Randomized, controlled interventional trial to investigate the efficacy of amiloride for the treatment of oedema in human nephrotic syndrome (AMILOR)

Statistical Analysis Plan (SAP)

Version: final

Valid since date of last signature (Sponsor)

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Confidentiality statement

Note

This SAP was performed according to the SOP BI03_V02 (valid since 11.02.2022) of the ZKS Tübingen. The analysis tables and listings will be independently validated by a second statistician according the SOP BI06 of the ZKS Tübingen.

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Abbreviations

AE	Adverse Event
BCM	Body Composition Monitor, Fresenius Medical Care AG & Co
CRF	Case Report Form
ECW	Extracellular water
ENaC	Epithelial sodium channel
FCBP	Females of childbearing potential
FSH	Follicle stimulating hormone
GFR	Glomerular filtration rate
HbA1c	Haemoglobin A1c
HCT	Hydrochlorothiazide
IKEAB	Institut für Klinische Epidemiologie und angewandte Biometrie Tübingen
KDIGO	Kidney Disease – Improving Global Outcomes
NKCC2	Sodium-potassium-chloride cotransporter
NYHA	New York Heart Association
OH	Overhydration
RAAS	Renin-angiotensin-aldosterone system
SAP	Statistical Analysis Plan
SAE	Serious Adverse Event
SOP	Standard Operating Procedure
TMF	Trial Master File
WOCBP	Women of childbearing potential
ZKS	Zentrum für Klinische Studien Tübingen

1 Introduction

This SAP is based on the study protocol Version 1.1 as of 10. January 2020 with amendment 1 as of 01. March 2021 (EudraCT-No: 2019-002607-18). Text citation directly form the study protocol is given in *Italic letters*.

1.1 Preface

The nephrotic syndrome is characterized by a high ("nephrotic") proteinuria> 3.5 g / day with resulting hypoalbuminemia, as well as by oedema and hyperlipidaemia. Affected patients suffer from massive generalized oedema involving the face and eyelids, effusions into the body cavities pleura, peritoneum and rarely pericardium as well as increased body weight.

In addition to the disease specific treatment commonly involving immunosuppression, supportive therapy of patients with acute nephrotic syndrome includes reduction of proteinuria by the renin-angiotensin-aldosterone-system (RAAS) blockade, blood pressure control, lipid lowering, thromboembolic prophylaxis and the correction of volume overload, which is achieved by a dietary salt restriction and diuretic therapy.

Currently, loop diuretics are preferred as they have the highest diuretic potency. However, the diuretic effect of loop diuretics is lower in nephrotic syndrome than in other forms of oedema or in healthy volunteers. A possible cause of this resistance to loop diuretics could be the activation of the epithelial sodium channel (ENaC) that is located distally to the sodium-potassium-chloride cotransporter (NKCC2), the target of loop diuretics. Sodium retention via ENaC may therefore counteract the inhibition of NKCC2.

To overcome refractory oedema, either higher doses of loop diuretics or combination with thiazide diuretics (e.g., hydrochlorothiazide, HCT) for sequential nephron blockade or with distally acting diuretics such as the ENaC inhibitors amiloride and triamterene blockers are conceivable.

Additionally*, our investigations in patients with protein uric kidney disease and nephrotic syndrome show that activation of ENaC by serine proteases also appears to be of central importance as a mechanism of oedema formation in humans*

1.2 Purpose of the Study

Treatment of resistant oedema and overhydration in nephrotic syndrome can be challenging and we successfully use ENaC inhibitors (in combination with hydrochlorothiazide) in these patients. However, most clinicians treat nephrotic oedema with higher doses of loop diuretics, or combination therapy with thiazide diuretics. Worldwide, ENaC inhibitors are rarely used in nephrotic syndrome. This is due to lack of evidence of the role of activated ENaC and the effectiveness of ENaC inhibitors in human nephrotic syndrome. In this clinical trial, we aim to investigate the therapeutic effect of amiloride on the reduction of oedema and overhydration in the nephrotic syndrome.

2 Study Objectives

In the *study, patients with nephrotic syndrome will be randomized to medication with the investigational drug amiloride (study arm 1) or the comparator furosemide (study arm 2) for a study period of 16 days. In case of treatment resistant oedema, hydrochlorothiazide (HCT) is added at day 8 in both study arms. To prevent bias, patients included in the study are randomized to start treatment with amiloride or furosemide.*

2.1 Primary Objectives and Endpoint

In the light of our current results on ENaC activation by serine proteases aberrantly filtered into urine in nephrotic syndrome, we hypothesize that targeting ENaC with amiloride will lead to a more effective reduction of overhydration in nephrotic syndrome than standard treatment with the loop diuretic furosemide.

Primary objective of the study is therefore to prove the efficacy and superiority of amiloride for reduction of oedema and overhydration (OH) in human nephrotic syndrome in comparison to standard medication with furosemide. Primary endpoint of the study is the decrease of overhydration after 8 days, compared to baseline. OH is measured using bioimpedance spectroscopy with the device Body Composition Monitor, Fresenius Medical Care AG & Co; BCM) and expressed as percent of extracellular water (% ECW).

2.2 Secondary Objectives and Endpoints

Secondary endpoints represent further parameters to evaluate course of overhydration and regulation of body volume status after initiation of study medication with amiloride or furosemide. Secondary endpoints include

1. *decrease of OH after 16 days, measured using bioimpedance spectroscopy and expressed as % ECW*
2. *decrease of body weight after 8 and 16 days*
3. *decrease of oedema circumference after 8 and 16 days, measured as the largest extent on the lower leg*
4. *decrease of systolic and diastolic blood pressure after 8 and 16 days*
5. *increase of urine volume and natriuresis after 8 and 16 days, determined from urine collection over 24 hours*
6. *course of plasma renin activity and serum aldosterone concentration after 8 and 16 days*
7. *number of required changes of dose of study medication*
8. *need for co-medication with HCT after 8 days*
9. *occurrence of adverse events*

3 Study Methods

3.1 General Study Design and Plan

The study design is a phase IIIb, monocentre, interventional, two-arm, randomized, open-label controlled clinical trial. We decided for a study type with randomization as method against bias and have omitted blinding, as laboratory results required for safety reasons and dose adjustments will inevitably reveal to which study group patients have been randomized.

Overall study design is shown in figure 1.

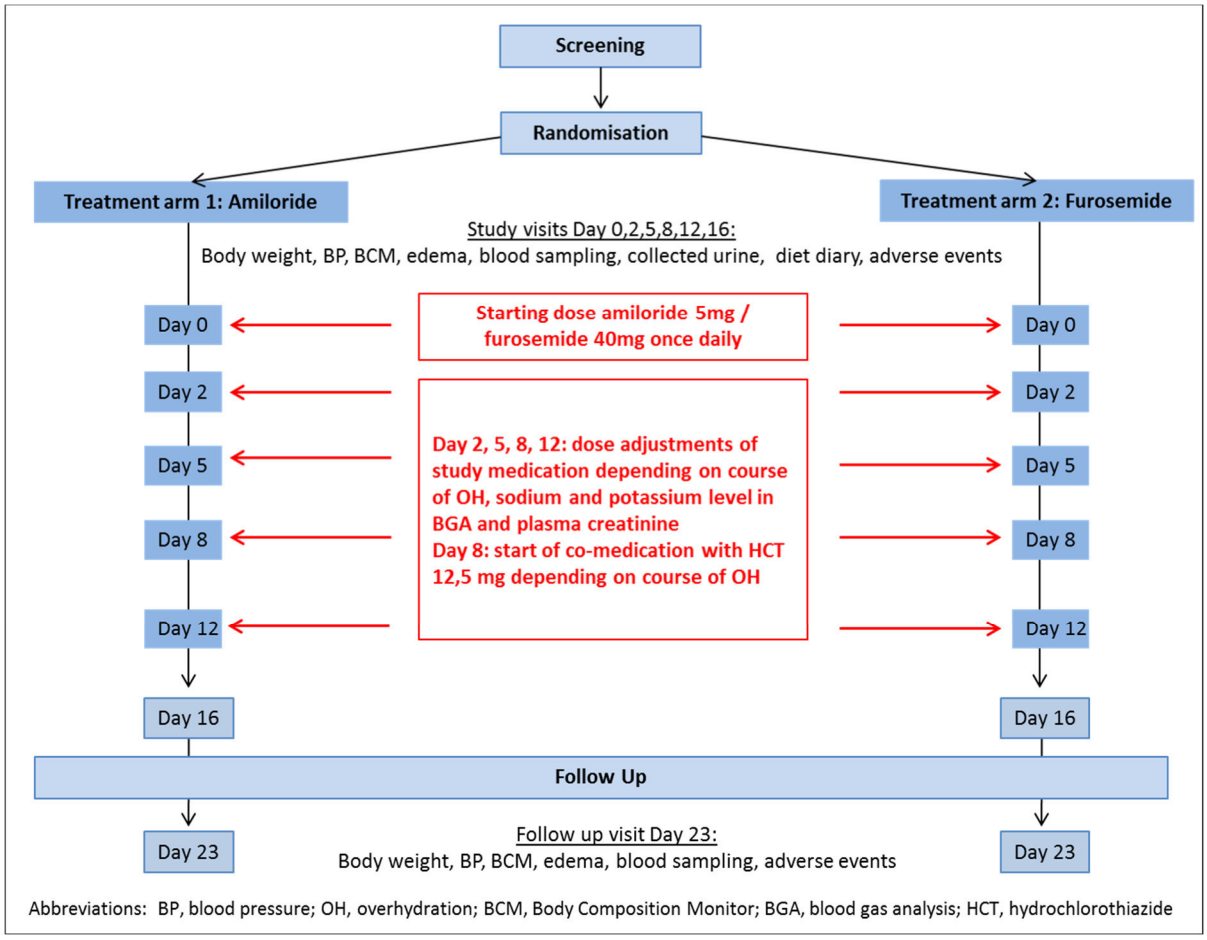

Figure 1 Overall study design (according to study protocol)

3.2 Study Treatment

Participants are randomized to start medication with amiloride 5 mg (trial arm 1) or furosemide 40 mg (trial arm 2) at day 0. Dose adjustments depending on course of overhydration, sodium and potassium level in blood gas analysis and plasma creatinine are performed on day 2, 5, 8 and 12. In case of therapy-refractory overhydration, co-medication with HCT 12,5 mg is started at day 8.

3.3 General Study Population and Inclusion/Exclusion Criteria

This study will include patients with acute nephrotic syndrome (definition see below) due to different underlying diseases (e.g. diabetic nephropathy, membranous nephropathy, minimal change disease, focal segmental glomerulosclerosis). The patients will be recruited from the nephrological outpatient department of the University Hospital Tuebingen or after referral from local nephrological practices.

Adult male and female patients with acute nephrotic syndrome and fulfilling the below outlined inclusion criteria will be enrolled into the study.

Trial population will consist of both genders. Gender distribution in the trial is supposed to reflect the distribution in the real patient's population, i.e. there will be no prior defined quantitative ratio between females and males. No statistical analysis based on genders or differences between genders will be performed.

3.3.1 Inclusion Criteria

According to the study protocol *subjects meeting all of the following criteria will be considered for admission to the trial:*

1. *Acute nephrotic syndrome with proteinuria > 3 g/day and formation of oedema.*
2. *Age ≥ 18 years at the time of signing the informed consent.*
3. *Understand and voluntarily sign an informed consent document prior to any study related assessments/procedures.*
4. *Ability to adhere to the study visit schedule and other protocol requirements.*
5. *Use of adequate thrombosis prophylaxis due to the increased risk of thrombosis in nephrotic syndrome and the expected fluctuations in volume balance during study participation*
6. *Subject (male or female) is willing to use highly effective methods of contraception according to the "Clinical trial fertility group" recommendations (http://www.hma.eu/fileadmin/dateien/Human_Medicines/01About_HMA/Working_Groups/CTFG/2014_09_HMA_CTFG_Contraception.pdf) during treatment and for 28 days (male or female) after the end of treatment (adequate: intrauterine device, intrauterine hormone-releasing system, bilateral tubal occlusion, vasectomized partner[1], sexual abstinence[2]; due to the increased risk of thrombosis in nephrotic syndrome, no hormonal contraceptives are recommended).*
7. *Female Patients of childbearing potential (WOCBP)[3] must agree to pregnancy testing before inclusion in the study.*
8. *Female Patients must agree to abstain from breastfeeding during study participation and 28 days after study drug discontinuation.*
9. *All subjects must agree not to share medication.*

[1] *Vasectomized partner is a highly effective birth control method provided that partner is the sole sexual partner of the WOCBP trial participant and that the vasectomized partner has received medical assessment of the surgical success*

[2] In the context of this guidance sexual abstinence is considered a highly effective method only if defined as refraining from heterosexual intercourse during the entire period of risk associated with the study treatments. The reliability of sexual abstinence needs to be evaluated in relation to the duration of the clinical trial and the preferred and usual lifestyle of the subject
[3] For the purpose of this document, a woman is considered of childbearing potential (WOCBP), i.e. fertile, following menarche and until becoming post-menopausal unless permanently sterile. Permanent sterilisation methods include hysterectomy, bilateral salpingectomy and bilateral oophorectomy. A postmenopausal state is defined as no menses for 12 months without an alternative medical cause. A high follicle stimulating hormone (FSH) level in the postmenopausal range may be used to confirm a post-menopausal state in women not using hormonal contraception or hormonal replacement therapy. However, in the absence of 12 months of amenorrhea, a single FSH measurement is insufficient.

3.3.2 Exclusion Criteria

Subjects presenting with any of the following criteria will not be included in the trial:

1. *Severe reduction of kidney function: Creatinine clearance or calculated GFR < 30 mL/min/1.73m² or acute kidney injury KDIGO stage 2 or 3 or anuria.*
2. *Hypovolemia or dehydration.*
3. *Uncontrolled diabetes mellitus.*
4. *Hypotension, systolic blood pressure < 90 mm Hg.*
5. *Hyperkalaemia, plasma potassium concentration > 4.8 mmol/l.*
6. *Hypokalaemia, plasma potassium concentration < 3.3 mmol/l.*
7. *Hyponatremia, plasma sodium concentration < 128 mmol/l.*
8. *Hypercalcemia, ionized calcium > 2.0 mmol/l or total albumin corrected calcium > 3.0 mmol/.*
9. *Signs of cardiac decompensation (orthopnoea, dyspnoea NYHA IV).*
10. *Hepatic coma or precoma.*
11. *Symptoms of gout.*
12. *Current therapy with potassium-sparing diuretics (e.g. spironolactone) or potassium supplements.*
13. *Women during pregnancy and lactation.*
14. *History of hypersensitivity to the investigational medicinal product, comparator or co-medication or to any drug with similar chemical structure or to any excipient present in the pharmaceutical form of the investigational medicinal product, comparator or co-medication.*
15. *Any other clinical condition that would jeopardize the patient's safety while participating in this clinical trial.*
16. *Active participation in other clinical trials or observation period of competing trials.*

3.4 Randomisation

The randomization will follow a randomization scheme of a 1:1 (amiloride and furosemide). Subjects will be assigned to their respective treatment by randomization. A randomisation number will be assigned (= Random. number). Subjects will then receive their study medication during their personal visit at day 0 of study participation.

3.5 Blinding

The principal investigator and the study team *have opted against blinding, as laboratory results required for safety reasons and for definition of dose adjustments will inevitably reveal to which study group each patient was randomized (hypokalaemia – furosemide, hyperkalaemia – amiloride).*

3.6 Study Procedures

This Study consists of the following consecutive phases:

- *Study entry*
- *Treatment*
- *Follow-up.*

Time-points and trial procedures are listed in Table 1.

Table 1 Table of Events

	Study entry	***Treatment***						***Follow-up***
Visit number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Day	*-3 to -1*	*0*	*2*	*5*	*8*	*12*	*16*	*23*
Study Entry								
Informed Consent	*X*							
Demographics, including sex, body weight, high, age	*X*							
Inclusion/Exclusion criteria: anamnesis, clinical examination, blood pressure, pre-existing medication, proteinuria in 24 hours collected urine, general laboratory control (including blood count, creatinine, urea, liver enzymes, lactate dehydrogenase, c-reactive protein, electrolytes (potassium, sodium, calcium), glucose, HbA1c, pregnancy test in females)	*X*							
Safety Assessments								
Adverse events		*X*	*X*	*X*	*X*	*X*	*X*	*X*
Concomitant medication	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Check of the study medication		*X*	*X*	*X*	*X*	*X*	*X*	
Return of any remaining study medication							*X*	
Plasma creatinine	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Potassium and sodium level in blood gas analysis	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*

Continued Table 1 Table of Events

	Study entry	***Treatment***						***Follow-up***
Visit number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Day	*-3 to -1*	*0*	*2*	*5*	*8*	*12*	*16*	*23*
Efficacy Assessment								
Body weight	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Bioimpedance spectroscopy with BCM	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Oedema circumference	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Blood pressure	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Plasma renin activity, serum aldosterone concentration	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
24 hours urine volume and natriuresis	*X*		*X*	*X*	*X*	*X*	*X*	
Diet diary during urine collection	*X*		*X*	*X*	*X*	*X*	*X*	
Check for dose adjustment		*X*	*X*	*X*	*X*	*X*		

4 Sample Size

Primary endpoint of the study is decrease of overhydration (measured using bioimpedance spectroscopy) after 8 days, compared to baseline. Based on data from a baseline sample (n = 14) and clinical experience on reduction of overhydration baseline overhydration is expected to be 26.5 % of extracellular water (standard deviation 12.3; normal distribution is assumed) and it is expected that the overhydration will be reduced by 90 % in the amiloride group and by 50 % in the furosemide group. For the final t-test for two groups, a one-tailed significance level of 0.05 and a power of 80 % should be ensured. From these conditions results a sample size of n = 18 patients per group (total n = 36; calculated for a t-test with the nQuery® Advisor 7.0 program). In order to take dropouts into account, the sample size is defined as n = 22 per group (total n = 44).

5 General Considerations

5.1 Timing of Analyses

The analysis will be performed after finalization and approval of this SAP. The analysis will be performed on a database-snapshot with clean data regarding the primary endpoint, in- and exclusion criteria and a few demographic variables. After the close-out visits took place the final analysis will be performed on the data which have been declared clean according to ZKS-SOP DA05. All changes to the database after the snap-shot will be documented in the TMF.

5.2 Analysis Population

5.2.1 Intention-to-treat Population (ITT)

All statistical analyses will be based on the Intention-to-Treat Population (ITT). The ITT includes all randomized patients with exception of patients who withdraw their informed consent for the analysis of their data during the study.

5.2.2 Safety Population (Safety)

The safety population includes all subjects who received any study treatment.

6 Data and Data Management

6.1 Case Report Form

The trial case report form (CRF) is the primary data collection instrument for the trial. All data requested on the CRF must be recorded. All missing data must be explained.

For this project, paper Case Report Forms (CRFs) will be used. The investigator is responsible for ensuring that all sections of the CRF are completed correctly and that entries can be verified against source data. The correctness of entries in CRFs will be confirmed by dated signature of the responsible investigator.

6.2 Data Handling

After first check for plausibility by eye, all data will be entered in a database as recorded in the CRF. To ensure data quality a double data entry will be performed. After completion of data entry, checks for plausibility, consistency, and completeness of the data will be performed. Based on these checks, queries will be produced combined with the queries generated by visual control.

6.3 Missing Data

All variables included in the CRF are mandatory. The monitoring will assure quality of the assessments. Thus, missing values are to be expected only due to refusal by patients. In the analysis of the primary endpoint missing values will be imputed using multiple imputation approaches, complete case and last observation carried forward analyses will be performed as sensitivity analyses. All other missing data will be given in the descriptive tables but excluded from the statistical test.

6.4 Derived Variables

Endpoints that are derived variables, i.e. not collected via the CRF:

- Change of overhydration from baseline to day 8 (see primary endpoint)
- Change of overhydration from baseline to day 16
- Change of body weight, oedema circumference, systolic and diastolic blood pressure after 8 and 16 days
- Change of urine volume, natriuresis, plasma renin activity and serum aldosterone concentration after 8 and 16 days

7 Statistical Analyses

7.1 Study Information

For all study subjects the disposition during the study and the duration including reasons (death, toxicity, treatment failure, withdraw consent) for pre-termination will be shown. All data for the CONSORT 2010 flow diagram will be given, i.e. the allocation to the analysis population and the number of subjects dropped out.

7.2 Protocol Deviations

The specific protocol deviations during the randomized treatment phase will be reviewed in the blind data review regarding the impact for the confirmatory analysis and categorized into major and minor. For all major protocol deviations summary statistics will be produced.

7.2.1 Major Protocol Deviations

The following events were classified as major protocol deviations:

- Deviation against inclusion or exclusion criteria
- Missing visit 5 (day 8) → no calculation of primary endpoint possible
- Late visit until day 8

7.2.2 Minor Protocol Deviations

The following events were classified as minor protocol deviations:

- Replacement of missing laboratory values from clinical patient record
- Late follow-up visit (1 – 5 days)
- Due to practical reasons blood and urine samples were taken at the routine ambulance visit which was declared as inclusion visit, and took place before the formal informed consent → no consequences for patient safety or data integrity.
- Stop of increasing the dose.

According to the blind data review (finalized 02.11.2023) no major protocol deviations occurred. Note-to-files were documented during the course of the study for all minor protocol deviations.

7.3 Descriptive Statistics

For all study data descriptive summary statistics will be produced*. Descriptive analyses will include absolute and percentage frequencies for categorical variables, means, medians, standard deviations, quartiles and ranges for quantitative variables and medians, quartiles and ranges for ordinal variables.*

7.4 Efficacy Analyses

7.4.1 Primary Endpoint

The primary endpoint variable is the *change of overhydration (OH) after 8 days compared to baseline, measured by bioimpedance spectroscopy using the Body Composition Monitor and expressed in percent of extracellular water (% ECW). The primary endpoint variable will be compared between the two study arms, which are patients with amiloride treatment and patients with furosemide treatment.*

The null-hypothesis is that there is equal or greater decrease of OH after 8 days in the group of patients with furosemide treatment compared to the group of patients with amiloride treatment. The alternative hypothesis is that there is a greater decrease of OH after 8 days in the group of patients with amiloride treatment compared to the group of patients with furosemide treatment. For analysis of the primary endpoint variable a one-sided t-test for two groups will be performed to test the null hypothesis against the alternative hypothesis. Effect size and 95%-confidence interval for effect size will be estimated. Only the rejection of the null hypothesis will be interpreted as statistical evidence for the efficacy of the amiloride treatment.

7.4.2 Secondary Endpoints

Secondary endpoint variables are listed in section 2.2. All secondary endpoints will be compared and statistically assessed for descriptive purposes and not in a confirmatory sense. The aim of the analysis is explorative data analysis, not hypothesis testing or generation of evidence for efficacy and no attempt will be made to adjust the p-values of statistical tests of the secondary endpoints for multiple testing. If adequate, secondary endpoints will be compared and statistically assessed using t-tests for two groups or covariance techniques with baseline values as covariates in case of quantitative variables. Depending on distribution, non-parametric tests may be indicated. Dichotomous data will be compared and statistically assessed using Mantel-Haenszel chi-squared tests including relative risks and 95%-confidence intervals for relative risks.

7.5 Safety Analysis

Safety will be assessed by frequency tabulations and line listings for AEs and SAEs*.*

8 Reporting Conventions

P-values ≥ 0.001 will be reported to 3 decimal places; p-values less than 0.001 will be reported as “< 0.001”. The mean, standard deviation, and any other statistics other than quantiles, will be reported to one decimal place greater than the original data. Quantiles, such as median, or minimum and maximum will use the same number of decimal places as the original data or one decimal place greater than the original data. Estimated parameters, not the same scale as raw observations (e.g. regression coefficients) will be reported within tables or figures.

9 Technical Details

At the time of writing this SAP the study specific documents used data base will be stored at Koordobas 5.1.0, the software package for statistical analysis will be SAS 9.4, and the operating system of the computer will be Windows Server 2019.

11 Time Table

Actual for data analysis and statistical report the following time table is planned

Approval of the SAP (signed)	11/2023
Final clean file for data analysis	11/2023
Submission of the final statistical report	12/2023

12 Planned Tables, Figures and Listings

Actual the following tables, figures and listings are planned for the statistical report:

Table 2 Planned tables, figures and listings

No	Title/Item
1	**Study information**
1.1	Consort 2010 statement (figure)
1.2	Inclusion-/exclusion criteria, informed consent
1.3	Study duration
1.4	Termination for subjects
1.5	Protocol deviations (major)
2	**Descriptive Analysis (ITT population)**
2.1	Study entry
2.1.1	Demographic data
2.1.2	Clinical examination
2.1.3	Blood analysis
2.1.4	Urine analysis
2.1.5	Additional laboratory data
2.1.6	Medical history
2.1.7	Previous medication
2.1.8	Study medication
2.2	Treatment phase
2.2.1	Clinical examination
2.2.2	Blood analysis
2.2.3	Urine analysis
2.2.4	Additional laboratory data
2.2.5	Dose of study medication
2.2.6	Concomitant medication

Continued Table 2 Planned tables, figures and listings

No	Title/Item
3	**Statistical Analysis (ITT population)**
3.1	Primary endpoint
3.2	Secondary endpoints
4	**Safety (safety population)**
4.1	Extend of exposure
4.2	AEs
4.3	SAEs
5	**Listings (ITT population)**
5.1	Study information
5.2	Inclusion-/exclusion criteria
5.3	Demographic and anamnestic parameters
5.4	Clinical examination
5.5	Pre-existing, concomitant and study medication
5.6	Laboratory parameters and pregnancy test
5.7	Bioimpedance spectroscopy (BCM)
5.8	Items of the diet diary
5.9	AEs and SAEs