Observational Study Protocol for

PENTAGON

AN OBSERVATIONAL, NON-INTERVENTIONAL, MULTICENTER, PROSPECTIVE STUDY TO EXPLORE THE ASSOCIATION OF MS-RELATED DISABILITY WORSENING AND LOSS OF THALAMUS VOLUME.

DOCUMENT HISTORY

Summary of change: not applicable.

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SYNOPSIS

Observational Study Protocol PENTAGON

Protocol Title:

PENTAGON: An observational, non-interventional, multicenter, prospective study to explore the association of MS-related disability worsening and loss of thalamus volume.

Department: Not applicable

Objective:

To demonstrate that the change of MS-related disability during a period of 24 months is inversely associated with change of thalamus volume during the same period.

Secondary Objectives

(S1) To demonstrate that worsening of MS-related disability is associated with loss of thalamus volume when loss of whole brain volume is within the normal range.

(S2) To demonstrate that the change of thalamus volume during 24 months is predictive of the change of disability during the following 12 months (months 24-36).

Study Design:

PENTAGON is a non-interventional, multicenter, prospective, observational study of active multiple sclerosis patients either already receiving or about to receive first-line oral therapy. Primary data will be collected for each study participant over a 24-month period. MS-related disability is characterized by the EDSS. The EDSS is determined at inclusion (baseline), 24 months after inclusion, and at additional visits at intervals of approx. every 6 months in between. Each study subject will undergo three brain MRI examinations using a standardized acquisition protocol following the 2021 MAGNIMS-CMSC-NAIMS consensus recommendations and including a high-resolution T1w MRI at affiliated radiology centers. The study will continue for an additional 12 months only if sample size requirements (minimum number of study patients at month 24) are met that indicate that the secondary outcome (S2) is sufficiently likely to be achieved. In a 6-month period preceding the primary data collection secondary data will be explored to confirm the validity of the statistical design and of the methodology.

Study Population:

The study population is defined by 600 study subjects with a diagnosis of relapsing-remitting MS aged 18 – 55 years, who either already receive or are about to receive first-line oral immune therapies with more than 10 T2 lesions or with active disease as defined by clinical (relapses) or imaging features (new/enlarging T2 lesions, Gd-enhancing lesions).

Data Collection Methods:

Three types of data will be collected: (1) clinical status, such as therapy change and relapse activity, (2) clinical scores, such as EDSS, and brain MRIs using a standardized protocol including a high-resolution T1w-MRI, at 20 study centers (neurology centers) and affiliated radiology centers in Germany. No procedures are mandatory, and no additional procedures are required beyond the standard examination of MS patients in routine clinical practice (standard-of-care). The EDSS will be assessed in a standardized manner. Evaluators at the study sites record clinical data and information required for the study after each patient visit and enter it into case report forms that are provided by the investigator. Affiliated radiology centers perform brain MRI scans according to standardized protocols, implemented together with the investigator, and transmit the image data to the investigator. Data privacy and security are ensured. Patient data are pseudonymized. The investigator can rely on an established infrastructure and a dense network of study centers and affiliated radiology centers. The processes and procedures are embedded in the clinical routine and have been successfully practiced since 2015.

Data Analyses:

The year-to-year change in EDSS during the 24-month period is calculated by linear regression of EDSS against time, accounting for all visits. The slope of the regression line is the annual change in EDSS. For the correlation analysis, the annual change in EDSS and annualized TVL are considered continuous variables. Three high-resolution T1w MRI scans will be available for all study participants. TVL will be calculated for each pair of two consecutive MRI scans using an advanced and validated method from Opfer et al (2020) that is less susceptible to noise and therefore can estimate TVL in individual subjects. The annualized TVL between the baseline scan (BL) and the last follow-up scan (FU) is calculated by multiplying the TVL between the 2 consecutive scans and dividing the resulting TVL by the time interval between the BL and FU scans. A Pearson correlation analysis is used to test for a significance level of $p = 0.05$.

Sample Size/Power:

The primary objective is to demonstrate whether the correlation between the change in MS-related disability and the change in thalamic volume is significantly different from zero. Both the change in MS-related disability and the change in thalamic volume are continuous variables. Pearson's correlation analysis is performed assuming an expected correlation coefficient of 0.15. Assuming a type I error of 0.05 and a type II error of 0.20, the required sample size is 347, 24 months after the baseline EDSS measurement. Considering an annual dropout rate of 15% to 20%, the total sample size required is approximately 500 to 600 subjects to be enrolled in the study.

Limitations/Strengths:

The PENTAGON study is relatively long due to the inclusion of longitudinal parameters of brain atrophy to ensure that these parameters are reliably measured in individual subjects.

If the PENTAGON study is successfully completed, it will relate the results of the Phase III clinical trials (RADIANCE and SUNBEAM) regarding the effect of current and future disease modifying drugs on brain volume and, in particular, focal brain volume loss, to disability progression in a real-world setting. The number of disabling events required to meet the study endpoints is critical. The study population is enriched by careful definition of inclusion criteria. However, it remains a challenge to find the right balance between overly stringent inclusion criteria, the ability to enroll a sufficient number of study participants, and the risk of too many patients switching therapies during the course of the study. This balance requires continuous monitoring by the reviewers and the study team, which must determine timely actions. Once pathological TVL is confirmed, it could be considered a risk factor for disability worsening. This would then justify the inclusion of TVL in the spectrum of treatment targets in MS.

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1. INTRODUCTION

Study Rationale

The clinical course of multiple sclerosis (MS) is heterogeneous between patients and as of yet largely unpredictable on the single subject level. In addition to inflammatory demyelination and astrogliosis, neurodegeneration – a term that refers to structural axonal and neuronal damage - plays a crucial role in the accrual of disability over time. An increasing body of evidence suggests that whole brain volume loss is indicative of disability progression (Sastre-Garriga et al. 2020). The mounting evidence prompted the MS community to add whole brain volume loss as a marker of tissue damage to the no-evidence-of-disease-activity (NEDA) concept. Since 2015 Germany-wide projects, such as QUANTUM, PANGAEA, MAGNON, have helped to establish a network of radiologists and neurologists for which accurate measurement and usage of whole brain volume loss (BVL) has become a "de-facto standard-of-care" (Schuh et al. 2020). Meanwhile, a solid connection between radiology centers, neurology centers and the investigator has developed. Through this network, thousands of MS patients in Germany recently received standardized MRI procedures for disease and therapy monitoring in routine clinical practice, providing hundreds of neurological centers in Germany with regular quantitative information on BVL and lesion activity.

The proposed study has the upside to build on this unique, large network and contribute to better understanding to what extent thalamus volume loss (TVL) appears as an independent driver of disability progression in early MS patients. Once proven and confirmed independently, TVL could serve as a potentially complementary marker of disease activity and a treatment target in future clinical practice, should surrogacy for disability progression be proven. Previous work has provided some important evidence rendering such ambitious goals within sight:

1. In the SUNBEAM and RADIANCE phase III trials, MS patients in the OZANIMOD groups showed a significant slowing of cortical gray matter and thalamic volume loss as compared to an active comparator (Cohen et al. 2019; Comi et al. 2019).
2. Opfer et al. (2020) demonstrated that TVL and BVL in MS patients do not necessarily go hand in hand (Fig. 1). This means, an MS patient with pathological TVL does not necessarily show a pathological BVL. It was hypothesized that TVL and BVL are "distinct" features of neurodegeneration, potentially driven by different underlying mechanisms that would warrant further research.
3. Results by Hänninen et al. (2019) suggest that thalamic atrophy rather than whole brain atrophy is a driver for disability worsening based on an assessment of 24 relapsing MS and 36 secondary-progressive MS patients.
4. Eshaghi et al. (2018) demonstrated that specifically deep gray matter volume loss is associated with disability progression, however, the technological pipeline which generated these results is susceptible to noise and thus is not yet applicable to decision making in single subjects under clinical routine conditions. This was addressed by Opfer et al. (2020) who presented and validated a technology which can identify pathological thalamic and deep gray matter volume

loss on a single subject level with a type II error of 5%. Furthermore, cut-offs were provided for application to individual patients in routine patient care.

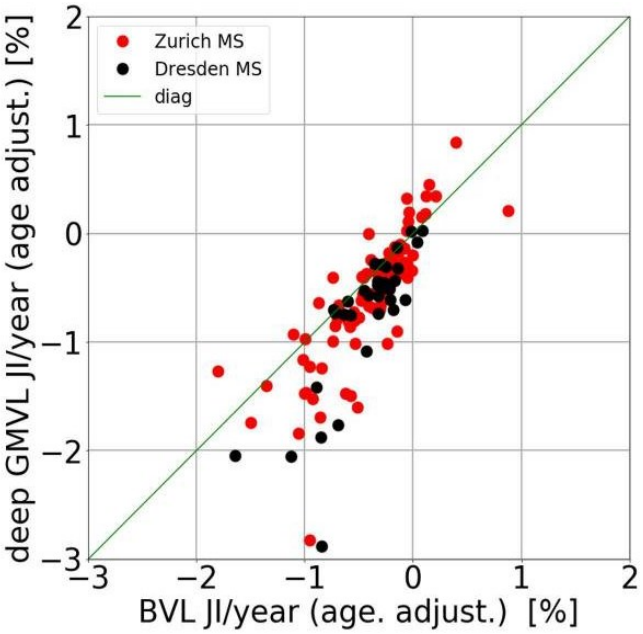

Figure 1. *Scatter plot of deep gray matter brain volume loss representing TVL as a function of BVL (Opfer et al. 2020).*

However, despite the evidence generated so far and outlined above, the scarcity of data and the limited number of subjects in the respective studies do not yet allow a firm conclusion that pathological and non-pathological TVL of MS patients with normal BVL generate distinct subgroups or "endophenotypes" of MS. Once confirmed in a sample size powered for this analysis in a true longitudinal setting, pathological TVL could be considered a risk factor for disability worsening. This would then justify inclusion of TVL into the spectrum of treatment targets in MS.

Research Question

The research question is whether TVL is a driver of disability progression in MS patients. If this hypothesis were proven to be true this would qualify pathological TVL to be suitable as a treatment target for slowing disability progression.

2. OBJECTIVES

Primary Objective

To demonstrate that the change of MS-related disability during a period of 24 months is inversely associated with the change of thalamus volume during the same period.

Secondary Objectives

(S1) To demonstrate that worsening of MS-related disability is associated with loss of thalamus volume when loss of whole brain volume is within the normal range.

(S2) To demonstrate the change of thalamus volume during 24 months is predictive of the change of disability during the following 12 months (months 24-36).

Exploratory Objectives

We will explore deep gray matter volume loss (DGMVL) in addition to TVL along the same objectives.

3. STUDY DESIGN

Overview of Study Design

Overall description

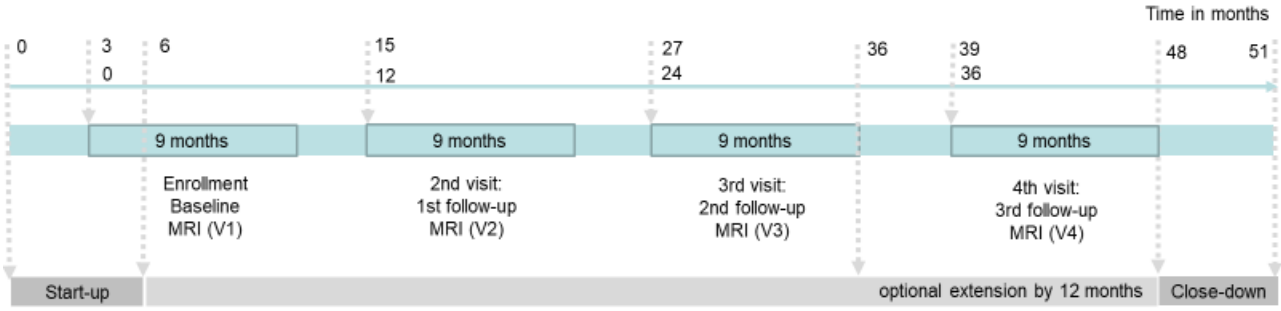

Figure 2. *Study schedule and composition.*

The PENTAGON study is an observational, non-interventional, multicenter, prospective, cohort study of active multiple sclerosis patients who either already receive or are about to receive first-line oral immune therapies with a preceding 6-month retrospective period for assessing the validity of the statistical design and of the methodology using secondary data.

Start-up phase

A suitable MS cohort of secondary data, in particular those with MRI and EDSS scores, will be created using proprietary databases and databases collected from 2 to 3 collaboration partners in Germany and Switzerland. The data is heterogeneous in nature, comprising relapsing-remitting and secondary progressive MS patients, treated with multiple therapy regimens and of all ages. We will ensure that the MRIs included into the secondary data collection adhere to a minimum quality standard so that we are able to successfully apply and advance the technology developed in Opfer et al. (2020) and that there are sufficient EDSS data points for each individual patient available to test the validity of the inclusion criteria and the statistical design:

Among others, we are going to explore the following questions:

- Is sampling of the SDMT for a subgroup feasible in the context of this observational study and would this clinical score add value to help accomplish the secondary objective (S2)?
- Would the status of brain volume (whole brain, thalamus) as additional inclusion criterion help to enrich the patient population with disability events?
- How valuable is to consider total T2 lesion load as an inclusion criterion or should it be removed as a inclusion criterion in order to make the potential results of the study more applicable to the real world?

In parallel preparations will start for submission of the necessary documents for approval by ethics committees.

The following table provides checkmarks and / or milestones for the startup phase:

ID	checkmark / milestone description	Type (mandatory / optional)	Due date (in months after official launch)
M-1	Contract in place	mandatory	0
M-2	Secondary data set of minimum 150 MS patients assembled	mandatory	+2
M-3	Ethics committee's approval in place	mandatory	+3
M-4	Data exploration completed and conclusions regarding statistical design drawn	mandatory	+5
M-5	Interim analysis completed (go / no-go-decision)	mandatory	+5
M-6	Technology sufficiently advanced to warrant application on primary data; final report ready	mandatory	+6
M-7	Manuscript prepared for submission to a clinical /technical journal	optional	+8

Main phase

Once ethics committee approval has been granted, enrollment of the first patients in the prospective observational study will begin. Enrollment of the first patients is planned 3 months after the official project launch. The enrollment period will last 9 months. Each study subject will be followed for 24 months. The EDSS is collected at inclusion (baseline), 24 months post baseline, and at additional visits at intervals of approx. every 6 months in between. Each study subject will undergo three brain MRI examinations using a standardized acquisition protocol following the 2021

MAGNIMS-CMSC-NAIMS consensus recommendations and including a high-resolution T1w MRI at affiliated radiology centers.

The following table provides checkmarks and / or milestones for the main phase including the extension period:

ID	checkmark / milestone description	Type (mandatory / optional)	Due date (in months after official launch)
M-8	Launch of the prospective observational study at the investigator's own risk; first patient enrolled	optional	+3
M-9	Last patient enrolled; baseline brain MRI examination (V1) for approx. more than 500 enrolled study subjects completed	Mandatory	+12
M-10	V2 completed for approx. more than 430 enrolled study subjects	Mandatory	+24
M-11	Interim analysis completed; formal go / no-go decision	Mandatory	+30
M-12	V3 completed for approx. more than 350 enrolled study subjects	Mandatory	+36
M-13	V4 completed for approx. more than 350 enrolled study subjects with an sufficient percentage number of disability events at a level of approx. 10% to 15%	Optional	+48

Period of enrollment

Recruitment of 600 eligible study subjects should last no longer than 9 months.

600 MS patients from 20 centers in Germany will be included. During the period of concept development (February and March 2021), we queried the interest of neurological centers in Germany. An overwhelming 83% (25 of 30) of the centers contacted returned a letter of intent indicating their willingness to participate in the study, suggesting recruitment of more than 1,200 study participants. Letters of intent from participating neurological centers, including patient numbers/recruitment estimates, are available upon request.

Statement that enrolment is independent of therapeutic decision

Patients meeting the inclusion and exclusion criteria are offered to take part into the study by the participating center. Prior to offering participation the therapeutic decision has already been made.

Process of enrolment of a patient (therapeutic decision occurring first, invitation to participate, informed consent, etc)

The invitation to participate is issued as soon as the therapeutic decision has been made. The selected patient is informed by the physician. When the conditions and safety concerns have been sufficiently clarified and there are no longer any objections to participation, verbal or written informed consent is obtained. Verbal or written consent is documented.

Process of data collection at baseline

If verbal or written informed consent is given the evaluator of the participating center completes the first paper-based case report form (CRF) for the baseline visit (V1) and sends the report via fax or email to the investigator.

Length of follow-up.

The prospective observational study runs for 24 months plus the additional 9 months enrollment period. In total a baseline visit (V1) and two follow-up visits (V2 and V3) per study subject are planned. The main purpose of these visits is to prescribe and schedule a brain MRI examination with the affiliated radiology center. It is important that brain MRIs are performed annually with a minimum of 11 months apart. If the decision is made to extend the study for another 12 months the individual study subject will be followed for 36 months adding another visit (V4) to the study schedule.

Statement that no visit or procedures are made mandatory, description of usual care of patient and definition of time points for study data collection

In Germany many MS patients regularly see their physicians on a three-month basis. The EDSS score is frequently taken in normal clinical routine. Annual brain MRI examinations are recommended by national and international guidelines for MS therapy monitoring. The procedures required for this study are part of the standard workup of MS patients in Germany. No visit or procedure is made mandatory. With the schedule of data collection we adhere to the best practices for MS patient management in Germany. No extra procedures are required.

Overview of Sampling Frame (sites, patients)

In total 600 eligible study subjects shall be sampled at approximately 20 clinical sites across Germany.

Part C (closing down of observational prospective study and primary data analysis)

The following table provides checkmarks and / or milestones for Part C:

ID	checkmark / milestone description	Type (mandatory / optional)	Due date (in months after official launch)
M-14	Missing data are collected and databases curated	mandatory	+37 (+49 in case of an extension)
M-15	Analysis of primary data completed and	mandatory	+39 (+51 in case of an

	conclusions drawn; final observational study report ready		extension)
M-16	Manuscript prepared for submission to a clinical /technical journal	optional	+42 (+54 in case of an extension)

Study Population

The source population comprises patients with a relapsing-remitting form of MS who are regularly seeing a neurologist (on a three-months basis). In Germany, an estimated 220,000 to 250,000 people currently have MS. MS incidence, i.e. the number of people newly diagnosed with MS in a year, is about a quarter higher in the West than in the East of Germany, with 19 versus 15 new cases per 100,000 inhabitants.

The study shall be conducted in Germany by neurologists who mainly operate in an outpatient setting.

The study population is defined by patients with a diagnosis of relapsing-remitting MS aged 18 – 55 years, who either already receive or are about to receive first-line oral immune therapies with more than 10 T2 lesions or with active disease as defined by clinical (relapses) or imaging features (new/enlarging T2 lesions, Gd-enhancing lesions) according to the Lublin criteria (Lublin et al. 2014).

The reasoning behind targeting this study population is that

1. MS patients receiving first-line oral therapies are most likely early-stage patients. Despite undisputed compensatory mechanisms, pathology studies have shown that features of neurodegeneration like axonal transection are quantitatively prominent at early stages of the disease (Kuhlmann et al. 2002).
2. MS patients with more than 10 T2 lesions or with active disease are more likely to show disability progression (Tintore et al. 2015)

Inclusion Criteria

- MS, as diagnosed by the revised 2017 McDonald criteria
- Exhibiting a relapsing clinical course and history of brain MRI lesions consistent with MS
- Ages 18 to 55 years, inclusive
- Meet one of the following disease activity criteria
    a. more than 10 T2 lesions at the time of the first visit OR
    b. at least one documented relapse with the last 12 months prior to the first visit OR
    c. documented evidence of at least 1 Gd-enhancing lesion on brain MRI within the last 12 months prior to the first visit OR
    d. documented evidence of at least one new or one enlarging T2 lesion within the last 12 months prior to the first visit.

- Either receive or about to receive first-line oral immune therapies, such as teriflunomide, ozanimod, dimethylfumarate, ponesimod, and diroximethylfumarate.
- No intention to evaluate the patient to switch from first-line oral immune therapies to other therapy regimens.

Exclusion Criteria

- Primary or secondary progressive MS at time of enrollment
- Patients who are not eligible to receive MRI

Data Source/Data Collection Process

Three types of data will be collected in the study

1. clinical status, such as therapy change and relapse activity
2. clinical scores, such as EDSS
3. imaging data (brain MRI) using a standardized image acquisition protocol including a high-resolution T1w-MRI.

Study procedures

- Expanded Disability Status Scale (EDSS) examination

  The EDSS is a standardized, widely accepted, numerical scale method used to evaluate disability in people with MS (Kurtzke 1983). The EDSS is evaluated according to signs and symptoms observed during a standard neurological examination. Based on a neurological examination, 8 functional systems (visual, brain stem, pyramidal, cerebellar, sensory, bowel & bladder, cerebral, and other functions) are scored from 0 (no disability) to 5 or 6 (more severe disability). Ambulation is assessed based on distance the patient is able to walk, whether assistance is required, or restrictions are present. The EDSS score ranges from 0 (normal) to 10 (death due to MS) in 0.5 unit increments. A template or an adaptation of this template, which can be found in the appendix, shall be used for documentation. The study will require the same evaluator to perform all EDSS assessments for an individual patient when possible. In addition, EDSS raters will be certified using the Neurostatus Standardized Examination and Assessment (www.neurostatus.net/training) prior to study initiation and examiners will be recertified every two years throughout the conduct of the study.

- Relapse activity assessment

  A relapse is defined as the occurrence of new or worsening neurological symptoms attributable to MS and immediately preceded by a relatively stable or improving neurological state of at least 30 days. The new or worsening neurological symptoms must be accompanied by objective neurological worsening consistent with an increase of at least half a point on the EDSS, or 2 points on one of the appropriate FS scores, or 1 point on two or more of the appropriate FS scores. The change must be documented by the evaluator at either scheduled or unscheduled visits and must affect the FS scales that correspond to the

patient's symptoms (e.g., pyramidal, gait, cerebellar, brainstem, sensory, or visual). Symptoms must persist for > 24 hours and should not be attributable to confounding clinical factors (e.g. fever, infection, injury, adverse reactions to concomitant medications).

- Brain MRI examination

  Brain MRIs will be acquired using a standardized image acquisition protocol including high-resolution T1w-MRIs. Standardized means that the protocol complies with the recently published 2021 MAGNIMS-CMSC-NAIMS consensus recommendations on the use of MRI in patients with MS (Wattjes et al. 2021). Gadolinium is administered depending on the judgement of the physician or radiologist. The same or a similar brain MRI protocol will be used across all sites. All participating radiology centers have to go through a protocol qualification procedure orchestrated by the investigator. The qualification status is continuously monitored by the investigator.

Time and events schedule

Event	Enrollment / baseline visit (V1)	Additional visits to the study center	Follow-up: 2nd visit (V2)	Follow-up: 3rd visit (V3)	Follow-up: 4th visit (V4) (extension)
Time [first patient in - last patient out]	0 - 9	Approx. every 6 months	12 – 21	24 - 33	36 - 45
Documentation of medical history	X				
Assessment of inclusion and exclusion parameters	X				
Assessment of clinical status	X	X			
EDSS	X	X			
Brain MRI examination(1)	X		X	X	X

(1) There is no strict requirement that brain MRI examination shall fall within +/- 3 months of the assessment of clinical scores.

Data entry and coding procedures

Together with information on the study, eligible patients will be informed about data capture, transmission and analyses processes. Once a patient is eligible and has given his/her informed consent to study participation and data collection, the evaluator will assign the patient a unique patient identification code which is always noted on the CRF. The code comprises the

identification number of the study center, the initials of the patient's name and the month and year of the patient's birthday. After each visit evaluators in the participating study centers will initially collect and document all necessary clinical data in due time on paper-based CRFs. CRFs are emailed or faxed to a centralized location of the investigator where trained members of the study team transcribe or transform the information on the CRFs to a central database. Patient data will be recorded in pseudonymized form (i.e. without reference to the patient's name) using exclusively the patient's identification code. Affiliated radiology centers transfer imaging data of study subjects via a secure data transfer service to the investigator. Prior to transfer of the imaging data to the investigator via an upload mechanism provided by the investigator, imaging data will be automatically pseudonymized.

For phase IV clinical trials (post-marketing) and the collection of patient data as part of routine clinical practice, the investigator can rely on an established infrastructure and a dense network of study centers and affiliated radiology centers. The processes and procedures are well established and have been supported by large Germany-wide research projects such as QUANTUM, PANGAEA and MAGNON since 2015. Meanwhile, a solid connection between radiology centers, neurology centers and the investigator has developed. Through this network, thousands of MS patients in Germany recently received standardized MRI procedures for disease and therapy monitoring in routine clinical practice, providing hundreds of neurological centers in Germany with regular quantitative information on brain volume loss and lesion activity.

Data validation procedures

Data validation methods shall be appropriately applied as defined in the investigator's standard operating procedures (SOPs), which are part of an ISO 9001:2015-certified quality management system (QMS) operated and regularly updated by the investigator and audited by sponsors.

Definitions of Study Variables

Event	Study variable	Operational definition
Documentation of medical history	Age	Age at index date
	Gender	trivial
	Therapy	Documentation of previous and current treatments and medications related to MS
	Disease duration	Documentation of time since first MS diagnosis
Assessment of	Relapse activity	Number of relapses within the last 12 months prior to enrollment

inclusion and exclusion parameters	T2 lesion load	Number of T2 lesions at time of assessment as defined by MRI
	New / enlarging T2 lesions	Number of new or one enlarging T2 lesions within the last 12 months prior to enrollment as defined by MRI
	Gd-enhancing lesion	Number of Gd-enhancing lesions within the last 12 months prior to enrollment as defined by MRI
Assessment of clinical status	Therapy change	Therapeutic regimen at the time of visit; therapy is subject to change
	Relapse activity	Number and time of relapses since last visit; assessment and documentation whether EDSS worsening is caused by relapses
	Diagnosis	Documentation of current diagnosis
Assessment of clinical scores	Disability	EDSS is used to quantify disability and disability progression over time in MS. Based on a neurological examination, 8 functional systems (visual, brain stem, pyramidal, cerebellar, sensory, bowel & bladder, cerebral, and other functions) are scored from 0 (no disability) to 5 or 6 (more severe disability). Ambulation is assessed based on the distance the patient is able to walk, whether assistance is required or restrictions are present.
	Disability progression	Disability progression is defined as 1.0-point increase for patients with a baseline EDSS of 0 to 5.0, and 0.5 points for scores equal or higher than 5.5.
Brain MRI examination	Thalamic volume	CNN-based estimation of thalamic volume, adjustment for head size and age and normalization to a z score
	Deep gray matter volume	CNN-based estimation of deep gray matter volume, adjustment for head size and age and normalization to a z score
	Whole brain volume	CNN-based estimation of whole brain volume, adjustment for head size and age and normalization to a z score.
	T2 lesion load	CNN-based detection of T2 lesion with a minimum volume of 0.01 ml
	New / enlarging T2 lesions	CNN-based detection of new (minimum volume of 0.01 ml) and enlarging T2 lesions

Outcomes/Endpoint Variables

Primary hypothesis and primary endpoint

The change of MS-related disability during a period of 24 months is inversely associated with the change of thalamus volume during the same period.

MS-related disability will be characterized by the Expanded Disability Status Scale (EDSS). The EDSS will be determined at inclusion (baseline), at 24 months after inclusion, and at additional visits at time intervals approx. every 6 months in between. The annual change of EDSS during the 24-month time period will be computed by linear regression of EDSS versus time including all visits. The change of thalamus volume will be determined in consecutive high-resolution T1w-MRI using a deep-learning based method developed and validated by JDX (Opfer et al. 2020). TVL will be calculated for each pair of two consecutive MRI scans using an advanced and validated method from Opfer et al (2020). The primary endpoint is the Pearson coefficient of the correlation between the annual change of EDSS and the annual change of the thalamus volume.

Secondary hypotheses and secondary endpoints

(S1) Worsening of MS-related disability is associated with loss of thalamus volume also if loss of whole brain volume is within the normal range.

Patients will be dichotomized with respect to the annual EDSS change during 24 months ('stable disability' versus 'progression of disability'), with respect to the annual change of thalamus volume ('stable thalamus volume' versus 'decline of thalamus volume'), and with respect to the annual change of brain parenchymal volume ('stable parenchymal volume' versus 'decline of parenchymal volume'). The parenchymal volume will be determined from the same T1w-MRI as the thalamus volume using a deep-learning based method developed and validated by JDX. The annual change of the parenchymal volume will be computed by linear regression of parenchymal volume versus time. Predefined cutoffs will be used for dichtomization. The association between dichotomized disability change and dichotomized thalamus volume change will be tested in the subgroup of patients with stable parenchymal volume using the chi-square statistic.

(S2) The change of thalamus volume during 24 months is predictive of the change of disability during the following 12 months (months 24-36).

EDSS will be determined at additional visits at 30 and 36 months. The annual change of EDSS between months 24 and 36 will be determined by linear regression of EDSS versus time including 24, 30 and 36 months. The annual change of EDSS between months 24 and 36 will be dichotomized using a predefined cutoff. The association between dichotomized disability change between 24 and 36 months and dichotomized thalamus volume change between baseline and 24 months will be tested using the chi-square statistic.

Exposure/Independent Variables of Interest

Not applicable

Other Co-variates/Control Variables

Total intracranial volume (TIV)	most regions-of-interest (such as the thalamus) in the brain, MRI-based volume estimates are associated with TIV. Careful adjustment for TIV is required to improve the statistical power and to avoid spurious effects and misinterpretation of actual effects.

Age and sex	Regional brain volumes are strongly correlated with age and sex.
Disease duration	EDSS and MRI-based volume estimates are associated with disease duration (time since diagnosis). Treatment may affect TVL, new lesions, and disability worsening. Mitigation may be to use treatment as a covariate in statistical analysis.
Baseline parameters used to define inclusion, such as T2 lesion load, Gd-enhancing lesions, new/enlarging T2 lesions	All these parameters could have a confounding effect and are thus to be controlled.
EDSS at baseline	EDSS at baseline is also a predictor for disability progression and should be controlled.
Treatment regimen	EDSS and MRI-based volume estimates are associated with disease duration. Treatment may affect TVL, new lesions, and disability worsening. Mitigation may be to use treatment as a covariate in statistical analysis. Treatments are first-line oral immune therapies such as teriflunomide, ozanimod, dimethylfumarate, ponesimod, and diroximethylfumarate.

4. STATISTICAL ANALYSIS

Statistical Analysis Methods

In the following the statistical analysis methods to be used are discussed:

Primary objective: To demonstrate that the change of MS-related disability during a period of 24 months is inversely associated with change of thalamus volume during the same period.

In a previous project sponsored by Celgene, the investigators in this study developed and validated a technique that allows reliable and robust calculation of change in regional volumes based on a longitudinal approach in high-resolution T1w MRIs (Opfer et al. 2020). Such an approach allows percentage estimation of volume change of corresponding regions in successive high-resolution T1w MRIs and is much less susceptible to noise than conventional methods based on calculation of absolute volumes at different time points and a regression model to estimate volume change. It has the potential to overcome the technological limitations that hindered the work of Eshaghi et al. (2018).

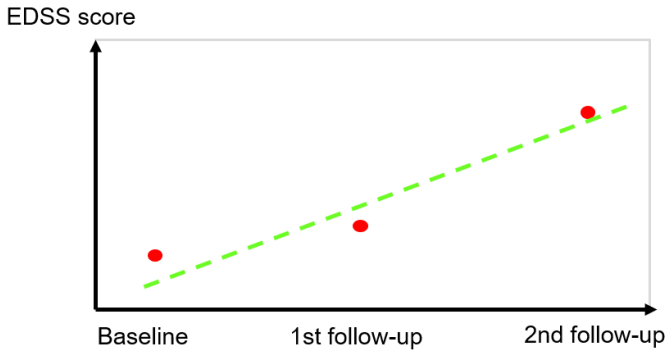

Figure 3. *Linear regression of all EDSS measurements*

With this approach, it is methodologically feasible to assess TVL and deep GMVL for individual MS patients.

MS-related disability will be characterized by the Expanded Disability Status Scale (EDSS). The EDSS will be determined at inclusion (baseline), at 24 months after inclusion, and at additional visits at time intervals approx. every 6 months in between. The annual change of EDSS during the 24 months' period will be computed by linear regression of EDSS versus time including all visits (Fig. 3). The slope of the regression line will be the annual change of EDSS.

For the correlation analysis annual change of EDSS and annualized TVL will be considered as continuous variables. For all study subjects three high-resolution T1w-MRI scans will available. TVL will be calculated for each pair of two consecutive MRI scans. Annualized TVL between the baseline (BL) and last follow-up (FU) scan will be calculated by multiplying the TVL between the two consecutive scans and dividing the resulting TVL with the time interval between BL and FU scan.

A Pearson correlation analysis will be used to test for with a significance level of $p = 0.05$. Outcomes can be modelled both as dichotomous (CDP – yes, no) and continuous (raw EDSS score) variables. In case of dichotomous variables, a logistic regression will be used.

Secondary objectives:

(S1) To demonstrate that worsening of MS-related disability is associated with loss of thalamus volume also if loss of whole brain volume is within the normal range.

Based on the individual results for TVL and BVL derived from three routine MRIs, the study population will be divided into four distinct subgroups as suggested by Opfer et al. (2020) and shown in Figure 4.

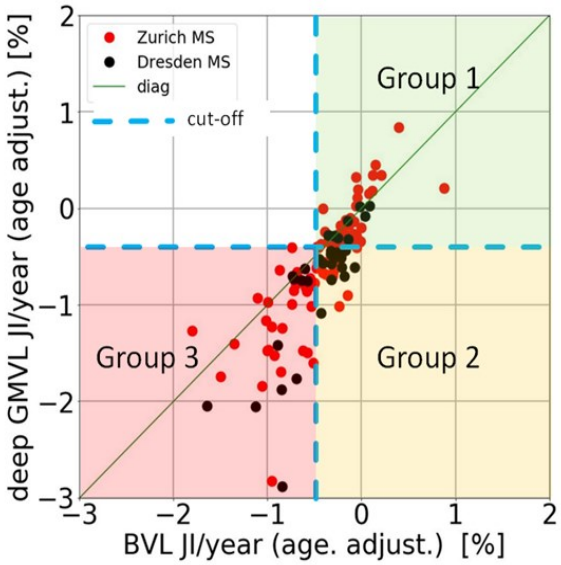

Figure 4. *Clinical characteristics of group 2 (~25% of all patients) are of particular interest and whether group 2 is statistically distinct with regard to clinical and imaging data.*

The probable frequencies for these groups were also taken from this paper:

Group 1: normal BVL, normal TVL (estimated frequency ~50% of the total relapsing-remitting MS population); **Group 2**: normal BVL, pathological TVL (~25%); **Group 3**: pathological BVL, pathological TVL (~25%); **Group 4**: normal TVL, pathological BVL (~0%)

We will use the cut-offs for normal and pathological TVL presented in Opfer et al. (2020) and corresponding cut-offs for BVL as published in Opfer et al. (2018a and 2018b).

At month 24 study subjects shall be grouped based on their individual EDSS increase in patients with (confirmed) disability progression and patients without disability progression.

Dichotomization is based on the following cut-offs[1]:

Group A (with evidence of disability progression / confirmed disability progression)

–1.0 increase for study subjects with baseline EDSS scores from 1.0 to 5.0

–0.5 increase for study subjects with baseline EDSS scores equal or higher than 5.5

Group B (no evidence of disability progression)

We will perform a chi-square test to see if there is a significant difference between the frequency of patients with progressive disability (group A) and without evidence of progressive disability (group B).

(S2) To demonstrate that the change of thalamus volume during 24 months is predictive of the change of disability during the following 12 months (months 24-36).

We will build a statistical model based on clinical and MRI (estimated from three MRI measurements sampled at baseline (BL), at month 12 and at month 24) parameters to predict disability progression between month 24 and month 36. EDSS will be determined at additional visits at 30 and 36 months. The annual change of EDSS between months 24 and 36 will be determined by linear regression of EDSS versus time including 24, 30 and 36 months. The annual change of EDSS between months 24 and 36 will be dichotomized using the same predefined cutoff as deployed for (S1) (see above). The association between dichotomized disability change between 24 and 36 months and baseline (BL) parameters and TVL between baseline and 24 months will be tested using the chi-square statistic.

The following parameters will be used:

- Clinical scores / characteristics @ BL:
  - Age and sex

[1] Guideline on clinical investigation of medicinal products for the treatment of Multiple Sclerosis from EMA as of 26 March 2015

- disease duration (DD)
- EDSS
- Therapy

– MRI @ BL
  - z-scores of thalamus and whole brain volume
  - T2 lesion load

– MRI @ BL, month 12 and month 24
  - TVL and BVL

The model will be based on multivariate regression or support vector machines. The performance of the model will be characterized using a receiver operating characteristic (ROC) curve. The ROC will be evaluated with respect to accuracy, area under curve (AUC), sensitivity (true positive rate) and specificity (true negative rate). Within each classifier including both clinical and neuroradiological features, permutation importance will be calculated for each feature and for the random variable: only parameters whose importance are higher than the random variable's importance, will be considered as relevant in the classifier.

Baseline volumes for thalamus and whole brain will be estimated using a novel convolutional neural network-based approach, recently submitted for publication (Opfer et al. 2021).

For most regions-of-interest (ROI) in the brain, MRI-based volume estimates (ROIV) are associated with total intracranial volume (TIV) and age. Thus, TIV and age are confounders of no interest (nuisance covariate) for between-subject comparisons. Careful adjustment for TIV and age is required in order to improve the statistical power to detect effects of interest, but also to avoid spurious effects and misinterpretation of actual effects. We will use the residuals method, to regress TIV and age in a large (n = 5,059) independent group of normal control subjects (normal database). The resulting regression model will be used to compute the residuals of ROIV relative to the model in the subjects of interest (not included in the normal database). The residuals quantitatively characterize the extent to which each individual subject's ROIV deviates from that predicted for normal control subjects with the same TIV and age. Residuals will be transformed to z-scores by scaling them to the standard deviation of the residuals in the normal database.

To avoid data redundancy and reduce variance in classifier performances, we will investigate co-linearity of clinical and imaging features using a partial correlation analysis. Specifically, a partial correlation between each pair of features controlling for all the remaining features will be done. This analysis will allow us the removal of confounding effects.

T2 lesion (accumulation) will be assessed by a novel convolutional neural network-based approach (Krüger et al. 2021).

<u>Secondary data analysis</u>

The investigators have access to a large brain MRI database of MS patients sampled over the past 3 to 4 years from multiple centers across Germany. We will select MS patients from the brain MRI database whose data match best the requirements of the prospective observational study. Since EDSS scores are sparsely available, missing clinical data will be retrospectively collected by the investigators from the associated neurology centers. Then, we will compare three alternative methods to measure TVL and associations with change in EDSS: The Jacobian method as described in (Opfer et al. 2020) uses a longitudinal registration technique which is based on a pairwise inverse-consistent highly elastic diffeomorphic alignment of baseline (BL) and the follow-up (FU) scans to the halfway image space of the subject. The approach incorporates rigid registration into a halfway image space (an image space between BL and FU image) and correction for intensity inhomogeneities. The output Jacobian is the composition of two Jacobians: the Jacobian of the transformation field from the halfway space image to the FU scan and the negative Jacobian from the halfway image to the BL scan. Each voxel of the Jacobian therefore describes the percentage volumetric change between BL and FU image for that particular voxel location. To estimate the volumetric change of certain subregions of the brain the signal of the Jacobian needs to be integrated over a region of interest. The above approach is based on an elastic registration of the whole brain image. As an alternative technique the above approach will be deployed for a region around the structure of interest (ROI) (the thalamus for example). The two images will be registered into a halfway space. In both images a bounding box around the ROI will be extracted from the whole image. The above-described steps will be performed using the bounding boxes instead of the whole brain image. The third approach uses a segmentation of the ROI in the baseline image. The highly elastic diffeomorphic registration will be used to compute a displacement field of the boundary of the ROI. From the displacement fields at the boundary a volume change can be determined. The three methods will be compared with respect to stability and sensitivity using the cohorts and methods described in Opfer et al. (2020). The most suitable method will qualify for application to the primary data. Important conclusions regarding the statistical design can be drawn from the results.

Sensitivity analysis

A third category for group A „1.5 increase for study subjects with a baseline EDSS score of 0“ shall only be used in a sensitivity analysis as performed in the SUNBEAM and RADIANCE trials.

Power/Sample Size

Primary objective

We want to show whether the correlation between the change in MS-related disability and the change in thalamic volume is different from zero. Both the change in MS-related disability and the change in thalamic volume are continuous variables. We will use Pearson's correlation analysis. The expected correlation coefficient is 0.15, which seems to be a reasonable assumption at this point because the correlation coefficient between change in MS-related disability and change in total brain volume published recently by Uher et al. (2021) is of the same order of magnitude.

Assuming a type I error of 0.05 and a type II error of 0.20, we estimate the required sample size to be 347 (24 months after baseline EDSS measurement). Considering an annual dropout rate of 15% the total required sample size is approximately 600 study participants.

Secondary objectives

For addressing the secondary objectives groups 1 and 2 as defined in the statistical analysis methods are particularly relevant, but differences between all groups will be equally looked at. We assume an annual dropout rate of 15%. 24 months after baseline EDSS measurement study subjects shall be grouped based on their individual EDSS progress: group A comprises study subjects with (confirmed) disability progression and group B study subjects without disability progression. We assume that the incidence of study subjects in group 1 who belong to group A (with (confirmed) disability progression) is 5%. We further assume that the incidence of study subjects in group 2 who belong to group A (with (confirmed) disability progression) is 15%. The difference between groups 1 and 2 is significant (accepting a type-I error of 0.05 and a type-II error of 0.20) if the size of group 1 and group 2 is 196 and 98, respectively, 24 months after baseline EDSS measurement. Taking the annual dropout rate of 15% into account approximately 600 study subjects should be included.

The basic figures for incidence rates of disability progression in early MS patients for short and longer clinical trials were derived from Röver et al. (2015).

Similarly, we assume that 5% of patients in groups 2 and 3 (pooled) show disability progression between month 24 and month 36, whereas in group 1 the incidence is approximately zero. The difference between the pooled group and group 1 is significant (assuming a type I error of 0.05 and a type II error of 0.20) when the size of each of group 1 and the pooled group is 152.

Overview patient attrition

Timline	No of patients @ 15% drop out (annual)	Minimum No. of patients required to meet all endpoints	Sample size requirement for PE	Sample size requirement for S1	Sample size requirement for S2
Baseline	600	**>496**			
Month 12	510	**>422**			
Month 24 (end of study)	433	**>358**	347	392	
Month 36 (end of extension)	368	**>304**			304

Missing data handling

Three MRIs will be available per patient. TVL will be calculated for each pair of two consecutive MRI scans. Annualized TVL between the baseline (BL) and last follow-up (FU) scan will be calculated by multiplying the TVL between the two consecutive scans and dividing the resulting

TVL with the time interval between BL and FU scan. If for a patient only two MRIs are available, the TVL can still be computed in the same way. If only one MRI is available, TVL cannot be computed, and the patient will be excluded from the study.

As explained above EDSS scores will be measured approximately every 6 months. The annual change of EDSS during the 24 months' time period will be computed by linear regression of EDSS versus time including all visits (see Fig. 3). This approach still works if EDSS measurements are missing. At least two EDSS measurement are needed otherwise the patient needs to be excluded from the study.

5. STUDY LIMITATIONS/STRENGTHS

Generalizability of study results

The PENTAGON study if pursued over the full length is able to link results of the phase III clinical trials (RADIANCE and SUNBEAM) regarding the effect of ozanimod on brain volume, and in particular TVL to disability progression in a real world setting. Once confirmed pathological TVL could be considered an independent risk factor for disability worsening. This would then justify inclusion of TVL into the spectrum of treatment targets in MS.

Validity of measurement

The PENTAGON study is relatively long due to the involvement of longitudinal brain atrophy parameters: The length is required to warrant that these parameters are measured reliably in single subjects.

Bias/confounding and effect modification

A potential difficulty is the influence of different treatment regimens during the study. The treatment can impact TVL, new lesions as well as disability worsening. If the groups 1-3 are unbalanced with respect to different treatments results can be biased. A mitigation can be to use the treatment as a covariate in the statistical analysis.

Patients with recent disease activity are included into the study. There should be no intention to switch from first-line oral immune therapies to other therapy regimens when enrolling the patient into the study. Nevertheless, some patients might switch from first-line oral immune therapies to a second line therapy. For these patients the phenomenon of pseudo-atrophy might occur as it was described in several studies (generally assumed to be due to the resolution of inflammatory edema). Measures TVL needs to be interpreted with caution for these patients. MRI data needed to be excluded if the therapy switch happened less than 6 months before the MRI.

6. STUDY CONDUCT

This study will be conducted in accordance with International Society for Pharmacoepidemiology (ISPE) Guidelines for Good Pharmacoepidemiology Practices (GPP) and applicable regulatory requirements.

Ethics Committee Review and Informed Consent

Ethics Committee Review

The investigator ensures that the required approvals from Ethics Committees, Independent Review Committees, Regulatory Authorities, and/or other local governance bodies are obtained before study initiation at the site.

Informed Consent

In accordance with local regulations, subjects should provide either written or oral consent before enrollment into the study. The participating centers must ensure that patients, or, in those situations where consent cannot be given by patients, their legally acceptable representatives, are clearly and fully informed about the purpose of the study, potential risks, the patient's rights and responsibilities when participating in this study. If local regulations do not require an informed consent document to be signed by the patient, the site staff should document key elements of the informed consent process in the patient's chart.

Responsibilities within the Study

The study shall be conducted as described in this approved protocol.

External Advisory/Steering Committee

Prof. Dr. med. Carsten Lukas; Direktor Institut für Neuroradiologie, St. Josef-Hospital Bochum; D-44791 Bochum; Email: carsten.lukas@rub.de; phone: +49 - (0)234 509 3311 / 3381.

Confidentiality of Study Data

The confidentiality of records that could identify patients within the database must be protected, respecting the privacy and confidentiality rules in accordance with the applicable regulatory requirement(s).

Quality Control

Quality control measures shall be appropriately applied as defined in the investigator's standard operating procedures (SOPs), which are part of an ISO 9001:2015-certified quality management system (QMS) operated and regularly updated by the investigator and audited by sponsors.

Database Retention and Archiving of Study Documents

The investigator retains all study records and source documents for the maximum period required by applicable regulations and guidelines, or institution procedures. Location of database and supporting documentation will be outlined in the final observational study report.

Registration of Study on Public Website

This study will be registered on Deutsches Register Klinischer Studien (https://www.drks.de/drks_web/).

7. ADVERSE EVENT REPORTING

Not applicable

8. GLOSSARY OF TERMS AND LIST OF ABBREVIATIONS

List of Abbreviations

Term	Definition
Investigator	Conducts and sponsors the study and is the warrantor of the study's integrity
Participant	Patient participating in the study
Study center	Clinical site participating in the study

9. SCIENTIFIC PUBLICATIONS

An active participation in scientific and clinical conferences is planned.

An early paper on the results of the secondary data analysis shall be published reporting on the identification of predictive factors for TVL.

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