Autoimmunity at the neuromuscular synapse: pathophysiology and disease course

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CHAPTER 8

Discussion and future perspectives

Discussion and summary

Lambert-Eaton myasthenic syndrome (LEMS) and myasthenia gravis (MG) are both autoimmune diseases causing fluctuating muscle weakness. In addition, LEMS is characterized by autonomic dysfunction and areflexia. Both diseases are antibody-mediated autoimmune diseases impairing the neuromuscular synapse. In MG, acetylcholine receptor (AChR) and muscle-specific kinase (MuSK) antibodies bind to the postsynaptic part of the neuromuscular synapse. Antibodies to several other antigens have been described in MG for which the pathogenicity and pathophysiological mechanism is currently not completely clear. In LEMS, voltage-gated calcium channels (VGCC) localized in the presynaptic motor nerve terminal and in the autonomic nervous system are targeted by antibodies. Overall, the immunopathogenesis of LEMS and AChR MG has several overlapping features.[1] In both autoimmune diseases, IgG autoantibodies are responsible for cross-linking and internalisation of cell surface protein complexes. Both can occur in the same immunogenetic background, i.e. association with HLA 8.1 haplotype, and as a paraneoplastic phenomenon in patients with an associated thymoma in MG or small cell lung cancer (SCLC) in LEMS.[2] In this thesis, an overview of several pathophysiological and clinical aspects of both LEMS and MG will be addressed.

In **chapter 1**, we summarized relevant knowledge concerning clinical characteristics, pathophysiology, tumour association and treatment of LEMS. LEMS is a rare autoimmune disease, which is probably under recognized, especially if patients present with predominantly proximal leg weakness. However, when considered by the clinician based on typical clinical manifestations (proximal muscle weakness, loss of tendon reflexes and autonomic dysfunction), a diagnosis is usually made relatively easy.[3,4] Pathogenic antibodies to P/Q-type VGCC can be found in about 90% of patients and the presence of decrement and increment upon repetitive nerve stimulation is also a highly sensitive diagnostic test. Rapid diagnosis is also important because of the association with SCLC in 50-60% of patients, which stresses the need for vigorous tumour screening after diagnosis. Treatment of the tumour as well as symptomatic treatment (3,4-diaminopyridine or 3,4-DAP) and immunosuppression can effectively control symptoms in the majority of patients.

Pathophysiology

Aside from pathogenic antibodies against VGCC, several immune responses against other antigenic targets have been described in LEMS patients, especially in patients with an associated SCLC. Antigenic targets include other presynaptic proteins involved in ACh release, such as synaptotagmin I, laminin ß2 and the presynaptic muscarinic acetylcholine receptor.[4,5] These antibodies do not necessarily have to be of pathogenic relevance, and could result from epitope spreading during the course of the disease. Antibodies to intracellular nuclear SOX1 protein in SCLC-LEMS patients indicate that the immune system initiates a response against multiple tumour antigens at the same time. Therefore, SOX antibodies can be used as a serological marker for SCLC-associated LEMS.[6,7]

In **chapter 2**, we aimed to identify new antibodies against LEMS associated SCLC antigens, by immunoprecipitation using a SCLC cell line. We discovered strong immunoreactivity

against the ERC1 protein (also known as ELKS) in one tumour-negative VGCC-positive LEMS patient. A recombinant ELISA assay and a cellular assay expressing GFP-tagged full length ERC1 were used to confirm the presence of autoantibodies against ERC1 in this patient. The pathogenic relevance of these antibodies is as yet unknown.

ERC1 is involved in several cellular functions, including formation of presynaptic active zones and a supportive role in synaptic transmission, promoting calcium-dependent acetylcholine (ACh) exocytosis.[8] ERC1 also associates with the ß4 subunit of the VGCC in the cerebellum of mice.[9] Antibodies to the intracellular protein ERC1 could be a consequence of disintegration of the presynaptic axon due to the immune response against the VGCCs. Structural damage of the presynaptic axon would be in contrast to current knowledge about the pathophysiological mechanism. LEMS IgG is thought to induce functional loss of VGCC, without involvement of complement deposition and membrane lysis.[10] We specifically screened sera of seropositive and seronegative LEMS and MG patients for immunoreactivity against ERC1, but found no other cases with reactivity. ERC1 is therefore a new, but rare, antigen in LEMS. As such it is not a relevant marker for seronegative LEMS. Around time of publication, another case has been described of ERC1 reactivity in an SCLC patient without LEMS, but with paraneoplastic cerebellar degeneration.[11] This paraneoplastic disease is also associated with VGCC antibodies, which target Purkinje fibres in the cerebellum. Subsequent generation of ERC1 antibodies could result from a comparable mechanism such as epitope spreading as in our described LEMS patient.

In LEMS patients with an associated SCLC, the tumour is usually detected after diagnosis of LEMS. In **chapter 3**, we have summarized clinical and serological markers shown to predict the presence of SCLC in LEMS patients. SCLC is a very immunogenic tumour, frequently eliciting antibodies against tumour antigens.[12] Due to the neuroendocrine origin of SCLC, several of the targeted antigens are also expressed in the nervous system, constituting both intracellular and cell surface targets for an immune response.[13] Within the group of related autoantibodies found in SCLC patients, SOX1 and SOX2 antibodies are highly specific for SCLC-LEMS.[14] These SOX antibodies are, however, not sensitive enough for use in clinical practice and do not relate to specific symptoms or tumour survival. Several clinical markers can discriminate between SCLC-LEMS and non-tumour LEMS. A more progressive disease course over the first months has been described in patients with an associated SCLC.[15,16] Combining a set of clinical risk factors, the DELTA-P score was developed to determine the risk of SCLC after LEMS diagnosis. This clinical tool combines bulbar symptoms, erectile dysfunction, age at onset, smoking at onset of symptoms, weight loss and Karnofsky performance score, all within 3 months of disease onset with high sensitivity and specificity.[17] The DELTA-P score can be used to guide intensity of tumour screening in individual patients.[4,17] Recently, a prospective cohort study by our group confirmed the DELTA-P score as an effective tool for predicting SCLC, although the score performed slightly less effective as compared to the previous cohorts.[18]

Clinical manifestations

Several studies have reported clinical manifestations as well as survival data in LEMS patients.[3,15,16,19-23] Data on long-term follow-up, limitations in daily life and quality of life are however very limited. In **chapter 4**, we studied survival and characterized long-term

functional impairments as well as health-related quality of life (HRQOL) in patients with LEMS. We included 150 consecutive Dutch LEMS patients from 1998 to 2015, for whom survival and baseline data were available.

Our study showed that survival was similar to the general population in 65 LEMS patients without an associated tumour. Tumour survival was significantly longer in 81 SCLC-LEMS patients compared to non-LEMS SCLC patients (overall median survival 17 vs. 7.0 months, $p<0.0001$). Several previous smaller studies have reported this improved survival including a recent prospective cohort study of patients with SCLC with and without LEMS.[19-21,24] We showed that tumour survival is increased in all SCLC-LEMS patients both with limited and extensive disease. Interestingly, survival in SCLC patients (limited disease) without LEMS is comparable to SCLC-LEMS patients with extensive disease. There will be an inevitable lead-time bias, due to earlier diagnosis of SCLC because of neuromuscular symptoms, but this is unlikely to fully explain the survival difference. This additional improvement in survival after correction for tumour stage supports a biochemical or immunological cause, like an anti-tumour immune response or a direct effect of ion-channel blocking antibodies on tumour proliferation.[25-27]

A majority of patients in this cohort were independent for ADL activities at diagnosis, improving to 85% at 1-year follow-up. Patients treated symptomatically improve sooner after diagnosis than those treated with immunosuppressive drugs, but both groups ultimately reach a relatively stable level of limitations about 2 years after diagnosis. Maximum disease severity has already been reached before diagnosis in a majority of patients and within the first two years in about 80%, the latter of which is very similar to myasthenia gravis.[28,29] LEMS patients with associated lung cancer report more functional impairments over the entire disease course. Physical HRQOL was significantly lower than in the general population and comparable to myasthenia gravis, while mental HRQOL was normal. Overall, the detailed information obtained in this study on long-term prognosis and limitations in LEMS can guide expectations of doctors and patients, and can be of potential relevance for treatment choices.

Electrophysiology

Diagnosis of LEMS is based on fluctuating muscle weakness, decrease of tendon reflexes and autonomic symptoms, supported by either presence of antibodies to VGCC or abnormal decrement and increment upon repetitive nerve stimulation (RNS). RNS in LEMS patients typically shows a triad of abnormalities:[30,31]

- Low initial compound muscle action potential (CMAP) amplitude
- Abnormal decrement of CMAP amplitude (>10%) upon low rate RNS
- Abnormal increment of CMAP amplitude (>60-100%) upon high rate RNS or directly after voluntary contraction.

Presence of decrement is highly sensitive for LEMS in clinically affected patients, however for distinction from myasthenia gravis additional detection of increment is required. Since making a diagnosis can be challenging, an optimal cut-off value for abnormal increment

is highly relevant for improved recognition of this rare disease. One previous study reported a 60% cut-off for abnormal increment (instead of the previous standard 100% cut-off) to increase sensitivity of this test, while maintaining specificity when compared to myasthenia gravis.[30] However, since its publication, several studies have still variably used either a 60%[32,33] or 100%[21,34,35] cut-off in diagnostic criteria. In **chapter 5**, we aimed to confirm this finding in a second, independent cohort of patients. Sensitivity and specificity of 60% and 100% cut-off values were determined in all consecutive patients who underwent increment testing in our hospital from 1999 to 2016. In 63 patients with LEMS and 93 without, sensitivity of a 60% cut-off for increment testing was 77.8% and 58.7% for a 100% cut-off. Specificity was comparable at 98.9% and 100% using a threshold of 60% and 100%, respectively. Excluding treated LEMS patients resulted in higher sensitivity for both thresholds, but a comparable absolute difference in sensitivity. These findings confirm that a 60% threshold for increment greatly increases sensitivity while maintaining a high specificity in a large group of LEMS patients and a different control group. Given the results of these two heterogeneous studies, the threshold for abnormal increment can be standardised to 60%, as this will lead to improved diagnosis of patients with this rare disease.

In myasthenia gravis, RNS is a standard method for diagnosis as well. Initial CMAP amplitudes before repetitive stimulation can be used as a measure for the output of effectively innervated muscle fibers; and its relations with disease severity could help to improve our understanding of the mechanism underlying muscle weakness. In **chapter 6**, we retrospectively studied the relation between the CMAP amplitude and the Myasthenia Gravis Foundation Association (MGFA) scores or Quantitative Myasthenia Gravis (QMG) scores as markers for disease severity. We found that a lower initial CMAP amplitude is a marker of more severe disease in AChR MG, especially in the nasalis and trapezius muscles. Related but unpublished data from our group show the same relation between initial CMAP amplitude and disease severity in LEMS patients as well. This has been previously reported and has led to use of CMAP amplitude as an outcome measure in clinical trials in LEMS.[36-39]

Clinically severe weakness in MG is likely to be associated with a larger number of constantly non-functional neuromuscular synapses resulting in a low initial CMAP amplitude, which does not change during RNS and therefore does not contribute to an increment or decrement. This might be due to a functional impairment of a structurally intact synapse, or to structural damage resulting in denervation of the muscle fiber from complement activation or chronic acetylcholine depletion. Dysfunction of neuromuscular synapses can result in a decrease in the number of muscle fibres available for contraction, resulting in a fairly constant muscle weakness, which has in fact been described in quantified testing of muscle strength in AChR MG patients.[40,41] This hypothesis suggests part of the muscle weakness in severely affected patients might be related to widespread dysfunction of neuromuscular synapses, resulting in a decrease in the number of muscle fibres available for contraction. This could be a relevant mechanism to explain more constant muscle weakness in MG, in addition to fatigable weakness related to the previously described mechanism of worsening decremental response that can be shown after exercise (post-exercise exhaustion).[42] The presence of fixed or even irreversible muscle weakness in AChR

MG is supported by the observation that these patients are also at risk for developing muscle atrophy.[43,44]

Treatment in myasthenia gravis

Besides pyridostigmine and immunosuppression, other symptomatic treatments for MG have been described previously in case reports and series. These treatment options include 3,4-diaminopyridine[45,46] (which is also the mainstay of treatment for LEMS) and ephedrine[47] and are mostly based on anecdotal evidence, lacking randomized controlled trials. In **chapter 7**, we studied the effect and safety of ephedrine as add-on treatment for patients with MG with AChR antibodies. Four AChR MG patients with moderate disease severity were included in a placebo-controlled, double-blind, randomised, multiple crossover series of n-of-1 trials. Sequential treatment cycles of either ephedrine 50mg daily in 2 doses or placebo were compared. Our trial showed that add-on treatment with ephedrine compared with placebo improved QMG score by 1.0 point, which was significant for the group of trial patients as well as for the population treatment effect. Secondary outcomes including MG-Composite, MG-ADL and a VAS score for muscle strength also showed significant improvement with ephedrine. Although the effect was consistent, the effect size was small and therefore the clinical relevance is limited and debatable. However, using only symptomatic medication at limited risk for remaining symptoms in patients with moderate disease severity can still be of value in preventing or postponing high-risk immunosuppressive drugs. This study also showed that a series of n-of-1 trials can be a very effective study design to detect even a small effect in a small patient population, by replacing the large variance between patients in standard randomized controlled trials (RCTs) with smaller variance within individual patients. This advantage is especially important in rare diseases, making it feasible to detect a short-acting treatment effect at limited cost and sample size. Another recent example of an effective n-of-1 trial in a rare disease showed a relevant reduction of symptoms using mexiletine in patients with non-dystrophic myotonia.[48] In this trial, results from a previous RCT with comparable outcome parameters were replicated in a new patient cohort, requiring only half of the patients as compared to the previous standard design RCT. Despite of these successful examples as well as recognition by regulatory agencies[49] and available guidelines for study protocols[50] and reporting of clinical trials,[51] the n-of-1 trial design still remains relatively uncommon.

Future perspectives

Immunopathology

One of the most fascinating aspects of this model autoimmune disease is the increased tumour survival in SCLC-LEMS patients (**chapter 4**).[19-21,24] Treatment advances in tumour immunology have increased exponentially in recent years, with immune checkpoint inhibitors (ICI) leading the charge. This class of drugs targets key regulators in the T-cell immune response, allowing T cells to effectively attack the tumour. In a majority of cases this treatment also results in autoimmunity, which might be even more frequent in SCLC due to the immunogenicity of this tumour.[52,12] Two LEMS cases have been reported, with symptoms starting months after tumour diagnosis and initiation of ICI treatment.[53,54] The timing of symptom onset was suggestive for a causative role of this treatment, since pure paraneoplastic cases usually present before SCLC diagnosis. Paraneoplastic diseases can be a model for a clinically relevant anti-tumour immune response, if, like in the case of LEMS, aside from the secondary neuromuscular autoimmune diseases, it also leads to a significant increase in survival due to an immune attack towards the tumour. This survival difference of about 8 months in SCLC-ED patients with LEMS is longer than the 2-3 months survival benefit added by ICI treatment in this group, suggesting this specific paraneoplastic immune response to be more effective than generic T-cell mediated treatments.[55] A more detailed understanding of the underlying mechanism of this increased survival in SCLC-LEMS can open up new pathways for cancer immunotherapy. This would require further study of a T cell mediated response in SCLC-LEMS and possible macrophage activation in response to the tumour. Detailed immunocytochemistry of SCLC tumour biopsies or studies with patient-derived SCLC tumour cell lines might also help to characterize the anti-tumour immune response in LEMS compared to non-LEMS patients.[56]

Besides an effect of the immune system itself on the tumour, a direct effect of ion channel (VGCC) blocking antibodies on tumour proliferation is also possible. LEMS serum has been described to decrease calcium influx in SCLC cell lines through VGCCs.[57,58] In a mechanism similar to VGCC-dependent acetylcholine release at the neuromuscular junction, VGCCs and calcium currents have been shown to be required for exocytosis of serotonin from SCLC cells.[25-27] Serotonin[59] as well as calcium currents[60] have been shown to influence SCLC cell proliferation *in vitro*. Taken together, a direct blocking effect of VGCC antibodies on calcium currents and therefore exocytosis of neuropeptides involved in SCLC proliferation might explain the increased tumour survival in SCLC-LEMS patients. Several assays are available for testing tumour cell proliferation *in vitro*. Adding serum, purified IgG or antigen-specific antibodies from SCLC-LEMS patients (especially those with the most profoundly increased tumour survival) might inhibit tumour growth by (directly) blocking VGCCs or (more slowly) decreasing the number and organisation of VGCCs on SCLC cells.

Comparison of the pattern of tumour growth in SCLC patients with and without LEMS might also contribute to discovery of the underlying mechanism leading to increased survival. Serial imaging studies might either reveal a slower growth rate of the primary tumour, a decrease in metastatic growth, or both. A synergistic effect of classical anti-tumour therapies (chemoradiation), checkpoint inhibitors and the immune response might be possible, but would be more challenging to study.

Clinical manifestations

As a coincidental finding during patient interviews related to the disease course, several patients reported symptoms corresponding to respiratory muscle weakness, sometimes even chronic in nature. Up to now, respiratory muscle weakness in LEMS has usually been reported only in acute exacerbations or rapidly progressive at initial presentation. Pulmonary function testing could help in screening for respiratory muscle weakness previously unnoticed by the clinician, especially comparison of spirometry in supine and sitting/standing position, as well as maximal sniff, in- and expiratory pressures.[61] Due to frequent co-morbidity, especially in LEMS patients with associated SCLC or those at increased risk for respiratory infections, other pulmonary function tests would probably be required to distinguish multiple possible causes of dyspnoea. Ultrasound studies of the diaphragm could also be helpful in detecting respiratory muscle weakness, although this test might not be sensitive enough to detect mild weakness.[62] In patients with SCLC, cachectic myopathy unrelated to LEMS might contribute to this respiratory muscle weakness. Although frequently recognized by pulmonologists (personal communication), few clinical studies have focused on this disease entity, which could be studied further by muscle imaging or biopsies.

Diagnosis

Improvement of the assay for detecting VGCC autoantibodies would be very useful. The current radioimmunoassay still has limited sensitivity and requires radioactive isotopes, using an extract of rabbit brain. Thus, the assay detects all antibodies to epitopes within the complex VGCC molecule and therefore cannot discriminate between antibodies to VGCC components with and without clinical relevance.[63] This has been shown to be of high relevance for patients with antibodies against the voltage-gated potassium channel (VGKC) complex, in whom only those patients with more specific antibodies against LGI1 or Caspr2 had related symptoms as well as other evidence of autoimmune inflammation.[64]

In recent years, several advances have been made in cell-based assays for other autoimmune disorders and myasthenic syndromes.[65,66] These techniques could also be applied to improvement of a VGCC assay. Using a refined and more specific assay, VGCC antibody titres could possibly relate more closely to disease severity when using appropriate, disease-specific outcome parameters, as will be discussed below.

It would also be of interest to compare the diagnostic yield of abnormal decrement and 60% increment using a practical approach (hypothenar and possibly adding nasal muscle when clinically appropriate) and a more complete approach (several muscles tested, both after voluntary contraction and upon high-frequency stimulation). Several previous studies have described extensive protocols for detecting increment that are useful for research, but might be less realistic in clinical practice.[67,68] Sensitivity in the untreated LEMS patients described in **chapter 5** suggests a more limited approach, tailored to individual symptoms, might be just as sensitive.

Treatment

Several questions regarding symptomatic therapy remain in both LEMS and MG, for which the serial n-of-1 trial design described in this thesis could prove very helpful. A previous randomized controlled trial in LEMS patients performed by our group showed a significant effect of 3,4-diaminopyridine (3,4-DAP) on isometric muscle strength and CMAP amplitude, but not for pyridostigmine.[39] Contrary to these findings, in this thesis we report 67% of patients subjectively benefit from additional symptomatic treatment with pyridostigmine (**chapter 4**). Aside from the relatively low dose of pyridostigmine in this previous trial, this discrepancy could be partially explained by the requirement of 3,4-DAP as co-medication for pyridostigmine to have a relevant effect. Pyridostigmine might only be effective after 3,4-DAP ensures presence of enough acetylcholine release into the neuromuscular junction, after which pyridostigmine can reduce acetylcholine clearance. Interindividual variability in benefit of pyridostigmine treatment might also relate to differences in compensatory mechanisms optimizing synaptic transmission, secondary to the immune response. Alternatively, further investigation of a possible effect of pyridostigmine might require a more functional outcome parameter measuring limitations corresponding with daily symptoms, instead of an immediate parameter such as muscle strength.

A useful functional outcome measure for testing fatigable limb weakness in LEMS patients might be the triple time up-and-go test (3TUG) recently used in a trial (again) proving the efficacy of 3,4-diaminopyridine.[69] Re-analysis of this trial showed the 3TUG to be reproducible and reliable as a specific outcome measure for LEMS patients, as well as responsive to change in disease severity.[70] Alternative functional outcome measures could be the six-minute walk test[71-73] or the Timed Up and Down Stairs test[74] (TUDS). Limited pilot data have already been obtained in our centre comparing these functional outcome measures to other general or myasthenia-specific outcomes (unpublished data), which can be used for a sample size calculation. This thesis also reports patient-reported outcome measures (PROMs, **chapter 4**) for characterizing long-term functional impairments, such as the modified Rankin Scale. These outcome measures can be highly relevant for long-term disease fluctuations, but do not seem sensitive enough for short-term or relatively small clinical changes. This would preclude use of these PROMS from most clinical trials, except when studying long-term benefit of immunosuppressants. Pilot data in our centre might still be explored to detect which short-term disease markers relate to long-term PROMs and would therefore be most useful as surrogate markers for disease fluctuations.

Randomized controlled trials aimed to show a relatively small symptomatic effect of short-acting drugs can be difficult to perform in rare diseases such as LEMS. However, as we have shown in MG patients in **chapter 7**, a serial n-of-1 trial can be highly effective in showing a significant effect of short-acting symptomatic treatment in only a limited number of patients. A serial n-of-1 trial in LEMS patients comparing pyridostigmine to placebo might be the most suitable design to further study the efficacy of this add-on treatment. A serial n-of-1 trial design could also be used to study the efficacy of 3,4-diaminopyridine in patients with MG, which has been anecdotally reported as well.[45,46]

Treatment of congenital myasthenia patients with ephedrine shows an increasing effect of this drug over months, instead of a direct effect as shown in our serial n-of-1 trial in AChR MG (**chapter 7**).[75] Proper study of a long-term effect of ephedrine treatment in patients with autoimmune MG would require longer follow-up but also several more patients. An increase in sample size compared to our serial n-of-1 trial is also related to the variation of muscle weakness over months, as part of the natural disease course. Follow-up data might elucidate whether the full effect of ephedrine is directly symptomatic (e.g. related to fatigue), or more long-lasting e.g. related to a role in innervation or stabilisation of the neuromuscular junction.[76,77]

Concluding remarks

Autoimmune responses towards antigens at the neuromuscular synapse can cause rare neuromuscular disorders, with clinically well-defined disease subgroups, all of which result in fluctuating muscle weakness. Either an anti-tumour immune response or an immunogenetic predisposition seem to able to trigger an autoimmune response leading to LEMS, with the same spectrum of clinical signs and symptoms. Still much can be learned on autoimmunity and paraneoplastic disease mechanisms from these rare but well-defined clinical entities. The increased tumour survival in SCLC-LEMS patients reported in this thesis could form the basis of another model for the rapidly developing field of cancer immunotherapy. Understanding the triggers for eliciting the potent immune response and discovering how we can manipulate the underlying mechanisms could greatly advance our knowledge about autoimmunity as well as tumour immunology in general. Detailed clinical studies will remain very important for a correct diagnosis and subgrouping, as they form a necessary prerequisite for studying pathophysiology and an anti-tumour immune response.

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