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Acronym of the project: Estrogens and disease		
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PROJECT COORDINATOR		
Name: Paul van der Saag and Anja van de Stolpe	**Title** Dr	**Address:** NIOB, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
Telephone: +31-30-2121883	**Telefax:** +31-30-2516464	**E-mail address:** Paul@niob.knaw.nl anja@niob.knaw.nl
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List of participants

1. Dr. P. T. van der Saag, and Dr. A. van de Stolpe (coordinators)
Netherlands Institute for Developmental Biology
Uppsalalaan 8
3584 CT Utrecht
The Netherlands
E-mail: paul@niob.knaw.nl; anja@niob.knaw.nl
Tel: (31)302121883
Fax: (31)302516464

2. Prof. Dr. D. de Rooij
Faculty of Biology
University of Utrecht
P.O.box 80088
3504 TB Utrecht
The Netherlands
E-mail: d.g.derooij@bio.uu.nl
Tel: (31)302509280
Fax: (31)302541797

3.Prof. Dr. J. □. Gustafsson (contractor)
Department of Medical Nutrition and Unit for Preventive Nutrition
NOVUM
S-141 86 Huddinge
Sweden
E-mail: jan-ake.gustafsson@cnt.ki.se
Tel: (46)8-58583701
Fax: (46)8-7798795

4. Prof. Dr. A.G. Byskov (contractor)
Laboratory of reproductive Biology
Juliane Marie Center, Rigshospital
Blegdamsvej 9
2100 Copenhagen
Denmark
E-mail: agb.lrb@rh.dk
Tel: (45)35455820 or (45)35455897
Fax: (45)35455824

5. Prof. Dr. Chr. Behl (contractor)
Institute of Physiological Chemistry&pathobiochemistry
Joh. Gutenberg University Mainz
Duesbergweg 6, 55099 Mainz
Germany
E-mail: cbehl@uni-mainz.de
Tel: (49)6131-39.25890
Fax: (49)6131-39.25792

Objectives:

1. Modulate estrogen levels in pregnant mice and link this to phenotypic effects in the prostate, testis, mammary gland, brain and the ovary of the offspring, to get insight in tissue specificity of sensitivity to hormonal disruption by estrogenic chemicals.
2. When sensitive targets for endocrine disrupters are found under point 1, the relevant cell- and receptor type involved in the effects will be determined. These results will be further used to design tests for the most critical stages and tissues sensitive to endocrine disruption.
3. Identify molecular markers that can help to assist in the diagnosis of disturbances by estrogens. We will be focussing on prenatal exposure, being the phase when animals, and probably humans, are highly susceptible to changes in their hormonal environment. We will concentrate on those genes that remain induced through adult life after embryonal estrogen exposure, even when the hormonal pulse is gone.
4. Further develop the markers identified in the animal studies for use in a clinical setting, aiming at markers that provide information about estrogen exposure during critical stages of development.
5. Identification of the molecular pathways leading to prenatal disruption of hormonal imprinting, and get further insight in the possible impact of these effect on development of disease later in life. When possible, improve current testing strategies, and generate more generally applicable molecular markers of exposure.

Results and Milestones:

A tissue collection of estrogen-sensitive C57B1/6J mice developmentally exposed to different low (0.1 to 10 µg/kg) doses of estrogens has been completed (WP1), despite extensive breeding problems in the animals treated with a higher dose estradiol. Tissues have been distributed to the partners for detailed analyses of estrogen effects in prostate, ovary, mammary gland, brain, and testis (D1).

Analysis of the effects in tissues has been completed (M1, D2-6). No statistically significant morphological effects of estrogen exposure in testis (WP3), ovary (WP5), prostate (WP4) and brain (WP6) and mammary gland (WP2) were found. No effects were found on oocyte maturation, and number of germ cells (WP5). The highest dose of estradiol was associated with reduced gonocyte number at birth, however all maturation stages were normally present (WP4). A primary gonocyte cell line, expressing ER¤ and responsive to estradiol has been established and used to identify ER target genes: ER¤, PDGFR¤ and Hsp90¤. In ovary, connexin 43 was found to be an estrogen-sensitive target, however not influenced by intra-uterine exposure (WP5). Primary cultures of nerve cells have not been established due to the technical difficulties in generating sufficient animal tissue (WP6). With regard to the mammary gland: in an independent experiment where mice were exposed to much higher doses estradiol and Diethylstilbestrol (WP2), a reduction of the ductal length and volume was found, while the nipple sheet disappeared; however exposure to 0.1-10 µg estradiol did not induce changes. In vivo ER activity has been determined in an ERE-reporter mouse: liver, testis, pituitary, brain, prostate, bone and colon were identified as estrogen-target organs (WP2). Comparison between estradiol, DES and the environmental estrogen bisphenol A (BPA) revealed strongest *in vivo* estrogenic activity of DES. Surprisingly BPA had *in vivo* a much higher estrogenic effect (only slightly less than estradiol) then reported *in vitro*, stressing the importance of this *in vivo* mouse test model (WP2).

With respect to identification of marker genes for estrogen exposure (WP7, M2), *caveolin* expression and glycogen synthase kinase 3 activity were found to be decreased in respectively hypothalamus and hippocampus of estrogen-exposed mice (D11). Caveolin expression was very low in prostate (D9). *CRH-binding protein* was found to be an estrogen target gene, which was induced by estradiol in a hypothalamic cell line GTI-7, while the in utero exposure protocol caused a long term increase in the expression of CRHBP in hippocampus and hypothalamus in 4 week old male animals. *Connexin 43* was found to be an estrogen-sensitive target in granulosa cells, however no long-term changes in expression were seen after in utero exposure (D10). In testis ER¤, PDGFR¤ and possibly Hsp90¤ expression decreased at highest exposure concentration.

With regard to the mechanisms involved in hormonal imprinting (WP8, M3), activity of the CRHBP promoter activity appeared to be modulated by CpG methylation, while in an endometrial cell line HEC1B CRHBP expression was induced by demethylation, suggesting that estradiol/ER-mediated changes in promoter CpG methylation status might result in long-term effects on protein expression.

Up to now, caveolin, CRHBP, ER¤, PDGFR¤ and possibly Hsp90¤ seem the most promising human markers for intra-uterine estrogen-exposure (M4)

It has been proposed that increased cancer incidence, but also fertility problems in wildlife and humans, are in part caused by an increased pre-natal exposure to environmental compounds which mimic endogenous estrogens and cause endocrine disruption.
To characterize effects of environmental estrogenic compounds, endocrine disruption was mimicked *in vivo* in mice by intra-uterine exposure to low dose estrogen. Several estrogen target organs, which were identified using an ERE-reporter mouse, were analyzed for phenotypical effects. No *statistically significant* morphological effects of intra-uterine low dose estrogen exposure in testis, ovary, prostate and brain and mammary gland were found. In contrast, in an independent experiment where mice were exposed to much higher doses estradiol and Diethylstilbestrol (DES), morphological effects on mammary gland morphology were found, indicating that higher doses are likely to be required for morphological effects. At cellular level the only statistical significant finding was a reversible reduced gonocyte number in testis at birth, but not at four weeks any more. At molecular level, expression levels of several estrogen-responsive genes, i.e. caveolin, glycogen synthase kinase 3, CRH-binding protein (CRHBP), ER¤, PDGFR¤ and Hsp90¤ changed following intra-uterine exposure, suggesting subtle long-term effects of endocrine disruption at a molecular level. These aberrantly expressed genes may be suitable for development as markers to diagnose prenatal exposure to environmental estrogens and relate occurrence of common diseases to endocrine disruption.