The effect of red clover isoflavones on urinary microbiota composition and interaction with the urothelium in postmenopausal women

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Content

Abbreviations

ER-β = Estrogen receptor beta

ER-α = Estrogen receptor alpha

UTI = Urinary Tract Infection

UUI = Urgency Urinary Incontinence

NB = Neuropathic Bladder

RCE = Red Clover Extract

PL = Placebo

PI = Principal Investigator

IBD = Inflammatory bowel disease

IBS = Irritable bowel syndrome

RT-qPCR = reverse transcriptase quantitative polymerase chain reaction

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Objectives and aims

The natural decrease in estrogen level during and after menopause may result in several complications and uncomfortable symptoms for women, including hot flushes, vaginal dryness, and reduced bone mineral density. Moreover, after menopause women often suffer from bladder diseases such as recurrent urinary tract infections, urgency urinary incontinence, and overactive bladder. Oral hormone replacement therapy with estrogen has been shown to decrease many of the symptoms during menopause. Additionally, local administrated estrogen to the vagina is found to decrease the risk of bladder diseases, including recurrent urinary tract infection in postmenopausal women. However, it is not well known why women after menopause suffer from various symptoms from the urinary tract and the role of estrogen in that connection. Recent studies indicate that postmenopausal women have a different urinary bacteria composition compared to women before menopause. This point towards an association between estrogen, bacteria composition, and function of the urinary tract system.

Isoflavones are natural plant-derived estrogens, which are used as an alternative treatment to estrogen. Isoflavones have been found to have a beneficial effect on some symptoms related to menopause. However, the effect of isoflavones on the urinary bacteria composition has not yet been investigated.

It is our hypothesis that isoflavones may have a beneficial effect on the urinary microbiota composition in postmenopausal women and thereby on their bladder symptoms. Additionally, isoflavones may as well influence the microbiota in the vagina and gut. However, understanding the role of the microbiota in clinical conditions requires an equivalent understanding of the interactions between the epithelial cells, the bacteria, and estrogen.

Therefore, the primary objectives of this study are to investigate how isoflavones influence the urinary microbiota in postmenopausal women using advanced culture-independent sequencing techniques, and to functionally analyze how isoflavones change the human urothelial cell gene expression. In addition, the secondary objectives are to investigate how isoflavones influence the vaginal and gut microbiota.

Background

Isoflavones are naturally occurring plant-derived nonsteroidal phytoestrogens, which have beneficial effects regarding menopausal symptoms(1,2). During menopause, a decrease in natural estrogen levels triggers a wide range of uncomfortable symptoms, such as hot flushes, night sweats, sleep disturbances, headache, and vaginal dryness, which significantly affect the quality of life for women worldwide(1,3,4). Isoflavones have primarily been found to reduce vasomotor symptoms in menopause, including hot flushes and night sweats(1,5). Studies report that Asian women less frequently experience menopausal derived vasomotor symptoms than women from America or Europe, which could be caused by the isoflavone-rich diet in Asian countries(6,7). Moreover, phytoestrogens may also reduce vaginal symptoms, including vaginal dryness and dyspareunia(2,8). Besides reducing vasomotor symptoms and increasing vaginal health in women during menopause, isoflavones have been found to improve bone density(9,10).

The main sources of isoflavones are legumes from the *Fabaceae* family, mainly from soybeans, which are the most common source of isoflavones(11). Soybeans contain the isoflavones daidzein, genistein, and glycitein(11). Another source of isoflavones is red clover, which contains primarily formononetin and biochanin A(11). Naturally, isoflavones mostly (>80 %) occur as glycosides, which makes the bioavailability and bioactivity low(11). This is because glucosides are hydrophilic and have a higher molecular weight that prevent passive diffusion across the intestinal brush border. In the gastrointestinal tract, the isoflavone-glycosides are hydrolyzed by β-glucosidases, which releases the aglycons, which are more apolar, lipophilic, and has a lower molecular weight(11). The isoflavone-aglycons can then be absorbed in the intestinal epithelium. To increase the bioavailability and bioactivity, the isoflavones can be converted to aglycones by bacterial fermentation. The red clover isoflavones biochanin A and formononetin are O-methylated isoflavones, which are converted to genistein and daidzein, respectively(11). Equol is a metabolite from daidzein formed from anaerobic intestinal bacteri(11)a. Equol have been ascribed most of the benefits of soy consumption(12). However, in western countries only around 30 % of the population is capable of equol production and around 60 % in vegetarians(13).

The chemical structures of isoflavones are similar to that of estradiol (17-β-estradiol, E2)(11). However, they have differential affinities towards the estrogen receptors in the body. Isoflavones selectively target estrogen receptor beta (ER-β), as they have a strong binding affinity to the beta-receptor and weak affinity to the alpha-receptor, whereas estradiol strongly binds both receptors(14,15). The ER-β is expressed in tissues such as prostate, kidney, ovary, lung, bladder, bone, endothelial cells, and intestinal mucosa(14–16). Increased risk of cancer is associated with strong estradiol binding of the estrogen receptor alpha (ER-α) highly expressed in cancer sensitive tissues such as breast, uterus, and ovary(17). The selectivity of isoflavones for the ER-β entail a beneficial effect while reducing negative side effects such as cancer risk.

Besides experiencing multiple symptoms as described above, postmenopausal women often suffer from recurrent urinary tract infections (UTIs) and unspecific urinary tract symptoms, including urinary incontinence and overactive bladder(18–20). It is not fully determined why postmenopausal women have a higher risk of bladder diseases, however studies indicate that estrogen level play a role together with the fact that postmenopausal women have a different urinary microbiota compared to women before menopause(21).

Until recently, the urine has been considered sterile. The urinary microbiota, a residential bacterial community in the lower urinary tract, was first described in 2012 using metagenomic 16S-sequencing that

detects microbial DNA(22–24). These advanced culture-independent techniques have enabled detection of microbes that previously could not be identified by standard urine cultures. Studies investigating the urinary microbiota have found that the bladder contains a wide range of bacteria. The main genus found in women is *Lactobacillus*, followed by *Gardnerella, Corynebacterium, Streptococcus*, and *Staphylococcus*(25,26). The urinary microbiota is found to have different compositions in women with and without bladder diseases. A study by *Pearce et al 2014* found that the urinary microbiota in women with Urgency Urinary Incontinence (UUI) had increased relative abundances of *Gardnerella* and decreased *Lactobacillus* levels compared to healthy non-UUI controls(27). Consistent with this result *Suzanne et al 2016* reported that Lactobacillaceae was less abundant in females with Neuropathic Bladder (NB) compared with a non-NB group(28).

Bladder symptoms can be relieved using estrogen treatment. Studies have found that local administration of estrogen in the vagina decreases the risk of UTIs in postmenopausal women(29). It is presumed, that estrogen restores the atrophic vaginal mucosa and encourages colonization of beneficial *Lactobacillus* species in the vagina. This facilitates the production of lactic acid from fermentation of glycogen produced by the vaginal epithelial cells. The resulting low pH inhibits growth of many uropathogens and thereby prevents infection in the bladder from ascending pathogens. (20,30,31). In addition, the microbiota in the bladder resemble that of the vagina, which as well is dominated by *Lactobacillus*(32). The closely anatomical relation between the vagina and bladder most likely explain the similarity between the microbiome of the two places. Given the fact, that composition of the vaginal microbiota differs with age and estrogenisation(33), the bladder likewise may alter dependent on menopausal stage and estrogen status.

The role of the microbiota in health and disease has been investigated more intensively in the human gut. The bacteria are suggested to be involved in the neurological functions of the gastrointestinal tract(34). During evolution, the microbial agents have adapted themselves to the host environment creating a healthy symbiotic relationship between the bacteria and the intestinal host cells(34). These interactions form the immune response, resulting in an immune tolerance to the residential microbiota(34). A disturbance in this balance, termed dysbiosis, has been shown to affect the function of the gastrointestinal tract and found to be implicated in a wide range of diseases(34). The mechanisms by which the microbial agents interact with the host cells and the nervous system may also occur in the bladder. A study by *Lüthje et al 2013*, investigated the influence of estrogen on the interaction between host cells and pathogens(35). They found that estrogen appears to support urothelial defense mechanisms against uropathogenic bacteria by: 1) enhancing the production of antimicrobial peptides, 2) strengthening intercellular junctions in the urothelium preventing exfoliation of infected cells, thereby preventing bacterial invasion of underlying less-differentiated cells, which increases the possibility of persistent infections, and 3) limiting *E. coli* proliferation in a mouse model(35).

However, we do not know if and how urothelial estrogen signaling might alter the urinary microbiota(36). Moreover, the research regarding the mechanisms that are involved in estrogen signaling and urothelial biology is still in its infancy and more research is needed.

Therefore, we wish to investigate the effect of isoflavones, on the urinary microbiota composition with the future goal of utilizing estrogen alternatives as treatment against urinary tract symptoms. Moreover, we are interested in the interaction between isoflavones and the host urothelial cells in order to increase our knowledge of estrogen-receptor triggered cellular mechanism and gene expression in the bladder.

Study design

The effect of isoflavones on the urinary bacterial composition will be examined by conducting a double-blinded, randomized, placebo-controlled three-month clinical trial in postmenopausal women. Additionally, we secondary look at the effect of isoflavones on the bacterial composition in vagina and gut. The intervention group will receive a probiotic rich red clover extract (se formulation further down), shortened RCE, containing particularly the isoflavones Formononetin and Biochanin A. The control group will receive an equivalent placebo formulation, shortened PL. A schematic representation of the study design is shown below.

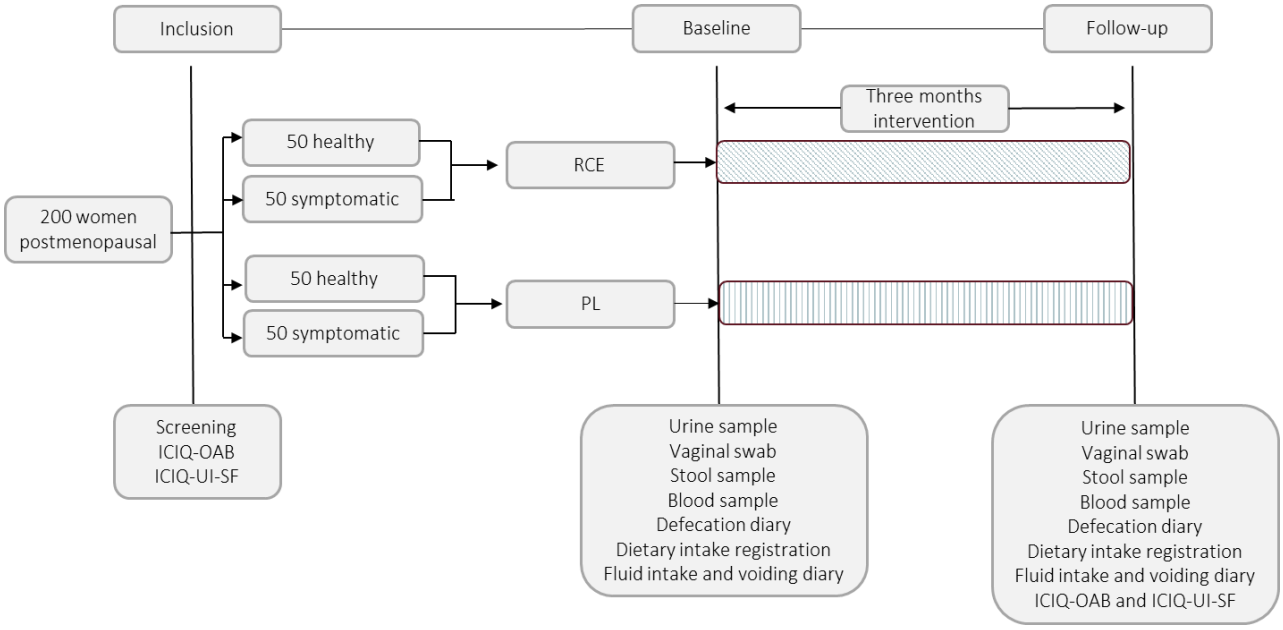

Figure 1 I Schematic presentation of the study design. In total, 200 postmenopausal women will be included in the study. The women will be screened according to the inclusion and exclusion criteria. The questionnaires ICIQ-OAB and ICIQ-UI-SF are used to identify women with urinary tract symptoms, which are allocated to the symptomatic group. Women meeting the inclusion criteria and who does not suffer from any urinary tract symptoms are allocated to the healthy group. Half of the women in the healthy group and half of the women in the symptomatic group will receive red clover extract (RCE) and the other half will receive placebo (PL). Samples will be collected at baseline and at follow-up after three months of intervention.

Participants and clinical material

Participants

Postmenopausal women will be recruited for this study. Two groups will be included – women without any bladder symptoms and women with symptoms of overactive bladder/UUI. Women with an acute urinary tract infection or recurrent urinary tract infections will not be included in the study. Women suffering from overactive bladder symptoms and/or UUI symptoms, evaluated using the ICIQ-OAB and ICIQ-UI-SF questionnaires (attached), are included in the study.

The following inclusion and exclusion criteria for the two groups will be applied:

Common Criteria	
Inclusion	- Postmenopausal (more than 5 years since last menstruation) - Read and understand Danish
Exclusion	- Participants receive treatment with estrogen both oral, transdermal, and topical within three months prior to inclusion in the study - Participants take estrogen-like compounds (isoflavones) three months prior to inclusion in the study - Participants take prebiotic and/or probiotic supplements within three months prior to study inclusion - Receive antibiotics (any) within three months prior to inclusion in the study - Recurrent urinary tract infections (defined as ≥ 2 infections in the last six months or ≥ 3 infections during the last year) - Acute urinary tract infection defined as positive urine culture and symptoms of acute cystitis - Previous or current diseases of the digestive and/or urinary systems evaluated by PI (including, but not limited to IBD and cancer) - Current or prior suffering from breast, ovary, and/or endometrial cancer - Use hormone spiral within the last 5 years if they are under 60 years - Hysterectomy before cessation of menstrual periods if the women are below the age of 60

Criteria for the individual study groups, in addition to the common criteria		
	Healthy	**Symptomatic**
Inclusion		- Positive score in ICIQ-OAB and/or ICIQ-UI-SF
Exclusion	- Positive score in ICIQ-OAB or ICIQ- UI-SF, symptoms of acute cystitis	- Stress incontinence as only symptom evaluated by ICIQ-UI-SF

Study groups

The women are divided into two different groups; 1) healthy women without any urinary tract symptoms (apart from stress incontinence), denoted healthy, 2) women suffering from overactive bladder and/or UUI or mixed incontinence symptoms evaluated using questionnaires, denoted symptomatic. In each group, half of the women are receiving RCE and the other half are receiving PL, resulting in the following four groups:

- Healthy RCE
- Healthy PL
- Symptomatic RCE
- Symptomatic PL

Number of participants to include

To our knowledge, no studies have analyzed the urinary bacterial composition in postmenopausal women treated with RCE or other isoflavone-containing products. Therefore, it is difficult to estimate the number of participants needed by power analysis to gain a sufficient statistical power. However, a study using the same RCE has investigated the effect of isoflavones on bone density using a sample size of 42-43 participants(10). Thus, a sample size of 50 women in each group would be reasonable. Therefore, we want to include 200 postmenopausal women, including 50 women in each group to ensure a high statistical power within groups. Former studies on postmenopausal women has experienced a very high inclusion rate and extremely low dropout rate(5,10,37). The mentioned studies have either originated from Center for Clinical research, North Denmark Regional Hospital or recruited participants from this hospital. Thus, it is expected that it is possible to obtain recruitment of 200 postmenopausal women from this hospital. To ensure inclusion of women in the durability period of the RCE and PL, women in the two groups (healthy and symptomatic) will be recruited in two steps. The healthy groups will be recruited first, from july 2019 followed by the symptomatic groups where recruitment will start no later than july 2020. To obtain the required number of participants for the study, we wish to continue recruitment until we have 50 participants in each group who have completed the protocol.

Clinical information and medicine

To investigate the effect of RCE on the microbiota in postmenopausal women, we will need to take into account potential clinical cofounders that may influence the microbiota and thus affect the results. This includes use of medicine and diseases associated with microbiota dysbiosis. Therefore, all women will be asked if they suffer from diseases associated with microbiota dysbiosis, including diseases of the digestive system. In addition, the women are asked to bring all medicine, nutritional and dietary supplements, vitamins etc. that they consume to the first sampling meeting. This is done to check if the participants take supplements or medicine that can influence the results of the study. Moreover, a medical history of all participants are obtained. Information from clinical journals (Elektronisk Patientjournal (EPJ) will be collected in case of hospitalization or occurrence of other unexpected adverse side effects of the participants in order to investigate if the event is related to the clinical trial. Information from the clinical journals will only be collected after signing informed consent. Consent includes access to the communication and processing of required information on the trial subject's health, other wholly personal matters and other confidential information as part of own control of the research project including quality control and monitoring which a sponsor or a possible monitor is under obligation to undertake. Information from clinical journals will be extracted by the clinical study responsible. The data will be stored in a REDCap file that the research responsible and clinical responsible has access to.

Procedure for participant inclusion and informed consent

Participants will be recruited through advertisements, including flyers and posters, in the local community of northern Denmark (e.g. convenience stores, sports centre, bingo hall, etc.) and on the North Denmark Regional Hospital, Aalborg University Hospital, and Aarhus University Hospital, and through postings on the North Denmark Regional Hospital Facebook profile, their homepage and Personalenet. Furthermore, women living in northern Denmark will receive the advertisement in e-boks (electronic mail). To be able to send the advertisement in e-boks, a list with cpr numbers will be passed on from IT-support. IT-support makes the list

based on women living in northern Denmark, who is going to mammography screening during 2020. These women fits the target group, because all women from the age of 50 will receive an invite to mammography screening. The healthy group will be recruited from July 2019, and the symptomatic group will be recruited from no later than July 2020. The advertisements will include study information, telephone numbers of the project team, and email contact information (see existing recruitment documents). Potential participants can contact the project team if they are interested in the project. Additionally, the project will be advertised in the waiting room at the gynecological outpatient clinic at the hospital.

If a potential participant is interested in the study, a project member will provide the women with the information material (see existing "Deltagerinformation", "Før du Beslutter dig", and "Forsøgspersoners rettigheder i et sundhedsvidenskabeligt forskningsprojekt") either in hand, by email or Quickpost if the participants do not use email. A prescreening over the phone or email will be conducted by a project member, at which point contact information and pre-eligibility information will be collected, including fulfillment of inclusion and none of the exclusion criteria. Data will not be collected or added to REDCap before signing informed consent.

Afterwards, the participants are invited to an oral information meeting given by a project member. The participants are informed that the meeting is a request for participating in the research study and the women can bring an accompanying person to the meeting. The meeting will take place in a separate room undisturbed. At the information meeting, the study will be thoroughly described, including all study procedures and the rights of the study participants. The study participants will receive an informed consent form. Signing of the informed consent may be done immediately, but the women are informed that they can have up to 24 hours of consideration, upon which a new meeting will be planned.

After consent, the women are asked to fil out the validated questionnaires ICIQ-UI-SF and ICIQ-OAB designed to measure frequency and bother of urinary incontinence and overactive bladder. The results from the questionnaires are manually inscribed in REDCap or directly entered by the participants.

Following signing of informed consent, the women will receive a three-day food diary (see existing "kostregistrering"), a three-day fluid intake and voiding diary (see existing "væske- og vandladningsskema"), a three-day defecation diary (see existing "afføringsdagbog"), equipment to collect stool samples and a mid-stream urine sample, and information material for collecting the samples. Moreover, data including weight and height are measured, and the women are asked basal questions including use of antibiotics, prebiotic, probiotic, symptoms of acute cystitis, and hormone estrogen treatment. If the women agree, a photo will be taken which will be placed in REDCap and connected to the participant's profile. A new meeting will be arranged in which baseline samples are collected (first sampling meeting). An appointment for blood sample collection is also arranged.

At the first sampling meeting at one of the sample collection facilities the participants are asked to bring the stool sample, the mid-stream urine sample, the food diary, fluid and voiding diary, and defecation diary. Moreover, the women are asked to fill out the validated questionnaire, Green Climacteric Scale (Danish version, see existing "GCS") designed to measure the degree of menopausal symptoms. Furthermore, the women are asked to fill out three validated questionnaires designed to measure IBS and gastrointestinal symptoms (see existing versions of "ROM IV kriterier", "IBS-SSS", and "GSRS"). The catheterized urine sample, vaginal swab, and blood samples are collected in a room used for clinical examinations and where the

sampling can be performed undisturbed. A project member, who has received proper training in the procedure, collects the samples. The women are randomly distributed in the two treatment cohorts (either RCE or PL), using computerized randomization. At the first sampling meeting, RCE or PL supplements are handed over to the participants and the women are instructed on how the supplements should be consumed. Group belonging is blinded for the participants and study group members. Moreover, the participants are given instructions on how and where to report potential side effects. The women will again receive the food diary, fluid intake and voiding diary, and defecation diary, which they are asked to fill out the last weeks of the intervention period. Moreover, equipment to collect the second stool sample and the second mid-stream urine sample is also handed over to the participants.

At three months post-intervention the second and last sampling meeting will be arranged, in which the same samples as at the first sampling meeting are acquired and questionnaires are collected. In addition, the women are asked to fill out the Green Climacteric Scale questionnaire, ICIQ-UI-SF and ICIQ-OAB questionnaires, and the questionnaires measuring IBS and gastrointestinal symptoms. A study member will contact the participants before sampling and meetings to ensure that they remember to fill out the questionnaires, deliver stool samples, and arrive at the arranged sampling meetings. In addition, a study member will contact the participants each month to ensure involvement in the project and it is possible to ask questions related to the project.

At any time during the entire study period, the participants can withdraw from the study without further explanations. In such case, the participant has to return unopened RCE or PL containers.

After a three months inclusion period, the inclusion process will be evaluated to determine if alterations in the procedure are needed. If that is the case, the ethical committee will be contacted to obtain permission for alterations in the recruitment of the study participants. The new procedures are not initiated until permission is granted from the ethical committee.

Procedure and clinical samples

Before sample collection, the women are asked to fill out a questionnaire covering demographic data, including age, smoking status, number of births, symptoms related to menopause, acute cystitis, and medicine. The participant's weight and height are measured before baseline sampling.

From all participants one catheter urine sample, one mid-stream urine sample, two vaginal swab samples, three samples from one stool, and three blood samples at baseline and after three-months of intervention are collected. Samples are collected at one of the sample collection facilities (North Denmark Regional Hospital or Aarhus University Hospital).

The catheter urine sample is collected to investigate the effect of RCE on the urinary microbiota and bladder symptoms. The mid-stream urine sample is collected to investigate the effect of RCE on the microbiota of the urinary tract compared to the catheter urine sample, which investigate the bladder microbiota. The vaginal swab are used to investigate if the RCE as well influence the bacterial composition and pH in the vagina. This is important, because it is presumed that bacteria are ascending from the vagina to the bladder influencing the bladder microbiome. In addition, we want to investigate if the RCE has an effect on the gut microbiota. The RCE is produced by fermentation using a mix of bacteria, mostly *lactobacilli*, and it has been speculated

that consumption of these bacteria may facilitate improved bowel functions, adding a possible secondary effect. This is based on our previous studies investigating the effect of RCE on postmenopausal symptoms(5). Several of the women expressed positive alterations to their bowel function after intake of RCE. This was however not systematically recorded. We therefore wish to investigate whether RCE influences the gut microbiota and thereby add additional health benefits.

The stool and mid-stream urine samples are collected at home, using standardized, easy-to-use collection kits. Blood, vaginal, and catheter urine samples will be collected by a member of the study team, which has received proper training in the procedure. The vaginal swab samples will be collected using a flocked swab before collecting the urine sample. The urine sample will be collected using sterile intermittent catheterization of the bladder. The procedure is as follows: sterile gloves are used and the urethra opening is cleaned with sterile water. A hydrophilic sterile catheter, size CH 10, is inserted into the urethra, past the sphincter muscle and into the bladder after which the urine is emptied into a sterile container. The first 10 mL of urine is discarded after which the urine is collected. 10 ml of urine is collected for standard urine culture, and if the participant has a positive urine culture in addition with symptoms of acute cystitis, she will be excluded from the study even though baseline samples has already been collected. These samples will then be destroyed.

The women are asked to make a three-day dietary registration, a three-day fluid intake and voiding diary, and a three-day defecation diary registration at baseline and post-intervention.

A schematic presentation of the clinical samples that are collected during the study are shown below:

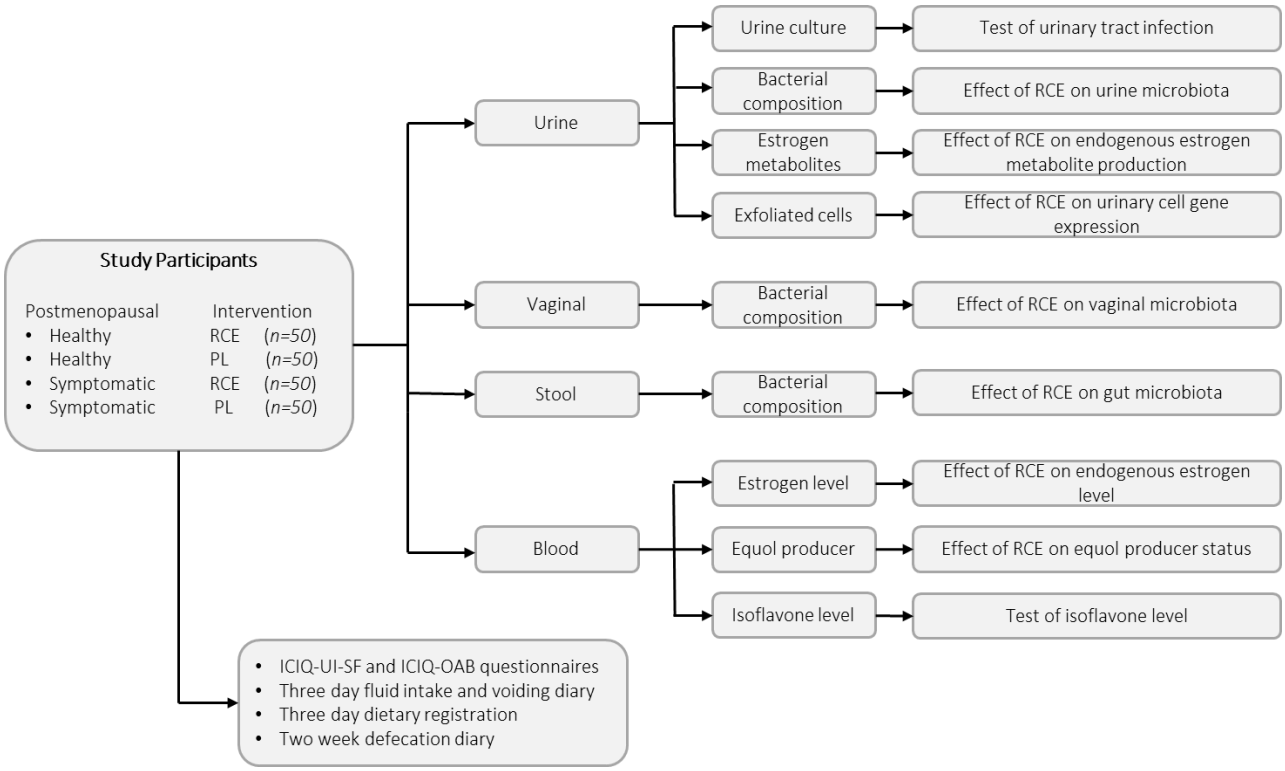

Figure 2 I Schematic presentation of clinical samples taken in the study. Samples are taken at baseline and after three months intervention with either red clover extract (RCE) or Placebo (PL).

Methods

Formulations

The RCE is produced by Herrens Mark ApS and contains a heterogeneous culture of probiotic lactic acid bacteria, which facilitate cold fermentation and improve the bioavailability of the isoflavones in the red clover. To mask the taste and appearance characteristics of the RCE, a natural sugar free raspberry/orange flavoring will be added. Standardization of postfermentation aglycone content will be determined by mass spectrometry by DB Laboratory A/S. Specifically, for each batch, 90 liters of either water (placebo) or RCE are sweetened with 6.3 liters of sugar free raspberry/orange flavoring. In the PL, the water is mixed with 250 gram brown food coloring (ammoniated caramel) (Kavli) to achieve likeness in appearance to the RCE. Both the RCE and PL will be sealed in identical sachets and marked with a code corresponding to either the RCE groups or the PL groups. The participants have to consume either the RCE or the PL twice daily with a meal (95 mL twice daily). The women are asked to bring containers that are not emptied to the second sampling meeting to monitor compliance. In addition, the women are asked to cross a check box every time they take RCE or PL. All participants and the research team will be blinded and have no knowledge of the content of the boxes. The actual content of the boxes with codes will be revealed at completion of the study by an independent third part (Herrens Mark ApS). DB Laboratory A/S will analyze quantitation and validation of isoflavones composition. Herrens Mark Aps has delivered RCE to a previous study concerning bone density in which the isoflavone amount was 57.45 mg isoflavones daily (55.84 mg aglycones daily)(10). We expect the RCE in our study will contain the same amount of isoflavones. In cases where the RCE has another amount of isoflavones, the amount of RCE the participants has to consume will be adjusted to ensure right intake of isoflavone content. The RCE and PL can be stored for up to 18 months.

Clinical samples

All clinical samples are collected from participants at baseline and after three months intervention.

Urine samples:

The catheterized urine sample is collected in sterile containers. The entire bladder is emptied for urine in order to measure the total urine volume. A 10 mL sample is send to the Clinical Microbiology Department at Aalborg University Hospital for urine culturing. A total of 120 mL urine is collected for analysis, whereas the rest of the urine is discarded. The urine is immediately transported to a sample collection facility. The pH is measured and the urine is distributed into tubes containing 10 mL each and stored at -80°C immediately until further downstream processing. Two tubes will be used for DNA purification for 16S rRNA sequencing and two tubes for 23S rRNA sequencing. Two tube will be used to harvest shedded urothelial cells for RNA extraction to investigate the urothelial cell gene expression and antimicrobial peptides in the urine. A tube is used to investigate concentrations of estrogen metabolites 16α-OH and 2-OH and 4-OH using ELISA. The last tubes are stored as back-up samples or for future projects.

The mid-stream urine sample is collected in a 50 mL container at home and transported frozen together with the stool samples to the sample collection facility. The urine sample will be stored at -80°C at Centre for Clinical Research until further downstream processing.

Stool sample: Participants will deliver a stool sample. The participant will collect three stool samples using the spoon provided in the feces container, which have been tested to yield 200-500 mg feces. The samples

will be stored in a -20°C domestic freezer in the homes of the participants for at maximum period of 3 days until delivery at one of the sample collection facilities. The samples are transported, in a cooler to ensure that the samples are not thawed, to the Centre for Clinical Research, where they will be stored at -80°C until further downstream processing.

Vaginal sample: A graft from the vagina is collected using a flocked swab. Moreover, vaginal pH is measured using the other flocked swab. The swab is immediately placed in a sterile tube and transported to one of the sample collection facilities. Afterwards, the samples will be transported to the Centre for Clinical Research, where it is stored at -80°C until further downstream processing.

Blood sample: Blood samples will be taken in tubes treated either with EDTA anticoagulant to yield plasma samples or tubes for collection of serum. We aim for five 6 mL samples. The tubes are stored at 4°C and transported to one of the sample collection facilities within 3 hours, where the samples are either directly processed or stored at -80°C. Afterwards, the samples will be transported to the Centre for Clinical Research, where it is stored at -80°C until further downstream processing. Blood samples are collected to investigate the concentrations of isoflavones and estradiol using mass spectrometry. Moreover, the equol-producer status of the women are determined using time-resolved fluoroimmunoassay.

If a study participant is unwilling to provide the stool or blood samples, the participant will not be excluded from the study. Instead, the obtainable data is analyzed for this study participant. However, the urine sample is necessary for study inclusion.

Methods

Bacterial DNA purification

DNA is extracted from the following samples: urine, stool, and vaginal swab. The DNA is extracted using standard DNA extraction kits. All extracted DNA samples will be aliquoted in smaller volumes and stored at -80°C.

16S rRNA and 23S rRNA sequencing of bacterial DNA

Anonymized purified DNA, from urine, vaginal, and stool samples, are transported to an external Next Generation Sequencing Facility, DNAsense, in Aalborg, Denmark for 16S rRNA gene sequencing (Illumina MiSeq, 2x 300 bp, bacterial ribosomal V4 gene region). Sequencing data will be analyzed using Usearch for amplicon processing and OTU clustering followed by bacterial taxonomic classification using MIDAS database v.2.1.3. The resulting bacterial composition is analyzed using R.

In addition to standard 16S rRNA gene sequencing as described above, we wish to evaluate the bacterial content of the urine samples at species level. This will be done by sequencing of the bacterial 16S and 23S rRNA genes using the MinION nanopore sequencing platform (https://nanoporetech.com/products/minion) in our laboratory.

16s rRNA and 23 srRNA sequencing is performed on bacterial DNA, without analyzing human DNA.

Harvest of exfoliated urinary cells and RNA extraction

A urine sample is collected to harvest human exfoliated urinary cells. After urine collection, the samples are immediately centrifuged to pellet exfoliated cells. Total RNA is extracted using standard RNA purification

protocols, aliquoted and stored at -80°C. Purified RNA will be used in target RT-qPCR and stored for future projects.

Urothelial cell gene expression using target RT-qPCR

Changes in human urothelial cell gene expression will be examined using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). The extracted RNA from exfoliated urinary cells are converted to complementary DNA and used in a PCR with primers targeting specific genes of interest found using cell culture experiments.

Plasma isoflavone concentration and Equol-producer status

A blood sample will be used to determine the blood isoflavone concentration and equol-producer status. The isoflavone concentration is analyzed using mass spectrometry before and after three months intervention. Equol-producer status is determined using time-resolved fluoroimmunoassay before and after three months intervention.

Statistics

For data analysis, IBM SPSS Statistics 22 (IBM), R (the R Foundation) and Microsoft Office Excel 2013 (Microsoft) will be used. Data for analysis will include: Participant descriptive data, microbiota composition, RNA gene expression, urinary estrogen metabolites, and plasma isoflavones concentration, estrogen level, and equol-producer status.

For continuous data, data distribution is tested using Shapiro-Wilk's test. Data will be expressed as either mean or median values for normally distributed or non-parametric data respectively. Continuous data will be compared using Student's t test or Mann-Whitney's test for normally distributed or non-parametric data respectively. Moreover, discrete variables will be analyzed using χ^2 or Fisher exact test. For all samples, a p value < 0.05 will be considered statistically significant.

Research Biobank

All biological samples, including isolated DNA and RNA, will be stored at the Centre for Clinical Research in a biobank for a maximum period of fifteen years. The biological samples will be stored according to existing guidelines, and approval will be obtained from the Data Protection Agency. If further studies on the clinical material are initiated, new approval from the regional ethical committee as well as new informed consent from the included participants will be obtained. In cases where new studies do not cause a risk or inconvenience to the study participants, approval to omit this second informed consent will be sought from the regional ethical committee. No experiments will take place before the ethics committee has approved the study. Additionally, in case of further storage of samples beyond fifteen years, an approval from the Data Protection Agency will be obtained. Following termination of the biobank, all samples will be safely eliminated.

Risks, side effects and inconveniences for participants

We do not expect this study to result in any serious risk or side effects for the participants.

Urine sampling using disposable sterile intermittent catheterization is connected with a quite small risk of subsequent urinary tract infection (1-5 %). Moreover, some women may experience mild and transient

discomfort in the area around urethra. The use of hydrophilic catheter reduces the risk of urinary tract infection substantially.
Vaginal swab sampling can cause transient discomfort for the women, but is not connected with any serious side effects.
The equipment and procedures allows study participants to collect stool samples easily and hygienically. Collection of stool samples may be mildly uncomfortable for some patients due to the stigmatized nature of stool, although overall, stool sampling is a non-invasive procedure. Collection of mid-stream urine samples is as well a non-invasive procedure and not connect with any side effects.
Venous punctures may lead to discomfort, pain, ecchymosis and a small risk of inflammation at needle entry. An instructed and trained member of the project group will place the intravenous catheter (IV) and blood will be drawn through the IV to minimize these risks. If wanted, the participant can have surface analgesia, in the form of prilocain crème, at the access site prior to blood sampling.
The risk of adverse effects using isoflavones have been investigated by Tempfer *et al* (38), which performed a meta-analysis on 92 randomized controlled trials, investigating the safety of phytoestrogens. The analysis included 9629 subjects and found no difference in overall incidence of side effects when comparing treatment groups with control groups. When looking at individual side effects, moderately elevated rates of gastrointestinal side effects, among subjects treated with phytoestrogens was found. When looking within the gastrointestinal side effect category for example abdominal pain, nausea, and vomiting, no significant increase was found. Moreover, no differences where seen in regard to gynecological, musculoskeletal, neurological and unspecific side effects. When investigating hormone-related side effects such as endometrial hyperplasia, endometrial cancer, and breast cancer, no effect was observed, which makes phytoestrogens a well-tolerated compound with a low side effect profile.(38) A single study from 2002 have found that self-reported high intake of soyfood was statistically significantly associated with increased risk of bladder cancer, but these results have not yet been confirmed in other studies(39). On the contrary, another study in Japan found a decreased risk of bladder cancer in self-reported high soy product exposed subjects(40). However, for safety´s sake women who has or previous has suffered from breast, ovary and/or endometrial cancer will be excluded from the study.

Adverse events and side effects

All events will be recorded during the entire study. In case of unexpected side effects from a group of participants, the project responsible personnel, including principal investigator, project initiator, and clinical response will determine if unblinding is necessary. In case of unblinding, the severity of the events/side effects will be judged and decided if the study should be stopped or continued single-blinded. In case of adverse side effects the project responsible personnel will decide within 24 hours if unblinding is necessary. In case of unblinding, the third part (Herrens Mark ApS) are contacted to unlock the identity of the affected women.

Ethical Considerations

The study will be conducted in accordance with the Helsinki Declaration. Before initiation of the study, the investigator must have received approval from the North Denmark Regional Committee on Health Research Ethics, for the research protocol. The North Denmark Regional Committee on Health Research Ethics must also approve any following changes in the protocol.

The RCE have been found to improve bone density in postmenopausal women(10), which will benefit the group of women receiving the RCE. Therefore, the women allocated to the PL groups will be offered three months RCE after the end of the study. Beyond that, this study will not directly benefit the study participants. However, we expect that postmenopausal women in the future will benefit from our study. We hope that this study will increase our knowledge about the effect of isoflavones on the microbiota in urine, gut, and vagina. Moreover, we hope to gain an increased understanding of the interaction between isoflavones, the urothelium, and bladder bacteria. Postmenopausal women often suffer from overactive bladder symptoms and urinary incontinence. The etiology of this is not determined, however new research raises the question whether changes in the urinary microbiota is the cause. We hope that this study can contribute to increase our knowledge about postmenopausal women may benefit from consuming RCE containing isoflavones. We think, that the potential beneficial outcome of this study, increased knowledge in the field, the frequency of bladder symptoms in postmenopausal women, and lacking causal explanation of these bladder symptoms compensate for the small risks and inconveniences that is connected with the study.

Protection of personal data

The study will be reported to the Danish Data Protection Agency through the North Denmark Region, and collection of clinical material will not take place until the study has been registered. All results, including sequencing results, will have assigned project identification numbers. The documents linking personal information with project identification numbers will be stored together with other personal data and clinical samples at the Centre for Clinical Research, according to existing guidelines (General Data Protection Regulation and law (databeskyttelsesforordning og -lov)). All data will be stored according to both Danish law and the General Data Protection Regulation.

Data management

All the study participants' data and documents related to this study, including all sequencing data, will be kept at the archive of the Centre for Clinical Research, North Denmark Regional Hospital, Denmark for 15 years after the end of the study. All non-electronic data (both clinical data and informed consent) will be kept in a safe storage locked with a key.

Insurance

Participants are insured through " patienterstatningsordningen" and "arbejdsskadeforsikringsloven".

Finances

The principal investigator of the study is paid in full by Centre for Clinical Research, North Denmark Regional Hospital/Department of Clinical Medicine, Aalborg University. Applications will be sent to public and private funds to cover all other expenses of the study. None of the involved study personnel has financial interests in the outcome of the study.

Herrens Mark ApS contribute the RCE and PL but has not any interests in the outcome of the study. The study is fully initiated by the project initiator from Centre for Clinical Research, North Denmark Regional Hospital.

The project has as of today (06.05.2019) received the following funding:
From Ulla og Mogens Folmer Andersens Fond: DKK. 100.000 to sampling and laboratory costs

From Niels Jensen Forskningslegat: DKK. 50.000 to sampling and laboratory costs
Marie Pedersen og Jensine Heibergs Legat: DKK. 50.000 to sampling, urine culture, and DNA purification

All donations are disbursed to a research account connected the project and not the study responsible.

Economic compensation of participants

Study participants will in general not be offered any economic payments to participate in the study. All women are offered three months RCE after the last sampling meeting, which has a value of approx. 2691 DKK, so the women allocated to the PL groups also gets the opportunity to get the RCE

Publication

Positive, negative and inconclusive results from the study will be published after the data analysis is finished. The results will mainly be published in scientific journals, but data will also be published in public media and for clinical personnel for whom the results may be of clinical or scientific interest. Publication of results is thus not dependent on publication in a scientific journal. The study participants will be anonymized.

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