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Hydroxychloroquine for Prevention of Recurrent Miscarriage:

Study Protocol for a Multicenter Randomized Placebo Controlled Trial

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ABSTRACT

Introduction Recurrent miscarriage (RM), defined by >=3 consecutive losses during the first trimester of pregnancy, affects 1 to 2% of fertile couples. Standard investigations fail to reveal any apparent cause in ~ 50% of couples. However, on the basis of animal models and clinical studies, several hypotheses have been put forward concerning underlying mechanisms of RM: altered ovarian reserve, progesterone defect, thrombotic and/or endothelial dysfunction, immunological disturbances. Nonetheless, no study has yet reached conclusive beneficial clinical evidence for a potential treatment in unexplained RM. Hydroxychloroquine (HCQ) is a molecule with extensive safety data during pregnancy. HCQ pharmacological properties (e.g. anti-thrombotic, vascular protective, immunomodulatory, improved glucose tolerance, lipidlowering, antiinfectious) could be effective against some mechanisms of unexplained RM. Furthermore, eventhough clinical benefit of HCQ is suggested in prevention of thrombotic and late obstetric events in antiphospholipid (APL) syndrome, there is no data suggesting the benefit of HCQ in RM in the presence of APL antibodies.

Methods and analysis Taken all together and given the low cost of HCQ, the aim of this multicenter, randomized placebo-controlled, double-blind study is to investigate whether HCQ would improve the live-bith rate in women with RM, irrespective of maternal thrombophilic status: i) no known thrombophilia, ii) inherited thrombophilia or iii) APL antibodies. The primary end-point is a live and viable birth. After confirming eligibility and obtaining consent, 300 non-pregnant women will be randomized in 2 parallel groups for a daily oral treatment (HCQ 400 mg or placebo), initiated before conception and stopped at 10 weeks' gestation. If pregnancy doesn't occur after one year, the treatment will be stopped.

Ethics and dissemination Agreement from the French National Public Health and Drug Security Agency (160765A-22) and ethical approval from the Committee for the Protection of Persons of NORD-OUEST I (2016-001330-97) have been obtained.

Trial registration number ClinicalTrials.gov: NCT0316513

STRENGTHS AND LIMITATIONS OF THIS STUDY

- Despite numerous fundamental research publications and clinical trials, the only recommendation that applies to follow-up of child bearing women suffering from RM relies on cocooning.
- This is the first randomized placebo-controlled study which aims to investigate whether oral hydroxychloroquine would improve the live-bith rate in women with recurrent miscarriage irrespective of maternal thrombophilic status.
- As a possible limitation, this study is based on the hypothesis that recurrent miscarriage often results from combined failures which could benefit from the pleiotropic effects of hydroxychloroquine.
- As hydroxychloroquine should probably be administrated at least 2 menstrual cycles before conception to have an effect, women will be advised not to get pregnant during this period, but we do not plan to exclude those who will conceive too early.

INTRODUCTION

Background

RM is a common public health reproductive burden causing both physical and emotional distress. To date there is no treatment whose benefit has been clearly proved, even in the presence of well-known risk factors of RM. Most importantly, the absence of benefit has been clearly demonstrated for some treatments that are yet empirically proposed.

Unexplained Recurrent Miscarriage (RM)

A sporadic miscarriage is clinically detected in approximately 10% to 15% of pregnancies. Fetal development usually stops before 10 weeks.[1]Given the recurrent miscarriage (RM) frequency among fertile couples (1% to 2%) being significantly higher than the expected random one (153 = 0.34 %), RM is most often defined as ≥ 3 consecutive losses. Apart from the detection of a lethal chromosomal abnormality on products of conception, the underlying mechanism of loss remains unknown in most cases. The rate of normal embryonic karyotypes in RM steadily increases from the third loss, suggesting alternative mechanisms than meiotic aberrations.[2]Standard investigations fail to reveal any apparent cause in ~50% of the women. However, On the basis of animal models and clinical studies, several hypotheses have been put forward. Here, we focus on thrombosis and both endothelial and immune dysfunctions. Those could be targeted by pharmacological properties of hydroxychloroquine (HCQ).

An association with some inherited thrombophilia has been reported (factor V Leiden, mutation G20210A of the prothrombin and protein S deficiency), although the odds ratio were most often ≤ 2.[3] Animal models have demonstrated that some actors of the haemostatic system may participate in normal implantation and placental development regardless of the

coagulation process.[4,5] In humans, a basal prothrombotic state outside of pregnancy has been measured in women with previous RM and without known thrombophilia.[6-8]This relative prothrombotic state, measured at distance of any obstetrical event, could reflect chronic endothelium damage in those women.[9,10]Notwithstanding, the clinical trials that have assessed antithrombotic treatments (aspirin initiated before or after conception, eventually combined with LMWH, or LMWH alone initiated after pregnancy diagnosis) did not show any benefit in prevention of further loss.[11-14]Likewise, no benefit of LMWH has been shown in the subgroup of women with inherited thrombophilia (TIPPS study).[15]However, we emphasize that the subcutaneous route of LMWH administration does not allow assessing this treatment at the critical time of implantation onset in fertile women. Indeed, the injections cannot be routinely initiated before 5 weeks' gestation.

Regarding immune dysfunction, apart from the detection of many auto-antibodies (antiphospholipid, anti-thyroid),[16] attention is now focused on natural killer cells, whose temporal and spatial distribution in the uterine mucosa suggests that they contribute to control trophoblast invasion and cytokine response at the maternal-fetal interface (balances Th-1 / Th-2 and Th-17 / T-reg). Th-2 and T-reg preponderance in normal pregnancy shifts to Th-1 and Th-17 predominance in RM.[17,18] Otherwise, an overexpression of TOLL receptors in decidua was observed in women with RM, especially TLR4 and TLR9 receptors.[19] Immunomodulatory treatments have therefore been proposed and assessed without conclusive results (paternal leukocyte immunization,[20] intravenous immunoglobulins,[21] Small doses of corticosteroids.)[22]

Despite numerous fundamental research publications and clinical trials, the only current recommendation for follow-up of child bearing women suffering from unexplained RM relies on cocooning.[17,23]

RM in the presence of antiphospholipid (APL) antibodies

Antiphospholipid syndrome (APS) is diagnosed in the presence of persistent, above the 99th percentile, APL levels and of at least one clinical manifestation (obstetrical, arterial or venous thrombosis). «Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded »[24] delineates one of the possible manifestation of APS. Even though RM is one accepted clinical manifestation of APS, its specificity is low, as compared to the occurrence of fetal death after 10 weeks of amenorrhea.[24] The benefit of low dose aspirin and/or LMWH or heparins for secondary prevention of RM has not been demonstrated in women who present with APL antibodies and no other clinical manifestation of APS.[25] The HEPASA study found no benefit of combination therapy of LMWH and low dose aspirin in women who presented with APL, without arterial or venous thrombosis history, and who had at least two fetal losses before 32 weeks of amenorrhea.[25] As a consequence, no clinical evidence has been drawn for an optimal management of these women.

Rationale for hydroxychloroquine (HCQ)

Chloroquine and HCQ are used for centuries to treat malaria. Because of a more favorable usage profile and immunomodulatory properties, HCQ use became common for treatment of autoimmune and inflammatory diseases. Other therapeutic properties of HCQ have since been discovered, or rather rediscovered, and assessed in a more consistent manner. The following HCQ properties could be effective against mechanisms of RM.

Since 30 years, benefit of HCQ to prevent venous thromboembolism (VTE) has been suspected[26-35] i) among patients with SLE[26,31] and ii) among all-comer patients, free of autoimmune disease, after orthopedic surgery.[27-30]Thus, before the use of LMWH, HCQ was assessed in the 1970s in the prevention of VTE events after orthopedic surgery. The results of randomized trials,[28-30] (around 10 000 patients included) led to the conclusion that HCQ was beneficial in preventing VTE after orthopedic surgery.[28] In patients with SLE, in the presence or absence of APL antibodies, studies have suggested the benefit of HCQ for the primary prevention of both arterial and venous thrombotic events.[26,31-33] In primary APS, HCQ reduced the thrombus size after vessel injury in mice[34] and would improve the secondary prevention of VTE in humans, in comparison to oral anticoagulants used alone.[35]The antithrombotic action of HCQ would be inherent to some biological effects (in the absence[36-38]or in the presence[39-40] of APL antibodies). Otherwise, much data support an endothelium protective action of HCQ via anti-diabetic,[41]lipidlowering,[42]anti-oxidant[43]effects or a direct endothelial protection, via ERK5 protein kinase activation.[44]

Regarding immunomodulation, HCQ has an impact on the innate immunity by inhibition of the activation of some ToLL receptors (3,7,9).[45-47]HCQ decreases the circulating levels of interleukine 1, 2,[48] 6,[49] TNF[49,50] and, interferon-γ,[51] promoting the TH2 processes that prevail in a “normal pregnancy». Otherwise, HCQ decreases APL plasma levels[52] and interferes with both endothelial cell activation and TNF-α production, 2 major key pathways involved in APS.[53-55]

We also outline the well-known anti-infectious action of HCQ which originates from the alkalinization of intracellular acidic vesicles and might inhibit the growth of intracellular microorganisms. This could act against chronic endometritis, additional mechanism suspected in RM.

Remarkably, the use of HCQ (and closely related molecules) as anti-malarial or in auto-immune diseases during pregnancy[56,58]and lactation[57] have provided extensive safety data during pregnancy and even during breast-feeding.

Otherwise, oral administration of HCQ facilitates its prescription early, before conception, thus enabling fetal exposure from the very beginning of time period at risk for activation of RM underlying mechanisms. Noteworthy, HCQ has never been evaluated in the indication of RM with or without APL antibodies.

Taken all together and given that RM is a stereotyped clinical entity whatever the maternal thrombophilic status, we aimed at evaluating the role of HCQ in prevention of RM, with or without APL antibodies or inherited thrombophilia.

STUDY OBJECTIVES

Primary objective

The aim of this placebo-controlled, double-blind trial is to investigate whether HCQ (orally administrated before conception until 10 weeks of gestational age) would improve the live-birth rate of 15% or more, in women with previous RM, irrespective of their biological thrombophilic status.

Secondary objectives

We aim to investigate whether:

- HCQ effect would be different among women with inherited thrombophilia or APL antibodies
- HCQ would have an impact on the occurrence of some pregnancy events (early miscarriage, intrauterine fetal death, placental vascular disease)
- The impact of HCQ would be different in subgroups of women at higher or lower risk of recurrence according to the number of previous miscarriages (= 3 ou > 3), the maternal age (≥ 35 ou < 35 ans), the parity: previous live-birth, a previous late fetal death after 10 weeks' gestation.

We aim to confirm that HCQ has no negative impact on the chance of getting pregnant, the newborn (gestational age, birth weight, survival at 28 days, congenital abnormalities), the child at 6 months.

METHODS

Study design

This is a prospective, randomized 1:1 placebo-controlled, double-blind, and French multi-centre trial of phase III, with 2 parallel-groups (HCQ 400 mg or placebo). At randomization, the minimization method will be used to balance between the 2 groups and 2 main determinants of miscarriage recurrence: maternal age (≤ 35 or >35 years) and number of previous losses (3 or ≥ 4).

Population

The study population consists of women trying to conceive and who had experienced at least 3 consecutive miscarriages at the first trimester of pregnancy (normal parental caryotypes, no uterine cavity abnormality that might explain the losses). Those women are followed-up by their gynecologist or general practitioner who refers them to one of the RM specialized unit participating in the study for screening. At the inclusion visit, the medical investigator checks inclusion and exclusion criteria of eligible women.

Inclusion criteria

- Women aged between 18 and 37 inclusive,
- Women trying to conceive,
- Women with at least 3 previous consecutive miscarriages in the first pregnancy trimester, of unknown origin defined as:
    - Normal parental karyotypes,
    - No uterine cavity abnormality that might explain RM (ultrasound scan, hysteroscopy or hysterography),

- In case of persistent positive APL antibodies according to the biological criteria of Myakis:[24]no previous thrombotic or obstetrical event defined in APS[24], except for RM in the first trimester of pregnancy, .

➢ Women who have given their informed consent.

Exclusion criteria

➢ Ongoing pregnancy,

➢ Normal pregnancy (live and viable birth) since the last miscarriage,

➢ Abnormal parental karyotype,

➢ Uterine cavity abnormality that might explain RM in the first trimester of pregnancy,

➢ Antiphospholipid Syndrome defined as both:

- persistent positive APL antibodies: lupus anticoagulant and/or APL (anticardiolipin or anti béta2 GPI, IgG or IgM) titers> 99th percentile or >40 with at least 12 weeks interval between two positive determinations (persistent antibodies) AND,
- a specific clinical setting of APS (thrombotic or obstetrical, apart from RM in the first trimester of pregnancy) according to Myakis criteria.[24]

➢ Known contraindication to a treatment by HCQ (retinopathy, hypersensitivity to chloroquine or HCQ, G6PD deficiency, acute intermittent porphyria, chronic liver or kidney insufficiency, extensive cutaneous psoriasis not controlled by local treatment, significant chronic digestive or hematologic disease) or known rare disorder of lactose metabolism (excipient),

➢ Past history of epilepsia or psychotic disorders,

➢ Indication to a treatment by HCQ according to the MA (rheumatoid arthritis, Lupus, solar eczema),

- Previous exposure >4 years to chloroquine or HCQ,
- Previous inclusion in this study,
- Woman unable to consent, protected under the terms of the law, or woman deprived of liberty by judicial or administrative decision,
- non affiliation to the social security system,
- Impossible follow-up.

Allocation, randomization and blinding

After inclusion and obtaining written consent, an identification number is assigned to the patient through a secure server providing access to electronic case report forms generated by the CIC Brest (via the "Capture System" software). The randomization is done using the "Capture System" software with implementation of a randomization with minimization to balance between the 2 treatment groups according to the main determinants of miscarriage recurrence: age (≤ 35 or >35 years) and number of miscarriages (3 or ≥ 4).

The patient, the investigator and all medical and paramedic professionals taking care of the patient will be blinded to the treatment group. The packaging of placebo and HCQ will be provided in the form of capsules with identical external shape and packaging. Information on the correspondence between treatment group and the patient number will be held by both the central pharmacy of Brest CHRU and the Data Management Unit.

Intervention

Included patients are assigned to receive either 400 mg of oral HCQ per day, administered in 2 divided doses of 200 mg or 2 daily doses of oral placebo. Thus, treatment is initiated before

conception and stopped at 10 weeks' gestation (weeks of amenorrhea) or after one year of treatment in the absence of pregnancy.

Rationale for choosing oral 400 mg daily HCQ

It is the usual route of administration and dose for indications specified by the Marketing authorization (MA). This choice of dose comes from the evaluation of the risk-benefit balance in the MA indications and, over all, from the fact that data of good tolerance have been compiled to this (usual) dosage in literature. Otherwise, in most cases, fetal development stops before 10 weeks of amenorrhea. Treatment exposure is essential from the very beginning of implantation, a developmental stage that can potentially be disturbed by the mechanisms responsible for miscarriage. The oral administration allows starting the treatment before conception. This drug has a long half-life (from 30 to 60 days). Its full effect is obtained only after certain duration of exposure. That is why its administration is required before conception and its discontinuation after 10 weeks of amenorrhea, i.e. before the end of the first trimester of pregnancy which is at the highest risk for RM.

Treatments frequently given in combination

- Folic acid and other vitamin supplements,
- Aspirin at low dose, which could be given by some investigators to prevent the recurrence of vasculo-placental disease (preeclampsia, intra-utero growth restriction) or as part of a primary prevention of vasculo-placental events in women with high levels of persistent APL antibodies.

Study plan and procedures

Before pregnancy, women are monthly contacted by phone. The non pregnant women are systematically seen in consultation after 6 and 12 months of treatment. In the absence of pregnancy after one year, the treatment will be withdrawn and patients will be contacted by phone at 14 months and 18 months (End-of-Study visit).

During pregnancy, women are monthly seen in consultation (4 first months) or contacted by phone. The treatment is withdrawn at 10 weeks' gestation or earlier in case of pregnancy loss.

After delivery, the women are seen in consultation at~2 months, and contacted by phone at 6 months (End-of-Study visit).

Four tubes of 2.7 ml with sodium citrate and 4 tubes SST of 5 ml (for plasma and serum bank, respectively) are collected at both the inclusion visit and the visit of pregnancy onset or the visit planned after one year of treatment in the absence of pregnancy. Additionally 2 tubes EDTA of 5 ml are collected for DNA bank at inclusion. The biological collection of plasma is carried out within 3 hours after receipt of tubes. DNA extraction is carried out within one week of collection (and at the latest within a month if the sample is frozen at -80 ° C pending extraction). Those specimens are designed to identify prognostic factors (including thrombophilic work-up) for efficacy of the study treatment.

End points

The primary end point is a live and viable birth.

The secondary end points are:

- A live and viable birth, for the subgroup analyses,

- The occurrence of miscarriage, in-utero fetal death, placental vascular disease, premature delivery,
- Gestational age at miscarriage,
- Concerning the child: gestational age and weight at birth, survival at 28 days, safety data at 6 months of life, congenital abnormality.

Safety considerations, safety monitoring and AE reporting

Apart from, adverse events (AE) and serious adverse events (SAE), some safety parameters will be systematically recovered: CBC, visual and neurological symptoms for women and reports of ultrasound and post-natal medical visits for children. Adverse events (AE) are defined as any untoward medical occurrence in a person who consents to biomedical research whether this event is related or not to the research or experimental drug on which this research addresses. The gravity of serious adverse events (SAE) is defined by one of the following findings: death, life-threatening situation, significant or sustained incapacity or disability, hospitalization, prolongation of hospitalization, deformity or birth defect, potentially serious event. Any SAE or event of interest, regardless of its causal relationship with the trial treatment or the research, must be reported to the sponsor, as soon as possible and the latest 24 hrs after their occurrence for SAE. The investigator is in charge of noting and reporting all SAE occurring during the study, from the date of the signed informed consent, during the study's expected duration of follow up (6 months after the treatment cessation for women and at age 6 months for newborns). Furthermore, regardless of the event occurrence deadline after the end of the study, all SAE that may be due to the research must be declared to the sponsor when there is no other cause and effect accounted for (such as serious events that may occur long after exposure to the drug, such as cancers or congenital abnormalities).

Some AE and SAE are expected and can be linked to i) pregnancy, ii) the disease (occurrence of another miscarriage) or study treatment (benign, adverse reaction AR, as digestive symptoms or severe, serious adverse reaction SAR as skin reaction, which requires to immediately stop the treatment).

Independent Data and safety monitoring Board (IDSMB)

The IDSMB comprises 8 members without competing interests, not directly involved in other aspects of the trial and independent from the sponsor: 7 voting members, experts in complementary fields of the pathology and clinical trials (internal medicine, methodology, specialist of teratogenic agents, neonatology, hematology, ophthalmology) and a non-voting independent statistician. IDSMB monitors the data that affects patient safety, provides independent expertise for the evaluation of adverse events (AE) occurring during the study, expresses an opinion concerning the benefit / risk ratio and provides recommendations in order to help the steering committee to take decisions on protocol modification or early termination of the study. The members of the IDSMB will have to meet every 20 inclusions. The IDSMB will transmit its recommendations to the steering Committee, which decides whether or not to stop the study. Given the safety data and the treatment benefit, several different recommendations can be provided by the IDSMB: i) continuation of study without protocol modification, ii) continuation of study with modification of protocol, iii) temporarily discontinuation of inclusions, iiii) early termination of the study

Over the course of study, the IDSMB will have an advisory role in reassessing the benefit/risk ratio of the study.

Number of patients

The total number of scheduled patients to be recruited is 300, on the basis of the following hypotheses:

- 80% of the included women should get pregnant (ALIFE study).[11]
- Among women who will get pregnant, we suppose that the rate of achieved pregnancy will be of 70% in the placebo group (PREFIX study).[14]

To demonstrate a minimal clinically important difference of 15% in live-birth rates on HCQ (i.e. a 50% relative risk reduction in pregnancy loss), we need to enroll 300 women for a power of 80%, and a two-tailed alpha risk of 5%.

The anticipated duration of recruitment is 3 years and the duration of participation of each patient: from 7 to 29 months.

Analyses plan

Analyses will be firstly conducted on an intention to treat basis for all women who have started pregnancy. Hierarchical analysis including three steps is planned :comparison of the number of live and viable births, between the two treatment groups i) among all the women, ii) among women without known APL antibodies, iii) among women without known thrombophilia (APL or inherited thrombophilia). We plan to adjust for age and number of previous losses if the difference between the two treatment groups is statistically significant ($p < .05$).

Secondary analyses will be conducted on an intention to treat mode:

For all included women, comparison of the number of live and viable births between the two treatment groups.

Among women who will get pregnant:

- Measure of treatment effect in women at higher risk of further miscarriage, according to the presence of thrombophilia, past history of intrauterine fetal death after 10 week's gestation, past history of miscarriage, > 3 previous miscarriage or, no previous newborn baby,
- Comparison between the two treatment groups of the occurrence of several obstetrical events (miscarriage and intrauterine fetal death, vasculo placental disease, premature birth),
- Comparison between the two treatment groups of newborn clinical data (gestation time at delivery, birth weight adjusted to the gestation time, number of newborn babies living at 28 days and congenital abnormalities.

A "per-protocol analysis" (primary endpoint) will include only patients properly exposed to the study treatment, that is i) at least during a whole menstrual cycle before pregnancy onset and until 10 weeks of amenorrhea ii) compliance≥ 80%.

No unblinded interim analysis is planned.

The biological collection will be carried out by the CRB of the CHRU of BREST and integrated into the collection "thrombosis" declared to the Ministry (2008-214/ DC-2009-925).

DISCUSSION

To date, there has not yet been an optimal therapy with conclusive clinical benefit for unexplained RM, in the absence or presence of inherited thrombophilia. Providing some psychological support seems to be the only available therapy for women with unexplained RM. Although the probability of a further normal pregnancy seems high, (~70% at age 32 after 3 consecutive miscarriages),[59] the proposed therapeutic interventions are sometimes excessive, (e.g. possible side effects) and costly (e.g. intravenous immunoglobulins, assisted procreation). Therefore, it is of utmost importance to investigate other therapeutic options.

In RM women with high titers of APL antibodies but without any other previous clinical event listed in APS (thrombotic or obstetric event), benefit of antithrombotic treatment (aspirin or LMWH) remains controversial. Clinical benefit of HCQ has not been demonstrated in prospective studies with robust methodology.[60]

In conclusion, the benefit/risk ratio and low cost are strong incentives for assessment of HCQ in unexplained RM irrespective of maternal thrombophilic status.

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This study was sponsored by Brest University Hospital.

PATIENT AND PUBLIC INVOLVEMENT

Patients were not involved in the design of the study.

ETHICS, REGULATORY APPROVALS AND DISSEMINATION

Agreement from the French National Public Health and Drug Security Agency (160765A-22) and ethical approval from the Committee for the Protection of Persons of NORD-OUEST I () have been obtained on November 4th 2016 and March 2nd 2017, respectively.

This trial is registered at www.ClinicalTrials.gov as # NCT0316513. and the French National Health and Drug Safety Agency (EudraCT # 2016-001330-97).

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Contributors

EP conceived and designed the study. EP drafted the original grant proposal and trial protocol. LDSM provides methodological and statistical expertise. GM has assisted in developing the protocol, helped with implementation and has responsibilities for day-to-day running of the trial including participant recruitment, data collection and liaising with other sites. JH and PM have helped with protocol implementation. CB, CC, FB, VLS, GPB, DM

have participated in the design of the study. VC coordinates treatment production and dispensation. All authors critically reviewed and approved the final version of the manuscript.

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Competing interests None declared.

Patient consent Obtained.

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Hydroxychloroquine for Prevention of Recurrent Miscarriage:

Study Protocol for a Multicenter Randomized Placebo Controlled Trial

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ABSTRACT

Introduction Recurrent miscarriage (RM), defined by >=3 consecutive losses during the first trimester of pregnancy, affects 1 to 2% of fertile couples. Standard investigations fail to reveal any apparent cause in ~ 50% of couples. However, on the basis of animal models and clinical studies, several hypotheses have been put forward concerning underlying mechanisms of RM: altered ovarian reserve, progesterone defect, thrombotic and/or endothelial dysfunction, immunological disturbances. Nonetheless, no study has yet reached conclusive beneficial clinical evidence for a potential treatment in unexplained RM. Hydroxychloroquine (HCQ) is a molecule with extensive safety data during pregnancy. HCQ pharmacological properties (e.g. anti-thrombotic, vascular protective, immunomodulatory, improved glucose tolerance, lipidlowering, antiinfectious) could be effective against some mechanisms of unexplained RM. Furthermore, eventhough clinical benefit of HCQ is suggested in prevention of thrombotic and late obstetric events in antiphospholipid (APL) syndrome, there is no data suggesting the benefit of HCQ in RM in the presence of APL antibodies.

Methods and analysis Taken all together and given the low cost of HCQ, the aim of this multicenter, randomized placebo-controlled, double-blind study is to investigate whether HCQ would improve the live-bith rate in women with RM, irrespective of maternal thrombophilic status: i) no known thrombophilia, ii) inherited thrombophilia or iii) APL antibodies. The primary end-point is a live and viable birth. After confirming eligibility and obtaining consent, 300 non-pregnant women will be randomized in 2 parallel groups for a daily oral treatment (HCQ 400 mg or placebo), initiated before conception and stopped at 10 weeks' gestation. If pregnancy doesn't occur after one year, the treatment will be stopped.

Ethics and dissemination Agreement from the French National Public Health and Drug Security Agency (160765A-22) and ethical approval from the Committee for the Protection of Persons of NORD-OUEST I (2016-001330-97) have been obtained.

Trial registration number ClinicalTrials.gov: NCT0316513

STRENGTHS AND LIMITATIONS OF THIS STUDY

- Despite numerous fundamental research publications and clinical trials, the only recommendation that applies to follow-up of child bearing women suffering from RM relies on cocooning.
- This is the first randomized placebo-controlled study which aims to investigate whether oral hydroxychloroquine would improve the live-bith rate in women with recurrent miscarriage irrespective of maternal thrombophilic status.
- As a possible limitation, this study is based on the hypothesis that recurrent miscarriage often results from combined failures which could benefit from the pleiotropic effects of hydroxychloroquine.
- As hydroxychloroquine should probably be administrated at least 2 menstrual cycles before conception to have an effect, women will be advised not to get pregnant during this period, but we do not plan to exclude those who will conceive too early.

INTRODUCTION

Background

RM is a common public health reproductive burden causing both physical and emotional distress. To date there is no treatment whose benefit has been clearly proved, even in the presence of well-known risk factors of RM. Most importantly, the absence of benefit has been clearly demonstrated for some treatments that are yet empirically proposed.

Unexplained Recurrent Miscarriage (RM)

A sporadic miscarriage is clinically detected in approximately 10% to 15% of pregnancies. Fetal development usually stops before 10 weeks.[1]Given the recurrent miscarriage (RM) frequency among fertile couples (1% to 2%) being significantly higher than the expected random one (153 = 0.34 %), RM is most often defined as ≥ 3 consecutive losses. Apart from the detection of a lethal chromosomal abnormality on products of conception, the underlying mechanism of loss remains unknown in most cases. The rate of normal embryonic karyotypes in RM steadily increases from the third loss, suggesting alternative mechanisms than meiotic aberrations.[2]Standard investigations fail to reveal any apparent cause in ~50% of the women. However, On the basis of animal models and clinical studies, several hypotheses have been put forward. Here, we focus on thrombosis and both endothelial and immune dysfunctions. Those could be targeted by pharmacological properties of hydroxychloroquine (HCQ).

An association with some inherited thrombophilia has been reported (factor V Leiden, mutation G20210A of the prothrombin and protein S deficiency), although the odds ratio were most often ≤ 2.[3] Animal models have demonstrated that some actors of the haemostatic system may participate in normal implantation and placental development regardless of the

coagulation process.[4,5] In humans, a basal prothrombotic state outside of pregnancy has been measured in women with previous RM and without known thrombophilia.[6-8]This relative prothrombotic state, measured at distance of any obstetrical event, could reflect chronic endothelium damage in those women.[9,10]Notwithstanding, the clinical trials that have assessed antithrombotic treatments (aspirin initiated before or after conception, eventually combined with LMWH, or LMWH alone initiated after pregnancy diagnosis) did not show any benefit in prevention of further loss.[11-14]Likewise, no benefit of LMWH has been shown in the subgroup of women with inherited thrombophilia (TIPPS study).[15]However, we emphasize that the subcutaneous route of LMWH administration does not allow assessing this treatment at the critical time of implantation onset in fertile women. Indeed, the injections cannot be routinely initiated before 5 weeks' gestation.

Regarding immune dysfunction, apart from the detection of many auto-antibodies (antiphospholipid, anti-thyroid),[16] attention is now focused on natural killer cells, whose temporal and spatial distribution in the uterine mucosa suggests that they contribute to control trophoblast invasion and cytokine response at the maternal-fetal interface (balances Th-1 / Th-2 and Th-17 / T-reg). Th-2 and T-reg preponderance in normal pregnancy shifts to Th-1 and Th-17 predominance in RM.[17,18] Otherwise, an overexpression of TOLL receptors in decidua was observed in women with RM, especially TLR4 and TLR9 receptors.[19] Immunomodulatory treatments have therefore been proposed and assessed without conclusive results (paternal leukocyte immunization,[20] intravenous immunoglobulins,[21] Small doses of corticosteroids.)[22]

Despite numerous fundamental research publications and clinical trials, the only current recommendation for follow-up of child bearing women suffering from unexplained RM relies on cocooning.[17,23]

RM in the presence of antiphospholipid (APL) antibodies

Antiphospholipid syndrome (APS) is diagnosed in the presence of persistent, above the 99th percentile, APL levels and of at least one clinical manifestation (obstetrical, arterial or venous thrombosis). «Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded »[24] delineates one of the possible manifestation of APS. Even though RM is one accepted clinical manifestation of APS, its specificity is low, as compared to the occurrence of fetal death after 10 weeks of amenorrhea.[24] The benefit of low dose aspirin and/or LMWH or heparins for secondary prevention of RM has not been demonstrated in women who present with APL antibodies and no other clinical manifestation of APS.[25] The HEPASA study found no benefit of combination therapy of LMWH and low dose aspirin in women who presented with APL, without arterial or venous thrombosis history, and who had at least two fetal losses before 32 weeks of amenorrhea.[25] As a consequence, no clinical evidence has been drawn for an optimal management of these women.

Rationale for hydroxychloroquine (HCQ)

Chloroquine and HCQ are used for centuries to treat malaria. Because of a more favorable usage profile and immunomodulatory properties, HCQ use became common for treatment of autoimmune and inflammatory diseases. Other therapeutic properties of HCQ have since been discovered, or rather rediscovered, and assessed in a more consistent manner. The following HCQ properties could be effective against mechanisms of RM.

Since 30 years, benefit of HCQ to prevent venous thromboembolism (VTE) has been suspected[26-35] i) among patients with SLE[26,31] and ii) among all-comer patients, free of autoimmune disease, after orthopedic surgery.[27-30]Thus, before the use of LMWH, HCQ was assessed in the 1970s in the prevention of VTE events after orthopedic surgery. The results of randomized trials,[28-30] (around 10 000 patients included) led to the conclusion that HCQ was beneficial in preventing VTE after orthopedic surgery.[28] In patients with SLE, in the presence or absence of APL antibodies, studies have suggested the benefit of HCQ for the primary prevention of both arterial and venous thrombotic events.[26,31-33] In primary APS, HCQ reduced the thrombus size after vessel injury in mice[34] and would improve the secondary prevention of VTE in humans, in comparison to oral anticoagulants used alone.[35]The antithrombotic action of HCQ would be inherent to some biological effects (in the absence[36-38]or in the presence[39-40] of APL antibodies). Otherwise, much data support an endothelium protective action of HCQ via anti-diabetic,[41]lipidlowering,[42]anti-oxidant[43]effects or a direct endothelial protection, via ERK5 protein kinase activation.[44]

Regarding immunomodulation, HCQ has an impact on the innate immunity by inhibition of the activation of some ToLL receptors (3,7,9).[45-47]HCQ decreases the circulating levels of interleukine 1, 2,[48] 6,[49] TNF[49,50] and, interferon-γ,[51] promoting the TH2 processes that prevail in a “normal pregnancy». Otherwise, HCQ decreases APL plasma levels[52] and interferes with both endothelial cell activation and TNF-α production, 2 major key pathways involved in APS.[53-55]

We also outline the well-known anti-infectious action of HCQ which originates from the alkalinization of intracellular acidic vesicles and might inhibit the growth of intracellular microorganisms. This could act against chronic endometritis, additional mechanism suspected in RM.

Remarkably, the use of HCQ (and closely related molecules) as anti-malarial or in auto-immune diseases during pregnancy[56,58]and lactation[57] have provided extensive safety data during pregnancy and even during breast-feeding.

Otherwise, oral administration of HCQ facilitates its prescription early, before conception, thus enabling fetal exposure from the very beginning of time period at risk for activation of RM underlying mechanisms. Noteworthy, HCQ has never been evaluated in the indication of RM with or without APL antibodies.

Taken all together and given that RM is a stereotyped clinical entity whatever the maternal thrombophilic status, we aimed at evaluating the role of HCQ in prevention of RM, with or without APL antibodies or inherited thrombophilia.

STUDY OBJECTIVES

Primary objective

The aim of this placebo-controlled, double-blind trial is to investigate whether HCQ (orally administrated before conception until 10 weeks of gestational age) would improve the live-birth rate of 15% or more, in women with previous RM, irrespective of their biological thrombophilic status.

Secondary objectives

We aim to investigate whether:

- HCQ effect would be different among women with inherited thrombophilia or APL antibodies
- HCQ would have an impact on the occurrence of some pregnancy events (early miscarriage, intrauterine fetal death, placental vascular disease)
- The impact of HCQ would be different in subgroups of women at higher or lower risk of recurrence according to the number of previous miscarriages (= 3 ou > 3), the maternal age (≥ 35 ou < 35 ans), the parity: previous live-birth, a previous late fetal death after 10 weeks' gestation.

We aim to confirm that HCQ has no negative impact on the chance of getting pregnant, the newborn (gestational age, birth weight, survival at 28 days, congenital abnormalities), the child at 6 months.

METHODS

Study design

This is a prospective, randomized 1:1 placebo-controlled, double-blind, and French multi-centre trial of phase III, with 2 parallel-groups (HCQ 400 mg or placebo). At randomization, the minimization method will be used to balance between the 2 groups and 2 main determinants of miscarriage recurrence: maternal age (≤ 35 or >35 years) and number of previous losses (3 or ≥ 4).

Population

The study population consists of women trying to conceive and who had experienced at least 3 consecutive miscarriages at the first trimester of pregnancy (normal parental caryotypes, no uterine cavity abnormality that might explain the losses). Those women are followed-up by their gynecologist or general practitioner who refers them to one of the RM specialized unit participating in the study for screening. At the inclusion visit, the medical investigator checks inclusion and exclusion criteria of eligible women.

Inclusion criteria

- Women aged between 18 and 37 inclusive,
- Women trying to conceive,
- Women with at least 3 previous consecutive miscarriages in the first pregnancy trimester, of unknown origin defined as:
    - Normal parental karyotypes,
    - No uterine cavity abnormality that might explain RM (ultrasound scan, hysteroscopy or hysterography),

- In case of persistent positive APL antibodies according to the biological criteria of Myakis:[24]no previous thrombotic or obstetrical event defined in APS[24], except for RM in the first trimester of pregnancy, .

➢ Women who have given their informed consent.

Exclusion criteria

➢ Ongoing pregnancy,

➢ Normal pregnancy (live and viable birth) since the last miscarriage,

➢ Abnormal parental karyotype,

➢ Uterine cavity abnormality that might explain RM in the first trimester of pregnancy,

➢ Antiphospholipid Syndrome defined as both:

- persistent positive APL antibodies: lupus anticoagulant and/or APL (anticardiolipin or anti béta2 GPI, IgG or IgM) titers> 99th percentile or >40 with at least 12 weeks interval between two positive determinations (persistent antibodies) AND,
- a specific clinical setting of APS (thrombotic or obstetrical, apart from RM in the first trimester of pregnancy) according to Myakis criteria.[24]

➢ Known contraindication to a treatment by HCQ (retinopathy, hypersensitivity to chloroquine or HCQ, G6PD deficiency, acute intermittent porphyria, chronic liver or kidney insufficiency, extensive cutaneous psoriasis not controlled by local treatment, significant chronic digestive or hematologic disease) or known rare disorder of lactose metabolism (excipient),

➢ Past history of epilepsia or psychotic disorders,

➢ Indication to a treatment by HCQ according to the MA (rheumatoid arthritis, Lupus, solar eczema),

- Previous exposure >4 years to chloroquine or HCQ,
- Previous inclusion in this study,
- Woman unable to consent, protected under the terms of the law, or woman deprived of liberty by judicial or administrative decision,
- non affiliation to the social security system,
- Impossible follow-up.

Allocation, randomization and blinding

After inclusion and obtaining written consent, an identification number is assigned to the patient through a secure server providing access to electronic case report forms generated by the CIC Brest (via the "Capture System" software). The randomization is done using the "Capture System" software with implementation of a randomization with minimization to balance between the 2 treatment groups according to the main determinants of miscarriage recurrence: age (≤ 35 or >35 years) and number of miscarriages (3 or ≥ 4).

The patient, the investigator and all medical and paramedic professionals taking care of the patient will be blinded to the treatment group. The packaging of placebo and HCQ will be provided in the form of capsules with identical external shape and packaging. Information on the correspondence between treatment group and the patient number will be held by both the central pharmacy of Brest CHRU and the Data Management Unit.

Intervention

Included patients are assigned to receive either 400 mg of oral HCQ per day, administered in 2 divided doses of 200 mg or 2 daily doses of oral placebo. Thus, treatment is initiated before

conception and stopped at 10 weeks' gestation (weeks of amenorrhea) or after one year of treatment in the absence of pregnancy.

Rationale for choosing oral 400 mg daily HCQ

It is the usual route of administration and dose for indications specified by the Marketing authorization (MA). This choice of dose comes from the evaluation of the risk-benefit balance in the MA indications and, over all, from the fact that data of good tolerance have been compiled to this (usual) dosage in literature. Otherwise, in most cases, fetal development stops before 10 weeks of amenorrhea. Treatment exposure is essential from the very beginning of implantation, a developmental stage that can potentially be disturbed by the mechanisms responsible for miscarriage. The oral administration allows starting the treatment before conception. This drug has a long half-life (from 30 to 60 days). Its full effect is obtained only after certain duration of exposure. That is why its administration is required before conception and its discontinuation after 10 weeks of amenorrhea, i.e. before the end of the first trimester of pregnancy which is at the highest risk for RM.

Treatments frequently given in combination

- Folic acid and other vitamin supplements,
- Aspirin at low dose, which could be given by some investigators to prevent the recurrence of vasculo-placental disease (preeclampsia, intra-utero growth restriction) or as part of a primary prevention of vasculo-placental events in women with high levels of persistent APL antibodies.

Study plan and procedures

Before pregnancy, women are monthly contacted by phone. The non pregnant women are systematically seen in consultation after 6 and 12 months of treatment. In the absence of pregnancy after one year, the treatment will be withdrawn and patients will be contacted by phone at 14 months and 18 months (End-of-Study visit).

During pregnancy, women are monthly seen in consultation (4 first months) or contacted by phone. The treatment is withdrawn at 10 weeks' gestation or earlier in case of pregnancy loss.

After delivery, the women are seen in consultation at~2 months, and contacted by phone at 6 months (End-of-Study visit).

Four tubes of 2.7 ml with sodium citrate and 4 tubes SST of 5 ml (for plasma and serum bank, respectively) are collected at both the inclusion visit and the visit of pregnancy onset or the visit planned after one year of treatment in the absence of pregnancy. Additionally 2 tubes EDTA of 5 ml are collected for DNA bank at inclusion. The biological collection of plasma is carried out within 3 hours after receipt of tubes. DNA extraction is carried out within one week of collection (and at the latest within a month if the sample is frozen at -80 ° C pending extraction). Those specimens are designed to identify prognostic factors (including thrombophilic work-up) for efficacy of the study treatment.

End points

The primary end point is a live and viable birth.

The secondary end points are:

- A live and viable birth, for the subgroup analyses,

- The occurrence of miscarriage, in-utero fetal death, placental vascular disease, premature delivery,
- Gestational age at miscarriage,
- Concerning the child: gestational age and weight at birth, survival at 28 days, safety data at 6 months of life, congenital abnormality.

Safety considerations, safety monitoring and AE reporting

Apart from, adverse events (AE) and serious adverse events (SAE), some safety parameters will be systematically recovered: CBC, visual and neurological symptoms for women and reports of ultrasound and post-natal medical visits for children. Adverse events (AE) are defined as any untoward medical occurrence in a person who consents to biomedical research whether this event is related or not to the research or experimental drug on which this research addresses. The gravity of serious adverse events (SAE) is defined by one of the following findings: death, life-threatening situation, significant or sustained incapacity or disability, hospitalization, prolongation of hospitalization, deformity or birth defect, potentially serious event. Any SAE or event of interest, regardless of its causal relationship with the trial treatment or the research, must be reported to the sponsor, as soon as possible and the latest 24 hrs after their occurrence for SAE. The investigator is in charge of noting and reporting all SAE occurring during the study, from the date of the signed informed consent, during the study's expected duration of follow up (6 months after the treatment cessation for women and at age 6 months for newborns). Furthermore, regardless of the event occurrence deadline after the end of the study, all SAE that may be due to the research must be declared to the sponsor when there is no other cause and effect accounted for (such as serious events that may occur long after exposure to the drug, such as cancers or congenital abnormalities).

Some AE and SAE are expected and can be linked to i) pregnancy, ii) the disease (occurrence of another miscarriage) or study treatment (benign, adverse reaction AR, as digestive symptoms or severe, serious adverse reaction SAR as skin reaction, which requires to immediately stop the treatment).

Independent Data and safety monitoring Board (IDSMB)

The IDSMB comprises 8 members without competing interests, not directly involved in other aspects of the trial and independent from the sponsor: 7 voting members, experts in complementary fields of the pathology and clinical trials (internal medicine, methodology, specialist of teratogenic agents, neonatology, hematology, ophthalmology) and a non-voting independent statistician. IDSMB monitors the data that affects patient safety, provides independent expertise for the evaluation of adverse events (AE) occurring during the study, expresses an opinion concerning the benefit / risk ratio and provides recommendations in order to help the steering committee to take decisions on protocol modification or early termination of the study. The members of the IDSMB will have to meet every 20 inclusions. The IDSMB will transmit its recommendations to the steering Committee, which decides whether or not to stop the study. Given the safety data and the treatment benefit, several different recommendations can be provided by the IDSMB: i) continuation of study without protocol modification, ii) continuation of study with modification of protocol, iii) temporarily discontinuation of inclusions, iiii) early termination of the study

Over the course of study, the IDSMB will have an advisory role in reassessing the benefit/risk ratio of the study.

Number of patients

The total number of scheduled patients to be recruited is 300, on the basis of the following hypotheses:

- 80% of the included women should get pregnant (ALIFE study).[11]
- Among women who will get pregnant, we suppose that the rate of achieved pregnancy will be of 70% in the placebo group (PREFIX study).[14]

To demonstrate a minimal clinically important difference of 15% in live-birth rates on HCQ (i.e. a 50% relative risk reduction in pregnancy loss), we need to enroll 300 women for a power of 80%, and a two-tailed alpha risk of 5%.

The anticipated duration of recruitment is 3 years and the duration of participation of each patient: from 7 to 29 months.

Analyses plan

Analyses will be firstly conducted on an intention to treat basis for all women who have started pregnancy. Hierarchical analysis including three steps is planned :comparison of the number of live and viable births, between the two treatment groups i) among all the women, ii) among women without known APL antibodies, iii) among women without known thrombophilia (APL or inherited thrombophilia). We plan to adjust for age and number of previous losses if the difference between the two treatment groups is statistically significant ($p < .05$).

Secondary analyses will be conducted on an intention to treat mode:

For all included women, comparison of the number of live and viable births between the two treatment groups.

Among women who will get pregnant:

- Measure of treatment effect in women at higher risk of further miscarriage, according to the presence of thrombophilia, past history of intrauterine fetal death after 10 week's gestation, past history of miscarriage, > 3 previous miscarriage or, no previous newborn baby,
- Comparison between the two treatment groups of the occurrence of several obstetrical events (miscarriage and intrauterine fetal death, vasculo placental disease, premature birth),
- Comparison between the two treatment groups of newborn clinical data (gestation time at delivery, birth weight adjusted to the gestation time, number of newborn babies living at 28 days and congenital abnormalities.

A "per-protocol analysis" (primary endpoint) will include only patients properly exposed to the study treatment, that is i) at least during a whole menstrual cycle before pregnancy onset and until 10 weeks of amenorrhea ii) compliance≥ 80%.

No unblinded interim analysis is planned.

The biological collection will be carried out by the CRB of the CHRU of BREST and integrated into the collection "thrombosis" declared to the Ministry (2008-214/ DC-2009-925).

DISCUSSION

To date, there has not yet been an optimal therapy with conclusive clinical benefit for unexplained RM, in the absence or presence of inherited thrombophilia. Providing some psychological support seems to be the only available therapy for women with unexplained RM. Although the probability of a further normal pregnancy seems high, (~70% at age 32 after 3 consecutive miscarriages),[59] the proposed therapeutic interventions are sometimes excessive, (e.g. possible side effects) and costly (e.g. intravenous immunoglobulins, assisted procreation). Therefore, it is of utmost importance to investigate other therapeutic options.

In RM women with high titers of APL antibodies but without any other previous clinical event listed in APS (thrombotic or obstetric event), benefit of antithrombotic treatment (aspirin or LMWH) remains controversial. Clinical benefit of HCQ has not been demonstrated in prospective studies with robust methodology.[60]

In conclusion, the benefit/risk ratio and low cost are strong incentives for assessment of HCQ in unexplained RM irrespective of maternal thrombophilic status.

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This study was sponsored by Brest University Hospital.

PATIENT AND PUBLIC INVOLVEMENT

Patients were not involved in the design of the study.

ETHICS, REGULATORY APPROVALS AND DISSEMINATION

Agreement from the French National Public Health and Drug Security Agency (160765A-22) and ethical approval from the Committee for the Protection of Persons of NORD-OUEST I () have been obtained on November 4th 2016 and March 2nd 2017, respectively.

This trial is registered at www.ClinicalTrials.gov as # NCT0316513. and the French National Health and Drug Safety Agency (EudraCT # 2016-001330-97).

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Contributors

EP conceived and designed the study. EP drafted the original grant proposal and trial protocol. LDSM provides methodological and statistical expertise. GM has assisted in developing the protocol, helped with implementation and has responsibilities for day-to-day running of the trial including participant recruitment, data collection and liaising with other sites. JH and PM have helped with protocol implementation. CB, CC, FB, VLS, GPB, DM

have participated in the design of the study. VC coordinates treatment production and dispensation. All authors critically reviewed and approved the final version of the manuscript.

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Hydroxychloroquine for Prevention of Recurrent Miscarriage: Study Protocol for a Multicenter Randomized Placebo Controlled Trial BaBy hydroxychloroQuine (BBQ) study

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Hydroxychloroquine for Prevention of Recurrent Miscarriage:

Study Protocol for a Multicenter Randomized Placebo Controlled Trial

BaB**y** hydroxychloro**Q**uine (BBQ) study

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ABSTRACT

Introduction Recurrent miscarriage (RM), defined by >=3 consecutive losses during the first trimester of pregnancy, affects 1 to 2% of fertile couples. Standard investigations fail to reveal any apparent cause in ~ 50% of couples. However, on the basis of animal models and clinical studies, several hypotheses have been put forward concerning underlying mechanisms of RM: altered ovarian reserve, progesterone defect, thrombotic and/or endothelial dysfunction, immunological disturbances. Nonetheless, no study has yet reached conclusive beneficial clinical evidence for a potential treatment in unexplained RM. Hydroxychloroquine (HCQ) is a molecule with extensive safety data during pregnancy. HCQ pharmacological properties (e.g. anti-thrombotic, vascular protective, immunomodulatory, improved glucose tolerance, lipidlowering, antiinfectious) could be effective against some mechanisms of unexplained RM. Furthermore, eventhough clinical benefit of HCQ is suggested in prevention of thrombotic and late obstetric events in antiphospholipid (APL) syndrome, there is no data suggesting the benefit of HCQ in RM in the presence of APL antibodies.

Methods and analysis Taken all together and given the low cost of HCQ, the aim of this multicenter, randomized placebo-controlled, double-blind study is to investigate whether HCQ would improve the live-bith rate in women with RM, irrespective of maternal thrombophilic status: i) no known thrombophilia, ii) inherited thrombophilia or iii) APL antibodies. The primary end-point is a live and viable birth. After confirming eligibility and obtaining consent, 300 non-pregnant women will be randomized in 2 parallel groups for a daily oral treatment (HCQ 400 mg or placebo), initiated before conception and stopped at 10 weeks' gestation. If pregnancy doesn't occur after one year, the treatment will be stopped.

Ethics and dissemination Agreement from the French National Public Health and Drug Security Agency (160765A-22) and ethical approval from the Committee for the Protection of Persons of NORD-OUEST I (2016-001330-97) have been obtained.

Trial registration number ClinicalTrials.gov: NCT0316513

STRENGTHS AND LIMITATIONS OF THIS STUDY

- Despite numerous fundamental research publications and clinical trials, the only recommendation that applies to follow-up of child bearing women suffering from RM relies on cocooning.
- This is the first randomized placebo-controlled study which aims to investigate whether oral hydroxychloroquine would improve the live-bith rate in women with recurrent miscarriage irrespective of maternal thrombophilic status.
- As a possible limitation, this study is based on the hypothesis that recurrent miscarriage often results from combined failures which could benefit from the pleiotropic effects of hydroxychloroquine.
- As hydroxychloroquine should probably be administrated at least 2 menstrual cycles before conception to have an effect, women will be advised not to get pregnant during this period, but we do not plan to exclude those who will conceive too early.

INTRODUCTION

Background

RM is a common public health reproductive burden causing both physical and emotional distress. To date there is no treatment whose benefit has been clearly proved, even in the presence of well-known risk factors of RM. Most importantly, the absence of benefit has been clearly demonstrated for some treatments that are yet empirically proposed.

Unexplained Recurrent Miscarriage (RM)

A sporadic miscarriage is clinically detected in approximately 10% to 15% of pregnancies. Fetal development usually stops before 10 weeks.[1]Given the recurrent miscarriage (RM) frequency among fertile couples (1% to 2%) being significantly higher than the expected random one (15*15*15% = 0.34 %), RM is most often defined as ≥ 3 consecutive losses. Apart from the detection of a lethal chromosomal abnormality on products of conception, the underlying mechanism of loss remains unknown in most cases. The rate of normal embryonic karyotypes in RM steadily increases from the third loss, suggesting alternative mechanisms than meiotic aberrations.[2]Standard investigations fail to reveal any apparent cause in ~50% of the women. However, On the basis of animal models and clinical studies, several hypotheses have been put forward. Here, we focus on thrombosis and both endothelial and immune dysfunctions. Those could be targeted by pharmacological properties of hydroxychloroquine (HCQ).

An association with some inherited thrombophilia has been reported (factor V Leiden, mutation G20210A of the prothrombin and protein S deficiency), although the odds ratio were most often ≤ 2.[3] Animal models have demonstrated that some actors of the haemostatic

system may participate in normal implantation and placental development regardless of the coagulation process.[4,5] In humans, a basal prothrombotic state outside of pregnancy has been measured in women with previous RM and without known thrombophilia.[6-8]This relative prothrombotic state, measured at distance of any obstetrical event, could reflect chronic endothelium damage in those women.[9,10]Notwithstanding, the clinical trials that have assessed antithrombotic treatments (aspirin initiated before or after conception, eventually combined with LMWH, or LMWH alone initiated after pregnancy diagnosis) did not show any benefit in prevention of further loss.[11-14]Likewise, no benefit of LMWH has been shown in the subgroup of women with inherited thrombophilia (TIPPS study).[15]However, we emphasize that the subcutaneous route of LMWH administration does not allow assessing this treatment at the critical time of implantation onset in fertile women. Indeed, the injections cannot be routinely initiated before 5 weeks' gestation.

Regarding immune dysfunction, apart from the detection of many auto-antibodies (antiphospholipid, anti-thyroid),[16] attention is now focused on natural killer cells, whose temporal and spatial distribution in the uterine mucosa suggests that they contribute to control trophoblast invasion and cytokine response at the maternal-fetal interface (balances Th-1 / Th-2 and Th-17 / T-reg). Th-2 and T-reg preponderance in normal pregnancy shifts to Th-1 and Th-17 predominance in RM.[17,18] Otherwise, an overexpression of TOLL receptors in decidua was observed in women with RM, especially TLR4 and TLR9 receptors.[19] Immunomodulatory treatments have therefore been proposed and assessed without conclusive results (paternal leukocyte immunization,[20] intravenous immunoglobulins,[21] Small doses of corticosteroids.)[22]

Despite numerous fundamental research publications and clinical trials, the only current recommendation for follow-up of child bearing women suffering from unexplained RM relies on cocooning.[17,23]

RM in the presence of antiphospholipid (APL) antibodies

Antiphospholipid syndrome (APS) is diagnosed in the presence of persistent, above the 99th percentile, APL levels and of at least one clinical manifestation (obstetrical, arterial or venous thrombosis). «Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded »[24] delineates one of the possible manifestation of APS. Even though RM is one accepted clinical manifestation of APS, its specificity is low, as compared to the occurrence of fetal death after 10 weeks of amenorrhea.[24] The benefit of low dose aspirin and/or LMWH or heparins for secondary prevention of RM has not been demonstrated in women who present with APL antibodies and no other clinical manifestation of APS.[25] The HEPASA study found no benefit of combination therapy of LMWH and low dose aspirin in women who presented with APL, without arterial or venous thrombosis history, and who had at least two fetal losses before 32 weeks of amenorrhea.[25] As a consequence, no clinical evidence has been drawn for an optimal management of these women.

Rationale for hydroxychloroquine (HCQ)

Chloroquine and HCQ are used for centuries to treat malaria. Because of a more favorable usage profile and immunomodulatory properties, HCQ use became common for treatment of autoimmune and inflammatory diseases. Other therapeutic properties of HCQ have since been discovered, or rather rediscovered, and assessed in a more consistent manner. The following HCQ properties could be effective against mechanisms of RM.

Since 30 years, benefit of HCQ to prevent venous thromboembolism (VTE) has been suspected[26-35] i) among patients with SLE[26,31] and ii) among all-comer patients, free of autoimmune disease, after orthopedic surgery.[27-30]Thus, before the use of LMWH, HCQ was assessed in the 1970s in the prevention of VTE events after orthopedic surgery. The results of randomized trials,[28-30] (around 10 000 patients included) led to the conclusion that HCQ was beneficial in preventing VTE after orthopedic surgery.[28] In patients with SLE, in the presence or absence of APL antibodies, studies have suggested the benefit of HCQ for the primary prevention of both arterial and venous thrombotic events.[26,31-33] In primary APS, HCQ reduced the thrombus size after vessel injury in mice[34] and would improve the secondary prevention of VTE in humans, in comparison to oral anticoagulants used alone.[35]The antithrombotic action of HCQ would be inherent to some biological effects (in the absence[36-38]or in the presence[39-40] of APL antibodies). Otherwise, much data support an endothelium protective action of HCQ via anti-diabetic,[41]lipidlowering,[42]anti-oxidant[43]effects or a direct endothelial protection, via ERK5 protein kinase activation.[44]

Regarding immunomodulation, HCQ has an impact on the innate immunity by inhibition of the activation of some ToLL receptors (3,7,9).[45-47]HCQ decreases the circulating levels of interleukine 1, 2,[48] 6,[49] TNF[49,50] and, interferon-γ,[49] promoting the TH2 processes that prevail in a "normal pregnancy». Otherwise, HCQ decreases APL plasma levels[51] and interferes with both endothelial cell activation and TNF-α production, 2 major key pathways involved in APS.[52-54]

We also outline the well-known anti-infectious action of HCQ which originates from the alkalinization of intracellular acidic vesicles and might inhibit the growth of intracellular microorganisms. This could act against chronic endometritis, additional mechanism suspected in RM.

Remarkably, the use of HCQ (and closely related molecules) as anti-malarial or in auto-immune diseases during pregnancy[55-57]and lactation[56] have provided extensive safety data during pregnancy and even during breast-feeding.

Otherwise, oral administration of HCQ facilitates its prescription early, before conception, thus enabling fetal exposure from the very beginning of time period at risk for activation of RM underlying mechanisms. Noteworthy, HCQ has never been evaluated in the indication of RM with or without APL antibodies.

Taken all together and given that RM is a stereotyped clinical entity whatever the maternal thrombophilic status, we aimed at evaluating the role of HCQ in prevention of RM, with or without APL antibodies or inherited thrombophilia. We therefore initiated a multicenter placebo-controlled trial sponsored by Brest University hospital and supported by a grant from the French Ministry of Health (PHRCN-17-0573).

STUDY OBJECTIVES

Primary objective

The aim of this placebo-controlled, double-blind trial is to investigate whether HCQ (orally administrated before conception until 10 weeks of gestational age) would improve the live-birth rate of 15% or more, in women with previous RM, irrespective of their biological thrombophilic status.

Secondary objectives

We aim to investigate whether:

- HCQ effect would be different among women with inherited thrombophilia or APL antibodies
- HCQ would have an impact on the occurrence of some pregnancy events (early miscarriage, intrauterine fetal death, placental vascular disease)
- The impact of HCQ would be different in subgroups of women at higher or lower risk of recurrence according to the number of previous miscarriages (= 3 ou > 3), the maternal age (≥ 35 ou < 35 ans), the parity: previous live-birth, a previous late fetal death after 10 weeks' gestation.

We aim to confirm that HCQ has no negative impact on the chance of getting pregnant, the newborn (gestational age, birth weight, survival at 28 days, congenital abnormalities), the child at 6 months.

METHODS

Study design

This is a prospective, randomized 1:1 placebo-controlled, double-blind, and French multi-centre trial of phase III (, with 2 parallel-groups (HCQ 400 mg or placebo). At randomization, the minimization method will be used to balance between the 2 groups and 2 main determinants of miscarriage recurrence: maternal age (≤ 35 or >35 years) and number of previous losses (3 or ≥ 4).

Study setting

Women are currently being enrolled in university hospitals (gynecology units: Besançon, Brest, Clermont Ferrand, Lille, Marseille, Nantes, Paris Cochin, Paris Bichat, Rennes, Saint-Etienne; internal medicine units: Brest, Paris Saint-Antoine) or in general hospitals (gynecology units: Auch, Quimper, Mont de Marsan, Pau).

Population

The study population consists of women trying to conceive and who had experienced at least 3 consecutive miscarriages at the first trimester of pregnancy (normal parental caryotypes, no uterine cavity abnormality that might explain the losses). Through medical meetings, emails and letters, all obstetricians and internal medicine practitioners working in each participating centre's catchment area have been informed and trained on BBQ study. All of those are asked to refer potentially eligible women to the unit participating in the study for screening. In each centre, patient recruitment is ensured by already in place settings such as specific RM consultations or other OBS/GYN patient management units. In addition to this recruitment

approach, poster information in consultation waiting rooms is being used to reach-out to more patients.

At the inclusion visit, the medical investigator checks inclusion and exclusion criteria of eligible women.

Inclusion criteria

- Women aged between 18 and 37 inclusive,
- Women trying to conceive,
- Women with at least 3 previous consecutive miscarriages in the first pregnancy trimester, of unknown origin defined as:
    - Normal parental karyotypes,
    - No uterine cavity abnormality that might explain RM (ultrasound scan, hysteroscopy or hysterography),
    - In case of persistent positive APL antibodies according to the biological criteria of Myakis:[24]no previous thrombotic or obstetrical event defined in APS[24], except for RM in the first trimester of pregnancy, .
- Women who have given their informed consent.

Exclusion criteria

- Ongoing pregnancy,
- Normal pregnancy (live and viable birth) since the last miscarriage,
- Abnormal parental karyotype,
- Uterine cavity abnormality that might explain RM in the first trimester of pregnancy,

- Antiphospholipid Syndrome defined as both:
  - persistent positive APL antibodies: lupus anticoagulant and/or APL (anticardiolipin or anti béta2 GPI, IgG or IgM) titers> 99th percentile or >40 with at least 12 weeks interval between two positive determinations (persistent antibodies) AND,
  - a specific clinical setting of APS (thrombotic or obstetrical, apart from RM in the first trimester of pregnancy) according to Myakis criteria.[24]
- Known contraindication to a treatment by HCQ (retinopathy, hypersensitivity to chloroquine or HCQ, G6PD deficiency, acute intermittent porphyria, chronic liver or kidney insufficiency, extensive cutaneous psoriasis not controlled by local treatment, significant chronic digestive or hematologic disease) or known rare disorder of lactose metabolism (excipient),
- Past history of epilepsia or psychotic disorders,
- Indication to a treatment by HCQ according to the MA (rheumatoid arthritis, Lupus, solar eczema),
- Previous exposure >4 years to chloroquine or HCQ,
- Previous inclusion in this study,
- Woman unable to consent, protected under the terms of the law, or woman deprived of liberty by judicial or administrative decision,
- non affiliation to the social security system,
- Impossible follow-up.

Allocation, randomization and blinding

After inclusion and obtaining written consent, an identification number is assigned to the patient through a secure server providing access to electronic case report forms generated by the CIC Brest (via the "Capture System" software). The randomization is done using the "Capture System" software with implementation of a randomization with minimization to balance between the 2 treatment groups according to the main determinants of miscarriage recurrence: age (≤ 35 or >35 years) and number of miscarriages (3 or ≥ 4).

The patient, the investigator and all medical and paramedic professionals taking care of the patient will be blinded to the treatment group. The packaging of placebo and HCQ will be provided in the form of capsules with identical external shape and packaging. Information on the correspondence between treatment group and the patient number will be held by both the central pharmacy of Brest CHRU and the Data Management Unit.

Intervention

Included patients are assigned to receive either 400 mg of oral HCQ per day, administered in 2 divided doses of 200 mg or 2 daily doses of oral placebo. Thus, treatment is initiated before conception and stopped at 10 weeks' gestation (weeks of amenorrhea) or after one year of treatment in the absence of pregnancy.

Rationale for choosing oral 400 mg daily HCQ

It is the usual route of administration and dose for indications specified by the Marketing authorization (MA). This choice of dose comes from the evaluation of the risk-benefit balance in the MA indications and, over all, from the fact that data of good tolerance have been compiled to this (usual) dosage in literature. Otherwise, in most cases, fetal development

stops before 10 weeks of amenorrhea. Treatment exposure is essential from the very beginning of implantation, a developmental stage that can potentially be disturbed by the mechanisms responsible for miscarriage. The oral administration allows starting the treatment before conception. This drug has a long half-life (from 30 to 60 days). Its full effect is obtained only after certain duration of exposure. That is why its administration is required before conception and its discontinuation after 10 weeks of amenorrhea, i.e. before the end of the first trimester of pregnancy which is at the highest risk for RM.

Treatments frequently given in combination

- Folic acid and other vitamin supplements,
- Aspirin at low dose, which could be given by some investigators to prevent the recurrence of vasculo-placental disease (preeclampsia, intra-utero growth restriction) or as part of a primary prevention of vasculo-placental events in women with high levels of persistent APL antibodies.

Study plan and procedures

Before pregnancy, women are monthly contacted by phone. The non pregnant women are systematically seen in consultation after 6 and 12 months of treatment. In the absence of pregnancy after one year, the treatment will be withdrawn and patients will be contacted by phone at 14 months and 18 months (End-of-Study visit).

During pregnancy, women are monthly seen in consultation (4 first months) or contacted by phone. The treatment is withdrawn at 10 weeks' gestation or earlier in case of pregnancy loss.

After delivery, the women are seen in consultation at~2 months, and contacted by phone at 6 months (End-of-Study visit).

Four tubes of 2.7 ml with sodium citrate and 4 tubes SST of 5 ml (for plasma and serum bank, respectively) are collected at both the inclusion visit and the visit of pregnancy onset or the visit planned after one year of treatment in the absence of pregnancy. Additionally 2 tubes EDTA of 5 ml are collected for DNA bank at inclusion. The biological collection of plasma is carried out within 3 hours after receipt of tubes. DNA extraction is carried out within one week of collection (and at the latest within a month if the sample is frozen at -80 ° C pending extraction). Those specimens are designed to identify prognostic factors (including thrombophilic work-up) for efficacy of the study treatment.

End points

The primary end point is a live and viable birth.

The secondary end points are:

- A live and viable birth, for the subgroup analyses,
- The occurrence of miscarriage, in-utero fetal death, placental vascular disease, premature delivery,
- Gestational age at miscarriage,
- Concerning the child: gestational age and weight at birth, survival at 28 days, safety data at 6 months of life, congenital abnormality.

Safety considerations, safety monitoring and AE reporting

Apart from, adverse events (AE) and serious adverse events (SAE), some safety parameters will be systematically recovered: CBC, visual and neurological symptoms for women and reports of ultrasound and post-natal medical visits for children. Adverse events (AE) are defined as any untoward medical occurrence in a person who consents to biomedical research whether this event is related or not to the research or experimental drug on which this research addresses. The gravity of serious adverse events (SAE) is defined by one of the following findings: death, life-threatening situation, significant or sustained incapacity or disability, hospitalization, prolongation of hospitalization, deformity or birth defect, potentially serious event. Any SAE or event of interest, regardless of its causal relationship with the trial treatment or the research, must be reported to the sponsor, as soon as possible and the latest 24 hrs after their occurrence for SAE. The investigator is in charge of noting and reporting all SAE occurring during the study, from the date of the signed informed consent, during the study's expected duration of follow up (6 months after the treatment cessation for women and at age 6 months for newborns). Furthermore, regardless of the event occurrence deadline after the end of the study, all SAE that may be due to the research must be declared to the sponsor when there is no other cause and effect accounted for (such as serious events that may occur long after exposure to the drug, such as cancers or congenital abnormalities).

Some AE and SAE are expected and can be linked to i) pregnancy, ii) the disease (occurrence of another miscarriage) or study treatment (benign, adverse reaction AR, as digestive symptoms or severe, serious adverse reaction SAR as skin reaction, which requires to immediately stop the treatment).

Independent Data and safety monitoring Board (IDSMB)

The IDSMB comprises 8 members without competing interests, not directly involved in other aspects of the trial and independent from the sponsor: 7 voting members, experts in complementary fields of the pathology and clinical trials (internal medicine, methodology, specialist of teratogenic agents, neonatology, hematology, ophthalmology) and a non-voting independent statistician. IDSMB monitors the data that affects patient safety, provides independent expertise for the evaluation of adverse events (AE) occurring during the study, expresses an opinion concerning the benefit / risk ratio and provides recommendations in order to help the steering committee to take decisions on protocol modification or early termination of the study. The members of the IDSMB will have to meet every 20 inclusions. The IDSMB will transmit its recommendations to the steering Committee, which decides whether or not to stop the study. Given the safety data and the treatment benefit, several different recommendations can be provided by the IDSMB: i) continuation of study without protocol modification, ii) continuation of study with modification of protocol, iii) temporarily discontinuation of inclusions, iiii) early termination of the study

Over the course of study, the IDSMB will have an advisory role in reassessing the benefit/risk ratio of the study.

Number of patients

The total number of scheduled patients to be recruited is 300, on the basis of the following hypotheses:

- 80% of the included women should get pregnant (ALIFE study).[11]
- Among women who will get pregnant, we suppose that the rate of achieved pregnancy will be of 70% in the placebo group (PREFIX study).[14]

To demonstrate a minimal clinically important difference of 15% in live-birth rates on HCQ (i.e. a 50% relative risk reduction in pregnancy loss), we need to enroll 300 women for a power of 80%, and a two-tailed alpha risk of 5%.

The anticipated duration of recruitment is 3 years and the duration of participation of each patient: from 7 to 29 months.

Analyses plan

Analyses will be firstly conducted on an intention to treat basis for all women who have started pregnancy. Hierarchical analysis including three steps is planned :comparison of the number of live and viable births, between the two treatment groups i) among all the women, ii) among women without known APL antibodies, iii) among women without known thrombophilia (APL or inherited thrombophilia). We plan to adjust for age and number of previous losses if the difference between the two treatment groups is statistically significant ($p < .05$).

Secondary analyses will be conducted on an intention to treat mode:

For all included women, comparison of the number of live and viable births between the two treatment groups.

Among women who will get pregnant:

- Measure of treatment effect in women at higher risk of further miscarriage, according to the presence of thrombophilia, past history of intrauterine fetal death after 10 week's gestation, past history of miscarriage, > 3 previous miscarriage or, no previous newborn baby,

- Comparison between the two treatment groups of the occurrence of several obstetrical events (miscarriage and intrauterine fetal death, vasculo placental disease, premature birth),
- Comparison between the two treatment groups of newborn clinical data (gestation time at delivery, birth weight adjusted to the gestation time, number of newborn babies living at 28 days and congenital abnormalities.

A "per-protocol analysis" (primary endpoint) will include only patients properly exposed to the study treatment, that is i) at least during a whole menstrual cycle before pregnancy onset and until 10 weeks of amenorrhea ii) compliance≥ 80%.

No unblinded interim analysis is planned.

The biological collection will be carried out by the CRB of the CHRU of BREST and integrated into the collection "thrombosis" declared to the Ministry (2008-214/ DC-2009-925).

DISCUSSION

To date, there has not yet been an optimal therapy with conclusive clinical benefit for unexplained RM, in the absence or presence of inherited thrombophilia. Providing some psychological support seems to be the only available therapy for women with unexplained RM. Although the probability of a further normal pregnancy seems high, (~70% at age 32 after 3 consecutive miscarriages),[58]the proposed therapeutic interventions are sometimes excessive, (e.g. possible side effects) and costly (e.g. intravenous immunoglobulins, assisted procreation). Therefore, it is of utmost importance to investigate other therapeutic options.

In RM women with high titers of APL antibodies but without any other previous clinical event listed in APS (thrombotic or obstetric event), benefit of antithrombotic treatment (aspirin or LMWH) remains controversial. Clinical benefit of HCQ has not been demonstrated in prospective studies with robust methodology.[59]

In conclusion, the benefit/risk ratio and low cost are strong incentives for assessment of HCQ in unexplained RM irrespective of maternal thrombophilic status.

ETHICS, REGULATORY APPROVALS AND DISSEMINATION

Agreement from the French National Public Health and Drug Security Agency (160765A-22) and ethical approval from the Committee for the Protection of Persons of NORD-OUEST I (2016-001330-97) have been obtained on November 4th 2016 and March 2nd 2017, respectively. This trial is registered at www.ClinicalTrials.gov as # NCT0316513. and at the French National Health and Drug Safety Agency as EudraCT # 2016-001330-97.The necessary trial insurance is provided by HDI Gerling Industrie Versicherung (PARIS). Protocol version V3.0, November 30, 2017

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PATIENT AND PUBLIC INVOLVEMENT

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Contributors

EP conceived and designed the study. EP drafted the original grant proposal and trial protocol. LDSM provides methodological and statistical expertise. GM has assisted in developing the protocol, helped with implementation and has responsibilities for day-to-day running of the trial including participant recruitment, data collection and liaising with other sites. JH and PM have helped with protocol implementation. CB, CC, FB, VLS, GPB, DM have participated in the design of the study. VC coordinates treatment production and dispensation. All authors critically reviewed and approved the final version of the manuscript.

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Funder and sponsor had no role in study design; collection, management, analysis and, interpretation of data; writing of the report and decision to submit the report for publication.

Competing interests for all trial investigators None declared.

Patient consent Obtained.

The data that will support the findings of this study will be available when the findings will be published in a peer-review journal, from the corresponding author, upon reasonable request.

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SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description
Administrative information		
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym reported on page 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry reported on pages 3, 21
	2b	All items from the World Health Organization Trial Registration Data Set
Protocol version	3	Date and version identifier reported on page 21
Funding	4	Sources and types of financial, material, and other support reported on p 9, 23
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors reported on p 1, 22
	5b	Name and contact information for the trial sponsor reported on p 21
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities reported on p 23
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) reported on the study protocol (supplementary file) on pages:15-17, 58-61
Introduction		
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention reported on pages 5-9
	6b	Explanation for choice of comparators reported on p 6,7
Objectives	7	Specific objectives or hypotheses reported on page 10
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) reported on page 11

Methods: Participants, interventions, and outcomes

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained reported on pages 11, 22
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists) reported on pages 12-13
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered reported on pages 14
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease) reported on page 17 and on page 48 of the study protocol (supplementary file)
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests) reported on page 46 of the study protocol (supplementary file)
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial reported on page 15
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended reported on pages 16 and, on pages 33-34 of the study protocol (supplement)
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure) reported on page 15, 16 and on pages 34-44 of the study protocol
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations reported on page 18-19
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size reported on page 48 and 70 of the study protocol (supplementary file)

Methods: Assignment of interventions (for controlled trials)

Allocation: reported on page 13-14 and on page 44 of the study protocol (supplementary file)

Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions

Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
reported on page 14 and on page 44 of the study protocol (supplementary file)	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial

Methods: Data collection, management, and analysis

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol
reported on pages 68, 69 of the study protocol (supplementary file)		
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols
reported on p 48-49 of the study protocol		
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol
reported on pages 68-69 of the study protocol (supplementary file)		
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol
reported on pages 19-20 and on pages 62-64 of the study protocol		
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)

Methods: Monitoring

Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed
reported on page 17-18 and on page 61 of the study protocol.		

	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial reported on page 18 and on page 62 of the study protocol (supplementary file)
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct reported on pages 16-17 and on pages 55-60 of the study protocol (supplementary file)
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor reported on page 66 of the study protocol (supplementary file)

Ethics and dissemination

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval reported on pages 3, 21 and on pages 46,59,61 of the study protocol
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) reported on page 66 and 70 of the study protocol (supplementary file)
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) reported on page 67 of the study protocol
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial reported on page 65 of the study protocol
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site reported on page 23
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators reported on pages 64-65 of the study protocol (supplementary file)
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation reported on page 67 of the study protocol (supplementary file)
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions reported on page 70 of the study protocol (supllementary file)
	31b	Authorship eligibility guidelines and any intended use of professional writers reported on page 70 of the study protocol (supplementary file)
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code

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Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates given as a supplementary file
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable reported on p 35, 39 of the protocol

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.