

# Eomesodermin-expressing CD4<sup>+</sup> Th cells and association with pregnancy in multiple sclerosis

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## Abstract

**Background:** Pregnancy in patients with multiple sclerosis (MS) is accompanied by a decline of relapse activity with increased risk of relapses 3 months post-partum, for unknown reasons. Eomesodermin<sup>+</sup> T-helper cells (Eomes<sup>+</sup> Th cells) are known to mediate neuroinflammation and disease progression in MS and are induced by prolactin-secreting cells.

**Objectives:** Here, investigated immune cell alterations and the pathophysiological role of Eomes<sup>+</sup> Th cells for disease activity during pregnancy and post-partum in MS.

**Methods:** We enrolled  $n=81$  pregnant patients with relapsing-remitting MS (RRMS),  $n=27$  post-partum RRMS and  $n=26$  female RRMS control patients under the umbrella of the German Multiple Sclerosis and Pregnancy Registry. Clinical data were collected and immune cell alterations were analysed using flow cytometry.

**Results:** While CD3<sup>+</sup>CD4<sup>+</sup> Th cells were unaffected, CD3<sup>+</sup>CD8<sup>+</sup> cytotoxic T-cells were elevated post-partum ( $p=0.02$ ) with reduced B-cell frequencies ( $p=0.01$ ) compared to non-pregnant RRMS patients. NK cells were elevated during first trimester ( $p=0.02$ ) compared to the third trimester. Frequencies of Eomes<sup>+</sup> Th and Eomes<sup>+</sup> Tc cells did not differ. There was no correlation of prolactin release and expression of Eomes<sup>+</sup> Th cells. However, Eomes<sup>+</sup> Th cells correlated with lower frequencies of regulatory T-cells during second ( $r=-0.42$ ;  $p<0.05$ ) and third trimester ( $r=-0.37$ ;  $p<0.05$ ). Moreover, Eomes<sup>+</sup> Th cells correlated with frequencies of B-cells during third trimester ( $r=0.54$ ;  $p=0.02$ ). Frequencies of Eomes<sup>+</sup> Th cells were not associated with the number of relapses before pregnancy, during pregnancy or post-partum. However, Eomes<sup>+</sup> Th cells strongly correlated with disability post-partum as assessed using the EDSS ( $r=0.52$ ;  $p=0.009$ ).

**Discussion:** Pregnancy in MS is associated with robust immunological alterations. Eomes<sup>+</sup> Th cells are capable of inducing immune cell alterations during the course of pregnancy, most evident during the second and third trimester as shown with a correlation of reduced Treg cells and a significant increase of B-cells. Importantly, Eomes<sup>+</sup> Th cells correlate with disability post-partum. In summary, during late pregnancy in MS an inflammatory, cytotoxic and dysregulated immunological environment is primed gaining function post-delivery. This may be responsible for post-partum disability accumulation.

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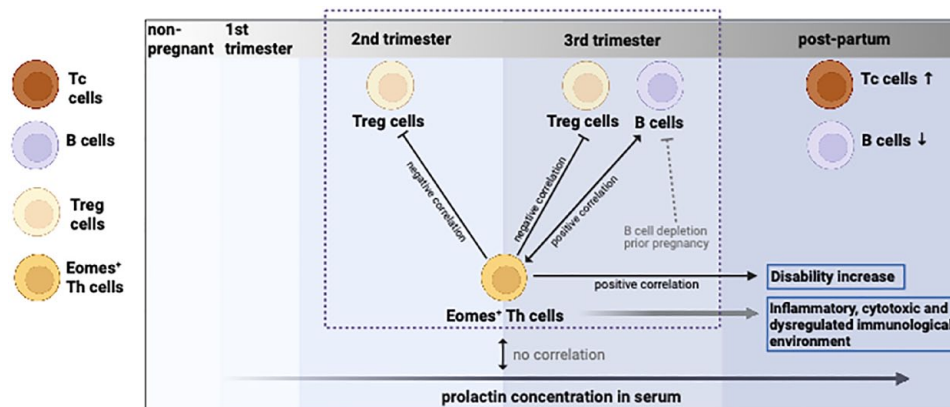
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## Graphical abstract

### The pathophysiological role of Eomes<sup>+</sup> Th cells during pregnancy and post-partum



Source: Created with Biorender.com.

**Keywords:** Eomesodermin<sup>+</sup> Th cells, multiple sclerosis, pregnancy, progression independent of relapse activity

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## Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by demyelination, axonal damage and neurodegeneration.<sup>1</sup> As the disease affects increasingly women during early adulthood, a phase often associated with the wish to build a family and become pregnant,<sup>2,3</sup> knowledge about the interaction of pregnancy with MS is crucial. The relapse rate decreases during pregnancy with the lowest risk during the third trimester, increasing frequency during the post-partum period and normalization to pre-pregnancy rates approximately 3 months after delivery.<sup>4</sup> Patients planning pregnancy need to be individually adjusted to disease-modifying therapies (DMTs) in order to decrease the risk of relapses during and after pregnancy.<sup>5,6</sup> Treatment with DMTs and low annualized relapse rates before pregnancy have a positive impact on the frequency of post-partum relapses.<sup>7</sup> Besides, pregnancy in MS has a normal course and is not associated with foetal abnormalities or miscarriage<sup>8</sup> and pregnancy does not influence secondary progression of MS, especially in patients without significant increase in post-partum clinical and MRI disease activity.<sup>9</sup> Breast feeding was shown to be slightly protective against post-partum relapses.<sup>10</sup> However, the protective

effect of breast feeding for post-partum disease activity is controversially discussed. At the moment, there is still lacking knowledge about immunological pathomechanisms driving disease activity during pregnancy and the post-partum period. Identifying patients with an increased risk of relapses during or after pregnancy using biomarkers could potentially be an important approach towards future personalized MS therapy during and after pregnancy. The expression of cytotoxic Th cells expressing Eomesodermin (Eomes) were associated with a higher risk of disease progression.<sup>11</sup> B-cells and non-B cell antigen-presenting cells (APCs) upregulate the hormone prolactin in the progressive phase of experimental autoimmune encephalomyelitis (EAE), a mouse model for MS.<sup>12</sup> Those prolactin producing APCs can induce the expression of Eomes on Th cells *ex vivo*.<sup>12</sup> Prolactin in general is mainly produced by the anterior pituitary gland and regulates, in particular, lactation in pregnant women. Additionally, prolactin elicits an important role for immune system regulation, for example, through the generation of autoreactive lymphocytes.<sup>13,14</sup> The role of prolactin in MS has been investigated in several studies with divergent results. Prolactin was found in higher concentration in female MS patients compared to healthy

control persons but did not correlate with disease activity, disease duration or active lesions in the CNS.<sup>15</sup> Furthermore, prolactin is associated with the generation of autoreactivity *via* B-cells in MS.<sup>16</sup> Contrarily, prolactin promotes proliferation of oligodendrocyte precursor cells and white matter repair and is therefore discussed as therapeutic target for remyelination.<sup>17</sup>

We hypothesized that prolactin release during pregnancy and post-partum might induce the expression of Eomes<sup>+</sup> Th-cells, potentially associated with an increased risk of disease activity. We assessed disease activity of pregnant MS patients in relation to alterations of immune cell subsets and prolactin concentration.

## Materials and methods

### *Enrolment of patients*

Participants were recruited from February 2021 to September 2022 in a prospective study design at the outpatient clinic at the Department for Neurology, Ruhr-University Bochum, St. Josef Hospital. Included patients were enrolled in the German Multiple Sclerosis and Pregnancy Registry (DMSKW), founded in 2006.<sup>18</sup> Written informed consent was signed by the subjects before study inclusion. Patients had a diagnosis of definite MS according to McDonald criteria 2017.<sup>19</sup> The study included in total  $n=26$  female relapsing-remitting MS (RRMS) patients without current pregnancy,  $n=25$  RRMS patients during the first trimester of pregnancy,  $n=24$  patients during the second trimester of pregnancy,  $n=32$  patients during the third trimester of pregnancy and  $n=27$  patients during the post-partum period (Figure 1). Exclusion criteria were competing diagnoses such as active cancerous disease or acute inflammatory diseases.

### *Clinical parameters*

Neurological examination was performed by attending consultant Neurologists. Demographic, disease-specific data and information about immunomodulating therapies were collected from all patients [Table 1(a)]. Disability was assessed using the Expanded Disability Status Score (EDSS). Recent relapse was defined as relapse within the last 8 weeks. Patients 'in remission' were clinically stable without recent exacerbations. Clinical disease activity post-partum was

routinely assessed under the umbrella of the DMSKW in telephone interviews.<sup>18</sup>

### *Blood collection and PBMC isolation*

Serum and EDTA (ethylenediaminetetraacetic acid) whole-blood were collected before intravenous administration of medications. Serum was used for the analysis of prolactin concentration. EDTA blood was used for flow cytometric analysis of peripheral blood mononuclear cells (PBMCs).<sup>20</sup> PBMCs were isolated with density gradient centrifugation using ROTI<sup>®</sup>Sep 1077 human lymphocyte separation medium.

### *Flow cytometry*

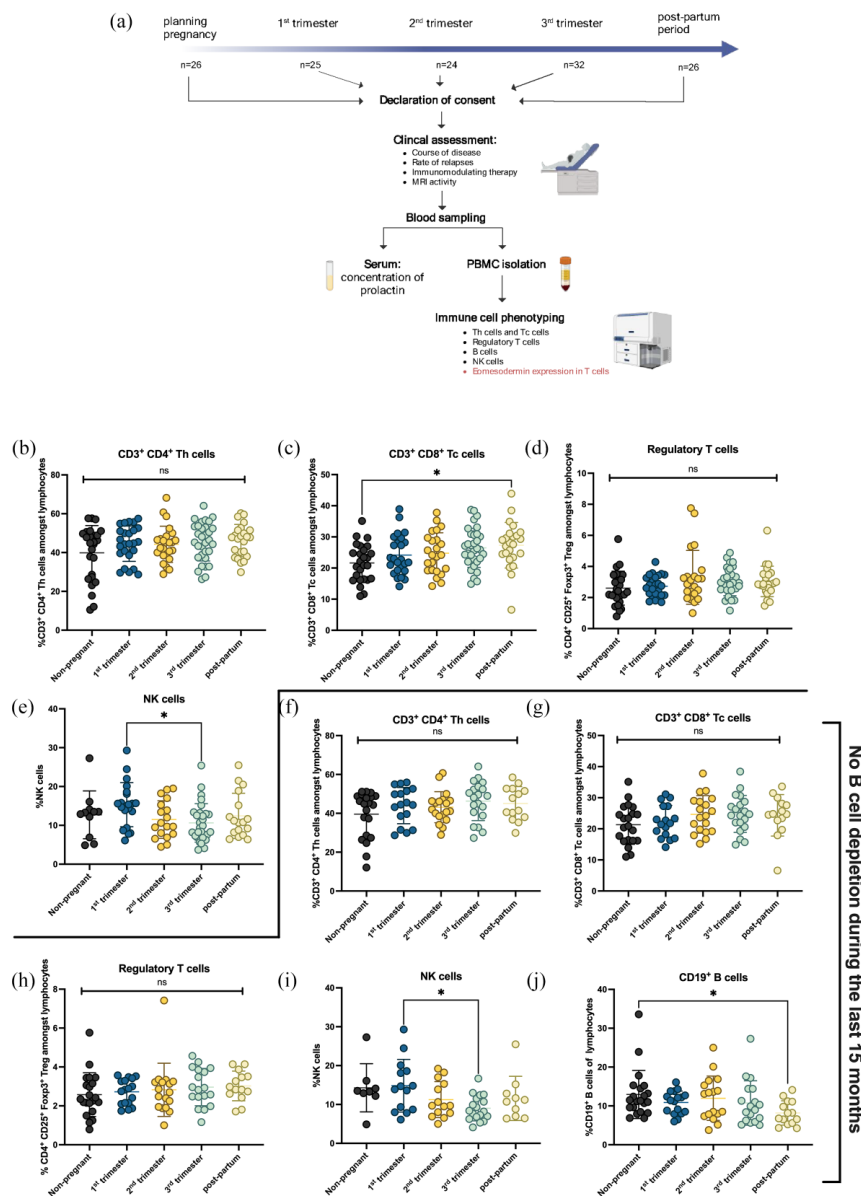
PBMCs were incubated with fluorochrome-conjugated antibodies against surface antigens (eBioscience<sup>™</sup> and BioLegend<sup>®</sup>, Supplemental Table S1). For intracellular staining we permeabilized and fixed cells using eBioscience<sup>™</sup> Foxp3/Transcription Factor Staining Buffer Set according to manufacture's instructions. Flow cytometry data were obtained using a FACSCanto<sup>™</sup> II (BD Biosciences, Franklin Lakes, NJ, USA) (gating: Supplemental Figure S2). Cells were analysed directly after staining. Data analysis was performed with FlowJo v.10.7.2 (FlowJo, Becton, Dickinson and Company, Ashland, OR, USA).

### *Prolactin concentration*

Concentration of prolactin in serum was measured using electrochemiluminescence immunoassay (Roche, Elecsys Prolactin II; range 4.79–23.3 ng/mL).

### *Statistical analysis*

Data were analysed using Prism software V.9 (GraphPad Software, San Diego, CA, USA). Normality tests were applied for all data sets using Kolmogorov-Smirnov test. Comparison of two groups with non-parametric data was performed with Mann-Whitney *U* test. More than two non-parametric data sets were compared using Kruskal-Wallis test with Dunn's multiple comparison. Correlation was measured with non-parametric Spearman correlation. Data are presented as mean values with standard deviation (SD). Correlations are depicted with linear regressions. A *p*-value <0.05 was considered as significant.



**Figure 1.** Immune cell subsets during pregnancy in MS. (a) RRMS patients were included at different time points before, during or after pregnancy. After written informed consent, demographic clinical data were collected. Blood was drawn in order to analyse immune cell alterations *via* flow cytometry and concentration of prolactin in serum. Patients were clinically assessed during the course of pregnancy and post-partum. (b) CD4<sup>+</sup> Th cells did not differ in pregnant women while (c) CD8<sup>+</sup> Tc cells were elevated during post-partum period compared with non-pregnant RRMS patients. (d) Proportions of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T-cells amongst lymphocytes did not differ. (b–d) Data are derived from pregnant RRMS patients (first trimester: n = 25, second trimester: n = 24, third trimester: n = 32), RRMS patients during post-partum period (n = 24) and RRMS patients with childbearing preferences (n = 26). (e) Patients during first trimester expressed higher levels of NK cells than patients during third trimester (first trimester: n = 21, second trimester: n = 18, third trimester: n = 29, post-partum period: n = 17, non-pregnant RRMS patients: n = 11). (f–j) Immune cell subsets were shown for patients who had not received B-cell depleting therapy during the last 15 months [first trimester (n = 17), second trimester (n = 17), third trimester (n = 18), post-partum period (n = 14), non-pregnant RRMS patients (n = 21)]. (j) B-cells were reduced during post-partum period compared to non-pregnant RRMS patients (p < 0.05). Shown is a mean ± SD for each patient group. Data were analysed using non-parametric Kruskal-Wallis test with *post hoc* Dunn's multiple comparison test. \*p < 0.05.

Source: (a) Created with BioRender.com.

MS, multiple sclerosis; RRMS, relapsing-remitting MS.

**Table 1a.** Demographic and clinical characteristics of recruited pregnant and non-pregnant RRMS patients.

Parameter	Non-pregnant	First trimester	Second trimester	Third trimester	Post-partum
Number	26	25	24	32	27
Age $\pm$ SD (range)	30.7 $\pm$ 5.1 (18–42)	32.4 $\pm$ 4.3 (25–42)	31.9 $\pm$ 3.6 (26–41)	31.9 $\pm$ 4.2 (26–42)	30.7 $\pm$ 4.5 (20–42)
Age median (IQR)	31.0 (27.75–33.5)	33.0 (29.0–35.5)	32.0 (30.25–34.0)	31.0 (29.0–35.0)	30.5 (28.0–33.25)
EDSS $\pm$ SD (range)	2.0 $\pm$ 1.1 (0–6)	2.2 $\pm$ 1.2 (1–6)	2.0 $\pm$ 0.8 (0–3)	2.0 $\pm$ 0.8 (0–3.5)	2.2 $\pm$ 0.8 (0–4)
EDSS median (IQR)	2.0 (1.38–2.13)	2.0 (1.25–2.5)	2.0 (1.5–2.5)	2.0 (1.0–2.5)	2.0 (2.0–2.5)
Age onset $\pm$ SD (range)	26 $\pm$ 5.1 (15–35)	24.9 $\pm$ 5.2 (15–34)	22.8 $\pm$ 4.7 (14–31)	24.1 $\pm$ 5.5 (15–38)	23.3 $\pm$ 5.4 (13–36)
Disease duration $\pm$ SD (range)	4.6 $\pm$ 4.4 (0–15)	7.0 $\pm$ 4.7 (0–18)	8.5 $\pm$ 5.0 (0–20)	7.2 $\pm$ 5.6 (0–21)	6.7 $\pm$ 4.0 (0–14)
Disease activity					
Remission (%)	16 (62%)	19 (76%)	18 (75%)	31 (97%)	25 (93%)
Recent relapse (%)	10 (38%)	6 (24%)	6 (25%)	1 (3%)	2 (7%)
Relapse after pregnancy	–	2 (8%)	2 (8%)	2 (6%)	5 (19%)

Age, disease duration, age onset (in years) and EDSS are presented as mean. Recent relapse is defined as an episode that has occurred no longer than 8 weeks ago. EDSS, Expanded Disability Status Score; IQR, interquartile range; RRMS, relapsing-remitting MS; SD, standard deviation; SEM, standard error of mean.

**Table 1b.** Overview of therapies according to different pregnancy periods including all follow ups.

Disease modifying therapy	Non-pregnant	First trimester	Second trimester	Third trimester	Post-partum
No therapy (%)	8 (31%)	3 (12%)	2 (8%)	4 (13%)	3 (11%)
Therapy before pregnancy (%)	–	14 (56%)	11 (46%)	20 (63%)	17 (63%)
Ocrelizumab (%)	–	7 (28%)	5 (21%)	11 (34%)	8 (29%)
Teriflunomide (%)	–	2 (8%)	–	1 (3%)	–
GA (%)	–	1 (4%)	–	2 (6%)	–
DMF (%)	–	2 (8%)	1 (4%)	3 (9%)	3 (11%)
Fingolimod (%)	–	1 (4%)	3 (12%)	–	1 (4%)
IFN- $\beta$ 1a (%)	–	–	1 (4%)	1 (3%)	1 (4%)
Cladribine (%)	–	1 (4%)	1 (4%)	2 (6%)	4 (15%)
Current therapy (%)	18 (69%)	8 (32%)	11 (46%)	8 (25%)	7 (26%)
Natalizumab (%)	1 (4%)	2 (8%)	5 (21%)	2 (6%)	1 (4%)
Ocrelizumab (%)	5 (19%)	–	–	1 (3%)	1 (4%)
Ofatumumab (%)	–	–	–	–	1 (4%)
IFN- $\beta$ 1a (%)	1 (4%)	2 (8%)	3 (12%)	2 (6%)	2 (8%)
DMF (%)	4 (15%)	1 (4%)	–	–	–
GA (%)	3 (11%)	–	–	2 (6%)	1 (4%)
Azathioprine (%)	–	–	–	1 (3%)	1 (4%)
Alemtuzumab (%)	1 (4%)	–	–	–	–
Cladribine (%)	3 (11%)	3 (12%)	3 (12%)	–	–

DMF, dimethyl fumarate; GA, glatiramer acetate; IFN- $\beta$ 1a, interferon-beta-1a.

## Results

### Study population

The study included  $n=134$  samples of which 59 patients were recruited at different time points during pregnancy. 29.6% ( $n=8$ ) of post-partum participants were breast feeding at sampling. Non-pregnant RRMS patients, used as controls, matched regarding age (mean  $\pm$  SD:  $30.7 \pm 5.1$ ) and EDSS ( $2.0 \pm 1.1$ ) [Table 1(a)]. Patients received different disease modifying therapies (DMTs) before or during pregnancy. Most patients (59%) paused DMT intake before pregnancy. Ten percent of the patients did not receive DMTs before pregnancy. Six patients (10%) had received a cycle of cladribine at least 6 months prior to pregnancy. Thirty-one percent were treated with DMTs during pregnancy (Supplemental Table S2). Therapies applied during pregnancy include glatiramer acetate ( $n=2$ , 2%), azathioprine ( $n=1$ , 1%), natalizumab ( $n=9$ , 11%), interferon-beta ( $n=7$ , 9%), dimethyl fumarate ( $n=1$ , 1%) and ocrelizumab ( $n=1$ , 1%) [Table 1(b)].

### Immune cell alterations during pregnancy

We analysed different immune cell subsets before, during and after pregnancy (Figure 1).  $CD3^+CD4^+$  T-helper cells did not differ between non-pregnant RRMS patients, during different trimesters of pregnancy and during the post-partum period, respectively [Figure 1(b)].  $CD3^+CD8^+$  cytotoxic T-cells were elevated during the post-partum period ( $p=0.02$ ) compared to non-pregnant RRMS [Figure 1(c)]. Frequencies of regulatory T-cells did not change during or after pregnancy [Figure 1(d)]. We analysed B-cells in a proportion of patients who had not received B-cell depleting therapies so far. Interestingly, B-cell frequencies declined after pregnancy compared to non-pregnant RRMS [ $p<0.05$ ; Figure 1(j)]. Tc cells did not change in patients without B-cell depleting therapies in the last 15 months [Figure 1(g)]. NK cells were elevated during first trimester ( $p=0.02$ ) compared to the third trimester [Figure 1(i)]. Since most patients were untreated during pregnancy, there were only minor treatment-related effects on immune cells subsets detectable. Frequencies of B-cells were significantly higher in natalizumab treated patients compared to patients without therapy, while frequencies of  $CD4^+$  T-cells were lower in natalizumab treated patients compared to interferon  $\beta$ 1a treated patients (Supplemental Figure S3).

### Eomes expressing Th cells

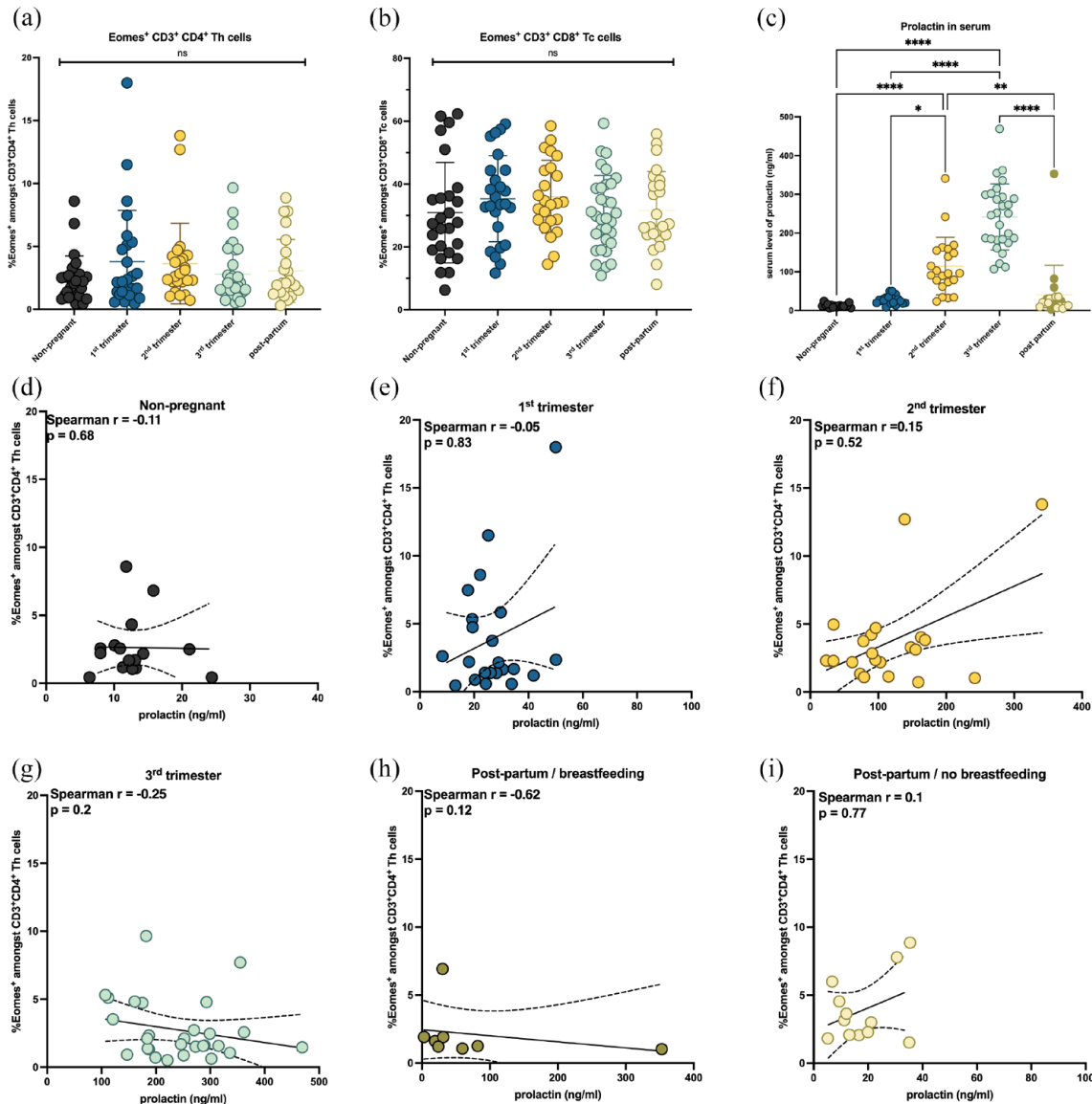
Analysis of Eomes<sup>+</sup> Th cells showed no differences between non-pregnant RRMS, pregnant RRMS or post-partum RRMS patients [non-pregnant RRMS:  $2.4 \pm 1.9$  mean  $\pm$  SD; first trimester:  $3.8 \pm 4.1$ ; second trimester:  $3.6 \pm 3.2$ ; third trimester:  $2.8 \pm 2.2$ ; post-partum:  $3.1 \pm 2.5$ ; Figure 2(a)]. Since  $CD3^+CD8^+$  cytotoxic T-cells were elevated in post-partum RRMS compared to non-pregnant RRMS patients we analysed the expression of Eomes expressing  $CD3^+CD8^+$  cells. Eomes<sup>+</sup> Tc cells did not differ between respective groups (non-pregnant RRMS:  $30.9 \pm 16.0$ ; first trimester:  $35.3 \pm 13.7$ ; second trimester:  $35.9 \pm 11.6$ ; third trimester:  $30.8 \pm 12.0$ ; post-partum:  $31.6 \pm 12.4$ ). The majority of patients did not receive therapies during pregnancy. However, immunomodulating therapies applied during pregnancy did not influence the frequency of Eomes<sup>+</sup> Th cells in contrast to patients with halted DMTs during pregnancy (Supplemental Figure S3).

### No association of prolactin levels and Eomes<sup>+</sup> Th cells

In order to analyse an effect of prolactin release during pregnancy on immune cell alterations, we investigated whether there was an association of prolactin and Eomes<sup>+</sup> Th cells. As expected, the concentration of prolactin in serum rose continuously during the course of pregnancy and dropped post-partum [ $p<0.0001$ ; Figure 2(c)]. There was no evident correlation of prolactin release and expression of Eomes<sup>+</sup> Th cells in non-pregnant patients and during the different trimesters of pregnancy as well as in post-partum patients [Figure 2(d)–(i)]. Breastfeeding with consequently high concentrations of prolactin showed no influence on the expression of Eomes in Th cells [Figure 2(h)]. The expression of Eomes<sup>+</sup> Tc cells was not associated with the levels of prolactin (Supplemental Figure S4).

### Eomes<sup>+</sup> Th cells correlate with reduction of regulatory T-cells during second and third trimester

We then set out to investigate how Eomes<sup>+</sup> Th cells and regulatory T-cells (Tregs) interact during pregnancy. In non-pregnant RRMS patients there was no association of Eomes<sup>+</sup> Th cells and Tregs [Figure 3(a)]. This held also true during the first trimester of pregnancy ( $r=-0.08$ ;  $p=0.71$ ). There was, however, a negative



**Figure 2.** Expression of Eomesodermin (Eomes) in Th cells and correlation with prolactin in serum.

Frequencies of (a) Eomes<sup>+</sup> Th cells amongst CD3<sup>+</sup>CD4<sup>+</sup> Th cells and (b) Eomes<sup>+</sup> Tc cells amongst CD3<sup>+</sup>CD8<sup>+</sup> Tc cells did not differ. (c) Prolactin in serum rose continuously during the course of pregnancy and decreased after delivery. Breastfeeding patients are marked in green colour. There was no correlation between prolactin in serum and Eomes<sup>+</sup> Th cells in (d) non-pregnant RRMS patients, (e) during first trimester, (f) second trimester, (g) third trimester, (h) in breastfeeding patients and (i) non-breastfeeding patients. (a, b) Data are derived from pregnant RRMS patients [first trimester:  $n=25$ , second trimester:  $n=24$ , third trimester:  $n=32$ , RRMS patients during post-partum period:  $n=24$  and non-pregnant RRMS patients:  $n=26$ ]. (c–i) Data are derived from pregnant RRMS patients [first trimester:  $n=22$ , second trimester:  $n=28$ , RRMS patients during post-partum period (breastfeeding:  $n=8$ ; no breastfeeding:  $n=12$ ) and non-pregnant RRMS patients ( $n=17$ )]. Shown is (a–c) mean  $\pm$  SD and (d–i) linear regression with 95% confidence interval. Data were analysed using (a–c) non-parametric Kruskal-Wallis test and (d–i) non-parametric Spearman test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ . RRMS, relapsing-remitting MS.

correlation between % of Tregs and the level of Eomes<sup>+</sup> Th cells during both second ( $r = -0.42$ ;  $p < 0.05$ ) and third trimester [ $r = -0.37$ ;  $p < 0.05$ ; Figure 3(c) and (d)]. During the post-partum period, the trend was similar, but missed significance [ $r = -0.42$ ;  $p = 0.07$ ; Figure 3(e)].

#### *Eomes<sup>+</sup> Th cells are associated with higher B-cell frequencies during third trimester*

B-cells are strongly involved in the pathophysiology of MS by providing an inflammatory environment through the activation of T-cells and the production of antibodies and pro-inflammatory cytokines.<sup>21</sup> Since B-cell frequencies were lower in post-partum RRMS patients compared to non-pregnant RRMS patients, we assessed whether there was an association between Eomes<sup>+</sup> Th cells and B-cells. B-cells showed no correlation with Eomes<sup>+</sup> Th cells in non-pregnant RRMS [Figure 3(f)]. There was, however, a positive trend of Eomes<sup>+</sup> Th cells and B-cells during second trimester ( $r = 0.42$ ;  $p = 0.08$ ), which reached significance during third trimester ( $r = 0.54$ ;  $p = 0.02$ ) [Figure 3(h) and (i)]. Since there was a substantial amount of patients having been treated with B-cell depleting therapies prior to pregnancy (29.6%), we assessed whether frequencies of Eomes<sup>+</sup> Th cells might differ depending on prior anti-CD20 therapy. There was a trend of lower Eomes<sup>+</sup> Th cells in anti-CD20 treated patients, which however did not reach significance [Eomes<sup>+</sup> Th cells mean  $\pm$  SD: with B-cell depletion:  $2.8 \pm 2.4$ , no B-cell depletion  $3.7 \pm 3.5$ ,  $p = 0.12$ ; Figure 3(k)].

#### *Eomes<sup>+</sup> Th cells and disease activity during and after pregnancy*

We then set out to analyse whether there might be an association between frequencies of Eomes<sup>+</sup> Th cells and disease activity in order to characterize their role during pregnancy. The number of relapses during the course of the disease before pregnancy did not correlate with the frequencies of Eomes<sup>+</sup> Th cells (number of relapses during the course of the disease before pregnancy: non-pregnant RRMS patients:  $3.8 \pm 3.0$ ,  $p = 0.35$ ; first trimester:  $3.1 \pm 2.6$ ,  $p = 0.69$ ; second trimester:  $3.5 \pm 2.8$ ,  $p = 0.73$ ; third trimester:  $3.3 \pm 2.4$ ,  $p = 0.84$ ; post-partum:  $3.6 \pm 2.2$ ,  $p = 0.15$ ) (Supplemental Figure S5). We then analysed if Eomes<sup>+</sup> Th cells might be associated with relapse activity during or after pregnancy. We documented  $n = 13$  relapses during pregnancy of which

$n = 6$  were in the first trimester (46%),  $n = 6$  were in the second trimester (46%) and  $n = 1$  was in the third trimester (8%) [Table 1(a)].

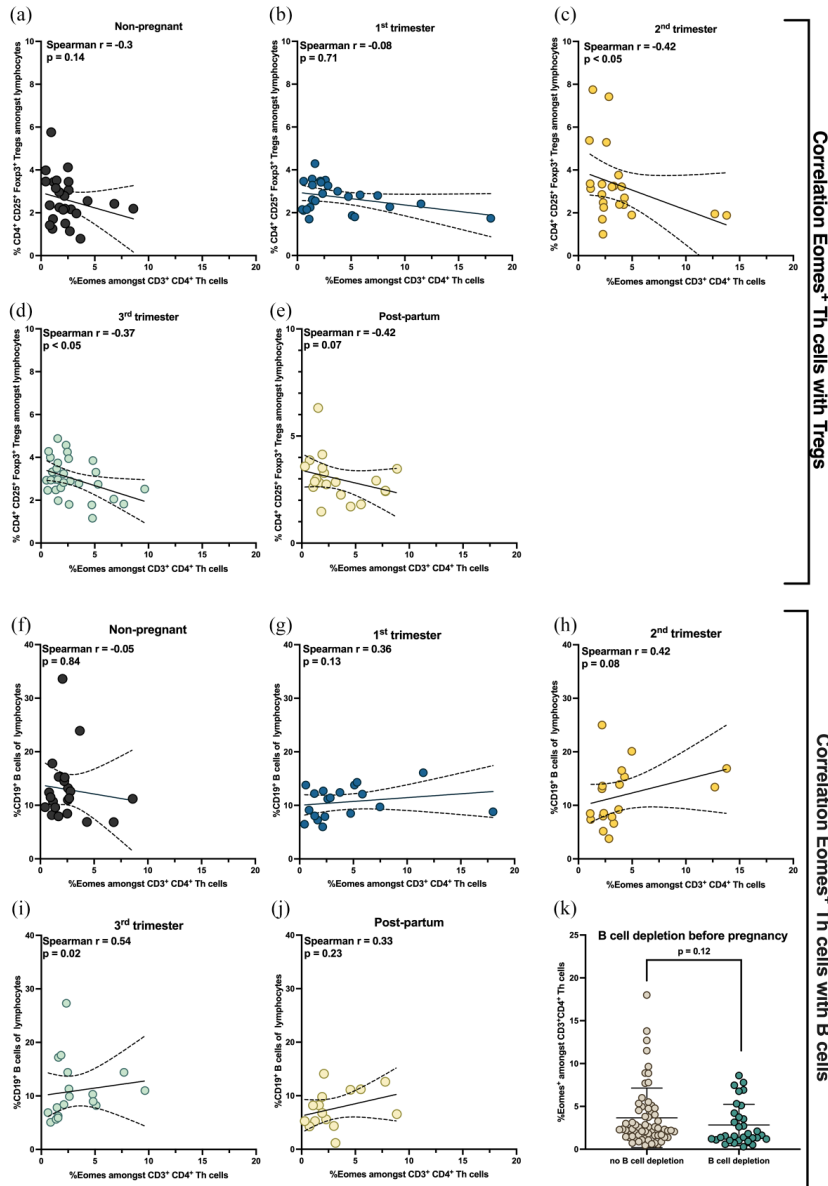
Patients with a relapse during pregnancy did not show higher levels of Eomes<sup>+</sup> Th cells than patients without relapse activity [Eomes<sup>+</sup> Th cells: relapse during pregnancy:  $2.9 \pm 1.9$ ; no relapse during pregnancy:  $3.3 \pm 3.2$ ; Figure 4(a)]. Because of the relation between Eomes<sup>+</sup> Th cells and Treg cells, we analysed changes of Treg cells during relapses as well, which did not differ [Treg cells: relapse during pregnancy:  $2.7 \pm 0.9$ ; no relapse during pregnancy:  $3.0 \pm 1.4$ ; Figure 4(c)]. We documented  $n = 7$  relapses after pregnancy. Patients with a relapse after pregnancy also showed no differences in the expression of Eomes<sup>+</sup> Th cells [relapse after pregnancy:  $4.9 \pm 4.6$ ; no relapse after pregnancy:  $3.2 \pm 3.0$ ; Figure 4(b)] or Treg cells [relapse after pregnancy:  $3.3 \pm 2.1$ ; no relapse after pregnancy:  $2.9 \pm 1.1$ ; Figure 4(d)] in contrast to patients without relapse activity.

The neurologic function of patients was characterized using the EDSS [Table 1(a)]. The EDSS did not correlate with frequencies of Eomes<sup>+</sup> Th cells in non-pregnant RRMS patients or during the course of pregnancy [Figure 4(e)–(h)]. However, we observed a highly significant positive correlation between Eomes<sup>+</sup> Th cells and EDSS in post-partum patients ( $r = 0.51$ ,  $p = 0.009$ ) [Figure 4(i)].

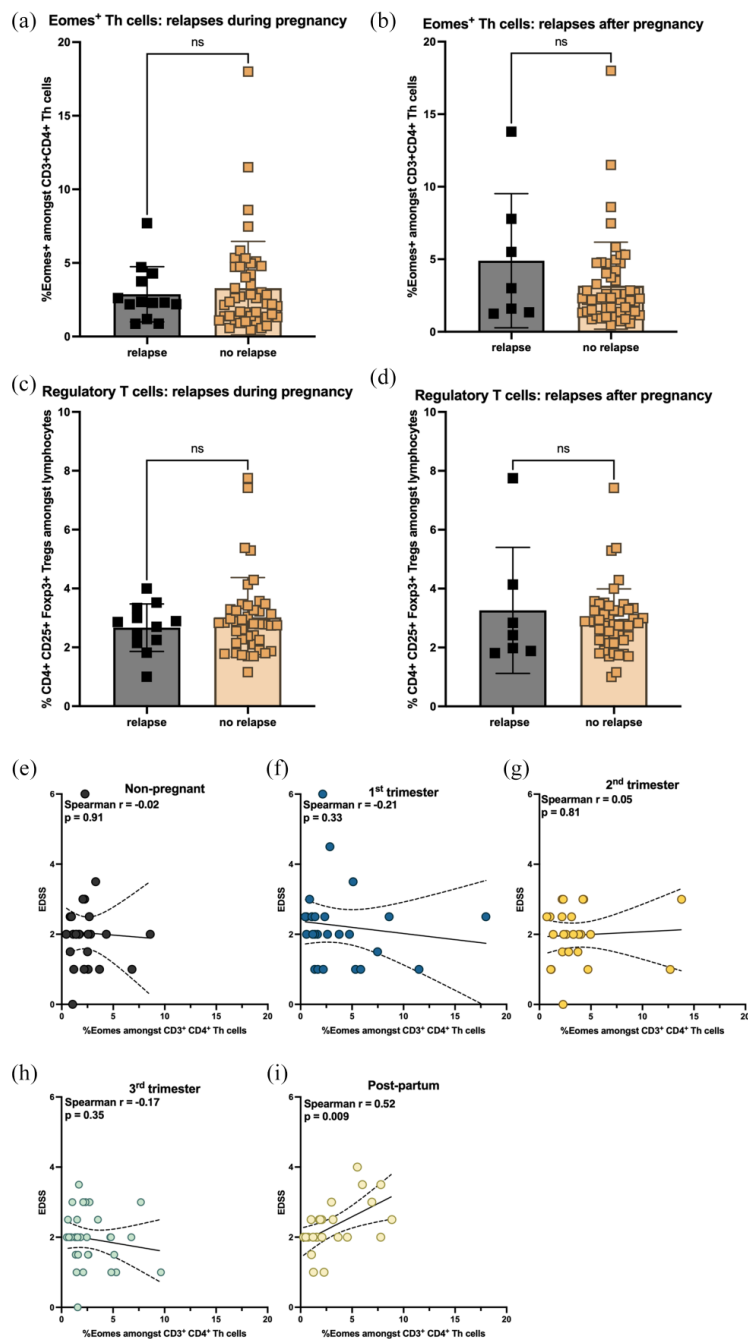
## Discussion

The interactions between MS and pregnancy are driven by complex immunological pathways. Here, we investigated immune cell alteration in pregnant RRMS patients with particular focus on Eomes expressing T-cells. We discovered immune cell alterations especially at the end of pregnancy and post-partum. Cytotoxic T-cells were elevated during third trimester and the post-partum period. Additionally, frequencies of B-cells were lower after pregnancy compared to non-pregnant patients. Mean frequencies of Eomes<sup>+</sup> Th cells and cytotoxic T-cells did not differ between groups. There was, however, a correlation of Eomes<sup>+</sup> Th cells with lower frequencies of regulatory T-cells in the second and third trimester. The effect was also present during the post-partum period but less pronounced.





**Figure 3.** Correlation of Eomes<sup>+</sup> Th cells and regulatory T-cells and B-cells. The frequency of Eomes<sup>+</sup> Th cells and frequencies of regulatory T-cells did neither correlate in (a) non-pregnant RRMS patients nor in (b) pregnant RRMS patients during first trimester and (e) patients during post-partum period. The frequency of Eomes<sup>+</sup> Th cells and regulatory T-cells correlated negatively in pregnant RRMS patients during (c) second trimester ( $r = -0.42$ ;  $p = 0.05$ ) and (d) third trimester ( $r = -0.37$ ;  $p < 0.05$ ). Expression of Eomes in Th cells and frequencies of B-cells did not correlate in (f) non-pregnant RRMS patients, pregnant RRMS patients during (g) first trimester, (h) second trimester and (j) patients during post-partum period. Eomes<sup>+</sup> Th cells and B-cells correlated positively in pregnant RRMS patients during (i) third trimester. (k) Pregnant and post-partum patients with B-cell depleting therapy before pregnancy ( $n = 32$ ) showed a trend towards lower frequencies of Eomes<sup>+</sup> Th cells compared to patients with no B-cell depleting therapy before pregnancy ( $n = 64$ );  $p = 0.12$ . (a–e) Data are derived from pregnant RRMS patients (first trimester:  $n = 25$ , second trimester:  $n = 22$ , third trimester:  $n = 30$ , RRMS patients during post-partum period:  $n = 20$  and RRMS patients with childbearing preferences:  $n = 26$ ). (f–k) Data are derived from pregnant RRMS patients (first trimester:  $n = 19$ , second trimester:  $n = 18$ , third trimester:  $n = 18$ , RRMS patients during post-partum period:  $n = 15$  and RRMS patients with childbearing preferences:  $n = 21$ ). Patients did not receive B-cell depleting therapy within the last 12 months. Shown is (a–j) linear regression with 95% confidence interval and (k) mean  $\pm$  SD. Data were analysed using (a–j) non-parametric Spearman test and (k) non-parametric Mann-Whitney  $U$  test. Eomes, Eomesodermin; RRMS, relapsing-remitting MS.



**Figure 4.** Frequencies of Eomes<sup>+</sup> Th cells correlate with disability post-partum. (a, b) Eomes<sup>+</sup> Th cells and (c, d) regulatory T-cells did neither differ between pregnant patients with current relapse activity and pregnant patients without relapse activity nor between pregnant patients with relapse activity after pregnancy and pregnant patients with stable disease activity. Frequencies of Eomes<sup>+</sup> Th cells and EDSS did neither correlate in (e) non-pregnant RRMS patients nor in pregnant RRMS patients during (f) first trimester, (g) second trimester or (h) third trimester. The frequency of Eomes<sup>+</sup> Th cells and EDSS strongly positively correlated, however, in (i) post-partum patients ( $r=0.52$ ;  $p=0.009$ ). (a, c) Data are derived from  $n=10$  patients with relapse during pregnancy and  $n=41$  patients without relapse during pregnancy. (b, d) Data are derived from  $n=7$  patients with relapse post-partum and  $n=52$  patients without relapse post-partum. (e–i) Data are derived from pregnant RRMS patients [first trimester ( $n=25$ ), second trimester ( $n=24$ ), third trimester ( $n=33$ )], RRMS patients during post-partum period ( $n=24$ ) and RRMS patients with childbearing preferences ( $n=26$ ). (a–d) Shown is a mean  $\pm$  SD for each patient group and (e–i) linear regression with 95% confidence interval. Data were analysed using (a–d) non-parametric Mann-Whitney test and (e–i) non-parametric Spearman test.

It is well established that, in general, pregnancy outcome is not negatively influenced by MS.<sup>6</sup> Pregnancy reduces the risk of relapses with yet higher activity post-partum.<sup>4</sup> This protective effect is, however, not sufficient to protect women with highly active disease who withdraw from DMTs such as natalizumab or fingolimod.<sup>22,23</sup> Of note, modern MS therapy since 2010 has led to a reduction of the post-partum relapse rate from 31% to 14% within 3 months post-partum.<sup>6</sup> This is in line with the data shown in our cohort with 22% of the patients with a relapse post-partum, potentially related to the good disease management in post-partum patients.

During pregnancy, the maternal immune system is reprogrammed to create tolerance towards the embryo. This leads to a shift in the immunological balance of CD4<sup>+</sup> T-cells with an increase of Th2 cells and a decrease of Th1 cells.<sup>24</sup> Th2 cells secrete anti-inflammatory cytokines like interleukin (IL)-4 and IL-10, which support stability of pregnancy, while inflammatory cytokines tumour necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$  and IL-2 released by Th1 cells have a negative effect on the foetus development both in mouse and in human.<sup>25</sup> These immunological changes are considered as a possible explanation for the stabilization of MS during pregnancy.<sup>25</sup> Another influencing factor are changes in the hormonal balance during pregnancy. In fact, the decrease of relapse rate during late pregnancy correlates with the increase of the oestrogens estradiol and estriol.<sup>26</sup> Estriol in combination with glatiramer acetate compared to monotherapy has a protective effect on the relapse rate with a risk reduction of 37% over 24 months.<sup>27</sup> However, the link between immunological changes and hormonal changes remains largely unknown to date.

To understand dynamics and potential associations of prolactin release and immune cell alterations we performed a conclusive immunological analysis at different time points of pregnancy. Tregs were reduced with rising Eomes<sup>+</sup> Th cells. Foxp3 is an important transcription factor of regulatory T-cells and is therefore essential in regulatory pathways of the immune system. We found a strong negative correlation of Eomes<sup>+</sup> Th cells and Tregs during the second and third trimester of pregnancy and a trend post-partum. The negative feedback between induction of Eomes<sup>+</sup> Th cells and Tregs might hence be responsible for the termination of an anti-inflammatory milieu

established during pregnancy to protect the unborn baby. In a murine model of colitis, Eomes<sup>+</sup>CD4<sup>+</sup> T-cells accumulated in old mice and deficiency of Eomes favoured the accumulation of Foxp3<sup>+</sup> cells.<sup>28</sup> Moreover, Eomes-deficient CD4<sup>+</sup> T-cells have a protective effect in accordance with enhanced frequencies of Foxp3<sup>+</sup> cells in EAE, a mouse model of MS.<sup>28</sup> This is in line with the data presented here, showing a suppression of Tregs by Eomes<sup>+</sup> Th cells, most evident during the final phase of pregnancy.

While frequencies of Eomes<sup>+</sup> Th cells were not associated with relapse activity during pregnancy and post-partum, there was a strong correlation between frequencies of Eomes<sup>+</sup> Th cells and disability. This is in line with findings in patients with SPMS having shown that frequencies of Eomes<sup>+</sup> Th cells are responsible for progression and the transition from RRMS to secondary-progressive multiple sclerosis (SPMS).<sup>11</sup> The underlying mechanisms in the CNS driving neurotoxicity and disability progression remain so far elusive. One potential mechanism might be related to progression independent of relapse activity (PIRA) which can be found in 25% following a first demyelinating event as shown in a longitudinal cohort of 1128 patients and which is associated with unfavourable long-term outcome.<sup>29</sup> RMS patients with PIRA exhibit accelerated brain atrophy especially in the cerebral cortex.<sup>30</sup> Of note, the post-mortem analysis in a SPMS patient showed, that Eomes<sup>+</sup> Th cells are present in cortical lesions but most abundantly in frontal and occipital normal appearing white matter.<sup>11</sup> Magnet resonance tomography studies showed that women with MS have higher T2 lesion volume, greater annualized T2 lesion volume increase, lower brain parenchymal fraction and greater annualized brain volume loss post-partum compared to the pre-pregnancy period.<sup>31</sup> It might hence be speculated that Eomes<sup>+</sup> Th cells, characterized by a cytotoxic phenotype, might support the formation of a neurotoxic environment associated with neuronal degeneration and disability progression, also post-partum. The initial hypothesis that pituitary prolactin might be linked to relapse activity could not be supported by the data presented here. This might, however, also be related to the sample size included in this study.

B-cells were lower in post-partum patients compared to non-pregnant patients, yet positively correlated with Eomes<sup>+</sup> Th cells. B-cells are

important for humoral immunity and may elicit both pro-inflammatory and regulatory functions.<sup>32,33</sup> In MS, B-cells have more pro-inflammatory functions including increased release of pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$ , reduced release of anti-inflammatory IL-10,<sup>34</sup> the stimulation of T-cell proliferation<sup>35,36</sup> and the formation of lymphoid follicular structures in the meninges.<sup>37</sup> Since B-cell activation has been linked to disability accumulation in MS, the altered Eomes<sup>+</sup> Th cell–B-cell axis might be a potential contributor to the enhanced risk of disability post-partum.

*Limitations* of the data presented here include the sample size and the missing power calculation prior to conducting the study, since the possible effect size of immunological changes was unclear. Moreover, recruitment of patients was conducted at different time points during pregnancy. Most patients could not be followed longitudinally, mostly related to the fact that patients usually presented only once or twice for a specialized consultation.<sup>18</sup> A strength of the dataset presented here is the inclusion of both a pregnant RRMS cohort and a non-pregnant cohort, conclusive immunophenotyping at different time points and the assessment of standardized clinical and immunological data with high accuracy.

### Conclusion

In summary, we provide substantial evidence of immunological alterations during pregnancy in MS and show that a subgroup of Th cells with cytotoxic properties expressing Eomesodermin are associated with disability post-partum. Eomes<sup>+</sup> Th cells might serve as surrogate for disease activity and could inform about the importance to optimize treatment in MS. Future work should focus on the functional characterization of those immune cell subsets and plasma cytokine levels to better understand pathogenic mechanisms.

### Declarations

#### *Ethics approval and consent to participate*

The study was approved by the local ethics committee of the Faculty of Medicine, Ruhr-University Bochum (Register-number: 20-6827) on 2 March 2020. Patients provided written informed consent.

#### *Consent for publication*

All patients provided written consent for publication.

#### *Author contributions*

**Simon Faissner:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

**Marielena Bongert:** Data curation; Formal analysis; Investigation; Methodology; Writing – original draft.

**Paulina Trendelenburg:** Data curation; Formal analysis; Investigation; Methodology; Writing – original draft.

**Sandra Thiel:** Data curation; Formal analysis; Investigation; Writing – review & editing.

**Takashi Yamamura:** Writing – review & editing.

**Kerstin Hellwig:** Conceptualization; Data curation; Funding acquisition; Investigation; Resources; Supervision; Writing – review & editing.

**Ralf Gold:** Conceptualization; Funding acquisition; Resources; Writing – review & editing.

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#### *Competing interests*

SF has received speaker's and/or scientific board honoraria from AstraZeneca, Biogen, BMS, Celgene, Janssen, Merck, Novartis and Roche; and grant support from Ruhr-University Bochum, DMSG, Stiftung für therapeutische Forschung, Lead Discovery Center GmbH and Novartis. MB and PT have received funding from the FoRUM-program of the Medical Faculty of Ruhr-University Bochum. ST has nothing to disclose.

TY has received speaker honoraria from Biogen, Novartis, Chugai, Takeda, Mitsubishi-Tanabe, Miyarisan and Sumitomo Pharma; serves on the editorial boards of *Therapeutic Advances in Neurological Diseases* and *Clinical and Experimental Neuroimmunology*; receives research support from Novartis and Chiome Bioscience; and receives royalties from EA Pharma. KH has received travel grants from Biogen, Novartis and Merck; and received speaker and research honoraria from Biogen Idec Germany, Teva, Sanofi Genzyme, Novartis, Bayer Health-Care, Merck Serono and Roche. RG serves on scientific advisory boards for Teva Pharmaceutical Industries Ltd., Biogen, Bayer Schering Pharma and Novartis; has received speaker honoraria from Biogen, Teva Pharmaceutical Industries Ltd., Bayer Schering Pharma and Novartis; serves as editor for *Therapeutic Advances in Neurological Diseases* and on the editorial boards of *Experimental Neurology* and the *Journal of Neuroimmunology*; and receives research support from Teva Pharmaceutical Industries Ltd., Biogen Idec, Bayer Schering Pharma, Genzyme, Merck Serono and Novartis. Because RG is Editor-in-Chief of this journal, the peer review process was managed by alternative members of the Board and the submitting Editor was not involved in the decision-making process.

#### Availability of data and materials

All data are available from the corresponding author SF upon reasonable request.

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#### Supplemental material

Supplemental material for this article is available online.

#### References

1. Reich DS, Lucchinetti CF and Calabresi PA. Multiple sclerosis. *N Engl J Med* 2018; 378: 169–180.
2. Orton SM, Herrera BM, Yee IM, *et al.* Sex ratio of multiple sclerosis in Canada: a longitudinal study. *Lancet Neurol* 2006; 5: 932–936.
3. Confavreux C, Aimard G and Devic M. Course and prognosis of multiple sclerosis assessed by the computerized data processing of 349 patients. *Brain* 1980; 103: 281–300.
4. Confavreux C, Hutchinson M, Hours MM, *et al.* Rate of pregnancy-related relapse in multiple sclerosis. Pregnancy in Multiple Sclerosis Group. *N Engl J Med* 1998; 339: 285–291.
5. Krysko KM, Bove R, Dobson R, *et al.* Treatment of women with multiple sclerosis planning pregnancy. *Curr Treat Options Neurol* 2021; 23: 11.
6. Krysko KM, Dobson R, Alroughani R, *et al.* Family planning considerations in people with multiple sclerosis. *Lancet Neurol* 2023; 22: 350–366.
7. Hughes SE, Spelman T, Gray OM, *et al.* Predictors and dynamics of postpartum relapses in women with multiple sclerosis. *Mult Scler* 2014; 20: 739–746.
8. Hellwig K. Pregnancy in multiple sclerosis. *Eur Neurol* 2014; 72(Suppl. 1): 39–42.
9. Koch M, Uyttenboogaart M, Heersema D, *et al.* Parity and secondary progression in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2009; 80: 676–678.
10. Krysko KM, Rutatangwa A, Graves J, *et al.* Association between breastfeeding and postpartum multiple sclerosis relapses: a systematic review and meta-analysis. *JAMA Neurol* 2020; 77: 327–338.
11. Raveney BJE, Sato W, Takewaki D, *et al.* Involvement of cytotoxic Eomes-expressing CD4. *Proc Natl Acad Sci U S A* 2021; 118:e2021818118.
12. Zhang C, Raveney BJE, Hohjoh H, *et al.* Extrapituitary prolactin promotes generation of Eomes-positive helper T cells mediating neuroinflammation. *Proc Natl Acad Sci U S A* 2019; 116: 21131–21139.
13. Brand JM, Frohn C, Cziupka K, *et al.* Prolactin triggers pro-inflammatory immune responses in peripheral immune cells. *Eur Cytokine Netw* 2004; 15: 99–104.
14. Saha S, Gonzalez J, Rosenfeld G, *et al.* Prolactin alters the mechanisms of B cell tolerance induction. *Arthritis Rheum* 2009; 60: 1743–1752.

15. Markianos M, Koutsis G, Evangelopoulos ME, *et al.* Serum and cerebrospinal fluid prolactin levels in male and female patients with clinically-isolated syndrome or relapsing-remitting multiple sclerosis. *J Neuroendocrinol* 2010; 22: 503–508.
16. Correale J, Farez MF and Ysrraelit MC. Role of prolactin in B cell regulation in multiple sclerosis. *J Neuroimmunol* 2014; 269: 76–86.
17. Gregg C, Shikar V, Larsen P, *et al.* White matter plasticity and enhanced remyelination in the maternal CNS. *J Neurosci* 2007; 27: 1812–1823.
18. Thiel S, Ciplea AI, Gold R, *et al.* The German Multiple Sclerosis and Pregnancy Registry: rationale, objective, design, and first results. *Ther Adv Neurol Disord* 2021; 14: 17562864211054956.
19. Thompson AJ, Banwell BL, Barkhof F, *et al.* Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol* 2018; 17: 162–173.
20. Faissner S, Heitmann N, Plaza-Sirvent C, *et al.* Immune response in ofatumumab treated multiple sclerosis patients after SARS-CoV-2 vaccination. *Front Immunol* 2022; 13: 980526.
21. Faissner S, Plemel JR, Gold R, *et al.* Progressive multiple sclerosis: from pathophysiology to therapeutic strategies. *Nat Rev Drug Discov* 2019; 18: 905–922.
22. Alroughani R, Alowayesh MS, Ahmed SF, *et al.* Relapse occurrence in women with multiple sclerosis during pregnancy in the new treatment era. *Neurology* 2018; 90: e840–e846.
23. Hellwig K, Tokic M, Thiel S, *et al.* Multiple sclerosis disease activity and disability following discontinuation of natalizumab for pregnancy. *JAMA Netw Open* 2022; 5: e2144750.
24. Raghupathy R and Kalinka J. Cytokine imbalance in pregnancy complications and its modulation. *Front Biosci* 2008; 13: 985–994.
25. Qiu K, He Q, Chen X, *et al.* Pregnancy-related immune changes and demyelinating diseases of the central nervous system. *Front Neurol* 2019; 10: 1070.
26. Gold SM and Voskuhl RR. Pregnancy and multiple sclerosis: from molecular mechanisms to clinical application. *Semin Immunopathol* 2016; 38: 709–718.
27. Voskuhl RR, Wang H, Wu TC, *et al.* Estriol combined with glatiramer acetate for women with relapsing-remitting multiple sclerosis: a randomised, placebo-controlled, phase 2 trial. *Lancet Neurol* 2016; 15: 35–46.
28. Lupar E, Brack M, Garnier L, *et al.* Eomesodermin expression in CD4<sup>+</sup> T Cells restricts peripheral Foxp3 induction. *J Immunol* 2015; 195: 4742–4752.
29. Tur C, Carbonell-Mirabent P, Cobo-Calvo Á, *et al.* Association of early progression independent of relapse activity with long-term disability after a first demyelinating event in multiple sclerosis. *JAMA Neurol* 2023; 80: 151–160.
30. Cagol A, Schaedelin S, Barakovic M, *et al.* Association of brain atrophy with disease progression independent of relapse activity in patients with relapsing multiple sclerosis. *JAMA Neurol* 2022; 79: 682–692.
31. Uher T, Kubala Havrdova E, Vodehnalova K, *et al.* Pregnancy-induced brain magnetic resonance imaging changes in women with multiple sclerosis. *Eur J Neurol* 2022; 29: 1446–1456.
32. Sebina I and Pepper M. Humoral immune responses to infection: common mechanisms and unique strategies to combat pathogen immune evasion tactics. *Curr Opin Immunol* 2018; 51: 46–54.
33. Hampe CS. B cell in autoimmune diseases. *Scientifica (Cairo)* 2012; 2012: 215308.
34. Häusser-Kinzel S and Weber MS. The role of B cells and antibodies in multiple sclerosis, neuromyelitis optica, and related disorders. *Front Immunol* 2019; 10: 20.
35. Fletcher JM, Lalor SJ, Sweeney CM, *et al.* T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol* 2010; 162: 1–11.
36. Van Langelaar J, Rijvers L, Smolders J, *et al.* B and T cells driving multiple sclerosis: identify, mechanisms and potential triggers. *Front Immunol* 2020; 11: 760.
37. Pikor NB, Prat A, Bar-Or A, *et al.* Meningeal tertiary lymphoid tissues and multiple sclerosis: a gathering place for diverse types of immune cells during CNS autoimmunity. *Front Immunol* 2015; 6: 657.