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Antibodies to β adrenergic and muscarinic cholinergic receptors in patients with Chronic Fatigue Syndrome

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ABSTRACT

Infection-triggered disease onset, chronic immune activation and autonomic dysregulation in CFS point to an autoimmune disease directed against neurotransmitter receptors. Autoantibodies against G-protein coupled receptors were shown to play a pathogenic role in several autoimmune diseases. Here, serum samples from a patient cohort from Berlin ($n = 268$) and from Bergen with pre- and post-treatment samples from 25 patients treated within the KTS-2 rituximab trial were analysed for IgG against human α and β adrenergic, muscarinic (M) 1–5 acetylcholine, dopamine, serotonin, angiotensin, and endothelin receptors by ELISA and compared to a healthy control cohort ($n = 108$). Antibodies against β2, M3 and M4 receptors were significantly elevated in CFS patients compared to controls. In contrast, levels of antibodies against α adrenergic, dopamine, serotonin, angiotensin, and endothelin receptors were not different between patients and controls. A high correlation was found between levels of autoantibodies and elevated IgG_{1-3} subclasses, but not with IgG_4. Further patients with high β2 antibodies had significantly more frequently activated HLA-DR+ T cells and more frequently thyreoperoxidase and anti-nuclear antibodies. In patients receiving rituximab maintenance treatment achieving prolonged B-cell depletion, elevated β2 and M4 receptor autoantibodies significantly declined in clinical responder, but not in non-responder. We provide evidence that 29.5% of patients with CFS had elevated antibodies against one or more M acetylcholine and β adrenergic receptors which are potential biomarkers for response to B-cell depleting therapy. The association of autoantibodies with immune markers suggests that they activate B and T cells expressing β adrenergic and M acetylcholine receptors. Dysregulation of acetylcholine and adrenergic signalling could also explain various clinical symptoms of CFS.

1. Introduction

With an estimated prevalence of 0.2–0.3% Chronic Fatigue Syndrome (CFS) is a frequent and severe chronic disease. Patients suffer from persistent exhaustion, cognitive dysfunctions, pain and flu-like symptoms, leading to a substantial reduction of life quality (Carruthers et al., 2011). A hallmark of CFS is delayed aggravation of symptoms by exertion. There is ample evidence of dysregulation of the autonomic sympathetic and parasympathetic nervous system, including vasomotor and gastrointestinal dysfunction, and increased sensitivity to pain and sensory stimuli (Felten et al., 1985; Freeman and Komaroff, 1997; Gur and Oktayoglu, 2008). In the majority of patients CFS onset is triggered by an infection with EBV or other intracellular pathogens (Faulkner and Smith, 2008). Persistent immune dysregulation with elevated or diminished antibody levels, T cell activation and diminished NK cell

Abbreviations: AChR, acetylcholine receptor; AdR, adrenergic receptors; ANA, anti-nuclear antibodies; CFS, Chronic Fatigue Syndrome; GPCR, G-protein coupled receptor; POTS, Postural Orthostatic Tachycardia Syndrome; RRTI, Recurrent respiratory tract infections; TG, thyreoglobulin; TPO, thyreoperoxidase.

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function were shown in many studies (Brenu et al., 2011; Klimas et al., 1990; Loebel et al., 2015; Ogawa et al., 1998; Skowera et al., 2004). In a recent study we had analysed 468 CFS patients and 25% had decreased serum levels of immunoglobulin mostly of IgG_3 subclass, while elevated immunoglobulin levels with an excess of IgM and IgG_2 in particular were found in another 25% (Loebel et al., 2015) and unpublished data. Further, the presence of autoantibodies was discussed in several small studies (Konstantinov et al., 1996; Onouchi et al., 1999; Tanaka et al., 2003; von Mikecz et al., 1997; Wheatland, 2005; Yamamoto et al., 2012). Autoantibodies against the M1 acetylcholine receptor (AChR) were shown in CFS patients and were associated with reduced binding of a M AChR ligand in brain detected by PET (Tanaka et al., 2003; Yamamoto et al., 2012). Recently, autoantibodies against β1 and β2 adrenergic receptors (AdR) were described in postural orthostatic tachycardia syndrome (POTS) and in patients with orthostatic hypotension (Li et al., 2014, 2015; Yu et al., 2012). This finding is of relevance for CFS, as an estimated subgroup of 10–20% of CFS patients concurrently suffers from POTS (Hoad et al., 2008; Jones et al., 2005; Reynolds et al., 2014). First evidence for a pathogenic role of autoantibodies in CFS comes from two clinical trials with the monoclonal anti-CD20 antibody rituximab (Fluge et al., 2011, 2015). Upon depletion of CD20+ B cells with rituximab approximately 60% of patients experience a partial or complete, and in some patients sustained clinical remission. The delayed onset of response with a median of approximately 5 months in both trials suggests that clinical effects are not mediated by direct depletion of CD20+ B cells, but by the short-lived antibody-producing plasma cells arising from CD20+ memory B cells, followed by subsequent wash-out of autoantibodies.

In the present study we analysed a total of 293 CFS patients for serum antibodies against various G-protein coupled receptors (GPCRs) including adrenergic, M acetylcholine, dopamine, and serotonin receptors and correlated their levels with parameters of immune dysregulation and response to rituximab.

2. Patients and methods

2.1. Human blood samples

Patients were diagnosed at the Charité outpatient clinic for immunodeficiencies at the Institute for Medical Immunology at the Charité Universitätsmedizin Berlin between 2011 and 2014. Diagnosis of CFS was based on Canadian Criteria (Carruthers et al., 2003) and exclusion of other medical or neurological diseases which may cause fatigue. Patients with systemic steroid or immunosuppressant therapy or a diagnosis of primary immunodeficiency were excluded from this study. Controls were recruited from staff and did not suffer from CFS. However, neither clinical nor laboratory assessment was performed for controls. The study was approved by the Ethics Committee of Charité Universitätsmedizin Berlin in accordance with the 1964 Declaration of Helsinki and its later amendments. All patients and healthy controls gave informed consent. Pre- and post-treatment samples from CFS patients treated within the rituximab trial KTS-2 (Clinical Trials: NCT01156909) were provided from the Haukeland university Hospital, Bergen Norway (Fluge et al., 2015).

2.2. Quantification of autoantibodies by ELISA

Serum IgG against GPCRs were determined by ELISA by CellTrend GmbH, Luckenwalde, Germany. Recombinant GPCR proteins were expressed in CHO cells. Pre- and post-treatment samples from the rituximab trial were analysed in the same assay plate, and these samples were analysed blinded to group allocation (CFS patients versus controls, responder versus non-responder, pre- versus post-treatment).

2.3. Laboratory data

Serum C3c and immunoglobulins were determined at the Charité routine diagnostics laboratory, Labor Berlin GmbH by immunological turbidity test (Roche Diagnostics). IgG subclasses were determined by Hitachi COMBI kit (The Binding Site). T cell activation was measured by quantitation of HLA-DR expression on human peripheral blood T cells by flow cytometric analysis (Quantibryte, BD Bioscience). The following mouse anti-human fluorescence-labelled monoclonal antibodies were used for quantification of T cells and subsets: cluster of differentiation (CD)3 Allophycocyanine-Alexa Fluor 750 (APC-A750), CD8 APC, HLA-DR Phycoerythrine (PE), CD45 Krome-Orange (KrO) (all from Beckman Coulter). Stained samples were acquired on a ten-colour Navios flow cytometer and analysed using Navios Software (Beckman Coulter). Thyreoperoxidase/thyreoglobulin and ANA antibodies were measured by immunofluorescence.

2.4. Statistical analysis

Statistical data analyses were done using the software GraphPad Prism 6.0. Nonparametric statistical methods were used. Continuous variables were expressed as median and interquartile range (IQR). Univariate comparisons of two independent groups were done using the Mann–Whitney-*U* test. Contingency analysis was done by Fisher's exact test. Comparisons of two dependent groups were done using the Wilcoxon matched-pairs signed-rank test. Correlation analysis was performed by nonparametric Spearman coefficient *r*. A two-tailed *p*-value of <0.05 was considered statistically significant.

3. Results

Antibodies against various GPCRs were analysed in serum samples from 268 patients with CFS diagnosed at Charité. Detailed information on the patients is given in Table 1. Control group included 108 sex- and age matched healthy individuals from Charité staff. We found significantly higher autoantibody levels against M3 and M4 AChR and β2 AdR in CFS patients compared to controls (Fig. 1A and B). Crossreactivity between all 5 M and β1 and β2 AdR but also between M and β receptor was seen in a substantial portion of CFS patients as shown for M4 in Fig. 1C.

Table 1
CFS patient characteristics.

Variable	CFS (n = 268)	Controls (n = 108)
Age (median ± STD)	40 ± 11	34 ± 9
m/f (%)	39/61	44/56
Bell score	40 ± 10	n.a.
RRTI (%)	44.6	n.a.
Dizziness (%)	35.7	n.a.
Fukuda – minor criteria (%)		n.a.
Post-exertional malaise lasting more than 24 h	100	
Unrefreshing sleep	100	
Multi-joint pain without swelling or redness	64	
Muscle pain	82	
Headaches of a new type	65	
Substantial impairment in short-term memory or concentration	82	
Tender lymph nodes	38	
Sore throat	35	

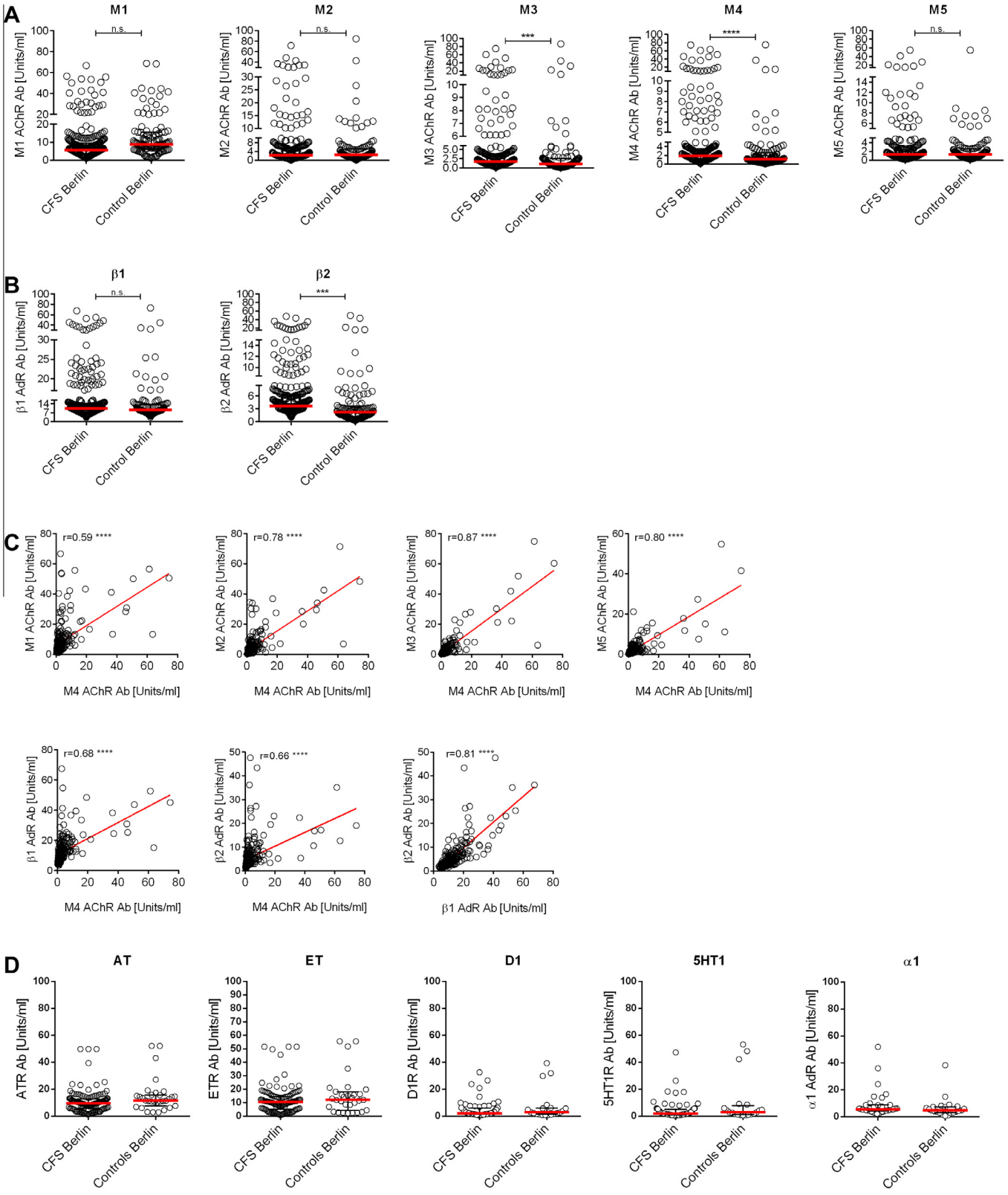

Fig. 1. Autoantibodies against GPCRs in Berlin patients with CFS ($n = 268$) and Berlin cohort of healthy controls ($n = 108$) determined by ELISA for (A) M1–5 muscarinic AChR, and (B) β1 and β2 AdR. (C) Correlation of M4 AChR antibodies with M1, M2, M3, and M5 AChR and β1 and β2 AdR antibodies, and correlation of β1 and β2 AdR antibodies in CFS patients. (D) Antibodies against endothelin (ET), angiotensin (AT), dopamine D1, serotonin 5HT1A and α1 adrenergic receptor in a subgroup of CFS patients and controls of the Berlin cohort. Statistic analysis was performed by Mann–Whitney *U* test and Spearman correlation with a two-tailed *p*-value of $^{***}p < 0.001$ and $^{****}p < 0.000$.

The highest correlation was found between M3 and M4 receptor antibodies ($r = 0.87$), the lowest for M1 and M4 receptor antibodies ($r = 0.59$). Analysis of patient and control samples for antibodies against α adrenergic, dopamine receptors D1, D2S, D3, D4.2, D4.4 and D4.7 and serotonin receptors 5HT1A, 5HT2A, 5HT2B, 5HT2C, 5HT5A, 5HT6, 5HT7 as well as the endothelin (ET) and angiotensin

Table 2
(A) Comparison of patients with and without elevated β AdR and M AChR antibodies (above the 90th percentile of the control group) for immunoglobulin levels shown as *p*-values ($n = 268$, Berlin cohort). (B) Correlation of β AdR and M AChR antibody levels with immunoglobulin levels shown as *r*-values ($n = 268$, Berlin cohort).

n with elevated antibodies	β1 ($n = 47$)	β2 ($n = 41$)	M1 ($n = 16$)	M2 ($n = 27$)	M3 ($n = 35$)	M4 ($n = 48$)	M5 ($n = 28$)
(A)							
IgM	0.0508	0.1409	0.2196	0.2302	0.6022	0.3729	0.1456
IgG	0.0001	0.0002	0.0008	0.0042	0.001	0.023	0.0065
IgG_1	0.0019	0.0098	0.0028	0.0049	0.0123	0.0590	0.0250
IgG_2	0.037	0.0323	0.1377	0.1606	0.0039	0.0162	0.1026
IgG_3	0.0002	0.0319	0.0086	0.0179	0.1846	0.0504	0.1944
IgG_4	0.4096	0.8775	0.9147	0.8953	0.2317	0.4589	0.6858
	β1	β2	M1	M2	M3	M4	M5
(B)							
IgM	0.046	0.081	0.068	0.070	0.108	0.086	0.097
IgG	0.311 (***)	0.277 (***)	0.328 (***)	0.283 (***)	0.261 (***)	0.262 (***)	0.254 (***)
IgG_1	0.239 (***)	0.167 (**)	0.267 (***)	0.228 (***)	0.188 (**)	0.209 (***)	0.199 (**)
IgG_2	0.249 (***)	0.246 (***)	0.207 (***)	0.203 (**)	0.216 (***)	0.225 (***)	0.201 (**)
IgG_3	0.316 (***)	0.304 (***)	0.318 (***)	0.219 (***)	0.178 (**)	0.173 (**)	0.160 (*)
IgG_4	0.055	−0.022	0.028	0.041	−0.010	0.007	0.017

* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

receptor (AT) did not reveal immunoreactivity neither in patient nor control cohort. Fig. 1D shows data for AT, ET, D1, 5HT1A, and α1 AdR.

We observed diminished serum levels of at least one immunoglobulin class or subclass in a total of 27.1% of CFS patients according to the reference range of the Charité diagnostic laboratory, and elevated serum levels in 20.9%, respectively. The most prominent finding was a single or concomitant IgG_3 (10.1%) deficiency in CFS patients. Activated HLA-DR+ CD8+ T cells were present in 14.3% of CFS patients. We therefore correlated levels of M and β receptor antibodies with levels of IgM, IgG and IgG subclasses. We observed a remarkably strong correlation of higher autoantibodies for all M and β subtypes with higher IgG_{1-3} subclasses, but not IgG_4 (*r*-values shown in Table 2). No association of M and β autoantibodies with diminished immunoglobulins was found. For comparative analysis we defined elevated β and M receptor antibodies as above the 90th percentile of the control group. The association of immunoglobulin levels with autoantibodies is shown in Table 2 and illustrated for β2 AdR antibodies in Fig. 2.

Based on this definition, we performed a comparative analysis with T cell activation, diminished levels of complement C3c, and elevated thyreoperoxidase (TPO)/thyreoglobulin (TG) and anti-nuclear antibodies (ANA). Patients with high β2 antibodies had significantly more frequently activated HLA-DR+ CD8+ T cells. There was also an association between M1 and β2 antibodies and elevated TPO/TG and ANA antibodies (Table 3). Both β1 and β2 ($r = 0.81$; Fig. 1C) and M1 and β2 ($r = 0.71$; not shown) showed a strong correlation.

We further correlated elevated receptor autoantibodies with various clinical symptoms including disease severity, muscle pain, susceptibility to infection and infection triggered disease onset. The only association we observed was of M1 AChR antibodies with dizziness ($p = 0.05$). Data on POTS was available in 8 patients only. Of these 4 patients fulfilled the criteria for POTS and 4 did not. Elevated β AdR antibodies were present only in two of the latter.

In the cohort of 25 CFS patients treated within the rituximab trial KTS2 at the Haukeland University Hospital, autoantibodies against receptors β1, β2, M1 and M4 were analysed in pre- and post-treatment samples. Patient characteristics for Bergen patients are reported elsewhere (Fluge et al., 2015). 15 patients had clinically significant response and 10 patients were non-responder to B-cell depletion therapy. In the KTS-2 study, Rituximab infusions were pre-planned to be given at 0 and 2 weeks, then maintenance at 3, 6, 10 and 15 months. According to the protocol, if there were no signs of response at the 10-months clinical visit, rituximab infusions at 10 and 15 months could be omitted. Also, according to an approved amendment for the study, patients in slow and gradual improvement after 12-months follow-up could receive further rituximab infusions with prolonged B-cell depletion period. Among 10 non-responder, three patients received four infusions (last at 6-months), five patients received five infusions (last at 10-months), one patient received six infusions (last at 15-months), and one patient with some improvement yet not a significant clinical response received in total eight infusions (last at 22 months). Among 15 clinical responder, 10 major responder received the pre-planned six rituximab infusions, one major responder had five infusions, and four moderate responder received additional infusions (Fluge et al., 2015).

Elevated antibody levels were detected in pre-treatment samples against β1, β2, M1 and M4 receptors in a subset of patients (Fig. 3). Remarkably, elevated autoantibody levels had normalised in the majority of clinical responder post-treatment. In responder post-treatment levels for all four antibodies were significantly lower compared to pre-treatment. In 10 responding patients post-treatment samples analysed were from 24 months after initiation of treatment and 9 months after the last rituximab infusion, when B cells had partially normalised. Post-treatment samples from the 10 non-responder were obtained at months 6–24. For statistical analysis, two samples harvested at 6 months follow-up were omitted, thus leaving serum samples harvested between months 10 and 24, from 8 out of these 10 non-responder to be included and compared to pre-treatment. In the samples from non-responder there were significantly lower levels for β1 AdR antibodies but no obvious difference between β2, M1 and M4 levels pre- and post-treatment. Total IgG, IgA and IgM levels post-treatment were somewhat lower (mean 1.04, 0.28, and 0.27 g/l lower, respectively) as compared to pre-treatment levels, but the reductions were not significantly different between responder and non-responder (manuscript submitted).

4. Discussion

We found significantly elevated levels of antibodies against β2 AdR, M3 AChR and M4 AChR in a subset of patients with CFS. Some controls had elevated antibodies as well. In contrast, no difference between CFS patients and controls were found in antibody levels neither against α AdR described in hypertension (Dandel et al., 2013; Wallukat and Schimke, 2014) dopamine receptors found in schizophrenia and movement disorders (Balint and Bhatia, 2013; Dale et al., 2012; Steiner et al., 2015), serotonin receptors found in antiphospholipid syndrome (Frauenknecht et al., 2013), nor

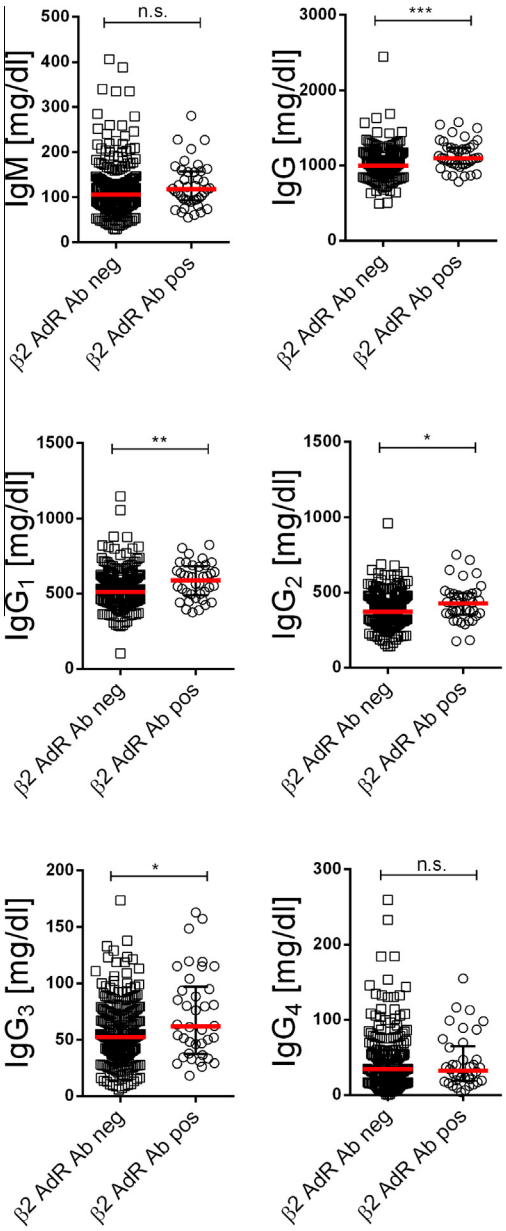

Fig. 2. Immunoglobulin levels in CFS patients with and without elevated β2 AdR antibodies. 268 CFS patients of the Berlin cohort were grouped as either antibody positive or negative according to the 90th percentile of the healthy control group and compared for IgM, IgG and IgG subclass levels. Statistics analysis of two independent groups was performed by nonparametric Mann–Whitney *U* test with a two-tailed *p*-value of $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.01$ and n.s. – not significant.

against endothelin or angiotensin receptor as in systemic sclerosis (Gunther et al., 2014), and thromboangiitis obliterans (Klein-Weigel et al., 2014). A previous study from Japan reported serum autoantibodies against the M1 AChR in 31 of 60 CFS patients (Tanaka et al., 2003). In a more recent PET study, evidence of M AChR autoantibody binding in the brain was provided by showing that antibody-positive patients had reduced binding of a labelled M AChR ligand as compared to antibody-negative CFS patients and healthy controls (Yamamoto et al., 2012).

Autoantibodies against β AdR and M AChR have already been described for more than two decades in cardiovascular and more recently in various autoimmune and neurological diseases (Wallukat and Schimke, 2014). β1 AdR and M2 AChR antibodies were first described in patients with protozoa-induced Chagas heart disease (Davila et al., 2008; Labovsky et al., 2007; Ribeiro et al., 2010; Thiers et al., 2012). β AdR autoantibodies were further shown in patients with dilated cardiomyopathy, orthostatic hypotension, and POTS (Dandel et al., 2012; Li et al., 2014, 2015; Wallukat and Schimke, 2014; Yu et al., 2012). Antibodies against M AChR were described in autoimmune diseases including M1 in Lambert Eaton (Suzuki, 2010), myasthenia gravis (Takamori et al., 2007) and schizophrenia (Jones et al., 2014), and M1 and M3 in Sjögren's Syndrome (Schegg et al., 2008; Sumida et al., 2013), and β1 and M2 in Graves' disease (Galloway et al., 2014; Stavrakis et al., 2009). These diseases share similarities with CFS including fatigue and autonomic dysfunction. A study in schizophrenia showed binding of M1 antibody positive patients IgG in brain tissue especially in hippocampus, but not in M1 negative patients (Jones et al., 2014). M1 AChR knockout mice have severe deficits in learning and memory (Gould et al., 2015). Similar to our findings, in most other diseases autoantibodies are found in only a subset of patients with a wide overlap of levels in patients and controls.

Various studies analysed function and binding of antibodies against β AdR and M AChR. In fact most studies on β AdR antibodies in cardiovascular disease used a cardiomyocyte contraction assay (Wallukat and Schimke, 2014). Autoantibodies against β1 AdR in cardiomyopathy were shown to affect ligand binding and cardiomyocyte function similar to agonists (Herda et al., 2012). However, the impact of β1 AdR antibodies on receptor function is quite complex as antibody binding to different epitopes and heterogeneous effects on receptor traffic and activity including antagonist effects were observed (Schulze et al., 2005; Turki and Liggett, 1995). In addition, non-functional antibodies were described mostly in healthy individuals (Bornholz et al., 2014). Further studies suggest that conformational epitopes are recognised by β1 AdR autoantibodies, requiring a three-dimensional receptor structure to detect them, which is often not preserved when recombinant protein or peptide-based assays were used (Bornholz et al., 2013). Assays using living cells are, however, difficult to standardise and not well suited for clinical diagnostics. In the present study we used recombinant receptors as antigens which are stabilized to maintain their conformation.

Further we observed more than half of the patients having antibodies against more than one receptor of the respective family. This is not surprising as there is a 40–70% sequence homology within the five M AChR and within the two β AdR and an approximately 30% overlap of β AdR and M4 AChR. Furthermore, the presence of both M AChR and β AdR autoantibodies is reported in other diseases as well (Galloway et al., 2014; Pei et al., 2012; Stavrakis et al., 2009; Talvani et al., 2006; Zhao et al., 2006). Our correlation studies suggest that the prime targets of antibody responses in CFS are β2 and possibly M4 receptors. This, of course, does not exclude other autoantibodies (not investigated in the present study) with functional activity in CFS.

What are potential mechanisms leading to induction of β AdR and M AChR antibodies in CFS patients? There is evidence that a subset of patients experienced major distressing life events before CFS onset and that CFS is frequently triggered by an infection. Thus it is tempting to speculate that chronic adrenergic stimulation may lead to conformational changes of receptors resulting in more immunogenic epitopes and that infection-triggered immune activation induces the autoantibody response. It is also conceivable that low level non-pathogenic β AdR and M AChR antibodies exerting regulatory functions are already present in healthy subjects.

We do not know yet, what the significance and functional relevance of β AdR and M AChR specific antibodies in CFS is. To get some evidence we correlated autoantibody levels with disease symptoms and other immunological alterations found in CFS. Remarkably, we observed a significant correlation of levels of

Table 3
Contingency analysis by Fishers' exact test (*p*-values) for elevated β AdR and M AChR antibodies (above the 90th percentile of the control group) and T cell activation (defined as HLA-DR high CD8+ T cells >30%), or diminished C3c levels (<900 mg/l), or elevated TPO (>35 kU/l)/TG (>100 kU/l) antibodies, or elevated ANA titres (>1:160).

n with elevated markers	*n* with elevated antibodies						
	β1 (47)	β2 (41)	M1 (16)	M2 (27)	M3 (35)	M4 (48)	M5 (28)
HLA-DR high CD8 (*n* = 37/259)	0.64 (*n* = 8)	0.05 (*n* = 10)	0.70 (*n* = 3)	0.55 (*n* = 5)	1.0 (*n* = 5)	0.49 (*n* = 5)	0.09 (*n* = 7)
TPO/TG high (*n* = 34/166)	1.0 (*n* = 10)	0.07 (*n* = 9)	0.03 (*n* = 5)	0.12 (*n* = 6)	0.39 (*n* = 6)	0.63 (*n* = 7)	0.14 (*n* = 6)
C3c low (*n* = 46/227)	0.54 (*n* = 11)	0.52 (*n* = 10)	1.00 (*n* = 3)	1.0 (*n* = 5)	0.17 (*n* = 4)	0.84 (*n* = 9)	1.0 (*n* = 5)
ANA high (*n* = 51/225)	0.11 (*n* = 15)	0.06 (*n* = 14)	0.02 (*n* = 9)	0.21 (*n* = 9)	0.51 (*n* = 6)	1.0 (*n* = 11)	0.8 (*n* = 7)

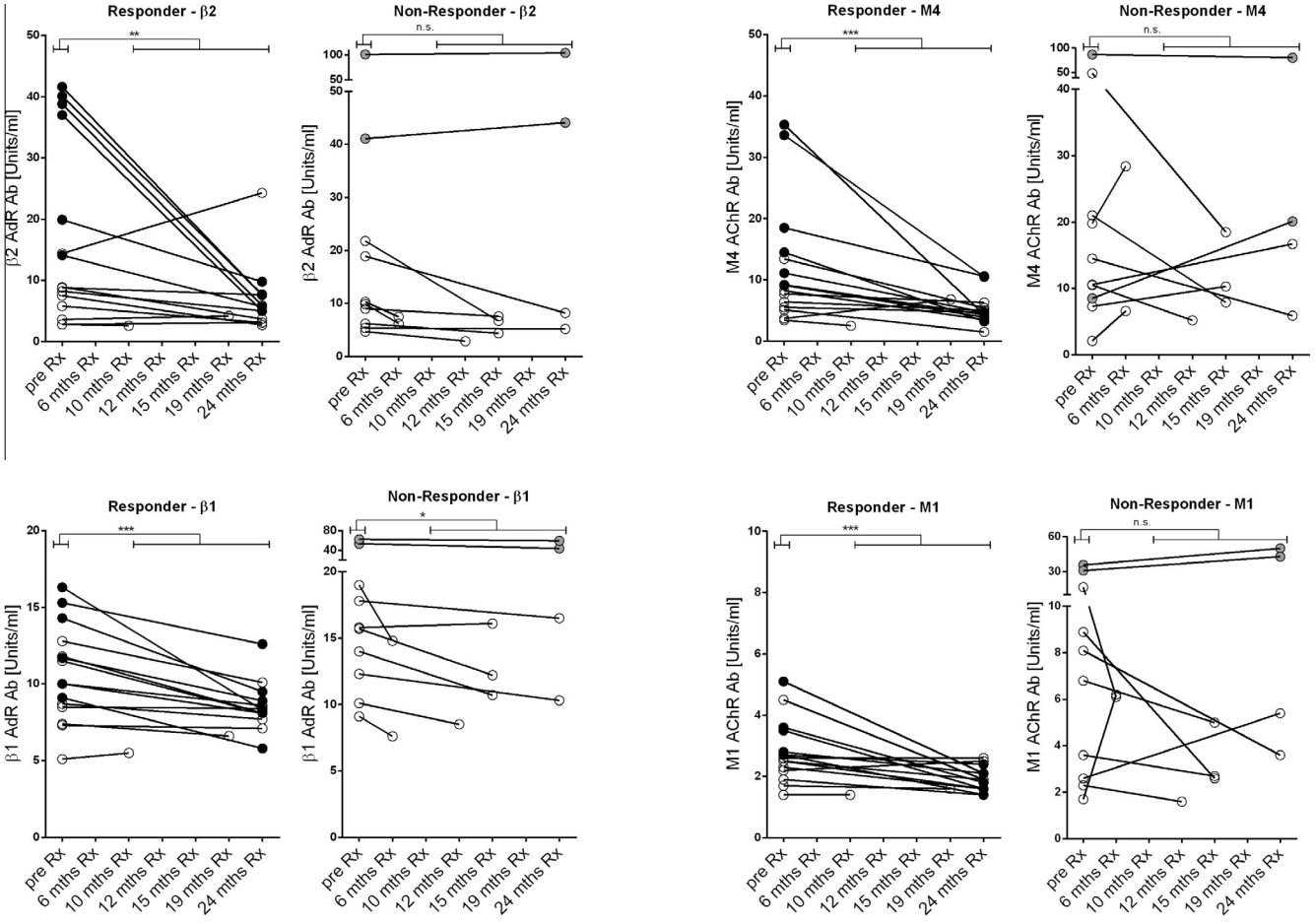

Fig. 3. Pre- and post-treatment autoantibodies against M1 and M4 AChR and β1 and β2 AdR in CFS patients from KTS-2 rituximab (Rx) study (*n* = 25) including 15 patients with clinically significant response (four moderate and 11 major responder) and 10 non-responder. Black circles (responder) and grey circles (non-responder) indicate patients with elevated β2 pre Rx levels. In patients with non-elevated β2 pre Rx levels circles are white. Statistics analysis of pre Rx and post Rx was performed by nonparametric comparisons of two dependent groups by Wilcoxon matched-pairs signed-rank test. A two-tailed *p*-value of $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.01$ and n.s. – not significant.

β AdR and M AChR antibodies with immunoglobulin levels, T cell activation, and elevated ANA and TPO antibodies. This fits well with findings in other autoimmune diseases. Increased IgG, mostly of IgG_1 and IgG_3 subclasses, but also IgG_2 were reported in SLE, Sjögrens Syndrome and primary biliary cirrhosis (Cakalaroski et al., 2000; Lin and Li, 2009; Liu and Li, 2011; Zhang et al., 2015; Zhao and Han, 2012). T cell activation is frequently present in autoimmune disease, as well as elevated ANA titres and TPO antibodies (Gilmour et al., 2000; Perilloux et al., 2000; Strioga et al., 2011). Interestingly, β AdR and M AChR are expressed by lymphocytes, too, regulating activation, migration, and antibody production (Fujii et al., 2003; Grisanti et al., 2010). Thus a direct effect of autoantibodies on the observed skewed immune parameters is conceivable. The effect of adrenergic stimulation on immune cells is complex depending on the activation status of the immune cell and type of stimulant and both immune stimulation and suppression were reported. Recent studies showed that while β2 AdR agonists reduce IFN-γ and IL-2 production in memory T cells, IL-1 and TNF production were enhanced (Slota et al., 2015; Zalli et al., 2015). Further β2 AdR stimulation inhibited lymphocyte proliferation and resulted in lymphopenia by inhibiting egress from lymph nodes (Nakai et al., 2014; Slota et al., 2015). In contrast agonistic β1 autoantibodies enhanced the proliferation of T lymphocytes (Du et al., 2012). In monocytes β2-adrenergic stimulation has inhibitory effects on LPS-induced monocyte TNF production. Interestingly, this inhibitory effect was less in CFS patients compared to healthy controls (Kavelaars et al., 2000). Further, enhanced production of IgG_1 upon β2 AdR stimulation of B cells was shown (Podojil

and Sanders, 2003). Thus, the association of autoantibodies with enhanced IgG levels we observed suggests agonistic effects of β2 AdR antibodies on B cell receptors. *In vitro* functional assays in CFS patients are required to clarify the direct effect of these autoantibodies on immune cell function. Also, autoantibodies to these GPCR may disturb function of other cells, like endothelial cell function, which was shown to be impaired in CFS patients (Newton et al., 2012).

Further evidence for a pathogenic relevance of such autoantibodies comes from clinical trials removing autoantibodies by immune apheresis. Both, non-selective immunoadsorption as well as selective β1 autoantibody removal led to improvement of haemodynamic parameters and long-term outcome in inflammatory cardiomyopathy (Dandel et al., 2013; Dorffel et al., 1997; Klein-Weigel et al., 2014; Muller et al., 2000). In autoantibody-mediated autoimmune diseases depletion of memory B cells with rituximab can be effective if the autoantibody production is mainly by short lived "early" plasma cells that to some extent express CD20. Recent studies from Norway showed that about 60% of CFS patients responded to rituximab treatment. Onset of clinical remission is delayed for 3–8 months after B-cell depletion suggesting an indirect effect on autoantibody production. Indeed, we could observe a significant decline of β2 AdR and M4 AChR receptor antibodies following rituximab treatment in clinical responder. This finding provides further evidence for a possible pathogenic role of these autoantibodies in CFS. Our observation of a decrease of M and β receptor autoantibodies in patients responding to rituximab, in whom levels pre-treatment were within the normal range of control subjects, suggests that we may miss functionally pathogenic antibodies by just assessing quantitative levels by ELISA. Out of the 25 CFS patients with blood samples pre- and post-rituximab treatment, eight had received two infusions rituximab two weeks apart in the KTS-1 study, with a minimum of 17 months time interval, and with recovered B cells in peripheral blood, before inclusion in the KTS-2 study. A presumed effect of previous B-cell depletion period would be further lowering of autoantibody levels. Further, these eight patients were divided between four responder and four non-responder, and should therefore not have a major influence on the interpretation.

In conclusion, there is evidence for elevated autoantibodies against β2 AdR and M AChR in a subset of patients with CFS. Although the function of these antibodies in CFS at present is unclear, the association of β2 AdR and M AChR antibodies with immune activation markers and their decline in CFS patients responding to B-cell depletion may support a pathogenic role and warrants their testing as potential biomarkers in clinical trials of B-cell/antibody depleting therapy. It is conceivable that various symptoms of CFS including cognitive deficits, autonomic dysregulation and immune activation could be partly mediated by autoantibodies against these receptors in a subset of patients.

Authorship

M.L. and C.S. conceived and designed the experiments. M.L., C.S., O.M., Ø.F. and H.D.V. wrote the manuscript. P.G., L.G.H., K.W., P.R., O.M. and Ø.F. recruited patients and controls. H.H. and S.B. performed the experiments, M.L., C.S., P.G., O.M. and Ø.F. analysed the data. C.M. and H.H. contributed reagents/materials/analysis tools.

Conflict-of-interest disclosure

CellTrend GmbH holds a patent on the use of β adrenergic receptor antibodies in diagnosis of CFS. Haukeland University Hospital has patents and pending patent applications on the issue of B-cell depletion therapy for Myalgic Encephalopathy/Chronic Fatigue Syndrome (ME/CFS). Family members of WO2009083602 A1 are pending and some of them are granted, including US 7.914.785. The two authors Ø.F. and O.M. are named as inventors in these applications and patents.

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