Coverpage

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THE IMPACT ON THE BACTERIAL COMPOSITION IN URINE, VAGINA AND FACES AFTER TREATMENT WITH LACTOBACILLI AND ITS INFLUENCE ON RECURRENT CYSTITIS IN POSTMENOPAUSAL WOMEN Protocol

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Purpose

Background

At menopause, a decrease in natural estrogen levels occurs with well-known symptoms such as hot flashes, headache, and vaginal dryness[1,2]. Among post-menopausal women, 10% said they had cystitis/urinary tract infection (UTI) within the last year[3]. Women also suffer from recurrent UTI (rUTI)*, and non-specific bladder symptoms including urinary incontinence. Studies have shown that some types of estrogen located in the vagina (topical estrogen), decrease the risk of rUTI in postmenopausal women[4,5,6].

Until menopause, estrogen levels are high. Estrogen maintains the amount of collagen in the epithelium and the connective tissue. It ensures a high content of mucopolysaccharides and hyaluronic acid and thus a thick, moist, and elastic vaginal wall with high cell-turnover of epithelial cells.
The high turnover of epithelium increases the amount of glycogen. Glycogen is the primary nutrient source for the bacteria lactobacillus. The greater the amount of glycogen, the greater the number of lactobacilli. The Lactobacillus bacterium is naturally occurring, and the most dominant bacterium in the vagina and urinary bladder. Lactobacillus lowers the pH because of the lactate production, thus maintaining a low pH between 3.5-5 in the vagina. The acidic environment is not attractive to pathogenic microorganisms/unwanted bacteria. Likewise, some strains of lactobacillus produce hydrogen peroxide, which in several studies has been shown bacteriostatic and inhibit fungal growth.

The decrease in estrogen levels at menopause causes the epithelium in the vagina to become thinner and with en less turnover of the epithelium. The glycogen mass decreases, which leads to a decrease in the number of lactobacilli and an increase in pH[7]. Vaginal mucosa can more easily be colonized with unwanted bacteria from the skin around the introitus, e.g. the intestinal bacteria E. coli.

In 1863, Louis Pasteur demonstrated that urine was sterile. This has later turned out to be incorrect. In 2012, the urinary microbiome, the bladder's naturally occurring bacteria, was described. In a healthy urinary bladder, more than 150 different bacteria have been found[8]. The urine microbiome cannot be grown with the usual culture methods, but is found by 16s rRNA sequencing, where the bacteria's DNA is purified and sequenced

The microbiome in the bladder is similar to the microbiome in the vagina[9]. The vaginal microbiota changes with age and estrogenization[9], and it has been shown that the microbiome of the bladder also changes in relation to menopause[9]. Regarding UTI, it is unknown whether it is the decrease in estrogen levels that is decisive, or whether it is primarily the consequences of the low estrogen level. It is quite possible that it is a smaller number of lactobacilli and thus a higher pH that makes the bladder environment more attractive to unwanted bacteria.

From research on the gut microbiome, it is known that probiotic bacteria can induce the production of glycoproteins/mucins, which are formed and excreted from the epithelial cells in the gut. The ability of glycoproteins to bind pathogenic bacteria is an important defense mechanism. The mucin layer that covers the epithelium is also an important part of intestinal barrier function[10]. In vitro studies have found that probiotics such as L. rhamnosus and L. plantarum inhibit the binding of entero-pathogenic E. coli due to increased expression of mucins at the gene level[11]. E. coli is the most frequently encountered bacterium that causes cystitis.
It has also been shown that many probiotic bacteria increase the level of butyrate, even if they do not produce it themselves. Probiomes promote the presence of gut-microbiome, which can break down the

* Defined as 2 or more cultured UTIs in 1/2 year and 3 or more cultured UTIs in 1 year

hard-to-digest fiber into short-chain fatty acids such as butyrate. Butyrate contributes to the maintenance of a healthy intestinal epithelium, induces apoptosis in cancer cells and positively affects the immune system.

There is still only limited knowledge about the urinary microbiome. It is expected that the urinary microbiome has the same type of function and importance for bladder health as the gut microbiome for gut health. There is a great potential for research into the urinary microbiome and its influence on exogenously infused products such as probiotics.

When treating women with rUTI, it is recommended to exclude physiological and anatomical gynecological and urological cause[10] initially. Fluid-and voiding diaries and bladder emptying problems are investigated. After this, further treatment is assessed.

Prophylactic antibiotics is not recommended for rUTI. It is only shown to work while it is being taken, and the risk of resistance is high[14,11,12]. Women with rUTI are often treated with prophylactic antibiotics for extended periods of time anyway. However, a study in which the diagnosis of rUTI was not ensured by inclusion, has shown that there is no better effect of prophylactic treatment with antibiotics than that of lactobacilli[13].
One study, in which the women included were defined as young and healthy, found that some lactobacilli had a better effect than placebo at rUTI[14].
In a Cochrane study, two of the studies showed, depending on type, that topical estrogen may have a preventive effect on rUTI[15].

Many women are concerned about the use of estrogen[16], including topical treatment, and do not want this treatment. Likewise, it is not known for certain whether it is estrogen itself or its consequences with an increased number of lactobacilli in the women's urinary microbiome that is the main cause of the possible influence demonstrated in the few studies that are available.
In a study in which 75% of women were postmenopausal, it has been shown that women with UTI have a different composition of their urinary microbiome than women without UTI[17]. The background for this has not been clarified, and whether it is due to a change in the urine microbiome or not is unknown.

Femidur® is a Danish product that contains vaginal lactic acid bacteria in vegetable capsules. Femidur capsules should be swallowed and not used locally in the vagina. The capsules contain 2 vaginal lactic acid bacteria, Lactobacillus rhamnosus GR-1® and Lactobacillus reuteri RC-14®, both of which are part of the normal urinary microbiome in women, and able to colonize the vagina after oral ingestion.
The lactobacilli are absorbed via the intestinal cells, and the intestine has, as described, its own microbiome. It is therefore important to monitor any changes in intestinal microbiome during treatment with vaginal lactobacilli. Vagina is not sterile and is also home to its own bacteria. The physical relationships between the bladder and vagina are close, and it is therefore important to be able to assess any possible impact of the vaginal microflora on the urinary microbiome.

Once the possible physiological, gynecological, and urological causes have been clarified and if necessary treated, there is currently no evidence-based well-documented treatment options for women with rUTI.

Our hypothesis is that postmenopausal women with rUTI have a decreased amount of lactobacillus in their bladder, and that treatment with lactobacillus rhamnosus and Lactobacillus reuteri, in the form of capsule Femidur, will affect the urinary microbiome with an increased number of Lactobacilli in the bladder and vagina and thus decrease the number of UTI. Monitoring both the urine and vagina microbiome is therefore necessary.

We also want to monitor the gut microbiome to see if they are altered by the uptake of lactobacilli, and whether this change follows the eventual change in urinary microbiome change and whether this might have an impact on the number of UTI.
The urinary microbiome between individuals is very different, whereas in the same woman it is stable over time unless she gets a urinary tract infection. It is therefore important to be able to compare the urinary microbiome of the same woman both during placebo and active treatment. In addition, a more accurate study of the bacterial composition on the individual women is possible when the women develop a urinary tract infection.

The women's urine microbiome is checked already after a month and a half. This is to get an idea of how quickly any change in the urine microbiome occurs and whether there is a difference between the individual women participating in the study.

Purpose of the project

The primary objective of the study is to describe the composition of the urinary microbiome in postmenopausal women with rUTI before and after treatment with Lactobacillus rhamnosus and Lactobacillus reuteri.
Secondarily to investigate whether treatment with Lactobacillus rhamnosus and Lactobacillus reuteri has a preventive effect on rUTI in postmenopausal women. In addition, whether the intestinal microbiota is affected by treatment with Lactobacillus rhamnosus and Lactobacillus reuteri and whether differences in the vaginal microbiome have an impact on the above

Method

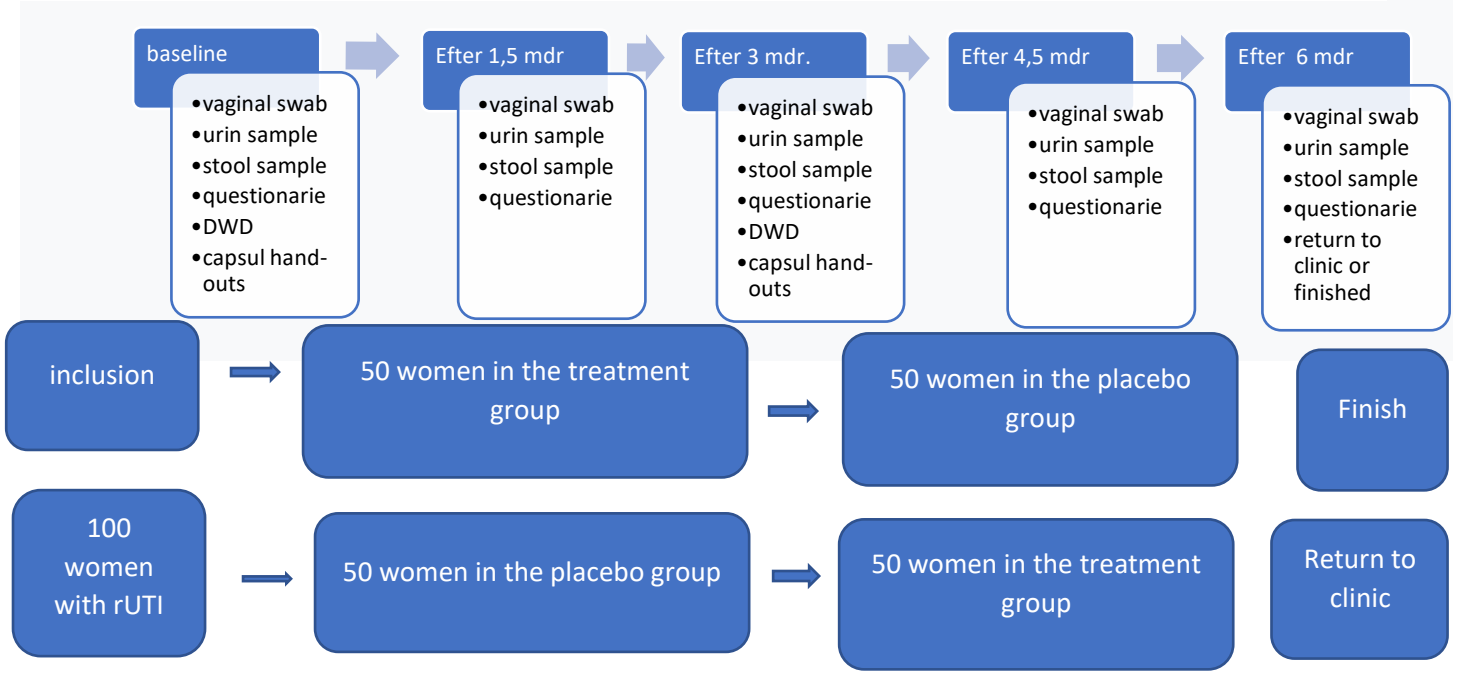
Study design

The study program is designed as a prospective, double-blind, randomized case-cross over study. The study program last 6 months.
The women are randomized to the treatment group (capsule Femidur containing two types of lactobacilli) and to the control group (placebo capsule). After three months, the women cross, and the group that received treatment now receives a placebo and vice versa. The women remain blinded throughout the period for both themselves and the staff.

Randomizing

Randomization of the women takes place by extraction in the electronic system RedCap where the woman's grouping in the study is determined. The woman is anonymized in the study by linking her CPR number to a study number. The tests she submits during her studies are linked to this number. After randomization and submission of samples and questionnaire, the capsules (Femidur® or placebo) are dispensed.

Overview of project progress:



Study Setup and intervention

The included women are divided into two groups. Women in both groups fill out the drinking and water-diary (DWD) over 3 days as well as the ICIQ-OAB and ICIQ-UI-SF questionnaires. A freshly loaded urine sample, a vaginal swab from the top of the vagina and a stool sample are then delivered. The vaginal swab can be taken by the patient himself, or by the doctor/nurse. The urine sample is freshly loaded mid-stream urine. The stool sample can be taken at home and stored up to 7 days in a regular freezer.

The women are then given the capsules, which they take every day for 3 months.

After 1.5 months, all the women show up in the outpatient clinic where urine samples and vaginal swabs are made, and stool samples are delivered. The women fill out the ICIQ-OAB and ICIQ-UI-SF questionnaires.

After 3 months, the women meet again. They hand in a stool sample they bring from home and do a urine sample and a vaginal swab in the outpatient clinic. They completed questionnaires like those filled in at the beginning, but only DWD for a period of one day. Capsules (Femidur or placebo) are again dispensed for the next three months. There has now been cross-over in the study, and the women who used to receive placebo now receive Femidur and vice versa. The women are still blinded, and it is not known who gets what.

The women meet again after 4.5 months, and a urine sample and a vaginal swab are performed. Stool sample is handed in. They fill out the ICIQ-OAB and ICIQ-UI-SF questionnaires.

After six months, the women meet for the last time. They bring a stool sample and a DWD for one day. In the outpatient clinic, they do a urine test and a vaginal swab. They fill out the ICIQ-OAB and ICIQ-UI-SF questionnaires. The effect of the treatment with capsule Femidur is assessed, and it is agreed with the woman whether she has finished treatment for the problem rUTI or whether she should return to the gynecological outpatient clinic for further examination.

If the women develop symptoms of UTI during the project, the women should contact their general practitioner or the emergency medical service to have their urine examined (regular U-stix and culture). In connection with the study, the women are given a card for display, which says that they are participating in a trial, and we ask that the urine is not only tested with regular U-sticks but is also sent for cultivation. If the women develop culture-verified UTI during the study period, the women are treated based on the urine culture response. The treatment is initiated by a doctor or emergency physician. Does the woman have UTI when she comes to the outpatient clinic, the treatment is initiated there.

Implementation, examination and extend of study

All women who are referred to the gynecological outpatient clinic or urological outpatient clinic with rUTI receive the examination usually done for their specific condition, at the first visit to the outpatient clinic.

Some of the women will already be treated with topical estrogen initiated by their general practitioner. For those women who are already being treated with topical estrogen, this is continued. For the women with a wish to participate in the study, it is noted in the project papers whether the woman is taking estrogen and whether it is topical or systemic treatment. If the woman enters the trial and are not already undergoing estrogen therapy, a possible start-up is awaited until after the end of the trial.

At the inclusion data on height, weight, tobacco and alcohol consumption, drug status, parity, previous cancers, diseases, and operations in the abdomen as well as constipation is collected. Information from the journal of the women is not used for the study.

If the woman has rUTI, she is informed about the trial and offered written material as well as a call for further information.

Standards

Femidur® is produced by Pharmaforce, a Danish company founded in 2005. Femidur® capsules are made from gelatin from plants, as well as maltodextrim. Femidur® contains 2 vaginal lactic acid bacteria; Lactobacillus rhamnosus GR-1® and Lactobacillus reuteri RC-14®, both of which can colonize the vagina after oral ingestion. Femidur® contains 5 billion vaginal lactic acid bacteria per capsule of Femidur®. Femidur® capsules should be swallowed whole.

The women are given capsules Femidur® once. It is either at startup or after three months, dependent on the randomization.

The placebo capsules will match Femidur® in appearance. The placebo capsules consist primarily of gelatin from plants and maltodextrim.

The distribution of capsules is planned to avoid the case where the women have capsules with both placebo and Femidur at home at the same time and may mix the two.

Statistical considerations

Number of patients for inclusion

To our knowledge, no previous studies have been conducted on lactobacilli and their possible impact on the urinary microbiome. The number of women to be included to achieve a sufficient number in relation to strength calculation is therefore difficult to estimate. There are other studies available on the vaginal microbiome and disease, and based on these [18], it is planned to include 100 women in total.

Previous studies on postmenopausal women have shown a very small drop-out rate and a high inclusion rate[19], which is why it is expected that the required number of women can be included within a limited period. The inclusion period will continue until the 100 women are included. If there are drop out during the first 3 months, a similar number to the drop out will be included until there is a total population of 100 women.

Statistical variables and data

Variables that are compared between the groups are operational taxonomic unit (OTU) richness (number of unique bacteria) and Shannon Index (the distribution of the bacteria in the sample) and expressions of alpha diversity. Beta diversity, which expresses how bacterial composition varies between samples, is assessed using PCA plots with Hellinger transformation or Principal Bray-Curtis. Heatmaps are used for further assessment of differences and similarities.

Numerical parametric data is given as mean +/- SD while non-parametric data is given as median and range. IBM SPSS Statistics 22 and R (the R foundation) will be used for statistical analysis. The data material will contain demographic data (age, smoking status, births, etc.) and the composition of the microbiome in the vagina, bladder and intestines will be used. Data that will undergo analysis is descriptive patient data, the urine-microbiome and its composition, vaginal and gut microbiome composition and results around rUTI. For all variables, p-values will be calculated and values <0.05 are significant.

Subjects

The women included in the study must be postmenopausal and with a minimum of 3 years of menostasis. If the women have undergone hysterectomy before menopause, the woman must be at least 60 years old when they are included. The women must be diagnosed with rUTI.

The diagnosis of rUTI is made by either:

(a) Three cultivation-verified UTI in a year, or

(b) Two cultivation-verified UTI over the course of six months.

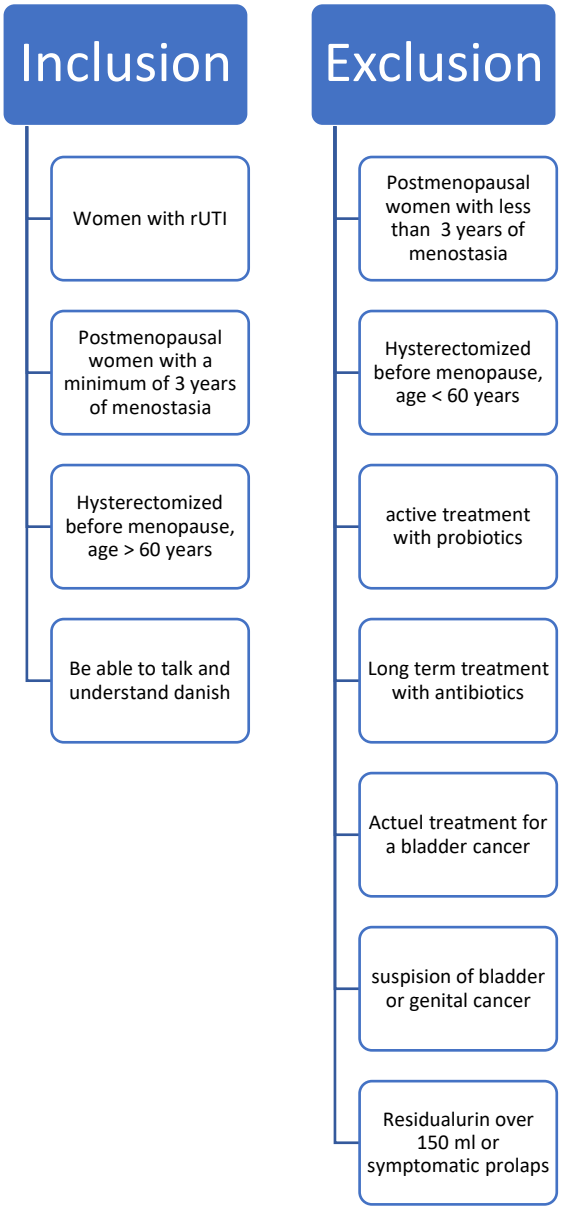
If the woman is diagnosed with rUTI, a positive culture response will be checked before the woman arrive at the outpatient clinic. If there are no culture responses in the hospital's medical record system, contact is

made prior to the outpatient clinic visit with the women's general practitioner to confirm that the UTI is culture-verified. The same is done if it is during the woman's visit to the outpatient clinic that the suspicion of rUTI arises. In such cases, the woman can only be offered final inclusion once the diagnose rUTI have been confirmed by cultivation as earlier described. The women can instead be informed about the study and provided with written material.

The included women are allowed to take regular medication, except for long-term treatment with prophylactic antibiotics. They are not allowed to take probiotics.

If the women have a current UTI, this is treated in relation to culture responses. After that, the women can be included in the study. It is recorded if the women use systemic or topical estrogen, and the specific type is noted.

Inclusion and exclusion



Risks and side effects

The women are not expected to experience serious side effects or disadvantages in the study. Nor that any damage, short or permanent is caused.

Femidur® is a probiotic and not a medical product. This means that it consists of well-defined microorganisms. Femidur contains two lactobacilli; Lactobacillus rhamnosus GR-1® and Lactobacillus reuteri RC-14®, which are part of the normal flora in humans. There are no known side effects for Femidur®.

Femidur® is a dietary supplement consisting primarily of plant gelatin, maltodexrim and lactobacilli (Lactobacillus rhamnosus GR-1® and Lactobacillus reuteri RC-14®). Maltodextrim are carbohydrates in the form of short and medium starch chains. It is found both in ordinary groceries and used in the pharmaceutical industry. None of the substances are likely to cause allergic reactions or side effects. Should discomfort or hypersensitivity reactions occur against the capsules, this will be recorded. If the women wish to leave their studies, they can always do so.

Passing a urine and stool sample is not considered associated with pain. The very act of handing in a sample, especially with feces, can for some be associated with discomfort and modesty. The treatment staff acts professionally and tries to ease this situation for the patient. If it is a major burden on the patient, it is expected that the patient will not sign up for the trial. Performing vaginal swabs can be uncomfortable for some women but not painful or dangerous. The women can take the swab themselves or have it taken by a healthcare professional, which one is associated with the least discomfort for the women.

Clinical tests, sampling of biological material.

All clinical samples are collected at baseline, after 1.5, 3, 4.5 and 6 months.

Urine samples

At each visit, the women deliver a freshly voided urine sample in a sterile container, 50 ml each time. The women must have physical attendance when the urine sample is made, as this must be done in comparable rooms each time and be fresh. Urine is taken to normal culture at the department of clinical microbiology (10 ml) and 40 ml of the remaining urine is divided into four tubes and frozen to -80 grader Celsius. There are two freezers, one is placed at the Department of Clinical Microbiology at Aalborg University Hospital, and the other at the North Jutland Regional Hospital. The frozen urine is stored until analysis. Urine is examined using 16s rRNA sequencing to identify bacterial DNA in the urine and thus describe bacterial types and distribution.

Vaginal swab

At each visit, all women deliver a vaginal swab that is placed in a sterile container. The inoculation is subject to the same conditions as urine, and it must therefore be made at the hospital every time. The container with vaginal swab is then stored in a freezer at the department of microbiology, Aalborg University Hospital or North Jutland Regional Hospital, at -80 degrees Celsius. The sample is examined using 16s rRNA sequencing to identify bacterial DNA in the vagina and thus describe the bacterial types and distribution.

Stool samples

At each visit, all women deliver a stool sample that is placed in a sterile container. The container with the stool sample is then stored in a freezer at the department of microbiology, Aalborg University Hospital or Regional Hospital North Jutland, at -80 degrees Celsius. The sample is examined using 16s rRNA sequencing to identify bacterial DNA in the vagina and thus describe the bacterial types and distribution. The sample can be made from home and stored in a regular freezer for up to 7 days.

Finishing of samples:

Urine

Urine is centrifuged and bacterial DNA purified from the pellet using QIAamp viral RNA Mini Kit (Qiagen), with the protocol set out by the manufacturer for purification of bacterial DNA, automated using a QIAcube. The protocol has an extra pre-processing step that involves 2 min. bead beating, 30 Hz treatment with a TissueLyser LT with a single steel ball.

Vaginal swab

Containers with swabs are frozen to -80 degrees. When the sample is to be analyzed, it is thawed, and PBS (Phosphate-Buffered Saline, physiological salt buffer) is added. Bakterie DNA is purified with the Allprep Power Viral DNA/RNA kit (Qiagen) automated using QIAcube. The resulting DNA is stored at -80 °C until further analysis.

Faeces

Pseudo anonymized DNA samples are transported on dry ice to an external Next Generation Sequencing facility (DNA sense, Aalborg) for 16s rRNA gene sequencing (Illumina MiSeq, 2x 300 bp, bacterial ribosomal V4 region). Usearch is then used for amplikon treatment and subsequent OTU clustering. The MIDAS database (V. 2.1.3) is used for taxonomy.

Biobank

All biological material will be stored at the Centre for Clinical Research, North Jutland Regional Hospital for a maximum of 15 years. For all types of sample material, purified DNA is stored, and frozen original samples are stored – 10 ml of urine and vaginal samples. The samples will be stored in accordance with current guidelines and after approval from the Danish Data Protection Agency. Further studies on the samples in the biobank can only be carried out after new approval from the Scientific Ethics Committee and obtaining new consent from the included women. In the event that a new analysis of the samples will not result in a risk to the women, the Research Ethics Committee is applied for permission to analyze the samples without the consent of the included women. The biological material shall be stored for possible subsequent analysis of bacteria or fungi. None of the material is sent abroad.

Research ethics section

The project follows the ethical rules described in the Helsinki Declaration.

The project is initiated by Consultant Karin Glavind and Department Physician Louise Arenholt

The project is considered a public research project and is notified via the region. Patient consent is obtained after oral and written information to the patient.

The purpose of the study is to look at the change of the urinary microbiome when influenced by lactobacilli and whether this has an impact on the prevention of rUTI. To ensure that the link being investigated is between lactobacilli and the urinary microbiome, samples of the gut and vagina's microbiome are also taken.

The study is set up as a cross-over study, so that all the women receive both placebo and active treatment. By presenting the study this way, the possible effect of lactobacilli on the urinary microbiome in women with rUTI could be found by comparing the urinary microbiome in the treatment group and the placebo group. By letting the groups cross treatment after three months, they become additional control group for

themselves. This is important as the urinary microbiome between individuals is very different, whereas in the same woman it is stable over time unless she gets a urinary tract infection.

There are no known disadvantages to adding an increased amount of the body's own lactobacilli and no documented effect on the urine microbiome yet. Smaller studies have shown effect of treatment with lactobacilli on rUVI[18]. It is therefore the expectation that the women (regardless of the influence of the urinary microbiome) will have a beneficial effect of the treatment with lactobacilli on their rUTI.

Relapsing UTI is debilitating for the individual woman, but also affects her surroundings. Frequent toilet visits are often needed, which can keep the woman and her family at home or limit their activities. Currently, there is no good treatment, and many end up with long-term use of antibiotics that increase the risk of developing resistance in the individual and in society in general.

In view of the challenges and problems, both in the individual woman, but also in society, that rUTI creates, the few annoyances created by the above study must be considered acceptable, considering the information that the study can provide.

Data protection

The study is reported to the North Denmark Region's research review for projects with personally identifiable data. Notification to the Danish Data Protection Agency via Region Nordjylland's own notification. The study will not be initiated until approval from this and from the Research Ethics Committee is available. The General Data Protection Regulation and the Data Protection Act will be complied with. All documents and test results will be affixed to project ID. Documents linking project ID with personally identifiable data including data material will be stored according to the law.

Economic relationship

The project is financially supported by Pharmaforce, which supplies capsule Femidur® and placebo capsules. Pharmaforce does not provide financial support to any extent other than this. Pharmaforce has had no impact on study design or data. The capsules are dispensed as a donation and without clause or conditions.

Support is sought from external funds. The financial support will be used for laboratory tests, collection kits and remuneration of laboratory technicians. None of the members of the project team have financial interests in the project.

Currently, 40,000 people have been granted from the Department of Clinical Research, Aalborg University for the above.

Remuneration and/or other benefits of the subjects

It is not possible to give remuneration or financially compensate participants.

Recruitment

The primary recruitment takes place when the patients are referred to gynecological outpatient clinics at either Aalborg University Hospital or Regional Hospital Nordjylland, Hjørring. Women referred with rUTI referred to the urology department in Frederikshavn can also be included.

The women may be referred with the problem rUTI, or the diagnosis is made in connection with the woman meeting in the outpatient clinic with other problems. If the woman has rUTI, she is offered to participate in the study in connection with her attendance at the outpatient clinic, and if she has an interest, the written patient material is provided. She is then contacted by the project manager and invited to a meeting and further information.

At the start of the study, contact will be made via the regional practice coordinator to the general practitioners to inform about the study. During the study, it will be possible to refer women with rUTI to gynecological outpatient clinics in Aalborg earlier than usual. In addition, the private gynecologists in the pre-hospital setting will be informed of the project and the opportunity of referring to it. It will be possible, in agreement with the private gynecologist, to provide small patient booklets with information on the study in the gynecological practice.

In the waiting rooms of the gynecological outpatient clinic, there will also be pamphlets with information material so that women who come for other reasons can be informed of the study. If this happens, the outpatient doctor will be able to refer the woman for rUTI examination to the uro-gynecological team in the usual way. After that, the woman will be included as previously described.

Two smaller papers and a more detailed description of the project have therefore been prepared for this purpose. The latter is only dispensing s if the patient meets the inclusion criteria and does not fall for the exclusion criteria.

The women are informed by the doctor or nurse in the outpatient clinic about the possibility of participating in the study, and the written project material is provided. The women are contacted by telephone after the written material has been completed. Here is the opportunity to ask further questions. If the woman is interested in participation, she is invited to an information meeting within 5 working days. She is informed about the possibility of having relatives with her while the information is given, and she is informed about max. 48 hours of reflection time from oral and written information is given. Consent is obtained when the patient indicates that there is no need for further reflection time. The conversation takes place in closed conversation rooms in the outpatient clinic at one of the two gynecological departments.

The women may withdraw their consent at any time and without further explanation. In this case, the delivery of capsule Femidur® or placebo is discontinued.

Publication of results

Publication of the results in international journals is planned. Both positive, negative, and inconclusive results will be published in anonymized form.

Information on compensation scheme

Participants in the project are covered by the Patient Compensation Scheme and the Workers' Compensation Act.

Project responsible

Responsible for the project is Caroline Juhl, department physician at the gynecological-obstetric department AAUH. Supervisor is department doctor at the department of Gynecology and obstetrics, Regional Hospital North Jutland, Louise Thomsen Schmidt Arenholt and consultant at the gynecological-obstetric department AAUH Karin Glavind.

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