Clinical features in Guillain-Barré syndrome with anti-Gal-C antibody.

Makoto Samukawa¹, Yukihiro Hamada¹, Motoi Kuwahara¹, Kazuo Takada¹, Makito Hirano¹, ², Yoshiyuki Mitsui¹, Masahiro Sonoo³, Susumu Kusunoki¹* and the GBS epidemiological Study Group#.

¹ Department of Neurology, Kinki University Faculty of Medicine, Osaka-sayama, Japan
² Department of Neurology, Sakai Hospital Kinki University Faculty of Medicine, Sakai, Japan
³ Department of Neurology, Teikyo University Faculty of Medicine, Tokyo, Japan

# Apendix

*To whom correspondence should be addressed at:
Department of Neurology, Kinki University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka Japan, 589-8511
E-mail: kusunoki-tky@umin.ac.jp
Tel: 072-366-0221 ext.3552; Fax: 072-368-4846

Running title: GBS with anti-Gal-C antibody

Key words: galactocerebroside, Guillain-Barré syndrome, electrophysiological study, demyelinating neuropathy, axonal neuropathy, anti-glycolipid antibody, mycoplasma pneumoniae

All authors reviewed the final report and consented to publish.

Word count: abstract 146 words, main text 2423 words.
4 figures are included.

Acknowledgements

The authors thank Mayumi Motoyama and Satoko Aoto for their technical assistance
Study Funding

This study was partly supported by Health and Labour Sciences Research Grants from Ministry of Health, Labor, and welfare of Japan (Research on intractable diseases, H23-017) to SK and Grants-in-Aid for Scientific Research (24390225 and 24110518 to SK) from Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure

None of the authors and group has conflicts of interest related to this report.
Abstract

Introduction:
Guillain-Barré syndrome (GBS) has often been associated with antibodies to glycolipids, such as galactocerebroside (Gal-C), a component of myelin. Whether patients who have GBS with anti-Gal-C antibody (Gal-C-GBS) more often have demyelinating neuropathy or axonal neuropathy remains controversial. Their clinical features have also been unestablished.

Methods:
We enrolled 47 patients with Gal-C-GBS. Their clinical and electrophysiological data were retrospectively reviewed and compared to 119 patients with GBS without anti-Gal-C antibody (non-Gal-C-GBS).

Results:
Demyelinating polyneuropathy occurred 4 times more frequently than axonal polyneuropathy in patients with Gal-C-GBS, but without statistical significance compared to patients with non-Gal-C-GBS (2.2 : 1). Patients with Gal-C-GBS had more frequent sensory deficits, autonomic involvements, and antecedent Mycoplasma pneumoniae (MP) infection than patients with non-Gal-C-GBS.

Conclusions:
This is the largest study clarifying the clinical and electrophysiological findings that more frequent sensory deficits, autonomic involvements, and antecedent MP infection are associated with Gal-C-GBS.
**Introduction**

Guillain-Barré syndrome (GBS) has often been associated with anti-glycolipid autoantibodies, such as antibodies against galactocerebroside (Gal-C), a component of myelin. Consistent with its localization, sensitization with Gal-C caused demyelinating neuropathy in rabbits\(^1\),\(^2\). In addition, a small-scale clinical study suggested that patients with GBS with anti-Gal-C antibodies (Gal-C-GBS) tended to have demyelinating neuropathy\(^3\), without statistical analyses. In contrast, a patient with axonal neuropathy and anti-Gal-C antibodies have also been reported\(^4\).

The pathogenesis of GBS is thought to involve an autoimmune mechanism induced by infections\(^5\),\(^6\), such as *Campylobacter jejuni*, cytomegalovirus and *Mycoplasma pneumoniae* (MP)\(^7\),\(^8\). Anti-glycolipid antibodies, probably produced by molecular mimicry, are considered an important factor in the pathogenesis of a subset of GBS. GBS with anti-Gal-C antibodies are frequently preceded by an MP infection\(^6\). MP has recently gained attention as a pathogen since outbreaks of MP pneumonia emerged in rural areas and in a Navy ship in the US\(^9\),\(^10\). Specific symptoms or severity of GBS have been associated with certain anti-glycolipid antibodies. For example, anti-GD1b antibody is associated with ataxic sensory neuropathy\(^11\),\(^12\). Anti-GM1 and anti-GalNAc-GD1a antibodies are positive in pure motor GBS\(^13\). Anti-GQ1b antibody is associated with oculomotor palsy and ataxia and is a factor predictive of mechanical ventilation\(^14\),\(^15\),\(^16\). Whether or how anti-Gal-C antibody is related with specific symptoms or electrophysiological findings remains unknown.

We investigated clinical and electrophysiological features of patients with Gal-C-GBS compared with the data of patients with GBS without Gal-C antibody (non-Gal-C-GBS). This is the largest study to assess Gal-C-GBS.

**Patients and Methods**

**Patients**

We collected clinical information and serum samples from 47 consecutive patients with Gal-C-GBS at several medical institutions including our own hospitals in response to requests to measure anti-glycolipid antibodies from July 2006 to July 2012. All patients signed an informed consent for utilization of personal data, storage and assay of biological materials for research purposes and were tested for anti-glycolipid antibodies.
in our hospital.

We also collected clinical information and serum samples from 119 patients with non-Gal-C-GBS from a GBS epidemiological study group of Japan for comparison. This study consisted of a multi-center cohort prospective survey of GBS conducted by the Research Committees of Neuroimmunological Diseases sponsored by the Ministry of Health, Labor and Welfare, Japan. Twenty-three hospitals collected all patients with GBS between August 2007 and July 2010. In this period, 222 patients with GBS were treated at these hospitals. One hundred and eighty-eight of all 222 patients signed an informed consent for utilization of personal data, storage and assay of biological materials for research purposes. Serum anti-Gal-C antibody was examined in 121 of those 188 patients. We used 119 patients with GBS who were confirmed to be negative for Gal-C antibody.

Forty-seven patients with Gal-C-GBS (25 men and 22 women, age 48.3 ± 25.3 yr [mean ± SD]) and 119 patients with non-Gal-C-GBS (70 men and 49 women, age 44.8 ± 17.8 yr [mean ± SD]) fulfilled the clinical criteria for GBS\(^{[17]}\). The difference in regional population between patients with Gal-C-GBS and non-Gal-C-GBS was statistically analyzed, but it was not significant (p=0.952).

**ELISA**

Anti-glycolipid antibodies in sera (40 times dilution) were examined as described previously, using GM1, GM2, GM3, GD1a, GD1b, GalNAc-GD1a, GD3, GT1b, GQ1b, and Gal-C as antigens\(^{[6]}\), \(^{[18]}\). Because this study focused on anti-Gal-C antibody, antibodies against GM3, GD3, or GT1b, of which positive ratio in total was less than 5\%, were not analyzed in 10 of 119 patients with non-Gal-C-GBS. In our previous study, the cut-off value of anti-Gal-C antibody was set at 0.2. However, small part of controls or patients with non-immunological diseases had been found to have the value of 0.2 – 0.4. Thus, we defined 0.4 or more of Gal-C antibody titer (corrected optical density: cOD) to be positive in this study.

**Clinical and Electrophysiological features**

We investigated clinical features of 47 patients with Gal-C-GBS to compare with 119 patients with non-Gal-C-GBS. We compared age, gender, cranial nerve involvement, sensory involvement, ataxia, autonomic disorders, disabilities, previous infection and
anti-MP antibody levels in serum between both groups. MP antibody was also measured with the use of a particle agglutination (PA) method (normal <40), which mainly detect IgM class antibodies indicative of a recent infection. We confirmed 320 times or more of this antibody titer to be positive. Disabilities were evaluated using the functional grading scale established by Hughes et al\[19\]. The Hughes grade (HG) at the time of peak disability was calculated. Nerve conduction studies (NCS) were performed using conventional procedures. Motor NCS were performed on the median, ulnar, posterior tibial and deep fibular nerves; sensory NCS included stimulation of the median, ulnar, and sural nerves. Compound muscle action potential (CMAP), motor nerve distal latency (MDL), motor nerve conduction velocity (MCV), sensory nerve conduction velocity (SCV) and sensory nerve action potential (SNAP) were measured. We defined the value as abnormal if it was outside the normal laboratory range, corrected for age. We assessed the patients on the basis of the electrodiagnostic criteria reported previously\[20], [21].

**Statistical analyses**

Differences in proportions were tested using the \( \chi^2 \) test or Fisher’s exact test. Differences in each nerve between patients with Gal-C-GBS and non-Gal-C-GBS were examined by the Mann-Whitney \( U \) test, because the data were not normally distributed. Linear regression analyses were performed to determine the relation between cOD of Gal-C antibodies and severity. We also compared severities between patients with different subclasses of anti-Gal-C antibodies. We considered P values <0.05 to be significant. Statistical calculations were performed using SPSS V.2.0 (IBM Japan).

**Standard protocol approvals, registration and patient consent.**

This study was approved by the institutional review board of Kinki University. All individuals enrolled in this study gave written informed consent.
Results

Results of ELISA

Of 47 patients with Gal-C-GBS, six had single IgM anti-Gal-C antibody, 38 patients had single IgG anti-Gal-C antibody, three had IgM and IgG anti-Gal-C antibodies.

We found 12 patients with other glycolipid antibodies in patients with Gal-C-GBS. One had IgM anti-GM1 antibody, three of them had IgG anti-GM1 antibodies, two had IgG anti-GD1a antibodies, two had IgG anti-GD1b antibodies, one had IgG anti-GD3 antibody, two had IgG anti-GT1b antibodies, four had IgG anti-GQ1b antibodies, and one had IgG anti-GalNAc-GD1a antibody (some had multiple antibodies).

Of 119 patients with non-Gal-C-GBS, 77 patients (65%) had at least one of the anti-glycolipid antibodies (28 were positive for multiple antibodies): 10 had IgM anti-GM1 antibodies, 24 had IgG anti-GM1 antibodies, six had IgM anti-GM2 antibodies, five had IgM anti-GD1a antibodies, 17 had IgG anti-GD1a antibodies, five had IgM anti-GD1b antibodies, 13 had IgG anti-GD1b antibodies, three had IgG anti-GD3 antibodies, one had IgM anti-GT1b antibody, two had IgG anti-GT1b antibodies, 12 had IgG anti-GQ1b antibodies, 11 had IgM anti-GalNAc-GD1a antibodies, and 24 had IgG anti-GalNAc-GD1a antibodies.

Subtype of neuropathy

We analyzed the electrophysiological data between 41 patients with Gal-C-GBS (the electrophysiological data of six patients were not available) and 119 patients with non-Gal-C-GBS. The electrophysiological subtypes were summarized in Figure 1A and B. Gal-C-GBS is more frequently associated with demyelinating neuropathy than axonal neuropathy on either criterion. The rates of demyelinating neuropathy are higher and the rates of axonal neuropathy are lower in Gal-C-GBS than in non-Gal-C GBS, although there were no significant differences. We could not identify any significant correlations of the subclass and the titer of Gal-C antibody with electrophysiological subtype.

We compared electrophysiological recordings of different nerves between patients with Gal-C-GBS and non-Gal-C-GBS. In the motor nerve conduction study, MDLs were more prolonged in patients with Gal-C-GBS than in those in non-Gal-C-GBS at the median, ulnar and deep fibular nerves. No significant differences were observed in CMAPs and MCVs. In the sensory nerve conduction study, SCVs and SNAPs
decreased more in patients with Gal-C-GBS than in those with non-Gal-C-GBS at the median, ulnar and sural nerves. The representative electrophysiological recordings were shown in Figure 2.

As mentioned above, 12 patients with Gal-C-GBS had other glycolipid antibodies. The electrophysiological data were available in nine patients. Five patients (56%) had a demyelinating condition, four (44%) had equivocal, none had axonal or a normal category based on Hadden’s criteria in these patients. When applying Ho’s criteria, five patients (56%) had AIDP, and four (44%) had unclassified, none had AMAN or normal and inexicitable. There were no significant differences compared to the Gal-C antibody single positive group in clinical and electrophysiological features on either criterion.

**Clinical features**

We analyzed the clinical features between the 47 patients with Gal-C-GBS and 119 patients with non-Gal-C-GBS. We summarized clinical data in Figure 3. There were no significant differences in the age and sex between both groups. Sensory nerve involvement was observed in 41/47 (87%) of patients with Gal-C-GBS but in 79/119 (66%) of patients with non-Gal-C-GBS (p=0.012). Autonomic disorders were observed significantly more often 13/47 (28%) in patients with Gal-C-GBS than in those with non-Gal-C-GBS 10/119 (8.4%) (p<0.01). For patients with Gal-C-GBS, the Hughes grade was 3.43±1.18 [mean ± SD] at the peak time of the clinical course. For patients with non-Gal-C-GBS, the Hughes grade was 3.35±1.12 [mean ± SD] at the peak time. We did not observe significant differences in disability between both groups. A linear regression analysis between cOD of Gal-C antibodies and severity revealed no significant relation. Severities did not differ between patients with different subclasses of anti-Gal-C antibodies (not shown). We summarized infection data in both groups in Figure 4. Respiratory infection was observed significantly more often 35/47 (74%) in patients with Gal-C-GBS than in those with non-Gal-C-GBS 32/119 (27%) (p<0.01). There were more MP infections in 20/47 (43%) of patients with Gal-C-GBS than that in 2/103 (1.9%) of patients with non-Gal-C-GBS (Data of only in 103 patients with non-Gal-C-GBS were available).

Of patients with Gal-C-GBS with autonomic disturbances, five patients had bladder and rectal disturbance, two had dyshidrosis, one had hypertension, one had fluctuating blood pressure and six had orthostatic hypotension (some had multiple disorders).
Discussion

This study demonstrates that demyelinating polyneuropathy occurs 4 times more frequently in patients with Gal-C-GBS than axonal polyneuropathy based on the Ho’s criterion. This ratio was higher than the ratio (2.2 : 1) in patients with non-Gal-C-GBS. In addition, no case in Gal-C-GBS was axonal neuropathy based on the Hadden’s criterion. It is consistent with the idea that anti-Gal-C antibody causes damage to myelin, where Gal-C is present. However, the difference is not statistically significant because demyelination is a major electrophysiological feature in GBS as a whole. Nevertheless, a small part of patients still had AMAN by Ho’s criterion. The reason for development of AMAN in Gal-C GBS remains unknown. A previous report by others suggested that the concomitant presence of other anti-glycolipid antibodies such as anti-GM1 antibody might have been more pathogenic in GBS than that of Gal-C\[^4\]. In this study, nine patients with Gal-C-GBS also had other glycolipid antibodies including anti-GM1 antibody in three patients. None of the nine patients had axonal neuropathy, electrophysiologically. Additional unknown factors may contribute to determination of AIDP or AMAN in such cases.

Examination of signs and symptoms in this study revealed that sensory deficits were significantly more frequent in patients with Gal-C-GBS. One earlier study by others also described that Gal-C-GBS tended to show more sensory deficits and paresthesia as compared to non-Gal-C-GBS\[^3\]. In this study we newly provided the positive ratio of the sensory deficits and their supportive electrophysiological findings with statistical significance. Electrophysiological data shown here indicate that variables of all tested sensory nerves were worse in patients with Gal-C-GBS than in those with non-Gal-C-GBS. By contrast, most variables in motor nerves did not significantly differ between patients with Gal-C-GBS and those with non-Gal-C-GBS. These results are consistent with the reported experimental finding that serum from rabbits inoculated with Gal-C damaged the dorsal root ganglia and caused sensory involvement\[^22\]. This idea seems in conflict with the predominant occurrence of demyelination in Gal-C-GBS, because damage in the dorsal root ganglia may cause axonal neuropathy, but not demyelinating neuropathy. However, the current electrophysiological diagnostic criteria place importance on the presence of demyelination. They do not define axonal neuropathy even with apparent axonal damage when demyelination is found in two or more nerves\[^20\],\[^21\]. Thus, damage in the
dorsal root ganglia might be concomitantly present with demyelinating neuropathy as in the rabbit model\textsuperscript{[22]}. Further studies are needed to clarify the mechanism for these clinical and electrophysiological abnormalities in human conditions.

We also found more frequent autonomic disturbances in patients with Gal-C-GBS than in those in non-Gal-C-GBS, though their detailed information was not available in this retrospective study. Autonomic manifestations are generally present in cardiovascular, sudomotor, gastrointestinal and other systems involving both sympathetic and parasympathetic fibers\textsuperscript{[23], [24], [25]}. An earlier study by others about AIDP, the most common subtype in Gal-C-GBS, demonstrated cardio-sympathetic hyperactivity, excessive or reduced sudomotor function and preserved skin vasomotor function. In contrast, AMAN did not have marked autonomic involvement without sudomotor hypofunction observed in severely impaired patients\textsuperscript{[26]}. The vagal nerve, which has a long myelinated course within the preganglionic parasympathetic system, may be susceptible to damage induced by demyelinating disorders such as AIDP unlike the sympathetic nerve, which are unmyelinated except for the short preganglionic segment\textsuperscript{[25]}. Because impairment of vagal efferent fibers shifts the sympato-vagal balance toward the predominantly sympathetic, AIDP frequently have cardio-sympathetic hyperactivity. Because autonomic disturbance is a critical factor in managing GBS patients, clinicians should be aware of the differences in the patterns of autonomic involvement versus the subtype of GBS and provide appropriate care.

Previous reports by us and others described the presence of anti-Gal-C antibody in sera from patients with GBS subsequent to MP infection\textsuperscript{[3], [4], [6], [8], [18]}. A Gal-C-like antigen is suggested to be present in MP, indicative of molecular mimicry between a major myelin glycolipid, Gal-C and MP\textsuperscript{[6]}. Our data show that MP as an antecedent infection occurred much more frequently in patients with Gal-C-GBS than in those with non-Gal-C-GBS. Our results support the relationship between MP infection and production of Gal-C antibody. On the other hand, 57% of patients with Gal-C-GBS did not have an MP infection. Mechanisms of antibody production in patients without MP infection are unclear, but we speculate the presence of unknown pathogenic organism with a Gal-C-like structure may be involved. Other possibilities include low sensitivity of the MP antibody measurement, since previous report describe that the sensitivities of MP antibody measured with the use of PA method were 56.1%, when cut off values for PA titers is set at 1:320\textsuperscript{[27]}. Thus, we might have overlooked false-negative patients.
Further serological studies are needed to clarify the mechanism of antibody production in patients without MP infection.

Anti-Gal-C antibody is associated with other neurological disorders, such as acute disseminated encephalomyelopathy (ADEM)\textsuperscript{[28]}. Our previous work demonstrates that the presence of anti-Gal-C antibody is a marker of steroid resistance and poor outcomes in patients with ADEM. We therefore evaluated outcomes of Gal-C-GBS. The results show no significant difference in outcomes as compared to non-Gal-C-GBS. We speculate that different roles of anti-Gal-C antibody in ADEM and GBS or host factors may account for distinct outcomes in these diseases.

In conclusion, Gal-C-GBS is more frequently associated with demyelinating neuropathy than axonal neuropathy. Sensory involvement and autonomic disturbances occurred significantly more frequently. We provided additional evidence that MP infection is more frequent in Gal-C-GBS. Further studies should be performed to clarify the pathogenetic roles of anti-Gal C-antibody in GBS.
References


[10] Sliman JA, Metzgar D, Asseff DC, Coon RG, Faix DJ, Lizewski S. Outbreak of acute respiratory disease caused by Mycoplasma pneumoniae on


Figure legends

Figure 1.
A. Electrophysiological subtypes of Gal-C-GBS and non-Gal-C-GBS based on Hadden’s criteria
   No patient had axonal and normal, 22 (54%) had demyelinating, 19 (46%) had equivocal category in patients with Gal-C-GBS, whereas 10 patients (8%) had axonal, 58 (49%) demyelinating, 46 (39%) equivocal, five (4%) normal in patients with non Gal-C GBS. No significant differences were observed on this criterion.

B. Electrophysiological subtypes of Gal-C-GBS and non-Gal-C-GBS based on Ho’s criteria
   Of Gal-C-GBS, five patients (12%) had AMAN, 20 (49%) had AIDP, and 16 (39%) had unclassified, none patients had normal and inexicitable. In non-Gal-C-GBS, 23 patients (19%) were classified as AMAN, 51 (43%) as AIDP, 45 (38%) as unclassified, none as normal and inexicitable. We did not observe no significant differences on this criterion.

Figure 2. Electrophysiological recordings of different nerves in Gal-C-GBS and non-Gal-C-GBS
   We compared the electrophysiological recordings of different nerves between Gal-C-GBS and non-Gal-C-GBS. In motor nerve conduction study, MDLs were more prolonged in Gal-C-GBS than in the non-Gal-C-GBS at the median nerve. No significant differences were observed in CMAPs and MCVs at the median and tibial nerves. In sensory nerve conduction study, SCVs and SNAPs decreased more in Gal-C-GBS than in non-Gal-C-GBS at the median and sural nerves.

Figure 3. Clinical features of Gal-C-GBS and non-Gal-C-GBS
   We analyzed the clinical features between the 47 Gal-C-GBS and 119 non-Gal-C-GBS. Sensory nerve involvements were observed in 41/47 (87%) of the Gal-C-GBS but in 79/119 (66%) of the non-Gal-C-GBS (p=0.012). Autonomic disorders were observed significantly more often 13/47 (28%) in the Gal-C-GBS than in the non-Gal-C-GBS 10/119 (8.4%) (p<0.01). No significant differences were observed
in the remaining findings.

**Figure 4. Summary of antecedent symptoms and *Mycoplasma pneumoniae* infection in Gal-C-GBS and non-Gal-C-GBS**

Respiratory infection was observed significantly more often 35/47 (74%) in the Gal-C-GBS than 32/119 (27%) in the non-Gal-C-GBS (p<0.01). There were more MP infections in 20/47 (43%) of the Gal-C-GBS than that in 2/103 (1.9%) of the non-Gal-C-GBS (p<0.01. Of non-Gal-C-GBS group, only 103 patients’ data were available).
Author contributors

1. Design or conceptualization of the study.
2. Acquisition of data.
3. Analysis or interpretation of the data.
4. Drafting or revising the manuscript for intellectual content.

Dr. Makoto Samukawa has contributed to 1, 2 and 3.
Dr. Yukihiro Hamada has contributed to 2 and 3.
Dr. Motoi Kuwahara has contributed to 2 and 3.
Dr. Kazuo Takada has contributed to 2.
Dr. Makito Hirano has contributed to 4.
Dr. Yoshiyuki Mitsui has contributed to 3 and 4.
Dr. Masahiro Sonoo has contributed to 2 and 3.
Dr. Susumu Kusunoki has contributed to 1 and 4.
The GBS epidemiological Study Group contributed to 2.
Appendix

Members of GBS epidemiological study group of Japan

1. Tomihiro Imai MD,PhD. (Sapporo Medical University Department of Neurology Site Investigator)
2. Yoshikazu Ugawa MD,PhD.(Fukushima Medical University Department of Neurology, Site Investigator)
3. Yuriko Yoshikawa MD,PhD. (Japanese Red Cross Narita Hospital Department of Neurology, Site Investigator)
4. Haruyuki Hiraga MD,PhD.(Chiba Rosai Hospital Department of Neurology, Site Investigator)
5. Ken-ichi Kaida MD,PhD. (National Defense Medical College Department of Neurology, Site Investigator)
6. Takanori Yokota MD,PhD (Tokyo Medical and Dental University Hospital of Medicine Department of Neurology, Site Investigator)
7. Yasuo Terao MD,PhD (The University of Tokyo Department of Neurology, site investigator)
8. Sousuke Takeuchi MD,PhD (National Center for Global Health and Medicine Center Hospital Department of Neurology, Site Investigator)
9. Mieko Ogino MD,PhD (Kitazato University Department of Neurology, Site Investigator)
10. Gen Sobue MD,PhD (Nagoya University Hospital Department of Neurology, Site Investigator)
(Haruki Koike)
11. Yasuhiro Kojima MD (Ijinkai Takeda General Hospital Department of Neurology, Site Investigator)
12. Yusaku Nakamura MD,PhD (Kinki University Sakai Hospital Department of Neurology, Site Investigator)
13. Nobuo Kohara MD,PhD (Kobe City Medical Center General Hospital Department of Neurology, Site Investigator)
14. Kazuhide Ochi MD,PhD (Hiroshima University Department of Neurology, Site Investigator)
15. Sadatoshi Tsuji MD,PhD (University of Occupational and Environmental Health Department of Neurology, Site Investigator)
16. Jun-ichi Kira MD,PhD (Kyushu University Department of Neurology, Site investigator)
17. Kaoru Matsunaga MD,PhD (Juryo Group.Kumamoto Kinoh Hospital Department of Neurology, Site Investigator)