increased in patients with CNS infections compared to healthy controls (Hagberg *et al* 1993). Plasma neopterin could originate from the CNS although pteridines have a relatively low permeability across the blood brain barrier (BBB) (Hagberg *et al* 1993). It is also possible that the activated cells of the immune system pass from the brain into the periphery where they generate neopterin. The BBB is no longer believed to be impermeable to immunocompetent cells. The BBB may be absent or weak in certain brain areas (Balin *et al* 1986). Furthermore, activated T-cells may pass through the BBB and secrete mediators that increase BBB permeability (Savion *et al* 1984, Leibowitz and Hughes 1983). It is also possible that in PD damage to the BBB occurs.

The role of the immune response in the pathogenesis of PD is unclear. An immune attack directed against the dopaminergic neurons could be responsible for degeneration of the substantia nigra. The neopterin itself could be a factor in the degeneration by acting as an oxidant (Arai *et al* 1994). It is also possible that the immune response is secondary to neuronal damage occurring due to some other cause. In this case, the immune response could still exacerbate the degenerative process. Alternatively, a third factor could result in neuronal degeneration and stimulation the immune system independently.

5.4.2 WHY IS AN IMMUNE RESPONSE GENERATED IN PARKINSON'S DISEASE?

As previously suggested immune activation occurring within the Parkinsonian brain could be secondary to degeneration within the substantia nigra resulting from some other cause.

It is also possible that the immune response is a primary event and could be generated against the dopaminergic neurons in PD for a number of reasons. Auto-immunity refers to a loss of tolerance to self-antigens and this could be the primary factor responsible for the degeneration of substantia nigral neurons in PD. An auto-immune response could occur due to a number of circumstances. Breakdown of the BBB could result in the release of antigens, normally prevented from contact with immune cells present in the circulation, with consequent stimulation of the immune system. Alternatively, auto-antigens may be altered by a virus, toxin or trauma so they are no longer recognised as self and thus result in the generation of auto-antibodies or auto-reactive T-cells. It is also possible that an antigen generated against an infectious agent or environmental toxin may cross react with the bodies own antigens. Auto-immunity may also occur due to loss of suppressor cell function, formation of a B-cell clone that recognises self-antigens or an increase in T-helper cell activity with the inappropriate activation of B-cells. Polyclonal activators such as endotoxins may activate B-cells nonspecifically leading to a nonspecific increase in antibodies, some of which may react with self antigens. Finally, expression of MHC molecules by cells that do not normally poccess these antigens could lead to presentation of antigen and induction of the immune response. It is also possible that the immune response observed in PD could be a chronic inflammatory disorder, similar to rheumatoid arthritis, in which an unknown aetiological trigger leads to an inflammatory response. Inflammation is the process whereby blood cells and proteins enter the tissue in response to infection or injury. This inflammation may be self propagating or could continue if the initial trigger persists.

CHAPTER 6: CIRCULATING IMMUNE COMPLEXES IN PARKINSON'S DISEASE

6.1 INTRODUCTION

The involvement of immune factors in PD is well documented. Anti-neuronal antibodies have been detected within the serum and CSF of PD patients (Husby *et al* 1977, Pouplard and Emile 1984, McRae-Deguerce *et al* 1986) suggesting that a humoral immune response may occur in this disorder. In addition, activation of the classical complement pathway has been demonstrated within the Parkinsonian brain (Yamada *et al* 1992).

Immune complexes are macromolecules formed by the interaction of antibody and antigen as part of the humoral immune response. Immune complexes are produced continually in healthy subjects and are usually removed from the circulation by a number of biochemical, enzymatic and cellular processes without any detrimental effect to the individual. However, the deposition of immune complexes may result in complement dependent tissue injury. The fixation of complement by immune complexes initiates a series of events that may damage the hosts own tissue due to anaphylatoxin production, leukocyte stimulation, macrophage activation and cell lysis (McDoudal and McDuffie 1985).

Increased levels of circulating immune complexes (CICs) have been detected in auto-immune diseases, malignancies and certain bacterial, viral and parasitic infections (Theofilopoulos and Dixon 1980). Furthermore, the formation of CICs has been implicated in a number of neurological diseases including, multiple sclerosis (Tachovsky *et al* 1976, Cojocaru *et al* 1992), amyotrophic lateral sclerosis (ALS) (Oldstone *et al* 1976, Westarp *et al* 1993), Alzheimer's disease and Down's syndrome (Heinonen *et al* 1993, Soininen *et al* 1993).

During the present study the levels of IgG containing CICs were investigated within the plasma of PD patients using the C1q solid phase binding assay, a form of enzyme linked immunosorbent assay (ELISA). The C1q solid phase binding assay is based upon the

principle that IgG containing CICs will bind to the complement component C1q which is attached to microwells to form a solid phase.

6.1.1 AIMS OF THE CHAPTER

The objective of this study was to establish whether a humoral immune response is involved in the aetiology of PD by investigate the levels of CICs with the plasma of controls, untreated and treated PD patients.

6.2 METHODS

Two different studies were made of CICs within Parkinsonian plasma, both involved the solid phase C1q binding assay. Study A utilised the Sigma immunoassay kit and study B the Quidel immunoassay kit.

6.2.1 CONTROL AND PATIENT STUDY GROUPS

The control groups comprised healthy volunteers living in the community.

Plasma from untreated PD patients was obtained from Dr. H. Pall (consultant neurologist, Queen Elizabeth Hospital, Birmingham) at the time of clinical diagnosis and before the start of drug therapy. All patients in the experimental group displayed at least two of the three classic symptoms of PD (tremor, rigidity and bradykinesia) and subsequently responded to levodopa therapy. The severity of the disease varied between stages 1-4 according to the Hoehn and Yahr's classification (Hoehn and Yahr 1967).

Blood samples were also obtained from treated PD patients, who were receiving anti-Parkinsonian drugs, from an outpatient clinic courtesy of Dr. H. Pall. For study A, 12 of the treated patients were receiving L-Dopa therapy (sinemet or madopar), 5 of these were also receiving a MAO B inhibitor (selegiline). In addition, one patient was receiving selegiline alone. For study B, 20 of the treated patients were receiving L-Dopa therapy

(sinemet or madopar), 10 of these patients were also receiving selegiline. Finally, one patient was receiving selegiline alone and another was receiving benzhexol alone.

The study excluded individuals with any condition known to result in abnormal levels of CICs such as auto-immunity, malignancy or infection. Tables 6.1 and 6.2 show details of the patient groups used in study A (using the Sigma kit) and study B (using the Quidel kit).

Table 6.1 Patient details for study A using the Sigma kit.

patient group	number of subjects	male: female	age (mean ±SD)	age range
controls	10	5:5	35.2±15.2	17-56
untreated PD	15	5:10	62.9±10.5	43-76
treated PD	13	6:7	66.8±11.4	49-84

Table 6.2 Patient details for study B using the Quidel kit.

patient group	number of subjects	male: female	age (mean ±SD)	age range
controls	5	1:4	26.0±10.32	21-44
treated PD	22	12:10	67.5±10.6	43-89

6.2.2 PLASMA PREPARATION

Blood samples were collected in lithium heparin tubes and plasma obtained by centrifugation at 2000g for 15 minutes at room temperature (MSE Minor centrifuge, MSE Ltd, Crawley, UK). All plasma samples were stored at -70°C prior to use.

6.2.3 MEASUREMENT OF CICS IN PLASMA

The C1q solid phase binding assay involves the binding of IgG containing immune complexes to immobilised C1q. Two immunoassay kits based upon this principle were employed.

6.2.3.1 STUDY A

Study A employed the Sigma immunoassay (SIA) immune complex kit (Sigma Diagnostics, Poole, UK). For each run the positive and negative controls, calibrator and plasma specimens were assayed in duplicate. The positive control and calibrator consisted of heat aggregated human IgG. The negative control consisted of human serum with no detectable immune complexes. A reagent blank was used consisting of sample diluent. A 1:41 dilution of all samples was carried out prior to the assay. The samples were incubated in microwells coated with goat C1q for 20 minutes followed by washing to remove any unbound material. Antibodies to human IgG labelled with alkaline phosphatase (conjugate) were then added to each well for 20 minutes. A second washing stage was carried out and P-nitrophenyl phosphate (substrate) was added to the wells. After 20 minutes, the absorbance of the end product was measured at 405nm (Anthos reader 2001, Anthos Labtec Instruments). A 620nm differential reading was automatically subtracted from the 405nm reading to maximise optical precision. The absorbance of the reagent blank was subtracted from the absorbance of each sample. The CIC concentration of the samples was calculated according to the following equation:

$$\text{CICs in } \mu\text{g Eq/mL} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the calibrator}} \times \text{Value of calibrator } (\mu\text{g Eq/mL})$$

Results are expressed as µg equivalents per mL (µg Eq/mL) of heat aggregated human IgG.

6.2.3.2 STUDY B

Study B employed the Quidel CIC-C1q enzyme immunoassay kit (Quidel, San Diego, USA). For each run the standards and plasma specimens were tested in duplicate. The standards consisted of a known quantity of heat aggregated human gamma globulin. The specimen diluent was used as a reagent blank. Plasma specimens were diluted 1:50 prior to the assay. The samples were incubated in microwells coated with C1q for 1 hour. The wells were then washed to remove any material not bound to C1q. Horseradish-peroxidase

conjugated goat anti-human IgG (conjugate) was added to each well for 30 minutes. This was followed by a second wash stage. A chromogenic substrate solution containing 2-2-Azino-di-(3-ethylbenzothiazoline sulphonic acid) diammonium salt was added to the wells according to the suppliers directions. After 30 minutes, the absorbance of the end product was measured at 405nm (Anthos reader 2001, Anthos Labtec Instruments). The absorbance of the reagent blank was subtracted from the absorbance of each sample. A standard curve was generated by plotting the concentration of the standards against the absorbance values obtained. The concentration of immune complexes in the plasma specimens was determined by reference to the standard curve. Results are expressed as μg of heat aggregated human gamma globulin equivalents per mL (μgEq/mL).

6.2.4 STATISTICAL ANALYSIS

Data were presented as the mean of each patient group ± standard deviation (SD). Data analysis for study A was by analysis of variance (ANOVA) followed by a Fisher's t-test to compare the differences between patient groups. For study B, an unpaired Student's t-test was employed for the comparison of treated PD patients with controls. The variance ratio was used to test the difference in variance between patient groups. The degree of correlation between CICs and age was tested by regression methods.

6.3 RESULTS

6.3.1 INTER-ASSAY VARIATION IN CICS

The variation in the measurement of CICs between assay runs (i.e. inter-assay variation) within positive and negative controls and a treated PD patient is shown in table 6.3 for the 3 Sigma kits (A, B and C) used in study A. The positive and negative controls were provided by the supplier. Different positive controls were used for each kit with varying concentrations of CICs.

Table 6.3 Inter-assay variation in the concentration of CICs within the positive and negative controls and a treated PD patient in study A (Sigma immunoassay kit).

	mean plasma CICs	interassay % CV
+ control for kit A	89.9±6.1 (n=5)	6.8
+ control for kit B	53.1±2.1 (n=5)	3.9
+ control for kit C	58.8±3.7 (n=2)	6.3
- control for kit A	7.3±1.4 (n=5)	18
- control for kit B	5.32±1.1 (n=5)	20
- control for kit C	7.3±0.46 (n=2)	6.1
treated PD patient	21.8±5.6 (n=3)	25.7

Values represent mean ± SD, n=number of runs. % CV= % coefficient of variation.

Table 6.4 shows the inter-assay variation in CICs within the positive control, a treated PD patient and a control patient tested using the Quidel kit in study B.

Table 6.4 Inter-assay variation in the concentration of CICs within a positive control, a treated PD patient and a control patient in study B (Quidel immunoassay).

	mean CICs (μgEq/mL)	interassay % CV
+ control	7.32±1.06 (n=3)	14.5
treated PD patient	11.0±2.15 (n=3)	19.6
control patient	1.07±0.56(n=4)	52.8

Values represent mean ± SD, n=number of runs. % CV= % coefficient of variation.

6.3.2 COMPARISONS BETWEEN PATIENT GROUPS

For the comparison between patient groups the level of CICs in study A are expressed as the concentration of CICs minus the negative control value. The results for study B refer to the absolute value of CICs.

Table 6.5 CICs in the plasma of controls, untreated and treated PD patients in study A.

	control	untreated PD	treated PD
CICs minus neg control (μgEq/mL)	2.79±5.29 (n=10)	0.36±5.52 (n=15)	1.98±5.66 (n=13)

Values represent the mean ± SD of the concentration of CICs minus the CIC value of the negative control, n=number of observations.
Statistical analysis: ANOVA (1 way): Effects of treatment, F=0.804, p>0.05.

Table 6.6 CICs in the plasma of controls and treated PD patients in study B.

	control	treated PD
CICs μgEq/mL	0.80±0.57(n=5)	*1.93±2.28 (n=22)

Values represent the mean ± SD, n=number of observations. *p=0.05 compared to control.
Statistical analysis: Unpaired Student's t-test: Control vs treated PD t=2.06, p=0.05.

Differences in mean plasma CICs in study A (using the Sigma kit) were not significant over the study population in table 6.5 (i.e. controls, untreated and treated PD) (p>0.05).

Plasma CIC levels in study B (using the Quidel kit) were found to be increased in treated PD patients (1.93±2.28, n=22) compared to controls (0.80±0.57, n=5) at a level that was just significant (P=0.05) (Table 6.6). However, the mean level of plasma CICs in treated PD patients was below 4 μgEq/mL and is therefore considered to be negative for significant levels of CICs according to the suppliers directions. Furthermore, the level of CICs in treated PD plasma (1.93±2.28, n=22) was comparable to the average CIC concentration in normal asymptomatic subjects (2.1±1.9, n=106) reported by the supplier.

In addition, individual patients were classified according to whether their CIC levels exceeded the normal limit stated in the suppliers instructions (Table 6.7). The concentration of CICs measured by both the Sigma and Quidel immunoassays was within the normal range in all controls. 1 of 15 (6.7%) untreated PD patients tested using the Sigma assay had abnormal CIC levels. In the treated PD patients, 1 out of 13 (7.7%)

patients assessed by the Sigma assay and 2 out of 22 (9.1%) patients assessed by the Quidel assay had CICs above the normal limit.

Table 6.7 The % of individuals in studies A and B with CICs above the normal limit.

	control	untreated PD	treated PD
STUDY A(> 20μgEq/mL)	0%	6.7%	7.7%
STUDY B(> 4μgEq/mL)	0%	—	9.1%

6.3.3 VARIATION WITHIN PATIENT GROUPS

As shown in Figures 6.1 and 6.2, the majority of individual had CIC levels within the normal range, although there was some variation within the patient groups. In study A, variance did not differ significantly in the untreated ($F=1.09$, $p>0.05$) or treated PD ($F=1.15$, $p>0.05$) patients compared to the controls or between the untreated and treated PD groups ($F=1.05$, $p>0.05$). However in study B, the treated PD patients had significantly different variance compared to the controls ($F=15.99$, $p<0.05$).

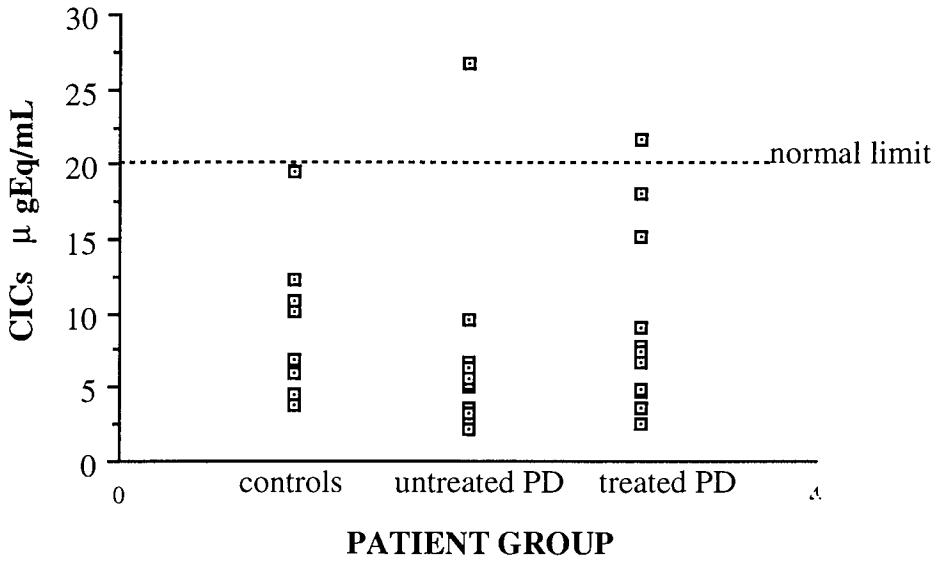


Figure 6.1 Variation in plasma CICs in study A.

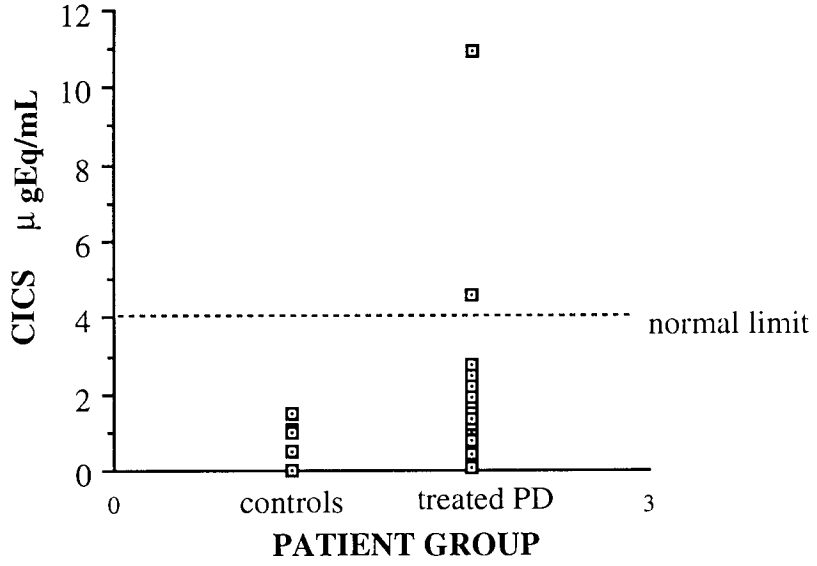


Figure 6.2 Variation in plasma CICs in study B.

6.3.4 EFFECT OF AGE UPON PLASMA CIC LEVELS

Plasma CIC levels were not correlated with age in controls, untreated or treated PD patients in studies A or B. The relationship between plasma CICs and age is shown in figures 6.3 to 6.6.

Figure 6.3 The relationship between plasma CICs and age in control patients in study A.

CIC values refer to the CIC level in plasma minus that in the negative control.

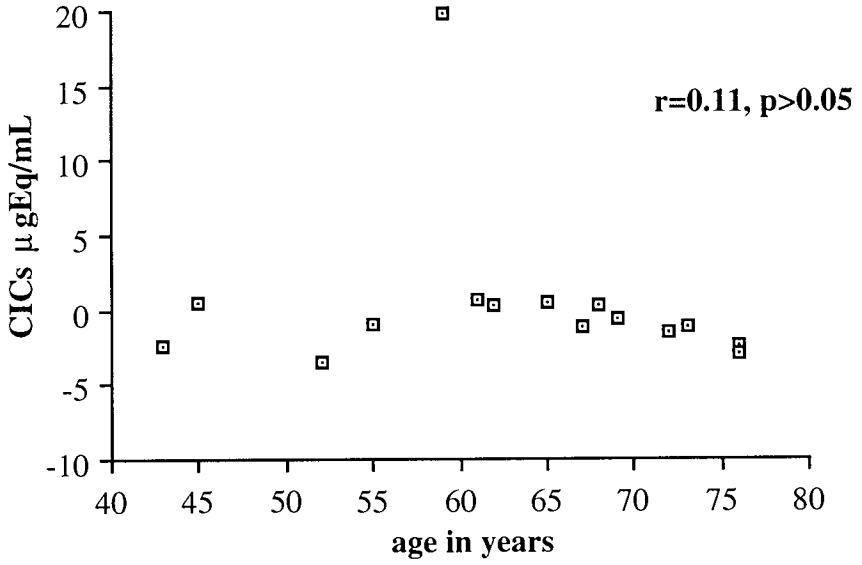


Figure 6.4 The relationship between plasma CICs and age in untreated PD patients in study A.

CIC values refer to the CIC levels in plasma minus that in the negative control.

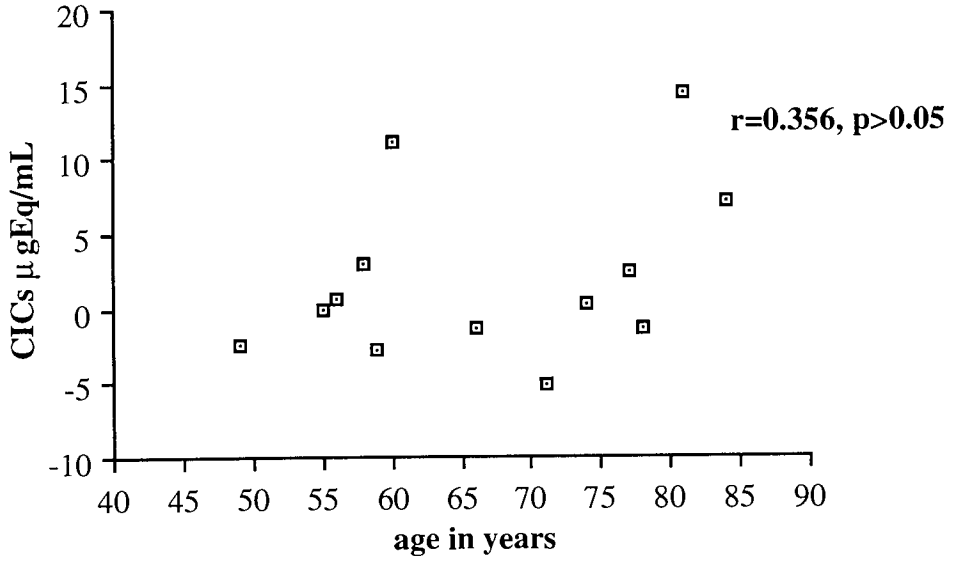


Figure 6.5 The relationship between plasma CICs and age in treated PD patients in study A

CIC values refer to the CIC levels in plasma minus that in the negative control.

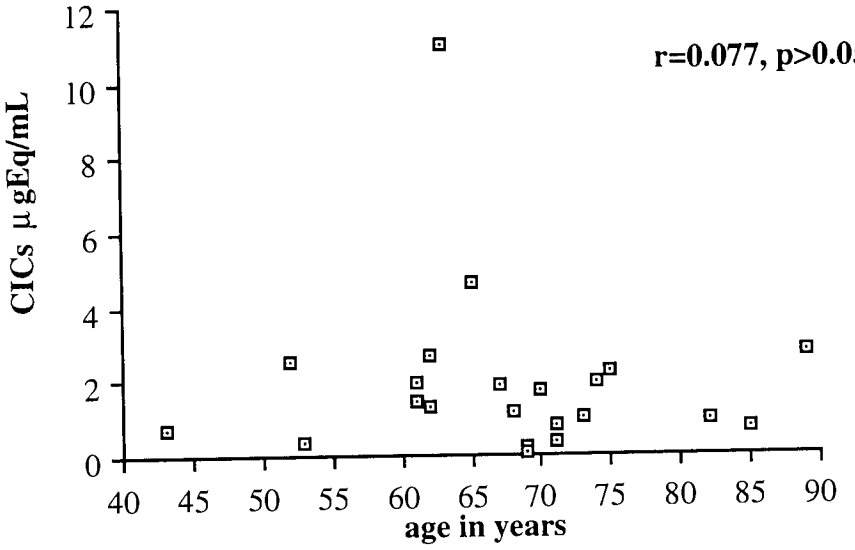


Figure 6.6 The relationship between plasma CICs and age in treated PD patients in study B

6.4 DISCUSSION

CICs have been detected in a number of conditions such as auto-immune disorders, infections and malignancies (Theophilopoulus and Dixon 1980). It was therefore necessary to screen the controls and PD patients for any of these condition which could result in abnormally high levels of CICs.

The results demonstrated some inter-assay variation in CICs. However, the concentrations of CICs in a plasma sample remained within the same range and results were consistent between assays. To account for inter-assay variation within study A (using the Sigma immunoassay), the CIC concentration within the plasma minus the negative control was used for comparisons between patient groups and for the correlations between CICs and age.

The concentration of plasma CICs, as measured by the Sigma immunoassay, did not differ significantly between controls, untreated and treated PD patients.

In study B, the mean plasma CICs in treated PD patients were similar to the level of CICs in PD patients measured using the same Quidel immunoassay kit by Yamada *et al* (1994b).

An increase in plasma CICs in the treated PD patients compared to controls was demonstrated in study B. However, the mean level of CICs within the treated PD patients was within the normal range (i.e. below 4μgEq/mL) and was similar to the average CIC concentration reported by the supplier for normal subjects (2.1±1.94μgEq/mL).

The percentage of individuals with abnormal levels of plasma CICs was lower in the untreated (6.7%) and two sets of treated PD (7.7% and 9.1%) patients compared to 149 blood donors assessed by Sigma (10.1%).

Age was not correlated with the concentration of plasma CICs in any of the study groups. Previous studies have also reported that there was no correlation between age and CIC levels in plasma of PD patients (Yamada *et al* 1994) and sera of controls, Alzheimer's disease and Down's syndrome patients (Heinonen *et al* 1993).

In this study, abnormal levels of CICs were not observed in PD patients. The results indicate that the excessive production of CICs is unlikely to be involved in PD, at least during the stages of the disease represented by our patients. As the results suggest, there is little evidence for the generation of CICs within PD patients. Yamada *et al* (1994b) reported that there was no significant difference in the levels of plasma or CSF CICs (as measured by the Quidel immunoassay kit) in PD patients compared to patients with progressive supranuclear palsy and cervical spondylosis. Furthermore, none of the 14 PD patients tested had CIC values above 4μgEq/mL which is considered to be the normal limit by the supplier. Elevated levels of CICs have previously been observed in ALS (Westarp *et al* 1993) and multiple sclerosis patients (Cojocaru *et al* 1992). In addition, CICs have been reported to occur more frequently in the sera of Down's syndrome and Alzheimer's disease patients with severe dementia compared to controls (Heinonen *et al* 1993, Soininen *et al* 1993). However, the frequency of CICs in AD patients with mild to moderate dementia was similar to control values (Soininen *et al* 1993).

CHAPTER 7: THE RELATIONSHIP BETWEEN TRANSFERRIN BINDING AND THE IMMUNE RESPONSE

7.1 INTRODUCTION

A reduction in the binding of gallium (Ga) to transferrin (Tf) and an elevation of serum neopterin (as a marker of a cellular immune response) has previously been reported in PD patients compared to controls.

Two possibilities may explain the relationship between Tf binding and neopterin. Firstly, a reduction in metal binding could lead to an increase in a low molecular weight metal species capable of stimulating a cellular IR and consequently increasing neopterin levels. For example, it has been hypothesised that a metal could induce an immune response within the brain via the mutual histocompatibility system in AD patients (Armstrong *et al* 1995). A number of metals have been shown to stimulate the immune system. Aluminium is used in vaccine adjuvants to enhance the immune response (Edelman 1980), exposure to beryllium is associated with an inflammatory lung disorder known as chronic beryllium disease (Mroz *et al* 1991) and injections of mercury can induce antibodies against renal antigens in experimental animals (Kosuda *et al* 1993). In addition, a number of metal haptens such as nickel, chromium, beryllium and mercury can generate a type of delayed hypersensitivity reaction (Druet 1994).

Secondly, the immune response itself might interact with the Tf molecule resulting in a reduction in the binding of metals. It has been proposed that in PD patients, the activation of macrophages could increase oxidation within the periphery with a subsequent decrease in metal binding to Tf (Winsper *et al* 1994). To investigate these effects, first, the relationship between Tf binding and the cellular immune response (using neopterin as a marker) was studied and second, the effect of oxidation upon Ga-Tf binding was tested by the addition of oxidising agents to the plasma. In addition, the effect of activated monocytes, stimulated with phorbol myristic acetate (PMA) to induce a respiratory burst, was investigated.

7.1.1 AIMS OF THE CHAPTER

The objectives of this study were to determine whether a relationship existed between the immune response and Tf binding and to investigate whether oxidative attack could induce a reduction in Tf binding in control plasma.

7.2 METHODS

7.2.1 MATERIALS

All chemicals used were of general grade and were supplied by: Sigma Chemical Company Ltd (Poole, UK); Aldrich Chemical Company (Gillingham, UK); BDH Chemicals Ltd (Poole, UK). Radio-isotopes were supplied by Amersham International PLC (Aylesbury, UK). Distilled water was used to make up all solutions.

7.2.2 PLASMA PREPARATION

Blood samples were collected in lithium heparin tubes and plasma obtained by centrifugation at 2000g for 15 minutes at room temperature (MSE Minor centrifuge, MSE Ltd, Crawley, UK). All plasma samples were stored at -20^{o}C prior to use.

7.2.3 CONTROL AND PATIENT STUDY GROUPS

7.2.3.1 CORRELATION STUDIES

The control group comprised 11 healthy volunteers living in the community.

Plasma from untreated PD patients was obtained from Dr. H. Pall (consultant neurologist, Queen Elizabeth Hospital, Birmingham) at the time of clinical diagnosis and before the start of drug therapy. All patients in the experimental group displayed at least two of the three classic symptoms of PD (tremor, rigidity and bradykinesia) and subsequently responded to levodopa therapy. The severity of the disease varied between stages 1-4 according to the Hoehn and Yahr's classification (Hoehn and Yahr 1967).

Blood samples were also obtained from treated PD patients, who were receiving anti-Parkinsonian drugs, from an outpatient clinic courtesy of Dr. H. Pall. Twenty one of the 23 treated patients were receiving L-Dopa therapy (sinemet or madopar), 11 of these were also receiving a MAO B inhibitor (selegiline). In addition, 2 patients were receiving benzhexol alone).

The details of the study groups used to investigate the relationship between Tf binding and neopterin are shown in table 7.1.

Table 7.1 Composition of the study group used for the correlation between % Ga-Tf binding and neopterin.

patient group	number of subjects	male: female	age (mean ±SD)	age range
controls	11	3:8	28.54±11.04	17-50
untreated PD	24	12:12	63.5±10.64	40-80
untreated PD high binders	12	7:5	65.25±10.98	40-77
untreated PD low binders	12	5:7	61.75±10.47	43-80
treated PD	23	8:15	67.09±10.69	43-89

7.2.3.2 OXIDATION STUDIES

To investigate the effects of oxidation upon % Ga-Tf binding, plasma from healthy volunteers living in the community was employed. Details of the controls used in the oxidation studies are shown in table 7.2.

Table 7.2 Composition of the study groups used for the oxidation studies.

patient group	number of subjects	male: female	age (mean ±SD)	age range
control oxidising agents	6	1:5	30.83±13.08	17-50
controls monocytes	3	1:2	39.0±14.18	23-50

7.2.4 GA-TF BINDING

% Ga-Tf binding within plasma samples was determined by gel filtration chromatography as described in section 4.2.4.1.

7.2.5 MEASUREMENT OF NEOPTERIN

Oxidised serum neopterin was measured by radioimmunoassay (Fuchs *et al* 1992) courtesy of Professor Fuchs (Institute of Medicinal Chemistry and Biochemistry, University of Innsbruck, Austria).

7.2.6 OXIDATION STUDIES

7.2.6.1 CHEMICAL OXIDISING AGENTS

Plasma from 6 control subjects was incubated with various concentrations of manganese dioxide, activated manganese dioxide and potassium permanganate for two hours at 37°C in a shaking water bath prior to incubation with Ga^{67} and application to a G75 column as described in section 4.2.4.1. The manganese dioxide which is sparingly soluble was prepared as a suspension. The activated manganese dioxide was purchased from Sigma Chemical Company (Poole, UK) and was designed specifically to take part in oxidation reactions.

7.2.6.2 ACTIVATED MONOCYTES

The human monocyte cell line U937 was employed during this study. The cell line was grown in R10 media (RPMI 1640 medium with phenol red plus 10% calf serum, 5% penicillin-streptomycin, 1% L-glutamate, 0.5% gentamycin). The cells were passaged to maintain a density of between 1×10^5 to 7×10^5 cells per ml. Cells were incubated with various concentrations of phorbol myristic acetate (PMA) for 72 hours in order to induce the respiratory burst. Plasma from 3 control subjects was incubated with a final approximate concentration of 2×10^3 activated and non-activated monocyte. Following

incubation with the monocytes for two hours at 37°C in a shaking water bath, the plasma samples were incubated with Ga^{67} and applied to a G75 column as described in section 4.2.4.1.

7.2.7 STATISTICAL ANALYSIS

Data were presented as the mean of each patient group ± standard deviation (SD). The degree of correlation between Ga-Tf binding and neopterin was assessed by correlation and regression methods. The effect of oxidising agents and monocytes upon Ga-Tf binding was tested via paired Student's t-tests.

7.3 RESULTS

7.3.1 THE RELATIONSHIP BETWEEN GA-TF BINDING AND PLASMA NEOPTERIN

Ga-Tf binding was not significantly correlated with plasma neopterin in controls, untreated or treated PD patients (table 7.3). However, in all PD groups the correlation coefficient was negative, as neopterin increased Ga-Tf binding was reduced.

Table 7.3 The relationship between Ga-Tf binding and plasma neopterin in controls, untreated and treated PD patients.

Patient Group	N	R	P
controls	11	0	>0.05
untreated PD	24	-0.27	>0.05
untreated PD high binders	12	-0.62	<0.05
untreated PD low binders	12	-0.737	<0.01
treated PD	23	-0.324	>0.05

It was possible to subdivide the untreated PD patients into high and low binding groups as previously shown by Hodgkins (1992). At low neopterin values, i.e. <10nmol/l, individuals with Ga-Tf binding of 60% or above were classified as high binders, those below 60% as

low binders. At high neopterin values, i.e. >10nmol/l, individuals with binding of 50% or more were selected as high binders and those with values below 50% as low binders. Plasma neopterin was found to be negatively correlated with % Ga-Tf binding in both the untreated PD high binders ($P<0.05$) and the untreated PD low binders ($p<0.01$) (Figure 7.1).

The two regression lines fitted to the high and low binders had significantly different elevations ($p<0.001$) but did not differ in slope ($P>0.05$).

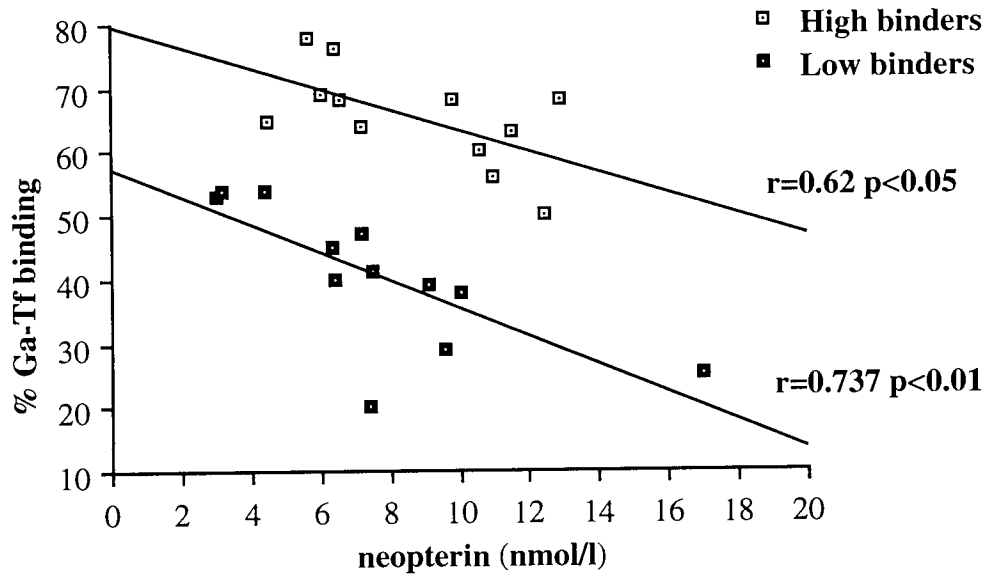


Figure 7.1 The relationship between Ga-Tf binding and plasma neopterin in untreated PD patients, high and low binders.

7.3.2 THE ADDITION OF OXIDISING AGENTS TO PLASMA

7.3.2.1 CHEMICAL OXIDISING AGENTS

To investigate the effect of oxidation upon Ga-Tf binding the oxidising agents $KMnO_4$ and activated MnO_2 (formulated to take part in oxidation reactions) were added to control plasma (table 7.4). MnO_2 was also added to the plasma to ensure the effects of the activated MnO_2 were not due to the Mn ion competing with Ga for Tf binding. The results indicate that the MnO_2 had little effect upon Ga-Tf binding. Ga-Tf binding did not differ significantly in control plasma compared to plasma with the addition of 10^{-3} or 10^{-2}

MnO_2. The presence of 10^{-3} activated MnO_2 had no significant effect upon Ga-Tf binding in control plasma. However, the addition of 10^{-2} activated MnO_2 was found to reduce Ga-Tf binding significantly ($P<0.05$). The oxidising agent $KMnO_4$ was found to greatly reduce the binding of Ga to Tf at a concentration of $10^{-4}M$ ($p<0.01$).

Table 7.4 The effect of oxidising agents upon Ga-Tf binding in control plasma.

	control	MnO_2		activated MnO_2		$KMnO_4$
		10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-4}
control 1	60	-	-	50	15	-
control 2	75	69	73	67	54	2.34
control 3	80	-	68	-	63	-
control 4	73	-	72	-	62	8.6
control 5	79	-	-	78	72	-
control 6	75	70	65	77	67	2.2
mean	73.7	69.5	69.5	68	55.5*	4.38#
± SD	7.2	0.7	3.7	13.0	20.7	3.66

Statistical analysis : Paired Student's t-test: Control vs 10^{-2} MnO_2 $t=2.25$, $p>0.05$; Control vs 10^{-3} activated MnO_2 $t= 1.5$ $p>0.05$; Control vs 10^{-2} activated MnO_2 $t= 3.13$ $p<0.05$*; Control vs $KMNO_4$ $t=25.19$ $p<0.01$#

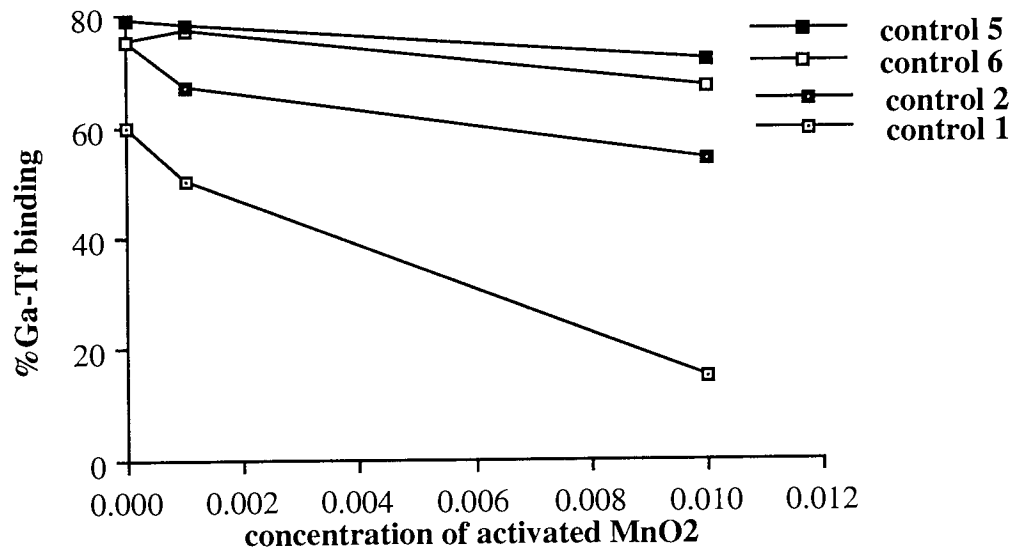


Figure 7.2 The effect of activated MnO_2 upon Ga-Tf binding in four control patients.

The results also indicate that certain subjects were more susceptible to a reduction in Ga-Tf binding due to the effects of oxidation. Figure 7.2 shows the effects of activated MnO_2 upon Ga-Tf binding within the plasma of 4 individual controls. All subjects demonstrated a reduction in binding with the addition of activated MnO_2. However control subject 1 demonstrated a greater reduction in binding than the other subjects. It is apparent that this individual had relatively low binding before the addition of oxidising agents.

7.3.2.2 ACTIVATED MONOCYTES

Table 7.5 shows the effect of monocytes and monocytes + PMA upon Ga-Tf binding in control plasma. The PMA was added to the monocytes in order to stimulate the respiratory burst thereby producing oxidising species. The addition of monocytes or monocytes stimulated with 6μg PMA was found to have no significant effect upon Ga-Tf binding in control plasma. However, in one subject (control 1) a reduction in Ga-Tf binding within the plasma was observed on incubation with monocytes stimulated with 6μg PMA. The same individual was found to demonstrate the greatest reduction in binding following incubation with activated MnO_2.

Table 7.5 The effect of the addition of monocytes and monocytes stimulated with PMA upon Ga-Tf binding in control plasma.

	control	monocytes	monocytes + PMA		
			2μg PMA	4μgPMA	6μgPMA
control 1	60	61		59	53
control 2	68	66	64		72
control 3	80	77			78
mean	69.3	68.0	64	59	67.7
± SD	10.1	8.2			13.1

Statistical analysis: Paired Student's t-test; Control vs monocytes $t=1.11$ $p>0.05$; Control vs 6μg PMA $t=0.52$ $p>0.05$

7.4 DISCUSSION

Individuals with conditions that could activate the immune system or receiving medication that could affect the immune response, such an anti-inflammatory drugs, were included in the study population. It is valid to include those individuals with elevated neopterin due to some other condition as when investigating the relationship between neopterin and % Ga-Tf binding the origin of the cellular immune response should not determine the effect upon Ga-Tf binding.

An inverse correlation between % Ga-Tf binding and plasma neopterin was observed when the untreated PD patients were subdivided into "high" and "low" Tf binders. As Ga-Tf binding is dependent upon neopterin levels, high and low binders were defined in relation to the level of neopterin. At low neopterin values, i.e. <10nmol/l, individuals with binding of 60% or above were classified as high binders, below 60% as low binders. At high neopterin values, i.e. >10nmol/l, high binders were defined as having binding of 50% or more, low binders as below 50%. The finding that the elevations of the two regression lines were significantly different confirms the presence of two distinct populations of high and low binders as reported by Hodgkins (1992).

The results indicate that in untreated PD patients there is an inverse relationship between Tf binding and serum neopterin, Ga-Tf binding declining as plasma neopterin increases. An associated between immune activation and disturbances in iron metabolism has also been reported by Fuchs *et al* (1993b), neopterin being inversely correlated with Tf levels.

A significant correlation between Ga-Tf binding and plasma neopterin was not observed in controls or treated PD patients. In the control group, all individuals demonstrated Ga-Tf binding values above 60% and neopterin levels below 10μgEq/mL. The relatively small range of values might explain why a relationship between the two parameters was not observed. In the treated PD group, drug therapy may have altered either Tf binding or affected the level of neopterin resulting in no significant correlation between the two.

The addition of oxidising agents to control plasma demonstrated that oxidation is capable of liberating metals from Tf. A reduction in Tf binding following the addition of oxidising agents to plasma has been previously reported by Hodgkins (1992). The results indicated that in certain subjects, oxidising agents induced a greater reduction in Ga-Tf binding. It is possible that individuals with lower levels of binding lose metals from Tf more easily than high binders. In the oxidative studies, the control with the lowest % Ga-Tf binding demonstrated the greatest reduction in binding following the addition of oxidising agents to the plasma.

The addition of activated monocytes to plasma aimed to produce an oxidative environment resembling that within the body during an immune response. In order to induce the respiratory burst, the monocytes were stimulated with PMA. However, the activated monocytes only produced a small reduction in Ga-Tf binding in one control subjects. This may be due to the fact that the monocytes were incubated for an insufficient time to affect binding. Within the body, oxidative stress due to immune activation may occur over a greater time period. Furthermore, the cell line employed in this study generates a relatively small amount of oxygen metabolites in comparison to macrophages or neutrophils.

The results indicate that in untreated PD patients there is an inverse relationship between Tf binding and serum neopterin. Three possibilities exist. Firstly, defects in Ga-Tf binding are responsible for an increase in immune activation and therefore the elevation of neopterin. Secondly immune activation results in the liberation of metals from Tf. Finally, a third factor could result in both a reduction in Ga-Tf binding and stimulation of the immune response.

7.4.1 DOES REDUCED METAL TF BINDING LEAD TO IMMUNE ACTIVATION?

An increase in free metal, due to reduced binding to Tf, could result in the stimulation of the immune system by a metal or metal complex. Metals are known to be capable of initiating a cellular immune response and inducing antibody formation. Metals also have the ability to activate complement. Al compounds have a long history of use as vaccine

adjuvants such as in the diphtheria toxoid (Edleman 1980). Adjuvants are substances that non-specifically enhance the immune response to an antigen. Al containing adjuvants have been found to induce antibody production (Reiotella and Orasy 1969), prime T-helper cells (Kishimoto and Ishizaka 1973) and activate complement proteins (Ramanathan *et al* 1979). The disorder known as chronic beryllium disease (CBD) is a lung disease arising due to industrial exposure to beryllium dust. In this condition, a cell mediated immunity to beryllium occurs with the formation of granulomas or clusters of immune cells surrounding beryllium particles in the walls of the alveoli (Mroz *et al* 1991). In addition, a number of metal haptens such as nickel, chromium, beryllium and mercury can induce contact dermatitis (Druet 1994), a type of delayed hypersensitivity reaction. Finally, mercury has been found to induce antibodies against renal antigens in experimental animals (Kosuda *et al* 1993).

It has been hypothesised that a metal induced immune disorder within the brain may play a role in the pathogenesis of AD (Armstrong *et al* 1995). Metals can generate an immune response in a number of ways. Firstly, metals are known to be capable of inducing a hypersensitivity reaction. In this case, a cellular or humoral immune response is mounted against the metal antigen which is overtly aggressive with pathological consequences for the host. Recognition of metal haptens by T-cells is believed to be MHC restricted (Sinigaglia 1994) and could occur under a number of circumstances. Nickel has been found to bind MHC associated peptides, modifying the structure of the MHC-peptide complex, and resulting in the presentation of the hapten to T-cells. By contrast, gold was demonstrated to bind directly to the MHC molecules accounting for the production of gold specific T-cells and the generation of the delayed type hypersensitivity reaction seen in some individuals (Sinigaglia 1994).

Secondly, metals are believed to be capable of inducing auto-immunity in which an immune response, involving auto-reactive T-cells or auto-antibodies, is directed against an auto-antigen. In this case, metals may modify self-proteins which then become auto-antigenic. For example, in rodents, gold and mercury salts have been found to stimulate an

autoimmune response which is believed to occur because the metals modify the MHC-peptide complex so it is recognised by auto-reactive T-cells (Druet 1994).

Furthermore, the MHC molecule itself may be important in determining whether a metal is recognised by the immune system. In CBD, certain MHC alleles were found to be associated with the disease (Richeldi *et al* 1993). In addition, T-cell clones from individuals with contact dermatitis only responded to nickel in association with a certain HLA isotype (Sinigaglia 1994). The influence of the MHC molecule could explain why some individuals could be susceptible to metal induced immune responses.

7.4.2 DOES IMMUNE ACTIVATION LEAD TO REDUCED METAL TF BINDING?

It is also possible that increased immune activation, as indicated by the elevation in neopterin, is responsible for the reduction in Ga-Tf binding demonstrated in PD patients. The effect of oxidising agents upon Tf binding *in vitro* demonstrated that oxidation within the plasma is capable of liberating Ga from Tf. Furthermore, those metals with lower stability constants than Ga, such as Al, would be more likely to be lost from the Tf molecule. A potential source of oxidising species is the immune response. It has been suggested that increased oxidative activity, due to the activation of macrophages, results in a reduction in Ga-Tf binding in PD patients (Winsper *et al* 1994). Furthermore, neopterin itself may act as an oxidant (Arai 1994) and thus contribute to the reduction in Tf binding. Alternatively, a specific immune attack of the Tf molecule could occur as in the case of auto-immune atransferrinaemia. This disorder involves the production of auto-antibodies specific for the Tf molecule resulting in the generation of a circulating immune complex of Tf and IgG (Westerhausen and Meuret 1977).

CHAPTER 8: GENERAL DISCUSSION

8.1 OBJECTIVES OF THE STUDY

This study had a number of objectives:

1) To determine whether the binding of Fe and Ga (and by implication other metals) to Tf was defective in plasma from patients with Parkinson's disease and to investigate the effect of drug therapy upon Ga-Tf binding.

2) To extend preliminary studies that demonstrated an elevation of neopterin in untreated PD patients (Winsper *et al* 1994) and to determine whether the levels of neopterin were influenced by treatment.

3) To investigate whether the humoral immune response is activated within the plasma of PD patients by measuring the levels of CICs.

4) To establish the relationship between Ga-Tf binding and plasma neopterin (as a marker of a cellular immune response) and to demonstrate the ability of oxidation to reduce Ga-Tf binding *in vitro.*

8.2 MAJOR CONCLUSIONS

Fe-Tf binding was found to be 100% within the plasma of all controls and PD patients indicating that a defect in the binding of Fe to Tf is not involved in the aetiology of PD.

The binding of Ga to Tf was found to be significantly reduced within the plasma of both untreated and treated PD patients compared to controls. Furthermore, treatment was found to increase Ga-Tf binding towards control values, treated PD patients having significantly higher levels of binding than untreated PD patients. Ga-Tf binding may reflect the binding of Tf to a series of other metals. A reduction in metal Tf binding could have several consequences. A decrease in Tf binding may result in an increase in a low molecular weight species which may be readily transported across the BBB leading to the accumulation of the metal within the brain. Alternatively, a decrease in Tf binding could limit the entry of an essential metal into the brain via the Tf receptor system. It is also possible that Ga-Tf binding is secondary to some other process, such as immune activation.

The study also demonstrated a significant elevation in neopterin within the plasma of untreated PD patients compared to controls indicating the activation of a cellular immune response. Treatment was found to influence neopterin levels, plasma neopterin being lower in treated PD patients compared to untreated PD patients, although the difference was not significant. By contrast, there was no evidence for the activation of the humoral immune response in untreated or treated PD patients as measured by CIC levels within the plasma.

An inverse relationship was found to exist between Ga-Tf binding and neopterin within the plasma of untreated PD patients. A number of possibilities may explain this relationship. Firstly, reduced metal Tf binding could result in the release of a low molecular weight form of the metal which is capable of stimulating an immune response thus increasing neopterin levels. Secondly, the activation of the cellular immune system may be responsible for the reduction in Tf binding. The immune response provides a source of oxidants which could damage the Tf molecule thereby reducing its ability to bind to metals. This hypothesis is supported by the finding that the addition of oxidising agents to control plasma reduced Ga-Tf binding *in vitro* demonstrating that oxidation is capable of inducing the release of metals from Tf.

Substantial evidence suggests that an immune response occurs within the Parkinsonian brain (Yamada *et al* 1992, McGeer *et al* 1988, McRae-Deguerce *et al* 1986, Mogi *et al* 1994) however, the origin of this response is unknown. One possibility is that an auto-immune attack is generated against the dopaminergic neurons resulting in the degeneration of the substantia nigra. Auto-immunity occurs when self-antigens are recognised by the immune system as foreign, resulting in the production of auto-antibodies and/or auto-reactive T-cells against the bodies own tissues. An auto-immune response may occur for a number of reasons. Firstly, damage to the BBB could result in brain antigens, coming into contact with immune cells in the plasma, thus stimulating an immune reaction. Secondly, an auto-antigen may be modified, for example in response to a virus, toxin or trauma, so that it would be recognised by the immune system as foreign. Thirdly, an antigen generated against an infectious agent or environmental toxin may cross-react with the bodies own antigens. Fourthly, autoimmunity may arise due to a defect within the immune

system itself for example by a loss of suppressor cell function, formation of a B-cell clone that recognises self-antigens or an increase in T-helper cell activity with the inappropriate activation of B-cells. Finally, expression of MHC molecules by cells that do not normally possess these antigens could lead to the abnormal presentation of antigen and generation of an auto-immune response.

It has been suggested that some forms of Alzheimer's disease could be a metal-induced auto-immune disorder (Armstrong *et al* 1995) and it is possible that such a process could be involved in the aetiology of PD. The metals Al and Fe are known to be increased within the Parkinsonian brain (Yasui *et al* 1992a, Dexter *et al* 1989b, Sofic *et al* 1991) and are present within Lewy bodies (Hirch *et al* 1991). Metals can induce two types of immune reaction. Metals such as nickel, chromium, beryllium and mercury are capable of stimulating a hypersensitivity reaction in which the immune system is overtly reactive to an exogenous antigen resulting in injury to the hosts tissue (Druet 1994). The recognition of metals during the hypersensitivity response appears to be MHC restricted (Sinigaglia 1994). Nickel has been found to bind directly to MHC associated peptides to modify the structure of the MHC-peptide complex (Sinigaglia 1994). By contrast, gold binds directly to the MHC molecule leading to the generation of metal specific T-cells. In addition, the metals mercury and gold have been demonstrated to induce an auto-immune response in rats. The mechanism involved is believed to be the modification of the MHC/MHC-peptide complex by the metal leading to the recognition of normal class II MHC molecules by auto-reactive T-cells with the polyclonal activation of B cells (Druet 1994).

It is also possible that the immune response is a more general inflammation occurring in response to the degeneration of the substantia nigra due to some other cause. However, even if the immune response is secondary it may play a role in the pathogenesis by causing further neuronal damage.

8.3 PROPOSALS FOR FUTURE WORK

1) To identify the low molecular weight Ga species present in both controls and PD patients and to establish whether patients with low levels of Tf binding posses different low molecular weight species than those with high levels of binding.

2) To investigate further the relationship between Ga-Tf binding and plasma neopterin in individuals over a prolonged period of treatment with Parkinsonian drugs and to extend the studies to include the effects of duration and dosage of treatment.

3) To establish whether the cellular immune response (as indicated by elevated neopterin) demonstrated within the plasma of PD patients is also present within the brain perhaps by investigating a marker of immune activation within the cerebrospinal fluid.

4) To establish whether immune activation precedes reduced Ga-Tf binding in PD patients by studying the parameters within the same individuals at different stages of the disease.

5) To establish whether PD patients are more susceptible to a reduction in Tf binding following oxidation within the plasma by investigating the effects of oxidising agents upon Ga-Tf binding in the plasma of PD patients.

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APPENDICES

APPENDIX 1

THE UNIFIED PARKINSON'S DISEASE RATING SCORE (UPDRS)
version 3.0 - February 1987 (courtesy of Dr H Pall)

I. MENTATION, BEHAVIOUR AND MOOD

1. Intellectual impairment
0 none.
1 Mild. Consistent forgetfulness with partial recollection of events and no other difficulties.
2 Moderate memory loss with disorientation and moderate difficulty handling complex problems. Mild but definite impairment of function at home with need of occasional prompting.
3 Severe memory loss with disorientation for time and often to place. Severe impairment of handling problems.
4 Severe memory loss with orientation preserved to person only. Unable to make judgements or solve problems. Requires much help with personal care. Cannot be left alone at all.

2. Thought disorder
(due to dementia or drug intoxication)
0 None.
1 Vivid dreaming.
2 Benign hallucinations with insight retained.
3 Occasional to frequent hallucinations or delusions; without insight, could interfere with daily activities.
4 Persistent hallucinations, delusions or florid psychosis, not able to care for self.

3. Depression
0 Not present.
1 Periods of sadness or guilt greater than normal, never sustained for days or weeks.
2 Sustained depression (1 week or more).
3 Sustained depression with vegetative symptoms (insomnia, anorexia, weight loss, loss of interest).
4 Sustained depression with vegetative symptoms and suicidal thoughts or intent.

4. Motivation/Initiative
0 Normal.
1 Less assertive than usual, more passive.
2 Loss of initiative or disinterest in elective (non-routine) activities.
3 Loss of initiative or disinterest in day to day (routine) activities.
4 Withdrawn, complete loss of motivation.

II. ACTIVITIES OF DAILY LIVING

5. Speech
0 Normal.
1 Mildly affected. No difficulty being understood.
2 Moderately affected. Sometimes asked to repeat statements.
3 Severely affected. Frequently asked to repeat statements.
4 Unintelligible most of the time.

6. Salivation	0 Normal. 1 Slight but definite excess of saliva in mouth, may have night time drooling 2 Moderately excessive saliva, may have minimal drooling. 3 Marked excess of saliva with some drooling. 4 Marked drooling, requires constant tissue or handkerchief.
7. Swallowing	0 Normal. 1 Rare choking. 2 Occasional choking. 3 Requires soft food 4 Requires NG tube or gastrotomy feeding.
8. Handwriting	0 Normal. 1 Slightly slow or small. 2 Moderately slow or small, all words are legible. 3 Severely affected, not all words are legible. 4 The majority of words are not legible.
9. Cutting food and handling utensils	0 Normal 1 Somewhat slow and clumsy but no help needed. 2 Can cut most foods although clumsy and slow, some help needed. 3 Food must be cut by someone but can still feed slowly. 4 Needs to be fed.
10. Dressing	0 Normal 1 Somewhat slow but no help needed. 2 Occasional assistance with buttoning, getting arms in sleeves. 3 Considerable help required but can do some things alone. 4 Helpless.
11. Hygiene	0 Normal 1 Somewhat slow but no help needed. 2 Needs help to shower or bathe, or very slow in hygienic care. 3 Requires assistance for washing, brushing teeth, combing hair, going to bathroom. 4 Catheter or other mechanical aids.
12. Turning in bed and adjusting bed clothes	0 Normal 1 Somewhat slow and clumsy but no help needed. 2 Can turn alone or adjust sheets but with great difficulty. 3 Can initiate but not turn or adjust sheets alone. 4 Helpless.
13. Falling	0 None 1 Rare falling 2 Occasional falls, less than once per day. 3 Falls on average of once per day. 4 Falls more than once daily.
14. Freezing when walking	0 None. 1 Rare freezing when walking, may have start hesitation. 2 Occasional freezing when walking. 3 Frequent freezing. Occasional falls when freezing. 4 Frequent falls from freezing.

15. Walking

0 Normal.
1 Mild difficulty, may not swing arms or may tend to drag leg.
2 Moderate difficulty but requires little or no assistance.
3 Severe disturbance of walking, requiring assistance.
4 Cannot walk at all, even with assistance.

16. Tremor

0 Absent.
1 Slight and infrequently present.
2 Moderate, bothersome to patient.
3 Severe, interferes with many activities.
4 Marked, interferes with most activities.

17. Sensory complaints related to Parkinsonism

0 None
1 Occasionally has numbness, tingling or mild aching.
2 Frequently has numbness, tingling or aching, not distressing.
3 Frequently painful sensations.
4 Excruciating pain.

III MOTOR EXAMINATION

18. Speech

0 Normal.
1 Slight loss of expression, dictation and/or volume.
2 Monotone, slurred but understandable, moderately impaired.
3 Marked impairment, difficult to understand.
4 Unintelligible.

19. Facial expressions

0 Normal
1 Minimal hypomimia could be normal "poker face'.
2 Slight but definitely abnormal diminution of facial expression.
3 Moderate hypomimia, lips parted some of the time.
4 Masked or fixed facies with severe or complete loss of facial expression, lips parted.

20. Tremor at rest

0 Absent.
1 Slight and infrequently present.
2 Mild in amplitude and persistent or moderate in amplitude but only intermittently present.
3 Moderate in amplitude and present most of the time.
4 Marked in amplitude and present most of the time.

21. Action or postural tremor of the hands

0 Absent.
1 Slight, present with action.
2 Moderate in amplitude, present with action.
3 Moderate in amplitude, present most of the time.
4 Marked in amplitude, interferes with feeding.

22. Rigidity

(judged on passive movement of major joints with patient relaxed in sitting position)
0 Absent
1 Slight or detectable only when activated by mirror or other movements.
2 Mild to moderate.
3 Marked but full range of motion easily achieved.
4 Severe, range of motion achieved with difficulty.

23. Finger taps

(Patient taps thumb with index finger in rapid succession with widest amplitude possible)
0 Normal
1 Mild slowing and/or reduction in amplitude.
2 Moderately impaired. Definite and early fatiguing, may have occasional arrests in movement.
3 Severely impaired, frequent hesitation in initiating.
4 Can barely perform the task.

24. Hand movements

(Patient opens and closes hands in rapid succession with widest amplitude possible)
1 Mild slowing and/or reduction in amplitude.
2 Moderately impaired. Definite and early fatiguing, may have occasional arrests in movement.
3 Severely impaired, frequent hesitation in initiating movements or arrests in ongoing movement.
4 Can barely perform the task.

25. Rapid alternating

(Pronation-supination movements of hands, vertically or horizontally with large an amplitude as possible, both hands simultaneously)
1 Mild slowing and/or reduction in amplitude.
2 Moderately impaired. Definite and early fatiguing, may have occasional arrests in movement.
3 Severely impaired, frequent hesitation in initiating movements or arrests in ongoing movement.
4 Can barely perform the task.

26. Leg agility

(Patient taps heel on ground in rapid succession, picking up entire foot, amplitude should be about 3 inches)
1 Mild slowing and/or reduction in amplitude.
2 Moderately impaired. Definite and early fatiguing, may have occasional arrests in movement.
3 Severely impaired, frequent hesitation in initiating movements or arrests in ongoing movement.
4 Can barely perform the task.

27. Arising from chair

0 Normal
1 Slow may need more than one attempt.
2 Pushes self up from arms of seat.
3 Tends to fall back and may have to try more than one time but can get up without help.
4 Unable to rise without help.

28. Posture

0 Normal erect
1 Not quite erect, slightly stooped posture, could be normal for older person.
2 Moderately stooped posture, definitely abnormal, can be slightly leaning to one side.
3 Severely stooped posture with kyphosis, can be moderately leaning to one side.
4 Marked flexion with extreme abnormality of posture.

29. Postural stability

(Response to sudden posterior displacement produced by pull on shoulders while patient erect with eyes open and feet slightly apart., Patient was prepared).
0 Normal
1 Retropulsion but recovers unaided.
2 Absence of postural response would fall if not caught.
3 Very unstable, tends to lose balance spontaneously.
4 Unable to stand without assistance.

30. Gait

0 Normal.
1 Walks slowly, may shuffle with short steps but no festination or propulsion.
2 Walks with difficulty but requires little or no assistance, may have some festination, short steps or propulsion.
3 Severe disturbance of gait, requiring assistance.
4 Cannot walk at all, even with assistance.

31. Body bradykinesia and hypokinesia

(Combining slowness, hesitancy, decreased arm swing, small amplitude and poverty of movement in general).
0 None
1 Minimal slowness giving movement a deliberate character , could be normal for some persons, possibly reduced amplitude.
2 Mild degree of slowness and poverty of movement which is definitely abnormal, alternatively some reduced amplitude.
3 Moderate slowness, poverty or small amplitude of movement.
4 Marked slowness, poverty or small amplitude of movement.

APPENDIX 2

FE-TF BINDING STUDIES

CONTROL RESULTS

I.D	Sex	Age	condition	treatment	%Fe-Tf binding
D	F	36	diabetic	insulin	100
HD	F	41	none	nil	100
PH	M	24	none	nil	100
DS	M	23	none	nil	100
ST	F	36	none	nil	100
SW	F	22	none	nil	100

PD PATIENT RESULTS

I.D	Sex	Age	condition	treatment	%Fe-Tf binding
WB	M	62	untreated PD hypertension gout	Hydralazine oxyprenolol allopurinol	100
MH	M	64	untreated PD	asparin	100
WH	M	71	untreated PD	nil	100
JS	M	61	untreated PD	naproxen	100
MT	M	58	untreated PD	amilodipine	100
MW	F	73	hypertension untreated PD thyroid disease depression	atenolol prothiaden	100
PA	M	59	treated PD	selegiline	100
WH	M	72	treated PD osteo-arthritis	sinemet sinemet plus nitrazepan	100
LH	F	57	vascular disease treated PD	sinemet selegiline phenobarbitone	100
SP	M	62	treated PD	sinemet 110	100

APPENDIX 3

CONTROL RESULTS FOR GA-TF BINDING AND NEOPTERIN

I.D	Sex	Age	condition	treatment	% Ga-Tf binding	neopterin (nmol/L)
RA	M	44	none	nil	59.5	5.1
TB	F	40	retroperitoneal-leimyosarcoma	50 mg MTX fortnightly	76	*7.6
HD	F	41	none	nil	82	6.0
SD	F	22	none	nil	67	6.0
PD	M	22	none	nil	-	3.8
BE	F	59	ulcerative-colitis	salazopyrinine	-	*6.4
GG	M	49	none	nil	-	7.8
DH	F	57	infection	doxycycline-metionidazole	-	*8.7
FH	F	70	bronchitis	nil	-	*15.5
PHi	M	53	none	nil	-	4.7
PHo	M	24	none	nil	80	3.6
DH	F	55	bronchitis thrombosis	brufen	-	*9.1
MH	F	50	none	nil	-	13.7
PHu	M	47	acute nephritis kidney stones	nil	-	*6.2
SK	F	24	none	nil	79	3.6
BM	M	52	infection	fludoxacilin	-	*11.4
SM	F	55	asthma	ventiline becotide asparin	-	*7.9
CP	M	56	none	nil	-	6.4
VP	F	71	non-Hodkins lymphoma (in remission)	thyroxine	77	5.3
SR	F	44	none	nil	-	7.0
JR	F	27	none	nil	75	6.9
MR	F	50	cancer of breast	hydrocorticosone maxolon stemetil cyclophosphamide MTX flourouracil folinic acid	74	*3.0
BS	F	53	adenosarcoma of breast	prednisolone	64	*5.4
DS	M	25	none	nil	-	3.6
RS	F	50	hypertension	bendoflourazide zesteril	-	*12.1
SS	M	81	cancer of prostate	none	73	3.0
ST	F	36	none	nil	-	4.3
TT	M	21	none	nil	69	2.3
YW	F	46	?	?	74	-
CW	F	21	none	nil	75	6.4
ClW	F	17	none	nil	71.3	4.9
MW	F	50?	none	nil	76.5	4.8
SW	F	23	none	nil	71.5	6.7

* individuals with conditions or receiving medication that can effect the immune response and where therefore excluded from the neopterin study.

APPENDIX 4

UNTREATED PD RESULTS FOR GA-TF BINDING AND NEOPTERIN

I.D	Sex	Age	additional conditions	treatment	% Ga-Tf binding	neopterin (nmol/L)
IB	F	68	diabetes depression	lofepramine	25	17
SB	M	67	hyperthyroidism	carbimazole amiodarone	33	-
CB	F	40	asthma anaemia	pulmicort ferrous sulphate	56	*11
JC	M	72	none	co-dydramol	38	10
LC	M	64	angina	prothiaden carace nitrolingual spray	41	7.5
DC	M	72	none	nil	68	6.6
EC	M	77	asthma cardiovascular-disease	duovent becotide asparin	68	*9.8
BF	F	62	thyroid disease	diazepam paracetamol	29	9.6
MG	F	55	none	nil	20	7.4
MH	F	61	hypertension	bendrofluazide rampril	78	*5.6
CH	F	68	none	multi-vitamins	65	4.5
DJ	F	65	depression	lorazepan clomipramine	40	6.4
MJ	F	80	none	nil	39	9.1
MM	F	76	none	nil	68	12.9
DN	M	45	angioneuritic oedemia	nil	54	4.4
SN	F	43	none	noriday	47	7.2
LO	M	76	arthritis	none	50	*12.4
DP	M	52	ulcer	omeprazol	53	-
IS	F	59	osteoarthritis	none	45	6.3
JS	M	67	diverticular-disease arthritis	fybogel	54	*3.2
HS	M	67	depression hiatis hernia	prothiaden zantac	60	10.6
JS	M	61	stiff shoulder	naproxen	53	*3.0
OT	F	67	none	asparin	69	6.0
MTa	M	58	hypertension	amilodipine atenolol	64	*7.2
MTr	F	69	anxiety arthritis	coproxamol	76	*6.4
MW	F	73	depression thyroid disease	prothiaden		5.6
CY	M	52	depression	imipramine temazepan diazepan	63	11.5

* individuals with conditions or receiving medication that can effect the immune response and where therefore excluded from the neopterin study.

APPENDIX 5

TREATED PD RESULTS FOR GA-TF BINDING AND NEOPTERIN

I.D	Sex	Age	additional conditions	treatment	% Ga-Tf binding	neopterin (nmol/L)
DA	M	62	none	sinemet plus fitd	-	6.0
SA	M	89	none	sinemet plus ttd	59	12.4
TA	F	61	none	sinemet plus od	72	-
PA	M	61	none	sinemet plus td sinemet CR ttd selegiline 10mg	67	5.6
SB	M	76	heart disease	sinemet std selegiline 10mg digoxin frusamide	67	23.8
MB	F	75	arthritis	madopar ttd selegiline 10mg amitriptyline volterol zantac	72	*7.3
BB	F	56	none	sinemet benzhexol	55	6.2
LB	M	69	none	sinemet plus ttd	-	5.6
TB	M	70	ulcer	sinemet plus ftd	65	*4.7
JC	F	49	none	sinemet plus ttd selegiline 10mg	64	4.3
MC	F	71	none	sinemet plus ftd selegiline	72	-
RD	M	59	none	selegiline	-	4.2
SF	M	71	hiatus hernia epilepsy	sinemet ttd selegiline amitriptyline phenytoin chlorodiazepoxide carbamazepine	66	9.4
EG	M	71	psoriasis	sinemet LS fitd sinemet CR selegiline	74	5.1
HG	F	65	none	sinemet plus ftd nitrazepan 2mg dispirin		8.6
RG	M	67	neurosis urinary infection	madopar sinemet 110 selegiline 10mg diazepan ciproxin	60	*8.5
EG	F	61	none	sinemet plus ttd selegiline 10mg	76	7.1
PG	F	68	depression	sinemet CR amitryptyline	64	5.4
EG	F	62	fluid retention	sinemet LS ttd selegiline 2 asparin multi-vitamins	75	6.9
WH	M	72	osteoarthritis vascular disease	sinemet plus ttd nitrazepan	65	11.1
CH	F	52	none	sinemet plus	72	5.7

I.D	Sex	Age	additional conditions	treatment	% Ga-Tf binding	neopterin (nmol/L)
TJ	M	58	none	sinemet ftd selegiline 10mg	67	-
DJ	F	66	none	sinemet selegiline 10mg amitryptyline	32	9.2
JK	M	46	none	sinemet ftd selegiline 5mg benzhexol domperidone apomorphine	50	-
HK	F	74	heart disease	madopar selegiline digoxin frusamide	66	5.9
JM	M	82	none	sinemet	-	7.7
MM	F	84	none	sinemet CR sinemet 110 benzhexol	69	7.1
GO	F	77	none	sinemet plus std disipal	56	7.9
GR	M	43	none	benzhexol	70	4.0
MR	M	53	none	sinemet plus ttd selegiline 10mg	-	3.4
FS	M	78	none	sinemet ftd selegiline 10mg	-	4.9
CT	M	69	hypertension	selegiline	-	*7.4
MT	F	71	depression	sinemet 110 ttd amitryptyline	-	3.4
DT	M	55	none	sinemet plus ftd	-	7.0
VS	F	63	asthma hypertension hiatis hernia rheumatoid-arthritis	benzhexol salbutamol co-amilozide co-drydramol cimetidine	69	*7.4
MW	F	74?	none	sinemet	72	3.9
GWh	F	66	hypothyroidism depression	sinemet plus thyroxine	70	8.4
GWo	F	73	none	sinemet ftd benzhexol 125mg digoxin	68	10.2
GWy	M	63	none	sinemet plus ftd amitriptyline 25mg	-	5.6

* individuals with conditions or receiving medication that can effect the immune response and where therefore excluded from the neopterin study.

od = once daily td = twice daily ttd = three times daily
ftd = four times daily fitd = five times daily std = six times daily

APPENDIX 6

STUDY A (SIGMA KIT)

CIC LEVELS IN CONTROLS

ID	Sex	Age	condition	treatment	CICs	CICs - neg control
HD	F	41	none	nil	10.83	3.89
PD	M	22	none	nil	19.5	11.35
GG	M	49	none	nil	3.72	-1.68
PHi	M	53	none	nil	10.2	4.23
PH	M	24	none	nil	12.3	6.33
CP	M	56	none	nil	19.5	10.3
YW	F	46	none	volterol	6.04	-0.1
CW	F	21	none	nil	4.44	-2.56
ClW	F	17	none	nil	3.75	-3.25
SW	F	23	none	nil	6.89	-0.74

CIC LEVELS IN UNTREATED PD PATIENTS

ID	Sex	Age	additional conditions	treatment	CICs	CICs - neg control
IB	F	68	diabetes depression	lofepramine	6.2	0.23
JC	M	72	none	co-drydramol	6.73	-1.42
BF	F	62	thyroid disease	diazepan paracetamol	6.4	0.3
MG	F	55	none	nil	5.2	0.9
MH	F	61	hypertension	bendrofluazide rampril	6.71	0.74
DJ	F	65	depression	lorazepan clomipramine	9.65	0.45
MM	F	76	none	nil	2.23	-2.37
DN	M	45	none	nil	5.0	0.4
SN	F	43	none	noriday	3.67	-2.5
LO	M	76	arthritis	none	3.28	-2.89
DP	M	52	ulcer	omeprazol	2.48	-3.53
IS	F	59	osteoarthritis	none	26.67	19.73
HS	M	67	depression hiatus hernia	prothiaden zantac	3.4	-1.2
MT	F	69	anxiety arthritis	coproxamol	5.64	-0.53
MW	F	73	depression thyroid disease	prothiaden	3.5	-1.1

CIC LEVELS IN TREATED PD

ID	Sex	Age	additional conditions	treatment	CICs	CICs - neg control
PA	M	60	none	sinemet fitd selegiline 10mg domperidom	18	11.06
BB	F	56	none	sinemet benzhexol	6.72	0.71
JC	F	49	none	sinemet plus ttd selegiline 10mg	3.65	-2.5
RD	M	59	none	selegiline 10mg	4.86	-2.77
DJ	F	66	none	sinemet selegiline 10mg amitriptyline	4.69	-1.32
TJ	M	58	none	sinemet ftd selegiline 10mg	8.95	2.98
MM	F	84	none	sinemet CR sinemet 110 benzhexol	15.23	7.08
JM	M	81	none	madopar fitd	21.75	14.33
GO	F	77	none	sinemet plus std disipal	7.8	2.4
FS	M	78	none	sinemet selegiline	6.63	-1.13
DT	M	55	none	sinemet plus ftd	7.48	-0.15
MT	F	71	none	sinemet 110 ttd amitryptyline	2.58	-5.05
MW	F	74	none	sinemet otd	7.79	0.22

APPENDIX 7

STUDY B (QUIDEL KIT)

CIC LEVELS IN CONTROLS

ID	Sex	Age	additional conditions	treatment	CICs
RA	M	44	none	nil	0
SW	F	23	none	nil	1.07
CW	F	21	none	nil	1.02
ClW	F	18	none	nil	0.47
SK	F	24	none	nil	1.45

CIC LEVELS IN TREATED PD

ID	Sex	Age	additional conditions	treatment	CICs
DA	M	62	none	sinemet plus fitd	1.33
SA	M	89	none	sinemet plus ttd	2.73
PA	M	61	none	sinemet fitd selegiline 10mg	1.96
MB	F	75	arthritis	domperidom madopar ttd selegiline 10mg amitriptyline volterol zantac	2.22
TB	M	79	none	sinemet plus ftd	1.73
AF	F	69	none	sinemet ftd seroxat	0.08
SF	M	71	hiatus hernia epilepsy	sinemet ttd selegiline amitriptyline phenytoin chlorodiazepoxide carbamazepine	0.8
EG	M	71	psoriasis	sinemet LS fitd sinemet CR selegiline???	0.345
HG	F	65	none	sinemet plus ftd nitrazepan 2mg	4.62
RG	M	67	neurosis	dispirin madopar sinemet 110 selegiline 10mg diazepan	1.9
EGr	F	61	none	sinemet plus ttd selegiline 10mg	1.47
PG	F	68	depression	sinemet CR amitryptyline	1.18
EGu	F	62	fluid retention	sinemet LS ttd selegiline 10mg asparin multi-vitamins	2.71
CH	F	52	none	sinemet plus	2.5
HK	F	74	heart disease	madopar std selegiline 10mg digoxin frusamide	1.93
JM	M	82	none	sinemet	0.95
MM	F	85	none	sinemet CR sinemet 110 benzhexol	0.74
GR	M	43	none	benzhexol	0.71
MR	M	53	none	sinemet plus ttd selegiline 10mg	0.39
CT	M	69	hypertension	selegiline	0.22
GWo	F	73	none	sinemet ftd benzhexol 125mg digoxin	1.02
GWy	M	63	none	sinemet plus ftd amitriptyline 25mg	10.97