SUPPLEMENTAL MATERIAL

Article Title: Impact of Dietary Intervention on Serum Neurofilament Light Chain in Multiple Sclerosis

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Supplemental Methods

Clinical Trial Design: This study was a three-armed parallel group, single center, controlled and randomized clinical trial. The permuted-block randomization was generated online at http://randomization.com. An investigator blind to the randomization plan determined the patients' randomization number before they underwent randomization. This study was registered at http://www.clinicaltrials.gov as NCT01538355.

Patients: Multiple sclerosis (MS) patients were randomly allocated to three different experimental groups: i) adapted ketogenic diet (AKD) for 6 months, ii) caloric restriction (CR) for 7 days followed by common (control) diet for 6 months, or iii) CD (control diet) for 6 months. Before starting the dietary treatment, blood was drawn from all participants for intra-group control purposes. The time interval of the initial blood sampling did not exceed 2 months prior to starting the dietary intervention and after the initial blood draw the patients stayed on their regular diet. After the start of the dietary intervention, blood was drawn again within the first month after changing the diet. This occurred on day eight for the CR group and between day nine and day

17 for the AKD and CD patients. Due to patient dropouts (n=12), and exclusions due to internal quality standards or sera losses (n=8) analyses for 40 patients (9 CD, 14 CR and 17 AKD) could be performed (**Scheme 1**). The patients recruited originally met the following criteria: between 18 and 67 years of age, stable disease modifying therapy (DMT) for at least six months prior to inclusion or no DMT for at least six months or naive to therapy, Expanded Disability Status Scale (EDSS) $\leq$ 6.5 and body mass index (BMI) between 18 and 45. Exclusion criteria were primary or secondary progressive forms of MS, clinically relevant heart, lung, liver, or kidney diseases, pregnancy or breastfeeding, other neurologic disorders, cancer, weight loss therapy in the month prior to screening, relapse or steroid pulse therapy < 30 days prior to screening, diabetes or other metabolic defects, bulimia, anorexia and drug abuse.

Study Settings. The local ethics committee approved the study and all participants gave informed written consent according to the 1964 Declaration of Helsinki. Relapsing-remitting MS patients fulfilling the panel criteria of 2010[1] were prospectively recruited from throughout Germany and their health status was assessed at the Experimental and Clinical Research Center (ECRC).

Interventions: MS patients who met the inclusion criteria (n=60) were randomly assigned to three study dietary interventions (n=20 per group). To evaluate food intake, we used a 115-item dietary self-record with additional free text entries for unlisted foods to track individual foods or liquids and quantities prospectively over a period of 7 days before baseline and between all other visits (Optidiet software Version 5.1 GOE mbH, Büro Linden, Linden, Germany). Participants meeting a discontinuation criterion such as unwilling to continue dietary obligations during study were offered the chance to attend the remaining study visits for follow up outside the study protocol.

Control Diet (CD): Patients on CD met the criteria of a common diet in German population as described in the "National Nutrition Survey II" (https://www.mri.bund.de/de/institute/ernaehrungsverhalten/publikationen/forschungsprojekte/nvsii/). We advised patients to continue their regular diet.

Caloric Restriction / Intermittent Fasting (CR): As previously described, a single cycle of 7-day CR (200-350 kcal/day) was performed at study outset; a 3-day stepwise reintroduction to an isocaloric common diet was performed starting on day eight.[2] Compliance during the fasting period was measured via ketosis in urine (≥ 8000 µmol/L acetoacetate), self-measured once a day (Ketostix, Bayer Consumer Care AG) and via ketosis in blood (≥ 2000 µmol/L ß-hydroxybutyrate), self-measured once a day (FreeStyle Precision, Abbott Diabetes Care Ltd.). The common diet was then maintained until study end.

Adapted Ketogenic Diet (AKD): Patients followed an AKD for 6 months from study outset. The established therapeutic models of ketogenic diet in children were adapted by Dr. Bock in regard to increased feasibility of traditional ketogenic diet in adult patients. AKD was designed to achieve i) a modest ketosis in blood (≥ 500 µmol/L ß-hydroxybutyrate), self-measured after dinner twice a week (FreeStyle Precision, Abbott Diabetes Care Ltd.), ii) a modest ketosis in urine (≥ 500 µmol/L acetoacetate), self-measured after dinner once a week (Ketostix, Bayer Consumer Care AG) and iii) to maintain patient compliance. Patients received a booklet (Dr. Bock protocol) with meal suggestions over 28 balanced days and were encouraged not to limit fat ingestion. We recommended an average daily intake of < 50 g carbohydrates, > 160 g fat (omega 6 vs omega 3 ratio 2:1) and average protein intake ≤ 100 g per day. Patients received detailed information about nutritional facts, glycemic load and how to handle carbohydrates from an experienced nutritional coach.

Primary clinical outcome measures: To judge the clinical outcome of this study, we quantified the Multiple Sclerosis Quality of Life-54 questionnaire (MS-54), which consists of 54 items. Two composite scores quantifying physical and mental health were calculated from the outcome parameters. The diet-induced alterations on the MS-54 index, which constituted the primary outcome measure of clinical trial, have been reported before.[3] The study presented here involves a secondary outcome measure; these data have recently been generated and were not available when the original report was published.[3]

Secondary outcome measure: As secondary outcome measure the sNfL concentration was quantified in 2020.

sNfL measurements: In the course of the clinical trial, we collected whole blood from all patients in 10 mL Serum-Vacutainer-tubes (Becton Dickinson,USA). We allowed the blood to clot for 45 min after sampling. Next, samples were spun at 1300 g also at room temperature for 15 min. Directly after centrifugation, the serum was evenly transferred (1 mL/tube) in 1 mL polypropylene tubes and locally stored at -80°C. sNfL was measured in several rounds by SiMoA HD-1 (Quanterix, USA) using the NF-Light Advantage Kit (Quanterix) from the same batch according to manufacturer's instructions. Resorufin-b-D-galactopyranoside (RGP) was incubated at 33°C for 60 min prior to running the assay. Samples were measured in duplicates. The coefficient of variation (CV, as a percentage) of each sample was obtained by dividing the standard deviation of both replicates by the mean of both replicates multiplied by 100. Since the range of sNfL concentrations in serum is smaller than in cerebrospinal fluid (CSF), some samples with a sample CV above 20% (or missing replicate result) were measured twice, as in previous publications.[4,5] Finally, the mean intra-assay CV of 7.6% was obtained by averaging all individual sample CVs. The two same low and high controls, consisting of recombinant human NfL antigen,

were run in duplicates with each sample run to monitor plate-to-plate variation. The mean concentration over all runs was 2.9 pg/ml for the low control and 139 pg/ml for the high control. We obtained inter-assay CVs of 4.4% and 0.5% for the low and high control, respectively. sNfL measurements were performed in a blinded fashion without information about clinical data.

Statistical Analysis: To test uniformity of the variables, baseline characteristics of the three intervention groups (AKD, CR, CD) were first compared using the non-parametric Kruskal-Wallis test. Owing to the small size of the CD group (n=9), robust statistical analysis was not possible. To overcome this problem, we combined the CD (n=9) and CR (n=14) to a combined common diet group (n=23) for analysis at baseline and study end (month six). Both groups were on a common diet throughout the 6-month study period, with the difference that the CR group fasted for the first 7 days. Therefore, a group difference at study outset and at the end of the study is not plausible. Thus, CD and CR groups served as a combined common diet group at baseline and month six. Combination of the data from the two groups increased the statistical power of our pilot study. All differences between the pooled common diet group and the AKD group were also controlled for baseline and BMI dependencies using analyses of covariance (ANCOVA). ANCOVA was reported as the most reliable method in trials with baseline and follow-up measurements. Additional we used Mann-Whitney test for inter-group comparison and Wilcoxon-matched-pairs test for intra-group comparison of non-parametric data. Baseline associations between variables were assessed using Spearman's rank correlation coefficient (r_s). To correct for outliers, samples exceeding Δ cutoff >5.5 or <−5.5 pg/ml (Δ = difference sNfL study outset – study end) were excluded from the analyses (CD n=3, CR n=2, and AKD n=1). All tests should be understood as constituting exploratory data analysis, as no prior power calculation or adjustments

for multiple testing were made. Data is presented as mean ± standard error of the mean (SEM), unless stated otherwise. The test level for statistical significance of differences between (inter-group comparisons) and within (intra-group comparison) the treatment groups was defined as $p = 0.05$ (two-sided) for all tests. For statistical analyses the following software was used: SPSS, version 26 (IBM, Armonk, New York, US) and Graph Pad Prism, Version 5.04 (GraphPad Software, CA, US).

Supplemental Tables & Figures

Table 1 Intra-group study results - Data were available for 40 patients (CD=common diet, CR= caloric restriction, AKD= adapted ketogenic diet). Data are mean sNfL ± standard deviation (SD) and represent results before pooling the common diet groups (CD+CR) and before statistical adjusting was performed. *Wilcoxon matched- pairs signed rank test was performed between baseline and month 3 and baseline and month 6.

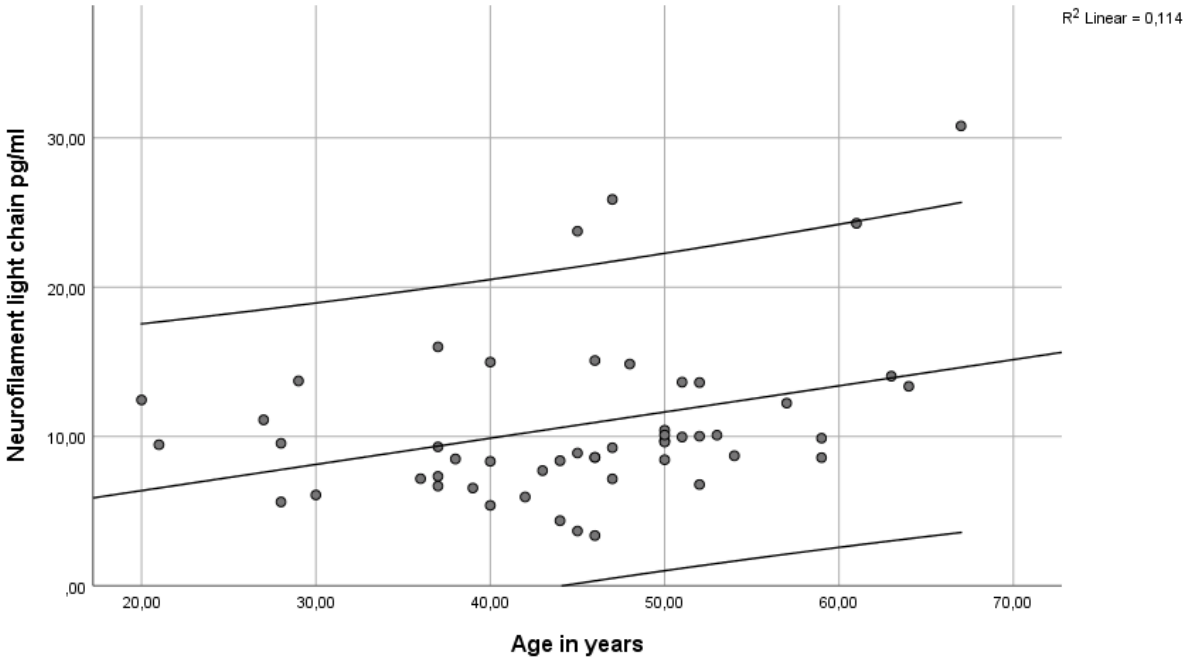
Group	Time-point	sNfL pg/ml	SD	*p-value
AKD n=17	Baseline	8.5	2.0	
	Month 3	11.6	4.6	***0.0039***
	Month 6	7.1	1.9	***0.0106***
CR n=14	Baseline	10.3	3.6	
	Month 3	12.3	3.9	***0.0353***
	Month 6	10.4	3.6	0.8077
CD n=9	Baseline	8.9	3.6	
	Month 3	12.0	4.4	***0.0039***
	Month 6	8.4	2.3	0.5703

Table 2 Inter-group study results - Adapted Ketogenic Diet (AKD) reduces sNfL levels in comparison to Common Diet (CD). Adjusted comparison between AKD and controls. Data were available for 40 patients. **analysis of covariance (ANCOVA) to adjust for baseline, relapse rate and BMI dependencies.

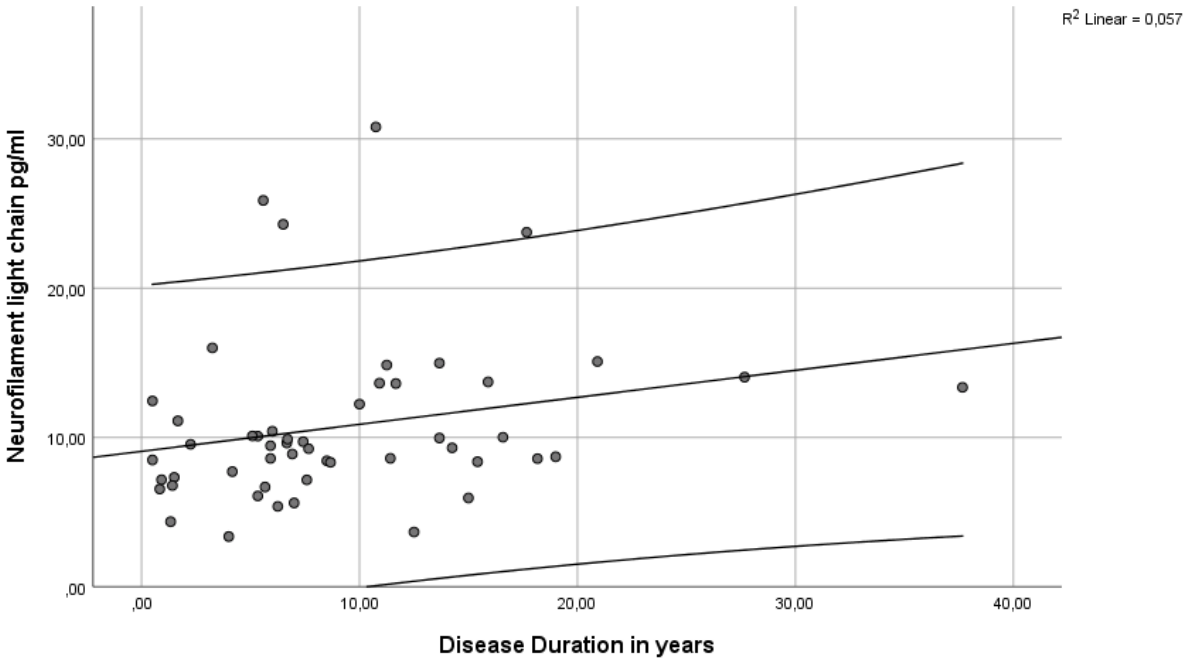
Para-meter	Time-point	CD (n=9)	SD	CR (n=14)	SD	AKD (n=17)	SD	**p-value
sNfL pg/ml	Baseline	8.9	3.6	10.3	3.6	8.5	2.0	
	Month 3	12.0	4.4	12.3	3.9	11.6	4.6	**0.7**
	Pooled Common Diet (n=23)							
		CD (n=23)	SD			AKD (n=17)	SD	
	Baseline	9.7	3.6			8.5	2.0	
	Month 6	9.6	3.3			7.1	1.9	***0.001***

Figure 1-5 Scatter plots depicting correlations between sNfL levels and patient or disease related outcomes. Included regression lines, 95% confidence intervals and R-squares refer to the total sample's data. Correlation coefficients and levels of significance are available in table 1 of the main manuscript.

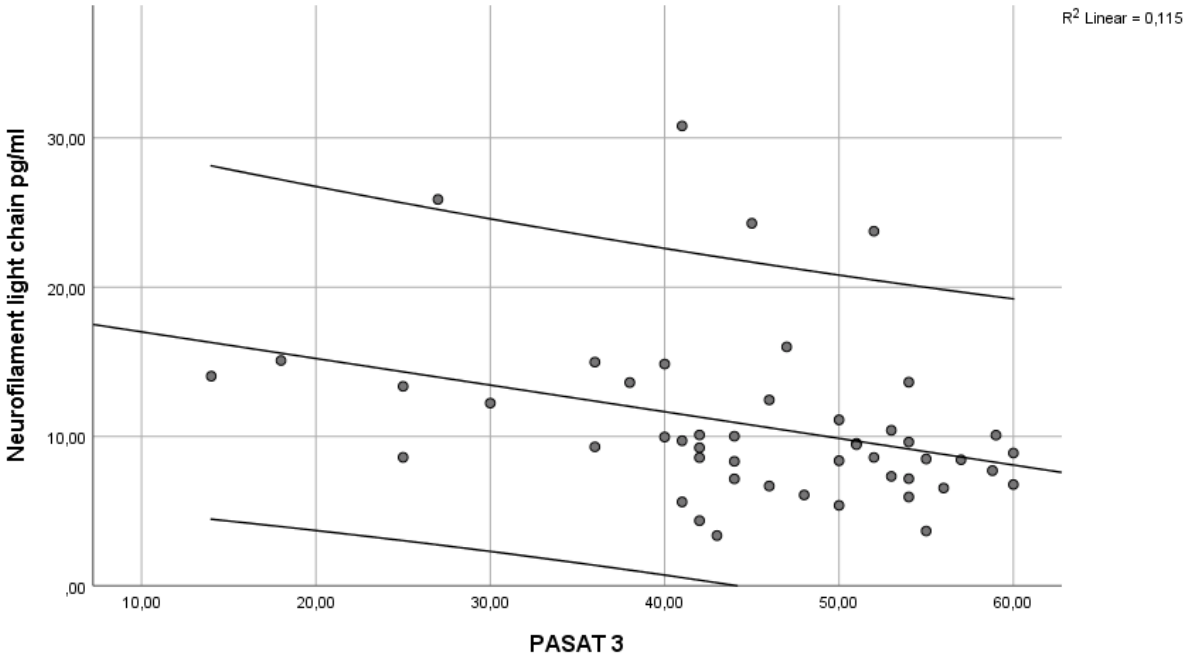
1) Association between sNfL and age of MS patients.



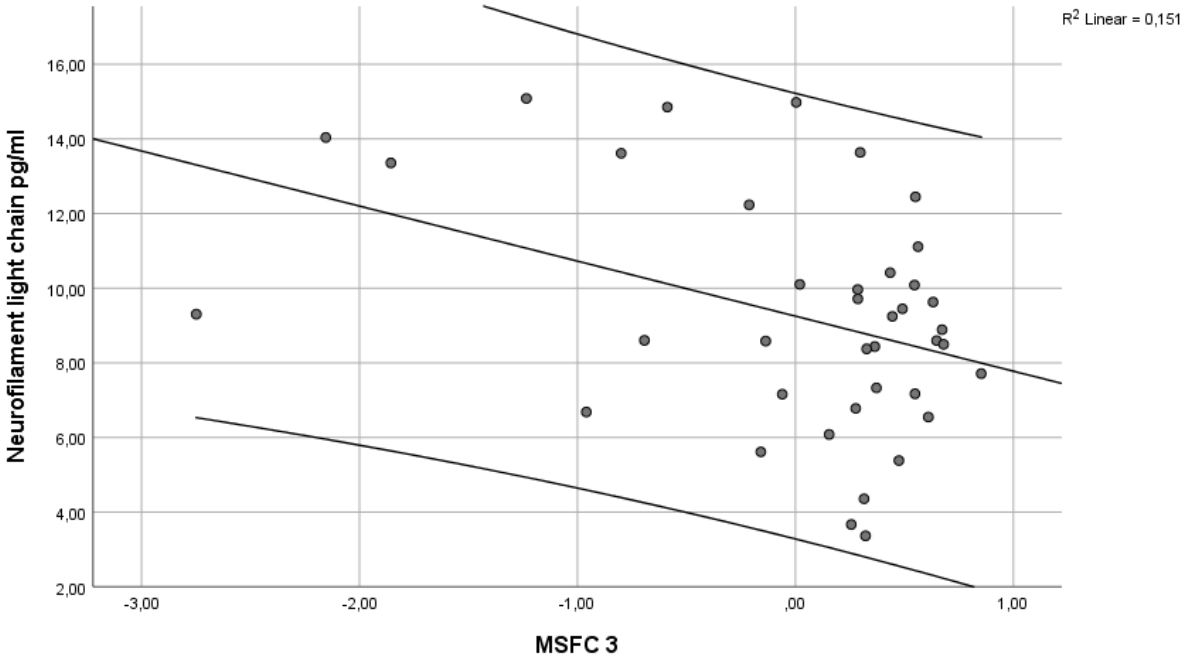
2) Association between sNfL and disease duration.



3) Association between sNfL and Paced Auditory Serial Addition Test.



4) Association between sNfL and Multiple Sclerosis Functional Composite 3



5) Association between sNfL and Multiple Sclerosis Functional Composite 2

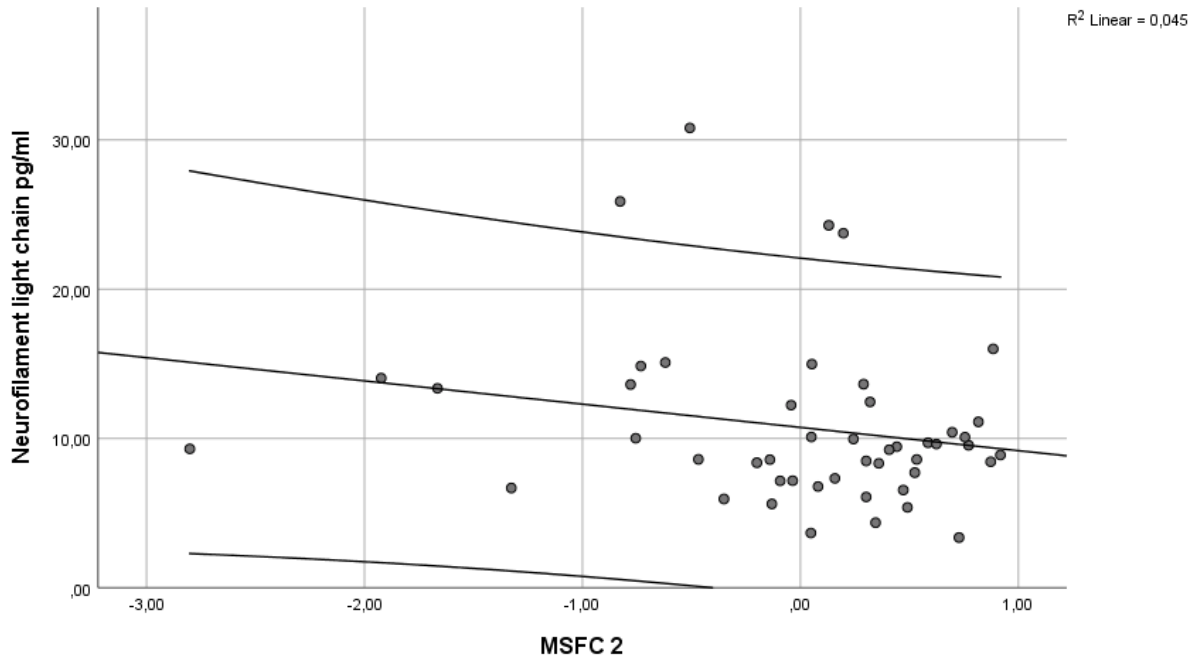


Figure 6

sNfL dynamics over the course of the study with patients with relapse activitiy

- sNfl levels increased at study month 3. This figure reflects all analyzed participants inclusively those with relapse activity. All reported relapses occurred before study visit 3. Data represent mean ± standard error of the mean (SEM) and were measured at baseline and retested at 3 and 6 months for all groups. *p-values are described in supplemental table 1

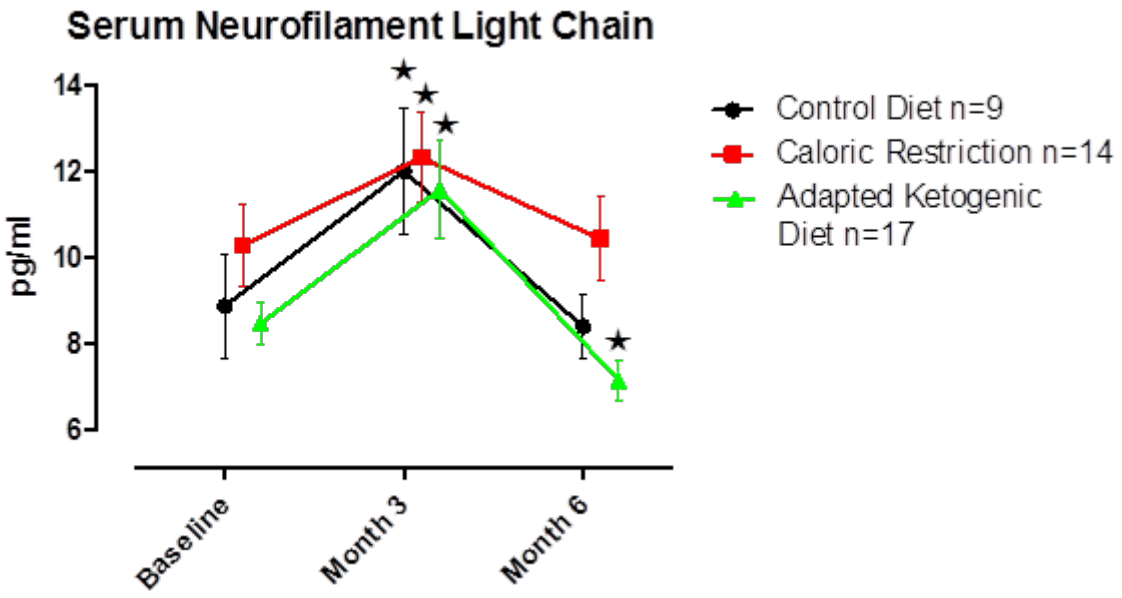
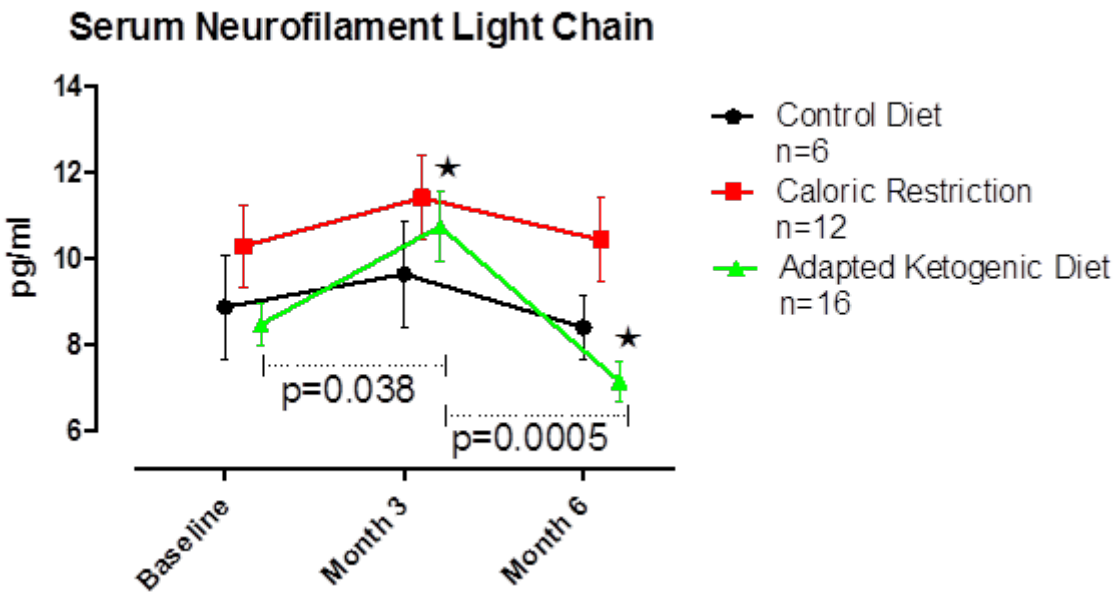


Figure 7

sNfL dynamics over the course of the study without patients with relapse activitiy – After excluding the values of patients with relapse activity, no significant change from baseline was observed in the CD and CR groups at study month 3or 6. However, sNfL levels remained elevated in the KD group in patients without relapse activity at month 3, but declined below baseline at the end of the study. Data represent mean ± standard error of the mean (SEM). *Wilcoxon matched- pairs signed rank test.



Reference

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4. Bittner S, Steffen F, Uphaus T, et al. Clinical implications of serum neurofilament in newly diagnosed MS patients: A longitudinal multicentre cohort study. *EBioMedicine*. 2020;56:102807. doi:10.1016/j.ebiom.2020.102807

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Figure 1 Intra-group comparison – Adapted Ketogenic diet lowers the serum concentration of neurofilament light chain. II. Alterations of sNfL levels induced by caloric restriction and adapted ketogenic diet. Data represent mean ± standard error of the mean (SEM) and were measured at baseline and retested at 6 months for all groups. p-values indicate Wilcoxon matched-pairs signed rank test analysis, *p<0.05.

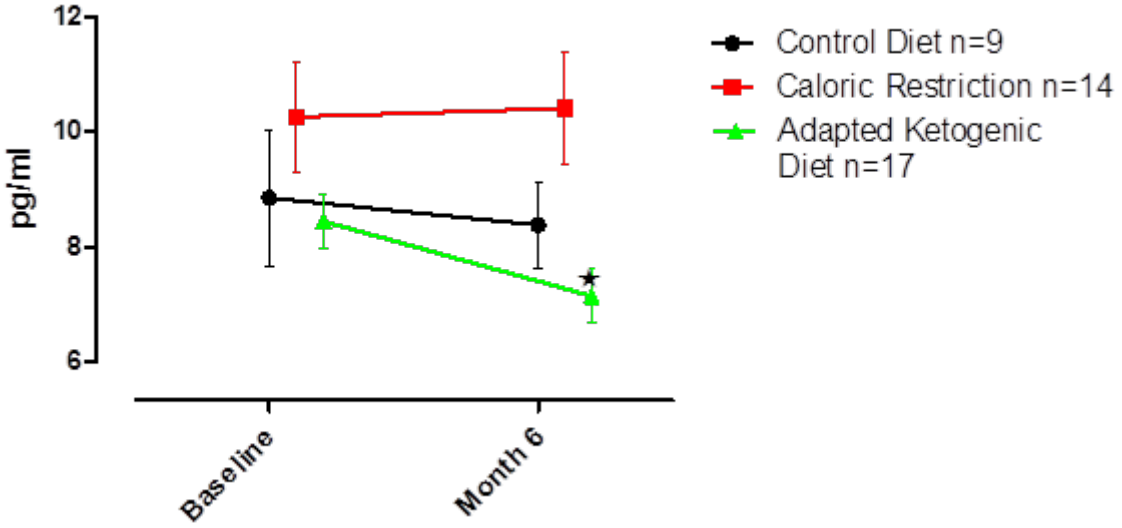

Figure 2 Comparison of sNfL levels in MS patients on adapted ketogenic diet (AKD) with common diet (CD). After 6 months of adapted ketogenic diet, the sNfL concentration declined significantly compared to the common diet group. Data were measured at baseline and retested at 6 months. Data represent mean ± standard error of the mean (SEM). **p<0.01, Analysis of Covariance (ANCOVA) adjusted for relapse rate, BMI and baseline dependencies.