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**Autoimmune antibodies to collagen XIII in myasthenia gravis patients**

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### **Autoimmune antibodies to collagen XIII in myasthenia gravis patients**

**ABSTRACT:** *Introduction:* Myasthenia Gravis (MG) is a neuromuscular junction (NMJ) disorder caused by autoantibodies against NMJ proteins. Collagen XIII is a muscle-derived transmembrane protein required for NMJ maturation. The objective of this study is to explore existence of autoantibodies to collagen XIII in MG patients. *Methods:* Seventy MG patient sera and 61 human healthy controls were screened for collagen XIII autoantibodies by enzyme-linked immunosorbent assay (ELISA). The collagen XIII autoantibody-positive sera were further analyzed by a cell-based assay together with western blotting and immunofluorescence staining of cells expressing recombinant collagen XIII. *Results:* Five of the 70 MG patients were found to have autoantibodies against collagen XIII. All the five patients were young women with no or low levels of autoantibodies to acetylcholine receptor (AChR). *Discussion:* Collagen XIII is associated with MG but it is unknown if the collagen XIII autoantibodies are pathogenic.

**Key words:** autoantigen; autoimmune antibody; autoimmune disease; collagen XIII; myasthenia gravis; neuromuscular junction

## Autoimmune antibodies to collagen XIII in myasthenia gravis patients

### INTRODUCTION

Myasthenia Gravis (MG) is an autoimmune disease mainly caused by antibodies to the neuromuscular junction (NMJ) components.<sup>1,2</sup> These antibodies influence the neuromuscular transmission by three mechanisms: they damage the postsynaptic membrane with the help of complement,<sup>3</sup> they cause antigenic modulation resulting in NMJ protein endocytosis<sup>4</sup> and more rarely, they directly block the effect of acetylcholine.<sup>5</sup> These effects lead to weakness and fatigue in skeletal muscles. Autoimmune antibodies against muscle AChR are detected in 80-90% of MG patients.<sup>6</sup> In the remaining patients ~50% have autoantibodies to muscle specific kinase (MuSK), lipoprotein-related protein 4 (LRP4), agrin, titin, or ryanodine receptor (RyR).<sup>7-11</sup> A small number of patients are seronegative for these known autoimmune antibodies.

Collagen XIII is a member of a collagen subgroup termed MACITs (Membrane-Associated Collagens with Interrupted Triple-helices), composed of a short cytosolic domain, a single transmembrane domain and a largely collagen ectodomain.<sup>12-14</sup> The ectodomain can become proteolytically shed and it has been shown to interact with other extracellular matrix (ECM) molecules.<sup>15</sup> Genetically-engineered mouse models have suggested requirements for collagen XIII in the maturation of NMJ.<sup>16</sup> Mutations in *COL13A1* cause congenital myasthenic syndrome (CMS) type 19 showing ptosis, limb hypotonia, neck and bulbar weakness, and dysmorphic features.<sup>17</sup>

The objective of this study was to identify autoimmune antibodies against collagen XIII in unselected MG patients.

### MATERIALS AND METHODS

Seventy MG patients were recruited consecutively in Karolinska University Hospital Solna, Sweden. Six patients had their blood samples collected twice after the onset of MG, and two patients had three blood samples. The MG diagnosis was based on abnormal muscle fatigability tests according to the International Classification of Diseases (ICD) code ICD-10:G70.0 and a decrement

on 3 Hz repetitive nerve stimulation. AChR antibodies and human leukocyte antigens (HLA) were tested.<sup>6,18</sup> Nineteen of 70 patients were AChR-antibody-seronegative. Written informed consent was obtained according to hospital ethics and the study was approved by the hospital Ethics Committee. Sera from 61 controls were collected in Oulu University Hospital, Finland. These individuals did not have MG symptoms defined in ICD-10 code G70.0, thyroid-associated ophthalmopathy, multiple sclerosis, rheumatoid arthritis or cancer. They were age and gender matched with the MG patients.

Collagen XIII autoantibodies were screened by a modified ELISA protocol. Recombinant collagen XIII ectodomain,<sup>15</sup> 0.2 µg/ml in Tris-buffered saline (TBS), was coated onto 96-well MaxiSorp plates (Thermo Scientific) at 4°C overnight. Thereafter the plates were blocked with 5% fat-free milk in TBS at room temperature for 3 hours. After removing the blocking solution, human serum pre-diluted 1:10 in the blocking solution were incubated with the coated protein at 4°C overnight. Horseradish peroxidase (HRP)-conjugated anti-human IgG (Jackson ImmunoResearch) together with its chromogenic substrate TMB X-tra (KEM-EN-TEC Diagnostics) were used for detection at 450 nm with Infinite M1000 PRO (Tecan). Different batches of testing were normalized with a collagen XIII monoclonal antibody.<sup>15</sup> The data were analyzed with GraphPad Prism 7.02 (GraphPad Software).

All the ELISA-positive sera (ColXIII<sup>+</sup>) and a few of the negatives and controls were further studied with a cell-based assay and western blotting. Sera diluted 1:50 in TBS containing 0.01% Tween-20 (TBS-T) and 5% fat-free milk were incubated in Protein A/G coated plates (Thermo Scientific) at 4°C overnight. Conditional media from Chinese hamster ovary (CHO) cells stably expressing collagen XIII tagged with enhanced green fluorescence protein (EGFP)<sup>14</sup> were then incubated with the protein A/G-captured serum IgG proteins at 37 °C for 1 hour. Cells expressing EGFP were used as negative controls. The collagen XIII-EGFP captured on the plates was quantified by green fluorescence detection with an Infinite M1000 PRO (Tecan). The data were analyzed with GraphPad Prism 7.02 (GraphPad Software). After fluorescence measurement, all the proteins captured on the plate were extracted with SDS-PAGE loading buffer (Bio-Rad), and applied to SDS-PAGE and western blotting detected by a rabbit anti-GFP antibody (Thermo Scientific) followed with

HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) and Western Bright ECL reagents (Advansta). Digital imaging was performed with LAS-3000 (GE Life Sciences).

CHO cells over-expressing collagen XIII-EGFP were used for immunofluorescence staining. Cells cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (Biowest) were fixed with methanol at -20°C for 10 min and blocked with 5% milk/PBS for 2 hours followed by 2% donkey serum/PBS for 1 hour. Human sera were diluted 1:10 in 2% donkey serum and incubated with the specimens at 4°C overnight. Goat anti-human Fab (Jackson ImmunoResearch) and AlexaFluor 568-conjugated donkey anti-goat IgG (Thermo Scientific) were used subsequently for immunofluorescence detection. The cell nuclei were stained with Hoechst 33342 (Thermo Scientific). Specimens were imaged with a FluoView FV 1000 (Olympus) confocal microscope using a 60X objective.

## RESULTS

ELISA screening of collagen XIII antibody in MG patients and controls is summarized in Fig. 1 A. By using the cut-off from mean of controls + 3x standard deviation (SD), 5 patients (all women) but none of the 61 controls were collagen XIII-antibody-seropositive (Fig. 1 A, Table 1). The average age at MG onset of the 5 positive patients was 25 years and for the 65 ELISA-negative 45 years. Four of the five patients had been thymectomized, all showing microscopically thymic hyperplasia. Four of them had HLA-B8 antigen. Three of them were AChR-antibody-seronegative and two had low levels of AchR-antibody. Four patients had generalized MG and one had mainly ocular symptoms. One woman had had severe MG including swallowing difficulty and breath shortness. None had immunomodulating treatments. In the whole cohort of patients 7.1% (5/70) were collagen XIII-antibody-seropositive, and 2.9% (2/70) were double positive for AChR- and collagen XIII-antibodies. Conversely, 15.8% (3/19) of AChR-antibody-seronegative patients in this series were collagen XIII-antibody-seropositive. There were no differences in phenotype of the myasthenics who were collagen XIII-antibody-seropositive compared to those who were AChR-antibody-seropositive only or double seronegative.

In order to study the specificity of the autoantibodies to collagen XIII, we next tested the sera with collagen XIII-EGFP shed in the conditional CHO cell medium. All the five collagen XIII-antibody-positive sera were able to capture collagen XIII-EGFP showing a band at ~100 kDa in western blotting (Fig. 1 B). One sample with a lower titer (between mean + 2x SD and mean + 3x SD) also showed affinity to collagen XIII. No visible bands were detected from the control and one collagen XIII-antibody-seronegative sample (Fig. 1 B).

Immunofluorescence staining showed that the collagen XIII autoantibodies in the MG patient sera (ColXIII<sup>+</sup>/AChR<sup>-</sup> and ColXIII<sup>+</sup>/AChR<sup>+</sup>) recognized mostly the mature collagen XIII located on the cell membrane and in the ECM (Fig. 2 A & C). The control (ColXIII<sup>-</sup>/AChR<sup>-</sup>) did not detect collagen XIII in the staining (Fig. 2 B). The sample with collagen XIII-antibody-seronegative but AChR-antibody-seropositive (ColXIII<sup>-</sup>/AChR<sup>+</sup>) showed some non-specific signals localized mostly inside the cells, but the mature collagen XIII in the medium was not stained (Fig. 2 D).

## DISCUSSION

Collagen XIII has been detected in various human tissues but with generally low expression levels.<sup>19-24</sup> Early studies report collagen XIII autoantibodies in patients with thyroid-associated ophthalmopathy, in whom autoantibodies target orbital fat, extraocular muscles and/or connective tissues.<sup>25-27</sup> The present data provide information highly relevant in view of the reported occurrence and functional significance of collagen XIII in mouse models and human CMS.<sup>16,17</sup> In addition, our recent studies suggested that collagen XIII is located at the functional unit of NMJ, binds acetylcholinesterase-associated collagen (ColQ) and contributes to the distribution pattern of acetylcholine esterase at the NMJ.<sup>28</sup> Therefore we speculate that a putative pathogenic mechanism of collagen XIII autoantibodies might be hindering the interaction between collagen XIII and other synaptic basal lamina components and consequently destabilizing the neuromuscular synapse.

Collagens have a tendency to aggregate and show ambiguous avidity.<sup>15,29</sup> To avoid false positive results, the ELISA conditions were optimized by reducing the amount of collagen XIII used

for coating and by improving the blocking efficiency. Due to the low amount of coating protein the detection signal was relatively low, which might cause false negatives. We noticed that a serum under the cut-off in ELISA was positive in a cell-based assay with Protein A/G coated plates combined with western blotting.

MG patients may develop other autoimmune diseases such as systemic lupus erythematosus, Sjögren's syndrome, thyroiditis, or multiple sclerosis.<sup>30</sup> Earlier reports indicated that autoantibodies against collagen XIII were detected in patients affected with thyroid-associated ophthalmopathy.<sup>25-27</sup> Based on these, one would suspect that collagen XIII antibodies are related to multiple autoimmune diseases or linked to ocular symptoms. In the present study, only one patient with collagen XIII-antibody-seropositive had mainly ocular MG. Studies with a larger number of patient samples are needed to draw further conclusions.

This study demonstrated collagen XIII as a putative antigen in MG patients. In the future studies measuring collagen XIII autoantibodies with a larger number of samples from patients with MG, other autoimmune and neurological diseases, and concomitant testing of MuSK-, LRP4-, agrin-, titin-, and RyR- autoantibodies should be considered to allow for identification of patient groups with distinct combinations of autoantibodies. In addition, follow-up studies should be performed for the patients who are collagen XIII-antibody-seropositive vs seronegative, in order to clarify whether the presence of the autoantibodies is correlated with immune system malfunction or with structural muscle damage.

## **Abbreviations**

AChR, acetylcholine receptor; CHO, Chinese hamster ovary; ColQ, acetylcholinesterase-associated collagen; CMS, congenital myasthenic syndrome; ECM, extracellular matrix; EGFP, enhanced green fluorescence protein; ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen; HRP, horseradish peroxidase; ICD, International Classification of Diseases; LRP4, lipoprotein-related protein 4; MACITs, Membrane-Associated Collagens with Interrupted Triple-helices; MG, myasthenia gravis; MuSK, muscle specific kinase; NMJ, neuromuscular junction; PBS, phosphate-buffered saline; RyR, ryanodine receptor; SD, standard deviation; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing Tween-20



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## Figure Legends

**FIGURE 1.** Detection of collagen XIII autoimmune antibodies in MG patient sera. **(A)** ELISA test for sera from MG patients (n = 70) and healthy controls (HC, n = 61) using collagen XIII-coated plates. The solid lines present the mean values of the MG and control samples respectively. The dashed line indicates mean of control sera + 3x SD, and the dotted line presents mean of control sera + 2x SD. **(B)** Immuno-recognition of EGFP-tagged collagen XIII (ColXIII-EGFP) by its autoantibodies. The positive control band ColXIII-EGFP is detection of recombinant EGFP-tagged human collagen XIII with an anti-GFP antibody. The ELISA results of collagen XIII autoimmune antibodies are presented with a detection value above mean of controls + 3x SD (+), mean + 2x SD (+/-), or under mean + 2x SD (-). The serum antibody titer to AChR was listed as “+” or “-”.

**FIGURE 2.** Detection of collagen XIII in CHO cells overexpressing collagen XIII-EGFP by autoantibodies in sera. Representative immune-fluorescence staining results are shown. **(A)** Seropositive for collagen XIII but negative for AChR in ELISA. **(B)** Seronegative for both collagen XIII and AChR. **(C)** Seropositive for both collagen XIII and AChR. **(D)** Seronegative for collagen XIII but positive for AChR. Green = EGFP signals from recombinant collagen XIII-EGFP fusion protein. Red = collagen XIII recognized by human serum autoantibodies and detected with goat anti-human Fab and Alexa Fluor 568-conjugated donkey anti-goat IgG. White arrowheads indicate collagen XIII in the cell and ECM. Blue = cell nuclei staining with Hoechst 33342. Bars = 40  $\mu$ m.

**Table 1.** Information on MG patients with collagen XIII autoantibodies

Sample ID	Gender	Age of onset (years)	AChR ab (nmol/L)	MG type	Thymic histology	Other autoantibodies
MG2320*	woman	21	0	generalized	hyperplasia	N.A.
MG2374*	woman	28	0	generalized	hyperplasia	N.A.
MG2552*	woman	33	0	mainly ocular	N.D.	N.A.
MG3056*	woman	21	3	generalized	hyperplasia	N.A.
MG3059*	woman	22	1.8	generalized	hyperplasia	anti-thyroglobulin ab

\*antibody titer > mean of healthy controls + 3x SD, no immunomodulating treatments before serum testing

N.D. not done

N.A. data not available

Figure 1

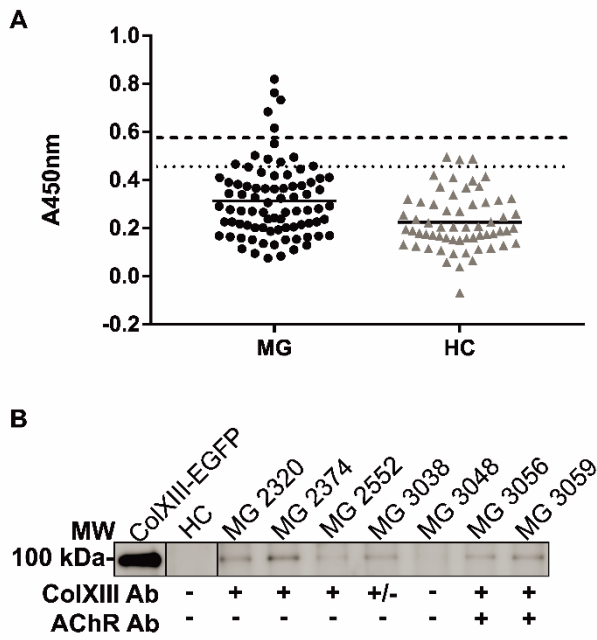


Figure 2

