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Inner nuclear layer and olfactory threshold are interlinked and reflect inflammatory activity in multiple sclerosis

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Abstract

Background: Retinal inner nuclear layer (INL) and olfactory threshold (OT) are associated with inflammatory activity in multiple sclerosis (MS).

Objective: The study aims to investigate (a) whether there is an association of INL and OT in MS and (b) if changes in INL and OT follow a time pattern in relation to MS relapse.

Methods: We assessed INL by optical coherence tomography and OT by Sniffin' Sticks in three different cohorts: a cross-sectional MS cohort ($n = 260$), a longitudinal, 3-year cohort of MS ($n = 141$) and healthy controls ($n = 30$), and a longitudinal, 24-weeks cohort with acute MS relapse ($n = 28$) and stable MS controls ($n = 27$).

Results: Cross-sectionally, INL and OT were strongly correlated with number but not localization of relapse in the previous 12 months and INL correlated with OT. Longitudinally, INL was thicker and OT score was lower short term in times of relapse activity, but not long term and independent of relapse localization. In acute MS relapse, INL and OT were altered compared with stable MS, again, independent of relapse localization resolving over 12–24 weeks with faster approximation to stable MS after escalation of disease-modifying treatment.

Conclusions: INL and OT are interlinked markers of short-term inflammatory activity, following a nearly congruent time pattern and independent of relapse localization, possibly reflecting a proinflammatory state within the central nervous system.

Keywords: Multiple sclerosis, optical coherence tomography, inner nuclear layer, olfactory threshold, relapse

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Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) pathophysiologically comprising both autoimmune-mediated inflammation and neurodegeneration.[1] Correspondingly, demyelination and axonal degeneration are present in olfaction-related brain regions in about 70% of investigated brains of patients with MS.[2] After having been disregarded for over three decades, dysfunction of different qualities of the olfactory sense is increasingly recognized in MS with reported prevalence rates of up to 75%.[3] Of these qualities, olfactory threshold (OT), i.e. the capacity to detect odours even at low concentrations, has recently gained specific interest: OT was found to be impaired in early, active MS, to predict short-term relapse activity and to resolve in the absence of relapse and following disease-modifying treatment (DMT) for MS, while other olfactory qualities such as odour identification and discrimination were not

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associated with these parameters of inflammatory activity in MS.[4–7] Also, thickening of the retinal inner nuclear layer (INL) measured by optical coherence tomography (OCT) is associated with MS disease activity,[8–10] while reduction of INL volume is reported in patients successfully treated with DMT.[8–11] INL can be either measured as mean thickness of multiple scans or as the complete volume in the perifoveal area, with both methods used in the literature.

Thus, both INL and OT are hypothesized to reflect the level of inflammation in MS.[5,12] However, the underlying pathophysiology remains unclear.[3,12] Specifically, the question arises whether OT impairment and INL thickening are independent local phenomena, i.e. 'relapse', as opposed to occurring interlinked reflecting a common process, possibly 'CNS inflammatory state'. Therefore, the aims of the present study were to investigate (a) whether there is an association of INL and OT in MS, and (b) if changes in INL and OT follow a certain time pattern in relation to MS relapses.

Materials and methods

We investigated three different cohorts, all recruited from the MS outpatient clinic of the Department of Neurology at the Medical University Innsbruck. The study was approved by the ethics committee (ethical approval number: AM3743-281/4.3) of the Medical University Innsbruck and all patients gave written informed consent according to the Declaration of Helsinki before inclusion.

Cohort 1 includes a cross-sectional group of 260 patients with MS diagnosed according to 2010 McDonald criteria and aged between 18 and 55 years.[6,13] Cohort 2 consists of 141 MS patients diagnosed according to 2010 McDonald criteria (age 18–55 years) and 30 age- and sex-matched healthy controls (HC) from a prospective, observational, 3-year study.[5,14] Cohort 3 comprises 28 relapsing MS (RMS) patients with an acute relapse and 27 clinically stable RMS patients followed for 24 weeks from another prospective, observational, study.[7]

Exclusion criteria in all three cohorts were cognitive impairment defined as a score of 26 or lower in the Mini Mental State Examination (MMSE) because it is required for conduction of olfactory testing, a history of current or chronic oto-pharyngeal-laryngeal disease or surgery, head trauma, toxic exposures, previous radiation, or other diseases known to be associated with olfactory disturbances. Other exclusion criteria were previous diagnoses of ophthalmological (i.e. myopia greater than –4 diopters, optic disc drusen), neurological, or drug-related causes of vision loss or retinal damage not attributable to MS to avoid confounding of OCT results.[12]

At every visit (cohort 1: at day of olfactory testing and OCT; cohort 2: at baseline and every 6 months over 3 years; cohort 3: at baseline and after 4, 12 and 24 weeks) a structured questionnaire regarding demographic data, smoking habits, neurological and pharmacological history including relapses and DMT, use of drugs and hormonal contraceptives was obtained from each patient. A relapse was defined as patient-reported symptoms or objectively observed signs typical of an acute CNS inflammatory demyelinating event, current or prior to the visit, with duration of at least 24 h in the absence of fever or infection, separated from the last relapse by at least 30 days.[13] All relapse symptoms were classified as either sensory/pyramidal, brainstem/cerebellar, optic neuritis (ON) or other.[15] In cohorts 1 and 2, patients were classified as 'no relapse activity' or 'relapse activity' ($\geq$1 relapse in any symptom category). For cohort 1 this classification was done before baseline (within the previous 12 months) and for cohort 2 in the timeframes from baseline to year 1 (B-Y1), year 1 to year 2 (Y1-Y2) and year 2 to year 3 (Y2-Y3). Patients in the 'relapse activity' group were subclassified according to type of relapse symptoms as 'monofocal' ($\geq$1 relapse in only one functional category of the Expanded Disability Status Scale (EDSS)) or 'polyfocal' ($\geq$1 relapse in $\geq$2 functional categories). Clinically stable MS was defined as absence of relapse for at least 12 months prior to inclusion in the study. In all three cohorts, patients were only included if DMT has not been changed $\geq$3 months prior to the baseline visit. Change of DMT during the observation period was explicitly allowed in all three cohorts, except for the stable MS group in cohort 3. In the relapse group of cohort 3, patients were subdivided into two groups: 'relapse – escalation' (DMT was initiated or escalated within 8 weeks from baseline) and 'relapse – no escalation' (no change of DMT within 8 weeks from baseline).[7] Every relapse was treated with a single course of standard intravenous high dose methylprednisolone (1000 mg per day over 3 days, followed by 500 mg/d over 2 days) without oral tapering. The onset of relapse symptoms had to occur no longer than 7 days prior to baseline. Patients were excluded from cohort 3 if they suffered from a separate relapse or received an additional course of corticosteroids during the

observation period. EDSS was obtained at every visit.[15] The Beck Depression Inventory (BDI) was performed to screen for depression. Depression was defined as a score of 18 or higher on the BDI.[16]

Olfactory threshold

OT was assessed at each visit in all three cohorts using the extended version of the Sniffin' Sticks test (Burghart Medizintechnik, Wedel, Germany) according to the manufacturer's instruction including change of testing sticks every 6 months.[17] In brief, the Sniffin' Sticks test is based on pen-like odour-dispensing devices. OT is assessed using n-butanol as a single odorant. Three sticks are presented to each subject in a randomized order, two containing solvent and the third containing n-butanol at a certain dilution. The subject is repeatedly asked to identify the stick with the odorant using a single-staircase, three-alternative forced-choice procedure.[17] The maximum score is 16 points and reflects optimal olfactory function. Lower scores are associated with an increased threshold for odour perception. The age-specific normative values are based on data from 3000 healthy subjects.[18]

In cohorts 1 and 2, olfactory testing was postponed for 4 weeks if the patient had received corticosteroids within 4 weeks or if upper respiratory tract infections were present at the time of assessment. In cohort 3, olfactory testing was postponed for 1 week in case of upper respiratory tract infections. In the relapse group of cohort 3 OT was always tested before corticosteroids were applied. Investigators performing the olfactory testing were blinded to clinical information and OCT results.

Optical coherence tomography

OCT imaging was performed at each visit by two experienced technicians using the same spectral-domain OCT (Spectralis, Heidelberg Engineering, Heidelberg, Germany; software Heidelberg eye explorer software version 5.4.8.0) without pupil dilatation in a dark room on both eyes of each patient. A $20^\circ \times 20^\circ$ macular volume scan (512 A-scans, 257 B-scans, vertical alignment, ART 16) automatically centred around the fovea was performed. INL thickness (μm) was calculated as the mean value of the inner four quadrants of the grid (corresponding to the 3-mm ring as defined by the Early Treatment Diabetic Retinopathy Study[19]). INL volume was calculated by the Spectralis software's segmentation algorithm (6 mm diameter circle around the fovea). Image processing was conducted semiautomated with manual correction of obvious errors. Longitudinal examinations were performed using the follow-up mode. All examinations were checked for sufficient quality using OSCAR-IB criteria.[20] Patients with a history of unilateral ON within 6 months before baseline or a history of bilateral ON were excluded from all three cohorts in order to minimize the confounding carry-over effect of acute or past ON. Also, eyes suffering ON or displaying microcystic macular edema (MME) during the observation period were excluded from all three cohorts, with data being censored from the occurrence of ON. In patients without a history of ON, OCT parameters were calculated as the means of the values for both eyes, while in patients with a history of unilateral ON only the values for the eye without ON were used in the analyses. The investigators performing the OCT were blinded to clinical parameters and vice versa.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 (SPSS Inc, Chicago, IL). Categorical variables were expressed in frequencies and percentages, continuous variables as mean and standard deviation (SD) or median and range as appropriate. Bivariate comparisons were conducted by *t*-test, ANOVA, Mann–Whitney *U* test, Kruskal–Wallis test or chi-square test as appropriate. Bivariate correlations were calculated by Pearson or spearman rho test as appropriate. In cohort 1, we performed multivariate linear regression models to predict INL/OT adjusting for number and type of relapses with correction for sex, age, disease duration, depression (no depression vs. depression), smoking status (non-smokers vs. smokers) and DMT status (no DMT vs. DMT). Also, interdependence of INL and OT was tested by multivariate linear regression models regarding OT modelling for INL volume/thickness adjusted for sex, age, disease duration, depression, smoking status and DMT status. Repeated measures in cohort 2 and 3 were analysed by repeated measurement ANOVA corrected for multiple testing. Intra-subject stability of repeated measures was tested by intraclass correlation coefficients (ICC). In cohort 2, we also investigated effects of number of relapses and relapse type (mono- vs. polyfocal) on OT, INL thickness and INL volume in short term (within the same follow-up period) and long term (within subsequent follow-up periods) using multivariate linear regression modelling for covariates. Missing values were handled by multiple imputation using the missing not at random (MNAR) approach.[21] Statistical significance was set at $p < 0.05$.

Results

The screening process and inclusion flow chart are provided in Figure 1. Characteristics of all three study cohorts are given in Table 1. OT and INL parameters are shown in Table 2 for all cohorts. Analyses performed in cohort 2 and cohort 3, respectively, showed that OT scores were significantly lower in MS compared with HC and in MS relapse compared with stable MS (Table 2). Similarly, both INL thickness and INL volume were higher in MS than in HC and in MS relapse than in stable MS.

Cross-sectional correlations of OT and OCT parameters

Cross-sectional correlations were conducted in cohort 1. Age was negatively correlated with OT ($\rho = -0.138$, $p < 0.001$), INL thickness ($\rho = -0.147$, $p < 0.001$), and INL volume ($\rho = -0.211$, $p < 0.001$), while there were no differences regarding sex. As well, disease duration was significantly negatively correlated with OT ($\rho = -0.295$; $p < 0.001$), INL thickness ($\rho = -0.269$, $p < 0.001$) and INL volume ($\rho = -0.283$, $p < 0.001$). With increasing number of relapses in the year prior to assessment, OT scores were lower, while INL was significantly thicker (Figure 2(a–c)). In the univariate analyses, the polyfocal relapse activity group displayed lower threshold scores than the monofocal group (4.1 vs. 5.3; $p = 0.007$), higher INL thickness (46.0 µm vs. 43.8 µm; $p = 0.081$) but comparable INL volume (1.04 mm^3 vs. 1.04 mm^3; $p = 0.996$). In patients on DMT, we found higher OT scores (6.5 vs. 5.5; $p = 0.012$), lower INL thickness (41.5 µm vs. 42.9 µm; $p = 0.032$) and lower INL volume (0.97 mm^3 vs. 1.00 mm^3; $p = 0.028$) in comparison to patients without DMT. Median OT scores were higher in non-smokers (6.5 vs. 5.8; $p = 0.023$) and patients without depression (6.8 vs. 6.0; $p = 0.020$), whereas INL was not significantly different. Neither INL thickness nor volume was correlated with smoking, depression, EDSS or MMSE. The multivariate analyses showed that number of relapses but not type of relapse was associated with OT, INL thickness and INL volume (Table 3, part A). Regarding interdependence of OT and INL in multivariable models, OT correlated negatively with both INL thickness ($B = -0.31$; $p < 0.001$; Nagelkerke R^2: 0.581) and INL volume ($B = -16.1$; $p < 0.001$; Nagelkerke R^2: 0.533) (Figure 2(d, e)).

Longitudinal changes in OT and INL depend on occurrence and timing of MS relapse

To further validate whether OT and INL reflect disease activity, changes in these parameters were

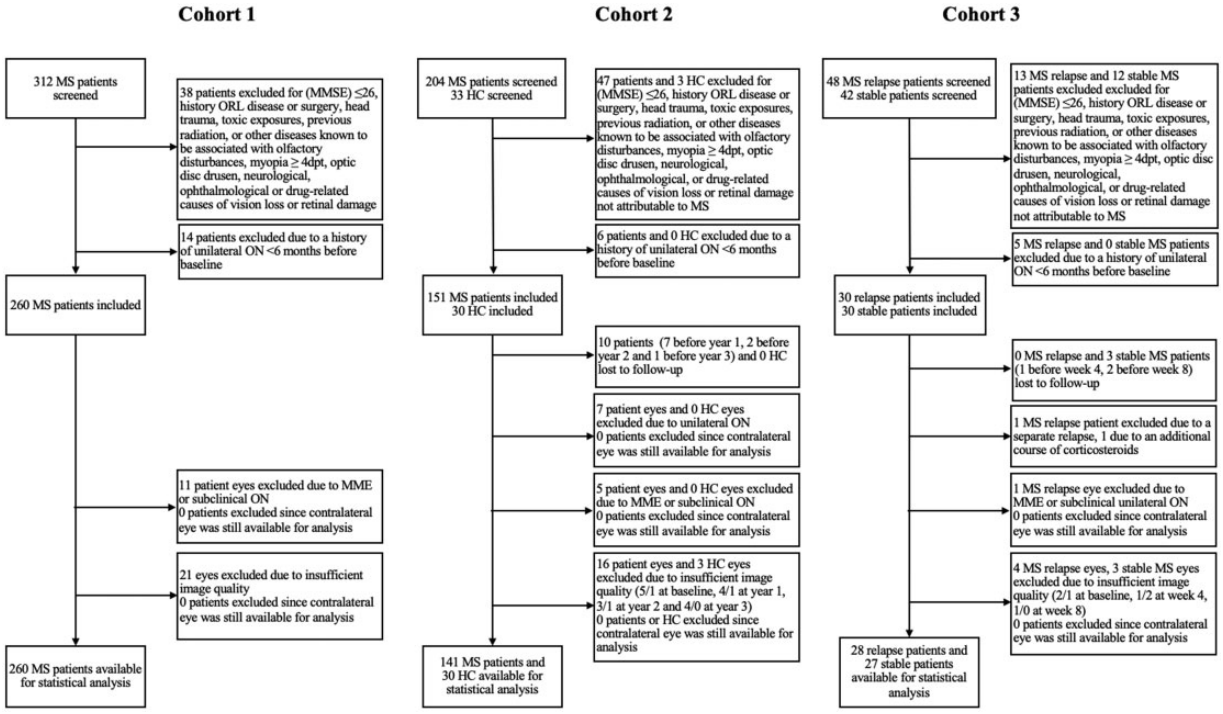

Figure 1. STROBE flow chart.
Dpt: diopters. HC: healthy controls. MMSE: Mini Mental Status Examination. MS: multiple sclerosis. ON: optic neuritis. ORL: oto-pharyngeal-laryngeal.

Table 1. Cohort characteristics.

	Cohort 1	Cohort 2		Cohort 3	
	MS ($n=260$)	MS ($n=141$)	HC ($n=30$)	MS Relapse group ($n=28$)	MS Stable group ($n=27$)
Females[a]	191 (68.7)	112 (79.4)	22 (73.3)	20 (71.4)	21 (77.7)
Age[b] (years)	35.9 (9.4)	34.9 (9.1)	34.5 (9.9)	34.6 (8.4)	33.7 (9.0)
Disease duration[b] (years)	6.9 (6.4)	6.3 (6.1)	NA	5.8 (5.7)	6.1 (6.0)
Diagnosis[a]					
RMS	204 (78.5)	128 (90.8)	NA	28 (100)	27 (100)
SPMS	31 (11.9)	8 (5.6)	NA	0 (0)	0 (0)
PPMS	25 (9.6)	5 (3.6)	NA	0 (0)	0 (0)
OCB positive[a]	256 (98.5)	137 (97.2)	NA	28 (100)	27 (100)
DMT[a]	221 (85.0)	82 (58.1)	NA	19 (67.9)	20 (74.1)
Interferon beta	66 (25.5)	41 (29.1)	NA	5 (17.9)	6 (22.2)
Glatiramer acetate	46 (17.7)	19 (13.5)	NA	5 (17.9)	5 (18.5)
Fingolimod	23 (8.8)	9 (6.4)	NA	2 (7.1)	1 (3.7)
Dimethyl fumarate	27 (10.4)	1 (0.7)	NA	6 (21.4)	7 (25.9)
Teriflunomide	18 (6.9)	0 (0)	NA	1 (3.6)	1 (3.7)
Natalizumab	38 (14.6)	12 (8.5)	NA	0 (0)	0 (0)
Alemtuzumab	3 (1.2)	0 (0.0)	NA	0 (0)	0 (0)
Number of relapses in last year[b]	0.52 (0.84)	0.47 (0.87)	NA	0.50 (0.42)	0.12 (0.20)
EDSS[c]	2.0 (0–6.5)	1.5 (0–6.5)	NA	2.0 (0–6.5)	1.5 (0–6.5)
MMSE[b]	30 (27–30)	30 (27–30)	30 (28–30)	30 (27–30)	30 (27–30)
BDI-Score[b]	5.4 (5.6)	5.8 (4.7–7.0)	5.0 (3.2–6.9)	5.0 (5.3)	4.3 (5.1)
Depression (BDI$\geq$18)[a]	11 (4.2)	7 (5.0)	1 (3.3)	1 (3.6)	2 (7.4)
Smokers[a]	110 (42.3)	43 (30.5)	10 (33.3)	9 (32.1)	9 (33.3)
Unilateral optic neuritis before baseline[a]	91 (35.0)	46 (32.6)	NA	9 (32.1)	8 (29.6)

[a]absolute number and percentage; [b]mean and standard deviation; [c]median and range.
BDI: Beck Depression Inventory. DMT: disease-modifying treatment. EDSS: Expanded Disability Status Scale. HC: healthy controls. MMSE: Mental State Examination. MS: multiple sclerosis. OCB: oligoclonal bands. PPMS: primary progressive MS. RMS: relapsing MS. SPMS: secondary progressive MS.

Table 2. Olfactory threshold and INL at baseline in MS and healthy controls.

	Cohort 1	Cohort 2			Cohort 3		
	MS ($n=260$)	MS ($n=141$)	HC ($n=30$)	p-value	MS Relapse ($n=28$)	MS Stable ($n=27$)	p-value
Olfactory threshold[a]	6.3 (1.6)	6.2 (1.9)	8.1 (1.9)	<0.001[b]	4.3 (2.0)	7.8 (1.5)	<0.001[b]
INL thickness (μm)[a]	42.2 (3.8)	42.5 (2.9)	37.4 (3.3)	<0.001[b]	47.5 (4.1)	41.3 (3.1)	<0.001[b]
INL volume (mm^3)[a]	0.98 (0.07)	0.99 (0.06)	0.94 (0.05)	<0.001[b]	1.08 (0.08)	0.96 (0.06)	<0.001[b]

[a]mean and standard deviation.
[b]p-value calculated by independent t-test.
HC: healthy controls. INL: retinal inner nuclear layer. MS: multiple sclerosis.

monitored annually over the course of three years (Cohort 2).

Neither OT nor INL changed in HC during the observation period, showing high intra-subject stability (ICC>0.9). In patients with MS, mean OT scores and INL measures also did not change. However, intra-subject stability was low for threshold (ICC = 0.29), INL thickness (ICC = 0.31) and INL volume (ICC = 0.33). When analysing

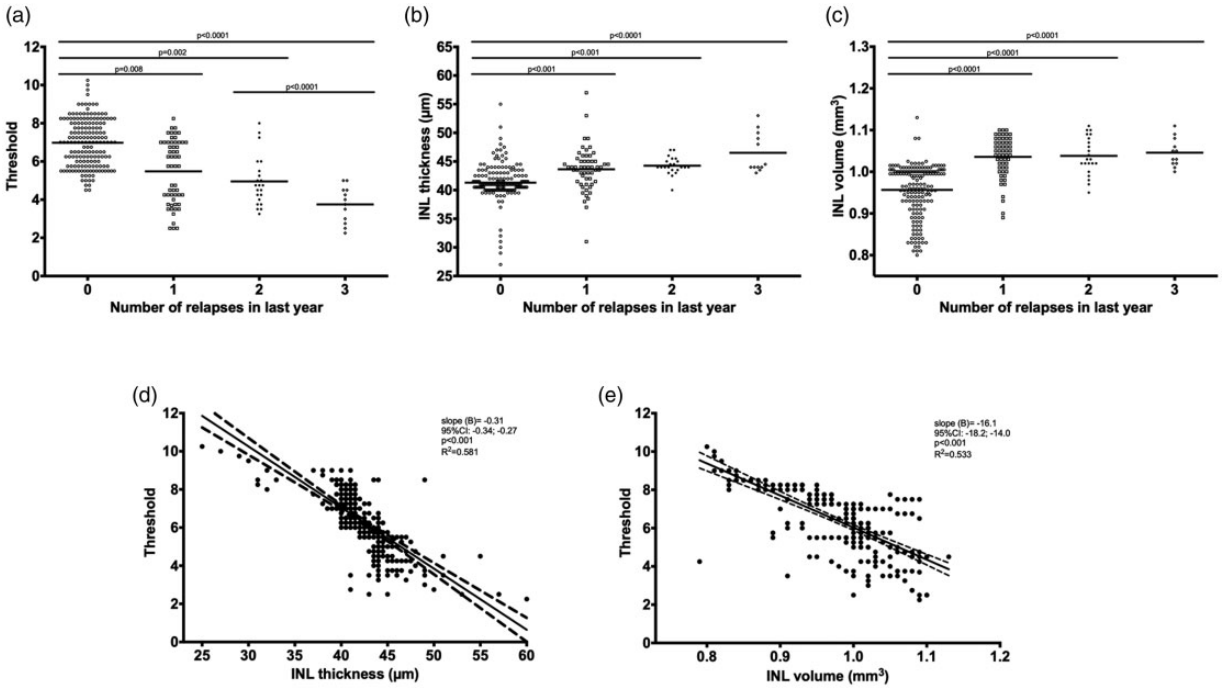

Figure 2. Olfactory threshold associated with clinical relapses and INL.
Panel a–c: *p*-values calculated by ANOVA. Panels d, e: Slope of change (Regression coefficient B) with 95% confidence interval of B and *p*-values calculated by multivariate linear regression models correcting for sex, age, disease duration, depression (no depression vs. depression), smoking status (non-smokers vs. smokers) and DMT status (no DMT vs. DMT). INL: retinal inner nuclear layer.

according to relapse activity within the predefined timeframes, we found that OT (Figure 3(a–c)), INL thickness (Figure 3(d–f)) and INL volume (Figure 3 (g–i)) were significantly altered in patients with relapse activity at testing points marking beginning and end of the respective timeframe, but not at more distant time testing points.

The multivariate regression models confirmed that the number of relapses in a 1-year period was associated with OT, INL thickness and INL volume at the end of the same timeframe (i.e. short term, Table 3, part B), but not in the long term (Table 3, part C). The type of relapse did not significantly influence threshold or INL, neither short term nor long term.

Regarding multivariate analyses of interdependence of OT and INL, change of OT score was dependent on change of INL thickness (Figure 3(j–l)) and INL volume (Figure 3(m–o)) within the same timeframe but independent of other timeframes.

INL and threshold in acute MS relapse

To further evaluate how OT and INL develop in acute MS relapse, changes in these parameters were monitored over 24 weeks (Cohort 3).

At baseline, threshold scores were significantly lower in acute MS relapse (mean difference: 3.5, $p < 0.001$, Figure 4(a)) than in clinically stable MS, while INL thickness (mean difference: 6.3 µm, $p < 0.001$, Figure 4(c)) and INL volume (mean difference: 0.12 mm^3, $p < 0.001$, Figure 4(e)) were significantly higher. There was no difference between types of relapse.

Over the observation period, OT and INL parameters significantly changed in acute MS relapse (all *p*-values < 0.001) but not in stable MS (all *p*-values > 0.9). Differences in OT and INL thickness gradually decreased but remained significant at week 4 (2.5 and 3.7 µm; $p < 0.001$, respectively) and week 12 (1.5; $p = 0.008$ and 2.2 µm; $p = 0.004$). INL volumes approximated faster, as the group difference was 0.06 mm^3 at week 4 ($p = 0.049$) and 0.03 mm^3 at week 12 ($p = 0.421$). At week 24, we did not find any significant differences between the groups. Again, there was no difference between types of relapse. In neither group was there a difference in OT scores and INL between patients with and without DMT at baseline. After relapse, DMT was initiated or escalated in 16/28 patients (relapse-escalation). The relapse-escalation group displayed

Table 3. Cross-sectional (A), short-term (B) and long-term (C) effects of number and type of relapse on olfactory threshold and INL.

	Olfactory threshold		INL thickness		INL volume	
A (cross-sectional, cohort 1)	B (95% CI)	*p*-value[a]	B (95% CI)	*p*-value[b]	B (95% CI)	*p*-value[b]
Number of relapses (per relapse)	−0.7 (−1.2; −0.3)	0.005	0.9 (0.3; 2.1)	<0.001	0.05 (0.01; 0.08)	0.005
Type of relapse (reference category: polyfocal)	−0.5 (−1.2; 0.3)	0.249	0.8 (−0.8; 2.8)	0.296	0.03 (−0.01; 0.05)	0.383
Nagelkerke R^2	0.606		0.426		0.522	
B (short-term, cohort 2)	B (95% CI)	*p*-value[c]	B (95% CI)	*p*-value[d]	B (95% CI)	*p*-value[d]
Number of relapses (per relapse)	−1.0 (−1.4; −0.5)	<0.001	3.7 (2.2; 5.2)	<0.001	0.07 (0.03; 0.10)	<0.001
Type of relapse (reference category: polyfocal)	−0.3 (−1.4; 0.6)	0.364	1.2 (−1.0; 3.4)	0.553	0.02 (−0.01; 0.05)	0.107
Nagelkerke R^2	0.753		0.446		0.497	
C (long-term, cohort 2)	B (95% CI)	*p*-value[a]	B (95% CI)	*p*-value[b]	B (95% CI)	*p*-value
Number of relapses (per relapse)	−0.1 (−1.0; 0.7)	0.739	1.4 (−0.3; 3.1)	0.102	0.03 (−0.02; 0.07)	0.246
Type of relapse (reference category: polyfocal)	0.2 (−0.6; 1.0)	0.634	−1.0 (−2.5; 0.6)	0.215	−0.03 (−0.07; 0.01)	0.172
Nagelkerke R^2	0.298		0.118		0.042	

[a]calculated by multivariate linear regression models correcting for sex, age, disease duration, depression (no depression vs. depression), smoking status (non-smokers vs. smokers) and DMT status (no DMT vs. DMT).
[b]calculated by multivariate linear regression models correcting for sex, age, disease duration, and DMT status (no DMT vs. DMT).
[c]calculated by multivariate linear regression models correcting for threshold baseline, sex, age, disease duration, depression (no depression vs. depression), smoking status (non-smokers vs. smokers) and DMT status (no DMT vs. DMT).
[d]calculated by multivariate linear regression models correcting for INL thickness/volume, sex, age, disease duration, and DMT status (no DMT vs. DMT).
INL: retinal inner nuclear layer. B: Regression coefficient. 95% CI: 95% confidence interval of B.

faster approximation to the values of the stable MS group than the relapse-no escalation in OT, INL thickness and INL volume (Figure 4(b, d, f)).

Discussion

In this study we showed that there is an association of INL thickening and OT impairment in MS correlating with number but not type of relapses. Longitudinally, both INL and OT were significantly altered short term in timeframes of relapse activity, but not long term and independent of relapse type. Changes in INL were dependent on changes of OT. In acute MS relapse, INL was significantly thicker and OT scores lower compared with stable MS, again, independent of relapse type. Alterations in INL and OT present in acute relapse resolved over 12–24 weeks, with faster approximation to stable MS occurring after escalation of DMT.

Thickening of the retinal INL, which can be robustly measured by OCT, has been repeatedly reported as a marker of inflammatory MS disease activity independent of local inflammation, i.e. ON.[8–10] These findings are corroborated by our study. Novel, we show that INL thickening is a transient feature of MS relapse activity which resolves in phases of clinical stability. A reduction of INL volume was previously reported to reflect DMT response within 6–9 months of treatment.[11] Our study adds to this evidence, specifically by showing that this effect can be seen both in INL volume and thickness, even though INL volume seems to be more sensitive. Also, INL seems to reflect response to change of DMT after acute MS relapse. However, the sample size in our study was not large enough to investigate differences between DMT regimens. It remains to be elucidated whether this effect is clinically relevant as the effect sizes are moderate.

The underlying pathophysiology of INL thickening is discussed with controversy. Initially MME, occurring in 1–5% of patients with MS, was believed to be the main mechanism.[8,22] However, more recent

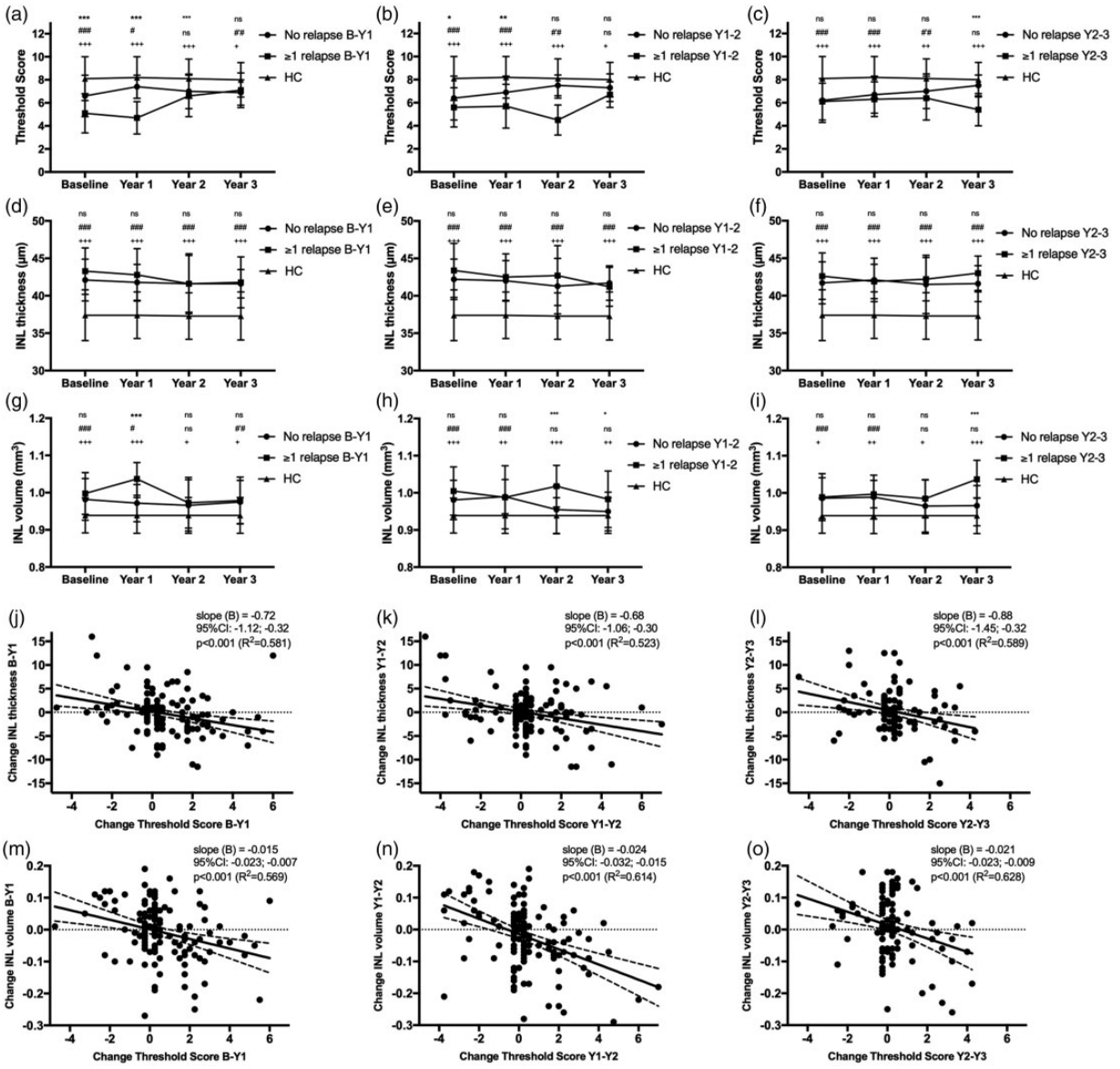

Figure 3. Longitudinal development of olfactory threshold and retinal inner nuclear layer depending on occurrence and timing of MS relapse over 3 years.

Panels a–i: comparison of olfactory threshold, INL thickness and INL volume in HC and MS patients with and without a relapse in the time frames between baseline and Year 1 (panels a, d, g), Year 1 and 2 (b, e, h), Year 2 and 3 (c, f, i). First line: HC vs. MS patients without a relapse (*$p<0.05$; **$p<0.01$; ***$p<0.001$ or non-significant). Second line: HC vs. MS patients with a relapse ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ or non-significant). Third line: MS patients without a relapse vs. MS patients with a relapse ($^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ or non-significant). All *p*-values calculated by repeated measurement ANOVA corrected for multiple testing.

Panels j–o: Comparison of change of olfactory threshold and change of INL thickness/volume in the timeframes between baseline and Year 1 (j, m), Year 1 and 2 (k, n), Year 2 and 3 (l, o). Slope of change (Regression coefficient B) with 95% confidence interval of B and *p*-values calculated by multivariate linear regression models correcting for sex, age, disease duration, depression (no depression vs. depression), smoking status (non-smokers vs. smokers) and DMT status (no DMT vs. DMT).

HC: healthy controls. INL: retinal inner nuclear layer. MS: multiple sclerosis.

studies found INL thickening in MS even after adjusting or completely excluding MME eyes.[9,11] To rule out confounding, we excluded all eyes with MME and also all eyes with acute ON. Other proposed mechanisms include direct retinal inflammation or inflammation-related dynamic fluid shifts, possibly related to the existence of a retinal glymphatic system with a prominent role for the INL.[22,23] Intriguingly, OT is impaired in active MS, predicts short-term relapse activity, and

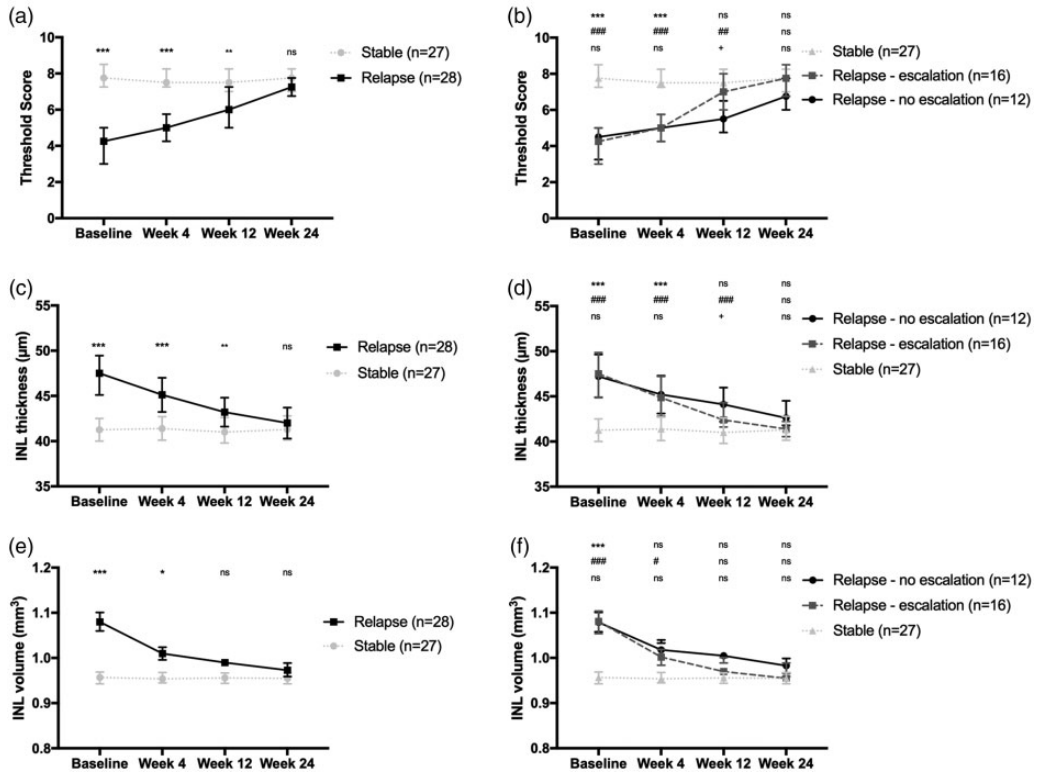

Figure 4. Longitudinal development of olfactory threshold and retinal inner nuclear layer in stable and acutely relapsing MS patients.
Panels a, c, e: Comparison of olfactory threshold, INL thickness and INL volume in stable and acutely relapsing MS patients.
Panels b, d, f: Comparison of olfactory threshold, INL thickness and INL volume in stable and acutely relapsing MS patients with and without escalation of DMT after relapse. First line: Stable MS vs. acutely relapsing MS with escalation after relapse (*$p<0.05$; **$p<0.01$; ***$p<0.001$ or non-significant). Second line: Stable MS vs. acutely relapsing MS without escalation after relapse ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ or non-significant). Third line: acutely relapsing MS with vs. without escalation after relapse ($^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ or non-significant). All p-values calculated by repeated measurement ANOVA corrected for multiple testing. INL: retinal inner nuclear layer. MS: multiple sclerosis.

resolves in the absence of relapse and following DMT for MS.[4–7] Impairment of OT has also been found in other autoinflammatory diseases with CNS affection, such as neuromyelitis optica spectrum disorders or systemic lupus erythematosus, with even more prominent impairment in active CNS disease.[24–26] Thus, OT is hypothesized to reflect the level of inflammation in MS.[5,27] The transient and reversible nature of threshold impairment underlines this concept.[4,5,7] However, the underlying pathophysiology is unclear: it has been argued that the peripheral parts of the olfactory system, which are essential for OT, might be affected through a 'bystander' inflammation during phases of clinical disease activity, possibly via humoral mechanisms such as cytokine release or antibody-mediated inflammation.[28] Others have speculated that OT impairment might be due to a 'olfactory neuritis' similar to ON or other relapse symptoms of MS.[27] Hence, INL thickening and OT impairment in MS pose a crucial question: are they separate, independent local phenomena and, thus, the expression of a localized inflammatory process, i.e. a 'relapse'? Or are they interlinked, reflecting a common process, possibly a general autoinflammatory state within the CNS?

We found that INL thickening and OT impairment occur together in the presence of inflammatory activity and resolve nearly simultaneously in inflammatory remission. In the short term, changes in INL were strongly correlated to changes in OT. Finally, INL and OT alterations responded similarly to change of DMT. Importantly, the type of relapse symptoms, i.e. the topography of inflammation, did not influence INL or OT. Thus, our data suggest that INL thickening and OT impairment are both indicators of a general proinflammatory state within the CNS rather than separate focal 'relapses'.

As a limitation, excluding patients with ON both before and during study period means that we cannot determine whether ON is itself associated with reduced OT. However, as we were primarily interested in determining whether both phenomena are interlinked or occurring separately, we had to rule out the confounding effect of acute ON which is much larger regarding effect size in OCT measures and, thus, obviously would have disguised the more subtle effects of systemic inflammation. As a further limitation of this study, we did not have magnetic resonance imaging (MRI) or body fluid biomarkers available for correlation with INL or OT which might further elucidate the underlying pathophysiology. Investigating MRI might show (a) whether there are associations between OT/INL and subclinical signs of inflammation (i.e. new T2 lesions or contrast-enhancing lesions) and (b) whether there is an association with certain structures within the CNS. These aspects are considered important future directions.

The proportion of primary progressive MS (PPMS)/secondary progressive MS (SPMS) patients included differs between the cohorts. Although we could not compare different MS phenotypes (RMS vs. PPMS vs. SPMS) as the low number of PPMS/SPMS patients included did not provide sufficient power for such analyses, we conducted sensitivity analyses excluding PPMS/SPMS patients which did not yield significantly different results.

In conclusion, INL and OT are interlinked markers of short-term inflammatory activity following a nearly congruent time pattern and independent of relapse localization. Alterations to INL and OT might together reflect a proinflammatory state within the CNS.

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