

## 4.2 Human milk and maternal blood as a biological indicators

### 4.2.1 Introduction

Human milk as well as blood have been used as markers of exposure of humans to certain persistent organic pollutants (POPs). Both these human sample media can show comparable temporal trends in a particular population because they integrate environmental exposure as well as dietary exposure related to different consumption habits. Furthermore, they provide relevant information on POPs transfer to infants and potential health effects.

#### Human milk

Human milk has been used on a global basis for monitoring of human body burdens of POPs for several decades. The purpose of these programmes has been to assess the body burden of contaminants in newborns, using breast milk as a tool without performing invasive sampling techniques on the newborns. An important concept in human milk studies is also that they reflect the integration of all contamination at a high trophic level. Thus, human milk samples reflect the intake in different regions: the extent of contamination and different consumption habits. Furthermore, such studies are also used as general biological monitoring tools. Human milk monitoring programmes have hence been designed for assessing levels of environmental pollution by lipophilic substances in different areas within and between countries. Trends in levels and effectiveness of regulations have been evaluated by comparing these assessments with earlier investigations.

Organized monitoring programmes have been implemented by WHO (human milk). Few countries have systematic human milk monitoring programmes that have tested considerable numbers of women over time using consistent sampling methods. WHO organised three rounds of exposure studies in 1987-1988, 1992-1993 and 2000-2001, on levels of specific POPs in human milk (WHO 1989, 1996, van Leeuwen and Malisch 2002, Malisch and van Leeuwen 2003). The main objectives of these studies were: 1) to produce more reliable and comparable data on concentrations of PCBs, PCDDs and PCDFs in human milk for further improvement of health risk assessment in infants, 2) to provide an overview of exposure levels in various countries and geographical areas, 3) to determine trends in exposure levels. Nineteen European countries participated in the second round, in which concentrations of PCBs, PCDDs/PCDFs were determined in milk samples collected in a total of 47 areas. The third round of the WHO-coordinated exposure study was initiated in 2000. In order to collect data in more countries, also beyond the European region, the study was organised in collaboration with the International Programme on Chemical Safety (IPCS) and WHO Global Environmental Monitoring System/Food Contamination Monitoring and Assessment (GEMS/Food). In this last round of exposure studies 18 countries participated and milk samples from 62 different areas were analysed. Historical trend data exist for PCDDs/PCDFs and PCBs in some of these countries (e.g. Becher *et al.* 2002). For some countries a pilot study of concentrations of other POPs than PCBs and PCDDs/PCDFs was included in the latter study. In these studies pooled human milk samples were used. A fourth round of exposure studies is being planned by WHO European Centre for Environment and Health (WHO-ECEH), IPCS and GEMS/Food. The main objective of the fourth round will be to produce reliable and comparable data on levels of POPs in human milk which will serve as a basis to determine time trends in exposure to POPs.

#### Human blood

The Arctic Monitoring and Assessment Programme (AMAP) has organized comprehensive human maternal blood monitoring with standardized protocols for specimen collection and analysis in the Arctic since the early 1990s. Maternal blood, supplemented with some human milk data have been used in assessing POPs and human health (AMAP 1998, 2004). Through this programme an international QA/QC program for blood analyses has been developed, with systematic ring tests of

reference standards, allowing many laboratories in the Arctic to produce reliable data on human maternal plasma as well as cord blood (CTQ, Quebec, Canada).

### **Some general methodological considerations with regard to the choice of sample medium**

Historically GEMS/Food and more recently UNEPs workshops have recognized human milk as a preferred matrix for POPs based on the fact that human milk is non-invasive and can be readily obtained from lactating women whose infants are considered a vulnerable population group. There are however three main tissues where the POP levels are normally measured in order to assess the exposure to the child; mothers blood, cord blood and mothers milk. Several studies have shown correlation between the levels of contaminants in these compartments (Jarrell et al. 2005, Muckle et al. 2001). Recently a comprehensive study of the relationship between breast milk, maternal blood and cord blood from a group of mother and child pairs from Chukotka, Russia, has been completed (Anda et al, submitted). Some of the results of this paper are summarized here:

1. Levels of POPs in maternal blood correlated well with the corresponding levels in human milk. Thus milk and maternal blood can be used for biomonitoring and monitoring of POPs on an equal basis.
2. Maternal blood contaminants provide the exposure index for fetal circulation; maternal blood contaminant levels are most likely also the general driving force for human milk concentrations of POPs and thus also of the exposure of the baby. Maternal blood reflects both recent and passed exposure and shows more rapid temporal response while milk is considered more a storage compartment. Mothers milk is the most sensitive exposure medium for the baby in terms of diversity and levels of contaminants.
3. To extract information about the contaminant situation in a population, some general epidemiological principles must be applied. Individual samples and individual analytical data must be obtained. Pooled samples might be considered for special, expensive analytes, such as the dioxin group, but that will provide limited information for statistical assessment of the material.

Sample size is crucial for the statistical power of a study. The analytical procedures are becoming more sensitive and less expensive. Many programmes (e.g. AMAP and their QA/QC program) are assisting to develop the laboratory capacity in the new independent states of Eastern Europe, as well as in many developing countries, such as Vietnam, South Africa, and Brazil. There is a continuing need for education of personnel, as well as implementation of new technology. This is a unique opportunity to increase international comparability of contaminant data by expansion of existing international GA/QC programmes in those new laboratories willing to join an educational programme for their staff and regular ring tests.

With regard to sample size, power calculations must be applied carefully (see the chapter on statistical considerations}. Based on known sources of contaminants, both long transport and local point sources, we should apply population stratification criteria for sampling as a way to achieve better comparability and reduce variability. To this end consideration should be given to certain more critically exposed populations (not occupational exposure) that could be the subject of a more detailed study including rural, urban and or fish eating populations. Indigenous populations of the Arctic have been shown to have different contaminant problems; e.g., Greenlandic Inuites have especially high Hg-levels, while the Chuchi of the Russian Far East have relatively higher PCB-levels; South African populations rely more on local sources, possibly except coastal, fish eating populations.

Selection of populations likely to be exposed, globally, regionally, and locally, will provide an opportunity for trend studies, as those populations are also most vulnerable to changes in exposure.

## Overall comments with regard to choice of sampling medium, study group and number of samples

Human milk and maternal blood are both equally good sample media for assessing POPs exposure in humans<sup>1</sup>. Furthermore, both these media can be used to demonstrate possible temporal trends in levels, and thus show effectiveness of regulations of the use of POPs. It should be encouraged to sample both milk and blood of the same mother.

1. Human milk sampling is non-invasive and milk can generally be obtained from lactating mothers. In certain populations it may however be difficult to obtain human milk samples in the required time period, 2 to 4 weeks post partum. For some mothers human milk sampling may also be considered stressful.
2. Blood sampling is invasive but sampling of mothers prior to giving birth may readily be achieved. However, blood sampling may not be acceptable in certain cultures.
3. All biological samples of human origin, including blood and milk, should be regarded as infected. Necessary precaution procedures should be applied in both sampling and handling of all samples not only in situations where you may expect a problem, e.g. HIV-positive serology and hepatitis.
4. The limit of detection for POPs will in general be lower in milk than in blood. The reason for this is partly the difference in lipids between the media and the fact that larger volumes of milk as compared to blood can normally be obtained. When the limit of detection is approached the analytical precision will decrease.
5. An important consideration in the choice of human milk and maternal blood as biological indicators is that we will only obtain information from a specific part of the population both with regard to gender and age. As for future trend studies a careful evaluation should be done to explore alternative representative groups in a population, e.g. men (specified age groups), youth groups of both gender, school children or infants.
6. A population study must be based on sampling and analyses of individual samples; human milk or maternal blood. Pooled samples might be considered for certain contaminants, such as the dioxins which are expensive to analyze and need larger sample volumes.
7. In order to reduce sample variance and facilitate comparability a stratified sample design could be adopted. This should be based on demographic information collected in specific questionnaires i.e. age, residence, occupational history, smoking habits, current and past diet etc.
8. Selection of study groups should be based on known likely exposure pattern, global or local. The groups with known high exposure levels are more sensitive to changes in the environment and will provide better indications in trend analyses. Even in countries with very limited background information one might be able to select population groups of interest, such as rural versus urban; fish eating populations versus rural agricultural populations with high exposures to pesticides; populations living in areas with re-introduction of DDT for malaria prophylaxis etc.
9. Sample size will depend on the circumstances, and to estimate the number of samples needed a number of factors have to be considered to achieve representative samples, see the chapter on statistical considerations. In the Arctic groups of 50 mother/child pairs have provided good documentation of the contaminant situation in the small communities. However, new technologies and new, certified laboratories will provide the opportunity to begin epidemiological studies with individual results on a larger scale.

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<sup>1</sup> Maternal blood is the best compartment for measuring of toxic metals and elements, such as Hg, Cd and Pb. As for future screening of populations, with individual blood sampling, the metals will have increasing importance when reproductive health aspects and fetal and child developmental effects are to be addressed. The question of including blood for metal analyses should be addressed in the evaluation of the monitoring programme.

10. The choice of milk or blood is not crucial for the assessment of contaminant levels. The choice should depend very much on the practical implementation regionally or locally. Two examples:

- a. In the Arctic many indigenous women deliver their babies and go home to the tundra before they have started their milk production. To collect colostrum provides a very different medium than the fully developed breast milk 2-3 weeks after delivery. It is not possible to trace the women at the right time for breast milk collection. A blood sample will solve that problem.
- b. In certain areas of Africa sampling of maternal blood might be very problematic. In those cases breast milk is the best matrix. Comparable information out of both media can then be obtained.

11. Trained personnel is just as crucial at the sampling stage as in the laboratory. Standardized protocols, equipment and education of field personnel as well as laboratory personnel must be implemented.

## 4.2.2 Objective of human milk monitoring within the GMP

The human milk monitoring within GMP will mainly aim at identifying temporal and as appropriate, spatial trends in levels of POPs exposure in humans.

It will also aim at regional capacity building in developing countries focused to ensure a capability to detect regional trends of such chemicals in human milk.

## 4.2.3 Sampling and sample preparation methodology

### 4.2.3.1 Sample matrices

#### Human milk

The GMP programme will use human milk as one matrix for biological monitoring. (See the proceedings of the GMP workshop for more information on the recommendation for the selection of human milk as matrix suited for temporal trend studies in GMP.)

As mentioned before, human milk is an attractive medium because it is non-invasive and relatively large volumes of samples can be easily collected in a more or less standardized manner. A disadvantage of using human milk is that of biological sample as explained above. Another disadvantage is of course that only one gender constituting a limited age group is monitored. On the other hand, as the main aim of the GMP is to determine a temporal trend in exposure to POPs, the restriction of concentrating only on a small, but well defined part of the population, can be considered to be an advantage. However, in certain areas there are social or ethical difficulties to overcome in the collection of human milk samples.

The GMP will mainly rely on data from pooled human milk samples. The analyses of pooled human milk samples represent a cost effective method for comparing POPs levels between and within countries and to elucidate time trends. A disadvantage with pooling is of course that information on individual variation is missed. In the Fourth WHO protocol (WHO, 2006), provision is made for individual samples to be analysed for pesticide POPs, in addition to the one (or perhaps two) pooled samples that will be analysed for all analytes.

Additional studies can, of course, be implemented within countries to answer questions that are country specific.

#### Maternal blood

Maternal blood is used by AMAP, as the prime matrix to determine human exposure (AMAP, 2002). Although it is an invasive procedure, in some cases it may be the matrix of choice, based on local infrastructure, customs and existing activities. **???? More background required**

### 4.2.3.2 Experimental design

#### Human milk

Under WHO, a protocol has been developed for sampling and sample preparation methodology for exposure studies of Persistent Organic Pollutants (Malish & Moy, 2006; WHO, 2006), and is based on the afore-mentioned three previous rounds of WHO coordinated studies (1987-1988, 1992-1993 and 2000-2001). This protocol will form the basis for the human milk component of the GMP.

The State Institute for Chemical and Veterinary Analysis of Food [country] has met all the criteria for analyses of PCDD, PCDF, dioxin-like PCB, marker PCB and fat in human milk and was selected as a reference laboratory for the third round of the WHO exposure study (WHO 2000, Malisch and van Leeuwen 2002), and will continue to do so for the fourth round as well (WHO, 2006).

The revised WHO protocol gives guidance on the number of samples/sampling locations and selection of donors. It also contains information on questionnaires, transport, storage, sample preparation and analysis. It contains annexes with questionnaires, summary information for a sample, an informed consent template, guidance for mothers, and an estimated timeline and budget. The WHO Research Ethics Review Committee has endorsed the project, but each country will also have to follow their own procedures. It should be noted that a country might have to adjust the volume to be collected per mother, if they do not intend to analyse individual samples.

#### Maternal blood

AMAP has developed a protocol..REF...

#### *Number of samples/sampling location*

##### Human milk

The Fourth WHO-Coordinated Survey (Malish & Moy, 2006; WHO, 2006) requires milk samples from 50 individuals. However, current experience shows that some countries may not be able to recruit that many, and the numbers might be adjusted downwards. However, the final volume of the pooled sample should still be 500 ml.

The Fourth WHO-Coordinated Survey (WHO, 2006) also makes provision for a country to stratify the participants such that it represents the presumed exposure profile of each country. Elements that need to be considered here typically include diet, agriculture, occupational exposure, rural and urban residence, and proximity to potential POPs releasing industries or activities (such as waste sites). This stratification will need to be the same for following rounds, so that changes/trends can be followed. However, since the exposure profiles in most developing countries are not well characterised, assumptions need to be made, but these would have to be documented and form part of the information package. The Fourth WHO-Coordinated Survey (WHO, 2006) also makes provision for countries with adequate resources to submit two pooled samples of 25 sub-samples each, with a final volume of 500ml for each pooled sample.

Although the Fourth WHO-Coordinated Survey (WHO, 2006) addresses countries, it may be feasible that consideration for stratification, and even sample collection, could be done on a regional level. However, the effort for this round should be focused on the participation of as many countries and regions as possible, to enable a good baseline to be set.

##### Maternal blood

Samples from 50 individuals are needed. Some countries may not be able to recruit that many, and the numbers might be adjusted downwards. However, the final volume of the pooled sample should still be 500 ml.

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POPs releasing industries or activities (such as waste sites). This stratification will need to be the same for following rounds, so that changes/trends can be followed. However, since the exposure profiles in most developing countries are not well characterised, assumptions need to be made, but these would have to be documented and form part of the information package. Countries with adequate resources can also submit two pooled samples of 25 sub-samples each, with a final volume of 500 ml for each pooled sample, as for the milk study.

Again as for the milk sampling, it may be feasible that considerations for stratification, and even sample collection, could be done on a regional level. However, the effort for this round should be focused on the participation of as many countries and regions as possible, to enable a good baseline to be set.

### ***Selection criteria for mothers***

#### **Human milk**

There are many factors explaining the variation in concentrations of POPs found in human milk and it is important to define selection criteria for the mothers to be included in the study (Harris *et al.* 2001; Loveday *et al.*, 2002). The Fourth WHO-Coordinated Survey (WHO, 2006) lists the criteria for selection of mothers, and this should be followed as closely as possible.

#### **Maternal milk**

Criteria for selection same as for milk?? If so, the adapt above #

### ***Questionnaire and informed consent***

#### **Human milk**

It is strongly recommended that the questionnaires developed for the Fourth round (WHO, 2006) be followed, but additional questions might be added if exposure profiles need to be better characterised. The questionnaires need to be translated into local languages, or **and?** administered by competent health or science professionals. This is especially the case in developing countries, where some questions might need to be aligned with local knowledge and customs.

The first part of the questionnaire is intended to recruit mothers by mail, at pregnancy. These mothers will then be selected and notified. However, in many developing countries, means of communication might not support such an approach, and selection and recruitment may therefore have to be done at clinics or other centres, as appropriate. The protocol also allows mothers to collect samples themselves and store them in a fridge, but this might also not be possible in areas with no electricity or means of cooling. Active collection by local teams might then be the only option.

The informed consent template must also be considered by each country or region, and aligned according to local practice, custom and experience.

#### **Maternal blood**

[The same questionnaire and approach as for milk may be applied. However, the informed consent form needs to be updated, to take the invasive nature of the procedure be taken into account.]

The informed consent template need also be considered by each country or region, and aligned according to local practice, custom and experience.

### ***Sampling and sample handling***

#### **Human milk**

Since there are many factors that may affect the composition of human milk (Harris *et al.*, 2001; Harris *et al.*, 2002; Loveday *et al.*, 2002), note should be taken of the guidance in the Fourth WHO-Coordinated Survey (WHO, 2006).

Sample handling is particularly important for obtaining homogeneous samples of human milk for analyses and to ensure sample integrity (Loveday *et al.*, 2002). Therefore, the guidelines on

handling of samples as laid down in the protocol should be strictly followed. Qualified personnel must be available to undertake the sampling and training may be required

During sampling of human milk from one mother the sample may be stored at 4 °C for a maximum of 72 hours. In countries where temperature control is not possible, the collection of milk samples should be done in bottles to which a tablet of potassium dichromate has been added. This method of preservation of the milk sample was applied successfully by some countries at the third round of WHO-coordinated exposure studies (van Leeuwen and Malisch, 2002; Schechter *et al.*, 2003).

When pooling samples from a number of mothers each sample must be heated to 38 °C and inverted gently several times to mix the cream layer. Thereafter a predetermined aliquot from each sample is pooled. The pooled sample is treated similarly and aliquots are divided into separate vials to minimize freeze-thaw cycle during analyses. The samples can be stored at -70 °C for an infinite length of time. When the sample is ready to analyze, it must be thawed and warmed to 38 °C. It can then be mixed by gentle inversion and the entire sample extracted. The container should be rinsed with solvents. Procedures for sample handling during storage, transport to analytical laboratory and handling by analyst etc must be developed to take into account both cross-contamination by chemicals and transfer of disease between people.

### **Maternal blood**

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Conditions of sample handling after sampling: the current protocol states that serum samples can be kept for 5 days at room temperature. In the tropics the temperature is quite high, and it should not be stored for longer than 1 day, and be kept out of sunlight.

Pooling procedure same as for milk – antibody response can cause precipitation, and therefore cause phase separation?.

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### **Ethics**

#### **Human milk**

The Fourth WHO-Coordinated Survey (Malish & Moy, 2006; WHO, 2006) has been endorsed by the WHO Research Ethics Review Committee, but each country will also have to follow their own procedures. Any variation from the WHO protocol, based on local ethical considerations, should be noted, and this should be included in the information package that accompanies the samples. Evidence of such approval should accompany the information package.

#### **Maternal blood**

Each country will have to ensure that their protocols be approved by the relevant ethical committees. Evidence of such approval should accompany the information package.

### **HIV/AIDS**

#### **Human milk**

The Fourth WHO-Coordinated Survey (WHO, 2006) excludes mothers that are HIV/Aids positive. In many countries though, discrimination based on infective status is not allowed, and often the status would not be known. In addition, ethical consideration in some cases prevents the HIV status from being determined. The current questionnaire also does not refer to HIV status. An unknown proportion of women in many developing countries are HIV positive, but they are also part of the breastfeeding community, and their exclusion might therefore not be justifiable. Exclusions should, however, be considered when the mother is unwell, and that may therefore include conditions such as clinical hepatitis, malaria, AIDS and others, as exclusion for such a region can be justified on a scientific basis.

Although the infectivity of human milk that contains HIV may be low when ingested by babies (Newell, 1998; Iliff *et al.*, 2005), human milk remains infectious. Sample collection should take this into account and samples from areas where HIV is prevalent should be handled and labeled as such, up to and including extraction. The extracts may be considered non-infective, but any waste materials should also be considered as a bio-hazard, and must be treated as such.

### **Maternal blood**

In many countries, discrimination based on HIV infective status is not allowed, and often the status would not be known. In addition, ethical consideration in some cases prevents the HIV status from being determined. Exclusions should, however, be considered when the mother is sick, and this may therefore include conditions such as clinical hepatitis, malaria, AIDS and others, as exclusion on this basis can be justified on a scientific basis.

Since blood collection is an invasive procedure using needles, existing and well established preventative procedures should be in place to preclude any accidental infections, even by cutaneous contact with infected blood (Radecki, Abbot & Eloi, 2000). Samples from areas where HIV is prevalent should be handled and labeled as such, up to and including extraction. The extracts may be considered non-infective, but any waste materials should also be considered as a bio-hazard, and must be treated as such.

### **4.2.3.3 Transporting of samples**

Shipping of milk and blood samples to the selected analytical laboratories should be done in accordance with instructions given by the responsible receiving party. Given the general prevalence of HIV and other infective diseases such as hepatitis, all human milk and blood samples should be labeled and handled as a bio-hazard, as a precautionary procedure.

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