Multi-modal Biomarkers Quantify Recovery in Autoimmune Autonomic Ganglionopathy

Running head: Multi-modal biomarkers quantify recovery in AAG

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Abstract

Objective: To evaluate patients with ganglionic acetylcholine receptor antibody (gAChR-Ab) positive autoimmune autonomic ganglionopathy using a multi-modal testing protocol to characterise their full clinical phenotype and explore biomarkers to quantify immunotherapy response.

Methods: Cohort study of thirteen individuals (seven female; 21-69 years) with autonomic failure and gAChR-Ab>100pM identified between 2005-2019. From 2018, all patients were longitudinally assessed with cardiovascular, pupillary, urinary, sudomotor, lacrimal and salivary testing, and COMPASS-31 autonomic symptom questionnaires. The orthostatic intolerance ratio was calculated by dividing change in systolic blood pressure over time tolerated on head-up tilt. Eleven patients received immunotherapy.

Results: At first assessment, all 13 patients had cardiovascular and pupillary impairments, 7/8 had post-ganglionic sudomotor dysfunction, 9/11 had urinary retention and xeropthalmia, and 6/8 had xerostomia. After immunotherapy, there were significant improvements in orthostatic intolerance ratio (33.3[17.8-61.3] to 5.2[1.4-8.2], *P*=.007), heart rate response to deep breathing (1.5[0.0-3.3] to 4.5[3.0-6.3], *P*=.02), pupillary constriction to light (12.0[5.5-18.0] to 19.0[10.6-23.8]%, *P*=.02), saliva production (0.01[0.01-0.05] to 0.08[0.02-0.20]g/min, *P*=.03) and COMPASS-31 scores (52 to 17, *P*=.03). Orthostatic intolerance ratio correlated with autonomic symptoms at baseline (r=0.841, *P*=.01) and following immunotherapy (r=0.889, *P*=.02). Immunofluorescence analyses of skin samples from a patient 32 years after disease onset showed loss of nerve fibres supplying the dermal autonomic adnexa and epidermis, with clear improvements following immunotherapy.

Interpretation: Patients with autoimmune autonomic ganglionopathy demonstrated objective evidence of widespread sympathetic and parasympathetic autonomic failure, with significant improvements after immunotherapy. Quantitative autonomic biomarkers should be used to define initial deficits, guide therapeutic decisions, and document treatment response.

Keywords: Autoimmune Autonomic Ganglionopathy - Ganglionic Antibody – Biomarkers – Immunotherapy - Skin Biopsy

Introduction

Autoimmune autonomic ganglionopathy (AAG) is an uncommon but treatable disease presenting with subacute pandysautonomia. Patients develop disabling symptoms reflecting orthostatic hypotension, pupillary, gastrointestinal, genitourinary, sudomotor and secretomotor dysfunction. Fifty percent of patients have a detectable antibody to the ganglionic nicotinic acetylcholine receptor (gAChR-Ab) which mediates fast synaptic transmission at sympathetic, parasympathetic and enteric autonomic ganglia.[1] Higher antibody levels have been associated with a greater degree of autonomic dysfunction;[2] while low titres <200pM are non-specific in the absence of autonomic failure.[3] Passive and active immunisation studies have provided strong evidence for the pathogenicity of the ganglionic antibody.[4, 5] Previous experimental models have shown that the ganglionic antibody reversibly impairs synaptic transmission through internalisation of the ganglionic acetylcholine receptor, but the autonomic ganglia remain structurally intact.[5, 6] Studies in patients with AAG have shown postganglionic sudomotor dysfunction[7, 8] and pathological evidence of postganglionic autonomic and somatic nerve fibre loss on both sural nerve and punch skin biopsies, suggesting long-term immune attack against the autonomic ganglia may lead to post-ganglionic denervation.[9, 10]

Patients with AAG can respond to immunotherapy, but the response of individual patients varies and multiple immunomodulatory agents may be needed.[11-14] Previous studies have largely utilised cardiovascular and sudomotor assessments when attempting to objectively quantify the severity of autonomic failure and treatment response in patients with AAG, failing to capture the pupillary, urinary and secretomotor deficits that are prominent in this disease.[1, 3, 12, 13, 15] For example, Iodice and colleagues described a patient with low Composite Autonomic Severity Score (CASS),

derived from cardiovascular and sudomotor testing, that poorly reflected her multiple prominent autonomic symptoms, measured by the COMPASS (Composite Autonomic Symptom Score) questionnaire, and a mismatch in CASS and COMPASS changes after immunotherapy.[12] We therefore investigated a cohort of thirteen individuals with seropositive AAG with a multi-domain autonomic function testing protocol including cardiovascular, pupillary, urinary, sudomotor, lacrimal and salivary assessments to characterise their full clinical phenotype and repeated assessments following immunotherapy to try establish objective biomarkers to quantify treatment response that correlated with patient reported outcome measures. In addition to quantitative markers of autonomic function, we performed immunofluorescence analyses on skin samples collected from one of the affected individuals to compare intraepidermal nerve fibre density and innervation of the autonomic adnexa before and after immune therapy.

Materials and methods

We studied patients referred to the Autonomic Unit at the National Hospital for Neurology and Neurosurgery, a national referral centre, with documented cardiovascular autonomic failure and elevated gAChR-Ab >100pM identified from February 2005 to August 2019. Consent was obtained according to the Declaration of Helsinki. The study was approved by the local Research Ethics Committee and Health Research Authority.

Ganglionic-AChR Antibody Testing

Ganglionic AChR-Ab levels were measured by a radioimmunoprecipitation assay using solubilised antigen from a human neuroblastoma (IMR-32) cell line bound to ^{125}I-epibatidine as previously described,[1] and performed by the University of Oxford Neuroimmunology lab since

2005. Samples testing positive (>100 pM) were then serially diluted and antibody concentrations expressed as pM (pmoles of ^{125}I-epibatidine precipitated per litre of serum).

Quantitative Multi-domain Autonomic Testing (Fig. 1)

Beat-to-beat measurements of blood pressure and heart rate were recorded and analysed with Labchart 8 Pro software (AD Instruments) as previously described.[16] Sympathetic function was evaluated by blood pressure response to the Valsalva manoeuvre, including calculation of the blood pressure recovery time (PRT),[17, 18] and head up tilt. Patients were passively tilted to 60° for up to 10 minutes. Tilt was terminated early if blood pressure fell significantly causing symptoms of cerebral hypoperfusion (e.g. loss of vision/hearing/reduced responsiveness). The orthostatic intolerance ratio was calculated by dividing the change in systolic blood pressure in mmHg by the maximum time tolerated in minutes on tilt. If a patient tolerated a full 10 minutes of tilt, 10 was used as the denominator for the calculation. Parasympathetic function was assessed by heart rate response to deep breathing and the Valsalva manoeuvre. Blood samples were collected in the supine and tilted position for measurement of plasma noradrenaline (NA) using high performance liquid chromatography. Nerve conduction studies and EMGs were performed as part of routine clinical care.

Baseline pupillary dark diameters and responses to stimulation with white light and topical pharmacological agents were recorded with a custom built infrared pupillometer as previously described[19] or commercially available devices (Procyon P3000, Konan RAPDx, or Neuroptics DP2000). Impaired pupillary constriction to light and cholinergic supersensitivity (pupillary constriction with dilute 0.125% pilocarpine) indicated parasympathetic dysfunction. Delayed

pupillary redilation following a light impulse to ¾ of baseline diameter, a lack of response to 4% cocaine, or adrenergic supersensitivity (abnormal dilation with 0.5% apraclonidine) indicated sympathetic dysfunction.[20] From May 2019, patients were also examined with a prolonged light stimulus to assess for pupillary fatigue, a unique phenomenon previously reported only in patients with seropositive autoimmune autonomic ganglionopathy.[21]

Urinary flow when voiding with the sensation of a full bladder was assessed by uroflowmetry (Albany Medical SmartFlow) and post-void residual volume measured using a bladder ultrasound scanner (Bardscan Realtime). Dynamic sweat testing was performed at both forearms and distal legs using 1% pilocarpine ionotophoresis.[22] Pilocarpine, directly stimulates M3 cholinergic receptors on sweat glands allowing assessment, by inference, of peripheral sweat gland innervation. , Lacrimal production was measured using Schirmer's test and average for both eyes calculated.[23] Salivary production was measured using the unstimulated salivary production test.[24]

Questionnaires

The COMPASS-31 was used to assess orthostatic intolerance, vasomotor, secretomotor, gastrointestinal, urinary and pupillomotor symptoms.[25] The SF-36 was used to assess eight components affecting quality of life including physical functioning, role limitations due to physical and emotional health, energy/fatigue, emotional well-being, social functioning, pain and general health.[26]

Longitudinal Assessments following Immunotherapy

Eleven patients received immunotherapy. From August 2018, patients were re-assessed with our full multi-domain autonomic testing protocol between 14-29 days following plasma exchange or intravenous immunoglobulin (IVIg), and between 4-6 weeks and three months after commencing corticosteroids. Prior to August 2018, testing was performed at various time frames as part of clinical care.

Skin Biopsy Analysis

Skin biopsies were obtained before and after immunotherapy from one of the patients. Specimens were fixed for 4-6 hours in Zamboni solution, cryoprotected in 20% sucrose in PBS and sent in a refrigerated package to the skin biopsy laboratory in Telese. Samples were cut into 50µm thick sections using a freezing slide microtome (Leica 2000R) and free-floating sections were processed for indirect immunofluorescence using antibodies to stain nerve fibres and vascular structures. Nerve fibres were marked with primary mouse and rabbit antibodies against the pan-neuronal marker protein gene product 9.5 (PGP; AbD Serotec, 1:800: Biogenesis; 1:400), mouse antibody against myelin basic protein (MBP; Santa Cruz Biotechnology; 1:800, to assess myelinated fibres), mouse and rabbit antibodies against vasoactive-intestinal-peptide (VIP; Santa Cruz Byotechnology, 1:300: Immunostar, 1:1000, to assess cholinergic fibres), and rabbit antibodies against dopamine-beta-hydroxylase (DβH, Chemicon, 1:1000, to assess noradrenergic fibres). Vascular bed and basal membranes were marked with mouse antibody against collagen IV (COLIV; Chemicon; 1:800) and endothelia and epidermis were marked with Ulex Europaeus agglutinin 1. Species-specific secondary antibodies coupled with cyanine 2 and cyanine 3 fluorophores were used to visualise the structures of interest. Skin sections were mounted on

coverslips with agar, dehydrated in alcohol, clarified in methylsalicylate and finally mounted in DPX. Digital images were acquired using a non-laser confocal microscope (Apotome; Zeiss).

Statistical Analysis

GraphPad Prism V.8 was used for statistical analysis. Data was tested for normality by the Shapiro-Wilk test. Summary data is provided as median (inter-quartile range) for simplicity as some data was not normally distributed. Baseline characteristics in idiopathic and paraneoplastic groups were compared with unpaired two-tailed t-tests/Mann-Whitney tests as appropriate. Baseline and follow up parameters after immunotherapy were compared with paired two-tailed t-tests/Wilcoxon signed-rank tests as appropriate. Spearman's rank/Pearson correlations were used to assess correlations between autonomic function testing and COMPASS-31 and SF-36 scores as appropriate. Two-sided $P<.05$ was considered significant.

Results

From February 2005 to August 2019, 168 patients with documented cardiovascular autonomic failure had blood samples sent to Oxford University for analysis of the ganglionic antibody (see Fig. 2 for a summary of the patients' diagnoses at most recent clinical review). After subtraction of healthy control values, antibody levels ranged from -30 to 5990pM (Fig. 1E). Only fifteen patients had positive values >100pM (all >200pM). Two patients were excluded from this study due to concomitant diseases affecting the autonomic nervous system and the remaining thirteen included. By August 2018, five patients were deceased. For these patients, retrospective data was extracted from our patient databases and individual patient records and supplemented with direct

correspondence with local physicians. The remaining eight patients were prospectively recruited to undergo our full panel of autonomic testing and questionnaires at baseline and after treatment.

Clinical Presentation

Of the thirteen patients, seven (54%) were female. Median (IQR) age at onset was 54 (31-63) years. They all presented with pandysautonomia (Table 1). Eight (62%) had other autoimmune conditions including hypothyroidism (n=6), inflammatory bowel disease (n=3), psoriasis/eczema (n=3), Addison's disease (n=2), pernicious anaemia (n=2) and alopecia totalis (n=1). Four (31%) had antecedent infections and two (15%) had surgical procedures before developing autonomic symptoms (Table 2).

Autonomic Function Testing and Neurophysiology

All patients had widespread sympathetic and parasympathetic cardiovascular autonomic failure with orthostatic hypotension, reduced/absent heart rate response to deep breathing, and impaired heart rate and blood pressure responses to the Valsalva manoeuvre (Table 3). One patient had very low levels of supine noradrenaline (<100pg/ml), eight patients had low-normal levels (100-200pg/ml) and four had normal levels (200-500pg/ml), with no significant rise on tilt. None of the patients had a large fibre neuropathy. Five had absent sympathetic skin responses (four both upper / lower limbs, two lower limbs only), two had elevated thermal thresholds in both upper / lower limbs, and one had prolonged cutaneous silent periods in the lower limbs.

Twelve patients (92%) had impaired pupillary constriction to light; all five tested with dilute 0.125% pilocarpine showed cholinergic supersensitivity. All thirteen patients had evidence of

bilateral sympathetic deficits; four (31%) had clinically apparent ptosis and all eleven (100%) tested with 0.5% apraclonidine or 4% cocaine demonstrated bilateral Horner's syndrome. Three patients were tested with 1% hydroxyamphetamine: two patients tested within a year of disease onset showed normal pupillary dilation; the third, tested after 3 years of disease, had no response, indicating post-ganglionic sympathetic dysfunction. All seven patients (100%) examined with a prolonged 2-second bright light impulse demonstrated premature pupillary redilation during the light stimulus (Table 3).

Most patients (9/11; 82%) had urinary retention when first assessed and five (38%) required indwelling or intermittent catheterisation. Six out of eight patients (75%) who underwent uroflowmetry had an abnormal profile with prolonged void times, intermittent flow, and evidence of straining (Fig. 3D). The two patients with normal profiles had received immunotherapy prior to first uroflowmetry.

Most patients (7/8; 88%) had impaired sweat production on dynamic sweat testing. The only patient with normal results was tested on maintenance immunotherapy, having first received immunotherapy within three months of disease onset. In 4/8 (50%) patients, sweat production was lower in the forearm compared to distal leg on one or both sides, in keeping with a non-length dependent, ganglionic pathology. Most patients had reduced lacrimal production (9/11; 82%) and salivary production (6/8; 75%). The two patients with normal saliva production (>0.1gram/min) were on oral steroids when first tested.

Comparison of Paraneoplastic and Idiopathic AAG

Five patients (38%) were found to have malignancies including rectal carcinoma (n=2), lung carcinoma, ovarian teratoma and chronic lymphocytic lymphoma (n=1 each) (Table 2). Clinical features and autonomic testing at presentation did not distinguish between idiopathic and paraneoplastic AAG. Three patients had malignancies discovered during their initial investigations for autonomic failure. Two patients had unremarkable paraneoplastic screening on presentation and responded well to immunotherapy, but later deteriorated and were found to have malignancies after seven and eleven years respectively (Table 5). Median duration of follow up was 8.5 years (IQR 6-12.5).

Immunotherapy Response and Complications of Treatment

One patient was found to have a locally advanced rectal carcinoma, deteriorated rapidly and died before planned treatment. Another historical patient treated symptomatically for pure autonomic failure was retrospectively found to have elevated gAChR-Ab after death. The other eleven patients had immunotherapy. The majority had plasma exchange (n=10). Other treatment included intravenous and/or oral corticosteroids (n=5), IVIg (n=3), rituximab (n=2), mycophenolate mofetil (n=5), azathioprine, methotrexate, and cyclophosphamide (n=1 each) (Table 5).

Plasma exchange was associated with a rapid clinical improvement, beginning within days. Greater improvements were seen in patients with early disease. Of note, some patients developed significant complications after repeated exchanges. One patient had disrupted and incomplete treatment due to problematic vascular access after nine courses. Two patients developed deep vein thromboses after five and nine courses, the latter further complicated by a massive saddle pulmonary embolus requiring intra-arterial thrombolysis. One patient who initially received one

course of IVIg (2g/kg) reported no immediate benefit but had a dramatic improvement with subsequent plasma exchange a month later. Two patients who improved with plasma exchange reported a less rapid but more sustained effect with regular IVIg; one patient developed a mild rash managed with topical steroid treatment and switching IVIg brand . Three patients treated with high dose oral prednisolone (1mg/kg) had striking improvements in multiple domains. One had intolerable adverse effects with weight gain, hair loss, low mood and anxiety, and the other had supine hypertension, which both improved with dose reduction.

Four patients commenced on mycophenolate mofetil for maintenance immunotherapy had stabilisation of disease with no major adverse effects; one patient had to discontinue treatment due to anaemia. One patient started on azathioprine developed drug-induced hepatitis, was switched to methotrexate and remained well for five years without other treatment.

Quantitative Autonomic Biomarkers to Monitor Treatment Response and Correlations with Patient Reported Outcome Measures

Following immunotherapy, there were significant improvements in ΔSBP (56[49-94] to 46[14-71], *P*=.03), time tolerated on head up tilt (2[1-5] to 10[10-10], *P*=.006, orthostatic intolerance ratio (33.3[17.8-61.3] to 5.2[1.4-8.2], *P*=.007), heart rate response to deep breathing (1.5[0.0-3.3] to 4.5[3.0-6.3], *P*=.02) pupillary constriction to light (12.0[5.5-18.0]% to 19.0[10.6-23.8]%, *P*=.02), saliva production (0.01[0.01-0.05]g/min to 0.08[0.02-0.20]g/min, *P*=.03). There were improvements in Valsava ratio (1.02[1.00-1.05] to 1.12[1.00-1.23], *P*=.05 and blood pressure recovery time (28.3[25.4-31.9] to 21.8[2.6-29.4], *P*=.07), although statistical significance was not reached at the 0.05 level. Supine NA levels, Δ NA on tilt, uroflowmetry parameters, post-void

residual volume, sweat production and lacrimal production were not significantly different in the cohort as a whole, although individual patients did show improvements. Total COMPASS-31 autonomic symptom scores improved significantly (52[34-64] to 17[8-31], *P*=.03), as well as secretomotor and pupillomotor subscores. SF-36 subscores showed improvements but did not reach significance (Table 4).

At baseline, the orthostatic intolerance ratio correlated with COMPASS-31 total scores (r= 0.841, *P* =.01), COMPASS-31 orthostatic tolerance subscores (ρ= 0.792, *P*=.03), SF-36 physical function subscores (r =-0.716, *P* =.046) and SF-36 role limitations due to physical health (ρ=-0.784, *P* =.048). Following treatment, improvements in the orthostatic intolerance ratio correlated with changes in total COMPASS-31 scores (r=0.889, *P*=.02) and orthostatic tolerance subscores (r=0.896, *P*=.02).

Illustrative Case

A 21-year-old woman presented in the 1980s with subacute pandysautonomia. Two decades later, after the discovery of the ganglionic antibody by Vernino et al,[1] historic banked frozen plasma was sent to Oxford University for gAChR-Ab testing and high levels were found (4185pM). Initial plasma exchange treatment resulted in transient changes in urinary and salivary symptoms only and she declined further immunotherapy. Ten years later, she represented with worsening symptoms. She required a mobility scooter due to severe orthostatic intolerance, dentures following dental caries caused by xerostomia, digital stimulation for bowel evacuation and experienced frequent pre-syncopal symptoms when straining to void her bladder. Quantitative multi-domain autonomic testing demonstrated widespread autonomic failure. As before, plasma

exchange initially produced improvements in salivary and urinary function only, but after four courses, a return of reflex tachycardia on tilt and Valsalva manoeuvre was observed. After a fifth course, she had a modest transient improvement in orthostatic tolerance but unfortunately developed a deep vein thrombosis treated with 3 months of anticoagulation. We then started high dose oral prednisolone (1mg/kg) and she reported marked symptomatic improvement in orthostatic tolerance, urinary, lacrimal, salivary and pupillomotor symptoms within weeks. Repeat testing at six weeks and three months demonstrated remarkable sustained improvements in orthostatic intolerance ratio (51 to 0.4), heart rate response to deep breathing (0 to 6), pupillary light response (8 to 25% constriction), uroflowmetry profile, saliva production (0.01 to 0.24g/min), average sweat production (7 to 121nL/cm^2/min), total COMPASS-31 scores (50 to 10), SF-36 physical function scores (10 to 100) and gAChR-Ab levels (506 to 220pM) (Fig. 2). She subsequently remained well on a tapering prednisolone regimen with maintenance mycophenolate mofetil with no orthostatic symptoms reported at her latest review 12 months later.

Punch skin biopsies were collected from the left forearm, left thigh and both distal legs prior to her 3rd course of plasma exchange in August 2018, 32 years after disease onset. There was a complete loss of intra-epidermal nerve fibres in all sites sampled, with a very poor subepidermal neural plexus (Fig. 3A). Innervation of the dermal adnexa including the arrector pili muscles, (Fig. 3B, 3C), sweat glands (Fig. 3D) and blood vessels was also markedly reduced. All visualised PGP-immunoreactive fibres showed a strikingly abnormal, fragmented pattern, suggesting ongoing active degeneration in the remaining nerves (Fig. 3A and 3B). There were few fragments of VIP-ir cholinergic (Fig. 3D) and DβH-ir noradrenergic fibres (Fig. 3C). Follow up samples were collected from the left thigh and distal leg, a few millimetres from the original biopsy sites, in

November 2019, 6 months after commencing oral prednisolone therapy. These showed a clear improvement in cutaneous innervation with several new fibres in the subepidermal neural plexus and few fibres reaching the epidermis. Intra-epidermal nerve fibre density was still below 5% cut-off for age and sex, but improved from 0.2/mm in the leg and 0.3/mm in the thigh to 3.9/mm and 4.1/mm.[27] In addition, the morphology of the visualised PGP-ir nerve fibres appeared improved, with resolution of the previously observed fragmentation and derangement of the neural network around the autonomic adnexa (Fig. 3A1 and 3B1). Sudomotor, pilomotor, and vasomotor innervation assessed on sections immunostained with the pan-neuronal marker PGP and selective cholinergic and noradrenergic markers VIP and DβH by a semiquantitative method (4: normal innervation, 3: mild loss of fibres, 2: severe loss of fibres, 1: rare surviving fibres) showed a mean density of 2.5 compared to 1 at the first assessment.

Discussion

We present the most comprehensively phenotyped cohort of patients with seropositive AAG and the largest longitudinal series studying immunotherapy effect to date. Our novel multi-domain autonomic function testing protocol allowed us to comprehensively quantify the breadth of autonomic deficits in patients with AAG and identified key biomarkers measuring cardiovascular, pupillary and salivary function that improved significantly following immunotherapy. Immunotherapy can be associated with significant and potentially life-threatening complications, so quantitative biomarkers are important to objectively assess the effect of immune therapy and guide clinical decision making. Assessing multiple domains increases the sensitivity of detecting early changes with immune treatment, which may reflect potential for more widespread recovery. In our illustrative case, cardiovascular and sudomotor testing was unchanged with initial plasma

exchange, but reproducible improvements in salivary and urinary domains suggested potential reversibility despite her prolonged history. Introduction of high dose oral prednisolone followed by maintenance mycophenolate mofetil resulted in remarkable widespread improvements on autonomic function testing, patient reported outcome measures, and cutaneous innervation. The case highlights that even in longstanding disease, with significant abnormalities of postganglionic innervation, AAG is potentially treatable with adequate immunotherapy.

The striking fragmented pattern of the visualised epidermal and dermal nerve fibres on the baseline skin biopsies has been seen in a previous case report on another patient with longstanding seropositive AAG,[9] who had a limited response to initial immunotherapy (a trial of IVIg) and declined further immune treatment. Larger studies examining skin biopsies in AAG are not currently available, but a study in demyelinating Guillain-Barre Syndrome found 11/20 (55%) patients had abnormal cutaneous innervation at the distal leg 27.0 ± 5.7 days after symptom onset, with reduced epidermal innervation, fragmented subepidermal nerve plexuses and a beaded appearance of dermal nerves suggesting active nerve degeneration.[28] Longitudinal skin biopsy studies suggest interventions in patients with impaired glucose tolerance can be associated with evidence of cutaneous axonal regeneration,[29] but this was uncommon in patients with longstanding diabetes (mean duration 29 ± 9 years), where most individuals had reduced or absent subepidermal plexuses from which intra-epidermal nerve fibres would be expected to regrow.[30] In AAG, the presence of reduced numbers of morphologically abnormal, apparently fragmented dermal nerves, rather than complete denervation, may indicate potential for recovery even with prolonged disease.

While our patients all presented with pandysautonomia, they commonly reported orthostatic intolerance was their most disabling autonomic symptom. Prior to treatment, all patients had marked symptomatic falls in blood pressure on tilt necessitating rapid return to a supine position. After immunotherapy, the fall in blood pressure decreased and time tolerated on tilt increased. Incorporating both these variables, the orthostatic intolerance ratio improved significantly following treatment. The orthostatic intolerance ratio correlated with the severity of orthostatic intolerance and total autonomic symptoms, and physical limitations reported by patients at baseline. Furthermore, following immune treatment, improvements in orthostatic intolerance ratio correlated with improvements in patient reported autonomic symptoms, suggesting it is a responsive and relevant biomarker and potentially useful outcome measure in future treatment trials.

Our multi-domain testing protocol revealed several other clinically important insights relevant to the management of patients with AAG. Prior to immunotherapy, patients commonly had urinary retention and abnormal uroflowmetry profiles reflecting voiding dysfunction and evidence of straining. Straining should be discouraged as it can cause precipitous falls in blood pressure by reducing venous return and may also lead to upper urinary tract damage. Urinary retention increases risk of infections that may exacerbate autonomic failure symptoms. Catheterisation should be considered to ensure regular emptying if post-void residual volumes are persistently >100ml. All our patients demonstrated pupil fatigue, premature redilation during a prolonged light impulse, which appears to be a unique phenomenon only seen in patients with gAChR-Ab positive AAG.[21, 31] Only four (31%) of our cohort had clinically apparent ptosis, but all 11 (100%) who underwent pharmacological pupillary testing demonstrated subclinical bilateral sympathetic

deficits. Symmetrical sympathetic pupillary deficits can be difficult to detect clinically, especially if there are co-existing parasympathetic deficits.[20] With a quantitative pupillometry protocol utilising physiological and pharmacological stimuli, we identified sympathetic and parasympathetic deficits in 12/13 (93%) patients. The profound impairments across cardiovascular, pupillary, urinary, sudomotor and secretomotor domains consistently documented in our patient cohort appears to be a phenotypic signature for AAG patients with high titres of ganglionic antibodies. The characteristic phenotype of cholinergic autonomic failure with orthostatic hypotension and other manifestations of sympathetic failure is consistent with the underlying pathophysiology of antibody-mediated impairment of synaptic transmission at sympathetic and parasympathetic autonomic ganglia.[4, 32, 33] Furthermore, we found preserved postganglionic pupillary function in assessments performed early in the disease course and normal dynamic sweat testing in a patient who had received prompt immunotherapy, suggesting earlier immunotherapy may help reduce postganglionic denervation. We advocate initiating induction immunotherapy regimens using plasma exchange, IVIg and/or steroids to achieve disease remission as soon as possible, and then quickly introducing maintenance therapies to reduce requirements for ongoing invasive treatments.

We found a high incidence of other autoimmune diseases (62%) including inflammatory bowel disease (23%), antecedent infections (31%) and surgical instrumentation (15%) in our cohort, as previously reported.[1, 34] Abdominopelvic instrumentation and inflammation may expose splanchnic autonomic ganglia and induce an aberrant immune response in susceptible individuals. Following our patients up over several years allowed us to identify occult malignancies in two patients several years after presentation, and may have contributed to the higher rate of

malignancies observed in our series compared to previous reports (38% v. 17-20%).[1, 34] A deterioration after previously well-controlled disease should prompt repeat screening for possible malignancy.

Five patients were deceased at the time of this study, but we were still able to gather comprehensive retrospective clinical and autonomic data and supplement information by direct correspondence with local physicians. The remaining eight patients were all prospectively evaluated with our full autonomic protocol. We only had longitudinal skin biopsy samples before and after immune therapy for one patient in our cohort but aim to prospectively study more patients with longitudinal samples to assess for changes in autonomic and somatic cutaneous innervation. As the largest Autonomic Unit in the UK, we were in an ideal position to study this rare disease, but it is possible that some patients with milder disease were solely managed by local teams and not referred to our centre. A referral bias may mean that the patients we studied were on the more severe end of the disease spectrum.

In summary, we found objective evidence of severe and widespread autonomic failure in multiple domains in patients with seropositive AAG, with significant improvements after immunotherapy. Quantitative testing with validated autonomic biomarkers should be used to define initial deficits, guide clinical management and monitor treatment response. Early improvements in some domains may reflect potential for more widespread recovery with modifications in immunotherapy. Clinically meaningful functional and pathological recovery is possible even in longstanding disease. The orthostatic intolerance ratio shows promise as a responsive and relevant quantitative

biomarker in AAG and may also be useful in other autonomic diseases with emerging treatment options.

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Author Contributions

SK, EV, FB, JNP, BL, MPL, LW, MN, AV, CJM and VI contributed to the conception and design of the study. SK, EV, FB, JNP, BL, LW, LJ, VP, MN, AV and VI contributed to acquisition and analysis of data. SK, EV, FB, JNP, BL, LW, GTI, EMH, PM, LJ, VP, MN, AV, CJM and VI contributed to drafting the text and preparing the figures.

Potential Conflicts of Interests

The authors report no conflicts of interests.

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Figure Legends

Figure 1. Quantitative multi-domain autonomic function testing and ganglionic antibody testing. Patients had A) cardiovascular autonomic testing, B) pupillometry, C) urinary, D) sudomotor, lacrimal and salivary testing and E) ganglionic AChR-Ab testing performed before and after immune treatment. A) Patients underwent passive head up tilt to 60^{o}. Top panel: stable blood pressure and heart rate profile in a healthy individual. Bottom panel: rapid fall in blood pressure with no compensatory heart rate rise in a patient with autonomic failure. B) Pupil diameters were recorded with infrared pupillometry, capturing dark diameters and responses to light and pharmacological stimuli. C) Patients were asked to void into a uroflowmeter when they had a sensation of a full bladder. Post-void residual volume was then measured with bladder ultrasound scan. D) Dynamic sweat testing was used to assess post-ganglionic sudomotor function. Left panel: even distribution of active sweat glands in a patient with AAG who received immunotherapy within 3 months. Right panel: significantly impaired sweat production and markedly reduced numbers of active sweat glands in a patient with AAG who first received immunotherapy 23 years after disease onset. Schirmer's test and the unstimulated salivary production test were used to measure lacrimal and salivary production (not illustrated). E) A radioimmunoprecipitation assay was used to test for ganglionic AChR-Abs at the University of Oxford. This graph illustrates gAChR-Ab levels for 108 referred sera showing the 15 positive sera (all >200 pM, indicated by dotted line); 60 additional sera were reported <100 pM but not quantified further.

Figure 2. Serial multi-domain autonomic testing in a patient with AAG illustrating response to high dose oral prednisolone therapy. Before treatment, when the patient's gAChR-Ab level was 506pM, her blood pressure rapidly fell with head up tilt (A), which had to be terminated early after 1 minute (*) due to symptoms of cerebral hypoperfusion. Three months after oral prednisolone therapy (gAChR-Ab titre 220pM), she was able to tolerate a full 10 minutes of head up tilt without a fall in blood pressure. There was a marked increase in heart rate response to deep breathing (B). Uroflowmetry profile (C) before treatment was abnormal with a prolonged void time and intermittent flow. After treatment, urine flow was smooth and void time reduced. Saliva production (D) and pupillary constriction to a light impulse (E) both increased greatly after treatment.

Figure 3. Punch skin biopsies demonstrating severe cutaneous somatic and autonomic denervation (A, B, C, D) with improvement following immunotherapy (A1, B1, C1 and D1). Initial biopsies revealed a loss of cutaneous nerve fibres, with a highly abnormal and distinctive fragmented staining pattern. The subepidermal neural plexus was poor and there was a lack of intraepidermal nerve fibres (A). A severe loss of nerves fibres supplying arrector pili muscles was evident using protein gene product 9.5 (PGP) as pan-neuronal marker (B) and a complete denervation is shown using dopamine-beta-hydroxylase (DβH) as specific noradrenergic marker (C). A severe loss of vasoactive-intestinal-peptide-immunoreactive (VIP-ir) cholinergic fibres around a sweat gland was evident (D). After immune therapy, there was marked improvement in both nerve fibre density and morphology (A1, B1, C1 and D1). Arrows in B, B1, C, C1 point to arrector pili muscles. Arrowheads in D and D1 point to sweat glands. Scale bar = 100 μm.

Supplementary Figure 1. Summary of diagnoses for patients with cardiovascular autonomic failure and ganglionic antibody testing performed between February 2005-August 2019. This diagram illustrates the clinical diagnosis at the most recent review (up to January 2020) for the 168 patients with documented cardiovascular autonomic failure and ganglionic antibody testing performed between February 2005-August 2019.