Microglia and Chitotriosidase in Multiple Sclerosis

a report by

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Initial biochemical investigations led to the unexpected discovery that plasma samples of patients with Gaucher disease (GD), a lysosomal storage disease, had a several hundred-fold elevated ability to hydrolyse chitin, a polymer of beta 1,4-linked N-acetylglucosamine that is largely diffuse in nature.[1] Later, the same investigators observed that lipid-laden activated macrophages accumulating in GD tissues were able to secrete exceedingly high levels of an enzyme able to cleave chitin and artificial chitotrioside substrates, therefore named chitotriosidase (*Chit*).[2,3] The measurement of plasma *Chit* activity has now also found an application in the clinical monitoring of patients with Fabry disease.[4]

Chit is a member of the mammalian chitinase family. It is mainly synthesised by activated macrophages and also by immature neutrophils[5] as a 50kDa protein, proteolitically cleaved and predominantly secreted. Its natural substrate, chitin, is known for being an integral part of an arthropod's exoskeleton.[6–9] Its content is variable within species and is reported to account for up to 20% of the fungal cell wall.[10,11] So far, no definitive mammalian chitin synthetase has been documented, although a pathogenic role of this oligosaccharide in vertebrates has been reported. Chitin is utilised in the enhancement of the inflammatory roles of macrophages and neutrophils[11,12] and, in such instances, may also modulate the cerebral microglial activation documented in neuro-inflammatory and neurodegenerative diseases.[13,14]

An important question arises when considering that chitin is absent in humans. However, the fact that the *Chit* gene is present and conserved in rodents and primates[15] argues in favour of a biological role of this enzyme.

Chit, Chitin and the Central Nervous System

In pathological conditions *Chit* has achieved an increasingly important role as a marker of some neurological diseases,[16] perhaps by virtue of the peculiar immune condition that characterises the brain. Nonetheless, physiological conditions such as ageing have also been found to correlate with a progressive increase of plasmatic *Chit* activity.[17,18] This might suggest that the innate immune system is involved in protecting the healthy human organism from cell damage that occurs during ageing, perhaps linked to oxidative processes.[19]

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Given its peculiar immune 'privilege', healthy central nervous system (CNS) status is tightly regulated and maintained at a low functional level in order to prevent immune-mediated damage from occurring. Among brain cells, microglia and a few other cell types such as astrocytes represent components of the innate immune system that promptly activate a complex immune cascade soon after specific or unspecific stimuli – either ischaemic–oxidative or inflammatory – face the brain. Microglia are the resident macrophages in the CNS. They exert important functions such as phagocytosis of cellular debris and/or neuronal signal processing when activated through communications with neurons, immune cells and other glial cells.[20–22] Activation of microglia occurs in most pathological processes. This activation is accompanied by changes in morphology, upregulation of immune surface antigens and production of cytotoxic or neurotrophic molecules.[20,23,24]

Despite the fact that chitin is absent in humans, *Chit* has been found to be elevated in some brain diseases, as described below. However, chitin-like substances have recently been found to accumulate within the brain in certain circumstances. Several lines of evidence exist that implicate impaired glucose metabolism in several CNS diseases.[25–27] It is known that brain hypoperfusion and several inflammatory conditions lead to increased glucose metabolism, which in turn strongly increases hexosamine pathway activity in endothelial cells, leading to synthesis of glucosamine polymers.[28,29] Glucosamine has various physiological properties: it inhibits pro-inflammatory cytokines from antigen-presenting cells (APCs), suppresses T-cell response by interfering with functions of APC and shows a direct inhibitory effect on CD3-driven T-cell proliferation.[30,31] Glucosamine administration in the animal model of multiple sclerosis (MS), known as experimental autoimmune encephalomyelitis (EAE), significantly reduces macrophage infiltration within the inflamed brain and reduces microglial activation and production of nitric oxide (NO) and inflammatory cytokines, such as interferon (IFN)-γ, interleukin (IL)-17 and tumour necrosis factor (TNF)-α, resulting in resistance to acute EAE.[31] However, glucosamine polymers are also the building blocks of chitin and chito-saccharides.[28,32–34] Thus, the markedly augmented glucose metabolism that occurs during inflammation or hypoperfusion within the brain could induce increased formation of chitin-like substances.[33,34]

Chit in Stroke

It is now very well established that after an acute brain ischaemia, early activation of microglia and endothelial cells and their transcription of TNF-α and IL-6 are able to induce a cascade of inflammatory pathways that transforms local endothelia into a pro-thrombotic state and allows peripheral mono- and poly-morphonuclear cytotoxic chemoattraction into the lesion site.[35,36] This early event correlates with the worsening of the cerebral damage, as a clear relationship between the extent of

brain damage and the early increase of TNF-α plasma level is generally reported.[37] Besides the increase of innate immune cytokines, we and others[38,39] have also found increased *Chit* activity in stroke, suggesting a relevant role for infiltrated macrophages, or resident microglia activated by the ischaemic event, inside the brain. Our results also suggest that *Chit*, TNF-α and other pro-inflammatory cytokines are markers of microglia activation occurring during a stroke, which is independent of pre-existing inflammatory or infectious conditions of the patients.[38]

Chit in Alzheimer's Disease and Ageing

Amyloid plaques, senile plaques and amyloid angiopathy of Alzheimer's disease (AD) brains have been described to co-localise with chitin-like glucosamine polymers. Such polymers are suggested to induce the formation of pathogenic amyloid fibrils.[33,34] Based on this original finding, we first conducted an association study of *Chit* in the plasma of people with AD,[18] analysing *Chit* activity in AD patients and healthy individuals. Results were unexpectedly interesting and indicated that *Chit* values in the AD group were significantly higher than those obtained from individuals of comparable age. Moreover, plasma *Chit* activity level correlated with the individual's age in the whole control group, confirming an age-related physiological *Chit* increase (see *Figure 1*).

It is thought that β-amyloid brain deposition is involved in the AD neurodegenerative cascade, perhaps through direct neurotoxicity or an immune network.[33,34,40,41] In this light, the high *Chit* expression – as demonstrated also by Di Rosa and collaborators[42] – may have a dual role: it either represents a mere epiphenomenon of a strong macrophage–microglia activation due to β-amyloid deposition, or reflects a scavenging protective immune activity against potentially pathogenic chitin-like glucosamine polymers.[33] Thus, encouraged by these findings, we performed an immunocytochemistry study in order to explore the presence of chitin-like substances within AD, MS and healthy control (HC) brains. On the one hand, we could confirm the presence of abundant chitin-like substances in the brains of AD patients, which may well relate to the detection of peripheral *Chit* activity. Conversely, we failed to demonstrate the deposition of chitin-like substances in both MS and normal brains.[43]

Chit in Multiple Sclerosis

MS is a chronic inflammatory/degenerative disease of the CNS. Most studies in humans, principally based on the EAE animal model, claim that an adaptive T- and B-cell antigen-specific response is patho-physiologically characteristic of this disease.[44] However, other studies indicate that the innate immune response predominates in most MS lesion subtypes; this response is initiated by both resident (microglia) and peripheral infiltrating macrophages.[45,46] TNF-α, IL-6, NO, reactive oxygen species (ROS) and other macrophage-derived products unfortunately show only a modest correlation with clinical activity in MS, and are as yet of very limited usefulness in clinical routine.[47] Through a case-control study, we demonstrated that *Chit* activity is increased in the blood and, particularly, in the cerebrospinal fluid (CSF) of MS patients,[48] and that this activity correlates well with the extent of CNS damage as scored by the Extended Disability Status Scale (EDSS) at follow-up[49] (see *Figures 2* and *3*).

Considering that with the normal immunoglobulin G (IgG) index found in these patients the *Chit* activity was of intrathecal rather than peripheral

Figure 1: *Chit* in Ageing and Alzheimer's Disease
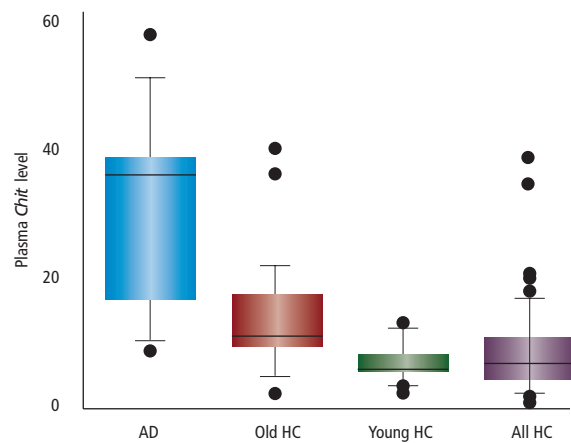
Chit activity in plasma of 30 Alzheimer's disease (AD) patients (blue) and 69 healthy controls (HC); whole HC (purple) comprise a group of 40 younger (green) and 29 older individuals (brown). Chit level in AD is significantly higher compared with the other groups (p<0.01). Also, plasma Chit correlates with ageing (r=0.72; p<0.05). Source: Sotgiu et al., 2007.[18]

Figure 2: *Chit* in Plasma and Cerebrospinal Fluid of Multiple Sclerosis Patients
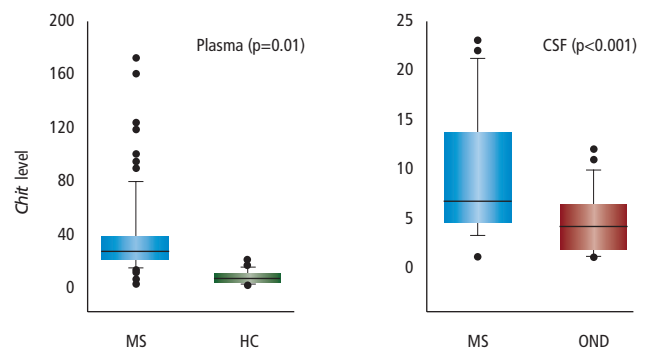

Chit activity differentiates multiple sclerosis (MS) (blue) from healthy controls (HC) (green) and other neurological disease (OND) subjects (brown) in both plasma and the cerebrospinal fluid (CSF). Chit level expressed as nmol/ml/h on the y-axis is significantly higher in the plasma of MS patients (77 subjects) compared with matched healthy controls (HC; 309 subjects). Chit level is also significantly higher in the CSF of MS patients (23 subjects) compared with patients with OND (36 subjects). Mann-Whitney test p-values are indicated. Source: Sotgiu et al., 2006.[48]

origin, we also calculated what we call the 'Chit ratio' (CSF Chit/plasma Chit) and compared the EDSS score of the individual patients, obtained after a mean follow-up of five years (range two to eight years), with both oligoclonal bands (OCB) number and *Chit* ratio at the time of CSF withdrawal. While there was no correlation between EDSS and OCB, there was a significant correlation between EDSS and the *Chit* ratio (p<0.001). Higher disease severity (EDSS ≥5.5 at follow-up) was restricted to those patients with initial higher intrathecal *Chit* level and *Chit* ratio (see *Table 1*).

This study[48] not only confirms the important role of the innate immune response in MS phenomenology, but also allows *Chit* determination in CSF and plasma to be proposed as a diagnostic and monitoring method in an MS laboratory. As a result of our analysis, plasma and, to a larger extent, CSF *Chit* levels better correlate with the extent of CNS damage compared with the previously proposed macrophage-derived markers such as TNF-α, IL-6, IL-1, NO and ROS. As *Chit* CSF production is unrelated to plasma level and albumin quotient, we argued that *Chit* activity is compartmentalised in MS.

## Figure 3: Cerebrospinal Fluid/Plasma *Chit* Ratio and Disability in Multiple Sclerosis Patients
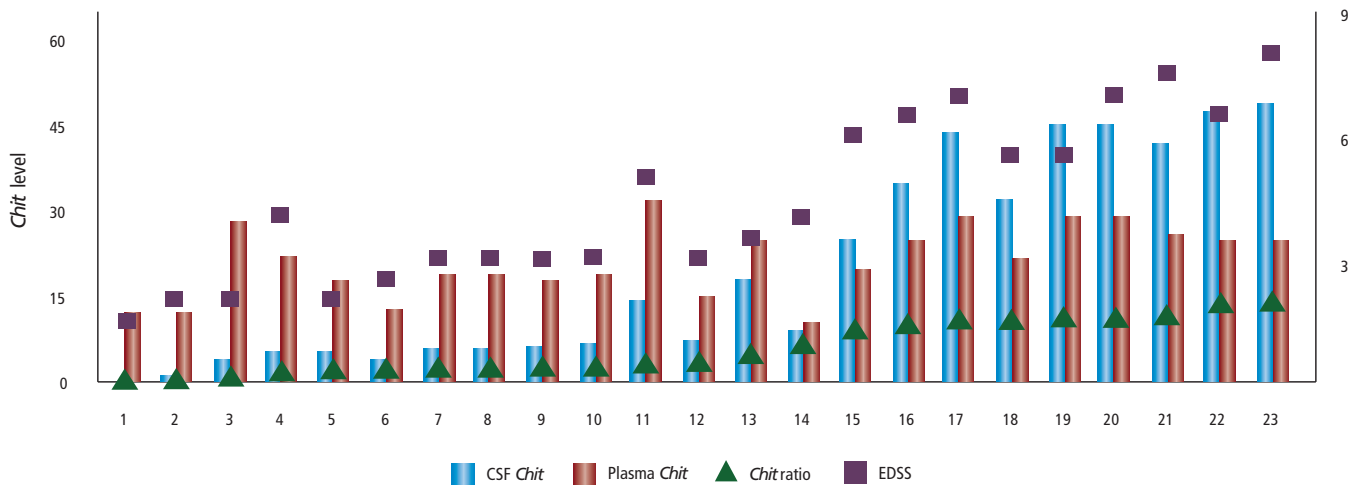
Correlation between Chit *levels in cerebrospinal fluid (CSF) (blue) and plasma (brown) on left y axis (Chit activity expressed as nmol/ml/h), Extended Disability Status Scale (EDSS) (purple squares) and* Chit *ratio (green triangles) on right y-axis in 23 patients (x-axis).*
Source: Sotgiu et al., 2006.[48]

Table 1: Correlation Between *Chit* Ratio (X Variable) and Cerebrospinal Fluid/Clinical Findings (Y Variables) in the Multiple Sclerosis Patients Series

Variables		Statistics	
X	Y	r	p
Chit ratio	Albumin quotient	-0.12	NS
"	EDSS at entry	0.24	NS
"	EDSS at follow-up	0.91	<0.001

Correlation coefficient (r) and significance (p) values are indicated. EDSS = Extended Disability Status Scale; NS = not significant.
Source: Sotgiu et al., 2006.[48]

Figure 4: *In Vitro Chit* Production from Murine Microglial Cells
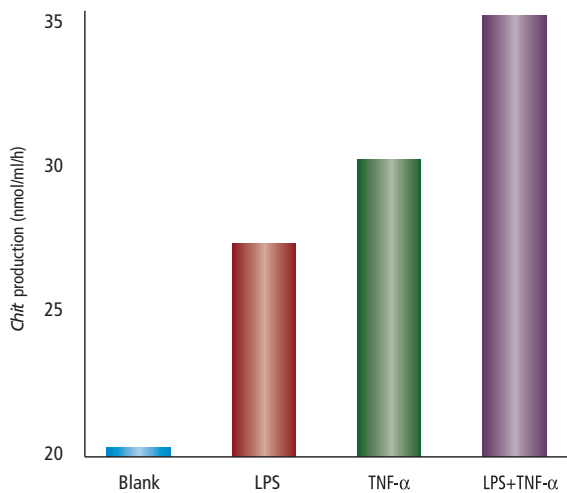
In vitro Chit *production from murine microglial cells incubated for 24 hours in the absence (blank) or presence of lipopolysaccharide (LPS) 100ng/ml, tumour necrosis factor (TNF)-α 100U/ml or in combination (see legend of the x-axis).* Chit *activity is expressed as nanomoles of the substrate hydrolysed per ml per hour (nmol/ml/h).*

Despite the fact that this enzymatic activity likely is derived from infiltrating macrophages, we thought that resident microglia might be induced to produce *Chit* as these cells can gradually transform phenotypically into lipid-laden activated macrophages.[50] This intrathecal, possibly microglia-derived *Chit* activity found in MS patients could counterbalance the naturally occurring glucosamine aggregation and its transformation into possibly detrimental chitin-like polymers.

Microglial Production of *Chit*

For this reason we have performed a new study with the aim of documenting a microglia-derived *Chit* production (Sotgiu et al., submitted). To this purpose, murine microglial cell line N9 was plated in 96-well tissue-culture plates with Royal Park Memorial Institute (RPMI) supplemented with 10% foetal bovine serum at 4×10^4 per 100μl per well and incubated in the presence or absence of lipopolysaccharide (LPS) 100ng/ml and TNF-α 100U/ml, or in combination. Different conditions were performed in quadruplicate.

Chit was determined according to published methods[38,48] in the supernatant of microglial cell cultures: briefly, after 24 hours of culture, cell-free supernatants were collected; 30μl were incubated with 22μlmol/l 4-methylumelliferyl-β-d-*N',N'',N'''*triacetylchitotriose in 0.5M citrate-phosphate buffer at pH 5.2, and fluorescence was measured on a fluorimeter. *Chit* activity was calculated as nanomoles of substrate hydrolysed per ml per hour (nmol/ml/h). In *Figure 4*, *Chit* production from microglial cells is shown; this production is susceptible to modification by changing experimental conditions.

Conclusions

Until recently, *Chit* was known to be produced only in lysosomal storage diseases.[1] Thanks to the contribution of other groups, including ours, this enzyme has progressively acquired new associations with other previously unrelated diseases, particularly neurodegenerative and neuroinflammatory diseases (reviewed in reference 16). In such circumstances, and by analogy to storage diseases, it has long been thought that the increase of *Chit* activity in the plasma of neurological patients, as well as in normal ageing, likely depends on macrophage activation at a peripheral level, perhaps linked to the known scavenging role of macrophages.[19] For the first time, we described that such enzymatic activity is also present in the CSF of patients with MS and that it is compartmentalised within the CNS, and therefore is of intrathecal origin.[48]

The question remained open for a couple of years as to whether such *Chit* production was of macrophage or microglial origin. Macrophages are known to rapidly and diffusely infiltrate the MS lesion sites[47] through the blood–brain barrier and are also known to produce *in loco*

a huge amount of inflammatory products.[45,46] However, microglia within the MS lesion are also known to give rise to continuous transformation into macrophages under facilitating circumstances such as digestion of myelin products.[50] Thus, the question as to whether intrathecal *Chit* production is autochthon (i.e. microglial-derived) or exogenous (from infiltrating macrophages) has remained unsolved for some time.

We now document that murine microglia can produce *Chit* under certain favourable *in vitro* circumstances, i.e. pro-inflammatory stimuli, and suggest that intrathecal *Chit*, recently found in MS patients,[48] is of microglial origin. It is unknown whether *Chit* activity has direct influences on the CNS or whether it simply reflects a relic of an archaic macrophage response to chitin-containing pathogens.[5,16,48] Thus, another important issue is whether *Chit* is determinant in the pathogenesis of MS. Perhaps an even more important issue involves the cerebral accumulation of chitin or chitin-like polymers and the role, if any, that they play. New histopathological studies on the AD brain[33,34,43] allow us to argue in favour of a protective role of *Chit* in the CNS by avoiding brain accumulation of chitin-like oligosaccharides.

Several contradictory reports in the literature document that β-amyloid plaques may be either protective or pathogenic elements in AD.[13,14,27,51,57] If β-amyloid has a pathogenic and not only a pathognomonic role in AD, the role of chitin may be that of favouring and accelerating its detrimental deposition.[33] Recently, Castellani and colleagues[34] suggested that chitin-like polysaccharides within the AD brain could provide a scaffolding for β-amyloid deposition and that glucosamine may therefore facilitate the process of amyloidosis. This finding may support the hypothesis that an imbalance between glucosamine polymerisation and

degradation in AD favours fibril formation. Thus, the different histochemical content of chitin-like substances recently found between MS and AD brain samples may be a reflection of different pathophysiological mechanisms.[33,34,43] The markedly augmented glucose metabolism occurring during inflammation in the MS brain could induce a spontaneous increase of glucosamine-derived chitin-like substances. In this context, the microglia-derived *Chit* activity[48] could counterbalance the naturally occurring glucosamine aggregation as well as its transformation into detrimental chitin, thus having a protective rather than a detrimental role within the CNS. In contrast, deposition of chitin-like substances in the AD brain could result from a reduced immune response that characterises the most severe clinical expression of disease,[58] or from an impaired chitin cleavage in relation to the advanced age of AD patients.

In conclusion, the role of *Chit* in MS is intriguing for its possible protective function. Much remains to be done in this area, but, even if it is still unclear whether *Chit* is a protagonist or a bystander, it could well represent a simple and sensible marker for monitoring MS, and as such deserves further attention. ■

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