# Determination of estrogenic (ERα CALUX®), androgenic (AR CALUX®) and dioxin-like (DR CALUX®) activity in human plasma from mother-child cohorts.

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## Introduction

Within the NewGeneris program (European Community 6$^{th}$ Framework Program, Priority 5 Food Quality and Safety), the hypothesis that maternal exposure to dietary compounds with carcinogenic and immunotoxic properties results in *in utero* exposure and molecular events in the unborn child leading to increased risk of cancer and immune disorders in later childhood, will be tested. Therefore, a variety of biomarkers will be developed, validated and used among which the DR, ERα and AR CALUX® bioassays by BDS. The DR, ERα and AR CALUX® bioassays for the analysis of dioxins and dioxin-like compounds, estrogenic compounds and androgenic compounds can be used for large scale screening of clinical samples and large cohorts. All CALUX® bioassays have been validated for the analysis of small amounts of human plasma[1,2] and are currently being used for screening of maternal and cord plasma from different cohorts. Here, the first results from these analyses are presented.

## Methods and materials

*Sample preparation.* Maternal blood and cord blood was collected from women and newborns in Denmark, Greece and Spain. Human blood was collected using heparinised blood-tubes. Following blood collection, the blood-tubes are centrifuged at 650 g for 10-15 min at room temperature and the plasma fraction is collected and frozen in aliquots at minimal -20$^0$C. Plasma samples from 67 mother-child pairs from Denmark, 41 from Greece and 34 from Spain were received for analysis of DR, ERα and AR CALUX® analysis.

*DR CALUX®*
*Extraction of plasma samples*. One gram maternal plasma or 3 grams of cord plasma were extracted by shake-extraction using hexane:diethylether (97:3 v/v) as extraction solvent. Extracted fat was used for clean-up on an acid silica column (20% and 33% $H_2SO_4$), topped with sodium sulphate. Cleaned extracts were dissolved in DMSO (8 µl).
*DR CALUX® bioanalysis*. The DR CALUX® bioassay was performed using a rat hepatoma H4IIE cell line stably transfected with an AhR-controlled luciferase reporter gene construct. Cells were

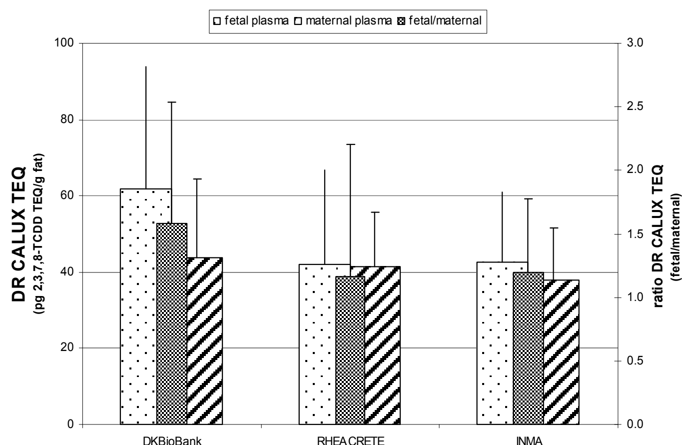


Fig.1 DR CALUX® TEQ in maternal and cord plasma and the ratio between cord plasma and maternal plasma CALUX® TEQ in Danish (DKBioBank), Greek (RHEACRETE) and Spanisch (INMA) cohorts.

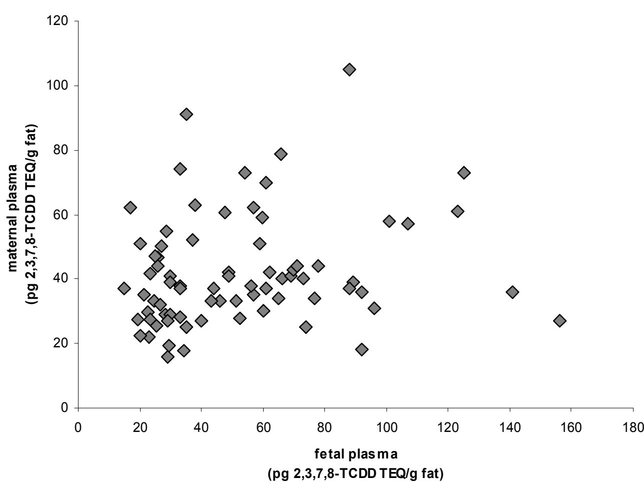


Fig.2 Relation between DR CALUX® TEQ in maternal and cord plasma (mother-child pairs).

cultured in α-MEM culture medium supplemented with 10% ($^{v}/_{v}$) FCS under standard conditions (37°C, 5% $CO_2$, 100% humidity). Following fat extraction and clean-up on an acidic silica column, samples were re-dissolved in DMSO. Cells were exposed in triplicate to cleaned extracts for 24 hours in 96-well microtiter plates. After incubation, the cells were lysed. A luciferine containing solution was added and the luciferase activity was measured using a luminometer equipped with 2 dispensers. Each 96-well microtiter plate contained a 2,3,7,8-TCDD calibration range (0 – 3 pM 2,3,7,8-TCDD per well). Total DR CALUX® TEQ in the samples analyzed was determined by interpolation from the fitted 2,3,7,8-TCDD calibration curve and corrected for procedure blank.

*ERα and AR CALUX®*

*Extraction of plasma samples*. 0.5 ml of maternal and cord plasma was used for extraction for both the *ERα and AR CALUX®* bioassay. Following addition of water, sodium phosphate buffer, $K_2CO_3/KHCO_3$ buffer and methyltertiairbutylether (MTBE), the plasma is shake-extracted (vortex) for 2 minutes. The organic MTBE layer is collected following centrifugation. MTBE is evaporated under a gentle stream of nitrogen after which the remaining extract is redisolved in 40 µl of DMSO.

*ERα CALUX® bioanalysis* The ERα and AR CALUX® bioassay was performed using a human U2-OS cell line stably transfected with an estrogen-controlled luciferase reporter gene construct together with the human ERα or an androgen-controlled luciferase reporter gene construct together with the human AR. ERα and AR CALUX® cells were cultured in DF medium supplemented with 7.5% FCS under standard conditions (37°C, 7.5% $CO_2$, 100% humidity). ERα CALUX® cells were plated in 96-well plates (8000 cells/well) with phenol red-free DF medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS) at a volume of 200 µl per well. Two days later, the medium was refreshed, and cells were incubated with the extracts to be tested (dissolved in DMSO; 0.1% DMSO) in triplicate. After 24 h the medium was removed, and cells were lysed in 30 µl Triton-lysis buffer and measured for luciferase activity using a luminometer (Lucy2; Anthos Labtec Instruments, Wals, Austria) for 0.1 min/well.

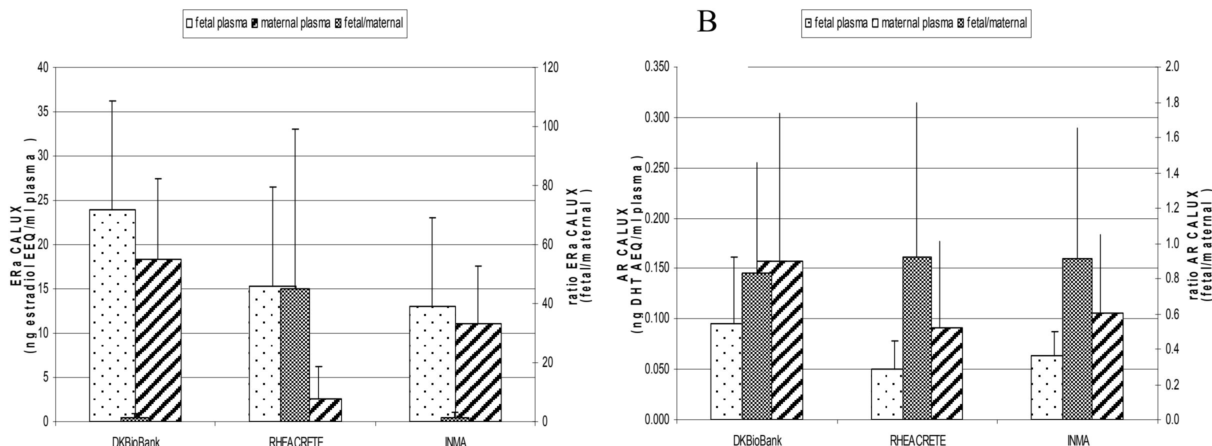


Fig.3 ERα CALUX® EEQ (A) and AR CALUX® AEQ (B) in maternal and cord plasma and the ratio between cord plasma and maternal plasma CALUX® EEQ and AEQ in Danish (DKBioBank), Greek (RHEACRETE) and Spanisch (INMA) cohorts.

**Results and Discussion**

DR CALUX® analyses were performed using 1ml of maternal plasma and 3 ml of cord plasma. Using these amounts of plasma, a detection limit of approximately 10 pg 2,3,7,8-TCDD TEQ/g fat is achievable. The low amount of maternal plasma necessary makes the bioassay suitable for screening of large biobanks with limit amounts of plasma stored. Since the fat content of cord plasma is approximately a factor of 3 lower as compared to the fat content of maternal plasma, 3 ml of cord plasma is required to obtain a detection limit of 10 pg 2,3,7,8-TCDD/g fat. In figure 1, the average DR CALUX® TEQ is given for maternal and cord plasma from the 3 countries from which plasma was received. In addition, the ratio between maternal and fetal DR CALUX® TEQ is given. The determined fetal DR CALUX® TEQ from the Danish cohort was higher as compared to the DR CALUX® TEQ from the Greek and Spanish cohorts. This difference was not observed for maternal plasma. For the later cohorts, the ratio of maternal and fetal DR CALUX® TEQ was approximately 1 indicting similar concentrations of dioxins and/or dioxin-like compounds in mother and newborn. In figure 2, the DR CALUX® TEQ of mother-child pairs are given. Although no significant relation between DR CALUX® TEQ in mother and child could be found, higher dioxin-like activities in the mother seem to relate to higher dioxin-like activity in the newborns.

For ERα and AR CALUX® analysis in human plasma, a total of 0.5 ml plasma is required. Using this amount of plasma, a limit of detection of 0.01 ng 17β-estradiol EEQ/ml plasma and 0.03 ng DHT AEQ/ml plasma was obtained. As for the dioxin analyses, slightly higher levels of estrogenic and androgenic activity in cord plasma from the Danish cohort was observed as compared to the Greek and Spanish cohorts (figure 3A and 3B). The estrogenic activity but not the androgenic activity in maternal plasma from the Greek cohort was lower as compared to the levels found in the Danish and Spanish cohort.

In the present paper, no relation between either DR, ERα and AR CALUX® activity and for example food intake, smoking etc was studied. This information is currently being evaluated by partners within the NewGeneris project. Such relations could shed a light on the difference observed between the various cohorts. Furthermore, the present results are the first results from screening additional cohorts.

**Acknowledgements**

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**References**

[1] Besselink H, Heijningen M van, Sonneveld E, Brouwer B. 2007; *Organohalogen Compounds*. 69:3006-3009 (Validation of the ERα CALUX® bioanalysis for the determination of estrogenic activity in human plasma)

[2] Brouwer, B., Sonneveld, E., Botschuijver, S., Besselink, H. and Burg van der, B. (2004). A low volume method for the analysis of dioxins and dioxin-like compounds in serum and whole blood using BDS' DR CALUX® bioassay. *Organohalogen Compounds*, 66:687-690