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The effect of metabolic alterations on regeneration in the central nervous system

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1. Zusammenfassung

a. Abstrakt

Einleitung: Die vorliegende Arbeit zum Thema Multiple Sklerose gliedert sich in drei Teile. Im ersten Teil haben wir den Einfluss von metabolischen Veränderungen des reduktiven und oxidativen (Redox-) Mileus auf die Differenzierung neuronaler Progenitorzellen (NPZ) im Mausmodell untersucht. Im zweiten Teil haben wir den Effekt von metabolischen Veränderungen durch mehrfach ungesättigten Fettsäuren (polyunsatured fatty acids; PUFAs) auf Remyelinierung bei transgenen *fat-1* Mäusen untersucht. Im dritten Teil entwickelten wir ein Protokoll für die Analyse von Lipiden und ihren Metaboliten aus Serumproben.

Methodik: Um den Effekt des Redox-Milieus auf die neuronale Entwicklung von Mäusen zu analysieren, haben wir kortikale NPZ isoliert und setzten sie entsprechenden oxidativen oder reduktiven Substanzen aus. Die Ergebnisse haben wir im Anschluss *in vivo* bei Jungtieren und autoimmun-bedingten Demyelinisierung überprüft. Im zweiten Teil haben wir das transgene *fat-1* Mausmodell genutzt, um zu vergleichen, ob es nach Cuprizone-induzierter Demyelinisierung in *fat-1* Mäusen durch erhöhte omega (n)3-PUFA Spiegel zu einer schnelleren Remyelinisierung kommt. Im dritten Teil haben wir ein Protokoll entwickelt, um durch Flüssigchromatographie gekoppelt mit Elektrospray-Ionisierung und Tandem-Massenspektroskopie die Konzentration von PUFAs und ihren Metaboliten in humanen und murinen Blutproben zu messen.

Ergebnisse: Im ersten Teil konnten wir zeigen, dass das Redox-Mileu die Differenzierung von NPZ via Histon-Deacetylase Sirt1 beeinflusst. Im oxidativen Mileu wird Sirt1 aktiviert und bindet an den Transkriptionsfaktor Hes1, der wiederum den Transkriptionsfaktor Mash1 hemmt. Durch Hemmung von Mash1 differenzieren NPZ nicht zu Neuronen, sondern zu Gliazellen. Im zweiten Teil konnten wir zeigen, dass es nach Cuprizone-induzierter Demyelinisierung durch ein erhöhtes Angebot an n-3 PUFAs zu einer leicht erhöhten Remyelinisierung kommt. Im dritten Teil konnten wir zeigen, dass wir durch die Aktivierung von humanem und murinem Vollblut mittels Calcium Ionophore A 23187 erhöhte PUFA Metabolite messen konnten.

Schlussfolgerung: In dieser Arbeit konnten wir im Mausmodell zeigen, dass metabolische Veränderungen des Redox-Mileus einen signifikanten Einfluss auf die Differenzierung von NPZ haben und metabolische Veränderungen der PUFA-Konzentration Remyelinisierung unterstützen könnte. Außerdem konnten wir zeigen, dass man Vollblut mittels A23187 stimulieren kann um die Kapazität der Zellen Lipidmediatoren zu produzieren zu messen; in unstimuliertem Vollblut sind die Konzentrationen oft unter der Nachweisgrenze.

b. Abstract

Introduction: The present work on Multiple Sclerosis consists of three parts. In the first part we looked at the effect of metabolic alterations of the reductive and oxidative (redox) state on the differentiation of neuronal progenitor cells (NPCs) in a mouse model. In the second part we evaluated the effect of metabolic alterations of polyunsaturated fatty acids (PUFAs) on remyelination of transgenic *fat-1* mice. In the third part we developed a protocol for analysing lipids and their metabolites in serum probes.

Methods: In order to assess the effect of alterations of the redox environment on neuronal development in mice, we isolated cortical NPCs and exposed them to reductive and oxidative substances. We then tested the *in vitro* results *in vivo* in mouse pups and in autoimmune-mediated demyelination. In the second part we used the transgenic *fat-1* mouse model that allows mice to endogenously convert n-6 into n-3 PUFAs in order to compare, whether remyelination following cuprizone-induced demyelination is improved by higher n-3 PUFA levels in *fat-1* mice. In the third part we developed a liquid chromatography coupled with electrospray ionisation and tandem mass spectroscopy (LC/ESI-MS/MS) protocol to measure PUFAs and their metabolites in human and murine blood samples. We analysed naive serum and compared it to samples activated by calcium ionophore A23187.

Results: In the first part we showed that the redox state influences the differentiation of NPCs via the histone-deacetlyase Sirt1. Under oxidative conditions Sirt1 is activated and binds to the transcription factor Hes1 which then inhibits the transcription factor Mash1. The inhibition of Mash1 alters the differentiation of NPCs away from neurons to glia cells. In the second part we showed that there was a slight increase in remyelination following cuprizone-induced demyelination when there was an abundance of n-3 PUFAs present. In the third part we showed that activation of human and murine whole blood by A23187 enables us to measure increased levels of PUFA metabolites.

Conclusion: In the first part we showed that metabolic alterations such as alterations of the redox state have a significant influence on the differentiation of NPCs. Similarly, we found that an abundance of n-3 PUFAS might enhance CNS remyelination. Lastly, we showed that when stimulating whole blood by A23187, we are able to measure cells' capacity to produce lipid mediators; in unstimulated whole blood such differences were mostly below detection threshold.

c. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS. In the Western world it is the main cause of disability in young adults [1] surpassed only by trauma, yet its aetiology remains elusive. Recent research has acknowledged that even at its onset MS is a neurodegenerative disease with irreversible damage to axons and neurons [2, 3]. Although the precise mechanism responsible for neuronal loss is not known, the two main risk factors for injurious processes are inflammation and demyelination.

However, recent evidence suggests that the inflammatory response to demyelination is not just a risk factor for neuronal loss, but also a necessary condition for remyelination [4-6] and neurogenesis [7-9]. It has been shown that inflammation enhances remyelination via direct effects on oligodendrocyte precursor cells' (OPCs') survival, migration and differentiation [10, 11]. Also, the presence of myelin debris in CNS tissue inhibits remyelination by OPCs [12, 13]. Hence the presence of activated macrophages that clear this debris is essential for remyelination. Furthermore, activated macrophages are also capable of inducing OPCs [14, 15]. Similarly, proinflammatory cytokines can promote neurogenesis. Interferon gamma induces neurite outgrowth [7, 8] and CXCL12 supports germ cell migration and survival [7, 9]. However, Interferon gamma has also been shown to inhibit NPC proliferation and survival [16]. This Janus-face of inflammation has resulted in the distinction between destructive and protective inflammation, where protective inflammation is associated with enhanced remyelination and reduced neuronal loss.

There are some suggestions that diets which are rich in antioxidants [21], rich in n-3 PUFAs and low in saturated fatty acids [22, 23] might stabilize disease in MS patients. The rationale behind this approach is that antioxidants might exert immunosuppressive effects [24, 25], protect from neurotoxic reactive oxygen species [24] and stabilises the blood-brain barrier [25]. N-3 PUFAs via their lipid mediators that are also known as prostanoids, isoprostanes and eicosanoids or resolvins and protectins [26], might modulate the destructive autoinflammatory response. However, empirical evidence supporting this advice is limited. In a prospective case-control study Zhang et al. could not find any evidence that higher intakes of antioxidants reduces the risk of developing MS in women [27]. In a Cochrane review of randomized trials of dietary interventions in MS, Farinotti et al. could not find a significant effect of neither n-6 nor n-3

PUFAs on disease progression nor relapse rate [28]. By contrast, a recent epidemiological study suggests that the risk for CNS demyelination is reduced when intake of n-3 PUFA is high [29].

Looking at methods to measure PUFA-derived metabolites LC/ESI-MS/MS is a well-established method to determine prostanoids, isoprostanes and eicosanoids in different tissues and body fluids [30-35]. While most assays examining the formation of lipid mediators in human blood have focused on isolated mononuclear cells [34] or neutrophils [36], some studies have described a procedure by which formation of these mediators can be stimulated in whole blood samples, using the calcium ionophore A23187 [37-40]. Such an activation protocol would also allow us to assess the generation capacity of blood cells for these compounds after activation with A23187 when lipid metabolite levels would be below detection limit in unstimulated blood samples.

d. Methods

Cell culture Cerebral cortical NPCs were obtained from C57BL/6 mice embryos (Charles River) and Sirt1$^{-/-}$ mice or wild-type littermates according to standard procedures [51]. Cells isolated from E17.5 cortices were seeded onto uncoated T75 tissue culture flasks in serum-free neurobasal medium supplemented with 2% B27 and 1% GlutaMAX-I, and then induced to proliferate as free-floating neurospheres by addition of 10 ng/ml recombinant bFGF.

Immunocytochemistry, immunoprecipitation and western blotting NPCs were cultured for 48 h in the presence of bFGF (10 ng ml^{-1}) to perform immunocytochemistry, Sirt1 immunoprecipitation and subsequent western blotting for Sirt1 and Hes1. To determine the acetylation level of histones H3 and H4, total histones were isolated by acidic extraction. Resulting precipitates equivalent to 20 µg protein were fractionated by 15% SDS–PAGE and incubated overnight at 4 °C with anti-acetyl-H3K9 (1:1000), anti-acetyl-H4K16 (1:1000), anti-acetyl-H3 (1:1000), anti-acetyl-H4 (1:1000), anti-H3 (1:500) and anti-H4 (1:500) antibodies (Biomol). Protein bands were detected using secondary antibody coupled to horseradish peroxidase (Dako) and the ECL-plus system (Amersham Biosciences).

Chromatin immunoprecipitation (ChIP) NPCs were grown for 48 h in the presence of bFGF (10 ng/ml) on poly-D-lysine-coated 6-well plates. Cells were washed and fresh NBM without bFGF was added for 24 h to induce differentiation. BSO (5 µM) was continuously present in the

culture. Cells were treated for 10 min with 1% formaldehyde at room temperature. The crosslinking reaction was quenched using glycine (125 mM), cells were washed several times in ice-cold PBS and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.0 with protease inhibitors). The lysate was ultrasonicated under conditions yielding fragments ranging from 200–600 bp and then diluted in immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl at pH 8.0). The lysate (300 µg) was used for a 12-h immunoprecipitation with 3 µg of specific antibody at 4 °C. ChIPs were performed with the appropriate antibodies. Complexes were collected for 2 h by using recombinant protein A–agarose beads (Sigma) pre-absorbed with salmon-sperm DNA (500 µg/ml) and bovine serum albumin (100 µg/ml). After the washing and elution steps, samples were incubated with RNAse (500 µg/ml) for 15 min and Proteinase K (500 µg/ml) for 1 h. Formaldehyde crosslinking was reversed by adding NaCl (200 mM) with a 12 hour incubation at 65 °C. Samples (100 µl) were purified with GFX PCR purification kit columns (Amersham Pharmacia) and used as a template in PCRs to detect the Mash1 promoter region between the -268 to -71 bps upstream from ATG start codon and placed directly downstream from CACGCG (-287 to -282) and CACGCG (-276 to -271). Control reactions using primers amplifying promoter regions in the mouse β-actin gene detected no PCR product. Quantification of ChIP analysis was performed by real-time (RT-) PCR.

Experimental Autoimmune Encephalomyelitis (EAE) induction, Sirt1 modulation and BSO treatment Animal experiments evaluating the role of the redox state on neuronal differentialtion were performed in accordance with a protocol approved by the local Charité Ethics Committee. SJL/J mice were killed by intraperitoneal injection of ketamine (Ketavet, Upjohn) and xylazine (Rompun, Bayer) 12 days after induction of EAE according to a standard protocol [52]. To examine the effects of Sirt1 activation in EAE, mice were treated with either resveratrol (50 mg/kg) or vehicle solution (Cremophore EL and ethanol; Sigma) from day 6 to day 12 after immunization. BrdU (60 mg/kg; Sigma) was administered by daily intraperitoneal injections on the last two days before animals were killed. To determine the effects of oxidation alone, BSO (2.5 mmol/kg; prepared from 1M stock in isotonic saline at pH 7.4) or vehicle alone was administered subcutaneously to mouse pups twice daily. Pups were then killed, perfused transcardially with saline and 4% paraformaldehyde, and brains were embedded in TissueTek. The Sirt1 inhibitor cambinol (100 mg/kg) or vehicle solution alone (Cremophor EL and ethanol) was administered subcutaneously to mouse pups twice daily. Pups were killed after two days of

treatment, subventricular zone (SVZ) tissue was isolated from 350 mm frontal sections and Mash1 gene expression was analysed by RT-PCR.

Short interfering (si)RNA transfection Transfections were performed with 1 μg ml^{-1} cationic lipid β-Argfectin35-plus (Atugen). Synthetic Silencer Sirt1 siRNA (30 nM; Ambion, siRNA ID 174220) specific for exon 5, Sirt1 siRNA (30 nM; Ambion, siRNA ID 174219) specific for exon 2, Hes1 siRNA (30 nM; Ambion, siRNA ID 100393) specific for exon 2, Hes1 siRNA (30 nM; Ambion, siRNA ID 158034) specific for exon 5, TLE1 siRNA (30 nM; Ambion, siRNA ID 187335) specific for exon 20 and TLE1 siRNA (30 nM; Ambion, siRNA ID 187336) specific for exon 9 were used.

In utero electroporation The in utero electroporation experiments were carried out as described previously [53]. We used time-pregnant C57BL/6 mice at embryonic day 15.5 - 17.5 post coitum. After anaesthesia with ketamine (10 mg/ml) and xylazine (1 mg/ml), the uterine horns were exposed. DNA solution (1–1.5 μl per embryo) coloured with Fast Green was injected through the uterine wall into the lateral ventricle of two of the pups by pulled-glass capillaries (World Precision Instruments). Electric pulses were delivered to embryos by holding the injected brain through the uterine wall with forceps-type electrodes (CUY650P5; Unique Medical Imada) connected to a square-pulse generator (CUY21 EDIT; Nepagene). Five pulses (38 V, 50 ms) were applied at 950 ms intervals. The uterine horns were carefully replaced into the abdominal cavity before the muscle wall and the abdomen were sutured.

***fat-1* animals** Transgenic *fat-1* mice were engineered as previously described [45]. They were subsequently backcrossed onto a C57BL/6 background at least four times. Generations of female heterozygous *fat-1* mice and male wt mice were then mated to obtain wt and transgenic mice from the same offspring. In this study, all transgenic *fat-1* mice used were heterozygous. Animals were kept under specific pathogen-free conditions in standard cages and were maintained in an air-conditioned atmosphere with a controlled 12 hour light-dark cycle. They were fed a special semi-purified diet (AIN-76A containing 10% corn oil) high in n-6 and low in n-3 fatty acids. Sterile drinking water was given *ad libitum*. Each cage housed four to six weight-matched female mice. Mouse studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Mice were divided into a *fat-1* group and a wt group according to their phenotype. For phenotyping the tip of mouse-tails (100 mg) was cut off from six-week-old mice and subjected to gas chromatographic analysis. The ratio between n-6 PUFAs

(arachidonic acid (AA) and gamma-linolenic acid (GLA n-6)) and n-3 (alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA n-3) and docosahexaenoic acid (DHA)) was determined for each individual mouse to distinguish between wt and transgenic phenotypes. Mice that had an n-6/n-3 ratio ≤ 5 were assigned to the *fat-1* group and those with an n-6/n-3 ratio > 10 were assigned to the wt group.

Chemically induced demyelination with cuprizone Six experimental groups were set up to investigate the impact of endogenously altered n-3/n-6 PUFA status on remyelination following cuprizone-induced demyelination in mice. At nine weeks of age control animals (four *fat-1* and four wt mice) were maintained on a normal pulverized diet for five weeks, while cuprizone animals received a pulverized diet containing 0.2% cuprizone (w/w) (Bis(cyclohexanone)oxaldihydrazone, Sigma-Aldrich, St. Louis, MO, U.S.A.), also for five weeks. Animals in the cuprizone demyelination group (seven *fat-1* and five wt mice) were sacrificed at the end of these five weeks on the cuprizone diet and their tissue was used to assess whether there were any differences in demyelination between wt and *fat-1* animals. Animals in the cuprizone remyelination group (seven *fat-1* and five wt mice) were first fed the cuprizone diet for five weeks and were then allowed to recover for another two weeks on a normal diet. They were then sacrificed in order to see whether there were any differences in remyelination between wt and *fat-1* animals. Animal weight was recorded throughout the entire experiment. All animals were sacrificed by anaesthesia with isoflurane (IsoFlo®, Abbott Laboratories, Abbott Park, Illinois, USA).

Histology The rostral 2/3 of the brains were first fixated in 4 % formaldehyde (Arcos Organics, Fairlawn, NJ, U.S.A.) at 4 ºC for 24 hours and subsequently quenched in a sucrose gradient (Sigma-Aldrich, St. Louis, MO, U.S.A.) over two days before they were fresh-frozen in Shandon M-1 Embedding Matrix® (Thermo Fisher Scientific, Waltham, MA, U.S.A.) for cryosectioning. Coronal brain sections were cut at 10 µm thickness corresponding to slice 205 of the Harvard High Resolution Mouse Brain Atlas[54]. Sections were then air-dried and stored at -20 ºC until further processing. Luxol Fast Blue (LFB) staining was used to quantify myelin. For this staining, sections were first hydrated in aqua bidest. and then rinsed in 70% ethanol and placed in 0.1% LFB (Solvent Blue 38®, Sigma-Aldrich, St. Louis, MO, U.S.A.) staining solution for 6 hours at 60 ºC. Following the 6 hours of staining, sections were then rinsed in 95% ethanol, followed by aqua bidest. In the next step sections were postfixed in 1% lithium carbonate solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) and subsequently rinsed in 70%

ethanol and aqua bidest. This step was repeated until there was a clear differentiation between white and grey matter staining. Sections were then counterstained in 0.1% Cresyl Violet acetate solution (Sigma-Aldrich, St. Louis, MO, U.S.A.). Eventually, sections were rinsed in aqua bidest followed by 95% and 100% ethanol, then xylol and then they were coverslipped. Stainings were evaluated with a LSM 5 Pascal confocal microscope (Carl Zeiss AG, Oberkochen, Germany). Of each brain a photo was taken of the corpus callosum at slice 205 of the mouse brain atlas. First, equal volumes (300,000 square pixels) of corpus callosum were selected. Within this volume the area that stained positive for myelin was then selected using ImageJ open source software and the size of the myelinated area was recorded.

Gas chromatography For PUFA analysis, brain or tail tissues frozen in liquid nitrogen were ground into powder in liquid nitrogen. The powder was subjected to extraction of total lipids and fatty acid methylation by heating them at 100 °C for 1 hour in a solution containing 2.5 ml hexane and 2.5 ml 14% boron trifluoride in methanol. Once the samples had cooled down to room temperature they were vortexed. Next, 1 ml water was added to the solution. In order to ensure that the concentration between the aqueous and the lipophilic phase was in equilibrium the samples were shaken by hand for four minutes. The phases were then separated by centrifugation and the lipophilic hexane phase containing fatty acid methyl esters was removed and dried under nitrogen. The fatty acid methyl ester residues were redissolved in 50 µl hexane and transferred into an autosampler vial. They were then analysed by GC using a fully automated Hewlett Packard 5890 system equipped with a flame ionization detector. Peaks of resolved fatty acids were identified by comparison with a fatty acid standard and the area under those resolved peaks represented their relative concentrations. The size of the areas was measured using a Perkin Elmer M1 integrator.

Liquid Chromatography Mass Spectrometry / Mass Spectrometry (LC/ESI-MS/MS)
For analysis of monohydroxy lipid metabolites thirty milligrams ground and frozen brain tissue from wt (n=7) and *fat-1* (n=4) mice was mixed with methanol and internal standard (LTB4-d4) and hydrolysed with 300 ml of 10 M sodium hydroxide for 30 min at 60 °C. The solution was neutralized with 60% acetic acid and pH was adjusted to 6.0 with sodium acetate buffer. For human blood samples, 4.5 ml blood was collected in heparinized containers and either incubated for 30 min at 37 °C with 50 µM A23187 or left untreated. Mouse blood was collected by cardiopuncture after mice were anesthetized with isoflurane and incubated with 50 µM A23187 for 30 min at 37 °C immediately afterwards. Plasma was then obtained by centrifugation for 10

min at 4 °C and 3000 rpm. Next an internal standard consisting of 15-HETE-d_8 (10 ng), LTB_4-d_4 (10 ng), PGE_2-d_2 (5 ng) and ice-cold methanol containing 0.1% BHT was added to the blood samples. In both brain and blood samples the pH was then adjusted with 1 M sodium acetate buffer containing 5% v/v methanol at pH = 6. After centrifugation, the obtained supernatant from all samples was added to the SPE-columns, which were preconditioned with 3 mL methanol, followed by 3 mL of 0.1 mol/L sodium acetate buffer containing 5% methanol (pH 6). The SPE-columns were then washed with 3 mL methanol/H_2O (50/50, v/v). For elution 2.0 mL of n-hexane:ethyl acetate 25:75 with 1 % acetic acid was used. The extraction was performed with a SUPELCO Visiprep manifold. The eluate was evaporated on a heating block at 40 °C under a stream of nitrogen to obtain a solid residue and stored at -20 °C until LC/ESI-MS/MS analysis was performed. A solid phase extraction was performed with an anion exchange column (Bond Elute Certify II, Agilent, Santa Clara, CA) as described previously [25]. For elution, an n-hexane:ethyl acetate extraction mixture 25:75 with 1% acetic acid was used. The eluate was evaporated on a heating block at 40°C under a stream of nitrogen to obtain a solid residue. Residues were then dissolved in 70 µl acetonitrile. An Agilent 1200 high performance liquid chromatography (HPLC) system and a solvent system consisting of acetonitrile/0.1% formic acid in water was used. The gradient elution was started with 15% acetonitrile, this was increased within 10 min up to 90% and held for 10 min. The HPLC system was coupled with an Agilent 6410 Triplequad mass spectrometer with electrospray ionization source. Analysis of lipid mediators was performed using Multiple Reaction Monitoring in negative mode and converted into an electrical signal by an electron multiplier dynode. The resulting signals were identified and quantified with the help of previously analysed standards, all from Cayman Chemical (Ann Arbor, MI, U.S.A.).

Statistical analysis Statistical analysis of the results was performed in Graph Pad Prism 6 (GraphPad Software, Inc., La Jolla, U.S.A.) calculating the mean values of group sizes ± standard error of the mean (SEM) or standard deviation (SD). The Mann Whitney U test or Student's t-test were performed to test for significant differences between groups at a certain point in time where appropriate. To compare differences in weight changes over time, a repeated measurements ANOVA was used. Statistical significance was set at a level of $p < 0.05$.

e. Results

In our first study we investigated whether metabolic alterations of the redox system affect the multipotentiality of NPCs. Under pro-oxidative conditions the proportion of astrocytes (GFAP(+)) increased by 40% under BSO ($p < 0.05$), whereas the proportion of neurons (NeuN(+)) decreased by 40% ($p < 0.05$). Reducing conditions had the opposite effect: lipoic acid increased the proportion of NeuN(+) cells by 70% and decreased the proportion of GFAP(+) cells by 17% ($p < 0.05$). Looking for a mechanism of action, we found that in NPCs exposed to pro-oxidative conditions the expression of Sirt1 was markedly upregulated, whereas levels of the neural transcription factors NeuroD, Hes1, Hes5, Olig1 and Olig2 were not altered. We then targeted Sirt1 in NPCs by adding the Sirt1 activator resveratrol to proliferating NPCs. Resveratrol increased the proportion of astrocytes and reduced the proportion of neurons in a Sirt1-dependent fashion, thus mimicking the effects of mild oxidative conditions. To confirm the direct contribution of Sirt1 to redox-mediated differentiation processes, we silenced Sirt1 in NPCs before BSO challenge, using two siRNAs designed against different exons, and indeed, oxidation-mediated effects were prevented. To analyse the downstream effects of Sirt1 upregulation, we performed immunoprecipitation experiments using an anti-Sirt1 antibody. Under mild pro-oxidative conditions the Sirt1–Hes1 complex was activated and acetylation of the histone residue H3K9, a putative target of Sirt1, was downregulated. Also, both pro-oxidative conditions inducing Sirt1 and direct Sirt1 activation using resveratrol decreased Mash1 gene expression. To determine whether the Sirt1–Hes1 complex mediates Mash1 repression during a pro-oxidative shift, we depleted Sirt1 and Hes1 with siRNAs and found that oxidation-mediated Mash1 repression did not occur when Hes1 or Sirt1 were silenced, nor in Sirt1$^{-/-}$ progenitor cells. Consequently, we hypothesized that, under conditions of oxidative challenge, Sirt1 cooperates with Hes1 as part of the co-repressor complex that inhibits Mash1 transcription. We performed ChIP analysis and found that, under pro-oxidative conditions, Sirt1 was markedly upregulated in the Mash1 promoter region.

To determine the relevance of our findings *in vivo*, we first performed an immunohistological analysis of Mash1 and Sirt1 patterning in the postnatal day 2 CNS. At this stage, Mash1 was detected in progenitors residing in germinal brain regions and was shown to contribute to the specification of both neurons and oligodendrocytes. Next, we investigated whether the fate of NPCs *in vivo* is also modulated by a pro-oxidative shift. Mouse pups received BSO by daily

subcutaneous injections and were pulsed with BrdU before histological analysis of the SVZ. Expression of Sirt1 was increased in the SVZ and mainly confined to GFAP(+) cells; however, the number of Mash1(+) cells decreased. At the same time, the number of proliferating cells, identified by BrdU staining decreased. To investigate the direct contribution of Sirt1 and Hes1 to oxidation-mediated effects *in vivo*, we performed knockdown of these genes using in utero electroporation. Animals were electroporated at embryonic day 17.5 with enhanced green fluorescent protein (EGFP) plasmids containing Sirt1, Hes1 or control short-hairpin (sh) RNA. As expected, Sirt1 or Hes1 knockdown resulted in a higher proportion of Dcx(+) cells in mice subjected to oxidation, indicating increased neurogenesis *in vivo*. Our *in vivo* knockdown strategy demonstrates the hitherto unknown contribution of Sirt1 to neural-fate decision in the oxidative milieu. Moreover, depletion of Sirt1 in animals electroporated in utero caused an increase in the proportion of Mash1(+) cells in the EGFP(+) population, indicating increased neurogenesis. Consistently, subcutaneous treatment of pups with cambinol, a small-molecule Sirt1 inhibitor, caused upregulation of Mash1 in the SVZ. Finally, we investigated Sirt1 expression in autoimmune demyelination. Brain pathology in mice with EAE induced by transfer of encephalitogenic T cells into naive recipients showed a lack of Sirt1 expression in non-inflamed brain regions. In contrast, areas affected by perivascular leukocyte infiltration and reactive astrogliosis showed marked Sirt1 upregulation in GFAP(+) cells with astroglial morphology, but very low Mash1 expression. In these cells, Sirt1 expression was mainly intranuclear, suggesting its involvement in chromatin silencing. Moreover, application of the Sirt1 activator resveratrol to EAE animals revealed an increase of GFAP(+), BrdU(+) early progenitors in lesions, suggesting enhanced gliogenesis through increased Sirt1 activity.

In our study on the effect of n-3 PUFAs on CNS remyelination in mice GC was used to analyse the lipid profile of brain tissue from wt and *fat-1* mice. While lipid profiles of mouse-tails showed significant differences for the PUFAs AA, EPA and DHA, these differences were significant only for EPA in brain tissue, which was detectable only in *fat-1* mice. This was also reflected in the amounts of monohydroxy lipid metabolites derived from AA, EPA and DHA: While amounts of AA-derived HETEs and DHA-derived HDHAs were not significantly different, levels of EPA-derived HEPEs were significantly higher. More specifically, there were no significant differences for 8-/9-HETE, as well as 5-HETE, 12-HETE and 15-HETE, which are derived from 5-, 12-, or 15-lipoxygenase action. Similarly, differences for the corresponding DHA metabolites 4-/7-HDHA, 14-HDHA and 17-HDHA were not significantly different between wt and *fat-1* animals. In contrast, while present only in much smaller amounts, the

corresponding EPA derived metabolites were all significantly increased in *fat-1* mice: Most notably high amounts of 18-HEPE were found. Given that 18-HEPE is a pathway marker of anti-inflammatory and pro-resolution mediators such as resolvin E1 and E2 [26], and an anti-inflammatory compound itself [55], we hypothesized that the difference between n-3 PUFA in wt and *fat-1* mice (while only clearly discernible for EPA and its metabolites) might lead to an anti-inflammatory and/or pro-resolution phenotype in the context of cerebral inflammation.
In the next step we thus evaluated the effect of cuprizone feeding in wt and *fat-1* mice. We observed a suppressive effect on their metabolism with weight loss in wt and *fat-1* mice (-11 and -13 %, respectively). There was no significant difference between those two groups indicating that expression of the *fat-1* gene and the changed n-3 PUFA tissue content in *fat-1* mice did not interfere with the uptake and toxic metabolic effect of cuprizone in this study.
Next, mice were sacrificed at two time points to evaluate the effect of different n-3 PUFA status on demyelination and remyelination. LFB staining was used to quantify myelin and the mean volume and the SEM were calculated for each treatment group. For the control groups, there were no significant differences between wt animals and *fat-1* animals (wt control: mean 274,257 pixels^2 (± 4,078), *fat-1* control: mean 287,886 pixels^2 (± 7,219), $p = 0.23$). This was true also for the demyelination group where wt animals had a mean volume of myelin in their corpus callosum that was similar to the values observed in *fat-1* animals (wt demyelination 104,459 pixels^2 (± 14,398), *fat-1* demyelination 121,534 pixels^2 (± 18,059, $p = 0.51$). At the same time the cuprizone-treated groups had both significantly lower myelin than the control groups ($p < 0.001$). In the remyelination phase there was a close miss of statistical significance between remyelinated wt animals with a lower mean volume in wt animals (156,414 ± 25,717) versus higher values in *fat-1* animals (239,981 ± 24,764) ($p = 0.07$).

In our last study, in which we developed a protocol to measure PUFAs and PUFA-derived lipid mediators in whole blood, A23187 activation increased the levels of nearly all tested metabolites significantly with combined amounts of 1299.3 + 234.2 ng/mL HETEs, 52.2 + 8.6 ng/mL HEPEs and 54.4 + 7.6 ng/mL HDHAs. Again there was a predominance of the 12-LOX products at 1179.1 + 226.1 ng/mL for 12-HETE, 45.7 + 7.9 ng/mL for 12-HEPE and 38.4 + 5.0 ng/mL for 14-HDHA. The 5-LOX (particularly LTB_4) and the 15-LOX products as well as PGE_2 and TXB_2 also increased significantly. However, even after activation with 9-HETE, 9-HEPE and 8-HDHA as well as the potent bioactive lipid mediators 10,17-DiHDHA, lipoxin A_4 and resolvin D_1 were not detectable in the assayed samples. The results in human samples were then compared with

measurements from activated mouse blood. Whole blood activation with A23187 was performed in blood samples obtained by cardiopuncture from healthy wild-type C57Bl6 mice. The results in this set of experiments were similar to those observed in human blood. There were high levels particularly of the HETEs and a predominance of 12-LOX products with values of 3654.0 + 523.6 ng/mL for 12-HETE, 19.8 + 2.1 ng/mL for 12-HEPE and 72.6 + 8.7 ng/mL for 14-HDHA. As in the human blood samples lipoxin A_4, resolvin D_1 and 10,17-DiHDHA were not detectable. However, there were several differences in the amounts of lipid metabolites detectable in mouse blood samples with generally higher levels of HETEs and of some higher levels of the HDHAs formed, while the HEPEs were lower. Notably, 9-HETE was detectable in mouse samples only, while not present in activated human plasma. On the other hand 7-, 11-, and 13-HDHA were not detectable in mouse samples but only in human ones.

f. Discussion

Our study on the redox-dependent fate of NPCs shows that non-toxic redox alterations affect the self-renewal capacity of NPCs. Here, oxidative conditions favour astrocyte expansion at the expense of neurogenesis, whereas reducing conditions have the opposite effect. The oxidation-mediated increase of astrocytes does not seem to be linked to either an enhanced proliferation of astrocyte precursors or to restriction of the multipotentiality of NPCs. Rather, oxidation directs differentiation of uncommitted NPCs towards the astroglial lineage by altering their development programme. Moreover, our data suggest that Sirt1 is the mediator of the observed redox effects, as mild oxidation in NPCs led to higher Sirt1 deacetylase activity at the Mash1 promoter. On the basis of our observations, oxidation does not induce a global histone deacetylation, as the acetylation status of whole histone H3 or whole histone H4 was not reduced under these conditions. On the contrary, oxidation reduced acetylation of the H3K9, but not of the H4K16 residue, both known to be regulated by Sirt1 [56]. This effect was reversed when we applied the functional Sirt1 blocker splitomicin. In contrast to these observations, trichostatin A, which inhibits class I and class II HDACs, induced a global increase in acetylation. There was no modulation of other potentially redox-dependent Sirt1 targets, such as UCP2 or cIAP2. We may conclude from these data that the observed loss of H3K9 acetylation at the Mash1 promoter is not part of a general phenomenon in the context of oxidation-mediated Sirt1 activation, but rather, that oxidation results in a stronger association of Sirt1 to the Mash1 promoter, leading to a targeted and local deacetylation of H3K9 and subsequently to Mash1 inhibition. As Mash1 has

a crucial role in neurogenesis [57], Sirt1 was considered to contribute to NPC fate *in vivo*. Indeed, analysis of the developing mouse brain revealed a predominantly non-coincident distribution of Sirt1 and Mash1, suggesting that they have opposing roles in commitment and differentiation of neural progenitors. Furthermore, to clarify the roles of Sirt1 and Hes1 under oxidative conditions *in vivo*, we performed gene knockdown using *in utero* electroporation. This technique allows a selective and transient manipulation of gene function *in vivo* in a subset of cells. Combined with the fluorescence tagging of the silenced cells, the selective and transient knockdown of these genes enabled *in vivo* clonal analysis and confirmed our *in vitro* findings.

Our study assessing the effect of n-3 PUFAs on CNS remyelination in *fat-1* mice showed that n-3 PUFAs might support remyelination. In a first step we compared lipid profiles of tails and brain from healthy wt and *fat-1* mice. We found only small differences between wt and *fat-1* mouse brain tissue. A possible explanation for this difference is that in the CNS DHA might play such a crucial role, so that they are enriched here even in the context of low n-3 PUFA supplementation. This has been shown previously by Jeffrey et al. for DHA in retinal cells [58] and it has been suggested that a similar principle might regulate trafficking of certain n-3 PUFAs to the brain [59]. In rats that are depleted of dietary n-3 PUFAs there is an increase in DHA synthesis from ALA in the liver [60, 61]. Hence one possible scenario would be that in DHA-depleted dietary states there is preferential trafficking of liver-derived DHA to the brain, amongst others through lipoprotein receptors [60]. There was, however, a significant difference of the amounts of EPA present, leading to increased amounts of potentially anti-inflammatory and anti-fibrotic 18-HEPE [24] in *fat-1* brains. At the same time, levels of anti-inflammatory DHA metabolites, while much higher than the EPA metabolites, were unchanged between wt and *fat-1* groups.

Following cuprizone-induced demyelination there was no significant difference in the amount of demyelination between *fat-1* and the wt animals. However, we saw improved remyelination in *fat-1* mice; but this difference missed statistical significance at $p = 0.07$. Given our relatively small sample size with an average group size of 5 animals statistical significance might be reached by increasing case numbers. Thus the data presented here suggest that an increased n-3 PUFA tissue status might lead to a mild beneficial effect of n-3 PUFAs on *in vivo* CNS remyelination in mice. An important point is that we quantified actual differences in PUFA tissue status both in the tail and in the CNS. Interestingly, despite small differences in the FA profile of wt and *fat-1* animal brains, we were able to see differences in the degree of remyelination between these groups. One possibility that would explain this finding is that

despite similar AA and DH profiles in the brain, there might be differences in the EPA-derived lipid mediators in the course of cuprizone-induced de- and remyelination. In this study we only established baseline measurements of hydroxyl metabolites, in which there was no difference for the DHA-derived metabolites but significant differences for the EPA-derived 18-HEPE.

Our finding of increased levels of EPA-derived 18-HEPE in the brain of *fat-1* versus wt mice could be interpreted in the light of other recent studies of 18-HEPE. Endo et al. [62] showed that in *fat-1* mice there is a selective enrichment of EPA in *fat-1* transgenic bone marrow cells and EPA-metabolite 18-HEPE in *fat-1* transgenic macrophages. Bone marrow transplantation experiments revealed that an 18-HEPE-rich environment through transplantation of *fat-1* transgenic bone marrow prevented macrophage-mediated cardiac remodelling via cardiac fibroblasts. This anti-fibrotic effect of 18-HEPE on cardiac fibroblasts was reproduced *in vivo* and *in vitro*. Similarly, in a study on murine macrophages 18-HEPE was able to significantly decrease macrophages' TNF-alpha formation *in vitro* [25]. While there might be a tight regulation of DHA levels in the CNS, differences in EPA levels might thus lead to significant changes in anti-inflammatory lipid metabolites in the brain. Therefore, a possible explanation for the protective effect of an n-3 PUFA-enriched tissue status in CNS demyelinating disease could be a difference particularly in EPA-derived metabolites such as 18-HEPE. Based on the data from Torkildsen et al. [44], these could affect mainly macrophage function, although further experiments might be necessary to assess also the role of astrocytes in this model. Future studies will now have to assess the activity and function of macrophages/microglia found at the sites of CNS injury in the cuprizone model and to understand the effect of EPA on activity of these cells.

The data on our protocol for measuring PUFAs and their metabolites presented here show potent activation of lipid metabolite formation in plasma from activated whole blood samples treated with A23187. This demonstrates the lipid mediator generation capacity in blood from healthy volunteers and C57Bl6 mice, and offers an analytical approach to test for the profiles of lipid metabolites in physiological and pathophysiological processes. At the same time, this approach cannot distinguish between the different pathways leading to lipid metabolite formation, as it cannot discriminate between de-novo synthesis and release of preformed compounds from phospholipids, and between enzymatic or autooxidation pathways leading to mediator formation. It is noteworthy that particularly the formation of 12-HETE, 12-HEPE and 14-HDHA was triggered by A23187, arguing towards an important contribution of 12-LOX to the observed effect. The activation is not limited to the 12-LOX, though, as the 5-LOX and 15-LOX metabolites as well as PGE_2 and TXB_2 also increased highly significantly. However, A23187

activation did not lead to the formation of detectable amounts of the biologically highly potent lipoxin A_4 or resolvin D_1 in samples from humans or mice. The data presented here demonstrate widespread A23187-triggered n-6 and n-3 PUFA utilisation for the generation of bioactive lipid metabolites and present a protocol that allows for the determination of a wide range of lipid mediators and metabolites derived from AA, EPA and DHA in human plasma. Particularly with regard to resolvins and lipoxins it could be possible that certain lipid mediators might only become detectable in peripheral blood of patients with acute or chronic inflammatory processes. This hypothesis requires further testing now and could lead to more precise descriptions of lipid mediator profiles in health and disease. Future work will also be necessary to understand the effect of underlying PUFA concentrations in the blood on the formation of lipid metabolites, as different ratios of n-6/n-3 PUFA could contribute to differences in the levels of the respective lipid mediators and metabolites that are formed upon activation with A23187.

In the present three studies we assessed the effect of metabolic alterations on regeneration in murine models of CNS regeneration. Firstly, we found that a pro-oxidative shift induces Sirt1 expression in neural progenitors both *in vitro* and *in vivo*. This in turn promotes astrogenesis and inhibits neurogenesis through the interaction of Sirt1 with Hes1 and subsequent repression of Mash1. By providing an explanation for downregulation of neurogenesis in inflammation, our data suggest new targets for the regulation of neural cell-fate in neurological disorders. Targeting these mechanisms may minimize undesirable aspects of reactive astrogliosis and improve the success of therapeutic neural stem-cell implantation. Secondly, we developed a protocol to measure PUFAs and their metabolites from A23187 activated blood samples that allows us to detect these substances that are below detection limit in unstimulated blood. It remains to be shown whether this activation corresponds, quantitatively and qualitatively, to physiological stimuli. Lastly, we found that in *fat-1* mice, which endogenously form n-3 PUFA, there was a trend towards increased CNS remyelination after cuprizone damage in the context of higher EPA levels in the brain. The differences in remyelination – in the face of CNS lipid profiles differing only in EPA levels and not in DHA levels – might be due to EPA-derived metabolites such as 18-HEPE, for which anti-inflammatory and anti-fibrotic effects have been described. While DHA is more abundant in the CNS, this finding puts an emphasis on the possible role of EPA and its metabolites in CNS remyelination. Given that our observation of increased remyelination due to increased n-3 PUFA tissue content narrowly missed significance, future mouse studies are also warranted to further establish this effect in a larger cohort of mice in the cuprizone model, as well as in other experimental MS models.

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2. Eidesstattliche Versicherung und ausführliche Anteilserklärung

„Ich, Elise Helen Dorothee Siegert, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „The effect of metabolic alterations on regeneration in the central nervous system“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe. Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet. Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet. Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum

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Unterschrift

Elise Helen Dorothee Siegert hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: **Siegert, E.**, Paul, F., Rothe, M., Weylandt, K.H., **The effect of omega-3 fatty acids on central nervous system remyelination in *fat-1* mice,** BMC Neuroscience, 2017.
Beitrag im Einzelnen: Konzeption und Durchführung der Experimente (Gewinnung von Proben, Histologie, Gaschromatographie), Verfassung des Manuskriptes und der Revision.

Publikation 2: Gomolka, B., **Siegert, E.**, Blossey, K., Schnuck, W.H., Rothe, M., Weylandt, K.H., **Analysis of omega-3 and omega-6 fatty acid-derived lipid metabolite formation in human and mouse blood samples,** Prostaglandins and other lipid mediators, 2011.
Beitrag im Einzelnen: Experimentelle Arbeit (Gewinnung von Proben, Aufarbeitung der Proben und Messung von Lipiden und Metaboliten) und Mitarbeit bei der Verfassung des Manuskripts.

Publikation 3: Prozorovski, T., Schulze-Topphoff, U., Glumm, R., Baumgart, J., Schröter, F., Ninnemann, O., **Siegert, E.**, Bendix, I., Brüstle, O., Nitsch, R., Zipp, F., Aktas, O., **Sirt1 contributes to the redox-dependent fate of neural progenitors,** Nature Cell Biology, 2008.
Beitrag im Einzelnen: Experimentelle Arbeit (Histologische Färbung und deren Quantifizierung) und Korrekturlesen des Manuskripts.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers

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Unterschrift des Doktoranden/der Doktorandin

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3. Druckexemplare der ausgewählten Publikationen

Die ausgewählten Publikationen sind aus rechtlichen Gründen in der elektronischen Version meiner Arbeit nicht enthalten.

Publikation I

Siegert E, Paul F, Rothe M, Weylandt KH: **The effect of omega-3 fatty acids on central nervous system remyelination in fat-1 mice**. *BMC neuroscience* 2017, **18**(1):19.

https://doi.org/10.1186/s12868-016-0312-5

Publikation II

Gomolka B, **Siegert E**, Blossey K, Schunck WH, Rothe M, Weylandt KH: **Analysis of omega-3 and omega-6 fatty acid-derived lipid metabolite formation in human and mouse blood samples**. *Prostaglandins & other lipid mediators* 2011, **94**(3-4):81-87.

https://doi.org/10.1016/j.prostaglandins.2010.12.006

Publikation III

Prozorovski T, Schulze-Topphoff U, Glumm R, Baumgart J, Schroter F, Ninnemann O, **Siegert E**, Bendix I, Brustle O, Nitsch R, Zipp F, Aktas, O: **Sirt1 contributes critically to the redox-dependent fate of neural progenitors**. *Nature cell biology* 2008, **10**(4):385-394.

https://doi.org/10.1038/ncb1700

4. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen
in der elektronischen Version meiner Arbeit nicht veröffentlicht.

5. Komplette Publikationsliste

1. Prozorovski T, Schulze-Topphoff U, Glumm R, Baumgart J, Schroter F, Ninnemann O, Siegert E, Bendix I, Brustle O, Nitsch R *et al*: **Sirt1 contributes critically to the redox-dependent fate of neural progenitors**. *Nature cell biology* 2008, **10**(4):385-394.
2. Siegert E, Paul F, Rothe M, Weylandt KH: **The effect of omega-3 fatty acids on central nervous system remyelination in fat-1 mice**. *BMC neuroscience* 2017, **18**(1):19.
3. Gomolka B, Siegert E, Blossey K, Schunck WH, Rothe M, Weylandt KH: **Analysis of omega-3 and omega-6 fatty acid-derived lipid metabolite formation in human and mouse blood samples**. *Prostaglandins & other lipid mediators* 2011, **94**(3-4):81-87.

4. Gunther J, Kill A, Becker MO, Heidecke H, Rademacher J, Siegert E, Radic M, Burmester GR, Dragun D, Riemekasten G: **Angiotensin receptor type 1 and endothelin receptor type A on immune cells mediate migration and the expression of IL-8 and CCL18 when stimulated by autoantibodies from systemic sclerosis patients**. *Arthritis research & therapy* 2014, **16**(2):R65.
5. Riemekasten G, Siegert E: **[Sex-specific differences of the immune system]**. *Zeitschrift fur Rheumatologie* 2014, **73**(7):600-606.
6. Gunther J, Rademacher J, van Laar JM, Siegert E, Riemekasten G: **Functional autoantibodies in systemic sclerosis**. *Seminars in immunopathology* 2015, **37**(5):529-542.
7. Humrich JY, von Spee-Mayer C, Siegert E, Alexander T, Hiepe F, Radbruch A, Burmester GR, Riemekasten G: **Rapid induction of clinical remission by low-dose interleukin-2 in a patient with refractory SLE**. *Ann Rheum Dis* 2015, **74**(4):791-792.
8. Rademacher J, Kill A, Mattat K, Dragun D, Siegert E, Gunther J, Riemekasten G: **Monocytic Angiotensin and Endothelin Receptor Imbalance Modulate Secretion of the Profibrotic Chemokine Ligand 18**. *The Journal of rheumatology* 2016, **43**(3):587-591.
9. von Spee-Mayer C, Siegert E, Abdirama D, Rose A, Klaus A, Alexander T, Enghard P, Sawitzki B, Hiepe F, Radbruch A *et al*: **Low-dose interleukin-2 selectively corrects regulatory T cell defects in patients with systemic lupus erythematosus**. *Ann Rheum Dis* 2016, **75**(7):1407-1415.

10. Ahrens HC, Siegert E, Tomsitz D, Mattat K, March C, Worm M, Riemekasten G: **Digital ulcers score: a scoring system to assess digital ulcers in patients suffering from systemic sclerosis**. *Clinical and experimental rheumatology* 2016, **34 Suppl 100**(5):142-147.
11. Siegert E, Riemekasten G: **Are we too lenient with immunosuppression in severe cases of Systemic Sclerosis?** *Rheumatology (Oxford)* 2016, **55**(10):1914-1916.
12. Grund D, Siegert E: **[Pulmonary fibrosis in rheumatic diseases]**. *Zeitschrift fur Rheumatologie* 2016, **75**(8):795-808.
13. Michelfelder M, Becker M, Riedlinger A, Siegert E, Dromann D, Yu X, Petersen F, Riemekasten G: **Interstitial lung disease increases mortality in systemic sclerosis patients with pulmonary arterial hypertension without affecting hemodynamics and exercise capacity**. *Clinical rheumatology* 2017, **36**(2):381-390.
14. Valentini G, Iudici M, Walker UA, Jaeger VK, Baron M, Carreira P, Czirjak L, Denton CP, Distler O, Hachulla E *et al*: **The European Scleroderma Trials and Research group (EUSTAR) task force for the development of revised activity criteria for systemic sclerosis: derivation and validation of a preliminarily revised EUSTAR activity index**. *Ann Rheum Dis* 2017, **76**(1):270-276.
15. Moinzadeh P, Riemekasten G, Siegert E, Fierlbeck G, Henes J, Blank N, Melchers I, Mueller-Ladner U, Frerix M, Kreuter A *et al*: **Vasoactive Therapy in Systemic Sclerosis: Real-life Therapeutic Practice in More Than 3000 Patients**. *The Journal of rheumatology* 2016, **43**(1):66-74.

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