

**Applicability of non-invasively collected matrices for human
biomonitoring**

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Abstract

With its inclusion under Action 3 in the Environment and Health Action Plan 2004-2010 of the European Commission, human biomonitoring is currently receiving an increasing amount of attention from the scientific community as a tool to better quantify human exposure to, and health effects of, environmental stressors. Despite the policy support, however, there are still several issues that restrict the routine application of human biomonitoring data in risk assessment, one of the most restricting issues being the need to routinely collect human samples for large-scale surveys. Particularly for small children and babies, the collection of invasive samples suffers from ethical and practical limitations which hamper the implementation of human biomonitoring as an instrument for risk assessment and management.

Unfortunately, these are the very populations most susceptible to the influence of environmental stressors, and for whom invasive sampling should be minimised. Children, pregnant women, elderly, or chronically-ill people are among those that would benefit the most from non-invasive, repeated or routine sampling. Because of these concerns, it can be argued that the use of non-invasively collected matrices for human biomonitoring should be promoted as an ethically appropriate, cost-efficient and toxicologically relevant alternative for many biomarkers that are currently determined in invasive matrices. The current paper provides a review of the most applicable non-invasively collected matrices that are currently utilised in human biomonitoring. For several non-invasively collected matrices, an overview of existing biomarkers of exposure and effect is presented, which are compared to biomarkers in other matrices. The main aim of the publication is to provide readers with an insight into the current state-of-the-art on the use of non-invasively collected matrices for human biomonitoring, and to illustrate how they could be further developed as matrices in environment and health research.

Background

The recent adoption of human biomonitoring (HBM) as Action 3 in the Environment and Health Action Plan 2004-2010 of the European Commission [1, 2] has motivated the implementation and application of HBM in European environment and health research. Projects within the Sixth Framework Programme of DG Research, such as the ESBIO project (Expert team to Support biomonitoring in Europe [3]) or the INTARESE project (Integrated Assessment of Health Risk of Environmental Stressors in Europe [4]) have further improved the visibility of human biomonitoring as a relevant instrument in environment and health research and integrated risk and health impact assessment, and have stressed the need for a harmonized European approach to HBM. The current paper reflects the work that was done in the context of the INTARESE project, a Sixth Framework Project that brings together a broad array of scientists in the areas of epidemiology, environmental sciences and biosciences to collaborate on the development and application of integrated environmental health impact assessment. This is defined as a methodology for assessing health-related problems deriving from the environment, and health-related impacts of policies that affect the environment, in ways that take into account the complexities, interdependencies and uncertainties of the real world.

Within the context of the INTARESE project, we perceived an overall lack of knowledge on the general methodology and potential application of HBM data in integrated health impact assessment. Therefore, a literature review was undertaken on past and current developments, potential and applicability of non-invasively collected matrices for HBM, and its relationship with integrated risk or health impact assessment.

While policy support for HBM and its applications in environment and health research is strong, there are still many issues that currently restrict the routine application of HBM data in integrated risk or health impact assessment. One of the most important issues is the

obvious need to collect human samples, often invasively. This suffers from ethical and practical constraints, particularly for small children and babies, from whom blood samples typically need to be collected. The European Commission's SCALE Initiative (Science, Children, Awareness, EU Legislation and Continuous Evaluation) has, however, specifically identified children as a main target population, so including this subpopulation in any HBM project is a priority [5]. Likewise, non-invasively sampling is preferable in susceptible groups as well, such as pregnant women, elderly, or chronically-ill people.

Additionally, repeated or even routine biomonitoring may be desirable for the efficiency evaluation of risk management options and efficacy of environment and health policies. In the case of chromium biomonitoring for occupational exposure for example, an expert panel suggested that a second, or if necessary, third spot (or 24-hour) urine sample was needed before it could be concluded that a person may be routinely overexposed [6]. For short-lived chemicals such as volatile organic compounds or agricultural pesticides, average exposures may not reflect peak exposures arising through infrequent exposure episodes. Repeated sampling of high-exposure subjects provides more insight into the true nature of these episodes and of their toxicological consequences [7]. Increased HBM efforts may also prove useful to identify areas of high exposure and delineate so-called hot-spots. Additional sampling may provide further information on the specific cause of exposure, and increasing the array of biomarkers measured may further clarify the link between exposure, dose and response [8, 9].

Finally, because non-invasively collected matrices need less specialized personnel for sampling, costs associated with large sampling designs may be significantly reduced. While blood sampling generally requires the contribution of medical doctors and trained nurses, for example, non-invasively collected matrices often do not require this level of training. Even more, Rockett et al [10] investigated the applicability of home-based collection of

non-invasive bio-specimens as an alternative sampling option for the United States' National Children's Study. While they concluded that there were a number of caveats that need to be taken into account in the study protocol, home-based collection of bio-specimens is certainly a viable option, and offers the additional advantage that participant rates would be higher as it would be less troublesome for participants to donate samples.

Because of these advantages, there is a strong case for non-invasively collected matrices for human biomonitoring to be promoted as an ethically appropriate, cost-efficient and toxicologically relevant alternative for many of the biomarkers that are currently determined in invasive matrices. This paper reviews the most applicable non-invasively collected matrices that are currently utilised in human biomonitoring. For all matrices discussed, existing biomarkers of exposure and effect are presented, and their relationship with other biomarkers, both invasive and non-invasive, is discussed. The main aim of the publication is to provide readers with an overview of the available information on the use of non-invasively collected matrices for human biomonitoring, in order to highlight their potential as routine matrices in environment and health research.

Urine

Relevance as a non-invasive biomarker

Urine is probably the most frequently used matrix in humans to quantify the degree of environmental or occupational exposure to environmental pollutants, especially for substances with short biological half-lives [11]. The collection and analysis of urine samples carries no associated risk, and large volumes of up to 800ml at once can be gathered per individual [12]. Whilst spot or grab (untimed) samples can easily be collected, 24-hr urine voids can be cumbersome to collect, often resulting in improper or incomplete sampling [11]. Therefore, spot collection of samples is most frequently used in biomonitoring

programmes, especially for surveys where large numbers of samples need to be gathered. The major disadvantage of using spot samples is the variability in the volume of urine and the fact that the concentration of many analytes in urine is related to the extent of diuresis. Hence, there is a need to standardize the results of biomarkers measured in spot urine samples. Urinary creatinine concentrations, gravity, and osmolality are methods that are commonly used to account for these variations in urinary analyte concentrations, and to determine whether a spot urine sample is valid for assessing chemical exposure:

- Frequently, urinary analyte concentrations are standardised by expression per gram of creatinine. This method is the standard method according to Bradman and Whyatt [13]. Urinary creatinine levels may vary by age, sex, ethnicity or body mass index and may not be appropriate for pregnant women or children [11]. These authors also suggested that in order to improve the interpretability of urinary biomarkers, both creatinine adjusted and unadjusted concentrations should be reported. WHO [14] has developed guidelines to determine whether spot urinary samples are valid, based on their creatinine concentration, with samples with creatinine concentrations < 30 mg/dL or > 300 mg/dL being either regarded as too diluted or too concentrated. However, these guidelines have been questioned recently based on detailed assessment of the role of age, gender and ethnicity [11];
- Another commonly used method to standardise biomarker measurements in urine is to take account of the gravity or relative density of urine: analyte concentrations are standardised based on the ratio between the density of the urine under investigation and average urine density [12].

Biomarkers of exposure

Urine has been used as a matrix for a wide variety of both organic and inorganic compounds for both occupational and environmental exposure. The presence of reference materials and

the identification of adequate techniques for the determination of organic xenobiotics and their metabolites in urinary samples have been discussed extensively (see [11], [15], [16] and references therein for more information). Urine is widely used in the occupational setting but also more recently to study impact of environmental contamination. Metals and pesticides with short half lives in the body and compounds that are rapidly excreted have been frequently measured in urinary samples of different age groups of the general population. However due to their short half life, the presence of these compounds or their metabolites in urine often only reflects recent exposure. To obtain information on day-to-day variability in exposure, repeated sampling is needed. Table 1 provides an overview of some biomarkers of exposure measurable in urine. Due to the specific characteristics of sampling, urine is particularly well-suited for repeated sampling of study participants, on a weekly or daily basis, and even at different times during the day. This advocates the use of urine as matrix for substances with relatively short half-lives, for which multiple samples need to be taken in a short period to adequately describe kinetics. Recently, monitoring of phthalate diesters and their metabolites using urine as a non-invasive matrix has received a lot of attention. These compounds are emerging as ubiquitous compounds that are found throughout the general population [17], and recent human biomonitoring data have shown that the tolerable intake of children is exceeded to a considerable degree [18], and increasing the number of repeated urine samples was found to be essential to adequately describe exposure and reduce misclassification [19]

Nevertheless, use of urine as a biomonitoring matrix is complicated by the circumstance that urinary biomarkers primarily focus on metabolites of substances, and it is generally not possible to distinguish between the uptake of parent compounds and environmental metabolites. For example, chlorpyrifos is broken down in the body into a number of metabolites which can be detected in urine. However, these same metabolites also occur as

natural products of environmental chlorpyrifos degradation, so it is not possible to distinguish exposure to chlorpyrifos from exposure to its environmental degradates [20].

Biomarkers of effect

Urine has a longstanding and well-documented history as a matrix in routine clinical health assessment, where basic urinary analysis includes specific gravity, colour, transparency, pH, protein, glucose, ketones and bile pigments [15]. Alpha and beta(2)-microglobulin excretion and retinol-binding proteins in urine have frequently been described as a sensitive biomarker of renal disfunctioning due to environmental exposure to e.g. cadmium and other contaminants [21]. Additionally, urine has been used as a non-invasive matrix for the presence of base DNA adducts as biomarkers for carcinogenesis- for example in smokers [22] – while urinary 8-hydroxy-deoxyguanosine (8-OHdG) has been used as a biomarker of the DNA repair response to oxidative stress and DNA damaging compounds [23].

Recently, there has been increased research interest in the metabolic profiling of biological fluids, for which urine can easily be used. This relatively novel technique, which is generally referred to as metabonomics or metabolomics, encompasses the systematic profiling of metabolite levels and their systematic and temporal changes through effects from diet, lifestyle, environment and genetics, using analytical techniques that are based on NMR spectroscopy and mass spectrometry [24]. Although metabonomics currently is not yet sufficiently developed to be used in large-scale biomonitoring studies, it remains a promising tool in the future for the non-invasive screening of reactive metabolites that are involved in many toxic processes [25].

Correlation with other (non-invasive) biomarkers

While urinary biomarkers of effect are well-documented in literature, most attention on the use of urine as a matrix for biomonitoring still is directed to biomarkers of exposure. Typical concentrations of some metals and organic compounds in urine are presented in

Tables 2 and 3. Compared to other bodily fluids such as cord blood and exhaled breath condensate, urine shows relatively higher levels of cadmium and cotinine, while concentrations for arsenic and total mercury are lower than those generally observed in cord blood.

Several research projects have found excellent correlations between the presence of metabolites in urine and related health effects: for example the correlation between hippuric acid (biomarker for toluene) or 1-OH pyrene (biomarker for exposure to PAHs) and DNA damage [26, 27]. For phthalate metabolites, urinary concentrations have been found to be more informative than blood, serum or milk concentrations in the Swedish population, as urinary concentrations showed lower day-to-day variability and were detected at much higher concentrations than in other matrices [17]. However, others [18] showed that caution is needed when using urine samples from pregnant women to screen for phthalate metabolites, as metabolism and excretion of phthalates varies significantly with changing exposure and/or physiological status during pregnancy.

As mentioned earlier, urinary biomarkers generally reflect metabolites of compounds rather than the pure compound itself. This makes it more difficult to correlate urinary biomarkers with biomarkers of exposure in other matrices. For non-metabolised compounds such as cadmium, correlations between levels in urine and other matrices such as blood appear to be good [28]. The same reasoning holds for correlations between urinary biomarkers and external exposure concentrations. Wang et al [29] have reported good correlations between unmetabolized volatile organic compounds in urine and concentrations in indoor environments, but correlating metabolite concentrations in urine to pure compounds in environmental compartments remains more challenging [30]. If positive correlations are found among different matrices, these are generally improved if urinary biomarkers are corrected for creatinine level or density.

Validation status

Urine as a matrix has widespread use, for both biomarkers of exposure and biomarkers of effect. Urinary biomarkers have been fully validated for a wide variety of substances, reference materials and internationally recognised standard methodologies are often available, and sampling procedures are well-documented.

Cord blood and placenta

Relevance as a non-invasive biomarker

The use of cord blood or placenta as a non-invasive matrix in biomonitoring is one of the most relevant non-invasively collected matrices with regard to the protection of infants and children from the early adverse effect of environmental contaminants. Although the female uterus is a very safe and protective place for unborn children, the fetus can prenatally come in contact with potentially hazardous substances through the placenta. The placenta can however also act as a barrier by concentrating specific toxicants and thereby to some extent reduce exposure of the foetus [31, 32, 33]. Since it has been widely illustrated that developing organisms, human as well as others, are particularly sensitive towards toxicological insults, the unborn and newborn child deserves specific protection and attention [34, 35]. This has also been addressed by policy makers, who have identified the need to better protect newborns and children from the negative effects from environmental pollution in for example the PINCHE (Policy Interpretation Network on Children's Health and Environment) or SCALE initiatives. Using placenta and/or cord blood as a matrix for biomonitoring can at the same time provide a picture of the substance exposure history of the mother and the early exposure of the newborn infant.

One problem associated with the use of the placenta as a non-invasive matrix for biomonitoring is the acquisition of a homogeneous tissue sample. Placenta is a complex

mixture of blood vessels, chorionic villi and membranes, and metals for example are not uniformly distributed in the different compartments. This raises the question of representativeness of the samples material. Iyengar and Rapp [33] provide an overview of different methodologies to gather placenta samples, but argue that no harmonised approach is practised. The main disadvantages of using cord blood or placenta as a matrix for biomonitoring is obviously that only women are sampled and that the timing of births is largely unpredictable. Additionally, the collection of cord blood during delivery is a secondary process, and it will not always be practicable to collect standardised, uncontaminated and representative samples.

Biomarkers of exposure

Iyengar and Rapp [36] describe how the placenta can be used to detect the presence of toxic trace elements such as arsenic, cadmium, mercury and lead. Table 2 provides an overview of the typical range of a number of these trace elements. These authors [36] additionally provide data indicating that concentrations of trace elements can vary 5- to 10-fold due to specific exposure situations, such as living in the vicinity of coal and metal mining and smelting [31].

The placenta may also be a useful matrix to investigate exposure to organic compounds, such as chlorinated hydrocarbons, PAHs, dioxins and pesticides [37, 38]. Table 3 gives an overview of typical concentrations of some organic compounds in cord blood and placenta. Shen et al [35] documented the accumulation of a wide variety of organic compounds including DDT, hexachlorobenzene, or dieldrin in the placenta. Wang et al [36] detected average PCDD/PCDF and PCB levels in placental tissue of 12.8 pg WHO-TEQ/g lipid (95% CI 11.5-14.1 pg/g lipid).

Cord blood has been extensively used to monitor substance exposure of newborns, in combination with the mother's exposure profile. Metals and organic compounds such as

pesticides and PCBs have all been extensively measured in cord blood samples. Basically, all biomarkers of exposure, effect or susceptibility that have been developed and standardised for venous blood samples can also be applied to cord blood samples, taking into account the specific nature of gathering cord blood and the transfer of compounds through the placental barrier. Cord blood was identified as the primary matrix to measure exposure to methylmercury, with also the dried umbilical cord tissue correlating well with cord blood and hair concentrations [40].

Biomarkers of effect

Because of its particular nature, measuring potentially harmful effects of substances in the placenta is of the utmost importance for the protection of the newborn child. In both placental tissue and cord blood, biomarkers for DNA damage have been measured using different methods, including ³²P-postlabeling and ELISA methods [41, 42]. CYP1A1 induction and placental glutathione status have also been proposed as biomarkers of environmental chemical stress [35]. It is alarming that observations have shown that although the estimated PAH dose to the foetus may be 10-fold lower than in the mothers, the PAH adduct levels in the newborns are similar to, or higher than, those in their mothers. This may imply that the foetus may be 10 times more susceptible to DNA damage than its mother: hence the urgent need to protect the unborn child and to closely monitor pollutant levels in placenta and cord blood [40].

Wang et al [41] demonstrated in utero effects of placental dioxins and PCBs on thyroid function, with significantly increased T₃, T₄ and thyroid-binding globulin (TBG) concentrations in cord blood correlating to upper-median exposure groups for female but not male infants. Other studies on the other hand found an inverse relation between thyroid hormone concentrations and levels of organochlorine compounds in cord blood. Maervoet et al [42] concluded that, while there are still many gaps in the understanding of the

relationship between environmental contaminants in cord blood and the functioning of the thyroid system, it is clear that any interference may adversely affect neurodevelopment early in life. Recently, epidemiological evidence has emerged that shows PFOS and PFOA concentrations in cord blood are related to altered physiological parameters in newborns, such as head circumference, birth weight or size, or ponderal index [43, 44]

The discovery that placental nucleic acids can be used as a marker for prenatal screening, using real-time quantitative PCR, has recently opened the door for the application of rapidly developing technologies such as toxicogenomics and proteomics, and the development of molecular markers for non-invasive prenatal gene expression profiling of the foetus using placenta and cord blood [45].

Correlation with other (non-invasive) biomarkers

For persistent organic contaminants, good correlations are generally found between paired cord blood or placenta samples and other matrices such as maternal blood, human milk or amniotic fluid. Shen et al [35] however illustrated how metabolites of less persistent organic compounds are found in higher concentrations in placenta than in human milk, which highlights the metabolization potential of the placenta. A range of typical concentrations of selected metals and organic compounds is provided in Tables 2 and 3.

Cotinine levels are generally significantly higher in newborn cord blood plasma than in paired maternal plasma [46]. Van Oostdam et al [47] found excellent correlations between cord blood and maternal blood samples for pesticides and PCBs in the Arctic. For metals, good correlations are usually observed when metal content of cord blood is compared to paired maternal blood concentrations, human milk or other tissues [40, 48].

Whyatt et al [46] found that the levels of DNA damage from PAHs were higher in cord blood white blood cells than in paired maternal white blood cells, which may be an

illustration of the reduced detoxification capabilities or decreased DNA repair capacity during foetal development.

Validation status

Biomarkers of exposure and effect are generally well validated for cord blood and placenta measurements, at both a methodological and an epidemiological level. Epidemiological evidence on the effects of transplacental exposure of environmental pollutants on birth outcomes has highlighted that for example levels of PAH-DNA adducts in cord blood are associated with lower birth weight or smaller head circumference [34]. Also oestrogen or progesterone metabolism have been associated with environmental tobacco smoke, dioxins and PCBs [38, 39].

Although the measurement of placental nucleic acids has been established [45], Myllynen et al [38] argue that it is currently too soon to estimate fully the potential of these techniques as validation of these biomarkers is still lacking. Optimal and appropriate biomarkers of placental toxicity are still being sought.

Human milk

Relevance as a non-invasive biomarker

Human milk is a sensitive matrix for the assessment of individual or population exposure to pollution. It is obtained non-invasively and contains a high proportion of lipids. In fact, human milk offers a number of exclusive nutritional and protective properties, which makes it a unique food source for newborn development [49]. Currently human milk is considered one of the most acceptable matrixes for monitoring persistent bio-accumulating toxicants (PBTs). In the lactation period, human milk is a more effective decontamination route than placenta due to its more fatty character [50], especially for primigravidae mothers [51]. Human milk is easy to collect, enriched in lipophilic compounds and represents the main

exposure source for breast feeding infants [52]. Exposures to chemicals around the home and in processed foods are also easily measured in human milk. As with placental materials, the main disadvantage of using human milk as a matrix for biomonitoring is that only women can be included in a biomonitoring study, and only at certain periods in their lives. The collection of human milk during lactation is a secondary process, and it will not always be practicable to collect standardised, uncontaminated and representative samples. Additionally, not all chemicals in a mother's body are transferred to human milk, so while human milk reflects exposure to the newborn child, it does not necessarily reflect complete exposure of the mother.

Biomarkers of exposure

Many persistent environmental pollutants, such as polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) are widespread and can be found in blood, milk and adipose tissue of human (see Tables 2 and 3 for typical ranges). Chemicals that are lipophilic and resistant to metabolic degradation have a tendency to accumulate with increasing levels of the food chain and to distribute to fatty tissue and human milk within the human body. Thus, human milk might be a useful matrix to investigate exposure of organic compound, such as POPs, which are lipophilic and persistent organic pollutant. They bioaccumulate via the food web and cause adverse effects to human health and the environment, and transfer from mother to infant through breastfeeding. High levels of POPs were measured in human milk samples from northern Russia [53]. Anderson and Wolff [54] documented increased serum levels of polybrominated biphenyl, PCBs, hexachlorobenzene, mirex, and lindane and DDE with increasing length of breast feeding in children. Human milk thus seems to be a major source of these pollutants during this period. Alarming, the median daily intake of dioxin levels is 77 pg I-TEQ/kg bw for a nursed 5 kg baby, which is about 50 times higher than the average daily PCDD/F intake for an adult [55]. Also, it has

been indicated that the mean daily intake of these compounds by babies is 10-38 times higher than the average WHO tolerable daily intake for lifelong exposure [56]. The exposure of the nursed infant to PAHs via milk has been shown to depend on the daily cigarette consumption of the mother [57]. Not only persistent organic compounds are present in human milk. Several toxic metals and their derivatives (eg. mercury and methyl mercury, lead, cadmium) also occur [58, 59]. El-Nezami et al [60] illustrated the presence of aflatoxins in human milk and its transfer to the infants.

Despite the detection of a variety of potentially hazardous substances in human milk, it still needs to be stressed explicitly that human milk is by far the best food source for an infant, and the established benefits of breastfeeding by far outweigh the risks of exposure to chemicals [49].

Biomarkers of effect

To our knowledge, no biomarkers of effect have been measured in human milk. However, exposure markers in human milk have frequently been associated with health effects in newborns. The persistent environmental pollutants in human milk samples from general populations have led to concerns that these chemicals may have detrimental effects on the health or development of breast-fed children. Several epidemiological studies reported a relationship between frequent dietary consumption of fish by child bearing-aged women and deficits in the neurological development of their children [61, 62]. Another study observed that bio-persistent chemicals in human milk, (e.g. hexachlorobenzene, p,p'-DDE, methylmercury, and PCBs) are associated with mild neurodevelopment deficits as a possible health hazard in infants, neurological interference was increased with the increasing level of contaminants in human milk, and the plasma level of thyroid stimulation hormone were increased [63].

Correlation with other biomarkers

The evaluation of persistent organic compounds in human milk has shown good correlations with biomarkers in other matrices such as placenta maternal blood and amniotic fluid. The serum level of several persistent organic compounds in children increases with the length of breast feeding and point to human milk as the main source of the pollutants during this period. Nair et al [51] reported that human milk may be the main source of chlorinated pesticide contamination to the newborn as levels typically found in maternal serum and cord serum generally are much lower than in human milk.

When concentrations of pollutants are expressed on a lipid basis, good correlations are generally reported between whole blood, human milk and adipose tissue level [52]. Shen et al [35] documented the accumulation of a wide variety of organic compounds such as DDT, hexachlorobenzene and dieldrin in the placenta. Wang et al [39] detected average PCDD/PCDF and PCB levels in placental tissue of 12.8pg WHO_TEQ/g lipid (95% CI 11.5-14.1 pg/g lipid). Some persistent PBT concentrations in placenta, such as p, p'-DDE, b-HCH, HCB, dieldrin, p,p0-DDT or mirex, can be predicted from milk concentrations, or vice versa, because of their linear relationship. For these compounds, both milk and placenta samples are suitable for biomonitoring. However, prenatal exposure to some less persistent PBTs cannot be predicted as the placenta probably metabolizes them. For these compounds, placenta appears to be more suitable for biomonitoring than milk to assess prenatal exposure [39].

Validation status

Human milk as a matrix has widespread use for biomarkers of exposure. It is easily collectable and fully validated for a wide variety of substances and reference materials. Internationally recognised standards for analysis are often available, and sampling procedures are well documented. The WHO coordinates a regular exposure study to using human milk to quantify the global presence and distribution of persistent organic pollutants.

Within the context of this repeated human biomonitoring program, guidelines for developing a national protocol for sampling human milk have been developed [64, 65].

Exhaled breath

Relevance as a non-invasive biomarker

The air that we breathe can be a major exposure route for a wide variety of potentially hazardous substances, mainly depending on their physico-chemical properties such as partitioning coefficients. Hence, it should not be surprising that a large number of substances that are detectable in ambient air can also be present in expired air in concentrations of biological and toxicological relevance. An advantage of using exhaled breath as a matrix for biomonitoring, particularly for volatile organic compounds, is that this matrix does not generally integrate exposure through a variety of routes, as is the case with blood, urine, or hair, but isolates exposure through air as the sole pathway. For other, less volatile compounds, this may be less restrictive due to the potential rapid diffusion of substances at the blood-gas barrier in the lungs. Measuring exposure and effect in exhaled air may be highly suitable for the characterization of dose at the target organ level, in this case the lungs and respiratory system. Because for most collection systems only tidal breathing is needed, the samples can be collected in a broad range of subjects: samples can also be collected from very young children, or individuals with airway diseases.

In general, exhaled breath can be subdivided into two clearly distinct fractions, a gaseous fraction and a fluid, condensate fraction. As will be illustrated further, both fractions have applicability in biomonitoring, though their biological consequences may be very different. On the one hand, the gaseous fraction of exhaled breath is more often used to measure biomarkers of exposure, particularly for volatile organic chemicals (VOCs) such as benzene. The fluid fraction, generally referred to as exhaled breath condensate (EBC), can be

obtained by cooling exhaled air and is most frequently used to measure biomarkers of effect, including for example protein or metabolite profiles. However, this division is by no means fixed and examples of biomarkers of exposure and effect will be given for both gaseous and fluid fractions.

Biomarkers of exposure

Probably the best known biomarker measured in exhaled breath is ethanol, which is frequently applied in the context of traffic safety control. In this routinely applied technique, ethanol concentrations in exhaled air are quantified and assumed to be correlated with blood levels, hence indicating potential alcohol intoxication in drivers. The gaseous fraction of exhaled breath can likewise be a good biomonitor for a wide variety of environmental substances. For example, 2,5-dimethylfuran is a good biomarker for smoking status of constituents. Also benzene has been proposed as a suitable indicator for active smoking [66], though there are generally too many other sources of benzene for it properly to function as an indicator of passive smoking [67]. Situations in which benzene proves to be a good indicator of exposure include exposure to automobiles, petrochemical products and combustion processes [68].

The monitoring of metals in exhaled breath is less well documented, although Mutti et al [69] show that toxic metals and transition elements are detectable in EBC, and may have potential in the assessment of the target tissue dose for substances with potential pneumotoxic activity, such as Cd, Co or Ni [69, 70].

A disadvantage of biomarkers in the gaseous phase of exhaled air is that the residence time of substances are generally rather short, in the order of minutes or hours. Hence they only represent short term exposure [66].

Biomarkers of effect

Exhaled breath has received much attention as a suitable clinical matrix for the early detection of pulmonary and respiratory diseases. Generally, the methods used for the detection and evaluation of diseases such as asthma and chronic obstructive pulmonary disease (COPD) are clinical in nature, and of limited value in environment and health research. However, a number of biomarkers of effect have been identified that may offer better insight between the presence of hazardous substances and associated health effects. It has extensively been demonstrated that fractional exhaled nitric oxide (FeNO) levels in exhaled air are higher in people suffering from various pulmonary diseases, including asthma and COPD [71, 72]. A significant amount of research shows that FeNO is a very sensitive and specific marker of pro-inflammation and oxidative stress in the lungs, with good reproducibility, sensitivity, specificity, and a well-documented dose-response association with different respiratory health effects.

Other biomarkers, such as exhaled breath temperature, pH of EBC, and the presence of cytokines (e.g. IL-4, IL-6, IL-8 and TNF- α), 8-isoprostane or hydrogen peroxide may also be biomarkers of lung inflammation and oxidative stress [71, 73]. Furthermore, it was demonstrated that a combination of biomarkers in exhaled breath (e.g. a combination of biomarker measurement in EBC and FeNO in the gaseous fraction) may increase the applicability, sensitivity and specificity of biomarkers in exhaled breath [71].

Most studies use ELISA kits to measure specific cytokines in EBC, but a very promising tool is the use of proteomics, in which a broad range of proteins can be measured simultaneously. In this approach, protein patterns can be studied instead of a single biomarker, which might be more useful in clinical practice or environmental biomonitoring. In a relatively new development, the profiling of low-molecular weight endogenous macromolecules and metabolites in both EBC and exhaled air may have useful applications in terms of a rapid screening or early warning tool [71, 74]. Using biochemical profiles of

these compounds through for example metabonomics in EBC or the use of an “electronic nose” for exhaled air may provide sensitive, rapid and easy-to-use screening techniques [71, 74]. The use of metabonomics may offer additional tools for EBC analysis, not only in the detection and quantification of known metabolites, but also in the prediction and identification of new markers and unknown metabolites, which may provide more insight into disease mechanisms. Carraro et al [74] have already illustrated how EBC metabonomics can define the airway biochemical phenotype in patients with asthma and argue that NMR metabonomics may have a multitude of further applications in pneumonology. Di Natale et al [75] were able to identify lung cancer patients by the analysis of the volatile substances in exhaled air using biosensor technology, usually referred to as an “electronic nose”. Others have illustrated how the VOC profile in exhaled breath of patients with lung cancer [76], pneumonia [77] or diabetes mellitus [78] have distinct characteristics that can be identified with an electronic nose biosensor.

Correlation with other (non-invasive) biomarkers

With regards to biomarkers of exposure, the Total Exposure Assessment Methodology Studies have established strong relationships between exhaled breath concentrations of VOCs and associated personal exposure, indicating that biomarkers in exhaled breath can provide information on previous exposure as well as current body burden [66, 67]. Nieuwenhuijsen et al [79] also illustrated that the concentration of water disinfection by-products correlated well with concentrations in other matrices such as blood serum or urine. The main problem associated with the correlation between alveolar chloroform and plasma chloroforms for example, is the relatively short biological half-life of chloroform in exhaled air, emphasising the need to take into account time since last exposure for volatile organic compounds.

Biomarkers of effect in EBC did not correlate well with those measured in broncho-alveolar lavage (an invasive method to sample the lining fluid of the lower respiratory tract), although this lack of correlation may reflect the differences in sampling strategy, as EBC samples reflect a much larger portion of the respiratory tract [73]. However, biomarkers of effect measured in EBC did correlate well with clinical observations in patients suffering from a range of inflammatory diseases including asthma and COPD, highlighting the health relevance of the biomarker measurements.

Validation status

A major advantage of measuring biomarkers in EBC and exhaled air is that an immediate quantification of relevant pneumotoxic substances is available, which provides an accurate estimate of the active dose at the level of the target organ. Methods of collecting and sampling exhaled breath biomarkers include Tedlar bags, canisters, or even portable real-time detectors. These methods are generally well validated and documented, as are the measurement techniques. The circumstance that sampling matrices are very similar to air and water, and do not show extensive matrix effect due to the presence of potentially interfering solutes, limits confounding matrix effects and strengthens the ease of use, sensitivity and efficiency of quantification [70]. Both the European Respiratory Society (ERS) and American Thoracic Society (ATS) have developed recommendations for measuring FeNO, and measurements are highly reproducible if careful attention is paid to technique [71, 72]. ERS and ATS also developed recommendations for the collection and measurements in EBC [80]. However, this method still needs further research (reproducibility of the markers, mechanisms of particle formation, dilution factor, etc) before this technique can be routinely used in clinical practice.

Hair

Relevance as a non-invasive biomarker

Hair as a non-invasive biomonitoring matrix has been successfully used to measure both internal and external exposure to a wide variety of organic and inorganic pollutants [81, 82, 83, 84]. With recent analytical developments, it is currently feasible to measure concentrations of metals such as Zn, Cu, Cr, Hg or Pb in individual hair strands (Table 2) [85, 86]. Also analysis of organohalogenated and other organic pollutants in hair samples has become more and more popular in recent years (Table 3). The attractiveness of using hair as a biological non-invasive matrix is in the fact that there generally are no ethical restrictions in collection with respect to age or gender. Even in newborns, hair samples can be collected in a standardised fashion, without needing to correct for confounding factors, as is the case for creatinine in urine samples for example. As hair grows about one centimeter per month, analysis of hair of different length may reflect cumulative exposure over several months.

Potential constraints on the use of hair include the difficulty in differentiating between internal and external sources of contaminant, as the contaminant load may be a result of uptake inside the hair or adsorption to the outside of the hair strand. However, this factor can also be a benefit. Schramm et al [87, 88] indicated that, through a successive washing process, fat and sweat as well as exogenous substances is removed from the surface of hair. Thus, the short-term external and long-term internal contamination can be potentially separated during sample preparation.

Biomarkers of exposure

Environmental organic contaminants have been proven to reach hair via two major routes: endogenous (xenobiotics reach the hair matrix through blood) and exogenous (atmospheric deposition) [83; 89]. Schramm et al [87] first reported the occurrence of PCDD/F on hair, and subsequently identified fast adsorption and a slow desorption of relevant compounds on

hair [90]. These authors also reported partition coefficients between ambient air and hair. Thus, gaseous exposure can potentially be predicted from hair concentrations. Findings by Nakao et al [91] and Stupar et al [92] also show that hair can be used as an indicator for detection of atmospheric exposure and in some cases even for the estimation of corresponding air concentrations.

Under the appropriate washing steps, exogenous contamination can be removed, allowing endogenous contamination levels to be successfully analyzed. As mentioned earlier, refined analytical techniques allow for the detection of metals in single hair strands, while a wide variety of organic contaminants can also be detected in hair. Table 2 provides an overview of typical concentrations of contaminants detected in hair. Probably the best-known use of hair as a non-invasive matrix is in the biomonitoring of organic and inorganic mercury, as hair is by far the best integrator of past exposure [93].

Hair samples have also extensively been used in non-invasive ecosurveillance monitoring, for example in the monitoring of contaminant levels in both domestic animals [94] and wild animals such as hedgehogs [95] or bird feathers [96].

Biomarkers of effect

To our knowledge, there are no examples of biomarkers of effect measured in hair samples. Recently however, RNA isolation from hair follicles has opened the possibilities to use hair for gene expression analysis, which may in the future have relevance for developing signatures of toxicant exposure [97].

Correlation with other (non-invasive) biomarkers

Many authors have addressed the correlation between contaminants in hair and other matrices. Nakao et al [98] found moderate to strong correlations between the contamination levels of PCDD/Fs and Co-PCBs in hair and blood, collected from six donors. Recently, Altshul et al [99] collected human hair samples from 10 volunteers and analyzed the

exposure level of organochlorine pesticides (OCPs) and 57 individual PCB congeners. Strong hair-to-blood correlation ($r=0.8$) of p,p'-DDE and moderate correlations between hair and blood levels of PCB congeners 28, 74, 99, 170, 180 and 194 were found. o,p'-DDE levels were negatively correlated between hair and blood. The other PCB congeners and OCPs showed no or only weak correlations between the two matrices.

For inorganic compounds, Mehra and Juneja [100] reported significant correlations between hair and nail levels of Cd and Pb, Stupar et al [92] found significant correlations between Pb in hair and blood, and Ng et al [101] reported significant correlations between hair mercury and mercury in blood, 24h urine or cord blood samples following a meta-analysis of mercury biomonitoring studies.

Validation status

A review by the Agency for Toxic Substances and Disease Registry highlighted some of the shortcomings of hair as a non-invasive biomonitoring matrix [102]. Although the review recognised that hair is a very useful matrix for identifying historical exposure to contaminants and may have predictive value towards health effects, a large amount of uncertainty remains regarding sampling procedures, quality assessment and control issues, and the lack of reference ranges and dose-response outcomes. In a recent review however, Schramm [88] provides a detailed overview of past research on the development of hair analysis for organic contaminants. This includes analytical procedures, successful applications and their limitations along with sampling requirements, sample processing, extraction procedures, clean-up and analysis methods for organic contaminants. Also, a broad overview of potential confounding factors is presented, including factors such as age, gender, smoking behaviour or the effect of hair treatment and colouring.

Schramm [88] concludes that hair analysis provides an ethically acceptable, inexpensive and easily applicable biomonitoring system for human and animal samples. Furthermore, the

special structure and position of hair makes the elaboration of short-term and long-term exposure of individuals possible. However, there are still some restrictions, which limit hair analysis as a valid monitoring tool for risk assessment. Therefore, it encourages further studies in the following fields:

- Establishment of standardized analytical process, including sampling, washing procedure, extraction, analytical methods and quality control/quality assurance.
- Study of the kinetic and mechanisms by which organic contaminants are incorporated into hair and their retention in the hair matrix, and identification of their metabolites in hair
- Establishment of a database of POPs in human hair
- Exploration of possible dose-response relationships
- Better understanding of hair biology (variations of hair composition with age, gender, race, etc)

Other relevant matrices

Relevance as a non-invasive biomarker

Apart from the matrices discussed above, there are a number of other non-invasively collected matrices that offer great potential for routine application in human biomonitoring. Although their use and applicability is much less documented in literature than the ones previously discussed, some examples and potential future applications are given below.

Biomarkers of exposure

Finger/toe nails: metals have frequently been monitored using fingernails or toenail clippings. Mehra and Juneja [100] reported concentrations of cadmium and lead in toenails from both occupationally exposed and environmentally unexposed subjects, and [103] used the concentration of arsenic in fingernails to confirm chronic exposure of a northern Chilean

population to arsenic in drinking water. Recently, [104] determined 14 different metals in toenail clippings from Arab-Americans in the Detroit area, Michigan, illustrating how toenail clippings could provide insight into lifestyle and demographic factors influencing exposure to metals.

Meconium/feces: meconium is the earliest stool from an infant, and can be used to check for in utero exposure to substances. Meconium is often used to quantify the neonate's exposure to harmful substances such as alcohol [105], environmental tobacco smoke [106], or other illicit drugs [107]. Meconium has also been used to quantify neonatal exposure to environmental contaminants, mainly organochlorine and organophosphate pesticides [108, 109, 110] and metals [111, 112,113] . For later stages of life, feces has been found to represent a suitable matrix for current gastrointestinal exposure to e.g. lead [114].

Saliva: Saliva sampling has been applied for a variety of contaminants and applications. Yuan et al [115] used saliva to determine arsenic speciation and found the concentrations to correlate well with drinking water arsenic. Moreover, participants with increased saliva arsenic concentrations also showed increased odds ratios for skin lesions, an important clinical symptom of chronic arsenic exposure and poisoning. Also other metals, such as lead [116], mercury [117] or several other metals [118] have been detected in saliva, and were often correlated with identified exposure sources. The presence of non-persistent organic chemicals in saliva is well documented, often following occupational exposure of pesticide applicators. Methods to determine chlorpyrifos [119], diazinon [120] or atrazine [121] have been described in literature, and were generally very sensitive in reflecting pesticide exposure and recovery after application. Also phthalates and several of its metabolites have been detected in saliva [122], and could used to estimate oral exposure to phthalates through mouthing of soft PVC baby toys [123].

Biomarkers of effect

Nail clippings: We have not found any examples of nail clippings used for effects monitoring.

Meconium/feces: No clear examples of the use of meconium or feces as a non-invasively collected matrix to determine health effects have been found in literature. However, exposure to metals and pesticides determined in meconium has previously been shown to be reflective of the neonate's health status, reflected in low birth weight or prematurity [112]. While feces has repeatedly been used as a non-invasively collected matrix for medical purposes, screening for diseases of the digestive tract such as inflammatory bowel diseases [124] or colorectal cancer [125], their application in environment and health research is not documented.

Saliva: Saliva as a non-invasively collected matrix has been used to determine health endpoints following exposure to environmental toxicants. Particularly the quantification of cholinesterase activity in saliva, a biomarker for potential neurotoxic effects, has been identified as potential useful indicator associated with exposure to organophosphorus and carbamate pesticides [126, 127]. Additionally, saliva specimens have been used in combination with the Ames test or a Chinese hamster V79 lung fibroblast cell line to investigate the genotoxic effects of smoking and alcohol consumption. The study showed that saliva showed a genotoxic and cytotoxic response, and may be predictive of increased risk for squamous cell carcinomas in the upper aerodigestive tract [128, 129].

Associated with the collection of salivary samples, exfoliated buccal cells have been used to monitor genetic damage in humans using the micronucleus test [130, 131]. This test has been used to illustrate the effects of a broad range of environmental and occupational contaminants, including petroleum derivatives such as benzene [132], ozone [133], or arsenic [134].

Correlation with other (non-invasive) biomarkers

Nail clippings: Mehra and Juneja [100] reported significant correlations between cadmium and lead concentrations in hair and nail clippings. In Nigerian auto-mechanics however, fingernail lead content failed to distinguish among exposed and unexposed subjects, while hair and blood lead levels were significantly different among these participants [135].

Meconium/feces: In many cases, meconium has been shown to be the most sensitive matrix to analyse fetal exposure to environmental contaminants. Ramirez et al [111] illustrated how the prevalence of mercury in meconium was higher than in maternal fluids, making this a very relevant non-invasively collected matrix to be measured to determine the load of fetal Hg. The authors illustrated that for 78 mother-infant pairs in a community with high Hg pollution, 46.1% of all meconium samples showed detectable amounts of Hg, while this was only 31.6%, 16.7% and 6.4% for respectively infant's hair, cord blood and human milk. Hg in meconium correlated well with levels in the mother's blood and cord blood. Also for a variety of pesticides, meconium showed the highest exposure rates compared to cord blood and infant hair [136].

Saliva: When paired blood and saliva samples were taken, pesticide concentrations generally correlated well among both matrices [119, 120, 121]. For metals, correlations are generally weaker. Urinary mercury levels correlated better with blood levels than with saliva levels [117], and also for lead, only weak correlations between saliva and blood levels were observed [116].

Validation status

Nail clippings: Slotnick et al [135] evaluated the effect of demographic characteristics and nutritional measures on the association between drinking water and toenail arsenic concentrations. Apart from toenail iron concentration, no demographic or nutritional parameters affected the biomarker response. In Kenya however, [136] reported that socio-economic background, health conditions, dietary habits and urban or rural living had a

significant effect on lead, cadmium, zinc and iron concentrations in the fingernails of school children. For arsenic, [137] illustrated that different ethnic groups showed significantly different levels of total arsenic in fingernails, suggestive of a different pattern of arsenic metabolism in different ethnic groups.

Meconium/feces: Because of the sensitivity of meconium for illicit drug screening, structured screening protocols have been developed [140, 141]. Also for environmental exposure to potentially harmful substances, validation studies have been performed and confounding factors have been defined and described [112, 142].

Saliva/buccal swabs: Because concentrations of toxicants in saliva are often very low compared to other matrices [115, 118], exposure monitoring using saliva is most documented for occupational exposure. The use of buccal cells to detect DNA damage using the micronucleus assay has been the subject of the HUMN project (Human Micronucleus Project), which aims at exploring sources of variability in the assay and resolve key technical issues. This will allow for a reliable comparison of data among populations and laboratories, taking into account inter- and intraindividual variability [130].

Conclusions

The use of non-invasively collected matrices can be a valuable alternative to, or addition for, invasive matrices such as peripheral blood sampling for most contaminants discussed. Generally, there is good agreement between invasive and non-invasive biomarker values. However, the applicability of non-invasively collected matrices is sometimes hampered by incomplete knowledge on, for example, toxicokinetics and validated sampling, sample treatment and analysis procedures. On the other hand, non-invasively collected matrices can offer substantial advantages for practical and routine implementation, such as increased

participation rates, repetitive sampling, more efficient inclusion of susceptible and vulnerable populations, and improved cost-efficiency.

Particularly within the context of risk and health impact assessment, the use of repeated sampling of non-invasively collected matrices to evaluate the efficiency of risk management options and efficacy of environment and health policies offers great potential.

Abbreviations

HBM: human biomonitoring; ES BIO: Expert team to Support biomonitoring in Europe; INTARESE: Integrated Assessment of Health Risk of Environmental Stressors in Europe; SCALE: Science, Children, Awareness, EU Legislation and Continuous Evaluation; 8-OHdG: 8-hydroxy-deoxyguanosine; NMR: Nuclear Magnetic Resonance; PAHs: polycyclic aromatic hydrocarbons; PINCHE: Policy Interpretation Network on Children's Health and Environment; PCDD/PCDF: polychlorinated dibenzodioxins/furans; TEQ: toxic equivalent; ELISA: enzyme-linked immunosorbent assay; PCB: polychlorinated biphenyls; TBG: thyroid-binding globulin; T₃: triiodothyronine; T₄: thyroxine; PFOS: Perfluorooctanesulfonic acid; PFOA: Perfluorooctanoic acid; PCR: polymerase chain reaction; PBTs: persistent bio-accumulating toxicants; POPs: persistent organic pollutant; DDE: Dichlorodiphenyldichloroethylene; HCH: hexachlorocyclohexane; HCB: hexachlorocyclobenzene; VOCs: volatile organic chemicals; EBC: exhaled breath condensate; COPD: chronic obstructive pulmonary disease; IL: interleukine; ERS: European Respiratory Society; ATS: American Thoracic Society; OCPs: organochlorine pesticides; PVC: polyvinylchloride

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors have taken responsibility for reviewing the use and applicability of different matrices, as described in the different chapters of the review. All authors read and approved the final manuscript.

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