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Thema: The Effect of the Patients Nutritional Status on Immune Alterations Induced by Ischemic Stroke

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List of Abbreviations

BIA	bioelectrical impedance analysis
CD	cluster of differentiation
CRP	C-reactive protein
CT	computed tomography
DWI	diffusion weighted imaging
FACS	fluorescence activated cell sorting
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gpt/l	gigaparticle per litre, 10^9/l
IL-1 receptor antagonist	interleukin-1 receptor antagonist
IL-1b	interleukin-1β
IL-2	interleukin-2
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-12	interleukin-12
IL-17	interleukin-17
IL-17F	interleukin-17F
IL-18	interleukin-18
IQR	interquartile range
MCAO	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein-1
MCP-4	monocyte chemoattractant protein-4
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MIP-3β	macrophage inflammatory protein 3β
MIP-3α	macrophage inflammatory protein 3α
MRI	magnetic resonance imaging

NIHSS	National Institute of Health Stroke Scale
PAI-1	plasminogen activator inhibitor-1
rtPA	recombinant tissue plasminogen activator
sIL-6R	soluble interleukin-6 receptor
TNF-α	tumor necrosis factor α

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1 Introduction

Worldwide, ischemic stroke is a major cause of morbidity and disability [1]. In 2015 ischemic stroke was one of the leading causes for loss of health as measured with disability-adjusted life years, a measure that combines premature death and the number of years lived with a disability, surpassed only by ischemic heart disease [2]. To prevent this loss of life and health, it is not only necessary to prevent strokes but also to decrease complications after stroke and to improve outcomes of stroke treatment.

Understanding the pathophysiological changes immediately after stroke is a major component towards a better treatment of stroke. Immunological changes after stroke play an important role in the progression and complications after stroke and facilitate one particularly relevant complication after stroke, namely post stroke infections [3, 4, 5, 6]. Adipositas leads to pro inflammatory changes in the immune system in humans [7] and in rodent stroke models, where obese mice had increased levels of circulating pro inflammatory markers as a sign of immune system activation [8, 9].

1.1 Ischemic Stroke

An ischemic stroke is defined as

> *"an episode of neurological dysfunction caused by focal cerebral [...] infarction. CNS infarction is brain [...] cell death attributable to ischemia, based on 1. pathological, imaging, or other objective evidence of cerebral [...] focal ischemic injury in a defined vascular distribution; or 2. clinical evidence of cerebral [...] focal ischemic injury based on symptoms persisting $\geq$24 hours or until death, and other aetiologies excluded [10]"*.

The major causes of ischemic stroke are cardioembolic stroke, mainly caused by atrial fibrillation, large vessel stroke caused by macroangiopathy of large brain-feeding vessels and small vessel disease, often resulting in lacunar stroke. A sizeable number of stroke patients, between 20 % and 40 % suffer cryptogenic strokes, where no likely cause can be found despite extensive clinical investigations [11]. Risk factors for stroke include smoking, obesity, dyslipidemia, alcohol consumption, hypertension and diabetes mellitus [11].

An ischemic stroke is a medical emergency, not only due to its severe consequences but also because there is a narrow therapeutic window for revasculariza-

tion therapies. Emergency evaluation of stroke patients include a focused clinical examination and the documentation of standardized stroke scores. Further diagnostic tests include standard laboratory studies and cerebral imaging studies, either by means of cerebral computed tomography (CT) scan or cerebral magnetic resonance imaging (MRI) to localize the lesion and exclude cerebral hemorrhage [12].

Primary goal of the treatment of ischemic stroke is to restore blood flow to the affected brain areas. With the publication of the National Institute of Neurological Disease and Stroke Tissue Plasminogen Activator Trial in 1995, the systemic thrombolysis with recombinant tissue plasminogen activator (rtPA) was established as the first acute treatment for ischemic stroke [13]. Since 2015 mechanical thrombectomy was established as another evidence based treatment option. In the first 4.5 hours after stroke, an appropriate patient can be treated with recombinant tissue plasminogen activator, a potent activator of the endogenous fibrinolytic system. In the first 6-12 hours after a stroke a patient with a large vessel occlusion can receive endovascular treatment with stent retriever devices [14]. Selected patients can be treated with thrombectomy up to 24 hours after stroke onset [15]

1.2 Stroke and the Immune System

Cerebral immunological injury, inflammatory and infectious challenges and changes in the immune system play an important role in the progression of stroke in the hours and days after stroke onset [5]. A major complication of strokes are infections and especially pulmonary infections and pneumonia. Stroke patients have several risk factors contributing to an increased risk to develop infections, namely immobility, indwelling catheters and dysphagia [16] but a major contributor to the risk of severe infections among these patients is a post-stroke immunodepressive syndrome [4, 5, 17, 18]. Consequently, stroke patients are likely to develop infections and a meta analysis of 87 studies found a pooled infection rate of 30% [6].

Infections and especially pneumonia do not only prolong the hospital stay and require additional therapy but lead to increased mortality and worse functional outcomes [6, 19, 20]. In one meta analysis the Odds ratio for in hospital mortality for stroke patients with pneumonia compared to those without pneumonia was 3.62 (95% confidence interval: 2.8-4.68) [6]. Infections after stroke lead to worse functional outcome, the Odds ratio for an unfavourable outcome after 90 days, defined as an inability to continue all pre stroke activities, was 1.71 (95% confidence interval: 2.46 - 3.42) in a recent trial [20] and even in young patients (18-49 years) pneumonia is associated with an increased risk of an unfavourable 3 month outcome (Odds ratio 2.26; 95% confidence interval 1.08 - 4.76) [19].

Preventing post stroke infection is key in improving the outcome of stroke and lowering the risk of mortality after a stroke. While some risk factors can be monitored and treated, e.g. with routine dysphagia screening and training by ergo therapists, others cannot be treated sufficiently. Understanding immunological changes after stroke and influencing post stroke immunosuppression offer appealing options to prevent post stroke infections [18].

1.3 Overview of the Immune System

The immune system is the body's defence against infections [21]. It can be divided in two interlinking parts. The innate immune system is the rapidly acting part, which detects the presents of pathogens by recognition of danger signals and pathogen associated molecular patterns and initiates an immune response. The adaptive immune system mounts a slower but targeted immune response in the presence of pathogens but requires activation through the innate immune system [21, 22].

The major cells of the innate immune system are monocytes, that differentiate into macrophages, responsible for initiating an immune response after recognition and phagocytosis of a pathogen, and granulocytes, the most numerous immune cells, rapidly produced in the face of an immunological challenge. Natural killer cells (NK cells) are lymphoid like cells, that can induce apoptosis in cells lacking certain surface markers, especially major histocompatibility complex (MHC)-molecules [21].

T-lymphocytes and B-cells form the major components of the adaptive immune systems. T-cells are generally divided into two major forms, distinguished by the presence of membrane proteins, so called clusters of differentiation (CD), namely CD4 or CD8, both of which are co-receptors of the main T-cell receptor responsible for interactions with other cells. B-cells develop into antibody-producing plasma cells after activation of T-helper cells [22, 23, 24].

CD4 positive (CD4+) T-lymphocytes recognize their cognate antigen in context of the MHC class II protein complex on the surface of other immune cells. They have been classically considered T-helper cells but are now known to harbour several subsets with functional distinct properties. CD8 positive (CD8+) T-lymphocytes recognize their cognate antigen in context of MHC class I protein complex on the surface of most body cells and are traditionally called cytotoxic T-cells. Their main function is to recognize cells infected by intracellular pathogens or tumour cells and induce apoptosis in these cells.

Immune cells communicate with each other and other cells in the body by the interaction of surface proteins and by the secretion of cytokines, small proteins that enable immune cells to communicate locally as well as systemically and chemokines,

Table 1: Major Cytokines

Cytokine	Producing Cells	Action
GM-CSF	macrophages, T-cells	stimulates development of myelomonocytes
IFN-β	body cells	pro inflammatory
IFN-γ	T-cells, NK-cells, neutrophiles	macrophage activation
IL-1β	macrophages	T-cell activation, macrophage activation
IL-1RA	monocyte, macrophages, neutrophiles	inhibits actions of IL-1 family
IL-2	T-cells	T-cell differentiation and proliferation
IL-4	T-cells	B-cell activation, Induces type 2 CD4+-T-cell response
IL-5	T-cells	granulocyte differentiation
IL-6	T-cells, B-cells, macrophages	T- and B-cell differentiation, acute phase protein production
IL-10	macrophages, T-cells, B-Cells	suppresses macrophage activation
IL-12	macrophages	NK-cell activation, Type-1 T-helper cell response
IL-17	CD4+T-cells , CD8+-T-cells, neutrophiles	pro inflammatory, increases cytokine production
IL-17F	CD4+T-cells, CD8+-T-cells, neutrophiles	pro inflammatory, increases cytokine production
IL-18	macrophages	increases IFN-γ, type 1 CD4+- response
TNF-α	macrophages, T-cells, NK-cells	pro inflammatory
MIF	T cells	inhibits macrophage migration, activates macrophages

Table modified according to [25]

another set of small proteins that attract immune cells to the site of inflammation [21, 23]. An overview of major cytokines, especially interleukins (IL), chemokines and other proteins associated with immunological function can be found in Table 1, Table 2 and Table 3, respectively.

CD4+-T-cells can be further distinguished according to the cytokines they produce and the type of immunological response they facilitate.

The type 1 CD4+-T-cells are characterized by the production of interferon-γ, tumor necrosis factor-α (TNF-α) and granulocyte and macrophage colony stimulating factor (GM-CSF). They facilitate a macrophage dominated immune response against intracellular bacteria, like e.g. tuberculosis.

The type 2 CD4+-T-cells are characterized by IL-4, IL-5 and IL-13. Additionally, they produce eotaxin, TGF-β and IL-10. Type 2 T-helper cells facilitate the immune

Table 2: Major Chemokines

Chemokine	Affected Cells
IL-8	neutrophiles, CD8-T-cells
MCP-1	T-cells, monocytes, NK cells
MCP-4	CD4+-T-cells, monocytes
MIP-3α	T-cells, B-cells
MIP-3β	T-cells, monocytes, B-cells
Eotaxin	granulocytes, CD4+-T-cells

Table modified according to [26]

response against extracellular parasites and play a role in allergies and asthma. CD4+-T-cells producing IL-17, IL-17F and IL-22 enhance neutrophils and their response against extracellular bacteria. 5-10% of CD4+-T-cells can be distinguished by the expression of the transcription factor FoxP3. They are so called regulatory T-cells and are able to suppress the function of other T-cells and express immunosuppressiv cytokines like IL-10 and TGF-β. They form an important check against excessive immune activation and autoimmunity [23, 24, 27].

Table 3: Biomarkers

Biomarker	Function
CRP	acute phase protein, pathogen recognition molecule, binds to bacteria and activates the complement system [$]
PCT	marker of bacterial infection, proinflammatory [#]
soluble IL-6-Receptor	binds to IL-6, part of the IL-6 trans-signaling, pro inflammatory [&]
E-selectin	integrin, initiales leukocyte rolling, recruits leukocytes to site of infection [$]
P-selectin	integrin, initiales leukocyte rolling, recruits leukocytes to site of infection [$]

$ according to [25]
# according to [28, 29]
& according to [30]

1.4 Stroke Induced Immune Alterations

As early as 1979 Czlonkowska et al. observed pronounced changes in the immune system immediately following cerebral stroke, with relative lymphopenia, especially T-cell-lymphopenia and reduced delayed-type hypersensitivity skin-reaction [31]. Since then different immunological changes after stroke have been described.

Lymphocyte, T-cell, B-cell and NK-cell numbers are decreased almost immediately after experimental stroke in mice and in human stroke patient and cell counts remain depressed for several days after the ischemic stroke [17, 32, 33, 34, 35, 36].

In human stroke patients CD3+-T-cell-, CD4+-T-cell-counts [17, 35, 36] and CD8+-T-cell counts [17, 36] are markedly decreased. But in the same stroke patients higher leukocyte, granulocyte [35] and monocyte counts [17, 37] can be observed. These changes are most pronounced within the first days after the stroke and can persist up to several days to weeks after stroke.

Spleen weight and size are reduced after experimental stroke in rodents [33, 38, 39], the cell count in the spleen is reduced and there is an increase in apoptotic splenocytes [33, 34, 38]. In humans a decreased spleen size after stroke has been reported as well [40, 41]. The immunological changes precede the occurrence of post stroke infections and are more pronounced in stroke patients who develop nosocomial infections in the first two weeks after stroke [17, 35, 36] and in patients with large strokes compared to small strokes [36, 42, 43]. In experimental stroke, mice developed spontaneous pneumonia in the first 72 hours after stroke [34].

In rodents large cerebral infarcts lead to a pronounced lympho- and leucopenia compared to smaller ischemic strokes, produced by shorter occlusion times or coagulation of the middle cerebral artery distal to the lenticulostriate arteries [32].

Despite the signs of immunodepression and especially lymphopenia, the remaining T-cells in human stroke patients are activated, as measured by an increased proportion of IL-2-receptor CD25 and human leukocyte antigen-DR (HLA-DR) expression [44], while human monocytes are deactivated, with decreased levels of HLA-DR expression [17, 35].

While phagocytosis remained unaffected in neutrophils and monocytes of stroke patients, the frequency and efficacy of oxidative burst that is the ability to kill phagocytosed bacteria is reduced compared to healthy controls. Additionally, the efficacy of neutrophil NETosis, the ability of neutrophils to trap bacteria in nets of chromatin is reduced early after stroke [45].

In mice and humans the data on cytokine changes after stroke are mixed. While MCAO induced strokes lead to increased serum concentrations of the pro inflammatory cytokines interferon-γ , TNF-α and the anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β) in one study [32], other authors found a decrease in the production of Interferon-γ and increased production of IL-4 after *ex vivo* endotoxine stimulation. [34].

In human stroke patients lower serum levels of the pro inflammatory cytokines interferon-γ, TNF-α, IL-2 and IL12p70 [44] have been described. In contrast to this, in the same study the authors found increased release of the pro inflammatory

cytokines IL-1β, TNF-α, IL-6 and TGF-β from *ex vivo* stimulated T-cells in human stroke patients, while interferon-γ secretion was unchanged compared to control patients [44]. According to this study reduced levels of circulating cytokines are a consequence of reduced cell numbers and not impaired cytokine release from individual cells.

In mouse and human studies, TNF-α production in monocytes is decreased following stroke [34, 37]. The expression of CD107a, a marker for cell degranulation, on the surface of CD8+ cells, and the intracellular expression of interferon γ and TNF-α were decreased in stroke patients indicating a reduced cytotoxic function of CD8+-cells [46].

Mechanistically stroke induced immune alterations and immune depression are linked to a stress response with activation of the hypothalamic-pituitary-adrenal stress axis. And indeed, stroke patients have higher metanephrine levels in the first days after stroke, associated with a marked lymphopenia and subsequent infections and increased three month mortality [43, 47]. In mice metanephrines and cortisol are increased hours after stroke and levels remain elevated for days. Both hormones are capable of inducing apoptosis in leukocytes *in vitro* [33].

Spleen weight and splenocyte counts as well as circulating T-cell counts normalized in stroke mice treated with glucocorticoid-receptor-blockers, [33] while there are conflicting results whether beta-blockade prevents apoptosis of splenocyts in mice [33, 34]. Beta-receptor- and glucocorticoid-receptor-inhibition increase stimulated *ex vivo* TNF-α and interferon-γ secretion in stroke mice. Propranolol normalizes interferon γ to IL-4 ratio and prevents blood and pulmonary infections and improves survival in mice after stroke [34]. β2-receptor blockade in stroke mice restored *ex vivo* stimulated interferon-γ-synthesis to normal values [33].

1.5 Obesity, Adipokines and the Immunology of Adipose Tissue

The adipose tissue serves multiple roles in the human organism. It stores energy and lipids and serves as a protective tissue, preventing mechanical stress by acting as a cushion for exposed body areas , but one of the key functions of adipose tissue is the regulation of the energy balance and nutritional homoeostasis of the human body [7]. Increases in weight in the face of over-nutrition leads to hypertrophy and hyperplasia of adipocytes [48]. While adipocytes make up more than 90% of the adipose tissue volume, they form only a subsection of the overall cell numbers, a large section being immune cells [7]. With increasing obesity immune cells, including macrophages [49, 50] and T-lymphocytes [51] infiltrate the adipose tissue. The infiltrating macrophages are aggregating in clusters around adipocytes,

forming so called 'crown like structures' [52]. Residential macrophages are mainly type M2 macrophages, which secrete predominantly anti-inflammatory cytokines like IL-10 and IL-1 receptor antagonist, infiltrating macrophages are dominated by M1 type cells, producing pro-inflammatory cytokines among them TNF-α and IL-6 [49, 53, 54]. This leads to an increasing M1/M2-ratio, which together with the infiltration of other immune cells results in a pro-inflammatory milieu in the adipose tissue with increasing obesity [55, 56]. The systemic effects of obesity, like insulin resistance leading to diabetes mellitus are at least partially mediated by inflammatory cytokines like TNF-α and interferon-γ [51, 55, 56, 57]. Interestingly weight loss leads to a transient increase in adipose tissue M1 type macrophages and the increase correlates with markers of lipolysis [58].

Adipose tissue secrets a number of peptides, so called adipokines, which have systemic effects. Leptin, the major adipokine, and resistin are secreted proportionally to the mass of adipocytes. Leptin modulates satiety and energy expenditure [59, 60]. Leptin and resistin have pro-inflammatory effects, they induce IL-6 and TNF-α secretion in macrophages and Interferon-γ secretion in TH1-cells. Resistin and TNF-α lead to insulin resistance [60].

Adiponectin is unusual for an adipokine insofar as adiponectin levels are decreased in obesity. Adiponectin has anti-inflammatory properties and inhibits TNF-α and IL-6 secretion [60]. Because leptin is positively and adiponectin is negatively correlated with obesity and both have opposite effects on insulin resistance the leptin/adiponectin-ratio is strongly associated with insulin resistance [61, 62]. PAI-1, a major regulator of fibrinolysis is secreted by adipocytes and increased in obesity [63] and is associated with diabetes mellitus [64]. Because leptin, resistin and PAI-1 have pro-inflammatory effects [59, 60, 63] and adipose tissue in obese patients is infiltrated by pro-inflammatory cells [55, 56], consequently, obesity and increases in adipose tissue are associated with increased markers of systemic inflammation, especially CRP [65, 66]. Of note, while obesity is associated with a pro inflammatory state, there is evidence that obese patients are especially susceptible for infections [67].

1.6 Obesity and Stroke: The Adipositas Paradox

While obesity is a relevant risk factor for ischemic stroke and cardiovascular diseases [68], there is conflicting data concerning its role during and after the ischemic stroke. In rodent models of ischemic stroke obesity seems to increase stroke severity, stroke volume and worsen neurological outcome [69], but in obese human stroke patients improved functional outcomes and reduced mortality have been described,

the so-called obesity paradox [69, 70, 71]. While critics argue that the obesity paradox is indeed a statistical artifact [72], it has been found repeatedly in stroke studies [73] and similar results have been obtained in other diseases that is a similar paradox has been described in heart failure or sepsis [74, 75].

Different explanations have been suggested. One is that obesity protects against weight loss and catabolic metabolism after stroke [76], which is associated with poor outcome. In a Korean study 9 % stroke patients lost more than 0.1 kg/BMI−Unit in the first week after stroke. These patients had a poorer outcome than patients who did not lose weight or gained weight [77]. While weight loss is a phenomenon often observed in rodent stroke studies [78, 79], there is only the aforementioned study [77] on acute weight loss in human stroke patients. Another intriguing possible explanation for the obesity paradox is that obesity modulates the immunological changes after ischemic stroke. And indeed, studies in humans [80] and rodent stroke models [8, 9] have shown increases in circulating pro-inflammatory cytokines in obese stroke subjects. In rodent stroke models, obese animals have higher post stroke plasma levels of IL-6, GM-CSF and monocyte chemoattractant protein 1 (MCP-1), all of which are pro-inflammatory, compared to lean mice [8, 9]. In contrast adiponektin and resistin plasma levels are decreased post stroke [9]. Additionally, in these models obesity is associated with larger strokes [8, 9]. Human stroke patients with metabolic syndrome had increased plasma levels of pro-inflammatory cytokines CRP, IL-6 and TNF-α but decreased levels of the anti-inflammatory IL-10 compared to stroke patients without metabolic syndrome and non-stroke patients [80]. Among human stroke patients some trials show no change in leptin values between stroke patients and control patients [81, 82], while others show an increase in leptin values [83] in stroke patients. Those trials that did not find an increased leptin value in stroke patients in general, did find increased leptin values in either cardioembolic [81] or macroangiopathic [82] stroke. Adiponectin seems to be decreased in stroke patients [82, 84], while at least one trial found a differential effect of stroke subtype on the adiponectin level [85] with decreased values in atherothrombotic stroke and increased levels in cardioembolic stroke. Resistin levels were unchanged in those studies analysing it [81]. While it is plausible that these pro-inflammatory changes increase cerebral injury by worsening post stroke inflammation, they might also ameliorate post stroke immunodepression and thereby improve the outcome.

1.7 Aims of the LIPS Trial

The LIPS study was designed to test the following hypotheses:

(a) That human stroke patients lose a relevant amount of weight. We aimed to measure the acute weight changes in human stroke patients in the first week after an ischemic stroke and identify the tissues responsible for this weight loss. We hypothesized that the major contribution of the weight loss would be adipose tissue due to stress induced lipolysis of this tissue. This part of the study will be reported in greater detail in the thesis of Christin Heuer.

(b) That obese patients would have a pro-inflammatory immune response after stroke compared to lean patients. We expected that obese stroke patients have a modified and possibly ameliorated post stroke immunosuppression.

(c) That effective treatment of ischemic stroke with rapid clinical improvement immediately after the stroke will not only improve the functional outcome but will modify and ameliorate the post stroke immune alterations. We predicted, that patients with a marked early clinical improvement will have a milder form of post stroke immunodepression.

1.8 LIPS Trial organization

Patients were screened for possible eligibility by the stroke physician on call. If potential patients were identified the study recruitment team consisting of Christine Heuer and myself was informed. Patient enrolment and data acquisition was performed by both. Christin Heuers thesis will give a detailed analysis of weight and fat metabolism in the study cohort while this thesis details the data on the immune alterations.

2 Methods

The trial was approved by the ethics committee at the Universitätsmedizin Greifswald (BB 050/15).

2.1 Patient selection

We enrolled consecutive patients admitted to our tertiary stroke unit, certified according to the guidelines of the DSG at the department of neurology at Universitätsmedizin Greifswald, which presented with acute anterior circulation stroke syndrome, who met the following inclusion and exclusion criteria.

Inclusion criteria:

- First anterior circulation stroke, that is an ischemic stroke in the vascular territory of the A. carotis interna within the last 24 hours
- NIHSS Score on admission ≥ 8
- Signed consent form, either by the patient or by a legal guardian if the patient is not able to consent
- Age $>$18

Exclusion criteria:

- Clinically significant anemia, Hb < 3.7 mmol/l
- Acute infection on admission, either clinical diagnosis or CRP > 50 mg/l or PCT > 0.5 ng/ml
- Treatment with immunosuppressive drugs
- Non-curatively treated malignoma in medical history
- Severe cerebral disorder in medical history (e.g. ischemic or haemorrhagic stroke, meningitis or encephalitis, severe cerebral trauma or epilepsy)
- Any contraindication against a MRI, e.g. pacemakers.

All patients were treated by a team of experienced stroke neurologists according to local and national guidelines. On admission they were screened by the stroke unit neurologist on call and the study team was informed.

On admission the stroke unit neurologist performed a detailed neurological examination and the initial NIHSS-score was calculated. When appropriate a MRI or CT-scan was performed and additional examinations, especially vascular diagnostics, e.g. CT-angiography were performed. Eligible patients received acute stroke therapy, either systemic thrombolysis with rtPA, mechanical thrombectomy or both. The initial study blood sample was drawn after the clinical examination but before diagnostic or therapeutic procedures were performed.

Control patients of similar age, without a history of stroke were recruited from patients receiving treatment in the Department of Opthalmology at the Universitätsmedizin Greifswald. Exclusion criteria applied to control patients as well.

2.2 Trial Outline

Enrolled patients were examined by the study team on the day of admission and then on days one to five and on day seven of their hospital stay. Examinations included a neurological examination to determine the NIHSS-Score, a brief physical examination and chart review to note signs of infection and changes in medication, weight measurements with a calibrated scale, body-impedance-analysis and skin fold thickness measurement. Blood was drawn on admission day, on days 1 to 5 and on day 7. If possible a cerebral and abdominal MRI were performed on the first 3 days after stroke and then repeated before discharge (See figure 1).

Control patients were examined once, their weight and height were recorded, the skinfold thickness measured and an abdominal MRI performed. In control patients blood was drawn once, on the examination day.

All patients were requested to return to our department for follow up visits after 30 days, 3 months and 6 months. During these follow up visits a neurological examination to determine the NIHSS-score, weight measurements, body-impedance-analysis and skin fold thickness measurements were performed as in the first part of the trial. Additionally, during follow up visits the modified Rankin Scale score and Barthel Index were determined for each patient.

2.3 Clinical Scales

2.3.1 National Institute of Health Stroke Scale

The NIHSS is a scale developed to quantify neurological deficits after stroke. In its current iteration it consists of 15 items testing the level of consciousness (items 1a-c, 1a: 0-3 points, 1b, 1c: 0-2 points), the gaze (item 2, 0-2 points), the visual

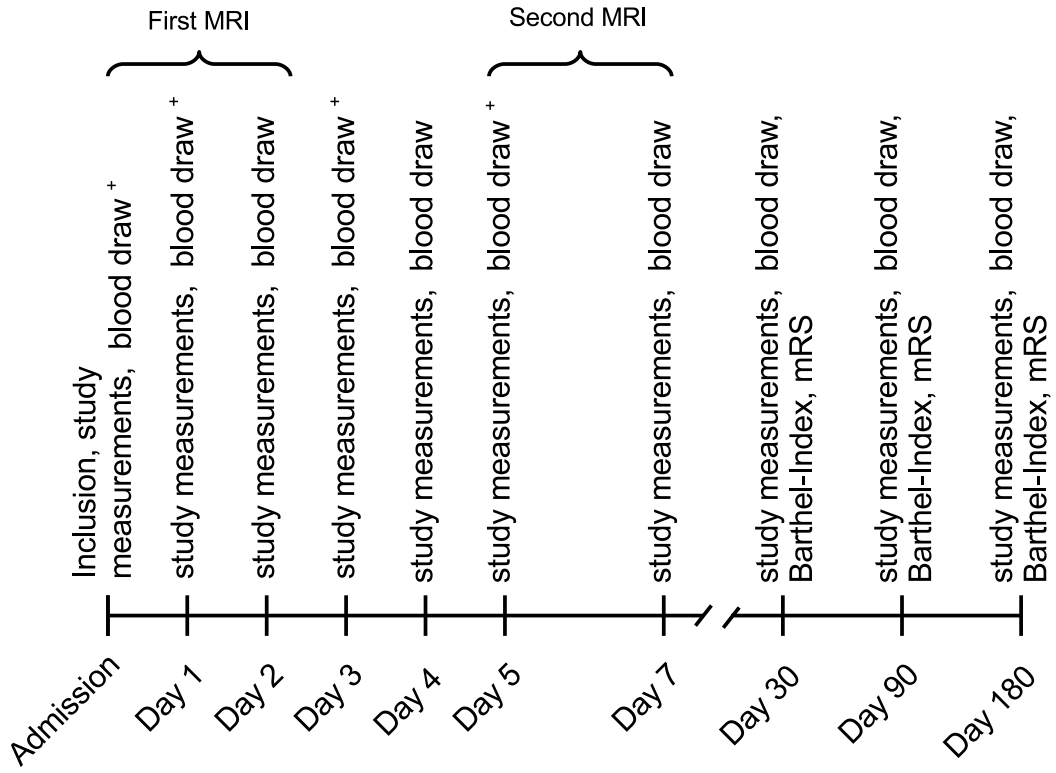

Figure 1: Outline of the LIPS trial. Patients were included in our trial on admission day within 24 hours after their index stroke. Study measurements included a physical examination, the NIHSS-score, weight measurement, BIA-analysis and measuring the triceps skinfold thickness. The first MRI was performed on any of the days one to three, the second MRI was performed after day four. MRI included a cerebral MRI and an abdominal MRI. Blood draws included routine clinical measures and FACS analysis, +-signs denote days with cytokine and adipokine measurements.

field (item 3, 0-3 points), testing for facial palsy (item 4, 0-3 points), the extremity strength (items 5a, b, 6a, b, 0-4 points each), for limb ataxia (item 7, 0-2 points), sensory deficits (item 8, 0-2 points), language (item 9, 0-3 points), speech (item 10, 0-2 points) and extinction and inattention (item 11, 0-2 points) [86, 87]. The scale ranges from values between 0 and 42, with 0 meaning no detectable deficit and 42 the worst value possible. For the purpose of this study we classified an NIHSS between 8 and 15 as a moderate Stroke and NIHSS above 15 as a severe stroke [88].

2.3.2 Modified Rankin Scale

The Rankin Scale (mRS) is a 6-point scale measuring disability and functional outcome after stroke. It uses key questions in a short, structured interview to assign a level of disability. The scale ranges from 0 to 5, with 0 meaning no symptoms at all and 5 meaning severe disability, patients with minor symptoms score 1 point, patients with severe symptoms, who can walk score 3 points, bed bound patients score 4 points, if they can be left unattended or 5 if they need constant nursing assistance [89].

2.3.3 Barthel Index

The Barthel Index measures the ability to perform the activities of daily living, e.g. bathing, cooking or dressing. Patients are rated in 10 activities and points are scored depending on how well the patient performs the activity and depending on how much help and supervision the patient needs, tested activities are feeding (0, 5 or 10 points), walking (0, 5, 10 or 15 points) and changing from bed to a wheelchair (0,5, 10 or 15 points), personal hygiene (0 or 5 points) and washing (0 or 5 points), going to the toilet (0, 5 or 10 points), using stairs (0, 5 or 10 points), dressing (0, 5 or 10 points), urinary and stool incontinence (both 0, 5 or 10 points). The Barthel Index can take values between 0 and 100. The patient scores 0 points if he cannot perform any of the scored activities. A patient with a Barthel Index of 100 points is continent, can feed and dress himself and walk at least a block and ascend and descend stairs [90].

2.4 Weight Measurement

Because Stroke Patients are often bed bound due to paresis, the study subjects were weighed using an in-bed scale SECA 985 (Krauth & Timmermann, Hamburg,

Deutschland). During weighing Patients were dressed in hospital gowns and sheets, blankets and pillows were removed as much as possible. The weight of the beds was pre-recorded or was measured after discharge of the patients. Remaining pillows, blankets etc. were measured afterwards and their weight subtracted as well. If patients were ambulatory their weight was measured using a calibrated Soehnle Professional type 7700 scale (Soehnle Industrial Solutions GmbH, Backnang, Germany). Control subjects were weighed using a calibrated Soehnle Professional type 7700 scale (Soehnle Industrial Solutions GmbH, Backnang, Germany). On the admission day patients were weighed before any treatment on the emergency department stretchers, using the in-bed scale. When patients were still dressed, their clothes were weighed afterwards and their weight subtracted. The Patients height was recorded on admission. The Body Mass Index was calculated using the following formula BMI $= weight/height^2$. We used the following subgroups for further analysis. Underweight: $BMI \leq$ 18.5, Normal weight: $BMI >$ 18,5, $\leq$ 25, Overweight: $BMI >$ 25, $\leq$ 30 Obesity BMI: $>$ 30.

2.5 Laboratory Analysis

Blood was drawn between 6:30 and 7:30 in the morning. Differential blood cell counts (XN9000, Sysmex, Norderstedt, Germany) and the values for PCT (Adivia Centaur XPT, Siemens Healthcare Diagnostics, Eschborn, Germany), CRP, cholesterol and triglycerides (all measured with the Dimension Vista, Siemens Healthcare Diagnostics, Eschborn, Germany) were measured in the central laboratory facility of the Universitätsmedizin Greifswald. Fluorescence activated cell sorting was used to measure cell counts for T-Cells, CD4+-T-Cells, CD8+-T-Cells, NK-cells and B-cells. Cell sorting was performed on a FC500 (Beckmann Coulter, Krefeld) flow cytometer in the Department of Oncology of theUniversitätsmedizin Greifswald. For cytokine and adiopokine measurements patient sera on admission day, day 1, day 3 and day 5 were frozen at -80 degrees and processed later. Analysis was performed at the Multiplex Facility at University Leiden using a multiplex approach utilizing the multi-analyte profiling (xMAP) technology (Luminex, Austin, USA). This approach allows to analyze a large number of cytokines quickly in small volumes of serum. For these measurements blood drawn on the admission day, day 1, day 3 and day 5 was used. Table 4 lists all measured cytokines and adipokines. Measured values were expressed as pg/ml. If values exceeded 1000pg/ml or 1000000 pg/ml, they are expressed as ng/ml or µg/ml respectively.

Table 4: Analyzed Biomarkers

IL-1 Receptor Antagonist	IL-1β	IL-2	g IL-5
IL-6	IL-10	IL-12	IL-17
IL-17F	IL-18	TNF-α	Interferon-β
Interferon-γ	MIF	MCP-1	Eotaxin
MCP-4	MIP-3β	MIP-3α	IL-8
GM-CSF	P-Selectin	E-Selectin	Leptin
Resistin	PAI-1	Adiponectin	Soluble IL-6 receptor

2.6 Additional Study Measurements

If clinically possible, stroke patients received an magnetic resonance imaging (MRI) with a cerebral diffusion weighted imaging (DWI) and an abdominal MRI within the first 3 days after stroke and one before discharge. To measure the body composition of our patients and its changes, body impedance analysis was performed daily and the triceps skinfold thickness was measured. These results will be presented by Christine Heuer in a separate thesis.

2.7 Definition of Systemic Infection

For our study, we used the same definition of stroke-associated infection as we used in previous trials [45, 44]. A patient was considered to have a stroke-associated infection if all the following criteria were met on any day in the first week after stroke:

- Clinical signs of infection, e.g. fever, cough, radiographic evidence of pulmonary infiltrations etc.
- Serum CRP concentration $>$50 mg/l
- Serum procalcitonin concentration $>$0.5 ng/ml

If none of the criteria were met within the first week, the patient was considered to have no stroke-associated infection. If one or two criteria were met but not all were met within the first week, then the patient had an undetermined infectious status.

2.8 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism Version 5.01 (GraphPad Software, Inc., La Jolla USA). Data are expressed as median and interquartile

range(IQR) or as mean $\pm$ standard error of the mean. Normality was tested using the D'Agostino-Pearson omnibus K2 test and statistical significance was tested either with a Student's T-Test, or an ANOVA or Kruskal-Wallis-Test, as appropriate. For statistical significance testing between different days in the stroke group repeated measure ANOVA or Friedman's Test were used. For group comparisons either a Newman-Keuls or Dunn Post Test were used. For ordinal or nominal scaled data Fisher's exact test was used. We assumed statistical significance, if p was below or equal to 0.05. For statistical purposes, if values were below the detection limit of a given test, they were set to zero. If patients were discharged after day 4 or 5, missing data was handled with the last observation carried forward method.

3 Results

3.1 Subjects and Baseline Statistics

Between July 2015 and July 2016, 50 patients were enrolled in the study. 10 patients had to be excluded from this analysis (see figure 2). The remaining 40 patients form our stroke cohort and were included in our analysis. Additionally we recruited 16 control patients.

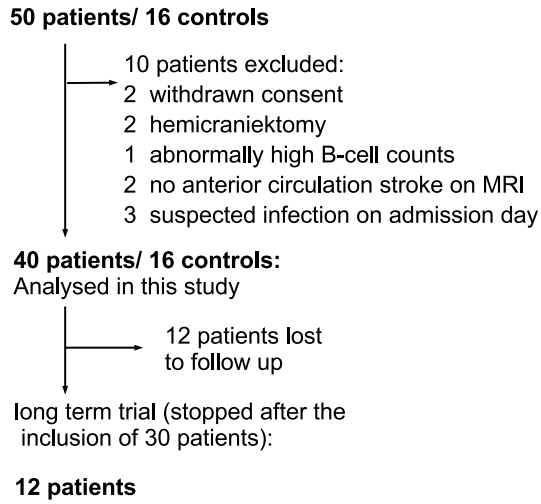

Figure 2: Overview of included and excluded patients throughout the LIPS trial

The mean age of our patient group was 71 $\pm$14 years, the mean age of the control group was 71 $\pm$8 years, 55 % of our patients and 44 % of our controls were female.

In the patient group 37.5 % were obese, 35.0 % were overweight and 27.5 % had a normal weight. In the control cohort 18.75 % were obese, 56.25 % were overweight and 25.00 % were normal weight (additional comparisons can be found in Table 5).

The median interval between stroke and inclusion in our trial was 3 hours 52 minutes (IQR 3:00 hours-8:15 hours).

In our cohort 30 out of 40 patients received any hyper-acute stroke treatment, that is mechanical thrombectomy or systemic thrombolysis with rtPA. 13 patients received a combination of both treatments, 12 patients received only systemic thrombolysis and 5 patients received only a mechanical thrombectomy. The stroke aetiologies were large-artery atherosclerosis in 25.0 % of the cases, cardioembolism in 47.5 % of the cases, stroke of other determined aetiology in 5.0 % of the cases and stroke of undetermined aetiology in 22.5 % of our stroke patients according to the TOAST-classification [91]. In the group of stroke patients of undetermined aetiology the most likely pathomechanism was embolic stroke, but despite an extensive work up no likely embolic source could be found.

Table 5: Baseline Statistics

Variable	Patient Group (N=40)	Control Group (N=16)
Age [Years, Mean±Std.]	71 ± 14	71 ± 8
Sex [as % female]	55%	44%
BMI [Mean±Std.]	28.45 ± 5.13	27.30 ± 3.42
Weight Categories		
Obese [n (%)]	15 (37.5)	3 (18.8)
Overweight [n (%)]	14 (35.0)	9 (56.3)
Normal [n (%)]	11 (27.5)	6 (15.0)
Co-morbidities		
Hypertension [n (%)]	31 (77,5)	8 (50)
Diabetes mellitus [n (%)]	6 (15)	5 (31.3)
Stroke Characteristica		
Aetiology		
Large-artery artherosklerosis [n(%)]	10 (25)	NA[$]
Cardioembolism [n(%)]	19 (47.5)	NA[$]
Stroke of other determined aetiology [n(%)]	2 (5)	NA[$]
Stroke of undetermined aetiology [n(%)]	9 (22.5)	NA[$]
1. MRI Stroke Size [ml^3, Median (IQR)]	31.0 (7.5-78.0)	NA[$]
2. MRI Stroke Size [ml^3, Median (IQR)]	41.2 (8.2-89.6)	NA[$]
Initial NIHSS [Median (IQR)]	15 (11.25-21.00)	NA[$]
Infarct side [n (%)left sided infarcts]	20 (50.0)	NA[$]
Treatment [n (%)]	30 (75.0)	NA[$]
Systemic Thrombolysis [n (%)] [&]	25 (62.5)	NA[$]
Mechanical Thrombectomy [n (%)][&]	18 (45.0)	NA[$]
Combined Treatment [n (%)]	13 (32.5)	NA[$]

[&] The numbers of systemic thrombolysis and mechanical thrombectomies are the total number of patients receiving the treatments and include patients receiving a combination of both.
[$] NA: Not applicable.

3.2 Trial Overview

3.2.1 National Institute of Stroke Scale and Stroke Severity

In this study 21 out of 40 patients had a moderate stroke with an initial NIHSS between 8 and 15. 19 patients had a severe stroke with an NIHSS greater than 15. After admission most patients improved neurologically, with corresponding decreasing median NIHSS scores (see figure 3). While the initial median NIHSS score was 15 (IQR: 11-21, minimum: 8, maximum: 28), on day seven, the median NIHSS was 9 (IQR: 4-14, minimum: 0, maximum 25). Between Admission and day one 13 patients (32.5 %) had a decrease of their NIHSS score by more than 25 %. The median improvement in NIHSS score in these patients was 10 (IQR 5.5-12). All these patients had received some form of treatment, either systemic thrombolysis, mechanical thrombectomy or both. While most patients improved clinically as measured by decreasing NIHSS scores, 9 patients had worsening neurological symptoms, with a corresponding increase in NIHSS by 2 or more points. The majority, 8 of these 9 patients worsened in the first three days (Admission-day two), but worsening neurological symptoms and increasing NIHSS were seen up to day seven (see figure 3).

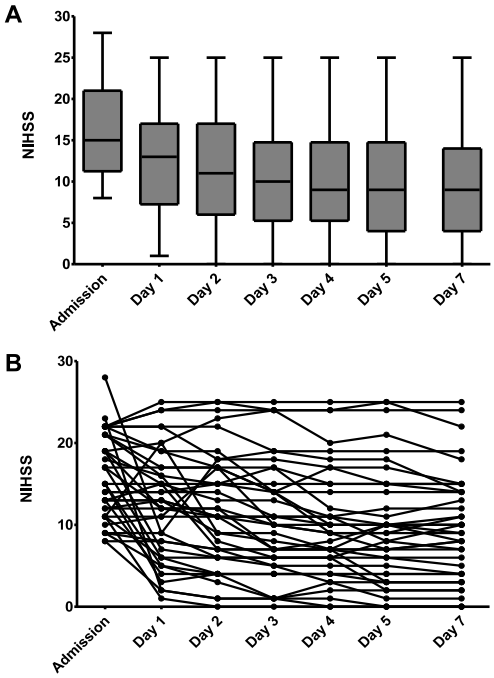

Figure 3: A: Box Plot of the National Institute of Health Stroke Scale score (NIHSS) for all stroke patients, shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values, B: NIHSS Scores for single patients, points mark the measured scores, lines connect all scores of a single patient.

3.2.2 Total Body Weight Changes

The mean weight of stroke patients decreased from 81.2 ± 14.9 kg on the day of admission to a nadir of 79.7 ± 14.9 kg on days one and two (see Figure 4). After day two, the weight remained fairly constant with a mean weight of 80.2 ± 16.2 kg on day seven. The largest change in weight occurred between the day of admission and

day one, with a drop of 1.5 kg, a phase were all patients treated with thrombolysis (*n* = 25 or 62.5 % of all stroke patients) were not permitted to eat or drink per protocol.

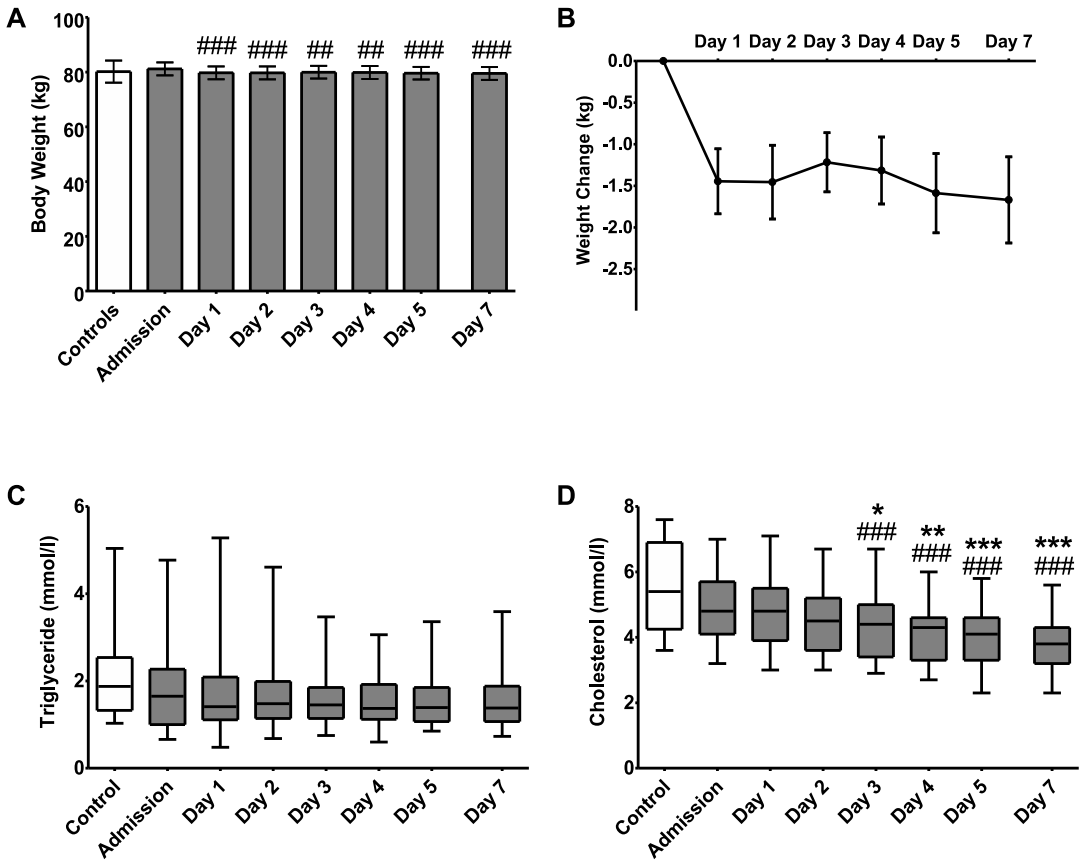

Figure 4: A: Body weight in kilogram (kg) for control (white bar) and stroke patients (grey bars). Body weight in stroke patients and controls were similar, but in stroke patients weight loss between admission and days 1-7 was statistically significant. Significant differences between admission and the following days are marked with $### = p < 0.001$, $## = p < 0.01$, $# = p < 0.05$; B: Weight change after stroke, calculated as the difference between weight on the given day and admission weight for stroke patients. A, B: Bars in A and points in B represent mean values, error bars represent standard error.

C, D: C: Median triglyceride and D: cholesterol values; Comparison between stroke patients (grey boxes) and controls (white box). Statistical differences between patients and controls are denoted as follows: $*** = p < 0.001$; $** = p < 0.01$; $* = p < 0.05$. Statistical significant differences between admission and days one to seven were marked as following . Shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values.

The weight differences between admission weight and the weights on days one to seven were statistically significant (see figure 4, repeated measures ANOVA, with Bonferroni's multiple comparison test $p < 0.0001$). While the mean weight in the stroke group declined after admission and most patients lost some weight after

stroke, 5 patients had an increased weight and gained 2.2 ± 2.4 kg between Admission and day one.

3.2.3 Serum Lipid Concentrations

On admission 11 (27.5 %) patients were treated with HMG-CoA reductase inhibitors, after day 1 all patients received either atorvastatin 40-80mg or simvastatin 40mg daily. Triglyceride values among stroke patients and control subjects were similar on admission but in stroke patients serum values decreased slightly but these changes were not statistically significant (see figure 4, comparison between control and stroke values: Kruskal-Wallis test, $p = 0.4253$, comparison with admission values in stroke patients: Friedmann test, $p = 0.8786$). Median admission values were 1.65 (IQR 1-2.27) mmol/l among stroke patients versus 1.88 (IQR 1.33-2.54) mmol/l in controls, on day seven the median triglyceride value in stroke patients was 1.38 (IQR 1.07-1.88) mmol/l.

The median cholesterol admission value among stroke patients and the median value in control subjects were similar, 4.80 (IQR 4.10-5.70) mmol/l versus 5.40 (IQR 4.25-6.90) mmol/l. In stroke patients median cholesterol values declined up to day seven, to a value of 3.80 (IQR 3.20-4.60) mmol/l. This decline was statistically significant compared to the admission value and compared to the mean control value (see figure 4, comparison versus control: Kruskal-Wallis test with Dunn's multiple comparison test, $p < 0.0001$; comparison versus stroke admission values: Friedmann test with Dunn's multiple comparison test, $p < 0.0001$).

3.2.4 Serum CRP and PCT and Infectious Complications

On admission median CRP concentration in the stroke patients was 3.3 (IQR 0-7.9) mg/l, on the following days the median CRP concentration increased and reached a maximum on day seven with 18.6 (IQR 4.6-34.7) mg/l. Control subjects had a median CRP concentration of 0 (IQR 0-5.2) mg/l. Compared with control values and compared with admission values, CRP concentrations on the days one through seven were significantly higher in stroke patients (see figure 5, One way ANOVA with Bonferroni multiple comparison test; $p < 0.0001$ for comparison with control subjects, Friedman test with Dunn's multiple comparison test; $p < 0.0001$ for comparison with admission values).

Median procalcitonin (PCT) concentrations in stroke patients on admission and in control patients were both 0.08 (IQR 0.06-0.11) ng/l. PCT concentrations in stroke patients increased on the following days and were highest on days three and four

(day 3: 0.11 (IQR 0.078-0.153) ng/ml, day 4: 0.10 (IQR 0.07-0.16) mg/l). Compared with control subjects the increase in PCT values was not statistically significant (see figure 5, Kruskal-Wallis test, p= *0.0227*; Dunn's multiple comparison test: no group differences). Compared with admission values, the increase in PCT values on the days one through five were significant in stroke patients (see Figure 5, Friedman test with Dunn's multipe comparison test; $p < 0.0001$).

While PCT and CRP concentrations increased after stroke and 8 patients showed clinical signs of infection only one patient with a pneumonia fulfilled all our predefined criteria for a systemic infection and was considered to have a stroke associated infection. 22 patients showed no signs of infection between admission and day seven. The remaining patients met only one or two predefined criteria and therefore their infectious status was classified as undetermined. While only one patient met our criteria for a stroke-associated infection, in total 15 patients were treated with antibiotics during the trial at the discretion of the stroke team. The indications, as documented by the treating physicians, were pneumonia in 5 patients, urinary tract infection in 8 patients, including one patient diagnosed with urosepsis and suspected infection without a focus in 2 patients.

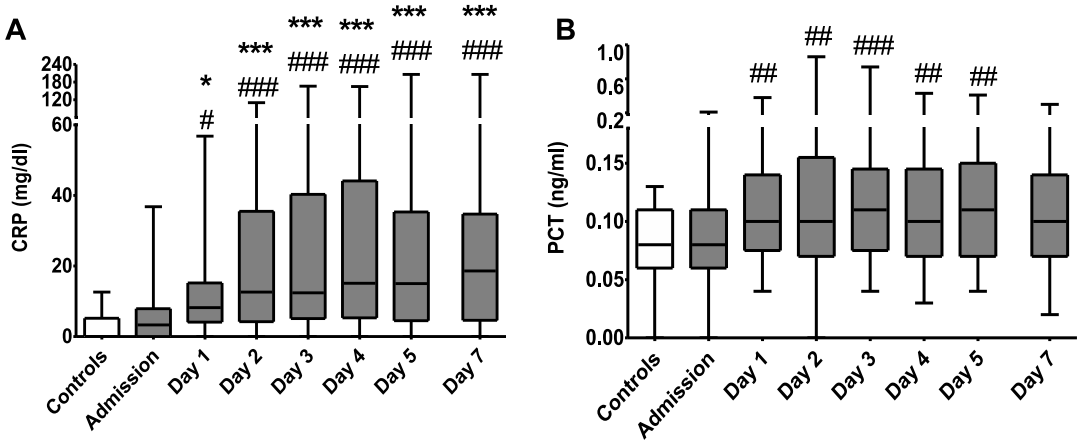

Figure 5: Median A: CRP and B: PCT values for stroke patients (grey boxes) and controls (white box) (for clarity purposes PCT values of one patient, which were higher than 1.0 ng/l are omitted. These values were 3.0 ng/ml on day 2, 3.6 ng/ml on day 3, 2.8 ng/ml on day 4, 1.7 ng/ml on day 5). Statistical differences between patients and controls are denoted as follows: $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$. Statistical significant differences between admission and days one to seven were marked as following $\#\#\#$ = $p < 0.001$, $\#\#$ = $p < 0.01$, $\#$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. The y-axis in A and B are split and both segments use different scaling.

3.2.5 Changes in Peripheral Blood Immune Cells

Among our stroke patients we found the changes in immune cells that have been described previously in other stroke trials [17, 35, 36]. We could reproduce the changes that have been described as typical for post stroke immune alterations and post stroke immunodepression.

3.2.6 Peripheral Blood Leukocyte Values

After stroke, patients had a trend toward increased median leukocyte counts on admission and days one through seven compared to control subjects, though the difference between controls and stroke patients did not reach statistical significance (see figure 6, comparison between patients and controls: Kruskal-Wallis Test, $p > 0.0561$; comparison between admission values and the following days: Friedman test, $p = 0.3310$). The median leukocyte count on admission for stroke patients was 9.61 (IQR 7.10-10.70) Gpt/l and then declined to 8.44 (IQR 6.90-10.80) Gpt/l on day seven. Control subjects had a median leukocyte count of 6.48 (IQR 5.51-7.60) Gpt/l.

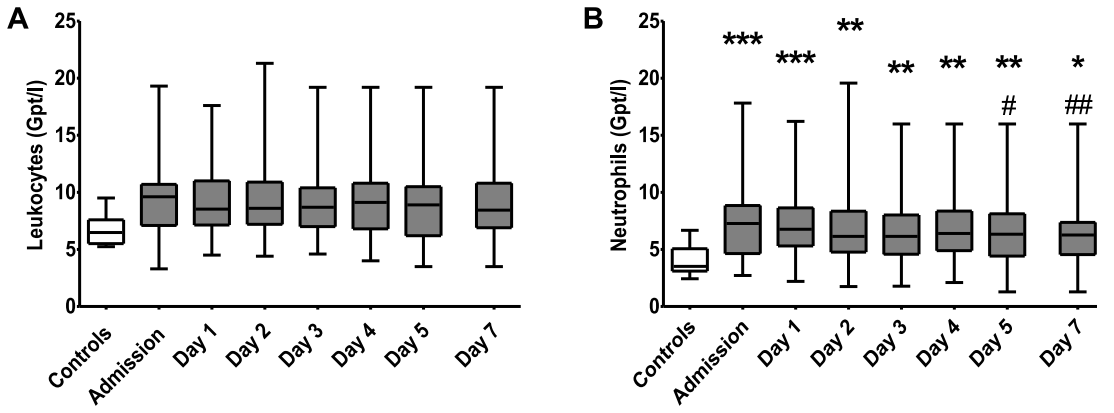

Figure 6: Median A: leukocyte and B: neutrophil values for stroke patients (grey boxes) and controls (white box). Statistical differences between patients and controls are denoted as follows: $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$. Statistical significant differences between admission and days one to seven, were marked as following $\#\#\#$ = $p < 0.001$, $\#\#$ = $p < 0.01$, $\#$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values.

3.2.7 Peripheral Blood Neutrophil Values

Median neutrophil counts were increased among stroke patients compared to control subjects (see figure 6, Kruskal-Wallis test with Dunn's multiple comparison test,

$p < 0.0041$). The median neutrophil count for stroke patients on admission was 7.28 (IQR 4.64-8.85) Gpt/l and decreased between admission and day seven to a median count of 6.26 (IQR 4.55-7.37) Gpt/l. The median neutrophil count of control subjects was 3.52 (IQR 3.10-5.06) Gpt/l. For stroke patients the difference between median neutrophil counts on admission and median values on the following days were significant for the days five and seven (Friedmann test with Dunn's multiple comparison test, $p = 0.0080$) .

3.2.8 Peripheral Blood Lymphocyte Values

Median CD4+-T-lymphocyte values were significantly lower among stroke patients immediately after stroke compared to control patients (see figure 7, Kruskal-Wallis test with Dunn's multiple comparison test, $p < 0.0024$, for comparison with controls, Friedmann test with Dunn's multiple comparison test, $p = 0.0024$, for comparison between admission and days one to sevenin stroke patients). The Median CD4+-T-lymphocyte count for stroke patients was 0.55 (IQR 0.37-0.90) Gpt/l on admission day and 0.64 (0.44-0.84) Gpt/l on day one. The median counts increased up to 0.69 (0.55-0.90) Gpt/l on day seven, control subjects had a median CD4+-T-lymphocyte count of 0.92 (IQR 0.75-1.09) Gpt/l.

Median CD8+-lymphocyte counts did not differ between stroke patients and controls or between admission and the following days(see figure 7, Kruskal-Wallis test, *p ¿ 0.53* for comparison between stroke patients and controls, Friedmann test, $p = 0.1922$ for comparison between admission and days one to seven in stroke patients). The median CD8+-T-lymphocyte count was 0.26 (IQR 0.20-0.38) Gpt/l on admission, which increased to 0.34(IQR 0.26-0.41) Gpt/l on day seven after stroke. Control subjects had a median value of 0.37(IQR 0.23-0.56) Gpt/l.

Median NK-cell values did not differ between stroke patients and controls or admission and the following days(see figure 7, Kruskal-Wallis test, $p > 0.18$, for comparison between stroke patients and controls, Friedmann test, $p = 0.7652$ for comparison between admission and days one to seven in stroke patients). The median values of stroke patients ranged between 0.23 (IQR 0.13-0-31) Gpt/l on admission to 0.17 (IQR: 0.13-0.27) Gpt/l on day three. The median value of control subjects was 0.33 (IQR 0.16-044) Gpt/l.

While there were differences in the lymphocyte subtypes, median total lymphocyte values on all days after stroke and control subject values were similar. On admission median total lymphocyte count in stroke patients was 1.50 (IQR 0.99-2.03) Gpt/l and the total lymphocyte counts on all other days were similar with median values between 1.50 (IQR 1.11-1.89) Gpt/l on day one and 1.62 (IQR 1.26-

2.08) Gpt/l on day three. Control subjects had a median total lymphocyte count of 1.76 (1.56-2.26) Gpt/l (see figure 7, Krustal-Wallis test, $p > 0.48$, for comparison between stroke patients and controls, Friedmann test, $p = 0.0586$, for comparison between admission and days one to seven in stroke patients).

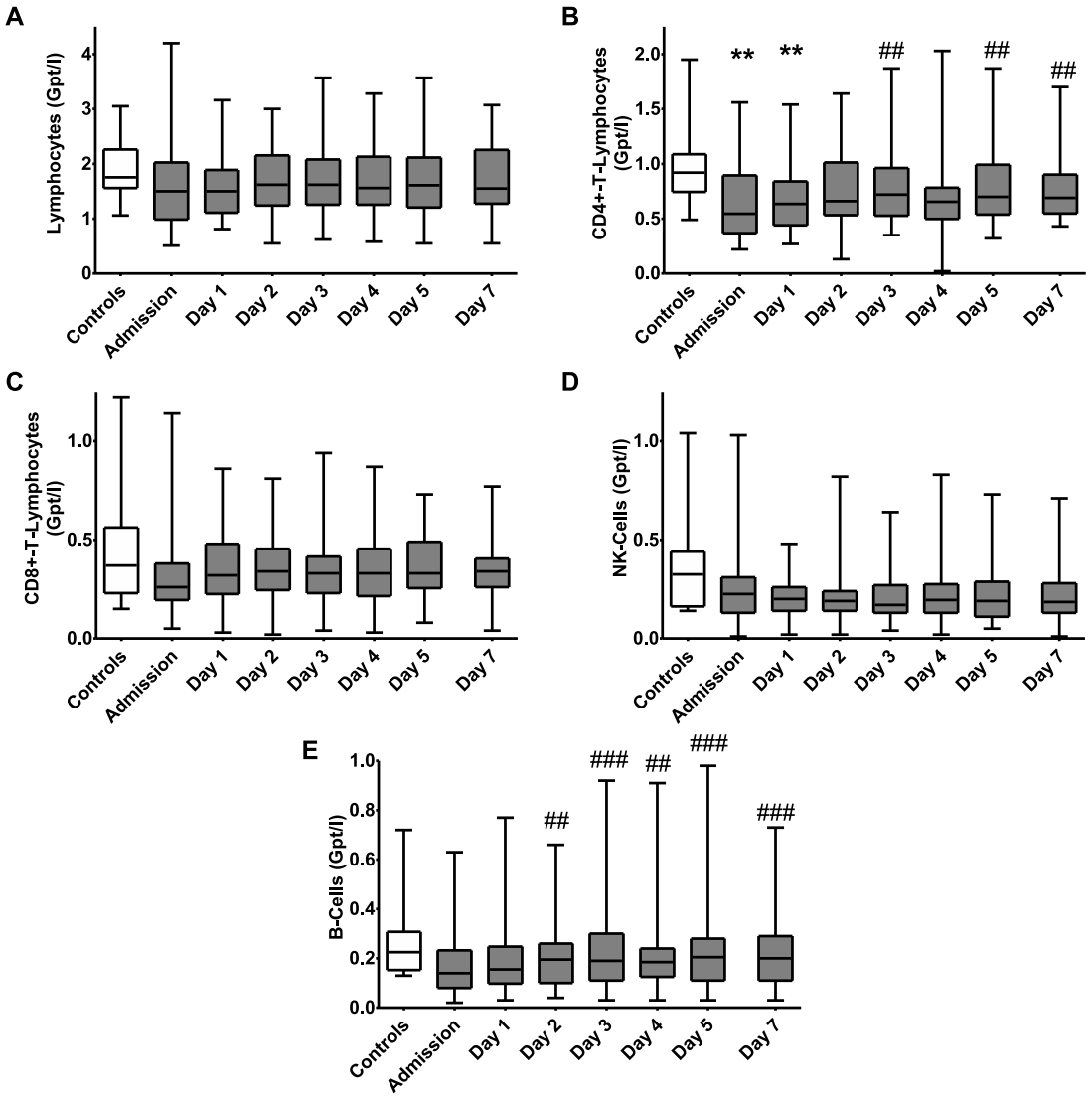

Figure 7: Median A: total lymphocyte, B: CD4+-T-cell, C: CD8+-T-cell, D: NK-cell and E: B-cell values for stroke patients (grey boxes) and controls (white box). Statistical differences between patients and controls are denoted as follows: $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$. Statistical differences between admission and days one to seven were marked as following $\#\#\#$ = $p < 0.001$, $\#\#$ = $p < 0.01$, $\#$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values.

There was a trend towards lower median B-cell values in stroke patients compared to control subjects early after stroke (see figure 7, Kruskal-Wallis test $p >$

0.15). The median admission value in stroke patients was 0.14 (IQR 0.08-0.23) Gpt/l, the median control value was 0.23 (IQR 0.15-0.31) Gpt/l . Median B-cell counts increased in stroke patients on the days following stroke up to a value of 0.20 (IQR 0.11-.029) Gpt/l on day seven, after day two, the difference between these values and the admission values were statistically significant (Friedmann test with Dunn's multiple comparison test, $p < 0.0001$).

3.3 Serum Cytokine Values

Cytokines were measured on the luminex system, which allow for a multiplex approach. Four samples gave positive readings on the negative control beads, one sample was hemolyzed. These five samples were excluded from further analysis.

3.3.1 Serum Adipokine Values

Median admission values for leptin, a marker for obesity and major regulator of energy expenditure, were similar among controls (6.04 (IQR 3.65-8.94) ng/ml) and stroke patients (5.38 (IQR 2.96-11.06) ng/ml) (see figure 8, Kruskal-Wallis test, $p = 0.7585$). In stroke patients median leptin values increased slightly from day one (8.22 (IQR 1.72-11.04) ng/ml) to day three (8.61 (IQR 2.66-16.09) ng/ml). The difference between Admission and day one and day three were statistically significant (see figure 8, Friedman test with Dunn's multiple comparison test, $p = 0.0008$). Leptin values returned to baseline values on day five (6.55 (IQR 2.56-14.40) ng/ml). Median values for resistin, a pro-inflammatory adipokine, did not differ between stroke patients and controls or between admission and the following days(see figure 8, Kruskal-Wallis test, $p > 0.1070$ for comparison between stroke patients and controls, Friedmann test, $p = 0.0996$ for comparison between admission and days one to seven in stroke patients).

Median values for adiponectin, an adipokine which negatively correlates with obesity and is implicated in improving insulin resistence, did not differ between controls (284.7 (IQR 165.5-415.0) µg/ml) and stroke patients (admission: 212.0 (IQR 144.0-343.0) µg/ml) (see figure 8, Kruskal-Wallis test with Dunn's multiple comparison test, $p = 0.88$, for comparison between stroke patients and controls, Friedmann test, $p = 0.9983$ for comparison between admission and days one to seven in stroke patients).

The ratio between leptin and adiponectin was slightly increased among stroke patients and increased on day one and day three after stroke to return to baseline on day five. While there was no statistical difference between control and

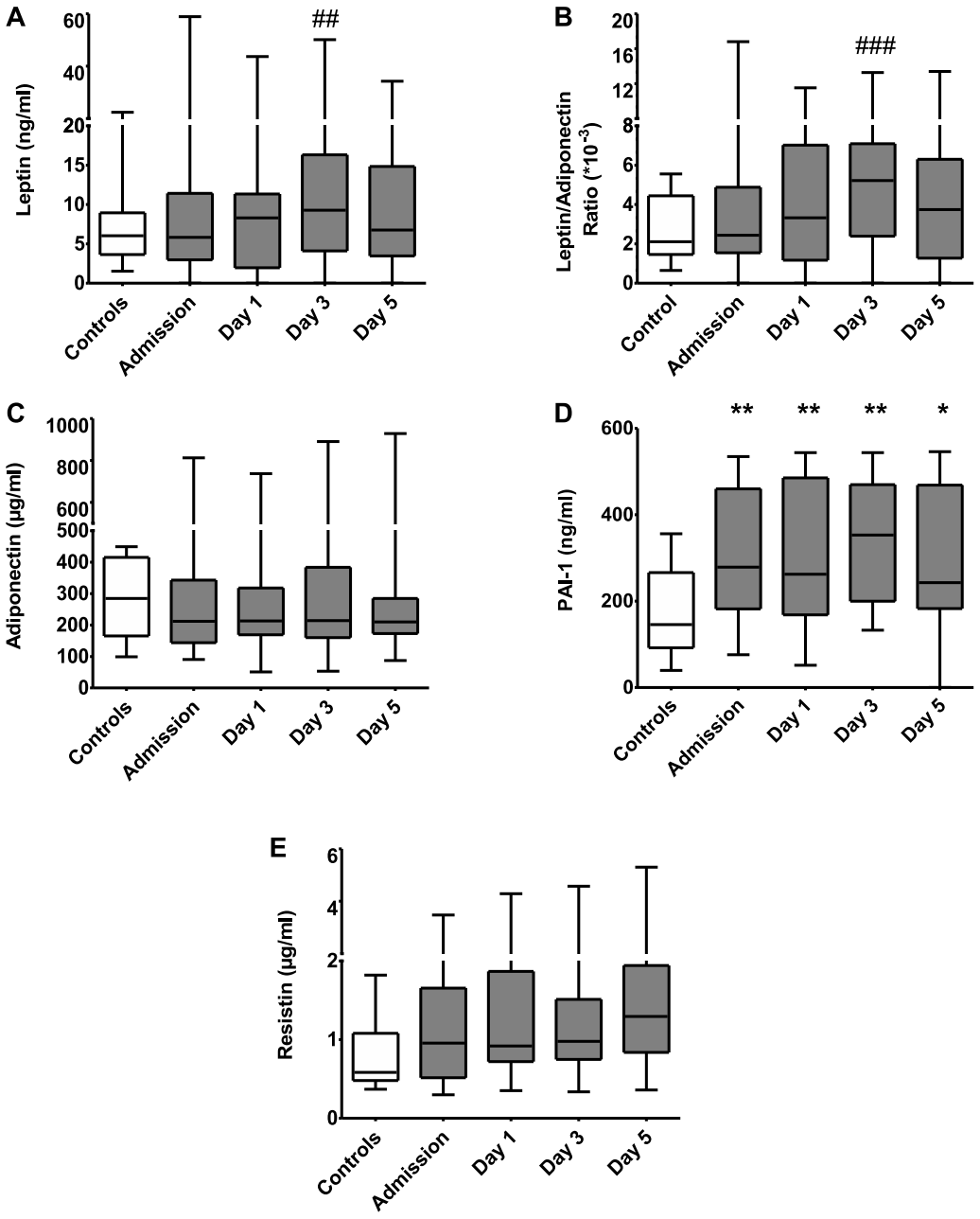

Figure 8: Median serum values of A: leptin, C: adiponectin, D: PAI-1, E: resistin and B: the leptin/adiponectin ratio for stroke patients (grey boxes) and controls (white box). Statistical differences between patients and controls are denoted as follows: $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$. Statistical significant differences between admission and days one to seven were marked as following $\#\#\#$ = $p < 0.001$, $\#\#$ = $p < 0.01$, $\#$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. The y-axis in A, B, C and E are split and both segments use different scaling.

stroke subjects (Kruskal-Wallis test, $p = 0.2815$), among stroke patients the median leptin/adiponectin-ration was higher on day three ($5.04 \cdot 10^{-5}$ (IQR $1.43 \cdot 10^{-6}$ - $6.83 \cdot 10^{-5}$)than on admission day ($2.42 \cdot 10^{-5}$ (IQR $4.19 \cdot 10^{-6}$ - $4.64 \cdot 10^{-5}$) (see figure 8, Friedman test with Dunn's multiple comparison test, $p < 0.0001$). Median values for PAI-1, a marker for obesity and a major regulator of the fibrinolytic system in stroke patients, were significantly increased compared to control subjects (see figure 8, Kruskal-Wallis test with Dunn's multiple comparison test, $p<0.0106$ for comparison between stroke patients and controls, Friedmann test, $p = 0.6448$ for comparison between admission and days one to seven in stroke patients). Median value in stroke patients on admission was 278.8 (IQR 182.0-459.8) ng/ml, compared to 145.8 (IQR 92.0-266.1) ng/ml in control subjects. Median PAI-1 values in stroke patients remained high compared to control subjects on days one, three and five.

3.3.2 Serum Pro- and Anti-Inflammatory Cytokine Values

Pro inflammatory cytokines were regulated differentially after ischemic stroke. IL-6 was increased in the stroke group in the first week after stroke. On admission the median IL-6 value (3.45 (IQR 2.02-7.13) pg/ml) was slightly larger than the control value (1.82 (IQR 0.23-3.30) pg/ml). Median IL-6 values increased on the following days up to day five (9.34 (IQR 2.04-15.76) pg/ml). On days one to five median values were significantly larger compared to control values (see figure 9, Kruskal-Wallis test with Dunn's multiple comparison test, $p < 0.0067$ for comparison between stroke patients and controls, Friedmann test with Dunn's multiple comparison test, $p = 0.0004$ for comparison between admission and days one to seven in stroke patients). The soluble IL-6 receptor (sIL-6R) was not regulated after stroke (Kruskal Wallis Test, $p = 0.6582$, for comparison between stroke patients and controls, Friedmann test, $p = 0.0872$ for comparison between admission and days one to seven in stroke patients).

The median eotaxin value decreased on all days among stroke patients, compared to control subjects (see figure 10, Kruskal-Wallis test with Dunn's multiple comparison test, $p <0.0001$ for comparison between stroke patients and controls, Friedmann test with Dunn's multiple comparison test, $p = 0 .0065$ for comparison between admission and days one to seven in stroke patients).

On admission median eotaxin levels were 59.41 (IQR 41.18-78.29) pg/ml compared to 104.7 (IQR 85.80-158.6) pg/ml among controls, the lowest median eotaxin value was on day three with 45.20 (IQR 26.15-74.84) pg/ml.

The selectins, platelet (P) and endothelial (E) selectin were decreased among stroke patients on all days compared to control subjects.

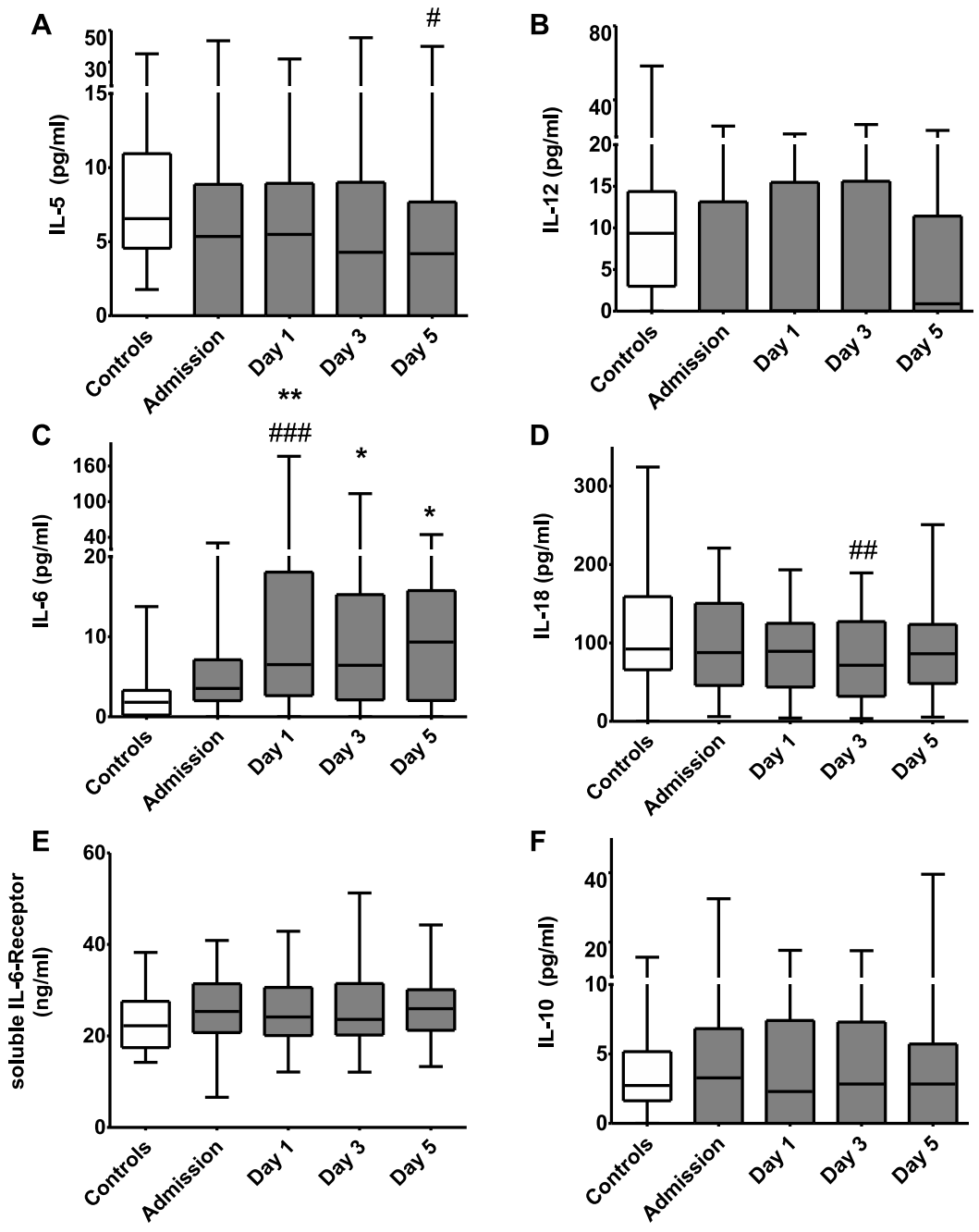

Figure 9: Median serum values of A: IL-5, C: IL-6, E: soluble IL-6-receptor, B: IL-12, D: IL-18 and F: IL-10 for stroke patients (grey boxes) and controls (white box). Statistical differences between patients and controls are denoted as follows: $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$. Statistical significant differences between admission day and days one to seven were marked as following ### = $p < 0.001$, ## = $p < 0.01$, # = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values, the y-axis for A, B, C and F are split and both segments use different scaling.

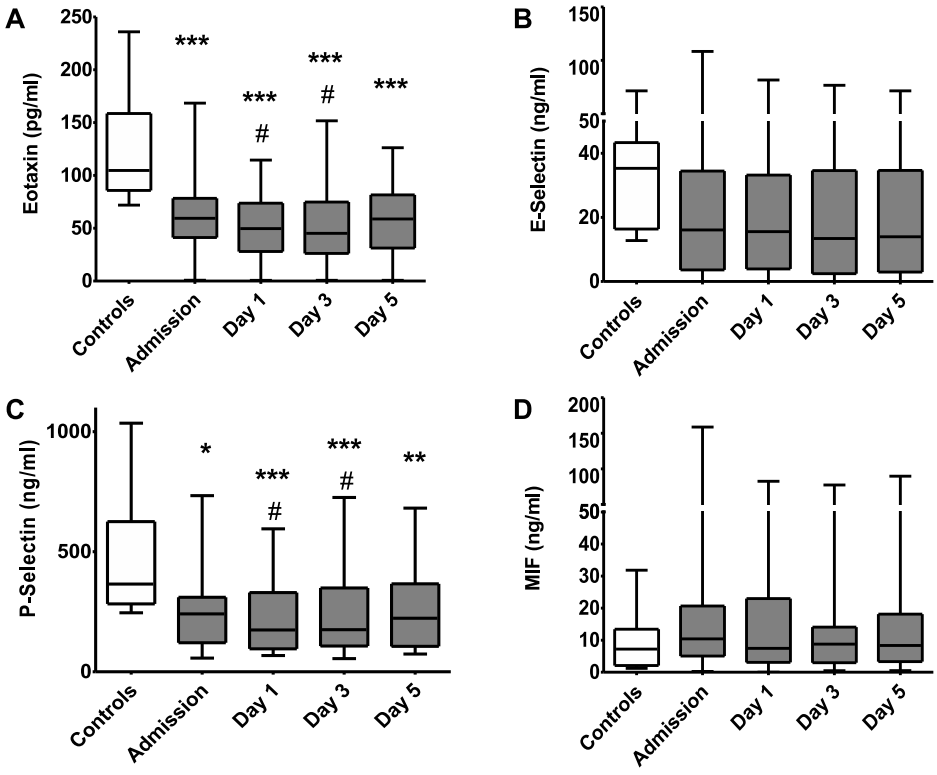

Figure 10: Median serum values of A: eotaxin, B: E-selectin, C: P-selectin and D: MIF for stroke patients (grey boxes) and controls (white box). Statistical differences between patients and controls are denoted as follows: $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$. Statistical significant differences between admission and days one to seven were marked as following $\#\#\#$ = $p < 0.001$, $\#\#$ = $p < 0.01$, $\#$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values, the y-axis for A, B, C and F are split and both segments use different scaling.

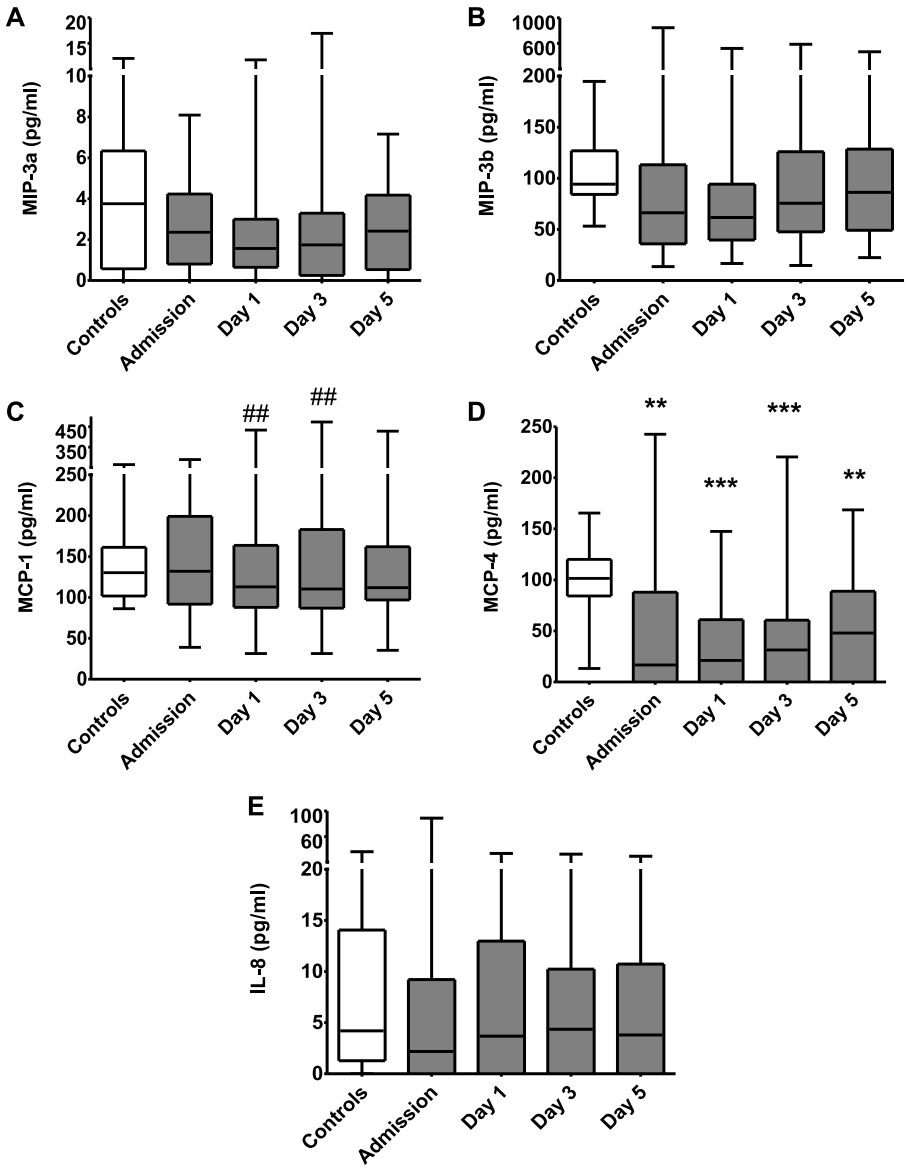

Figure 11: Median serum values of A: MIP-3a, B: MIP-3b, C: MCP-1, D: MCP-4 and E: IL-8 for stroke patients (grey boxes) and controls (white box); Statistical differences between patients and controls are denoted as follows: $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$. Statistical significant differences between admission and days one to seven were marked as following $\#\#\#$ = $p < 0.001$, $\#\#$ = $p < 0.01$, $\#$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values, the y-axis for A, B, C and E are split and both segments use different scaling.

This difference was statistically significant for P-selectin but only a trend for E-selectin (see figure 10, P-selectin: Kruskal-Wallis test with Dunn's multiple comparison test, *p = 0.0399* for comparison between stroke patients and controls, Friedmann test, with Dunn's multiple comparison test, *p = 0.0399* for comparison between admission and days one and seven in stroke patients, E-selectin: Kruskal-Wallis test, *p = 0.1095* for comparison between stroke patients and controls, Friedmann test, *p = 0.0815* for comparison between admission and days one and seven in stroke patients). The median admission value for P-selectin was 240.90 (IQR 121.03-310.22) ng/ml compared with 365.24 (IQR 282.64-625.52) ng/ml in control subjects. P-selectin values remained significantly decreased on all days after stroke.

While MCP-1 did not differ between stroke patients and controls (see figure 11, Kruskal-Wallis test, *p = 0.7143*), median MCP-1 values decreased slightly between admission and days one and three in stroke patients (Friedmann test with Dunn's multiple comparison test, *p = 0.0050*). MCP-4 is as well decreased among stroke patients immediately after stroke and in the first week after stroke compared to control subjects (see figure 11, Kruskal Wallis test with Dunn's multiple comparison test *p = 0.0009* for comparison between stroke patients and controls, Friedmann test, *p = 0.1276* for comparison between admission values and values on days one to seven in stroke patients). The median admission value in stroke patients was 16.65 (IQR 0-61.1) pg/ml, compared to 101.5 (IQR 84.3-120.1) pg/ml in control subjects. Median MCP-4 values in stroke patients increased slightly to 47.9 (IQR 0-88.9) pg/ml on day five.

The chemotactic cytokines IL-8, MIP-3α and MIP-3β remained unchanged among stroke patients. (see figure 11, IL-8: Kruskal-Wallis test, *p = 0.7494* for comparison between stroke patients and controls, Friedmann test, *p = 0.5494* for comparison between admission and days one to seven in stroke patients, MIP-3α: Kruskal-Wallis test, *p=0.4102*, for comparison between stroke patients and controls, Friedmann test, *p=0.2183* for comparison between admission and days one to seven in stroke patients, MIP-3β: Kruskal-Wallis Test, *p=0.0813* for comparison between stroke patients and controls, Friedmann test, *p=0.0006* but no statistical differences in Dunn's multiple comparison test for comparison between admission and days one to seven in stroke patients). The anti-inflammatory cytokine IL 10 was not regulated after stroke. Median values were 3.28 (IQR 0-6.85) pg/ml and 2.735 (IQR 1.64-5.17) pg/ml for stroke patients and for control patients respectively (see figure 9, Kruskal-Wallis-Test, *p = 0.9933*) and did not change on the following days for stroke patients (Friedmann test, *p = 0.7948*). Interestingly, several key inflammatory cytokines were not detectable in most stroke patients and control subjects. This group included the IL-1 receptor antagonist (with only 1 patient and 2 controls with detectable levels),

IL-1β (7 patients, 3 controls), IL-2 (1 patient, 2 controls), IL-17 (7 patients, 7 controls) and IL-17F (9 patients, 7 controls), TNF-α (5 patients, 4 controls), interferon-β (3 patients, 2 controls), interferon-γ (6 patients, 3 controls) and GM-CSF (19 patients, 4 controls with detectable levels).

3.3.3 Long Term Results

As part of the study design long term follow up visits up to three months were planned. After enrolling 30 patients in total, we stopped the long term follow up visits, because patients who attended follow up visits were those only mildly affected at hospital discharge, while patients lost to follow up were those that had severe symptoms at hospital discharge. A preliminary analysis showed that patients attending (n = 12) and missing the visits (n = 12) were not comparable, resulting in a skewed patient selection. Patients that attended the follow up visits had less severe infarcts with lower NIHSS scores and NIHSS scores decreased faster during the early phase after the stroke (see figure 12). Accordingly patients attending the follow up visits had a median Bartel Index score of 100 (IQR 92.5-100; Minimum: 30) on day 30 and 100 (IQR 100-100; Minimum 45) on days 90 and 180. Only 3 Patients had low Bartel Index scores and all but one of those patients were lost to follow up after the 30 day study visit as well.

According to the data for the first 30 patients median NIHSS values were higher for patients lost to follow up. NIHSS on admission for patients attending follow up was 14 (IQR 11-21.25) compared to 17.5 (IQR 13-21) for patients lost to follow up. On day 1 and later patients lost to follow up had significantly higher NIHSS values (see figure 12, Kruskal-Wallis test with Dunn's multiples comparisons test, $p < 0.0001$). The median NIHSS Score on day one was 6.5 (IQR 2-12.75) compared to 15.5 (IQR 12.25-19.75) and on day seven the NIHSS scores were 2.5 (IQR 0.25-5.75) compared to 10.0 (IQR 8.25-14.75) for patients who attended follow up and patients who were lost to follow up respectively. Immunological parameters were different as well. Patients lost to follow up were older (61.1 ± 17.3 versus 74.5 ± 10.7) tended to have a higher BMI and CRP values, higher neutrophil counts and lower CD4+T-lymphocyte counts, though these differences failed to reach statistical significance (Data not shown, Age and BMI: two tailed T-Test, Age: $p = 0.0746$, BMI: $p = 0.6805$; CRP, neutrophiles and CD4+-T-lymphocytes Kruskal Wallis Test, CRP: $p = 0.4155$, neutrophils: $p = 0.0446$ Dunn's multiple comparisons test: no significant difference for values on the same day, CD4+-T-lymphocytes: $p = 0.6308$). For the 24 patients that attended the follow up visits there was a trend to higher CRP values for the days two to seven after stroke and a trend towards higher neutrophile counts

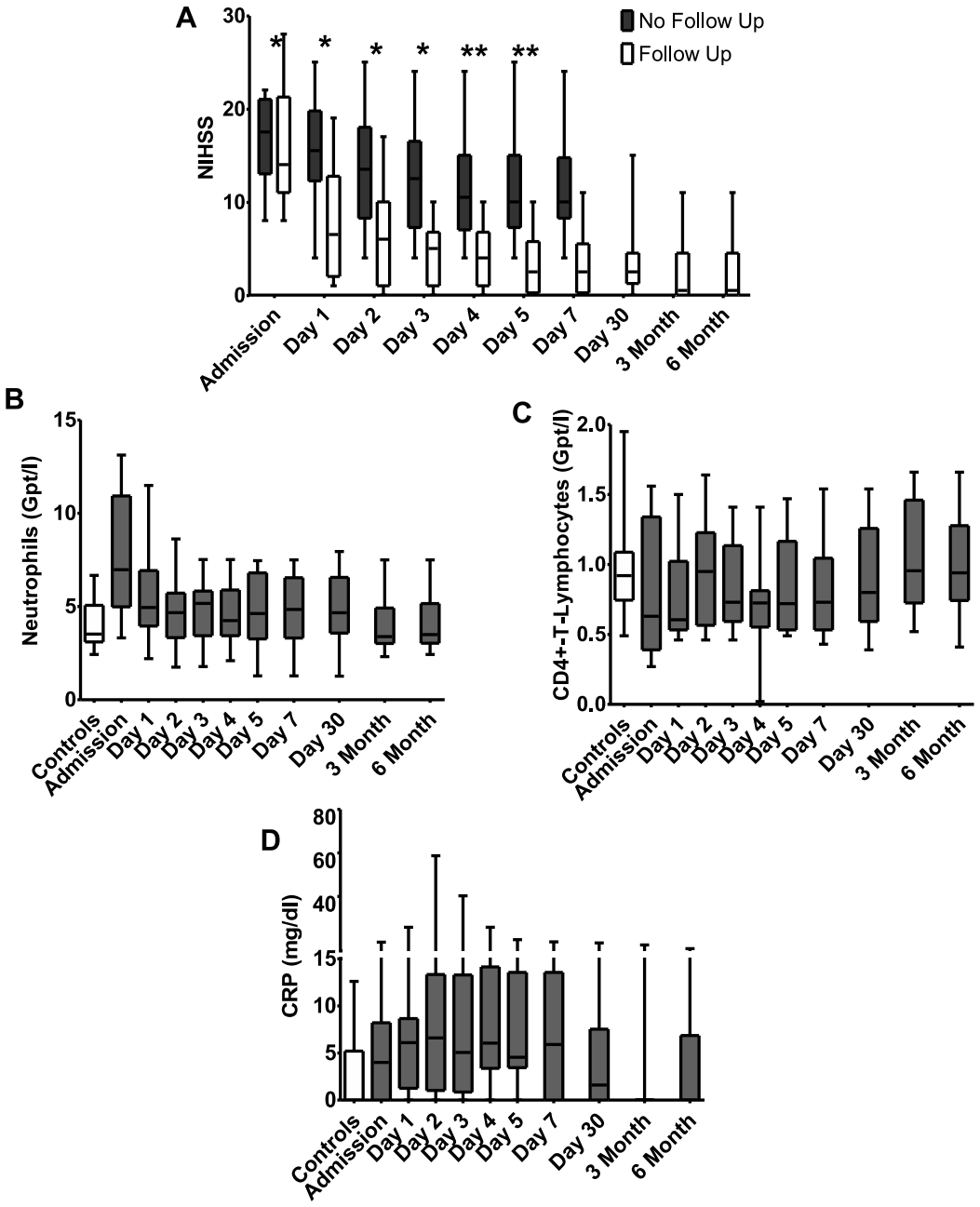

Figure 12: Overview of the results of the follow up examinations. A: Patients who attended the planned follow up examinations recovered faster and had less symptoms on day seven as measured by the NIHSS. Statistical differences on each day are annotated with $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$. Median B: Neutrophil values, C: CD4+-T-lymphocyte counts and E: CRP values for patients who attended the long term follow up visits (n=24, grey boxes) compared with control subjects (n=16, white box), there were no statistically significant differences between stroke patients and control subjects; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values, the y-axis for figure E is split and both segments use different scaling.

immediatly after stroke compared to control patients, but these changes were not significant. CD4+-T-cells were similar for stroke patients and control subjects (see figure 12, Kruskal-Wallis test with Dunn's multiple comparison test, CRP: *p = 0.0151*, Dunn's test: no statistical difference between control values and stroke patients, neutrophiles: *p = 0.0667*, CD4+-T-lymphocytes: *p = 0.4597*).

3.4 Influence of Weight and BMI on Immunological Changes

To test the hypothesis that body weight and especially obesity influences the immunological changes after stroke, we analyzed the immunological parameters stratified to the body mass index of our stroke patients. Patients were grouped into three groups. Normal weight patients (*n*=11) were those with a BMI below 25 , overweight patients (*n*=14) were those with a BMI between 25 and 30 and obese patients (*n*=15) were those with a BMI equal to or greater than 30. An overview of their baseline statistics can be found in table 6. Subjects of normal weight were older, had a higher rate of hypertension and were least likely to receive thrombolysis or mechanical thrombectomy. Overweight subjects had slightly higher NIHSS stroke scale scores compared with subject of normal weight or obese subjects (see table 6).

Table 6: Baseline statistics of BMI Subgroups

Variable	Normal weight (*n* = 11)	Overweight (*n*= 14)	Obese (*n* = 15)
Age [Years, Mean±Std.]	76 ± 17	72 ± 11	66 ± 14
Sex [as % female]	55%	57%	53%
BMI [kg/m^2, Median, (IQR)]	22.8 (22.1-24.4)	27.0 (25.9-29.1)	32.0 (30.8-36.8)
Co-morbidities			
Hypertension [n (%)]	10 (90.9)	11 (78.6)	10 (66.7)
Diabetes mellitus [n (%)]	1 (9.1)	2 (14.3)	3 (20.0)
Stroke Characteristica			
Initial NIHSS [Median (IQR)]	15 (12-21)	18 (12.75-22)	13 (11-18)
Infarct Side [n (%) left sided infarcts]	8 (72.7)	6 (42.9)	6 (40.0)
Treatment [n (%)]	6 (54.6)	11 (78.6)	13 (86.7)

3.4.1 Influence of BMI on Acute Phase Proteins in Stroke Patients

While CRP and PCT values where higher among patients of normal weight on days two to four after stroke, the difference failed to reach statistical significance (see figure 13, CRP: Kruskal-Wallis test, *p = 0.0051*; Dunn's multiple comparison

test: no statistical significant difference between groups; PCT: Kruskal-Wallis test, *p = 0.0013*; Dunn's multiple comparison test: No statistical significance between groups). The only patient fulfilling our criteria for certain infection had a normal weight (BMI 24.1 kg/m^2). On admission patients of normal weight had a median CRP value of 0.0 (IQR 0-7.29) mg/l, overweight patients had a median CRP value of 4.6 (IQR 0-15.9) mg/l and obese patients had a median CRP value of 4.0 (IQR 0-6.1) mg/l. These values increased up to a maximum on day four. On day four, the median CRP value of patients of normal weight was 34.2 (IQR 5.5-75.3) mg/l, the median CRP value of overweight patients was 13.7 (IQR 5.5-47.3) mg/l and the CRP value of obese patients was 11.2 (IQR 4.4-22.1) mg/l .

Median PCT values on admission were 0.07 (IQR 0.04-0.09) ng/dl, 0.085 (IQR 0.053-0.173) ng/dl and 0.08 (0.06-0.1) ng/dl for patients of normal weight, overweight and obese patients respectively. These values increased slightly up to day three, with median values of 0.12 (IQR 0.11-0.4) ng/dl, 0.11 (0.075-0.185) ng/dl and 0.09 (IQR 0.06-0.11) ng/dl for patients of normal weight, overweight and obese patients respectively and stayed there up to day seven .

3.4.2 Effect of BMI on Immunological Changes in Stroke Patients

No statistically significant differences in immune cell counts after stroke between the BMI subgroups were found. CD4+-T-cells and neutrophils, which were the cell types with the largest changes after stroke in our patients compared to control subjects, showed no significant differences between the different BMI subgroups. If anything, overweight and not obese patients had the highest neutrophil and lowest CD4+-T-cell counts counts compared to patients with normal weight (see figures 13 and 14).

3.4.3 Peripheral Blood Leukocyte Values

Median leukocyte counts were not statistically different between BMI subgroups on any day after stroke (see fig 13, Kruskal-Wallis Test, *p = 0.9991*). Median admission values were 8.89 (IQR 7.90-11.40) Gpt/l, 9.96 (IQR 6.49-12.65) Gpt/l and 9.61 (IQR 7.00-10.40) Gpt/l for patients with normal weight, overweight and obese stroke patients respectively.

3.4.4 Peripheral Blood Neutrophil Values

On admission overweight patients had a slightly higher median neutrophil count, with 8.79 (IQR 4.00-11.15) Gpt/l compared to either patients of normal weight or obese patients with 6.91 (IQR 5.42-9.58) Gpt/l and 7.22 (IQR 5.05-7.74) Gpt/l respectively.

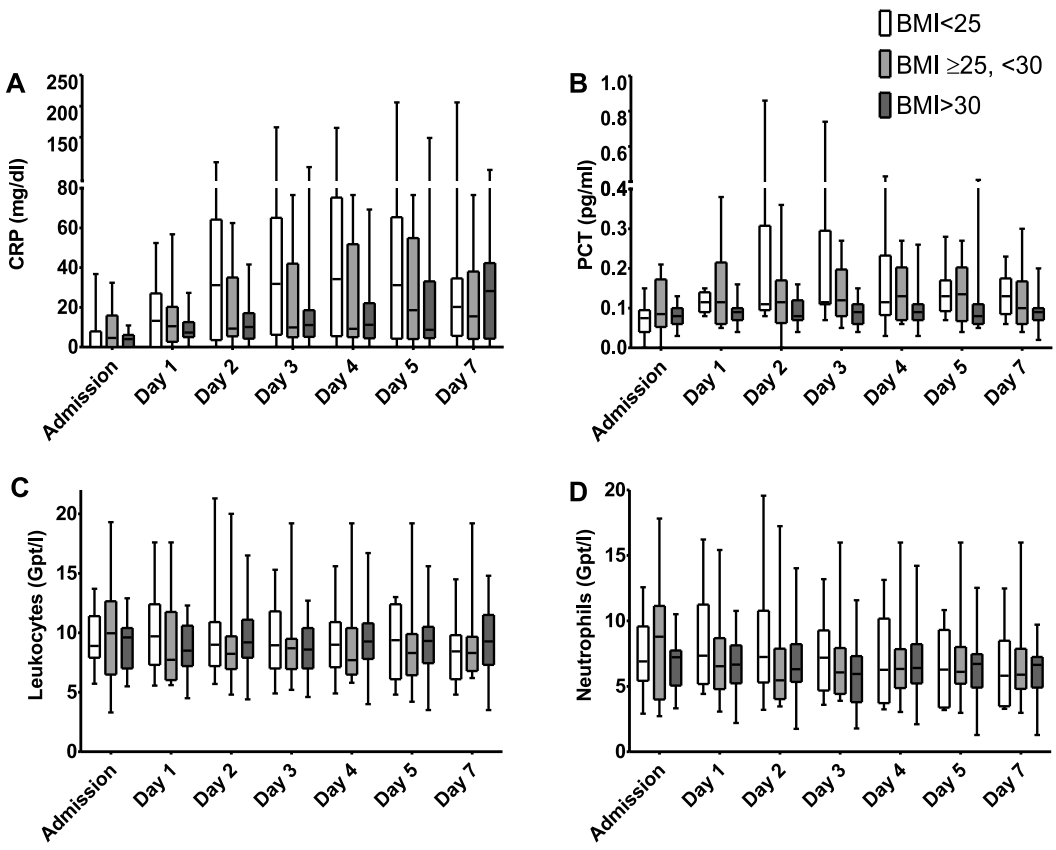

Figure 13: Median A: CRP and B: PCT Serum values for different BMI-subgroups (BMI<25, BMI above 25 but below 30, BMI >30). C: leukocyte and D: neutrophil counts for different BMI-subgroups. There were no statistical differences between BMI subgroups. Shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. In A and B the y-axis is split and both segments use different scaling.

But these values converged up to day seven, where they were nearly identical with values of 5.49 (IQR 3.53-7.89) Gpt/l, 5.52 (IQR 4.67-7.57) Gpt/l and 6.64 (IQR 4.90-7.24) Gpt/l for patients of normal weight, overweight and obese patients respectively. These differences in neutrophil counts never reached statistical significance (see fig 13, Kruskal Wallis test, *p = 0.9819*).

3.4.5 Peripheral Blood Lymphocyte Values

Overweight patients had slightly lower median CD4+-T-lymphocyte counts (0.41 (IQR 0.27-0.73) Gpt/l for overweighted versus 0.74 (IQR 0.36-0.97) Gpt/l for patients of normal weight and 0.61(IQR 0.45-1.26) Gpt/l for obese subjects) and CD8+-T-lymphocyte counts (0.20 (IQR 0.14-0.29) Gpt/l for overweighted versus 0.26 (IQR 0.12-0.53) Gpt/l for normal weighted and 0.35 (IQR 0.25-0.43) Gpt/l for obese patients) on admission compared to patients of normal weight or obese patients, but these differences were likewise not statistically significant (see figure 14, CD4+: Kruskal Wallis test, *p = 0.5848*; CD8+: Kruskal-Wallis test, *p = 0.0240*; Dunn's multiple comparison test: No significance between groups).

On admission median NK cell counts were non-significantly higher in normal weighted patients compared to overweighted or obese patients, 0.36 (IQR 0.23-0.74) Gpt/l for normal weigthed patients versus 0.16 (IQR 0.10-0-24) Gpt/l and 0.18 (IQR 0.13-0.28) Gpt/l for overweighted and obese patients, respectively (see figure 14, Kruskal-Wallis test, *p = 0.2787*). Initial B-cell counts were slightly but not significantly lower in overweighted patients compared to normal weighted or obese patients, with a median B-cell counts of 0.11(IQR 0.07-0.17) Gpt/l for overweighted, 0.13 (IQR 0.07-0.31) Gpt/l for normal weighted and 0.17 (IQR 0.12-0.24) Gpt/l for obese patients (see figure 14, Kruskal-Wallis test, *p = 0.8842*).

3.5 Influence of BMI on Cytokines in Stroke Patients

3.5.1 Adipokines

Median leptin values were non-significantly higher in overweight and obese patients on all days after stroke, but obese and overweight patients had similar leptin values on all days. On admission median leptin values were 3.19 (IQR 0.39-6.71) ng/ml for patients of normal weight, 7.74 (IQR 2.96-12.60) ng/ml for overweight and 6.08 (IQR 4.07-13.02) ng/ml for obese patients (see figure 15, Kruskal-Wallis test, *p = 0.2091*). When compared over all BMI values, on admission leptin values correlated moderately with the BMI (r^2 = 0.26; *p = 0.0012*). Median resistin values were similar in all patient groups and on all days after stroke. On admission median resistin

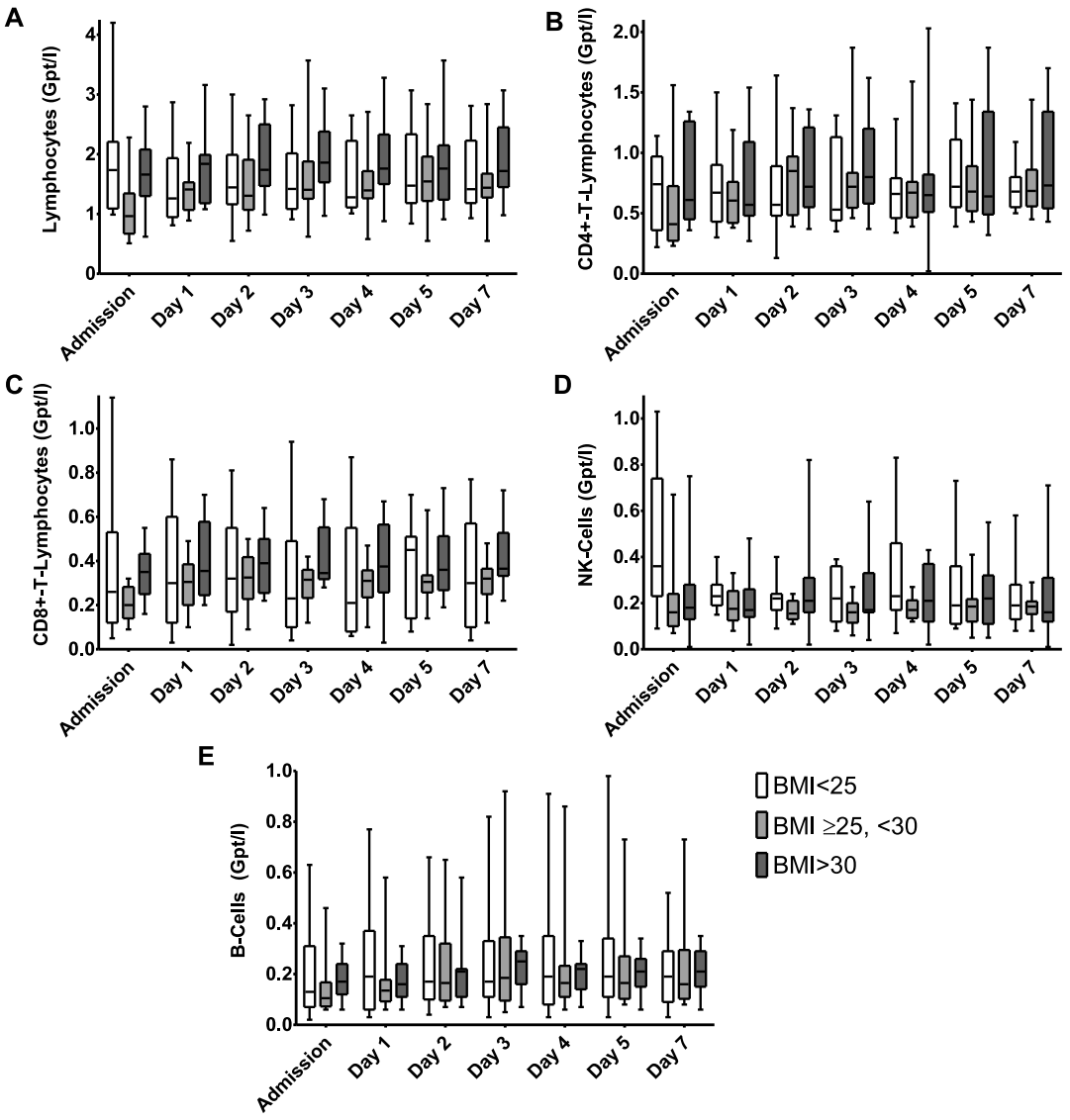

Figure 14: Median cell counts for A: total lymphocytes, B: CD4+-T-lymphocytes, C: CD8+-T-lymphocytes, D: NK-cells and E: B-cells for different BMI-subgroups (BMI$<$25, BMI above 25 but below 30, BMI $>$30). There were no statistical significant differences between BMI subgroups. Shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values.

values were 1.29 (IQR 0.49-1.94) µg/ml, 0.87 (IQR 0.49-1.50) µg/ml and 0.95 (IQR 0.58-1.22) µg/ml for patients of normal weight, overweight and obese patients respectively (see figure 15, Kruskal Wallis test; *p=0.7397*), accordingly resistin values did not correlate with BMI values on admission or on any other day.

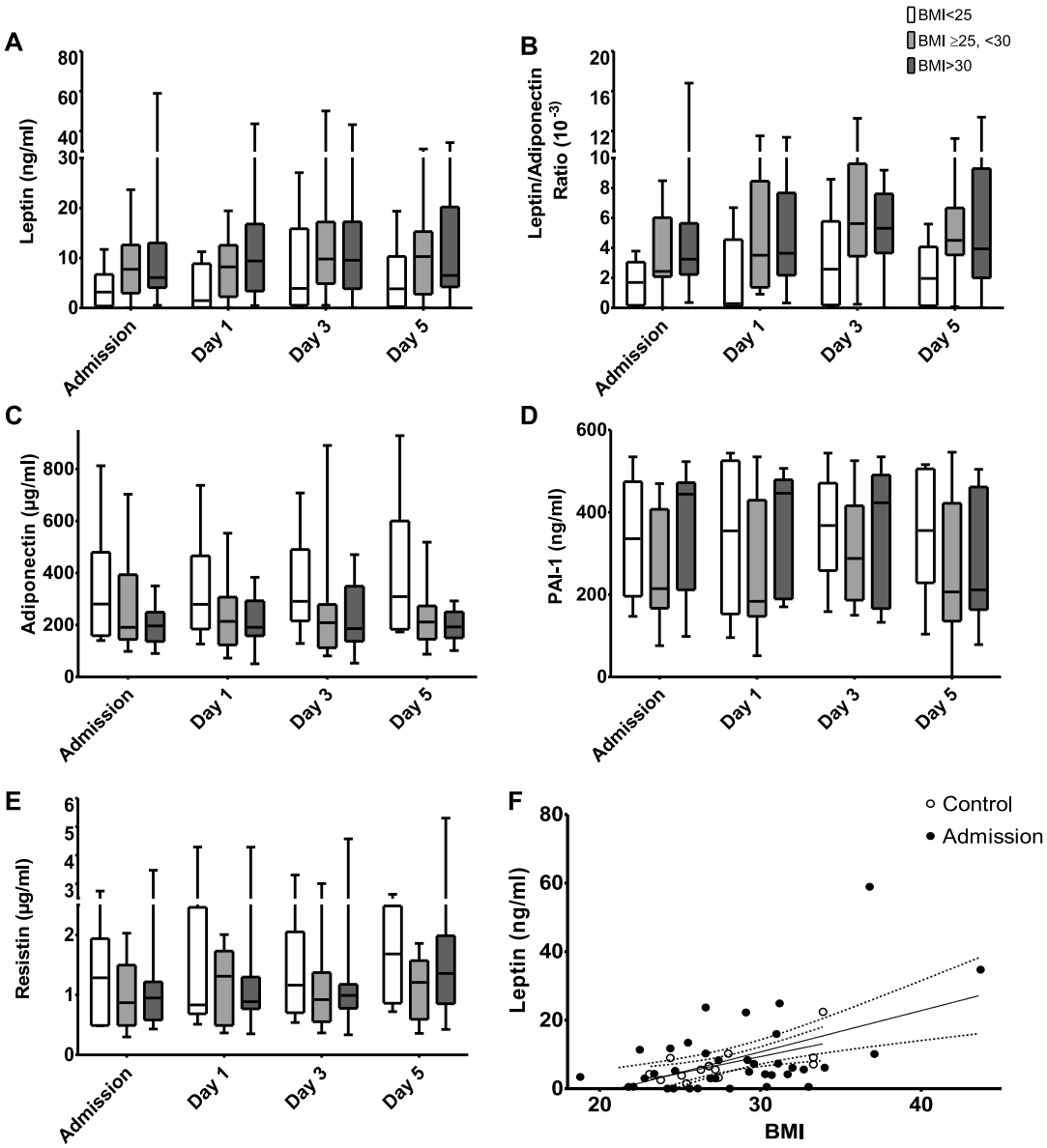

Figure 15: Serum values of A: leptin, C: adiponectin, D: PAI-1, E: resistin and B: the leptin/adiponectin ratio for different BMI-subgroups (BMI$<$25, BMI above 25 but below 30, BMI $>$30). There were no significant differences between BMI subgroups. Shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. The y-axis of A, B, C and E were split and both segments use different scaling. F: Correlation between admission BMI and leptin. Stroke patients: r^2 = 0.26, for control patients r^2 = 0.44.

Median adiponectin values did not differ between normal weighted, overweighted or obese patients. Overweighted and obese patients had similar median adiponectin values on all days after stroke, on admission the median values were 280.60 (IQR 158.70-479.60) µg/ml, 190.80 (IQR 144.40-393.40) µg/ml and 196.20 (IQR 136.50-249.10) µg/ml, respectively (see figure 15, Kruskal Wallis test; *p = 0.2641*). Admission values of adiponectin did not correlate with BMI values (r^2 = 0.096). On all days after stroke median PAI-1 values were similar for all BMI subgroups. Median PAI-1 values on admission were 335.88 (IQR 196.12-474.28) ng/ml, 214.57 (IQR 166.77-407.18) ng/ml and 444.09 (IQR 211.72-471.96) ng/ml for normal weighted, overweighted and obese patients, respectively (see figure 15, Kruskal-Wallis test; *p = 0.5059*).

3.5.2 Pro- and Anti-Inflammatory Cytokines

The pro- and anti-inflammatory cytokines in our panel did not show statistically significant differences between BMI subgroups. Median IL-6 values were not different early after stroke. On admission the median IL-6 value for patients of normal weight was 3.97 (IQR 1.99-10.03) pg/ml, 4.19 (IQR 2.34-12.58) pg/ml for overweighted and 3.54 (IQR 1.21-4.85) pg/ml for obese patients. On day five overweighted patients had slightly higher median IL-6 values than either patients of normal weight and obese patients, with 13.45 (IQR 5.45-19.89) pg/ml versus 6.42 (IQR1.42-22.48) pg/ml and 4.03 (IQR 0-12.46) pg/ml for patients of normal weight and obese patients. None of these differences were statistically significant (see figure 16, Kruscal Wallis test, *p = 0.076*).

Similarly median sIL6-R values did not differ between the BMI-subgroups. On admission the median sIL-6R value for patients of normal weight was 22.69 (IQR 20.45-32.40) ng/ml, 26.02 (IQR 19.37-31.07) ng/ml for overweighted and 25.98 (22.96-33.17) pg/ml for obese patients. These values did not change much during the course of this trial (see figure 16, Kruskal Wallis test, *p = 0.8969*).

Median eotaxin values did not show significant differences between BMI subgroups. Patients of normal weight had a median admission value of 77.9 (IQR 21.5-113.8) pg/ml, overweighted median admission value was 61.56 (IQR 56.7-83.2) pg/ml and obese patients median admission value was 46.9 (IQR 24.2-70.1) pg/ml (see figure 17, Kruskal-Wallis test, *p = 0.0089*, no significant group differences in Dunn's multiple comparison test).

Likewise the selectins P-selectin and E-selectin did not show significant differences between the BMI subgroups and median values stayed fairly constant during the trial.

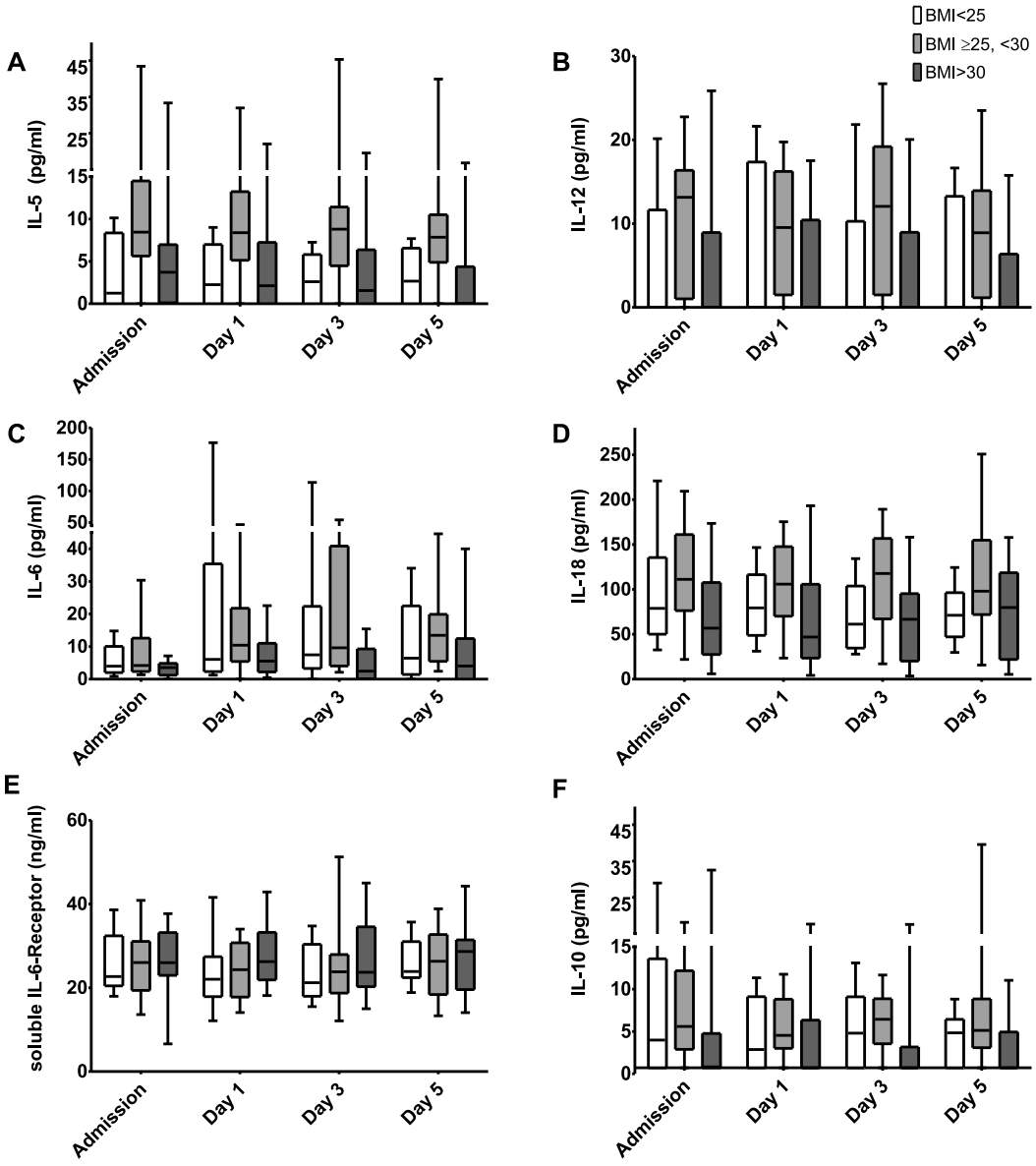

Figure 16: Median serum values of A: IL-5, B: IL-12, C: IL-6, D: IL-18, E: sIL-6R and F: IL-10 for different BMI-subgroups (BMI$<$25, BMI above 25 but below 30, BMI $>$30). There were no significant statistical differences between BMI subgroups. Shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values, the y-axis of A, C and F were split and both segments use different scaling.

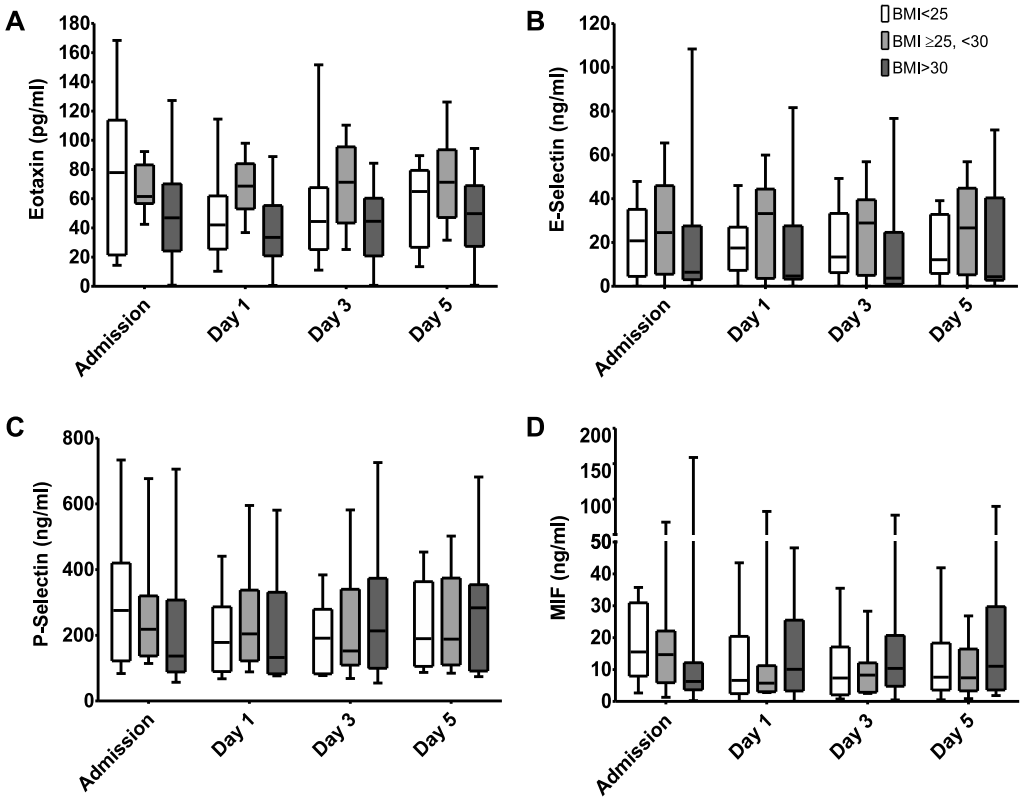

Figure 17: Serum Values of A: eotaxin, B: E-selectin, C: P-selectin and D: MIF between different BMI-subgroups (BMI<25, BMI above 25 but below 30, BMI >30). There were no significant statistical differences between BMI subgroups. Shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values, the y-axis of figure F is split and both segments use different scaling.

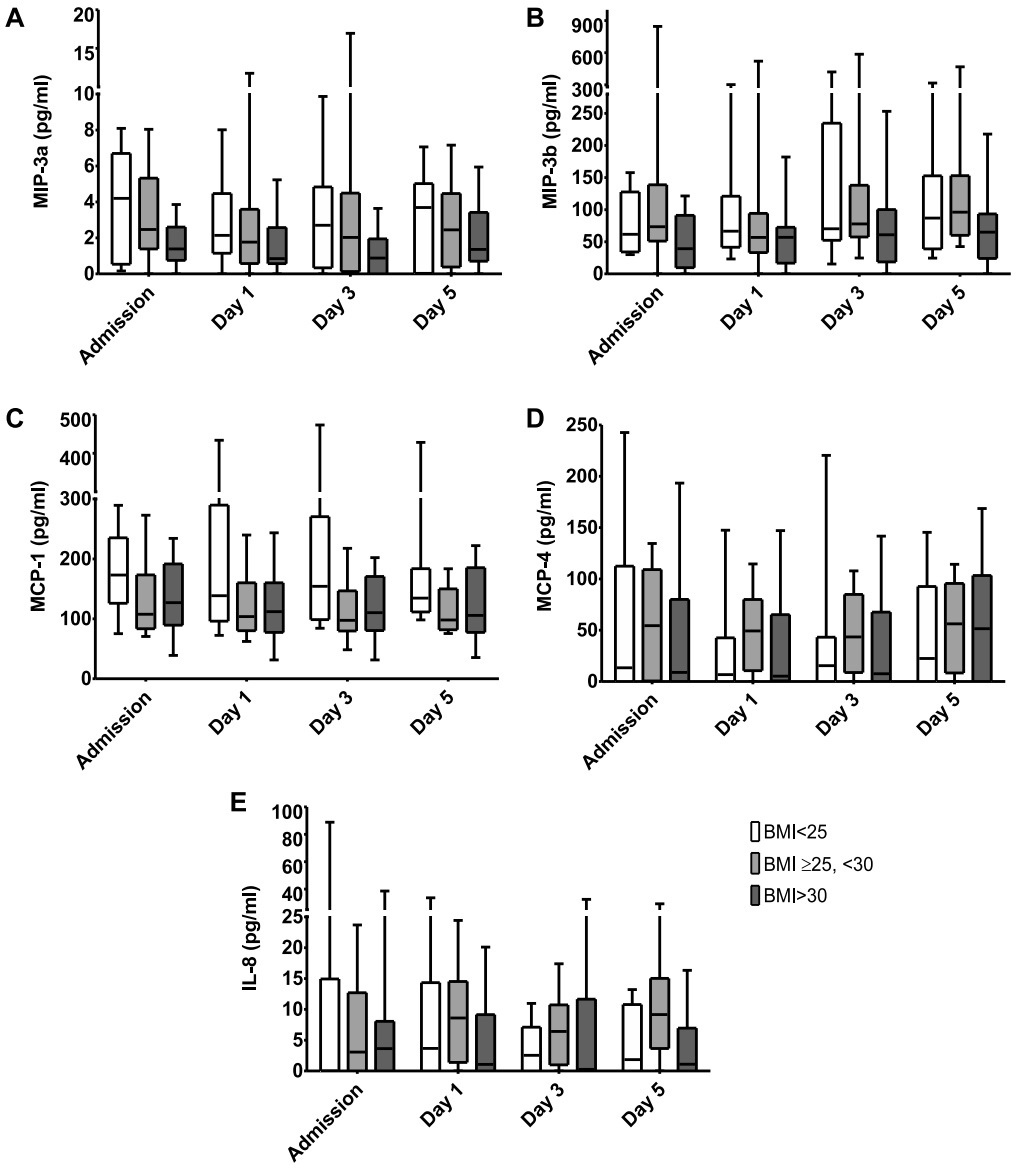

Figure 18: Serum values of A: MIP-3a, B: MIP-3b, C: MCP-1, D: MCP-4 and IL-8 for different BMI-subgroups (BMI$<$25, BMI above 25 but below 30, BMI $>$30). There were no statistically significant differences between BMI subgroups. Shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values, the y-axis in A, B, C and E are split and both segments use different scaling.

Median admission P-selectin values were 275.55 (IQR 121.92-419.95) ng/ml, 218.27 (IQR 137.09-319.98) ng/ml and 136.90 (IQR 88.47-307.42) ng/ml for patients of normal weight, overweighted and obese patients, respectively. For E-selectin median admission values were 20.76 (4.52-35.12) pg/ml, 24.54 (IQR 5.48-45.97) pg/ml and 6.39 (3.02-27.57) pg/ml for patient of normal weight, overweighted and obese patients, respectively (see figure 17, P-selectin: Kruskal-Wallis test, $p = 0.9406$; E-selectin: Kruskal-Wallis test, $p = 0.7488$).

There were no statistically significant differences in median IL-10 values and IL-10 values remained relatively stable during the trial. On admission median IL-10 values were 3.3 (IQR 0-12.9) pg/ml, 4.9 (IQR 2.2-11.5) pg/ml and 0.1 (IQR 0-4.1) pg/ml (see figure 16, Kruskal-Wallis test, $p = 0.1141$). The median values for the chemokines MIP-3a, MIP-3b, MCP-1, MCP-4 and IL-8 were not statistically different between BMI subgroups on any day after stroke (see figure 18, MIP-3a: Kruskal-Wallis test: $p = 0.3013$, MIP-3b: Kruskal-Wallis rest: $p = 0.1379$, MCP-1: Kruskal-Wallis test: $p = 0.2622$, MCP-4: Kruskal-Wallis test: $p = 0.9794$, IL-8: Kruskal-Wallis test: $p = 0.4713$).

3.6 Clinical Improvement and Immunological Changes

As an additional part of this trial, we studied the effect of rapid clinical improvement after stroke on the immunological consequences of stroke, as a surrogate parameter for effective stroke treatment. We used a reduction of the NIHSS score of more than 25 % between admission and day one as an operational definition of rapid clinical improvement after stroke. We then compared patients with and without rapid clinical improvement. In our group of 40 stroke patients 13 patients fulfilled our criterion of rapid improvement. A compilation of the basic statistics of both groups can be found in table 7. Notably all patients who improved rapidly after the stroke according to our criteria, received some form of treatment, either mechanical thrombectomy or systemic thrombolysis with rtPA or both, while in the groups without rapid improvement only 63 % of patients received some form of treatment and this difference was statistically significant (Fisher's exact test, *p = 0.0164*).

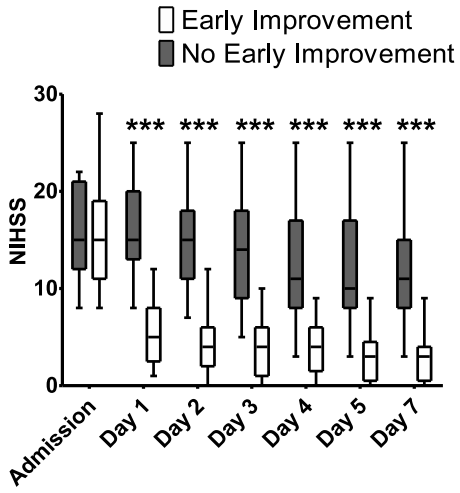

Figure 19: Comparison of NIHSS scores of patients with and without clinical improvement. Statistical differences on each day are annotated with $*** = p < 0.001; ** = p < 0.01; * = p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values.

While both groups had nearly identical median NIHSS Scores on admission, 15 (IQR 11-19) for the rapidly improving group versus 15 (IQR 12-21), median NIHSS scores were significantly different on all other days (see figure 19, Kruskal Wallis test with Dunn's multiple comparison test, $p < 0.0001$). On day seven the median NIHSS value for the rapidly improving group was 3 (IQR 0.5-4) compared to a median NIHSS of 11 (IQR 8-15) in the patient group without rapid improvement.

3.6.1 Influence of Clinical Improvement on Acute Phase Proteins

In the subgroup of patients with clinical improvements after stroke median values of the acute phase proteins CRP and PCT were lower after day two compared to patients without improvement. These differences were not statistically significant

Table 7: Baseline Statistics of Patients With and Without Rapid Clinical Improvement

Variable	Improvement (n = 13)	No Improvement (n= 27)
Age [Years, Mean±Std.]	65 ±16	73±13
Sex [as % female]	69%	49%
BMI [Mean±Std.]	30.43±5.87	27.48±4.53
Co-morbidities		
Hypertension [n (%)]	8 (61.5)	23 (85.2)
Diabetes mellitus [n (%)]	0 (0)	6 (22.2)
Stroke Characteristica		
Initial NIHSS [Median (IQR)]	15 (11-19)	15 (12-21)
1. MRI [cm^3, Median (Range)]	11,8 (2,4-63,4)	61,5 (1,7-322,0)
Infarct Side [n (%)left sided infarcts]	6 (46.2)	14 (51.9)
Any Treatment [n (%)]	13 (100.0)[$]	17 (63.0) [$]
Systemic Thrombolysis [n (%)]	10 (76.9)	15 (55.6)
Mechanical Thrombectomy [n (%)]	11 (84.6)	7 (25.9)

[$] Statistical different, Fisher's exact test, $p = 0.0164$,

(see figure 20, CRP: Kruskal-Wallis test; $p < 0.0001$, Dunn's multiple comparison test: no difference between groups; PCT: Kruskal-Wallis test; $p = 0.0169$; Dunn's multiple comparison test: No difference between groups).

On admission median CRP values were 3.3 (IQR 0.0-8.2) mg/l versus 2.0 (IQR 0.0-7.5) mg/l for patients with and without improvement respectively. In patients without improvement the maximum median CRP value were reached on day five, with 31.2 (IQR 4.5-57.0) mg/l, the corresponding value for patients with improvement was 7.2 (IQR 4.3-17.7) mg/l. The maximum median CRP value for patients with improvement was reached on day two with 9.7 (IQR 5.7-13.7) mg/l, the corresponding value for patients without improvement was 22.6 (IQR 5.8-51.4) mg/l.

Median admission PCT values were 0.07 (IQR 0.06-0.10) ng/ml versus 0.08 (IQR 0.06-0.12) ng/ml for patients with and without improvement. As with CRP the maximum value was reached on day five for patients without improvement, with a median value of 0.13 (IQR 0.08-0.17) ng/ml, with 0.09 (IQR 0.07-0.11) ng/ml as the corresponding value for patients with improvement, who reached their maximum median value on day 3, with 0.10 (IQR 0.06-0.11) ng/ml and a corresponding median value of 0.12 (IQR 0.08-0.20) ng/ml for patients without improvement.

3.6.2 Changes in Peripheral Blood Immune Cells

While patients with and without rapid clinical improvement had similar median cell counts on admission, leukocyte and neutrophil values decreased faster and lympho-

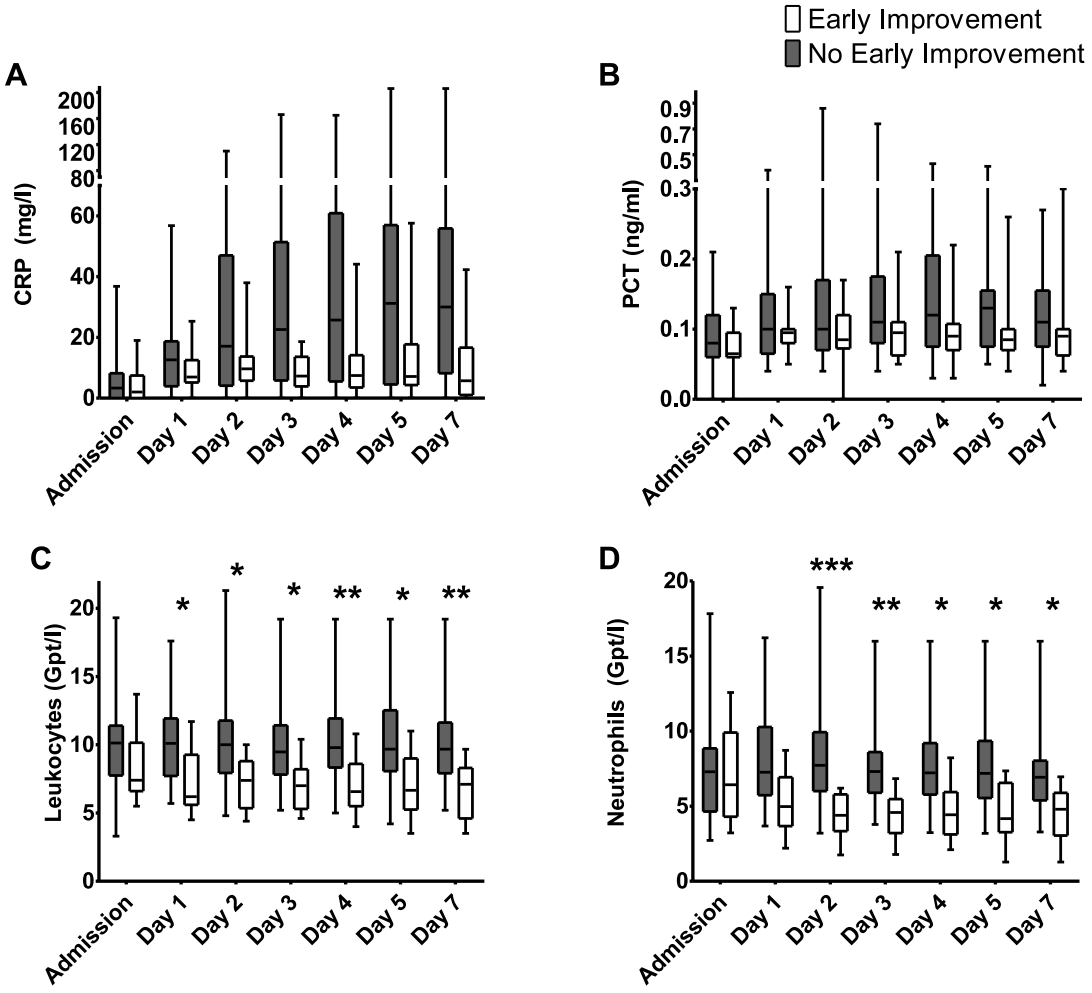

Figure 20: Median A: CRP and B: PCT values during the course of the trial were higher in patients without early improvement, but these differences were not statistically significant on any day. Median C: leukocyte and D: neutrophil counts were similar on admission but lower after day one and day two, respectively in patients with early improvement. Statistical differences on each day are annotated with $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. The y-axis in B and C are split and both segments use different scaling.

cyte counts increased faster for patients with rapid clinical improvement compared to those without clinical improvement, that is these values normalized faster for patients with rapid clinical improvement.

3.6.3 Peripheral Blood Leukocytes

On admission the median leukocyte counts for patients with improvement and without improvement were similar, with 7.40 (IQR 6.61-10.15) Gpt/l and 10.13 (IQR 7.75-11.40) Gpt/l, respectively. On all other days patients with improvement had significantly lower median leukocyte counts compared to patients without improvement (see figure 20, Kruskal-Wallis test with Dunn's multiple comparison test; $p < 0.0001$). Median leukocyte values on day one were 6.20 (IQR 5.60-9.27) Gpt/l versus 10.10 (IQR 7.72-11.93) Gpt/l, on day four 6.57 (IQR 5.50-8.60) Gpt/l versus 9.97 (IQR 8.33-11.93) Gpt/l for patients with and without improvement, respectively. On the other days median values were similar.

3.6.4 Peripheral Blood Neutrophils

Median neutrophil values for patients with and without improvement were similar on admission (6.43 (IQR 4.30-9.91) Gpt/l versus 7.29 (IQR 4.64-8.85) Gpt/l), but while median neutrophil counts decreased after admission in patients with early improvement, the median neutrophil count in patients without improvement did not change. Group differences on and after day two were statistically significant, with median counts of 4.40 (IQR 3.33-5.79) Gpt/l versus 7.72 (IQR 6.00-9.93) Gpt/l on day two and similar values on all following days (see figure 20, Kruskal-Wallis test with Dunn's multiple comparison test; $p < 0.0001$).

3.6.5 Peripheral Blood Lymphocytes

Median total lymphocyte counts were nearly identical on admission with 1.49 (0.98-2.06) Gpt/l versus 1.50 (0.99-1.95) Gpt/l for patients with and without clinical improvement. On the following days median lymphocyte counts were slightly higher for patients with clinical improvement. On day three this difference was most pronounced and statistically significant (see figure 21, Kruskal-Wallis test; $p = 0.0306$; Dunn's multiple comparison test), with a median lymphocyte value of 1.89 (IQR 1.58-2.42) Gpt/l versus 1.38 (IQR 1.15-1.86) Gpt/l. Afterwards median lymphocyte values converged again, with 1.67 (IQR 1.53-2.31) Gpt/l versus 1.45 (IQR 1.24-2.23) Gpt/l on day seven.

CD4+-T-lymphocytes and CD8+-T-lymphocytes were nearly identical between both subgroups (see figure 21, Kruskal-Wallis test, CD4+: $p = 0.8081$; CD8+: $p = 0.7821$). On admission median CD4+-T-lymphocyte counts were 0.61 (IQR 0.38-0.92) Gpt/l versus 0.53 (IQR 0.36-0.89) Gpt/l and median CD8+-T-lymphocytes were 0.26 (IQR 0.21-0.36) Gpt/l versus 0.26 (IQR 0.18-0.46) Gpt/l for patients with and without clinical improvement.

Median B-cell values were slightly higher in patients with improvement on all days, but these differences were not statistically significant (see figure 21, Kruskal-Wallis test, $p = 0.09$). On Admission median counts were 0.18 (IQR 0.11-0.32) Gpt/l versus 0.12 (0.08-0.18) Gpt/l for patients with and without improvement, these values increased slightly up to day three, with median counts of 0.25 (IQR 0.16-0.38) Gpt/l versus 0.18 (IQR 0.11-0.28) Gpt/l.

3.6.6 Serum Pro- and Anti-Inflammatory Cytokine Values

Patients without improvement had higher pro- and anti-inflammatory cytokine and chemokine values than patients with clinical improvement.

Median IL-6 values were higher in patients without clinical improvement compared to patients with improvement. The difference between these groups was statistically significant on day three (see figure 22, Kruskal-Wallis test with Dunn's multiple comparison test; $p = 0.0017$). On admission median IL-6 values were 4.7 (IQR 2.7-9.3) pg/ml versus 2.2 (IQR 1.8-4.0) pg/ml for patients with and without improvement respectively and on day 3 median IL-6 values were 9.3 (IQR 4.0-26.7)pg/ml versus 2.1 (IQR 0.2-6.9) pg/ml.

Median sIL-6R values were similar in both patient groups and they did not change much during the trial (see figure 22, Kruskal Wallis test; $p = 0.2757$). Median admission values were 23.92 (IQR 19.86-30.12) ng/ml versus 26.69 (IQR 23.43-37.13) ng/ml for patients with and without improvement. Median IL-10 values were higher for patients without improvement, with median admission values of 4.0 (IQR 0.1-7.4) pg/ml versus 0.0 (IQR 0.0-4.6) pg/ml, but the difference between patients with and without improvement were not significant(see figure 22, Kruskal-Wallis test; $p = 0.3807$). These values on the following days were similar.

Median eotaxin values were significantly higher for patients without improvement for all measurement days (see figure 23, Kruskal-Wallis test with Dunn's multiple comparison test; $p < 0.0001$), admission values were 72.5 (IQR 56.0-90.4) pg/ml versus 40.8 (IQR 22.0-57.7) pg/ml . Chemokines were increased in patients without clinical improvement, this was most pronounced initially after stroke.

Median IL-8 values were higher for patients without early improvement, espe-

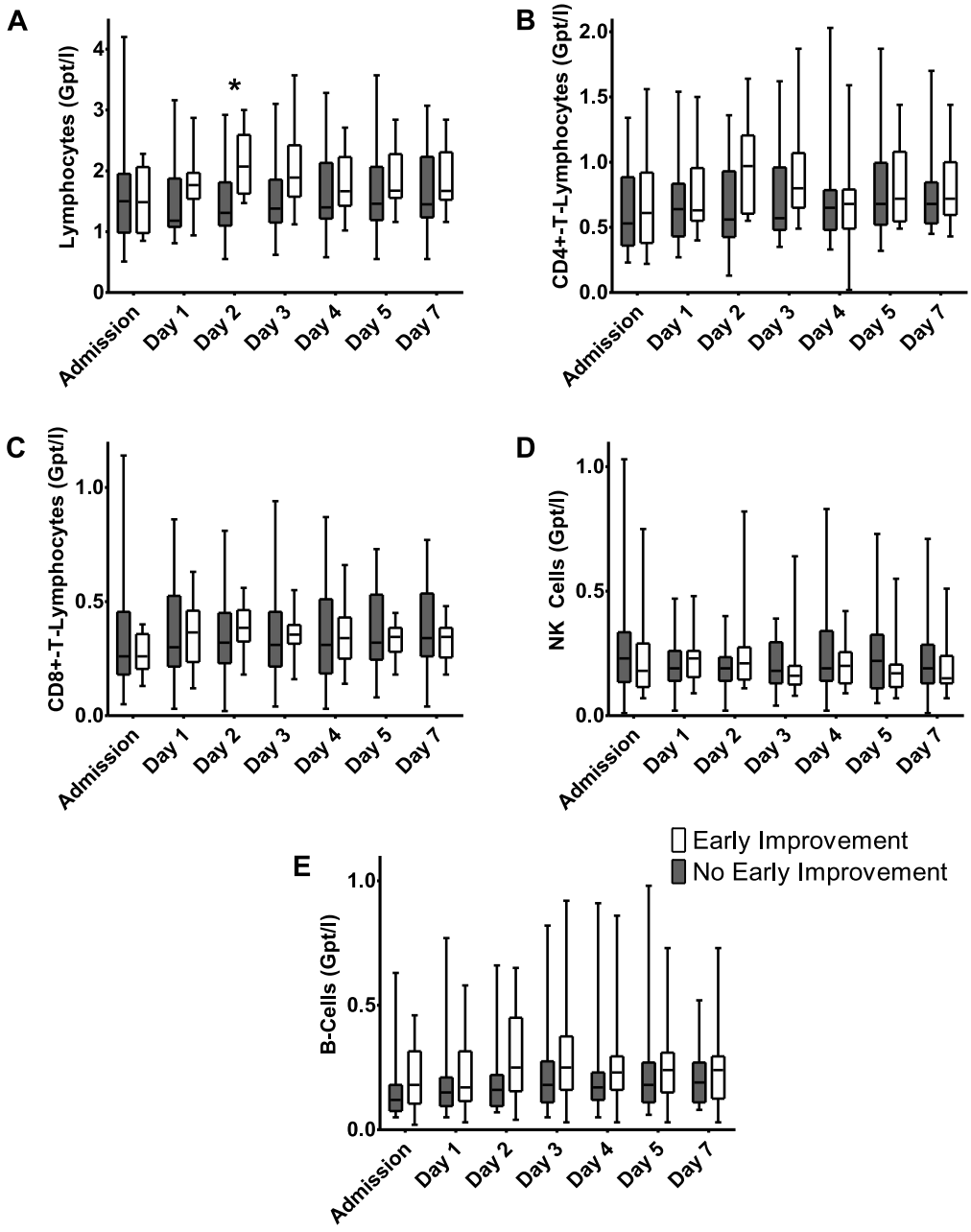

Figure 21: A: Lymphocyte, B: CD4+-T-lymphocyte, C: CD8+-T-lymphocyte, D: NK-cell and E: B-cell counts in patients with and without early clinical improvement after stroke. Statistical differences on each day are annotated with $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values.

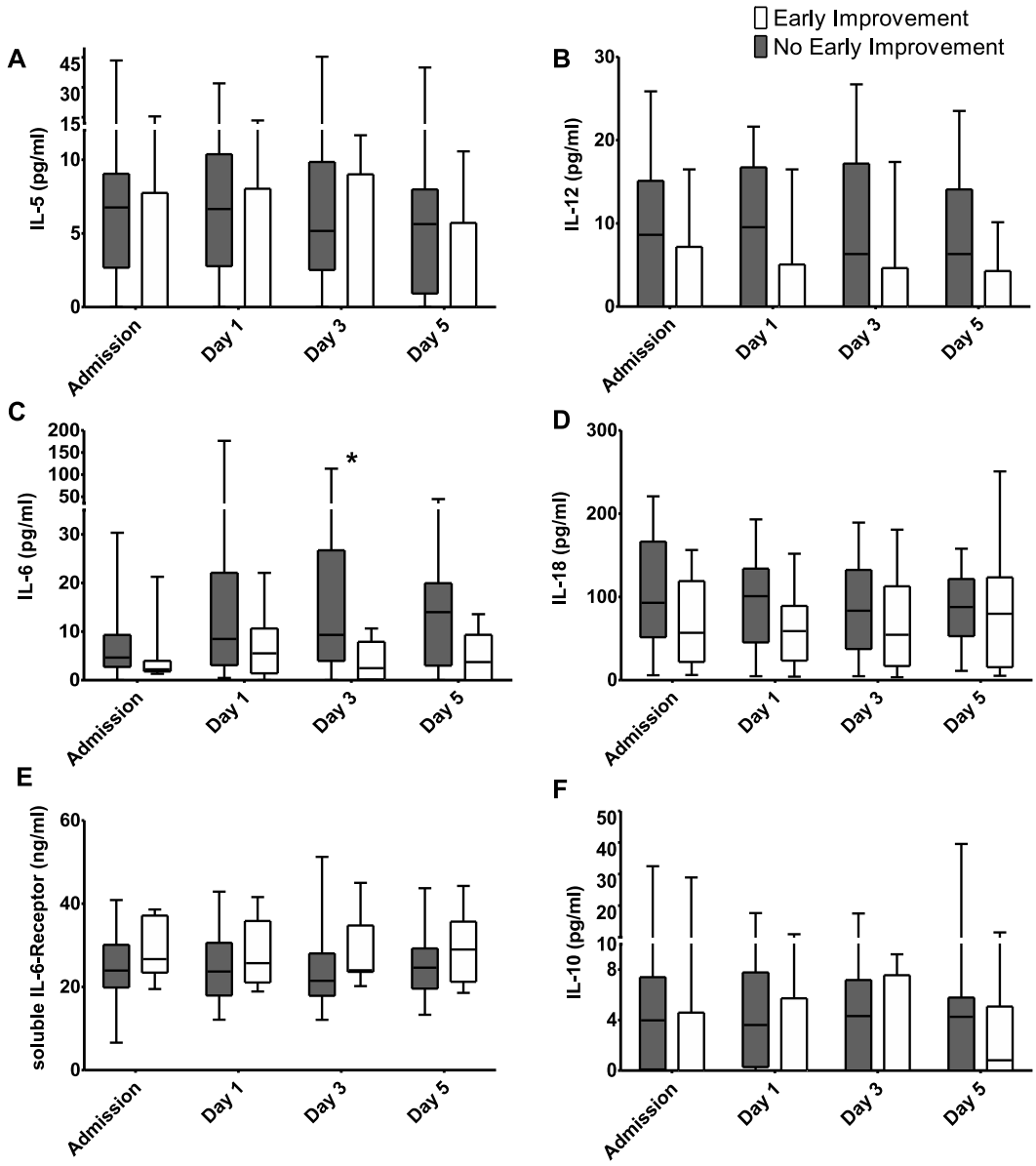

Figure 22: Median serum values of A: IL-5 B: IL-12, C: IL-6, D: IL-18, E: sIL-6R and F: IL-10 for patients with and without early improvement. Statistical differences on each day are annotated with $*** = p < 0.001$; $** = p < 0.01$; $* = p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. The y-axis in A, C and F are split and both segments use different scaling.

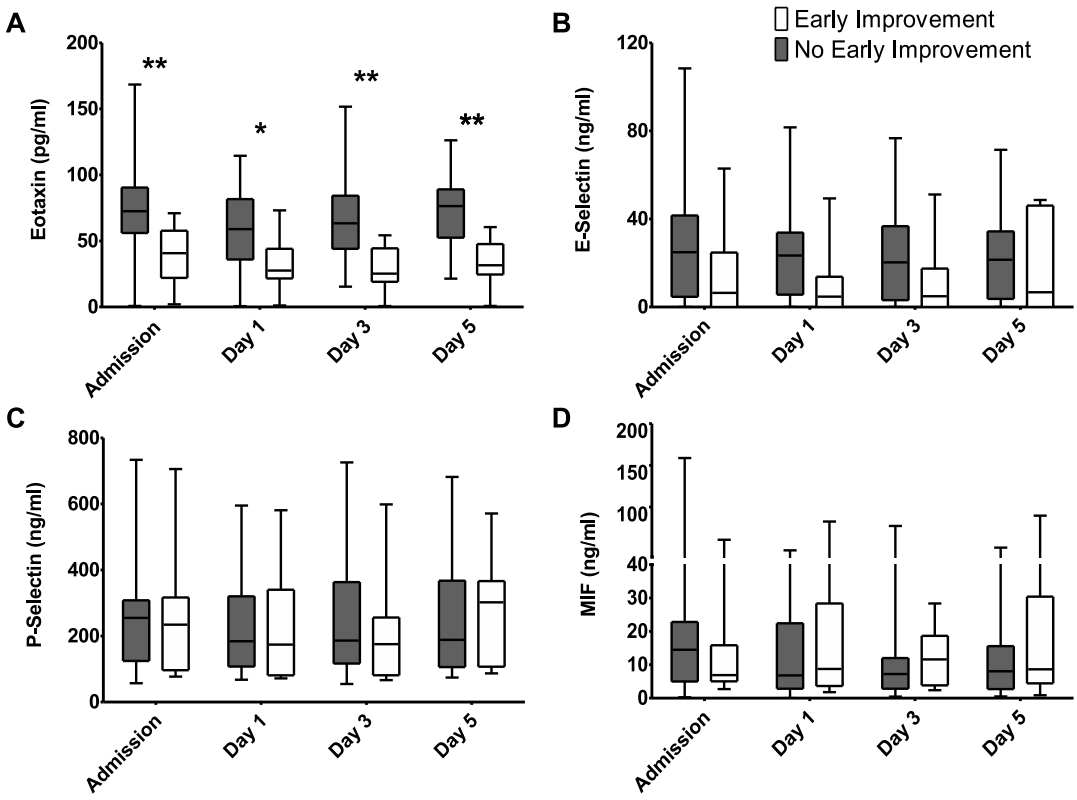

Figure 23: Median serum values of A: eotaxin, B: E-selectin, C: P-selectin and D: MIF for patients with and without early clinical improvement. Statistical differences on each day are annotated with $*** = p < 0.001$; $** = p < 0.01$; $* = p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. The y-axis in figure D is split and both segments use different scaling.

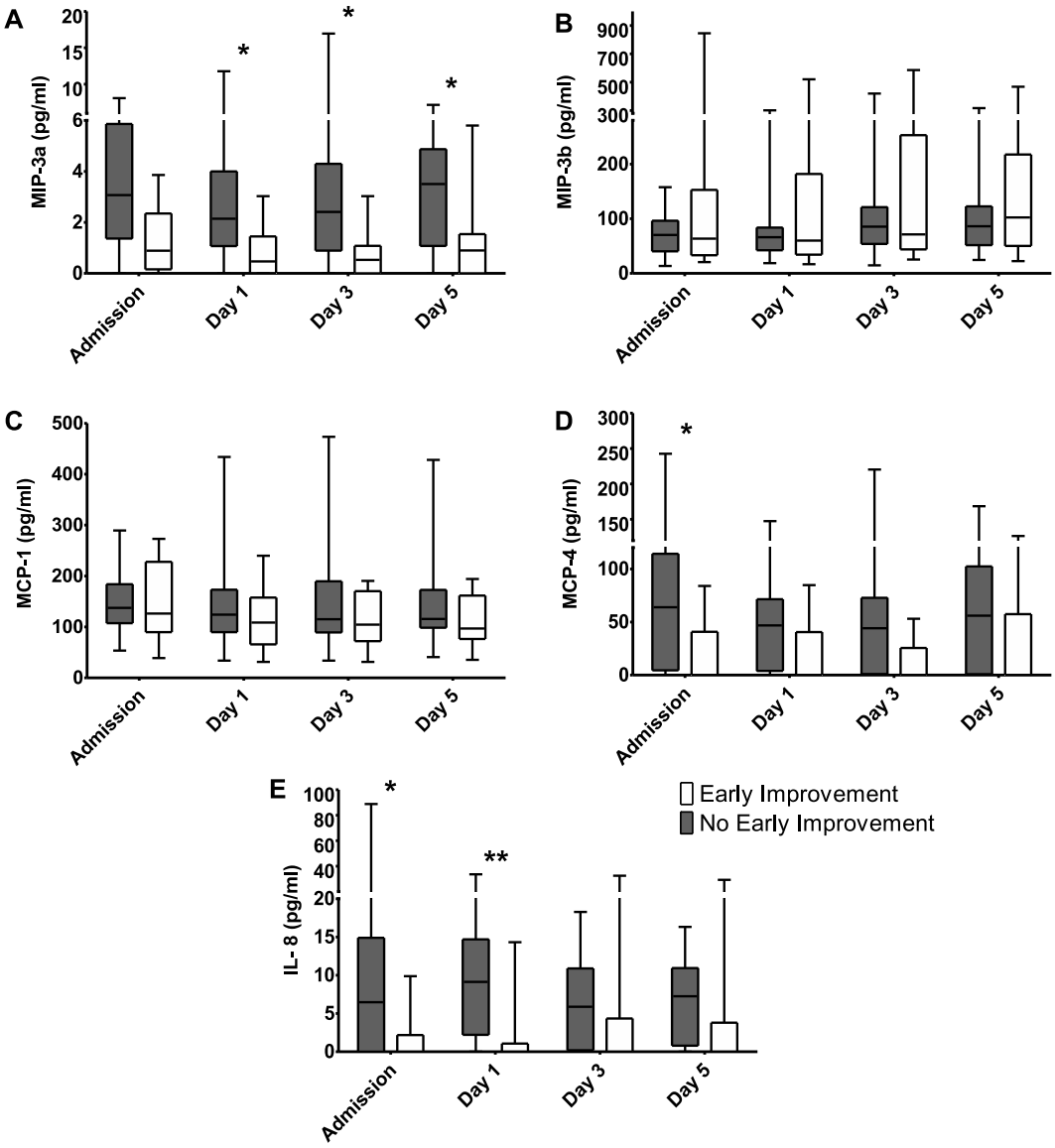

Figure 24: Median serum values of A: MIP-3a, B: MIP-3b, C: MCP-1, D: MCP-4 and E: IL-8 of patients with and without early improvement. Statistical differences on each day are annotated with $*** = p < 0.001$; $** = p < 0.01$; $* = p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. The y-axis in A, B, D and E are split and both segments use different scaling.

cially early after stroke, admission values were 6.9 (IQR 00.-18.6) pg/ml versus 0.0 (IQR 0.0-2.2) pg/ml, values on day one were 8.2 (IQR 2.7-14.4) pg/ml versus 0.0 (0.0-1.1) pg/ml for patients with and without improvement (see figure 24, Kruskal-Wallis test with Dunn's multiple comparison test; $p = 0.0028$). On day five median values were 5.7 (IQR 0.1-10.7) pg/ml versus 0.0 (IQR 0.0-3.8) pg/ml.

Median MCP-4 values were higher for patients without improvement on all days, a statistical difference was only detectable on admission, with median values of 63.9 (IQR 4.5-114.3) pg/ml for patients without improvement versus 0.0 (IQR 0.0-40.8) pg/ml for patients with improvement (see figure 24Kruskal-Wallis test with Dunn's multiple comparison test; $p = 0.0013$).

In contrast MIP-3a was especially high in patients without improvement on days one, three and five (see figure 24Kruskal-Wallis test with Dunn's multiple comparison test; $p = 0.0001$) with median values of 2.1 (IQR 1.1-4.0) pg/ml versus 0.5 (IQR 0.0-1.5) pg/ml on day one and 3.5 (IQR 1.1-4.9) pg/ml versus 0.9 (IQR 0-1.5) pg/ml on day five, while the difference on admission was not significant with values of 3.1 (IQR 1.4-5.9) pg/ml versus 0.9 (IQR 0.2-2.4) pg/ml for patients without and with clinical improvement.

Median P-selectin values were similar between both groups. Admission values were 254.87 (IQR 124.55-308.17) ng/ml versus 234.28 (IQR 96.43-316.85) ng/ml for patients without and with improvement (see figure 23, Kruskal-Wallis test; $p = 0.8206$). Median E-selectin values were slightly higher for patients without improvement on all days. On admission the values were 24.89 (IQR 4.61-41.57) ng/ml versus 6.39 (IQR 0-24.74) ng/ml for patients without and with improvement but these differences were not significant (see figure 23, Kruskal-Wallis test; $p = 0.0615$).

3.6.7 Influence of Clinical Improvement in rtPA-treated Patients.

There is evidence that rtPA acts as a regulator of the immune system [92] and patients treated by rtPA might be clinically different than patients not treated with rtPA. Because the proportion of patients treated with rtPA was different between our two clinical improvement subgroups, we compared the immunological effects of clinical improvement among those patients treated with rtPA. In total 25 patients were treated with rtPA after Stroke, 10 of those patients had an improvement of their NIHSS Score by more than 25 % and fulfilled our criterion for rapid clinical improvement. The differential immunological changes of these patients were similar to those of the complete group of stroke patients. In patients treated with rtPA those with a rapid clinical improvement had a decrease in leukocyte and neutrophil counts compared to patients without clinical improvement(see figure 25, Kruskal-Wallis test with

Dunn's multiple comparison test; $p < 0.0001$ for leukocytes and $p < 0.0001$ for neutrophils), while differences in lymphocytes were not statistically significant (see figure 25, Kruskal-Wallis test; $p = 0.3318$).

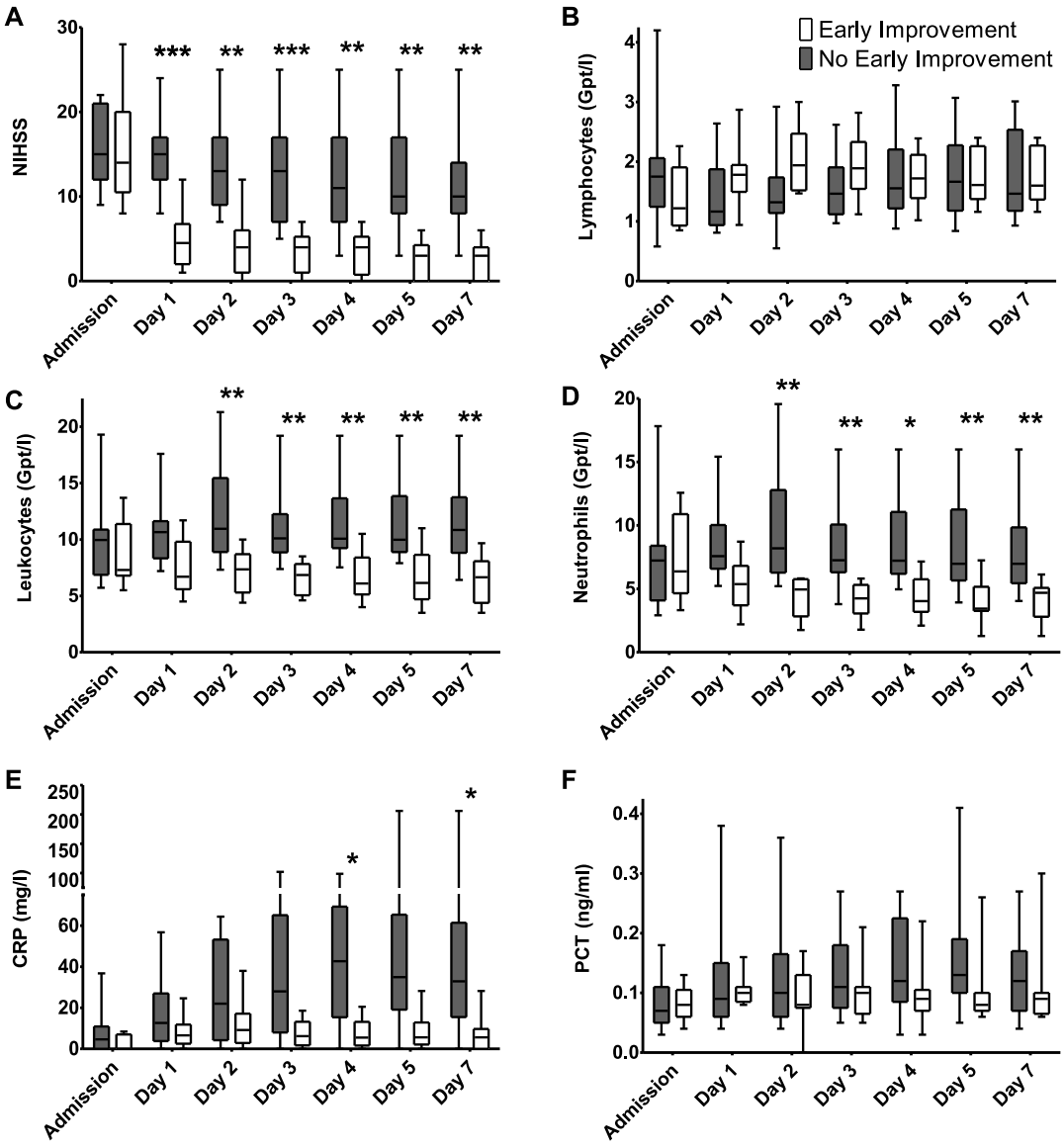

Figure 25: Comparison of patients treated with rtPA with (n=10) and without (n=15) early clinical improvement. A: NIHSS-scores for both groups, Median B: lymphocyte, C: leukocyte and D: neutrophil values. E and F: Median serum values for CPR and PCT; Statistical differences on each day are annotated with $*** = p < 0.001$; $** = p < 0.01$; $* = p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values. The y-axis in E and F are split and both segments use different scaling.

Pro inflammatory IL-6 was higher on days three and five in patients without clinical improvement (see figure 25, Kruskal-Wallis test with Dunn's multiple comparison

test; $p = 0.0003$), IL-12 was higher on days one and three in the same patients (see figure 25, Kruskal-Wallis test with Dunn's multiple comparison test; $p = 0.0002$), chemokines Eotaxin and MCP-4 were higher in patients without clinical improvement on all days after stroke (see figure 25, Kruskal-Wallis test with Dunn's multiple comparison test; Eotaxin: $p < 0.0001$; MCP-4: $p < 0.0001$). Anti-inflammatory interleukin 10 was not statistically different between patients with and without early improvement (see figure 25, Kruskal-Wallis test with Dunn's multiple comparison test; $p = 0.9248$).

Interestingly in rtPA treated stroke patients median PAI-1 values were higher among those patients with clinical improvement. This difference was significant on day one (see figure 25, Kruskal-Wallis test with Dunn's multiple comparison test; $p = 0.0049$).

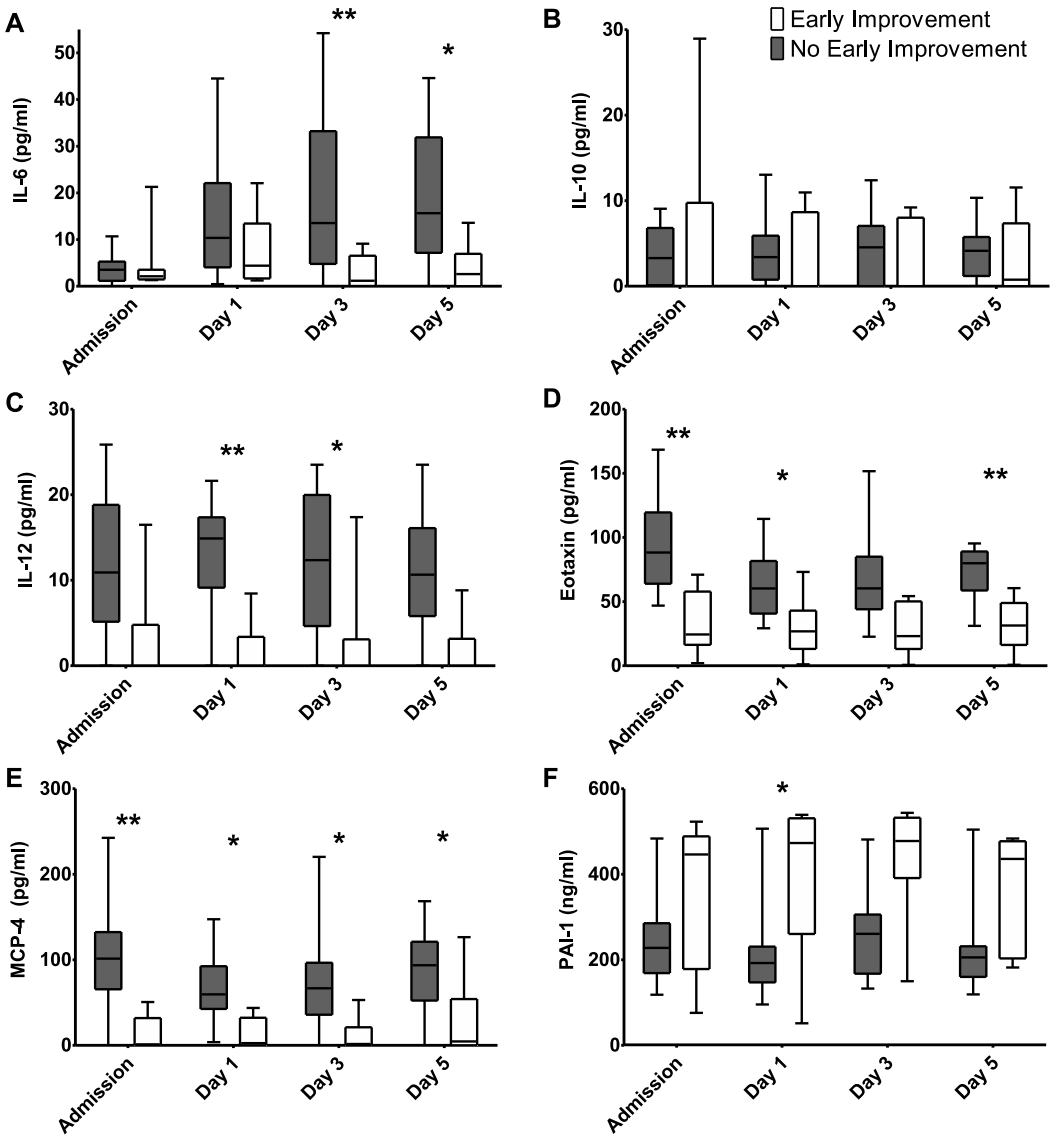

Figure 26: Median serum values of A: IL-6, B: IL-10, C: IL-12, D: eotaxin, E: MCP-4 and F: PAI-1 for patients, treated with rtPA, with and without early improvement. Statistical differences on each day are annotated with $***$ = $p < 0.001$; $**$ = $p < 0.01$; $*$ = $p < 0.05$; shown are median values, lower and upper box edges mark the 25% and 75% percentiles, whiskers mark the minimal and maximal values.

4 Discussion

In the LIPS trial our aim was to characterize changes in body weight after stroke and corresponding changes in adipokines and immunological function and their effects on post stroke immunosuppression. Additionally, we wanted to characterize the effects of overweight and obesity on stroke outcome and post stroke immunosuppression.

We recruited 50 stroke patients in total. After excluding patients with contraindications or those who were transferred, 40 stroke patients formed our stroke cohort. Initially we attempted to recruit 100 patients, but later on reduced our inclusion goal to 50, due to a rather slow inclusion process. This was mainly affected by our restrictive inclusion criteria, especially the requirement of a first ever stroke and severe strokes limiting the pool of potentially eligible patients. In our stroke cohort 30 patients or 75% received any form of hyper acute stroke treatment and 25 patients or 62.5% were treated with rtPA. This is a high rate compared to rates observed in unselected stroke patients, where treatment rates with rtPA vary between 6% and 14% [93, 94, 95]. In our stroke unit in the year 2015 15.4% and in the year 2016 18.1% of all stroke patients were treated with systemic thrombolysis. The difference can be explained by considering that our inclusion and exclusion criteria ascertain that our patients were often eligible for hyper acute stroke treatment. Additionally, the requirement of an NIHSS larger than 8 defines a stroke population with a high rate of intra cerebral large vessel occlusions [96], especially M1-Occlusion, which are uniquely suited for mechanical thrombectomy.

We could observe some of the typical immunological changes associated with the immediate post stroke period that have been described previously [17, 35, 36, 97, 98], namely increased granulocyte counts, reduced CD4+-T-lymphocytes and increased acute phase proteins in our stroke cohort. But some of these changes were only marginal in our patients. There was a trend towards increased leukocyte and decreased B-cell counts early after stroke but total lymphocytes, CD8+-T-lymphocytes and NK-cells did not differ from control values. In another study [42] that reported no or minor changes in leukocytes and lymphocytes, infarct volume was discussed as a relevant parameter determining the immunological changes but our median stroke volume of 31 cm^3 was well within the range of previously reported trials similarly to Hug et al. [42]. Additionally, we included only patients with moderate to severe strokes to increase the chance of observing immunological alterations, which correlate with stroke severity and NIHSS-score [35, 36].

Interestingly in a previous study from our group [17] with similar stroke patients treated at the same hospital, more pronounced changes where found. Compared

to our study, were only one patient showed an unambiguous systemic infection, in the study of Vogelgesang et al. [17] 11 out of 46 stroke patients had a systemic infection fulfilling our criteria. One possible reason for the lower percentage of clear cut infections in our trial might be the fact that patients with suspected infections were rapidly treated with antibiotics and consequently did not develop a severe infection. Indeed, all patients with indeterminate infectious status received antibiotic therapy in our trial.

In our trial adipokines were not changed in stroke patients compared to controls, the only exception being PAI-1 (plasminogen-activator-inhibitor-1), which was markedly increased on all days after stroke compared to controls. PAI-1, an adipokine synthesized both in adipose tissue and in the liver is a potent inhibitor not only of endogenous plasminogen activators but also of rtPA used in hyper acute stroke treatment [99, 100]. It has previously been shown to be increased in ischemic stroke [101], most likely as part of a regulatory response within the fibrinolytic system.

Leptin values changed after stroke, having a modest peak on day three. These results are in line with most previous studies that did show increases in leptin only in selected aetiological subgroups [81, 82]. The time courses of leptin [102] and adiponectin [85] in our study were similar to those previously reported .

Our stroke patients had increased levels of CRP compared to control subjects after admission and showed an increase of CRP and procalcitonin values compared to the admission day. This is in line with previous research showing increasing levels of these proteins in stroke patients [5, 103, 104] and likely represents the acute phase response to the cerebral injury. Indeed, previous studies found that CRP values in stroke patients correlated with infarct size [105]. However, increasing values might indicate developing post stroke infections in our cohort, with 17 patients receiving antibiotics for clinically diagnosed infections. In our stroke patients serum IL-6 levels were increased after admission, with a peak on day three, in line with other human stroke studies [5, 98, 106], which is further evidence for a systemic immune activation [107].

Interestingly, serum levels of several chemokines and cellular adhesion molecules were reduced in our stroke patients, compared to control values. Eotaxin, MCP-4 and platelet selectin serum levels were significantly reduced compared to controls on all days after stroke. For eotaxin this has been described previously [108]. Interestingly in this study lower eotaxin levels were predictive of worse outcomes and at odds with increased eotaxin levels in experimental stroke in mice [108].

For platelet selectin previous trials have shown increased serum values in stroke patients [106]. These findings contradict our own findings possibly reflecting differences among control subjects. Pusch et al. used patients with Parkinson disease

and asymptomatic carotid artery stenosis while our control cohort consisted of patients undergoing treatment for macular disease and indeed our control patients had markedly higher P-selectin values compared to Pusch et al..

To our knowledge MCP-4 has not been studied in acute stroke patients before, but it is known that serum levels are increased in patients with carotid stenosis and especially high in patients with a symptomatic carotid stenosis and a stroke or transitory ischemic attack within the last two months [109]. MCP-4 is a chemoattractant for monocytes, neutrophils and especially eosinophils and is implicated in the pathogenesis of asthma and multiple sclerosis [110].

For a number of important cytokines, which have been previously implicated in ischemic stroke pathophysiology, namely TNF-α, IL-1β and interferon-γ we could detect serum levels above the lower level of detection for a minority of stroke patients or control subjects. These might be an indication of a problem with our test assay, especially with the lower level of detection, although they are well within the range of previously reported values. Previous studies have reported TNF-α values between 21 and 26 pg/ml [47], IL-1β values between 1 and 2 pg/ml and interferon-γ values between 1000 to 2000 pg/ml [97] in stroke patients, while the lower limit of detection for our assays are 2 2pg/ml, 1 2pg/mland 8 8pg/ml respectively. The number of patients with detectable serum levels was within the range of a previously published study at least for TNF-α and interferon-γ [111]. One further aspect is that at least for TNF-α, GM-CSF and interferon-γ many studies publish cerebrospinal fluid levels or stimulated *ex vivo* values e.g. [35, 36, 97], while we measured serum levels *in vivo*.

In our stroke cohort weight loss after stroke was only 1.7 ± 0.5 kg on average on day seven. The weight loss occurred predominantly between admission and day one, a period were most patients were not allowed to eat or access to food was restricted due to stroke associated dysphagia according to local guidelines. And while weight measurements on the days one through seven were performed at a similar time point in the morning, admission weights were performed on any time in the day and due to the emergent nature of most encounters could not account for example for urinary bladder content or defecation. Additionally, before thrombolysis patient do not routinely get a urinary catheter, therefore even estimation of the size of this bias was not possible. While few studies have looked at weight loss immediately after stroke, one study [77] found that 9 % of stroke patients had a relevant weight loss, and that those patients did indeed have a poor prognosis. If we adopt the criteria of Kim et al. 45 % of our patients had a significant weight loss on day five or seven compared with the admission weight, defined as losing more than 0.1 kg per BMI-Unit of Body weight in this time interval. The higher rate of significant weight

loss might be partially explained by the severity of stroke in our study, because overall in the study of Kim et all. those patients with milder strokes did not lose weight [77]. As part of our trial body composition, skin fold thickness and MRI-based abdominal and liver fat measurements were performed. The results are reported in a separate thesis in more detail by Christin Heuer. As additional measurements we performed a body composition analysis with Body Impedance Analysis. Body fat loss was proportional to overall weight loss. Skinfold thickness, a clinical marker for the subcutaneous fat tissue did not change in our stroke patients, nor did abdominal or liver fat. Overall there was no indication for a stroke induced lipolysis.

These results contrast with experimental stroke models, where weight loss in stroke animals is often a significant fraction of their body weight [78, 112].

At least part of the discrepancy can be explained by the fact that a common variation of the middle cerebral occlusion model of ischemic stroke, in mice where the external carotid artery is litigated to gain access to the internal carotid artery [113] leads to an especially large weight loss, compared to other variants, where the common and the external carotid artery are spared [114, 115]. Another possible explanation are differences in adipose tissue biology and anatomy between humans and rodents and the differences in weight and brown adipose tissue and their regulation [116, 117, 118], which might explain differences in weight loss during episodes of food restriction and stress. Additionally, due to size differences metabolic requirements of humans and rodents are expected to be significantly different [119], which might contribute to the differences in weight loss after stroke as well. And indeed at least one study, which reported body weight after experimental ischemic stroke in pigs found no significant weight loss [120].

Another possibility is that while body weight is not routinely measured, the treatment of human stroke patients in specialised stroke units prevents dramatic weight loss.

While weight measurement is an established method, assessing weight and weight change in critically ill, bed bound patients posed several challenges. Getting an accurate admission weight was challenging especially for patients eligible for thrombolysis where time constraints limited the time span during which weighing was possible.

While we could show the typical immunological changes after stroke, there was no evidence for an effect of obesity on these changes. In the pre-specified subgroup analysis, patients with different BMI values had similar immune cell counts, acute phase proteins, cytokine and chemokine values. We found no evidence for a differential post stroke immune response in overweight or obese patients compared to patients of normal weight.

This is in contrast to Tuttolomondo et al. [80] who found increased inflammatory markers in stroke patients with metabolic syndrome, especially IL-6, TNF-α, E-selectin, P-selectin and PAI-1 compared to stroke patients without metabolic syndrome. Our results contrasts with experimental stroke studies [8, 9] as well, which also found an increase in inflammatory cytokines in obese compared to lean mice after ischemic stroke. One possible explanation for the different results between Tuttololmondo et al. [80] and our one results is that we only compared patient groups in different BMI ranges, while the aforementioned authors compared patients with and without metabolic syndrome. One link between obesity and other manifestations of metabolic syndrome, especially diabetes, is a state of chronic inflammation [56, 65].

Therefore, these patients are highly likely to have pro-inflammatory markers after stroke. Another explanation is that our obese patients were only moderately obese with a median BMI of 32 kg/m^2 and at least some pro-inflammatory markers correlate with high BMI values [121], implying that effects of obesity on post stroke immunological changes can be seen with higher BMI values. On the other hand, the BMI values in our study were in line with other reports [56, 65], which did show effects of obesity on systemic immune parameters.

To characterize the effects of rapid clinical improvement as a surrogate parameter for effective hyper-acute stroke treatment, we compared patients with a rapid clinical improvement after stroke with patients without such an improvement. We operationalized rapid improvement as a decrease of the NIHSS of more than 25 %. In total 11 patients in our cohort fulfilled this criterion of rapid improvement.

While it was theoretically possible that a patient with an Admission NIHSS of 8 could fulfil this criterion with a decrease of 2 points, which is clinically insignificant, all patients improved by at least 4 points.

In the course of this trial patients with rapid clinical improvement had lower values in a number of key inflammatory parameters in the course of this trial, namely leukocytes, granulocytes and IL-6. Additionally, a number of chemokines and cytokines were lower than in patients without rapid clinical improvement. These results could be confirmed in the subgroup of patients who were treated with rtPA, which is a more homogeneous group, which had to be included in our study 4.5 hours after stroke on-set. These results imply that the mechanisms that underpin the immunological alterations after stroke are not a one hit event, that once triggered is running its course. Rather it could be described as a process, which can be influenced and potentially reversed. Initially, on admission patients with and without improvement had identical stroke severity, as measured with the NIHSS score (15 (IQR 11-19) versus 15 (12-21)) which is a marker for tissue at risk. The clinical improvement, then correlates with salvaged tissue, i.e the penumbra. The observation that the MRI

lesion of patients with an improvement are markedly smaller than in patients without improvement supports this picture, because due to logistic reasons the MRIs were performed after the treatment in the first days after stroke and therefore the difference can be seen as a treatment effect. Among patients with rapid improvement, the treatment prevented the infarction of viable brain parenchyma in the penumbra of the stroke and thereby not only reduced the neurological injury but also prevented part of the systemic complications of the stroke. This is in line with previous studies showing that stroke size correlates with the extent of immunological changes after human stroke [42, 98] and in experimental stroke [32].

Our study has a number of limitations. We were not able to recruit the projected number of patients, because recruitment of eligible patients was slower than anticipated. The number of patients and controls is comparable to other studies [5, 35, 36, 47], but might be insufficient to detect small but relevant effects due to a limited statistical power. Due to logistic restraints the study protocol did not include cerebral and abdominal MRI-data before stroke treatment and therefore might miss relevant acute effects. Furthermore, one principal limitation of our study is that the immunological status of our patients before the stroke is unknown and therefore part of the observed effects might be pre-existing and unrelated to the stroke. This is especially true for values which remained stable throughout the course of our trial. Due to our small study size, subtle mismatches in patient and control characteristics might exist, despite our effort to match patients and controls.

In conclusion, while we could detect the typical immunological changes associated with stroke, namely a post stroke immunodepression and signs of immune activation, our data did not confirm the initial hypothesis that BMI significantly modulates the stroke induced immune response. In addition no significant lipolysis was observed in our stroke cohort. These results will be detailed in the thesis by Christin Heuer. Additional analysis of our data demonstrated that reversal of the neurological deficit is associated with a rapid reversal of stroke induced immune alterations. This deepens our insight into the pathophysiology of these stroke induce immune alterations: While stress hormones and HMGB1 have been implicated in triggering SIIA it was not known whether SIIA represents a cascade of events that is self-maintaining after triggering. Our data implies that SIIA requires a constant, yet to be identified signal to persist. We hypothesize that this signal is brain derived as rapid recovery of cerebral function results in the resolution of SIIA. This suggest that successful reversal of brain ischemia also reverses the stroke induced immune alterations. Additionally, identification of these signals might offer an additional treatment option to prevent post-stroke complications.

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Summary

Ischemic stroke is one of the leading causes of death and disability throughout the world. One important aspect of stroke pathophysiology are immunological changes after stroke, especially a combination of post stroke immunodepression, leading to infectious complications after stroke and an activation of the immune system, leading to cerebral injury. Adipose tissue has several immunological functions and obesity leads to immunological complications and is accompanied by a chronic immune activation. To study the effects of body weight and obesity on the immune system and measure weight and fat tissue changes after ischemic stroke we conducted the LIPS Trial and enrolled 50 stroke patients and 16 control subjects between July 2015 and July 2016. On the day of admission and on the days 1, 2, 3, 4, 5, 7, 30, 90 and 180 after admission stroke patients were weighed with an in-bed scale, body composition was measured with BIA, the triceps-skin fold thickness was measured, the NIHSS scale was obtained and blood was drawn. FACS-analysis was performed and triglycerides, cholesterol, CPR and PCT were measured at the central laboratory facility of the Universitätsmedizin Greifswald. Luminex-multiplex analysis for multiple cyto- and chemokines was performed at the Multiplex Facility at the University Leiden. A cerebral MRI and an abdominal MRI were performed shortly after admission and on days 5-7 for most patients and the infarct volume, abdominal fat and hepatic fat percentage were measured. On days 30, 90 and 180 after stroke Bartel Index and mRS were obtained. After stroke our patients showed the typical immunological changes described previously as stroke induced immune alterations, namely a post stroke immunodepression as well as signs of an activated immune system and an acute phase response. Our patients lost weight, but only 1.7 ± 0.5 kg. Skinfold thickness did not change during the course of our trial and abdominal fat measurement did not change in stroke patients. Immunological parameters (leukocytes, neutrophils, CPR, PCT, IL-6) did not differ between BMI subgroups (normal weight: BMI$<$25, overweight: BMI$\geq$25, $<$30, obese: BMI$>$30) and in this trial we could not detect a difference in patients with normal weight, overweight or obesity in the post stroke periode. In an additional analysis we could show that rapid clinical improvement did result in a rapid improvement of post stroke immune alterations, especially for leukocytes, neutrophils, IL-6 and CPR.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät, keiner anderen wissenschaftlichen Einrichtung vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Datum Unterschrift

Danksagung

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