



## **Newsletter September 2011**

The present newsletter contains a brief introduction to the work going on at the molecular biology laboratories involved in the CLEAR project at Lund University, Sweden and ENEA, Rome, Italy. Within this part of the project, the main aim is to identify possible interactions between the measured environmental chemicals and genetic factors. This includes identification of alleles of genes, which may confer different sensitivity towards exposure to environmental chemicals and studies of the epigenetic profile of sperm cells, which may be modified by exposure to environmental chemicals changes.

## *Lund University, Molecular Reproductive Genetic Medicine and Molecular Reproductive*

### *Medicine*

The Medical Faculty of Lund University has two campuses – one in Malmö and one in Lund – both closely related to a University Hospital. The Malmö campus is represented in CLEAR by two research groups:

1. Molecular Reproductive Genetic Medicine under lead of associate professor Yvonne Lundberg Giwercman;
2. Molecular Reproductive Medicine, lead by professor Aleksander Giwercman

*Yvonne Lundberg Giwercman's* main interest is to study the impact of small genetic variations (polymorphisms) on disease risk.

In the context of CLEAR, Yvonne is responsible for the molecular part of studying gene-environment interaction in relation to the male reproductive system and the health of the children studied within the frame of CLEAR.



The aim of our research is to enhance our understanding of diseases of the male reproductive system and the importance of genetic predisposition with respect to hormonal action and metabolism of pollutants or drugs. The hypothesis is that some individuals are carriers of genetic variants that could imply that they are either more sensitive or more resistant to these compounds than the general population.

An example of this work is the androgen receptor gene, where different populations, with varying degrees of exposure to pollutants, have been analyzed regarding exposure levels as well as mutations and polymorphisms in the androgen receptor gene. Since pollutants are known to interfere with endogenous hormonal action, genetic variants have then been related to outcomes such as genital malformations and prostate cancer that are androgen dependent disorders. The actions of the pollutants on androgen receptor function are currently studied in vitro.

We believe that our research will lead to a better understanding of the causes of diseases in the reproductive system and development of new therapeutic and preventive approaches.

These studies are carried out at the Clinical Research Centre, Lund University, Malmö.

*Leon Brokken*, PhD in endocrinology, is responsible for the statistical analysis related to gene environment interactions. Furthermore, Leon is organising sampling of DNA from the CLEAR children.



It is not feasible to collect blood samples from children for DNA analysis. We have, therefore, sought for a less invasive way to collect their DNA. In cooperation with DNA Genotek Inc., we have decided to use the Oragene-DNA collection kit, which uses sponges that are especially designed to collect saliva from infants and young children. DNA can then be purified from the saliva and analysed for genetic polymorphisms.



**Collecting saliva from an Inuit boy in Greenland**

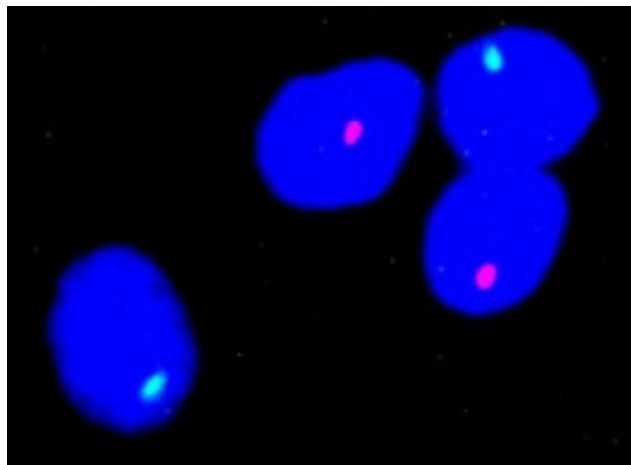
As candidate genes for studying gene-environment interaction we have selected genes that are known to interact with endocrine disruptors, such as steroid hormone receptors and steroidogenic enzymes and genes related to exposure and detoxification of toxicants. Since perfluorinated compounds are thought to interfere with development, we also included genes related to early growth. The CLEAR project offers a unique possibility to study how genetic differences between the children influences their susceptibility to environmental pollutants.

Another part of the CLEAR project performed in Malmö is to estimate Y:X chromosome ratio in sperms in relation to the exposure levels. The rationale behind this work are previous findings on skewed sex ratio among offspring of men who have been exposed to high levels of dioxin following the Seveso disaster in 1976. Also other research reports indicate that exposure to environmental toxicants may affect the ratio between male and female offspring. The mechanisms behind this phenomenon are unknown, but might be related to alterations in ratio between Y and X chromosome bearing sperms. As a part of the Inuendo project, in Swedish fishermen, we have previously found a positive association between the levels of CB153 and proportion Y sperms. Now, we will try to extend these analyses to the new compounds included in the CLEAR project.

*Linus Kvist* is a PhD student in molecular biology and the one performing analyses for Y and X chromosomes in sperm cells.



The technique I use to determine the Y:X chromosome ratio in sperm cells is called the FISH (fluorescent *in situ* hybridization) technique. It visualizes the chromosomes using differently coloured fluorescent probes, which enables us to establish a Y:X ratio by counting the differently labelled cells in a sample under the microscope.



**X- (red) and Y-chromosomes (green) in four human sperm nuclei (blue) are visualized by fluorescent *in situ* hybridization**

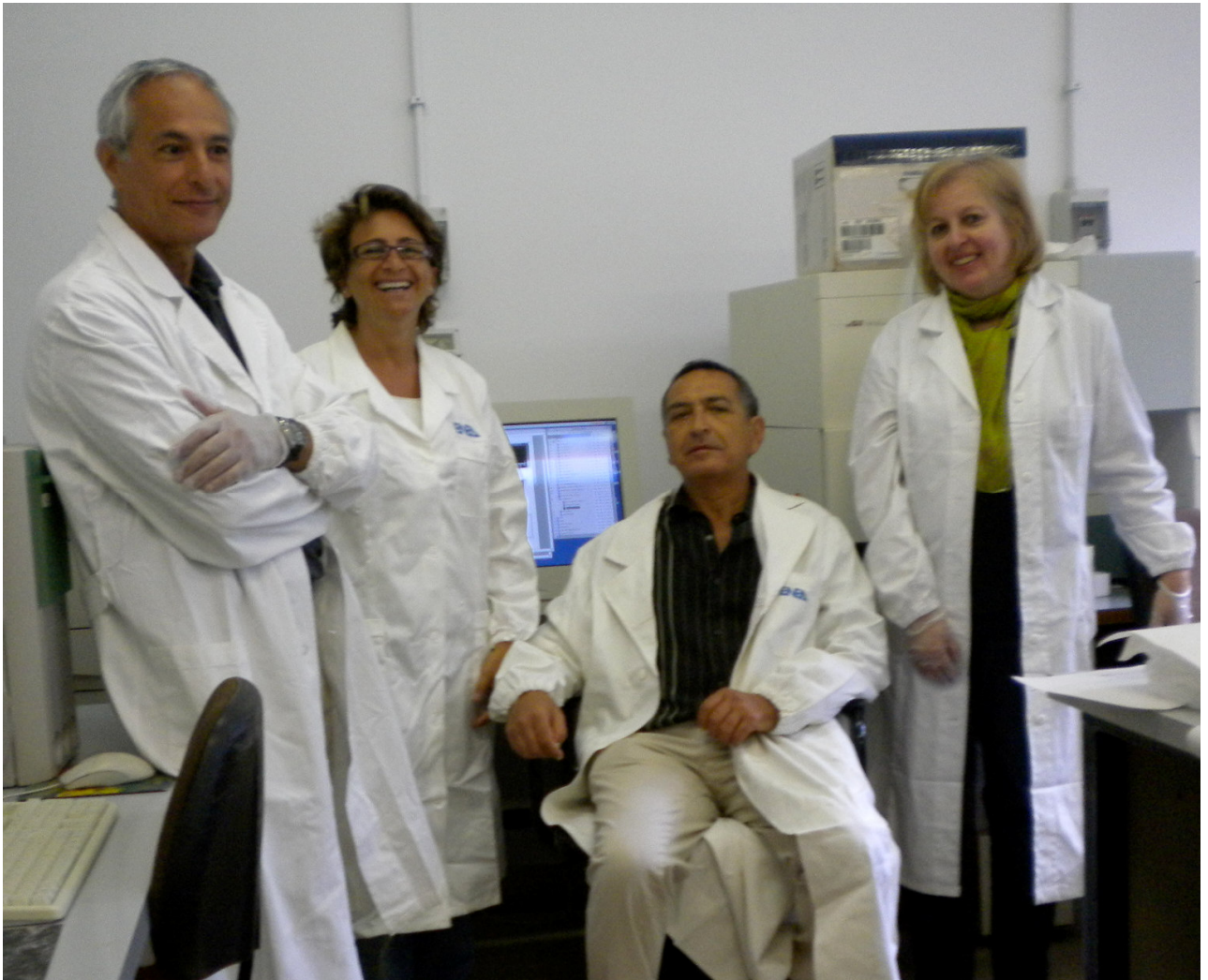
This is a fascinating technique, and although working in a dark room for long periods of time, counting red and green dots on glass slides can sometimes be tedious, it is rewarding work.

*Aleksander Giwercman* is chairman of the Reproductive Medicine Centre and clinician. His interest is clinical andrology and his input to the project is related to the clinical aspects of male reproductive function.



As a clinician it is fascinating to have a close collaboration with laboratory people. It gives a new dimension to understanding the mechanisms behind diseases. The issue of gene-environment interaction is widely discussed within other major areas of research as e.g. diabetes, cardiovascular disease and cancer. I am sure that this is also the key to understanding common conditions as infertility. CLEAR can provide important information on this issue and also on aspects related to the health of the offspring. The collaboration between Yvonne's and my research groups opens up for translational research tightly coupling epidemiological and clinical findings to the lab bench and vice versa.

*Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Technical Unit Radiation Biology and Human Health, Laboratory of Toxicology, Rome, Italy.*



*Part of the ENEA staff included in the CLEAR task force (from the left: Giorgio Leter, Claudia Consales, Marcello Spanò, Patrizia Eleuteri)*

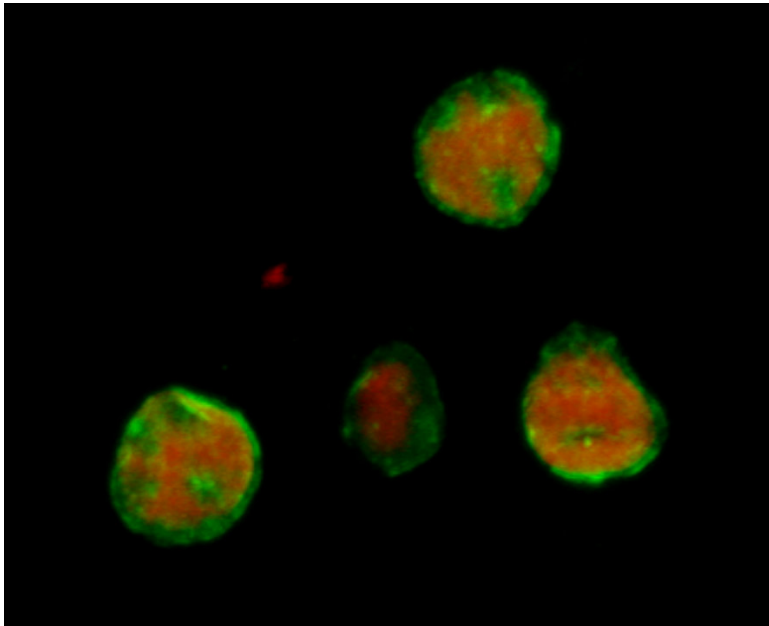
## **Epigenetics**

Epigenetics deals with heritable changes in gene expression that occur without changes in DNA sequence. Epigenetic changes encompass an array of molecular modifications to both DNA and chromatin, involving DNA methylation, histone modification, and non coding RNAs. DNA methylation is a covalent modification of DNA by addition of methyl residues to Cytosine (C) bases in DNA. In vertebrates, the CpG dinucleotide sequence is the main target of DNA methylation. It is estimated that 5-methyl C (5-mC) represent 2-5% of all genomic C. DNA methylation is involved in regulating many cellular processes, including transcriptional

gene silencing, tissue specific gene expression, chromatin structure and remodelling, X-chromosome inactivation in females, silencing of retroviral transposable elements, chromosome stability, and genomic imprinting. DNA methylation is vital during development and aberrant DNA methylation, both hypermethylation and hypomethylation, has been associated with ageing, cancer and other diseases.

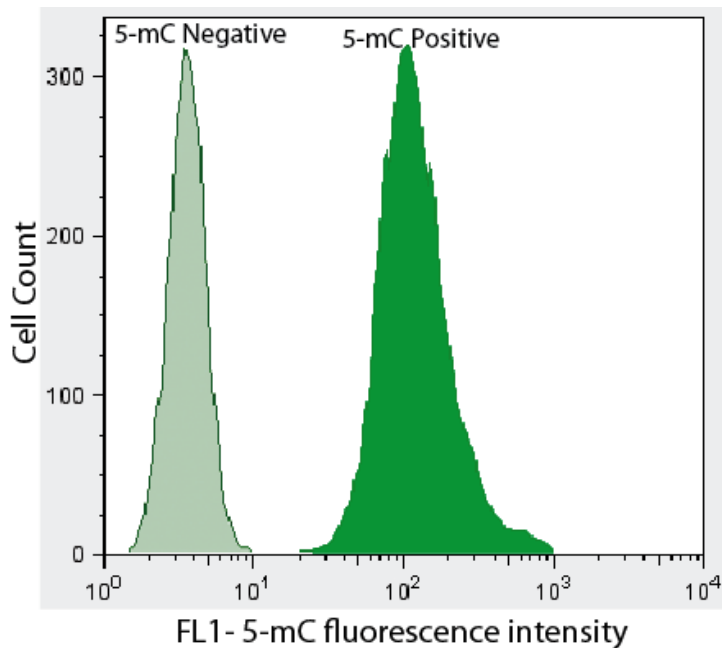
Imprinting defects have also been associated with disturbed spermatogenesis in humans. Recently, it has been reported that in-utero exposure to certain pesticides can induce epigenetic changes of methylation pattern in rodent sperm impairing their fertility. Once established, these epigenetic changes may be permanent and passed to the following generations. It is speculated that toxic not mutagenic compounds, like most of the endocrine disruptors (EDCs), can cause stable adverse phenotypic changes if they interfere with epigenetic processes. Actually, there are very recent studies reporting association between DNA global methylation level in somatic cells and exposure to a variety of environmentally relevant pollutants, Persistent Organochlorine Pollutants (POPs) included. The CLEAR project, which investigates the potential impact of global climate change on exposure to environmental contaminants and related reproductive health, will address the issue of DNA global methylation level in relation to blood levels of several POPs and EDCs on the most relevant cells for male reproduction, the sperm.

Measuring global DNA methylation levels, instead of looking at single, albeit biologically relevant loci, is potentially an important test to screen deleterious agents. There are a variety of strategies to measure DNA global methylation level. One common technical shortcoming of global methylation assays is represented by the fact that an average methylation level is defined. Thus, important changes involving increases in some regions of the genome coupled with decreases in other regions might not be detected simply looking at the average level. However, this approach has recently been used in epidemiology studies to tackle environmentally induced epigenetic changes.



*Microphotograph of human sperm nuclei stained with AlexaFluor488 Fab-IgG1 mouse anti 5-mC monoclonal antibody complex (green), counterstained with the DNA specific dye Propidium Iodide (red). Sperm nuclei appear swelled and the green fluorescence is quite specific and localized inside the nuclei.*

One of the quantitative methods to measure DNA global methylation levels uses fluorescently-labeled specific monoclonal antibodies to 5-mC. Within the CLEAR framework, the first application of this technology to field studies has been undertaken. We set up a sound flow cytometry (FCM) immunofluorescence method to measure the level of global DNA methylation on a sperm-by-sperm basis. During the first part of the project, we optimised a FCM technique which was tested on a variety of human samples. Now that the details have been worked out, according to the workplan, we are ready to measure the sperm DNA global methylation level in at least 200 human randomly selected semen samples from the INUENDO biobank. The first shipment took place during this summer 2011. The results from the FCM analysis will be transferred to the CLEAR database and will be statistically analysed and associated with the levels of pollutants' internal contamination.



*Flow cytometry 5-mC fluorescence intensity distribution histogram of a human semen sample stained with Alexa Fluor488-Fab-antibody complex (dark green). The corresponding negative control distribution is represented as light green. The mean, median and standard deviation of the fluorescence intensity distribution will be calculated as the main parameters describing the DNA global methylation level.*

At the ENEA laboratories, the level of sperm DNA integrity had been measured in the above samples by Sperm Chromatin Structure Assay (SCSA) within the framework of the previous INUENDO project. In that occasion, results suggested that human dietary PCB (but not DDT) exposure could have a weak negative impact on sperm chromatin integrity of European (but not Inuit) adult males and additional issues remained to be tackled and elucidated (Spanò et al., Hum Reprod 20: 3488–3499, 2005). Interestingly, another INUENDO partner (University of Modena and Reggio Emilia, Italy), by using an independent approach to address the issue of sperm DNA integrity, reached the very same biological conclusions (Stronati et al., Reproduction 132: 949–958, 2006). Noteworthy, within this CLEAR project, the SCSA results, which during the INUENDO project have been compared in some 700 individuals with blood concentrations of PCB 153 and DDE, will also be associated with a long list of new contaminants [hexachlorobenzene, polybrominated diphenylethers perfluorinated surfactants, phthalates, and metals (Pb and Hg)], whose blood levels are being measured by our Swedish partner in Lund.

As in the past, these analyses applied to our large biobank promise to hold a great statistical power to expand the existing knowledge on the environmental (epi)genetic effects on human sperm and, more at large, on human contaminant exposure in the Arctic and selected European populations.