CLINICAL STUDY PROTOCOL

Study Code: UNL074

Title: Comprehensive Biomarker Profiling of the IFN-α Pathway in Amyotrophic Lateral Sclerosis Patient Biofluids

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Study Identifier	UNL074
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Version	Version 02
Date	30 July 2025

Sponsor	Ulysses Neuroscience Ltd Pioneer Life Sciences Cherrywood - Building 10 Cherrywood Business Park Loughlinstown Co. Dublin, Republic of Ireland
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Principal Investigator	Angelo Quattrini, MD. UO neurology UO Experimental pathology-INSPE Tel 02-26435094 Email: quattrini.angelo@hsr.it

DISCLAIMER

VERSION HISTORY

Protocol version n.	Reason of changes	Date issued
01	First Version	12/05/2025
02	Response to clarifications requested by Ethical Committee	30/07/2025

PROTOCOL SIGNATURE PAGE

Study Title: *Comprehensive Biomarker Profiling of the IFN-α Pathway in Amyotrophic Lateral Sclerosis Patient Biofluids.*

Study Identifier: UNL074

Protocol Version and Date:

01 – 12/05/2025

02 – 30/07/2025

The undersigned has read and understood all the aspects of the protocol detailed within this document and agrees to supervise and conduct the study in accordance with the protocol, the Declaration of Helsinki, and all applicable regulatory requirements.

Massimiliano Bianchi		Ulysses Neuroscience Ltd	17/07/2025
Authorized Sponsor Representative Name	**Signature**	**Affiliation**	**Date**

Angelo Quattrini		UO neurology UO Experimental pathology-INSPE	17/07/2025
Principal Investigator Name	**Signature**	**Affiliation**	**Date**

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1. KEY STUDY CONTACTS

Sponsor	Ulysses-neuroscience Ltd Pioneer Life Sciences Cherrywood - Building 10 Cherrywood Business Park Loughlinstown Co. Dublin, Republic of Ireland
Authorized Sponsor Representative	Prof Massimiliano Bianchi - Ulysses-neuroscience Ltd Pioneer Life Sciences Cherrywood - Building 10 Cherrywood Business Park Loughlinstown Co. Dublin, Republic of Ireland Tel +353 87 600 4092 max.bianchi@ulysses-neuro.com
Principal Investigator	Angelo Quattrini, MD. UO neurology UO Experimental pathology-INSPE Tel 02-26435094 Email: quattrini.angelo@hsr.it
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Clinical Trial Center	Email: ctc.firstcontact@hsr.it; ctc.trialstartup@hsr.it; ctc.datamanagement@hsr.it; ctc.quality@hsr.it

2. ABBREVIATIONS AND DEFINITIONS

2.1 Abbreviations

AE	Adverse event
ALS	Amyotrophic Lateral Sclerosis
CRF	Case Report Form
CRO	Contract Research Organization
CSF	Cerebrospinal fluid
DPIA	Data Protection Impact Assessment
EC	Ethics Committee
ELISA	Enzyme-Linked Immunosorbent Assay
GCP	Good Clinical Practice
ICF	Informed Consent Form
ICH	International Conference on Harmonization
PI	Principal Investigator
SOP	Standard Operating Procedure
CTC	Clinical Trial Center
OSR	Ospedale San Raffaele

2.2 Definitions

MESO	ELISA Meso-scale Discovery Platform

3. STUDY DESCRIPTION AND RATIONALE

Amyotrophic lateral sclerosis (ALS) is an adult-onset progressive, fatal, neurodegenerative disorder that results in the gradual degeneration of the motor neurons in the motor cortex and spinal cord. ALS is the most common type of motor neuron disease with an incidence rate of 2.8 per 100,000 persons/year and a mean prevalence rate of 5.4 per 100,000 people in Europe. ALS is characterized by rapidly progressive paralysis and death typically occurs from respiratory failure within 2 to 4 years of symptom onset, although about 10% of ALS patients manifest a slow form of the disease with a survival of 10 years or longer (1). Clinical diagnosis of ALS is often a prolonged process due to heterogeneity in patient presentation and prognosis as well as a lack of specific diagnostic, prognostic and the diagnostic biomarkers. This is a recognized critical bottleneck for both ALS translational research and clinical applications. The importance of biomarker discovery for therapeutic advancement is underscored by the recent accelerated approval of QALSODY (Tofersen) for treatment of SOD1-ALS patients, a decision that was primarily based on reduction in plasma Nf-L in treated patients. Nevertheless, despite its value, an increase in circulating Nf-L (2,3) following neuro-axonal damage represents a late pathogenic event also found in other neurological disorders besides ALS. The ideal fluid biomarker for ALS would be capable to sensitively monitor early disease manifestation, treatment responses and dynamic pathogenetic events, features that are not met by Nf-L. Other potential biomarkers of neuronal damage in ALS include tau (associated with shorter survival), UCHL1 and TDP-43 (both increased in ALS patients). The involvement of microglia and astrocytes to ALS pathogenesis can be studied by measuring MCP-1 and GFAP respectively (4-6). Considering the mounting evidence implicating IFN-alpha signaling in the pathogenesis of ALS (7), we aim to comprehensively profile cytokines, neuroinflammatory markers, and neurodegeneration-related analytes in plasma and CSF from clinically characterized ALS patients and healthy controls. Biomarker investigation will be performed in a retrospective cohort of 31 ALS patients compared with 29 healthy subjects.

The samples to be analyzed in this study were obtained from a single visit and there is no planned longitudinal analysis component as part of this current study. Thus, this project is an initial exploratory biomarker study aimed to use established ALS fluid biomarkers as a benchmark to identify further potential hypothesis-driven candidate biomarkers within the IFN-alpha pathway. Promising candidate biomarkers will not be correlated against disease progression (since there are no follow-ups), but will be considered alongside the available clinical data and clinical outcome measures provided by the principal physician.

This study aims to support the initial validation of IFN-alpha pathway activation as a therapeutic target and explore its association with disease phenotype to provide insights into potential diagnostic and prognostic biomarkers for ALS, facilitating future therapeutic development.

4. OBJECTIVES AND ENDPOINTS

Objectives	Endpoints	Time point(s)
Primary Objectives To validate established ALS-related fluid biomarker levels within the sample cohort.	**Primary Endpoint** To evaluate levels of established fluid ALS biomarkers such as Nf-L, GFAP, and pTau (T181) in plasma and CSF of ALS patients vs. healthy controls.	Blood and CSF sampling at day 0
Secondary Objectives To identify hypothesis-driven novel fluid biomarkers in ALS related to the IFN-alpha pathway.	**Secondary Endpoints** To evaluate expression of candidate disease biomarkers in plasma in a cohort of ALS patients. To evaluate expression of candidate disease biomarkers in CSF in a cohort of ALS patients. To compare candidate disease biomarkers specificity and sensitivity with Nf-L, the benchmark biomarker for ALS	Blood and CSF sampling at day 0

5. INCLUSION/EXCLUSION CRITERIA

a. Inclusion Criteria

Inclusion criteria for ALS patients:

- Age equal or over 18 years old
- ALS patients, diagnosed accordingly to the revised El Escorial Criteria

Disease duration <24 months from symptom onset.

Inclusion criteria for controls

- Age equal or over 18 years old
- Subjects without a diagnosis of neurodegenerative disease or neuromuscular disorder.

b. Exclusion Criteria

Exclusion criteria for ALS patients:

- FVC <60%;
- nutritional or respiratory failure; Significant hepatic or chronic renal failure or any intervening infective **(pneumonia, flu-like syndromes, urinary tract infections)** or metabolic **(acute renal failure, hyperosmolar hyperglycaemic state, severe hyponatremia)** conditions **present at the time of assessment that could potentially affect** biomarker levels.
- ALS patients exhibiting any of these conditions at the time of biofluid sampling as per the Case Report From (CRF) will be excluded from sample selection.

Exclusion criteria for controls:

- Significant hepatic or chronic renal failure or any intervening infective **(pneumonia, flu-like syndromes, urinary tract infections)** or metabolic **(acute renal failure, hyperosmolar hyperglycaemic state, severe hyponatremia)** conditions **present at the time of assessment that could potentially affect** biomarker levels.
- Controls exhibiting any of these conditions at the time of biofluid sampling as per the Case Report From (CRF) will be excluded from sample selection.

6. INFORMED CONSENT

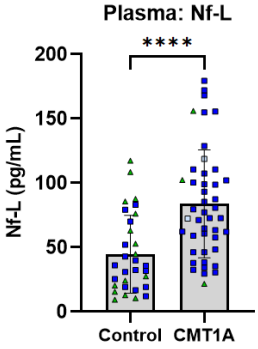
Plasma and CSF were collected accordingly to clinical practice indication (GCP). ALS patients whose clinical data (demographic, epidemiological, family history of disease and neurological history of the patient) and biological samples (plasma and CSF for biomarker dosage) have already been collected, after informed consent (protocollo Banca Inspe), from the creation of the INSPE BANK and stored as per specific regulation will be included retrospectively.

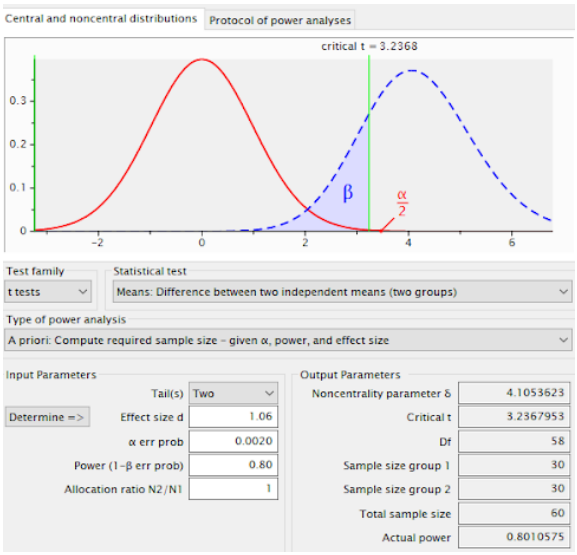
This research is aimed at improving the health of people belonging to the same age group or who suffer from the same pathology or who are in the same conditions and the research program is subject to a motivated favourable opinion from the EC. The research does not involve significant risks to the dignity, rights, and fundamental freedoms of the interested party.

7. STUDY OBJECT

PROCEDURE	
Procedure(s)	**<u>Biomarker analysis</u>** Ulysses Neuroscience Ltd. will perform electrochemiluminescence sandwich ELISA using the Meso-Scale Discovery platform (MESO QuickPlex SQ 120 instrument and analyzed by Discovery Workbench 4.0 software). Samples will be analyzed in parallel using the NULISAseq™ proteomic platform (Alamar Biosciences) for confirmatory analysis of analyte expression using a secondary platform. Data will be analysed as ALS vs healthy control as raw values of each analyte in the samples (pg/mL) and graphed using GraphPad Prism v.9. Specific analytes to be measured include: a) **U-Plex Immuno-Oncology Custom:** IFN-α2a, IFN-β, IL-12/IL-23p40, CXCL10/IP10, CCL2/MCP1. b) **U-Plex Immuno-Oncology Custom:** IFN-γ, IL-6, IL-8, IL-13, TNF-α, CCL5/RANTES. c) **R-Plex Human TDP-43:** TDP-43. d) **S-Plex Neurology Panel 1:** GFAP, Neurofilament L, Tau [Total]. e) **S-Plex Human Tau:** pTau T181 f) **S-Plex Human Tau:** pTau T217 g) **S-Plex Human Tau:** pTau T231 **h) NULISAseq™ CNS Disease Panel 120** i) **NULISAseq™ Inflammation Panel 250 AQ**

8. SAMPLE SIZE AND SUBJECT IDENTIFICATION

Sample Size	This proposed retrospective fluid biomarker study includes a sample size of 29 control and 31 ALS patient samples. Plasma and CSF samples were selected from healthy control individuals in order to ensure that two age-homogeneous groups were selected, based on the general age distribution of the ALS patient cohort (Healthy Control: 55 ± 12.47 years; ALS: 56.58 ± 13.46 years at time of sampling). Sample sizes were selected based on our previous work and current scientific literature. In a prior study completed in collaboration with Dr. Stefano Previtali's group at SRH investigating Charcot-Marie-Tooth disease type 1A (CMT1A), we used a comparable number of control and patient samples (n=31 and 45, respectively) to analyse neurofilament light chain (Nf-L) levels in plasma. This study yielded a highly significant increase in Nf-L levels in CMT1A patient plasma compared to healthy controls ($p<0.0001$), demonstrating the suitability of this sample size for detecting biologically meaningful effects in peripheral nervous system degenerative disease.  **Nf-L is increased in the plasma of CMT1A patients** - Plasma concentrations (pg/mL) of neurofilament light chain (Nf-L) in healthy control (n=31) and CMT1A patients (n=45) analysed by MSD electrochemiluminescence sandwich ELISA. Plasma Nf-L was significantly elevated in CMT1A patients compared to healthy controls (p=0.0001). Given this experience, as well as data from published ALS biomarkers studies, we are confident our current sample size is appropriate for detecting group differences in established biomarkers such as Nf-L, GFAP, and pTau (T181) in ALS (8-11). Additionally, we are using data from published literature to inform expectations for other analytes included in the proposed analysis panel, such as inflammatory cytokines, markers of neurological disorder, and different phospho-tau species. However, we acknowledge that published data on many of these biomarkers in ALS are limited or inconsistent, underscoring the importance of investigating these analytes within this study. Furthermore, sample sizes were established based on Cohen's d power analysis performed based on published meta-analyses of case-control studies reporting effect sizes, or mean or standardised mean difference (SMD) for the benchmark ALS biomarker, Nf-L, in ALS CSF and plasma compared to healthy controls. SMD is statistically equivalent to Cohen's d, in meta-analyses: CSF Nf-L = SMD 1.46, Plasma Nf-L = 1.35 (12). With an effect size (Cohen's d) of 1.06 employed combined with an alpha level of 0.002 (considering all 18 biomarkers) and a minimum power cutoff of 80%, the minimum sample size required to reach statistical power was given to be 30. Considering this power analysis, and that dropout rate is not a factor of consideration for these samples because they have been already collected, and there is no risk of in blood collection, sample volume, or people withdrawing from the study, a sample size of 31 was selected.

	 ***Power Analysis performed to inform sample size selection for this study.*** This study is designed as an exploratory biomarker investigation. Its goals are two-fold: (1) **to validate established ALS biomarkers using this cohort**, and (2) **to assess a broad panel of additional analytes that may be dysregulated in ALS**, with a view toward identifying promising candidates for further investigation. We believe that this approach is scientifically justified for an initial exploratory study and aligns with standard practice in early-phase biomarker research. Future studies based on the findings of this work will incorporate expanded cohorts and statistical power analyses derived from this foundational dataset.
Subject Identification	Pseudonymised: participants are identified by a unique coded identification number. This code maintains confidentiality and links the participant to their data without revealing their identity.

9. STATISTICAL DESIGN

Plasma and CSF samples were selected from healthy control individuals in order to ensure that two age-homogeneous groups were selected, based on the general age distribution of the ALS patient cohort (Control: 55 ± 12.47 years; ALS: 56.58 ± 13.46 years at time of sampling) to negate both age and gender as covariates when analyzing each individual biomarker expression in ALS vs control.

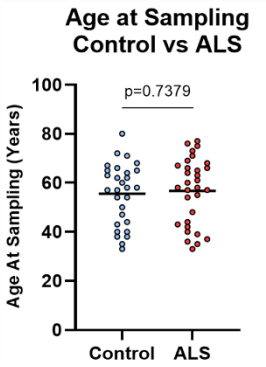
A.

	Control	ALS
Male + Female	n=29	n=31
Age at Sampling (Years)	55.55 ± 12.47	56.68 ± 13.46
Male	21 (72.4%)	21 (67.8%)
Age at Sampling (Years)	55.62 ± 13.44	55.76 ± 13.59
Female	8 (27.6%)	10 (32.2%)
Age at Sampling (Years)	55.38 ± 10.31	58.60 ± 13.69

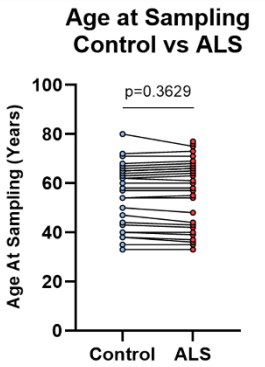
B.

	Welch's t test	
1	Table Analyzed	Control_ALS_Age 2
2		
3	Column B	ALS
4	vs.	vs.
5	Column A	Control
6		
7	**Unpaired t test with Welch's correction**	
8	P value	0.7379
9	P value summary	ns
10	Significantly different (P < 0.05)?	No
11	One- or two-tailed P value?	Two-tailed
12	Welch-corrected t, df	t=0.3362, df=58.00
13		
14	**How big is the difference?**	
15	Mean of column A	55.55
16	Mean of column B	56.68
17	Difference between means (B - A) ± SEM	1.126 ± 3.348
18	95% confidence interval	-5.576 to 7.828
19	R squared (eta squared)	0.001945
20		
21	**F test to compare variances**	
22	F, DFn, Dfd	1.164, 30, 28
23	P value	0.6887
24	P value summary	ns
25	Significantly different (P < 0.05)?	No
26		
27	**Data analyzed**	
28	Sample size, column A	29
29	Sample size, column B	31

C.



D.



***Distribution of ages in across Control and ALS sample cohorts**. **A**) Distribution of age at sampling with standard deviation of the total control and ALS patient cohort for this study, as well as breakdown of age at sampling with standard deviation for male and female samples. **B**) Unpaired two-tailed t-test with Welch's correction for age at sampling between control and ALS patient cohorts. No statistically significant differences were identified in age at sampling between groups (p=0.7379) or the variance within groups (p=0.6887). **C**) Distribution plot of age at sampling for control and ALS patient samples with lines indicating mean group ages. Analyzed by unpaired two-tailed t-test (p=0.7939). **D**) Distribution plot of age at sampling for control and ALS patient samples with lines connecting the nearest age match between sample cohorts. Analyzed by paired two-tailed t-test (p=0.3629).*

All statistical analyses will be performed using GraphPad Prism v.9. Each analyte will be analyzed on an individual analyte-by-analyte basis by unpaired two-tailed t-test, without multiple comparisons performed between analytes as is standard during early-phase exploratory biomarker research.

While several of the proposed biomarkers to be analyzed show strong correlation with age, selecting two age- and gender-homogenous populations for ALS and control samples will mitigate any non-pathological age-related deviations in specific biomarker levels.

10. DURATION OF THE STUDY, DISCONTINUATION AND WITHDRAWAL

a. Duration of the Study

Duration of enrolment: Plasma and CSF are already available in Biobanking-INSPE

Duration of total study period: 1 year

b. Discontinuation And Withdrawal

Not applicable

11. SAMPLE HANDLING

The samples were taken from each participant (accordingly to routine best clinical practice) as part of a standard clinical investigation and for future research. Upon collection, samples will be immediately processed to preserve their integrity. Blood samples were centrifuged to separate plasma and stored at -80C. CSF was collected in sterile conditions and aliquoted to avoid repeated freeze-thaw cycles that could degrade the samples. All samples were properly labeled with a unique identifier, devoid of any personal identifying information to maintain participant confidentiality. All samples are stored in the iNSPE-Biobank under controlled conditions until analysis.

OSR agrees to provide Ulysses neuroscience with the following biological samples:

- Plasma samples from 31 ALS patients
- CSF samples from 31 ALS patients

The samples are anonymized and do not contain any patient-identifiable information.

The samples are stored and transported under appropriate conditions to ensure their integrity. Shipment shall be carried out through Ulysses Neuroscience Ltd.'s partner company, Biorep, which specializes in the secure transport of biological materials. Biorep shall ensure that all necessary handling and storage conditions are met during transit. Ulysses Neuroscience Ltd. shall be responsible for covering all costs associated with the shipment of the samples. The samples shall be used by Ulysses Neuroscience Ltd. for biomarker research in its own facilities. Ulysses laboratories in Cherrywood (Dublin, Republic of Ireland) are fully equipped for analysis of central and peripheral biomarkers related to neuroinflammation, synaptic plasticity and cytoskeleton integrity. Samples are also to be shared with a collaborator laboratory for further comprehensive protein expression analysis within the scope of the study set out in this protocol. The Ulysses Neuroscience Ltd. team has now collected significant data and performed several studies that clearly underline the importance of biomarkers in psychiatric and rare CNS diseases in bridging preclinical and clinical research.

Ulysses Neuroscience Limited is an Irish CRO providing clinical and preclinical research. It is committed to highest ethical scientific practice for both clinical and preclinical studies. The clinical biomarker studies are ethically reviewed and performed by specialised trained staff.

12. DATA MANAGEMENT

a. Definition of source data and source documents

Clinical Data: Includes all medical history, treatment administration, collected through case report forms (CRFs)

Biomarker Data: Comprises biochemical and other molecular data collected from blood and CSF.

b. Documentation of data in Case Report Forms (CRFs)

All relevant data collected during the study for all the patients enrolled in the study shall be entered in the CRF by the principal investigator or someone authorized by the investigator in a timely manner (as soon as possible after the information is collected) to ensure that they are clear and legible. The physician shall confirm the completeness, correctness, plausibility, and compliance with the ICH guidelines and the institutional SOPs of the data by dated signature. An explanation must be provided for any and all missing data. The entries shall be made with black ballpoint pen.

c. Data Recording and Record Keeping

The Principal Investigator must maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be subsequently verified.

The investigator(s)/institution(s) should permit trial-related monitoring, audits, and regulatory inspection(s), providing direct access to source data/documents.

The investigator must keep the documents on file for at least 25 years after completion or discontinuation of the study. After that period, the documents may be destroyed, subject to local regulations. Before proceeding to documents' destruction, sites must inform the Sponsor Investigator in writing.

d. Data Protection

Data Anonymity and Confidentiality

Anonymity: participants are identified not by name, initials, or birth date but by a unique coded identification number. This code helps maintain confidentiality and links the participant to their data without revealing their identity.

Each investigator assures that patients' anonymity will be maintained and that their identities are protected from unauthorized parties. Particular attention should be paid whenever patient data are supplied to third parties and may be autonomously processed, or biological samples/materials are taken and kept for future research purposes.

The investigator should keep in a confidential way a patient identification log recording both patient code and name. The investigator should also maintain patients' written consent forms, in strict confidence (i.e. not for submission to the Sponsor Investigator).

Authorization and Data Entry

Authorized Personnel: Access to data is restricted to authorized personnel only. This access control is crucial for maintaining the integrity and confidentiality of the data.

13. ETHICAL AND REGULATORY CONSIDERATION

The Principal Investigator ensures that the study is conducted in agreement with this protocol, the Good Clinical Practice, the current version of Declaration of Helsinki and the applicable regulations.

The protocol and any amendments are subject to review and approval by the concerned Ethics Committee(s) and Health Authority(ies)

13.1 Responsibilities of the Investigators

The Investigator(s) undertake(s) the responsibility to perform the study in accordance with this Protocol, Good Clinical Practice, and the applicable regulatory requirements. The Investigator is required to ensure compliance with the investigational product schedule, visits schedule, and procedures required by the protocol. The Investigator agrees to provide all information requested in the Case Report Form (CRF) in an accurate and legible manner. The investigator may implement a deviation from, or a change of, the protocol to eliminate an immediate hazard(s) to study subjects without prior EC approval/favorable opinion. As soon as possible, the implemented deviation or change, the reasons for it, and, if appropriate, the proposed protocol amendment(s) should be submitted. The investigator must have available an adequate number of qualified staff and adequate facilities for the foreseen duration of the study to conduct the study properly and safely.

13.2 Ethics Committee (EC) Approvals

This clinical study protocol as well as the Informed Consent are to be submitted to the appropriate Ethics Committee for only notification or approval, in this case it is mandatory to obtain the written and dated approval, signed by the chairman with Ethics Committee(s) composition. The clinical study the documents reviewed, the list of voting members and their qualifications, and the date of the review should be clearly stated on the written Ethics Committee approval.

14. GENDER MEDICINE IN RESEARCH PROTOCOL

ALS predominantly affects males, with a male/female incidence rate ratio of 1.4, and people in their late adulthood, with median age at diagnosis of 65.2 years for men and 67.0 years for women. To avoid bias in the gender dimension we will implement appropriate protocols for treating male and female biological samples (equal proportion and indistinguishability with respect to gender).

15. QUALITY ASSURANCE AND CONTROL

Quality Assurance and Quality Control systems based on written SOPs are in place at the Sponsor Investigator site.

16. END OF CLINICAL STUDY

In accordance with applicable regulation, ICH GCP and SOPs, the PI shall notify the end of the clinical study within 15 days from the end of the clinical study and the reasons for such action.

17. INTELLECTUAL PROPERTY

A clinical observational study agreement will be stipulated with an ad hoc contract.

18. PUBLICATION POLICY

After completion of the study, the Sponsor Investigator prepares a draft manuscript containing final results of the study on the basis of the statistical analysis. The manuscript is delivered to the co-authors for comments and then sent to a scientific journal for publication.

All publications, abstracts, presentations, manuscripts and slides - issued by the Investigators of the collaborative sites and including data from the present study- should be submitted to and reviewed by the Sponsor Investigator at least 3 (three) weeks in advance the planned date for the submission to the scientific journal.

19. REFERENCES

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20. APPENDIX A: AMENDMENT HISTORY

List details of all protocol amendments here whenever a new version of the protocol is produced.

Amendment No.	Protocol Version No.	Date issued	Rationale	Study status	Details of Changes

21. APPENDIX B: LIST OF CLINICAL SERVICES / LABORATORIES

Fill in form the form CTC 021B.

22. APPENDIX C: LIST OF ENROLLING SITES

Fill in the form CTC 021C.