Monitoring the M-protein of multiple myeloma patients treated with a combination of monoclonal antibodies: the laboratory solution to eliminate interference

Abstract

Objectives: The therapeutic monoclonal antibody (t-mAb) daratumumab, used to treat multiple myeloma (MM) patients, interferes with routine, electrophoretic based M-protein diagnostics. Electrophoretic response assessment becomes increasingly difficult when multiple t-mAbs are combined for use in a single patient. This is the first study to address the analytical challenges of M-protein monitoring when multiple t-mAbs are combined.

Methods: In this proof-of-principle study we evaluate two different methods to monitor M-protein responses in three MM patients, who receive both daratumumab and nivolumab. The double hydrashift assay aims to resolve t-mAb interference on immunofixation. The MS-MRD (mass spectrometry minimal residual disease) assay measures clonotypic peptides to quantitate both M-protein and t-mAb concentrations.

Results: After exposure to daratumumab and nivolumab, both t-mAbs become visible on immunofixation electrophoresis (IFE) as two IgG-kappa bands that migrate close to each other at the cathodal end of the γ-region. In case the M-protein co-migrates with these t-mAbs, the observed interference was completely abolished with the double IFE hydrashift assay. In all three patients the MS-MRD assay was also able to distinguish the M-protein from the t-mAbs. Additional advantage of the MS-MRD assay is that this multiplex assay is more sensitive and allows quantitative M-protein-, daratumumab- and nivolumab-monitoring.

Conclusions: Daratumumab and nivolumab interfere with electrophoretic M-protein diagnostics. However, the M-protein can be distinguished from both t-mAbs by use of a double hydrashift assay. The MS-MRD assay provides an alternative method that allows sensitive and simultaneous quantitative monitoring of both the M-protein and t-mAbs.

Keywords: daratumumab; hydrashift; immunofixation electrophoresis; mass spectrometry; monoclonal antibody; M-protein; multiple myeloma; nivolumab; serum protein electrophoresis; therapeutic drug monitoring.

Introduction

Therapeutic monoclonal antibodies (t-mAbs) have become increasingly important in the treatment of hematological malignancies [1], including multiple myeloma (MM) [2]. Daratumumab is a fully human anti-CD38 IgG1-kappa mAb, and is approved for the treatment of newly diagnosed [3–5] and relapsed/refractory [6, 7] (RR) MM. Daratumumab has...
direct on-tumor effects, but also immunomodulatory effects through the elimination of CD38-positive immune suppressor cells such as regulatory T-cells [8]. The fully human IgG4-kappa t-mAb nivolumab binds to the immune checkpoint programmed death (PD)-1, which is present on the cell surface of T-cells and NK-cells. Blockade of PD-1 by nivolumab improves the host-anti-tumor immune response in several types of cancer. As a single agent, nivolumab induced durable stable disease in 67% of RRMM [9], but no objective responses were observed. Preclinical studies have shown that immune modulation through dual targeting of CD38 and PD-1 resulted in enhanced anti-MM activity, compared to single targeting [10]. Several phase 2 clinical trials investigating these combinations are ongoing in RRMM (NCT03184194, NCT01592370).

Therapeutic mAbs can appear on electrophoretic scans as small monoclonal bands [11, 12]. In routine diagnostics it may be challenging to distinguish the human(ized) t-mAb from the endogenous M-protein due to co-migration. As a result, the IMWG response criteria have been modified to account for the presence of t-mAb interference [13]. However, co-migration of t-mAb and the endogenous M-protein can result in the inability to accurately assess therapeutic complete responses [11, 14]. Complete response is used in clinical trials as a surrogate for improved outcome, since it is significantly correlated with survival after any given treatment [15, 16]. Electrophoretic interference of t-mAb can be overcome using an antibody targeting the t-mAb to alter the serum protein electrophoresis (SPEP) migration. For daratumumab, a so-called shift-assay has been realized [17, 18]. The commercial version is named the hydrashift assay (Sebia), which was recently FDA-approved to mitigate possible antibody interference [19]. However, this assay is specific for daratumumab and will not eliminate immunofixation electrophoresis (IFE) interference caused by other t-mAbs. Response assessment with electrophoretic M-protein diagnostics will become increasingly difficult when MM patients are treated with a combination of t-mAbs, a strategy that is explored in multiple clinical studies.

Mass spectrometry (MS) has recently been introduced as an alternative method to identify and monitor M-proteins with increased sensitivity compared to routine M-protein diagnostics [20]. Initial publications convincingly show that MS methodology to measure the endogenous M-protein is not affected by t-mAb interference [21–23]. However, this was never shown in patients exposed to more than one t-mAb.

In this proof-of-principle study we evaluate two different methods to monitor M-protein responses in three MM patients, who receive both daratumumab and nivolumab (NCT03184194). We demonstrate that both daratumumab and nivolumab, dosed at therapeutic blood levels, can be detected as two monoclonal bands in serum IFE. The endogenous M-protein can be distinguished from these two t-mAb bands using a double hydrashift assay. In addition, we show that the MS-MRD (mass spectrometry minimal residual disease) assay provides a method that allows sensitive and simultaneous quantitative monitoring of both the M-protein and the t-mAbs.

Materials and methods

Patient samples

Peripheral blood samples were collected from relapsed/refractory MM patients participating in the Nivo-Dara study (NCT03184194). In this study, patients received daratumumab at a dose of 16 mg/kg i.v., according to the approved schedule (weekly during the first eight weeks, then every two weeks for a period of 16 weeks, and every four weeks thereafter), in combination with nivolumab (240 mg i.v. every two weeks during the first six cycles and 480 mg i.v. every four weeks thereafter), with or without daily low-dose cyclophosphamide (50 mg p.o.). To monitor t-mAb fluctuations, blood samples were collected directly prior to the administration of both t-mAbs (pre-infusion) and at the end of the infusion (post-infusion). Serum was separated from whole blood and stored at −20 °C until further analysis. The study site Ethics Committee (METc VUmc, study registration number 2017.159/NL60544.029.17) approved the protocols, which were conducted according to the principles of the Declaration of Helsinki, the International Conference on Harmonization, and the Guidelines for Good Clinical Practice. All patients provided written informed consent.

M-protein diagnostics

Routine serum protein electrophoresis (SPEP) and IFE were performed on the Hydrasys 2 system using the Hydragel 4 IF kit (Sebia). The hydrashift (Sebia) procedure is similar to that of a normal IFE, except that the mouse monoclonal t-mAb antisera (5 g/L) is applied to the gel with an additional applicator. All electrophoretic methods were performed according to manufacturer’s protocol. All components of the hydrashift kit have a stability of at least 24 months. The anti-idiotype antibodies against daratumumab and nivolumab are chemically modified in order to achieve anodic electrophoretic mobility. The increased electrophoretic mobility directs the anti-ideotype antibodies to the 1-region, away from the regions where normal immunoglobulins migrate. The sensitivity and specificity of the hydrashift assay was previously shown by Caillon et al. [24]. Supplementary Figure 1 shows the performance of the nivolumab hydrashift in three sera from healthy controls spiked with nivolumab. For the double hydrashift assay that was performed in this study, an off-protocol procedure was developed in which both anti-daratumumab and anti-nivolumab antisera were simultaneously applied to the gel with the same aforementioned applicator. In addition, serum free light chain (sFLC) measurements were...
performed using FreeLite reagents (The Binding Site) on a BNII nephelometer (Siemens), according to the manufacturer’s protocol.

**Tryptic digestion and LC-MS**

Prior to digestion, samples were reduced for 30 min at 60°C in 60 μL 50 mM ammonium bicarbonate containing 15% v/v acetonitrile, 0.1% Rapigest (Waters, Milford, MA), 10 mM DTT, 10 mM of each stable isotope labelled internal peptide standards (Pepsan B.V., Lelystad, The Netherlands) and 0.12 μL patient serum. After cooling, iodoacetamide was added to 15 mM and incubated for 30 min at 25°C in darkness. After addition of 400 ng trypsin (Promega, Madison, WI) samples were incubated overnight at 37°C. Samples were then acidified with 1.5 μL 25% trifluoroacetic acid, incubated for 30 min at 37°C and centrifuged for 65 min at 20,000 g and 4°C.

**Mass spectrometry, LC-MS measurement**

The samples were measured on a nano-LC system (Ultimate 3000, Thermo Fisher Scientific, Munich, Germany). Five microliter sample was captured on a trap column and separated on a 75 μm ID × 250 mm C18 PepMap column (2 μm, 100 Å pore, Thermo Fisher Scientific). Peptides were eluted with a 30 min gradient of 4%–38% solvent B. Solvent A consisted of 0.1% formic acid in water and solvent B of 0.08% formic acid plus 80% acetonitrile. Peptides were ionized in a nanospray ESI source on an Orbitrap Fusion (Thermo Fisher Scientific, Bremen, Germany). Each sample was measured first with a data dependent acquisition method, then measured with targeted MS on selected peptides of the M-protein and monoclonal antibodies (Table 1) with the following parameters: isolation width of 1.4 m/z, maximum injection time of 246 ms and analyzer resolution of 120,000.

**Mass spectrometry, M-protein peptide selection**

M-protein peptides have been selected based on *de novo* sequencing information with PEAKS Studio (Bioinformatics Solutions Inc., version 6). For the *de novo* search in PEAKS an error tolerance of 5 ppm was applied, the search was performed on specific trypsin cleavages with a maximum of one missed cleavage. A database search was performed against a database of human germline immunoglobulin sequences (IMGT) [25]. With this search, unique clonal peptides are identified with amino acid alterations that are different compared to the immunoglobulin germline. Peptide quantity was assessed by labeled free analysis in Progenesis QI (version 2.0, Waters, Milford, MA). For the selection of M-protein peptides a number of criteria were evaluated: homology to the germline sequence, but at least one amino acid mutation compared to the germline sequence [26]. This is further verified with IgBlast [27]. The isobaric amino acids leucine and isoleucine cannot be distinguished in the dataset and were therefore not scored as mutations.

**Mass spectrometry, protein quantification**

The targeted MS data is imported into Skyline-daily (MacCoss Lab Software, version 20.1.1.175), that visualizes the identified peptide fragments and their contribution to the peptide peak signal. Supplementary Figure 2 shows an example of the chromatograms of one identified clonotypic peptide. Two control sera from a healthy serum were spiked with 5 g/L of intact t-mAb daratumumab or nivolumab. These sera with known concentration of t-mAb were used as calibrators for the quantification of daratumumab and nivolumab in all three patients. The concentration of daratumumab and nivolumab is calculated in every sample based on the ratio between the area of the endogenous peptide and and of the stable isotope labelled (SIL) peptide peak, calculated by Skyline. The M-protein concentration is calculated based on the known M-protein concentration in the baseline sample and the ratio between peptide areas of the baseline sample and the unknown sample of the patient.

**Results**

**Both daratumumab and nivolumab interfere with routine M-protein diagnostics**

To investigate whether the human IgG-kappa nivolumab at therapeutic dosing interferes with M-protein diagnostics using electrophoretic methods, we selected three patients from the Nivo-Dara study, who were treated with the combination of daratumumab and nivolumab. Patient characteristics are shown in Supplementary Table 1. The left panels of Figure 1 show the IFE’s at study entry (i.e. prior to t-mAb

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**Table 1: Selected mAb-specific peptides.**

| Protein          | Peptide sequence | Ig chain     | m/z         | z | HCD collision energy, % |
|------------------|------------------|--------------|-------------|---|-------------------------|
| Daratumumab      | GLEWVSASISSGGGTYYADSVK | IgG-HC       | 735.3551    | 3 | 30                      |
| Nivolumab        | ASGIFSNSGGMHWVR    | IgG-HC       | 825.3963    | 2 | 28                      |
| IgG-kappa       | APNLLYDASNLAEAGVPSR | IgG-HC       | 1,000.5260 | 2 | 28                      |
| M-protein pt 1   | EWVAVAVLYDGŠEIIHYVESVK | Kappa-LC     | 853.0797    | 3 | 28                      |
| IgG-kappa       | AAPPGQPSPSSLGYGASTR | IgG-HC       | 885.4809    | 2 | 28                      |
| M-protein pt 2   | WELGTLFFYDGDTHYADSVK | Kappa-LC     | 836.0397    | 3 | 28                      |
| FLC-kappa      | ASQSVGTNLAWLHPGQAPR  | Kappa-LC     | 761.0661    | 3 | 28                      |
| M-protein pt 3   | LDVMQTSPATLSVSPADR  | Kappa-LC     | 944.4777    | 2 | 28                      |

Mutations in the M-protein peptides compared to the germline sequence are underlined. HCD, higher-energy collisional dissociation; HC, heavy chain; FLC, free light chain; m/z, mass to charge ratio.
administration) for patients 2 and 3. For patient 1 the entry sample was not available and the earliest treatment sample (cycle 2) is shown. Patients 1 and 2 had an IgG-kappa M-protein migrating at the anodal end of the γ-region and patient 3 had an FLC-kappa M-protein migrating at the cathodal end of the γ-region. After exposure to daratumumab and nivolumab (see Table 2 for therapeutic details), two new IgG-kappa bands appear close to each other at the cathodal end of the γ-region in all three patients (Figure 1, right panels). Furthermore, in all three patients we observe therapeutic responses (Table 2).

With two t-mAbs migrating close to each other, the chance increases that the endogenous M-protein co-migrates with the t-mAbs. This hampers accurate quantification of the M-protein using SPEP. Additionally, if a patient with an IgG-kappa or FLC-kappa M-protein responds well to therapy, it is no longer possible to make valid clinical response calls that meet the IMWG criteria for (stringent) complete response ((s)CR). The latter is the case in patient 3 since the FLC-kappa M-protein comigrates with daratumumab and nivolumab. After treatment, it is not possible to properly interpret whether this

![Figure 1: Detection of daratumumab and nivolumab by serum immunofixation electrophoresis. Patients 1 (A) and 2 (B) both have an IgG-kappa M-protein (left panels). After exposure to daratumumab and nivolumab the endogenous M-protein band becomes less pronounced and two new IgG-kappa bands appear close to each other at the cathodal end of the γ-region. (C) At baseline an FLC-kappa M-protein is visible in patient 3 (left panel). Since the M-protein comigrates with daratumumab and nivolumab, it is no longer possible to distinguish the M-protein from the two t-mAbs. The M-protein, daratumumab and nivolumab are highlighted in each panel with respectively green, blue and red arrowheads.](image-url)
patient is IFE negative or positive because of the t-mAb interference.

**Double daratumumab/nivolumab hydrashift assay resolves interference**

A daratumumab-specific hydrashift assay was recently developed to abrogate daratumumab interference (Figure 2). The double hydrashift assay is similar to a normal IFE, except that antisera against daratumumab and nivolumab are applied to the gel using an additional applicator. The antisera bind to the t-mAb and shift both biologics towards the α-region, where they no longer interfere with the interpretation of the M-protein. A remaining band in the region of interest indicates that the IgG-kappa M-protein is still present (Figure 2D, representation of a positive double hydrashift). A negative result in a double hydrashift assay shows the complete disappearance of the M-protein from the γ-region (Figure 2E).

Proof-of-principle of the double daratumumab/nivolumab hydrashift assay is shown for all three patients in Figure 3. When a daratumumab hydrashift assay is performed in patients 1 and 2 (Figure 3A, B, middle panels), we observe that daratumumab-antisera complexes are shifted and two sharp bands, that represent the M-protein and nivolumab, remain visible. With the double daratumumab/nivolumab hydrashift assay (Figure 3A, B, right panels), only the M-protein band remains visible in the γ-region in serum samples from patients 1 and 2. Based on the comparison of the baseline data (Figure 1 left panels) and assessment of therapy response using the double hydrashift assay (Figure 3 right panels) it was concluded that patient 1 achieved a partial response and patient 2 a very good partial response. The added value of the double daratumumab/nivolumab hydrashift assay is most pronounced for patient 3, because of comigration of the M-protein and the t-mAbs (Figure 3B). With a daratumumab hydrashift assay (middle panel), IFE interpretation of the FLC-kappa is still masked by the comigrating nivolumab band. With the double daratumumab/nivolumab hydrashift (right panel) both interfering t-mAbs are shifted away from the γ-region and it is clear that the FLC-kappa M-protein is no longer visible in the IFE. These results, combined with a negative urine IFE and <5% MM cells in the bone marrow, indicate that this patient had achieved a CR.

**MS-MRD assay can simultaneously quantify t-mAbs and M-protein concentrations**

We further investigated whether our previously published MS-MRD assay in which we monitor clonotypic peptides of the M-protein [21, 28], could be expanded with simultaneous quantification of multiple t-mAb. Table 1 provides an overview of the clonotypic peptides and MS-characteristics of both the t-mAbs and the unique M-proteins from the three different patients. In all three patients the M-protein concentrations could be monitored independently of the t-mAb titers (Table 2). The M-protein and both biologics are all measured in one multiplex MS-assay. Additional advantage of the MS-MRD assay is the increased sensitivity.

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**Table 2: Patient-specific therapeutic details and overview of MS results.**

| Pt | Therapy cycle | Administered dose of Dara | Administered dose of Nivo | M-protein type | M-protein concentration | Timing blood sampling | MS results, g/L |
|----|---------------|----------------------------|---------------------------|----------------|-------------------------|----------------------|-----------------|
|    |               |                            |                           |                |                         |                      | M-protein | Dara | Nivo |
| 1  | C2D1          | 1,080 mg                   | 240 mg                    | IgG-κ          | 7.1 g/L                 | Pre                   | 7.1          | 0.43 | 0.13 |
|    | C9D1          | 1,080 mg                   | 480 mg                    | IgG-κ          | 2.4 g/L                 | Pre                   | 2.2          | 0.12 | 0.26 |
| 2  | Baseline      | –                          | –                         | IgG-κ          | 14.5 g/L                | Pre                   | 14.5         | n.d. | n.d. |
| 3  | Baseline      | –                          | –                         | FLC-κ          | ≤1 g/L                  | Pre                   | 0.075        | 0.39 | 0.20 |
| 4  | Baseline      | –                          | –                         | FLC-κ          | <450 mg/L               | Pre                   | 0.050        | 0.46 | 0.18 |
| 5  | Baseline      | –                          | –                         | FLC-κ          | 69 mg/L                 | Pre                   | 0.055        | 0.86 | 0.40 |

*Measured using FLC-assay. Pt, patient; Dara, daratumumab; Nivo, nivolumab; C, cycle; D, day; κ, kappa; n.d., not detected; MS, mass spectrometry.*
for M-protein quantification. The lower limit of electrophoretic M-protein quantification is ~2 g/L [29, 30], in Table 2 it is shown that MS-MRD can monitor mAb concentrations down to 0.12 g/L. This allowed quantitative monitoring of both the M-protein and the t-mAb well beyond the limit of quantification of conventional M-protein diagnostics. The t-mAb MS-titers were always higher in samples taken directly after t-mAb infusion as compared to the MS-titers measured prior to t-mAb infusion (Table 2). The M-protein concentration quantified using MS-MRD closely resembled the M-protein concentration measured with routine M-protein diagnostics. In the two baseline samples, we did not detect any t-mAb MS-signal which is in accordance with the fact that these patients were not yet exposed to daratumumab and nivolumab.

**Discussion**

We demonstrate that daratumumab and nivolumab, dosed at therapeutic blood levels, can both be detected by using serum IFE. Both t-mAbs migrate close to each other to form a broad band at the cathodal end of the γ-region. The combined use of t-mAbs further increases the chance for interference with routine M-protein follow-up that is part of the IMWG criteria to assess treatment response in patients with MM [31, 32]. Incorrect response assessment due to interference in SPEP/IFE assays, most often a misclassification between very good partial response (VGPR) and complete response (CR), may lead to underestimation of prognosis and possible overtreatment of individual patients.

**Figure 2:** Schematic presentation of double IFE Hydrashift Assay.
(A) The endogenous IgG-kappa M-protein (green clonal band) comigrates with the t-mAb’s daratumumab (blue) and nivolumab (red). Interpretation is impossible as all three IgG-kappa monoclonal antibodies accumulate into one broad band. (B) Single daratumumab hydrashift assay, only daratumumab is shifted towards the α-region. Nivolumab band still interferes with M-protein. (C) Single nivolumab hydrashift assay, only nivolumab is shifted towards the α-region. Daratumumab band still interferes with M-protein. (D) The double hydrashift assay causes both the t-mAb bands to shift towards the α-region. A remaining band in the region of interest indicates that the M-protein (green) is still present. (E) A hydrashift negative result shows the complete disappearance of the monoclonal IgG-kappa bands from the γ-region and indicates that the band visible on standard IFE was due entirely to t-mAbs. (F) anti-IgG IFE of daratumumab and nivolumab spiked in saline both at 1 g/L (lane G3); shift reagents only at regular concentration of 5 g/L, this provides no IFE signal since the IFE anti-sera detect only human IgG (G2); daratumumab and nivolumab spiked in saline both at 1 g/L in the presence of shift reagents (G1).
M-protein monitoring is most difficult in patients that respond well to the therapy and have an IgG-kappa or FLC-kappa M-protein that co-migrates with the t-mAb(s). In this situation the endogenous M-proteins do not shift and remain visible. A double hydrashift assay allows IFE M-protein interpretation even in the presence of two t-mAbs. Advantage of the double hydrashift assay is that it can be easily performed using analyzers already in place, since it involves only a simple modification of the routine IFE protocol. A limitation of the daratumumab/nivolumab hydrashift assay is that it will not abrogate interference of other t-mAbs. Therefore, with the introduction of novel (combinations of) t-mAbs, each time new hydrashift assays must be developed, and

![Figure 3: Double IFE Hydrashift Assay to assess therapy response.](image)

During treatment of patients 1 (A) and 2 (B) with daratumumab and nivolumab, the endogenous IgG-kappa M-protein is clearly visible as monoclonal band (left panels). During hydrashift of daratumumab (middle) and daratumumab plus nivolumab (right) the endogenous M-proteins do not shift and remain visible. (C) During treatment of patient 3, the endogenous FLC-kappa M-protein comigrates with the t-mAbs and can’t be distinguished as a separate monoclonal band (left panel). Only the double hydrashift (right) allows correct IFE-interpretation (IFE-negative) of the M-protein. The timepoint of serum sampling is indicated for each patient in the left panel. The M-protein, daratumumab and nivolumab are highlighted in each panel with respectively green, blue and red arrow-heads.
FDA-approved, using antisera specific for these novel t-mAbs. Currently, the daratumumab Hydrashift assay (Sebia) is the only commercially available FDA-approved IFE-assay to mitigate antibody interference [19].

Another limitation of the hydrashift assay is that it is a qualitative assay. In this manuscript we show proof-of-principle that one multiplex MS-MRD assay allows quantitative monitoring of the endogenous M-protein and all the t-mAbs that are dosed simultaneously. The MS-characteristics of t-mAbs that are used for the treatment of MM patients and that have previously been published, can be readily implemented in non-quantitative top-down MS and quantitative bottom-up MS methods [21, 22]. For quantification of M-protein-specific clonotypic peptides, a two-step approach is needed which affects the turn-around-time of the first measurement. From shotgun MS performed on a baseline sample, clonotypic peptides are first identified by aligning the M-protein primary sequence to the most homologous germ-line variable region of the IMGT reference directory (www.imgt.org). For all subsequent samples of that same patient, the M-protein clonotypic peptides and t-mAb specific peptides are measured and quantified using targeted MS [33]. Advantage of this bottom-up MS method is that it allows extremely sensitive mAb measurements down to 1 mg/L. Disadvantage of bottom-up MS is that it is currently not yet widely implemented in clinical laboratories. Because of improved anti-myeloma therapy an increased number of MM-patients reach a status of (stringent) CR [15], and more sensitive methods to measure M-protein are needed to be able to monitor these patients.

In conclusion, routine M-protein diagnostics becomes increasingly challenging now that the first myeloma patients are treated with combinations of t-mAbs. In this study we show that both daratumumab and nivolumab interfere with electrophoretic M-protein diagnostics. In addition, it is expected that combined t-mAb treatments may further improve the rates and depth of therapy-response which requires increased assay-sensitivity to monitor disease activity. To anticipate on possible interference of t-mAbs treatment in M-protein monitoring, it is important that the laboratory specialist is informed on the use of monoclonal antibodies in MM treatment. We here describe two different methods to successfully circumvent t-mAb interference, using double hydrashift assays and MS-MRD assays.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The study site Ethics Committee (METc VUmc, study registration number 2017.159/ NL60544,029.17) approved the protocols, which were conducted according to the principles of the Declaration of Helsinki, the International Conference on Harmonization, and the Guidelines for Good Clinical Practice.

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