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High mobility group box 1 in human serum - investigation of different analysis methods and possible associations with disease activity and fatigue in patients with systemic lupus erythematosus

by

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Ingeborg Kvivik

ABSTRACT

The *High mobility group box 1* (HMGB1) protein is a DNA-binding protein mainly located in the nucleus of cells. It can be passively or actively released to the extracellular environment in necrotic cells or activated cells in the innate immune system. Extracellular HMGB1 functions as a proinflammatory molecule and is important for activation of the innate immune system, acting alone or in complex with other molecules such as interleukin-1β, lipopolysaccharide and DNA. In cytosol HMGB1can bind foreign nucleic acids and enhance signalling through *damage associated molecular pattern* (DAMP) receptors, activate the innate immune system, and induce production of type I interferons, proinflammatory cytokines and chemokines.

Due to its strong proinflammatory properties, HMGB1 is a mediator of several different inflammatory diseases including rheumatoid arthritis, sepsis and autoimmune diseases such as primary Sjögren's syndrome and *systemic lupus erythematosus* (SLE).

Measuring HMGB1 in serum has proven to be difficult due to its "sticky nature", i.e. its ability to form complexes with other molecules. Also, anti-HMGB1 antibodies both in sera from healthy people and patients with SLE confer difficulties. The place for the HMGB1 *enzyme-linked immunosorbent assay* (ELISA) has therefore been questioned. Treating serum with *perchloric acid* (PCA) prior to the ELISA analysis has been proposed as interference modification to remove proteins, complexes and antibodies from the HMGB1 molecule itself.

The aim of this study has been to investigate available methods for HMGB1 analysis, including the PCA-ELISA, and to establish a valid and reliable assay for analyses of human sera. The methods have been applied to sera from SLE patients and healthy control subjects and HMGB1 levels have been associated with levels of disease activity and fatigue.

Our results show that serum HMGB1 concentrations measured with a conventional ELISA do not correlate with immunoblot results and the PCA-ELISA method do not improve the conventional ELISA. The preferred method for serum HMGB1 analysis is the immunoblot method.

HMGB1 levels were in our study not associated with SLE disease activity, but patients with high HMGB levels had more fatigue than those with low levels. This indicates a possible association between serum HMGB1 and fatigue in SLE patients.

ABBREVATIONS

aa	Amino acids
ACR	The American College of Rheumatology
CSF	Cerebrospinal fluid
CV	Coefficient of variance
DAMP	Damage associated molecular pattern
ELISA	Enzyme-linked immunosorbent assay
FSS	Fatigue severity scale
HC	Healthy control subjects
HMGB1	High mobility group box 1
IL	Interleukin
IFN	Interferon
LPS	Lipopolysaccharide
Mw	Molecular weight
NF-κB	Nuclear factor-kappa B
OD	Optical density
PAMP	Pathogen associated molecular pattern
PCA	Perchloric acid
pDC	Plasmacytoid dendritic cells
pSS	Primary Sjögren's syndrome
PVDF	Polyvinylidene difluoride
RAGE	Receptor for advanced glycation end products
rhHMGB1	Recombinant human HMGB1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity index
TBS	Tris-buffered saline
TLR	Toll like receptor
VAS	Visual analogue scale

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1. INTRODUCTION

During recent years it has become clear that *High mobility group box 1* (HMGB1) protein plays an important role in innate immunity. In several inflammatory diseases, including autoimmune diseases like *systemic lupus erythematosus* (SLE) and *primary Sjögren's syndrome* (pSS), the serum level of HMGB1 is increased and associated with disease activity and other disease features. Because of the interesting aspects of this protein, this study aimed to investigate if current methods for detecting HMGB1 were valid and reliable. Measuring HMGB1 in serum and *cerebrospinal fluid* (CSF) could later possibly be an important tool to explore mechanisms for cerebral manifestations in autoimmune diseases, such as cognitive disturbances and chronic fatigue.

1.1 The immune system

The immune system may arbitrary be divided in the innate and the adaptive immune system. The two parts are functionally different, but act together in the defence against invading pathogens and cell damage.

The innate immune system is the body's first line of defence and provides protection for the organism with physical barriers, such as skin and mucosa, phagocytic cells, *interferons* (IFNs), the complement system and other signalling- and effector-molecules. The innate immune system is nonspecific and relies on recognition of *pathogen associated molecular pattern* (PAMP) molecules by pattern recognition receptors on cell surfaces or in the cytoplasm of immune cells. *Damage associated molecular pattern* (DAMP) molecules is another term used to describe that the immune system can recognize a variety of molecules associated with danger to the organism. These danger signals originate from cell damage rather than from invading pathogens, for instance after tissue damage (1, 2).

The adaptive immune system is highly differentiated and able to recognize and remember specific pathogens. Receptors on the surface of B- and T-cells recognize foreign antigen specifically and after activation of the cells generate a long-lasting immunity. B-cells mediate the humoral immunity and when activated become immunoglobulin-producing plasma cells or memory B-cells. T-cells are part of the cell-mediated immunity that involves several subclasses of T-cells with different functions, such as T-helper cells, cytotoxic T-cells,

regulatory T-cells and natural killer cells. Cooperation between B- and T-cells is important during the immune reaction (3).

1.2 HMGB1

1.2.1 Structure

Almost 40 years ago, a group of proteins with high electrophoretic mobility was discovered and named *High Mobility Group* (HMG) proteins (4). One superfamily of HMG proteins includes HMGB1, -2, -3 and -4. They are DNA-binding proteins consisting of two HMG Box domains, A and B. The HMG Box domain is a well conserved functional region of the protein, approximately 75 *amino acids* (aa) long and forming three α-helixes folded into a L-shape (5, 6). HMGB1, -2 and -3 have a C-terminal acidic and negatively charged tail of 30 aa (mainly aspartic- and glutamic- acid).

HMGB1 is expressed in all vertebrate cells, yeast, plant and bacteria, and is well conserved between species with > 98 % sequence identity in mammals. The protein consists of 216 aa with a predicted *molecular weight* (Mw) of 28 kDa, Figure 1.1 (7). Several post-translational modifications of the protein such as methylation, phosphorylation, acetylation and oxidation has been reported, the latter two important for extraction to extracellular environment and inflammatory functions respectively (8-11).

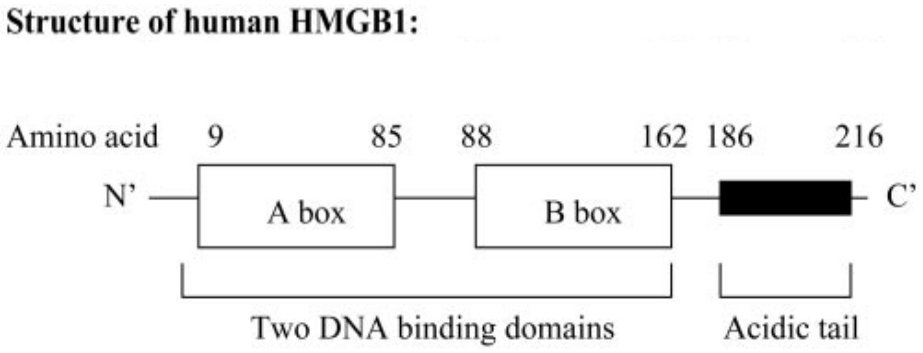

Figure 1.1: Schematic structure of human HMGB1 protein showing the position of the two HMG-Box domains A and B, and the C-terminal acidic tail (7).

An extracellular function of HMGB1 was discovered as a late mediator of endotoxin lethality in mice (12). Since then, the protein's role in inflammation has been extensively investigated. Possible extracellular functions for the other HMGB proteins have not yet been explored.

1.2.2 Intranuclear function

HMGB1 is abundant and mainly located in the nucleus of almost all human cells. Its intranuclear functions are regulation of gene transcription and maintenance of nucleosome structure. The L-shape of HMG Box-A and -B can bind to parts of DNA with limited or no sequence specificity. This binding will bend the DNA and lead to an allosteric transition which can promote the binding of other proteins to DNA (5, 13).

1.2.3 Extracellular function

Extracellular HMGB1 is a typical DAMP molecule and an important mediator of inflammation and tissue regeneration (14, 15). The protein can be released from cells in three different ways, Figure 1.2:

1. From cells of the innate immune system such as monocytes, macrophages and dendritic cells that are activated by inflammatory cytokines or *lipopolysaccharide* (LPS) (12, 16). This activity has also been demonstrated in other cells: hepatocytes, endothelial cells, glial cells and neurons (reviewed in (17)).
2. Necrotic cells will leak HMGB1 upon cell damage and permeabilized cell membrane (16).
3. Apoptotic cells that escape clearance by macrophage phagocytosis and reach late apoptosis (secondary necrosis) can leak HMGB1 to the extracellular milieu (18, 19). HMGB1 will either be oxidized or tightly bound to chromatin (16, 20).

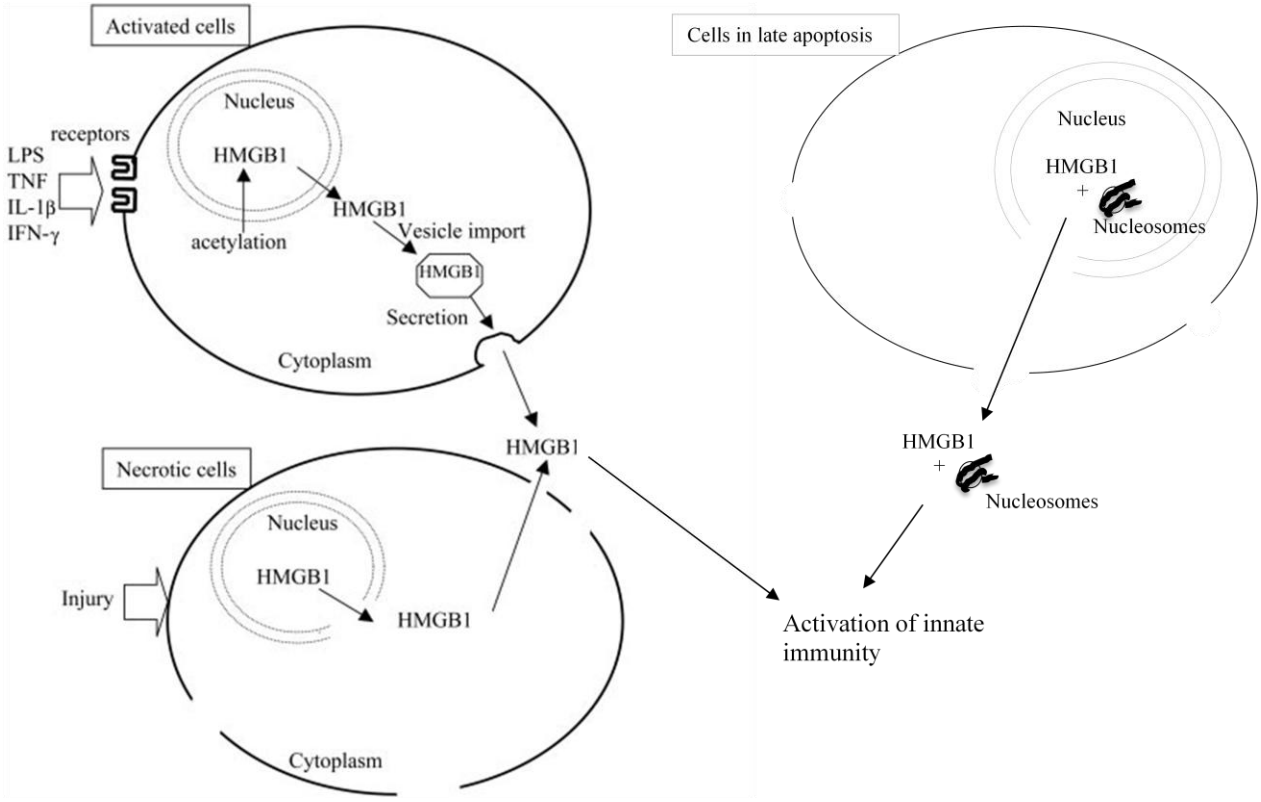

Figure 1.2: Extracellular release of HMGB1.
Upper left: Active release from stimulated cells of the innate immune system. Lower left: Passive release from necrotic or damaged cells. Right: Passive release from cells in late apoptosis. HMGB1 is oxidized and tightly bound to chromatin (Modified from Yang et al (7)).

Upon release from cells, HMGB1 will bind to cell-surface receptors on monocytes, macrophages and dendritic cells, such as the *receptor for advanced glycation end products* (RAGE), *toll like receptor* (TLR) 2 and TLR4 (14, 21), Figure 1.3. Receptor binding leads to activation of the transcription factor *nuclear factor-kappa B* (NF-κB) which induces production of multiple proinflammatory molecules such as *tumor necrosis factor* (TNF)-α, *interleukin* (IL)-1β, IL-6, IL-8, etc. (22).

Signalling through cell surface receptors cannot alone explain all the downstream effects of HMGB1. Different biological effects may be related to whether it is complexed or free. HMGB1 will easily form complexes with IL-1β, nucleosomes, LPS and DNA which interacts with receptors like IL-1R, TLR2, TLR4, and TLR9 respectively, Figure 1.3. HMGB1-

complexing has an enhancing effect on the activation of the receptors and the immune response (23, 24)**.** This complex-binding ability is probably the source of analytical difficulties in various immunological assays (15, 25).

1.2.4 Cytosolic function

HMGB1, -2 and -3 have recently been found to have a cytosolic function in cells of the innate immune system (26). They will bind foreign immunogenic nucleic acids and activate innate immune responses via endosome-based cytosolic receptors like TLR3, TLR7 and TLR9. RNA- and DNA- sensing receptors in the cytosol like *Rig-I like receptors* (RLRs), *melanoma differentiation-associated protein 5* (MDA5), *DNA-dependent activator of IFN regulatory factors* (DAI) and *absent in melanoma 2* (AIM2) will trigger the innate immune system and induce production of type I IFN, proinflammatory cytokines and chemokines, Figure 1.3 (27). It has been shown that absence of cytosolic HMGB-proteins will reduce induction of type I IFN and cytokines by foreign DNA and RNA (reviewed in (19, 28)).

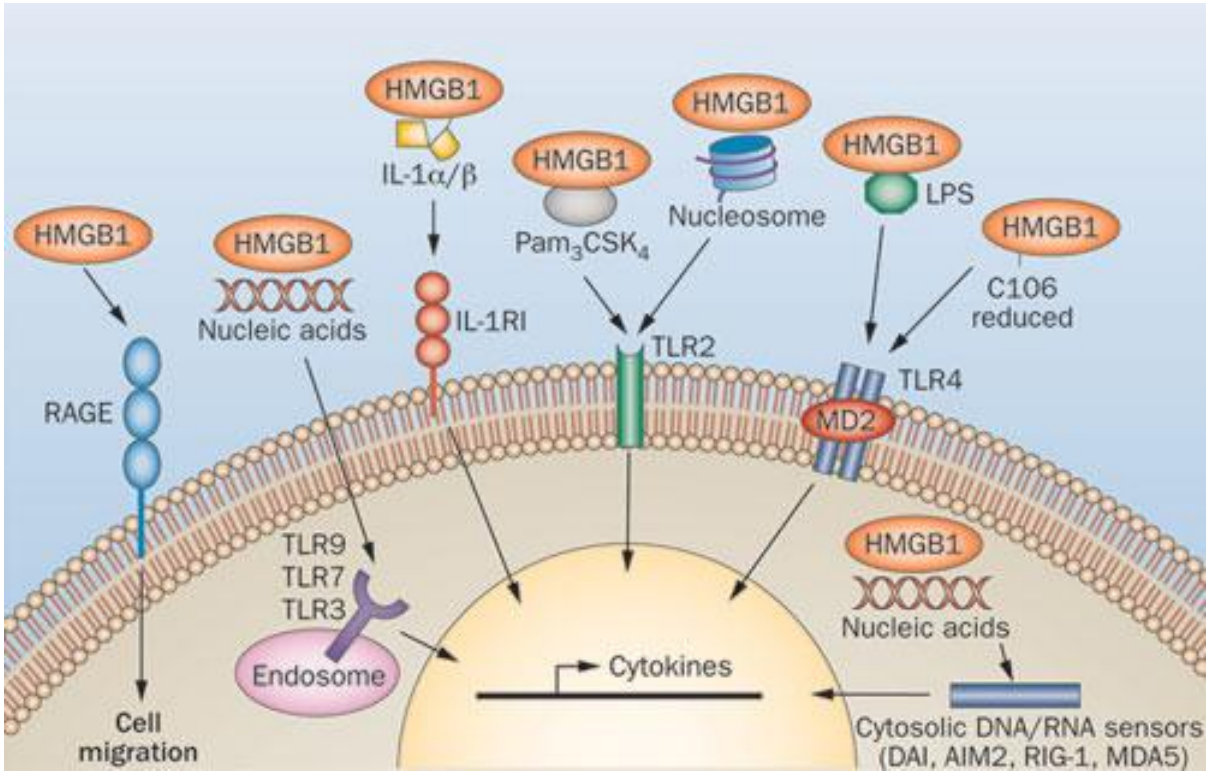

Figure 1.3: Signalling by HMGB1.
HMGB1 is multifunctional and mediates inflammation by signalling through a variety of receptors, alone or complexed with other proinflammatory signalling molecules (27).

1.2.5 Oxidation of cysteine residues

Activation of innate immune cells such as macrophages and granulocytes which can induce excessive generation of *reactive oxygen species* (ROS) can lead to imbalance between the physiological anti-oxidant defences and ROS. This condition of oxidative stress with excess ROS in the extracellular environment can influence the pro-inflammatory activity of HMGB1 by oxidizing the *cysteine in position 106* (C106). C106 has to be in the reduced form as a thiol group for HMGB1 to act alone and signal though TLR4 as a cytokine-inducing molecule. Two other cysteine residues in position 23 (C23) and 45 (C45), can form an intramolecular disulfide bond which is also required for HMGB1 to induce nuclear NF-κB translocation and TNF production in macrophages (11, 29). The unpaired C106 can theoretically form a disulphide bridge with another C106 residue on a second HMGB1 molecule. Such HMGB1 dimers have been observed in some preparations of recombinant HMGB1, but show no cytokine-stimulating activity (11). Redox status seems not important when HMGB1 is complexed to other molecules (23).

1.2.6 HMGB1 and diseases

HMGB1 plays an important role in the pathogenesis of acute and chronic inflammation. Increased levels are found in viral and bacterial infections, sepsis and septic shock, cancer and autoimmune diseases. In chronic inflammatory diseases such as rheumatoid arthritis, SLE and pSS, elevated HMGB1 levels has been found in both serum (30, 31) and in synovial fluid (32), skin lesions(33) and salivary glands (34), respectively. Stroke, epilepsy and atherosclerosis are conditions in which HMGB1 is reported to be operative, also (reviewed in (19)).

1.3 SLE

SLE is a chronic autoimmune disease characterised by systemic inflammation and damage in multiple organs such as skin and the renal, nervous, cardiovascular and haematopoetic system. In Scandinavia the prevalence is 60-70 per 100,000 (35). There is a predominance of women to men; 9-10:1 (36).

1.3.1 Diagnosis

The diagnosis of SLE is based on certain clinical and laboratory findings. *The American College of Rheumatology* (ACR) has established classification criteria for SLE mainly to be used in research (37), Appendix 1. One of the apparent clinical criteria is the malar rash/butterfly rash over the patient's nose and upper chin, Figure 1.4.

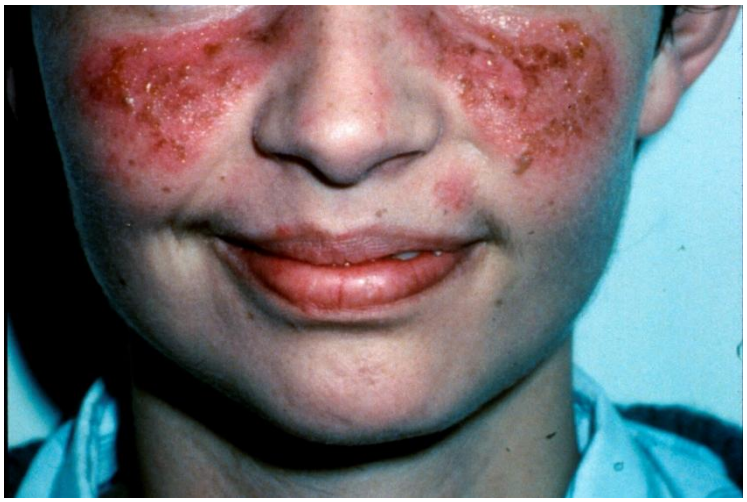

Figure 1.4: Malar rash/butterfly rash.
The picture shows a SLE patient with an apparent malar rash over the nose and upper chin.

Laboratory features are *antinuclear autoantibodies* (ANA) demonstrated in the majority of patients, and the more specific subgroup of ANA, anti-DNA antibodies (found in 60 % of the patients) (38). A variety of other autoantibodies also occur.

1.3.2 Pathogenesis

In autoimmune diseases the body's immune system loose "self-tolerance" and produces autoantibodies and/or cytotoxic T-cells directed against the body itself. This immune reaction leads to inflammation which eventually results in cell damage. Polyclonal activation of B-cells results in an abundance of autoantibodies. The activation and survival of the B-cells is among other factors enhanced by reduced T-regulatory cell function and increased production of B-lymphocyte stimulator (39).

Genetic factors are important for the pathogenesis of the disease, but additional factors like hormones and environment also contributes. The mechanisms involved in breaking tolerance against self components are not fully understood.

Many of the clinical manifestations in SLE are caused by antibodies directed to cell surface components in various tissues and immune complex formation. These complexes activate complement, macrophages and dendritic cells and cause tissue damage (reviewed in (19)). A prominent phenomenon in SLE is also increased cell death (apoptosis) and impaired clearance of apoptotic material which serve as a continuous source of autoantigens (40, 41). The apoptotic cell may undergo secondary necrosis and release autoantigens like DNA, core histones and chromatin (38).

1.3.3 Sickness behaviour and fatigue

The complex behaviour observed in animals exposed to infection or inflammation is called sickness behaviour. It is characterized by decreased activity, drowsiness, loss of appetite and social withdrawal (42). Sickness behaviour can be regarded an automated and beneficial behaviour that will serve to protect the sick and vulnerable animal by not being exposed to predators.

Fatigue in humans can be considered a component of sickness behaviour. It can be described as an overwhelming sense of tiredness, lack of energy and feeling of exhaustion (43). Patients with SLE frequently complain of fatigue having a significant impact on quality of life. Conflicting observations have been made on associations between fatigue and SLE disease activity (44-46). Our group previously revealed that cerebral white matter hyperintensities in SLE were associated with fatigue (47). The pathophysiological processes and the biological disease factors for fatigue in SLE are still largely unknown, and are most likely a result of a complex response to disease (48).

1.4 HMGB1 and SLE

Inflammation caused by immune complexes formed by nuclear antigens and autoantibodies is central to the pathogenesis of SLE. During SLE flares, a strong immune activation and inefficient clearance of apoptotic cells lead to free- and nucleosome-complexed HMGB1 that activate production of proinflammatory cytokines (40). The HMGB1-nucleosome complexes will also facilitate autoantibody formation against DNA and nucleosomes (40, 49).

Ongoing production of type I IFNs, especially IFN-α, by *plasmacytoid dendritic cells* (pDC) is central in the pathogenesis of SLE. Type I IFNs promotes maturation of DCs and activation and differentiation of B-cells (50). In immune cells like macrophages and pDCs, cytosolic- (RLR, MDA5, DAI and AIM2) and endosomal transmembrane-receptors (TLR3, TLR7 and TLR9) induces production of type I IFN when activated by DNA or RNA. The increased presence of DNA-containing HMGB1 complexes in SLE will enhance this activation. HMGB1-nucleosome complexes will also stimulate IFN-α production in pDC when incubated together with anti-DNA antibodies (reviewed in (19, 28, 49)).

Several studies have found increased levels of HMGB1 in the blood of patients with SLE (51-53), and correlation with SLE disease activity (41, 54, 55). Some of the recent results are summarized in Table 1.1.

The HMGB1 present in SLE serum is mainly oxidized and partially bound to nucleosomes, probably derived from cells in late apoptosis (20, 40). Oxidation might generate neo-epitopes, which could promote immune responses to HMGB1 (56). High levels of autoantibodies to HMGB1 (anti-HMGB1 antibodies) are frequently present in serum from SLE patients and patients with other autoimmune diseases (51). They seem to be associated with SLE disease activity and with levels of HMGB1 (41, 57). It has been demonstrated that multiple HMGB1 epitopes are recognised by autoantibodies, with the major epitopes mapping to box A and the joiner region (57). Lower concentrations of anti-HMGB1 antibodies occur in serum from healthy subjects, and preliminary data indicate that the antibodies play important regulative roles for cytokine release and inflammation induced by HMGB1 (51). Several groups have observed that the presence of anti-HMGB1 antibodies in serum interfere with HMGB1 detection by ELISA (41, 51). This makes serum HMGB1 detection especially difficult in patients with autoimmune diseases.

Table 1.1: Studies reporting HMGB1 levels in blood of SLE patients.

	SLE patients		Healthy subjects			
	HMGB1, ng/ml	**No. of subjects**	**HMGB1, ng/ml**	**No. of subjects**	**Association to SLEDAI**	**Method**
Zickert et al 2012 (53)	108.4 (SD ± 48.0) [a]	20	13 (SD ± 10)	48	NA	WB
Abdullahad et al 2011 (41)	6.2 (range 1.3-32.3) (SLEDAI < 4)	33	2.9 (range 0-7.7)	35	Yes (negative association)	ELISA [b]
	2.3 (range 0.95-12.5) (SLEDAI ≥ 4)[c]	19				
	1.2 (range 0-47.2) (SLEDAI ≥ 4)[d]	18				
Abdullahad et al 2011 (41)	51 (range, 28-121) [e] (SLEDAI < 4)	33	43 (range, 7-85) [e]	35	Yes	WB
	135 (range, 55 to 496) [e] (SLEDAI ≥ 4)	37				
Ma et al 2010 (55)	4.42 (SD ± 1.97) (SLEDAI ≤ 4)	11	4.45 (SD ± 1.59)	39	Yes	ELISA[b]
	6.44 (SD ± 4.18) (SLEDAI > 4)	26				
Li et al 2010 (54)	Median 27 , IQR 68	39	Median 0, IQR 0	24	Yes	WB
Urbonaviciute 2007 (51)	Range (0.4-190)[f]	27	Range (0.4-40)[f]	22	NA	ELISA[g]

NA, Not applicable; ELISA, Enzyme linked immune sorbent assay; WB, Western blot; SLEDAI, SLE disease activity index; IQR, interquartile range.
[a] Lupus nephritis; [b] HMGB1 ELISA from Shino-test (Tokyo, Japan); [c] Non-renal; [d] Renal; [e] Intensity-ratio; [f] Median not reported, [g] In-house ELISA

1.5 HMGB1 and sickness behaviour

Probably fatigue is induced by proinflammatory proteins in the *central nervous system* (CNS). LPS injections in the brain of mice and injections of IL-1β in rats upregulates the production of proinflammatory cytokines in the brain and induces sickness behaviour (reviewed in (42)). Interestingly, intracerebroventricular injections of HMGB1 induce an increase in IL-1β, IL-6 and TNF expression in the mouse brain and induce aphagia, taste aversion and fever (58, 59). This indicates that HMGB1 can be a mediator of sickness behaviour and fatigue. It has also been postulated that increased HMGB1 in the brain can impair learning and memory in mice through TLR4 and RAGE activation (60).

1.6 HMGB1 assays

1.6.1 Immunoblot

Immunoblot (Western blot) is widely used for detection of HMGB1 in serum or other biological fluids. Generally serum is diluted 1:20-50 and separated under reducing conditions. Some research groups use filtration and ultracentrifugation of serum, probably to reduce the amount of large proteins (61, 62). Proteins in serum are then separated according to Mw by *sodium dodecyl sulphate polyacrylamide gel electrophoresis* (SDS-PAGE) followed by electrophoretical transfer to a *polyvinylidene difluoride* (PVDF) membrane or a *nitrocellulose* (NC) membrane (63). The membrane is incubated with primary antibodies directed towards HMGB1 followed by incubation with an enzyme-labelled secondary antibody. When adding a substrate, HMGB1 can be visualized as a band with a specific Mw, Figure 1.5.

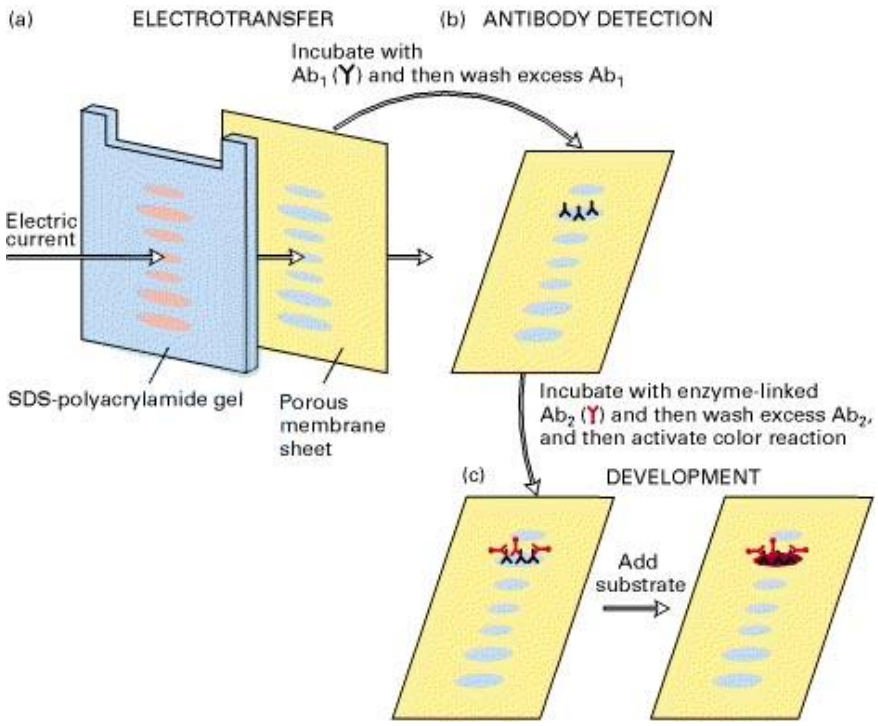

Figure 1.5: The principal steps of the immunoblot method.
Proteins are separated with SDS-PAGE, blotted on to a membrane and incubated with primary antibodies against the proteins of interest. When incubated with an enzyme-linked secondary antibody and a substrate is added, the proteins are visualized as bands at specific Mw (64).

1.6.2 Enzyme-linked immunosorbent assay (ELISA)

The other widely used method for measuring HMGB1 concentrations is a commercial HMGB1 ELISA (Shino-test, Tokyo, Japan). A purified anti-HMGB1 antibody is used to coat the wells of a microtiter plate. When samples are added to the wells, HMGB1 will bind specifically to the immobilized antibody. A secondary enzyme-labelled antibody will bind the HMGB1. When adding as substrate, the colour will change proportionally with the concentration of HMGB1. The colour intensity is measured with a photometer and related to a standard curve of known HMGB1 concentrations, Figure 1.6.

The use of HMGB1 ELISA for serum measurements has been questioned by several research groups (41, 51, 57, 65, 66) and it has been shown that the method is not able to detect complexed HMGB1 (41). It has also been implied that anti-HMGB1 antibodies interferes with HMGB1 detection by ELISA (51).

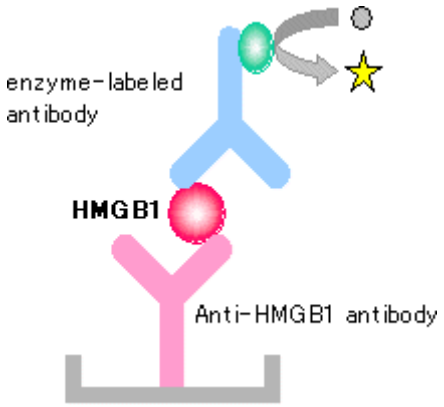

Figure 1.6: The reaction in the well of an HMGB1 ELISA plate.
Anti-HMGB1 antibodies immobilized in the well bind HMGB1 in the sample. A secondary enzyme-labelled antibody detects the captured HMGB1 protein and the enzyme will change the colour of an added substrate (67).

1.6.3 Perchloric acid (PCA)-ELISA

Recently, Barnay-Verdier et al (65) demonstrated that incubation of recombinant human HMGB1 with IL-1β, LPS or rabbit anti-HMGB1 antibodies significantly reduced the amount of protein detected by conventional ELISA. They reported to have developed an improved ELISA method for quantifying both free and complexed forms of HMGB1. HMGB1 stays soluble in strong acid due to its long acidic tale (68). HMGB1 complexes and HMGB1-bound

proteins are dissolved by 3% PCA and unbound HMGB1 can be measured by an ELISA method.

1.7 Objectives

The objectives of this study have been

1. Investigate the properties of available methods for measuring HMGB1 and establish a valid and reliable assay for human serum .
2. To investigate if the PCA-ELISA method is an improved, valid and reproducible method.
3. Apply the methods to analyze serum samples from patients with SLE and healthy controls.

The knowledge and skills obtained could later be used to explore a possible role of HMGB1 for cerebral manifestations in autoimmune diseases, such as cognitive disturbances and chronic fatigue.

2. MATERIALS AND METHODS

2.1 Subjects

The SLE patients and *healthy control subjects* (HC) in this study are from a larger cohort participating in a study of cerebral manifestations in autoimmune diseases conducted at Stavanger University Hospital. All clinical data and serum samples were collected from 2003 to 2005.

Sixty-seven SLE patients - 58 (87%) women and 9 (13%) men - fulfilling the revised ACR criteria for SLE (37) underwent clinical examination, fatigue registration and blood sampling. Mean (± SD) age was 43.9 (13.8) years (range 20-76 years) and mean (± SD) disease duration was 12.6 (8.6) years (range 1-32 years). Disease activity was measured with the *SLE disease activity index* (SLEDAI). This gives a composite measure of SLE disease activity during the last 10 days (69), Appendix 2. Patients' median SLEDAI score was 2 (range 0-26). Also, serum samples from 23 healthy control subjects – 20 (87%) women and 3 (13%) men - with a mean (± SD) age of 43.3 (12.9) years (range 24-64 years) were collected.

The study was approved by the *Regional ethics committee* (REK III nr. 22.02). All subjects gave informed consent for participation.

2.2 Sample material

Serum samples were collected from venous blood drawn into vacutainers with no additives. After coagulation for 30 min in room temperature the samples were centrifuged for 10 min at 1,300*g*. Serum was separated and stored in aliquots at -70 °C. None of the serum samples had visible haemolysis. Before analyzing HMGB1, the serum samples had been thawed once.

2.3 Routine laboratory analysis

Routine haematological, biochemical, and immunological tests were analysed in the hospital's laboratories. The HEp-2000 assay (Immunoconcepts, Sacramento, CA, USA) was used for detection of antinuclear antibodies (ANA) and Nova Lite dsDNA Crithidia luciliae 708200 indirect immunofluorescence assay (NOVA Diagnostics, San Diego, CA, USA) for anti-DNA antibodies.

2.4 Fatigue measurements

Fatigue was measured using the *Fatigue severity scale* (FSS) (70) and a fatigue *visual analogue scale* (VAS), Appendix 3 and 4. FSS is a questionnaire with 9 questions. Patients are asked to relate the questions to the last two weeks. The FSS score is the mean of the sum of scores in the 9 items, each rated from 1-7. A FSS score of 3 is commonly applied as a cut-off value for fatigue in SLE (70).

The fatigue VAS is a 100 mm horizontal line with anchoring lines, and with the statement "No fatigue" at the left (0 mm) and "Fatigue as bad as it can be" on the right (100 mm). The patients are asked to rate their fatigue at present with a mark on the horizontal line. The distance (mm) from the left anchoring line to the mark represents the patients VAS score.

2.5 HMGB1 measurements by immunoblot

All patient and HC samples were analyzed with the immunoblot method. The procedure had through several weeks been optimized in terms of membrane type, transfer method, wash buffer, blocking agent and incubation times. Different dilutions of primary and secondary antibody had also been evaluated. Serum treatments like filtration and ultracentrifugation as described by others had been tried without enhanced detection of HMGB1.

Samples from SLE patients and HC were randomly distributed for analysis on different gels.

2.5.1 Reagents

Chemicals used in this study are listed in the table below, Table 2.3.

Table 2.3: Chemicals used for immunoblot

Name	Supplier
Ammoniumpersulfate (APS) for electrophoresis, ≥ 98 %	Sigma-Aldrich (St. Louis, MO, US)
N,N,N′,N′-Tetramethylethylenediamine (TEMED), BioReagent, suitable for electrophoresis, ~99 %	
Glycine for electrophoresis	
Bromphenolblue sodium salt for electrophoresis	
β-Mercaptoethanol for electrophoresis, ≥ 98 %	
Albumin, from bovine serum	
Trizma® base	
Acrylamide-bis, ready-to-use solution 30%	Merck (Darmstadt, Germany)
Methanol	
Skim milk powder for microbiology	
Hydrochloric Acid (HCl), fuming 37%	
Tween® 20	
Sodium chloride (NaCl)	VWR International/BDH (Radnor, PA, US)
SuperSignal®WestFemto Maximum Sensitivity Substrate	Pierce (Thermo Fisher Scientific, Waltham, MA, US)
BupH ™ Phosphate Buffered Saline Packs	
Sucrose 99.7 %	DUCHEFA Biochemie B.V. (Haarlem, Nederlands)
Sodium dodecyl sulphate (SDS) solution	Fluka/ Sigma-Aldrich (St. Louis, MO, US)

2.5.2 Stock solutions

The following stock solutions were made:

6 X Loading buffer with 12 % β-mercaptoethanol (reducing conditions):

7.5 g	sucrose
0.3 ml	10 % Bromphenolblue
3 g	SDS
9.38 ml	1 M Tris-HCl, pH 6.8
3 ml	β-mercaptoethanol

Add H_2O up to 25 ml.

Separation gel (15 %), 40 ml

9.4 ml	H_2O
20 ml	30 % Acrylamide mix
10 ml	1.5 M Tris-HCl, pH 8.8
0.2 ml	20 % SDS
0.4 ml	10 % APS
0.016 ml	TEMED

Stacking gel (5%), 10 ml

6.85 ml	H_2O
1.7 ml	30 % Acrylamide mix
1.25 ml	1 M Tris-HCl, pH 6.8
0.05 ml	20 % SDS
0.1 ml	10 % APS
0.01 ml	TEMED

10 X Running buffer, 1000 ml

144 g	Glycine
30 g	Trizma base
10 g	SDS

Add H_2O up to 1000 ml.

Towbin Transfer Buffer (pH 8.3- 8.5), 1000 ml

14.4 g	Glycine
3.03 g	Trizma base
200 ml	Methanol

Add H_2O up to 1000 ml.

10 X *Tris-buffered saline (pH 7.6) with Tween20* (TBST), 1000 ml

61 g	Trizma base
88 g	NaCl
5 ml	Tween 20

Add H_2O up to 1000 ml.
Adjust pH with HCl to pH 7.6.

Phosphate buffered saline (PBS), 1000 ml
1 pack of BupH ™ Phosphate Buffered Saline was dissolved in a total of 1000 ml H_2O.
0.5 ml Tween20 was added to 1000 ml PBS to make PBST.

2.5.3 Gel electrophoresis

A 15 % SDS-PAGE gel was prepared with a 5 % stacking gel using gel casting-equipment from Bio-Rad (Hercules, CA, US). All samples and calibrators were separated on the gel with the Mini-PROTEAN® Tetra Cell system (Bio-Rad, Hercules, CA, US). HMGB1 calibrator (standard from the HMGB1 ELISA kit) was diluted 1:16 and 1:64 and used as positive control and reference. In initial testing of the procedure a *recombinant human HMGB1* (rhHMGB1) (R&D Systems Europe, Abingdon, England) was used as positive control. Serum samples were diluted 1:20 with H_2O before Loading buffer (6x) was added. The sample was then heated at 99°C for 5 min and a spun down quickly. 18 µl of sample with loading buffer was then added to each well in the SDS-PAGE gel. A serum sample from one SLE patient was analyzed on each gel as an internal control. A protein ladder (2.5 µl) was loaded into one of the wells (PageRuler™Plus Prestained Protein Ladder SM1811/SM1812, Thermo Scientific, Waltham, MA, US). The gel was run for 15 min at 50 V and 1 h at 150 V with 1X running buffer.

2.5.4 Immunoblotting

The separated proteins were blotted on to a PVDF membrane (Immunoblot ™, 0.2µm, Bio-Rad, Hercules, CA, US) using the Bio-Rad Mini Trans-Blot® electrophoretic transfer cell and Towbin transfer buffer. The membrane was soaked in methanol for 5 min and Towbin buffer for 10 min before blotting. The blotting was run with constant 100 V and maximum 350 mA for 1 h while the buffer was kept cool with a cooling element.

After transfer blotting the membrane was blocked with *5 % milk in TBST* (TBST-M) for 1 h. Primary antibody (Human HMGB1 Antibody Monoclonal Mouse IgG2B, MAB1690, R&D Systems Europe, Abingdon, England) was diluted to 1µg/ml in TBST-M. The membrane was incubated with the primary antibody for 1 h in RT and over night in 4°C, washed with TBST 3 x 10 min before 1 h incubation with secondary antibody (1:25 000) (mouse IgG HRP-linked whole antibody, GE Healthcare, Piscataway, NJ, US) in TBST-M. The membrane was then washed 3 x 5 min with TBST.

2.5.5 Scanning and analyzing

SuperSignal®WestFemto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, US) was added to the membrane for detection with *enhanced chemiluminescence* (ECL). ECL-labelled bands were visualized with a Gel-doc (Bio-Rad, Hercules, CA, US) with CCD camera.

The optical band intensity of each band on the blot was measured using the image analysis software ImageJ 1.45s (71). In each blot, a sample's level of HMGB1 was expressed as a ratio between the band intensity of the sample and one of the calibrators.

2.6 HMGB1 measurements by ELISA

A commercial HMGB1 ELISA was used for quantification of serum HMGB1 in 67 SLE patients and 23 healthy controls. The kit is manufactured by IBL-international (Hamburg, Germany) under the license of Shino-Test Corporation (Tokyo, Japan). The kit offers two measurement ranges; "Normal range" (2.5-80 ng/ml) and "high sensitive range" (0.313-10 ng/ml). The procedure for "normal range" was followed. Limit of detection (mean signal of zero-standard + 2.6 SD) was reported by the manufacturer to be 1.0 ng/ml.

2.6.1 Reagents and materials

Reagents and materials included in the HMGB1 ELISA kit are listed in Table 2.1. All reagents were allowed to reach room temperature before the analyses were performed. Dilutions of reagents were made according to the manufacturer's instructions (72). The reconstituted standard, positive control and enzyme conjugate were all aliquoted in polypropylene tubes and stored at -70 °C until analysis.

Table 2.1: Contents of the HMGB1 ELISA kit.

Name	Description	Preparation
96-well microtiter plate	Coated with anti-HMGB1 antibodies (polyclonal).	
Enzyme conjugate (lyophilized)	Anti-HMGB1 antibodies conjugated to peroxidase	Reconstituted in 12 ml enzyme conjugate diluent
Standard (lyophilized)	320 ng HMGB1(pig)	Reconstituted in 1 ml diluent buffer, final concentration 320 pg/ml.
Positive control (lyophilized)	9-26 ng HMGB1 (pig)	Reconstituted in 1 ml diluent buffer, final concentration 9-26 pg/ml
Diluent buffer	Buffer, 0.01 % NaN_3	
Enzyme conjugate diluent	Buffer	
Wash buffer, concentrate (5x)	Phosphate buffer, < 0.5 % Tween 20	Diluted 1:5 with H_2O
Colour reagent A	*3,3',5,5'-tetramethylbenzidine* (TMB) substrate	
Colour reagent B	Buffer with 0.005 M H_2O_2	
Colour Stop Solution	0.35 M H_2SO_4	

2.6.2 Procedure

Serum samples were analyzed in triplicates, and samples from SLE patients and HC were randomly distributed to different plates. The analyses were carried out following the manufacturer's instructions for a normal measurement range:

1. The standard curve was prepared according to Table 2.2

Table 2.2: Preparation of standard curve

HMGB1 concentration (ng/ml)	Dilution method	Standard
80	Add 100 µl of standard solution (320 ng/ml) to 300 µl of Diluent buffer and mix.	7
40	Add 100 µl of Standard 7 to 100 µl of Diluent buffer and mix.	6
20	Add 100 µl of Standard 6 to 100 µl of Diluent buffer and mix.	5
10	Add 100 µl of Standard 5 to 100 µl of Diluent buffer and mix.	4
5	Add 100 µl of Standard 4 to 100 µl of Diluent buffer and mix.	3
2.5	Add 100 µl of Standard 3 to 100 µl of Diluent buffer and mix.	2
0	Diluent buffer only.	1

2. The serum samples were thawed on ice, spun down quickly and kept on ice until analysis.
3. 100 µl of diluent buffer was added to the wells of the microtiter plate
4. 10 µl of standard, controls and serum samples were added in triplicate to the respective wells. The plate was shaken briefly on a plate shaker.
5. The plate was covered with adhesive foil and incubated for 22 h at 37 °C.
6. The plate was washed 5 times with 400 µl wash buffer.
7. 100 µl enzyme conjugate was added to each well and the plate was incubated for 2 h at 25 °C, covered with adhesive foil.
8. The plate was washed 5 times with 400 µl wash buffer.
9. 100 µl colour solution was added to each well. The colour solution was made by mixing equal amounts of colour reagent A and colour reagent B.
10. The plate was then incubated for 30 min at room temperature.
11. 100 µl of stop solution was added to stop the colour reaction. The plate was shaken gently.
12. *Optical density* (OD) was measured with a photometer (Multiskan ascent, Thermo Fisher Scientific, Waltham, MA, US) at 450 nm and at 630 nm for correction.

13. OD measured at 630 nm was subtracted from OD measured at 450 nm before the ODs were used in further calculations.
14. A standard curve was made by four-parameter logistic analysis of the calibrators and used to determine HMGB1 concentrations in the serum samples. Calculations were done with Ascent software (Thermo Fisher Scientific, Waltham, MA, US).

2.7 HMGB1 measurements by PCA-ELISA

The PCA-ELISA is an assay where the serum samples are pretreated with perchloric acid prior to analyses by the commercial ELISA described above (paragraph 2.5.2). The PCA pretreatment was performed according to the previously described method (65, 68).

2.7.1 Reagents

A working solution with 13.7 % PCA was prepared by adding 1.26 ml 70 % PCA (Fluka/ Sigma-Aldrich, St.Louis, MO, US) to 8.69 ml H_2O. 1 M *sodium hydroxide* (NaOH) (BDH, Poole England) was used for neutralization.

Four acidic solutions were prepared for alternative testing: 2.9 % PCA (0.25 ml 13.7 % PCA in 1 ml H_2O), 5.7 % PCA (0.5 ml 13.7 % PCA in 0.75 ml H_2O), 0.1 M citric acid ($C_6H_8O_7$, assay < 99.5 %, Merck, Damstadt, Germany) and 0.05 M citric acid.

2.7.2 Procedure

All serum samples, controls and standards were pretreated in the following way before ELISA measurements:

1. 25 µl 13.7 % PCA was added to 100 µl sample and mixed well (Total PCA content in samples was 3 %).
2. The samples were centrifuged at 13,000*g* for 5 min at 4 °C
3. 50 µl of the supernatant was pipetted off and 10 µl 1 M NaOH was added to neutralize the pH.
4. The acid and NaOH increased the total volume of the samples by 50%. To adjust for this increase, the volume of sample added to each well was increased by 5 µl and the volume of diluent was decreased by 5 µl. Hence, 15 µl sample, control or standard were added in triplicates to 95 µl diluent buffer in the wells of the microtiter plate. The plate was shaken briefly on a plate shaker.

5. At this point volume of each sample was increased by 50 %. This was adjusted by increasing the volume added to the wells on the plate.

The procedure was completed according to the manufacturer, as described in steps 5-14 in paragraph 2.5.2.

2.8 Statistics

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, US).

Results are reported as median and range, otherwise as mean ± *standard deviation* (SD) if data are normally distributed. Differences between two groups of quantitative data were analyzed with Mann-Whitney U test for non-parametric data, and Student t-test for normally distributed data. Correlation between two methods was assessed by simple correlation (Spearman) and agreement by Bland-Altman plot. Simple linear regression was used to test associations between one dependent and one independent quantitative variable. Kruskal Wallis test was used for one-way-analysis-of-variance between three groups of non-parametric data with Dunn's multiple comparison tests for post-hoc analysis. Statistical significance was defined by $p < 0.05$.

3. RESULT

3.1 HMGB1 measurements by Immunoblot

HMGB1 was visualized in serum samples with a band on an immunoblot at approximately 28 kDa, Figure 3.1. There were also signals from proteins at 50-55 kDa. The possibility that these bands resulted from unspecific binding by the secondary antibody was eliminated by performing an immunoblot without primary antibody. The bands were then not visible compared to the immunoblot with primary antibody, Figure 3.1. The intensity of the band at 28 kDa was used in the measurements.

Initially, rhHMGB1 (30-35 kDa) was used as a positive control, but the concentration of recombinant protein was lower than stated by the manufacturer, and it had to be added in large amounts (> 100 ng/well) to be detected, Figure 3.1. We found after several experiments that the HMGB1 calibrator from the ELISA kit performed better as antigen in our immunoblot compared to the recombinant protein.

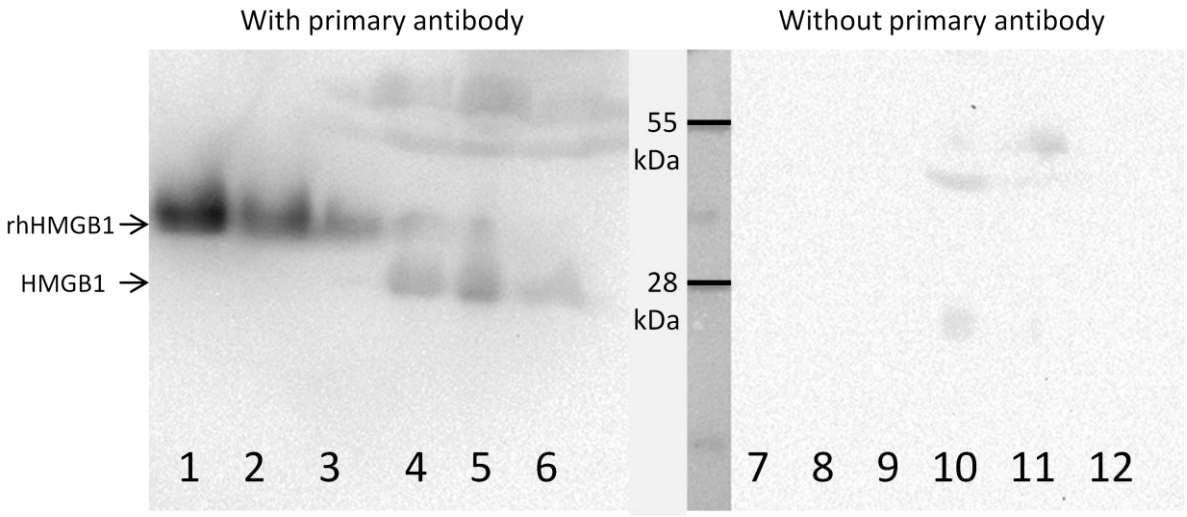

Figure 3.1: HMGB1 visualized on immunoblot.
The left immunoblot was incubated with primary antibody as described in the procedure, the right immunoblot was incubated without primary antibody. The respective samples in lane 1-6 and 7-12 are identical. Lane 1-3: rhHMGB1 (R&D systems) 400 ng, 200 ng and 100 ng. Lane 4: serum from SLE patient (JH). Lane 5: serum from SLE patient (ER). Lane 6: CSF from SLE patient (JH).

To assess inter-assay variance of the immunoblot assays, an in-house serum control was analyzed on each blot (mean 0.366, SD ± 0.03). The *coefficient of variance* (CV) was 7.7 %. Blots with an in-house control diverging more than 20% from the mean, were not considered valid.

3.1.1 Correlation between immunoblot and ELISA/PCA-ELISA.

HMGB1 in sera from 23 SLE patients and 9 HC were measured with immunoblot. HMGB1 levels were compared to concentrations measured with ELISA. No significant correlation was found (r = -0.04, p = 0.85), Figure 3.6-A. HMGB1 measured with immunoblot were also compared to HMGB1 measured by PCA-ELISA (n = 10), with no apparent correlation (r = − 0.49, p = 0.16), Figure 3.2-B.

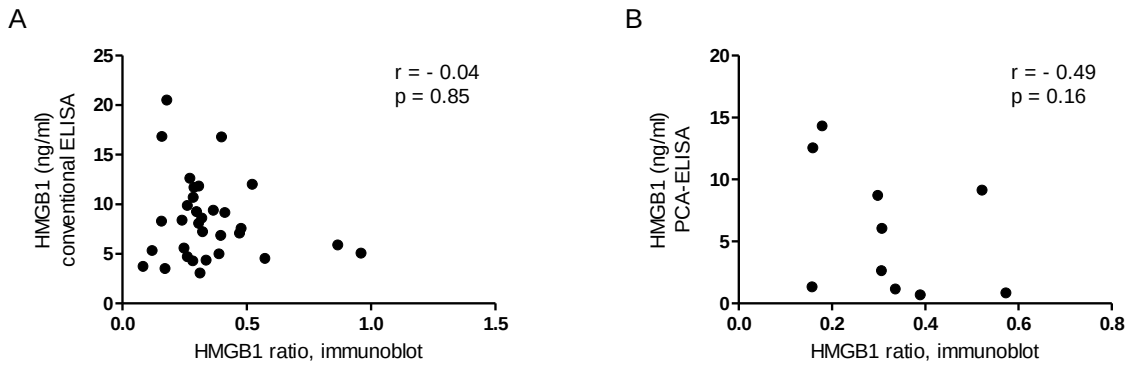

Figure 3.2: Correlation between immunoblot and ELISA/PCA-ELISA.
A. HMGB1 measured in 32 serum samples with immunoblot (x-axis) and conventional ELISA (y-axis). **B.** HMGB1 measured in 10 serum samples with immunoblot (x-axis) and PCA-ELISA (y-axis).

3.2 HMGB1 measurements by ELISA

Inter-assay variations were monitored with a positive control sample included in the ELISA kit was analyzed on each plate. A serum sample from one well defined SLE-patient (JH) was also analyzed as an in-house control. The commercial positive control had a mean HMGB1 concentration of 17 ng/ml as reported by the manufacturer. Acceptance range for the control was 9-26 ng/ml. In order to establish an acceptance range for the in-house control JH, the sample was analyzed on different plates on different days (n = 7). Mean (± SD) HMGB1 concentration was 5.7 ng/ml (2.0) and acceptance range was 1.7-9.7 ng/ml (mean ± 2SD). The

commercial positive control and the in-house control JH were within their respective ranges on all plates analyzed.

To assess reproducibility and plate to plate consistency for the method, inter-assay CV was calculated from analyzes of both controls. For the commercial positive control the inter-assay CV was 23.2 % (n = 8) and for the in-house control JH, inter-assay CV was 36 % (n = 7).

All samples, controls and calibrators were analyzed in triplicates. The CV % of these triplicates indicates the methods intra-assay variance. CV for triplicate measures of the samples on one plate ranged from 0.6-9.7 % (mean 4.1 %, SD ± 2.5).

A four parameter logistic curve fit of the results from the seven standards produced a calibration curve. A unique curve was made for each plate, an example is shown in Figure 3.3.

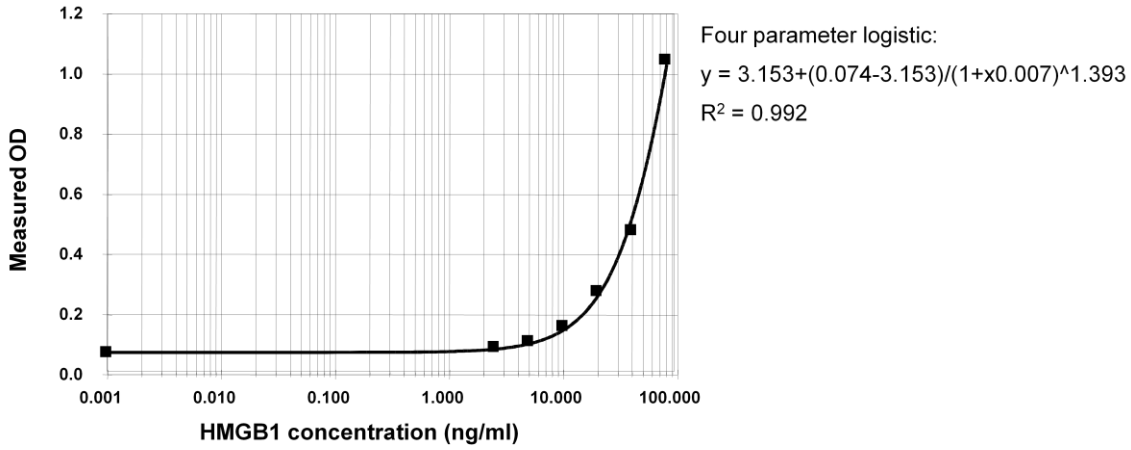

Figure 3.3: Calibration curve from a HMGB1 ELISA assay.
Optical density (OD) of seven standards was plotted against their respective known HMGB1 concentrations. A four-parameter logistic curve-fit was made.

3.3 HMGB1 measurements by PCA –ELISA

3.3.1 Correlation to conventional ELISA

Fourteen serum samples (9 SLE and 5 HC) were analyzed with both the conventional ELISA and PCA-ELISA method. The results correlated well (r = 0.83, p = 0.0002), Figure 3.4.

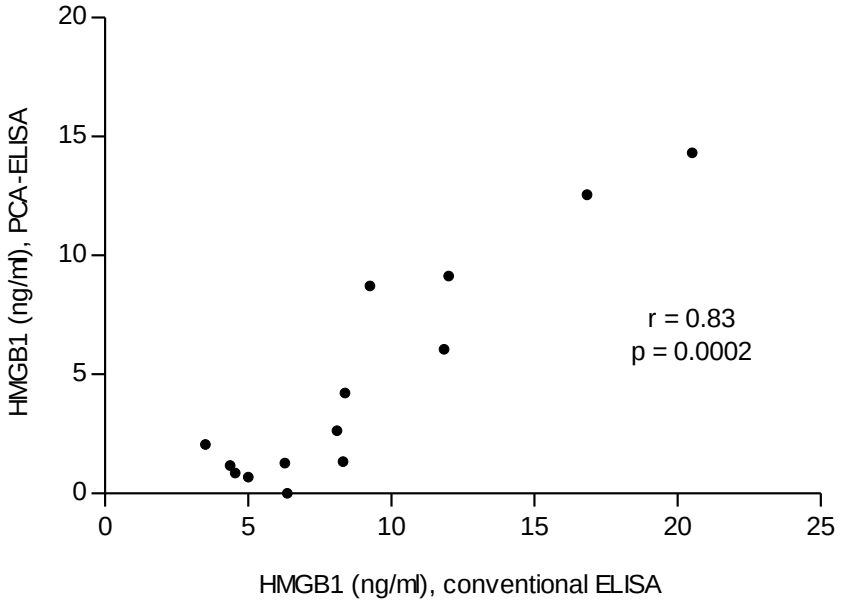

Figure 3.4: Correlation between conventional ELISA and PCA-ELISA.
Correlation (Spearman) between results from 14 serum samples measured with conventional ELISA (x-axis) and PCA-ELISA method (y-axis).

The PCA-ELISA method generally resulted in lower HMGB1 concentrations than the conventional ELISA method, demonstrated with a Bland-Altman plot where difference (ng/ml) between two measurements is plotted against the average (ng/ml) of the same two measurements. The mean difference ("bias") is 4.3 ng/ml (SD ± 1.96) and 95 % limits of agreement is 0.66-7.95, Figure 3.5. This is an acceptable agreement between the two methods.

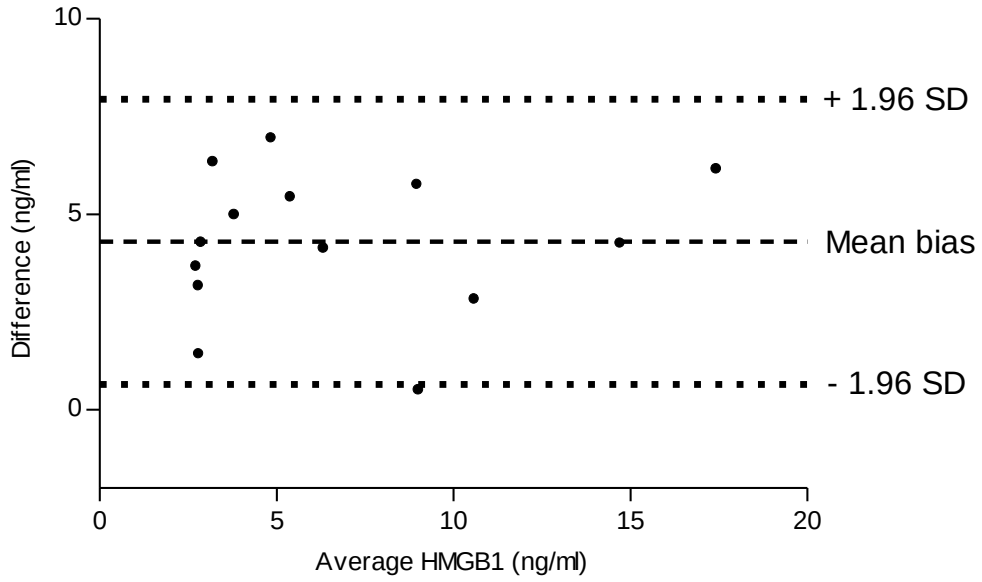

Figure 3.5: Bland-Altman plot showing the agreement between the conventional ELISA and PCA-ELISA.

3.3.2 Serum pretreatment

A precipitate was formed in the serum samples treated with PCA. It is possible that HMGB1 precipitated along with other proteins, thus giving rise to lower HMGB1 concentrations. Different acid solutions that did not precipitate proteins were tested; two lower concentrations of PCA (2.9 % and 5.7 %) and two concentrations of citric acid (0.1 M and 0.05 M), Figure 3.6. Serum samples and a calibrator (5 ng/ml) were pretreated with these acid solutions and ratios between the sample's OD and the respective calibrator's OD were calculated. The different pretreatments were compared and none of the acidifying agents increased the measured OD significantly compared with the sample without acid treatment.

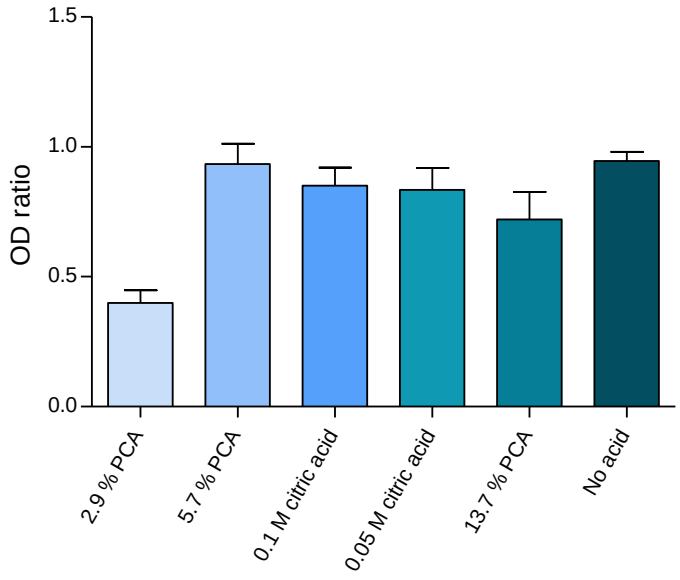

Figure 3.6: OD of sera with different pretreatments.
The graph shows OD of samples (n = 2) pretreated with different acidic solutions compared with a calibrator pretreated with the same agent. Horizontal lines indicate SD.

3.4 HMGB1 in SLE patients and healthy controls

3.4.1 Results from immunoblot

There were no differences in HMGB1 levels between patients and HC, Figure 3.7. The SLE patients (n = 23) had a median (range) ratio of 0.31 (0.08-0.96) compared to the healthy controls (n = 9) with a median (range) 0.32 (0.16-0.57) (p = 0.33).

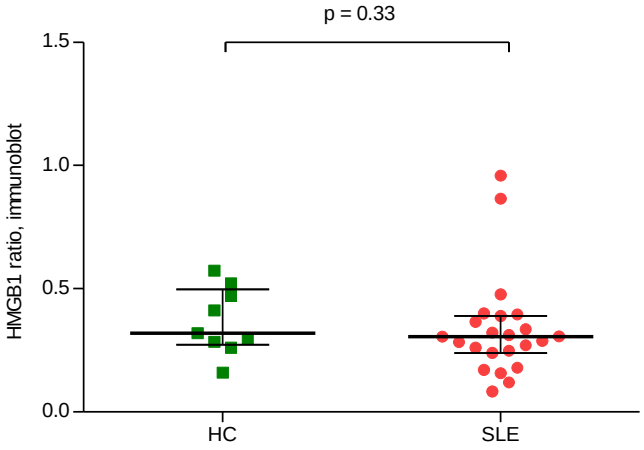

Figure 3.7: HMGB1 levels in SLE patients and HC.
HMGB1 levels in SLE patients (n = 23) and HC (n = 9), measured with immunoblot. The bold line represents the median, and the two horizontal lines 25% and 75% percentiles.

3.4.2 Results from ELISA

Sera from all 67 SLE patients and 25 HC were analyzed using the conventional ELISA. HMGB1 concentrations in SLE patients were significantly lower than in HC (median (range); 7.17 ng/ml (2.78-20.51) vs. 10.70 ng/ml (3.95-20.31), p = 0.0004), Figure 3.8. In contrast, HMGB1 levels in the two groups measured with PCA-ELISA were not significantly different; Median (range) for SLE patients (n = 9) was 1.34 ng/ml (0-14.33) and for HC (n = 5) 8.73 ng/ml (0.86-12.55), p = 0.19.

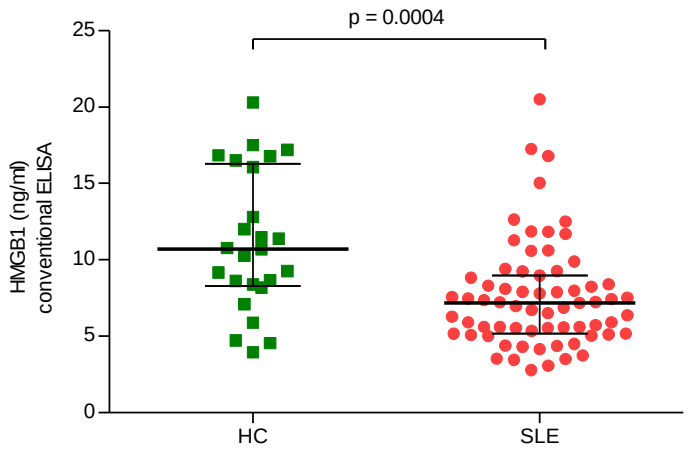

Figure 3.8: HMGB1 concentrations in SLE patients and HC.
HMGB1 levels in SLE patients (n = 67) and HC (n = 25), measured with conventional ELISA. The bold line represents the median, and the two horizontal lines 25% and 75% percentiles.

3.5 HMGB1 and anti-DNA antibodies

Using simple linear regression, no significant associations were found between HMGB1 measured with immunoblot and anti-DNA antibody titers ($R^2 = 0.02$, $p = 0.54$), Figure 3.9. Also, the HMGB1 concentrations measured with ELISA were not associated with anti-DNA antibody titers ($R^2 = 0.02$, $p = 0.20$).

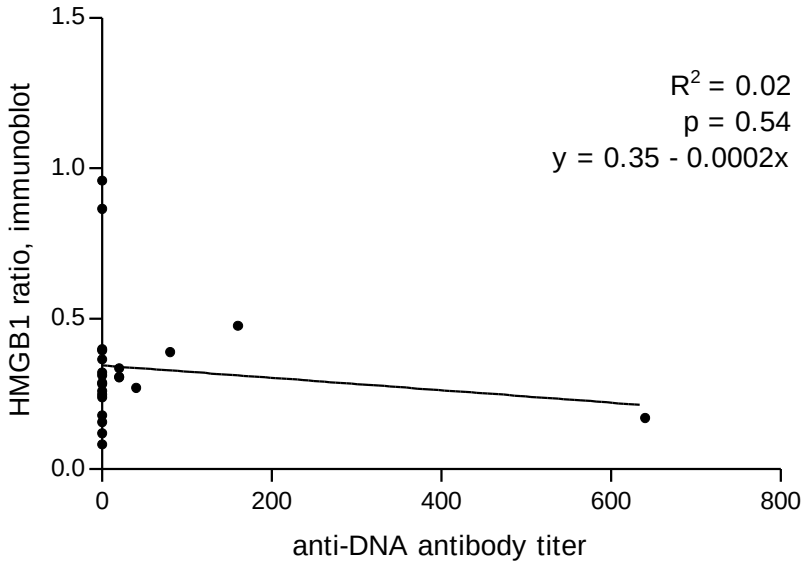

Figure 3.9: HMGB1 levels and anti-DNA antibodies.
HMGB1 levels in SLE patients (n = 23) in relation to anti-DNA antibody titers by simple linear regression analysis.

3.6 HMGB1 and disease activity

Linear regression was used to assess the possible association between HMGB1 concentrations and SLEDAI scores.

3.6.1 ELISA measurements and disease activity

HMGB1 concentrations measured with ELISA was associated with SLEDAI scores ($R^2 = 0.09$, $p = 0.01$, $y = 8.6-0.26x$), Figure 3.10. An apparent outlier was observed in the data set. However, this significance remained after removing the outlier from calculation.

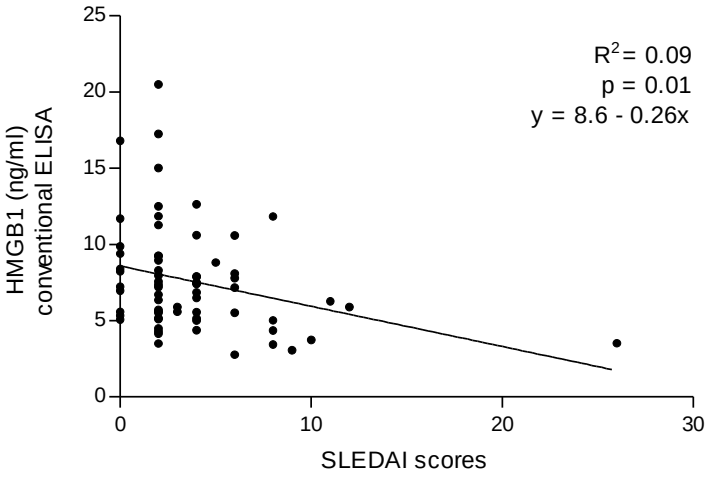

Figure 3.10: HMGB1 concentrations in association with SLE disease activity. Simple linear regression showing HMGB1 levels in SLE patients (n = 67) in relation to SLEDAI scores.

One-way-variance-of-analysis (Kruskal Wallis test) was used to compare three groups; patients with low disease activity (SLEDAI < 5), patients with high disease activity (SLEDAI ≥ 5) and HC, Figure 3.11. There were significant differences in median HMGB1 concentrations ($p = 0.0003$). Median (range) for patients with SLEDAI < 5 (n = 51) was 7.37 ng/ml (3.51-20.51) and 5.72 ng/ml (2.78-11.85) for patients with SLEDAI ≥ 5 (n = 16). Post-hoc analysis with Dunn's multiple comparison test showed that HC had significantly higher HMGB1 concentrations than the SLE patients ($p < 0.05$), but no difference between the two groups of patients.

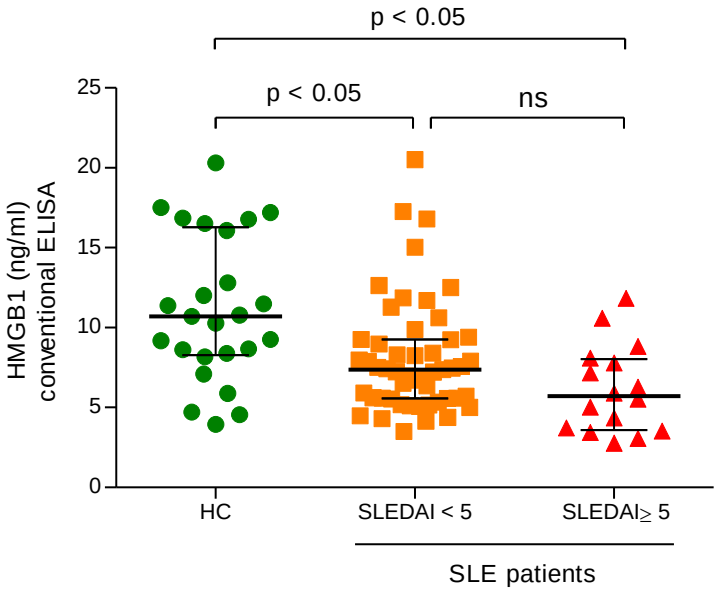

Figure 3.11: HMGB1 concentrations in HC and SLE patients with low and high disease activity.
The bold line represents the median, and the two horizontal lines 25% and 75% percentiles.

3.6.2 Immunoblot measurements and disease activity

No significant associations were found between HMGB1 levels and SLEDAI scores (R^2 = 0.06, p = 0.28), Figure 3.12.

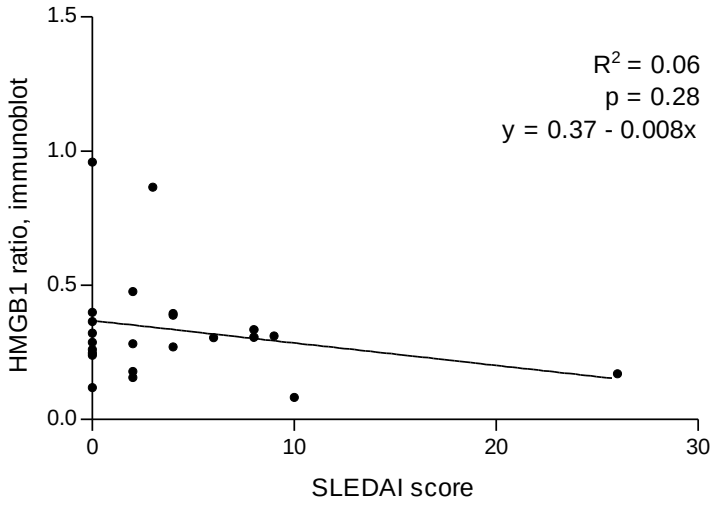

Figure 3.12: HMGB1 levels and SLE disease activity by simple linear regression analysis.
No. of SLE patients = 23.

3.7 HMGB1 and fatigue

Two methods were used to assess the degree of fatigue in the SLE patients: the FSS score and the fatigue VAS. No associations were found between HMGB1 measured by immunoblot and fatigue levels (FSS: $R^2 = 0.01$ and $p = 0.97$, fatigue VAS: $R^2 = 0.01$ and $p = 0.69$).

Patients were then divided into two groups, low fatigue (FSS < 5) and high fatigue (FSS ≥ 5) and HMGB1 levels were compared to HC with one-way variance of analysis with Kruskal Wallis test. There were no significant differences in HMGB1 levels between the three groups ($p = 0.11$), Figure 3.13. When other cut-off values for high fatigue was used, no differences were detected either.

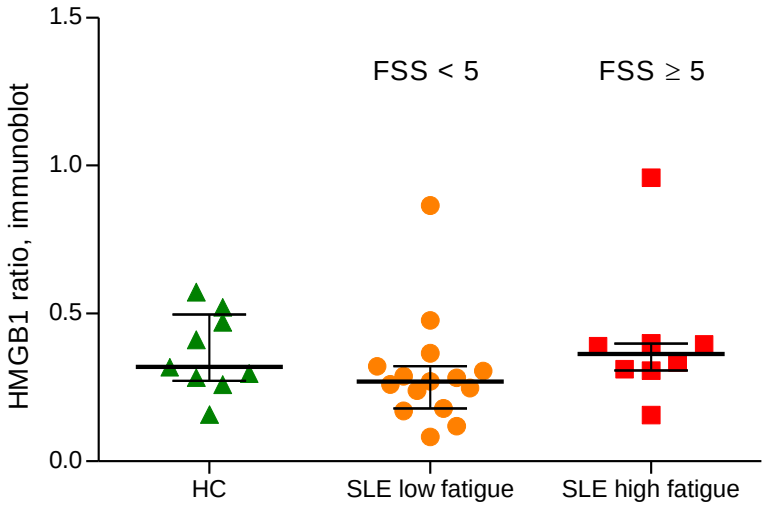

Figure 3.13: HMGB1 levels in HC and SLE patients with low and high fatigue. HMGB1 levels in SLE patients with FSS < 5 (n = 15), FSS ≥ 5 (n = 8) and HC (n = 9), measured with immunoblot. The bold line represents the median, and the two horizontal lines 25% and 75% percentiles.

The same calculations were performed with fatigue VAS. No significant differences were found between patients with fatigue VAS < 30 mm versus patients with fatigue VAS ≥ 30 mm or HC ($p = 0.36$), Figure 3.14.

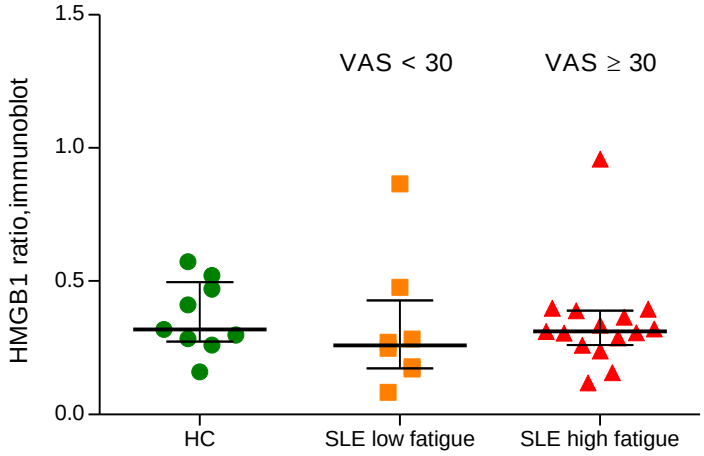

Figure 3.14: HMGB1 levels in HC and SLE patients with low and high fatigue. HMGB1 levels in SLE patients with VAS < 30 mm (n = 8), VAS ≥ 30 mm (n = 15) and HC (n = 9), measured with immunoblot. The bold line represents the median, and the two horizontal lines 25% and 75% percentiles.

Based on their HMGB1 levels, patients were then divided into two groups and mean fatigue VAS in the two groups was compared with a t-test. The group of patients (n = 11) with HMGB1 levels below median HMGB level (0.31) had fatigue VAS of 36 mm ± 23 (mean ± SD), and the patients (n = 12) with high HMGB1 levels had fatigue VAS of 64 mm ± 29, Figure 3.15. The difference between the two groups were significant (p = 0.02).

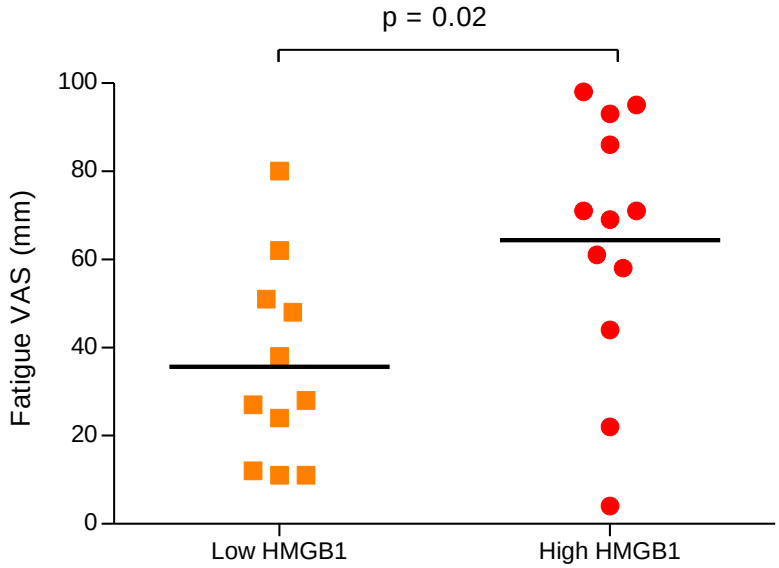

Figure 3.15: Fatigue VAS scores in SLE patients with low and high serum HMGB1 levels. Horizontal lines represent mean fatigue VAS scores in each group.

4. DISCUSSION

The objective of this study was to investigate different methods for measuring HMGB1 in serum and establish a valid and reliable method based on this. The methods would be used to measure HMGB1 in samples from SLE patients and HC. Three methods for determining HMGB1 levels in serum were evaluated. HMGB1 levels in patient sera were compared to levels found in HC, and associations to levels of disease activity and fatigue were explored.

4.1 Evaluation of methods

4.1.1 Fatigue measurements

Fatigue was evaluated by FSS and a fatigue VAS scale. These are generic and uni-dimensional fatigue instruments. The weaknesses of the methods are that they both rely on self-reporting and are highly subjective. They are also quite sensitive to the patient's different interpretation of the questionnaire and the explanation/instructions given to them. However, all methods for fatigue measuring are based on self-report.

4.1.2 Disease activity

The SLEDAI is a validated model of experienced clinicians' global assessments of disease activity in lupus. It represents the consensus of a group of experts in the field of lupus research (69). The anti-DNA antibody concentration can to some degree be regarded as a surrogate marker of disease activity.

4.1.3 Immunoblot

The immunoblot method is a basic, semi-quantitative immunological method, and HMGB1 has originally been detected in a number of different sample materials by this method. Serum HMGB1 detection by immunoblot described in literature varies regarding membrane, buffer solutions, antibodies etc. The process of establishing an immunoblot for HMGB1 in serum at our laboratory was time-consuming and challenging due to the rhHMGB1 that did not perform well as a positive control and due to disturbance by high protein levels in the samples.

Some blots were not considered valid due to too large variations of the in-house control, thus only 23 SLE patients and 9 HC were finally analyzed.

HMGB1 was visualized by immunoblot as a band of proteins at 28 kDa, similar to what is described in the literature. Additional strong bands at approximately 55 kDa were also observed. The primary antibody was a monoclonal antibody with documented good specificity and there were very little unspecific binding from the secondary antibody.

Immunoblot is probably the best method for determining levels of HMGB1 in serum because immune-complexes and antibodies do not interfere with the detection of HMGB1. SDS in loading buffer and heating of the sample will denaturize the proteins and dissociate any covalent bonds to other molecules. β-mercaptoethanol will break any disulfide bonds internally in the protein between C23 and C45 or between two C106 residuals on different HMGB1 molecules. In our experiments, there is a possibility that the reducing conditions of the SDS-PAGE may have been insufficient, resulting in the visual bands at 55 kDa.

However, immunoblot is time-consuming and labour-intensive and not suitable for large-scale studies. With only semi-quantification of HMGB1 it is also difficult to compare results between different studies.

4.1.4 ELISA

HMGB1 ELISA from Shino-test has frequently been used in studies with serum, plasma, cell culture and CSF, and is preferred for large-scale analyses due to its convenient protocol and good performance, as reported by Yamada et al (73) and the manufacturer.

We observed an intra-assay CV < 9.7% in our experiments. This was in accordance to what is reported by the manufacturer (5.5-13.7 %). The observed inter-assay CV % was very high compared to the manufacturer's data, especially in the lower range. We observed an inter-assay CV of 36 % in lower range and 23 % in mid-range. The manufacturer reports inter-assay CV from 7.6 to 13.7 %. Reproducibility was not good, but both controls (commercial and in-house) were within limits through the entire study.

Recovery in pooled sera is reported by the manufacturer to be 94.7-104.6 % and correlation between the ELISA and immunoblot method was demonstrated using sera from sepsis patients (74).

Serum HMGB1 levels measured with conventional ELISA did not correlate with HMGB1 levels measured with immunoblot. This is in accordance with other reports on SLE patients (41, 51, 57, 65). Also, with sera from patients with antineutrophile cytoplasmatic antibody (ANCA)-associated vasculitis there has been conflicting results (75, 76).

Sera from HC had significantly higher HMGB1 levels than sera from SLE patients measured by the conventional ELISA. HC had a median of 10.7 ng/ml, this is much higher than what has been reported in other studies, Table 1.1. One study reported the HMGB1 concentration in 626 healthy Japanese people to 1.65 ± 0.04 ng/ml (mean ± SD), measured with the commercial ELISA (77), and the manufacturer of the kit reports < 1.4 ng/ml as normal HMGB1 concentrations. Median HMGB1 level found in the SLE-patients (7.17 ng/ml) were similar to levels found in other studies using the same commercial ELISA test, Table1.1.

4.1.5 Immunoblot versus ELISA

Generally the conventional ELISA method underestimates the HMGB1 levels due to serum proteins and antibodies that interfere with HMGB1 in the ELISA system. The manufacturer of the kit reports good recovery in pooled sera, but this is probably sera from healthy individuals with low levels of antigens and immune-complexes. In our experiments we found a significant negative association between SLEDAI scores and concentrations of HMGB1 measured with ELISA, indicating that high SLE disease activity may be associated with more anti-HMGB1 antibodies. This is in accordance with Abdullahad et al (41). It has previously been shown that anti-HMGB1 antibodies increases with increased SLEDAI and the amount of HMGB1-complexes will most likely be increased due to increased cell death and immune activation. Consistent with the theory of interfering antibodies and complexes, sera from patients with high SLEDAI will inhibit the ELISA measurements more than sera from HC and patients with low SLEDAI. Obviously, one should be careful when interpreting results from SLE sera measured with HMGB1 ELISA.

The conventional HMGB1 ELISA may detect predominantly free, non-complexed HMGB1. This might not be the form of HMGB1 important in the pathophysiology of SLE. It has been demonstrated that HMGB1 is oxidized and partially bound to nucleosomes in SLE patients (20, 40). Complex forming with nucleosomes and proinflammatory cytokines is important because it enhances the immune stimulatory effect of HMGB1. The protein's redox status is

also important for its ability to bind to receptors and induce inflammation. The presences of anti-HMGB1 antibodies are probably also central, but their role is unclear. Anti-HMGB1 antibodies may have a pathogenic or a neutralizing effect, and may regulate the effect of HMGB1 on its targets. It could be that serum HMGB1 levels should be evaluated in parallel with serum levels of anti-HMGB1 antibodies. Interactions between HMGB1 and anti-HMGB1 antibodies in SLE pathogenesis needs further study.

4.1.6 PCA-ELISA

The previously reported improvement with PCA ELISA (65) was investigated by analyzing sera from 9 SLE patients and 5 HC. No improvement was found; the PCA-ELISA actually reported lower concentrations of HMGB1, but correlated well with conventional ELISA and was within limits of agreement as assessed by Bland-Altman plot.

It is possible that strong precipitation of proteins caused by PCA also precipitated the HMGB1. Thus lower concentrations of PCA and two different concentrations of citric acid were tried as alternative acidification agents. Citric acid is able to increase solubility of proteins without precipitation (78). No of the acidic solutions tested led to precipitates, and no enhanced detection of HMGB1 was observed.

The improvement with PCA pretreatment was reported in sera from patients with septic shock (65, 68). It is possible that the problems related to the SLE sera and ELISA evolve mainly from the presence of high levels of anti-HMGB1, not complexed HMGB1 and that PCA will not dissociate the anti-HMGB1 bound to HMGB1.

4.2 HMGB1 in SLE patients and healthy controls

No significant differences were found between HMGB1 levels in sera from SLE patients and HC by immunoblot. The two groups had similar median, but the patient group had a larger range. The numbers of patients and HC were relatively low (n = 23 and n = 9), and low statistical power could be an explanation.

4.3 HMGB1 and SLE disease activity

No associations between SLEDAI score and HMGB1 levels, or anti-DNA titers and HMGB1 levels could be demonstrated. Association with SLEDAI scores has been reported previously (41), and also - higher levels of HMGB1 has been demonstrated in patients with SLEDAI > 4 (55). In our experiments, no difference between the group with SLEDAI ≥ 5 and SLEDAI < 5 was found.

4.4 HMGB1 and fatigue

No associations between HMGB1 levels and FSS or HMGB1 and fatigue VAS was revealed. SLE patients with FSS > 5 or fatigue VAS > 30 mm had a tendency to higher HMGB1 levels than the patients with lower fatigue, but this was not significant. However, when dividing the patient group into low and high HMGB1, a significant difference in fatigue VAS was found. Patients with HMGB1 levels higher than median had significantly increased fatigue VAS.

To our knowledge, no other studies have investigated the association between HMGB1 and fatigue in SLE patients. Fatigue is closely related to sickness behaviour in animals, and animal studies indicate that HMGB1 is important in immune activation in the CNS and maybe one of the mediators of sickness behaviour. Levels of HMGB1 in CSF from patients with SLE and fatigue should be investigated. Measurements of CSF would probably be easier to perform due to less interfering autoantibodies and proteins in complex with HMGB1. Preliminary testing revealed lower levels of HMGB1 in CSF than in serum with both methods. Both ELISA from Shino-test and immunoblot has previously been applied for HMGB1 measurements in CSF (79, 80).

4.5 Conclusions

The preferred method for HMGB1 detection in patients with SLE is immunoblot. This method is without interference from serum components. Also, serum HMGB1 measured with ELISA do not correlate with immunoblot results.

Serum pretreatment with PCA did not improve the conventional ELISA as expected.

We were not able to demonstrate an association between HMGB1 and disease activity in SLE patients. Patients with high fatigue scores had a tendency to higher HMGB1 levels and there

was a significant difference in fatigue VAS between the groups with high and low HMGB levels. This indicates that there might be an association between serum HMGB1 and fatigue in SLE patients.

4.6 Future perspectives

Further research should be carried out regarding the association between fatigue and HMGB1 in serum as well as CSF. Improving the immunoblot procedure in our laboratory and analyze more samples from patients and HC. CSF samples from our biobank will in the future be measured with a method adapted for CSF.

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APPENDIX

Appendix 1:

ACR diagnostic criteria of SLE (37)

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	a) Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion *or* b) Pericarditis--documented by ECG or rub or evidence of pericardial effusion
7. Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed *or* b) Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurological disorder	a) Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance *or* b) Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	a) Hemolytic anemia--with reticulocytosis *or* b) Leukopenia--less than 4,000/mm<>3<> total on 2 or more occasions *or* c) Lyphopenia--less than 1,500/mm<>3<> on 2 or more occasions *or* d) Thrombocytopenia--less than 100,000/mm<>3<> in the absence of offending drugs
10. Immunologic disorder	a) Positive LE cell preparation *or* b) Anti-DNA: antibody to native DNA in abnormal titer *or* c) Anti-Sm: presence of antibody to Sm nuclear antigen *or* d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

Appendix 2:

SLE disease activity index (SLEDAI)

SLEDAI: DATA COLLECTION SHEET

Chart no.: _____ Date of Visit: _____

M.D.: _____ Patient's Name: _____

(Enter weight in SLEDAI Score column if descriptor present at the time of the visit or in the preceding 10 days.)

Weight	SLEDAI Score	Descriptor	Definition
8	_____	Seizure	Recent onset. Exclude metabolic, infectious, or drug causes.
8	_____	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behavior. Exclude uremia and drug causes.
8	_____	Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function, with rapid onset and fluctuating clinical features. Include clouding of consciousness with reduced capacity to focus, and inability to sustain attention to environment, plus at least 2 of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious, or drug causes.
8	_____	Visual disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serous exudate or hemorrhages in the choroid, or optic neuritis. Exclude hypertension, infection, or drug causes.
8	_____	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	_____	Lupus headache	Severe, persistent headache; may be migrainous, but must be nonresponsive to narcotic analgesia.
8	_____	CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	_____	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.
4	_____	Arthritis	More than 2 joints with pain and signs of inflammation (i.e., tenderness, swelling, or effusion).
4	_____	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/aldolase or electromyogram changes or a biopsy showing myositis.
4	_____	Urinary casts	Heme-granular or red blood cell casts.
4	_____	Hematuria	>5 red blood cells/high power field. Exclude stone, infection, or other cause.
4	_____	Proteinuria	>0.5 gm/24 hours. New onset or recent increase of more than 0.5 gm/24 hours.
4	_____	Pyuria	>5 white blood cells/high power field. Exclude infection.
2	_____	New rash	New onset or recurrence of inflammatory type rash.
2	_____	Alopecia	New onset or recurrence of abnormal, patchy or diffuse loss of hair.
2	_____	Mucosal ulcers	New onset or recurrence of oral or nasal ulcerations.
2	_____	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	_____	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation.
2	_____	Low complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.
2	_____	Increased DNA binding	>25% binding by Farr assay or above normal range for testing laboratory.
1	_____	Fever	>38°C. Exclude infectious cause.
1	_____	Thrombocytopenia	<100,000 platelets/mm^3.
1	_____	Leukopenia	<3,000 white blood cells/mm^3. Exclude drug causes.
TOTAL SLEDAI SCORE	_____		

Appendix 3:

Skala for tretthet og utmattelse (fatigue severity scale)

Dette skjemaet utfylles av forsøkspersonen under veiledning av intevjuer. Til høyre for hver påstand skal det skrives et tall. Velg et tall fra 1 til 7, der 1 betyr *helt uenig med påstanden* og 7 betyr *helt enig med påstanden*.

		Tall
1.	Mitt pågangsmot blir dårligere når jeg er utmattet	____
2.	Jeg blir fort utmattet ved anstrengelser	____
3.	Jeg har lett for å bli utmattet	____
4.	Utmattelse nedsetter min fysiske funksjonsevne	____
5.	Utmattelse skaper ofte problemer for meg	____
6.	Utmattelse fører til at jeg har dårlig fysisk utholdenhet over lengre tid	____
7.	Utmattelse virker negativt inn på mine gjøremål og forpliktelser	____
8.	Utmattelse er ett av mine tre mest plagsomme symptomer	____
9.	Utmattelse virker negativt inn på mitt arbeid, min familie og mitt øvrige sosiale liv	____

Ikke skriv under denne linjen
IKM/UiTø 1995

Mean: ____

Appendix 4:

Fatigue VAS

Tretthet og utmattelse

Dette spørreskjemaet spør om hvordan du har opplevd følelse av tretthet og utmattelse den siste uken.

Linjen nedenfor er et "mål" på hvor mye tretthet og utmattelse du har hatt den siste uken. Helt til venstre på linjen er "ingen problemer med tretthet og utmattelse", og helt til høyre er "så mye tretthet og utmattelse som det er mulig å ha."

Vi ber deg sette et merke med blyant eller penn på linjen nedenfor som best passer med den følelse av tretthet og utmattelse som du har.

Ingen problemer med tretthet og utmattelse

Så mye tretthet og utmattelse som det er mulig å ha