STATISTICAL ANALYSIS PLAN

Protocol Title:	**A Double-blind, Randomized, Controlled Clinical Study of the Pharmacokinetics, Pharmacodynamics, Tolerability, and Safety of Multiple Intravenous Injections of BCD-066 (JSC BIOCAD) and Aranesp® (Amgen Europe B.V., the Netherlands) in Healthy Volunteers)**
Protocol Number:	BCD-066-3
Protocol version	1.0
Version Date:	October 02, 2016
SAP version	1.0
SAP version date	October 02, 2016
Name and address of the clinical study sponsor	JSC BIOCAD, Russia Legal address: 34 A, Ulitsa Svyazi, Strelna, Petrodvortsoviy District, Saint Petersburg, Russian Federation, 198515 Postal Address: Petrovo Dalneye, Krasnogorskiy District, Moscow Region, Russian Federation, 143422. Tel.: +7 (495) 992-66-28 Fax: +7 (495) 992-82-98
Name and function of person(s), authorized to sign the protocol and its amendments for the sponsor:	Roman Alexeevich Ivanov, Vice President, Research & Development Address: Petrovo Dalneye, Krasnogorskiy District, Moscow Region, Russian Federation, 143422 Email: ivanov@biocad.ru Tel.: + 7 (495) 992-66-28 (ext. 154)
Name, position, address and telephone number of the Medical Expert assigned by the Sponsor for this Study	Bolsun D.D., Medical Expert, Category II JSC BIOCAD, 34 A, Ulitsa Svyazi, Strelna, Petrodvortsoviy District, Saint Petersburg, Russian Federation, 198515 Email: bolsun@biocad.ru Tel.: +7 (812) 380 49 33 (ext. 745); 7 (911) 137-89-29 e-mail: bolsun@biocad.ru

TABLE OF CONTENTS

1. INTRODUCTION

The Statistical Analysis Plan (SAP) provides a detailed analysis plan and steps of study report preparation for the clinical trial BCD-066-3.

2. GOALS AND OBJECTIVES

2.1. Purpose

To confirm the equivalent pharmacokinetics, pharmacodynamics, safety, and tolerability of multiple IV injections of BCD-066 and Aranesp® in healthy volunteers.

2.2. Objectives

Purpose:

To confirm the equivalent pharmacokinetics, pharmacodynamics, safety, and tolerability of multiple IV injections of BCD-066 and Aranesp® in healthy volunteers.

Study objectives:

- To evaluate and compare the key pharmacokinetic parameters of darbepoetin alfa in the serum after repeated dose intravenous injections of 1 μg/kg BCD-066 (JSC BIOCAD, Russia) and 1 μg/kg Aranesp® (Amgen Europe B.V., the Netherlands) in healthy volunteers.
- To evaluate and compare the key hemoglobin-based pharmacodynamic parameters after repeated-dose intravenous injections of 1 μg/kg BCD-066 (JSC BIOCAD, Russia) and 1 μg/kg Aranesp® (Amgen Europe B.V., the Netherlands) in healthy volunteers.
- To evaluate and compare the safety and tolerability characteristics after repeated-dose intravenous injections of 1 μg/kg BCD-066 (JSC BIOCAD, Russia) and 1 μg/kg Aranesp® (Amgen Europe B.V., the Netherlands) in healthy volunteers.

3. STUDY DESIGN

3.1. Design

Clinical study BCD-066-3 was a double-blind controlled, randomized, parallel-group study of the pharmacokinetics, pharmacodynamics, tolerability, and safety of repeated-dose intravenous BCD-066 and Aranesp®.

The study was planned to include 62 healthy male volunteers (56 active participants and 6 back up subjects to replace dropouts). Before inclusion in the study, all potential participants are

given full information about the study. This information is presented in the Participant Information Sheet. If the potential participant gives his consent to be in the study, he has to sign the Informed Consent Form for participation in a clinical study and then undergo a 14-day screening examination to confirm that he is eligible for the study.

After passing the screening and being considered eligible for the study, subjects are centrally randomized at a 1:1 ratio to one of two treatment groups (#1 and #2).

- Subjects in Group #1 will receive IV injections of 1.0 μg/kg BCD-066 on Day 1, Day 8, Day 15, and Day 22.
- Subjects in Group #2 will receive IV injections 1 μg/kg Aranesp® on Day 1, Day 8, Day 15, and Day 22.

This is a double blind study, which means that neither the investigator nor the subject will know what drug is used in each group.

Blood samples for evaluation of darbepoetin alfa concentration will be collected 30 min, 20 min, and 10 min before the injection, immediately before the injection (not more than 5 min before drug administration), and then 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h after the first and the fourth IV injection. There will be 4 blood collections before and 14 blood collections after the first and the fourth IV injection of darbepoetin alfa. The serum concentrations will be used to calculate the key PK parameters of darbepoetin alfa. Before the second and the third injection, blood samples will be collected only once. During the entire study, there will be 38 blood collections for PK evaluation.

Blood samples for evaluation of hemoglobin (the primary PD endpoint) and secondary PD parameters (reticulocytes, RBC, hematocrit) will be collected before each injection of the test drug/reference drug, then 1 day, 3 days, and 5 days after the first, second, and third injection, and 1 day, 3 days, and 7 days after the fourth injection. Two more blood collections will be performed for immunogenicity assessment (at screening and on Day 29).

This study includes a screening examination (not more than 14 days) and 16 study visits. After the first and fourth injection of the test/reference drug, all volunteers will be required to stay in the clinic for at least 24 h for monitoring purposes. The duration of the study from the first drug injection to the final visit is 29 days.

Special requirements to volunteers

Study subjects are required not to drink any alcohol for 24 h before and 72 h after each injection of BCD-066/Aranesp®; not to drink more than 10 units of alcohol per week (1 unit of alcohol is equivalent to 0.5 L of beer, 200 mL of wine, or 50 mL of a strong alcohol beverage); and not to smoke for 1 h before and 3 h after each injection of BCD-066/Aranesp® and for 1 h before each blood pressure measurement.

3.2. Time Points for Blood Sampling to Evaluate PK and PD

In this study, blood samples will be collected according to the schedule to measure the concentration of darbepoetin alfa, evaluate the hemoglobin-based PD parameters, and to evaluate additional characteristics of the red blood (reticulocytes, RBC, hematocrit). The study also involves procedures to monitor the safety.

Blood samples for darbepoetin alfa concentration will be collected before and after each IV injection of the test/reference drug.

Collections will be performed as follows:

- Visit 1 (Day 1 – Day 2): 30 min, 20 min, 10 min and immediately before the first injection of darbepoetin alfa and then 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h after the first injection.
- Visit 2 (Day 3): 48 h after the first injection.
- Visit 3 (Day 4): 72 h after the first injection.
- Visit 5 (Day 8): immediately before the second injection.
- Visit 9 (Day 15): immediately before the third injection.
- Visit 13, (Day 22 – Day 23): 30 min, 20 min, 10 min and immediately before the fourth injection of darbepoetin alfa and then 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h after the fourth injection.
- Visit 14 (Day 24): 48 h after the fourth injection.
- Visit 15 (Day 25): 72 h after the fourth injection.

Blood samples for PD study will be collected as follows:

- Visit 1 (Day 1 – Day 2): before the injection and 24 h (one day) after the first injection.
- Visit 3 (Day 4): 72 h (3 days) after the first injection.
- Visit 4 (Day 6): 120 h (5 days) after the first injection.
- Visit 5 (Day 8): 168 h after the first injection (immediately before the second injection).

- Visit 6 (Day 9): 192 h after the first injection (24 h after the second injection).
- Visit 7 (Day 11): 240 h after the first injection (3 days after the second injection).
- Visit 8 (Day 13): 288 h after the first injection (5 days after the second injection).
- Visit 9 (Day 15): 336 h after the first injection (immediately before the third injection).
- Visit 10 (Day 16): 360 h after the first injection (one day after the third injection).
- Visit 11 (Day 18): 408 h after the first injection (3 days after the third injection).
- Visit 12 (Day 20): 432 h after the first injection (5 days after the third injection).
- Visit 13 (Day 22 – Day 23): 504 h after the first injection (immediately before the fourth injection); 528 h after the first injection (one day after the fourth injection).
- Visit 15 (Day 25): 576 h after the first injection (3 days after the fourth injection).
- Visit 16 (Day 29): 672 h after the first injection (7 days after the fourth injection).

Safety monitoring procedures:

For safety evaluation the study team will monitor vital signs (BP, pulse, respiration rate, and body temperature), perform regular physical examinations including assessment of the injection site, and run hematology and chemistry tests, blood tests for anti-darbepoetin alfa binding and neutralizing antibodies, urinalysis, and ECG.

3.3. Selection of Study Population

1.3.1. Inclusion criteria

1. Signed informed consent form
2. Men from 18 to 45 years old (inclusive)
3. BMI within the normal limits (18.5 to 30 kg/m^2)
4. Hemoglobin from 120 g/L to 150 g/L and hematocrit 41% to 49% at screening (before the first injection)
5. Serum transferring 2.15 g/L to 3.6 g/L; serum ferritin from 20 µg/L to 250 µg/L
6. Vitamin B12 from 187 pcg/mL to 883 pcg/mL; folic acid from 3.1 ng/mL to 20.5 ng/mL
7. Endogenous serum erythropoietin < 30 mIU/mL at screening
8. The subject is verified as “Healthy” according to results of standard clinical, laboratory and instrumental tests
9. Subject’s ability (in the investigator’s opinion) to follow the protocol procedures

10. The subject and his sexual partner with retained childbearing potential consent to implement reliable contraceptive methods starting 2 weeks before inclusion in the study and up to 2 weeks after the last dose of the test/reference drug. This requirement does not apply to surgically sterile subjects. Reliable contraception methods mean one barrier method in combination with one of the following: spermicides, intrauterine device and/or oral contraceptives used by the subject's partner.
11. The subject agrees not to drink alcohol for 24 h prior to each injection of the test/reference drug and for 72 h after the injection.

3.3.2. Exclusion criteria

1. Psychiatric disorders or other conditions that can affect the ability of the subject to follow the study protocol
2. Acute infections within 4 weeks before the study start
3. Results of laboratory and/or instrumental tests are outside the normal range
4. Chronic cardiovascular, bronchial and/or pulmonary, neuroendocrine GI, liver, kidney, and blood diseases, including CAD, arterial hypertension, peripheral vascular and/or cerebral vascular disorders, and thrombocytosis
5. A history of chronic hemorrhages
6. Any malignancy (or a prior history of malignancy)
7. Prior treatment with any erythropoietin/darbepoetin product or other products promoting erythropoiesis received at any time before enrollment
8. Intravenous iron therapy within two years prior to enrollment
9. Treatment with any drugs (including over-the-counter drugs, herbal drugs, or nutritional supplements) within 14 days prior to the first administration of the investigational product. The restriction does not apply to the use of paracetamol (less than 3 g a day) or ibuprofen (less than 1 g a day)
10. Epileptic seizures within six months prior to the first administration of the investigational product
11. Extensive surgery within one month before enrollment

12. Impossibility to insert an intravenous catheter for blood sampling (e.g., because of a skin condition at the venepuncture site)
13. Hypersensitivity to any components of BCD-066 (JSC BIOCAD), Aranesp® (Amgen Europe B.V., the
14. Netherlands) or drug products of the same therapeutic category; intolerance to erythropoietins and/or other recombinant human proteins; intolerance to iron (III) products
15. Episodes of thrombosis and/or thromboembolism in the past (myocardial infarction, stroke, transient ischemic attacks, deep vein thrombosis, pulmonary embolism within 6 months before enrollment); increased risk of deep vein thrombosis
16. Known severe allergies (anaphylaxis or multiple drug allergy)
17. Antibodies to darbepoetin alfa at screening
18. Smoking of more than 10 cigarettes a day
19. The subject consumes more than 10 units of alcohol per week (1 unit equals to 0.5 L of beer, 200 mL of wine or 50 mL of a strong alcohol beverage) or has a history of alcohol, recreational drug or medication abuse
20. Acute hemorrhage or blood/plasma donation or blood transfusion within 2 months before enrollment
21. Participation in other clinical studies within 30 calendar days before enrollment in this study
22. Prior participation in this study.

4. EVALUATION CRITERIA

4.1 Pharmacokinetics endpoints

− $AUC_{(0-72)}$ (area under the plasma concentration curve from the moment of injection of darbepoetin alfa to 72 h after the first and the fourth IV injections of BCD-066 or Aranesp®

− C_{max} (the maximum concentration of darbepoetin alfa in the serum after the first and the fourth IV injection of BCD-066 or Aranesp®)

− The elimination half-life (T½)

− $AUC_{0-\infty}$ (area under the plasma concentration curve from 0 to infinity)

− T_{max} (the time to maximum drug concentration in the serum)

− The residual area

− k_{el} (the elimination constant)

− CL (the total clearance)

4.2. Pharmacodynamics Endpoints

- Primary endpoint:
  AUEC $_{(1-29)}$ (the area under effect curve) based on the change of hemoglobin level from the moment of injection to Day 29 after the repeated IV administration of the test/reference drug
- Secondary endpoints:
  AC-E_{max} (the maximum increase in hemoglobin level from baseline), from the first injection to Day 29 after the repeated IV administration of the test/reference drug
- Supplementary endpoints:
  T_{max} (the time to the absolute maximum increase of hemoglobin level from injection to Day 29 after the repeated IV administration of the test/reference drug).
  In addition, PD parameters (AUEC, AC-E_{max} and T_{max}) will be estimated based on the reticulocyte and RBC counts and on hematocrit values.

4.3. Safety assessment

- The rate of SAEs
- The rate of CTCAE grade 3/4 AEs
- The rate of withdrawals due to AEs
- The proportion of subjects who had injection site reactions
- The proportion of subjects who had antibodies to darbepoetin alfa on Day 29

THE PLANNED ANALYSIS

The study report will be made after the study completion (after all included volunteers will finish the study)

6. SAMPLE SIZE CALCULATION

The sample size was determined using the variation coefficients for hemoglobin increase (Hb AUEC) obtained by Cheung, 2001[1]. The estimated Type I error was 5% (α=0.05) and Type II error was 20% (β=0.2), with the test power of 80%.

The sample size was calculated with the "sample N.TOST" function of the "PowerTOST" package for R. The calculation used the CV values and the ratios of the mean Hb increases (AUEC).

$$CV_{AUC(Hb)}(\%) = \frac{\sigma_{AUC(Hb)}}{\bar{x}_{AUC(Hb)}} = \frac{12.92}{45.4} = 28.46\%,$$

Table 1. Test power and sample size

Index	Mean value ratio	CV (%)	Sample size (N)	Test power
AUEC(1-29) hemoglobin	$\frac{\mu_T}{\mu_C} = 1$	28,46	N AUC(Hb) = 56 volunteers. (28 volunteers per group)	80,95%

The study should include 28 subjects per group and 6 subjects as backups to replace potential early withdrawals (62 volunteers total).

7. STATISTICAL ANALYSIS OF POPULATION

Pharmacokinetics analysis

The pharmacokinetics (PK) analysis will include data of all volunteers who have received all 4 doses of BCD-066 or reference drug. If a subject has had more than two PK serum samples missed / lost / spoilt and skipped blood sampling time points more than twice, his data will not be included in the PK analysis.

Pharmacodynamics analysis

The pharmacodynamics (PD) analysis will include data of all volunteers who have received all 4 doses of BCD-066 or reference drug. If a subject has had more than two PD serum samples

[1] W.Cheung, N. Minton, K.Gunawardena. Pharmacokinetics and pharmacodynamics of epoetin alfa once weekly an three times weekly, 2001

missed / lost / spoilt and skipped blood sampling time points more than twice, his data will not be included in the PD analysis.

Safety analysis

The safety analysis will include all volunteers who have received one dose of the test/reference drug.

Immunogenicity analysis

The immunogenicity analysis will include data of all volunteers who have received one dose of BCD-066 or reference drug. If a subject has had serum samples taken on Day 1 and Day 29 missed / lost / spoilt, his data will not be included in the immunogenicity analysis.

8. ANALYSIS PLAN AND STATISTICAL METHODS

8.1. The software

For statistical analysis and to construct the tables and diagrams would be used the software STATISTICA 10 and statistical programming language R.

8.2. Description of the Statistical Methods to be Employed

Quantitative Data

The following quantitative data will be analyzed in the study:

Pharmacokinetics:

- Plasma concentration of drug,
- Area under plasma concentration curve,
- Time (e.g., maximum plasma concentration time, half-life)
- Elimination parameters (e.g. clearance, terminal rate constant)

Safety:

- RBC results,
- Blood chemistry results,
- Vital signs;
- ECG findings,
- Urea analysis results,
- Skin disorders size at the sites of venipuncture,

- Skin disorders lifetime at the sites of venipuncture.

Pharmacodynamics:

- Absolute reticulocyte count.
- Hb level.
- Area under effect curve «absolute reticulocyte count — time».
- Area under effect curve «Hb level — time».
- Time (e.g. the time of the maximum increase of absolute reticulocyte count vs. baseline). The statistical analysis will include two-tailed hypothesis testing; the chosen significance level is 0.05. Quantitative variables will be tested for normality using the Shapiro-Wilk test.

Normally distributed quantitative variables will be tested using the two-sample Student's t-test, Welch's t-test, and ANOVA.

Non-normally distributed quantitative variables will be tested using the Mann-Whitney U-test, the Wilcoxon test, the Kruskal-Wallis test, and the Friedman test.

Normally distributed quantitative data will be described using the following descriptive statistics: mean, SD, CV, min, and max.

Non-normally distributed quantitative data will be described using the following descriptive statistics: median, quartiles, CV, min, and max.

Categorical Data.

The following categorical data will be analyzed in the study:

Safety:

- Rate and severity grades of AEs/SAEs
- Rate of Grade 3-4 AEs,
- Incidence of administration site reactions.
- Frequency of local reactions (total and by skin disorder type).
- Frequency of early withdrawals due to AEs.

Categorical data will be processed using frequency tables, contingency tables, the Fisher's exact test, the test of equal frequencies, Pearson's χ^2 test, and the Cochran-Mantel-Haenszel test. Percentages or proportions will be used to describe categorical data. The Benjamini-Yekutieli correction for multiple testing will be used. Statistical methods will be chosen according to the

type of initial data and their distribution. Appropriate statistical tests will be established after the data collection has been completed because the type of data distribution, sample homogeneity, etc. are unknown before the study start. For appropriate data processing, the list of statistical methods used may expand during the analysis.

Descriptive statistics for the data on drug concentration in the serum, effect values, and protocol-specified PK and PD parameters will be performed using the following: mean values, geometric mean values, standard deviations, medians, upper and lower quartiles, min and max, and the coefficient of variation.

The statistical comparison involves the calculation of two-sided parametric 90% confidence intervals (CIs) for the ratios of the $AUEC_{(1-29)}$ means for hemoglobin and darbepoetin alfa following the IV injection of BCD-066 and Aranesp®, and comparison of these CIs with the PD equivalence interval: $AUEC_{(1-29)}$ 80-125%.

The study drugs will be considered equivalent if the estimated 90% CIs for the mean $AUEC_{(1-29)}$ ratio for darbepoetin in BCD-066 and darbepoetin in Aranesp® will fall in the said range.

8.3. Accounting for missing, unavailable or doubtful data, outliers

All information specified in the e-CRFs should be supported by relevant data in source documents.

After entering all data into the electronic database, a database specialist checks it for inconsistencies, errors, and missing data points. The Clinical Study Database Manager or Medical Expert of JSC BIOCAD generates queries to correct error data or to request missing data; the queries are site-specific and subject-specific (i.e., individual queries are generated for each subject). The Clinical Study Monitor will send queries to the study site by fax or email. The queries should be resolved by the investigator within five business days from the date they have been submitted to the study site. Copies of responses to queries must be kept at the study site; original responses must be stored at JSC BIOCAD.

When responses to queries are received from investigators, the database specialist checks it for inconsistencies, errors, and missing data points. When all data at all study sites have been collected and entered, the database is closed. Afterwards, statistical processing can be started.

Missing, unused, and spurious data will not be substituted.

Spurious and unevaluable data are revealed during the outlier analysis by examination of Mahalanobis or Cook distance, visual analysis of scatter plots and box plots.

All actions taken to handle missing, unevaluable, spurious data and outliers before/during the statistical analysis will be described in the Clinical Study Report.

8.4. Multivariate Comparison

Pharmacokinetic parameters

Based on determined concentrations in blood a number of the pharmacokinetic parameters will be calculated for the study drug:

1. C_{max} – maximum plasma concentration after the first and the fourth intravenous administration
2. T_{max} - time until C_{max} is reached
3. $AUC_{(0-72)}$ – area under the plasma concentration curve from administration to 72 h after the first and the fourth intravenous administration.

$$AUC_{(0-72)} = \sum_{p=2}^{15} \frac{(C_p + C_{p-1}) * (t_p - t_{p-1})}{2},$$

где C_p plasma concentration at the moment t_p (hours);

$t_p \in \{0; 0.083; 0.167; 0.25; 0.33; 0{,}5; 0.75; 1; 2; 4; 8; 16; 24; 48; 72\}$; p=1,…,15

4. $AUMC_{(0-72)}$ - the total area under the plasma concentration curve from administration to 72 h after the first and the fourth intravenous administration.

$$AUMC_{(0-72)} = \sum_{p=2}^{15} \frac{(C_p * t_p + C_{p-1} * t_{p-1}) * (t_p - t_{p-1})}{2},$$

где C_p – plasma concentration at the moment t_p (hours),

$t_p \in \{0; 0.083; 0.167; 0.25; 0.33; 0{,}5; 0.75; 1; 2; 4; 8; 16; 24; 48; 72\}$; p=1,…,15

5. Total clearence (CL) will be calculated as:

$$CL = \frac{DOSE}{AUC_{0-72}}$$

6. Mean Residence Time (MRT) will be calculated as:

$$\mathrm{MRT} = \frac{\mathrm{AUMC}_{0-72}}{\mathrm{AUC}_{0-72}}$$

7. Apparent volume of distribution (V_d) will be calculated as:

$$V_d = CL * MRT$$

8. Elimination rate constant (K_{el}) will be calculated as:

$$k_{el} = \frac{1}{MRT}$$

9. Plasma concentration half-life ($T_{1/2}$) will be calculated as:

$$T_{1/2} = \frac{\ln(2)}{k_{el}} = \ln(2) * MRT$$

10. Area under the plasma concentration curve from 0 to infinity ($AUC_{(0-\infty)}$) will be calculated as:

$AUC_{(0-\infty)} = AUC_{(0-72)} + \frac{C_{72}}{k_{el}}$, где

C_{72}- plasma concentration of the drug during the 72 hours after the administration of the drug

11. The residual area will be calculated as:

$$\frac{AUC_{(0-\infty)} - AUC_{(0-72)}}{AUC_{(0-\infty)}}$$

Pharmacodynamics parameters

Based on determined hemoglobin level, reticulocytes counts, RBC counts, hematocrit a number of the pharmacodynamics parameters will be calculated for the study drug:

1. **E_{max}** – the maximum hemoglobin level (reticulocytes, RBC, hematocrit)
2. **T_{max} -** the time to the absolute maximum increase of hemoglobin level (E_{max}) (reticulocytes counts, RBC, hematocrit)
3. **$AUEC_{(1-29)}$** – area under effect curve from the moment of injection to the day 29 after repeated IV injections test drug/reference drug based on the hemoglobin level (reticulocytes counts, RBC, hematocrit)

$AUEC_{(1-29)}$ will be calculated as:

$$AUEC_{(1-29)} = \sum_{p=2}^{16} \frac{(C_p + C_{p-1}) * (t_p - t_{p-1})}{2},$$

где C_p hemoglobin level (reticulocytes counts, RBC, hematocrit) in the time point t_p (days);

$t_p \in \{0; 1; 3; 5; 7; 8; 10; 12; 14; 15; 17; 19; 21; 22; 24; 28\}$; $_{p=1,\ldots,16}$

4. **AC-E_{max} –** absolute maximum increase in the test parameter vs. baseline based on the change in the hemoglobin level (absolute reticulocyte count, erythrocytes count)

8.7. Subgroup analysis, interaction and related variables

Not available.

9. OTHER PLANNED ANALYSES

No additional analyses are planned in this study.

10. DEVIATIONS FROM ANALYSIS METHODS DESCRIBED IN STUDY PROTOCOL

This Statistical Analysis Plan has no deviations from methods described in Study Protocol.