Technische Universität München
Klinik und Poliklinik für Strahlentherapie und Radioonkologie
Klinikum rechts der Isar
(Direktor: Prof. Dr. Stephanie E. Combs)

Heat shock protein 70 in multiple sclerosis and its potential as a biomarker for inflammatory processes

Patricia Zimmermann

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Vorsitzender:	Prof. Dr. Jürgen Schlegel
Prüfer der Dissertation:	1. Prof. Dr. Gabriele Multhoff
	2. Prof. Dr. Thomas Korn

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Summary

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by inflammatory and neurodegenerative processes. Inflammation induces the synthesis and release of heat shock protein 70 (Hsp70) into the extracellular space, which in turn can elicit immune responses. The aim of this study was to determine the level of extracellular Hsp70 in serum and cerebrospinal fluid (CSF) of patients with different MS subtypes and evaluate its potential as a biomarker to distinguish inflammatory and neurodegenerative processes in MS.

For this purpose, serum and CSF was obtained from patients with MS and, as controls, from patients with non-inflammatory neurological diseases (NIND), other inflammatory neurological diseases (OIND), tumors of the CNS, and furthermore, serum samples of healthy donors (HD). Hsp70 levels were quantified using the R&D enzyme-linked immunosorbent assay (ELISA), which determines free Hsp70 and the lipHsp70 ELISA, which additionally detects lipid-bound Hsp70.

Hsp70 serum levels in MS patients revealed significantly higher values than HD ($p < 0.001$) and significantly lower values than OIND ($p = 0.001$). An analysis of CIS/RRMS compared to progressive MS displayed significantly higher levels in patients with CIS/RRMS than with SPMS/PPMS ($p < 0.05$). Divided into the four different subtypes of MS, CIS and RRMS displayed the highest Hsp70 levels followed by SPMS and PPMS with the lowest Hsp70 levels. In CSF, Hsp70 occurred in lower concentrations compared to serum, and displayed, therefore, non significant results. A relation of the values to cell count and to blood brain barrier (BBB) disruptions could not be detected.

As potential influencing parameters, age ($p = 0.435$) and gender ($p = 0.422$) were analyzed, but displayed no effect on the Hsp70 serum levels. Furthermore, long-term stability of Hsp70 serum levels could be shown in patients diagnosed with RRMS. Active inflammatory processes, indicated by clinical symptoms ($p = 0.249$) or gadolinium (Gd) enhancing lesions in magnetic resonance imaging (MRI) of the CNS ($p = 0.357$), did not result in further elevated serum Hsp70 levels. Nevertheless, after an acute relapse the Hsp70 serum levels displayed a decrease under a high-dose methylprednisolone (MPS) therapy in patients, who responded to the treatment.

Within the group of patients with tumors of the CNS, the serum Hsp70 level indicated a correlation with the severity of the disease and displayed significantly higher levels than all other examined groups.

In summary, serum Hsp70 levels might be a useful biomarker to identify inflammatory processes and to differentiate inflammatory and neurodegenerative processes in MS. As a consequence, serum Hsp70 levels have the potential to become a useful diagnostic and prognostic biomarker in future MS therapy.

Zusammenfassung

Multiple Sklerose (MS) ist eine chronisch-entzündliche Erkrankung des zentralen Nervensystems (ZNS), die durch Entzündung und Neurodegeneration charakterisiert ist. Entzündung induziert die Synthese von Hitzeschockprotein 70 (Hsp70) und dessen Freisetzung in den extrazellulären Raum, was wiederum Immunreaktionen hervorrufen oder verstärken kann. Das Ziel dieser Dissertation war, erstmals Hsp70 im Serum und im Liquor von Patienten mit MS zu bestimmen und so sein Potential als Biomarker zur Unterscheidung von entzündlichen und neurodegenerativen Prozessen zu untersuchen.

Hierzu wurde Serum und Liquor von Patienten mit MS und als Vergleichsgruppen von Patienten mit anderen entzündlichen neurologischen (OIND) und nicht entzündlichen neurologischen Erkrankungen (NIND) gesammelt. Als weitere Vergleichsgruppen dienten Patienten mit Hirntumoren und gesunde Spender (HD). Die Hsp70-Werte wurden mit Hilfe des R&D enzyme-linked immunosorbent assay (ELISA), der freies Hsp70 detektiert und dem lipHsp70 ELISA, der zusätzlich lipidgebundenes Hsp70 messen kann, bestimmt.

Patienten mit MS zeigten signifikant höhere Werte von Hsp70 im Serum als HD ($p < 0.001$) und signifikant niedrigere verglichen mit OIND ($p = 0.001$). Eine Analyse der Untergruppen ergab, dass die Werte der Patienten mit CIS/RRMS signifikant höher waren als die von Patienten mit progredient verlaufender MS – SPMS/PPMS ($p < 0.05$). Mit Blick auf alle vier Untergruppen zeigten CIS und RRMS die höchsten Werte, gefolgt von SPMS und PPMS.

Als mögliche Einflussfaktoren wurden Alter ($p = 0.435$) und Geschlecht ($p = 0.422$) analysiert, es zeigten sich aber keine Effekte auf den Hsp70-Wert im Serum. Zusätzlich blieb der Hsp70-Wert über längere Zeiträume stabil. Ein Schub, angezeigt durch klinische Symptome ($p = 0.249$) oder Gadolinium anreichernde Bereiche in der Magnetresonanztomographie (MRT) des ZNS ($p = 0.357$), erhöhte den Wert nicht. Nichtsdestotrotz führte eine Hochdosis-Kortison-Therapie bei einem akuten Schub zu absinkenden Werten, wenn der Patient klinisch gut auf die Medikamente ansprach.

In der Gruppe der Patienten mit Hirntumoren deuteten die Hsp70-Werte auf einen Zusammenhang mit dem Schweregrad der Erkrankung hin und zeigten signifikant höhere Werte als in allen anderen untersuchten Patientengruppen.

Zusammenfassend zeigte der Serum Hsp70-Wert das Potential als Biomarker, um entzündliche Prozesse zu identifizieren und um diese von Neurodegeneration in MS abzugrenzen. In der Zukunft könnte Hsp70 in Serum daher als diagnostischer und prognostischer Biomarker in der MS-Therapie nützlich werden.

Contents

List of Abbreviations

AIF	Apoptosis-inducing factor
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APC	Antigen-presenting cells
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood brain barrier
BSA	Bovine serum albumin
CIS	Clinically isolated syndrome
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DIS	Dissemination in space
DIT	Dissemination in time
DMT	Disease-modifying therapy
EAE	Experimental autoimmune encephalomyelitis
EBV	Ebstein-Barr virus
EDSS	Expanded Disability Status Scale
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Gd	Gadolinium
GA	Glatiramer acetate
HD	Healthy donors
HLA	Human leukocyte antigen
HRP	Horseradish-peroxidase
HSF	Heat shock factor
HSP	Heat shock protein
IFN	Interferon
IFNb	Interferone beta
IgG	Immunoglobulin G
IL	Interleukin
IND	Inflammatory neurological diseases
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MPS	Methylprednisolone
MRI	Magnetic Resonance Imaging

MRT	Magnetresonanztomopgraphie
MS	Multiple sclerosis
N	Number
NIND	Non-inflammatory neurological diseases
NK	Natural killer
NT	No treatment
NZ	Natalizumab
OIND	Other inflammatory neurological diseases
OT	Other or unspecified treatment
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PLP	Proteolipid protein
PPMS	Primary progressive multiple sclerosis
ROC	Receiver operating characteristic
RRMS	Relapsing-remitting multiple sclerosis
QAlb	Albumin quotient
SD	Standard deviation
SPMS	Secondary progressive multiple sclerosis
Th1	T-helper Type 1
Th17	T-helper Type 17
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TUM	Technical University of Munich
WHO	World Health Organization
ZNS	Zentrales Nervensystem

1 Introduction

1.1 Multiple Sclerosis

Autoimmune diseases are a major health problem with approximately one in 30 individuals affected (Wanstrat and Wakeland, 2001). There are more than 80 different identified autoimmune diseases (Fox, 2007; Hayter and Cook, 2012), which have in common the underlying defect in the immune response, which induces an attack against the body's own structures (Fox, 2007). Multiple sclerosis (MS) is one of the six most common autoimmune diseases alongside rheumatoid arthritis, Graves' disease, diabetes mellitus, pernicious anemia and systemic lupus erythematosus (Wanstrat and Wakeland, 2001), and is the most frequent chronic disease of the central nervous system (CNS) in young adults (Gold et al., 2012; Hoffmann et al., 2009).

In this chapter, background information about MS including epidemiology, etiology and pathogenesis is presented. Furthermore, the clinical features and their different diagnostic procedures are explained, followed by an animal model for MS and therapeutic options.

1.1.1 Epidemiology

MS has an average prevalence of 33 per 100,000 and affects worldwide about 2.3 million people (*Atlas of MS Data* 2013). Its global distribution is inhomogeneous (cf. Figure 1.1) with a focus on Northern Europeans and their successors (Compston and Coles, 2008; Hoffmann et al., 2009). Therefore, Europe (108 per 100,000) and North America (140 per 100,000) have the highest prevalence for MS. In contrast, there are less than 5 people per 100,000 suffering from MS in East Asia and Southern Africa. Within Europe a difference depending on the latitude can be observed, since Sweden in the north has the highest

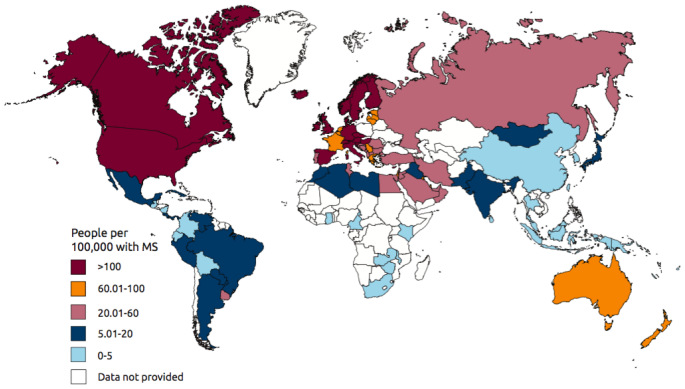


Figure 1.1: Prevalence of MS by country (2013) (*Atlas of MS Data* 2013)

(189 per 100,000) and Albania in the south the lowest prevalence (22 per 100,000). Germany lies in-between with 149 patients per 100,000 inhabitants and has an incidence of 5 per 100,000. Thus, the number of MS patients in Germany is estimated to be around 130,000 (*Atlas of MS Data* 2013).

Besides the global distribution, there are also gender-based differences with twice as many affected women as men (*Atlas of MS Data* 2013). Furthermore, the average onset of the disease is between the ages of 20 and 40 years with a peak around the age of 30 (Hoffmann et al., 2009). Manifestations at a young age (< 18 years, 2 - 5 %) (*Atlas of MS Data* 2013) as well as in older ages (> 50 years, 3 - 5 %) are possible but rare (Delalande et al., 2002; Polliack et al., 2001; Schmidt et al., 2015).

1.1.2 Etiology

Currently, it is presumed that MS has a multifactorial genesis (Hoffmann et al., 2009). Genetic and environmental factors are commonly seen to be involved in the disease development (Compston and Coles, 2008). These two aspects are described below.

For first-degree relatives the risk of developing MS is increased from 0.1 (Sadovnick and Baird, 1988) to 3 % (Compston and Coles, 2008). In twins, the concordance rate for monozygotic twins is 25 % or five times higher than for dizygotic twins at just 5 % (Willer et al., 2003). Due to this higher incidence in twins a genetic involvement can be presumed (Compston and Coles, 2008).

A correlation between autoimmune diseases and variations in the human leukocyte antigen (HLA) alleles, was discovered in the 1960s. Less than a decade later, the association between HLA and MS was confirmed (Gourraud et al., 2012). The *HLA-DRB1*15:01* region has been identified as the major risk factor for MS (Patsopoulos et al., 2013). Beside the genes in the HLA regions, more than 100 genes in non-HLA regions have been specified in association with MS. Many of them are involved in the regulation of the immune system (Andlauer et al., 2016).

An additional factor is a variant of the CYP27B1 gene, which is associated with the metabolism of vitamin D and predisposing to MS, as more than 80 % of the genes related to MS – including *HLA-DRB1*15:01* – are presumed among other factors to be regulated by vitamin D (Gourraud et al., 2012). In this regard, not only the genetics, but also the supply of vitamin D and the exposure to the sun in early life are taken into account (Ascherio, 2013).

In conclusion, many different genetic factors, which are involved in the development of MS, have been detected. Nevertheless, the exact impact of genetics and epigenetics is still not completely understood. Due to advances in technology, more findings can be expected in the future (Andlauer et al., 2016; Gourraud et al., 2012).

In addition to genetics, environmental factors also have an influence on the development of

MS. The impacts of migration, tobacco smoking and infectious agents are described below.

As shown before, the prevalence is dependent on the geographical latitude (cf. Section 1.1.1). Migration studies, however, showed that if a person moves to a different latitude before age 15, they adopt the geographical risk of the new place – relocations afterwards show no effect. This indicates the environmental exposure as part of the genesis of MS (Gale and Martyn, 1995).

Tobacco smoking in general increases the risk of autoimmune diseases (Maghzi et al., 2011) and in the case of MS by the factor of two (Fragoso, 2014). In addition to being a predisposing factor, it also leads to a more severe clinical course of disease (Costenbader and Karlson, 2006) and to a faster disability progression (Manouchehrinia et al., 2013).

Furthermore, the infection with certain pathogens, such as measles (Malli et al., 2015), mumps, rubella and Epstein-Barr virus (EBV) (Compston and Coles, 2008), plays a role in the development of MS. In case of EBV, the risk for patients who were infected at a young age is 15-fold higher. Being infected in adolescence leads even to a 30-fold higher hazard (Ascherio, 2013). This underpins the hygiene hypothesis, which says that if a person is grown up in a clean environment, their immune system will have an aberrant reaction when infected at a higher age (Compston and Coles, 2008). In relation to EBV an additional thesis implies a molecular mimicry between viral components and myelin basic protein (MBP) (Serafini et al., 2007), which might lead to a cross-reaction (Compston and Coles, 2008).

Besides smoking and various diseases, other environmental impacts such as obesity, alcohol, diet (Fragoso, 2014; Malli et al., 2015), air pollution, geomagnetism and toxins (Compston and Coles, 2008) are also considered to have an influence on the development of MS.

1.1.3 Pathogenesis

MS is considered as a chronic autoimmune-mediated inflammatory disease of the CNS. It is characterized by inflammation, demyelination, gliosis and neurodegeneration (Frohman et al., 2006; Pasquali et al., 2015; Popescu et al., 2013) mainly located in the white matter but also occurring in the grey matter (Calabrese et al., 2010; Geurts et al., 2005). Those focal areas called plaques or lesions emerge periventricular, juxtacortical, infratentorial or within the spinal cord (Gold et al., 2012; Polman et al., 2011). The dominant cells within the plaques are monocytes (Brück et al., 1995; Hauser et al., 1986), CD4+, CD8+ (Hauser et al., 1986; Traugott et al., 1983) and $\gamma\delta$ T cells (Wucherpfennig et al., 1992). In the course of time, the inflammation and edema decline, macrophages and microglia disappear and finally astrogliosis becomes the prominent feature (Popescu et al., 2013).

Since the cause of MS is not entirely understood, two main theses – the outside-in and the inside-out model – have been established and are described below (Gleixner et al., 2013).

The outside-in model considers an inappropriate immune reaction of T cells as the cause of the inflammation (Turturici et al., 2014) with the suspected key actors CD4+ T-helper cells

Type 1 (Th1), Type 17 (Th17) (McFarland and Martin, 2007) and CD8+ T cells (Crawford et al., 2004). Those cells are activated in the peripheral blood and transmigrate across the blood brain barrier (BBB). Within the brain parenchyma, macrophages and microglia present self antigens, which trigger an inflammatory reaction including the release of cytokines and chemokines. This release causes the recruitment of additional inflammatory cells such as T cells, monocytes and B cells. Their attack is directed to the oligodendrocytes and their myelin sheath. Additional damage is caused by the release of toxic mediators, radicals and by an increased oxidative stress (Popescu et al., 2013).

In conflict with this theory is the fact that the lesions can also occur in the middle of a myelin sheath, not accessible for immune cells from the outside. Furthermore, there was no infiltration of T or B cells found in plaques obtained from patients in early active stages. Therefore, the inside-out model was proposed, which argues that MS is mainly a neurodegenerative disease with inflammation as a result (Turturici et al., 2014).

Despite those conflicting findings, the dominating opinion is that MS is an autoimmune disease as described by the outside-in model.

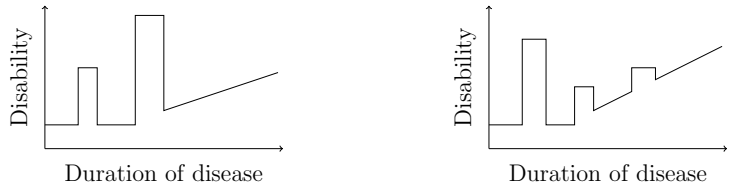
1.1.4 Clinical Features

MS is a heterogeneous disease with different courses and a variety of symptoms. For 80 % of the patients the onset is an acute episode (Compston and Coles, 2008). The presenting symptoms involve most commonly sensory (40 %), motor (39 %) and visual (30 %) systems. In addition, more rare symptoms as fatigue (30 %), balance problems (24 %), sexual dysfunction (20 %), micturition disturbance (17 %), pain (15 %) and cognitive issues (10 %) can be found (*Atlas of MS Data* 2013). If the manifestation indicates a demyelinating event and does not fulfill the criteria of dissemination in time (cf. Section 1.1.5), it is termed clinically isolated syndrome (CIS) (Filippi et al., 2016; Miller et al., 2004). If the criteria is met and the patient has episodes of relapse and remission, the subtype is called relapsing-remitting MS (RRMS) (cf. Figure 1.2a). At the beginning of the disease, the average relapse rate is about 1.8 relapses per year, but decreases over the course of time. After an attack, most patients show a gradual decline of symptoms within six to eight weeks (Gold et al., 2012). Nevertheless, the recovery can be incomplete and some impairments remain over time (Compston and Coles, 2008) (cf. Figure 1.2b).

(a) RRMS with complete remissions (b) RRMS with remaining disability

Figure 1.2: Course of RRMS (adapted from Lublin and Reingold, 1996)

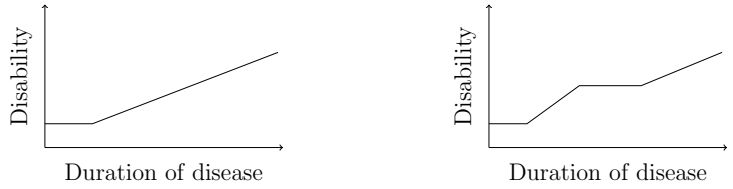
After a disease duration of 10 years about 50 % (Gold et al., 2012) and after 20 - 25 years about 90 % of untreated patients with RRMS develop a secondary progressive MS (SPMS) characterized by a constant worsening (Trojano et al., 2003) (cf. Figure 1.3a). The change from RRMS to SPMS is gradual and cannot be determined at a certain point in time (Lublin et al., 2014). Those patients can also have occasional relapses (cf. Figure 1.3b).



(a) SPMS with constant worsening (b) SPMS with occasional relapses

Figure 1.3: Course of SPMS (adapted from Lublin and Reingold, 1996)

Besides RRMS and SPMS, there is a disease course characterized by progression without relapses from the onset. It is termed primary progressive MS (PPMS) (cf. Figure 1.4a) and represents the most infrequent type with an incidence of 5 - 10 % of all MS patients (Miller and Leary, 2007). During the course of disease, temporary improvements and plateaus are possible (cf. Figure 1.4b).



(a) PPMS with constant worsening (b) PPMS with a plateau

Figure 1.4: Course of PPMS (adapted from Lublin and Reingold, 1996)

1.1.5 Diagnosis

For the diagnosis of MS nearly all countries (96 %) follow the McDonald criteria (*Atlas of MS Data* 2013). They are based on anamnesis, dissemination of lesions in time (DIT) and space (DIS), and exclusion of other causes. An attack is characterized by an acute or subacute episode of focal neurological disturbances, which lasts more than 24 hours. Fever and infection need to be absent, as a change in body temperature can trigger a worsening of the symptoms – the so-called Uhthoff's phenomenon. In order to differentiate two relapses, 30 days have to pass after the occurrence of symptoms of the last attack (Gold et al., 2012; McDonald et al., 2001; Polman et al., 2011).

According to the McDonald criteria, DIT and DIS can be evidenced by magnetic resonance imaging (MRI). DIT is proved by either asymptomatic gadolinium (Gd)-enhancing and

non-enhancing lesions at the same time or a new T2 or Gd-enhancing lesion on a follow-up scan (Polman et al., 2011). For DIS the Swanton's criteria have to be satisfied, which means the evidence of one or more T2 lesions in at least two out of four typical regions of the CNS (periventricular, juxtacortical, infratentorial or spinal cord) are necessary (Swanton et al., 2007). If both disseminations – DIT and DIS – are determined, the diagnosis of RRMS can be made (Gold et al., 2012; Polman et al., 2011).

A disease progression of at least twelve months is necessary for the diagnosis of PPMS. Additionally, two of the following criteria have to be fulfilled: one or more MS typical T2 lesions (periventricular, juxtacortical or infratentorial), two or more lesions within the spinal cord or the proof of oligoclonal bands in the CSF (Gold et al., 2012; Polman et al., 2011).

The degree of neurologic impairments is quantified by a detailed clinical-neurological examination and is measured via the Expanded Disability Status Scale (EDSS). It contains grades from zero indicating no restrictions to ten indicating death due to MS. This score is also used to scale the disease progression (Kurtzke, 1983).

Besides the clinical-neurological examination and a MRI scan, additional tests are required, including blood analyses, lumbar puncture and evoked potentials. The expected results are described hereinafter.

Despite the fact that MS is an inflammatory disease, the common inflammatory serum markers such as C-reactive protein (CRP) (Giovannoni et al., 2001) occur to be normal. The main aim of the laboratory diagnostics is the exclusion of other diseases as for example Lyme disease, sarcoidosis, collagen, cerebrovascular and metabolic diseases (Gold et al., 2012).

Furthermore, an analysis of the CSF has to be carried out. In around 50 % of the patients a mild pleocytosis with more than 4 cells/µl (Andersson et al., 1994), mainly lymphocytes and plasma cells (Gleixner et al., 2013), can be observed. The albumin quotient, an indicator for a dysfunction of the BBB, is negative in around 88 % of the cases. Another sign for an immune response within the CNS is the intrathecal synthesis of immunoglobulin G (IgG). It can be quantified via the IgG quotient, which is increased in 70 - 80 % of the patients. The qualitative verification is done by isoelectric focusing for the presence of oligoclonal bands of IgG. For more than 95 % of the patients this test is positive and, therefore, highly sensitive (Andersson et al., 1994).

Evoked potentials are additionally measured along visually, auditory, somatosensory or motor pathways. The typical results for MS patients are delayed conductions and smaller amplitudes (Gleixner et al., 2013).

In summary, the MS diagnosis is a diagnosis by exclusion. Therefore, a variety of examinations have to be performed to exclude any other cause for the symptoms. Afterwards, the MRI diagnostic and the clinical appearance are decisive.

1.1.6 Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory neurological disease of laboratory animals with similar clinical symptoms as MS. It is triggered by an injection of different proteins, which are part of myelin such as MBP, proteolipid protein (PLP) (Constantinescu et al., 2011) and myelin oligodendrocyte glycoprotein (MOG). The resulting autoimmune response leads to inflammation, demyelinisation and axonal loss (Rao and Segal, 2004).

In contrast to MS, the lesions of EAE are mainly located within the spinal cord rather than in the cerebral and cerebellar cortex (Ransohoff, 2012). Some of the medications used to treat these conditions showed success either on MS or EAE (Constantinescu et al., 2011; Ransohoff, 2012). Nevertheless, EAE is a valuable animal model for the research of disease mechanisms and is useful in testing of new therapeutic compounds (Farooqi et al., 2010).

1.1.7 Therapy

Despite a considerable scientific effort, there is no causal therapy so far and MS remains as an incurable, chronic disease. The course of illness, however, may be influenced by a variety of medications to enhance the patients' quality of life. Depending on the stage, MS is currently treated with different medications: medication during an acute attack, disease-modifying therapy (DMT) and symptomatic treatment (Rosenzweig et al., 2010), which are described below.

1.1.7.1 Acute Attack

Currently, the therapeutic standard of an acute exacerbation is the treatment with glucocorticosteroids. The preferred substance is methylprednisolone (MPS) (500 - 1000 mg/d intravenous) over a period of three to five days. In several studies, this treatment showed a rapid regression of symptoms, but whether it has a positive impact on the progress of the disease in the long term is still not known. If a patient is unresponsive to the high-dose corticosteroids, a plasmapheresis as second-line therapy can be considered (Gold et al., 2012; Rosenzweig et al., 2010).

1.1.7.2 Disease-Modifying Therapy

Different DMTs are required according to the MS subtype. Detailed below are three different substances – interferone beta (IFNb), glatiramer acetate (GA) and natalizumab – and their indication are described.

IFNb is applied as a basic therapy for patients with CIS, RRMS or SPMS with additional relapses (Gold et al., 2012). While the precise mode of action of IFNb is still unknown, different theories have been proposed. Besides inducing regulatory T cells and inhibiting the

activation and proliferation of T cells, IFNb acts through cytokine modulation (Dhib-Jalbut and Marks, 2010). It also reduces the amount of inflammatory cells crossing the BBB and is presumed to have a positive regenerative effect within the CNS (Dhib-Jalbut and Marks, 2010; Kieseier, 2011). The impact of this treatment can be seen with fewer new lesions in MRI scans (Dhib-Jalbut, 1997; Jacobs et al., 1996).

For patients with CIS, who have a high risk to develop RRMS, and patients with RRMS, GA is approved (Gold et al., 2012). GA interacts with the immune response by suppressing the pro-inflammatory response with inhibition of T cell activity (Rosenzweig et al., 2010) and enhancing the anti-inflammatory pathway (Aharoni, 2013). The inflammatory process in the CNS is also down-regulated, as T-helper cells Type 2 are stimulated to migrate to the brain acting anti-inflammatory (Ziemssen and Schrempf, 2007). Furthermore, GA shows neuroprotective and neuroregenerative features (Lalive et al., 2011).

If the basic therapeutics are not effective, different medications for an escalation therapy are available. One of them is natalizumab, which is an integrin receptor antagonist (Rosenzweig et al., 2010). It acts at the BBB and hinders the adherence of T cells to vascular walls and, as a result, their migration to the CNS (Sellebjerg et al., 2016). Different studies have shown that natalizumab reduces the number of new lesions (Miller et al., 2003) and the progression of disability (Polman et al., 2006).

Eventually, the choice of the right DMT depends on the MS subtype, the disease activity, previous treatments and other requirements of the patient.

1.1.7.3 Symptomatic Treatment

In addition to the immunosuppressive and immunomodulative therapy, also the symptomatic treatment is of high value. In order to decrease encumbering symptoms such as spasticity, pain, bladder dysfunction, depression and dysphagia, a variety of medical and non-medical treatments exist. Those include among others physiotherapy, speech and psychological therapy (Gold et al., 2012).

1.2 Heat Shock Protein 70

The heat shock response, which leads to induction of so-called heat shock proteins (HSP), was described for the first time in 1962 by Ferruccio Ritossa (Ritossa, 1962). Those ubiquitously existing proteins are expressed in prokaryotic as well as in eukaryotic cells and can, therefore, be found in bacteria, plants and animals (Lindquist and Craig, 1988). Different families are defined and named according to their molecular weight: HSP100, HSP90, HSP70, HSP60, HSP40 (Li and Srivastava, 2004) and the small HSPs with a molecular weight of approximately 20 kDa (Gusev et al., 2005)

In the following, HSP70 and in particular the stress induced Hsp70 are described in terms of structure, function and appearance. Additionally, the role of Hsp70 in MS and tumor diseases is presented.

1.2.1 Structure and Occurrence

The HSP70 family comprises a variety of isoforms, which are sized between 66 kDa and 78 kDa (Tavaria et al., 1996). These proteins are incredibly highly conserved among various species such as bacteria and humans (Lindquist and Craig, 1988). Two major representatives can be specified: the constitutively expressed Hsc70 (73 kDa) and the mainly stress induced Hsp70 (72 kDa) (Miller et al., 1991; Minota et al., 1988). The Hsp70 consists of two major functional components: the N-terminal nucleotide binding domain (44 kDa), which can hydrolyze adenosine triphosphate (ATP) to adenosine diphosphate (ADP), and the C-terminal substrate binding domain (28 kDa) (Flaherty et al., 1990).

Under physiological conditions Hsp70 is expressed constitutively (Jindal, 1996). It is localized predominantly intracellular (Tavaria et al., 1996) – in the cytosol, nucleus, mitochondria (Radons, 2016) and in the endoplasmatic reticulum (ER) (Haas, 1994) – but is also found in the extracellular space (Pockley et al., 2014; Radons, 2016). In malignantly transformed tumor cells and virally infected cells, Hsp70 can additionally be found on the plasma membrane (Moseley, 2000; Multhoff et al., 1995). A wide variety of stress stimuli upregulates the expression of Hsp70. Those include thermal (Ritossa, 1962) and oxidative stress (El Golli-Bennour and Bacha, 2011), anoxia, ethanol, heavy metals (Lindquist and Craig, 1988), inflammation, infection, nutrient deprivation and tissue injury (Jindal, 1996). Furthermore, the simple presence of abnormal proteins can trigger an increased expression (Ananthan et al., 1986).

The heat shock factor (HSF) is essential for the transcription of Hsp70. It regulates the transcription by binding to the heat shock element after nuclear translocation (Lis and Wu, 1993; Mosser et al., 1988). Four different factors (HSF1-4) are described in the literature, but only HSF1 and HSF2 are ubiquitously expressed. Since HSF1 is more potent than HSF2 (Mathew et al., 2001), it is determined as the key factor in activating the transcription of Hsp70 (Cwiklinska et al., 2010).

Hsp70 is actively secreted into the extracellular space by a non-classical pathway and released from dying cells (De Maio, 2011). Additionally, the protein was found to be inserted into the plasma membrane and released within vesicles, which act as a danger signal (De Maio, 2011; Vega et al., 2008).

1.2.2 Function

As a molecular chaperone, the function of Hsp70 is to protect cells against the lethal effects of stress. It plays a role in protein synthesis by supporting the proper folding of newly

synthesized polypeptides, the prevention and refolding of misfolded proteins and assists their transportation (Hartl, 1996; Mayer and Bukau, 2005; Pelham, 1990; Tavaria et al., 1996). If the refolding is impossible, Hsp70 supports the proteasomal degradation (Pelham, 1990). In case of aggregated proteins, Hsp70 can also bind to the hydrophobic surface and as a result support the dissolution of those complexes (Hartl, 1996; Lewis and Pelham, 1985; Mayer and Bukau, 2005). Since misfolded and aggregated proteins play a role in neurodegenerative diseases such as Alzheimer's and Parkinson's Disease, Hsp70 is presumed to have a positive effect on those conditions (Wyttenbach and Arrigo, 2009).

Another cytoprotective function is the induction of antiapoptotic mechanisms, as it interacts directly with the apoptosis-inducing factor (AIF). As a result, the AIF is prevented from translocation to the nucleus, which would lead to DNA fragmentation (Benn and Woolf, 2004; Ravagnan et al., 2001). Hsp70 can additionally influence AIF independent processes in the apoptosis pathway (Jäättelä et al., 1998).

Concerning the immune response, Hsp70 also performs important functions. It can activate or enhance the immune defense in different ways. Three major aspects are explained below.

The first facet comprises the ability of Hsp70 to form antigen-Hsp70-complexes (Srivastava, 1994). Professional antigen-presenting cells (APCs), such as dendritic cells or macrophages, can initiate an adaptive immune response. These can recognize antigen-Hsp70-complexes via Hsp receptors and phagocytose them. After processing, they can be presented by major histocompatibility complex (MHC) class I molecules (Arnold-Schild et al., 1999; Basu and Srivastava, 2000; Blachere et al., 1997) to the CD8+ cytotoxic T cells (Blachere et al., 1997; Singh-Jasuja et al., 2000). Since MHC class I molecules are originally known to present intracellular proteins, this process is called antigen cross presentation (Murshid et al., 2012). Apart from this, APCs present external antigens via their MHC class II molecules to CD4+ T cells after processing in lysosomes (Cresswell, 1994).

For the second facet of immune system activation, the fact that Hsp70 occurs on the cell membrane of tumor cells (Multhoff et al., 1995) and virally infected cells is important (Moseley, 2000). This protein expression correlates with an increased sensitivity to lysis by natural killer (NK) cells resulting in the killing of these abnormal and infected cells (Botzler et al., 1996; Multhoff et al., 1997).

Thirdly, Hsp70 is involved in the production of cytokines. It can trigger the secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, Interferone (IFN)-γ (Breloer et al., 1999; Todryk et al., 1999), interleukin (IL)-1β, IL-6 (Asea et al., 2000) and IL-12 (Todryk et al., 1999) by monocytes and macrophages (Tsan and Gao, 2004). Another family of receptors capable of activating lymphocytes are important: the Toll-like receptors (TLR). TLR2 and TLR4 are able to recognize Hsp70, which also results in the synthesis of several cytokines (Turturici et al., 2014).

In summary, the functions of Hsp70 can be differentiated according to its location. When located intracellular, it protects against lethal damage induced by stress and is an important part of the synthesis and transportation of proteins. In contrast, it plays a substantial role in the immune response, if located in the extracellular space and on the cell surface. On the one hand, it can elicit specific immune responses via the pathway of antigen presentation and activation of NK cells. On the other hand, it is able to modulate the immune defense by trigger a pro-inflammatory immune response through cytokines.

1.2.3 Role of Hsp70 in MS

Hsp70 is presumed to affect the development of autoimmune diseases (Tsan and Gao, 2004) due to its immunmodulatory functions (Radons, 2016). In the following, the impact of Hsp70 in the development of MS is described. Furthermore, previous studies concerning Hsp70 in MS patients are presented.

Aberrant adaptive and innate immune responses are involved in the pathogenesis of MS (cf. Section 1.1.3 and Section 1.1.6). This is presumed to target – among others – proteins of the myelin such as MBP (Greer and Pender, 2008). If MBP is bound to Hsp70, its uptake by APC is significantly enhanced, which results in an intensified MBP-specific immune response. Thus, the overexpression of Hsp70 might enhance the already high immunogenic potential of MBP (Cwiklinska et al., 2003; Greer and Pender, 2008) and might lead to a pro-inflammatory environment (Cwiklinska et al., 2003).

Another facet, which indicates the harmful effect of Hsp70 on MS is that mice with a knock-out of the Hsp70.1 gen are more resistant to developing EAE (Mansilla et al., 2014a; Mycko et al., 2008).

Apart from this, Hsp70 is also recognized by TLR2 and TLR4 (cf. Section 1.2.2). The activation results in the release of cytokines, which are presumed to be responsible for CNS autoimmunity and neurodegenerative diseases. In peripheral mononuclear blood cells (PMBCs) and in mononuclear cells in the CSF of MS patients, TLR2 and TLR4 are significantly elevated. As a result, those receptors and Hsp70 seem to play a role in MS pathogenesis (Turturici et al., 2014).

Hsp70 is considered as a potential medication for MS, since its intracellular cytoprotective functions (cf. Section 1.2.2) may reduce cell death in the CNS (Mansilla et al., 2012). In contrast, those mentioned findings suggest that the negative effects are predominant and the downregulation of Hsp70 expression might be a promising target for medications (Mansilla et al., 2014a).

So far, studies concerning Hsp70 gene polymorphisms (Boiocchi et al., 2016), intracellular Hsp70 levels in PBMCs (Bomprezzi et al., 2003; Cwiklinska et al., 2010; Mandel et al., 2004; Mansilla et al., 2014b; Satoh et al., 2005) and Hsp70-specific autoantibodies in the serum of MS

patients have been carried out (Mansilla et al., 2012). Published results of intracellular levels in PBMCs show a great diversity ranging from low (Bomprezzi et al., 2003; Mandel et al., 2004; Satoh et al., 2005) to normal (Cwiklinska et al., 2010) to elevated levels (Mansilla et al., 2014b). Additionally, an altered expression of Hsp70 after stress has been observed. The results also vary between a lower (Mansilla et al., 2014b) and a higher expression (Cwiklinska et al., 2010).

In addition to studies of blood, also the brain and the CSF are under observation. Concerning the occurrence of Hsp70 in the brain tissue of MS patients, the amount of Hsp70 within the myelin cells is higher compared to healthy individuals (Aquino et al., 1997). Analyses of CSF showed that the level of anti-Hsp70 autoantibodies was increased compared to healthy donors (Chiba et al., 2006; Yokota et al., 2010).

Overall, already a lot of studies concerning Hsp70 in connection with MS have been carried out and showed a great diversity in outcomes. To date, neither free nor lipid-bound Hsp70 in the serum or CSF of MS patients has been determined.

1.2.4 Role of Hsp70 in Tumors

In contrast to healthy cells, Hsp70 is located on the cell membrane of malignantly transformed tumor cells (Multhoff et al., 1995). This has been observed in a variety of tumors such as head and neck (Kleinjung et al., 2004), pancreas (Gastpar et al., 2005), brain (Breuninger et al., 2014), gastric, colon, lower rectal and squamous cell lung carcinomas(Pfister et al., 2007). These cells release lipid vesicles with Hsp70 on their membrane and thereby enhance the targeted immune response mediated through NK cells (cf. Section 1.2.2) with stimulating migration and lytic activity against Hsp70 membrane-positive tumors (Gastpar et al., 2005). A result might be that the expression of membrane-bound Hsp70 correlates with a significantly improved survival in gastric and colon cancer. Nevertheless, it is also linked with a worse prognosis in lower rectal and squamous cell lung carcinoma (Pfister et al., 2007).

In the future, Hsp70 could be used as a tumor-specific target structure for theranostic purposes. On the one hand, it may be deployed for immunotherapy (Multhoff and Hightower, 2011; Stangl et al., 2011) and on the other hand, it may be used for tumor imaging (Gehrmann et al., 2015; Gehrmann et al., 2014c).

2 Aim of this Study

Precision medicine is a focus of research in health care with a constant rising number of annual publications. The common expectation is that precision medicine has the potential to make health care more effective and efficient (Schleidgen et al., 2013). Therefore, individual variability in genes, environment, lifestyle and metabolism are tried to integrate into disease prevention, diagnosis and therapy. Consequently, the development and application of biomarkers is meaningful (Gamulin, 2016; Schleidgen et al., 2013). Since MS is a heterogenic disease (Compston and Coles, 2008) with a multifactorial genesis (Hoffmann et al., 2009) and variable individual responses to the therapy, the establishment of precision medicine would be beneficial (Pravica et al., 2013).

The potential of Hsp70 as a biomarker has already been stated (Ogawa et al., 2007). As tumor cells frequently overexpress Hsp70, present it on their membrane and release it, Hsp70 is presumed to be a suitable biomarker for tumor detection and monitoring (Gehrmann et al., 2014b). Since its synthesis and release is induced by stress, it is also considered to be a sensitive biomarker for cellular stress, such as oxidative stress, inflammation and tissue injury (Ogawa et al., 2007).

Hsp70 is able to elicit immune responses and is therefore presumed to be part of the development of different autoimmune diseases (Tsan and Gao, 2004) and also of MS. So far, many studies have been carried out to evaluate the role of Hsp70 in MS and revealed a possible relation (Turturici et al., 2014). Those studies, however, focused on intracellular Hsp70 (Bomprezzi et al., 2003; Cwiklinska et al., 2010; Mansilla et al., 2014b), anti-Hsp70 antibodies (Mansilla et al., 2012) and genpolymorphism (Boiocchi et al., 2016).

The aim of this study was to investigate the role of extracellular Hsp70 protein levels in the serum and CSF of patients with different MS subtypes and inflammatory diseases as a potential biomarker. For this purpose, serum and CSF was collected and analyzed from patients with MS, other inflammatory neurological diseases (OIND), non-inflammatory neurological diseases (NIND) and tumors of the CNS, and serum of healthy donors (HD). The Hsp70 levels were determined using two different enzyme-linked immunosorbent assays (ELISA) – R&D ELISA, which determines free Hsp70 and lipHsp70 ELISA, which additionally detects lipid-bound Hsp70.

Specific aims were to explore whether serum and CSF Hsp70 levels are associated with inflammatory processes and to assess the different impact of inflammatory and tumor diseases of the CNS. Another objective was to examine Hsp70 levels in MS patients depending on their subtype, disease phase and medication. Finally, it was intended to identify the origin of elevated Hsp70 levels in serum.

3 Methods and Material

3.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is an immunoassay, which is suited for the determination of molecules in body fluids. In this study, two different ELISA setups – the R&D ELISA and the lipHsp70 ELISA – were used to determine the level of Hsp70. Both are able to detect free Hsp70, whereas the lipHsp70 ELISA also determines lipid-bound Hsp70. In the following, the two different protocols are described.

3.1.1 R&D ELISA

For the R&D ELISA the manufacturer's protocol was followed. On the day before the experiment, the solution IC Diluent # 4 (1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 % Triton in phosphate buffered saline (PBS)) and 1 % bovine serum albumin (BSA) in PBS were prepared. Those were stored in a rotator at + 4 °C in the refrigerator over night to prepare a homogeneous solution. For coating, the Capture Antibody was diluted to a working concentration of 2 µg/ml in PBS. The 96-well plate was immediately coated with 100 µl per well and incubated at room temperature over night.

During all incubations, the plate was sealed and stored in a plastic box to avoid contamination and direct light. All incubations hereinafter were at a temperature of + 27 °C in the incubator. Furthermore, all dilutions were prepared right before usage. Each washing step contained three repetitions with 250 µl Wash Buffer (PBS with 0.05 % Tween-20) per well.

On the day of the experiment, first a washing step was done and afterwards, the well was blocked with 250 µl per well of Block Buffer (1 % BSA in PBS) and incubated for 2 h. At the same time the samples, which had been stored at - 80 °C, were thawed on ice. Serum samples were diluted in a ratio of 1:5 and CSF samples with 1:3 in IC Diluent # 4. After washing, the diluted samples together with a control sample were added in duplicates with 100 µl per well. In order to apply an eight point standard curve with concentrations between 0 and 10 ng/ml, Total Hsp70 Standard in IC Diluent # 4 was used. Afterwards, the plate was placed in the incubator for 2 h, followed by another washing step. Then the Detection Antibody with a working concentration of 100 ng/ml in 1 % BSA in PBS, was incubated with 100 µl per well for 2 h. After washing, streptavidin conjugated to horseradish-peroxidase (HRP) was diluted 1:200 using 1 % BSA in PBS and 100 µl were added to each well and incubated for 1 h. Then a final washing step was done and the Substrate Solution (100 µl per well) was added. The reaction was stopped with 50 µl of the Stop Solution per well after 20 min of incubation time. Finally, the absorbance was read at 450 nm, corrected by absorbance at 570 nm, in a Microplate Reader. The measuring range of this ELISA setup is stated with 0.157 – 10 ng/ml (*Human/Mouse/Rat Total HSP70/HSPA1A ELISA*).

3.1.2 LipHsp70 ELISA

For the lipHsp70 ELISA following preparations were done on the day before the experiment. Block Buffer (2 % milk powder in PBS) and 1 % BSA in PBS were mixed and then stored in a rotator at + 4 °C over night. CrossDown Buffer, which had been stored at - 20 °C, was thawed in the refrigerator at + 4 °C. For coating, rabbit polyclonal antibody was diluted to a working concentration of 2 µg/ml in sodium carbonate buffer (0.1 M sodium carbonate, 0.1 M sodium hydrogen carbonate). This antibody is directed against human recombinant Hsp70. The 96-well plate was coated with 100 µl per well and incubated at room temperature.

During all incubations, the plate was sealed and stored in a plastic box to avoid contamination and direct light. All incubations hereinafter were at a temperature of + 27 °C in the incubator. Furthermore, all dilutions were prepared right before usage. Each washing step contained three repetitions with 250 µl Wash Buffer (PBS with 0.05 % Tween-20) per well, if not stated otherwise.

On the next day, the plate was washed and afterwards blocked with 300 µl Block Buffer for 1.5 h. Meanwhile the serum and CFS samples, which had been stored at - 80 °C, were thawed on ice. The serum samples were diluted 1:5 in CrossDown Buffer; the CFS samples were diluted 1:3 or added straight, when the result in an earlier experiment had been below the detection limit. After a further washing step, this time with 300 µl instead of 250 µl Wash Buffer per well, the diluted samples were added in duplicates, 100 µl per well. A control sample and a Hsp70 eight point standard was included into each ELISA test using between 0 and 50 ng/ml recombinant Hsp70 diluted in CrossDown Buffer. The well was incubated for 2 h. After another washing step, the wells were incubated with 4 µg/ml (later on: 3 µg/ml) detection antibody (biotinylated mouse monoclonal antibody cmHsp70.1) in 2 % milk powder in PBS, 100 µl were added per well and the plate was incubated for 2 h. Then the wells were washed and afterwards incubated with 0.2 µg/ml streptavidin-HRP in 1 % BSA (100 µl/well) for 1 h. After a final washing step the Substrate Solution was added (100 µl per well) and incubated for 30 min. The reaction was stopped with 50 µl Stop Solution per well. Finally, the absorbance was read at 450 nm and corrected by absorbance at 570 nm, in a Microplate Reader. The range of this ELISA setup is specified with 0.3 – 17 ng/ml (Breuninger et al., 2014).

3.2 Material

3.2.1 Plastic Material

Table 3.1: Plastic material

Article	Name	Producer
96 well plate	EIA/RIA Plate # 3590*	Costar, NZ, USA
96 well plate	MaxiSorp Nunc-Immuno plates**	Thermo Fisher Scientific, Roskilde, DK

*for the R&D ELISA **for the lipHsp70 ELISA

3.2.2 Chemicals, Reagents and Buffer

Table 3.2: Chemicals, reagents and buffer

Chemicals, reagents and buffer	Producer
BSA (A7030-100G)	Sigma-Aldrich, St. Louis, MO, USA
CrossDown Buffer**	Applichem, Chicago, IL, USA
EDTA (0.5 M)*	Ambion, Austin, TX, USA
Milk powder**	Carl Roth, Karlsruhe, DE
PBS	Sigma-Aldrich, MO, USA
Sodium carbonate**	Merck, Darmstadt, DE
Sodium hydrogen carbonate**	Merck, Darmstadt, DE
Substrate Reagent Pack (Color Reagent A&B)	R&D Systems, Minneapolis, MN, USA
Sulfuric acid	Sigma-Aldrich, St. Louis, MO, USA
Triton®X-100*	Sigma-Aldrich, St. Louis, MO, USA
Tween®20	Calbiochem, CA, USA

The chemicals and reagents were necessary for both ELISAs. If they were only used for one ELISA, it is characterized as following: *for the R&D ELISA **for the lipHsp70 ELISA

Abbreviations: BSA: bovine serum albumin; EDTA: ethylenediaminetetraacetic acid; PBS: phosphate buffered saline

BSA and the Substrate Reagent Pack were stored at + 4 °C in the refrigerator, the other chemicals and reagents were stored at room temperature.

3.2.3 Solutions

Table 3.3: Solutions

Solution	Production
Block Buffer (R&D ELISA)*	1 % BSA in PBS, pH 7.2-7.4
Block Buffer (lipHsp70 ELISA)**	2 % milk powder in PBS, ph 7.2-7.4
IC Diluent # 4*	1 mM EDTA, 0.5 % Triton®X-100 in PBS, pH 7.2-7.4
Sodium carbonate buffer**	0.1 M sodium carbonate, 0.1 M sodium hydrogen carbonate
Stop Solution	2 N H_2SO_4
Substrate Solution	1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)
Wash Buffer	0.05 % Tween®20 in PBS, pH 7.2-7.4

The solutions were necessary for both ELISAs. If they were only used for one ELISA setup, it is characterized as following: *for the R&D ELISA **for the lipHsp70 ELISA

Abbreviations: BSA: bovine serum albumin; EDTA: ethylenediaminetetraacetic acid; PBS: phosphate buffered saline

3.2.4 Antibodies and Proteins

Table 3.4: Antibodies and proteins

Antibody & Protein	Producer
Biotinylated mouse monoclonal antibody cmHsp70.1**	multimmune, Munich, DE
Rabbit polyclonal antibody**	Davids Biotechnologie, Regensburg, DE
Streptavidin-HRP (# 890803)*	R&D Systems, Minneapolis, MN, USA
Streptavidin-HRP**	Pierce Thermo, Rockford, IL, USA
Total Hsp70 Capture Antibody (# 841680)*	R&D Systems, Minneapolis, MN, USA
Total Hsp70 Detection Antibody (# 841681)*	R&D Systems, Minneapolis, MN, USA
Total Hsp70 Standard (# 841682)*	R&D Systems, Minneapolis, MN, USA

*for the R&D ELISA **for the lipHsp70 ELISA

Abbreviation: HRP: horseradish-peroxidase

Streptavidin-HRP (Pierce Thermo, Rockford, IL, USA) was stored at - 20 °C, all other antibodies and proteins were stored at + 4 °C in the refrigerator.

3.2.5 Kit

The used ELISA kit – IC Human/Mouse/Rat Total Hsp70 ELISA– was obtained from R&D Systems, Minneapolis, MN, USA. It was stored at + 4 °C in the refrigerator.

3.2.6 Measuring Equipment

In order to mesuare the Hsp70 levels, a Microplate Reader obtained from BioTek, Winooski, VT, USA was used.

3.2.7 Sample Collection

All samples were collected between 2008 and 2016 from patients of the Neuro-Kopf-Zentrum, Klinikum rechts der Isar, Technical University of Munich (TUM) and immediately stored at -80 °C until measurement. The research group of Professor Multhoff (Department of Radiation Oncology, Klinikum rechts der Isar, TUM) provided the Hsp70 levels of HD (Breuninger et al., 2014) and additionally the values of 41 patients with tumors of the CNS to increase the number of samples. Informed written consent was provided by all patients and healthy donors. The diagnoses, additional laboratory parameters of CSF and tumor volumes were received from the medical records. Furthermore, the approval of the study protocol was obtained by the ethical committee of the Klinikum rechts der Isar, TUM.

3.3 Statistics

Serum samples were provided in two separated collectives. Initially, the serum samples of a discovery cohort (n = 87) and afterwards an independent validation cohort (n = 90) were measured. Both cohorts displayed similar results (cf. Appendix Figure 5.1 and 5.2). Therefore, mean values of both were summarized and the combined data was used for further analyses. The second collective included additionally CSF samples (n = 87). All experiments were performed blinded, afterwards the patient characteristics were decoded and the statistical analyses were done. Each sample was measured in up to four independent experiments, depending on the provided amount of material, each in duplicates and the values were averaged. The result of the serum sample was only considered as valid when the relative standard deviation (SD) was smaller than 15 % between the duplicates within one experiment. CSF samples were included, if both duplicates displayed a valid measurement, also if the relative SD was higher than 15 %. Eleven patients had to be excluded, as the serum samples were haemolysed, which makes an effective measurement impossible (Breuninger et al., 2014). Another three patients were excluded because of invalid measurements. Those samples were eliminated before any analyses were done and are not mentioned in any diagram or table in this thesis.

During the last experiments instability of the standard curve occurred. As a consequence, the results were only used, if the standard curve had the right shape but with lower levels. In order to make those comparable to the results of the other experiments, two serum control samples, with a known amount of Hsp70 as determined in earlier experiments, were added

each time. All measured values of one well were corrected by the same factor depending on those control samples.

For statistical analyses and generation of figures MATLAB R2015a (MathWorks, Inc., Natick, Massachusetts, USA) was used. The results represent the means of up to four independent experiments each in duplicates $\pm$ SD. In order to compare two subgroups, the Student's t-test was performed. For comparisons between more than two subgroups an analysis of variance (ANOVA) with the Bonferroni method as post-hoc analysis was done. Multiple linear regression analyses with the diagnosis as an additional independent variable were used to identify potential correlations between Hsp70 levels, age, gender, relapse rate, EDSS and disease duration. The Spearman's rank correlation coefficients were calculated to identify correlations between two blood/CSF meters. A p-value of < 0.05 was defined as statistically significant. In regard of receiver operating characteristic (ROC) curve analyses, an area under the curve (AUC) of 1 represents a perfect test, whereas an AUC of 0.5 reflects a random guess (Bewick et al., 2004).

Within the dot plot diagrams, each dot represents one patient and the line indicates the median value. Statistical significant differences are marked with one, two or three stars, if $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ respectively.

4 Results

In the following chapter, the results of the performed analyses are presented in detail. First of all, the clinical characteristics of the patients and healthy donors are described. The second section focuses on Hsp70 levels in serum, whereas the third section is about Hsp70 levels in CSF. In the fourth section, the connection of Hsp70 levels with tumors of the CNS are presented and compared to the other subgroups. As the lipHsp70 ELISA measures free and lipid-bound Hsp70 and contains therefore more information, the shown data of serum are the results of experiments using the lipHsp70 ELISA. Analyses of CSF displayed very low Hsp70 levels, which is why in this case the shown data reflects the results of the R&D ELISA, due to its lower detection limit (cf. Section 3.1). In the last section the two different ELISA setups are compared.

4.1 Clinical Characteristics

In this study, serum samples of 177 patients were included and Hsp70 levels were quantified. Follow-up samples were provided from six of the patients diagnosed with RRMS. Three patients were attended during their high-dose MPS therapy over up to six days and blood samples were taken on each day. Additional the Hsp70 levels in CSF of 87 patients were determined.

The clinical characteristics of the healthy control group (n = 114) is presented in Table 4.1. Serum samples of the patients were provided in two separated collectives. Initially, the serum samples of a discovery cohort (n = 87) and afterwards an independent validation cohort (n = 90) were measured. Within the second cohort samples of patients with tumor diseases of the CNS and CSF samples of patients with MS, NIND, OIND and tumors were included. Those patient characteristics are presented separately hereinafter. The clinical characteristics of the patients divided into the discovery and validation cohort and also combined as one are summarized in Table 4.2. The group of NIND summarizes patients with epilepsy (n = 1), head pressure (n = 1), headache (n = 25), lumbosciatica (n = 2) and pseudotumor cerebri (n = 12). The OIND group is composed of patients with encephalitis (n = 2), herpes zoster neuritis (n = 2), meningitis (n = 15) and meningoencephalitits (n = 9).

Table 4.1: Clinical characteristics of healthy donors (Lechner et al., 2018, Table 1)

	HD
Number of patients[a]	114
Gender (M/F)[a]	67/47
Age[b c]	43 ± 15

[a] Number of patients, [b] Mean $\pm$ SD, [c] years

Table 4.2: Clinical characteristics of patients (Discovery and validation cohort of NIND and OIND: Lechner et al., 2018, Table 1; Combined cohort: Lechner et al., 2018, Table 2)

	MS	NIND	OIND
Discovery Cohort			
Number of patients[a]	50	21	16
Gender (M/F)[a]	15/35	7/14	9/7
Age[b c]	36 ± 11	33 ± 12	51 ± 18
Validation Cohort			
Number of patients[a]	44	20	12
Gender (M/F)[a]	16/28	7/13	10/2
Age[b c]	42 ± 13	39 ± 11	43 ± 15
Combined cohort			
Number of patients[a]	94	41	28
Gender (M/F)[a]	31/63	14/27	19/9
Age[b c]	39 ± 12	36 ± 12	47 ± 17

[a] Number of patients, [b] Mean $\pm$ SD, [c] years

The MS patients were further subclassified according to the four different types – CIS (n = 26), RRMS (n = 40), SPMS (n = 19) and PPMS (n = 9). The diagnosis of MS was based on the revised McDonald criteria from 2005 (Polman et al., 2005) and 2010 (Polman et al., 2011) (cf. Section 1.1.5). Detailed information about those subgroups is presented in Table 4.3.

Table 4.3: Clinical characteristics of MS patients (Combined cohort: number, age and gender distribution of CIS, RRMS, SPMS and PPMS: Lechner et al., 2018, Table 2)

	CIS	RRMS	SPMS	PPMS
Discovery cohort				
Number of patients[a]	13	27	10	–
Gender (M/F)[a]	4/9	6/21	5/5	–
Age[b c]	30 ± 9	34 ± 10	49 ± 6	–
Treatment (NT/IFNb/GA/NZ/OT)[a]	10/2/1/0/0	10/11/4/2/0	10/0/0/0/0	–
Relapse/remission[a]	3/10	7/20	–	–
Gd-enhancing lesions in MRI (yes/no)[a d]	5/0	3/3	–	–
Relapses[b e]	0.9 ± 0.3	1.6 ± 1.2	–	–
Disease duration from manifestation[b c]	0.9 ± 1.2	5.2 ± 4.4	17.4 ± 10.7	–
EDSS[b]	0.9 ± 0.8	1.4 ± 0.9	3.4 ± 2.5	–
Validation cohort				
Number of patients[a]	13	13	9	9
Gender (M/F)[a]	4/9	6/7	3/6	3/6
Age[b c]	35 ± 10	35 ± 8	52 ± 11	54 ± 8
Treatment (NT/IFNb/GA/NZ/OT)[a]	12/1/0/0/0	12/1/0/0/0	4/0/0/0/5	6/0/0/0/3
Relapse/remission/unspecified[a]	9/3/1	8/3/2	–	–
Gd-enhancing lesions in MRI (yes/no)[a d]	7/2	6/3	–	–
Relapses[b e]	0.9 ± 0.3	1.1 ± 0.5	–	–
Disease duration from manifestation[b c]	1.0 ± 2.6	5.0 ± 6.2	19.4 ± 10.4*	5.6 ± 3.6*
EDSS[b]	1.3 ± 1.3	1.5 ± 0.7	5.7 ± 1.6	3.7 ± 1.5
Combined cohort				
Number of patients[a]	26	40	19	9
Gender (M/F)[a]	8/18	12/28	8/11	3/6
Age[b c]	32 ± 10	35 ± 10	51 ± 9	54 ± 8
Treatment (NT/IFNb/GA/NZ/OT)[a]	22/3/1/0/0	22/12/4/2/0	14/0/0/0/5	6/0/0/0/3
Relapse/remission/unspecified[a]	12/13/1	15/23/2	–	–
Gd-enhancing lesions in MRI (yes/no)[a d]	12/2	9/6	–	–
Relapses[b e]	0.9 ± 0.3	1.4 ± 1.1	–	–
Disease duration from manifestation[b c]	0.9 ± 2.0	5.2 ± 5.0	18.2 ± 10.3*	5.6 ± 3.6*
EDSS[b]	1.1 ± 1.1	1.5 ± 0.8	4.5 ± 2.4	3.7 ± 1.5

GA: glatiameracetat; IFNb: interferone beta; NT: no treatment; NZ: natalizumab; OT: other or unspecified treatment.

[a] Number of patients, [b] Mean $\pm$ SD, [c] years, [d] MRI scans were not available from all patients, [e] Number of relapses during the two years before blood sampling, * information about 2 patients missing

According to the medical records, 91 MS patients did not suffer from any other acute or chronic inflammatory or tumor disease at the time point of blood sampling. Three MS patients had pre-existing inflammatory conditions such as Uveitis, Hashimoto's disease and erosive reflux disease. MRI scans were available from 29 patients at the time point of blood sampling.

In addition to the serum samples, the second cohort consisted of matching CSF samples of 87 patients. The clinical characteristics of the patients with MS, NIND and OIND are presented in Table 4.4. Furthermore, MS patients are summarized in more detail in Table 4.5.

Table 4.4: Clinical characteristics of patients – CSF samples

	MS	NIND	OIND
Number of patients[a]	42	20	12
Gender (M/F)[a]	14/28	7/13	10/2
Age[b c]	42 ± 13	39 ± 11	43 ± 15

[a] Number of patients, [b] Mean $\pm$ SD, [c] years

Table 4.5: Clinical characteristics of MS patients – CSF samples

	CIS	RRMS	SPMS	PPMS
Number of patients[a]	12	12	9	9
Gender (M/F)[a]	3/9	5/7	3/6	3/6
Age[b c]	35 ± 10	35 ± 8	52 ± 11	54 ± 8
Treatment (NT/OT)[a]	12/0	12/0	4/5	6/3
Relapse/remission/unspecified[a]	9/2/1	8/3/1	–	–
Gd-enhancing lesions in MRI (yes/no)[a d]	7/2	6/3	–	–
Relapses[b e]	1.0 ± 0.0	1.2 ± 0.4	–	–
Disease duration from manifestation[b c]	0.8 ± 2.7	5.2 ± 6.4	19.4 ± 10.4*	5.6 ± 3.6*
EDSS[b]	1.5 ± 1.3	1.5 ± 0.7	5.7 ± 1.6	3.7 ± 1.5

EDSS: Expanded Disability Status Scale; NT: no treatment; OT: other or unspecified treatment.

[a] Number of patients, [b] Mean $\pm$ SD, [c] years, [d] MRI scans were not available from all patients, [e] Number of relapses two years before blood sampling, * information of 2 patients missing

The last patient group consists of patients suffering of tumors of the CNS with World Health Organization (WHO) grading I ((n = 1), II (n = 3), III (n = 1) and IV (n = 9). If the tumor was classified between two graduations or a patient was diagnosed with two tumors, the patient was assigned to the higher grading. Additionally, 41 serum values of patients with tumors of the CNS were provided with WHO grading II (n = 2), III (n = 9) and IV (n = 30). Matching CSF

samples were available in 13 cases. Patient characteristics are presented in Table 4.6 and 4.7.

Table 4.6: Clinical characteristic of patients with tumors of the CNS

WHO grading	I	II	III	IV
Serum				
Number of patients[a]	1	3 + 2*	1 + 9*	39
Gender (M/F)[a]	0/1	2/1	1/0	18/21
Age[b c]	37	47 ± 8	79	59 ± 14
CSF				
Number of patients[a]	1	3	1	8
Gender (M/F)[a]	0/1	2/1	1/0	4/4
Age[b c]	37	47 ± 8	79	68 ± 11

[a] Number of patients, [b] Mean $\pm$ SD, [c] years, *number of provided values with missing information about gender and age

Table 4.7: Clinical characteristic of patients with known tumor volumes

WHO grading	I	II	III	IV
Number of patients[a]	1	3	1	6
Gender (M/F)[a]	0/1	2/1	1/0	3/3
Age[b c]	37	47 ± 8	79	67 ± 12
Volume of the tumor[b d]	0.4	13.2 ± 12.0	3.3	7.8 ± 12.5

[a] Number of patients, [b] Mean $\pm$ SD, [c] years, [d] cm^3

Regarding age and gender, the four main groups (HD, MS, NIND and OIND) showed similar distributions (cf. Table 4.1 and 4.2). Within the subgroups of MS, however, patients with SPMS and PPMS were older (Table 4.3) due to a later onset of the disease (Compston and Coles, 2008).

4.2 Serum

Initially, two different confounding variables – age and gender – were examined and afterwards, the impact of active inflammation with a special focus on MS was investigated. In this regard, additional variables, which reflect the disease activity, the effect of immunomodulative medications and observations over longer time periods, were analyzed.

4.2.1 Influence of Age and Gender

Age and gender were identified as possible confounding variables and further examined. Multiple regression analyses displayed no age-related differences in the Hsp70 serum levels ($p = 0.435$) (Lechner et al., 2018). For visualization separate scatter plots for each diagnosis with the values of Hsp70 depending on the age of the patients are shown in Figure 4.1.

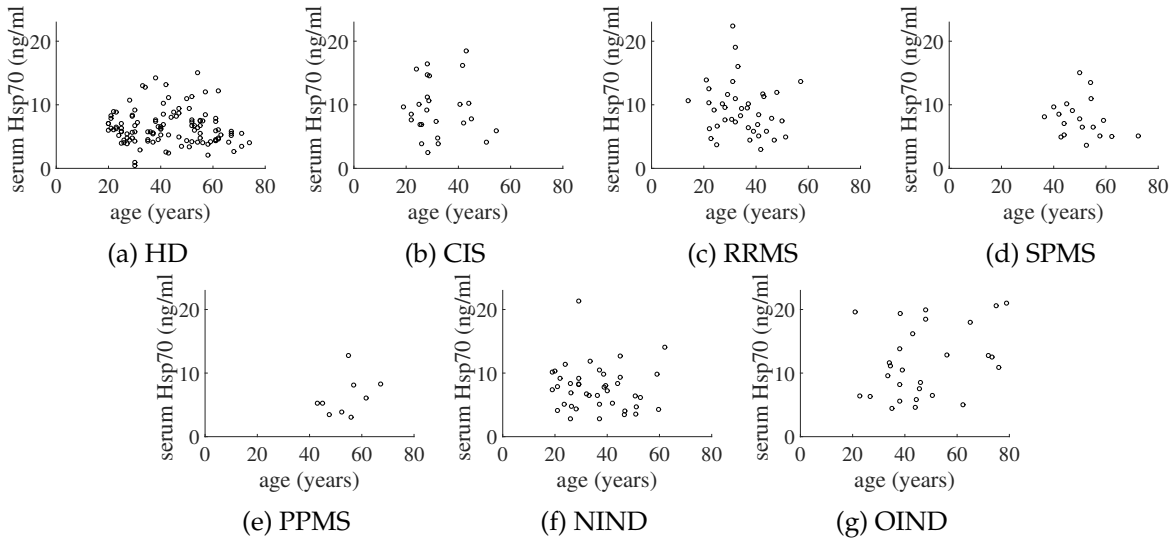


Figure 4.1: Influence of age on the serum Hsp70 level
HD: n = 114; CIS: n = 26; RRMS: n = 40; SPMS: n = 19; PPMS: n = 9; NIND: n = 41; OIND: n = 28.

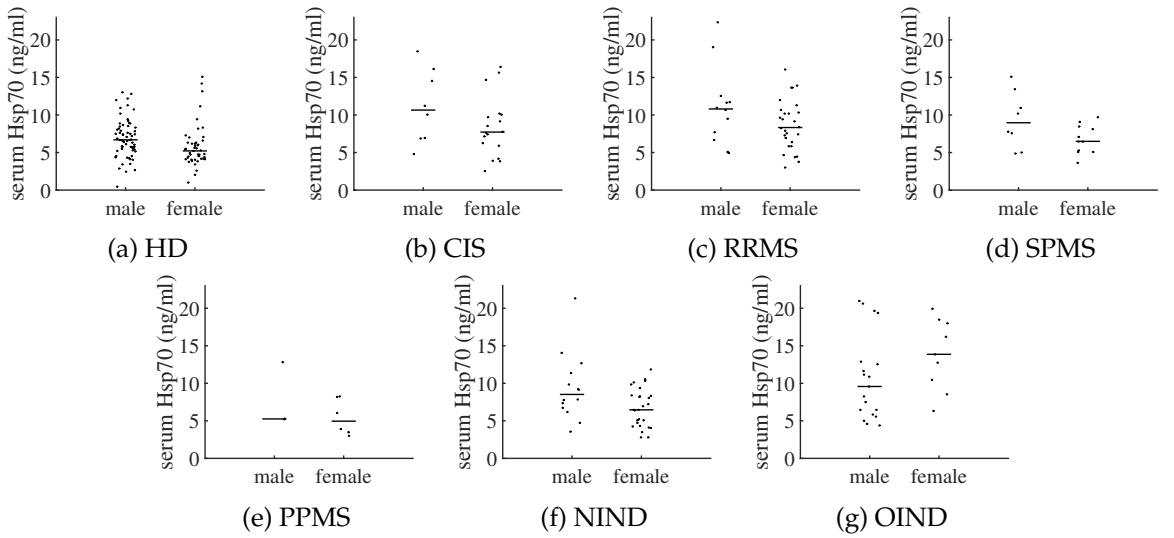


Figure 4.2: Influence of gender on the serum Hsp70 level
HD: $n_{male} = 67$, $n_{female} = 47$; CIS: $n_{male} = 8$, $n_{female} = 18$; RRMS: $n_{male} = 12$, $n_{female} = 28$; SPMS: $n_{male} = 8$, $n_{female} = 11$; PPMS: $n_{male} = 3$, $n_{female} = 6$; NIND: $n_{male} = 14$, $n_{female} = 27$; OIND: $n_{male} = 19$, $n_{female} = 9$.

The influence of gender on serum Hsp70 level was also evaluated by multiple regression analyses. Those displayed no gender-related differences ($p = 0.422$) (Lechner et al., 2018). Nevertheless, females showed in nearly all subgroups slightly lower Hsp70 serum levels than males (cf. Figure 4.2). Only within the subgroup of OIND, male patients displayed higher values (cf. Figure 4.2g). Since analyses for each gender separately resulted in a similar outcome, a combined data evaluation was preferred.

Summarizing, age displayed no influence on the serum level of Hsp70. In respect of gender, females presented slightly lower levels, but the differences were also not significant. Therefore, both parameters were neglected hereinafter.

4.2.2 Correlation between Inflammation and Serum Hsp70 Levels

In order to determine the influence of inflammatory processes on Hsp70, serum levels were measured comparatively in patients with NIND and OIND and further compared to HD (cf. Figure 4.3). Those measuring results are summarized in Table 4.8. Patients with OIND showed significantly higher Hsp70 serum levels compared to HD ($p < 0.001$) and NIND ($p < 0.001$). Between Hsp70 serum levels of HD and NIND ($p = 0.383$), no significant difference was determined (Lechner et al., 2018).

In conclusion, diseases with inflammation might lead to elevated serum Hsp70 levels.

Table 4.8: Serum Hsp70 levels of HD, NIND and OIND (Lechner et al., 2018, Table 2)

Diagnosis	Number	Serum level of Hsp70[a]	p-value[b]
HD	114	6.0 [3.1]	–
NIND	41	7.3 [4.3]	0.383
OIND	28	11.0 [10.2]	0.000

[a] Median (ng/ml) [interquartile range], [b] p-values refer to comparisons to HD

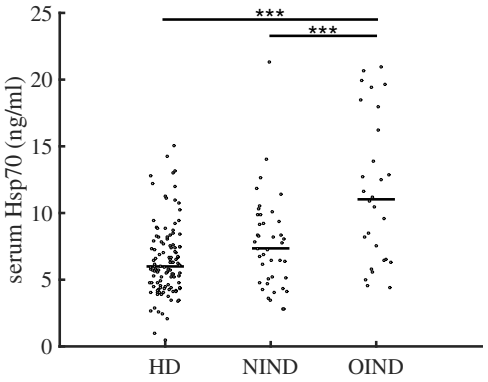


Figure 4.3: Influence of inflammation on serum Hsp70 level (Lechner et al., 2018, Figure 1)

4.2.3 Hsp70 Expression in MS Patients

In order to discover the impact of inflammatory processes in MS, serum Hsp70 levels of MS patients were analyzed in more detail. First the results depending on the pathomechanisms and subtypes are demonstrated, followed by further analyses concerning disease activity and immunomodulative medications. In addition, analyses regarding the stability of serum Hsp70 over time and course of disease are presented. Finally, the application of Hsp70 levels to distinguish between the different MS subtypes is investigated.

4.2.3.1 Influence of the Different Pathomechanisms and Subtypes

MS patients in total compared to HD displayed significantly elevated serum Hsp70 levels ($p < 0.001$, cf. Figure 4.4a) and compared to OIND significantly lower levels ($p = 0.001$) (Lechner et al., 2018). For a more detailed analysis, MS patients were divided into different subgroups and their measured serum levels are summarized in Table 4.9.

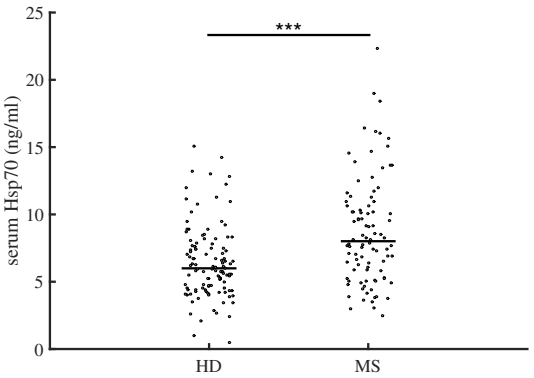
The first subdivision was based on the difference in their pathomechanism. As CIS/RRMS are presumed to be dominated by inflammatory processes and the progressive forms (SPMS/PPMS) by neurodegeneration (Lassmann et al., 2007), those two groups were contrasted (cf. Figure 4.4b). This revealed that serum levels of Hsp70 in CIS/RRMS were significantly elevated compared to HD ($p < 0.001$) and to SPMS/PPMS ($p < 0.05$) (Lechner et al., 2018).

Another subdivision was depending on the different MS subtypes. CIS and RRMS showed highest values followed by SPMS and PPMS. Hsp70 serum levels of CIS and RRMS patients were significantly higher than HD ($p < 0.001$, $p < 0.001$, respectively). Concerning SPMS and PPMS, the values were in a similar range as HD and reduced compared to CIS and RRMS (Figure 4.4c) (Lechner et al., 2018).

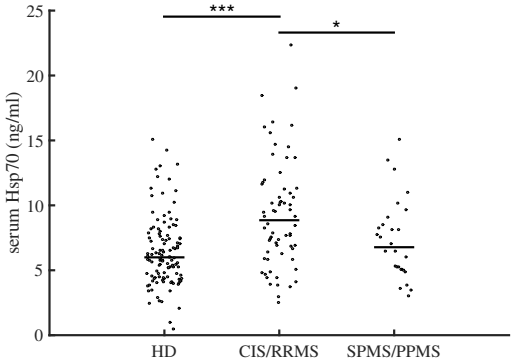
Table 4.9: Serum Hsp70 levels of MS patients (Results of CIS, RRMS, SPMS and PPMS: Lechner et al., 2018, Table 2)

Diagnosis	Number	Serum level of Hsp70[a]	p-values[b]
CIS/RRMS	66	8.9 [4.8]	< 0.001
SPMS/PPMS	28	6.8 [3.6]	< 0.05
CIS	26	8.2 [4.6]	0.001
RRMS	40	9.3 [4.8]	< 0.001
SPMS	19	7.5 [4.2]	0.831
PPMS	9	5.3 [4.3]	1.000

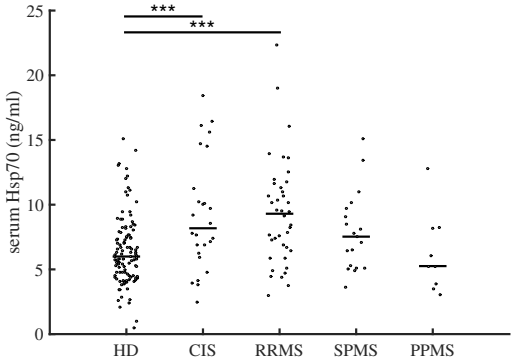
[a] Median (ng/ml) [interquartile range], [b] p-values refer to comparisons to HD



(a) MS patients in total



(b) MS in two groups (Lechner et al., 2018, Figure 2a)



(c) MS subtypes (Lechner et al., 2018, Figure 2b)

Figure 4.4: Serum Hsp70 levels of HD compared to MS patients (a) in total, (b) divided depending on the underlying pathomechanism and (c) the four subtypes

Since every inflammatory condition or tumor disease can enhance the Hsp70 serum levels (cf. Section 1.2.1), it was examined whether the patients were suffering from another inflammatory or tumor disease at time point of blood sampling. According to the medical records, 91 MS patients did not suffer from any acute or chronic inflammatory disease or from a tumor disease. Three patients had a pre-existing inflammatory condition (Uveitis, Hashimoto's disease and erosive reflux disease), but did not show extraordinary high serum Hsp70 levels (Lechner et al., 2018).

In conclusion, the elevated Hsp70 levels did not seem to be caused by another inflammatory condition, but were more likely the result of MS-induced inflammatory processes (Lechner et al., 2018).

4.2.3.2 Influence of Disease Activity

Active inflammation can be indicated by clinical symptoms or by Gd-enhancing lesions determined by MRI. A detailed clinical analysis of the CIS/RRMS group revealed that depending on this criteria 36 patients were in remission and 27 patients had suffered a relapse within the last 30 days at the time point of blood sampling. Between relapse and remission Hsp70 serum levels showed no significant difference ($p = 0.249$) (Lechner et al., 2018). The samples were selected in such a manner that only four patients had taken MPS in the last 40 days before blood sampling (6, 8, 9 and 40 days, respectively).

A comparison of patients with new Gd-enhancing lesions ($n = 21$) and patients with no observed new lesions ($n = 8$) displayed also no significant difference ($p = 0.628$) (Lechner et al., 2018). The group without new lesions consists of those patients, who displayed neither in the cranial, nor in the spinal cord MRI new Gd-enhancing lesions. Patients with just one of those two MRI scans were excluded from this analysis. The requirement to be included into the group with new lesions was that Gd-enhancing lesions had to occur in only one of the two MRI regions and, therefore, only one positive scan was necessary.

Furthermore, the Hsp70 serum levels in connection with the relapse rate were evaluated. The median number of relapses within the last two years of the patients with RRMS was 1.0, overall it ranged between 0 and 5. Multiple regression analyses displayed no significant correlation ($p = 0.161$).

Finally, in order to assess the influence of the severity of the disease course, the EDSS of the patients in correlation to the Hsp70 serum level was evaluated. Multiple regression analyses showed no significant correlation ($p = 0.333$).

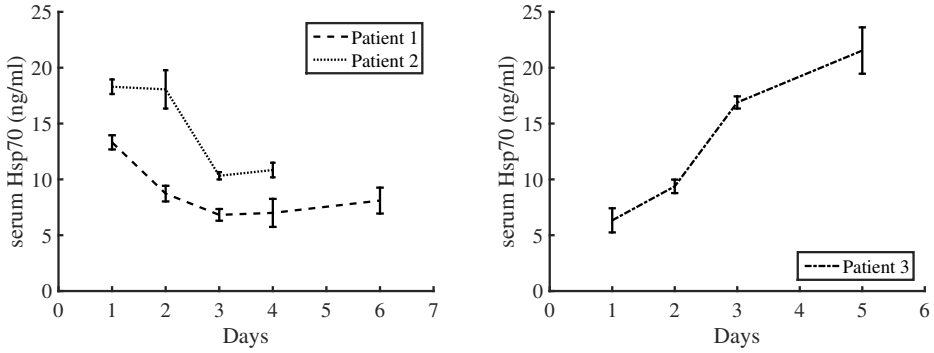
In conclusion, no correlation between disease activity and serum Hsp70 levels was seen.

4.2.3.3 Influence of Pharmacological Therapy

As the medication for MS patients modulates the immune system (cf. Section 1.1.7) and, therefore, might have an impact on the Hsp70 serum levels, two different kinds of pharmacological treatments are analyzed hereinafter – first the medication of an acute attack with high doses of MPS and second different DMTs.

Three patients were recruited during an acute attack to identify the influence of a high-dose MPS therapy (cf. Figure 4.5). The first blood sample was taken before start of the treatment. Afterwards, the first intravenous injection of MPS (1 g) was administered. All further blood samples were collected every morning after the daily drug administration. As a result, between the sample of day 1 and 2, two doses of MPS were administrated. The sample of patient 1 on day 5, and of patient 3 on day 4 had to be excluded, as the serum samples showed a high haemolysis, which is known to disturb the ELISA (Breuninger et al., 2014). Two patients, who responded to steroid therapy by a regression of symptoms, displayed also decreasing Hsp70 serum values (cf. Figure 4.5a). After the treatment cycle, Hsp70 levels decreased to

52 % in patient 1 and to 41 % in patient 2 compared to the values measured before start of the therapy. In contrast, the third patient showed only a minor response to the therapy according the medical records and the Hsp70 serum level was found to be increased (Figure 4.5b) (Lechner et al., 2018).



(a) Two patients with a good response to the therapy

(b) One patient with a minor response to the therapy

Figure 4.5: Serum Hsp70 level under high-dose cortisone therapy (Lechner et al., 2018, Figure 4)

The second pillar of MS therapy is DMT. 44 patients received no long-term medication contrasting to 22 patients with treatments consisting of IFNb (n = 15), GA (n = 5) and natalizumab (n = 2). The serum Hsp70 levels are summarized in Table 4.10. Between the two main groups – with and without DMT – no difference was detected (p = 0.171) (Lechner et al., 2018). A closer look at the different substances displayed also no difference (p = 1.000), however, the number of patients taking GA (n = 5) or Natalizumab (n = 2) was low.

Table 4.10: Influence of disease-modifying therapy (DMT) on serum Hsp70 levels of patients diagnosed with CIS or RRMS (Results of no DMT and DMT: Lechner et al., 2018, Table 3)

Therapy	Number	Serum level of Hsp70[a]
no DMT	44	7.7 [4.6]
DMT	22	9.9 [5.4]
IFNb	15	10.3 [5.3]
GA	5	10.2 [6.2]
Natalizumab	2	7.9 [0.3]

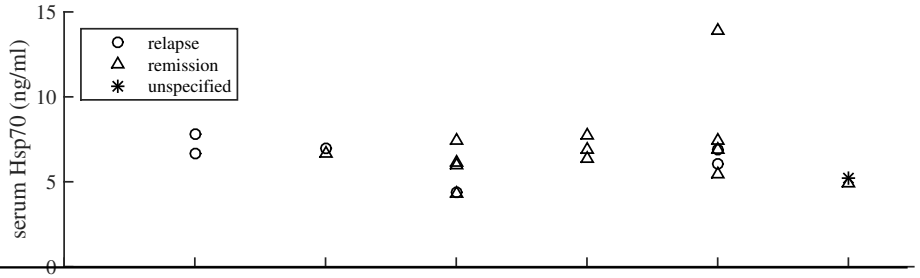
DMT: Disease-modifying therapy; IFNb: interferone beta; GA: glatiramer acetate.

[a] Median (ng/ml) [interquartile range]

Summarizing, steroid therapy led to decreasing Hsp70 levels, if the patient had a good clinical response to the medication. In contrast, DMT showed no effect on Hsp70 serum levels.

4.2.3.4 Stability over Time

In order to study the course of Hsp70 serum levels over time, blood was collected from six patients who were diagnosed with RRMS. Blood samples were taken during phases of relapse and remission. For none of the patients the diagnosis changed from RRMS to SPMS during the observation period. This ranged between 22 and 1216 days and samples were collected at two up to six different time points. Over the whole period, the Hsp70 serum levels remained stable within each individual patient (cf. Figure 4.6). Only one exception appeared within patient number 5 with one outlier out of its six samples. When the samples were collected, the patients were not suffering from any other mayor inflammatory condition, according to the medical records.



Patient number	1	2	3	4	5	6
Observation period[a]	22	48	402	1128	1192	1216
Number of blood samples	2	2	5	3	6	2
– during relapse/remission	2/0	1/1	1/4	0/3	2/4	0/1*
Serum Hsp70 level[b]	7.2 ± 0.8	6.8 ± 0.2	5.7 ± 1.3	7.0 ± 0.7	7.8 ± 3.1	5.1 ± 0.2

[a] days, [b] Mean $\pm$ SD (ng/ml), *one unspecified sample

Figure 4.6: Serum level of Hsp70 over the time (adapted from Lechner et al. 2018, Figure 3)

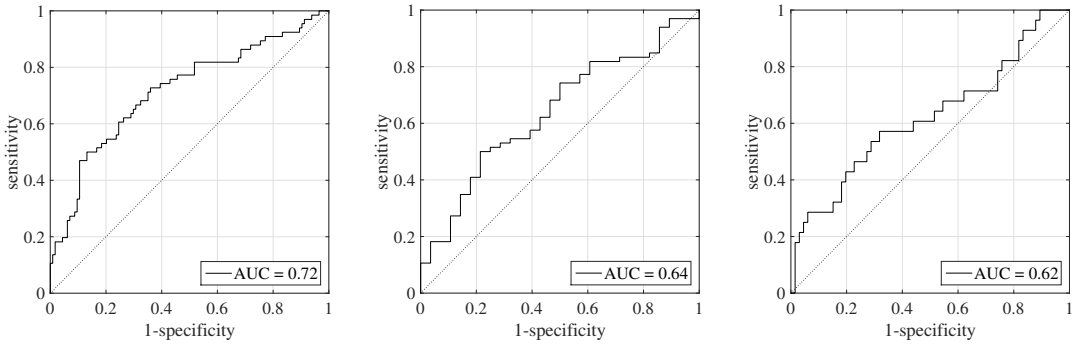
Additionally, the influence of the time period between the first manifestation of symptoms and blood sampling was evaluated. Regression analyses displayed no significant correlation between serum Hsp70 levels and disease duration ($p = 0.735$).

In conclusion, serum Hsp70 levels were stable over the time and course of disease.

4.2.3.5 Serum Hsp70 Levels to Distinguish between MS Subtypes

In order to evaluate the potential of serum Hsp70 levels as a measurement to differentiate CIS/RRMS from healthy individuals and other diseases, ROC curve analyses were performed.

The ROC curve distinguishing CIS/RRMS and HD via serum Hsp70 level showed a confident result with an AUC of 0.72 (cf. Figure 4.7a). In contrast, the ROC curve analyses of CIS/RRMS discriminated from the progressive forms (SPMS/PPMS) (cf. Figure 4.7b) and OIND (cf. Figure 4.7c) displayed poor results with AUCs of 0.64 and 0.62, respectively.



(a) Serum Hsp70 level to differentiate CIS/RRMS from HD

(b) Serum Hsp70 level to differentiate CIS/RRMS from SPMS/PPMS

(c) Serum Hsp70 level to differentiate CIS/RRMS from OIND

Figure 4.7: Serum Hsp70 levels as a measurement to differentiate CIS/RRMS from HD, other MS subtypes and OIND

Summarizing, on the basis of serum Hsp70 it might be possible to distinguish between CIS/RRMS and HD.

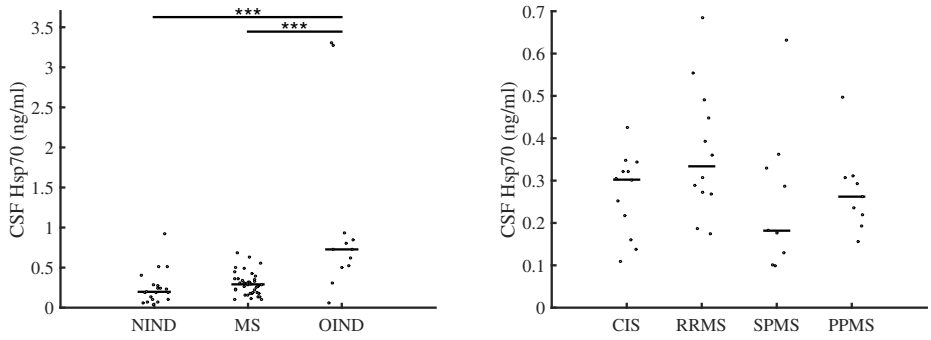
4.3 Cerebrospinal Fluid

Since a lumbar puncture is necessary to extract CSF, it was impossible to obtain a healthy control group. Nevertheless, the second collective included CSF samples with corresponding serum samples of 87 patients, which were collected at the same point in time. The first notable observation were the lower values of Hsp70 compared to the quantified levels in serum. As a result, 36 % of the values measured with the lipHsp70 ELISA were below the measuring limit and were, therefore, valued with 0.0 ng/ml Hsp70. As the measuring limit for the R&D ELISA is lower compared to the lipHsp70 ELISA (cf. Section 3.1) only one sample had insufficient protein concentrations. Hence, the depicted diagrams and tables display the values measured by the R&D ELISA.

Hereinafter, the analyses concerning inflammatory processes and MS subtypes are presented, followed by a combined evaluation of those sample pairs.

4.3.1 Correlation between Inflammation and CSF Hsp70 Levels

First the influence of inflammatory diseases on the CSF Hsp70 level was investigated. The analysis revealed that the amount of inflammation in OIND was reflected in the significantly higher values compared to NIND ($p < 0.001$) and MS ($p < 0.001$) (cf. Figure 4.8). No difference, however, could be seen between NIND and MS ($p = 1.000$). With a closer look at the subtypes of MS (cf. Figure 4.8b) also no difference was noticeable ($p = 0.143$).



(a) Comparison between NIND, MS and OIND

(b) Comparison between the four MS subtypes

Figure 4.8: Influence of inflammation on the CSF Hsp70 level
NIND: n = 20, MS: n = 42 and OIND: n = 12; CIS: n = 12; RRMS: n = 12; SPMS: n = 9; PPMS: n = 9.

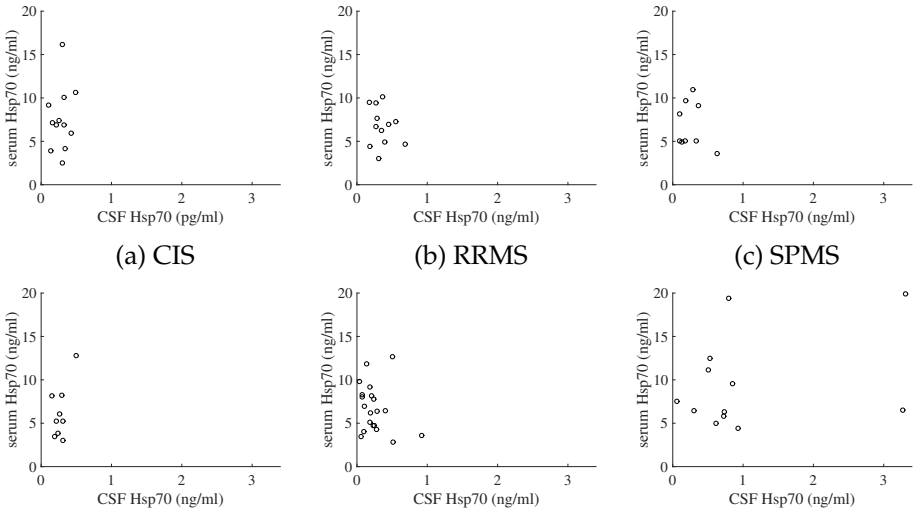
During a relapse, the active inflammation is presumed to be within the CNS. Therefore, the influence of this inflammation was analyzed. None of the patients was treated with MPS in the weeks before blood sampling. In order to be included into the group with new Gd-enhancing lesions, either a spinal or a cranial MRT was necessary. In contrast, only patients with no new lesions in both scans were included into the second group.

Patients with relapses indicated by clinical symptoms in the last 30 days (n = 17) were compared to patients in remission (n = 5). Additionally, patients with relapses indicated by Gd+ lesions in MRI scans (n = 13) were contrasted to patients with no Gd+ lesions (n = 5). Both analyses displayed values ranging in a similar scope and non significant differences ($p = 0.574$ and $p = 0.329$, respectively).

Summarizing, the disease activity displayed no influence on the CSF Hsp70 levels.

4.3.2 Correlation between Serum and CSF Hsp70 Levels

In order to gather more information about the origin of enhanced serum Hsp70 levels, a connection between the Hsp70 in serum and CSF was evaluated. With the purpose to investigate, whether a high serum level corresponds to a high CSF level, correlation analyses were performed. The Spearman's rank correlation coefficients displayed no significant correlation. For visualization the associated scatter plots are presented in Figure 4.9.



(a) CIS (b) RRMS (c) SPMS

(d) PPMS (e) NIND (f) OIND

Figure 4.9: Correlation between serum und CSF level of Hsp70
CIS: n = 12; RRMS: n = 12; SPMS: n = 9; PPMS: n = 9; NIND: n = 20; OIND: n = 12

In conclusion, neither the correlation coefficients nor the scatter plots indicated a correlation between serum and CSF Hsp70 levels.

4.3.3 Serum and CSF Hsp70 Levels Depending on BBB Disruption

Since the previous analyses revealed no correlation and due to the fact that interactions between CSF and serum are limited by an intact BBB, an analysis dependent on BBB disruptions was performed. As a measurement of the BBB function, the albumin (CSF/serum) quotient (QAlb) can be used. This quotient changes physiologically during life and the threshold was, therefore, age-adjusted using following formula: QAlb = (4 + age (years)/15) x 10^{-3} (Berlit, 2011; Reiber et al., 2001). Depending on this quotient, the BBB function was determined either as intact or as disrupted. Afterwards, the relation of CSF Hsp70 levels to serum Hsp70 levels was compared in each subgroup (cf. Table 4.11).

The described analysis had the aim to specify the origin of the measured Hsp70. If the proportion of CSF to serum Hsp70 is greater, it can be concluded that an increased release of Hsp70 within the CNS did occur. This would be expected in patients with inflammatory processes within the CNS. If this proportion is lower, the origin of Hsp70 can be concluded to be the blood. Furthermore, if only one compartment – blood or CSF – is the main origin of the quantified Hsp70, the relation would change considerable due to BBB disruption, which makes an exchange between the two compartments possible.

Table 4.11: Relation of CSF Hsp70 level to serum Hsp70 level depending on BBB disruption

	BBB disruption		no BBB disruption		
	Number	CSF/serum Hsp70 level[a]	Number	CSF/serum Hsp70 level[a]	p-value[b]
CIS	7	0.072 [0.029]	5	0.099 [0.085]	0.936
RRMS	6	0.082 [0.082]	6	0.128 [0.040]	0.958
SPMS	5	0.061 [0.066]	4	0.053 [0.043]	0.577
PPMS	1	0.170	8	0.092 [0.053]	–
NIND	9	0.056 [0.095]	11	0.059 [0.064]	0.396
OIND	11	0.264 [0.224]	1	0.149	–

[a] Median [interquartile range], [b] p-values refer to comparisons between BBB disruption and no BBB disruption done by a Student's t-test

The measurement results displayed the highest proportion within OIND followed by RRMS and CIS, however, those differences were small. Additionally, no significant difference between BBB disruption and intact BBB could be seen (cf. Table 4.11).

4.3.4 Correlation between Cell Count and Hsp70 Levels in CSF

As the mononuclear cells within the CSF may be the origin of Hsp70, correlation analyses between cell count and Hsp70 in CSF were performed. Therefore, the Spearman's rank correlation coefficient for each diagnosis subgroup was calculated (cf. Table 4.12). Within the group of OIND a significant correlation ($p = 0.049$) could be seen. In contrast, no coherence could be detected within the other groups.

Table 4.12: Spearman's rank correlation coefficients about a correlation between the level of Hsp70 and cell count in CSF

Diagnosis	Number	Cell count[a]	CSF Hsp70 level[b]	Correlation coefficient	
				ρ	p-value
CIS	12	6 [10]	0.3 [0.1]	-0.109	0.737
RRMS	12	4 [3]	0.3 [0.2]	0.324	0.304
SPMS	9	2 [1]	0.2 [0.2]	0.460	0.215
PPMS	9	1 [2]	0.3 [0.1]	0.308	0.416
NIND	20	1 [2]	0.2 [0.2]	-0.357	0.123
OIND	12	103 [163]	0.7 [0.3]	0.587	0.049

[a] Median (n/µl) [interquartile range], [b] Median (ng/ml) [interquartile range]

Furthermore, the proportion of Hsp70 to total protein in CSF was analyzed. Therefore, Hsp70 levels were divided by the total protein amount measured in CSF. The summarized results are shown in Table 4.13. An ANOVA test revealed that there was no difference between the subgroups (p = 0.456). OIND and RRMS, however, depicted the highest proportion.

Table 4.13: CSF Hsp70 level in proportion to total protein

Diagnosis	Number	CSF Hsp70 level/total protein[a]
CIS	12	3.9 [2.3]
RRMS	12	6.5 [4.0]
SPMS	9	4.1 [3.5]
PPMS	9	4.4 [1.4]
NIND	20	4.3 [6.7]
OIND	12	6.0 [8.3]

[a] Median $*10^6$ [interquartile range]

All in all, no conclusion concerning the compartment from which the Hsp70 originates could be drawn.

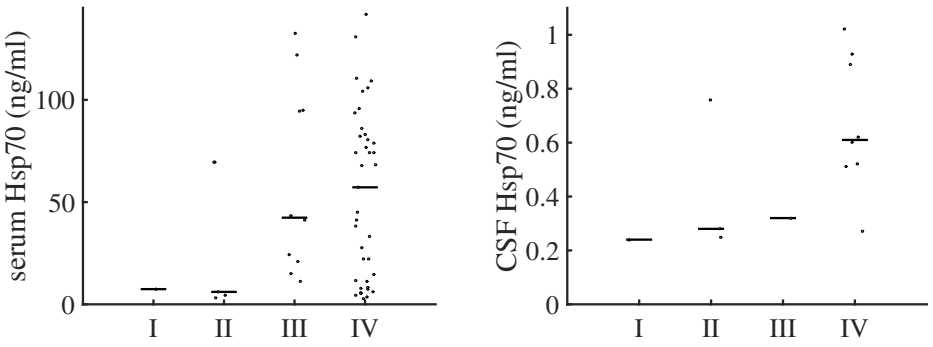
4.4 Hsp70 Levels of Patients with Tumors of the CNS

In this study, a group of 55 serum samples of patients with tumors of the CNS were analyzed. This group consisted of tumors with WHO grading I (n = 1), II (n = 5), III (n = 10) and IV (n = 39). Table 4.14 shows the measured Hsp70 levels. An analysis of the serum samples revealed no significant differences between the WHO gradings (p = 0.409). From 13 patients also CSF samples were available and measured, but also no significant differences were detectable (p = 0.282). Nevertheless, a drastic downgrading from grade IV to I were in both – serum and CSF Hsp70 values – apparent (cf. Figure 4.10).

Table 4.14: Serum Hsp70 level of patients with tumors of the CNS

WHO grading	Number	serum Hsp70 level[a b]	Number	CSF Hsp70 level[a c]
I	1	7.4	1	0.2
II	5	6.1 [65.1]	3	0.3 [0.3]
III	10	42.4 [72.8]	1	0.3
IV	39	57.2 [71.2]	8	0.6 [0.4]

[a] Median (ng/ml) [interquartile range], [b] measured by lipHsp70 ELISA, [c] measured by R&D ELISA



(a) Serum Hsp70 levels depending on their WHO grading, measured by lipHsp70 ELISA

(b) CSF Hsp70 levels depending on their WHO grading, measured by R&D ELISA

Figure 4.10: Serum and CSF Hsp70 levels of patients with tumors of the CNS

If a MRI scan at the time point of blood sampling was available, the volume of the tumor was determined. It was examined whether tumors with a greater volume would release more Hsp70 than smaller ones. Therefore, the patients were divided into three groups according to their tumor volume. Within the serum no correlation between Hsp70 level and tumor volume could be seen ($p = 0.615$; cf. Table 4.15). The Hsp70 levels in CSF displayed higher values in tumors with a greater volume (cf. Table 4.15), however, those observed differences did not reach statistical significance ($p = 0.394$). This might be due to the small number of patients analyzed.

Table 4.15: Serum and CSF Hsp70 level of patients with tumors of the CNS in correlation with the tumor volume

Volume[a]	Number	serum Hsp70 level[b c]	CSF Hsp70 level[b d]
< 10	5	7.4 [5.1]	0.3 [0.3]
10 - 100	3	8.3 [6.1]	0.5 [0.4]
> 100	3	6.1 [2.5]	0.6 [0.2]

[a] cm^3, [b] Median (n/ml) [interquartile range], [c] measured by lipHsp70 ELISA, [d] measured by R&D ELISA

Tumor diseases were also compared to HD, NIND and inflammatory neurological diseases (IND), which combines the MS and OIND group. The analyses showed that the serum Hsp70 levels of tumors of the CNS patients were significantly higher than HD, NIND and IND ($p < 0.001$; $p < 0.001$; $p < 0.001$, respectively). Nevertheless, the values were spread over a great scope with a minimum at 2.7 ng/ml and a maximum at 141.9 ng/ml.

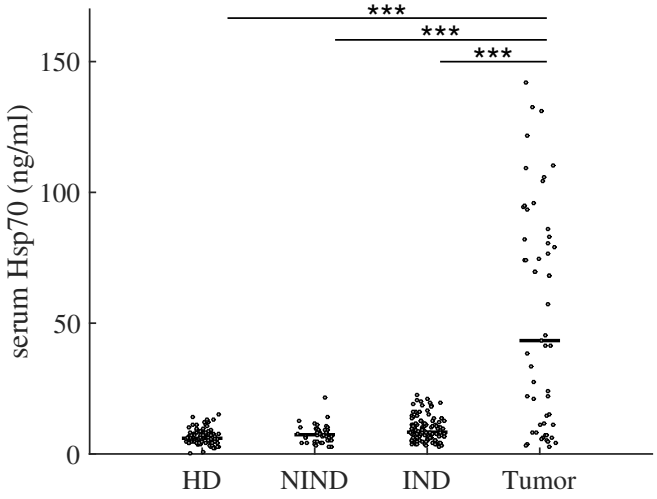


Figure 4.11: Hsp70 levels of patients with tumor of the CNS, divided depending on their volume

In this study, a sign for a possible correlation between the severity of the tumor, indicated by the WHO grading, and the amount of Hsp70 was seen. With respect to the tumor volume a correlation between Hsp70 levels and tumor volume is potentially possible, but due to the small number of samples a statistical significance was not observed. Overall, tumor diseases of the CNS enhanced the serum Hsp70 level significantly compared to all other subgroups.

4.5 R&D ELISA compared to lipHsp70 ELISA

Two different ELISA setups were used in this study. The R&D ELISA is able to determine free Hsp70, whereas the lipHsp70 ELISA can detect both free and lipid-bound Hsp70. All samples were measured with both setups. The overviews of the results of both ELISAs were compared (cf. Figure 4.12 and 4.13).

All in all, the results of the two ELISAs revealed a similar distribution. The lipHsp70 ELISA, however, displayed throughout higher values than the R&D ELISA. In both, OIND showed the highest serum Hsp70 levels followed by CIS and RRMS, whereas HD displayed the lowest values.

The difference between the values measured with lipHsp70 ELISA and R&D ELISA can be presumed as the amount of lipid-bound Hsp70. Analyses of the relation of lipid-bound Hsp70 to Hsp70 in total displayed mean proportions of 62 % (CIS), 63 % (RRMS) and 61 % (SPMS). PPMS had the lowest with 41 %.

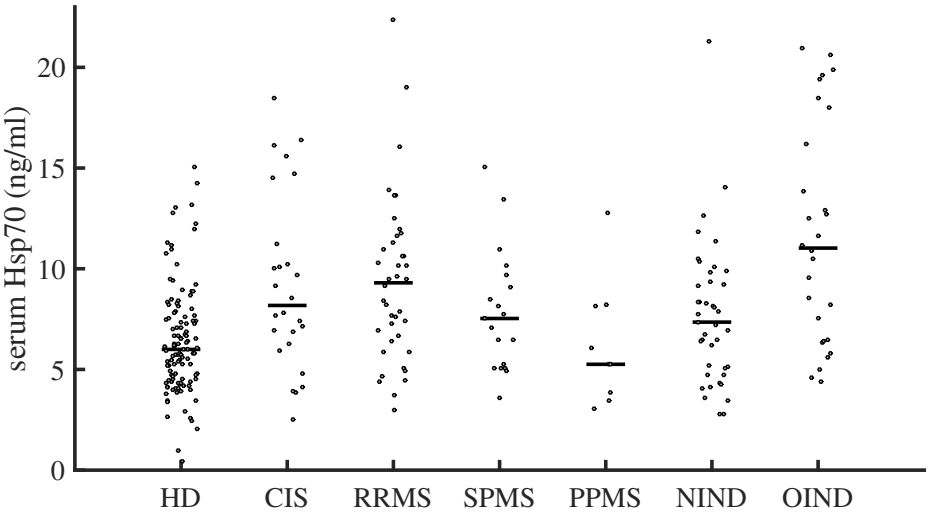


Figure 4.12: Overview of all samples measured with the lipHsp70 ELISA

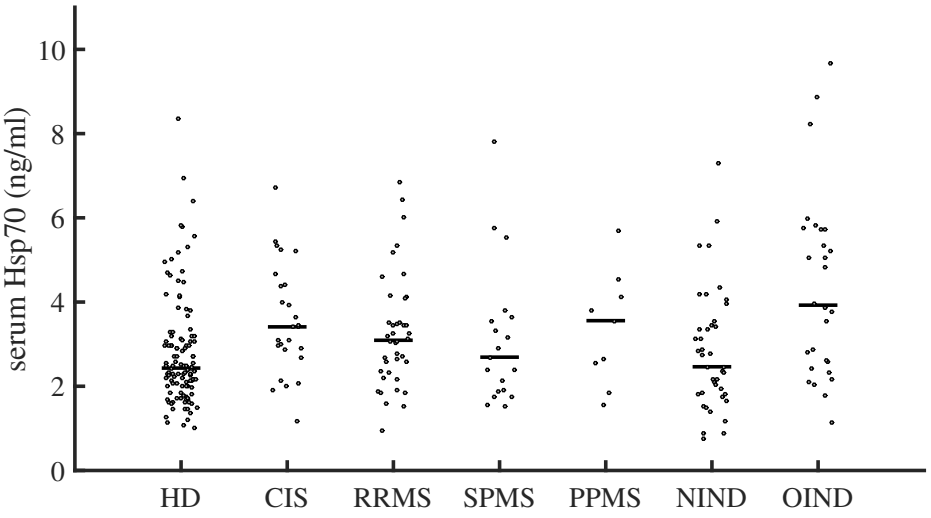


Figure 4.13: Overview of all samples measured with the R&D ELISA

5 Discussion

The aim of this study was to investigate the potential value of serum Hsp70 levels as a biomarker to distinguish inflammatory and neurodegenerative processes in MS patients. In order to evaluate this, serum samples of MS, NIND and OIND were collected and serum Hsp70 levels were measured. Samples of HD and patients with tumor diseases of the CNS served as comparison groups. Additionally, matching CSF samples were quantified with the aim to identify the source of Hsp70 in serum.

The potential of serum Hsp70 as a biomarker for inflammation in general and in MS subtypes is examined below. Additionally, the option to use serum Hsp70 levels as a monitoring tool during the course of MS and for the application of medication is argued. Furthermore, the results of the CSF measurements are demonstrated and finally, the strengths and limitations of this study together with future aspects are addressed.

5.1 Serum Hsp70 as a Potential Biomarker for Inflammation and Tumors

Hsp70 is well known to occur in the serum of healthy individuals (Pockley et al., 1998), which can either originate from living or from dead cells (Calderwood et al., 2007). In a stressful environment, such as inflammation, an increased release of Hsp70 can be observed (Mambula et al., 2007). As a result, elevated Hsp70 levels in serum were shown in a variety of diseases such as preeclampsia (Molvarec et al., 2009) and liver inflammation (Gehrmann et al., 2014a). In this study, significantly elevated levels were determined in the serum of patients with inflammatory neurological diseases – OIND ($p < 0.001$) and MS ($p < 0.001$) – compared to HD. A comparison of the two patient groups revealed lower Hsp70 serum levels in MS compared to OIND patients. This result can be explained by the fact that inflammation is generally less pronounced in MS compared to acute viral or bacterial infections of the CNS. In accordance with these findings, it could be shown that a therapy with high-dose MPS in MS patients during relapse resulted in a decrease in Hsp70 levels due to its anti-inflammatory effect. These results underpin the presumption that inflammation increases the amount of Hsp70 in serum (Lechner et al., 2018), however, this was only observed in a very small patient cohort and further studies are needed.

Gehrmann and colleagues demonstrated that inflammatory diseases of the liver result in elevated serum Hsp70 levels and that liver tumors depict even higher values (Gehrmann et al., 2014a). Instead of the liver, in this study inflammatory and tumor diseases of the CNS were compared. It could be confirmed that the inflammatory diseases showed elevated but still significantly lower levels compared to tumor diseases ($p < 0.001$). Within the tumor diseases, the amount of Hsp70 reflected the WHO grading. Lower values could be seen in

low grade tumors with benign tendencies. In contrast, the higher grade tumors, which have a poorer prognosis (Kleihues et al., 2002), showed higher serum levels. This is consistent with the findings of an in vitro study by Beaman and colleagues, which also suggested Hsp70 as a prognostic biomarker in glioma (Beaman et al., 2014). Nevertheless, these results were not significant and more studies with larger sample numbers are needed. Furthermore, no correlation between Hsp70 serum levels and the volume of the tumor was apparent. This finding, however, does not exclude a possible connection, as the investigated sample number was low (n = 11). In order to draw sensible conclusions, studies with a greater number of cases are necessary.

Recently, it was considered, that Hsp70 might be a useful biomarker for inflammation (Radons, 2016) as well as for tumor diseases (Gehrmann et al., 2014a; Qu et al., 2015). Since both groups are seen in connection with elevated serum Hsp70 levels, but rank in different scopes, this study underpins its possible potential as a biomarker for both of them.

5.2 Serum Hsp70 as a Potential Biomarker to Distinguish Inflammatory and Neurodegenerative Processes in MS

Relapsing and progressive subtypes are presumed to differ with respect to their underlying pathomechanisms. CIS and RRMS are associated predominantly with inflammation, whereas in the progressive forms – SPMS and PPMS – neurodegeneration seems to dominate (Lassmann et al., 2007). Within the group of progressive subtypes, PPMS goes along with less inflammation than SPMS (Revesz et al., 1994). In this study, CIS and RRMS showed the highest serum Hsp70 levels within the subtypes of MS followed by SPMS and PPMS with values in the same range as HD. This also reflects the amount of inflammatory components within those groups and supports the theory that the increase is triggered by the different pathomechanisms (Lechner et al., 2018).

During stress such as inflammation, PBMCs release both, free (Hunter-Lavin et al., 2004) and exosomal Hsp70 (Lancaster and Febbraio, 2005; Mambula et al., 2007). In contrast, cell death is presumed to lead to mainly free Hsp70 (Mambula et al., 2007). In this study, free Hsp70 was measured with the R&D ELISA, while the lipHsp70 ELISA detects both free and lipid-bound Hsp70. The values of both ELISA setups displayed similar distributions, but the lipHsp70 ELISA displayed, as expected, results with a greater extent. The results of one subgroup – the PPMS samples – are noticeable. Serum Hsp70 levels of PPMS displayed not only lower amounts but also smaller proportion of exosomal Hsp70 compared to the other MS subtypes. Therefore, it can be considered that Hsp70 in PPMS is released due to cell death rather than inflammatory processes. This finding is in line with the presumed dominating pathogenesis of PPMS – neurodegeneration.

Since there are differences in the age distribution between the subgroups of MS, this

influence has to be taken into consideration. Patients with SPMS and PPMS were on average older than patients with CIS and RRMS. A different sample selection could not avoid this age distribution as it is due to the later onset of the progressive forms of the disease (Compston and Coles, 2008). Since a negative correlation with age was postulated (Jin et al., 2004; Njemini et al., 2011; Rea et al., 2001), this could explain the lower levels in SPMS and PPMS compared to CIS and RRMS. Nevertheless, contrary results were seen in this study, as age indicated no impact on the serum levels, which is concordant with other reported studies (Breuninger et al., 2014; Gehrmann et al., 2014a; Molvarec et al., 2009). There is a consensus view that gender has no influence on serum Hsp70 levels (Gehrmann et al., 2014a; Jin et al., 2004; Njemini et al., 2011). As a result, age does not seem to be the reason for the lower levels in SPMS and PPMS. It is more likely to presume that the main cause for the elevated Hsp70 levels is the underlying pathomechanism (Lechner et al., 2018).

The heterogeneity of MS requests for precision medicine and therefore requires a more differentiated defining of the patients, which can be established by the development of biomarkers. Over the last decades, considerable efforts have been made to identify diagnostic and prognostic biomarkers for MS (Housley et al., 2015; Katsavos and Anagnostouli, 2013). In this regard, the involvement of pro- and anti-inflammatory cytokines and chemokines has been extensively investigated (Hagman et al., 2011; Imitola et al., 2005). As cytokines and chemokines interact closely, Hsp70 is affected as well as it influences others. IL-1, IFN-γ, TNF-α are just a selection that induce the synthesis and secretion of Hsp70 (D'Souza et al., 1994), which in turn, induces IL-1β, IL-6, TNF-α (Asea et al., 2000) and IL-12 (Todryk et al., 1999).

Pasquali and colleagues carried out a study in order to evaluate the cytokine profiles in different MS subtypes, showing that cytokine levels of patients with RRMS and SPMS differ. IFN-γ, which induces the synthesis of Hsp70, displayed significantly higher plasmatic levels in RRMS compared to SPMS. A closer look at active versus inactive and treated versus untreated patients revealed no significant differences (Pasquali et al., 2015). This is in accordance with the results of this study, in which RRMS displayed higher serum Hsp70 levels than SPMS and also no further elevation due to disease activity. In another study carried out by Stelmasiak and colleagues, IL-6 levels in serum and CSF of patients with SPMS and RRMS without an acute relapse were evaluated. The level of IL-6 was significantly elevated in both – serum and CSF (Stelmasiak et al., 2000). As Hsp70 interacts closely with IL-6, it reflects also the results of this study. Martins and colleagues investigated a great variety of cytokines in MS patients. His research group revealed significantly increased levels of IFN-γ, IL-1β and TNF-α in serum of MS patients compared to a healthy cohort. The difference of IL-6 between those two groups was, however, not significant. Those results were only seen in the total of MS patients and a division according to the subtypes revealed no significant differences (Martins et al., 2011). As a consequence, the findings of Martins and colleagues confirm in parts this study. Nevertheless,

it has to be taken in consideration that statements about altered cytokine profiles in MS patients are limited, as studies were enrolled mainly with a low number of patients and provided heterogeneous and conflicting results.

In order to evaluate Hsp70 as a biomarker for the different courses of MS, analyses of ROC curves were performed. The predictability of CIS/RRMS compared to HD showed confident results with an AUC of 0.72. In contrast, the AUC to differ due to the serum Hsp70 level between CIS/RRMS and progressive forms (SPMS/PPMS) or OIND showed less convincing results with an AUC of 0.64 and 0.62, respectively. As in daily clinical practice, the differentiation between those is not trivial, the inclusion of Hsp70 as an additional parameter might still be useful.

In summary, serum Hsp70 seems to reflect the inflammatory components of CIS and RRMS with higher levels and the predominant neurodegeneration of SPMS and PPMS with lower levels. Additionally on the basis of these findings, it can be presumed that the increased Hsp70 serum levels are not only induced by an acute exacerbation, but result from a chronic stressful environment. Therefore, serum Hsp70 could be considered as a potential biomarker for MS. On the one hand, it might be possible to differentiate on the basis of serum Hsp70 levels between MS patients, healthy individuals and patients with OIND. On the other hand, it might be useful to distinguish between the subgroups of MS. This serum level remained stable over time and was not affected by age, gender, DMT, active or inactive disease phases.

5.3 Serum Hsp70 as a Monitoring Tool for the Course of Disease

Hsp70 serum levels might also provide a useful biomarker to monitor patients' response to a steroid therapy and the course of their disease. Additionally, it could make a precision medicine with anti-Hsp70 medications possible. The potential of those two applications is discussed hereinafter.

In this study, a reduction of symptoms during MPS therapy was associated with descending Hsp70 serum levels. In contrast, the patient, who showed rising Hsp70 serum values, had a minor clinical response to the medication and needed two additional treatment cycles with MPS within the following three month. Increasing Hsp70 values during a steroid therapy might, therefore, be indicative either of a poor response or a severe course in the future. As a consequence, an increasing level might point out the need for DMT at an early stage or a premature escalation of the therapy. Since these findings were only observed in a low patient number, future studies with larger patient cohorts are urgently needed to prove this hypothesis.

During the course of a patient changing from RRMS to SPMS, first inflammation seems to be the main pathomechanism, which is later replaced by neurodegeneration (Lassmann, 2013). As patients with SPMS showed lower levels of Hsp70 compared to RRMS, an observation over

time might indicate the transition of a patient from RRMS to SPMS. Thus, the value might predict the time point in the course of the disease, when DMT might have no positive effect any more.

There is no consensus on whether Hsp70 plays a harmful or a beneficial role in MS. Hsp70 was considered as a potential medication for MS, as its cyto-protective functions, when located intracellular, may reduce cell death in the CNS (Mansilla et al., 2012). Conversely, it is also presumed that Hsp70 elicits and enhances inflammatory immune responses, especially if it is localized in the extracellular space (Arnold-Schild et al., 1999; Breloer et al., 1999; Cresswell, 1994; Singh-Jasuja et al., 2000; Tsan and Gao, 2004). Therefore, Hsp70 was proposed as a target for a potential treatment strategy for MS (Mansilla et al., 2014a). For a personalized therapy, basal Hsp70 serum levels might provide a useful marker to predict therapeutic efficiency. Patients with high levels might show a better response to Hsp70 reducing drugs, than patients with initially low levels (Lechner et al., 2018). During therapy, Hsp70 serum levels might be predictive for the outcome and might indicate a required adaptation of the dose.

In summary, serum Hsp70 levels might provide a useful biomarker in order to monitor inflammatory processes in MS and the application of anti-Hsp70 therapeutics (Lechner et al., 2018). Additionally, a more targeted application of DMT could reduce unnecessary drug administrations and, thereby, enrich patient's quality of life.

5.4 Efforts to Identify the Origin of Hsp70 in Serum by Measuring Hsp70 in CSF

In order to investigate the compartment from which the elevated serum Hsp70 originates, paired CSF and serum samples of 87 patients were analyzed. As the composition of CSF is mainly an ultrafiltration of blood, it was presumed that the ELISAs were able to detect Hsp70 in CSF in similar way to detection in serum. Notwithstanding, the lipHsp70 ELISA was shown to be not suitable for measuring low amounts of Hsp70 in the CSF due to its detection limit of 0.3 ng/ml. In contrast, the R&D ELISA with a measuring limit of 0.157 ng/ml delivered valid results. Therefore, only the results of the R&D ELISA are discussed.

The analyses displayed a similar result, but to a lesser extent when compared to those of serum Hsp70 levels. OIND showed again significant higher values compared to MS ($p < 0.001$) and NIND ($p < 0.001$). MS, however, displayed only slightly higher levels than NIND. Also, the analyses between MS subtypes showed no significant differences. Consequently, the low levels of Hsp70 might be the reason for those non significant results. Therefore, the utility of CSF Hsp70 levels for diagnostic or prognostic purposes underlies serum Hsp70 levels.

Since the location of inflammation in MS and OIND is the CNS, the origin of the released Hsp70 can be suspected there. Various theories were formulated, as the Hsp70 levels in CSF displayed unexpected low levels compared to serum and are discussed below.

Neurons are known to synthesize only low amounts of Hsp70 and show a rapid uptake of free Hsp70 from the extracellular space during stress (Mansilla et al., 2012). As a result, the inflammation in the CNS itself could be the origin of the elevated Hsp70 levels, but is not reflected in the measurements as Hsp70 has been taken up by other cells such as neurons. Also, complex formation of Hsp70 with other proteins such as MBP might be a reason why it was not possible to measure the real amount of Hsp70. Nevertheless, no correlation between CSF Hsp70 levels and cell count in CSF or BBB disruption was detectable. Further, also the possibility that CSF is not a suitable medium for the used ELISA setups has to be taken into consideration.

Since Hsp70 serum levels decrease in patients who respond to a high-dose MPS therapy it can be speculated that Hsp70 may be derived from activated immune cells in serum and CSF. As MS is predominantly mediated by activated T cells (Lassmann et al., 2007) and B cells (Gasperi et al., 2016), those can be considered as one source for elevated Hsp70 serum levels. The fact that liposomal Hsp70, which is believed to be actively released by living cells (Calderwood et al., 2007), is increased during inflammation, underpins this assumption. Nevertheless, the origin of the elevated Hsp70 levels in serum remains unknown and additional studies are needed (Lechner et al., 2018).

5.5 Strengths, Limitations and Future Aspects

In this section, the strengths, limitations and future aspects of this study are addressed.

First of all, the strong study setup has to be emphasized. Initially, the serum samples of a discovery cohort (n = 87) and afterwards an independent validation cohort (n = 90) were measured. Additionally, all experiments were performed blinded. In summary, this has generated a solid data base.

It also has to be highlighted that this study is the first to address the relationship between the level of Hsp70 in serum and MS.

Another special feature is the use of two different ELISA setups. On the one hand, the commercial R&D ELISA and on the other hand the established lipHsp70 ELISA, which is able to detect furthermore lipid-bound Hsp70. As the latter one was novel, the results of the R&D acted as control measurements and, as it displayed similar results, strengthened those of the lipHsp70 ELISA. All in all, the lipHsp70 ELISA showed reliable and reproducible measurement results.

Besides those strengths, there are also limitations, which require further research and are addressed hereinafter. The origin of Hsp70 in serum, particularly, could not be specified. Efforts to investigate this with measuring the Hsp70 level in CSF failed as the used ELISAs were possibly inappropriate. Therefore, experiments regarding the ability of both ELISA setups to measure Hsp70 in CSF would be necessary and other alternative experiment setups should be

evaluated.

Due to the relatively low number of samples, measurements with larger patient cohorts are necessary to finally evaluate the value as a biomarker for MS and for monitoring of outcome. Furthermore, the analyses regarding active and inactive disease courses are weakened by the fact that the two groups consisted of two sets of individuals. Also, the samples of patients observed over a long time period were in the majority either collected during phases of relapse or remission. Future analyses of the serum Hsp70 level during relapse and remission of the same patient would generate a more meaningful conclusion.

In the future, Hsp70 might not only be a protein measured in research laboratory, it may also take the decisive step into daily clinical practice in the treatment of MS. Determination of serum Hsp70 could have the potential to become a diagnostic and prognostic tool. It might predict the risk of developing MS and diagnose its presence at an early stage. Therefore, serum Hsp70 levels could be used for screening in populations and help in preventing severe courses of MS. During the disease course, it could potentially act as a monitoring tool to predict the progress and evaluate the efficacy of the medical treatment. In summary, the shown study is a promising approach in MS research and offers a variety of new concepts.

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Appendix

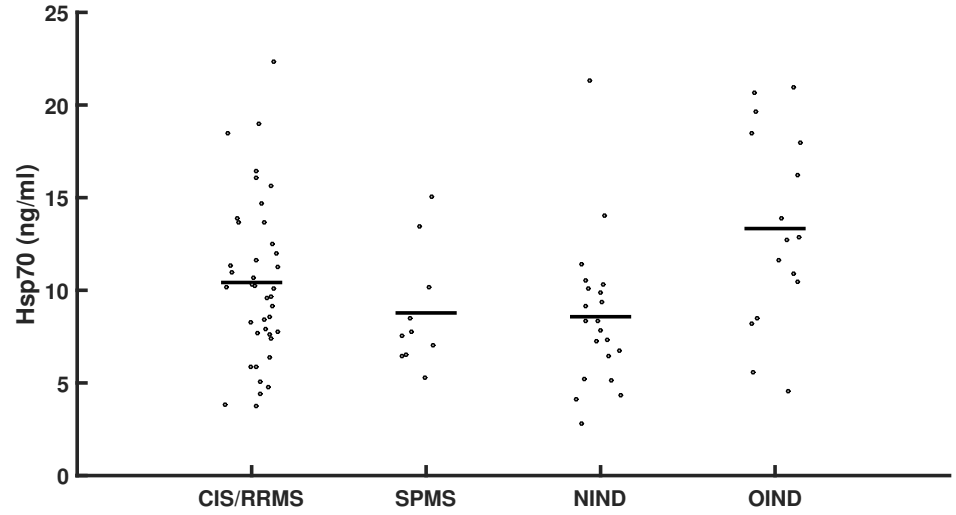

Figure 5.1: Serum Hsp70 levels of the patients of the discovery cohort (Lechner et al., 2018, Supplementary material)
CIS/RRMS: n = 40; SPMS: n = 10; NIND: n = 21; OIND: n = 16
Each data point represents one patient. The lines show the mean value.

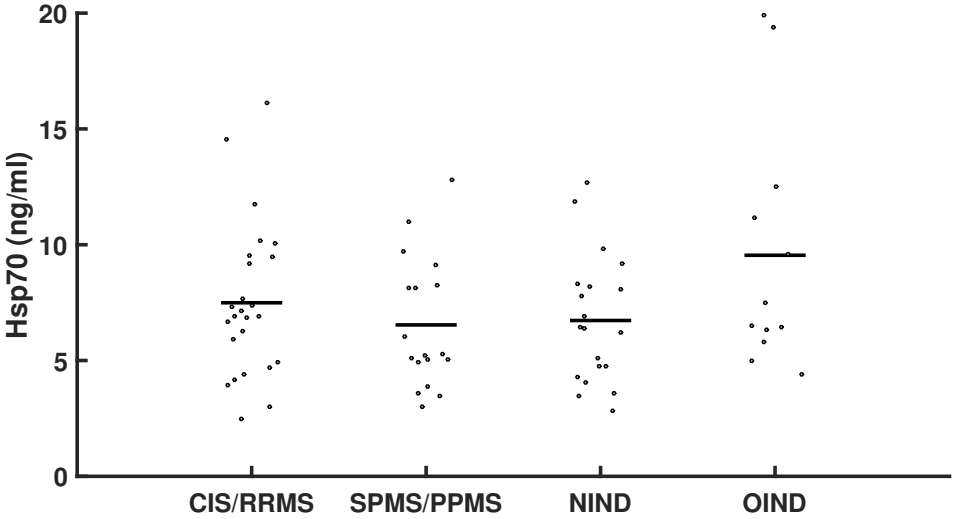

Figure 5.2: Serum Hsp70 levels of the patients of the validation cohort (Lechner et al., 2018, Supplementary material)
CIS/RRMS: n = 26; SPMS/PPMS: n = 18; NIND: n = 20; OIND: n = 12
Each data point represents one patient. The lines show the mean value.

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Serum heat shock protein 70 levels as a biomarker for inflammatory processes in multiple sclerosis

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Patricia Lechner, Dorothea Buck, Lisa Sick, Bernhard Hemmer and Gabriele Multhoff

Abstract

Background: Inflammatory and neurodegenerative processes are hallmarks of multiple sclerosis (MS). The synthesis of the major stress-inducible heat shock protein 70 (Hsp70) is induced by inflammation.
Objective: The purpose of this study is to determine whether Hsp70 in serum can serve as a potential biomarker to distinguish inflammatory and neurodegenerative processes in MS.
Methods: Serum was obtained from 94 patients: 26 clinically isolated syndrome (CIS), 40 relapsing–remitting MS (RRMS), 19 secondary progressive MS (SPMS), and nine primary progressive MS (PPMS). As controls, serum samples were collected from patients with non-inflammatory neurological diseases (NINDs, $n = 41$), other inflammatory neurological diseases (OINDs, $n = 28$) and healthy donors (HDs, $n = 114$). Serum levels of Hsp70 were quantified using the enzyme-linked immunosorbent assay detecting free and liposomal Hsp70 (lipHsp70 ELISA).
Results: Patients with MS displayed significantly higher Hsp70 serum levels than HDs ($p < 0.001$) and significantly lower levels than OINDs ($p = 0.001$). A subgroup analysis revealed that Hsp70 serum levels of CIS/RRMS patients are significantly higher than those of patients with progressive MS (SPMS/PPMS) ($p < 0.05$).
Conclusion: Inflammation causes the release of Hsp70 into the blood. As CIS/RRMS are associated with higher Hsp70 serum levels than progressive MS, serum Hsp70 levels might provide a marker for inflammatory processes.

Keywords: Multiple sclerosis (MS), inflammation, neurodegeneration, serum Hsp70, lipHsp70 ELISA, biomarker

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Introduction

MS is considered as a chronic inflammatory disease of the central nervous system (CNS) that is characterised by inflammation, demyelination, gliosis and neurodegeneration.[1] It is widely believed that the underlying pathomechanism of relapsing–remitting MS (RRMS), including clinically isolated syndrome (CIS) and progressive forms of MS (secondary progressive MS (SPMS)/primary progressive MS (PPMS)), differ. In RRMS, the disease seems to be driven by peripheral immune responses targeting the CNS. In progressive MS compartmentalised immune responses within the CNS and secondary neurodegeneration appear to play a key role in disease progression[2] whereas within the group of progressive subtypes, PPMS seems to go along with less inflammation than SPMS.[3]

Heat shock proteins (HSPs) are molecular chaperones which are highly conserved among different species from bacteria to humans.[4] Depending on their molecular weights, HSPs can be grouped into the following main families: HSP40, HSP60, HSP70, HSP90, HSP100[5] and the small HSPs with a molecular weight of approximately 20 kDa.[6] The human HSP70 family comprises a variety of different isoforms, with molecular weights ranging between 66 kDa and 78 kDa.[7] The main

Correspondence to:
Gabriele Multhoff, Center for Translational Cancer Research TU München (TranslaTUM) campus Klinikum rechts der Isar, Technische Universität München, Ismaninger Strasse 22, 81675 Munich, Germany.
gabriele.multhoff@tum.de

Patricia Lechner, Center for Translational Cancer Research TU München (TranslaTUM) campus Klinikum rechts der Isar, Technische Universität München, Munich, Germany

Dorothea Buck, Department of Neurology, Klinikum rechts der Isar,

Technische Universität München, Germany
German Competence Network Multiple Sclerosis (KKNMS), Germany

Lisa Sick,
Department of Neurology, Klinikum rechts der Isar, Technische Universität München, Germany

Bernhard Hemmer,
Department of Neurology, Klinikum rechts der Isar, Technische Universität München, Germany
German Competence Network Multiple Sclerosis (KKNMS), Germany
Munich Cluster for Systems Neurology (SyNergy), Germany

Gabriele Multhoff,
Center for Translational Cancer Research TU München (TranslaTUM) campus Klinikum rechts der Isar, Technische Universität München, Munich, Germany

representatives of the HSP70 family are the constitutively expressed Hsc70 (73 kDa) and the major stress-inducible Hsp70 (72 kDa).[8]

Hsp70 is predominantly localised in the cytosol[7] but is also found in the extracellular space.[9,10] In malignantly transformed tumour cells and virally infected cells,[11,12] Hsp70 is present on the plasma membrane. The expression of Hsp70 is highly upregulated upon a large variety of stress stimuli including thermal stress[13], anoxia, ethanol, heavy metals,[4] inflammation,[14] infection and tissue injury.[15] Depending on its intra- or extracellular location, Hsp70 fulfils different functions. Intracellular Hsp70 protects cells against lethal damage induced by stress, supports the synthesis and transport of other proteins, and prevents misfolding and aggregation.[7,8] Extracellular and cell surface-bound Hsp70 plays an essential role in eliciting immune responses. It can support specific immune reactions by assisting antigen presentation[16] and it has been shown to activate natural killer cells[17] in the presence of interleukin 2.[18] Furthermore, it can modulate the immune defence by triggering the transcription and release of pro-inflammatory cytokines.[19]

Hsp70 also has been assumed to play a role in the development of MS.[20,21] It is known that Hsp70 knock-out mice are better protected against the development of experimental autoimmune encephalomyelitis (EAE), which represents an animal model of MS.[22] In contrast to these findings, elevated intracellular Hsp70 levels can also exert beneficial effects for MS by protecting cells within the CNS against apoptotic cell death.[20]

To date only Hsp70 gene polymorphisms and intracellular Hsp70 levels in peripheral blood mononuclear cells (PBMCs) have been studied in MS patients.[23] Different publications show a great diversity of intracellular Hsp70 levels in PBMCs of MS patients ranging from low[24] to normal[25] to elevated levels.[26] In the extracellular milieu, only Hsp70-specific antibodies[20] but not Hsp70 proteins have been determined in the serum of MS patients.

The aim of this study was to measure Hsp70 protein levels in the serum of patients with different MS subtypes and inflammatory diseases. It was further assessed whether extracellular Hsp70 can serve as a potential biomarker to distinguish inflammatory and neurodegenerative processes in MS. For this purpose, serum of patients with MS, non-inflammatory neurological diseases (NINDs), other inflammatory neurological diseases (OINDs), and healthy control donors (HDs) was collected.

Materials and methods

Sample collection and enzyme-linked immunosorbent assay (ELISA) experiments

All blood samples of patients ($n = 163$) were collected between 2008 and 2016 by the Department of Neurology, Klinikum rechts der Isar, TU München (TUM). After centrifugation, serum samples were aliquoted and immediately stored at –80°C. The Hsp70 levels of an age- and gender-matched control group ($n = 114$) was provided by the Department of Radiation Oncology, Klinikum rechts der Isar, TU München (TUM). The data of this control group have been published before,[27] but several samples of HDs were re-analysed with identical results in parallel to the patient samples. Informed written consent was provided by all patients and healthy donors. The diagnoses of the patients were obtained from medical records. Approval of the study protocol was granted by the ethical committee of the Klinikum rechts der Isar, TUM.

Serum samples of a discovery cohort consisting of MS, NIND and OIND patients ($n = 87$) and an independent validation cohort ($n = 76$) were measured blinded. Since both cohorts showed similar results as illustrated in supplementary Figures 1 and 2, mean values of both cohorts were summarised and used for further analyses. Lipid-bound and free Hsp70 in the serum was quantified by using the lipHsp70 ELISA following the protocol described previously in detail.[27] The Hsp70 results represent the means of up to three independent experiments, each measured in duplicates ± standard deviation (SD). The mean intra- and inter-plate coefficients of variation were 5.1% and 16.3%, respectively.

Patient characteristics, therapy and magnetic resonance imaging (MRI)

The NIND group comprises patients with epilepsy ($n = 1$), head pressure ($n = 1$), headache ($n = 25$), lumbosciatica ($n = 2$) and pseudotumour cerebri ($n = 12$). The OIND group is composed of patients with encephalitis ($n = 2$), herpes zoster neuritis ($n = 2$), meningitis ($n = 15$) and meningoencephalitis ($n = 9$).

The group of MS patients ($n = 94$) can be subdivided into CIS ($n = 26$), RRMS ($n = 40$), SPMS ($n = 19$), and PPMS ($n = 9$). For diagnosis of MS the revised McDonald criteria from 2005[28] and 2010[29] were

applied. Three patients with CIS/RRMS received high-dose methylprednisolone (MPS) therapy over a period of five to six days. Five additional patients received glucocorticoids due to a relapse ($n = 4$) or an uncertain relapse ($n = 1$) within the last 30 days before blood sampling. Twenty-two patients diagnosed with CIS/RRMS received disease-modifying therapy (DMT) (interferon substances, glatiramer acetate or natalizumab).

MRI was performed at the time point of blood sampling in 29 patients. A new lesion is defined as a gadolinium enhancement in either spinal cord or brain, as detected by MRI scan. A patient with no new lesions is characterised by the absence of gadolinium enhancement in the spinal cord and brain, as detected by MRI scan.

Statistical analyses

Statistical analyses were performed using MATLAB R2015a (MathWorks Inc, Natick, MA, USA). A comparison of two subgroups was performed by using the Student's *t* test; a comparison of more than two subgroups was performed with the analysis of variance test using the Bonferroni method as post hoc analysis. In order to identify potential correlations between Hsp70 levels, age and gender multiple linear regression analyses with the diagnoses as an additional independent variable were performed. A p value of <0.05 was defined as statistically significant.

Results

Clinical characteristics of patients and HDs

In this study, Hsp70 protein levels were quantified in the serum of a total of 163 patients (MS, $n = 94$; NINDs, $n = 41$; OINDs, $n = 28$). Patients with MS were further subclassified into CIS ($n = 26$), RRMS ($n = 40$), SPMS ($n = 19$) and PPMS ($n = 9$). Table 1 summarises the clinical characteristics of patients in the discovery and validation cohort and HDs.

Regarding age distribution, the four main groups (HDs, MS, NINDs and OINDs) were in a similar age range. Nevertheless, within the subgroups of MS, patients with SPMS and PPMS were, as expected, older than those with CIS and RRMS (Table 1).

Hsp70 serum levels are not influenced by age and gender

The role of age and gender as potential parameters that could potentially influence Hsp70 serum levels was evaluated. Regression analyses displayed no age- ($p = 0.435$) or gender-related ($p = 0.422$) differences in Hsp70 serum levels.

Inflammation causes increased Hsp70 serum levels

In order to evaluate the general influence of inflammatory processes on Hsp70 serum levels, Hsp70 was measured in patients with NINDs and OINDs (Figure 1). Patients with OINDs displayed significantly higher Hsp70 serum levels compared to HDs ($p < 0.001$) and NINDs ($p < 0.001$), whereas no significant differences were determined in Hsp70 serum levels of HDs and NIND patients ($p = 0.383$).

Furthermore, we evaluated whether MS patients suffered from additional diseases (data not shown). As documented in the medical records, 91 out of 94 MS patients showed no additional inflammatory disease or cancer at the time point of blood sampling. Three MS patients with pre-existing inflammatory conditions several years ago had low serum Hsp70 levels. Therefore, we speculate that elevated Hsp70 serum levels are mainly caused by MS-induced inflammatory processes.

Hsp70 expression varies in different MS subtypes

Comparison of Hsp70 serum levels in HDs and MS patients revealed significantly elevated Hsp70 levels in the serum of MS patients ($p < 0.001$; Table 2). Furthermore, the Hsp70 serum levels of MS patients were significantly lower compared to patients with OINDs ($p = 0.001$). Regarding the different subtypes, CIS/RRMS displayed significantly higher levels than the progressive forms (SPMS/PPMS) ($p < 0.05$; Figure 2(a)). With respect to the four different subtypes of MS, CIS and RRMS showed highest Hsp70 serum levels followed by SPMS and PPMS patients (Figure 2(b)). Hsp70 serum levels of CIS ($p = 0.001$) and RRMS ($p < 0.001$) patients were significantly higher than those of HDs, whereas serum levels of SPMS, who displayed lower Hsp70 levels, did not show significant differences compared to HDs ($p = 0.831$). Regarding PPMS, the Hsp70 serum levels were similar to those of HDs (p=1.000) (Figure 2(b)).

Further analysis of disease activity parameters of the CIS and RRMS groups revealed that 36 patients were in remission and 27 patients had experienced a relapse within the last 30 days before blood sampling. A comparison of both groups showed no significant difference with respect to their Hsp70 serum levels ($p = 0.249$; Table 3). We also detected no significant differences in Hsp70 serum levels in

Table 1. Patient characteristics.

	Number *N*	Gender M/F	Age Mean ± SD, years (range)	EDSS Median (interquartile range)	Disease duration Median (years) (interquartile range)	Serum level of Hsp70 Median (ng/ml) (interquartile range)
Comparison group						
HDs	114	67/47	43 ± 15 (20–74)	–	–	6.0 (3.1)
Discovery cohort						
CIS/RRMS	40	10/30	33 ± 10 (14–57)	1.0 (1.3)	1.9 (4.4)	10.1 (5.1)
SPMS	10	5/5	49 ± 6 (42–59)	4.0 (4.0)	19.2 (15.8)	7.6 (3.1)
NINDs	21	7/14	33 ± 12 (19–62)	–	–	8.3 (3.7)
OINDs	16	9/7	51 ± 18 (21–79)	–	–	12.8 (8.1)
Validation cohort						
CIS/RRMS	26	10/16	35 ± 9 (22–54)	1.5 (1.0)	0.7 [3.2)	7.0 (4.3)
SPMS/PPMS	18	6/12	53 ± 10 (36–72)	4.5 (2.9)	7.6 [16.3)[a]	5.2 (3.3)
NINDs	20	7/13	39 ± 11 (22–60)	–	–	6.4 (3.6)
OINDs	12	10/2	43 ± 15 (23–73)	–	–	7.0 (5.3)

The disease duration is the time between initial manifestation and blood sampling.
[a]The information about initial manifestation was missing from four patients.
CIS: clinically isolated syndrome; EDSS: Expanded Disability Status Scale; F: female; HDs: healthy donors; Hsp70: heat shock protein 70; M: male; NINDs: non-inflammatory neurological diseases; OINDs: other inflammatory neurological diseases; PPMS: primary progressive multiple sclerosis; RRMS: relapsing–remitting multiple sclerosis; SD: standard deviation; SPMS: secondary progressive multiple sclerosis.

patients with newly detected gadolinium-enhanced lesions ($n = 21$) compared to patients with no detectable new lesions in MRI scans of the brain and spinal cord ($n = 8$) ($p = 0.628$; Table 3).

Stable Hsp70 levels in the serum were demonstrated in five out of six patients who were diagnosed with RRMS over a period of time ranging from 22 to 1216 days (Figure 3).

Responders to high-dose cortisone therapy show decreasing Hsp70 serum levels

Three patients with CIS/RRMS and an acute relapse were recruited to determine the influence of high-dose MPS therapy on Hsp70 serum levels. The first blood sample was taken immediately before start of therapy (day 1); the second blood sample was taken on the next day (day 2) after the first and second intravenous injection of MPS (1g). The following blood samples were taken every next day after the daily drug administration. As shown in Figure 4(a), the Hsp70 serum values of two patients who responded to MPS therapy by a regression of symptoms significantly decreased (41% and 52%, respectively) after the whole treatment cycle. However, the mean (8.1 ± 1.2 ng/ml, 10.8 ± 0.7 ng/ml) and median (8.2 and 10.7 ng/ml) Hsp70 values still remained elevated after therapy compared to those of HDs (mean 6.4 ± 2.7 ng/ml; median 6.0 ng/ml). In contrast, the Hsp70 serum level of a third patient showing only minor responses to steroid treatment increased (Figure 4(b)).

A cohort of 22 patients with CIS/RRMS receiving DMT displayed no significant differences in serum Hsp70 levels ($p = 0.171$) compared to CIS/RRMS

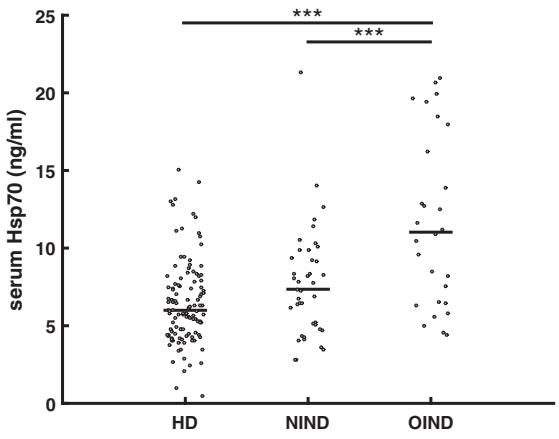


Figure 1. Serum Hsp70 levels of HDs compared to patients with NINDs and OINDs. Patients with OINDs ($n = 28$) display significantly higher Hsp70 serum levels than HDs ($n = 114$, $p < 0.001$) and patients with NINDs ($n = 41$, $p < 0.001$). HDs: healthy donors; Hsp70: heat shock protein 70; NINDs: non-inflammatory neurological diseases; OINDs: other inflammatory neurological diseases. Each data point represents one patient. The lines show the median value.

patients with no DMT ($n = 44$) (Table 3). Nevertheless, both subgroups showed significantly higher Hsp70 serum levels than HDs ($p < 0.001$, $p < 0.001$).

Discussion

In this study, the question was addressed whether serum Hsp70 levels, as determined by the lipHsp70 ELISA, can serve as a biomarker to distinguish inflammatory and neurodegenerative processes in MS patients. Patients with OINDs and MS displayed significantly higher Hsp70 serum levels than HDs. In patients with MS Hsp70 serum levels were found to be lower than in OIND patients. This finding can be explained by the fact that inflammation in MS is generally less pronounced compared to other inflammatory diseases of the CNS. In line with these findings, we were able to show that the anti-inflammatory effect of high-dose MPS therapy in a small cohort of MS patients with acute relapse was accompanied by a decrease in Hsp70 serum levels, although the mean and median Hsp70 values

Table 2. Serum Hsp70 levels related to the different diagnoses.

	Number *N*	Gender M/F	Age Mean ± SD, years (range)	EDSS Median (interquartile range)	Disease duration Median (years) (interquartile range)	Serum level of Hsp70 Median (ng/ml) (interquartile range)	*p* value
HDs	114	67/47	43 ± 15 (20–74)	–	–	6.0 (3.1)	–
MS	94	31/63	39 ± 12 (14–72)	1.8 (2.0)		8.0 (4.8)	<0.001
CIS	26	8/18	32 ± 10 (19–54)	1.0 (2.0)	0.2 (1.1)	8.2 (4.6)	0.001
RRMS	40	12/28	35 ± 10 (14–57)	1.5 (1.0)	2.7 (6.1)	9.3 (4.8)	<0.001
SPMS	19	8/11	51 ± 9 (36–72)	5.0 (2.5)	20.5 (17.2)[a]	7.5 (4.2)	0.831
PPMS	9	3/6	54 ± 8 (43–67)	3.5 (1.0)	4.7 (2.9)[a]	5.3 (4.3)	1.000
NINDs	41	14/27	36 ± 12 (19–62)	–	–	7.3 (4.3)	0.383
OINDs	28	19/9	47 ± 17 (21–79)	–	–	11.0 (10.2)	<0.001

The disease duration is the time between initial manifestation and blood sampling.
[a]The information about initial manifestation was missing from two patients.
P values refer to comparisons to HD and were calculated with the analysis of variance test using the Bonferroni method as post hoc analysis. A *p* value <0.05 is defined as statistically significant.
CIS: clinically isolated syndrome; F: female; HDs: healthy donors; Hsp70: heat shock protein 70; M: male; MS: multiple sclerosis; PPMS: primary progressive multiple sclerosis; RRMS: relapsing–remitting multiple sclerosis; SD: standard deviation; SPMS: secondary progressive multiple sclerosis.

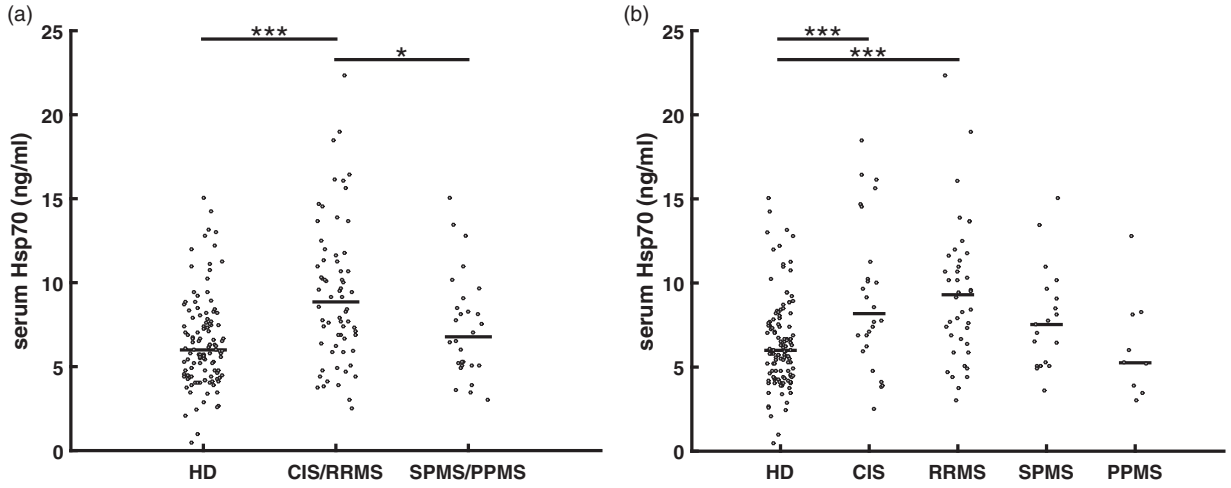


Figure 2. Serum Hsp70 levels of HDs compared to subgroups of MS patients. (a) Samples of patients with CIS/RRMS ($n = 66$) display significantly higher levels than HDs ($n = 114$, $p < 0.001$) and SPMS/PPMS ($n = 28$, $p < 0.05$). (b) Samples of patients with CIS ($n = 26$, $p = 0.001$) and RRMS ($n = 40$, $p < 0.001$) have significantly higher Hsp70 serum levels than HD ($n = 114$). SPMS ($n = 19$) and PPMS patients ($n = 9$) display a reduced trend toward lower levels compared to CIS and RRMS. Both showed no significant differences in the Hsp70 serum levels compared to HDs. CIS: clinically isolated syndrome; HDs: healthy donors; Hsp70: heat shock protein 70; MS: multiple sclerosis; PPMS: primary progressive multiple sclerosis; RRMS: relapsing–remitting multiple sclerosis; SPMS: secondary proressive multiple sclerosis. Each data point represents one patient. The lines show the median value.

Table 3. Serum Hsp70 level related to disease activity parameters and disease-modifying therapy.

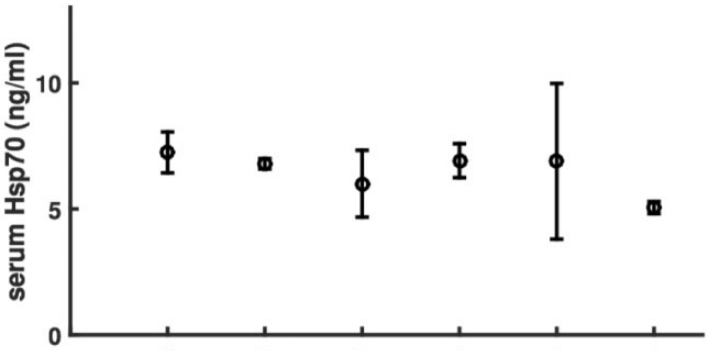
	Number *N*	Serum level of Hsp70 Median (ng/ml) (interquartile range)	*p* value
Relapse in the last 30 days	27	7.7 (3.8)	0.249
Remission	36	9.3 (5.3)	
New gadolinium-enhancing lesion	21	7.7 (4.2)	0.628
No new gadolinium-enhancing lesion	8	7.4 (3.1)	
No disease-modifying therapy	44	7.7 (4.6)	0.171
Disease-modifying therapy	22	9.9 (5.4)	

P values refer to comparisons between each pair and were calculated with the Student's *t* test. A *p* value <0.05 is defined as statistically significant.
Hsp70: heat shock protein 70.

remained elevated compared to HDs. Nevertheless, the decreased Hsp70 serum levels under MPS therapy were determined in only two patients and, therefore, further studies with larger patient cohorts are needed.

Since it appears that Hsp70 serum levels remain stably elevated over a longer period of time, we hypothesise that increased Hsp70 serum levels are not directly caused by an acute exacerbation, but may be the result of a chronically stressed peripheral immune system. This notion is supported by our results that acute clinical relapses and the detection of gadolinium-enhanced lesions by MRI in the brain and spinal cord were not associated with a further increase in serum Hsp70 levels.

Hsp70 serum levels did not change significantly with age and gender. Therefore, the lower Hsp70 levels in patients with SPMS and PPMS compared to CIS and RRMS cannot be explained by an unequal distribution of age in the different patient groups, but rather are triggered by different pathomechanisms. High Hsp70 serum levels in CIS and RRMS and low Hsp70 levels in SPMS and PPMS most likely reflect the degree of systemic inflammation.



Patient number	1	2	3	4	5	6
Observation period (days)	22	48	402	1128	1192	1216
Number of blood samples	2	2	5	3	6	2
Mean ± SD (ng/ml)	7.2 ± 0.8	6.8 ± 0.2	5.7 ± 1.3	7.0 ± 0.7	7.8 ± 3.1	5.1 ± 0.2

Figure 3. Serum heat shock protein 70 (Hsp70) levels over longer time periods. Follow-up samples over a period of time ranging from 22 to 1216 days were measured. Levels remained stable over time in five out of six patients diagnosed with relapsing–remitting multiple sclerosis. The graph represents mean values ± standard deviation (SD).

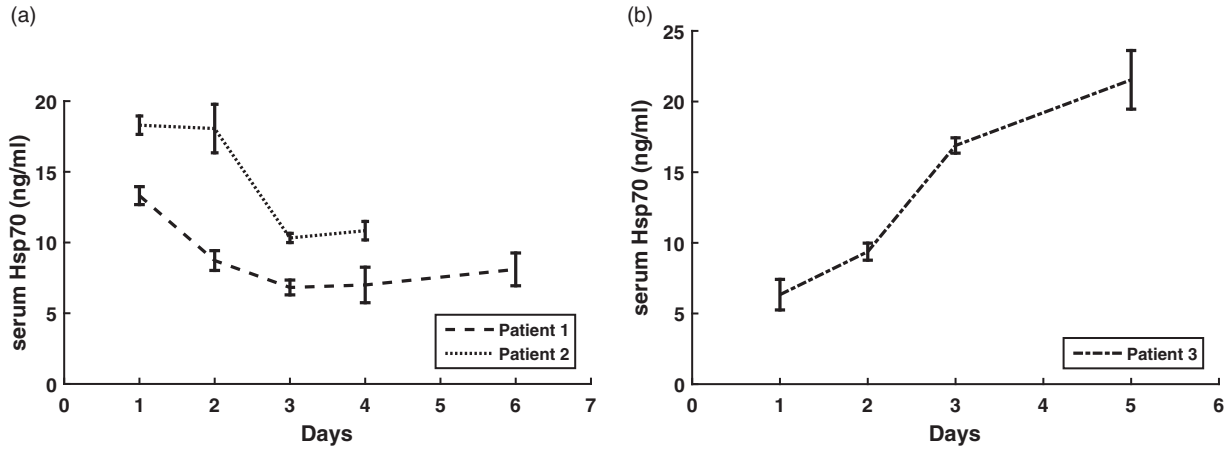


Figure 4. Serum heat shock protein 70 (Hsp70) levels of relapsed multiple sclerosis patients before and during high-dose cortisone therapy. Day 1 reflects the serum Hsp70 value of patients with acute relapse before start of the treatment. Directly thereafter the patient received a dose of 1 g methylprednisolone intravenously, which was repeated every day thereafter. The blood samples were collected after the daily drug administration. (a) Kinetics of serum Hsp70 levels of two patients showing good clinical responses to the steroid treatment. (b) Kinetics of serum Hsp70 levels of a patient showing only a minor clinical response to the steroid treatment. Data show the mean value of two independent experiments measured in duplicates ± standard deviation (SD).

However, future studies with larger patient cohorts are necessary to confirm this observation.

The origin of Hsp70 in the serum, however, could not be specified as it is released by both living and dead cells.[30] Nevertheless, since Hsp70 serum levels decreased in patients who responded to high-dose cortisone therapy, we speculate that Hsp70 might originate from activated immune cells such as T or B cells. As MS is believed to be predominantly mediated by T[31] and B cells,[32] we hypothesise that these cells might be considered as a possible source for elevated Hsp70 serum levels. The fact that liposomal Hsp70, which is believed to be actively released by living cells,[19] is increased during inflammation, underpins this assumption. However, additional studies are needed to identify the origin of extracellular Hsp70 in the serum of MS patients.

Concerning the role of Hsp70 in the pathogenesis of MS, presently there is no general consensus as to whether Hsp70 mediates beneficial or harmful effects. High cytosolic Hsp70 levels, which are known to exert cytoprotective functions, might

reduce cell death in brain cells and thus elevated cytosolic Hsp70 levels might be considered beneficial for the treatment of MS.[20] In the extracellular milieu, however, it is assumed that Hsp70 can activate and enhance inflammatory processes. Therefore, medications that can inhibit the production and release of Hsp70 might also provide a potential treatment strategy for MS.[22] In an effort to personalise this therapeutic approach, it is necessary to know the actual Hsp70 serum levels of an individual patient during the course of disease. Patients with high basal Hsp70 serum levels might respond better to Hsp70-reducing drugs than patients with low levels. As a result, serum Hsp70 levels could provide a useful biomarker in the future for monitoring inflammatory processes in MS and for an adaptation of therapy. In order to confirm serum Hsp70 levels as a diagnostic biomarker, further prospective studies are warranted.

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Conflicts of interest

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Supplementary material

Supplementary material is available for this article online.

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