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SHORT COMMUNICATION

Complement activation by IgM autoantibodies linked to immune-mediated neuropathies depends on C2

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Abstract

Background and purpose: Complement factor C2 is a potential therapeutic target in immune-mediated neuropathies. However, literature suggests that classical complement pathway activation may proceed to C3 in the absence of C2, a so-called "C2 bypass." Here, we evaluated a C2 bypass mechanism during complement activation by pathogenic human IgM from patients with immune-mediated neuropathies.

Methods: IgM autoantibodies from 51 patients with multifocal motor neuropathy (MMN) or anti-myelin-associated glycoprotein (MAG) neuropathy (AMN) were used to activate complement in ex vivo disease models. C2 bypass was evaluated using C2-depleted (C2D) serum and a therapeutic anti-C2 antibody.

Results: In two different disease models of MMN, IgM anti-GM1 and IgM anti-GM2 autoantibodies from MMN patients were bound to induced pluripotent stem cell-derived motor neurons and Schwann cells, respectively, and fixed C3 upon incubation with fresh serum. C3 fixation was inhibited by anti-C2 and did not occur with C2D serum. Similarly, in an AMN model, IgM anti-MAG antibodies were incubated with fresh serum fixed C3, which in all cases was abrogated in the absence of C2 or in the presence of anti-C2.

Conclusions: In ex vivo disease models of MMN and AMN, complement activation by IgM autoantibodies from 51 patients was in all cases dependent on C2 and was inhibited by an antihuman C2 antibody. No evidence of a C2 bypass mechanism was found.

KEYWORDS
antibodies, complement, IgM, immune-mediated neuropathies

INTRODUCTION

Multifocal motor neuropathy (MMN) and anti-myelin-associated glycoprotein (MAG) neuropathy (AMN) are prototypical immune-mediated peripheral neuropathies caused by IgM autoantibodies. In MMN, which is an inflammatory motor polyneuropathy resulting in progressive asymmetrical distal muscle weakness of the extremities, the ganglioside GM1 is proposed to be the main antigen for IgM autoantibodies. IgM anti-GM1 antibodies can be detected in serum in approximately 40%–60% of patients with MMN using current standard diagnostic procedures [1]. In AMN, which is a predominantly demyelinating sensory–motor neuropathy characterized by distal weakness and sensory ataxia, MAG is the main autoantigen [2].

The induced pluripotent stem cell-derived motor neuron (iPSC-MN) model for MMN confirmed patient-derived IgM anti-GM1 antibodies activate complement [3, 4]. Studies of sural and peripheral nerve biopsies of AMN patients also support that complement activation is important in the pathogenesis of IgM-mediated neuropathies [5, 6].

IgM activates complement via the classical pathway (CP) and possibly via the lectin pathway (LP) [7, 8]. Both pathways activate C4 and C2, which in turn activate C3. To prevent pathologic activation of CP and LP, C2 constitutes an attractive therapeutic target because of its relatively low plasma concentration and its position upstream of the complement factors that mediate inflammatory damage. Moreover, inhibition of C2 does not affect the alternative pathway (AP), thus leaving part of the antimicrobial defense capabilities of complement intact. However, several studies suggest that under certain conditions CP or LP activation can proceed to the activation of C3 in the absence of C2, a so-called "C2 bypass" [9–13]. It is unclear whether such a C2 bypass may occur during pathologic complement activation in human disease.

The aim of this study was to investigate a potential C2 bypass during complement activation induced by human pathogenic IgM autoantibodies.

METHODS

We used MMN or AMN as a model employing sera from 51 patients with documented IgM autoantibodies in ex vivo models. Serum samples were obtained from 33 MMN patients who fulfilled the diagnostic criteria for definite, probable, or possible MMN according to European Federation of Neurological Societies/Peripheral Nerve Society guidelines [14] (University Medical Center Utrecht [UMCU] Ethical Committee approval 14-528), and from 18 patients who met the criteria for AMN [15] (UMCU Ethical Committee approval 16-177). These patients were selected from larger cohorts based on seropositivity for circulating IgM anti-GM1, IgM anti-GM2, or IgM anti-MAG antibodies, and on availability of serum.

The iPSC-MN model for MMN [3, 4] was used to evaluate complement activation by IgM anti-GM1 autoantibodies from 25 MMN patients. Each serum was heat-inactivated and used to opsonize iPSC-derived MNs. Subsequently, opsonized cells were incubated with 15% IgG/IgM-depleted serum as complement source.

Previously, we have shown that some MMN sera contain IgM anti-GM2 autoantibodies that bind to Schwann cells (SCs), activate complement, and are associated with early onset of MMN [16]. We utilized this model to further investigate IgM-mediated complement activation in detail.

To investigate complement activation in a disease model of AMN, we adapted the ImmuGlo anti-MAG IFA kit. Primate peripheral nerve slides were incubated with heat-inactivated serum from AMN patients, and then with IgG/IgM-depleted serum as complement source to minimize background complement activation due to species incompatibility.

In all three models, C2 bypass was studied using human serum depleted for C2 (C2D serum) or human serum supplemented with a therapeutic anti-C2 antibody, which specifically inhibits the formation of the classical/lectin pathway C3 convertase [4, 17], as complement source. Complement activation was assessed at the level of C3 fixation using specific detection antibodies. Additionally, the complement source was supplemented with EDTA, which inhibits complement activation, to check for nonspecific binding of C3 to the cells, and with MgEGTA to assess the contribution of AP to C3 fixation.

RESULTS

In the iPSC-derived MN model for MMN, C3 fixation (representative images presented in Figure 1a) was higher with cells opsonized with MMN sera than with those opsonized with healthy control sera ($n = 26$; upper limit represented as dashed line in Figure 2a), and strongly correlated with their IgM anti-GM1 titers. C3 fixation was expressed as the ratio of C3 fixation to the cells with no inhibitors added to the complement source and that observed with EDTA added ($ratio_{C3/EDTA}$; Figure 2a). The AP did not contribute to the observed C3 fixation to cells opsonized with MMN serum, as there was no significant difference between the EDTA and MgEGTA controls (Figure 2b). Twenty-one patients were tested for a potential C2 bypass with anti-C2 supplementation, and eight with C2 depletion (four patients in both methods); these were selected because of serum availability. There was no difference in C3 fixation when the complement source was supplemented with anti-C2 compared to the negative EDTA control (Figure 2b), showing no C2 bypass. This was supported by results obtained with C2D serum as source of complement. Reconstitution of C2D serum with plasma-derived C2 restored C3 fixation (Figure 2c), although incompletely, reflecting that presumably the C2 depletion procedure had affected the intrinsic complement activation capacity of this serum.

Using this SC model, we investigated the C2 dependency of IgM anti-GM2-mediated C3 fixation. From the UMCU MMN cohort, sera from 10 MMN patients who were positive for IgM anti-GM2 antibodies were used to opsonize SCs, which were then incubated with pooled normal human serum and assessed for C3 fixation. C3 fixation to SCs opsonized with MMN sera correlated significantly with IgM binding (Figure 2d). C3 fixation was completely inhibited by EDTA, whereas in presence of MgEGTA some residual C3 fixation was found, suggesting a minor contribution of the AP to the observed activation. Addition of anti-C2 to the complement source abrogated C3 fixation to that in presence of MgEGTA, consistent with the notion that inhibition of C2 does not affect AP activation (Figure 2e). With C2D serum as complement source, C3 fixation was inhibited to a similar level as that by MgEGTA. Reconstitution of C2D serum with plasma-derived C2 restored complement activation to approximately 40%–50% (Figure 2f), similarly as observed in the iPSC-MN model (Figure 2c).

To further expand our findings in disease models for immune-mediated neuropathies, we investigated complement activation

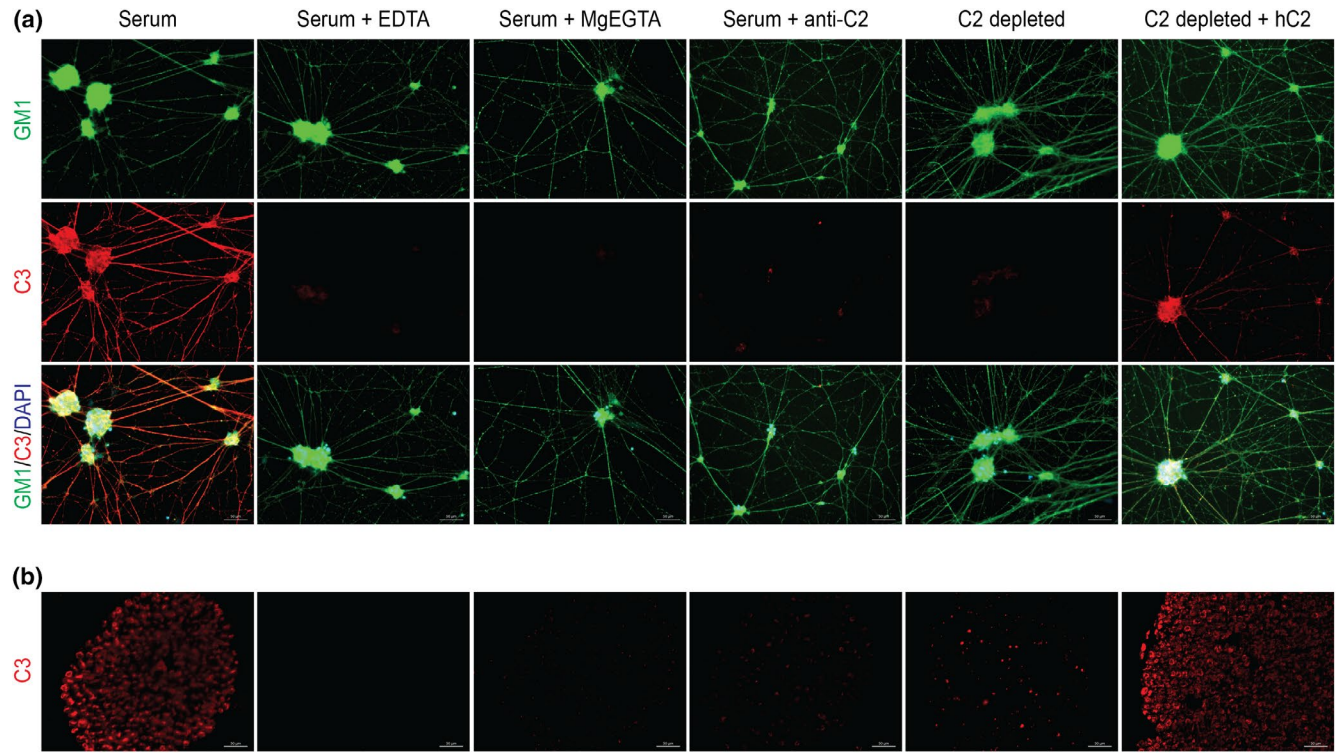

FIGURE 1 Complement activation in microscopy-based disease models for multifocal motor neuropathy (MMN) and anti-myelin-associated glycoprotein neuropathy (AMN). Representative images are shown for the complement-activation assays in disease models for MMN (a) and AMN (b). C3 fixation (red channel) is measured after opsonization with heat-inactivated patient sera and incubation with different complement conditions (complement active serum, serum + EDTA, serum + MgEGTA, serum + anti-C2, C2-depleted serum, or C2-depleted serum reconstituted with plasma-derived human C2 [hC2]). GM1 (green channel) was used as counterstain for the induced pluripotent stem cell-derived motor neuron experiments. The bottom row in panel a depicts the overlay of the individual channels (GM1, green channel; C3: red channel; 4,6-diamidino-2-phenylindole [DAPI], blue channel) depicted in the upper and middle rows. Scale bars indicate 50 μM.

in a disease model for AMN (representative images are depicted in Figure 1b). Increased C3 fixation was found when AMN sera ($n = 18$) were used for opsonization compared to healthy control sera ($n = 12$; upper limit depicted as a dashed line in Figure 2g), which correlated with IgM anti-MAG titers (Figure 2g). AP did not contribute to this fixation, as MgEGTA inhibited C3 fixation similarly as EDTA. Pretreatment of complement active serum with anti-C2 abrogated C3 fixation similarly as EDTA (Figure 2h), indicating that complement activation by IgM anti-MAG antibodies was dependent on C2. This was confirmed when C2D serum was used as complement source (Figure 2i).

DISCUSSION

MMN and AMN are immune-mediated neuropathies in which IgM autoantibodies are thought to play a major role in the pathogenesis of the disease. Not all MMN patients are seropositive for anti-GM1 antibodies, and large differences in seropositivity are described between MMN patient cohorts, presumably due to methodological differences in detection methods [1]. Interestingly, using the iPSC-MN model for MMN, patients negative for IgM anti-GM1 antibodies measured via enzyme-linked immunosorbent assay-based methods seem to have complement-activating IgM antibodies [3]. Additionally, in both MMN and AMN, evidence supports a role for CP-mediated complement activation, initiated by IgM autoantibodies upon binding to their respective targets, GM1 or MAG [1, 2]. For MMN, three clinical trials investigating CP inhibition are ongoing (NCT05225675, NCT05405361, and NCT06537999). A bypass at any level in CP-mediated complement activation could potentially result in a loss of therapeutic efficacy of the investigated drug. Therefore, we investigated in this study whether a C2 bypass, that is, direct activation of C3 by activated C1s or mannose binding lectin/ficolin-associated serine proteinases, could be triggered by human pathogenic IgM autoantibodies involved in MMN and AMN.

Most data on a C2 bypass have been found in the context of LP-mediated complement activation and often with purified proteins, specific activators, matrixes, or prolonged incubation times [9–13]. To what extent such a bypass mechanism occurs in human pathology remains to be established. We used ex vivo models that mimic presumed pathogenic in vivo mechanisms of these diseases as much as possible. Our data demonstrate that in these established ex vivo disease models of MMN or AMN, complement activation by IgM antiantibodies from in total 51 patients did in all cases depend on C2 and could be dose-dependently inhibited by an antihuman C2

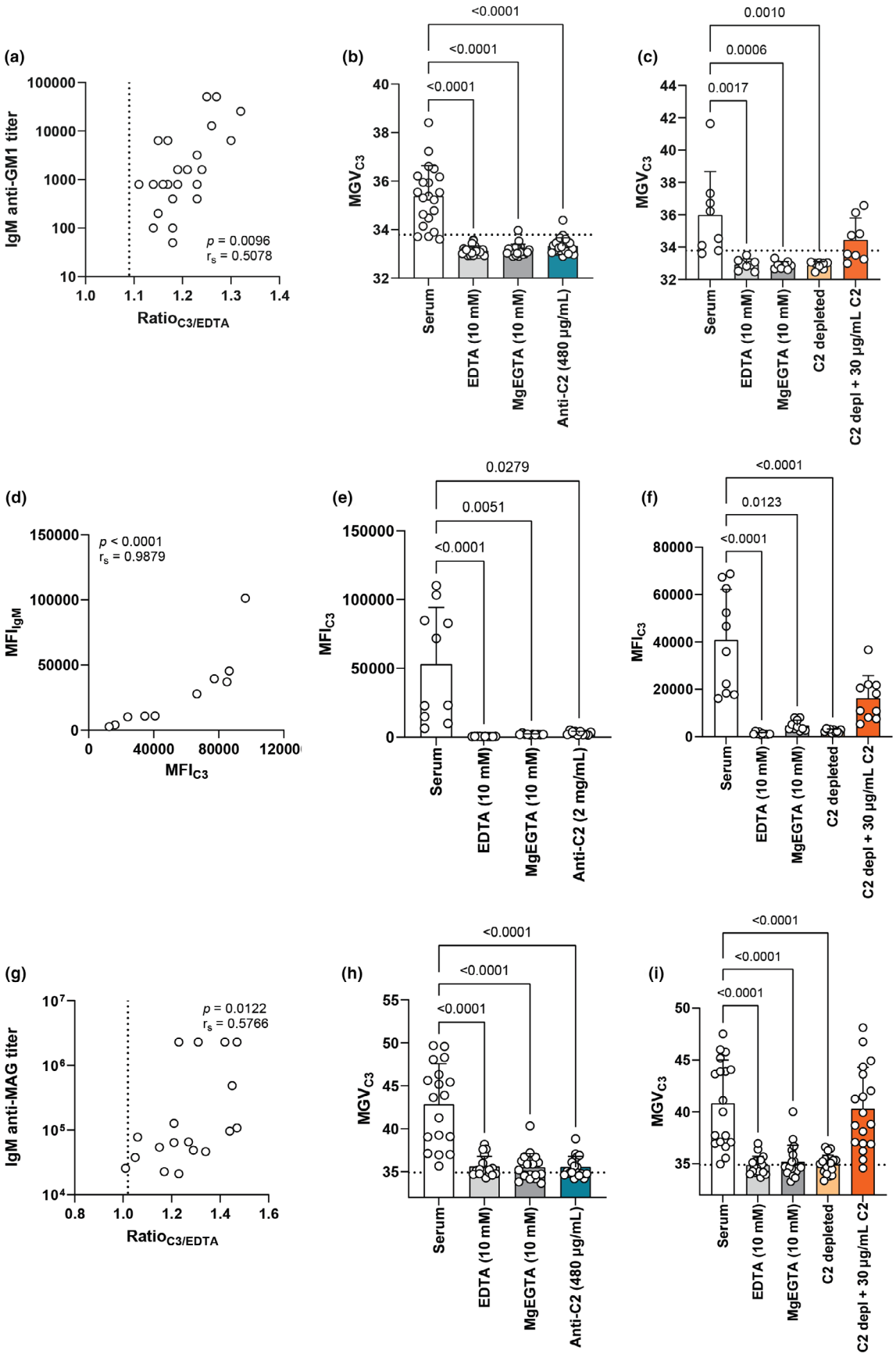

FIGURE 2 C2 dependency of complement activation in disease models for IgM-mediated neuropathies. (a) Correlation between C3 fixation on induced pluripotent stem cell-derived motor neuron opsonized with multifocal motor neuropathy serum (depicted as $Ratio_{C3/EDTA}$) and IgM anti-GM1 titer. Dashed line represents C3 fixation after healthy control (HC) serum opsonization. (b) C3 fixation is inhibited by anti-C2 (blue) to the levels of the EDTA (light gray) and MgEGTA (dark gray) control. (c) C3 fixation is dependent on C2 and does not occur in presence of C2D serum (light orange). Supplementation of C2D serum with purified C2 partially restores C3 fixation (dark orange). (d) IgM anti-GM2 binding correlates with C3 fixation. (e) IgM anti-GM2-mediated complement activation is inhibited by anti-C2 (blue) to the levels of the EDTA (light gray) and MgEGTA (dark gray) control. (f) C3 fixation is abrogated when C2-depleted serum (light orange) is used as complement source, which is partially restored by addition of purified C2 in physiologically relevant concentrations (dark orange). (g) Slides of primate peripheral nerve were incubated with heat-inactivated serum from anti-myelin-associated glycoprotein (MAG) neuropathy patients, and then with human serum as complement source. Results are expressed as $Ratio_{C3/EDTA}$. Dashed line represents C3 fixation when opsonized with HC sera. IgM anti-MAG units correlate with complement activation. (h) IgM anti-MAG-mediated complement activation is inhibited by anti-C2 (blue) to the levels of the EDTA (light gray) and MgEGTA (dark gray) control. (i) C3 fixation is completely abrogated upon use of C2D serum (light orange) as complement source. Reconstitution with C2 (dark orange) restores C3 fixation to the level of the serum control. Mean + SD. For a, d, and g: Spearman rank correlation. For b, c, e, f, h, and i: Kruskal–Wallis test with Dunn multiple comparisons test. MFI: mean fluorescent intensity, MGV: mean gray value.

antibody. No evidence for a C2 bypass mechanism that substantially contributed to the observed complement activation was found. We therefore conclude that complement activation in relevant disease models for IgM-mediated neuropathies is fully dependent on C2. Future studies should reveal whether these findings can be extrapolated to other pathologic conditions in which activation via CP or LP plays a role. Additionally, residual complement activation in clinical studies using complement inhibitors should be monitored closely, to identify potential bypasses in complement activation.

AUTHOR CONTRIBUTIONS

Kevin Budding: Conceptualization; formal analysis; writing – original draft. **Kim Dijkxhoorn:** Methodology; writing – review and editing. **Elisabeth de Zeeuw:** Methodology; writing – review and editing. **Lauri M. Bloemenkamp:** Methodology; writing – review and editing. **W. Ludo van der Pol:** Conceptualization; formal analysis; writing – original draft. **Nicolette C. Notermans:** Conceptualization; formal analysis; writing – review and editing. **Monique C. Minnema:** Conceptualization; formal analysis; writing – review and editing. **Jeanette H. W. Leusen:** Conceptualization; formal analysis; writing – review and editing. **C. Erik Hack:** Conceptualization; formal analysis; writing – original draft. **Inge Van de Walle:** Conceptualization; formal analysis; writing – original draft.

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CONFLICT OF INTEREST STATEMENT

K.B. was an employee of the UMCU Utrecht on a research collaboration agreement with argenx when this study was conducted. Currently, K.B. is an employee of and has equity ownership in argenx. K.D., E.d.Z., and L.M.B. are employees of UMC Utrecht on a research collaboration agreement with argenx. W.L.v.d.P. serves on the scientific advisory board for SAB SMA Europe; provides ad hoc consultancy for argenx, Biogen, and Novartis genetherapies; is the local principal investigator for the ARDA (NCT05225657) and ARDA+ (NCT05405361) trials; and receives research support from the Prinses Beatrix Spierfonds, Vriendenloterij, and Stichting Spieren voor Spieren. N.C.N. reports no disclosures. M.C.M. receives research support from Beigene and provides consultancy for Jansen Cilag. J.H.W.L. reports no disclosures. C.E.H. provides consultancy services to argenx. I.V.d.W. is employee of and has equity ownership in argenx.

DATA AVAILABILITY STATEMENT

Anonymized data and documentation of this study will be shared upon reasonable request from any qualified researcher. Standard data sharing agreements apply.

ETHICS STATEMENT

The Medical Ethical Committee (METC) of University Medical Center Utrecht approved the collection of patient sera as part of national cross-sectional studies for MMN (METC protocol 14-258) and AMN (METC protocol 16-177). Written informed consent was obtained from all study participants prior to inclusion in this study.

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