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14. ABSTRACT

Purpose and scope: we are conducting a large case-control study, nested within a prospective cohort, to estimate relative risk of breast cancer in post-menopausal women by levels of urinary estrogens and estrogen metabolites.

Progress report: in this third year of the project, a very sensitive gas-chromatography/mass-spectrometry method for the measurements of estrogens and estrogen metabolites in frozen urine samples from post-menopausal women has been set up to replace the method initially foreseen in collaboration with Dr. Mindy Kurzer (University of Minnesota). Some urine samples from the DOM cohort have already been analyzed, showing the applicability of the method for this study.

Conclusions: due to the lack of sensitivity of the mass spectrometry method initially foreseen for the measurement of the estrogen metabolites, and the need to replace this method with a more sensitive one, our study is substantially delayed. However, we have finally set up a very sensitive method that will allow the measurements of all estrogens and estrogen metabolites in the DOM urine samples. We ask for an extension of one year for the project, so as to allow us to complete the hormone assays in all urine samples, and perform statistical analyses.

15. SUBJECT TERMS
Breast Cancer, Epidemiology, Genetics, Urinary Estrogen Metabolites

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INTRODUCTION:

It has long been recognized that estrogenic steroid hormones, particularly 17 β-estradiol (E_2) can promote the development of breast tumors. Besides stimulating cell proliferation, there is increasing experimental evidence that estrogens may also be activated into genotoxic hydroxy metabolites that cause DNA mutations. In addition, some of the same metabolites may bind irreversibly to estrogen receptors, and thus stimulate cell proliferation permanently.

Major pathways through which hydroxy metabolites of estrogens (estrone [E_1] and estradiol [E_2]) are formed are the 16α-hydroxylation pathway – which leads to formation of 16α-hydroxy E_1 and estriol – and pathways that lead to 2- and 4-hydroxy ("catechol") estrogens. Preliminary epidemiological evidence suggests that estrogen metabolism via the 16α-hydroxy pathway is increased in breast cancer patients compared to controls, and an inverse relationship has been found between breast cancer risk and the ratio of urinary concentrations of 2-hydroxy/4-hydroxy or 2-methoxy/4-methoxy estrogens.

Amongst key enzymes involved in the natural conversion of estrogens to hydroxy estrogens and are CYP1A1, CYP1B1, and CYP3A4. Furthermore, catechol-*O*-methyl transferase (COMT) is a key enzyme in the methoxylation of 2- and 4- hydroxyl groups, thus leading to methoxy estrogens. Methoxylation is a major pathway for the inactivation of the chemically very reactive catechol estrogens. In addition, experimental studies indicate that the methoxy metabolites inhibit tumor formation and development by decreasing cell growth, and inhibiting the formation of blood vessels in tumors.

Given these various observations, it has been hypothesised that breast cancer risk would be lower in women who produce more 2- and 4-metyhoxy estrogens relative to the levels of the corresponding hydroxyl estrogens. To examine the above hypotheses, we have started a case-control study nested within a large prospective cohort (the 'DOM' cohort, the Netherlands), with the following specific aims:

1) examine relationships of post-menopausal breast cancer risk with absolute and relative prediagnostic urine levels of 2-hydroxy, 4-hydroxy, 16α-hydroxy, 2-methoxy and 4-methoxy metabolites of E_1 and E_2
2) examine relationship of polymorphic variants of genes encoding estrogen-metabolizing enzymes (CYP1A1, CYP1B1, CYP3A4 and COMT) to urinary levels of the various estrogen metabolites, as well as to breast cancer risk.

Our project is designed as a case-control study nested within a large prospective cohort, using urine and DNA samples collected from more than 50,000 women in the Dutch city of Utrecht and surroundings ("DOM" cohort). This cohort is unique, in that rather large volumes (50-100 ml) of urine were collected and stored for all study subjects. The majority of women in the cohort provided also a second (and even third) urine sample. The samples were stored in a large frozen warehouse. Relatively large volumes of urine (>10 ml) are needed to measure the estrogen metabolites, by gas chromatography coupled with mass spectrometry (GCMS). Cases and controls are selected among women who were post-menopausal at recruitment, and who did not use hormone replacement therapy. For about 60% of women who provided a

second urine sample within a time interval of about one year we also incorporated this second sample in our study, so as to improve exposure measurements.

BODY:

For year 3, our work plan was (as in the "Statement of Work" of the original grant application):

- Statistical analysis of nested case-control study on urinary estrogen metabolites and breast cancer risk, and writing of reports: **Task 6 (months 25-36)**

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We have not been able to start this task because, as stated in the Progress Report for year 2, our study encountered some major problems in the analyses of urinary estrogen metabolites in the DOM samples because of the lack of sensitivity of the gas chromatography/mass spectrometry (GC/MS) method used in Dr. Kurzer's laboratory. It is therefore substantially delayed. In this third year of the project, however, we have been able to develop a very sensitive method for the analyses of estrogen metabolites in blood based on negative chemical ionization gas chromatography/mass spectrometry (GC/ NCI-MS) as part of a parallel project that was funded independently by the National Cancer Institute (grant application nr 5RO3 CA096398). Our new method is based on enzymatic hydrolysis, solid phase extraction, purification by high performance liquid chromatography (HPLC), derivatization with fluorinated agents (essential to have a very sensitive detection by NCI-MS) and final injection on GC/ NCI-MS. The automation of the purification steps by HPLC, and of the injections on the GC/MS by an automatic injector allows the measurements of about 40 samples per week, so our method can be easily applied to medium sized epidemiological studies. The GC/ NCI-MS method gives very good results in terms of linearity, accuracy and precision for the measurements of all estrogen metabolites. The detection limits for each of the hormones are the following:

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E_2	50 pg/ml
E_3	50 pg/ml
16αOH-E_1	50 pg/ml
2OH-E_2	50 pg/ml
4OH-E_2	50 pg/ml
2OMe-E_2	125 pg/ml
4OMe-E_2	500 pg/ml
E_1	500 pg/ml
2OH-E_1	500 pg/ml
4OH-E_1	500 pg/ml
2OMe-E_1	125 pg/ml

4OMe-E_1 250 pg/ml

Our new method is 100 to 1,000 times more sensitive than the method used in Dr. Kurzer's laboratory, depending on the specific steroid being measured.

Although initially developed for analyses in blood, our method could be easily adapted to measurements in urine, and we have started analyzing some of the urine samples from the DOM cohort (using only 2 ml of urine). Contrary to the results obtained when applying the method used in Dr. Kurzer's laboratory (see our previous Progress Report), when using the GC/ NCI-MS method all steroids could be specifically identified and quantified on DOM cohort urine samples. All concentrations of the analytes were found to be much higher than the detection limit of the method. Some GC/ NCI-MS chromatograms of the urine samples tested have been included in the report (Figures 1 and 2).

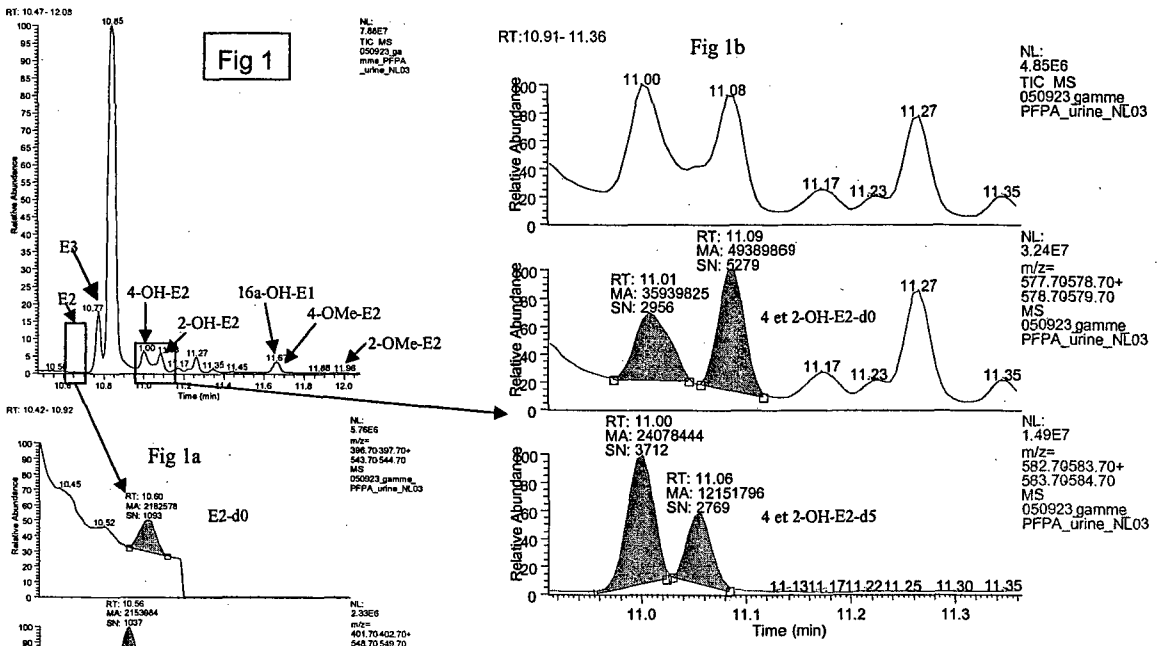

Fig 1. Chromatogram (single ion monitoring) of one urine sample from the DOM cohort obtained by GC/NCI-MS. Derivatizationagent:perfluoropropionic anhydride

Fig 1a. Ions monitored for E2 (*m/z* 397 + 544) and for E2-d5 (deuteratedinternal standard) (*m/z* 402 + 549).
Fig 1b. Ions monitored for 4 et 2-OH-E2-d0 (*m/z* 578 + 579) and for 4 et 2-OH-E2-d5 (deuteratedinternal standards) (*m/z* 583 + 584).

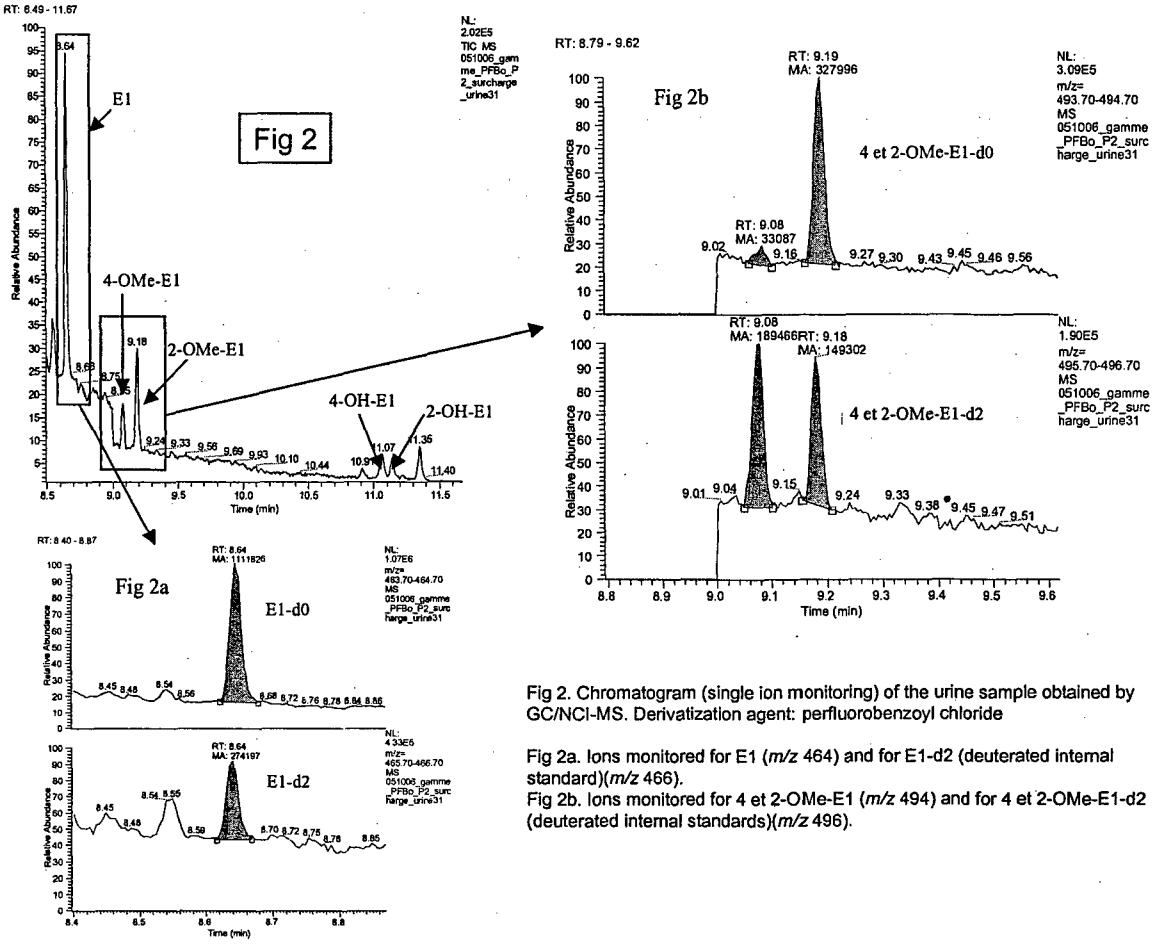

Fig 2. Chromatogram (single ion monitoring) of the urine sample obtained by GC/NCI-MS. Derivatization agent: perfluorobenzoyl chloride

Fig 2a. Ions monitored for E1 (*m/z* 464) and for E1-d2 (deuterated internal standard)(*m/z* 466).
Fig 2b. Ions monitored for 4 et 2-OMe-E1 (*m/z* 494) and for 4 et 2-OMe-E1-d2 (deuterated internal standards)(*m/z* 496).

The GC/ NCI-MS method allows the measurements of all estrogens and estrogen metabolites in the DOM samples without encountering problems of sensitivity, and it allows a substantial reduction in the volume needed for these measurements (2 ml rather than 30 ml). Given these very promising results, we plan to complete the measurements for the entire nested case-control study within the next 12 months.

KEY RESEARCH ACCOMPLISHMENTS

In year 3 of the study, we set up a very sensitive method for the measurements of estrogens and estrogen metabolites in urine samples to replace the method initially foreseen. Due to the delay in year 2, we have

not been able to measure all the samples from the nested case-control study. We therefore request to continue the project in year 4, without additional funds. We have left most of the funds for year 3 untouched, and we plan to use this balance for completion of the measurements in year 4.

REPORTABLE OUTCOMES

There are no reportable outcomes so far.

CONCLUSIONS

To overcome the problems of sensitivity of the method initially foreseen, a very sensitive method for the analyses of estrogen metabolites in frozen urine samples from post-menopausal women has been developed. We plan to complete the analyses of estrogens and estrogen metabolites in all urine samples within the next 12 months.

REFERENCES:

APPENDICES: